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Development of recombinant OPCML tumour suppressor protein as a therapy in ovarian cancer

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This thesis is submitted in
fulfilment of the requirements for the degree of Doctor of Philosophy
(PhD) in Clinical Medicine Research

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DEDICATION

This work is dedicated, first and foremost, to my lovely husband, Massimo, my lovely daughter, Elizabeth and the little one who is coming soon, Olivia. I would not have been able to do this work without you all. Thank you so much for your love. This work is also for my mum, Anna, and dad, Leandro. I hope this will make you proud.

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ABSTRACT

Ovarian cancer is the leading cause of death for gynaecological malignancies. Despite the recent introduction of new successful therapeutics such as PARP inhibitors, the mortality rate for ovarian cancer remains high, highlighting the need for new treatments.

In over 80% of ovarian cancers, OPCML is silenced by loss of heterozygosity and epigenetic mechanisms.

OPCML is a highly conserved glycosylphosphatidylinositol (GPI)-anchored protein that exhibits tumour-suppressive properties across various cancer types (e.g., ovarian and lung cancer), likely due to its inhibitory effects on specific receptor tyrosine kinases (RTKs), including HER2 and AXL.

Given OPCML's localization on the plasma membrane and its proposed mechanism of action at the external leaflet, we postulate that a targeted protein therapy delivering recombinant OPCML directly to the cell surface could serve as an effective anti-cancer treatment strategy.

Here, I produced a soluble form of recombinant OPCML (rOPCML) and an Fc-fusion derivate (rOPCML-Fc) in HEK-293 cells.

rOPCML +/- Fc effects were tested in ovarian cancer cell lines, lung cancer cell lines and cancer cells from ascites-derived spheroids of an ovarian cancer patient.

The results show that rOPCML +/- Fc treatment induces tumour-suppressive effects comparable to ectopic OPCML expression.

Mechanistically, the treatment effectively inhibited the activation of the AXL and HER2, along with the downstream phosphorylation of effector proteins AKT and ERK1/2. Consequently, this significantly reduced cell viability, colony formation capacity, migration, and invasion while promoting apoptosis.

Furthermore, an extensive PamGene kinase activity assay demonstrated that rOPCML treatment inhibits the phosphorylation of other RTKs and downstream pathway effectors involved in ovarian cancer survival and invasiveness.

In conclusion, rOPCML +/- Fc demonstrates significant tumour-suppressive effects, paving the way for a novel therapeutic approach for ovarian and other cancers that could be administered systemically or delivered to the tumour sites (e.g., intraperitoneally during peritoneal washing).

ABBREVIATIONS

ABL	Abelson (Gene)
AC	Affinity Chromatography
ACK	Acetyl-CoA Carboxylase Kinase.
AJCC	American Joint Committee On Cancer
AKT	v-Akt Murine Thymoma Viral Oncogene Homolog
ALK	Activin Receptor-Like Kinase
ARHI	Aplasia Ras Homolog Member
ARID1A	AT-Rich Interactive Domain 1A Gene
ASGPR	Asialoglycoprotein Receptor
ATCC	American Type Culture Collection
AXL	Greek Word Anexelekto, Which Means “Uncontrolled”
BC	Breast Cancer
BHK	Baby Hamster Kidney
BRAF	v-Raf Murine Sarcoma Viral Oncogene Homolog B1
BRCA1	BReast CAncer gene 1
BRCA2	BReast CAncer gene 2
Ca125	Cancer Antigen 125
CA19.9	Cancer Antigen 19.9
CCC	Clear Cell Carcinoma
CDDP	Cisplatin
CDM	Chemically Defined Medium
CEA	Carcinoembryonic Antigen
CHO	Chinese Hamster Ovary
CI	Confidence Interval
CICs	Cortical Inclusion Cysts
CK7	Cytokeratin 7
CM	Conditioned Medium
CMV	Cytomegalovirus
CpG	5'—C—Phosphate—G—3'
CT	Computed Tomography

CTNNB1	β-Catenin Disorders
D1	Domain 1
D2	Domain 2
D3	Domain 3
DMEM	Dulbecco's Modified Eagle's Medium
ECD	Extracellular Domain
EDTA	Ethylenediaminetetraacetic
EFS	Embryonal Fyn-Associated Substrate
EGFR	Epidermal Growth Factor Receptor
EMA	European Medicines Agency
EMT	Epithelial-To-Mesenchymal Transition
EOC	Epithelial Ovarian Cancers
EphA	Erythropoietin-Producing Hepatocellular Receptor A
EPHA2	Eph Receptor A2
EphB	Erythropoietin-Producing Hepatocellular Receptor B
EphB2	Ephrin Type-B Receptor 2
ErbB	Derived From Erythroblastic Leukemia Viral Oncogene
ERK	Extracellular Signal-Regulated Kinase
ERK1	Extracellular Signal-Regulated Kinase 1
ERK2	Extracellular Signal-Regulated Kinase 2
EV	Empty Vector
FAK	Focal Adhesion Kinase
FCS	Foetal Calf Serum
FDA	Food And Drug Administration
FGFR	Fibroblast Growth Factor Receptor
FIGO	Federation Internationale De Ginecologie Et Obstetrie
FRK	Fyn-Related Kinase
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GAS6	Growth Arrest Specific 6
GlcNAc	N-Acetylglucosamine
GPI	Glycosylphosphatidylinositol
HE4	Human Epididymis Protein 4

HEK293	Human Embryonic Kidney Cells 293
HER2	Human Epidermal Growth Factor Receptor 2
HER4	Human Epidermal Growth Factor Receptor 4
HGF	Hepatocyte Growth Factor
HGSOC	High-Grade Serous Ovarian Cancer
HNF1	Hepatocyte Nuclear Factor 1
HR	Hazard Ratio
hr	Hours
HRP	Horseradish Peroxidase
HAS	Human Serum Albumin
HSF	Heat-Shock Factor
Ig	Immunoglobulin
IgLON	Immunoglobulin Of LSAMP, OBCAM/OPCML, And Neurotrimin
InsR	Insulin Receptor
IRDB	Institute Of Reproductive And Developmental Biology
JAK	Janus Kinase (Aka Just Another Kinase)
LB	Luria-Bertani
LGSOC	Low-Grade Serous Ovarian Cancer
LMR	Lemur Tyrosine Kinase
LOH	Loss Of Heterozygosity
LSAMP	Limbic System-Associated Membrane Protein
MAPK	Mitogen-Activated Protein Kinase
MCS	Multi-Cloning Site
MEK	Mitogen-Activated Protein Kinase Kinase
MET	Mesenchymal Epithelial Transition Factor Receptor
MMP	Matrix Metalloprotease
MRI	Magnetic Resonance Imaging
mTOR	Mechanistic Target Of Rapamycin
MuSK	Muscle-Specific Kinase
NACT	Neoadjuvant Chemotherapy
NEGR1	Neuronal Growth Receptor 1

Neu5Gc	<i>N</i>-Glycolylneuraminic Acid
NFkB	Nuclear Factor Kappa B
NK	Natural Killer
NRTKs	Non-Receptor Tyrosine Kinases
NSCLC	Non-Small-Cell-Lung Cancer
OBCAM	Opioid-Binding Cell Adhesion Molecule
OC	Ovarian Cancer
OPCML	Opioid-Binding Protein Cell Adhesion Molecule
OR	Overall Response
OS	Overall Survival
OSE	Ovarian Surface Epithelium
PBD	Polybutadiene
PBS	Phosphate-Buffered Saline
PDGFR	Platelet Derived Growth Factor Receptor
PEG3	Paternally Expressed 3
PEI	Polyethylenimine
PFI	Platinum Free Interval
PFS	Progression Free Survival
PI3K	Phosphoinositide 3- Kinase
PKC	Protein Kinase C
PLAGL1	Pleiomorphic Adenoma Gene-Like 1
PTEN	Phosphatase And Tensin Homolog
PTK7	Protein Tyrosine Kinase 7
PTMs	Post-Translational Modifications
PTPRG	Lipid Domain-Localized Phosphatase
Raf	Rapidly Accelerated Fibrosarcoma
RAS	From Rat Sarcoma Virus
Ret	Derived From Rearranged During Transfection
rOPCML	Recombinant OPCML
ROS	Reactive Oxygen Species
RTKs	Receptor Tyrosine Kinases
SEER	Surveillance, Epidemiology, And End Results

SIR	Standardized Incidence Ratio
SP	Signal Peptide
SRC	Proto-Oncogene Tyrosine-Protein Kinase
STIC	Serous Tubal Intraepithelial Carcinoma
SYK	Spleen Tyrosine Kinase
TAM	Tumour-Associated Macrophages
TEC	Ec Protein Tyrosine Kinase
Tie	TGF-Beta Inhibitory Element
TKI	Tyrosine Kinase Inhibitor
TNM	Tumour, Nodes, Metastasis
Trk	Neurotrophic Tyrosine Kinase Receptor Type 1
TSG	Tumour-Suppressor Gene
uPA	Urokinase-Type Plasminogen Activator
uPAR	Upa Receptor
VEGFR	Vascular Endothelial Growth Factor Receptor
WT1	Wilms Tumor 1

CHAPTER 1

INTRODUCTION

1.1 Opcml

1.1.1 OPCML Structure and Function

The gene encoding opioid-binding protein cell adhesion molecule like (*OPCML*), also known as opioid-binding cell adhesion molecule (*OBCAM*), is located on human chromosome 11q2.5 and encodes a protein belonging to the IgLON family. IgLONs comprise OPCML, LSAMP (Limbic System-Associated Membrane Protein), Neurotrimin, NEGR1 (Neuronal Growth Receptor 1) and IgLON5 (Sellar et al., 2003; Czekierdowski et al., 2006; Ye et al., 2008; Sabater et al 2014).

IgLONs are glycosylphosphatidylinositol (GPI)-anchored cell adhesion-like molecules composed of three canonical Immunoglobulin (Ig)-like domains, an N-terminal cleavable signal peptide (SP) and a C-terminal GPI-anchor through which the IgLONs bind the outer leaflet of the plasma membrane preferentially clustering in the cholesterol-rich lipid domains (Figure 1.1) (Venkannagari et al., 2020).



Figure 1.1: Domain organisation of IgLon family

The IgLONs joint domain organisation is composed of an N-terminal cleavable signal peptide (SP) followed by three Ig-like domains and a C-terminal GPI anchor (GPI)

IgLONs tend to form homo and heterodimers called Diglons (Venkannagari et al., 2020; Reed et al., 2004).

OPCML is composed of 345 amino acids; the SP goes from methionine (Met, M) 1 to threonine (Thr, T) 27, the domain 1 (D1) goes from proline (Pro, P) 39 to serine (Ser, S) 126, D2 from Pro 136 to Thr 219, and D3 from Pro 223 to Thr 310 (Figure 1.2 A). Recently, the crystal structure of OPCML has been elucidated, and it has been confirmed that OPCML tends to create homodimers that resemble an inverted V-shape (Figure 1.2 B). A membrane-distal D1-D1 interface mediates the OPCML homodimer, and the OPCML dimer is anchored to the plasma membrane by two D3-linked GPI anchors. The membrane-distal D1-D1 interface is stabilised by reciprocal salt bridges between arginine (Arg, R) 65 of one monomer and aspartic acid (Asp, D) 80 of the other, together with a combination of hydrophobic contacts and hydrogen bonds. Of note, the D1-D1 dimerisation interface is highly conserved within the Ig-LONs. In fact, across the IgLON family members, five of the seven key residues between which salt bridges and hydrophobic contacts are created at the interface are conserved (i.e., Leu 69; Ile 74; Asp 80; Trp 82, and Thr 114) (Birtley et al., 2019).

A

```

MGVCGYLFLPWKCLVVSLRLLFLVPTGVPVRS GDA TF PKAMDNVTVRQGESATLRCTID
DRVTRVAWLNRS TI LYAGNDKWS IDPRVI ILVNTPTQYSIMIQNDVYDEGPYTC SVQTD
NHPKTS RVHLIVQVPPQIMNISSDITVNEGSSVTLLCLAIGRPEPTVTWRHLSVKEGQGF
VSEDEYLEISDIKRDQSGEYECSALNDVAAPDVRKVKITVNY PPYISKAKNTGVS VGQKG
ILSCEASAVPMAEFQWFKEETRLATGLDGMRIENKGRMSTLTFFNVSEKDYGNITCVATN
KLGNTNASIT LYGP GAVIDGVNSASRALACLWLSGTL LAHFFIKF

```

B

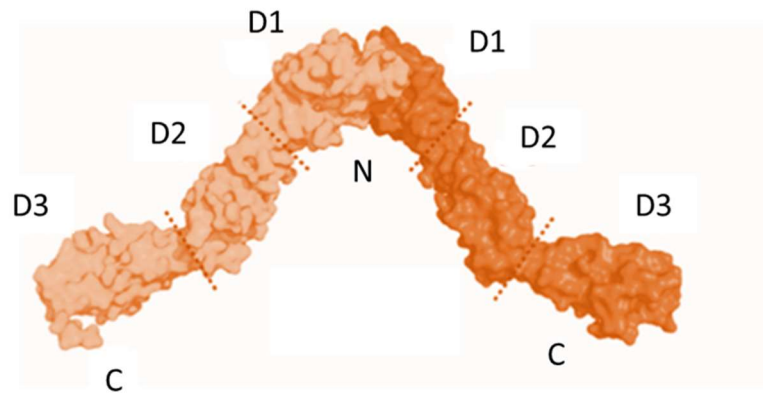


Figure 1.2: OPCML amino acids sequence and homodimer

- A. OPCML amino acids sequence. In blue is the SP amino acid sequence, in orange is the D1 amino acid sequence, in green is the D2 amino acid sequence and in yellow is the D3 amino acid sequence.
- B. Graphical representation of OPCML homodimer. In the figure, one molecule of OPCML is shown in light orange, and the dashed line separates the other in dark orange, and the boundaries of the domains. (Adapted from Birtley et al., 2019)

All the IgLONs are highly glycosylated, and glycosylation is fundamental to regulating their biological functions. For example, N-glycosylation sites near the N-terminus in OBCAM and in LSAMP and neurotrimin commonly contain di-sialic acids, which might play an important role in cell–cell interactions (Itoh et al., 2008).

Along with glycosylation, the OPCML protein undergoes several post-translational modifications (PTMs) reported in Table 1.1 (<https://www.uniprot.org/uniprotkb/Q14982>).

Table 1.1: OPCML PTMs

PTM Type	ID position (s)	Domain
N-Glycosylation	Asparagine (Asn, N) 44, 70 140 285,293,306	D1 D2 D3
Disulfide Bond	From Cysteine (Cys,C) to Cysteine 57-115 157-202 244-296	D1 D2 D3
Lipidation	N-322	GPI-anchor amidated asparagine

IgLONs are mainly expressed in the central and peripheral nervous systems, where they regulate cell adhesion, cellular communication, and axonal guidance (Eagleson et al., 2003; Miyata et al., 2003; Reed et al., 2007).

OPCML is widely expressed in the brain, moderately expressed in other adult and foetal tissues (e.g., kidney, spleen, stomach, trachea, testis, cervix, ovary, and prostate), and weakly expressed in the lung, breast, and bone marrow (OPCML available proteinatlas.org/ENSG00000183715-OPCML).

In the brain, OPCML seems to regulate opioid receptor function (Lane et al., 1992). In addition, functional studies have demonstrated that OPCML is necessary for neurite outgrowth and cell surface recognition events in brain development (Wilson et al., 1996; Faivre-Sarrailh et al., 1997; McNamee et al., 2002; Rougon et al., 2003). Furthermore, OPCML positively regulates synaptogenesis (Yamada et al., 2007) and is implicated in astrocytes' morphological changes and proliferation (Sugimoto et al., 2012). Zhang et al. also demonstrated that OPCML

regulates spine maturation and remodelling in hippocampal and cortical neurons by interacting with Ephrin type-B receptor 2 (EphB2), ultimately interfering with cognitive behaviours (Zhang et al., 2019). However, the molecular basis of these interactions has not been elucidated yet.

1.1.2 OPCML Role in Cancer

1.1.2.1 OPCML as a Diagnostic and prognostic biomarker

OPCML gene methylation levels and *OPCML* expression in cancer tissues are correlated with tumour grade and clinical outcomes, highlighting the possible role of *OPCML* as a prognostic biomarker. Specifically, low or absent *OPCML* expression in tumour tissues is associated with more aggressive tumours and lower overall patient survival (Duarte-Pereira et al., 2011; Zhou et al., 2014; Xing et al., 2017; Khamko et al., 2022).

In addition, several studies have suggested that *OPCML* could be a cancer epigenetic diagnostic biomarker. This is because *OPCML* hypermethylation appears to be quite specific for the presence of cancer, genomically stable, readily amplifiable, and easily detectable in patients' serum (Wasenang et al., 2019). Circulating methylated *OPCML* DNA has been detected in the serum of patients with ovarian cancer (OC) and was not detectable in healthy patients' serum. Additionally, *OPCML* CpG methylation has been detected in many tumour tissues but not in healthy ones (Zhang et al., 2016; Cui et al., 2008; Amornpisutt et al., 2015; Ye et al., 2008; Castaño-Rodríguez et al., 2018; Shao et al., 2021).

1.1.2.2 OPCML as a tumour-suppressor gene

In 1996, Gabra et al. first identified allele imbalance on chromosome 11 loci as a frequent event occurring in OC (Gabra et al., 1996). In the early 2000s, Sellar et al. identified a peak area of loss of heterozygosity (LOH) lying within the *OPCML* gene at 11q25, proposing *OPCML* as a tumour-suppressor gene (TSG). Subsequently, Sellar et al. demonstrated that *OPCML* somatic inactivation can mainly occur through CpG island methylation (Sellar et al., 2003).

Since then, a lack of *OPCML* expression has been demonstrated in many tumour types, including brain tumours (Reed et al., 2007), non-small cell lung carcinoma (Tsou et al., 2007), bladder cancer (Duarte-Pereira et al., 2011), cholangiocarcinoma (Sriraksa et al., 2011), nasopharyngeal, oesophageal, gastric, hepatocellular, colorectal, breast, cervical cancers, and OC (Cui et al., 2008). Although in most of these cancers, *OPCML* is lost by promoter methylation, Birtley et al. also described *OPCML* inactivating mutations occurring in different types of cancer (e.g., breast cancer, skin cancer, ovarian cancer) (Birtley et al., 2019). In addition, in glioblastoma and other brain tumours in which *OPCML* expression is lost, *OPCML* promoter methylation does not occur. This, therefore, suggests that *OPCML* could be transcribed from an alternative promoter or that other mechanisms of *OPCML* down-regulation could occur (Reed et al., 2007).

Following these discoveries suggesting the potential role of *OPCML* as a tumour-suppressor gene, different studies have evaluated its role outside the central nervous system.

Cui et al. demonstrated that OPCML has tumour cell growth inhibitory activities and suggested a potential role of OPCML as a modulator of stress response to several environmental stimuli. Therefore, speculating that epigenetic silencing of *OPCML* could be an early event that can promote cancer development by impairing normal cells' protective response to environmental stresses. They showed that after stress stimuli or in the presence of p53, the cell lines with unmethylated *OPCML* promoter responded by upregulating *OPCML*. In contrast, this mechanism was interrupted in cancer cells with the methylated promoter (Cui et al., 2008).

Mei et al. suggested that the oncogenic activation of *RAS* in some ovarian cancer cells also drives *OPCML* promoter hypermethylation and the consequent reduced expression of OPCML, a finding that underscores the urgency of understanding the role of OPCML in cancer. This suggests that OPCML inactivation could be essential for inducing RAS-mediated oncogenic transformation of human ovarian surface epithelial (HOSE) cells in OC cells (Mei et al., 2006).

Along with these findings, several other clinical and pre-clinical results have confirmed the functional properties of OPCML as a tumour suppressor (Sellar et al., 2003; Mei et al., 2006; Cui et al., 2008; McKie et al., 2012; Xing et al., 2017; Khanko et al., 2021). *In-vitro* and *in-vivo* studies showed that restoration of OPCML inhibits the growth and proliferation of ovarian, breast, gastric, and colon-rectal cancer and cholangiocarcinoma cells (Sellar et al., 2003; Mei et al., 2006; Cui et al., 2008; McKie et al., 2012; Xing et al., 2017; Khanko et al., 2021). OPCML restoration also significantly inhibits tumour growth in both subcutaneous and intraperitoneal models of xenograft nude mice (Sellar et al., 2003). In addition, OPCML determines cell cycle arrest,

which induces apoptosis because of the accumulation of cells in the G0/G1 state (Sellar et al., 2003; Xing et al., 2017).

McKie et al. were the first to decipher the potential mechanisms of action of OPCML as a TSG (McKie et al., 2012). They demonstrated that the tumour suppressive role of OPCML could be due to an impairment of a specific repertoire of receptor tyrosine kinases (RTKs), including Eph receptor A2 (EPHA2), FGFR1, FGFR3, HER2, and HER4 and AXL in OC and breast cancer (BC) cell lines (McKie et al., 2012; Zanini et al., 2017; Antony et al., 2018). At the same time, McKie et al. also demonstrated that OPCML does not regulate other RTKs, i.e., EGFR, EPHA10, FGFR2, HER3 and VEGFRs (McKie et al., 2012). However, the reasons and the mechanisms behind this selectivity of OPCML for some RTKs have not yet been clarified, nor has the question of whether the inhibition of all the RTKs mentioned above is due to a direct interaction of OPCML with all of them or to an interaction with only some of them.

To date, a direct interaction of OPCML has been demonstrated for HER2 EPHA2, FGFR1 and AXL (McKie et al., 2012; Zanini et al., 2017; Antony et al., 2018).

OPCML interacts with the extracellular domain (ECD) of FGFR1 or HER2 (McKie et al., 2012). Specifically, OPCML's interaction with HER2 causes a redistribution of HER2 to the cholesterol-rich, detergent-insoluble fraction of the plasma membrane, along with proteasomal degradation of HER2 through polyubiquitination (McKie et al., 2012). This interaction prevents the formation of the EGFR-HER2 heterodimer, resulting in reduced levels of phospho-HER2, HER2, and pEGFR, as well as the downregulation of their downstream

pathway, ultimately inhibiting tumour growth (McKie et al., 2012; Zanini et al., 2017).

The interaction between OPCML and AXL is triggered by AXL binding to its ligand GAS6 (Antony et al., 2018). Upon OPCML interaction, AXL is redistributed into cell membrane lipid domains, wherein it is deactivated by the lipid domain-localized phosphatase (PTPRG). This deactivation also determines a downregulation of AXL downstream pathways with a reduction in pERK and pAKT (Antony et al., 2018). Inactivation of AXL-mediated pathways seems to induce the Epithelial-to-Mesenchymal Transition (EMT) factor SLUG (Antony et al., 2016; Antony et al., 2019) that consequently prevents AXL-mediated motility and invasion determining primary tumour growth and metastasis spreading inhibition (Antony et al., 2016; Antony et al., 2018; Antony et al., 2019).

The restoration of OPCML not only reduces the phosphorylation of both HER2 and AXL but can sensitise HER2-overexpressing OC and BC cells to the dual HER2/EGFR tyrosine kinase inhibitor (TKI) lapatinib and anti-EGFR TKI erlotinib and OC cells to the AXL inhibitor BGB3425, sustaining a potential role of an OPCML recombinant therapy as a valuable sensitising approach in combination with TKIs (Zanini et al., 2017; Antony et al., 2018).

Along with OPCML tumour suppressive activity determined through RTK inhibition, OPCML's role as a negative modulator of EMT and metastatic dissemination has been further demonstrated in colon cancers, where this role seems to be mediated by TGF- β /SMAD pathway inhibition. In fact, OPCML restoration in colon cancer cells increased E-cadherin levels, decreased vimentin levels, and suppressed

the TGF- β /SMAD pathway by lowering total and phospho-SMAD2 and SMAD3. The deregulation of these promoters of motility, invasion and metastasis dissemination induced an EMT reversal and a less aggressive phenotype in colon cancers (Li et al., 2015).

Therefore, OPCML seems to act as a tumour suppressor by modulating specific pathways involved in cancer cell survival, dissemination, and EMT transformation. A recombinant OPCML treatment could represent an important therapy with cross-activity among different cancer types.

1.1.2.3 OPCML Mutations and their functional role

A recent analysis of DNA sequence data from TCGA and COSMIC databases has identified several *OPCML* mutations in different cancer types (Figure 1.3). Most are missense mutations, while only 3% were splice site variants and stop-codon-related alterations (Birtley et al., 2019).

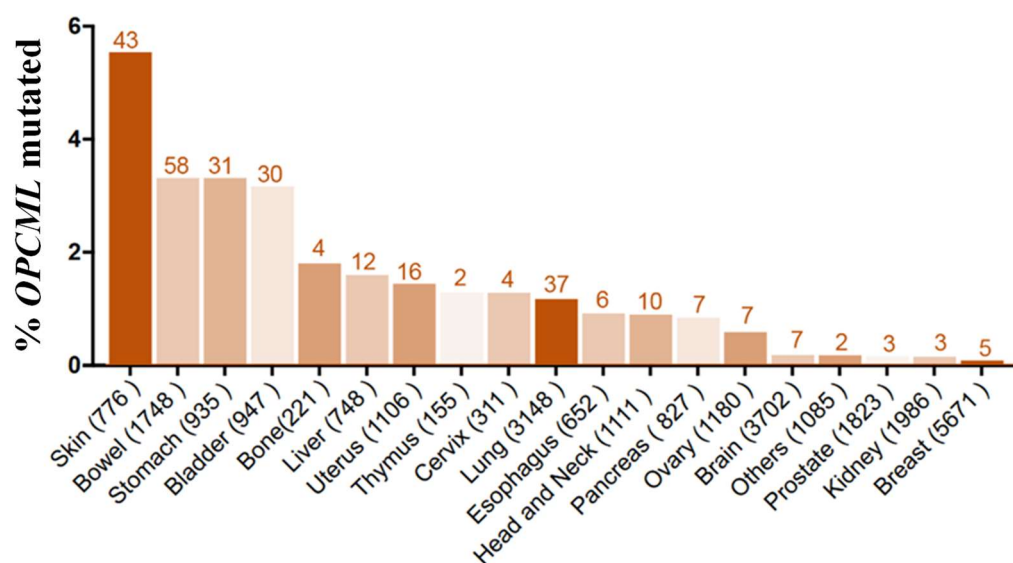


Figure 1.3: OPCML mutation frequency

Bar chart showing the frequency of OPCML mutations in different tumour types. The numbers in brackets on the x-axis indicate the total number of patients included in the dataset for each cancer type. In contrast, the number above the bars shows the number of patients with mutations. (Adapted from Birtley et al., 2019)

Although *OPCML* mutations are rare, the analysis of the clinically relevant *OPCML* point mutations *in-vitro* and *in-vivo* has allowed us to elucidate the functional characteristics of OPCML better (Birtley et al., 2019). OPCML mutations can occur in residues of all three domains of OPCML. However, the most studied are three mutations located within domain one: R65L, N70H, and P95R (Figure 1.4). OPCML carrying one of these mutations has a partial or complete loss of function (Figure 1.5).

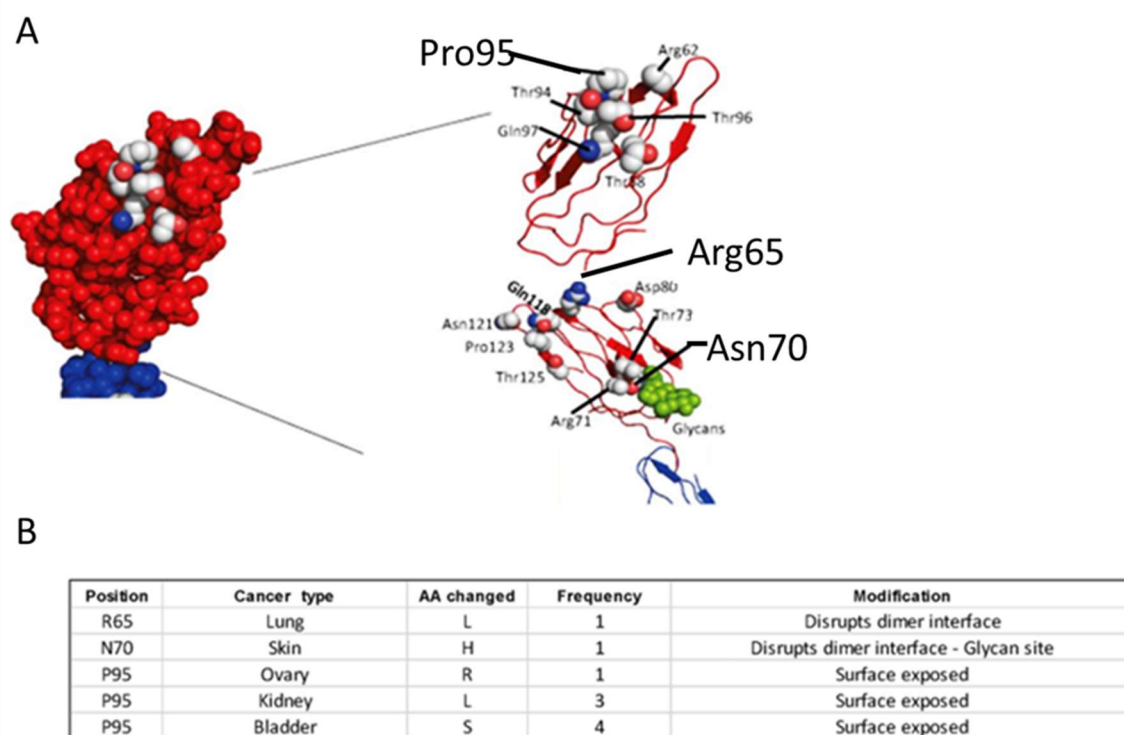


Figure 1.4: Domain 1 OPCML mutations:

Mapping of the OPCML mutations, discussed in the text and tested in the experiments, on the tertiary structure of OPCML (A). Summary table of the illustrated domain I mutations, showing mutation's position, cancer type, amino acid changed, and the possible modification occurring in OPCML protein (B) OPCML R65L, N70H and P95R mapped (underlined) on OPCML amino acids sequence (Adapted from Birtley et al., 2019)

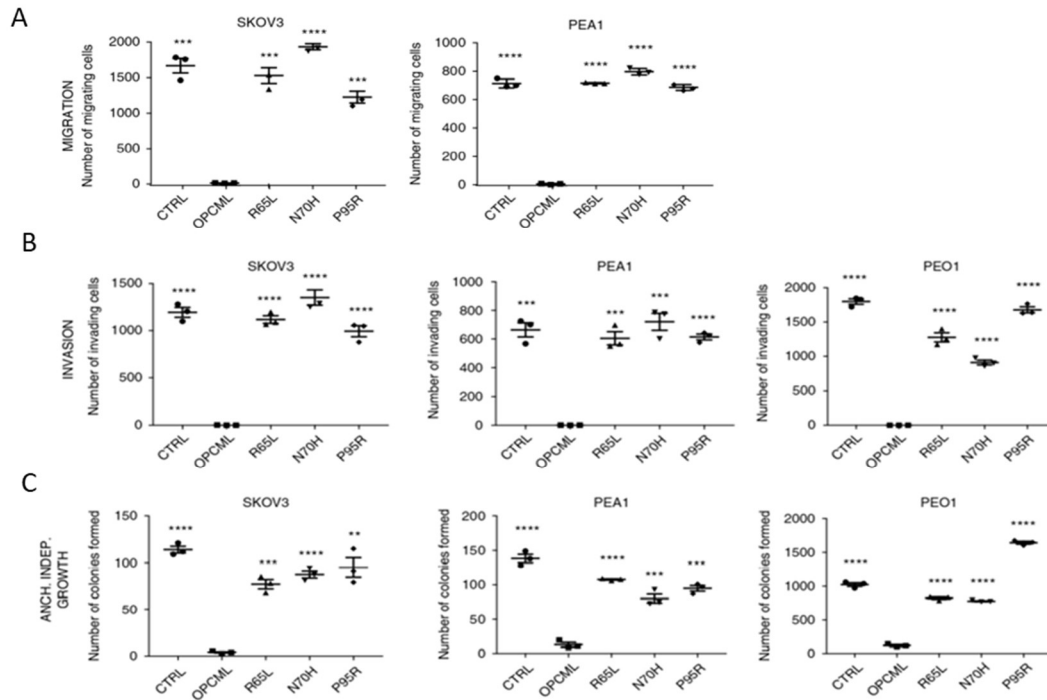


Figure 1.5: R65L, N70H, and P95R mutants show loss of tumour suppressor activity in-vitro

*SKOV3, PEA1 and PEO1 ovarian cancer cells transduced with wild-type OPCML (OPCML) showed a reduced ability to migrate (A), invade (B) and grow (C) in transwell migration (A), invasion (B) and agarose anchorage-independent (C) assays compared with the same cells transduced with empty vector (CTRL). This ability was lost when the cells were transduced with mutant OPCML R65L, N70H and P95R. *** $p < 0.001$, **** $p > 0.0001$ (Birtley et al., 2019)*

In particular, the R65L mutation determines the removal of a reciprocal salt bridge interaction with Asp80, resulting in a monomeric protein that is unable to interact with RTKs, to downregulate their downstreaming pathways, as well as is incapable of inhibiting migration, invasion, anchorage-independent growth and tumorigenicity. Therefore, the study of R65L mutation has shed light on the importance of OPCML dimerisation for its biological function.

The N70H mutation occurs at a glycosylation site in the OPCML dimer interface. This results in an unstable OPCML protein that retains the

ability to bind RTKs but has a partial to complete loss of function *in-vitro* and *in-vivo*. This has highlighted the importance of OPCML glycosylation for OPCML stability and function.

The P95R somatic missense inactivating mutation exposes the dimerisation interface on the surface of the OPCML protein. The resulting protein is homodimeric and stable, but it shows a differential interaction with RTKs; in fact, it can interact with FGFR1 but not with AXL. Therefore, the P95R mutation at domain 1 showed the importance of the dimer interface location for interaction with some RTKs and adhesion molecules.

Along with these, mutations in other residues of D1 and mutations in domains D2 and D3 affect the interaction of OPCML with Collagen I and IV, determining increased migration and invasion (Birtley et al., 2019). Therefore, these mutations seem to determine a partial or complete loss of tumour suppressive function of OPCML.

1.1.2.4 OPCML as a Therapeutic

Given OPCML's anti-proliferative activity in cancer cells, a synthetic and deliverable version of OPCML protein could represent a viable therapeutic option for cancer (Wu et al., 2012).

A recombinant OPCML protein (rOPCML) devoid of the GPI anchor was expressed and produced in *E. coli* to be tested. rOPCML treatment demonstrated a significant dose-dependent inhibition of cancer cell growth in OPCML-non-expressing ovarian cancer cells. In fact, rOPCML treatment mirrored the effects of ectopically expressed OPCML in these OC cells. rOPCML treatment down-regulated HER,

pHER2, and the same spectrum of RTKs down-regulated by OPCML, resulting in an abrogation of pERK and pAKT in both SKOV3 and A2780 cells. Furthermore, in orthotopic mouse models of ovarian cancer, intraperitoneal treatment with rOPCML showed a significant reduction in tumour weight, ascites volume and peritoneal dissemination compared to BSA control (McKie et al.,2012). In tumour tissues extracted from rOPCML-treated mice, there was a downregulation of the specific RTKs known to be regulated by OPCML and an inhibition of RTKs' downstream pathways (McKie et al.,2012).

However, during the production of this rOPCML in bacteria, it was observed that the recombinant protein tended to accumulate in inclusion bodies, requiring an additional refolding step. This ultimately resulted in the misfolding of the produced protein, leading to high variability in the protein bioactivity and a reduced reproducibility of experiments performed to test it. The accumulation in inclusion bodies and the need to perform refolding procedures are known limitations when non-mammalian expression systems are used for protein production, limitations that could be overcome using mammalian production systems.

These results underscore the potential of developing a reproducible and stable way to produce a GPI-anchor-less OPCML protein that can act as a cancer treatment to restore the tumour suppressor functions of the endogenous OPCML.

1.2 Production Of Recombinant Protein Therapeutics

1.2.1 Therapeutic Protein Production

Protein therapeutics are drugs used to replace deficient or abnormal proteins in a particular disease. Currently, several protein therapeutics are approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) as treatments for many diseases in different fields of medicine. Differently from small molecules (chemically synthesised), protein therapeutics are produced in living cells or organisms.

Recombinant protein therapeutics can be produced in non-mammalian host systems, which include bacteria, yeast, plants, insects, and cell-free expression systems, as well as in mammalian systems. Mammalian host systems can be divided into non-human hosts, which include Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, Sp2/0 murine hybridoma cells, NS0 murine myeloma cells and C127 murine mammary gland cells, and human host systems such as HT-1080 human fibrosarcoma cells and human embryonic kidney cells 293 (HEK293) (Dumont et al., 2016).

The choice of the host system is fundamental to ensure that the recombinant protein manufacturing characteristics are of high quality for use in humans. Such features are mainly represented by human-like post-translational modifications (PTMs), which are cellular processes that increase the complexity of proteins and their interactions. Among PTMs, protein glycosylation plays a fundamental role in ensuring stability, good serum half-life, efficacy and antigenicity of a

recombinant protein (Durocher et al., 2009; Ghaderi et al., 2012; Swiech et al., 2012). Glycosylation of a recombinant protein facilitates solubility and stability by preventing aggregate formation (Oh-Eda et al., 1990; Langer et al., 1998;). Glycosylation can also protect recombinant proteins from proteases by masking cleavage sites. This masking is mainly due to the creation of hydrogen bonds between the hydrophilic amino acids and glycans (Cantell et al., 1992; Wittwer et al., 1990). In addition, sialylated glycans in protein therapeutics prolong half-life. This is because the asialoglycoprotein receptor (ASGPR), located on the hepatocyte surface and responsible for the binding to, internalisation and ultimately degradation of a protein, does not recognise fully sialylated glycoproteins (Berger et al., 2012). Additionally, the presence of core fucosylation as well as of bisecting N-acetylglucosamine (GlcNAc) structure, a β 1,4-linked GlcNAc attached to the core β -mannose residue, has been reported to be involved in signal transduction and cell adhesion by regulating the function of glycoproteins (Wang et al., 2006; Shinkawa et al., 2013).

Regarding immunogenicity, it has been reported that specific glycans, such as those with a 1,3-Gal residue, are immunogenic in humans (Ezzelarab et al., 2005; Berger et al., 2011).

Mammalian host systems ensure more human-like PTMs. Among mammalian host systems, CHO cells represent the most utilised and validated system (Dumont et al., 2016). However, CHO cells have limitations related explicitly to the glycosylation of their recombinant products, which ultimately generate specific glycosylation patterns that differ from human glycosylation and confer increased immunogenicity and lower activity to the final recombinant products. In particular, it has been reported that CHO cells introduce terminal Galactose- α 1-

3Galactose group and *N*-glycolylneuraminic acid (Neu5Gc) into glycoproteins and that humans produce variable spectra of circulating anti-Gal and anti-Neu5Gc antibodies (Ghaderi et al., 2010). The interaction of these antibodies with the CHO-derived products leads to the production of immune complexes that could potentially fix complement and induce an inflammatory immune response. Furthermore, the interaction between CHO-derived proteins and circulating anti-Neu5Gc antibodies also increases the clearance rate of the protein itself, consequently reducing its half-life and efficacy. It has been shown that multiple injections of Neu5Gc-carrying substances, such as repeated administration of drugs containing Neu5Gc in patients, can lead to an accumulation of non-human sugars in human tissues. This accumulation specifically involves Neu5Gc in the tissues, which binds to anti-Neu5Gc antibodies. This process can contribute to chronic inflammation in the tissues, potentially aiding the progression of cancer (Hedlund et al., 2008; Ghaderi et al., 2010). Along with the generation of non-human glycosylation, another issue is the inability of CHO-cells to generate specific human glycans and to produce other PTMs, such as glutamic acid γ -carboxylation, both essential to ensure activity for some recombinant protein therapeutics (Berkner et al., 1993; Fliedl et al., 2015).

Therefore, aiming for more human-like protein therapeutics, pharmaceutical production has focused on developing novel human-derived cell lines. Among these, AGE1.HN, CAP, HKB-11, and PER.C6 are still in early development, while HEK293 and HT-1080 are well-established human cell lines for protein production (Chin et al., 2019). Several therapeutics approved by the FDA and EMA have been produced in HEK293 and HT-1080 (Chin et al., 2019).

HEK293 are widely used because, along with their ability to produce human-like PTMs, they are easy to transfect and transduce with genetic material encoding the protein of interest. In addition, many subtypes of HEK293 have been developed to increase their adaptability to serum-free media and suspension culture. Using a serum-free medium avoids the contamination of recombinant proteins with animal-derived materials, including Neu5Gc (Gadheri et al., 2010). Therefore, for these reasons, HEK293 cells were chosen in this work as a platform for rOPCML +/- Fc production.

Other crucial issues for protein biotherapeutic production are the choice of the expression vector, the transfection method and the culture medium.

The expression vector is essential for delivering the gene encoding the protein of interest into the target cell. Mammalian expression vectors must contain three essential elements: a promoter, a multi-cloning site (MCS) and a 3' untranslated region. The promoter drives transcription initiation, and the most commonly used promoters are CMV immediate early, SV40, CAG, and EF1 α (Dalton et al., 2014). The 3' untranslated (3'UTR) is essential for translation termination, and the most commonly used mammalian 3'UTRs include SV40, hGH, BGH and rbGlob polyA sequences (Gil et al., 1987; Lanoix et al., 1988; Goodwin et al., 1992; Schek et al., 1992; Ho et al., 2014). Along with these essential elements, the expression vector may include other elements, such as selection markers and enhancers, introns, viral amplifiers, and universal chromatin opening elements. All these elements act at different transcriptional levels to increase protein expression (Hunter et al., 2019).

Expression vectors can be plasmid or virally encoded, and based on that, the method used to introduce nucleic acids into the cells can be considered as mechanisms of eukaryotic transfection or viral transduction, respectively.

Transfection methods are mainly used for protein production in mammalian cells, whereas transduction is used more often in insect cells.

Transfection methods can be grouped into chemical methods and physical methods. The first consists of adding a chemical to the DNA of interest to introduce it into the cell. The most common chemical transfection method uses cationic lipids; it is frequently performed with high transfection efficiency and can be used in many cell lines (Hunter et al., 2019). Polyplexes are an alternative, economical transfection method with good efficiency and little cellular toxicity. However, they may not be compatible with all types of growth media (Ye et al., 2009; Rajendra et al., 2011). Other chemical methods are older, such as CaCl_2 (Graham et al., 1973), or unsuitable for stable transfection, such as diethylaminoethyl-dextran (Hunter et al., 2019). Physical transfection methods include electroporation, the most used method, direct injection, laser-based transfection, and biolistic particle delivery. Unlike chemical methods, physical transfection lacks scalability and can induce greater cell toxicity.

Another critical point in the recombinant protein production process is the choice of the culture medium, which is crucial to achieving high cell density and viability without affecting the final protein quality. The method of transfection to be used influences the choice of medium. Some medium components can inhibit chemical transfection, and

others can enhance chemical transfection (Rajendra et al., 2015; Ye et al., 2009). For example, it has been demonstrated that iron (III) citrate inhibits polyethyleneimine (PEI)-mediated transient transfection, although the mechanism behind this inhibition is not clear. It has been hypothesised that iron (III) citrate can prevent the release of DNA/PEI complexes from the endosomes. Conversely, adding EDTA may prevent iron (III) citrate formation and allow PEI-mediated transfection (Eberhardy et al., 2009). Also, polar solvents (N, N dimethylacetamide) can enhance PEI-mediated transfection (Rajendra et al., 2015).

The presence or absence of serum can also affect transfection. For instance, negatively charged molecules in serum could potentially affect transfection that uses cationic transfection reagents, such as HiperFect lipofectamine and endofectinmax (Diener et al., 2015; Wallenstein et al., 2010; Shi et al., 2020), by interacting with lipoplexes and altering the steps necessary for efficient transfection (Simoes et al., 2000; Misra et al., 2013). Conversely, the presence of 10% serum was found to increase transfection efficiencies in which cationic transfection reagents are involved (Yamano et al., 2010), and this can be because the presence of the serum modulates the surface charge density of the transfection complexes consequently improving their interaction with its host cell surface inter-surface. (Yamano et al., 2010)

However, the best way to achieve optimal production is to identify the optimal media conditions requiring titration of medium components.

In recombinant protein production, transient and stable gene expression can be chosen. The first ensures more rapid recombinant protein

production but on a small scale. These characteristics make this system suitable for structural analysis but not for experiments that require large-scale production in sufficient quantity to test a new drug's toxicity or efficacy. In contrast, stable transfection is a more complex process but ensures a large-scale production (Thomas et al., 2005; Meissner et al., 2001; Loignon et al., 2008; Spidel et al., 2016). It is preferred in experiments requiring a large quantity of protein, especially when protein quality and homogeneity control are essential.

Based on all the above, in this work, I used HEK-293 as a host system to ensure more human-like PTMs of the rOPCML produced. The soluble rOPCML was produced using stable cationic-lipid-based transfection.

These recombinants were produced as secreted proteins in the conditioned medium of HEK-293 cells, which were then collected and purified to obtain the purified recombinant proteins. I tested different media with different serum percentages to find the optimal production/purity rate.

In the production of secreted proteins, it is desirable to maximise the secretion of recombinant proteins into the extracellular space to increase protein production and with this aim, either a native or non-native signal sequence can be used to enhance the translocation of the protein into the ER lumen and then outside the cell (Zhang et al., 2005; Knappskog et al., 2007; Kober et al., 2013; Dalton et al., 2014; Dalton et al., 2014). Native and alternative signal peptides, such as human serum albumin (HSA), ceruloplasmin, prolactin, interleukin-2 and Immunoglobulin Kappa light chain, are highly heterogeneous with high variability in enhancing production and secretion of different

proteins (Dalton et al., 2014). Therefore, trials testing a panel of commonly used signal peptides may help select the most efficient signal peptides in enhancing the production of the protein of interest. In this study, three signal peptides (i.e., native OPCML, ceruloplasmin and HSA signal peptide) were tested to determine if foreign signal peptides could result in a higher rOPCML secretion/production.

Lastly, following the production of the protein, appropriate purification steps are essential to ensure the final product. Fusion tags, such as polyhistidine, facilitate protein purification (Carter et al., 2010; Zhang et al., 2017). A cleavable polyhistidine tag was used in this study in an attempt to ease protein purification.

1.2.2 Therapeutic Fc-fusion protein

The clinical potential of protein therapeutics is often hampered by their short serum half-life, which is secondary to renal clearance and proteolytic metabolism (Sato et al., 2006). Consequently, many protein therapeutics have a limited target exposure, and more frequent dosing intervals are required to maintain a clinically effective drug concentration (Wu et al., 2014; Strohl, 2015).

This issue led conceptually and practically to the development of Fc (crystallisable fragment) fusion proteins, a unique class of therapeutics that combine the pharmacological properties of biological ligands with the additional properties of an antibody's Fc-domain.

Fusion of the IgG-Fc domain to a protein therapeutic improves the clinical potential of active protein therapeutics by extending the plasma half-life of that biotherapeutic or by introducing an additional

mechanism of action through engaging immune-mediated effectors (Levin et al., 2015).

Among the antibodies that can be used as a basis for generating Fc-fusion proteins, the gamma immunoglobulin (IgG) isotype is the most used because of its favourable characteristics, such as increased plasma half-life and recruitment of effector function.

Most Fc-fusion proteins are produced by genetic fusion of the N-terminus of the IgG-Fc domain to the C-terminus of a biological moiety. The strong interaction of the IgG-CH3 domains creates a stable Fc-structure (Heath et al., 2018).

Renal clearance plays an essential role for proteins with a molecular weight of less than 60 kDa (Meibohm et al., 2012). Coupling an Fc-domain to an active protein therapeutic increases the size of the active protein itself; this can increase the circulating time of the active protein by bringing the construct above the threshold for kidney filtration (Pechtner et al., 2017).

Furthermore, adding an Fc-domain to an active protein can further prolong the circulation time via interaction with the neonatal Fc receptor (FcRn) in a pH-mediated recycling procedure. FcRn are receptors expressed on vascular endothelial cells. In those cells, IgG is internalised into endosomes by pinocytosis. At low pH, this molecule associates with membrane-bound FcRn and is transported back to the cell membrane, where it dissociates from the receptor, and this results in the protection of Ig and Fc-fused proteins from proteolytic degradation and leads to recycling (Ko et al., 2022).

In this work, I produced and tested the tumour suppressive activity of an Fc-fused-rOPCML to determine if the molecule was equally effective with a potentially higher half-life in comparison to non-Fc fused GPI-anchorless recombinant OPCML (rOPCML).

1.3 Ovarian Cancer

1.3.1 Incidence and Epidemiology

Ovarian cancer is the fifth most common type of cancer and the fourth most common cause of cancer deaths in women. The estimated risk for a woman getting ovarian cancer during her lifetime is about 1 in 78. (Ledermann et al., 2013)

Ovarian cancer mainly occurs in older women, with about half of ovarian cancer diagnosed in women aged 63 or above.

Globally, nearly 250,000 women are diagnosed with ovarian cancer each year, resulting in 140,000 deaths (Eurohealth, 2021). This high rate of death is related to late diagnosis at advanced stages when a 5-year relative survival rate is less than 30%. Only 15% of ovarian cancers are detected at an early stage (Stage I), when they can be cured with surgery and chemotherapy, with a 5-year relative survival rate of 92% in this stage (Reid et al., 2017).

1.3.2 Molecular subtypes and pathogenesis

Ovarian cancer can be divided into epithelial ovarian cancers (EOCs) and non-epithelial ovarian cancers based on the tissue from which they originate. EOCs are the most common, representing 90% of all ovarian cancers. Non-epithelial ovarian tumours account for approximately 10% of all ovarian cancers.

EOC represents a heterogeneous group of neoplasms with different morphological and biological characteristics. Five main groups of EOC have been identified based on histology, immunohistochemistry, and

molecular characteristics: high-grade serous carcinoma (HGSOC) (70%), endometrioid carcinoma (10%), clear cell carcinoma (10%), mucinous carcinoma (7%), low-grade serous carcinoma (LGSOC) (<5%) (fig.1.6) (Dion et al., 2020). These five groups differ in epidemiology and risk factors, dissemination pattern, genetic alterations, response to chemotherapy and prognosis.

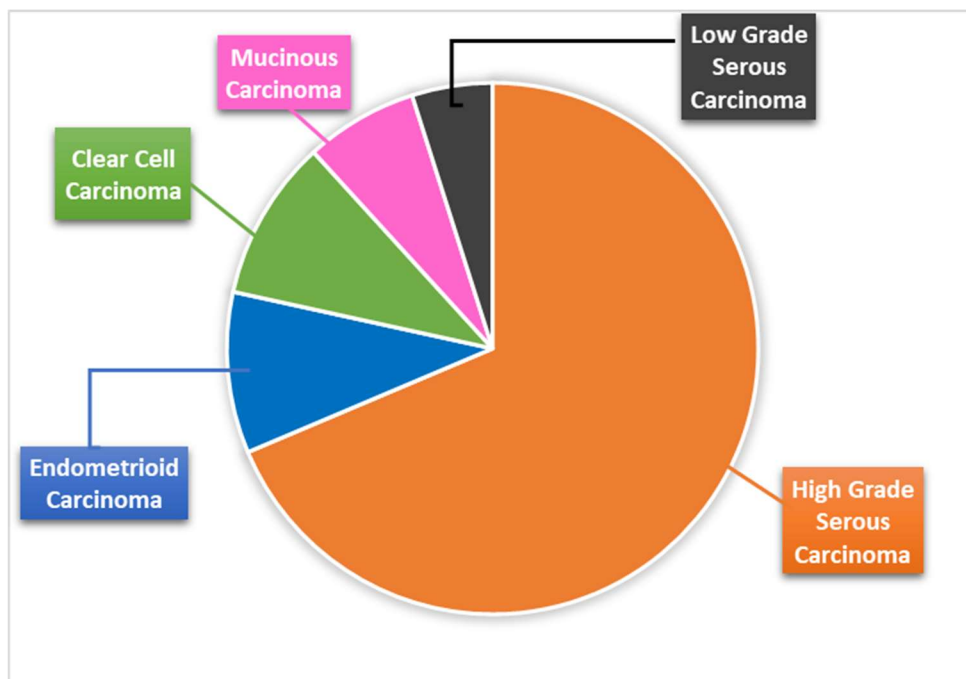


Figure1.6: Pie chart of incidence of EOC subtype

The immunohistochemical profile of high-grade serous carcinoma is characterised by high staining positivity for CK7, WT1, PAX8, and oestrogen receptors, and sometimes also positivity for progesterone receptors. From the genetic profile perspective, this histological subtype can have germline or somatic *BRCA1* and *BRCA2* mutations. Women carrying germline *BRCA1* or *BRCA2* pathogenic variants

(PVs) have a 30% to 70% risk of developing ovarian cancer during their lives (Paluch-Shimon et al., 2016).

Low-grade serous carcinoma, which may originate from a borderline serous tumour, usually retains its morphology even in recurrences (Gershenson, 2016). Low-grade serous carcinoma lacks *BRCA* and *p53* mutations, but in about 2/3 of the cases, they present mutations in *BRAF* and *KRAS* (Paluch-Shimon et al., 2016; Gershenson, 2016; Gadducci et al., 2020).

Primary ovarian mucinous carcinomas are sporadic (Paluch-Shimon et al., 2016), and they require a differential diagnosis with metastasis from mucinous carcinoma from other primary origins, such as colorectal cancer and gastrointestinal cancer. The presence of a unilateral large mass (>13 cm) is the main parameter to differentiate a primary ovarian mucinous carcinoma from a metastasis. Furthermore, primary mucinous tumours are usually confined to the ovary and do not extend to the ovarian surface or the peritoneum. Regarding the immunohistochemical profile, mucinous ovarian tumours are positive for cytokeratin 7 (CK7) in more than 80% of cases, while metastatic colorectal cancers are CK7 negative. Most mucinous ovarian tumours have negative immunohistochemical staining for WT1 and ER and positive for CK20. However, unlike mucinous tumours from the gastrointestinal tract, CK20 and CDX2 positivity in mucinous ovarian cancer is usually weak and focal. From a genetic point of view, *KRAS* mutations are the most frequent in mucinous tumours. Stage I mucinous ovarian cancers have an excellent clinical outcome, particularly if, histologically, the tumour has a pushing margin as opposed to an infiltrative margin. Conversely, in advanced stages, the clinical

outcome of mucinous ovarian cancers is unfavourable due to poor responsiveness to chemotherapy (Babaier et al., 2020).

Endometrioid cancers are usually low-grade (G1-G2) and are frequently associated with ovarian or pelvic endometriosis or borderline endometrioid tumours (Prat et al., 2018). The differential diagnosis between high-grade endometrioid carcinomas and high-grade serous carcinoma is often difficult. Both tumours, in fact, immunohistochemically express WT1 and have a similar gene expression profile (Rajendran et al., 2022). Approximately 30% of all ovarian endometrioid endometrial carcinomas are characterised by mutations in ARID1A (AT-rich interactive domain 1A gene) (Wiegand et al., 2010). These mutations have also been found in areas of endometriosis near the tumour, indicating the significant role of ARID1A in the development and progression of endometrial cancer (Matias-Guiu et al., 2018). On the other hand, ARID1A mutations have not been identified in high-grade serous carcinoma. Compared with endometrioid carcinomas of the uterus, endometrioid carcinomas of the ovary have a similar frequency of *CTNNB1* mutations (*β-catenin*: 38%) but a lower incidence of microsatellite instability (17.5%) and *PTEN* alterations (14%) (Matias-Guiu et al., 2018). These neoplasms are immunohistochemically positive for CK7 (97%) and CK20 (13%), as well as for epithelial membrane antigen, vimentin, oestrogen and progesterone receptors and negative for α-inhibin and calretinin.

Clear cell carcinoma (CCC) is closely associated with endometriosis and is characterised by mutations in ARID1A and PIK3CA (Wiegand et al., 2010; Prat et al., 2018). CCCs are immunohistochemically positive for CK7, epithelial membrane antigen, HNF1-beta, and napsin-A and negative for oestrogen, progesterone receptors, and WT1.

Additionally, CCCs are characterised by chemoresistance, leading to a poor prognosis when diagnosed at an advanced stage (Gadducci et al., 2021).

1.3.3 The origins of ovarian cancer

Several studies have raised the hypothesis that high-grade serous cancers can originate from a precancerous lesion located in the fallopian tubes. This precancerous lesion is named serous tubal intraepithelial carcinoma (STIC). STIC generally arise from a p53-signature lesion. A p53-signature lesion is a benign fallopian tube lesion with a somatic mutation in *TP53*. Both STIC and p53-signature lesion can lose their proliferation activity and become dormant, or conversely continue their proliferation process and acquire genomic instability, ultimately transforming into HGSOC. (Shih et al., 2011).

It has been suggested that the tubal fimbria is exposed to iron-induced oxidative stress caused by red blood cell lysis during retrograde menstruation (hypothesis of "incessant menstruation"). Iron's redox processes ($\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$) generate reactive oxygen species (ROS), which cause lipid peroxidation, DNA strand breaks, activation of oncogenes, and inhibition of tumour-suppressor genes, therefore increasing the risk of neoplastic transformation of the p53-signature lesion and STIC (Grzelak et al., 2020). However, the real etiopathogenesis of OC is not yet been fully elucidated.

1.3.4 Ovarian cancer risk factors

In the last decade, three main groups of risk factors for ovarian cancer development have been identified, and three possible processes through which these risk factors determine ovarian cancer have been hypothesised:

1. A positive family history of ovarian cancer increases the risk of ovarian cancer due to genetic alterations (familial genetic factors).
2. Incessant ovulation and gonadotropic stimulation can lead to the accumulation of deleterious mutations and promote neoplastic transformation, consequently increasing the risk of developing cancer (paracrine/endocrine factors)
3. The ovary can be exposed to carcinogens through the vagina and fallopian tubes. These carcinogens can lead to malignant transformation of ovarian or tubal cells (environmental factors)

A positive family history of ovarian and breast cancer is the greatest risk factor for ovarian cancer. However, only in 10 – 15% of ovarian cancer cases, there is an identifiable genetic mutation (*BRCA1* and *BRCA2* mutations, Lynch syndrome, PTEN mutations, RAD51C/D, PALB2 and BRIP1) (Hennessy et al., 2009). Germline *BRCA* pathogenetic variants are more frequently found in patients with high-grade serous ovarian cancer compared to patients with other subtypes of OC. In fact, *BRCA* pathogenetic variants occur in 17-20% of all HGSOC cases. The presence of these pathogenetic variants drives greater sensitivity to platinum-based treatment, resulting in a better prognosis for carriers (Alsop et al., 2012; Rish et al., 2001; Schrader et al., 2012; Soegaard et al., 2008).

Multiparity, breastfeeding and prolonged use of oral contraceptives reduce the risk of ovarian cancer (Babic et al., 2020; Karlsson et al., 2021; Schrijver et al., 2021). The protective effect of the prolonged use of oral contraceptives is maintained for more than 20 years after termination of their consumption. This chemo-preventive action has also been demonstrated in women carriers of *BRCA1/2* gene mutations. However, the data concerning hormone replacement therapy and the incidence of ovarian tumours are still conflicting (Lee et al., 2016). Some studies demonstrated that oestrogen-progestogen therapy is not associated with an increased risk of ovarian cancer (OR = 0.85, 95% CI = 0.72-1.0) (Menon et al., 2021), but conversely, other studies demonstrated that oestrogen-only replacement therapy had an increased risk of serous cancer (OR= 1.73, 95%CI= 1.26-2.38) and endometrioid carcinoma (OR =4.03; 95%CI= 1.91-8.49) of the ovary (Lee et al., 2016).

Malignant transformation of endometriosis is uncommon, occurring in 0.7-2.5% of cases (Gaia-Oltean et al., 2021). However, two cohort studies have demonstrated that endometriosis is correlated with an increased risk of specific subtypes of ovarian cancer. The risk is particularly high for endometrioid, clear cell and low-grade serous invasive ovarian cancers. No association was reported between endometriosis and the risk of mucinous or high-grade serous invasive ovarian cancer (Pearce et al., 2012; Gaia-Oltean et al.,2021)

1.3.5 RTKs in ovarian cancer

Receptor tyrosine kinases (RTKs) are a subclass of tyrosine kinases. To date, 58 RTKs have been identified in humans, and they can be

divided into 20 subfamilies: EGFR, InsR, PDGFR, VEGFR, FGFR, PTK7/CCK4, Trk, Ror, MuSK, MET, TAM, Tie, EphA/B, Ret, Ryk, DDR1/2, Ros, LMR, ALK and SuRTK106/STYK1 (Butti et al., 2018). The RTKs share a similar protein structure composed of an extracellular ligand binding domain, a single transmembrane helix, and an intracellular region that contains a juxtamembrane regulatory region, a tyrosine kinase domain, and a carboxyl (C-) terminal tail (Hubbard, 1999). Upon activation, RTKs recruit and activate various downstream signalling proteins and pathways. Phosphoinositide 3-kinase/Akt pathway/mechanistic target of rapamycin (PI3K/AKT/mTOR), the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway, the Raf/MEK/ERK1/2 pathway, and the protein kinase C (PKC) pathway are the most studied signalling pathway activated by RTKs and are involved in critical biological processes. In addition to RTKs, non-receptor tyrosine kinases (NRTKs) are involved in signal transduction cascades crucial for cell proliferation and migration. Based on their domain structure, the non-RTKs can be grouped into nine families: ABL, SYK, JAK, TEC, FAK, ACK, SRC, BRK/PTK6, and CSK (Kempner et al., 2013).

Dysregulation of RTKs, NRTKs and their mediated pathways is evident in many human diseases, including cancer development and progression.

Among the most studied RTKs in EOC are EGFR, HER2, MET, AXL and VEGFR.

The EGF receptor family comprises four members: EGFR (Epithelial Growth Factor Receptor, also known as HER1 and ErbB1), HER2 (Neu/ErbB2), HER3 and HER4 (ErbB3 and ErbB4, respectively). These receptors are composed of a glycosylated extracellular domain,

a single hydrophobic transmembrane portion, and an intracellular portion with a juxtamembrane segment, a protein kinase domain, and a carboxy-terminal tail (Yarden & Sliwkowski, 2001; Roskoski, 2014). Whilst EGFR, HER3 and HER4 are activated via ligand-induced dimerisation, HER2 is activated through ligand-independent heterodimerization.

In EOC, the rates of EGFR or HER2 overexpression or amplification are variable, ranging from 4% to 100% for EGFR and 2% to 66% for HER2 (Reyes et al., 2014; Hirte, 2013; Reibenwein et al., 2008; Serrano-Olvera et al., 2006).

Although EGFR expression does not correlate with disease aggressiveness, it is associated with poor clinical outcomes and prognosis (Reyes et al., 2014; Lafky et al., 2008; Skirnisdóttir et al., 2001; Tas et al., 2014). The role of HER2 as a prognostic factor is controversial, with some studies showing a correlation between HER2 overexpression and poor prognosis and some others demonstrating no relationship between HER2 expression and survival (Raspollini et al., 2006; Tuefferd et al., 2007; Lassus et al., 2004; Verri et al., 2005; Nielsen et al., 2004). This uncertainty may be correlated with high variability in EGFR and HER2 expression among the different subtypes of ovarian cancer.

Also, the rates of c-MET overexpression/amplification in EOC are variable and range between 11% and 60% (Ayhan et al., 2005; Di Renzo et al., 1994; Bu et al., 2011; Huntsman et al., 1999). c-MET immunohistochemical overexpression is associated with lymph node metastasis, high histologic grade, and low survival rate in patients with ovarian cancer (Ayhan et al., 2005; Sawada et al., 2007). Hepatocyte growth factor (HGF) is the ligand of c-MET. The activation of c-MET

upon HGF ligation plays an essential role in OSE regeneration during ovulation; however, in EOC, this HGF-mediated c-MET activation leads to an increase in proliferation, invasion through the stroma, and migration to other tissues of cancer cells.

In the normal ovary, HGF production seems to be under hormonal regulation, whereas it seems to be incessantly expressed by cancer-associated fibroblasts and tumour-associated macrophages in EOC (Kwon et al., 2017; Kim et al., 2022). Therefore, inhibition of the constitutively activated HGF-c-MET pathway in EOC is still a field of interest for future therapeutic development.

AXL is a TAM RTK family member expressed in HGSOE whose overexpression correlates with poor prognosis and chemoresistance (Sun et al., 2004; Antony et al., 2016). GAS6 is the only known ligand for AXL. Upon interaction with GAS6, AXL is activated and acts through the PI3K/AKT and MAPK/ERK pathways in promoting proliferation and inhibiting apoptosis in different cancer types (Elkabets et al., 2015; Sun et al., 2004; Antony et al., 2016). In addition, it has been demonstrated that inhibition of AXL prevents migration and invasion *in-vitro* and in mouse models and improves response to paclitaxel and carboplatin in EOC models (Antony et al., 2016; Quinn et al., 2019). A GAS6/AXL inhibitor (AVB-500) showed clinical benefit with favourable tolerability in a phase Ib trial in platinum-resistant ovarian cancer (Fuh et al., 2021). In the phase III AXLerate-OC/GOG-3059/ENGOT OV-66 trial, AVB-500 plus paclitaxel did not meet the progression-free survival (PFS) and overall survival (OS) endpoints in platinum-resistant recurrent ovarian cancer. However, there was a significant improvement in median PFS and median OS among patients with high AXL-expressing tumours (Fuh et al., 2024).

This underscores the importance of AXL as a potential target for treating ovarian cancer in a specific subgroup of patients.

The vascular endothelial growth factor family of receptors (VEGFRs) are RTKs composed of seven immunoglobulin-like domains, a trans-membrane domain, and a tyrosine kinase domain with a long kinase insert (Shibuya, 2013). VEGFRs ligands include VEGFA, VEGFB, VEGFC, VEGFD and VEGFE. In ovarian cancer, VEGF stimulates proliferation *in-vitro* and promotes angiogenesis *in-vivo*. In addition, high levels of VEGF, specifically of VEGF-C, are expressed in ascites of OC cancer patients and correlate with a poor prognosis (Bamias et al., 2012). Therefore, it is not surprising that the humanised anti-VEGF-A monoclonal antibody bevacizumab effectively improves the outcomes of ovarian cancer patients (Perren et al., 2011; Burger et al., 2011; Haunschild et al., 2019).

A therapy able to inhibit contemporary different RTKs and NRTKs involved in EOC development and evolution, such as rOPCML, which we hypothesise could be, would be beneficial for the patients.

1.3.6 Ovarian cancer and tumour suppressor genes

Many tumour suppressor genes (TSGs) are involved in EOC. TSGs may undergo various mutations, but both alleles must be mutated to be inactivated (the Knudson two-hit hypothesis). *TP53* is the most frequently altered TSG in ovarian cancer (Bernardini et al., 2010). Another mechanism of TSG inactivation includes epigenetic promoter-island methylation. This has been described for the two tumour-suppressor genes: RASSF1 and OPCML (Bast et al., 2009). Lastly, defects in TSG may occur because of genomic imprinting, an

epigenetic phenomenon by which only a silenced copy of a gene is inherited. This type of defect has been described for the adenoma gene-like 1 (PLAGL1), paternally expressed 3 (PEG3) and aplasia Ras homolog member (ARHI) (Feng et al., 2008).

1.3.7 Peritoneal Tumour Microenvironment

The peritoneal tumour microenvironment plays a fundamental role in the progression and spread of ovarian cancer, and a better understanding of the interplay between the tumour and peritoneal tumour microenvironment could be of great help for the definition of future therapeutic strategies (Ghoneum et al., 2018). For example, omental adipocytes release cytokines and adipokines, which contribute to neoplastic cell homing, invasion, and metastasis. Furthermore, the interaction between neoplastic cells and tumour-associated macrophages (TAM) stimulates the secretion of metalloproteinases (matrix metalloprotease, MMP) of the urokinase-type plasminogen activator (uPA), the uPA receptor (uPAR), and prostaglandin E2, which in turn facilitate the migration and invasion of neoplastic cells through the activation of NFkB (Said et al., 2007; Said et al., 2008). The increased concentration of TAM in the peritoneal microenvironment also exerts an immunosuppressive effect on T lymphocytes, dendritic cells and natural killer (NK) cells, promotes the onset of chemoresistance, and reduces sensitivity to anti-VEGF agents (Lau et al., 2014; Jammal et al., 2016; Worzfeld et al., 2017; Dalton et al., 2017). A meta-analysis of 12 retrospective cohort studies including 3346 ovarian cancer cases showed that a high lymphocyte/monocyte ratio before treatment correlates positively with both PFS (HR = 1.70,

95% CI = 1.49-1.94, $p < 0.001$) and with OS (HR = 1.85, 95% CI = 1.50-2.28, $p < 0.001$). The favourable prognostic effect of this variable is even more evident in women under 55. This higher correlation with younger patients may be due to the fact that ageing is characterised by a progressive increase in chronic subclinical inflammatory processes and chronic inflammatory diseases, which could reduce the positive effect of a high lymphocyte-monocyte ratio (Gao et al., 2019).

1.3.8 Diagnosis and Staging

1.3.8.1 Diagnostic Framework

Ovarian cancer is often diagnosed at an advanced stage because symptoms are generally present only in late stages. Consequently, the rate of ovarian cancer treatment failure is high. To date, no diagnostic procedures have adequate sensitivity and specificity for early diagnosis. In the absence of an effective screening strategy, the diagnostic procedures generally adopted for the diagnosis of ovarian epithelial tumours include clinical examination, transvaginal pelvic ultrasound and, in cases with suspected adnexal lesions, the determination of CA125, a tumour marker that is increased in ovarian cancer, along with other tumour markers to exclude other types of cancer (HE4, CEA, CA19.9). If the clinical suspicion is confirmed, further diagnostic procedures, such as chest X-ray, abdomen-pelvis CT with contrast medium, or abdomen-pelvis MRI with contrast medium, can be required to evaluate better the locoregional and distant dissemination of cancer (Ledermann et al., 2013).

1.3.8.2 Staging

Epithelial neoplasms are staged according to the FIGO classification (Federation Internationale de Gynecologie et Obstetrie) and the AJCC (American Joint Committee on Cancer) TNM (Tumour, nodes, metastasis) staging system (reported in table 1.2)

Table 1.2: American Joint Committee on Cancer (AJCC) TNM and International Federation of Gynaecology and Obstetrics (FIGO) Staging Criteria for Ovarian, Fallopian Tube, and peritoneum Cancer

AJCC Stage	Stage grouping	FIGO Stage	Stage description
I	T1N0M0	I	Tumour limited to the ovaries or fallopian tubes
IA	T1aN0M0	IA	Tumour limited to one ovary (capsule intact) or fallopian tube. No tumour on the ovary or fallopian surface, no malignant cells in the peritoneal washing. No ascites.
IB	T1bN0M0	IB	Tumour limited to both ovaries (capsule intact) or fallopian tubes. No tumour on the ovary or fallopian surface, no malignant cells in the peritoneal washing. No ascites.
IC	T1cN0M0	IC	Tumour limited to one or both ovaries but with one of the following: IC1: Surgical spill IC2: Capsule rupture before surgery or tumour on the ovary or fallopian surface IC3: Malignant cells in ascites or peritoneal
II	T2N0M0	II	Tumour involves one or both ovaries or fallopian tubes with pelvic extension or primary peritoneal cancer.

IIA	T2aN0M0	IIA	Extension or implants on uterus and/or fallopian tubes and/or ovaries.
IIB	T2bN0M0	IIB	Extension to other peritoneal tissues
III	T1-2N1M0 T3N0-1M0	III	Tumour involves one or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes.
IIIA	T1-2N1M0 T3aN0-1M0	IIIA	Positive retroperitoneal lymph nodes only (cytologically or histologically proven): IIIA1 (i): metastasis up to 10 mm in greatest dimension IIIA1 (ii): metastasis more than 10 mm in greatest dimension IIIA2: microscopic extrapelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes
IIIB	T3bN0-1M0	IIIB	Macroscopic peritoneal metastasis beyond the pelvis up to 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes
IIIC	T3cN0-1M0	IIIC	Macroscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes (includes extension of tumour to capsule of liver and spleen without parenchymal involvement of either organ)
IV	AnyT AnyN M1	IV	Distant metastasis, excluding peritoneal metastases,
IVA	AnyT AnyN M1a	IVA	Pleural effusion with positive cytology
IVB	AnyT AnyN M1b	IVB	Parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside the abdominal cavity)

The staging is pathological; therefore, surgery is functional for both the diagnosis and staging of ovarian cancer as it allows a more precise and accurate assessment of the disease extension.

1.3.9 Prognostic Factors

The most relevant prognostic factor in ovarian cancer is the stage of the disease at diagnosis. The 5-year OS of patients with malignant epithelial ovarian cancers is around 50%. However, 5-year OS widely differs in the different stages of the disease, being 85-90% for stage I ovarian cancer, 57-70% for stage II, 39-59% for stage III, and 17% for stage IV (Ovarian Cancer Prognosis - From NCI <https://www.ocrahope.org>). Within the same stage, other prognostic factors influence the patients' clinical outcomes, such as the grade of differentiation, the histological subtypes (Vergote et al., 2001; Pisano et al., 2005), the achievement of an optimal debulking surgery (Bristow et al., 2002) the presence or absence of pathogenic variants in *BRCA1/2* genes (Liu et al., 2012; Sun et al., 2014); Candido-dos-Reis, 2015; Lavie et al., 2019;) and the CA125 serum levels after radical primary surgery/adjuvant chemotherapy (Gadducci et al., 1995; Crawford et al., 2005; Kang et al., 2010; Mirza et al., 2016).

1.3.10 Treatment

1.3.10.1 Treatment for stage I and II ovarian cancer

The front-line treatment for early-stage ovarian cancer is cytoreductive surgery. The surgery aims are the histological confirmation of ovarian

cancer, its staging and debulking. The surgery staging is also essential to guide post-operative decision-making.

Post-operative adjuvant chemotherapy is recommended in most patients with early-stage EOC, especially those with high-risk diseases. A Cochrane meta-analysis evaluated four prospective randomised studies and the two largest randomised trials, ICON1 and ACTION. It confirmed the advantage of adjuvant platinum-based chemotherapy over observation alone and that it lasts for 10-year follow-up, particularly in high-risk patients who should receive chemotherapy regardless of staging (Lawrie et al., 2015).

In conclusion, there is broad consensus that adjuvant chemotherapy should be recommended in early-stage ovarian cancer (stage I–IIA), except for optimally debulked patients with stage IA LGSOC, stage IA grade 1-2 ovarian cancer and stage IA grade 1-2 mucinous ovarian cancer, mainly where there is a pushing rather than infiltrative margin (Colombo et al., 2019).

Platinum-based monotherapy or combination chemotherapy are the suggested regimes. The optimal duration remains controversial, but six cycles are generally recommended when patients are treated with single-agent adjuvant carboplatin. In comparison, a minimum of 3 cycles may be considered for the carboplatin and paclitaxel combination therapy. However, six cycles of combination therapy are recommended for HGSOC or stage IC (any histological type) (Colombo et al., 2019).

1.3.10.2 Treatment for stage III and IV ovarian cancer

The standard management of stage III and IV ovarian cancers involves the combination of cytoreductive surgery and chemotherapy. However, the role of surgery for patients with stage IV ovarian cancer is less clear. Generally, their outcomes are less favourable than those of patients with stage III ovarian cancer.

Primary cytoreductive surgery

Cytoreductive surgery followed or preceded by chemotherapy is an important step in treating patients with advanced ovarian cancer. Retrospective analyses indicate that the volume of residual disease after primary surgery is related to patient survival (Bristow et al., 2002; Allen et al., 1995). Complete macroscopic debulking (i.e., no residual disease) was associated with a median OS of 81.1 months versus a median OS of 34.2 months of patients with residual disease post-surgery (HR 0.36, 95%CI 0.31-0.42). (Du Bois et al., 2009). Among patients with residual disease following primary cytoreductive surgery, patients who received an “optimal” debulking (residual disease of 1-10 mm) had OS advantage compared with patients who received a "suboptimal" cytoreduction (residual disease >10 mm) (HR 0.80, 95%CI 0.70-0.91).

Therefore, complete resection of all macroscopic diseases is the goal of surgical treatment for advanced-stage ovarian cancer. Sometimes, removing parts of the gastrointestinal tract, spleen and diaphragm, if involved with the disease, may be required to achieve complete debulking. Consequently, primary surgery is recommended in patients amenable to complete surgical resection.

Neoadjuvant chemotherapy

For patients, where optimal cytoreduction is not possible in primary surgery, pre-operative chemotherapy (neoadjuvant chemotherapy (NACT)) followed by interval debulking surgery, is suggested. The main objective of this strategy is to reduce the neoplastic mass in advanced tumours to decrease the risk of perioperative complications.

The NACT followed by interval debulking surgery confirmed a non-inferiority in terms of survival outcomes of this approach compared to primary surgery. The main advantage of NACT followed by interval debulking surgery was, in fact, in terms of peri-operative complications and quality of life (Vergote et al., 2010; Kehoe et al., 2015; Tangjitgamol et al., 2010; Fagotti et al., 2016). However, in these trials, NACT was not inferior to primary surgery. Therefore, selecting patients as candidates for NACT is extremely important to ensure good clinical outcomes. The factors that may discourage primary surgery in favour of NACT are extensive intra-abdominal disease spread, a disease that involves the porta hepatis or mesentery, and poor performance status secondary to ovarian cancer-related symptoms, comorbidities and/or high anaesthetic risk.

Intraperitoneal chemotherapy

Intraperitoneal (IP) chemotherapy offers the possibility of direct exposure to high doses of chemotherapy in the peritoneum, where 80% of the disease takes place, minimising the effects of systemic toxicity. However, its use is questioned. Three randomised clinical trials,

GOG104/SWOG, GOG114 and GOG172, have demonstrated an advantage in terms of overall survival of intraperitoneal cisplatin compared to intravenous (IV) chemotherapy (Armstrong et al., 2006; Alberts et al., 1996; Markman et al., 2001; Jaaback et al., 2016). A pooled analysis of GOG-114 and GOG-172 data showed that the IP/IV regimen was associated with a lower risk of intraperitoneal relapse (Landrum et al., 2016) and significant long-term (> 10 years) PFS and OS benefit after adjusting for other prognostic factors (Tewari et al., 2015). Based on these results, NCCN panels of experts suggest IP chemotherapy as a valid option for patients with optimally debulked (<1 cm residual) stage III disease (Armstrong et al., 2022). However, in these trials, there has been low compliance with the treatment due to a high toxicity rate that precluded about 60% of the patients from completing the treatment.

In these trials, there was also some disagreement about the use of a high dose of IP chemotherapy (Cisplatin 100 mg/m²), as it was believed to have caused an imbalance between the treatment arms (IP/IV vs IV) and increased the side effects of the IP/IV regimen. To address these concerns, the trial GOG-0252 used a lower dose of cisplatin (75 mg/m² vs 100 mg/m²). However, in this case, the IP treatment did not improve PFS or OS compared to the IV control (Walker et al., 2019). This suggested that a lower dose of IP cisplatin reduces side effects but does not improve outcomes. Additionally, in the GOG-0252 trial, bevacizumab was added to chemotherapy and used as maintenance therapy. The trial results did not show an advantage in PFS and OS, suggesting that IP administration does not further improve outcomes when bevacizumab is added to the IV regimen during chemotherapy and maintenance. Therefore, it is crucial to accurately select patients

for IP candidacy, which should be performed by a multidisciplinary team.

Primary chemotherapy for advanced-stage ovarian cancer

The therapeutic standard in treating advanced-stage ovarian cancer is carboplatin plus paclitaxel, administered for six cycles. The choice of recommending this therapeutic regime derives from the results obtained over time from numerous clinical trials that have demonstrated the superiority of platinum-based chemotherapy over other types of chemotherapies, the equal efficacy of carboplatin compared to cisplatin, and the advantages in terms of OS and PFS obtained by the addition of paclitaxel to platinum-based regimes (Piccart et al., 2000; Neijt et al., 2000; Ozols et al., 2003; McGuire et al., 1996).

Up to 90% of patients with advanced-stage ovarian cancer treated with cytoreductive surgery and chemotherapy achieve complete remission. However, despite the initial effectiveness, 70-80% of those patients relapse within the first two years, requiring a second-line treatment. For this reason, numerous efforts have been made over the past 20 years to improve the effectiveness of first-line chemotherapy by adding a third non-cross-resistant drug (gemcitabine, topotecan, anthracyclines) to the carboplatin/paclitaxel combination, or modifying the dose-density of the standard treatment or adding maintenance therapy. The first strategy has not improved outcomes (Bookman et al., 2009; Bolis et al., 2010; Pignata et al., 2014; Chan et al., 2016). Delivering paclitaxel as a dose-dense weekly regime improved both PFS and OS in a study that recruited Japanese patients (Katsumata et al., 2009) but not in ICON8, which recruited predominantly Caucasian patients (Clamp et al., 2019).

With regards to the third strategy, different types of maintenance therapies have been tested in different trials aiming at maintaining response to chemotherapy and delaying disease recurrence (Markman et al., 2003; Markman et al., 2009; Pecorelli et al., 2009; Bolis et al., 2006; De Placido et al., 2004; Varia et al., 2003; Sorbe et al., 2003; Berek et al., 2009; Sabbatini et al., 2013; Vergote et al., 2014; du Bois et al., 2014). However, none has demonstrated a significant benefit in OS or a sufficiently good toxicity/clinical benefit ratio, except for bevacizumab (Burger et al., 2018; Haunschild & Tewari, 2020; Burger et al., 2011; Perren et al., 2011) and Olaparib.

Bevacizumab is a recombinant humanised monoclonal antibody that binds to VEGF and inhibits binding to its receptor on the cell surface. This inhibition prevents tumour angiogenesis.

Both phase III clinical trials investigating the role of bevacizumab in advanced-stage OC, GOG and ICON-7 demonstrated a significant increase in PFS when bevacizumab was added to standard adjuvant chemotherapy with carboplatin and paclitaxel (Burger et al., 2011; Perren et al., 2011). The advantage in these two trials was also confirmed in meta-analyses (Ruan et al., 2018).

However, an updated analysis of ICON-7 showed that the addition of bevacizumab did not give a statistically significant difference in PFS or OS in the overall patient cohort but only in the high-risk patient cohort. Furthermore, in the final analysis of the overall survival of the GOG-0218 trial, no statistically significant differences in OS were observed for patients who received bevacizumab compared with chemotherapy alone (Tewari et al., 2019).

Despite this discordant data, bevacizumab is currently indicated as a first-line treatment for ovarian cancer in stages IIIB and IV in addition to carboplatin-paclitaxel chemotherapy and as maintenance therapy (Colombo et al., 2019).

Poly ADP-ribose polymerase inhibitors (PARPi) have changed the clinical history and outcomes in HGSOC. PARPi are targeted cancer drugs that inhibit DNA repair pathways and cause cancer cell apoptosis. Recently, the phase 3 randomised SOLO1 trial showed a significant improvement in survival outcomes obtained with the addition of the PARPi Olaparib in Stage III/IV BRCA-mutant HGSOC and endometrioid ovarian carcinoma following a partial or complete response to primary chemotherapy (Banerjee et al., 2021; Moore et al., 2018; Disilvestro et al., 2023).

However, although the new maintenance treatments improved the clinical outcomes of patients with HGSOCs, more than 50% of these patients relapse within five years of diagnosis. Therefore, there is still an urgent need for finding more effective treatments for HGSOC.

1.3.10.3 Treatment of Recurrent/Relapsed Ovarian Cancer

Chemotherapy and targeted therapy

Treatment of recurrent or relapsed ovarian cancer is typically palliative. In rare cases of isolated recurrences, treatment with curative intent may be pursued. The choice of treatment is determined by the interval between the end of treatment with platinum and the onset of relapse (Platinum free interval-PFI), therapy received in the first line, *BRCA1/2*

mutational status, and the patient's suitability to receive platinum-based retreatment.

Patients are clinically divided into patients candidates for platinum retreatment vs patients not eligible for platinum therapy.

Among the patients not eligible for platinum retreatment, there are platinum-refractory patients who progressed during or within one month of the last platinum cycle or platinum-resistant patients who progressed or relapsed within six months of the last platinum treatment. In these two groups of patients, chemotherapies tested in phase III trials (i.e., topotecan, pegylated liposomal doxorubicin, gemcitabine, weekly paclitaxel, and trabectedin) demonstrated limited efficacy (Ferrandina et al., 2008; Pujade-Lauraine et al., 2014; Poveda et al., 2015). However, in patients with platinum-resistant BRCA-mutated HGSOc, therapy with the PARP inhibitors Olaparib or Rucaparib has shown a clinical benefit (Moore et al., 2019; Moore et al., 2018; Mirza et al., 2016).

In patients potentially eligible for platinum treatment (e.g., PFI > 6 months) but not candidates for platinum rechallenge for clinical reasons, a phase III study demonstrated the superiority of the pegylated liposomal doxorubicin and trabectedin combination over pegylated liposomal doxorubicin alone in prolonging PFS and OS (Monk et al., 2012).

In patients eligible for platinum retreatment, the therapeutic choice is to reuse platinum-based regimens. The addition of an anti-VEGF or a PARP inhibitor to platinum-based doublet in recurrent or relapsed ovarian cancer has demonstrated significant clinical advantages in many trials. The PARP-inhibitors Olaparib, Niraparib and Rucaparib

have been tested in three randomised phase III trials as maintenance therapy in platinum-responsive patients with platinum-sensitive relapsed ovarian cancer (Ledermann et al., 2012; Pujade-Lauraine et al., 2017; Mirza et al., 2016; Coleman et al., 2017).

All three of these PARP inhibitors were reported to have a PFS advantage. The benefit gained from PARP inhibitor maintenance treatment was reported in patients with *BRCA* mutations and patients with wild-type *BRCA* (Pujade-Lauraine et al., 2017).

Of note, despite the significant improvement in PFS obtained by new drug combinations in patients with relapsed HGSOV, the progression rate and the ovarian-cancer-related death rate are still high. The discovery of new therapeutics with a low toxicity profile may improve these rates further. Therefore, research aiming to develop new therapeutics for ovarian cancer is warranted as it may ultimately benefit patients affected by these tumours and improve their outcomes.

Secondary cytoreductive surgery

In general, most patients with ovarian cancer relapse are treated with chemotherapy, while the role of surgery is reserved for selected patients (Janco et al., 2016; da Costa et al., 2016; Eriksson et al., 2017). The main objective of secondary surgery remains cytoreduction with no tumour residual disease (Berek et al., 1983). Two phase III randomised trials affirmed the role of secondary cytoreductive surgery in relapsed platinum-sensitive ovarian cancer. The first, the German AGO-OVAR DESKTOP 3 / ENGOT trial, recruited only patients who met strict pre-specified criteria (complete debulking during first-line surgery, >6 months platinum-free interval, absence of ascites at relapse and showed

that secondary cytoreductive surgery produced a significant improvement in OS (53.7 months with surgery and 46.2 months without surgery; HR, 0.76; 95% CI, 0.59–0.97; $P = 0.03$), but exclusively among those patients in whom complete second resection was achieved (Du Bois et al., 2020). The second trial, the SOC1/SGOG-OV2 trial, demonstrated that secondary cytoreductive surgery and chemotherapy improved PFS compared to chemotherapy alone (PFS, 17.4 months versus 11.9 months; HR, 0.58; 95% CI, 0.45–0.74; $P < 0.001$) (Zang et al., 2020). These results are in contrast with the results of the randomised phase 3 trial, GOG-0213, which demonstrated that secondary cytoreductive surgery and chemotherapy do not lead to any advantage in terms of OS compared to chemotherapy alone (HR, 1.29; 95% CI, 0.97–1.72; $P = 0.08$) (Coleman et al., 2019). In this latter trial, patients did not have to meet the same strict inclusion criteria as DESKTOP3. In addition, the chemotherapy was administrated with concomitant and maintenance Bevacizumab, which may have levelled the difference between the two treatment arms. Together, these trials suggest that the clinical benefit of secondary cytoreductive surgery depends on the achievement of R0 and that adding Bevacizumab to standard second-line chemotherapy may determine an improvement in OS and level the advantage gained from the secondary cytoreductive surgery. They also suggest that effective drug treatment in relapsed ovarian cancer may be equivalent to complete resection of the relapsed tumour. Therefore, finding new treatments that can potentiate the current therapeutic armamentarium for OC is extremely important, avoiding the peri-operative risks of a surgical reintervention.

1.4 Project Aims

Ovarian cancer remains the leading cause of death for gynaecological cancers and the fourth most common cause of death for cancer in women in Western countries (Armstrong et al., 2022). In recent years, the significant advances made in developing and approving new treatments for ovarian cancer have not been reflected in an equally significant improvement in survival rates. The 5-year relative survival rate for EOC remains less than 50%. This poor survival is due to late diagnosis and ineffective second-line treatment for relapsed ovarian cancer. Therefore, there is a pressing need for new and original treatments for advanced ovarian cancer (Colombo et al., 2019).

OPCML is a GPI-anchored protein expressed in normal ovarian tissue, but OPCML expression is lost in ovarian cancer (Sellar et al., 2003; McKie et al., 2012; Reed et al., 2007), mainly through loss of heterozygosity (LOH) and epigenetic mechanisms, such as hypermethylation of the promoter of *OPCML* (Sellar et al., 2003). The ectopic expression of OPCML in ovarian cancer cells has a proven tumour suppressive activity both *in-vitro* and *in-vivo* (Mei et al., 2006; Cui et al., 2008; McKie et al., 2012; Zanini et al., 2017; Antony et al., 2017). The Gabra group investigated and confirmed that part of OPCML activity is due to its ability to interact with and impair downstream signalling of specific RTKs (Sellar et al., 2003; McKie et al., 2012; Zanini et al., 2017; Antony et al., 2017; Birtley et al., 2019). They further demonstrated that OPCML is located and interacts with target proteins in the outer layer of the plasma membrane, consequently generating the hypothesis that treatment with a recombinant OPCML delivered at the cancer cell surface could be a viable therapeutic option.

As a demonstration of this hypothesis, in 2012, McKie et al. showed that an rOPCML produced in bacteria and lacking the GPI-anchor caused EOC cell

growth inhibition *in-vitro* and *in-vivo* (McKie et al., 2012). However, the bacterial expression system led to some reproducibility issues regarding rOPCML production. In fact, rOPCML tended to accumulate in inclusion bodies, requiring additional steps of solubilisation and folding before obtaining the final recombinant, and this caused poor recovery of the correctly folded rOPCML. Furthermore, after elucidating the crystal structure of OPCML, it was demonstrated that post-translational modifications (PTMs) are important for the mature form of OPCML (Birtley et al., 2019).

Based on the above results, this project aimed to produce a stable recombinant OPCML (rOPCML) and its Fc-fusion derivative (rOPCML-Fc) in human HEK293 cells and evaluate their therapeutic potential in ovarian cancer models. The production in the human HEK293 cells was intended to avoid some of the refolding and PTM issues, particularly introducing human pattern glycosylation and facilitating the generation of less immunogenic proteins (Gharderi et al., 2012; Durocher et al., 2009; Swiech et al., 2012).

The rOPCML-Fc is engineered to have a biotherapeutic with a prolonged half-life for *in-vivo* intravenous injection.

The data obtained will allow the design of *in-vivo* pharmacodynamics experiments. They will serve as a proof of concept for the clinical implementation of rOPCML and rOPCML-Fc as a treatment for OC.

As an additional part, I have conducted preliminary experiments to assess the effect of rOPCML in lung cancer cells, chosen as a model of cancer strictly dependent on particular RTK signalling pathways, and to evaluate the potential role of the GPI anchor on the anti-tumour effect of OPCML. This latter group of experiments has been set up with a future plan to

incorporate OPCML protein or mRNA into extracellular vesicles (e.g., exosomes) as alternative delivery methods for cancer cells.

However, it is essential to highlight that protein production is expensive, and bridging from academic research to commercial is complex. So, some of the choices made for the experiments in this thesis come from a balance between scientific rationale and the cost-effectiveness of the materials and methods.

In detail, the aims of this project are:

1. Produce stable rOPCML and rOPCML-Fc in HEK293 cells
2. Evaluate the broader role and impact of rOPCML and rOPCML-Fc treatment on EOC cancer cells in proteomic and functional assays
3. Investigate the activity of rOPMCL in other cancer cell lines
4. Investigate rOPCML-Fc pharmacokinetics
5. Evaluate with preliminary experiments the role of OPCML GPI-anchor

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals, Reagents, And Drugs

Chemicals were obtained from Sigma Aldrich (Merck, Gillingham, UK) unless otherwise indicated. Some basic reagents and solutions were prepared by the Institute of Reproductive and Developmental Biology (IRDB) technical staff.

Soluble rOPCML and rOPCML-Fc were obtained through affinity coupled with size exclusion chromatography of the supernatant collected from transfected HEK-293 cells (see below 2.7).

Dr. James Birtley, PhD (University of Massachusetts), kindly provided three mutated recombinants of OPCML (rOPCML mut). Each rOPCMLmut carried a known inactivating single amino acid substitution in domain 1 of OPCML (i.e., N70H, R65L, P95R).

Cisplatin (CDDP) was obtained from Imperial College NHS Trust pharmacy, and osimertinib was purchased from Selleckchem (Selleck Chemicals GmbH, Cologne, Germany).

2.2 Cell Lines

Ovarian SKOV3, from the American Type Culture Collection (ATCC) cell bank, OVCAR8 and PEO4 cells were already available in Dr Gabra group. (Imperial College), and lung cancer PC9-ER cells, kindly provided by Dr Rajat Roy (Imperial College London), were cultured in RPMI 1640 medium

(Sigma-Aldrich, Merck, Gillingham, UK) supplemented with 10% foetal calf serum (FCS), 1% penicillin and streptomycin (Gibco, Montana, USA) and 1% L-glutamine (Gibco, Montana, USA) at 37°C in a 5% CO₂ incubator. The medium supplemented with 10% FCS has been used to stimulate RTKs in all the experiments. Ascites-derived tumour cells from 2 patients with HGSOC were kindly donated by Dr Darren Ennis and were cultured in Advanced Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (ADMEM-F12) supplemented with 20% patient clarified supernatant of the ascites at 37 °C in the presence of 5% CO₂ and 95% humidity. All cell lines, including the ones derived from patients' ascites, lacked OPCML expression as tested by Western Blot.

The sample from which ascites-derived tumour cells were derived was obtained under the authority of Imperial College Healthcare Tissue Bank (HTA licence 12275; REC approval no. 17/WA/0161; Project ID R18060). The patient gave written consent. HGSC cells were identified as being PAX8, WT1, and CK7 positive, with p53 either null or showing diffuse nuclear staining. The cells were obtained from ascites spheroids of a newly diagnosed stage IIIc HGSOC patient who did not carry germline pathogenic variants in BRCA1/2. This patient experienced disease recurrence after 12 months of platinum-based treatment.

HEK-293 cells were maintained in DMEM (Sigma-Aldrich, Merck, Gillingham, UK) supplemented with 1% FCS, 1% L-glutamine, and geneticin (Gibco) at a final concentration of 1mg/mL as the selection agent.

SKOV3 cells were used to replicate previous OPCML and rOPCML tumour suppressive function data (McKie et al., 2012; Anthony et al., 2018; Zanini et al., 2017). OVCAR8 and PEO4 cells were used as well-established ovarian cancer models (Beaufort et al., 2014; Domcke et al., 2013).

In lieu of in-vivo experiments, ascites-derived tumour cells were used as an ex-vivo model to demonstrate rOPCML and rOCPML-Fc function.

The cells donated were authenticated using STR profiling within three years before the experiments, and all cells were regularly checked for mycoplasma absence.

2.3 Plasmid Constructs

Dr Manuela Mura designed the following plasmid constructs, and Oxford Genetics performed the cloning. The GPI anchor signal has been removed to produce an anchorless and soluble protein.

OPCML (UniProt entry Q14982) was cloned into the mammalian expression vector pcDNA3.1/Geneticin 5.0 kb. Specifically, OPCML residues 36–316, corresponding to D1-D3, were cloned into the pcDNA3.1/Geneticin vector (Invitrogen, USA) downstream of an N-terminal Human Serum Albumin (HSA) signal peptide (MKWVTFISLLFLFSSAYS) and upstream of a C-terminal portion composed of 6-histidine residues for affinity purification. The GPI anchor at asparagine 322 was eliminated to generate a soluble protein. To develop a second construct of OPCML expressed as an Fc-fusion protein, an Fc-fragment of human IgG1 (Uniprot entry P01857, residues 119–330) with the insertion of a linker sequence (STKG) was added at the C-terminus, ahead the 6-histidine residues, in one of the plasmids.

The HAS-signal peptide sequence substituted the OPCML signal peptide in the N-terminus of both constructs to increase the protein secretion. HSA was chosen as the signal sequence based on an experiment that compared the secretion of recombinant OPCML with HSA signal peptide vs the one with

native OPCML or ceruloplasmin signal peptide. HSA signal peptide showed increased secretion of rOPCML compared to the other two signal peptides (experiment performed by Dr Manuela Mura).

In Table 2.1, the aminoacid sequences of rOPCML and rOPCML-Fc are reported.

Table 2.1: rOPCML and rOPCML-Fc construct amino acids sequence

Protein	rOPCML	rOPCML-Fc
Aminoacidic Sequence	MKWVTFISLLFLFSSAYSAT FPKAMDNV<u>TVRQGESATLRC</u> <u>TIDDRVTRVAWLNIRSTILYAG</u> <u>NDKWSIDPRVILVNTPTQYSI</u> <u>MIQNVDVYDEGPYTCSVQTD</u> <u>NHPKTSRVHLIVQVP</u><u>PQIMNIS</u> <u>SDITVNEGSSVTLLCLAIGRPE</u> <u>PTVTWRHLSVKEGQGFVSED</u> <u>EYLEISDIKRDQSGEYECAL</u> <u>NDVAAPDVRKVKIT</u><u>VNYPPYI</u> <u>SKAKNTGVSVGQKGILSCEAS</u> <u>AVPMAEFQWFKEETRLATGL</u> <u>DGMRIENKGRMSTLTFFNVSE</u> <u>KDYGNYTCVATNKLGN TNAS</u> <u>ITLYGPGGGG</u>LEVLFQGPRT GHHHHHH	MKWVTFISLLFLFSSAYSATFP KAMDNV<u>TVRQGESATLRC</u><u>TIDD</u> <u>RVTRVAWLNIRSTILYAGNDKWS</u> <u>IDPRVILVNTPTQYSIMIQNVDV</u> <u>YDEGPYTCSVQTDNHPKTS</u>RVH <u>LIVQVP</u><u>PQIMNISSDITVNEGSSV</u> <u>TLLCLAIGRPEPTVTWRHLSVKE</u> <u>GQGFVSEDEYLEISDIKRDQSGE</u> <u>YECALNDVAAPDVRKVKIT</u><u>VN</u> <u>YPPYISKAKNTGVSVGQKGILSC</u> <u>EASAVPMAEFQWFKEETRLATG</u> <u>LDGMRIENKGRMSTLTFFNVSEK</u> <u>DYGNYTCVATNKLGN TNASITL</u> <u>YGPGGGG</u>LEVLFQGPGGSTKGP SVFLFPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNK ALPAIEKTISKAKGQPREPQVYT LPPSRDELTKNQVSLTCLVKGFY PSDIAVEWESNGQPENNYKTTPP VLDSDGSFFLYSKLTVDKSRWQ

		QGNVFSCSVMHEALHNHYTQKS LSLSPGKRTGHHHHHH
Sequence key	UNDERLINED: <u>WILD-TYPE OPCML SEQUENCE</u> BOLD ON N-TERMINUS: HSA SIGNAL PEPTIDE RED PURPLE: OPCML DOMAIN1 GREEN: OPCML DOMAIN2 ORANGE: OPCML DOMAIN3 BOLD RED: HUMAN RHINOVIRUS 3C PROTEASE CLEAVAGE SITE PINK ON C-TERMINUS: FC FRAGMENT OF HUMAN IGG1 (UniProt ENTRY P01857) LIGHT BLUE: HIS TAG	

2.4 Plasmid Preparation and Purification

Transformed *E. coli* bacteria were grown overnight in Luria-Bertani (LB) medium supplemented with 100µg/ml ampicillin at 37°C on an orbital shaker. Maxiprep kit (Qiagen) was used to purify the plasmid following the manufacturer's instructions, and the plasmid's concentration was measured using the nanodrop.

2.5 Hek-293 Lipid-Based Transfection

Cells were seeded at 70% confluence in 6-well plates and transfected with DNA-lipid complexes using Effectene transfection reagent (Qiagen) following the manufacturer's instructions. The amount of DNA used was 0.4 µg, and the volume of Effectene Reagent was 10 µl. After 48 hours, medium containing 1mg/ml of Geneticin was added. After two weeks, positive clones were pooled and maintained in a medium containing Geneticin at 1mg/ml. The conditioned medium was regularly collected to

assess the positivity of the soluble rOPCML and rOPCML-Fc by western blotting.

Dr Manuela Mura generated stable clones for rOPCML and rOPCML-Fc.

2.6 Purification of rOPCML and rOPCML-Fc By Affinity Chromatography

The soluble rOPCML ($\pm$ Fc) in the transfected HEK-293 cells supernatant was purified by affinity chromatography. Affinity chromatography separates selected proteins using their specific interaction with transition metal ions immobilised on a resin matrix. The ion used for rOPCML-his-tag purification was Nickel (Ni^{2+}) in the form of Ni-NTA agarose beads (Superflow Qiagen, Manchester, UK). The his-tag has a high affinity for Ni ions and binds strongly to the matrix, whereas most other lysate proteins will not bind to the matrix or bind only weakly. The weakly bound proteins to the beads are eluted by the low concentration of Imidazole, which is typically added to the washing buffer. A higher concentration of Imidazole is needed to elute His-tagged proteins, which bind strongly to the matrix. Imidazole competes with the proteins and his-tag for binding to the metal-charged resin.

To purify rOPCML ($\pm$ Fc), 1.5×10^6 transfected HEK-293 cells were seeded in T75 flasks in 20 mL of DMEM (Sigma-Aldrich, Merck, Gillingham, UK) plus 1% of FCS, 1% of L-glutamine. The supernatant of transfected HEK-293 was collected every 5 days, centrifuged to eliminate cell debris, and then purified or frozen at -80° for storage. In the case of purification from frozen supernatant, the supernatant was thawed overnight, keeping it at 4°C to avoid thermic shock and to minimise the impact on protein stability and

bioactivity. Once collected or defrosted, the supernatant was filtered through a 0.2 μm Durapore™ sterile filter unit with a polyvinylidene fluoride membrane filter to eliminate further cell debris and separate possibly contaminating viral particles from the recombinant proteins. Following filtration, the pH of the supernatant was checked using pH test strips (Sigma Aldrich, Merck, Gillingham, UK) to ensure a pH of 7.5-8. If the pH was out of range, it was adjusted to avoid protonation of the histidine residues and ensure optimal binding on the Ni-NTA agarose beads. An aliquot of the conditioned medium was collected and labelled with an I for input. Subsequently, the supernatant was incubated for 1 hour on a magnetic stirrer at 4°C with Ni-NTA agarose beads, which were prewashed thrice with phosphate-buffered saline (PBS). The proportion between the volume of beads and the volume of supernatant was 1:1500.

The supernatant complexed with Ni-NTA agarose beads was pipetted into 20 ml gravity flow Columns (Biorad, Watford, Hertfordshire, UK), allowing the resin to ultimately settle by gravity at the bottom of the columns before opening the bottom cap of the columns to make pass through the supernatant without the beads. An aliquot of the medium discarded from this purification step was collected and labelled as flow-through (FT). Settled beads at the bottom of the purification columns were then washed with 80 ml wash buffer (50mM NaH_2PO_4 , 300mM NaCl , 10mM imidazole, pH 8.0) in a continuous flow to remove proteins non-specifically bound to the beads, an aliquot of the washing buffer passed through the beads was collected and labelled with a W. Subsequently, rOPCML +/- Fc was eluted from the beads with a total of 40 ml of elution buffer (50mM NaH_2PO_4 , 300mM NaCl , 100mM imidazole, pH 8.0), and an aliquot of elution buffer passed through the beads was collected and labelled with an E.

The eluted protein was concentrated using 20 ml vivaspin[®] ultrafiltration units (Sartorius, Göttingen, Germany) with molecular weight cut-off (MWCO) of 10kDa for rOPCML and 30kDa for rOPCML-Fc at 4000 RPM for 45mins at 4°C and subsequently desalted with PBD (polybutadiene) by size exclusion chromatography using PD SpinTrap G-25 (GE Healthcare, Chicago, Illinois, United States). Desalting was used to separate the rOPCML and rOPCML-Fc from small molecules such as salt and free residues of poly-his tag. For the desalting process, the columns were equilibrated by adding 400µL of equilibrium buffer (Milli-Q[™] water) and centrifuging the tube at 800 x g. Following the repetition of this step four times, 140 µL of sample containing the eluted and concentrated protein were added in the middle of the packed bed of the columns and eluted by centrifugation at 800 x g for 2 minutes. The protein solution was filter sterilised using 0.22 µm Corning[®] Costar[®] Spin-X[®] centrifuge tube filters (Millipore sigma, Merck KGaA, Darmstadt, Germany). As the protein purity was, on average, more than 90%, the concentration was measured by spectrophotometry at 280 nm using the nanodrop. The final preparation was stored in aliquots at -80°C.

To test the purification efficiency and check the final protein's purity, the aliquots collected at each stage of the purification process were loaded on two SDS-PAGE gels. After the running phase, one gel was stained with coomassie-blue following the manufacturer's instructions, whilst another gel was transferred on the membrane by western blotting and subsequently probed with an anti-OPCML antibody. The staining with coomassie-blue staining served to assess the purity of the final product. The western blotting with anti-OPCML antibody showed the presence of the recombinant OPCML. The purity of the protein was estimated by comparing specific bands on the western blot membrane to total bands in coomassie-blue

stained gels through the ImageJ software. Photos of a Coomassie blue and WB analysis are reported as an example in the appendix (Figure 7.1).

2.7 Western Blotting and Pamgene Kinase

Activity Assay

To evaluate the effect of OPCML on RTK signalling, proteins extracted after cell treatment were subject to an SDS page and western blotting analysis.

Ovarian cancer SKOV3, OVCAR8, PEO4 cells, and lung cancer PC9-ER cells were seeded at a cell density of 2.5×10^5 cells/well in 2 ml of complete medium (RPMI 1640 + 10% FCS) in 6-well flat-bottomed plates and allowed to attach and form a semi-confluent layer for 24 hours. Twenty-four hours later, cells were washed thrice with PBS and were serum starved for 16 hours by keeping them in RPMI 1640 + 0%FCS. After 16 hours, the medium was removed and replaced with RPMI 1640 + 0% FCS or RPMI 1640 + 10% FCS +/- treatment.

ASC-01 cells were seeded at 2.5×10^5 cells/well in 2 ml of ADMEM-F12 medium supplemented with 20% patient-clarified supernatant of the ascites in 6-well flat-bottomed plates and allowed to attach and form a semi-confluent layer for 24 hours. Then, cells were washed thrice with PBS and left to serum starve by keeping them in ADMEM-F12 medium for 16 hours. After 16 hours, the medium was removed and replaced with ADMEM-F12 medium supplemented with 2% patient-clarified supernatant of the ascites or ADMEM-F12 medium supplemented with 20% patient-clarified supernatant of the ascites +/- treatment.

SKOV3 cells were treated with 40 or 100 $\mu\text{g/ml}$ of rOPCML or rOPCML-FC, with 40 $\mu\text{g/ml}$ of rOPCML or rOPCML-R65L, rOPCML-N70H,

rOPCML-P95R or with 100 µg/ml of rOPCML or 100 µg/ml of rOPCML+ 25 µM of Cisplatin or 25 µM of Cisplatin alone for 3 hours before extracting proteins for evaluating phospho- and total AXL, HER2, ERK1/2, AKT.

OVCAR8 cells were treated with 40 or 100 µg/ml of rOPCML or rOPCML-FC or 100 µg/ml of rOPCML or rOPCML-R65L for 6 hours, and then proteins were extracted for phospho- and total AXL, HER2, ERK1/2, and AKT level evaluation.

PC9-ER cells were treated with 50, 100 and 150 µg/ml of rOPCML for 3 hours, and subsequently, protein extraction was performed to detect levels of phospho-ERK1/2 (T202/Y204), phospho-AKT-S473, total-ERK and pan-AKT.

ASC-01 were treated with 40 or 100 µg/ml of rOPCML and rOPCML-FC for 3 hours before performing protein extraction to evaluate phospho-AXL-Y779, total-AXL, phospho-ERK1/2 (T202/Y204), phospho-AKT-S473, total-ERK and pan-AKT levels.

Table 2.2 shows the drug concentration and the timing of treatment exposure used in WB assays before protein extraction.

Table 2.2: Doses and timing of the different drugs used in WB assay for other cell lines

CELLS	DRUGS	CONCENTRATION	HOURS OF TREATMENT
SKOV3	rOPCML	40 µg/ml	3 hours
		100 µg/ml	3 hours
	rOPCML-FC	40 µg/ml	3 hours
		100 µg/ml	3 hours
	rOPCML-R65L	40 µg/ml	3 hours
	rOPCML-N70H,	40 µg/ml	3 hours
	rOPCML-P95R	40 µg/ml	3 hours
	Cisplatin	25 µM	3 hours
	Cisplatin + rOPCML	25 µM + 100 µg/ml	3 hours
OVCAR8	rOPCML	40 µg/ml	6 hours
		100 µg/ml	6 hours
	rOPCML-FC	40 µg/ml	6 hours
		100 µg/ml	6 hours
	rOPCML-R65L	100 µg/ml	6 hours
ASC-01	rOPCML	40 µg/ml	3 hours
		100 µg/ml	3 hours
	rOPCML-FC	40 µg/ml 100 µg/ml	3 hours 3 hours
PC9-ER	rOPCML	50 µg/ml	3 hours
		100 µg/ml	3 hours
		150 µg/ml	3 hours

Proteins were extracted after 3 and 6 hours (depending on the cells and based on previous experiments) of treatment as follows: cells were washed once with cold PBS and protein extraction was carried out at 4°C using ice-cold lysis buffer (5M NaCl, 10% NP-40, 1M Tris (pH 8.0), supplemented with phosphatase inhibitor (Calbiochem™, Millipore sigma, Merck KGaA, Darmstadt, Germany) and cOmplete™ Protease Inhibitor Cocktail (Roche Diagnostics, Deutschland GmbH) and collected using cold sterile cells scrapers. Protein samples were collected in safe-locked Eppendorf tubes,

incubated in a rotator for 30 mins at 4°C, and centrifuged at 13000 rpm at 4°C for 15 before the supernatant was collected and either quantified immediately or stored at -80°C for later use. Protein concentration was estimated using the Thermo Scientific™ Pierce™ BCA Protein Assay Kits (Thermo Fisher Scientific, Rockford, Illinois, USA) according to the manufacturer's instructions.

Levels of phospho and total AXL, HER2, ERK1/2, and AKT were detected by Western Blotting. Proteins underwent wet transfer to nitrocellulose membranes. Membranes were blocked with 5% w/v dry milk in Tris-Buffered Saline supplemented with 1% Tween (TBST) or 5% BSA-TBST for 1 hour and incubated with primary antibodies according to the manufacturer's instructions. Species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Dako. All washes were performed in Tris-buffered saline (TBS) supplemented with 1% TWEEN. Blots were developed using the Clean Blot™ IP detection reagent (Pierce Biotechnology, Illinois, USA) or luminescence, visualised with X-ray films.

The protein tyrosine kinase Pamchip array® was performed following the manufacturer's instructions (PamGene International, 's-Hertogenbosch, the Netherlands). Lysates from OVCAR8 cells and PEO4 cells treated for 6 hours with rOPCML at 100 µg/mL or untreated were obtained using Halt Phosphatase Inhibitor Cocktail and Halt Protease Inhibitor Cocktail EDTA free diluted at 1:100 in M-PER Mammalian Extraction Buffer. Protein quantification was performed with the BCA (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. Then, the samples were processed and loaded on the Protein Tyrosine Kinase PamChip® following the manufacturer's instructions. Technical triplicates were analysed, and sequence-specific peptide tyrosine phosphorylation was

detected by the fluorescein-labelled antibody PY20 (Exalpha, Maynard, MA) and a CCD camera using the Evolve software (PamGeneInternational).

2.8 Antibodies

Polyclonal goat anti-OPCML (#2777-CM) and rabbit anti-phospho-Axl (Tyr 779) (#AF2228) antibodies were purchased from R&D Systems (Minneapolis, USA). Rabbit anti-calnexin was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Mouse Anti-Phospho-Erk1 (pT202/pY204) + Erk2 (pT185/pY187) antibody (ab50011) was purchased from Abcam (Cambridge, UK). Rabbit anti-Phospho-Akt (Ser473) (#4060), anti-pan AKT (#4685), anti-AXL (#5724) anti-phospho-HER2 (Tyr 1248) (#2247), Anti HER2/Erb2 (#2165), anti-GAPDH (#5174), and anti-ERK1/2 (# 9102) antibody were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). All the primary antibodies except anti-phospho-AXL were diluted at 1:1000 and incubated overnight at 4° C. Primary antibody against phospho-AXL was diluted at 1:250 and incubated overnight. Anti-Goat HRP, Anti-Mouse HRP, and anti-Rabbit HRP secondary antibodies (Agilent Dako, Santa Clara, USA) were diluted at 1:5000 and incubated for 1 hour at room temperature.

Tables 2.3 and 2.4 list the primary and secondary antibodies used in this work.

Table 2.3: List of primary antibodies used in western blotting, the molecular weight (MW) of their respective immunogens, concentration in dilution medium, and manufacturer

Antibody	MW (K Da)	Dilution Medium	Dilution	Manufacturer
Polyclonal goat Anti-OBCAM	55/75	5% milk TBST	1/1000	R&D Systems Minneapolis, USA
Rabbit anti-phospho-Axl	140	5% BSA TBST	1/250	R&D Systems Minneapolis, USA
Mouse Anti-Phospho-Erk1 (pT202/pY204) +Erk2 (pT185/pY187)	42/44	5% milk TBST	1/1000	Abcam Cambridge, UK
Rabbit anti-Phospho-Akt (Ser473)	60	5 % BSA TBST	1/1000	Cell Signaling Technology Danvers, Massachusetts, USA
Rabbit anti-pan AKT	60	5% BSA TBST	1/1000	Cell Signaling Technology Danvers, Massachusetts, USA

Rabbit anti-AXL	140	5% milk TBST	1/1000	Cell Signaling Technology Danvers, Massachusetts, USA
Rabbit anti-phospho-HER2 (Tyr 1248)	185	5% milk TBST	1/1000	Cell Signaling Technology Danvers, Massachusetts, USA
Rabbit anti-HER2/Erb2	185	5% milk TBST	1/1000	Cell Signaling Technology Danvers, Massachusetts, USA
Rabbit anti-GAPDH	37	5% milk TBST	1/1000	Cell Signaling Technology Danvers, Massachusetts, USA
Rabbit anti-ERK1/2	42/44	5% milk TBST	1/1000	Cell Signaling Technology Danvers, Massachusetts, USA
Rabbit anti-Calnexin	90	5% milk TBST	1/1000	Enzo life sciences Farmingdale, NY, USA

Table 2.4 List of HRP secondary antibodies used and their concentration

Antibody	Dilution	Manufacturer
HRP goat anti-mouse	1/5000	Agilent Dako, Santa Clara, USA
HRP goat anti-rabbit	1/5000	Agilent Dako, Santa Clara, USA
HRP mouse anti-goat	1/5000	Agilent Dako, Santa Clara, USA

2.9 MTT Cell Viability Assay

Viability was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Abcam, #211091, Cambridge, UK).

MTT is a colourimetric assay that estimates the viable cell number. The tetrazolium ring of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is cleaved by dehydrogenases in active mitochondria of living cells; this cleavage causes the formation of the formazan. The assay, therefore, detects the colour change from the yellow of the tetrazolium dye to purple due to the formation of the formazan.

To perform the MTT assay, 5,000 SKOV3, OVCAR8, ASC-01 or PC9/ER cells, and 8,000 PEO4 per well were seeded in triplicates in 96wells flat bottom plate (Corning®, Fisher Scientific, Rockford, Illinois, USA). Twenty-four hours post-seeding ovarian cancer cells (SKOV3, OVCAR8, PEO4, and ASC-01 cells) were treated with increasing doses (0, 40, 60, 80, 100 µg/mL) of rOPCML and rOPCML-Fc. OVCAR8 and PEO4 were also treated with 0, 6, 8, 12, 18 µM of cisplatin (CDDP) alone or in combination

with 100 µg/mL of rOPCML (OVCAR8 and PEO4 cells) for 72 hours. Lung cancer cells (PC9/ER cells) were treated with exponentially increasing doses of rOPCML (0, 1.18, 2.34, 4.68, 9.37, 18.75, 37.5, 75, 150, 300 µg/mL) or with Osimertinib alone (0, 0.75, 1.5, 3, 6, 12, 24 nM) or with Osimertinib in combination with rOPCML (150 µg/mL) for 72 hours. After 72 hours of treatment, the medium from the cell culture was carefully discarded, and 50 µL of serum-free medium and 50 µL of MTT solution (prepared per manufacturer's instruction) were added to each well. The plate was incubated at 37°C for three hours, and subsequently, 150 µL of MTT solvent was added to each well. The plate was then wrapped in foil on an orbital shaker, and the absorbance was read at an optical density of 590 nm.

MTT assay was also performed in SKOV3 cell lines expressing OPCML with and without GPI anchor and OPCML R65L at different time points (0-24-48-72-96 hours): 5,000 SKOV3-EV, SKOV3-OP, SKOV3-SOP, and SKOV3-R65L were seeded in triplicates in 96-well flat-bottom plates (Corning®, Fisher Scientific, Rockford, Illinois, USA). Viability was assessed as described above.

2.10 Caspase-Glo Apoptosis Assay

Apoptosis was assessed by Casapase-3/7 activity using the Caspase-Glo® 3/7 assay kit, which was used according to the manufacturer's instructions (Promega, Madison, Wisconsin, USA).

The Caspase-Glo® 3/7 assay provides a pro luminescent caspase-3/7 Asp-Gly-Val-Asp (DEVD)-amino luciferin substrate and a proprietary thermostable luciferase in a reagent optimised for caspase-3/7 activity, luciferase activity, and cell lysis.

DEVD is a caspase-3 (Cas-3) and caspase-7 (Cas-7)-cleavable peptide substrate. During apoptosis, caspases are activated, and the changes in Caspase activity are related to stages of apoptosis. In particular, Cas-3 and Cas-7 are cysteine-aspartic acid proteases that can directly execute apoptosis. Therefore, the addition of Caspase-Glo[®] 3/7 Reagent in cell wells results in cell lysis, and the cells with cas-3/7 active cause the cleavage of DEVD into the DEVD-amino luciferin substrate. This liberates free amino luciferin, consumed by the luciferase in the kit and generates a luminescent signal proportional to cas-3/7 activity. The luminescent signal is then detected by the LUMIstar OPTIMA plate reader (BMG Labtech, UK).

To perform the Caspase-Glo Apoptosis Assay, 5,000 SKOV3, OVCAR8, ASC-01, PC9/ER cells per well and 8000 PEO4 cells per well were plated in triplicate in full serum medium (RPMI 1640 + 10% FCS for SKOV3, OVCAR8, PC9/ER and PEO4 cells, and ADMEM-F12 + 20% patient clarified supernatant of the ascites for ASC-01) in 96-well, opaque flat bottom plates (Corning[®], Fisher Scientific, Rockford, Illinois, USA). Twenty-four hours later, ovarian cancer cells (SKOV3, OVCAR8, ASC-01, PEO4) were treated with increasing doses (0, 40, 60, 80, and 100 µg/mL) of rOPCML or rOPCML-Fc for 24 hours. PEO4 and OVCAR8 were also treated for 24 hours with rOPCML at 100 µg/mL +/- CDDP at 12 µM and 6 µM, respectively. The CDDP doses used in the Caspase-Glo apoptosis assay were the doses at which the combination of CDDP and OPCML had the strongest effect on decreasing cell viability.

Lung cancer cells (PC9/ER) were treated with rOPCML (150 µg/mL), Osimertinib (30 nM), or a combination of the two for 24 hours. Then, cells' caspase 3/7 activity was analysed with the Caspase-Glo assay. The concentration of rOPCML was decided based on the results obtained from

the MTT and WB assay. The dose of Osimertinib was chosen based on previous experiments by Dr Rajat Roy.

Following 24 hours of drug treatment, Caspase-Glo[®] 3/7 reagent was added to each well at 1:1 volume with the medium, and the plates were incubated for 1 hour at room temperature in the dark. The luminescence was measured using the LUMistar OPTIMA plate reader (BMG Labtech, UK).

2.11 Clonogenic Assay

A clonogenic assay is an in vitro cell survival assay that assesses the ability of single cells to survive and grow to produce colonies. In this study, the clonogenic assay has been used to study the effectiveness of rOPCML +/- Fc on cell survival and proliferation.

Clonogenic assays were performed by plating 1×10^3 SKOV3 cells per well in 6-well plates and 2×10^2 OVCAR8 cells in 24-well plates, leaving them to attach, and then treating them with 40 $\mu\text{g/ml}$ of rOPCML, rOPCML-Fc or rOPCML-R65L. Plates were then incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 7 days, the medium above the cells was discarded. Cells were rinsed carefully with PBS 3 times, then fixed with ice-cold methanol for 20 min at -20 °C, and subsequently stained with 0.4% crystal violet. After 30 minutes of staining, the crystal violet mixture was removed carefully and rinsed with tap water.

The same passages were taken with PEO4 cells, but their seeding density was 5×10^2 cells/well in twelve-well plates. PEO4 cells were treated with 40 and 100 $\mu\text{g/ml}$ of rOPCML and rOPCML-Fc, and the clonogenic assay was performed 8 days post-treatment.

The seeding density and the experimental endpoints were established based on the previous optimisation I performed for all three tested cells.

SKOV3-EV, SKOV3-OP, SKOV3-SOP, and SKOV3-R65L were seeded at 1×10^3 cells/well in six-well plates, and colonies were let grow in Incucyte® Live-Cell Analysis System. Pictures of SKOV3-EV, SKOV3-OP, SKOV3-SOP, and SKOV3-R65L were taken live by Incucyte® Live-Cell Analysis System using whole-well, label-free imaging automatically scanning for colonies starting from day 6 post-seeding. Ten days post-seeding, colonies were stained with 0.4% crystal violet, as described above.

2.12 Incucyte Wound Healing Assay

SKOV3 and OVCAR8 cells at a density of 25,000 cells/well (100 μ L/well) into the Incucyte® Imagelock Plate well. Cells were allowed to settle at room temperature for 15 minutes, and then the plate was incubated into a 37° C incubator at 5% CO² overnight. The day after, cells were wounded using the wound confluent cell monolayer using a 96-well woundmaker. This wound maker creates a wound simultaneously in all the wells. After wounding, the media from each well was aspirated and discarded, and the wells were washed twice with culture media (100 μ L/well). After washing, 100 μ L of culture medium $\pm$ 40 and 100 μ g/mL of rOPCML or rOPCML-Fc were added to each well. Subsequently, the plate was placed into the Incucyte® Live-Cell Analysis System and allowed to warm to 37° C for 30 minutes before scanning. Scanning was then taken with an objective at 10X, a channel selection corresponding to Phase Contrast, and with the scratch wound as defined scan type. Scans were taken every 2 hours for 24 hours.

Seeding densities were established based on previous optimisation experiments.

2.13 Tumour Spheroid Invasion in Matrigel[®]

Assay

5000 SKOV3 and OVCAR8 cells were seeded in 100 µl medium in triplicates in ultra-low attachment 96-well plates and allowed to form and grow for 5 days at 37°C in a humidified atmosphere containing 5% CO₂. On Day 5, the spheroids formed and were serum-starved for 16 hours. To avoid disturbing and disrupting the spheroid, 70% of the medium was removed and substituted with serum-free medium (RPMI 1640 + 0% FCS). This passage was repeated 7 times until the amount of serum was sufficiently reduced. Sixteen hours later, this process was reverted using the same method to replace the medium containing 10% FCS as a stimulant except for unstimulated and untreated control.

After 30 minutes, 50 µl of the medium was removed and replaced with medium containing 2X concentrated (final concentration 40 µg/ml) treatment (rOPCML, rOPCML-Fc, rOPCML-mut for SKOV3 cells and rOPCML and rOPCML-Fc for OVCAR8 cells) or PBS.

After 3 hours, 50 µl Matrigel[®] was added to the wells and allowed to solidify at 37°C for 30 min. 50 µl of full medium or medium containing 10% FCS +/- rOPCML or rOPCML-Fc or rOPCML-mut at the above concentration was added on top of the matrix.

Images of the spheroids were taken with the Nikon Eclipse TE2000 digital light microscope at 0h, 24h, 48h, 72h, and 120 h at 4x magnification, ensuring the focus captured the invading extensions from the spheroid. Images were analysed with Image J software.

In the result section, I reported the pictures of the time points at which the most significant differences were noticed between control, rOPCML +/- Fc, and rOPCML-mut treatment.

2.14 Tumour Spheroid Formation Assay

5000 SKOV3 cells transduced with empty-vector, OPCML with the GPI-anchor, OPCML without the GPI-anchor, and OPCML carrying R65L mutation were seeded in 100 µl of medium in triplicates in ultra-low attachment 96-well plates and allowed to form and grow for five days at 37°C, in Incucyte® Live-Cell Analysis System. Pictures of the neo-forming spheroids were taken from day one post-seeding to day 5. In the result section, only pictures captured on day five are shown. In the picture, the timepoint indicated is day 4.12h because I started to take photos from day one post-seeding; therefore, it was displayed as day 0 from the Incucyte® Live-Cell System.

2.15 Statistical Analyses

Data were analysed using GraphPad Prism software (USA) unless stated otherwise. All experiments were performed in three independent replicates unless stated otherwise. Results are expressed as mean $\pm$ SEM. Differences were analysed by Student t-test. $P < 0.05$ was considered significant unless otherwise specified.

CHAPTER 3

RECOMBINANT OPCML AND RECOMBINANT OPCML-FC SPECIFICITY AND BIOLOGICAL ACTIVITY IN PROTEOMIC ASSAYS

3.1 Introduction

Ectopic expression of OPCML and treatment with bacterially produced rOPMCL determined an inhibition of EGF-induced and GAS6-induced phosphorylation of HER2 and AXL, respectively, and an impairment of their downstream signalling pathway (i.e., MAPK and PI3K pathways) in ovarian cancer cells. This impairment was postulated to be one of the determinants of OPCML anti-tumour activity (McKie et al., 2012; Anthony et al., 2018). Therefore, to investigate the biological activity of the rOPCML and rOPCML-Fc that I produced, I tested both recombinants in proteomic assays in ovarian cancer cells (SKOV3, OVCAR8 and PEO4 cells). The aim was to evaluate whether rOPCML and rOPCML-Fc produced in human cells could reiterate the phenotype observed in previous experiments (McKie et al., 2012; Anthony et al., 2018). SKOV3 cells were used in previous experiments testing the ectopic expression of OPCML and rOPCML produced in bacteria. In addition, both SKOV3 and OVCAR8 cells express active forms of HER2 as well as high levels of AXL, two of the RTKs mainly influenced by OPCML expression (McKie et al., 2012; Zanini et al., 2017). Furthermore, both cell lines' proliferation and invasiveness are

profoundly affected by HER2 and AXL knockdown, respectively (Montero et al., 2015; Rankin et al., 2010). For these reasons, I used SKOV3 and OVCAR8 cells in the experiments to test the rOPCML and rOPCML-Fc I produced. I also tested rOPCML and rOPCML-Fc in cells derived from ascites of a patient with high-grade serous stage IIIc ovarian cancer that served as an *ex-vivo* model.

The time points for protein extraction and the doses of rOPCML+/-Fc used were decided based on previous experiments performed in our labs, which suggested that rOPCML +/- Fc have maximum effects in inhibiting phosphorylation of AKT and ERK at 3 hours in SKOV3 and 6 hours in OVCAR8, and that these effects start with the dose of 20 µg/mL to be evident at 40 µg/mL.

Subsequently, rOPCML and rOPCML-Fc activity were validated in proteomic assays by comparing the efficacy between treatment with rOPCML, rOPCML-Fc and rOPCML-mut.

These were recombinant forms of OPCML carrying specific point mutations (i.e., R65L, N70H, and P95R) in domain one and were produced by Dr. Birtley, who kindly donated some aliquots to me. These mutations resulted in a loss of function of OPCML/rOPCML ovarian cancer anti-tumour activity (Birtley et al., 2019). Therefore, I wanted to test the effects of rOPCML and rOPCML-Fc on inhibiting SKOV3 and OVCAR8 cell AKT and ERK phosphorylation compared to rOPCML-mut. For these purposes, I employed WB analysis.

It is important to note that due to the limited quantity of aliquots of each rOPCML-mut, I could not perform all the experiments with all the mutants. This limitation should be considered when interpreting the results.

In addition, to further widen the potential clinical application of recombinant OPCML, I tested rOPCML with the platinum agent cisplatin (CDDP). Platinum agents are the most effective cytotoxic chemotherapy used in clinics to treat ovarian cancer. The molecular mechanisms of resistance to platinum include activation of PI3K and MAPK pathways through overexpression of cell surface receptor tyrosine kinases (Agarwal et al., 2003; Kim et al., 2018; Marth et al., 1997; Pegram et al., 1997; Hengstler et al., 1999) and/or direct amplification of the pathway itself (Page et al., 2000; Mitsuuchi et al., 2000; Bellacosa et al., 1995). Therefore, I hypothesised that a strategy that combines the activity of rOPCML in impairing the PI3K and MAPK pathways and the cytotoxicity of platinum agents could be advantageous for both increasing rOPCML effects and sensitising ovarian cancer cells to platinum. The effect of CDDP and CDDP in combination with rOPCML on PI3K and MAPK signalling pathways was investigated by WB analysis in OVCAR8 cells, which are considered ovarian cancer cells highly resistant to CDDP (Schilder et al., 1990). Furthermore, I used PamChip® microarray, a high throughput kinomic profiling array, as wider kinases screening to test the activity of rOPCML and rOPCML-Fc in regulating the comprehensive tyrosine kinases activity. I used OVCAR8 and PEO4 ovarian cancer cells as a model in this experiment.

Lastly, I tested rOPCML in a non-ovarian cancer model to understand whether rOPCML's effect on PI3K/AKT and MAPK/ERK pathways is cancer-type independent. Lung cancer, in particular, non-small-cell lung cancer (NSCLC), was chosen as a model of cancer strictly dependent on RTKs, for which rOPCML treatment could have important future perspectives (Du et al., 2018).

Lung cancer, in particular, non-small-cell lung cancer (NSCLC), was chosen as a model of cancer strictly dependent on RTKs, and therefore, for which rOPCML treatment could have important future perspectives (Du et al., 2018).

The effect of rOPCML was investigated in PC9/ER cells. These cells are lung adenocarcinoma cells carrying an *EGFR* deletion of exon 19 and an insertion at exon 20. These rearrangements render PC9/ER resistant to Erlotinib, a first-generation EGFR-TKI. One mechanism of resistance to Erlotinib is the upregulation of other RTKs, such as HER2, MET, and EPHA2 (Amato et al., 2016; Romaniello et al., 2018), which are conversely inhibited by rOPCML treatment.

3.2 rOPCML and rOPCML-Fc treatment inhibit the phosphorylation of AXL and HER2, as well as ERK and AKT, two key effectors of the MAPK/ERK and PI3K/AKT pathways

To demonstrate that the rOPCML and rOPCML-Fc I produced were active and capable of recapitulating the inhibition of HER2 and AXL phosphorylation and the impairment of MAPK and PI3K pathways previously observed with OPCML transduction and bacteria-produced rOPCML treatment in SKOV3 cells, I performed Western blot analysis in SKOV3, OVCAR-8, and ASC-01 cells in the presence or absence of rOPCML and rOPCML-Fc treatment. The addition of 10% FCS or 20% patient-derived serum (for ASC-01) after 16 hours of serum starvation was used as a source of growth factors to stimulate RTK phosphorylation and

render the test as close as possible to what happens in an in-vivo biological model.

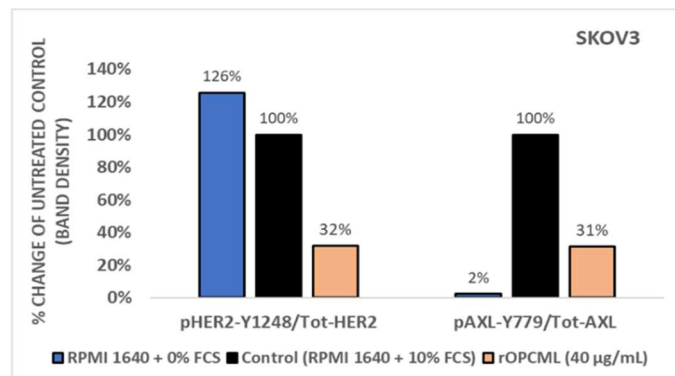
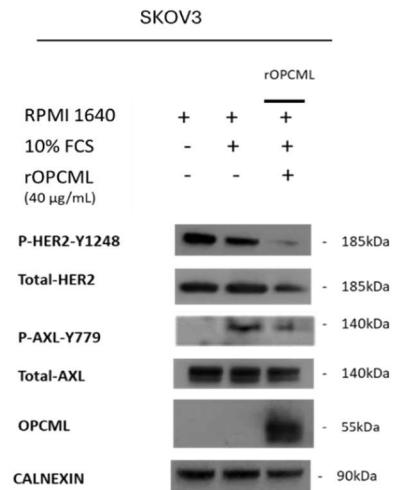
I demonstrated that rOCPML at the dose of 40 $\mu\text{g/mL}$ reduced by 68% the phosphorylation of both HER2 at tyrosine 1248 and 69% the phosphorylation of AXL at tyrosine 779 compared to the control in SKOV3 cells, as shown in Figure 3.1 A. The effects on these RTKs translated into impairment of the MAPK and PI3K pathways, as indicated by a reduction in pAKT and pERK levels. Specifically, treatment with 40 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ of rOCPML significantly reduced the phosphorylation of AKT at serine 473 by 24% ($p=0.006$) and 47% ($p=0.07$) compared to the untreated control, respectively (Figure 3.1 B). The levels of pERK1/2 at T202/Y204 and T185/Y187 were significantly decreased by the treatment with rOCPML, with reductions of 20% ($p=0.01$) and 32% ($p=0.05$) of the control after treatment with 40 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ of rOCPML, respectively (Figure 3.1 B).

Conversely, 40 $\mu\text{g/mL}$ of rOCPML-Fc did not inhibit HER2 phosphorylation; instead, this effect was achieved by increasing the dose of rOCPML-Fc to 100 $\mu\text{g/mL}$, resulting in a reduction in pHER2_Y1248 levels to 75% of the control (Figure 3.1 C). A dose-dependent effect of rOCPML-Fc was also observed in the decrease in pAKT and pERK levels. As depicted in Figure 3.1C, rOCPML-Fc at the doses of 40 and 100 $\mu\text{g/mL}$ reduced the phosphorylation of AKT at serine 473 by 45% and 56%, and the levels of pERK1/2 by 22% and 78% of the control, respectively (Figure 3.1 C).

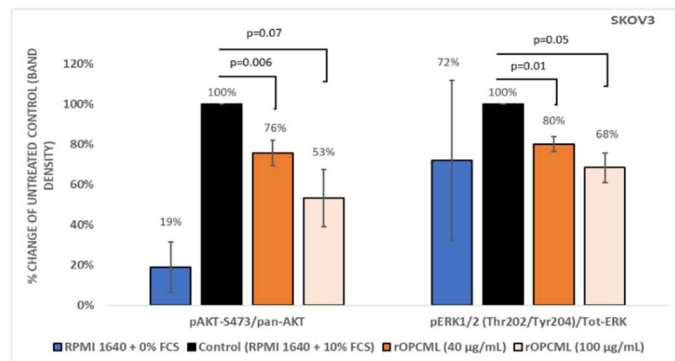
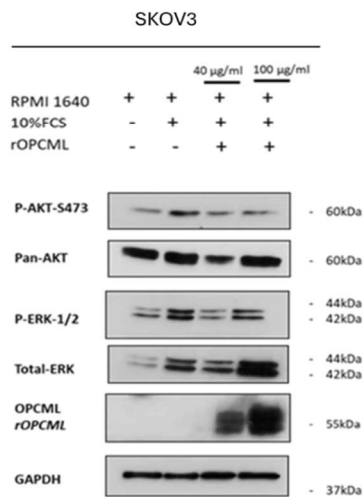
In these experiments, we observed that pHER2 levels are high regardless of the presence or absence of FCS (the stimulating agent) in the medium. This occurs because the cell line SKOV3 expresses active forms of HER2 that are not significantly influenced by the presence of serum in the medium (Montenero et al., 2015). This also justifies the validity of the 3 and 6-hour

time points used in these experiments, as the cell lines used the RTKs constitutively active independently from the stimulation of serum.

A



B



C

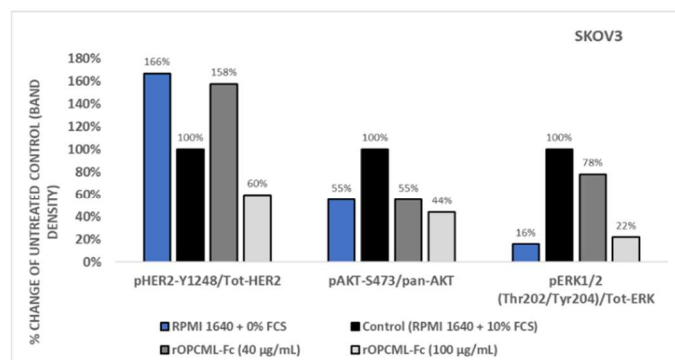
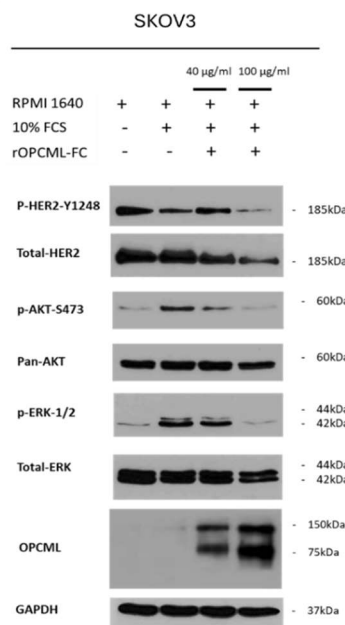


Figure 3.1: Effects of rOPCML and rOPCML-Fc on phosphorylation of HER2, AXL, AKT and ERK in SKOV3 cells:

- A. Western Blot analysis (LEFT) for total HER2 and AXL, and HER2 phosphorylated at Y1248 and AXL phosphorylated at Y779 in SKOV3 cells upon stimulation with 10% FCS and treatment with 40 µg/mL of rOPCML. Histogram (RIGHT), from 1 experiment (N=1) showing the quantification of pAXL and pHER2 band density normalised to total-AXL and total-HER2 and expressed as the percentage of the untreated control.
- B. Western Blot analysis (LEFT) for total AKT and ERK1/2, and AKT phosphorylated at S473 and ERK1/2 phosphorylated at Thr202 and Tyr204 in SKOV3 cells upon stimulation with 10% FCS and treatment with 40 and 100 µg/mL of rOPCML. Histogram (RIGHT), from 3 independent experiments (N=3), showing the quantification of pAKT and pERK band density normalised to pan-AKT and total-ERK and expressed as the percentage of the untreated control
- C. Western Blot analysis (LEFT) for total HER2 and HER2 phosphorylated at Y1248, and for total AKT and ERK1/2, and AKT phosphorylated at S473, and ERK1/2 phosphorylated at Thr202 and Tyr204 in SKOV3 cells upon stimulation with 10% FCS and treatment with 40 and 100 µg/mL of rOPCML-Fc. Histogram (RIGHT), from 2 independent experiments (N=2) showing the quantification of pHER2, pAKT, and pERK band density normalised to total HER2, pan-AKT, and total-ERK density and expressed as the percentage of the untreated control.
- In all the experiments, GAPDH or Calnexin was used as a loading control.

Similar effects in reducing AXL, AKT, and ERK phosphorylation were consistently demonstrated after treatment with rOPCML or rOPCML-Fc in OVCAR8 and ASC-01 cells.

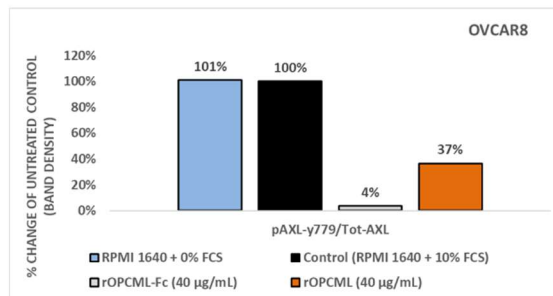
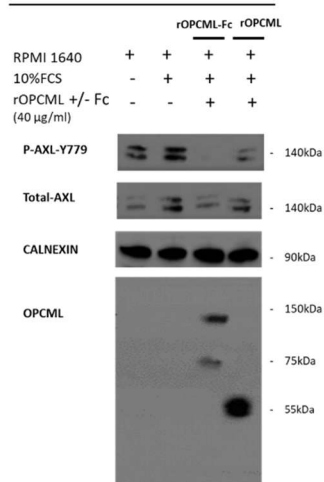
In both these cell lines, even when using different types of anti-HER2 antibodies at various dilutions and exposure times during WB film development, I could not detect the HER2 band. Therefore, it was not possible to evaluate the effects of rOPMCL +/- Fc on pHER2 in these cells.

In OVCAR8 cells, treatment with 40 µg/ml of rOPCML reduced pAXL levels by 63% of the control, and treatment with 40 µg/ml rOPCML-Fc resulted in the almost complete abolition of AXL phosphorylation at Y779, as shown in figure 3.2 A.

Significant inhibition of the phosphorylation of AKT at S473 and ERK1/2 was achieved upon treatment with rOPCML at 40 and 100 µg/ml. rOPCML treatment at 40 µg/ml resulted in a 54% reduction in pAKT levels ($p=0.02$) and a 63% reduction in pERK levels ($p=0.05$) compared to the control. Treatment with 100 µg/ml of rOPCML resulted in a 51% reduction in pAKT levels ($p=0.02$) and a 73% reduction in pERK levels ($p=0.02$) of the control (Figure 3.2 B). Similarly, rOPMCL-Fc significantly reduced the phosphorylation of AKT and ERK; however, the effect appeared less dose-dependent. At 40 µg/ml, rOPCML-Fc reduced pAKT levels by 57% and pERK levels by 69% ($p=0.01$) compared to the control, and at 100 µg/ml, rOPCML-Fc did not significantly amplify this effect. The higher dose of rOPCML-Fc at 100 µg/ml resulted in a 58% reduction in pAKT levels and a 70% reduction in pERK levels ($p=0.05$ and $p=0.07$, respectively) compared to the untreated control (Figure 3.2 C).

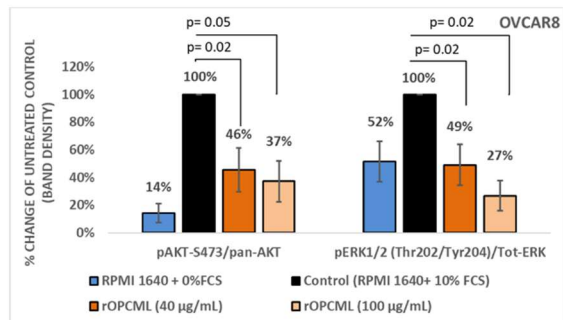
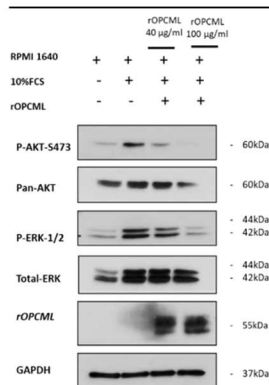
A

OVCAR8



B

OVCAR8



C

OVCAR8

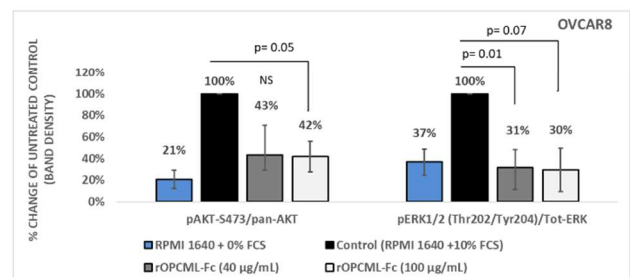
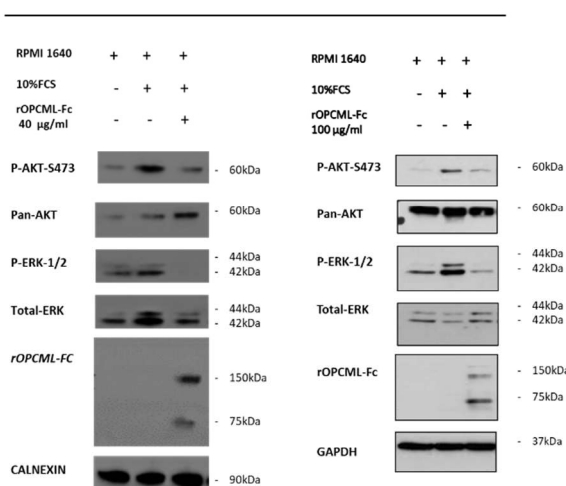


Figure 3.2: Effects of rOPCML and rOPCML-Fc on phosphorylation of AXL, AKT and ERK in OVCAR8 cells:

- A. Western Blot analysis (LEFT) for total AXL, and AXL phosphorylated at Y779 in OVCAR8 cells upon stimulation with 10% FCS and treatment with 40 µg/mL rOPCML-Fc and rOPCML. Histogram (RIGHT), from 1 experiment (N=1), showing the quantification of pAXL band density normalised to total AXL and expressed as percentage of untreated control
- B. Western Blot analysis (LEFT) for total AKT and ERK1/2, and AKT phosphorylated at S473 and ERK1/2 phosphorylated at Thr202 and tyr204 in OVCAR8 cells upon stimulation with 10%FCS and treatment with 40 and 100 µg/mL of rOPCML. Histogram (RIGHT), from 3 independent experiments (N=3), showing the quantification of pAKT and pERK band density normalised to pan-AKT and total-ERK and expressed as the percentage of the untreated control
- C. Western Blot analysis (LEFT) for total AKT and ERK1/2, and AKT phosphorylated at S473 and ERK1/2 phosphorylated at Thr202 and tyr204 in OVCAR8 cells upon stimulation with 10%FCS and treatment with 40 and 100 µg/mL of rOPCML-Fc. Histogram (RIGHT), from 3 independent experiments (N=3), showing the quantification of pAKT and pERK band density normalised to pan-AKT and total-ERK and expressed as the percentage of the untreated control

GAPDH or Calnexin was used as a loading control in all the experiments.

Cells isolated from the ascites of a patient with stage IIIc HGSOc (ASC-01) were used as an *ex-vivo* cancer model to validate further the activity of rOPCML and rOPCML-Fc in inhibiting AXL phosphorylation and impairing the MAPK and PI3K pathways.

AXL phosphorylation was completely inhibited by 100 µg/mL rOPCML-Fc. At this dose, there was also a deep reduction in pAKT and pERK levels by 99% and 78% of the untreated control, respectively. A reduction of pAXL, pAKT and pERK levels was also observed after treatment with rOPCML-Fc at 40 µg/mL. These reductions were minor compared to the reduction achieved by 100 µg/mL of the same molecule, reflecting a dose-dependent effect of the phosphorylation inhibition (Figure 3.3).

Also, rOPCML treatment reduced the phosphorylation of AXL, AKT and ERK. However, the effect of rOPCML seemed less pronounced and dose-dependent than the one achieved by rOPMCL-Fc. rOPCML at 40 and 100 µg/mL reduced pAXL levels to 89% and 61% of the untreated control, respectively. The pAKT levels were reduced to 73% and 71% of untreated control. Similarly, the effect achieved by rOPMCL in inhibiting ERK1/2 phosphorylation was not dose-dependent. However, it was more pronounced than the effect achieved in inhibiting AXL and AKT phosphorylation with a reduction of pERK levels to 49% and 41% of the untreated control upon treatment with rOPCML at 40 and 100 µg/mL.

These results are shown in Figure 3.3.

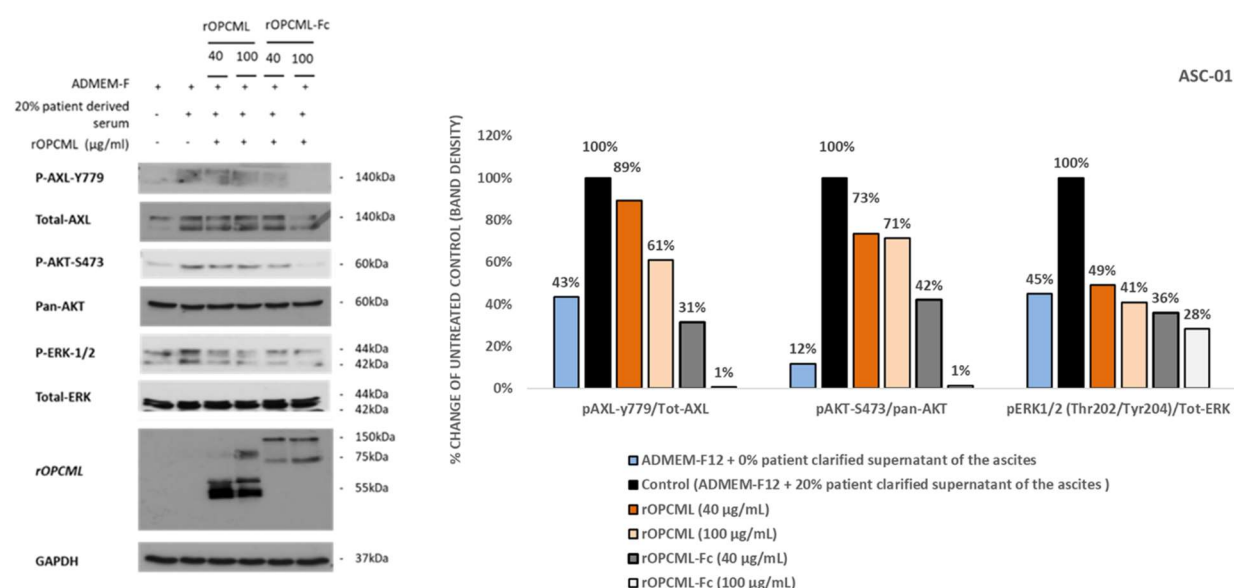


Figure 3.3: Effects of rOPCML and rOPCML-Fc on phosphorylation of AXL, AKT and ERK in ASC-01 cells (cells isolated from ascites of a patient with stage IIIc HGSOC): Western Blot analysis (LEFT) shows total and phosphorylated AXL, ERK and AKT in untreated and rOPCML/rOPCML-Fc treated ASC-01 cells. Histogram (RIGHT) shows the quantification of pAXL, pAKT and pERK band density normalised to total AXL, Pan-AKT and Total-ERK and expressed as a percentage of untreated control GAPDH was used as the loading control. N=1 experiment

3.3 rOPCML reduces the phosphorylation of ERK and reverses the increase in pAKT induced by Cisplatin (CDDP)

To evaluate whether a combination strategy of rOPCML with platinum agents could be advantageous in increasing rOPCML effects and sensitising ovarian cancer cells to platinum, I tested the effect of CDDP and CDDP in combination with rOPCML on the MAPK and PI3K signalling pathways by performing a WB analysis of AKT and ERK phosphorylation upon treatment with rOPCML, CDDP, or a combination of the two.

The WB analysis showed that consistent with the above results, rOPCML at 100 µg/mL reduced phosphorylation of both AKT at Ser 473 and ERK 1/2 by 40% and 75% of the control. Conversely, treatment with 25 µM CDDP increased AKT phosphorylation at Ser 473, as expected in a platinum-resistant cell line. However, this increase was counteracted by combining CDDP with rOPCML (100 µg/ml). This combination resulted in a 70% decrease in pAKT levels compared to the untreated control, as shown in Figure 3.4 A&B.

CDDP also reduces the phosphorylation of ERK 1/2 at Thr202 and Tyr204. The combination of CDDP and rOPCML yielded a more pronounced effect on this reduction: 41% compared to 98% of the untreated control (Figure 3.4 A&B).

Of note, I observed a higher OPCML level in cells treated with the combination, but the experiment was conducted only once; thus, no definitive conclusion can be drawn.

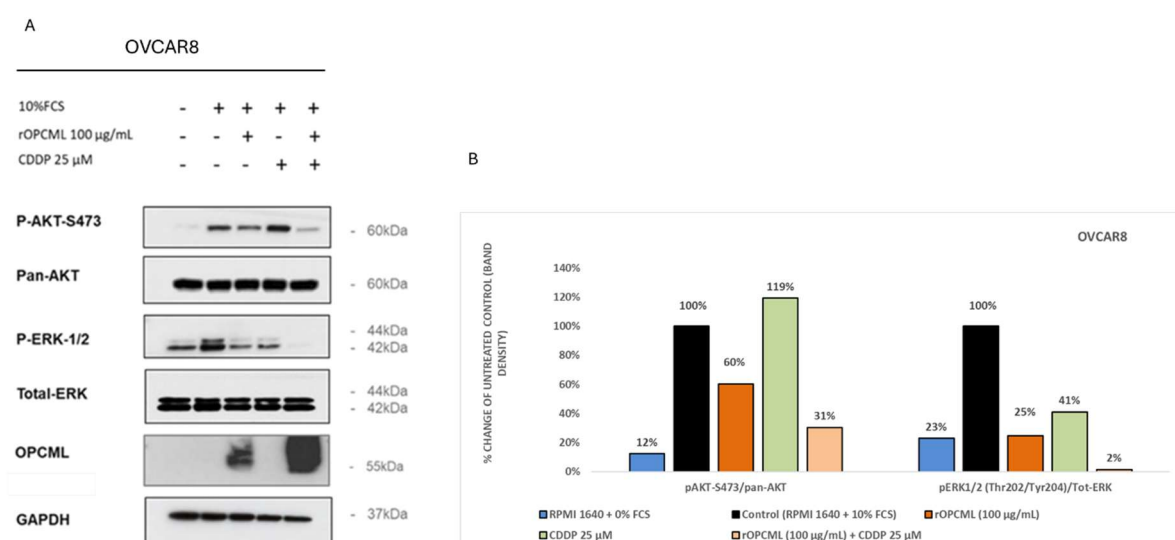


Figure 3.4 rOPCML +/- CDDP effects on phosphorylation of ERK pAKT in OVCAR8 cells:

- A. WB analysis showing expression and phosphorylation of AKT at S473 and ERK1/2 after stimulation with 10% FCS and treatment with rOPCML 100 µg/mL, or CDDP 25 µM or the combination of the two drugs.
- B. Histogram showing the quantification of pAKT and pERK level expressed as the ratio of Pan-AKT and Total-ERK, respectively, and normalised to control.
- In the experiment (N=1), GAPDH was used as loading, and all bands were firstly normalised to GAPDH band density.

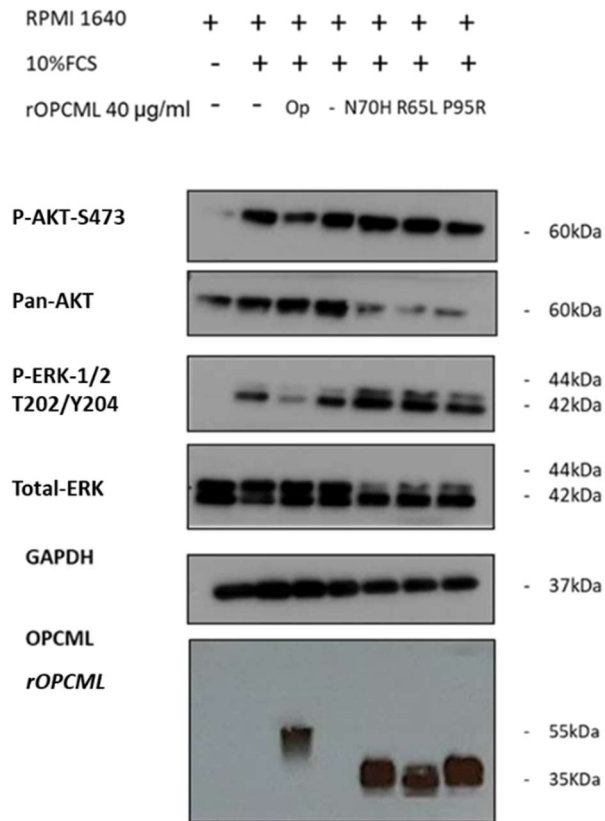
3.4 rOPCML but not rOPCML-mut impairs the PI3K/AKT and MAPK/ERK pathways

To demonstrate the specificity of rOPCML in downregulating the PI3K/AKT/mTOR and MAPK/ERK pathways, I compared its effects on AKT, ERK, and AXL phosphorylation with those of mutated rOPCML in both SKOV3 and OVCAR8 cells. In SKOV3 cells, I confirmed that rOPCML at 40 µg/mL reduces pERK and pAKT levels by 58% and 68%, respectively. However, this effect was not observed when SKOV3 cells were exposed to the same dose of rOPCML (40 µg/mL) carrying well-known

inactivating mutations—N70H, R65L, and P95R. Conversely, mutated rOPCML (rOPCML-N70H, rOPCML-R65L, and rOPCML-P95R) led to an increase in both pAKT-S473 and pERK1/2 levels. Despite various attempts using different primary and secondary antibody dilutions and film exposure times, pAXL-Y779 bands were not detected in this experiment. Furthermore, due to the lack of three independent experiments, assessing the significance of the observed differences was not feasible (Figure 3.5).

A

SKOV3



B

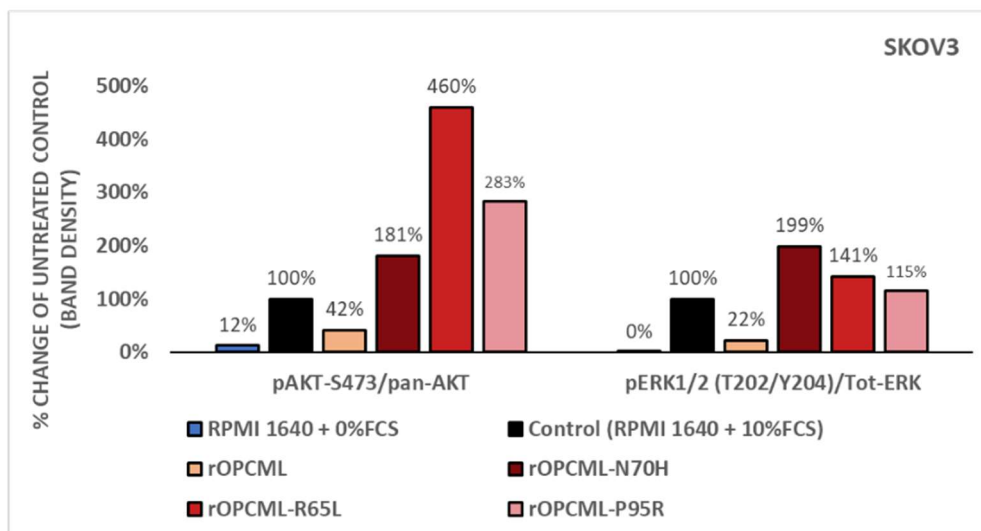


Figure 3.5: Effects of rOPCML and rOPCML-mut on phosphorylation of AKT and ERK in SKOV3:

- A. *Western Blot analysis shows phosphorylation and expression of AKT and ERK following rOPCML (Op) and rOPCML mutants (OPCML R65L, OPCML N70H, OPCML P95R) treatment at 40 µg/mL.*
- B. *Histogram (B) showing the quantification of pAKT and pERK band density normalised to pan-AKT and Total-ERK and expressed as the percentage of untreated control.*
- In the experiment (N=1), GAPDH was used as loading, and all bands were firstly normalised to GAPDH band density.*

In OVCAR8 cells, treatment with 40 µg/mL of rOPCML was compared with the same dose treatment of rOPCML-R65L and rOPCML-P95R. rOPCML-N70H was not used because the quantity Dr Bentley kindly provided us was insufficient to perform this experiment. I observed that rOPCML at 40 µg/mL inhibits AXL, AKT, and ERK phosphorylation by 16%, 23%, and 80%, respectively (Figure 3.6 A&B). The effect on AXL phosphorylation at Y779 is completely lost upon treatment with rOPCML-R65L and rOPCML-P95R, which instead increases by 43% and 78%, respectively. Conversely, the inhibition of AKT and ERK phosphorylation at S473 and Thr202/Tyr204 determined by rOPCML is only partially lost after treatment with rOPCML-R65L and rOPCML-P95R, resulting in a reduction of pAKT-S473 levels by 20% and 18% and a reduction of pERK1/2 levels by 62% and 72% (Figure 3.6 A&B). This experiment was only performed once; therefore, I could not evaluate the significance of the highlighted differences.

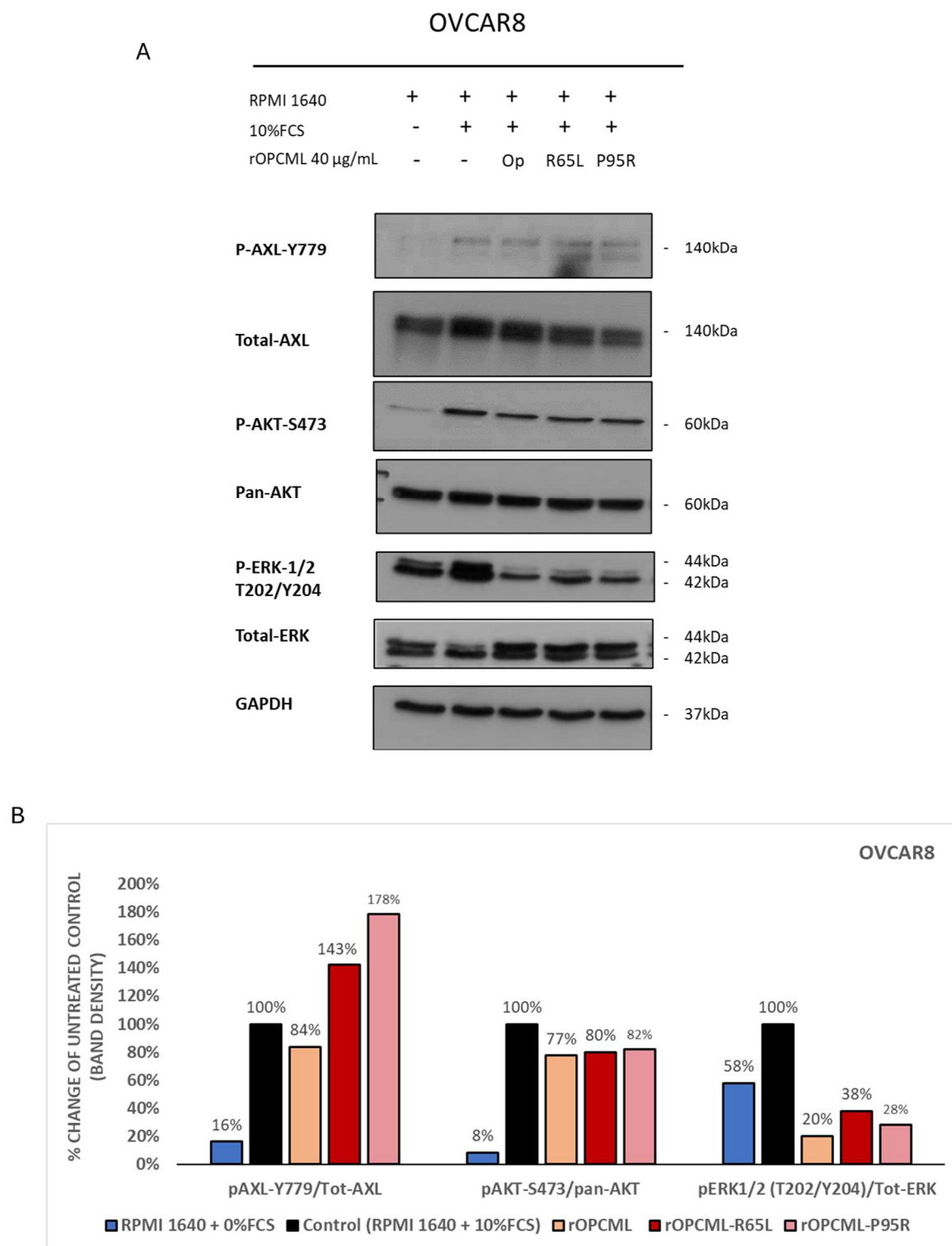


Figure 3.6: Effects of rOPCML and rOPCML-mut on phosphorylation of AXL, AKT, ERK in OVCAR8:

- A. Western Blot analysis shows phosphorylation and expression of AXL, AKT and ERK following rOPCML (Op) and rOPCML mutants (OPCML R65L and OPCML P95R) treatment at 40 µg/mL.
- B. Histogram (B) showing the quantification of pAXL, pAKT and pERK band density normalised to Total-AXL, Pan-AKT and Total-ERK and expressed as the percentage of untreated control.

In the experiment (N=1), GAPDH was used as a loading control, and all bands were first normalised to GAPDH band density.

3.5 rOPCML activity on PTK kinomic profile of ovarian cancer cells

To measure the Tyr-kinase activity in a more comprehensive manner than WB analysis, I used the Protein Tyrosine Kinase (PTKs) PamChip-96 microarrays[®] (catalogue # 86311, PamGene International BV, 's-Hertogenbosch, The Netherlands) in OVCAR8 and PEO4 cells treated with rOPCML at 100 µg/mL.

The PTK PamChip[®] Array, a flow-through microarray assay including 144 distinctive peptide sequences spotted on a porous membrane, was used to measure Tyr-kinase activity comprehensively. Each peptide on the chip represents a putative endogenous phosphorylation site that functions as a tyrosine kinase substrate, allowing us to identify the specific PTKs influenced by rOPCML and the sites of phosphorylation.

Therefore, I would use these experiments to evaluate the impact of rOPCML on PTKs' phosphorylation and determine the specific sites affected.

In OVCAR8, I demonstrated that rOPCML treatment resulted in a significant reduction of phosphorylation of several p-Tyr peptides over the control, with a significant decrease in phosphorylation at different sites of 36 of 82 PKs. Of these PKs, 14 were RTKs (ERBB2, PGFRB, EGFR, VGFR2, VGFR1, MET, EPHA7, EPHA2, EPHA1, EPHB1, MET, RET, FGFR3, TYRO) (Figure 3.7 A); five were members of MAPK family: Ras, FAK2, ARAF, RAF1, MK10 (Figure 3.7 B); four were PKs members of SRC family kinases: FRK, EFS (Fyn-related kinase and Embryonal Fyn-

associated substrate), LCK and Lyn (Figure 3.7 C); two were adaptor molecules LAT and ART (Figure 3.7 D); two were important intracellular signal transducers involved in multiple downstream pathways: PLCG1 and TEC; one transmembrane signalling transducer: PLCG2; one master kinase: TEC; two were responsible for immune regulation: ZAP10 and CD32; one was the key downstream component of the canonical Wnt signalling pathway: CTNB1 (Figure 3.7 C), and one was a phosphatase PTN6 (Figure 3.7 D). In addition, it was confirmed that ERK and AKT1 phosphorylation were reduced (Figure 3.7 B&C), suggesting a promising potential for rOPCML in cancer treatment.

Conversely, there was no significant downregulation of phosphotyrosine in FGFR1 and FGFR2, VEGFR3, ERBB4, and INSR, and no regulation of the phosphotyrosine of the other 41 PKs such as CDK7, EPHA4, CD70 and NCF1

In PEO4 cells, rOPCML significantly decreased the phosphorylation of the RTKs PDGFRB and MET, the phosphatase PTN11, c-jun, ERK1, PLCG1, FAK2, CD31, and Vinculin (Figure 3.7 E).

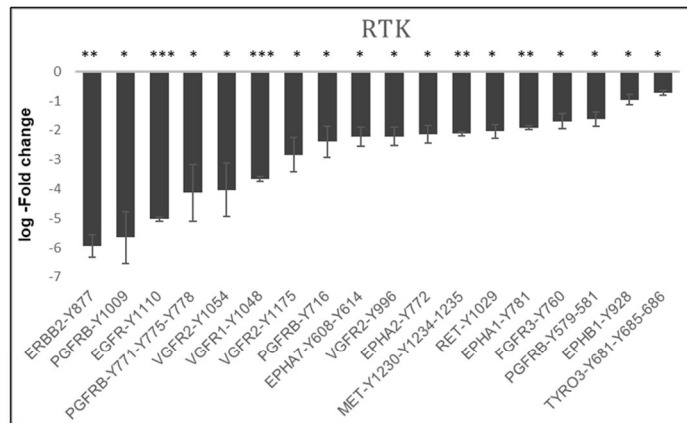
The differential regulation of phosphorylation of TKs in the two cell lines may be due to several factors. Firstly, OVCAR8 and PEO4 are different types of ovarian cancer cell lines. These differences in cell type may lead to different responses to rOPCML treatment. Secondly, OVCAR8 express a repertoire of constitutively active RTKs, whereas PEO4 have lost, in comparisons of their isogenic-paired cell lines PEO1 (platinum-sensitive), expression of some RTKs such as AXL. These differences in RTK expression may also contribute to the observed differences in phosphorylation regulation.

A GeneGo analysis was conducted using data generated with rOPCML-treated samples to visualise better and interpret these findings. The most

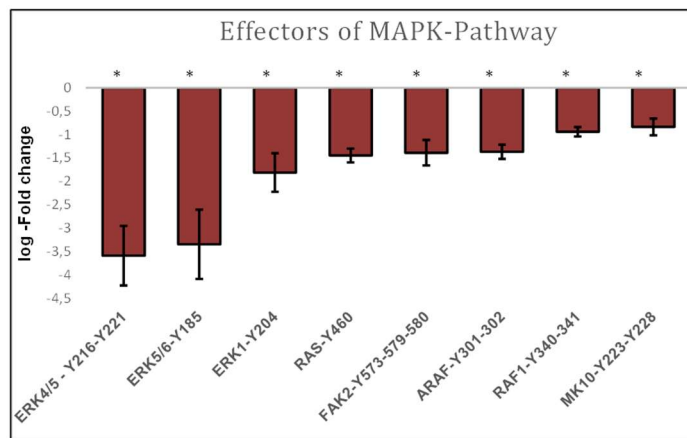
active cellular processes include developmental signalling, cell-cycle regulation and immune response (Figure 3.7 F). The latter is highlighted by the evidence of the significant impairment by rOPCML treatment of the phosphorylation of a group of kinases involved in immunoregulator pathways (Figure 3.7 G). This might suggest a possible role of OPCML in cancer immunomodulation that needs to be further investigated in more complex models such as co-culture (OC cell lines + immune cells).

OVCAR8

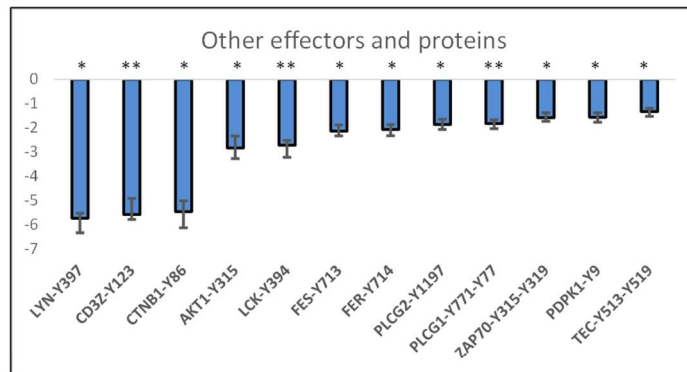
A



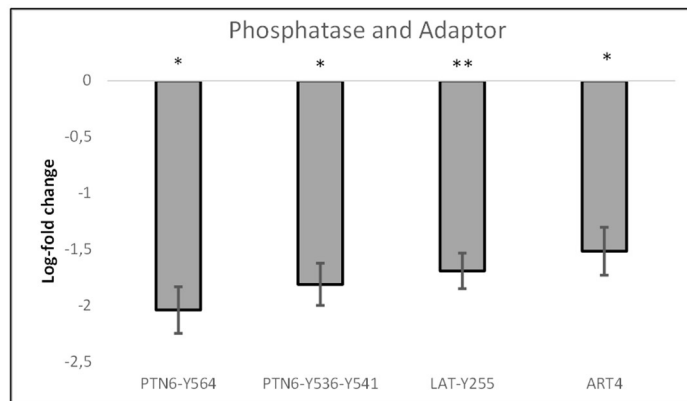
B



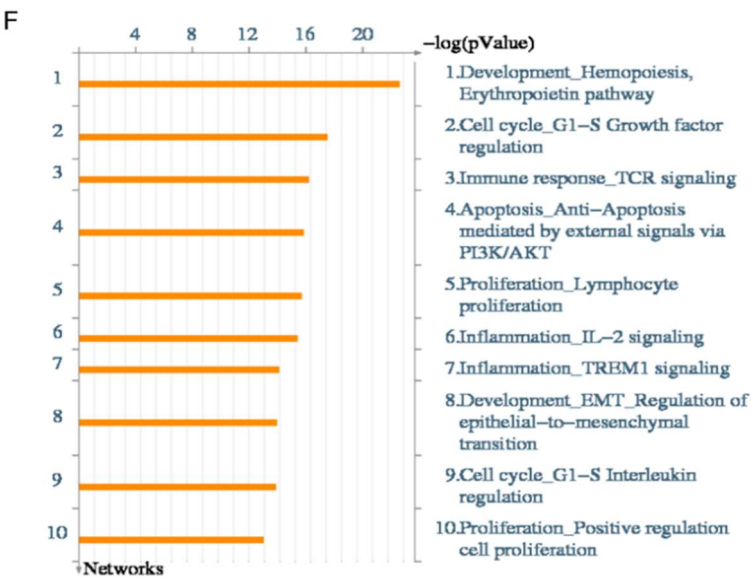
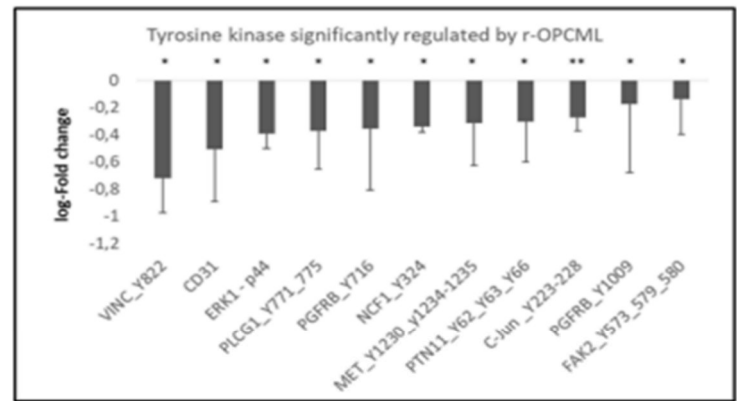
C



D



E PEO4



G OVCAR8

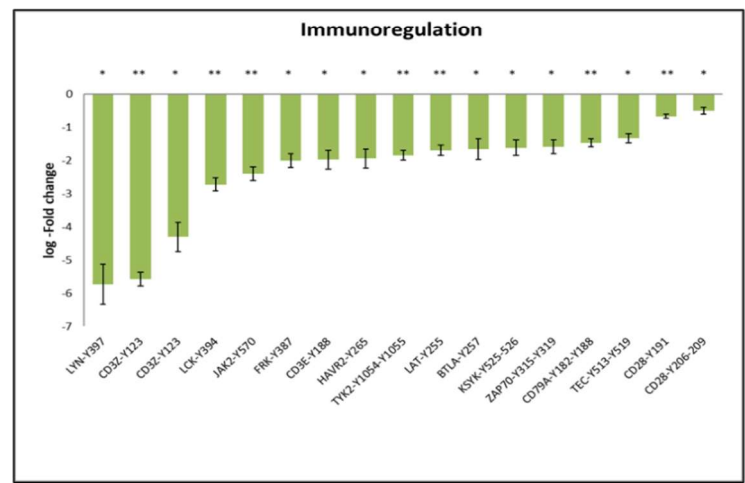


Figure 3.7: Effect of rOPCML on the phosphorylation of a specific panel of tyrosine kinases in OVCAR8 and PEO4 cells:

- A. Receptor tyrosine kinase showing a significant decrease in OVCAR 8 upon rOPCML treatment
- B. Effectors of MAPK pathway, in which rOPCML significantly reduces tyrosine phosphorylation in OVCAR8 cells
- C. Effectors of other signalling pathways whose tyrosine phosphorylation is significantly reduced by rOPCML in OVCAR8 cells
- D. Phosphatase and adaptor molecules whose tyrosine phosphorylation is inhibited by rOPCML in OVCAR8
- E. Proteins whose tyrosine phosphorylation is reduced by rOPCML in PEO4 cells
- F. Enriched GeneGo process networks following rOPCML treatment generated by the PamGene team. The top 10 most significantly enriched process networks are sorted by statistical significance based on manually curated objects generated by PamGene fold-change data. Data represent the negative log of the p-value determined by GeneGo (more significant processes appear nearer the top).
- G. Phosphoproteins involved in immunoregulation processes, whose tyrosine phosphorylation is reduced by rOPCML in OVCAR8 cells

The specific tyrosine of which phosphorylation is inhibited is indicated for each protein. Student t-test and ($p < 0.05$) were used for statistical analysis, and error bars depict mean $\pm$ SEM. Fold decrease is indicated as Log2.*

3.6 rOPCML impairs PI3K/AKT and MAPK/ERK pathways, inhibiting the phosphorylation of the two pathway effectors AKT and ERK in lung cancer PC9/ER cells

To evaluate the effects of rOPCML on the PI3K/AKT and MAPK/ERK pathways in another cell line, we decided to use the RTK-dependent lung cancer PC9/ER cells and test three doses of rOPCML on the phosphorylation of AKT and ERK, which are the two downstream effectors of these pathways. Treatment with rOPCML showed a dose-dependent effect on inhibiting the phosphorylation of both AKT and ERK. However, while AKT phosphorylation was slightly inhibited at 50 and 100 $\mu\text{g/ml}$, it became

significant at 150 $\mu\text{g/ml}$, resulting in a 72% reduction in pAKT-S473 levels compared to the control. In contrast, ERK1/2-T202/Y204 phosphorylation was inhibited at 50 $\mu\text{g/ml}$ of rOPCML, reducing pERK1/2 levels by 73%. This reduction increased further to 86% and 87% at the 100 $\mu\text{g/ml}$ and 150 $\mu\text{g/ml}$ doses, respectively. (Figure 3.8).

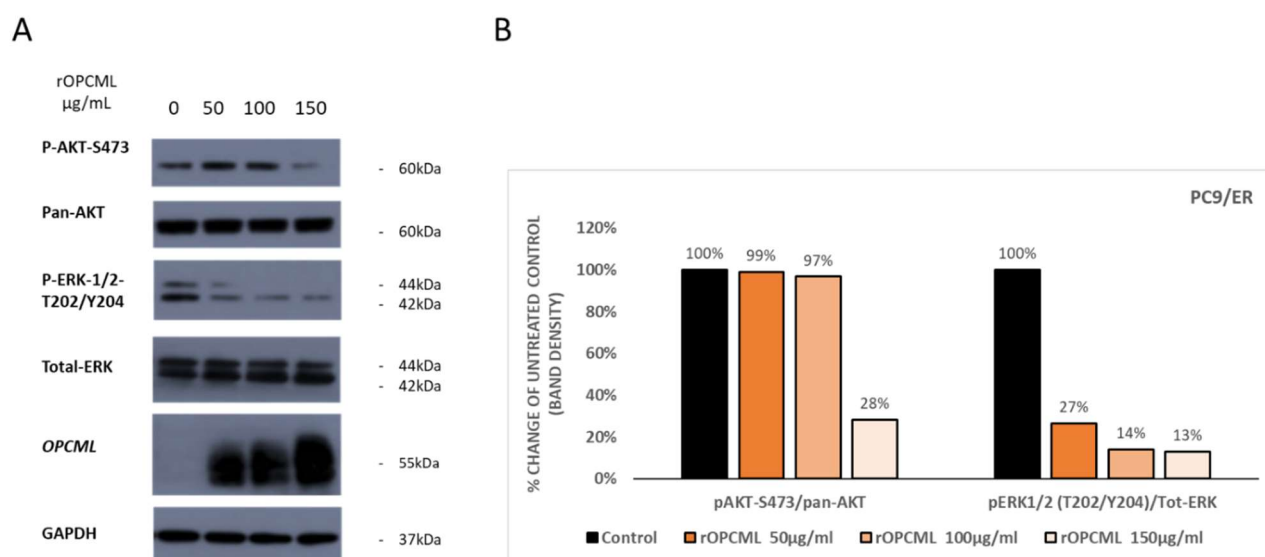


Figure 3.8: Effects of different doses of rOPCML in PC9/ER lung cancer cells:

- Western Blot analysis shows phosphorylation and expression of AKT and ERK upon stimulation with 10% FCS and treatment with 0, 50, 100, and 150 $\mu\text{g/ml}$ of rOPCML.
- Histogram showing the quantification of pAKT and pERK band density normalised to pan-AKT and Total-ERK and expressed as a percentage of the untreated control.

In the experiment ($N=1$), GAPDH was used as the loading control to normalise all the other density bands.

3.7 Summary

In this chapter, I have shown that both rOPCML and rOPCML-Fc reproduce the observed traits in transduced SKOV3 cells and in SKOV3 cells treated

with bacterial rOPCML by inhibiting AXL, HER2, AKT, and ERK phosphorylation in SKOV3, OVCAR8, and ASC-01 cells.

The levels of pAKT and pERK were reduced in all three cancer cell lines at various doses. The reduction was dose-dependent for both rOPCML and rOPCML-Fc in SKOV3 and ASC-01 cells. In OVCAR8 cells, the reduction in pAKT and pERK levels was dose-dependent when treated with rOPCML, but the dose-dependent pattern was not observed with rOPCML-Fc treatment.

Interestingly, and as further confirmation of rOPCML +/- Fc activity, I demonstrated here that rOPCML inhibits the phosphorylation of AXL, AKT, and ERK in ovarian cancer cells and that this effect is lost when rOPCML-mut is used as a treatment. Additionally, I observed that rOPCML reduces ERK phosphorylation and reverses AKT activation caused by CDDP. It is important to note that the experiment was only conducted once, so we cannot draw definitive conclusions. Still, it suggests potential directions for future research on using rOPCML +/- Fc treatment.

I also used the Pamgene multikinase assay to demonstrate that rOPCML changes the phosphokinase profile of OVCAR8 and PEO4 cells. The analysis of phosphorylation changes in the tested kinases confirmed the inhibition of phosphorylation of RTKs and effectors known to be involved in OPCML mechanisms of action but also suggested other phospho-inhibitions. The results I obtained here also suggested a potential role of rOPCML in immunomodulation, a field worthy of further research.

Finally, initial findings indicate that rOPCML might be effective in certain types of lung cancer. Here, I found that rOPCML decreases the

phosphorylation of both AKT and ERK in PC9/ER cells, indicating a possible role in lung cancer treatment.

CHAPTER 4

rOPCML AND rOPCML-FC ACTIVITY AND SPECIFICITY IN FUNCTIONAL ASSAYS

4.1 Introduction

To investigate whether the observed effect of rOPCML and rOPCML-Fc on PI3K/AKT and MAPK/ERK pathway correlates with an antiproliferative anti-invasive response in ovarian cancer cells, I evaluated the effect of rOPCML+/- Fc on ovarian cancer cells:

1. Viability assessed with MTT assay
2. Apoptosis assessed by caspase 3/7 assay
3. Invasiveness assessed by 3D-Spheroid invasion assay in Matrigel®
4. Clonogenicity assessed with clonogenic assay
5. Migration assessed through wound healing assay

In addition, I tested the effect of the combination of rOPCML and CDDP on OVCAR8 and PEO4 platinum-resistant ovarian cancer cell viability and apoptosis to examine the effects of the two drugs. I also tested the differential effects of rOPCML and the rOPCML-mut on ovarian cancer clonogenicity and invasion; this would prove the specificity of the rOPCML effects.

Furthermore, to corroborate whether the dose-dependent effect of rOPMCL on PC9/ER cells in reducing AKT and ERK phosphorylation corresponds to an antiproliferative effect, I tested the effects of escalating doses of rOPCML in cell viability assay in these cells. Then, I tested the rOPCML effect in combination with osimertinib on PC9/ER cell viability and apoptosis. Osimertinib is an irreversible EGFR TKI largely used in NSCLC, thanks to

its ability to irreversibly bind to mutated EGFR proteins, particularly to those with more common mutations such as the exon 19 *EGFR* deletion also carried in PC9/ER cells.

4.2 rOPCML and rOPCML-Fc reduce ovarian cancer cells' viability in a dose-dependent manner

To assess the effect of rOPCML and rOPCML-Fc on tumour cell viability and investigate their potential as cancer treatment, I used the MTT assay in SKOV3, OVCAR8, and PEO4 cells. Cells were seeded in 96-well flat-bottom plates, and 24 hours post-seeding, they were treated with 0, 40, 60, 80 and 100 $\mu\text{g/mL}$ of rOPCML and rOPCML-Fc. After 72 hours of treatment, the MTT assay was performed.

Both rOPCML and rOPCML-Fc significantly reduced the viability of all the ovarian cancer cells tested in a dose-dependent manner (Figure 4.1).

In SKOV3 treated with rOPCML, a significant reduction in cell viability was observed compared to the control. The reduction ranged from 21% ($p=0.008$) at the lowest dose tested of 40 $\mu\text{g/mL}$ to 38% ($p=0.0005$) at the highest dose tested of 100 $\mu\text{g/mL}$ (Figure 4.1 A). Similar and significant was the reduction obtained in the same cell lines after rOPCML-Fc treatment, with cell viability reduction that ranged from 23% ($p=0.03$) to 37% ($p=0.005$) of the relative control at the lowest (40 $\mu\text{g/mL}$) and highest (100 $\mu\text{g/mL}$) dose tested, respectively (Figure 4.1 B).

A significant reduction was also obtained at all doses tested of rOPCML (40-60-80 and 100 $\mu\text{g/mL}$) in OVCAR8 and PEO4 cells. Treatment with rOPCML reduced cell viability from 18% ($p=0.0001$) to 33% ($p=0.0002$) of the control in OVCAR8 cells and from 10% ($p=0.03$) to 28% ($p=0.0002$) of

the relative control in PEO4 (Figure 4.1 A). Analogue results were reported with rOPCML-Fc treatment that reduced OVCAR8 viability by 17% ($p=0.0001$) at 40 $\mu\text{g/mL}$ and 30% ($p=0.003$) at 100 $\mu\text{g/mL}$ and PEO4 viability by 12% ($p=0.02$) and 30% ($p=0.001$) respectively at 40 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ (Figure 4.1 B).

The IC₅₀ was not reached at the tested doses; however, the reduction achieved with both rOPCML and rOPCML-Fc in SKOV3, OVCAR8 and PEO4 cells viability was statistically significant compared with the viability of untreated cells, demonstrating a consistent inhibition of cell viability.

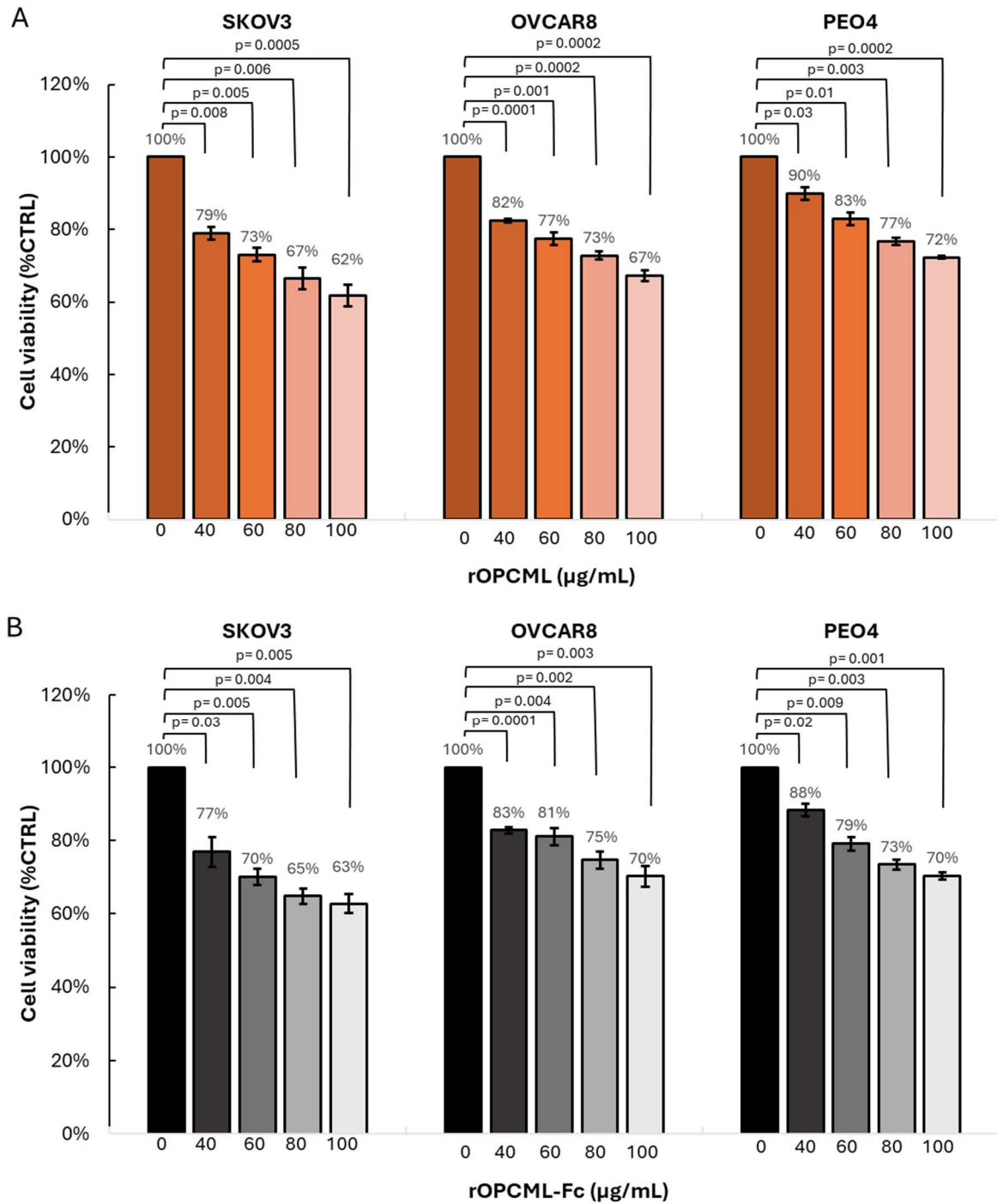


Figure 4.1: Effects of rOPCML and rOPCML-Fc on SKOV3, OVCAR8 and PEO4 cells viability: SKOV3, OVCAR8 and PEO4 cells were treated with 0, 40, 60, 80, 100 $\mu\text{g/mL}$ of rOPCML (A) and rOPCML-Fc (B) for 72 hours and then cells viability was analysed with MTT assay. The data represent the mean and standard deviation of three independent experiments ($N=3$) normalised for the mean of untreated control. Student t-test was used for statistical analysis (statistical significance as for $p < 0.05$), and error bars depict mean $\pm$ SEM.

4.3 rOPCML and rOPCML-Fc induce caspase 3/7 activity of ovarian cancer cells in a dose-dependent manner

To determine whether rOPCML and rOPCML-Fc may induce apoptosis in ovarian cancer cells, I measured caspase 3/7 activation by the Caspase-Glo assay (Promega) in SKOV3, OVCAR8, and PEO4 cells after 24 hours of treatment with rOPCML and rOPCML-Fc at increasing doses (0, 40, 60, 80, and 100 $\mu\text{g/mL}$).

The results showed that rOPCML and rOPCML-Fc induced caspase 3/7 activation in SKOV3, OVCAR8 and PEO4 cells and that the increase in caspase 3/7 activity is dose-dependent.

rOPCML significantly increased caspase 3/7 activity in SKOV3 by 2 ($p=0.002$), 2.4 ($p=0.004$), 3.1 ($p=0.001$) and 3.6-fold ($p=0.003$), compared to the control when used at 40, 60, 80 and 100 $\mu\text{g/mL}$, respectively. Similar results were obtained with increasing doses of rOPCML in OVCAR8. In these cells, caspase 3/7 activity increased significantly in a dose-dependent manner from 2 ($p=0.02$) at 40 $\mu\text{g/mL}$ to 3.4-fold ($p=0.0006$) at 100 $\mu\text{g/mL}$. In PEO4 cells, the increase in caspase 3/7 activity was still significant but less marked, with an increase that ranged from 1.5 ($p=0.005$) to 2.5-fold ($p=0.04$) at the lowest (40 $\mu\text{g/mL}$) and highest dose (100 $\mu\text{g/mL}$) of rOPCML tested (Figure 4.2 A).

The same trend was observed with rOPCML-Fc treatment, which significantly augmented caspase 3/7 activity in the three cell lines tested, with a more pronounced effect in SKOV3 and OVCAR8 than in PEO4 (Figure 4.2 B).

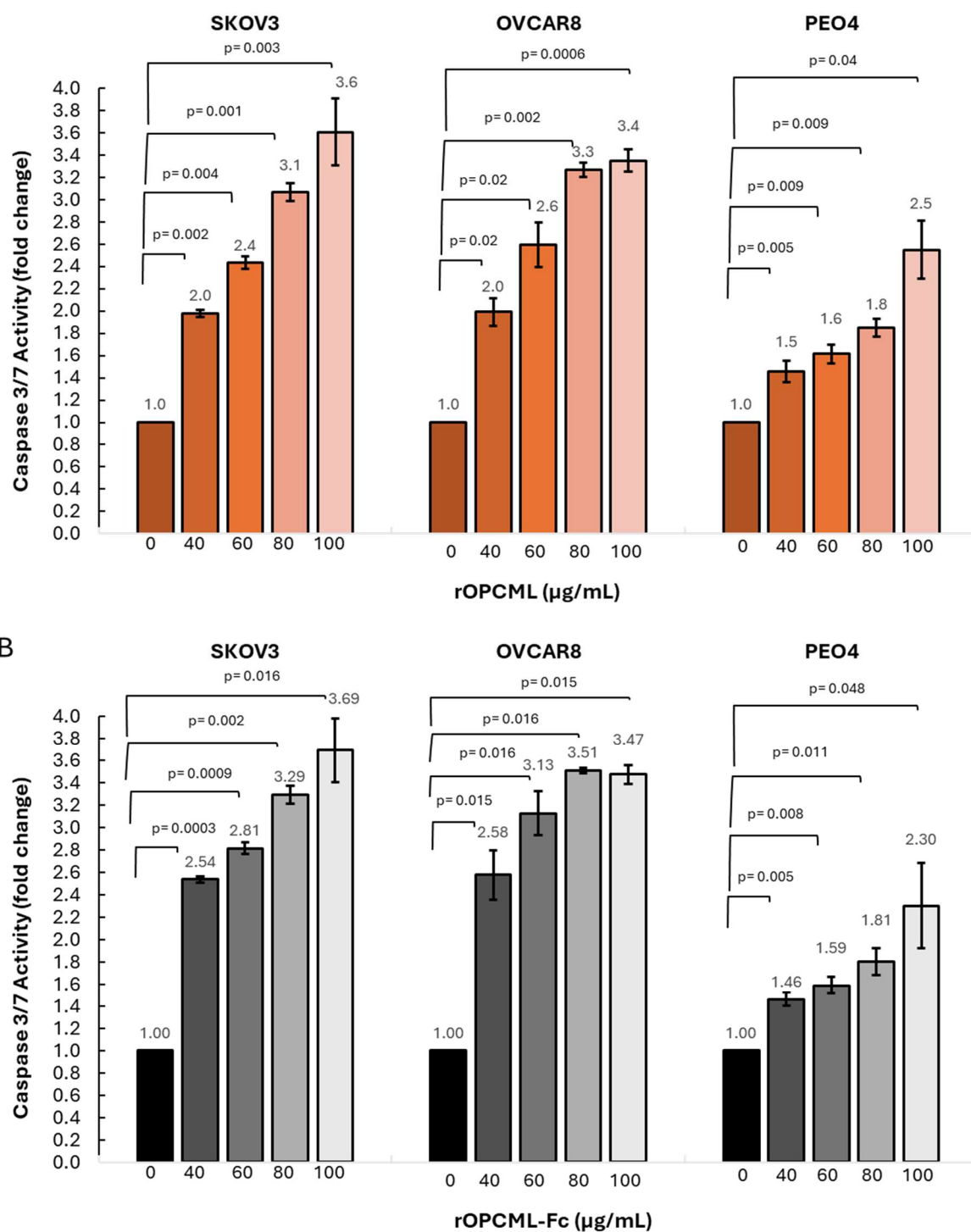


Figure 4.2: rOPCML and rOPCML-Fc effect on SKOV3, OVCAR8 and PEO4 caspase 3/7 activity: SKOV3, OVCAR8 and PEO4 cells were treated with 0, 40, 60, 80, 100 µg/mL of rOPCML (A) and rOPCML-Fc (B) for 24 hours and then cells caspase 3/7 activity was analysed with Caspase-Glo assay. The data represent the mean and standard deviation of three independent experiments (N=3) normalised to the mean of untreated control. Student t-test was

used for statistical analysis (statistical significance as for $p < 0.05$), and error bars depict mean $\pm$ SEM.

4.4 rOPCML and rOPCML-Fc exert their anti-tumour activity in an ex-vivo model

To investigate whether the effect observed using cell lines was retained when using patients' derived tumour cells, I tested rOPCML and rOPCML-Fc in ascites-derived cells from one patient with ovarian cancer (ASC-01) as an *ex-vivo* model.

ASC-01 cells were treated with 0, 40, 60, 80, 100 $\mu\text{g/mL}$ of rOPCML and rOPCML-Fc and then analysed for cell viability with MTT assay and cell apoptosis with the caspase 3/7 assay.

rOPCML and rOPCML-Fc, at 40 $\mu\text{g/mL}$, reduced cell viability by 10% of the untreated control. The reduction was then dose-dependent, reaching a decrease of 35% and 31% of the control at 100 $\mu\text{g/mL}$ of rOPCML and rOPCML-Fc, respectively. (Figure 4.3).

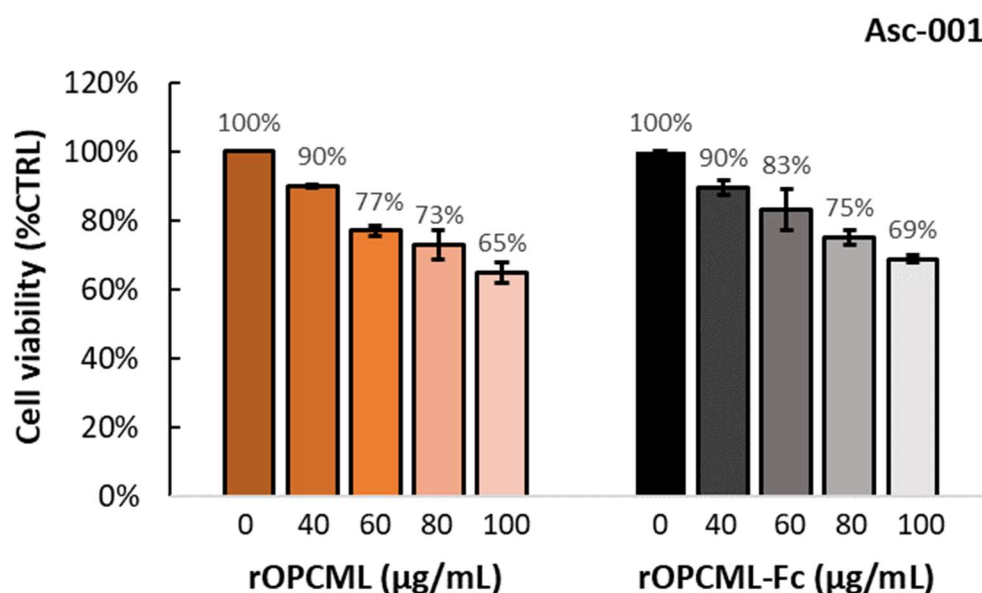


Figure 4.3: Effects of rOPCML and rOPCML-Fc on ASC-01 cell viability: ASC-01 cells were extracted from ascites of a patient affected by ovarian cancer and treated with 0, 40, 60, 80, 100 $\mu\text{g/mL}$ of rOPCML (left) and rOPCML-Fc (right) for 72 hours. Cell viability was analysed with MTT assay. The data represent the mean of three technical repeats to which the error bars are referred.

rOPCML and rOPCML-Fc at 40, 60, 80, and 100 $\mu\text{g/mL}$ achieved a dose-dependent increase in caspase 3/7 activity. At the highest dose of 100 $\mu\text{g/mL}$, rOPCML achieved a 4-fold increase and rOPCML-Fc a 3.7-fold increase in caspase 3/7 activity (Figure 4.4).

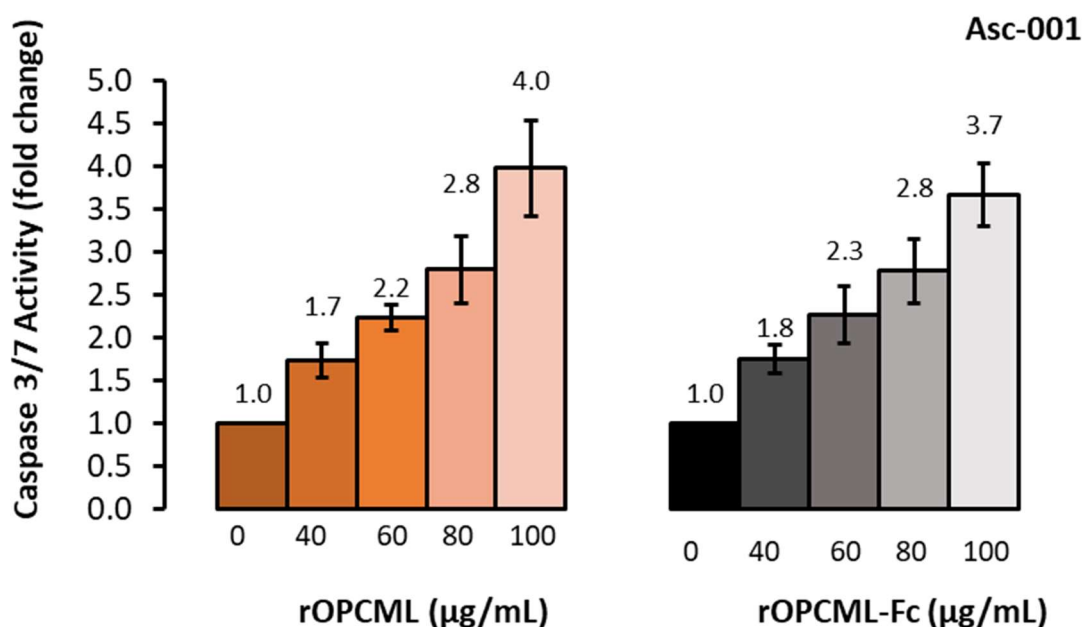


Figure 4.4: Effects of rOPCML and rOPCML-Fc on ASC-01 cells caspase 3/7 activity: ASC-01 cells were extracted from ascites of a patient affected by ovarian cancer and treated with 0, 40, 60, 80, 100 $\mu\text{g/mL}$ of rOPCML (left) and rOPCML-Fc (right) for 24 hours. Caspase 3/7 activity was analysed with Caspase-Glo assay. The data represent the mean of three technical repeats to which the error bars are referred.

The statistical significance of the reduction in ASC-01 cells was not calculated because only one experiment with three technical repeats was performed.

4.5 rOCPML increase CDDP-induced OVCAR8 and PEO4 cells viability inhibition and caspase 3/7 apoptosis induction

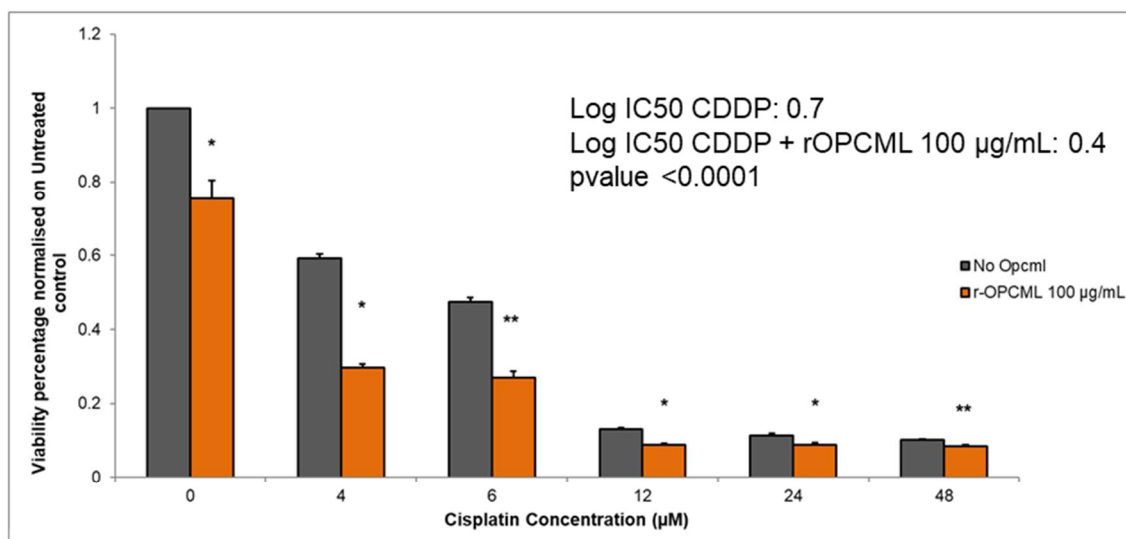
Following these results, which demonstrated that rOPCML and rOPCML-Fc reduced the viability of ovarian cancer cells and increased their caspase 3/7 activity, I questioned whether the addition of rOPCML treatment could increase CDDP's effects on viability and apoptosis in the CDDP-resistant OVCAR8 and PEO4 cells.

To achieve these aims, I tested two CDDP-resistant ovarian cancer cell lines, OVCAR8 and PEO4 cells, in the presence or absence of CDDP, rOPCML treatment, and a combination of the two in a cell viability MTT assay and a caspase 3/7 apoptotic assay.

Treatment with 100 µg/ml rOPCML almost halved ($p=0.0001$) CDDP IC₅₀ in OVCAR8 (Figure 4.5 A). No significant difference in viability was observed when PEO4 were treated with CDDP alone or in combination with 100 µg/ml rOPCML at any concentration studied (0 to 18 µM), even if a trend in reduced viability was noticed (Figure 4.5 B). Therefore, further repeats may increase the significance of these results.

A

OVCAR8



B

PEO4

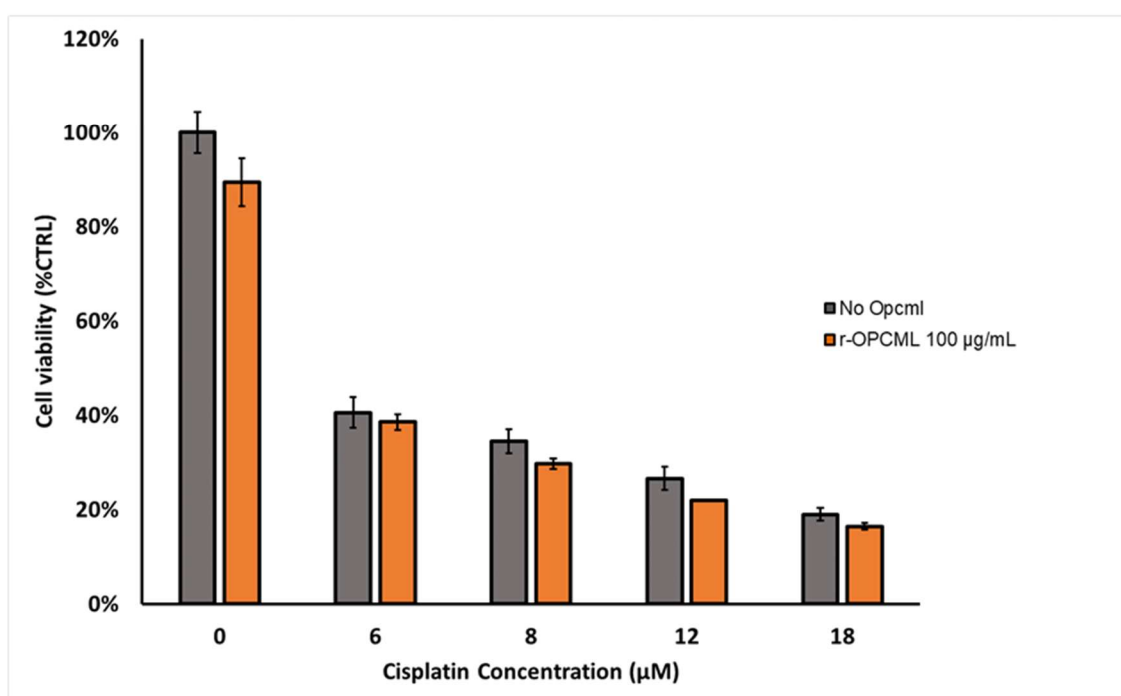


Figure 4.5: rOPCML reduces CDDP-IC₅₀ in OVCAR8 cells but not in PEO4 cells

A. OVCAR8 were treated with crescent doses of CDDP (0-18 µM) and 100µg/mL of rOPCML for 72 hours, and then cell viability was analysed with MTT assay. CDDP IC₅₀ curve with or without rOPCML was calculated on GraphPad.

B. PEO4 cells were treated with crescent doses of CDDP (0-18 µM) and 100µg/mL of rOPCML for 72 hours, and then cell viability was analysed with MTT assay.

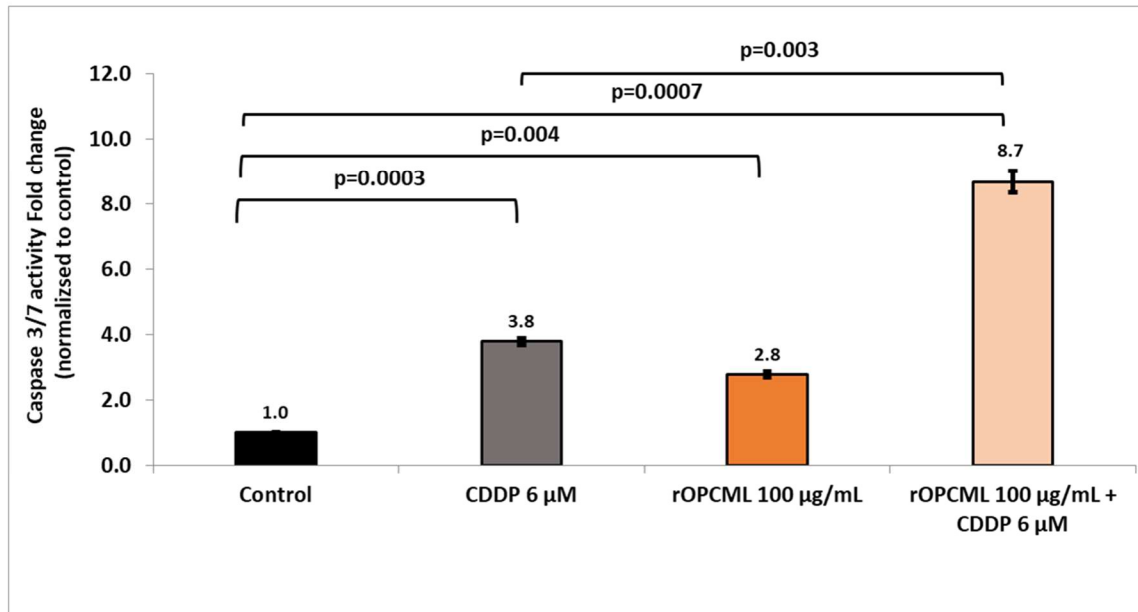
The data represent the mean and standard deviation of three independent samples normalised for the mean of the untreated control.

Student t-test was used for statistical analysis ($p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$) and error bars depict mean $\pm$ SEM*

In both cell lines OVCAR8 and PEO4, a significant increase in caspase 3/7 activity was induced by combining CDDP and rOPCML at 100 $\mu\text{g/ml}$ over rOPCML and CDDP alone or untreated control. In OVCAR8 cells, the addition of rOPCML to 6 μM CDDP significantly increased caspase 3/7 activity by 4.9-fold ($p=0.003$) compared to CDDP alone (Figure 4.6 A). In PEO4 cells, the combination of rOPCML (100 $\mu\text{g/ml}$) and CDDP significantly increased caspase 3/7, with an increase of 3.5-fold ($p=0.0149$) over CDDP alone (Figure 4.6 B).

A

OVCAR8



B

PEO4

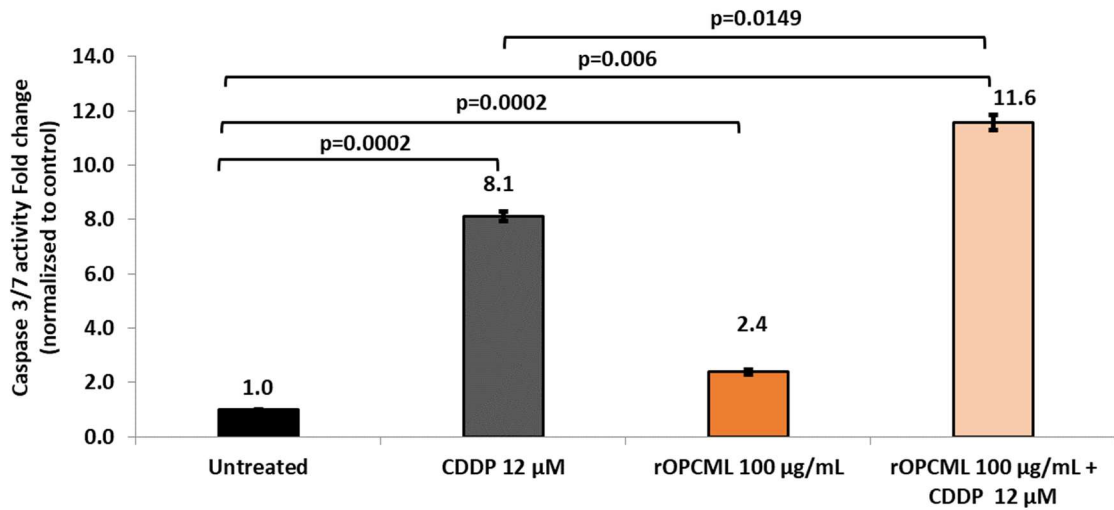


Figure 4.6: rOPCML enhances CDDP caspase 3/7 activity in OVCAR8 and PEO4 cells

OVCAR8 (A) and PEO4 (B) cells were treated with rOPCML, CDDP or a combination of the two for 24 hours, and then cell caspase 3/7 activity was analysed with Caspase-Glo assay. The data represent the mean and standard deviation of three independent experiments normalised for the mean of untreated control. Student t-test was used for statistical analysis significance defined as $p < 0.05$, and error bars depict mean $\pm$ SEM.

4.6 rOPCML and rOPCML-Fc inhibit clonogenicity in SKOV3, OVCAR8 and PEO4 cells

To further demonstrate the anti-tumour activity of rOPCML and rOPCML-Fc, I tested their effect on ovarian cancer cell survival and colony formation ability through the clonogenicity assay.

SKOV3 and OVCAR8 cells were treated for 7 days with 40 µg/ml of rOPCML and rOPCML-Fc and subsequently stained with 0.4% crystal violet to better detect colonies.

Treatment with both rOPCML and rOPCML-Fc significantly reduced colony formation by 4-fold ($p=0.03$; $p=0.01$, respectively) in SKOV3 and by 2 ($p=0.007$) and 3-fold ($p=0.008$) in OVCAR8 compared to the untreated control (Figure 4.7 A&B).

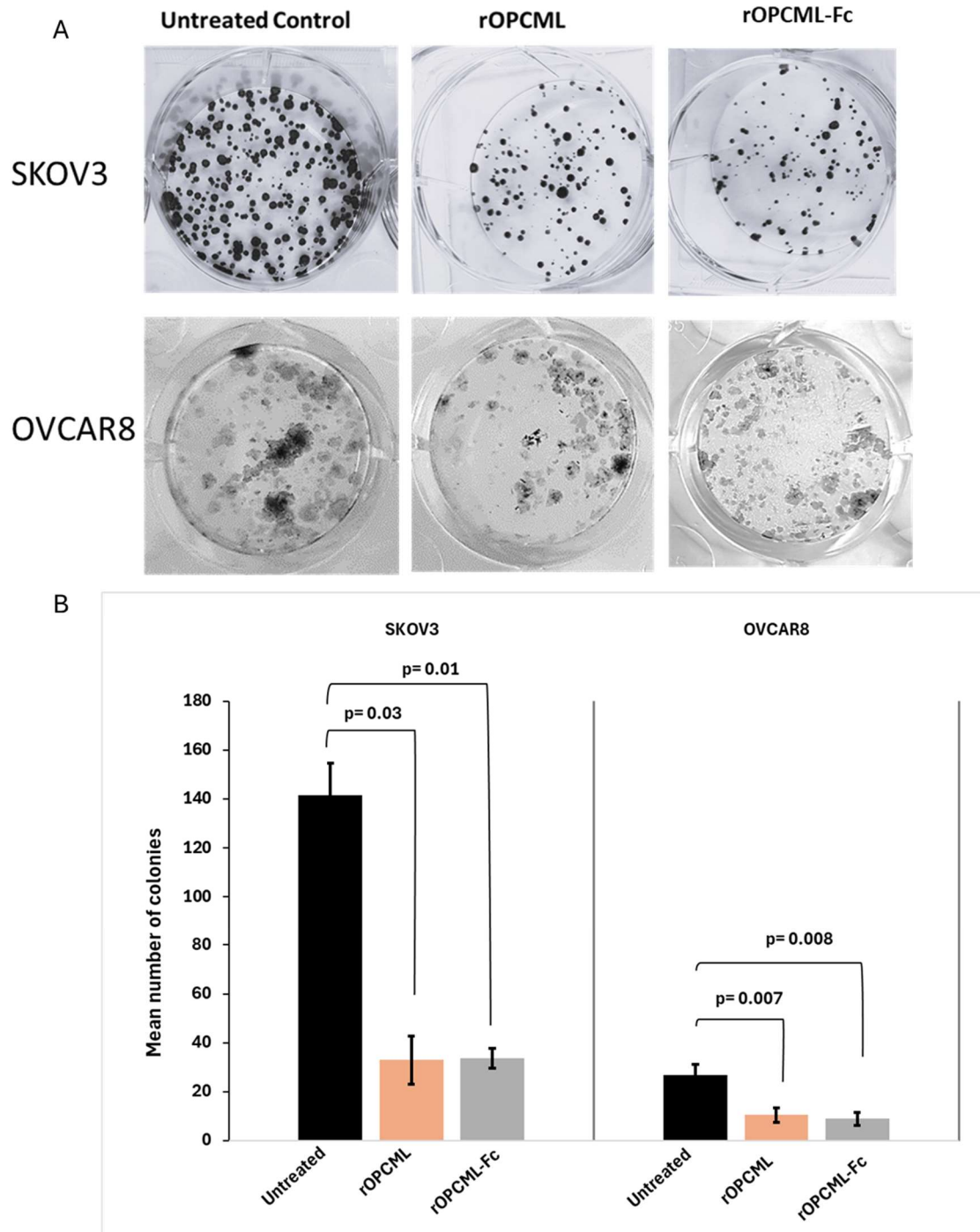


Figure 4.7: rOPCML and rOPCML-Fc inhibit SKOV3 and OVCAR8 clonogenicity:

Representative image of $N=3$ independent experiments of SKOV3 and OVCAR8 colony formation assay (A) and quantitation of the SKOV3 and OVCAR8 cell colonies in the presence or absence of rOPCML +/- Fc treatment (B). The data are expressed as mean ($N=3$) $\pm$ SEM of three independent experiments. Student *t*-test (significance defined as $p < 0.05$) was used for statistical analysis.

Treatment with rOPCML and rOPCML-Fc reduced PEO4 colony formation capability in a dose-dependent manner; however, the results were not statistically significant. At 40 $\mu\text{g/ml}$, rOPCML and rOPCML-Fc reduced colony number by 2-fold compared with the untreated control. At 100 $\mu\text{g/ml}$, both recombinants reduced the colony number 3-fold of the control (Figure 4.8 A&B).

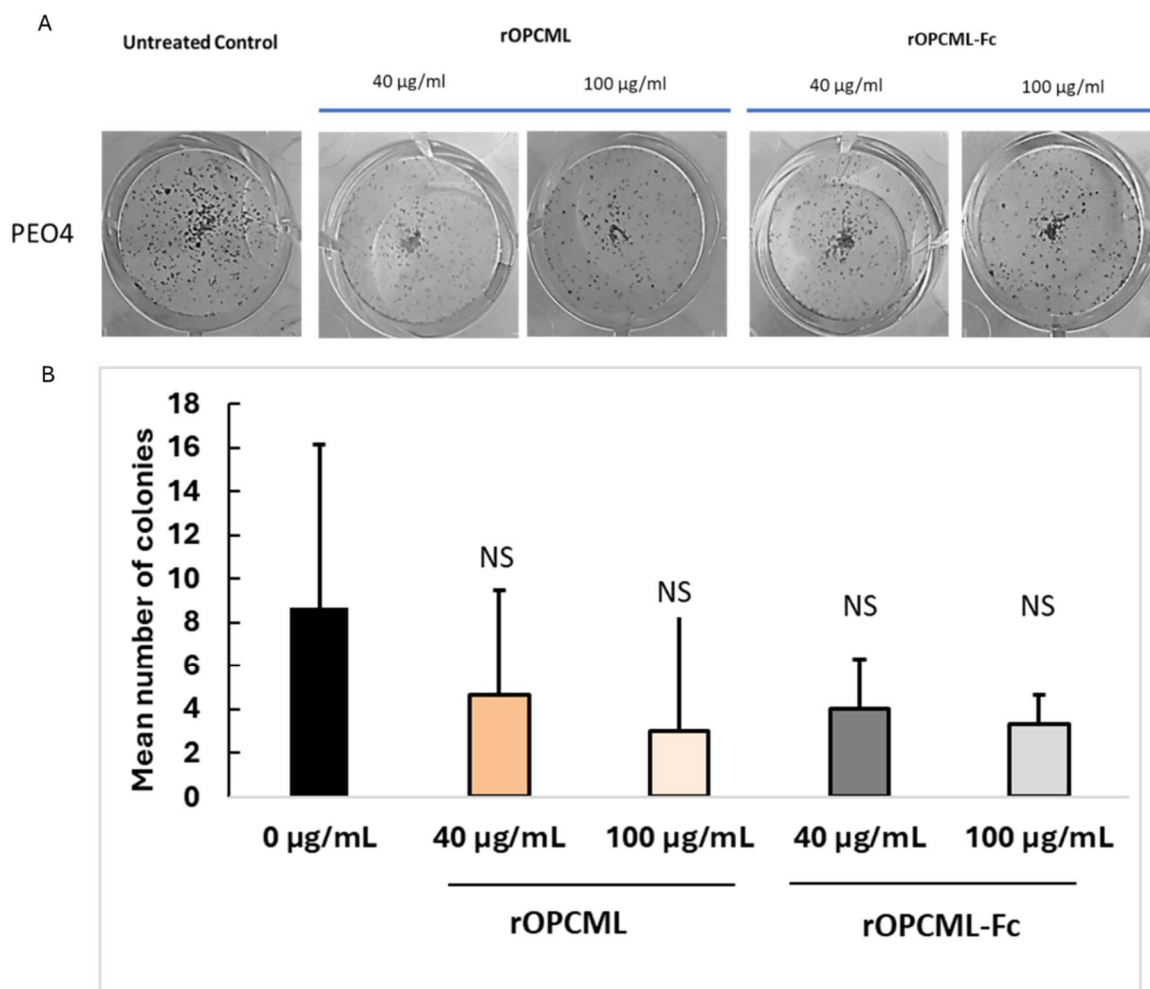


Figure 4.8: rOPCML and rOPCML-Fc reduce PEO4 clonogenicity activity

Representative image of $N=3$ independent PEO4 colony formation assay (A) experiments and quantitation of the PEO4 cell colonies in the presence or absence of rOPCML +/- Fc treatment at 40 and 100 $\mu\text{g/ml}$ (B). The data are expressed as mean ($N=3$) $\pm$ SEM of three independent experiments. Student t-test was used for statistical analysis (significance defined as $p < 0.05$).

4.7 rOPCML and rOPCML-Fc inhibit migration and invasion of SKOV3 and OVCAR8 cells

Ovarian cancer aggressiveness is linked to the dissemination of primary tumours as single cells or clusters that migrate to, adhere to, and invade the peritoneal organs and walls. Therefore, I evaluated the effects of rOPCML and rOPCML-Fc on ovarian cancer cell migration and invasive capacity through Incucyte® scratch wound assay and the 3D-Spheroid invasion assay in Matrigel®.

The Incucyte® scratch wound assay showed that rOPCML and rOPCML-Fc significantly inhibit SKOV3 and OVCAR8 cell migration ability.

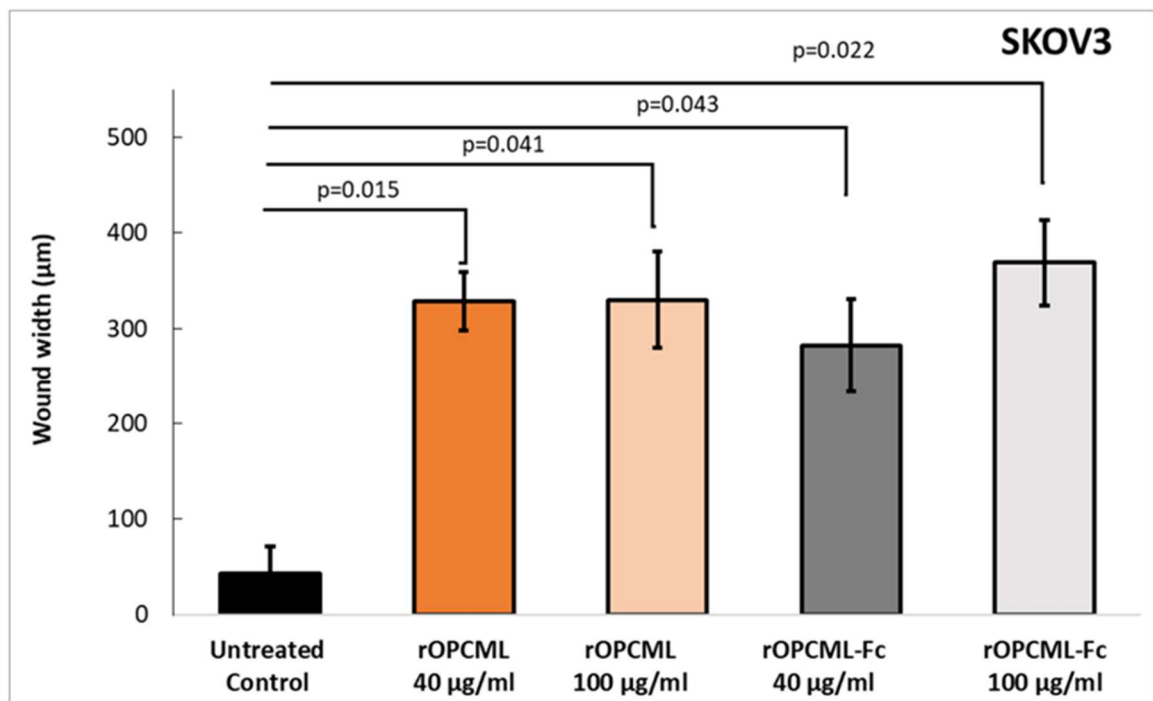
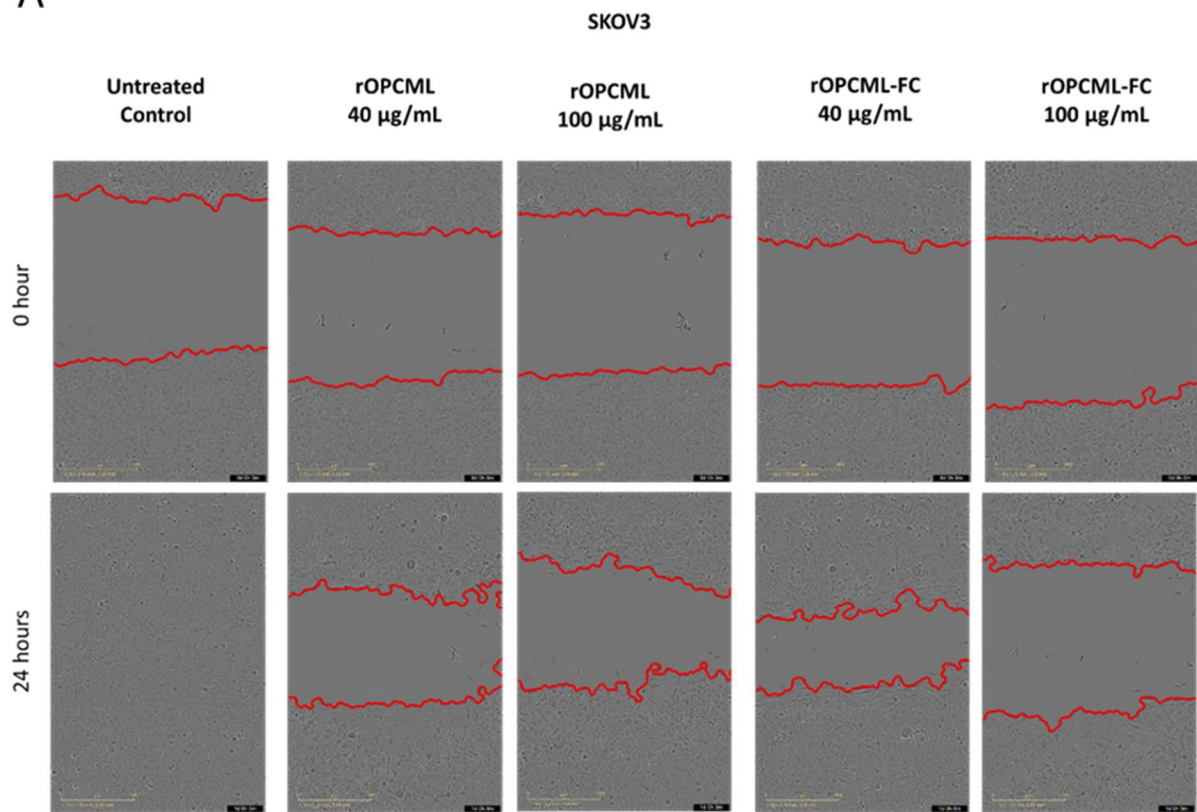
In these experiments, wound widths of untreated and treated SKOV3 and OVCAR8 were compared after 24h of rOPCML and rOPCML-Fc treatment at 40 and 100 µg/mL (Figure 4.9).

Treatment with 40 µg/mL of rOPCML significantly reduced SKOV3 migration by 7.6-fold ($p=0.015$) of the control; however, further increase of the dose to 100 µg/mL did not show a further decrease in migration ability being the reduction achieved with 100 µg/mL of rOPMCL equally of 7.6-fold ($p=0.04$) of the control (Figure 4.9 A). The same trend was observed in OVCAR8, in which both doses (40 and 100 µg/mL) significantly reduced cell migration by 16.4-fold of the control with a p-value of 0.041 and 0.0002, respectively (Figure 4.9 B).

Conversely, the inhibition of SKOV3 and OVCAR8 migration induced by rOPCML-Fc treatment was dose-dependent. In SKOV3, migration was reduced by 6.5 ($p=0.04$) and 8.5-fold ($p=0.02$) of the control after treatment with 40 and 100 µg/mL of rOPCML-Fc, respectively (Figure 4.9 A). OVCAR8 migration was reduced by 7.0 and 20.1-fold ($p=0.03$) of the

control after treatment with 40 and 100 $\mu\text{g/mL}$ of rOPCML-Fc (Figure 4.9 B).

A



B

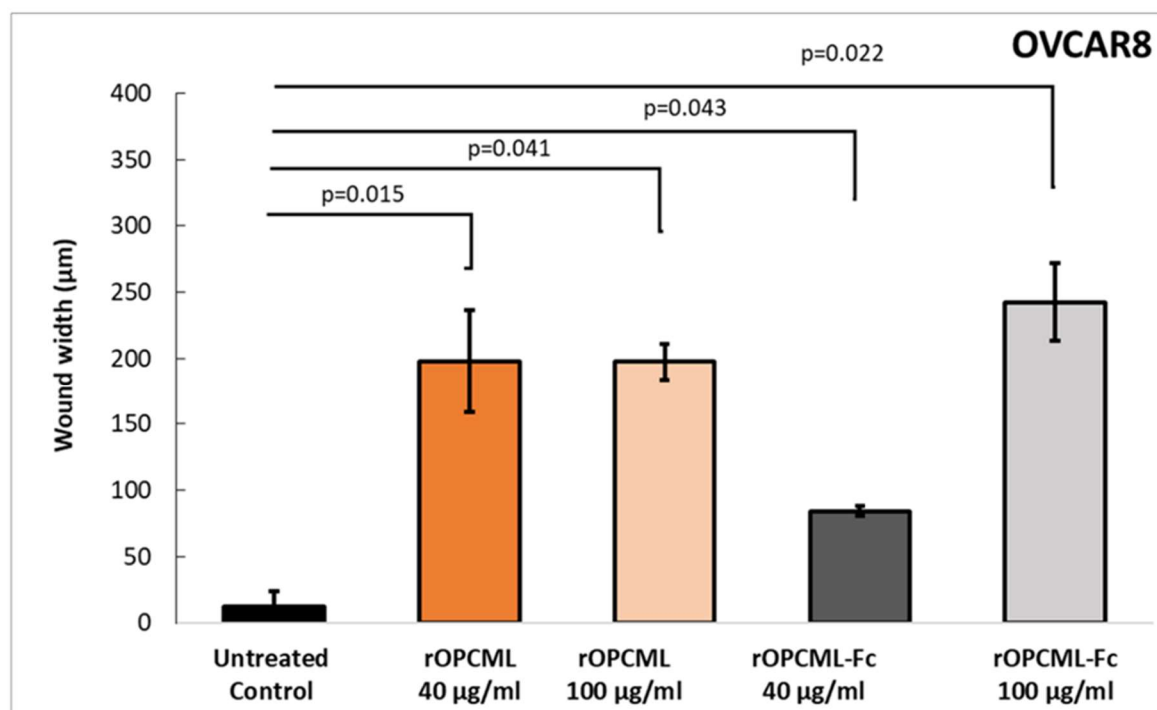
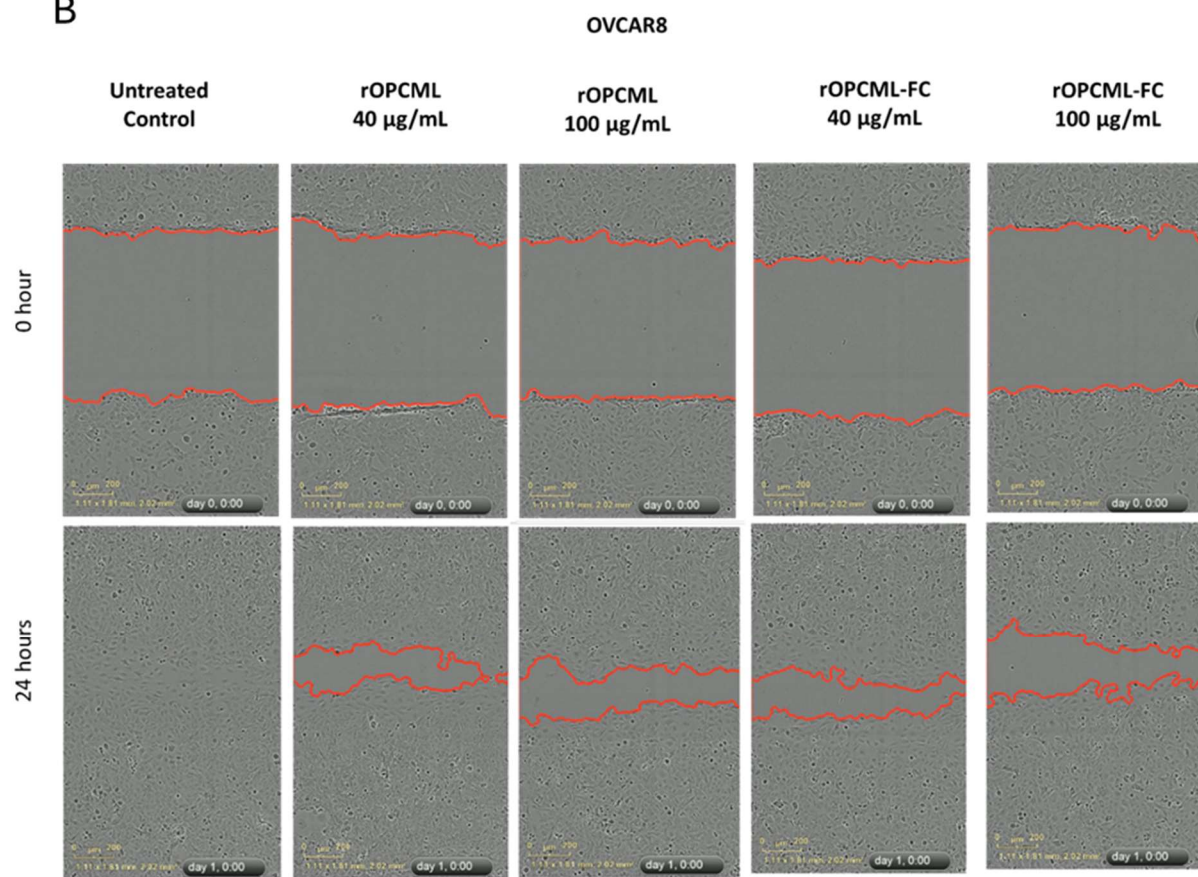


Figure 4.9: rOPCML and rOPCML-Fc reduce SKOV3 and OVCAR8 migration:

Representative image of N=3 independent experiments of SKOV3 (A) and OVCAR8 (B) cells migration assessed by Incucyte® Scratch Wound Assay and quantitation of wound width in the presence or absence of rOPCML +/- Fc treatment at 40 and 100 µg/ml. The data are expressed as mean (N=3) ± SEM of three independent experiments. Student t-test (significance defined as $p < 0.05$) was used for statistical analysis.

To further assess the effects of rOPCML and rOPCML-Fc on ovarian cancer cells, I used a 3D-spheroid invasion assay in Matrigel® to evaluate whether the rOPCML and rOPCML-Fc could affect cancer cells' invasion.

After 120 hours of treatment with 40 µg/mL of rOPCML and rOPCML-Fc, SKOV3 cell invasion in Matrigel® was significantly reduced by 67% ($p=0.05$) and 68% ($p=0.0001$) compared to the untreated control, respectively (Figure 4.10).

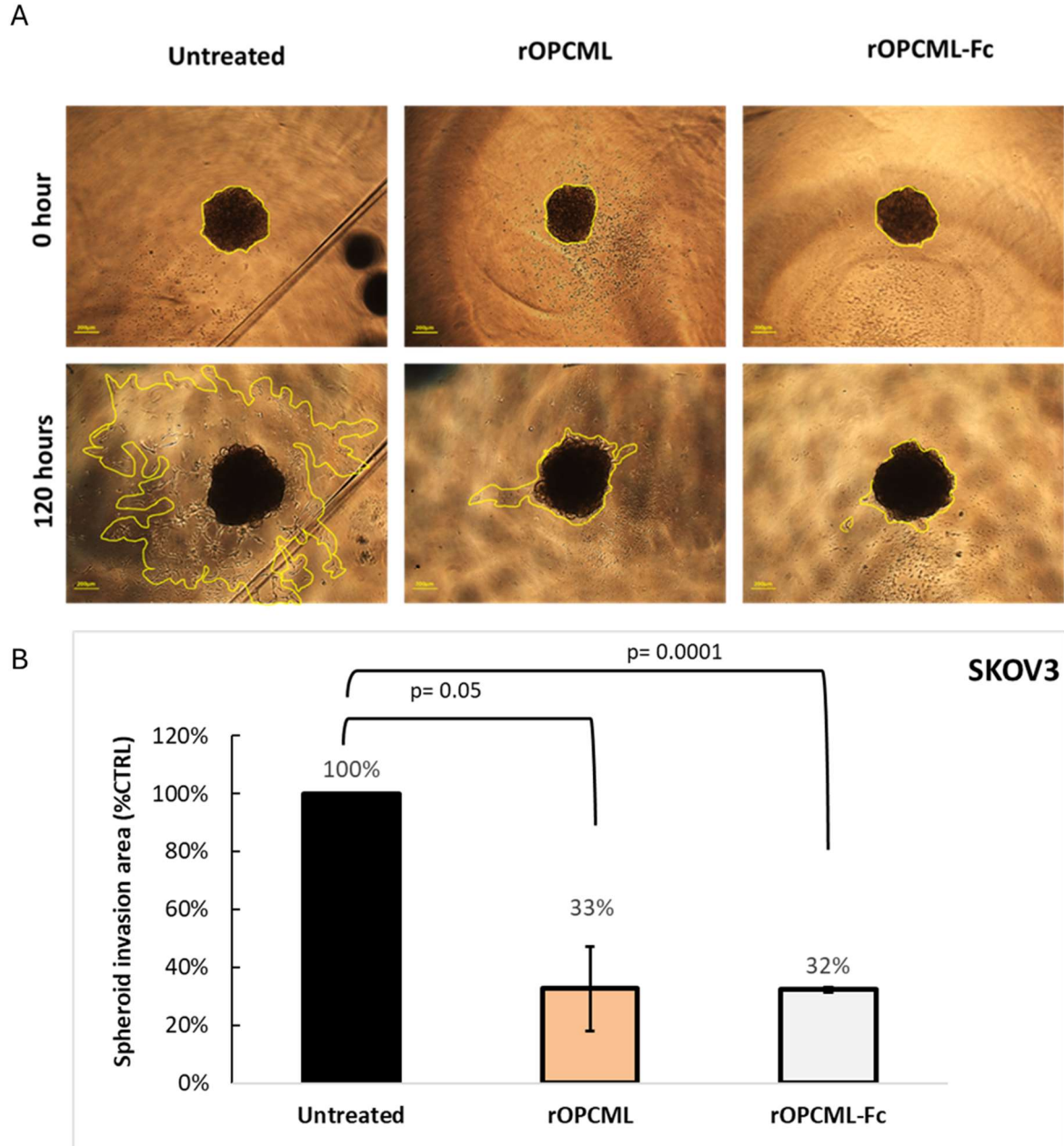


Figure 4.10: rOPCML and rOPCML-Fc suppress SKOV3 spheroids invasion in Matrigel®

Representative images of 3D spheroid invasion assay from SKOV3 (A) cells embedded in Matrigel® and treated with 40 µg/mL of rOPCML +/- Fc. Quantification of invasion relative to untreated control at 120 hours (B).

The data are expressed as the mean of three independent experiments. Student t-test was used for statistical analysis (significance defined as $p < 0.05$), and error bars depict mean $\pm$ SEM.

Similarly, rOPCML and rOPCML-Fc demonstrated a significant reduction in OVCAR8 invasion to 41% ($p=0.002$) and 68% ($p=0.033$) of the control when treated respectively (Figure 4.11).

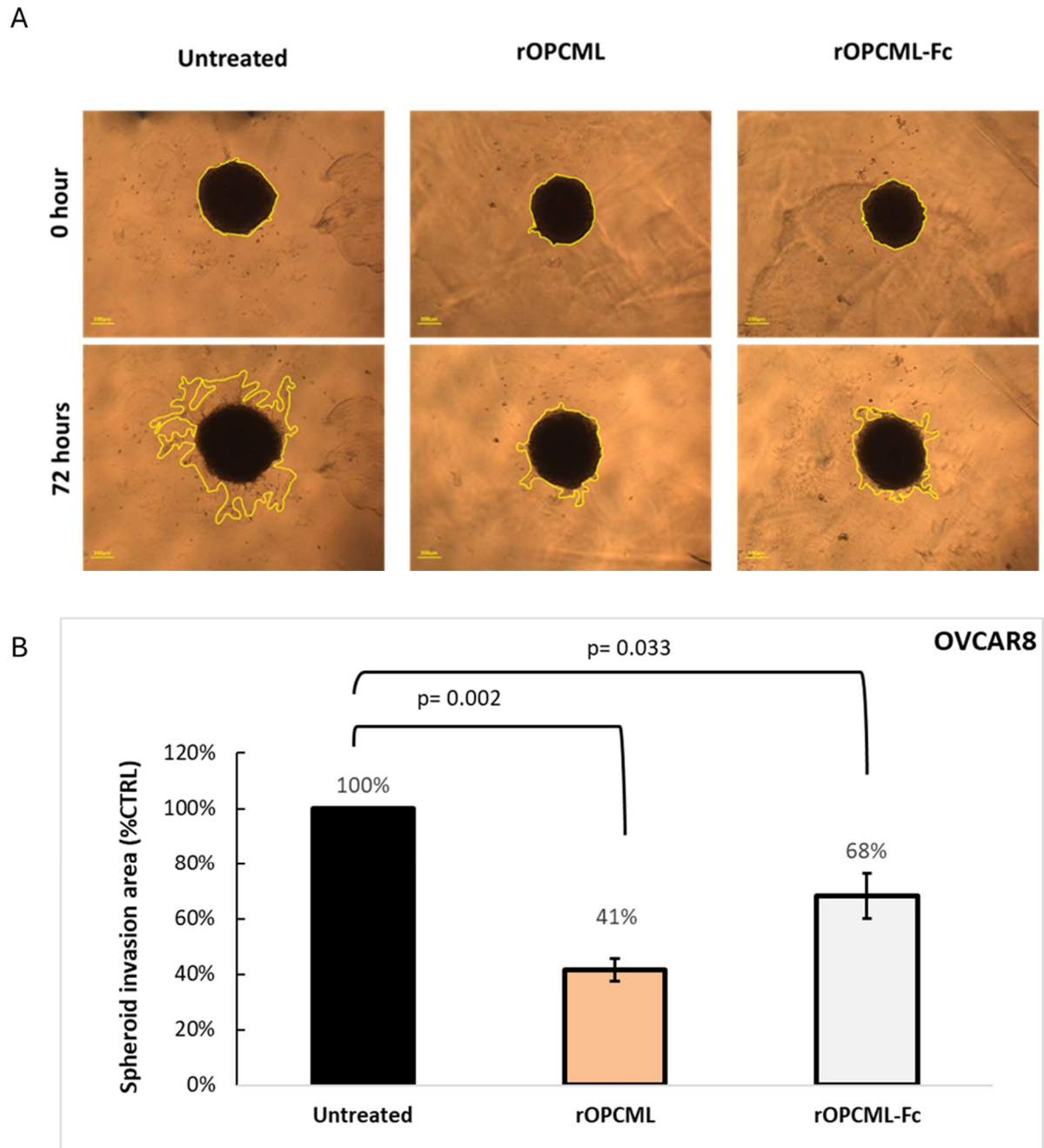


Figure 4.11: rOPCML and rOPCML-Fc reduce OVCAR8 spheroid invasion in Matrigel®: Representative images of 3D spheroid invasion assay from OVCAR8 (A) cells embedded in Matrigel® and treated with rOPCML +/- Fc at 40 $\mu\text{g}/\text{mL}$. Quantification of invasion reduction relative to untreated control at 72 hours (B).

The data are expressed as the mean of three independent experiments. Student t-test was used for statistical analysis (significance defined as $p < 0.05$), and error bars depict mean $\pm$ SEM.

4.8 rOPCML-mut, different from rOPCML and rOPCML-Fc, are unable to impair clonogenicity and invasion of SKOV3 and OVCAR8 cells

To further validate the rOPCML and rOPCML-Fc treatment specificity, I tested rOPCML+/- Fc versus rOPCML-mut effects in the clonogenic assay. SKOV3 and OVCAR8 cells were treated with 40 μ g/ml of rOPCML, rOPCML-Fc, and rOPCML-R65L and subsequently stained with 0.4% crystal violet to better detect colonies.

For these experiments, I only used rOPCML-R65L as rOPCML-mut because I did not have the same quantity of aliquots of each rOPCML-mut, and this did not allow me to perform all the experiments with all the mutants. As demonstrated in previous experiments, treatment with rOPCML and rOPCML-Fc significantly reduced colony formation in SKOV3 and OVCAR8 compared to the untreated control. Also, treatment with rOPCML-R65L, compared to the control, significantly reduced colony formation by 2.2-fold in SKOV3, whereas the reduction in OVCAR8 was not significant and less marked (1.3-fold; $p=0.1$). However, the reduction achieved by rOPCML-R65L was significantly lower than that achieved by rOPCML and rOPCML-Fc in both cell lines (Figure 4.12).

The mean number of SKOV3 cell colonies upon treatment with 40 μ g/ml of rOPCML-R65L was almost double the number of colonies formed upon treatment with rOPCML ($p=0.05$) and rOPCML-Fc ($p=0.05$) at the same dose. Similarly, OVCAR8 cells formed a significantly higher number of colonies upon rOPCML-R65L treatment than after rOPCML and rOPCML-

Fc treatment at 40 $\mu\text{g/ml}$, by respectively 1.9 times ($p=0.02$) and 2.2 times ($p=0.006$) (Figure 4.12 A&B).

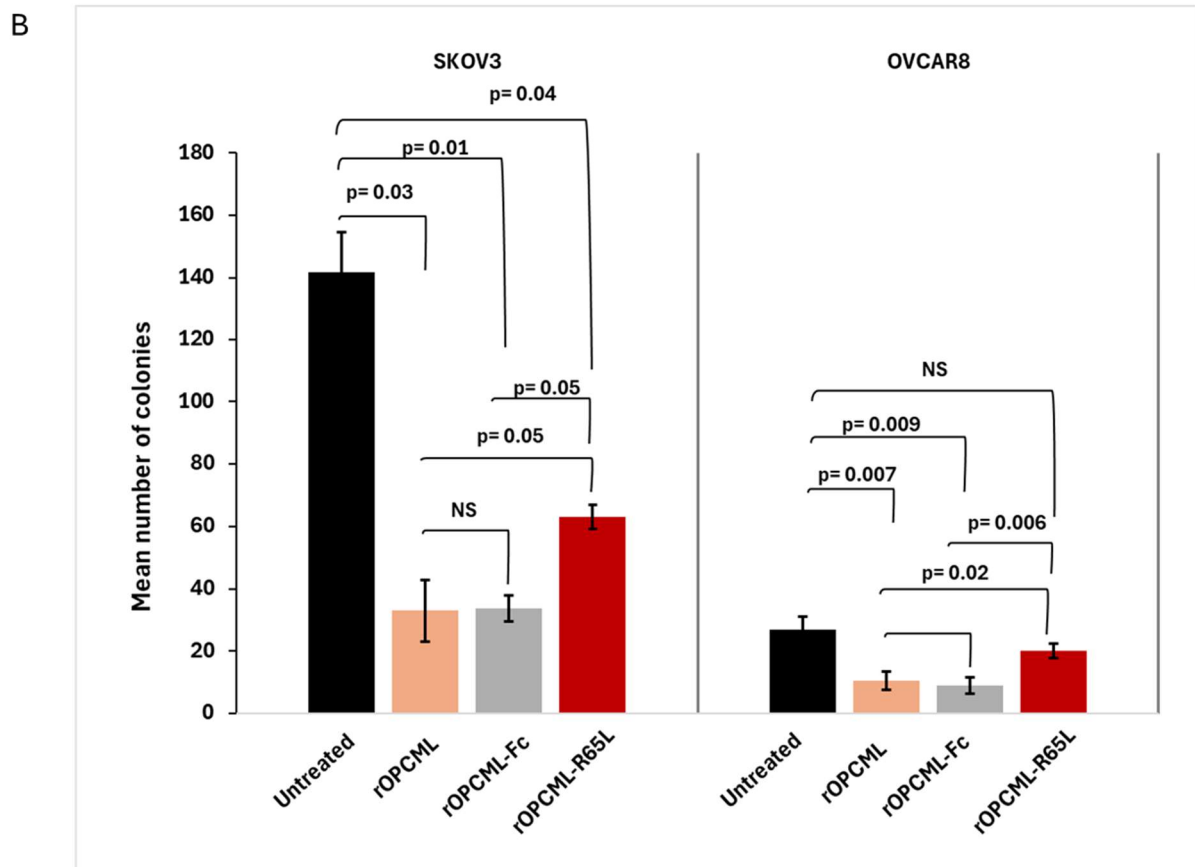
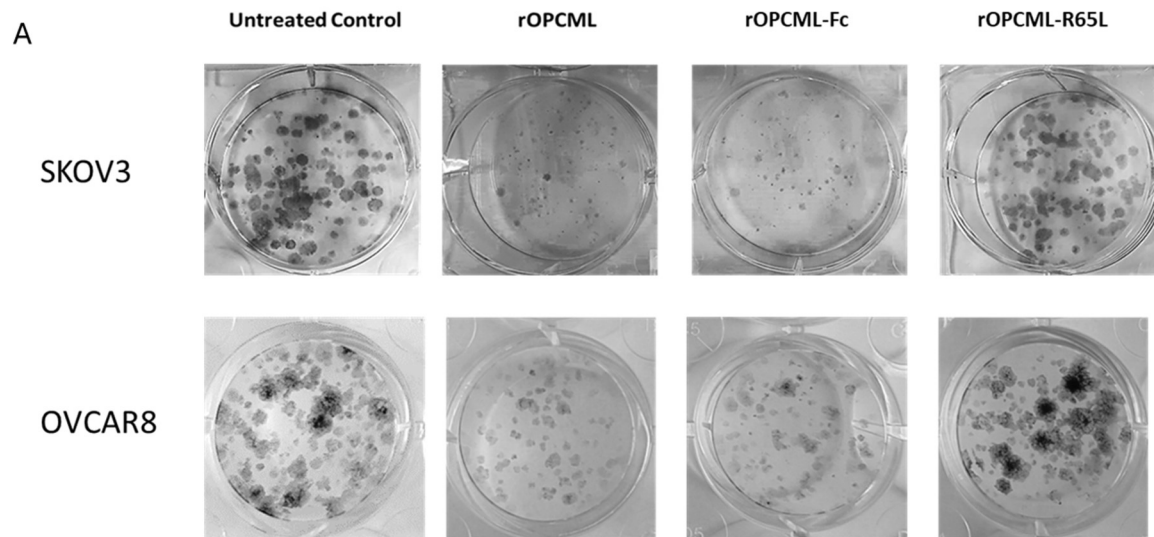


Figure 4.12: rOPCML-mut is less effective than rOPCML and rOPCML-Fc in inhibiting SKOV3 and OVCAR8 clonogenicity:

A. *Representative image of N=3 independent experiments of SKOV3 (top) and OVCAR8 (bottom) colony formation assay in the presence or absence of rOPCML +/- Fc and rOPCML-R65L treatment at 40 µg/mL*

B. *Quantitation of the SKOV3 and OVCAR8 cell colonies in the presence or absence of rOPCML +/- Fc and rOPCML-R65L treatment. The data are expressed as mean (N=3) ± SEM of three independent experiments. Student t-test (significance defined as $p < 0.05$) was used for statistical analysis.*

Then, to prove that the inhibition of invasion was due to rOPCML and rOPCML-Fc treatment, I assessed the rOPCML, rOPCML-Fc, rOPCML-R65L, rOPCML-N70H, and rOPCML-P95R effects on SKOV3 cell invasion using a 3D-spheroid invasion assay in Matrigel®.

SKOV3 cells were treated with 40 µg/mL of rOPCML, rOPCML-Fc, rOPCML-R65L, rOPCML-N70H, rOPCML-P95R. Treatment with rOPCML and rOPCML-Fc significantly reduced SKOV3 invasion in Matrigel® by 67% ($p = 0.05$) and 68% ($p = 0.0001$), respectively; instead, treatment with rOPCML-R65L increased SKOV3 invasion in Matrigel®, whereas rOPCML-N70H and rOPCML-P95R reduce SKOV3 invasion in Matrigel® by 2% and 5% respectively with no significant differences to untreated control and rOPCML. Statistical differences were instead observed between rOPCML-Fc reduction of 3D-Spheroid invasion compared with the effect of rOPCML-R65L ($p = 0.02$), rOPCML-N70H ($p = 0.01$) and rOPCML-P95R ($p = 0.02$) on the same assay (Figure 4.13 A&B).

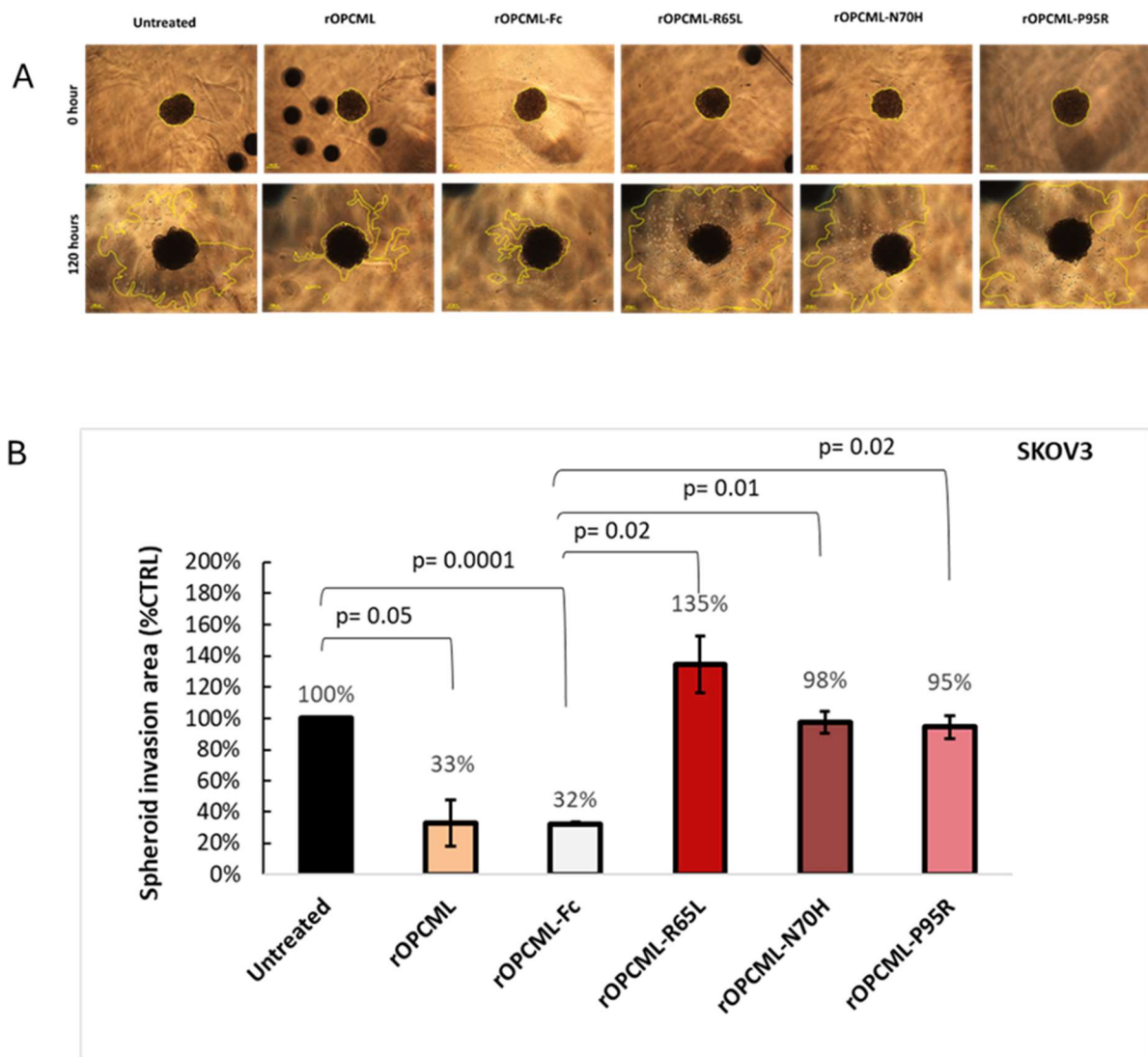


Figure 4.13: rOPCML-mut impairs the rOPCML and rOPCML-Fc ability to suppress SKOV3 spheroids invasion in Matrigel®:

A. Representative images of 3D spheroid invasion assay from SKOV3 (top) cells embedded in Matrigel® and treated with 40 µg/mL of rOPCML +/- Fc or rOPCMLmut (R65L; N70H; P95R). Quantification of invasion relative to untreated control at 120 hours.

B. The data are expressed as the mean of three independent biological repeats. Student t-test (significance defined as $p \leq 0.05$) was used for statistical analysis, and error bars depict mean $\pm$ SEM

4.9 rOPCML inhibits PC9/ER lung cancer cell viability and increases the effects of osimertinib

To further demonstrate the effects of rOPCML on cancer cell viability and apoptosis, I tested rOPCML on a different type of cancer cell: the PC9/ER lung cancer cells. These cells were chosen because they strongly depend on growth by the overactivation of many RTKs.

I treated PC9/ER cells with exponentially increasing doses of rOPCML (from 0 to 300 µg/ml) and assessed cell viability with the MTT assay. I observed that rOPCML inhibits PC9/ER cell viability in a dose-dependent manner, starting with a reduction by 3% of the control with 1.8 µg/ml and reaching a significant inhibition by 21 (p=0.017) and 22% (p=0.006) upon treatment with 150 µg/ml and 300 µg/ml of rOPCML (Figure 4.14 A).

Based on these results, I used the rOPCML dose of 150 µg/ml to evaluate its effects in combination with Osimertinib, a TKI broadly used in erlotinib-resistant lung cancer treatment.

rOPCML at 150 µg/ml was added to exponentially increasing doses of Osimertinib in an MTT viability assay. 150 µg/ml of rOPCML significantly reduced PC9/ER cell viability and significantly enhanced the reduction in viability in response to Osimertinib at every dose of Osimertinib tested. The decrease in viability went from an 18% (p=0.01) reduction of the control when PC9/ER cells were treated with only rOPCML to 36% (p=0.04) of the Osimertinib-treated control when 150 µg/ml of rOPCML were added to (Figure 4.14 B).

Then, treatment with rOPCML (150 µg/ml) was also tested alone or in combination with Osimertinib (30 nM) to examine their effects in a caspase 3/7 assay. rOPCML (150 µg/ml) alone resulted in a significant 2-fold increase (p=0.026) of PC9/ER cells apoptosis over the control and

significantly enhanced the Osimertinib (30 nM) induced apoptosis ($p=0.01$) (Figure 4.14 C).

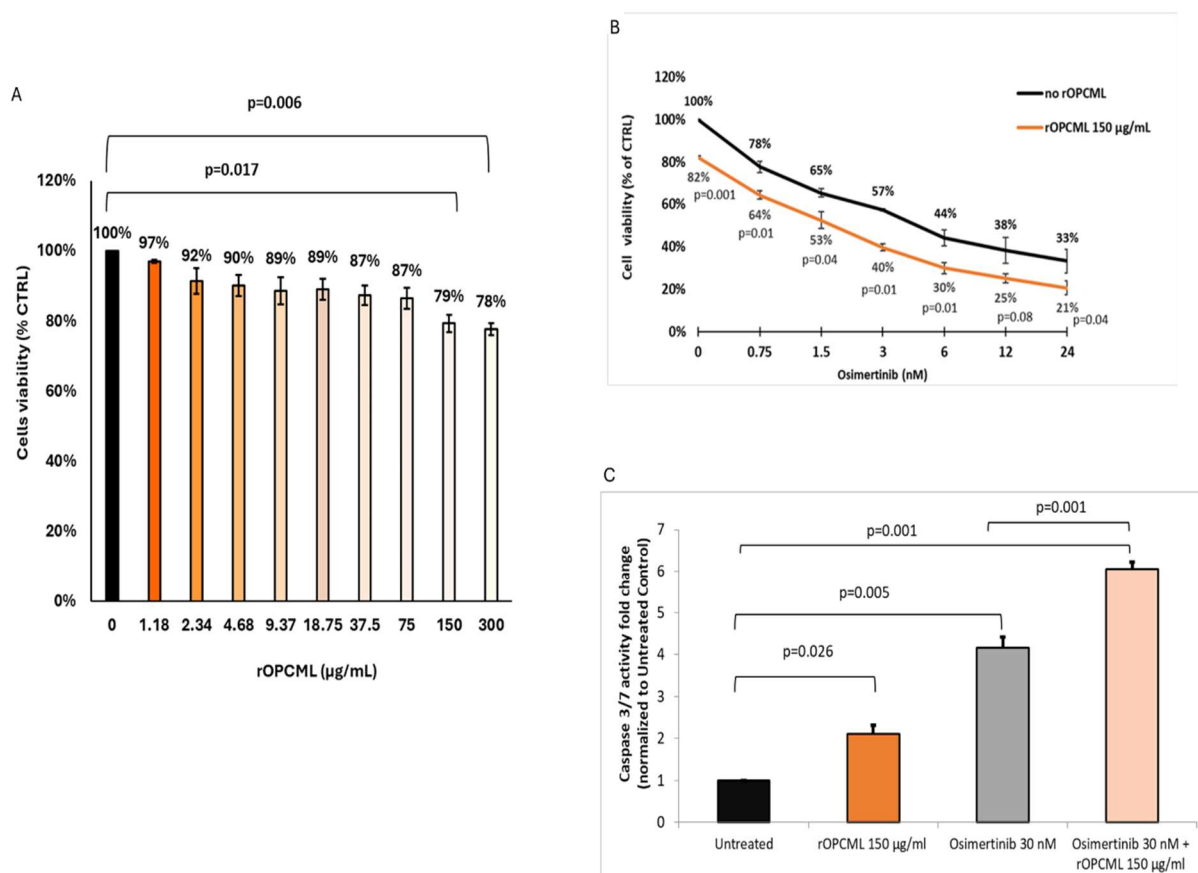


Figure 4.14: rOPCML inhibits PC9/ER lung cancer cell viability and increases the effects of osimertinib:

A. PC9/ER cells viability assessed by MTT under exponentially increasing doses of rOPCML (from 0 to 300 $\mu\text{g/mL}$) treatment. The data represent the mean and standard deviation of three independent experiments normalised for the mean of untreated control. Student *t*-test (significance defined as $p < 0.05$) were used for statistical analysis, and error bars depict mean $\pm$ SEM.

B. PC9/ER cell viability assessed by MTT viability assay under treatment with exponentially increasing doses of Osimertinib alone or in combination with rOPCML (150 $\mu\text{g/mL}$).

C. PC9/ER cells apoptosis assessed by Caspase-Glo assay upon treatment with rOPCML (150 $\mu\text{g/mL}$), Osimertinib (30 nM) or a combination of the two.

The data in A and B represent the mean and standard deviation of three independent experiments normalised for the mean of untreated control. Student *t*-test (significance defined as $p < 0.05$)

were used for statistical analysis, and error bars depict mean $\pm$ SEM. PBS was used for the untreated control.

4.10 Summary

To confirm that rOPCML and rOPCML-Fc produced in HEK293 cells mirror the anti-tumour activity of OPCML and r-OPCML produced in bacteria, I treated with rOPCML and rOPCML-Fc three ovarian cancer cell lines and one primary cell line derived from the ascites of an ovarian cancer patient.

I confirmed that rOPCML and rOPCML-Fc significantly reduce viability and induce caspase 3/7 activity in a dose-dependent manner in SKOV3, OVCAR8 and PEO4 cells. Furthermore, treatment with rOPCML and rOPCML-Fc significantly reduced clonogenicity in all three cell lines, as well as migration and spheroid invasion in Matrigel[®] in SKOV3 and OVCAR8 cells.

rOPCML and rOPCML-Fc were also demonstrated to reduce the viability of patient-derived cells and increase their caspase 3/7 activity.

I also showed that rOPCML significantly reduces CDDP IC₅₀ in OVCAR8 and significantly increases CDDP-induced caspase 3/7 activity in the CDDP-resistant OVCAR8 and PEO4 cells.

To further confirm rOPCML and rOPCML-Fc activity, I compared rOPCML +/- Fc effects on ovarian cancer cells clonogenicity and invasiveness with those of rOPCML carrying point mutations known to reduce/abolish OPCML anti-tumour activity.

Here, I demonstrated that rOPCML-R65L is significantly less active than rOPCML and rOPCML-Fc in inhibiting SKOV3 and OVCAR8 cell clonogenicity and that rOPCML-R65L, rOPCML-N70H, and rOPCML-P95R completely impair the inhibition of SKOV3 3D spheroid invasion in Matrigel[®], which rOPCML and rOPCML-Fc achieve.

In addition, as proof of concept to broaden the use of rOPCML in other cancer types, I tested rOPCML in the erlotinib-resistant lung cancer cell line PC9/ER. In this cell line, rOPCML reduces cell viability and increases apoptosis. In addition, rOPCML augments the effects of Osimertinib on PC9/ER cell viability and apoptosis.

CHAPTER 5

PRELIMINARY DATA ON THE ROLE OF OPCML-GPI ANCHOR FOR FUTURE POTENTIAL THERAPEUTICAL IMPLICATION

5.1 Introduction

This thesis aimed to produce recombinant OPCML +/- Fc as protein therapeutics. However, OPCML can also be delivered as therapeutic to cancer cells in different ways, such as through therapeutic vesicles carrying OPCML protein or OPCML mRNA.

In the constructs used for the recombinant OPCML, OPCML was devoid of a GPI anchor to facilitate the production/collection and purification process and for *in-vivo* delivery of the protein.

From the perspective of having a different delivery system with the possibility of carrying the full-length protein and/or the mRNA for OPCML re-expression in cancer cells, I wanted to investigate if the GPI anchor may have some role in OPCML's effects on cancer cell viability, clonogenicity, and tumour spheroid formation.

Therefore, in this chapter, I transduced SKOV3 cells with full-length OPCML with GPI anchor (SKOV-OP), OPCML without the GPI anchor (SKOV-SOP), OPCML carrying R65L mutation (SKOV3-R65L), and empty-vector (SKOV3-EV), and compared them in viability-MTT assay, clonogenic and spheroids formation assays.

5.2 SKOV3 cells expressing OPCML with and without GPI-anchor have reduced cell viability and clonogenicity, and form smaller single spheroids compared to SKOV3 not expressing OPCML

To verify how the absence of the GPI-anchor acts on the effects of OPCML, I transduced SKOV3 cells with full-length OPCML with GPI anchor (SKOV-OP), OPCML without the GPI anchor (SKOV-SOP), OPCML carrying R65L mutation (SKOV3-R65L), and empty-vector (SKOV3-EV) and tested in different assays.

The effectiveness of transduction in SKOV3 cells was confirmed by WB (Figure 5.1).

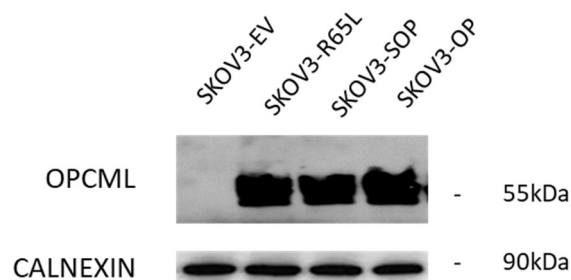


Figure 5.1: WB OPCML expression in SKOV3 cells transduced with different OPCML constructs

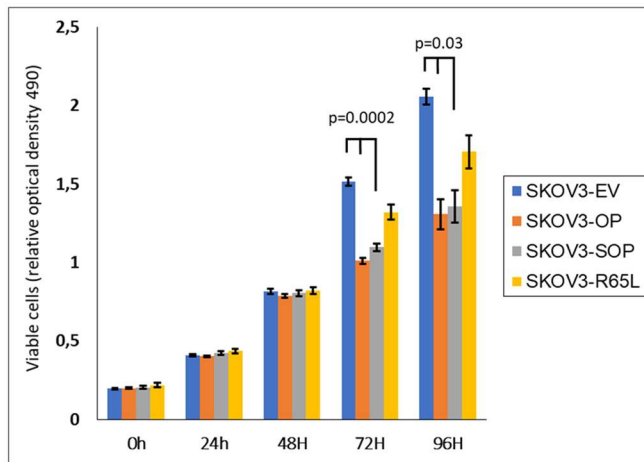
WB analysis for OPCML expression in SKOV3 cells transduced with empty vector (EV), OPCML carrying R65L mutation (R65L), OPCML without GPI-anchor (SOP), OPCML with GPI-anchor (OP)

Subsequently, cells were seeded to assess the viability with an MTT assay. SKOV3 cell lines expressing OPCML with and without GPI anchor had a significantly reduced cell viability by 33% ($p=0.0002$) and 28% ($p=0.0002$)

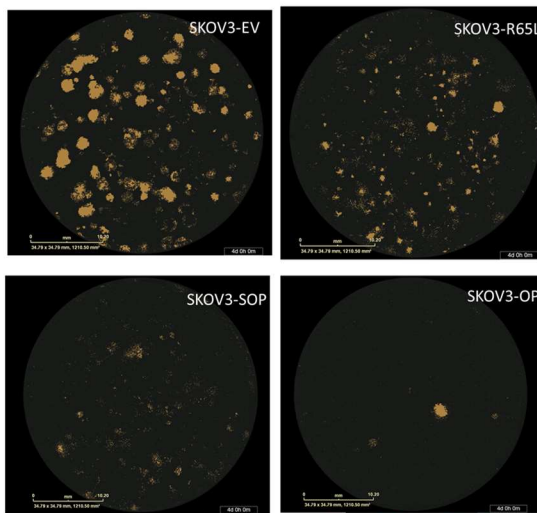
at 72 hours, and by 36% ($p=0.03$) and 34% ($p=0.03$) at 96 hours compared to the SKOV-EV. Conversely, SKOV3-R65L cells had a reduced viability compared to SKOV3-EV, but the reduction was not significant (Figure 5.2 A).

A significant reduction was also noticed in the clonogenicity of SKOV3-OP and SKOV3-SOP compared to SKOV3-EV, which corresponded to a reduction by 97% ($p=0.003$) and 87% ($p=0.0002$) in colonies number, respectively. Again, there were no statistically significant differences between SKOV3-OP and SKOV3-SOP cells. Although not significant, SKOV3-R65L cells showed reduced clonogenic activity compared to SKOV3-EV cells and higher clonogenic activity compared to SKOV3-OP and SKOV3-SOP cells (Figure 5.2 B-D).

A



B



C

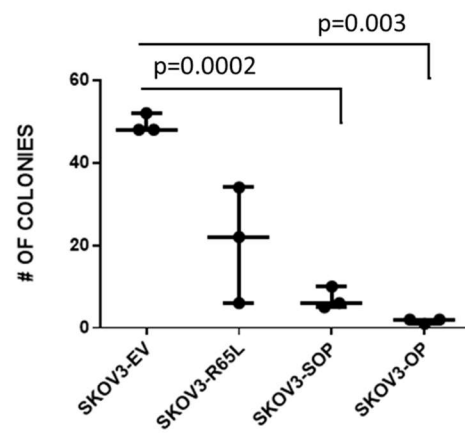
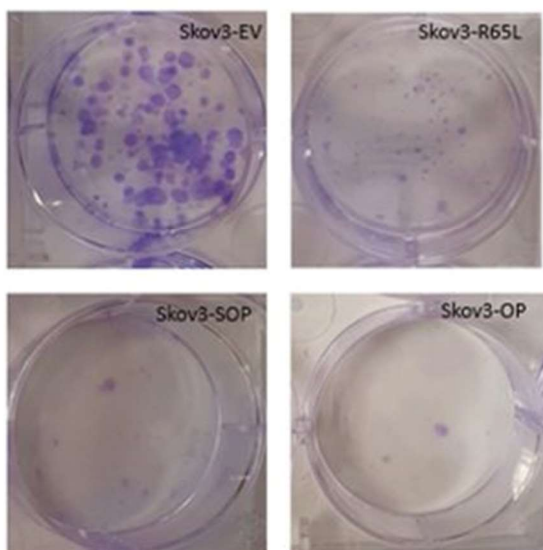


Figure 5.2: SKOV3 cells expressing OPCML with and without GPI-anchor have a significantly reduced cell viability and clonogenicity activity compared to SKOV3-EV

A. Viability of SKOV3-EV, SKOV3-OP, SKOV3-SOP, and SKOV3-R65L cells over time determined by an MTT assay. The graph shows the absorbance of MTT reagent at 490 nm; the results are representative of three independent experiments (N=3). Data are presented as a mean and standard error of the mean (SEM, error bars); the significance of the differences was determined by two-way ANOVA analysis using Prism 9 with Bonferroni correction.

B. Clonogenic assay of SKOV3-EV, SKOV3-OP, SKOV3-SOP, SKOV3-R65L cells. In B. images of colonies 10 days post-seeding as showed by Incucyte® mask (note: pictures were captured by day 6 post-seeding to day 10 by Incucyte®); in C. images of colonies ten days post-seeding as showed by Crystal Violet; D. Graphical quantification of n. of colonies formed by SKOV3-EV, SKOV3-OP, SKOV3-SOP, SKOV3-R65L cells in each biological-repeat. The results are representative of three independent experiments (N=3). The significance of the differences was determined by two-way ANOVA analysis using Prism 9 with Bonferroni correction.

In the single-spheroid formation assay, I showed that SKOV3-SOP and SKOV3-OP form smaller spheroids than SKOV3-EV and SKOV3-R65L cells. SKOV3-OP cells also form smaller spheroids than SKOV3-SOP cells. However, the significance of all these differences cannot be calculated because the experiment was performed once with seven technical replicates (Figure 5.3).

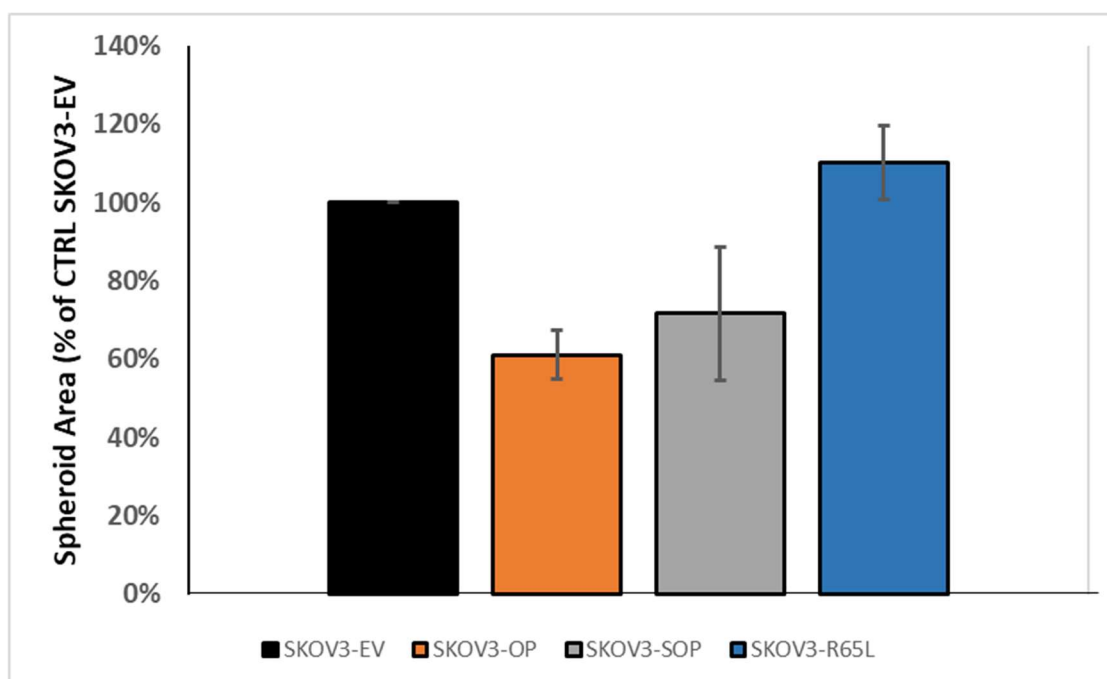
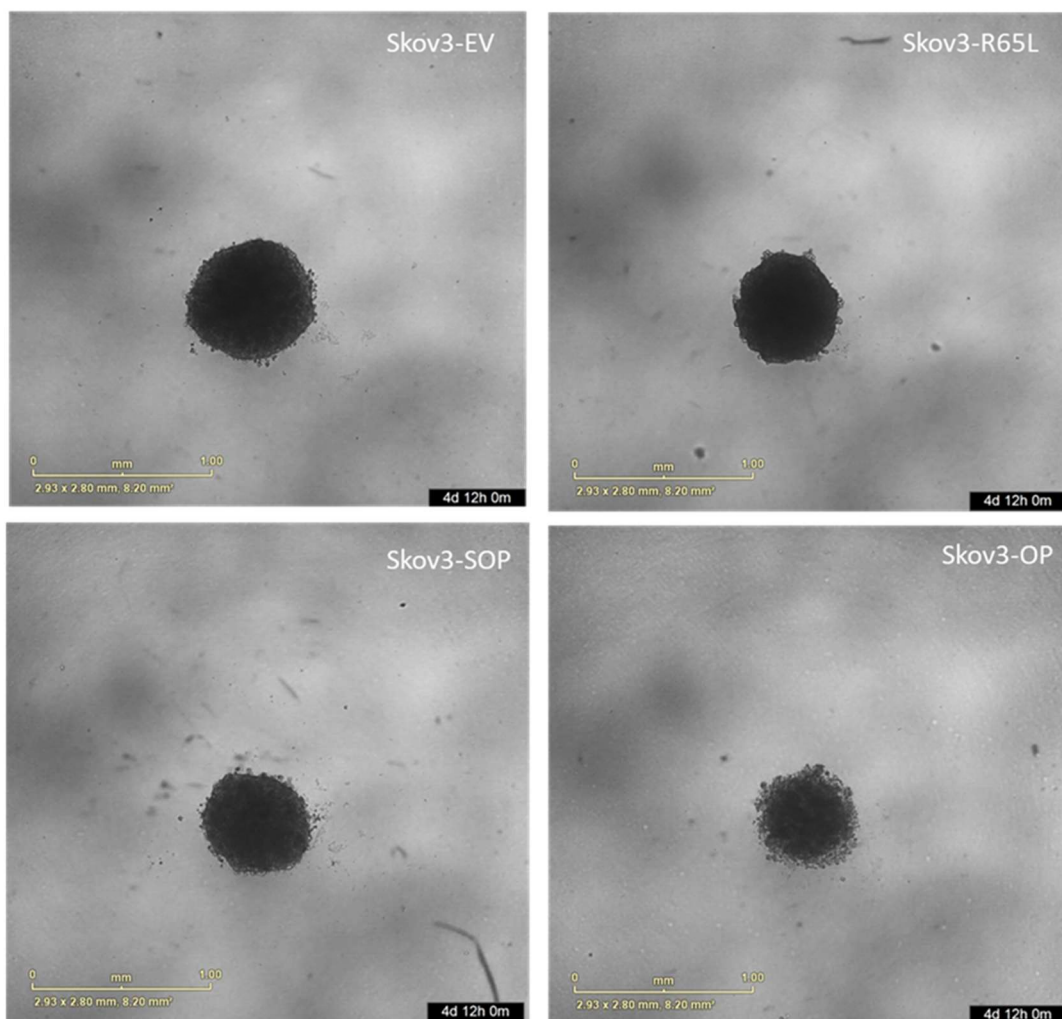


Figure 5.3: SKOV3-OP cells form smaller spheroid compared to SKOV3-EV, SKOV3-R65L, and SKOV3-SOP cells

Single spheroid formation assay for SKOV3-EV, SKOV3-OP, SKOV3-SOP, SKOV3-R65L cells (up). Graph representing the area of the spheroids normalised for the control (SKOV3-EV)(bottom). Results are representative of 7 technical repeats (N=7). Data are presented as a mean and standard error of the mean (SEM, error bars);

5.3 Summary

Here, I showed that SKOV3 transduced with OPCML expressing or not the GPI-anchor are less viable and clonogenic than SKOV3 cells that do not express OPCML; in addition, they also form smaller spheroids. No significant differences were noticed in the viability and clonogenicity ability of SKOV3 cells expressing OPCML with GPI-anchor vs. OPCML without GPI-anchor.

CHAPTER 6

DISCUSSION

6.1 Treatment with soluble rOPCML +/- Fc impairs the phosphorylation of a specific repertoire of RTKs and their downstream signalling, reiterating the phenotype of OPCML ectopically re-expressed in ovarian cancer cell lines.

Despite the development of new therapeutics for EOC treatment, the high mortality associated with this disease remains a pressing issue. Therefore, there is an urgent need for new therapeutics.

The most studied RTKs in EOC are EGFR and HER2, MET, AXL, and VEGFRs. In EOC, overexpression of all these RTKs, except HER2, correlates with poor clinical outcomes (Skirnisdóttir et al., 2001; Sun et al., 2004; Ayhan et al., 2005; Sawada et al., 2007; Lafky et al., 2008; Bamias et al., 2012; Tas et al., 2014; Reyes et al., 2014; Antony et al., 2016). The prognostic role of HER2 in EOC remains controversial (Lassus et al., 2004; Nielsen et al., 2004; Verri et al., 2005; Raspollini et al., 2006; Tuefferd et al., 2007; Zhao et al., 2013).

AXL activation, along with the activation of its downstream pathways, the PI3K/AKT and MAPK/ERK pathway, is associated with cancer cell proliferation, enhancement of epithelial-mesenchymal transition and apoptosis inhibition (Sun et al., 2004; Elkabets et al., 2015; Antony et al., 2016). Conversely, AXL inhibition prevents migration, EMT and invasion and enhances the responsivity to paclitaxel and carboplatin (Sun et al., 2004;

Elkabets et al., 2015; Antony et al., 2016). Also, c-MET activation increases proliferation, invasion, and migration in EOC.

Ectopic expression of OPCML and treatment with rOPCML produced in bacteria have demonstrated, in ovarian cancer, the inhibition of phosphorylation of HER2, AXL, and the two key effectors of MAPK and PI3K pathways, ERK and AKT. This inhibition resulted in tumour suppressive activity (McKie et al., 2012; Zanini et al., 2017; Antony et al., 2018; Birtley et al., 2019).

I conducted a series of proteomic assays to assess whether the soluble rOPCML+/- Fc I produced has the same effects on RTKs and their downstream pathways as OPCML. The results showed that the soluble rOPCML+/- Fc maintains the same inhibitory activity on the phosphorylation of RTKs and their downstream pathways as when SKOV3 cells were transduced with OPCML or treated with rOPCML produced in bacteria.

Zanini et al. showed that OPCML interacts with the HER2 receptor, preventing the formation of EGFR-HER2 heterodimers and homodimers, consequently inhibiting HER2 phosphorylation and inducing the proteasomal degradation of HER2 (Zanini et al., 2017). Consistent with this finding, I demonstrated that treatment with rOPCML in SKOV3 cells reduces both phospho- and total-HER2 levels. Similar effects were observed with rOPCML-Fc treatment, but the reduction was triggered at 100 µg/mL, unlike rOPCML, which triggered reduction at 40 µg/mL. It is important to note that the molecular weight of rOPCML-Fc is higher than that of rOPCML. Therefore, even though the dose is expressed in µg/mL, the dose in µM is different, with 100 µg/mL of rOPCML corresponding to 3µM and 100 µg/mL of rOPCML-Fc corresponding to 1.77 µM. However, since the comparison of the effect of rOPCML and rOPCML-Fc was not the focus of

this work, this difference will not affect the results or the conclusions drawn from them.

OPCML also interacts with AXL. OPCML resides in the cholesterol-rich lipid domains of the plasma membrane of the cancer cells; when OPCML interacts with AXL, it brings AXL close to the lipid domain-restricted phosphatase PTPRG, by which AXL is then deactivated (Shu et al., 2010; Antony et al., 2018). This AXL inactivation prevents ovarian cancer cell migration and invasion (Antony et al., 2018). Here, I demonstrated that treatment with both rOPCML and rOPCML-Fc at 40 and 100 µg/mL inhibits AXL phosphorylation at Y779 in SKOV3 cells, OVCAR8 and ASC-01 cells like the effect observed when OPCML is ectopically expressed. Notably, the phosphorylation of AXL at Y779 was almost completely abolished upon treatment with rOPCML-Fc at 40 and 100 µg/mL in OVCAR8 and ASC-01 cells.

The effects of rOPCML and rOPCML-Fc on AXL and HER2 resulted in downregulating two important downstream signalling pathways, PI3K/AKT and MAPK/ERK. Treatment with rOPCML and rOPCML-Fc significantly inhibited the phosphorylation of AKT and ERK in SKOV3 and OVCAR8 cells, as well as ASC-01. The inhibition of AKT and ERK phosphorylation showed dose-dependent kinetics upon treatment with rOPCML and rOPCML-Fc in SKOV3 and ASC-01. In OVCAR8, the effect of rOPCML-Fc on reducing pAKT and pERK was similar at the doses of 40 and 100 µg/mL. However, it is essential to note that the experiments with rOPCML-Fc were not part of a dose-escalating experiment but were performed as independent experiments.

I have further confirmed the tumour-suppressive activity of rOPCML and rOPCML-Fc by comparing them with mutated forms of rOPCML. Birtley et al. identified and tested the role and effects of different mutated forms of

OPCML. They demonstrated that OPCML carrying these mutations loses function in most *in-vitro* assays. Among all the tested mutations, the majority of data were generated using three mutations occurring in domain 1: N70H, R65L, and P95R. Three recombinant forms of OPCML carrying these specific mutations were produced in insect cells and tested here (Birtley et al., 2019).

These recombinant forms were used as a treatment control in this work to validate the rOPCML+/- Fc effects. I confirmed that treatment with rOPCML and rOPCML-Fc in SKOV3 cells reduced pAKT and pERK levels, colony formation, and 3D-spheroid invasion in Matrigel. Conversely, treatment with rOPCML-R65L, rOPCML-N70H, and rOPCML-P95R did not show these inhibitory effects. In OVCAR8, the effects of rOPCML in inhibiting phosphorylation of AXL, AKT, and ERK were confirmed. In contrast, treatment with rOPCML-R65L and rOPCML-P95R did not inhibit AXL phosphorylation, which is consistent with the loss of OPCML's ability to interact with AXL in the presence of R65L and P95R mutations (Birtley et al., 2019).

However, treatment with rOPCML-R65L and rOPCML-P95R inhibited AKT and ERK phosphorylation in OVCAR8 cells. This may be because the two mutated forms of OPCML can still inhibit other receptor tyrosine kinases (RTKs) that use AKT and ERK as downstream effectors. For example, OPCML-P95R has been shown to inhibit FGFR1 and RET, as well as their downstream pathways, which are crucial for regulating cell survival and proliferation in OVCAR8 cells (Birtley et al., 2019). The observed inhibition of AKT and ERK phosphorylation with rOPCML-P95R treatment may be due to the inhibition of these RTKs. Similarly, the inhibition observed with rOPMCL-R65L treatment may be due to the continued inhibition of other RTKs, such as MET, on which OVCAR8 cells depend.

However, this latter hypothesis must be validated for MET and OPCML-R65L.

To confirm this hypothesis, a duo-link and a co-IP assay could be performed in cells expressing and not expressing OPCML or OPCML-R65L or treated with rOPCML or rOPCML-R65L. Subsequently, a western blot analysis of MET or selected RTK downstream pathways could be conducted to determine the functional consequences of the confirmed or disconfirmed interaction.

However, the data obtained from rOPMCL should undergo additional validation through testing and comparing the effects of rOPCML with that of another protein known not to have oncosuppressive functions. This validation will help dispel any uncertainties regarding whether the rOPCML effects are attributed to the high protein load within the cells.

In this work, I also demonstrated that rOPCML reverts the increase in pAKT (S473) caused by CDDP and enhances the CDDP-induced reduction of pERK in OVCAR8 cells. These effects are important because the overactivation of PI3K is one of the mechanisms of platinum resistance (Agarwal et al., 2003; Kim et al., 2018). In addition, the activation of ERK1/2 protects ovarian cancer cells from cisplatin cytotoxicity, and its pharmacological inhibition enhances sensitivity to CDDP in ovarian cancer cells (Wang et al., 2007). Therefore, the effects of rOPCML may somehow increase the sensitivity to platinum agents. However, in this analysis, the western blot was also probed for the OPCML, and I oddly found a higher level (thicker band) of OPCML in the sample of cells treated with rOPCML and CDDP compared to the sample of cells treated only with rOPCML. Upon treated with CDDP, platinum interacts with lipids on the plasma membrane. This interaction leads to changes in the physical properties of the plasma membrane, resulting in the clustering of receptors in the lipid raft of

the plasma membrane (Martinho et al., 2019). The lipid raft is where OPCML is located and exerts its effects on the RTKs (McKie et al., 2012; Antony et al., 2018). Therefore, it is possible that the changes caused by CDDP treatment could increase the availability of OPCML/rOPCML targets on the cell membrane, ultimately leading to an increase in the amount of rOPCML that reaches and binds to its specific target. This finding is very interesting and requires further evaluation because this experiment was only repeated once, and a conclusion can only be drawn once it is confirmed that this increase in the rOPCML band is not due to operator error.

However, in this study, I also demonstrated that the combination therapy of CDDP and rOPCML caused a 1.9-fold shift in CDDP IC₅₀ in OVCAR8 and a significant increase in apoptosis in OVCAR8 and PEO4. These initial findings support the idea that rOPCML could be an effective treatment for a specific group of platinum-resistant patients or could be used in combination with platinum and as maintenance therapy.

The effects of rOPCML on regulating the RTK downstream pathway were also examined in non-OPCML-expressing lung cancer PC9/ER cells. These cells, which carry the *EGFR* T790M mutation and have an upregulation of many RTKs such as HER2, MET, and EPHA2 (Higo et al., 2019), are resistant to the EGFR-TKI Erlotinib. Treatment with rOPCML decreased the phosphorylation of AKT and ERK, which are important mediators of these RTKs. Furthermore, rOPCML can increase the sensitivity to Osimertinib, a drug currently used to treat erlotinib-resistant lung cancer. This effect is likely due to the inhibition of MET and HER2 phosphorylation determined by rOPCML, as both these receptors are involved and overexpressed in TKIs and osimertinib-resistant NSCLC cells (Ferro et al., 2024).

The effects of rOPCML in lung cancer cells were observed at higher doses than in ovarian cancer cells. This may be due to different OPCML target saturation in the two cell lines or a higher expression/activation of RTKs on one cell line compared to the other. These are speculative hypotheses.

In conclusion, even if preliminary and obtained on a single lung cancer cell line, these results could suggest that rOPCML can also act in cells resistant to other TK inhibitors and act synergistically or additively with some of them.

Lastly, to better understand possible other targets of rOPCML, I performed a large kinomic analysis using the TKs Pamgene kinomic assay. In OVCAR8, the Pamgene kinomic assay confirmed the reduction of HER2 phosphorylation also observed by Western blot and showed a reduction in phosphorylation of PDGFR, EGFR, VGFR2, VGFR1, MET, EPHA7, EPHA2, EPHA1, EPHB1, RET, FGFR3, and TYRO3.

In previous studies, when OPCML was ectopically expressed in ovarian cancer cells, a reduction in phosphorylation was observed for HER2, AXL, EPHA2, FGFR3, and MET. The effect on other RTKs was not evaluated except for EGFR (McKie et al., 2012; Antony et al., 2018). AXL and HER2 phosphorylation inhibition was attributed to a direct interaction between OPCML and these two RTKs (Zanini et al., 2017; Antony et al., 2018). Conversely, it is still unclear how OPCML inhibits the phosphorylation of the other RTKs and whether this effect results from a direct interaction or a feedback loop. Feedback loops and regulators play a significant role in RTK activation, either attenuating or amplifying their signalling pathway. These feedback regulators are categorised as early regulators (attenuators or amplifiers) if they are present and active before or immediately after receptor activation (Haglund et al., 2003; Neben et al., 2019) or as late regulators if they are induced by the pathways on which they act (Fiorini et

al., 2002; Tsang & Dawid, 2004; Korsensky & Ron, 2016; Neben et al., 2019). Therefore, rOPCML may act in inhibiting the known RTKs through direct interaction and then act through these effectors or a feedback loop to inhibit the other RTKs' phosphorylation. Evidence supporting the presence of a feedback loop can be found in the decrease in EGFR phosphorylation shown in Pamgene kinomic analysis. Despite previous experiments showing that OPCML does not directly interact with EGFR in ovarian and breast cancer cells, here I demonstrated that rOPCML somewhat regulates EGFR phosphorylation (McKie et al., 2012; Zanini et al., 2017). This leads us to hypothesise that some of the observed effects in this assay and previous reports are due to secondary regulation by a feedback loop involving receptor tyrosine kinases interaction (e.g., HER2-EGFR interaction).

Furthermore, the data on Pamgene kinase analysis showed that rOPCML also activates the Src homology phosphatase (PTN6, also known as SHP1); this phosphatase is involved in the downregulation of many RTKs activities, including EGFR-family and HER2, via its phosphatase function (Wu et al., 2016). Therefore, I can speculate that rOPCML dephosphorylates different RTKs by activating these phosphatases (PTN6 and PTPRG). Furthermore, I saw arrestin-related protein (ART) activation in the same assay. This type of protein targets specific plasma membrane proteins for endocytic downregulation (Mittal et al., 2009); we can, therefore, hypothesise that through this adaptor, rOPCML could mediate, as for HER2, the endocytosis and degradation of other RTKs. Lastly, the phosphorylation of different proteins responsible for kinase control or regulation of intracellular signalling seems to be influenced by rOPCML treatment (e.g., PLCG2, PDPK1 and TEC), suggesting that rOPCML may have a more complex mechanism of action than the one hypothesised until now (McKie et al., 2012; Zanini et al., 2017; Antony et al., 2018).

In the Pamgene kinomic assay, I found that treatment with rOPCML inhibits the phosphorylation of several proteins involved in the MAPK/ERK and SRC kinase pathways. These pathways play crucial roles in tumour invasion, adhesion, and resistance to paclitaxel in ovarian cancer (Xiao et al., 2015; Sun et al., 2016). Based on these findings, it would be beneficial to conduct experiments in paclitaxel-resistant cells to evaluate the potential of rOPCML in reversing this resistance. Additionally, considering the previously proposed hypothesis that rOPMCL functions at the plasma membrane leaflet (McKie et al., 2012; Antony et al., 2018), I can hypothesise that the effects of rOPCML on these pathways may be a result of inhibiting the upstream RTKs. These pathways are known to be activated by HER2, EGFR, PDGFR, FGFR, and HGFR (Yang et al., 2013; Bromann et al., 2004). However, I cannot rule out the possibility that rOPCML may be internalised by tumour cells and exert its effects at that level.

Similar effects but in a reduced number of RTKs and effectors were observed upon rOPCML treatment in PEO4 cells. rOPCML showed a significant reduction in phosphorylation of PDGFR, MET and different effectors of the MAPK/ERK pathway, such as ERK1. The discrepancy between the data obtained with OVCAR8 and PEO4 could be due to the activation of a different repertoire of RTKs in the two cell lines (Huang et al., 2016) or to a more delayed response of PEO4 to rOPCML that consequently was not captured at the 6 hours timepoint used for this experiment.

The data from Pamgene kinomic analysis suggest that rOPCML could effectively disrupt the downstream signalling of different RTKs. However, these findings need further validation through Western blotting (WB) or a high-throughput analysis such as Reverse-Phase Protein Microarrays (RPPA). The latter analysis can detect levels of total protein expression or

post-translational modifications, such as phosphorylation, as a measure of protein activity. If the results of the RPPA analysis are consistent with the Pamgene analysis, it would confirm and strengthen the data obtained by the Pamgene analysis.

The Pamgene kinomic analysis results have provided insights into potential future applications of evaluating rOPCML. The GeneGo analysis suggests that rOPCML may be involved in the immune response. According to the human protein atlas data, there is no expression of OPCML in immune cell types within peripheral blood mononuclear cells (PBMC). However, OPCML is expressed in some fibroblasts, T-cells, and endothelial cells in specific tissues, such as adipose visceral tissue, colon, kidney, pancreas, stomach, prostate, skin, and thyroid (Appendix Figure 7.4). It would be interesting to assess whether OPCML expression is lost in these cells in the case of a tumour and whether the use of soluble rOPCML +/- Fc in the tumour microenvironment could have a multifaceted function, inhibiting tumour cells while regulating the microenvironment.

6.2 Treatment with soluble rOPCML -/+ FC inhibits ovarian cancer cell viability, colony formation, migration and invasion and enhances apoptosis

The mitogen-activated protein kinase and the phosphatidylinositol-4,5-bisphosphate 3-kinase cascade, which are impaired by treatment with rOPCML +/- Fc, play crucial roles in several cancer processes, such as cell growth, spread, and resistance to drugs (De Luca et al., 2012; Hay et al., 2004). Therefore, I wanted to investigate whether treatment with rOPCML +/-Fc disrupts these processes.

Both rOPCML and rOPCML-Fc significantly reduced cell viability in a dose-dependent manner in SKOV3, PEO4 and OVCAR8 cells. The reduction in cell viability ranged between 28 and 38% at the maximum dose tested of both recombinants (100 µg/mL). Higher doses were not tested in this assay; however, it may be that we could reach and calculate an IC50 of rOPCML+/-Fc at higher doses.

Treatment with rOPCML and rOPMCL-Fc also promoted apoptosis in ovarian cancer cells, as assessed by caspase-3/7 activity, which significantly increased upon treatment with rOPCML and rOPCML-Fc, showing a dose-dependent effect.

Both effects were also confirmed in the ex-vivo model of ASC-01 cells. The cells were obtained from ascites spheroids of a newly diagnosed stage IIIc HGSOC patient who did not carry germline pathogenic variants in BRCA1/2. This patient experienced disease recurrence after 12 months of platinum-based treatment.

Ascite spheroids are considered 'metastatic units' of primary ovarian cancer that possess unique molecular characteristics, contributing to their highly invasive phenotypes and low sensitivity to traditional chemotherapy (Ding et al., 2021). Furthermore, they are emerging as a good translational preclinical model to study EOC because they represent inter and intra-tumour heterogeneity (Velletri et al., 2022). One limitation of the experiment in this work is that it has only been tested in one patient. However, the ability of rOPCML in such a model strengthens the obtained results, making them more translational and thus rendering further investigation in this model worthy of consideration.

rOPCML and rOPCML-Fc treatment significantly reduces the ability of SKOV3 and OVCAR8 cells to form colonies and their migration and

invasion in Matrigel. Additionally, they inhibit colony formation in PEO4 cells. These effects specifically result from rOPCML +/- Fc treatment, as treatments with mutated forms of OPCML (rOPCML-R65L, rOPCML-N70H, and rOPCML-P95R) did not have the same inhibitory effects. The mutated recombinant forms of OPCML completely lose the ability to inhibit tumour spheroid invasion in Matrigel. However, rOPCML-R65L can still inhibit colony formation in SKOV3 and OVCAR8 cells, albeit significantly less than rOPCML. This finding aligns with Birtley et al.'s demonstration that OPCML-R65L, OPCML-N70H and OPCML-P95R retain the activity of the wild-type OPCML in inhibiting ovarian cancer cell proliferation but with lower effects (Birtley et al., 2019).

In the wound healing migration assay, I observed that rOPCML and rOPCML-Fc do not have the same dose-dependent dynamics in inhibiting SKOV3 and OVCAR8 cell migration. When comparing the two, rOPCML caused a similar reduction in migration at both 40 and 100 µg/mL. On the other hand, rOPCML-Fc showed greater inhibition of cell migration at 100 µg/mL compared to 40 µg/mL. This difference is likely because the doses of the two recombinants are measured in different concentration units.

In conclusion, here, I confirmed that the downregulation of MAPK and PI3K pathways induced by rOPCML and rOPCML-Fc treatment also determined inhibition of ovarian cancer cell viability, clonogenicity, migration and invasion, and enhanced apoptosis reiterating the tumour-suppressive phenotype of OPCML ectopically re-expressed and the bacterially produced rOPCML in ovarian cancer cells (McKie et al., 2012).

6.3 Limitations of this project

Although new data have been published regarding OPCML's function as a tumour suppressor, little is known about how OPCML exerts this function, which has represented one limitation of this project.

OPCML protein consists of three domains and a GPI anchor. While we are aware that point mutations in domains 1, 2, and 3 could abolish or diminish the anti-tumour effects of OPCML (McKie et al., 2012; Birtley et al., 2019), we still do not fully understand the individual roles of the domains and the GPI anchor. Understanding this would be crucial for developing a more effective therapeutic by optimising the recombinant protein sequence to retain the functional parts and eliminate the non-functional ones. To address this, I conducted experiments to compare the anti-tumour activity of OPCML with a GPI anchor versus OPCML without a GPI anchor when transduced into SKOV3 cells. The experiments revealed no significant differences in the viability and clonogenic capacity of SKOV3 cells expressing OPCML with a GPI anchor compared to those expressing OPCML without a GPI anchor. To confirm the importance of the GPI-anchor, an assessment of the biodistribution of OPCML without the GPI-anchor on the plasma membrane of the cells and the consequent interaction with RTKs should be performed. OPCML is usually located in cholesterol-enriched lipid domains, and here, it exerts its function on different RTKs, such as AXL and HER2 (McKie et al., 2012; Anthony et al., 2018). The absence of a GPI anchor could affect this spatial restriction to the lipid draft of the plasma membrane. The localisation of the OPCML at the plasma membrane could be analysed by immunofluorescence microscopy, and the co-localisation of OPCML and RTKs could be confirmed using Förster resonance energy transfer, as well as *in-situ* proximity ligation assay Duolink. It is crucial to emphasise that the distribution of rOPCML lacking

a GPI-anchor may vary pharmacologically from OPCML lacking a GPI-anchor at a physiological level. Therefore, it would be particularly intriguing to investigate whether rOPCML, even in the absence of a GPI-anchor, can be spatially restricted to the lipid raft of the plasma membrane due to its high concentration in the proximity of cancer cells upon treatment. This could have significant therapeutic implications.

However, the challenge lies in producing a non-soluble GPI-anchored form of rOPCML, which could be a significant obstacle if the GPI-anchor is demonstrated to contribute substantially to the activity of rOPCML. In light of this, it is important to consider alternative drug delivery methods, particularly focusing on gene therapy approaches targeting OPCML expression and regulation or OPCML protein or mRNA incorporation into extracellular vesicles (e.g., exosomes) as alternative delivery methods for cancer cells.

Along with this and with the limitations of the single experiments already discussed above, another limiting factor, which reduced the number of experiments I could perform, was the amount of soluble recombinant protein I could produce in-house. A way to increase the yield and protein quality would be to use bioreactors, which favour a continuous renewal of the culture medium, generating a stable and favourable environment for the transfected-HEK293 cells to grow (Bielser et al., 2018; Gomez et al., 2019). In addition, using automated chromatography systems for purification would have also fastened the purification steps.

Lastly, another limitation is that *in-vivo* data on rOPCML activity are missing. Although I have obtained preliminary results on the effectiveness of rOPCML and rOPMCL-Fc in an *ex-vivo* model using ascites-derived tumour cells, which have recently been identified as a potent tool for

precision oncology in ovarian cancer (Velletri et al., 2022), it is essential to complement these findings with *in-vivo* data to bolster the outcomes obtained. For the *in-vivo* experiments, an intraperitoneal model with injections of SKOV3 and OVCAR8 cells in mice is recommended. The mice should be divided into two cohorts: one receiving intraperitoneal injections and the other receiving tail vein injections. The treatment groups to be compared include BSA in PBS as control and rOPCML and rOPCML-Fc as treatment arms. Based on previous experiments (McKie et al., 2012), the treatment administration should be repeated twice weekly.

6.4 Future directions

In conclusion, our work shows that rOPCML+/- Fc can potentially play an important role in the treatment of ovarian cancer.

The soluble rOPCML +/- Fc I developed demonstrated anti-tumour solid effects in ovarian cancer and could be worth exploring further for potential benefits in lung cancer.

Their pharmacodynamic and pharmacokinetic properties heavily influence the effectiveness of protein therapeutics. Pharmacodynamics refers to a therapeutic's impacts on the body, such as its binding to the target and any antagonistic effects. Pharmacokinetics, on the other hand, refers to how a medication travels through the body during treatment, encompassing absorption, distribution, metabolism, and elimination or clearance. In experiments conducted in the Jackson laboratory to study the pharmacokinetics of rOPCML-Fc compared to the monoclonal antibody Herceptin, it was found that rOPCML-Fc has a short half-life (18.43 ± 2.6 hours vs 275.5 ± 75.6 hours) and rapidly accumulates in the liver and kidney. These findings were based on *in-vivo* experiments performed in mice

without tumours, and only a single administration of rOPCML-Fc was tested. More consistent data on the biodistribution of rOPCML and rOPCML-Fc could be obtained by treating Tg32 SCID mice with these therapeutics in the presence or absence of a tumour. The presence of a tumour, which typically expresses high levels of the rOPCML targets (i.e., RTKs), may affect the biodistribution, leading to increased binding of rOPCML+/-Fc to the targets on the tumour cells and reduced accumulation in the liver and kidney. Additionally, this approach could determine whether the short half-life of rOPCML+/-Fc affects their *in-vivo* anti-tumour activity and whether the pharmacokinetics follow a linear or nonlinear pattern. These latter factors will influence the proposed administration schedule of rOPCML +/- Fc. For example, some FDA/EMA-approved anticancer drugs have a short half-life but are still effective. However, the administration schedule is different due to their different pharmacokinetic dynamics. For instance, blinatumomab, a monoclonal antibody, has a plasma half-life of 2.11 hours with linear pharmacokinetics, requiring a daily schedule for administration (Zhu et al., 2016). On the other hand, elotuzumab, another monoclonal antibody, has a plasma half-life of approximately 4.73 hours with nonlinear pharmacokinetics, characterised by a decrease in clearance when the dose is increased, which allows for a weekly administration schedule at a higher dose (Ide et al., 2022). Previous experiments demonstrated that bacterially produced rOPCML is effective *in-vivo* when administered twice weekly (McKie et al., 2012), making us hypothesise that this schedule is sufficient to achieve a good antitumour effect. However, of interest, especially if the short half-life of rOPCML-Fc might impact its effectiveness, would be to consider creating a form of OPCML using new techniques to improve its pharmacokinetics, such as coating it with polymeric nanoparticles or formulating it as sustained-release microspheres.

In addition, to further develop these recombinants as treatments, it is fundamental to optimise the recombinant's structure, understand the target for rOPCML treatment, and finally ensure that the recombinant OPCML has no potential issues for patients in terms of immunogenicity and safety.

We have already commissioned an in-silico assessment of the rOPCML-Fc sequence from Apollo therapeutics, and it was revealed that, as expected, the molecule carries a low immunogenicity risk, has a good isoelectric point (pI), and there were no predicted sites at risk of deamination (Table 6.1). However, the analysis flagged the six glycosylation sites in rOPCML as a protein sequence liability that could determine a batch-to-batch variability. In addition, the analysis identified the presence of 5 methionine (Met or M) residues at positions 66, 104, 216, 235 and 243 at risk of oxidation, and a potential Aspartate (Asp or D) isomerisation site at position 233 that can both interfere with long-term storage (Wang et al., 2011; Wakankar et al., 2007). Two possible cleavable motifs (Asp-Proline (P) and lysine (K)-threonine (T)) have also been identified. However, the cleavage at these sites is mostly pH-dependent (Hinterholzer et al., 2021; Vlasak et al., 2011). These characteristics are summarised in Table 6.1 and are all characteristics that need to be considered and, if possible, modified for the future development of a rOPCML+/- Fc treatment.

Table 6.1: Table reporting rOPCML-Fc molecular structure characteristics that can impact its stability as shown by in-silico analysis

	Findings	Impact on the final product
Deamidation	No deamidation risk predicted	
Oxidation	5 Met oxidation sites were predicted in the fusion protein at residue positions 66, 104, 216, 235 and 243	Impact on long-term storage conditions.
Asp Isomerisation	Potential Asp isomerisation site at position 233 in the fusion protein	Impact on long-term storage conditions.
N-Glycosylation	Six sites in the Fusion protein were predicted to have N-glycosylation sites, and 8 sites were predicted in the Fc fragment	Could potentially lead to a more significant number of isoforms and more batch-to-batch variability.
DP Motif	One predicted motif	Potential sites of cleavage. pH-driven.
KT Motif	1 predicted KT motif at position 89	Metal-ion-driven cleavage step.

pI	pI=7.7	pI of 7-8 is ideal for purification steps
Immunogenicity	The linker _ low risk OPCML is a human protein	Low risk of immunogenic reaction after administration

Further work would also implement our understanding of rOPCML+/-Fc interaction with RTKs and, in particular, clarify which RTKs are specific and direct targets and which others are regulated secondarily because of RTK heterodimerisation and transactivation. To assess this OPCML/rOPCML-RTKs interaction, immunoprecipitation coupled with mass spectrometry with OPCML-transduced cells and rOPCML-treated cells could be performed. Further, it would be fundamental to understand how the presence of the GPI-anchor interferes with the OPCML function and OPCML- RTKs interaction.

Based on the Pamgene® analysis results and the discussion above, it would be interesting to investigate the role of OPCML in the tumour microenvironment (TME). Exploring whether the re-expression of OPCML on tumour cells can also regulate TME cells would be highly significant for developing rOPCML +/-Fc.

Furthermore, it would be intriguing to study the impact of rOPCML+/-Fc on specific subpopulations of HGSOC (e.g., HGSOC carrying *BRCA* mutations or amplification of specific RTKs or *PTEN* mutations). This could be achieved by using ovarian cancer cell lines with these specific alterations or, ideally, by utilising patient-derived organoids or xenograft models derived

from patients' tumours with these molecular or genetic alterations. Such investigations would provide valuable insights into whether specific characteristics make ovarian tumours more susceptible to rOPCML+/-Fc treatment. Ultimately, this could lead to identifying patient subgroups that would benefit the most from rOPCML+/-Fc treatment and help identify potential markers for response to rOPCML+/-Fc. These points are crucial for designing clinical trials involving rOPCML.

Finally, as OPCML has been shown to inhibit AXL phosphorylation and downstream pathways in cholangiocarcinoma and ovarian cancer cells, and its re-expression has enhanced the effects of R428, a selective small molecule inhibitor of the AXL-kinase (Antony et al., 2018; Khamko et al., 2021; Khamko et al., 2022), it would be interesting to investigate if rOPCML also retains these effects. It would also be valuable to explore the role of rOPCML in other cancer types where OPCML has shown a tumour-suppressive function, such as breast cancer, gastric cancer, and colon-rectal cancer (Reed et al., 2007; Tsou et al., 2007; Duarte-Pereira et al., 2011; Sriraksa et al., 2011; Cui et al., 2008), and the effects of a combinational treatment with rOPCML and R428.

These aspects are crucial for the potential development of a cancer-therapeutic rOPCML.

Other important considerations involve creating a product that could be used in humans and clinical trials. This includes studying and optimising the stability of the recombinants, as well as investigating the pharmacokinetics and pharmacodynamics in-vivo. Furthermore, compliance with EMA and FDA regulations, including specific guidelines for immunogenicity assessment of protein therapeutics, is essential to move the rOPCML+/- Fc

towards use in humans (European Medicines Agency, 2017; US Food and Drug Administration, 2019).

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APPENDIX

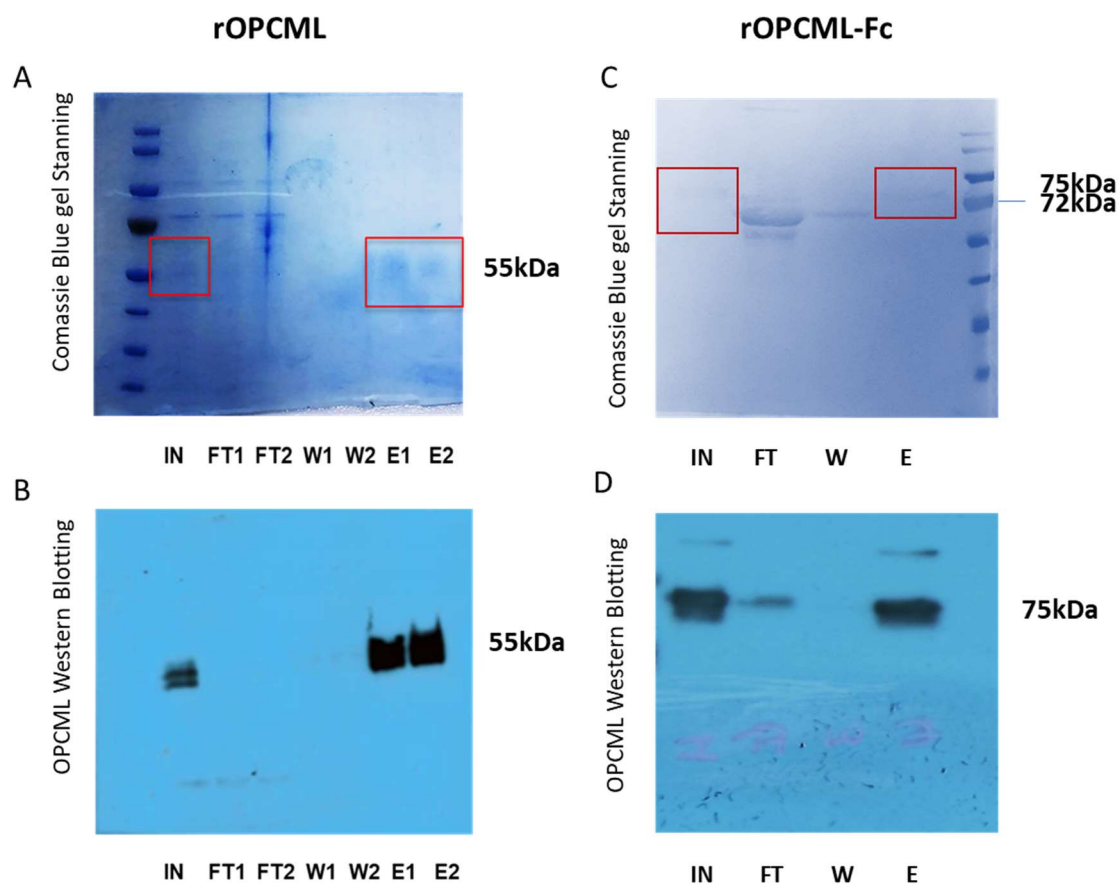


Figure 7.1: Coomassie blue and WB analysis of three purified transfections. Aliquots of conditioned medium (input), flow-through, last wash and elution protein were collected. Coomassie-stained gels (A; C) with red rectangular indicating the recombinants OPCML bands and green squares indicating contaminant proteins bands. Western blotting for OPCML of the single aliquots from recombinants purification (B, D); in C) OPCML is observed as a band at 55kDa in D) rOPCML-Fc is observed as a band at 75kDa; Molecular weight in kDa is shown on the left; IN – input, FT – flow through, W – wash, E – elution.

Pharmacokinetic Data

Pharmacokinetics (PK) experiments were performed by Jackson Laboratory in Farmington, Connecticut. A frozen aliquot of 500 µl of rOPCML-Fc at 1mg/ml concentration was sent to them to perform the PK experiments as summarised below.

Tg32 SCID mice (also called B6.Cg-Fcgr^{ttm1Dcr} Prkdc^{scid} Tg(FCGRT)32Dcr/DcrJ) expressing a human alpha chain *FCGRT* molecule in place of the endogenous mouse *Fcgrt* and a *scid* allele that makes the mice immunodeficient were used. These mice lack endogenous FcRn (Neonatal Fc receptor) and carry transgenic human FcRn (hFcRn). The hFcRn mouse model is a potentially promising and convenient surrogate for evaluating the pharmacokinetics of protein human Fc fusion and Ig based therapeutics (Roopenian et al., 2003; Proetzel et al., 2004).

Herceptin, an antibody protein therapeutic, was used as control. Briefly, rOPCML-Fc and Herceptin were respectively administrated intravenously in three 6-10-week old Tg32 SCID (B6.Cg-Fcgr^{ttm1Dcr} Prkdc^{scid} Tg(FCGRT)32Dcr/DcrJ, stock #18441) male mice at a dose of 5mg/kg.

Blood samples for PK analysis were collected from each mouse at 5 minutes, 8 hours, 1 day, 3 days, 5 days, 7 days, 10 days, 14 days, and 21 days, processed and stored as plasma. Plasma samples were assessed in triplicate by mouse anti-human IgG capture (Mab 145). Mouse anti-human IgG Fc-AP capture ELISA, previously tested by the service at Jackson Lab as the best match of ELISA antibody to detect rOPCML-Fc and Herceptin avoiding high confounding background using each test article as its standard to match what is in the plasma samples being assessed.

Liver, heart, and kidney tissue slides from the mice treated with rOPCML-Fc and Herceptin or untreated mice were sent to INT IRCCS Fondazione G.

Pascale, Naples, Italy. Here, these tissues have been analysed for macroscopic and microscopic signs of damage in hematoxylin and eosin. To assess rOPCML-Fc biodistribution, tissues were also analysed for the presence of human rOPCML-Fc at immunohistochemistry with monoclonal anti-human OPCML antibody (AF2777, R&D Systems Inc., Minneapolis, USA). Sections were examined by light microscopy, and staining was assessed for the presence and intensity of staining. Staining was reported as the percentage of OPCML expression. Since OPCML is expressed in normal mice tissue, and the human anti-OPCML antibody could interact with murine OPCML expressed in mice tissues, an untreated mouse was used as a control. The results of these experiments are reported in the Appendix (Figures 7.2 and 7.3).

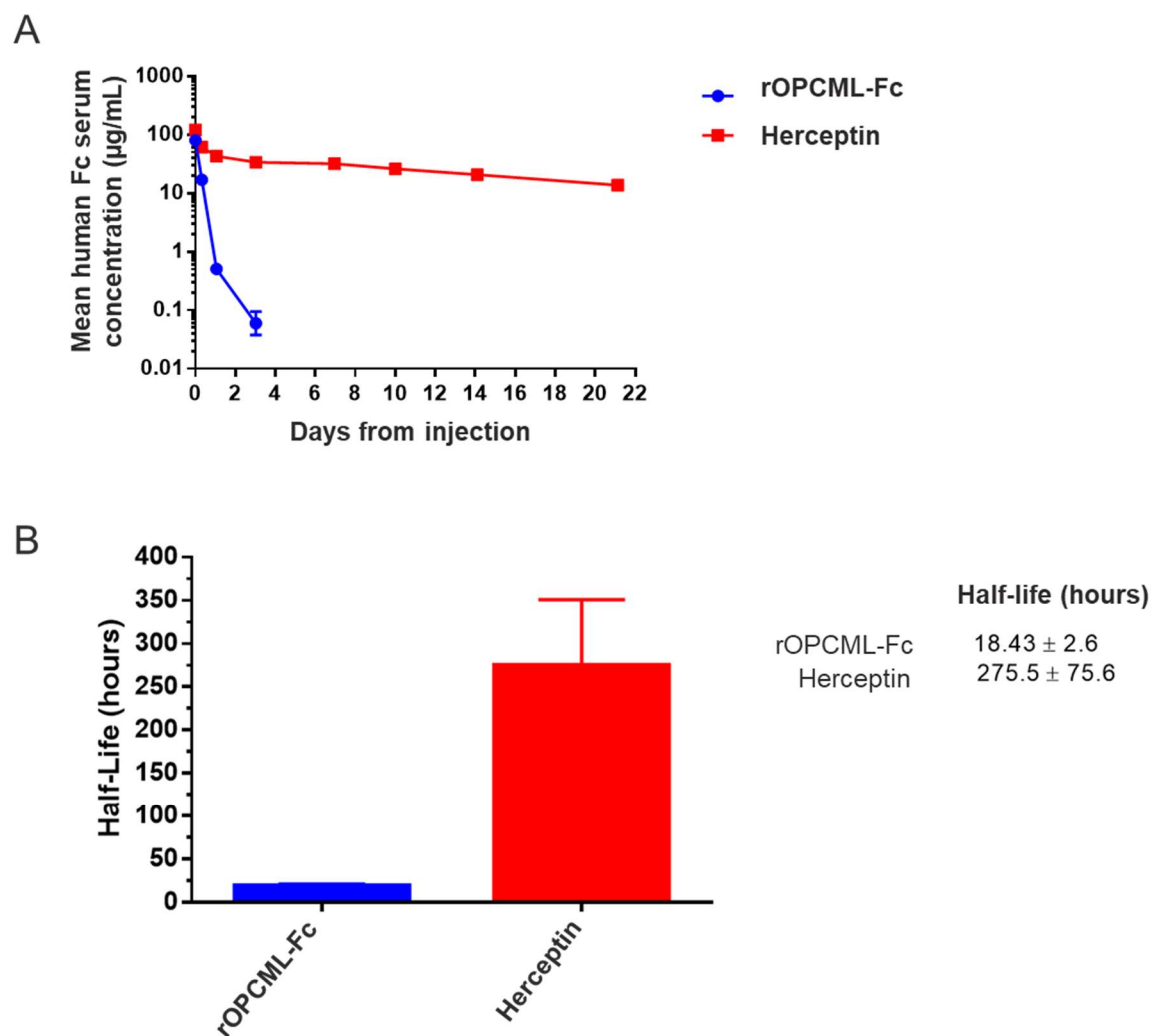


Figure 7.2: rOPCML and Herceptin pharmacokinetics

- A. rOPCML-Fc (blue) and Herceptin (red) distribution/elimination curves expressed as the concentration of hFc over time. Plasma samples were assessed in triplicate and at different time points using mouse anti-human IgG capture (Mab 145).
- B. rOPCML-Fc (blue) and Herceptin (red) half-life plotted graph.

The data in A and B represent the mean and standard deviation of the results obtained in three mice per treatment. Error bars depict mean $\pm$ SEM.

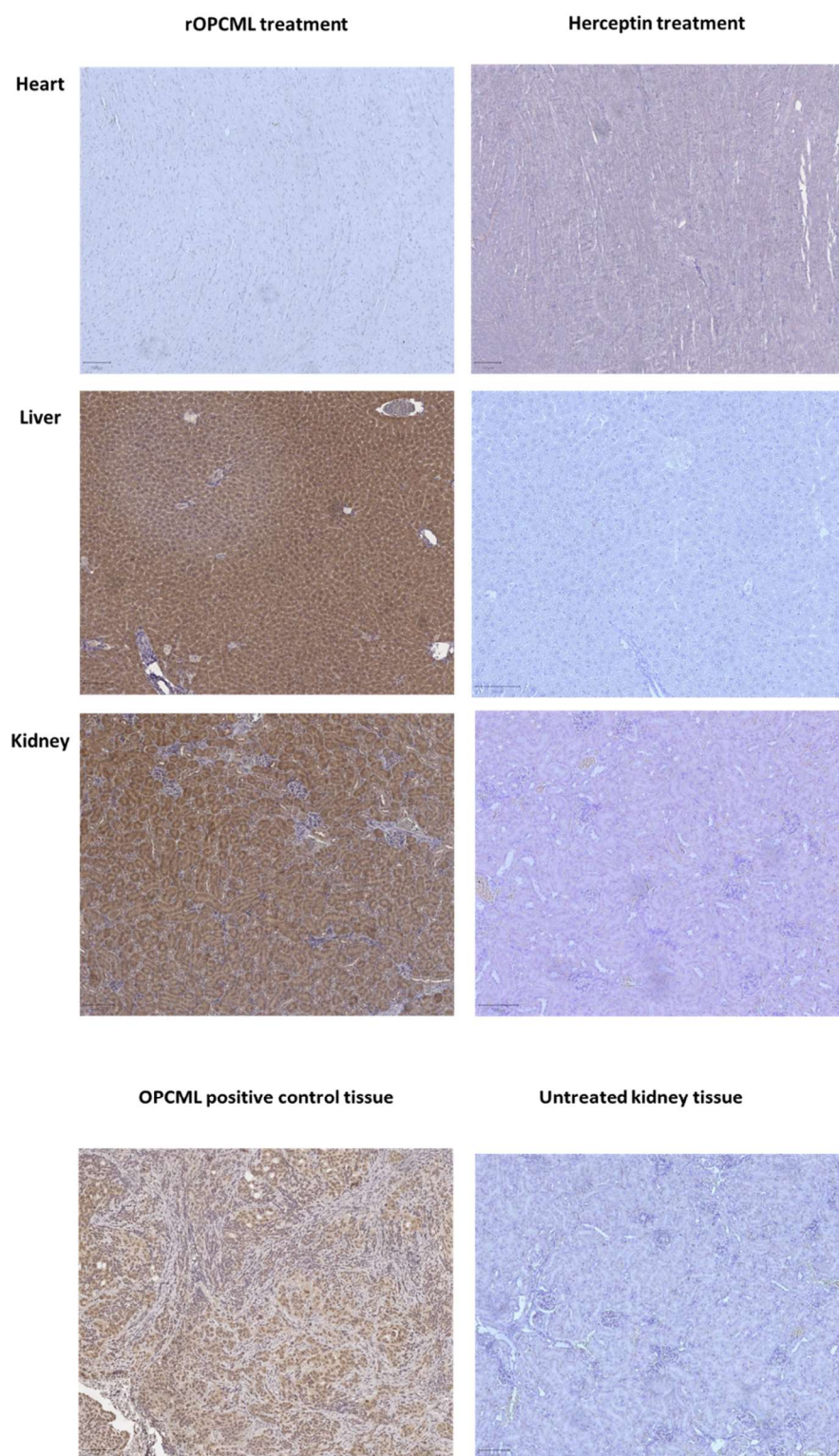
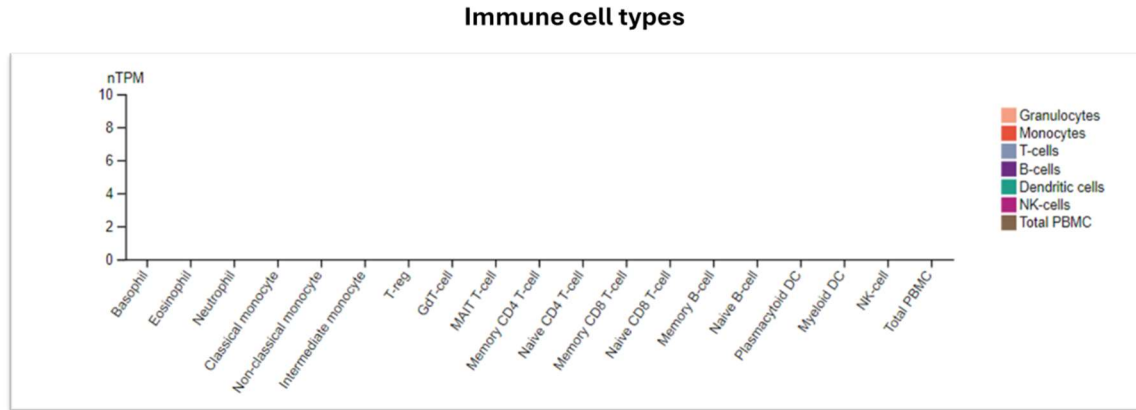


Figure 7.3: OPCML immunohistochemical expression in rOPCML and Herceptin treated and control mice tissues

Heart, Liver, and kidney tissue obtained from rOPCML-Fc treated, Herceptin treated, positive control and untreated control with OPCML staining (brown).

Treatment with rOPCML-Fc did not cause damage to heart, kidney or liver tissue either macroscopically or microscopically. However, an accumulation of OPCML was seen in the kidneys (77% of cells were positive for OPCML) and liver (87% of cells were positive for OPCML) of mice treated with rOPCML-Fc compared with an untreated mouse and Herceptin-treated mice.

A



B

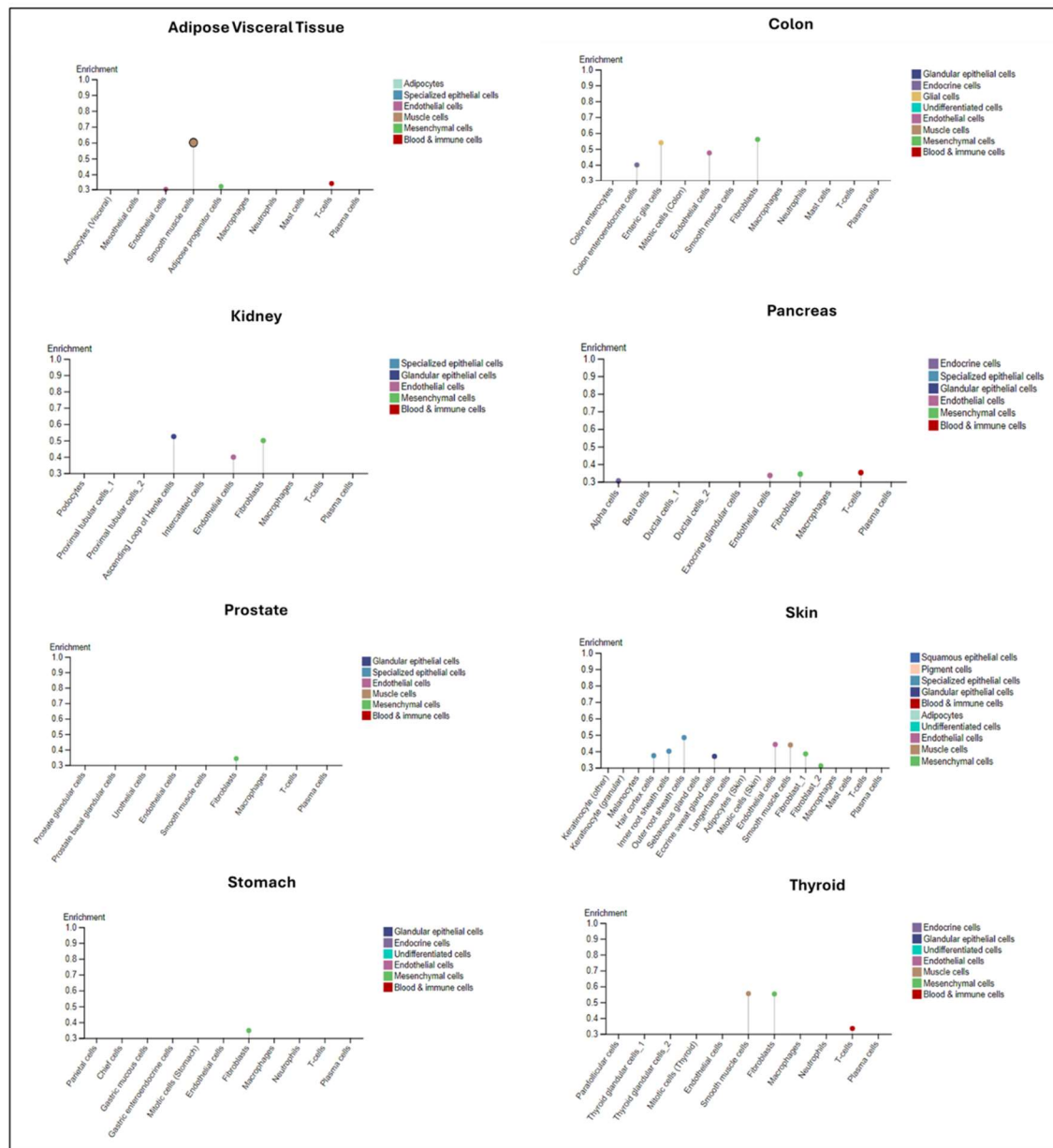


Figure 7.4: RNA expression of OPCML as for Human Protein Atlas

- A. *Transcript expression values calculated as nTPM, resulting from the internal normalisation for 18 immune cell types and total peripheral blood mononuclear cells (PBMC).*
- B. *Plots showing the enrichment prediction score for each cell type profiled in the specific tissue (indicated above each plot). The score is the mean correlation between the selected gene and the 3 reference transcripts selected to represent each cell type profiled within the tissue. An enlarged symbol indicates the classification of the OPCML gene as a cell type enriched in the corresponding cell type.*

Publications List

Publications related to the PhD thesis:

INACTIVATING MUTATIONS AND X-RAY CRYSTAL STRUCTURE OF THE TUMOR SUPPRESSOR OPCML REVEAL CANCER-ASSOCIATED FUNCTIONS.

Birtley JR, Alomary M, Zanini E, Antony J, Maben Z, Weaver GC, **Von Arx C**, Mura M, Marinho AT, Lu H, Morecroft EVN, Karali E, Chayen NE, Tate EW, Jurewicz M, Stern LJ, Recchi C, Gabra H. Nat Commun. 2019 Jul 17;10(1):3134. doi: 10.1038/s41467-019-10966-8.

Poster presentations at National meetings:

INVESTIGATION OF ANTICANCER ACTIVITY OF RECOMBINANT OPCML (ROPCML) IN OVARIAN CANCER AND GLIOBLASTOMA CELL LINES.

Claudia von Arx, Yuewei Xu, Nelofer Syed, Chiara Recchi, Kevin O'Neill, Manuela Mura, Hani Gabra, Poster section NCRI Cancer Conference abstracts 2018

Papers published during my PhD that are not related to the thesis:

- PREVENTION, DIAGNOSIS AND CLINICAL MANAGEMENT OF HEREDITARY BREAST CANCER BEYOND BRCA1/2 GENES.
- Calabrese, A., **von Arx, C***, Tafuti, A. A., Pensabene, M., & De Laurentiis, M. (2024). Cancer treatment reviews, 129, 102785. Advance online publication.
- *Co-First and corresponding
- A NEW SCHEDULE OF ONE WEEK ON/ONE WEEK OFF TEMOZOLOMIDE AS SECOND-LINE TREATMENT OF ADVANCED NEUROENDOCRINE CARCINOMAS (TENEC-TRIAL): A MULTICENTER, OPEN-LABEL, SINGLE-ARM, PHASE II TRIAL.
- **von Arx, C.**, Della Vittoria Scarpati, G., Cannella, L., Clemente, O., Marretta, A. L., Bracigliano, A., Picozzi, F., Iervolino, D., Granata, V., Modica, R., Bianco, A., Mocerino, C., Di Mauro, A., Pizzolorusso, A., Di Sarno, A., Ottaiano, A., Tafuto, S., & ENETs Center of Excellence in Naples, Italy and with the endorsement of the Italian Association for Neuroendocrine Tumor IT.A.NET (2024). ESMO open, 9(5), 103003.
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Cannella L; **Von Arx C***, Bracigliano A, Pizzolorusso A, Antonella Di S, Clemente O, Modica R, Di Marzo M, Tatangelo F, Tafuto S. * **Presenting author** _ ORAL PRESENTATION 19th Annual ENETS Conference, 10 - 11 March 2022 in Barcelona, Spain

- SELINEXOR AND THE SELECTIVE INHIBITION OF NUCLEAR EXPORT: A NEW PERSPECTIVE ON THE TREATMENT OF SARCOMAS AND OTHER SOLID AND NON-SOLID TUMORS.

Marretta AL, Di Lorenzo G, Ribera D, Cannella L, **von Arx C**, Bracigliano A, Clemente O, Tafuto R, Pizzolorusso A, Tafuto S. *Pharmaceutics*. 2021 Sep 20;13(9):1522. doi: 10.3390/pharmaceutics13091522. PMID: 34575598; PMCID: PMC8466603.

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