Investigating the effects of aspirin in high-grade serous ovarian carcinoma models *in vitro*

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Statement of originality

I, Brinda Lakshmi Varahan, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below, and my contribution indicated. Previously published material is also acknowledged below.

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Details of collaborations

- RNA-sequencing data was analysed by Dr. Eleni Maniati, Barts Cancer Institute, Queen Mary University of London, UK.
- HCT116, HT-29 and HCT-15 colorectal cancer cell lines were provided by Dr. Belinda Nedjai's group, William Harvey Research Institute, Queen Mary University of London, UK.
- Immortalised human fallopian tube epithelial cell lines (p53 mut FT318) were a kind gift from Professor Ronny Drapkin's laboratory, University of Pennsylvania, Philadelphia, USA.
- The AOCS-1 cell line were kindly given by Professor David Bowtell's group, based at the Peter MacCallum Cancer Centre, Melbourne, Australia.
- Prostaglandin profiling of aspirin-treated cells was performed by Professor Jesmond Dalli's group, based at the Lipid Mediator Unit, William Harvey Research Institute, Queen Mary University of London, UK.
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Abstract

Aspirin is a non-steroidal anti-inflammatory drug. It blocks prostaglandin (PG) production by inhibiting cyclooxygenase 1 (COX-1) and COX-2 activity. There is now significant evidence about the therapeutic cancer-preventative effects of aspirin, especially as inflammation is a critical hallmark of tumour progression. Nevertheless, most studies investigating the anti-cancer effects of aspirin are on colorectal cancer. There is limited research in high-grade serous ovarian carcinoma (HGSOC), even though the inflammatory ovarian tumour microenvironment (TME) is important in promoting HGSOC progression.

Aspirin treatment (≤7 days) did not inhibit the growth of two HGSOC cell lines, three colorectal cancer (CRC) cell lines, primary omental fibroblasts, and primary omental mesothelial cells. To ensure I was using preparing the drug correctly, I performed a prostanoid profile in aspirin-treated malignant cells. Aspirin reduced PG levels in malignant cells, primary omental fibroblasts, and mesothelial cells, but only when they were cultured in serum-free medium. Aspirin did not inhibit malignant or normal cell growth even in serum-free medium.

I then investigated the actions of aspirin in multi-cellular models (four cell types) of HGSOC omental metastases. Treating multi-cellular models with aspirin in serum-free medium did not alter cell viability. However, performing a cytokine analysis showed that aspirin significantly and specifically reduced IL-8 secretion in multi-cellular models of HGSOC, monocultures of primary omental fibroblasts and mesothelial cells, but not monocultures of malignant cells. There was no action of aspirin on another 10 cytokines (including IL-6) that I measured. Results from monocultures of primary omental fibroblasts and mesothelial cells suggest that aspirin may reduce IL-8 release via COX-2-PGE_2-EP2/EP4 pathway. Aspirin inhibition of IL-8 production by stromal cells in the TME via the COX-2-PGE_2-EP2/EP4 pathway could potentially contribute to its cancer-preventive actions.

Om Vinayaga

Maha Periyava Thunai

ISKCON (Radha London-isvara) Thunai

This thesis is dedicated to my wonderful parents, Indira Lakshmi Varahan and Chandrasekar Lakshmi Varahan.

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Abbreviations

2D	2 dimensional
3D	3 dimensional
µl	Microlitre
µM	Micromolar
5S-HETE	5-Hydroxyeicosatetraenoic acid
15-PGDH	15-hydroxyprostaglandin dehydrogenase
AA	Arachidonic acid
ADR	European Agreement Concerning the International Carriage of Dangerous Goods by Road
Akt	Protein kinase B
ANOVA	Analysis of variance
AOCS	Australian Ovarian Cancer Study
ARRIVE	Aspirin to Reduce Risk of Initial Vascular Events
ASA	Acetylsalicylic acid
ASCEND	A Study of Cardiovascular Events in Diabetes
Arg-Gly-Asp	Arginine-Glycine-Aspartic acid
A-SMA	Alpha-smooth muscle actin
ASPREE	Aspirin in Reducing Events in the Elderly
ATCC	American Type Culture Collection
AT-LX	Aspirin-triggered lipoxin
AT-RvD	Aspirin-triggered resolvin
AT-SPM	Aspirin triggered- specialised pro-resolving mediator
BGTB	Barts Gynae Tissue Bank
BRCA	Breast Cancer gene
BSA	Bovine Serum Albumin
CAA	Cancer-associated adipocytes

CAF	Cancer-associated fibroblast
CAPP	Colorectal Adenoma/Carcinoma Prevention Programme
CD44	Cluster of Differentiation 44
CI	Confidence interval
CK20	Cytokeratin 20
CO_2	Carbon dioxide
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
CVD	Cardiovascular disease
DAB	3,3'-diaminobenzidine
DdH_2O	Distilled water
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DPX	Distyrene Plasticizer Xylene
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme Linked Immunosorbent Assay
EOC	Epithelial ovarian cancer
EPA	Eicosapentaenoic acid
EpCAM	Epithelial cellular adhesion molecule
FAP	Fibroblast activation protein
FBS	Foetal bovine serum
FN1	Fibronectin

GI	Gastrointestinal
GPCR	G-protein coupled receptor
GSK3β	glycogen synthase kinase 3-beta
HCC	Hepatocellular carcinoma
HGSOC	High-grade serous ovarian carcinoma
HPLC	High Performance Liquid Chromatography
HPV	human papilloma virus
HR	Hazard ratio
HRP	Horseradish peroxidase
HS	Human Serum
HTA	Human Tissue Authority Codes of Practice
IDS	Interventional Debulking Surgery
IFN-γ	Interferon gamma
IL-1ß	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-23	Interleukin 23
ITS-1	Insulin-Transferrin-Selenium-1
JAK	Janus Kinase
LC-MS	Liquid chromatography–mass spectrometry
LTB_4	Leukotriene B4
M199	Medium 199
MAPK	Mitogen-activated kinase

mg	Milligrams
MHC	Major histocompatibility complex
mM	Millimolar
MMP	Matrix metalloprotease
MRM	Multiple reaction monitoring method
mRNA	Messenger RNA
MSD®	Mesoscale Discovery
NACT	Neo-adjuvant chemotherapy
NaOH	Sodium Hydroxide
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nM	Nanomolar
NSAID	Non-steroidal anti-inflammatory drug
OR	Odds ratio
OS	Overall survival
PAX-8	Paired-box-gene-8
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline- tween 20
PFS	Progression free survival
PGD_2	Prostaglandin D2
PGE_2	Prostaglandin E2
PGE-M	Prostaglandin E-metabolite
PGF_{2a}	Prostaglandin F2a
PGH_2	Prostaglandin H2
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3-kinase
PPARδ	Peroxisome proliferator-activated receptor-delta
P/S	Penicillin/streptomycin
PTGER (EP)	Prostaglandin E2 receptor

PTGES-2	Prostaglandin E synthase 2
PTGS1	Prostaglandin-Endoperoxide Synthase 1
PTGS2	Prostaglandin-Endoperoxide Synthase 2
RCT	Randomised control trial
RLH	Royal London Hospital
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
SEM	Standard error of the mean
Ser516	Serine 516
Ser529	Serine 529
STAT	Signal transducer and activator of transcription
STIC	Serous tubular intraepithelial carcinoma
TAM	Tumour associated macrophages
TGF-ß	Transforming growth factor beta
T_H1	Helper T cell 1
T_H2	Helper T cell 2
T_H17	Helper T cell 17
TME	Tumour microenvironment
TNF-α	Tumour Necrosis Factor alpha
TP16	Tumour protein 16
TP53	Tumour protein 53
T_{reg}	Regulatory T cells
TXA_2	Thromboxane A2
UK-TIA	United Kingdom- Transient Ischaemic Attack
USPSTF	U.S. Preventive Services Task Force
VCAN	Versican
VEGF	Vascular Endothelial Growth Factor

1 Introduction

The overall aim of my PhD is to investigate the potential cancer-preventative actions of aspirin by studying its effects on high-grade serous ovarian cancer cells *in vitro*.

1.1 Aspirin

1.1.1 History

Aspirin is one of the most widely consumed anti-inflammatory, anti-pyretic and analgesic drugs in the world[1,2,3]. Salicylate derivatives were first isolated from willow bark thousands of years ago and were used to treat non-specific pain and fevers. In the early nineteenth century, several scientists identified and extracted a bitter-tasting component of willow, which was named salicin[1]. Charles Frédéric Gerhart, a French chemist, used this information to elucidate the structure of salicylic acid. In 1897, Felix Hoffman developed a strategy to retain the therapeutic properties of salicylic acid while simultaneously reducing side effects associated with prolonged administration. This was done by acetylating the phenol hydroxyl group of salicylic acid to form acetylsalicylic acid (ASA)[2]. In 1899, Friedrich Bayer re-branded ASA as aspirin (Figure 1.1), which became a commercial success by the early 20th century[2,3]. Approximately 80 years later, Sir John Vane discovered that aspirin inhibited the production of prostaglandins from arachidonic acid. He was awarded a Nobel Prize for his work in 1982, along with Bengt Samuelsson and Sune Bergstrom[1-4]. Subsequent research revealed aspirin's potential as a cardio-preventive and cancer-preventive agent, making it a multi-purpose medication with an ever-expanding therapeutic repertoire [1,2,3].

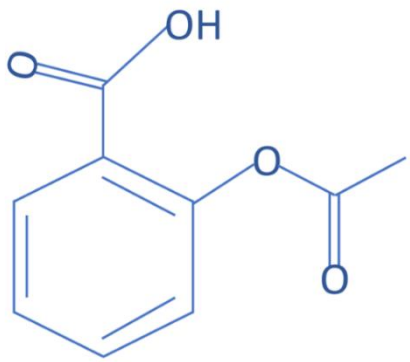


Figure 1.1. **Structural formula of acetyl salicylic acid (aspirin).** The chemical formula is $C_9H_8O_4$.

1.1.2 Background

Aspirin is a non-steroidal anti-inflammatory drug (NSAIDs)[5,6]. Other examples of NSAIDs include ibuprofen, indomethacin, piroxicam and sulindac. Similar to all NSAIDs, aspirin blocks the metabolism of arachidonic acid (AA) into prostaglandins by inhibiting the COX-1 (*PTGS-1)* and COX-2 *(PTGS-2)* enzymes[7]. However, a unique feature that distinguishes aspirin from other NSAIDs is its ability to irreversibly inhibit COX activity. It does so by acetylating critical serine residues (Ser529 and Ser516 for COX-1 and COX-2 respectively) of the enzymes[6].

Prostaglandins (PGs) are made from the liberation of membrane-bound arachidonic acid by cytosolic phospholipase A_2[8]. Phospholipase A_2 usually requires calcium for activation[8]. COX-1 and COX-2 then catalyse the formation of prostaglandin (PG)H_2, which serves as an unstable biologic intermediate for the production of several prostanoids. Prostanoids are lipid mediators which mediate the inflammatory response. Examples of prostanoids include prostaglandins, such as prostaglandin E2 (PGE_2) and thromboxanes, (e.g., TXA_2)[7,9] (Figure 1.2). COX-1 is constitutively expressed in many cells and is also found in platelets; it is associated with regulating homeostatic functions of prostaglandins, whereas COX-2 is an inducible isoform that is linked to inflammation[10]. Stimuli such as growth factors and pro-inflammatory cytokines (e.g., interleukin-6, IL-6 and interleukin-8, IL-8) induce COX-2 expression[5]. It is therefore not surprising that the overexpression of COX-2 is documented in various types of malignancies, e.g., breast[11], pancreas[12], and colon[13]. Elevated levels of COX-2 enhance the production of prostaglandins, especially PGE_2, which favour downstream pro-survival and anti-apoptotic pathways in malignant cells. PGE_2, can also contribute to an immunosuppressive tumour microenvironment (TME)[14].

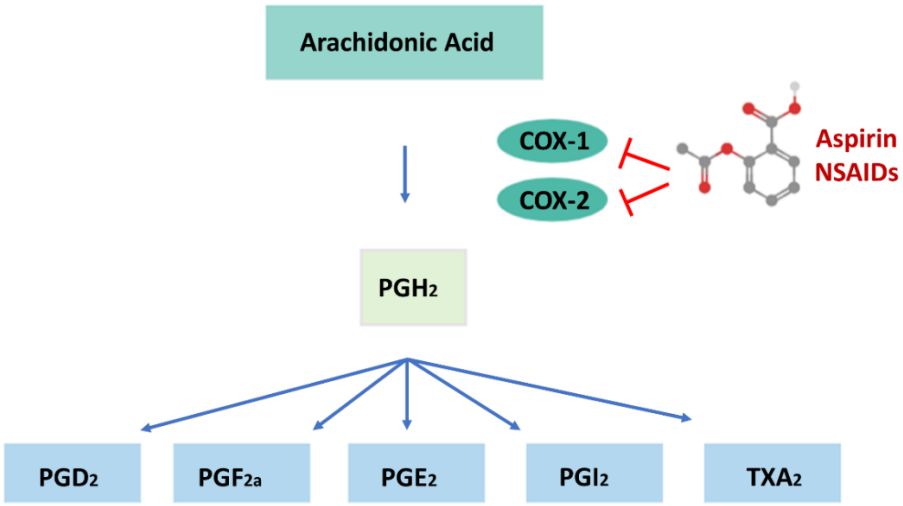


Figure 1.2. Metabolic pathway of arachidonic acid. The metabolism of arachidonic acid into prostaglandins (PG) is a two-step process. Cytosolic phospholipase A2 usually liberates membrane-bound arachidonic acid. Next, cyclooxygenase-1 (COX-1) and COX-2 catalyse the production of PGH_2, an intermediate molecule, which then forms the production of prostaglandins, including PGD_2, PGE_2, PGF_{2a}, PGI_2 and TXA_2. NSAIDs block the metabolism of arachidonic acid into prostanoids by inhibiting COX-1 and COX-2 enzymatic activity. Aspirin is the only NSAID that irreversibly inhibits COX-1 and COX-2 activity. It does so by acetylating critical serine residues (Ser529 and Ser516 for COX-1 and COX-2 respectively) of the enzymes.

1.1.3 Evidence for Aspirin as a Cancer Preventive Agent

There is compelling pre-clinical and epidemiological evidence about the cancer-preventive properties of aspirin. In 2016, the US Preventive Service Task Force (USPSTF) provided a grade B recommendation for using low-dose aspirin for the primary prevention of cardiovascular disease (CVD) and colorectal cancer (CRC) in adults aged 50-59, who have a ≥ 10% chance of developing CVD. Furthermore, these individuals should not be at increased risk for bleeding, are likely to have a life expectancy of at least 10 years, and willing to consume daily low-dose aspirin for at least 10 years[15]. A Grade B recommendation indicates that benefits outweigh risks[16]. Before the USPSTF, no organisation recommended aspirin for cancer prevention.

The cancer-preventive activities of aspirin were first reported as early as 1988. *Kune et al* used data from a large case-control study in Melbourne to reveal that aspirin reduced the risk of CRC[17]. The mechanisms underpinning this interesting finding were not, however, elucidated at that time, and are still not fully understood[17].

Twenty-three years later, Algra and Rothwell conducted a meta-analysis to examine the long-term effects of aspirin on cancer incidence and metastasis by comparing

evidence from observational studies and randomised controlled trials (RCTs). Case-control and cohort studies published from 1950 to 2011 were systematically reviewed. They found that aspirin decreased the risk of CRC (pooled odds ratio, OR= 0.62; 95% CI: 0.58-0.67, p<0.0001)[18]. These effects were also noticed in oesophageal, gastric, biliary and breast cancers (pooled OR= 0.62; 95% CI: 0.55-0.70; p<0.001)[18]. This trend is in accordance with a systematic analysis of individual patient data from two RCTs, the British Doctors Trial and the UK-Transient Ischaemic Attack (UK-TIA) Aspirin Trial. Here, Rothwell and colleagues demonstrated a 40% reduction in cancer incidence in aspirin-treated patients over a prolonged follow-up period of 20 years[19]. There was also a 20% reduction in cancer-associated mortality[19]. Similar outcomes were also seen in a meta-analysis of fifty-one randomised trials that were primarily designed to examine the effects of aspirin on the risks of vascular events (OR: 0.85, 95% CI: 0.76–0.96; P=0.008)[20].

Inferences from analyses of aspirin have also been observed in primary RCTs. Recently, results from the CAPP2 (Colorectal Adenoma/Carcinoma Prevention Programme) reported a 40% reduction in cancer incidence in individuals with Lynch Syndrome who had received 600mg of aspirin every day for a mean of 25 months[21]. Lynch Syndrome is caused by a defect in the mismatch repair pathway, predisposes to CRC and is implicated in approximately 5% of CRC cases[22]. The key clinical studies investigating the cancer-preventive potential of aspirin have been summarised in Table 1.

Table 1. Key clinical studies examining the cancer-preventive potential of aspirin.

Study	Number of participants	Aspirin dose used	Intervention and follow-up period	Outcome Odds ratio (OR) (95% CI)
Long-term follow-up of low-dose aspirin on colorectal cancer risk	7,588	300-1200mg per day	23 years - follow-up period 5 years intervention period	0.74 (0.56-0.97)
Pooled analysis of 6 trials of low-dose aspirin for the primary prevention of vascular events	35, 535	75-100 mg per day	Mean intervention and follow-up period : 4.8 years, dependent on trial	0.71 (0.57-0.89)
Pooled analysis of 3 UK-based trials on the long-term effect of aspirin on cancer	12,659	75-1200mg per day	20-year follow-up period (median intervention period of 4-7 years)	0.78 (0.70-0.87)
Analysis of 34 trials of daily aspirin for preventing vascular events	69,224	40-1500mg per day	Mean intervention and follow-up period of 1-8 years depending on the trial	Any dose- 0.63 (0.49-0.82) Low-dose (<200mg) 0.63 (0.46-0.86)

The odds ratio, OR, measures the association between exposure and outcome. In this instance, the OR quantifies the relationship between aspirin treatment and disease risk.

1.2 Inflammation and Cancer

The longstanding interest in the cancer-preventive potential of anti-inflammatory drugs such as aspirin is linked to the critical role played by inflammation in modulating tumourigenesis. Especially in light of recent evidence, it is not surprising that aspirin has sparked significant attention as a chemo-preventive agent.

Inflammation can be acute or chronic. Acute inflammation is a short-lived, self-limiting response to tissue injury, and is associated with the active clearance of cell debris and immune cells. Conversely, chronic, dysregulated, unresolved inflammation increases the risk of carcinogenesis[23]. Often referred to as the 'fuel' that 'feeds the cancer flame', tumour-promoting inflammation is an important hallmark of neoplastic progression[24].

The interplay between inflammation and cancer was first postulated in 1863 by Rudolf Virchow[24]. Over the years, there has been a substantial increase in our knowledge and understanding of the tumour microenvironment, TME, and its pivotal role in propagating carcinogenesis, as tumours are more likely to thrive in an aberrant, exuberant inflammatory milieu. There are several factors which can lead to chronic inflammation, including infection, contact with toxic substances such as asbestos, or autoimmune conditions[23]. 15-20% of cancer deaths worldwide are linked to underlying infection and inflammatory responses[23,25]. For example, exposure to human papilloma virus (HPV) increases the risk of cervical cancer, hepatitis B and C for hepatocellular carcinoma (HCC), ulcerative colitis for colorectal cancer, and *Helicobacter pylori* for gastric tumours[23,26].

There are two main pathways that lead to cancer-associated inflammation: the intrinsic pathway and the extrinsic pathway (Figure 1.3). The intrinsic pathway is associated with oncogenic changes that promote inflammation and neoplastic progression[25]. The extrinsic pathway is derived from pre-disposing inflammatory conditions that augment cancer risk (such as inflammatory bowel disease and colorectal cancer)[23]. These two pathways are not mutually exclusive.

It is now clear that during the process of malignant transformation, cancer cells produce inflammatory mediators that help establish the TME. In this

microenvironment, cancer-intrinsic or cancer-extrinsic inflammation leads to the constitutive activation of transcription factors in tumour cells which would otherwise be transiently expressed. One such example is NFκB, which drives the transcription of anti-apoptotic genes and pro-inflammatory cytokines and chemokines, including IL-6, IL-8 and TNF-α[27,28]. NFκB can also upregulate the expression of COX-2 and subsequent release of prostaglandins, especially PGE_2[29]. Furthermore, cytokines and chemokines can also recruit immune cells, such as tumour associated macrophages, myeloid cells, and regulatory T cells via autocrine and paracrine signalling, consequently orchestrating a complex, interconnected web of tumour promoting pro-inflammatory mediators. Collectively, this steers angiogenesis, invasion and metastatic spread [23].

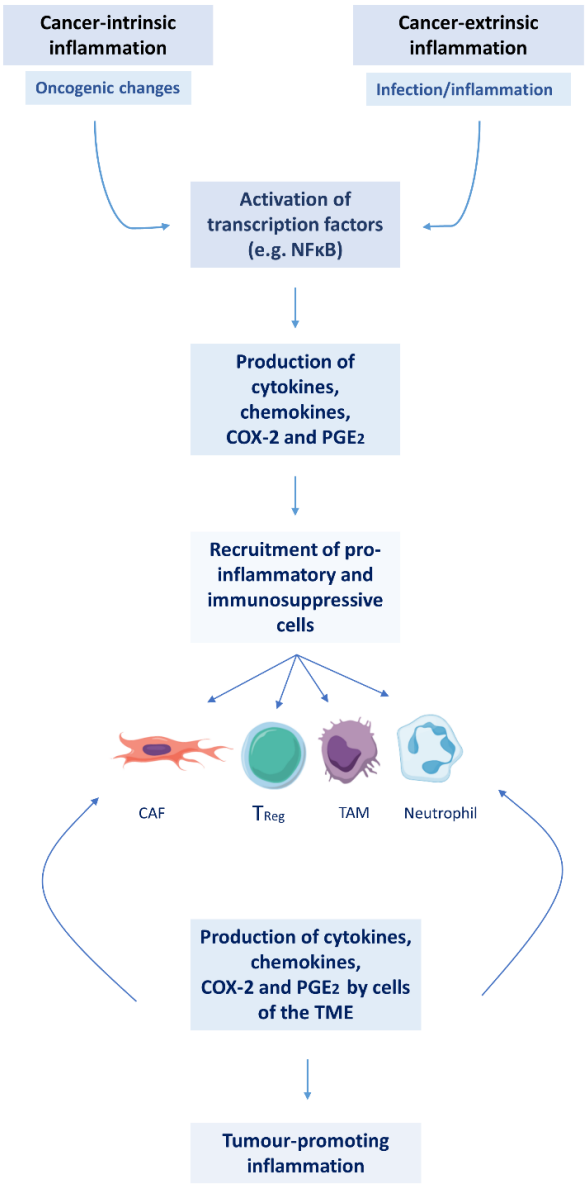


Figure 1.3. Cancer-related inflammation. There are two pathways associated with tumour-promoting inflammation: the intrinsic pathway and the extrinsic pathway. The former is linked to changes in oncogenes, whereas the latter correlates with predisposing conditions that increase neoplastic transformation. The two cascades converge, leading to the constitutive activation of transcription factors (e.g., NFκB) which would only be transiently activated in a normal physiological scenario. NFκB stimulates the production of pro-inflammatory mediators, including COX-2, leading to elevated levels of PGE_2 in the TME. Cytokines and chemokines can also recruit immune and stromal cells, such as $T_{Reg,}$ TAMs and CAFs. Moreover, these cells produce COX-2, and COX-2-induced-PGE_2, creating a positive feedback loop. Jointly, this creates a favourable microenvironment for malignant cells to thrive.

1.2.1 Prostaglandin E_2 and cancer

Prostaglandins are some of the earliest molecular responders to inflammatory stimuli. Out of all prostanoids, PGE_2 is the most abundant prostaglandin found in malignancies, especially in gastrointestinal tumours[30]. The roles of other prostaglandins (PGD_2, PGE_2, PGF_{2a}, PGI_2) and thromboxane (TXA_2) in cancer are not as well understood. Prostaglandins elicit their biological effects by binding to their cognate G-protein coupled receptor(s) (GCPR). In the case of PGE_2, it binds to one of four receptors: EP1 (also referred to as PTGER1), EP2 (PTGER2), EP3 (PTGER3) or EP4 (PTGER4)[31].

The level of PGE_2 in tumour tissue depends on the equilibrium between PGE_2 synthase and 15-hydroxyprostaglandin dehydrogenase (15-PGDH)[14,31]. PGE_2 synthase is responsible for the production of PGE_2. Conversely, 15-PGDH is responsible for the degradation of PGE_2 into its metabolite, PGE-M. 15-PGDH is present in normal tissue, but its expression is lost in cancer[32]. Professor Andrew Chan and colleagues have demonstrated that urinary PGE-M can be used as a biomarker to measure PGE_2 levels, and subsequently predict CRC risk and prognosis[33]. The same group has also shown that the regular use of aspirin in patients with high 15-PGDH confers a better overall survival compared to those with low 15-PGDH in the colonic mucosa[33].

PGE_2 directly induces the proliferation and survival of epithelial cancer cells through numerous signalling cascades, including the Wnt/βcatenin and mitogen-activated kinase (MAPK) pathways[31,34,35] (Figure 1.4). For instance, PGE_2 acts on the 'destruction complex' of the Wnt pathway by inhibiting glycogen synthase kinase 3-beta (GSK-3β)[36]. This prevents the phosphorylation and degradation of its downstream target, β-catenin. β-catenin then translocates to the nucleus and promotes tumour cell proliferation. PGE_2 can trans-activate epidermal growth factor receptor (EGFR), which results in the activation of the Ras-Erk cascade, as seen in HT-29 CRC cells *in vitro*[37]. In addition, PGE_2–activated EGFR stimulates PI3K-Akt, which promotes CRC cell migration and invasion[37].

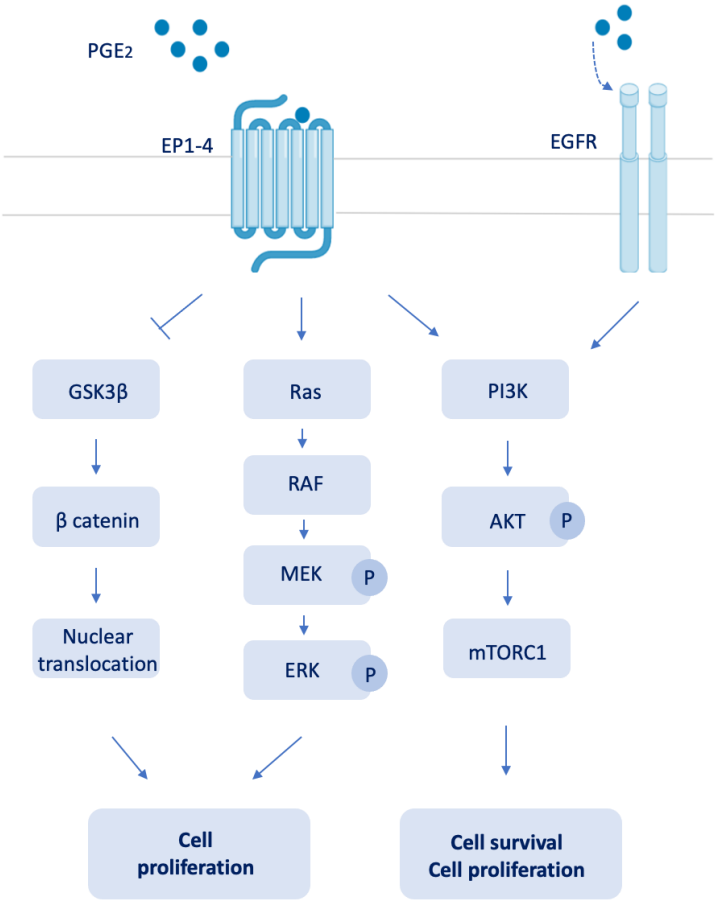


Figure 1.4. Effects of PGE_2 on epithelial cancer cells. PGE_2 binds to its cognate G-protein coupled receptor (EP1, EP2, EP3 or EP4) and promotes malignant cell proliferation and survival through several signalling cascades, including Wnt, MAPK and PI3K pathways. PGE_2 inhibits GSK3β through EP4 signalling, preventing the degradation of β-catenin. β-catenin then translocates to the nucleus and promotes the transcription of genes associated with malignant cell proliferation. The activation of the Ras-Erk cascade by EP signalling can also induce a hyperproliferative phenotype. Furthermore, PGE_2 can trans-activate EGFR, which results in downstream activation of the PI3K cascade, consequently promoting malignant cell survival and tumourigenesis.

Equally, PGE_2 is a powerful mediator in generating an immunosuppressive TME through several mechanisms (figure 1.5). For instance, PGE_2 inhibits anti-tumour helper T cell (T_H)1 responses by downregulating T_H1 cytokines (e.g. IL-2 and IFN-γ), and upregulating pro-tumourigenic, immunosuppressive T_H2 cytokines, including IL-4, IL-10 and IL-6[38]. PGE_2 can also directly suppress the activation of cytotoxic T cells and natural killer cells and induce the expansion of regulatory T cells (T_{reg})[31,38,39]. Additionally, PGE_2 is also believed to recruit myeloid cells which secrete high levels of IL-6 and CXCL1 in murine models of melanoma, as shown by Zelenay *et al* [40].

Furthermore, PGE_2 mediates the interactions between T cells and dendritic cells. It does so by altering the balance of the IL-12/IL-23 axis by favouring IL-23 production[30,31,38]. IL-23 enables the expansion and survival of T_H17 cells, which are pro-inflammatory in nature, whereas IL-12 inhibits T_H17 production and favours a T_H1 response. Moreover, PGE_2 can also impair antigen presentation in dendritic cells by switching its function from induction of immunity to T cell tolerance[41]. As a result, dendritic cells lose their capacity to cross-present tumour antigens with major histocompatibility class I molecules (MHC), which subsequently prevents the activation of an anti-tumour $CD8^+$ T cell response[31,38,41].

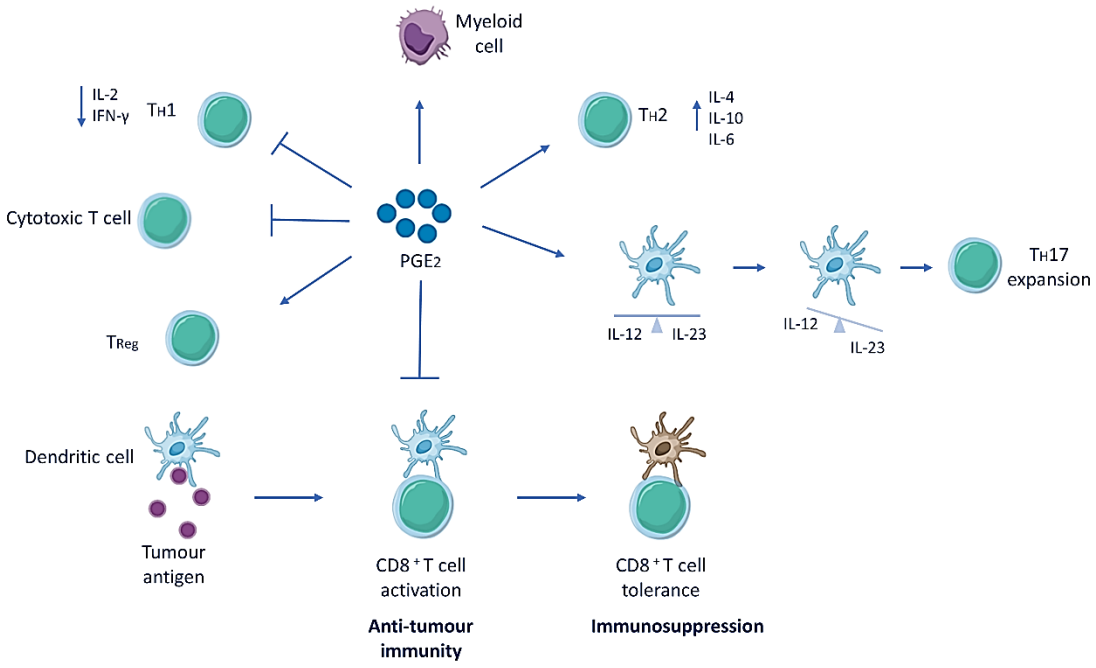


Figure 1.5. Effects of PGE_2 on the tumour microenvironment. PGE_2 inhibits T_H1 responses and upregulates T_H2-induced cytokine secretion, leading to elevated levels of IL-4, IL-10, and IL-6 levels. Furthermore, PGE_2 blocks cytotoxic T cell responses, and stimulates the upregulation of immunosuppressive T_{Reg} lymphocytes. Moreover, PGE_2 shifts the IL-12/IL-23 balance in dendritic cells by favouring IL-23 production. This promotes the expansion of pro-inflammatory T_H17 cells. PGE_2 also interferes with the interactions between T cells and dendritic cells by switching the function of dendritic cell from induction of immunity to T cell tolerance. Consequently, dendritic cells lose the capacity to activate $CD8^+$ T cells, which further promotes the generation of an immunosuppressive microenvironment.

Reversing COX_2-PGE_2-induced immunosuppression remains a major challenge. Nonetheless, by gaining a deeper insight into the diverse effects of PGE_2 in the tumour and its surrounding microenvironment, we can aim to exploit these targets

with anti-inflammatory agents, including aspirin, as well as EP antagonists, which are currently being investigated as therapies[42,43].

1.3 Potential Cancer Preventive Mechanisms of Action of Aspirin

Aspirin has a unique set of pharmacological properties which may be helpful in understanding its cancer-preventive mechanisms of action. Aspirin is rapidly absorbed in the stomach and gastrointestinal tract. Peak plasma levels occur 30-40 minutes after aspirin administration[44]. The peak plasma concentrations of aspirin after low-dose aspirin (75-100mg) is 7.31μM, 28-40 μM after medium dose aspirin (325-600mg/4-6h), and 142μM after high-dose aspirin (1200mg/4-6h)[44]. Notably, oral-dose aspirin has a short half-life of 15-20 minutes and a poor bioavailability of 40-50% of the dose given, due to rapid hydrolysis by hepatic and plasma esterases[1,45].

In view of the above information, low-dose aspirin (75-100mg) is likely to target cells which either completely lack or have very limited capacity of re-synthesising COX enzymes *de novo*[9,10]. Given this information, one possible mechanism by which aspirin may exert its cancer-preventive effects is by inhibiting platelet activation[6,9,10,45,46]. Furthermore, inhibition of COX-1 activity in platelets may also further suppress the induction of COX-2 in adjacent, nucleated cells, which could potentially reduce subsequent activation of pro-tumourigenic and anti-apoptotic pathways[6].

There is emerging evidence highlighting the important role played by platelets in propagating invasion and metastasis[9,10]. Platelets are small, anucleate cells generated from megakaryocytes in the bone marrow that are traditionally associated with regulating haemostasis[47]. Moreover, it is now recognised that they secrete extracellular vesicles, such as exosomes, as well as oncogenic microRNAs to augment cross-talk with cancer cells, which facilitates tumour progression[47]. Furthermore, a recent study published by Johnson *et al.* demonstrates that the cross-talk between platelets and tumour cells upregulates IL-8 levels in triple negative breast cancer cell lines, which potentiates malignant cell invasion[48].

Additionally, aspirin may exert its therapeutic effects by directly inhibiting COX-2 in malignant and stromal cells. This inhibits the production of PGs, including PGE_2, and

its downstream pro-tumourigenic pathways. I have summarised the possible ways by which aspirin may elicit its cancer-preventive effects in Figure 1.6.

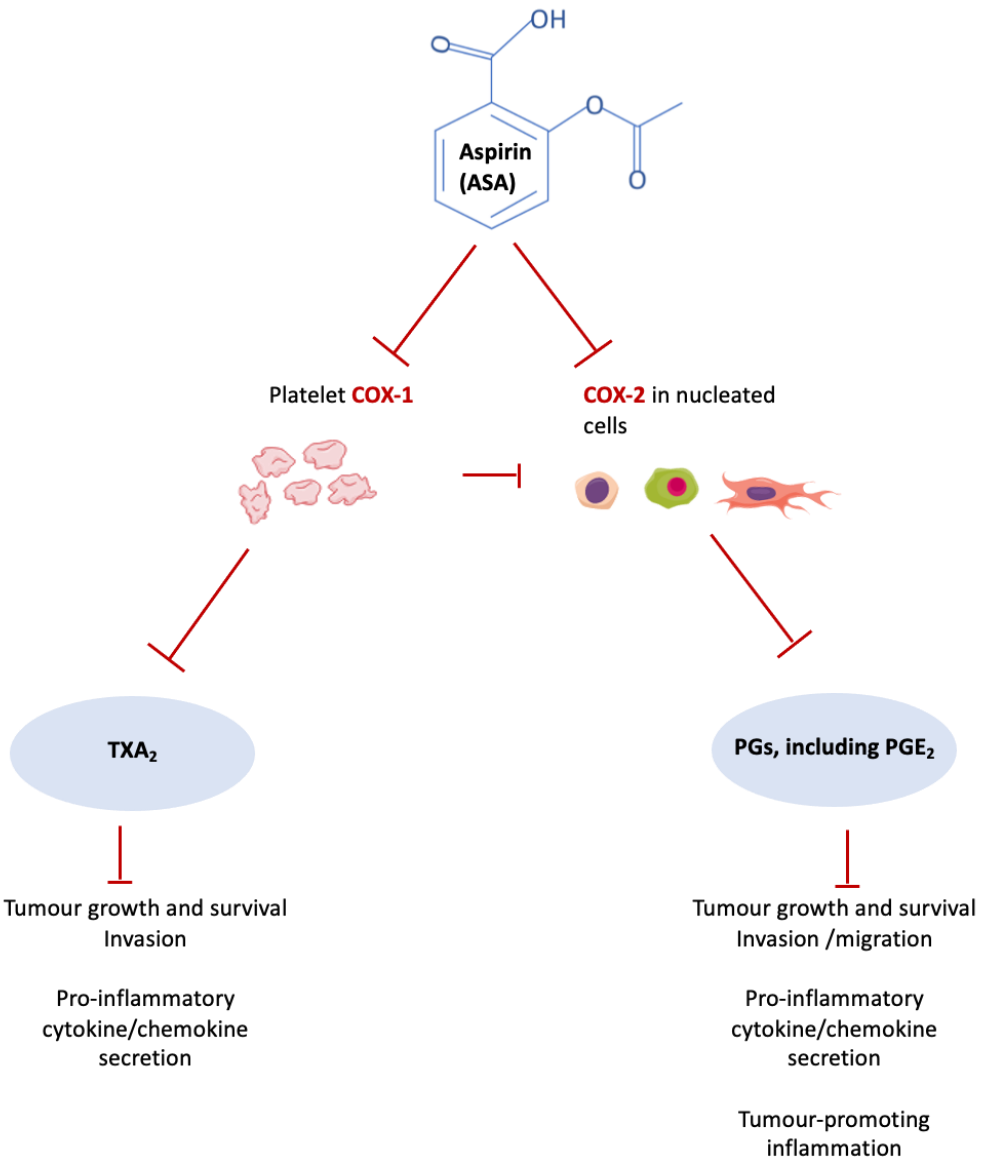


Figure 1.6. Possible therapeutic mechanisms of action of aspirin. There are several ways by which aspirin may exhibit its cancer-preventive properties. Firstly, aspirin can block platelet COX-1, which hampers cross-talks between platelets and tumour cells, and reduces tumourigenesis. Secondly, inhibiting platelet COX-1 can also inhibit COX-2 in adjacent nucleated cells, such as malignant cells and fibroblasts. Thirdly, aspirin may directly block COX-2 in nucleated cells. This inhibits the production of prostaglandins, such as PGE_2, and its downstream pro-tumourigenic effects.

1.4 Current Limitations of Aspirin as a Primary Cancer Preventive Agent

Recommendation of routine prescription of low-dose aspirin as a prophylactic agent has been controversial, as prolonged platelet inactivation by aspirin inhibits the coagulation cascade, which can lead to serious gastrointestinal bleeding and intracranial haemorrhage[7,16,49]. These concerns were raised at the European Society of Cardiology Congress in August 2018[50], after presenting findings from three major clinical trials (ASCEND[51], ARRIVE[52] and ASPREE[53]).

Results from the ASPREE (Aspirin in Reducing Events in the Elderly) study demonstrated an increase in all-cause mortality following aspirin administration compared to placebo (hazard ratio, HR 1.14, 95% CI 1.01-1.29)[50,53]. Although this difference is not statistically significant, the trial terminated early (median time 4.7 years) due to elevated levels of bleeding events in aspirin users compared to untreated individuals (HR 1.38, 95% CI 1.18-1.62)[53]. A grade I recommendation (i.e., insufficient evidence) has been indicated for individuals aged 70 and above[15,17]. However, it is not surprising that there was a 9-fold increase in bleeding events reported in the ASPREE study compared to other aspirin-RCTs, as the risk of bleeding increases substantially with age[54].

In 2022, the USPSTF revised its guidelines regarding the use of aspirin as a cancer-preventive agent against CVD and CRC[55]. They recommended against using low-dose aspirin for the primary prevention of CVD in individuals aged 60 years or older (grade 'D'), and also withdrew its recommendation against the prevention of CRC. However, as emphasised by Chan *et al.* , the USPSTF updated their guidelines largely based on the ASPREE trial, which is not representative of all studies examining the cancer-preventive effects of aspirin.[53] As an example, the ASPREE trial was conducted in people who were aged 70 or above, and the majority of individuals (85%) did not consume aspirin prior to the trial. The cancer-preventive benefits of aspirin are likely to be seen in individuals who start to take aspirin from a young age, as evidenced in a recent study by Professor Chan's group[56]. Here, they demonstrated that aspirin

reduced CRC risk in older adults aged 70 or above, but only in participants who took daily low-dose aspirin from a young age[56]. Furthermore, it is important to note that the cancer-preventive effects of aspirin are usually seen after a prolonged follow-up period of at least 10 years, as evidenced in the Women's Health Study[57]. Moreover, the USPSTF did not consider results from the CAPP2 study, which clearly exhibited the reduction of CRC incidence in people with Lynch syndrome who took aspirin for 10 years. Currently, a follow-up study from CAPP2, namely CAPP3, is exploring the optimal dose of aspirin for people with Lynch syndrome by allocating participants with 100, 300 or 600 mg of enteric-coated aspirin every day[58].

The current guidelines updated by the USPSTF may slow down research examining the use of aspirin for cancer prevention. Nevertheless, there are promising studies highlighting aspirin's cancer preventive properties, and future trials such as CAPP3 will shed light regarding the optimal use of aspirin required for long-term cancer prevention.

The next section of my introduction will focus on high-grade serous ovarian carcinoma (HGSOC), as I am interested to investigate the effects of aspirin in this cancer type.

1.5 High-grade serous ovarian carcinoma (HGSOC)

1.5.1 Background

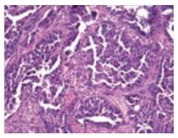
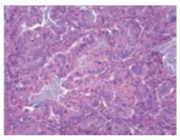
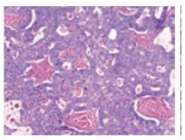
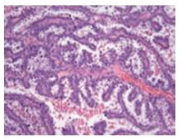
Ovarian cancer is one of the most lethal gynaecological malignancies, accounting for more than 140,000 deaths worldwide[59]. It confers a poor prognosis with a 5-year overall survival of only 44%[60]. Most patients are diagnosed with advanced stage disease (stages III/IV), often with extensive metastatic dissemination[61]. Five-year survival rates can be as high as 90% if detected early[62]. Nevertheless, this is confronted by the highly non-specific nature of early-stage symptoms, such as abdominal bloating or lower back pain[63]. There is therefore a need to improve early-stage detection strategies.

For a long time, the term 'ovarian cancer' was perceived as one disease. However, it is now acknowledged that ovarian cancer is heterogenous in nature. 90% of ovarian

cancers are epithelial in origin[63]. There are four distinct histological subtypes of epithelial ovarian cancers (EOC): serous, mucinous, endometrioid, and clear cell, as summarised in Table 2.[61]

Table 2. The different subtypes of epithelial ovarian cancers

(Histological images obtained from Vaughan *et al, 2011*[64])

EOC subtype	**High-grade serous**	**Mucinous**	**Endometrioid**	**Clear cell**
Possible origin of tumour	Fallopian tube (majority of cases) Ovary	Stomach Colon	Endometriosis-associated with retrograde menstruation from the endometrium	Endometriosis-associated with retrograde menstruation from the endometrium
Histological image				

Most EOCs (70%-80%) are high-grade serous in nature[61]. A majority of high-grade serous ovarian cancers (HGSOC) are now thought to arise in the distal fallopian tube from precursor lesions referred to as serous tubular intra-epithelial carcinoma (STICs), which shed malignant cells to the adjacent ovaries[65]. HGSOC cells bear mutations in p53 (*TP53)* p16 (*TP16), BRCA* mutations and other double stranded DNA repair deficiencies[66].

1.5.2 Treatment of HGSOC

A multitude of factors, such as disease stage, age, prior treatments, and co-morbidities are taken into consideration in order to provide the optimal therapeutic strategy for patients with HGSOC[61].

The standard mode of treatment relies on cytoreductive surgery combined with chemotherapy[67]. The main objective of surgery is to remove all macroscopically visible tumours with no residual disease left (termed as 'R0')[63]. Surgery is also used to classify the stage of the tumour using the International Federation of Gynaecology and Obstetrics (FIGO) guidelines[63]. Sometimes, surgery is postponed until after three

cycles of chemotherapy, known as neo-adjuvant chemotherapy, due to extensive disease present. This is referred to as interventional debulking surgery.

The majority of patients with HGSOC (up to 80%) respond well to initial chemotherapy, but disease progression is inevitable[68]. This has led to the incorporation of targeted agents, such as bevacizumab, into the treatment landscape of HGSOC[61,63]. Bevacizumab is an anti-angiogenic monoclonal antibody which targets vascular endothelial growth factor, VEGF. Bevacizumab is usually given in combination with chemotherapy, and then used as a single agent for up to a maximum period of 15 months[69]. It is administered as an intravenous infusion once every three weeks. According to the National Institute for Health and Care Excellence (NICE), the licensed dose of bevacizumab is 15mg/kg of body weight[70]. Bevacizumab has shown to significantly improve progression free survival in patients with ovarian cancer, as evidenced by two large scale phase III clinical trials: the Gynaecologic Oncology Group (GOG)-0218 and the International Collaboration on Ovarian Neoplasms (ICON-7, ISRCTN91273375)[71,72]. It is now routinely incorporated into the frontline management of HGSOC.

More recently, poly-ADP-ribose polymerase (PARP) inhibitors have revolutionised HGSOC treatment in some patients [64,73]. PARP enzymes (especially PARP1 and 2) play a crucial role in the base excision repair (BER) of single-stranded DNA breaks (SSBs)[74]. Therefore, inhibiting PARP enzymes prevents the repair of SSBs. Persistent SSBs lead to the collapse of replication forks, which eventually results in the accumulation of double-stranded breaks (DSBs)[74]. In healthy cells, DSBs can be repaired through homologous recombination (HR). However, in HR-deficient cells (e.g. those with mutations in *BRCA1/2),* DSBs cannot be repaired, leading to cell death[63,67].

HGSOC patients with mutations in *BRCA1/BRCA2* display sensitivity to PARP inhibitors, such as olaparib and niraparib[67,64]. These PARP inhibitors have been extensively analysed in phase II and III clinical trials, and are now being used as routine maintenance treatment for women with *BRCA1/2*-mutant advanced ovarian cancer[73,75]. Moore *et al.* conducted a phase III trial to evaluate the efficacy of olaparib in patients with newly diagnosed advanced ovarian cancers, including HGSOC. They demonstrated that the risk of disease progression was reduced by 70% in patients

who received olaparib treatment compared to placebo[73]. Similarly, Ledermann *et al* carried out a phase II study to examine olaparib maintenance treatment in patients with platinum sensitive, relapsed HGSOC[75]. They showed that progression-free survival (PFS) was significantly elevated in patients who received olaparib compared to placebo (median PFS 8.4 months vs 4.8 months, respectively).

1.5.3 Inflammation and HGSOC

Inflammation is a common mechanism underpinning epithelial ovarian cancer (EOC) development. In 1980, Lawrence Espey proposed that ovulation is comparable to an inflammatory reaction[76]. This is known as the 'incessant ovulation hypothesis,' which suggests that recurrent damage of the ovarian epithelium over time causes defective DNA repair, increased oxidative stress, elevated levels of pro-inflammatory mediators, and ultimately malignant cell transformation[77]. Pro-inflammatory cytokines such as IL-6 and TNFα, as well as lipid mediators, including PGE_2 and potentially PGF_{2a} are elevated within the first few hours of ovulation[77,78]. Perhaps, incessant ovulation could generate an inflammatory microenvironment that may favor fallopian tube epithelial cells to form STICs and subsequent malignant cell transformation.

In addition, the ovarian cancer microenvironment is instrumental in providing malignant cells with a pro-inflammatory milieu of stromal cells, immune cells, secreted factors and a dense extracellular matrix (ECM) to orchestrate distant metastasis and resistance[68]. Unlike the vast majority of solid tumours that spread haematogenously, high-grade serous ovarian cancers typically diffuse via the peritoneal cavity to the omentum and other sites in the peritoneum[79], as represented in Figure 1.7. The omentum is one of the sites of HGSOC metastasis, and is home to an abundance of energy-rich adipocytes. Anatomically, the omentum is a fold of visceral peritoneum and is subdivided into lesser and greater sections. It is host to a plethora of cell types that promote metastasis[61,79]. These include mesothelial cells, cancer-associated fibroblasts, adipocytes, and immune cells, such as tumour associated macrophages and regulatory T cells.

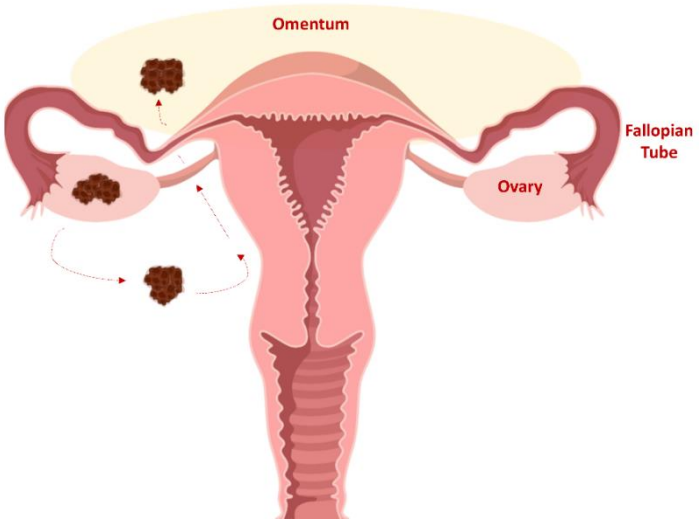


Figure 1.7. Spread of HGSOC cells from the ovary to the omentum. This is a simplified figure which illustrates primary HGSOC tumours metastasising into the omentum via the peritoneal cavity. The vast majority of high-grade serous ovarian cancers are believed to arise from fallopian tube epithelial cells, which can undergo malignant transformation to form serous tubular intra-epithelial carcinomas (STICs). STICs shed malignant cells to adjacent ovaries, which then diffuse into the omentum. HGSOC cells preferentially metastasise to the omentum, as it is host to adipocytes, a useful source of fuel for cancer cells. This figure was created using MindTheGraph.

1.5.3.1 Mesothelial cells

Mesothelial cells in the peritoneum are the first mechanical barrier faced by HGSOC cells, and have been previously referred to as a 'bystander' of invasion[68,80]. The mesothelium consists of a single layer of cells and expresses markers such as calretinin and mesothelin[81]. Mesothelial cells promote malignant cell invasion and migration. Kenny *et al* showed that mesothelial cells secreted fibronectin in the presence of ovarian cancer cells, which promoted malignant cell invasion via αvß5 signalling[80]. More recently, Quian *et al* showed that mesothelial cells are key drivers of chemo-resistance, and therapeutically blocking osteopontin, a protein secreted by mesothelial cells, could enhance sensitivity to chemotherapy[82]. Furthermore, mesothelial cells can secrete chemokines, such as interleukin 8 (IL-8), to promote malignant cell invasion[83]. IL-8 can then bind to its receptor CXCR1, to activate PDK1 in malignant cells[83]. This can promote the downstream signalling of integrins, enabling malignant cells to adhere to mesothelial cells, and eventually invade into the sub-mesothelial layer[83]. Furthermore, previous work in our laboratory by

Malacrida *et al.* demonstrated that mesothelial cells activated by platelets stimulate malignant cell invasion into the omentum in multi-cellular models of HGSOC[84].

1.5.3.2 Cancer associated fibroblasts, CAFs

Cancer associated fibroblasts (CAFs) are implicated in almost every type of solid tumour, and high densities correlate with a poor prognosis[85]. They are a heterogenous population of cells and are profusely present in the cancer stroma[86]. Traditionally, markers such as alpha-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP) have been used to define CAFs[85].

There are different subpopulations of CAFs, such as myCAFs (myofibroblast-like CAFs), iCAFs (inflammatory CAFs) and apCAFs (antigen-presenting CAFs). myCAFs usually express high levels of α-SMA (α-SMA^{high}), are in close contact with tumour cells, and contribute to aberrant ECM deposition[87]. They are activated by TGFβ in pancreatic cancer, and are associated with immunosuppression[88]. iCAFs usually express high levels of IL-6 (IL-6^{high}) and leukaemia inhibitory factor (LIF)[87]. Recently, a study showed that a the IL1α/hypoxia axis promoted an iCAF phenotype in pancreatic cancer[89]. apCAFs express major histocompatibility complex class II (MHC class II) molecules and are associated with regulating tumour immunity. Huang *et al.* showed that apCAFs are derived from mesothelial cells and promote the formation of regulatory T cells in pancreatic cancer[90].

CAFs are activated following various pro-inflammatory stimuli. Cytokines such as TNFα can activate CAFs and induce EGFR signalling, which stimulates malignant cell growth[27]. CAFs can also secrete immunosuppressive factors, including COX-2-induced PGE_2, to directly repress the anti-tumour activity of CD8+ T lymphocytes and natural killer, NK, cells[91]. They also modulate the remodelling of the ECM by upregulating the expression of MMPs[85]. This results in matrix degradation, which physically creates space for malignant cell growth and migration.

1.5.3.3 Adipocytes

Adipocytes are important in fuelling malignant cell growth in the omentum. Interactions between cancer cells and adipocytes result in the re-programming of adipocytes into cancer-associated adipocytes (CAA)[68]. This cross-talk also enhances

the production of growth factors, hormones (e.g. adiponectin) and pro-inflammatory cytokines, termed 'adipokines'[92]. This triggers the lipolysis of adipocytes, releasing glycerol and fatty acids[93]. These fatty acids are then used by tumour cells as a means to generate energy to meet the demanding physiological requirements for the hyperproliferative cancer cells.

1.5.3.4 Immune cells

Immune cells are an important part of the TME and play a crucial role in propagating tumour progression.

1.5.3.4.1 Tumour associated macrophages, TAMs

Macrophages are phagocytic cells which are derived from monocytes. Macrophages are heterogenous in nature, and can be broadly classified as 'classically activated' or 'alternatively activated.' TAMs usually resemble the 'alternatively activated' phenotype and express markers such as CD163 and CD206. TAMs are recruited to the tumour site via chemotactic signals, and a high infiltration of TAMs is linked to a poor prognosis[94,95]. TAMs interact closely with other components within the TME to drive tumour progression, such as CAFs, endothelial cells, cytokines/chemokines, and other immune cells[25]. For example, TAMs can stimulate angiogenesis by secreting VEGF and IL-8[96]. TAMs can also promote invasion and migration by stimulating the activation of MMP9/VEGF in a COX-2 dependent manner[97]. TAMs can also secrete growth factors, such as epithelial growth factor, EGF, which binds to EGFR and induces pro-tumourigenic signalling cascades[94,98]. Recent work from our group shows that CD163+ macrophages produces the TGFβ induced protein (TGFBI, an ECM protein), proximal to STIC lesions, which contributes further to an immunosuppressive microenvironment in HGSOC[99].

1.5.3.4.2 Tumour associated neutrophils, TANs

Tumour associated neutrophils (TANs) have also emerged as an important component of the TME. TANs can stimulate angiogenesis, ECM remodelling, metastatic spread, and immunosuppression. TANs can stimulate malignant cell proliferation by secreting a whole host of growth factors, including EGF, hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF)[100]. Neutrophils can also overexpress the fatty acid transport 2 protein (FATP2), which drives the

metabolism of arachidonic acid into PGE_2[101]. Moreover, the release of neutrophil extracellular traps (NETs) can trigger the production of MMPs, such as MMP9, which facilitates malignant cell invasion[102].

1.5.3.4.3 Tumour infiltrating lymphocytes

Tumour infiltrating lymphocytes (TILs) are an important part of the immune microenvironment. Examples of TILs include CD4+ and CD8+ T cells, regulatory T cells (T_{reg}), NK cells and B cells.

CD8+ intraepithelial TILs have been linked to a better prognostic outcome in HGSOC. Almost 20 years ago, Prof. Coukos's group analysed the relationship between CD3+ TILs and survival in advanced ovarian cancer using 186 frozen tumour samples[103]. They demonstrated that 102 samples with detectable levels of intraepithelial TILs had a 5-year overall survival of 38%, and patients whose samples had no TILs had a 5-year overall survival of 4.5%[103]. Similarly, Huang *et al.* performed a meta-analysis of 10 studies with 1815 patients with ovarian cancer[104]. They showed that increased CD8+ TILs conferred a better survival benefit and a lack of intraepithelial TILs was associated with a poorer survival (pooled hazard ratio, HR: 2.24, 95% CI; 1.71-2.91)[104]. CD4+ T cells are also important in anti-tumour immunity. Referred to as helper T cells, CD4+ T cells have historically known to help CD8+ T cells to produce a cytotoxic response[105]. However, it is now known that CD4+ TILs can also directly induce a cytotoxic effect on tumours, as demonstrated by Quezada *et al* in 2010[106].

Regulatory T cells (T_{reg}) are CD4+ T cells that play a crucial role in the ovarian TME. It is well known that T_{reg} favour the generation of an immunosuppressive microenvironment[31,107]. Curiel *et al* showed that the recruitment of T_{reg} to the tumour site confers a poorer overall survival in 106 patients with ovarian cancer[108]. A more recent study published in 2022 characterised the immune landscape of HGSOC tumours by examining multi-omics data from 495 TCGA HGSOC tumours and RNA-sequencing data from 1708 HGSOC tumours[109]. They showed that increased activation of CD8+ T cells and reduced T_{reg} were linked with a better prognosis[109]. Furthermore, as described in section 1.2.1, PGE_2 promotes the recruitment of T_{regs} into the microenvironment. Recently, a Cell Reports paper demonstrated that the PGE_2-EP2/EP4 signalling axis stimulated mature immuno-regulatory dendritic cells

(recently classified as 'mRegDC'), which promoted T_{reg} chemo-attractants, such as CCL17 and CCL22[110]. On the other hand, there is some evidence to suggest that high numbers of T_{Regs} can confer a good prognosis. For instance, Salama *et al.* investigated the prognostic significance of T_{Regs} in CRC. They demonstrated that FOXP3+ T_{Regs} infiltration in tumour tissue (but not in normal colonic mucosa) improved survival (HR=0.54;95%CI, 0.38-0.77)[111]. Similarly, Badoual *et al.* investigated the prognostic value of tumour-infiltrating T_{Regs} in 84 patients with head and neck cancers[112]. They deduced that increased levels of FOXP3+ T_{Regs} was linked with a favourable prognosis.

Natural killer (NK) cells are cytotoxic lymphocytes designed to recognise and mediate the killing of stressed cells, such as infected cells or malignant cells[113]. NK cells are part of the 'innate lymphoid cell' (ILC) family and produce cytokines such as IFN-γ [114]. NKG2D, NKp30 and NKp46 are examples of activating NK cell receptors which recognise and bind to ligands linked with stress pathways[115]. However, the TME can suppress NK cell activity. For instance, malignant cells and produce TGF-β, which can inhibit the activation of NK cell receptors[113]. Furthermore, Santiago Zelenay's lab showed that COX-2-induced PGE_2 secretion from malignant cells bind to EP2/EP4 receptors on NK cells, which hampers NK cell activity and facilitates immune cell invasion[116]. They also confirmed that NK cell mediated killing was seen in tumours deficient of EP2 and EP4 receptors. Similarly, NK cell-induced tumour lysis was seen in *PTGS2*-deficient tumours. This publication goes in line with their previous work published in 2015, where they highlighted the role of COX-2 in immune evasion and tumour progression[40]. It would be interesting to explore whether COXIBs (COX-2 inhibitors) and NSAIDs may potentially reverse PGE_2-mediated immunosuppression in NK cells.

B cells are also involved in mediating an anti-tumour response, although less is known about the role played by B cells in the TME. Higher densities of CD20+ B confer a better prognosis[107]. In 2017, Montfort *et al* showed that the presence of CD20+ B cells in omental metastases of HGSOC favoured the generation of an anti-tumour response[117]. Moreover, Santoeimma *et al* showed that the presence of CD20+ and CD8+ TILs positively correlated with a better overall survival in EOCs[118]. Similarly,

Edin *et al.* demonstrated that higher levels of CD20+ B cells in the stroma conferred a survival benefit in CRC (HR = 0.45, 95% CI 0.28–0.73, $P = 0.001$)[119].

1.5.3.5 Extracellular Matrix (ECM)

The ECM is a complex network of macromolecules that is responsible for maintaining tissue integrity and homeostasis. However, it is dysregulated in cancer and provides a supportive microenvironment to favour tumourigenesis[80,120].

Aberrant deposition of the ECM enhances the stiffness of tumour tissue and drives disease progression. Previous work in our laboratory showed that omentum from HGSOC metastases was up to 100 times stiffer than healthy omentum[120]. Stromal cells, such as CAFs, are major contributors to increased matrix stiffness. CAFs can secrete high levels of ECM molecules, such as fibronectin and collagen1[121]. Increased matrix stiffness can also stimulate the interaction between ECM components and cell-surface receptors on malignant cells, which can trigger integrin-mediated downstream signalling[122].

In 2018, Pearce and colleagues from our centre 'deconstructed' the metastatic microenvironment of HGSOC. They discovered the expression of 22 'matrisome' genes and proteins that were associated with a poor prognosis and overall survival[120]. The 'matrisome' is defined as the ensemble of genes encoding for ECM and ECM-associated proteins[120].The 'matrisome' gene signature seen in HGSOC was also shared with 12 other solid tumour types, including pancreatic, breast and colon cancers. Ultimately, deciphering the role played by the ECM in tumour progression is important to further our understanding of the TME and develop TME-targeting therapies.

1.5.4 Secreted factors in HGSOC

Cytokines, chemokines (chemo-attractant cytokines), and growth factors form an integral part of the TME. Over the last 40 years, there has been a profound interest to decipher the complex nature of cytokine networks in cancer progression, and develop effective anti-cytokine therapies[123]. Furthermore, previous work by Pearce *et al.* also examined the role of cytokine networks that regulate omental metastasis. They found five significant co-expressions at gene and protein level, including IL-6

and IL1-α, and IL1-β with IL-8[120]. There is also a growing interest to test whether anti-inflammatory agents, such as celecoxib and aspirin, could reduce circulating levels of pro-tumourigenic cytokines[48,124,125]. Below, I have discussed some of the most-studied cytokines and chemokines in the TME.

1.5.4.1 Interleukin 6, IL-6

IL-6 is one of the most important cytokines implicated in HGSOC and has been extensively studied in our laboratory[95,126,127,128]. It is highly expressed in the TME and confers a poor prognosis[28,123,129]. Many different cell types produce IL-6, including malignant cells, TAMs, CAFs and endothelial cells[23].

IL-6 typically stimulates the JAK/STAT3 signalling pathway, resulting in the downstream activation of genes associated with cell proliferation, such as c-myc and cyclin D1[126]. IL-6 can also induce the secretion of pro-inflammatory, pro-angiogenic factors (e.g., IL-8 and VEGF). Siltuximab is an example of an IL-6 antagonist which is commonly used in clinic to treat Castleman's disease.[130,123] In 2011, Coward *et al.* examined the potential of IL-6 as a therapeutic target in patients with platinum-resistant ovarian cancer, by using a combination of pre-clinical experiments and a phase II clinical trial[127]. Performing immunohistochemical staining from tissue microarrays of 221 ovarian cancer patients showed that high levels of IL-6 conferred with a poorer prognosis. Siltuximab treatment for six months led to a significant reduction in plasma levels of IL-6, as well as CCL2 and VEGF. Furthermore, siltuximab reduced genes associated with IL-6-induced inflammation, angiogenesis, and macrophage infiltration. Moreover, subjecting ovarian cancer cells with siltuximab treatment inhibited IL-6 signalling (e.g., p-STAT3), production of pro-inflammatory cytokines (IL-8, CCL2, IL-1ß, and TNF-α) and angiogenesis[127].

1.5.4.2 Interleukin 8, IL-8

IL-8 (otherwise referred to as CXCL8) is a well-documented pro-tumourigenic chemokine. It promotes the chemotaxis of macrophages and neutrophils to the tumour site, and can trigger the extrusion of NETs[102]. IL-8 is produced by both tumour cells and the surrounding TME[23,131]. Aberrant IL-8 expression is found in many cancer

types, and is linked with a high tumour burden, poor prognosis, and resistance to immune checkpoint inhibitors[123,132].

There are many studies which have investigated the biological effects of IL-8 in cancer. For instance, Larco and colleagues revealed that patients with metastatic disease had elevated IL-8 expression compared to those with localised tumours[133]. Shi Z, Yang WM, Chan LP *et al*. showed that multi-drug resistant breast cancer cell lines with several rounds of chemotherapy produced significantly higher levels of IL-8[134]. In 2019, Johnson *et al.* documented that tumour-derived IL-8 stimulates malignant cell invasion *in vitro*[48]. More recently, Olivera *et al* explored the pro-tumoural cytokine loop involving IL-8, IL-1ß, and TNF-α. They demonstrated that high levels of IL-8 expression positively correlated with IL-1ß and TNF-α. Furthermore, inhibiting TNF-α with infliximab, or IL-1ß with anakinra decreased circulating levels of IL-8. Overall, this study provided a deeper insight into targeting pro-tumourigenic cytokine networks[135].

IL-8 activates its downstream pro-tumourigenic effects by binding to one of its two cognate GPCRs: CXCR1 (IL-8RA) and CXCR2 (IL-8RB)[135]. It can activate the MAPK pathway by promoting the expression of cyclin D1 in malignant cells [136]. IL-8 can also promote malignant cell invasion via the PI3K-Akt pathway, as shown in breast cancer cell lines *in vitro*[48]. Similarly, IL-8 can transactivate EGFR, which is linked to cell proliferation and survival[137]. IL-8 signalling is also linked with the JAK2/STAT3 pathway, which promotes malignant cell invasion, survival, and angiogenesis[138].

1.5.4.3 CCL2

CCL2 (also known as monocyte chemoattractant protein 1, MCP-1) is a powerful, pro-inflammatory chemokine involved in the recruitment of monocytes and macrophages into the TME[139]. It is overexpressed in many types of malignancies and favours tumour progression and metastasis[123]. CCL2 is mainly produced by components of the TME, such as macrophages and CAFs, but may also be secreted by some tumour cells[94,139]. CCL2 has demonstrated to favour the polarisation of TAMs, which facilitates immune evasion and tumour progression[140].

1.5.4.4 Tumour necrosis factor alpha, TNF-α

TNF-α is a member of the TNF/TNFR cytokine superfamily. The 'dark side' of TNF-α was discovered over the years, as it plays a critical role in chronic inflammation, angiogenesis and chemoresistance[141]. Elevated levels of TNFα in biopsies of epithelial cancers have been linked to a poorer overall survival[123,141]. TNFα also upregulates COX-2 and PGE_2 in CAFs, consequently stimulating malignant cell invasion[142]. TNFα activates various downstream pathways associated with cancer progression. For example, it binds to TNFRI to promote the activation of the transcription factor AP-1, which activates NFκB and the BCL-2 family of anti-apoptotic proteins[95]. TNFα can also interact with TNFRII, which stimulates a whole range of signalling cascades, including p38 MAPK, PI3K and Erk.

1.5.4.5 Interleukin-1 beta, IL-1β

IL1-β is a well-established tumour-promoting cytokine. It is most notably produced by myeloid cells and CAFs[123]. IL1-β has been linked with angiogenesis and immunosuppression[95]. IL1-β from serum or tissue has been associated with a poor prognosis and survival, especially in non-small cell lung cancer[143]. Canakinumab is an example of an anti-IL1-β drug which was found to reduce lung cancer incidence in patients with cardiovascular disease. This was a secondary outcome of the large-scale Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS) [143,144].

1.5.4.6 Vascular Endothelial Growth Factor

As suggested by its name, VEGF is important in regulating angiogenesis. VEGF drives many processes, including endothelial cell proliferation, migration and sprouting[23]. VEGF can activate a plethora of pro- signalling cascades to drive vascular permeability and tumourigenesis. For instance, VEGF activates focal adhesion kinase (FAK) which promotes migration, angiogenesis and cancer cell survival[145]. Furthermore, CAFs can also upregulate VEGF via COX-2[146]. Recent work in our laboratory examined cediranib (a small molecule VEGF receptor inhibitor) resistance in three different murine models of HGSOC, where two/three models did not show a survival benefit with cediranib monotherapy. In one model which had high levels of IL-6, combining anti-IL-6 treatment with cediranib improved survival and T cell infiltration. In another model which had low levels of IL-6 but high levels of PD-1, combining cediranib with

an anti-PD1 agent improved survival. Overall, cediranib treatment in combination with anti-IL-6/anti-PD1 may help to improve response to anti-angiogenic inhibitors and extend progression-free survival[147].

1.5.4.7 Summary table showing the effects of COX-2 and PGE_2 on the TME

I have summarised the above-mentioned effects of COX-2 and PGE_2 on cells and mediators described in sections 1.5.3.1-1.5.4.6 (Table 3).

Table 3. Effects of COX-2 and PGE_2 on cells and mediators described in sections 1.5.3.1-1.5.4.6

Cell type	Effect of COX-2/ PGE_2 on cell type
Cancer associated fibroblasts (CAFs)	CAFS directly repress activity of CD8+ T cells and natural killer (NK) cells
Tumour associated macrophages (TAMs)	TAMs stimulate MMP9/VEGF via COX-2 and PGE_2
Tumour associated neutrophils (TANs)	TANs overexpress the fatty acid transport protein 2 (FATP2) which drives the metabolism of arachidonic acid into PGE_2
Regulatory T cells (T_{reg})	PGE_2 directly promotes the recruitment of T_{reg} to the TME PGE_2- stimulates mature immuno-regulatory dendritic cells (classified as 'mRegDC'), which promoted CCL22 (a T_{reg} chemoattractant)
Natural killer (NK) cells	PGE_2 secreted from tumour cells bind to EP2/EP4 receptors on NK cells, which blocks NK cell activity and facilitates immune cell evasion
Interleukin-6 (IL-6)	PGE_2 upregulates T_H2 cytokines (Figure 1.5), including IL-6. Increased levels of IL-6 can in turn drive COX-2 production
Interleukin-8 (IL-8)	PGE_2 may upregulate IL-8 through EP4 signalling (discussed more in results chapter 5). Increased levels of IL-8 can in turn drive COX-2 production
Tumour necrosis factor alpha, TNF-α	TNFα also upregulates COX-2 and PGE_2 in CAFs, which stimulates malignant cell invasion *in vitro*
Vascular Endothelial Growth Factor (VEGF)	CAFs promotes VEGF production via COX-2 PGE_2 produced by malignant cells can bind to EP1-4 receptors on endothelial cells, and stimulate the production of VEGF

1.5.5 Aspirin and HGSOC

As described in section 1.1.3, the vast majority of studies examining the cancer-preventive effects of aspirin are in colorectal and upper GI tumours. There is

relatively limited evidence in ovarian cancers, even though the pro-inflammatory ovarian TME is important in promoting HGSOC progression.

A meta-analysis by the Ovarian Cancer Consortium analysed pooled data from 12 case-control studies of ovarian cancer. Among three studies that reported information on dose, they documented that daily low-dose aspirin (<100mg/day) reduced incidence of invasive epithelial ovarian cancers (OR=0.66;95% CI=0.53-0.83)[59]. Similarly, in seven studies which provided information on frequency of aspirin use, daily aspirin intake was associated with a reduction in ovarian cancer risk (OR=0.80; 95% CI=0.67-0.96)[59]. Another large-scale study in Denmark showed some evidence of a cancer-preventative effect of aspirin in ovarian cancer patients, with 4103 cases and 58,706 healthy controls, although the strongest effects were seen with endometrioid and mucinous cancers[148]. There is also an ongoing randomised Phase II double-blind placebo-controlled trial of aspirin (STICs and STONEs) in the prevention of ovarian cancer in women that harbour *BRCA 1 /2* mutations sponsored by the Canadian Cancer Trials group. This could yield to interesting outcomes[149].

It was not until July 2022 that the largest meta-analysis to date on aspirin use and ovarian cancer risk was published in the Journal of Clinical Oncology[150]. This paper analysed data from 17 different studies to investigate the relationship between prolonged aspirin use and ovarian cancer risk. A 13% reduction in risk in all ovarian cancers was observed following frequent aspirin administration, and a 14% risk reduction was noted for HGSOC in particular[150]. This finding has shown to be promising, as aspirin may potentially be incorporated into existing chemo-preventive strategies to ultimately minimise risk of ovarian cancer incidence. It is important to note that earlier studies can be difficult to evaluate as they do not always record the different subtypes of ovarian cancer. As described in section 1.5.1, and as reviewed extensively, 'ovarian cancer' is a heterogenous disease[64,151]. Therefore, comparing mucinous and high-grade serous ovarian cancers, for example, is synonymous to comparing breast and renal cancers[64,151].

1.6 Experimental models of HGSOC

1.6.1 Cell lines used in HGSOC

Over the recent years, significant improvements have been made in terms of models of ovarian cancer. Domcke *et al.,* Beaufort *et al.,* and Barnes *et al.* performed extensive genomic profiling on a wide panel of ovarian cancer cell lines and identified those which possessed similarities with HGSOC tumour samples[152,153,154]. These studies were instrumental in revealing that the vast majority of *in vitro* literature studying the biology of HGSOC did not, in fact, use cell lines which were high-grade serous in nature. One such example is the SKOV-3 cell line. Overall, these publications demonstrated the importance of performing *in vitro* research using cell lines that possess genetic similarities with the disease (e.g., copy number alterations, ubiquitous *TP53* mutations/deletions). Recently, Dr. Sarah McClelland's laboratory examined mechanisms underpinning chromosomal instability in 7 HGSOC cell lines (including AOCS-1 and G164), and suggested that chromosomal instability (CIN) mechanisms could be used as functional biomarkers to predict response to different treatments[155].

1.6.2 3D human multi-cellular models of HGSOC

1.6.2.1 Background

For a long time, tumours were thought to arise purely from disorganised masses of hyperproliferative cells[156]. It was therefore widely accepted to carry out *in vitro* research using immortalised cell lines grown on tissue-culture plastic. However, tissue culture plastic is stiff, and cancers do not grow as monolayers *in vivo*. Furthermore, given the pivotal role played by the TME in driving carcinogenesis, it is important that research questions are investigated using 3D *in vitro* multi-cellular models[151].

3D multi-cellular models are becoming increasingly attractive tools to study cancer development, progression, metastasis, and response to novel therapies. There are several advantages to using human 3D models. Firstly, 3D cultures are particularly relevant for understanding the human cancer microenvironment, as the interplay between malignant and stromal cells is critical to facilitate invasion and metastatic spread[156,157]. Secondly, 3D models enable the co-culturing and interactions between

different cell types, such as stromal cells and immune cells. Thirdly, 3D multi-cellular models enable the integration of ECM components, such as collagen I, allowing a better understanding of tumour-ECM interactions[158]. Fourthly, from an ethical standpoint, using human 3D TME models will contribute to implementing the three 'R' principles, reduction, refinement, replacement, i.e. reducing the use of and dependence on animals in research[159].

3D models also help overcome some of the limitations associated with patient-derived xenografts (PDX), which have been the gold standard model to study tumour development and progression. PDXs are associated with a slow turnaround time, high costs, and low throughput[160]. It is also not possible to study the TME in immunodeficient mice that bear the PDXs. However, murine models which accurately mimic disease features can be beneficial to improve our understanding of tumour progression and patient response to different treatments. Colleagues in our laboratory have developed mouse models of HGSOC which recapitulate the human ovarian TME[161]. They found similarities in p53, DNA damage response pathways and NFκB signalling. The 22 'matrisome' gene signature identified in human HGSOC tumours were also expressed in the murine models. Furthermore, murine cell lines were injected intra-peritoneally, which mimics the peritoneal spread of cancer cells, especially into the omentum, in human disease.

It is also important to acknowledge some disadvantages of using 3D models. 3D models cannot recapitulate the true complexity of the human TME, as it is a model and not an organism. Furthermore, there is a lack of standard protocols to culture different types of 3D models, which can make it difficult to compare results from different studies[162]. In addition, some types of 3D models, such as patient-derived organoids, rely on components such as Matrigel, which has extensive variability across different batches[163]. It may therefore be beneficial to use both 3D models and disease-specific murine models to answer biological questions.

1.6.2.2 Types of 3D models used to study the tumour microenvironment *in vitro*

There are different types of 3D models which focus on interactions between malignant cells and stromal components of the TME, including the ECM and immune cells.

1.6.2.2.1 Spheroids

Spheroids are typically cultured in ultra-low attachment 96-well or 384-well plates. Alternatively, spheroids can also be generated using the hanging drop method[164,165]. Spheroids can comprise of one cell type (e.g., malignant cells only) or 2-3 cell types (e.g., malignant cells, fibroblasts, and monocytes/macrophages).

There are several advantages associated with spheroid cultures. To begin with, there is no need for specialised equipment to culture spheroids, making it relatively cheap and straightforward to do so. Spheroids also enable the close interaction between cells, which can influence drug response and delivery[164]. Co-culture spheroids can also help understand the role played by non-malignant cells in tumour progression, which is critical to further our understanding on the TME and develop TME-targeting therapies. Furthermore, spheroids allow the formation of an oxygen and nutrient gradient, with a hypoxic core in the middle of the mass[166]. This is representative of conditions *in vivo* and therefore serves as a useful model to test anti-cancer drugs[166]. Additionally, spheroids can be cultured using a relatively small number of cells, making it medium- to high-throughput in nature.

Spheroids have been useful in studying chemo-resistance and relapse in ovarian cancer. For instance, Raghavan *et al* studied the effects of chemotherapy in OVCAR3 cells cultured in 2D monolayers and 3D spheroids[167]. They showed that 3D tumour spheroids displayed more resistance to cisplatin treatment (70-80% viability) compared to 2D monocultures (30-50% viability)[167]. Similarly, Ward Rashidi *et al* investigated chemoresistance in serial passages of OVCAR3 spheroids[165]. They demonstrated that serial passages of spheroids increased malignant cell proliferation and the emergence of a platinum-resistant phenotype. Spheroids are therefore more appropriate models to study drug testing and delivery compared to 2D monocultures.

However, there are a few limitations associated with spheroids. There is usually no ECM present to recapitulate the biomechanical properties of the tissue[164]. This can also affect drug delivery and response, as the ECM can influence response and resistance to drug treatment. Furthermore, it is not always possible to control the uniformity of spheroids, as they form naturally by interacting with other cells[166].

1.6.2.2.2 Patient-derived organoids

Patient-derived organoids (PDOs) have emerged as an appealing model in cancer research to study tumour development, drug response and patient stratification[156,164]. PDOs are developed from a single cell type (e.g. cancer cells), which self-organises to form 'mini organs.'[162]. PDOs can be formed from healthy and diseased tissues. Organoids started to gain popularity roughly 14 years ago. In 2009, Sato *et al.* developed intestinal organoids from single sorted Lgr5(+) stem cells[168]. Two years later, the same lab adapted and modified culture conditions from their earlier study to develop human and murine colonic epithelial organoids, which was then used to study inflammation and colon cancer progression[169].

There are multiple studies examining the use of organoids in cancer. Nanki *et al* developed primary patient-derived ovarian cancer organoids to test different treatments[170]. They developed expandable organoids in under 3 weeks and had an 80% success rate. This is a faster turnaround time than a traditional patient-derived xenograft (PDX) model, [157,171]. Similarly, De Witte *et al* demonstrated the importance of stratified medicine and intra-tumour heterogeneity by developing thirty-six PDOs from twenty-three patients[172]. Here, they showed that tumour organoids from different tumour locations from the same patient exhibited different responses to drug treatments. Perhaps, testing PDOs from multiple tumour locations will help us gain a deeper understanding on intra-tumoural heterogeneity[172]. Furthermore, PDOs have more recently been used to understand the chemo-preventive effects of aspirin. , Drew *et al.* investigated aspirin-induced transcriptome-wide changes in human colonic epithelial organoids[173]. They treated organoids with 50uM aspirin for 72h, and identified differentially expressed genes which may shed light on the chemo-preventive mechanisms of action in CRC. As an instance, *FOXO3* and *TRABD2A* were upregulated in aspirin-treated organoids compared to untreated controls. Both genes are associated with Wnt inhibition, which alludes to the possibility of aspirin inhibiting Wnt signalling[173]. Moreover, aspirin increased the expression of *ACSL5*. Low levels of *ACSL5* is believed to be associated with CRC recurrence, suggesting that aspirin may reduce the recurrence of CRC[173].

However, there are disadvantages to using PDOs. For instance, Matrigel is usually used to culture organoids. Matrigel is poorly defined, expensive, derived from murine sarcoma cells and has been reported to have extensive lot-to-lot variation[163]. This can lead to the generation of mixed results and prevent the reproducibility of experiments. Moreover, PDOs do not have vascular networks. Furthermore, there is no standardised protocol to maintain PDOs. Developing universal protocols to culture PDOs and exploring alternatives to Matrigel (e.g., hydrogel-based scaffolds) will help improve the reproducibility and reliability of the culture technique[164].

1.6.2.2.3 Hydrogel-based scaffolds

Scaffolds are biomaterials which provide structural support for cell attachment and tissue development. Hydrogel-based scaffold cultures are particularly popular, as their biomechanical properties can closely mimic the ECM[164]. As described in section 1.5.3.5, the ECM plays a crucial role in propagating cancer progression. Therefore, developing models that recapitulate cell-ECM interactions could lead to the identification of relevant pathways that may be exploited to develop new therapies. Scaffolds can be made out of synthetic polymers, such as poly-ethene glycol (PEG), and native polymers, such as collagen I[164].

Synthetic polymers such as PEG-based hydrogels confer several advantages. For instance, they have adjustable mechanical properties, the ability for photo-polymerisation and are commercially available. Furthermore, the surface of synthetic polymers contains peptides (e.g., Arg-Gly-Asp, RGD), which are designed to promote cell adhesion, typically via integrin-mediated signalling[164,171]. In 2010, Loessner *et al.* developed a synthetic 3D PEG hydrogel model study complex cell-ECM interactions in ovarian cancer[174]. Here, they embedded EOC cell lines within a PEG hydrogel system and treated these models with paclitaxel. They showed that paclitaxel did not reduce cell proliferation and viability to the same extent as it did in 2D monolayers of malignant cells. Similarly, Taubenberger *et al.* investigated the role of integrins in modulating cell-ECM interactions by culturing cancer cells in PEG-hydrogels funtionalised with different peptide motifs: RGD, collagen I, or laminin-III[175]. They deduced that the PC-3 prostate cancer cell line displayed an invasive morphology when cultured with collagen-I or laminin-III compared to RGD and non-functionalised

controls. Similarly, Loessner *et al.* showed that ovarian cancer cells embedded in hydrogels functionalised with the RGD motif proliferated more compared to non-functionalised controls[174]. These papers highlight the ability to modify PEG hydrogels to answer specific research questions, making them useful models to study tumour development and progression.

Equally, native hydrogel models are of interest to study the TME. Natural scaffolds offer mechanical stability, enable cell-ECM communication, promote cell adhesion, growth, and migration[164176]. An example of a natural scaffold is collagen I, which is an ECM protein shown to be linked with tumour progression. Professor Umber Cheema's laboratory have developed a 'tumouroid' model whereby they cultured CRC cell lines (HT-29, HCT116) within hydrogels of collagen I[176,177]. This model comprises of an 'artificial cancer mass', which is embedded within a compartmentalised stromal compartment. Using different ECM densities, compositions, and stromal cells, they showed that mimicking tissue ECM composition and stromal cell composition enhanced CRC invasion. Moreover, Barbolina *et al.* developed a collagen-based hydrogel model to study cell-ECM communication in ovarian cancer metastases[178]. They cultured ovarian cancer cells on top of a collagen gel matrix, and discovered the upregulation of MT1-MMP, a protein which is linked with invasion. Furthermore, Delaine-Smith *et al.* developed a multi-cellular 'tri-culture' model of HGSOC to unravel signalling pathways that regulate the production of cancer-associated ECM molecules (previously referred to as the 'matrix index')[179,120]. To build the model, collagen gels of HGSOC cells and primary omental fibroblasts were cast, left to remodel for 7 days, and embedded within gels of primary omental adipocytes. They showed that cross-talks between TGFB and hedgehog (Hh) signalling pathways stimulated the production of the 'matrix index' molecules in tri-culture models, particularly by primary omental fibroblasts. Moreover, in January 2023, Estermann *et al.* developed a 3D collagen-based multi-cellular model of the omentum to study the formation of HGSOC metastasis[180]. This model was developed using patient-derived adipocytes and the Ea.*hy926* endothelial cell line, layered with immortalised cell lines of macrophages, fibroblasts, mesothelial cells, and OVCAR8 cells. They used this model as a platform to study malignant cell invasion, ECM re-

modelling and drug testing. They subjected the model to doxorubicin treatment for 48h and showed that doxorubicin reduced the viability of OVCAR8 cells, but not mesothelial and endothelial cells. However, this model was built with immortalised cell lines, which does not consider patient heterogeneity. Immortalised cell lines can also behave differently from primary cells. As an example, this paper showed that malignant cell invasion was limited when OVCAR8 cells were added apically to the model. However, malignant cell invasion was enhanced when patient-derived cancer cells were added instead.

Overall, hydrogel-based 3D cultures can be particularly useful to investigate cell-cell, and cell-ECM interactions and further understand the role of the ECM in tumour progression.

1.6.2.3 Tetra-culture model of HGSOC

Our laboratory has been studying the role of the TME in HGSOC development and progression. As mentioned earlier in paragraph 1.5.3.5, Pearce *et al.* 'deconstructed' the HGSOC TME in the omentum, and identified an ECM-signature linked with tumour progression and poor prognosis[120]. This work was then used as a guide to 're-construct' a multi-cellular model *in vitro* that aims to recapitulate the early stages of omental metastases in HGSOC[181].

Our multi-cellular model has been developed with four cell types (tetra-culture). It comprises of primary omental adipocytes, primary omental fibroblasts, primary omental mesothelial cells, and early-passage HGSOC malignant cell lines. Primary adipocytes, fibroblasts and mesothelial cells are freshly isolated from 'uninvolved' omental tissue, and HGSOC cells (namely G33 and G164) are established as cell lines from diseased omentum[84]. A schematic of this model is shown in Figure 1.8.

Recently, Malacrida *et al* used the tetra-culture model to examine the role of platelets in promoting early-stage metastasis, invasion of malignant cells into the omentum tissue, and the production of cancer-associated ECM molecules[181]. This paper highlighted the following points. Firstly, platelets are linked with a diseased ECM signature. This goes in line with previous literature highlighting the role of platelets in tumourigenesis[47,48]. Secondly, platelets activate mesothelial cells, which

disrupts the mesothelial barrier and facilitates cancer cell invasion into the artificial omentum. Thirdly, platelets stimulate mesothelial cells and malignant cells to produce key ECM proteins, the 'matrix index' (fibronectin, versican, cathepsin B, collagen 1A1), which are linked with disease progression and poor prognosis.

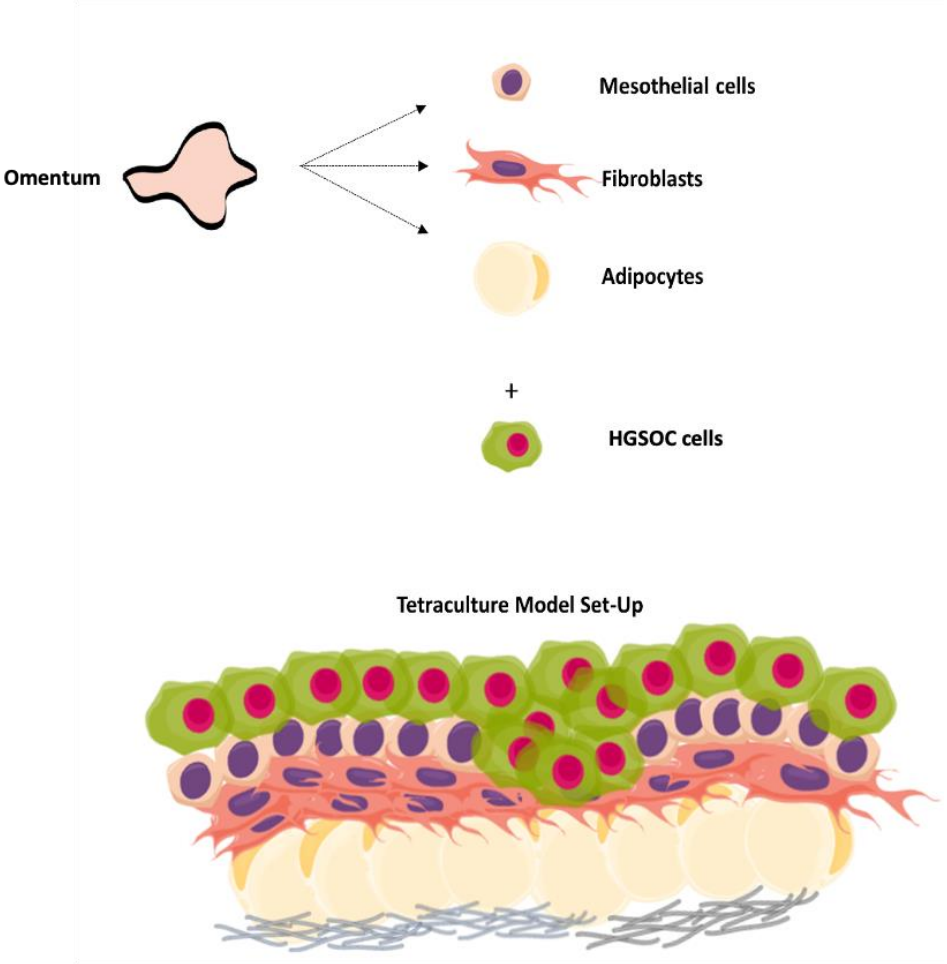
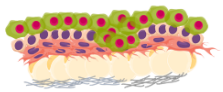


Figure 1.8. Schematic of our disease-specific 3D tetra-culture model. Primary omental adipocytes, fibroblasts and mesothelial cells are isolated from 'uninvolved' omentum tissue from patients. Adipocyte gels are made with rat tail collagen I, and are layered with fibroblasts, mesothelial cells, and early-passage HGSOC cells. Tetra-culture gels have a viability for up to 21 days.

There are several advantages to using our model, as summarised in Table 4. Firstly, it is partially self-assembling and mimics some aspects of HGSOC tumours *in vivo*. Secondly, our tetra-culture model recapitulates complex cell-cell and cell-ECM interactions, which is critical to gain a better understanding of the ovarian TME and screen different drugs, including aspirin. Thirdly, this model is developed using primary omental adipocytes, primary omental fibroblasts, and primary omental mesothelial cells, which is more representative of the disease. Fourthly, it is possible to increase the complexity of the model by adding platelets or immune cells (e.g., monocytes), or alternatively removing cells to answer specific research questions as highlighted by Malacrida *et al*[181]. From a technical standpoint, the tetra-culture model is developed in a 96 well-plate format, making it medium throughput in nature. Our model is also viable up to 21 days in culture, which is useful to answer questions pertaining to ECM remodelling or longer-term drug treatments.

Ultimately, working with models that closely recapitulate the disease is imperative to help overcome the gap in translating pre-clinical laboratory research into novel therapies in the clinic.

Table 4. Advantages and disadvantages of our tetra-culture model

Model	Advantages	Disadvantages
Tetra-culture model (4 cell types) 	All primary cells (adipocytes, fibroblasts, mesothelial cells) are derived from patient-tissue	No immune cells (e.g. monocytes/macrophages)- but currently being developed in the lab
	Partially self-assembling, mimicking conditions *in vivo*. This makes it an attractive model to test different drugs too.	
	>4 cell types involved, allowing for cell-cell interactions.	No vasculature- but currently being developed in the lab
	ECM produced by model Model allows for cell-ECM interactions	Tissue-dependent
	Cells can added into the model/ removed from the model to answer specific questions. This is especially useful to address mechanistic questions.	Malignant cell line is not patient-specific.
	Viable for up to 21 days in culture.	
	Assembled in a 96 well-plate format, making it relatively high-throughput	

1.7 Aims of the study

The overall aim of my PhD is to use *in vitro* models to understand the potential cancer-preventative role of aspirin in high-grade serous ovarian cancer. I am to do this by:

1. Investigating the short term and longer-term effects of aspirin on the growth and viability of high-grade serous ovarian cancer cells and stromal components of the TME.
2. Examining the effects of aspirin on the lipid mediator profile in malignant cells and components of the TME.
3. Investigating the effects of aspirin on the viability and pro-inflammatory cytokine profile in multi-cellular models of high-grade serous ovarian cancer.

2 Methods

2.1 List of antibodies used

I have tabulated a list of primary and secondary antibodies used for my project below.

Table 5. Primary Antibodies used for Immunohistochemistry.

Antibody	Species	Dilution	Cat no.	Company
PAX-8	Rabbit	1:1000	NBP1-32440	Novus Biologicals
FAP	Rabbit	1:500	ab207178	Abcam
Cleaved Caspase 3	Rabbit	1:100	CST 9664S	Cell Signalling Technologies
CK20	Mouse	1:500	M7019	Dako
Ki67	Mouse	1:50	M7240	Dako

Table 6. Unconjugated Antibodies used for Immunofluorescence.

Antibody	Species	Dilution	Cat no.	Company
FAP	Rabbit	1:500	ab207178	Abcam
a-SMA	Rabbit	1:100	Ab5794	Abcam

Table 7. Conjugated antibodies used for Immunofluorescence.

Antibody	Clone	Dilution	Cat no.	Company
AlexaFluor™488 anti-mouse CD326 (EpCAM)	G8.8	1:200	118210	Biolegend

Table 8. Secondary antibodies used for Immunofluorescence.

Antibody	Dilution	Cat no.	Company
Goat anti-rabbit AlexaFluor™ 488	1:1000	A11008	Invitrogen
Goat anti-rabbit AlexaFluor™ 633	1:1000	A21070	Invitrogen

2.2 Cell lines, primary cells, and culture conditions

All cell lines and primary cells used in this project are tabulated below.

Table 9. Cell lines and primary cells used in this project.

Cell type	Origin
G33	Omental metastases of HGSOC-previously generated in the laboratory
G164	Omental metastases of HGSOC-previously generated in the laboratory
AOCS-1	Silverberg grade 3 primary tumour, from ascites after relapse
P53 mutant FT318 (R273C)	Fallopian tube surface epithelium
HT-29	Grade II colon adenocarcinoma
HCT116	Colorectal carcinoma
HCT-15	Adenocarcinoma; Colorectal; Dukes' type C
Primary omental fibroblasts	Isolated from human omentum
Primary omental mesothelial cells	Isolated from human omentum

G33 HGSOC cells were grown in DMEM-F12 GlutaMAX™ (ThermoFisher Scientific, cat no. 10565018), 1% penicillin/streptomycin (P/S, Merck, cat no. 11074440001), 1% insulin transferrin selenium-1 (ITS-1, Gibco, cat no. 41400045), and 10% foetal bovine serum (FBS, Gibco, cat no. 17256336). G164 cells were maintained in DMEM-F12 GlutaMAX™ (ThermoFisher Scientific, cat no. 10565018), 1% P/S (Merck, cat no. 11074440001), 1% ITS-1 (Gibco, cat no. 41400045) and 4% human serum (HS, Merck, cat no. H4522-100ML). The AOCS-1 cell line was a kind gift from Professor David Bowtell's group (Peter MacCallum Cancer Centre, Melbourne, Australia). AOCS-1 cells were cultured in RPMI (Fisher Scientific, cat no. 21875034), supplemented with 1% P/S (Merck, cat no. 11074440001) and 10% FBS (Gibco, cat no. 17256336). Tamura *et al.* examined mechanisms underpinning chromosomal instability in 7 HGSOC cell lines, including AOCS-1 and G164 cell lines[155].

HT-29, HCT116 and HCT-15 CRC cell lines were cultivated in DMEM (ThermoFisher Scientific, cat no. 11965084), supplemented with 1% P/S (Merck, cat no. 11074440001), 1% glutamine (Gibco, cat no. 11539876) and 10% FBS (Gibco, cat no. 17256336).

P53 mutant (R273C) FT318 fallopian tube epithelial cells were kindly given by Professor Ronny Drapkin's laboratory (University of Pennsylvania, Philadelphia, USA). P53-mut(R273C) FT318 cells were grown in DMEM-F12 GlutaMAX™ (ThermoFisher Scientific, cat no. 10565018), supplemented with 1% P/S (Merck, cat no. 11074440001), 1% ITS-1 (Gibco, cat no. 41400045) and 10% FBS (Gibco, cat no. 17256336). These cells were maintained in 'Primaria™' tissue culture flasks (ThermoFisher Scientific, cat no. 10469222).

Primary omental fibroblasts and mesothelial cells were isolated from human omentum tissue (section 2.8.3.2). Primary omental fibroblasts were cultured in DMEM-F12 GlutaMAX™ (ThermoFisher Scientific, cat no. 10565018), supplemented with 1% P/S (Merck, cat no. 11074440001), 1% ITS-1 (Gibco, cat no. 41400045) and 10% FBS (Gibco, cat no. 17256336). Primary omental mesothelial cells were maintained in DMEM-F12, GlutaMAX™ (ThermoFisher Scientific, cat no. 10565018), supplemented with 1% P/S (Merck, cat no. 11074440001), 1% ITS-1 (Gibco, cat no. 41400045) and 15% FBS (Gibco, cat no. 17256336).

All cells were cultured in a humidified incubator of 5% CO_2 at 37°C and were routinely tested for mycoplasma contamination (Lonza, cat no. LT07-318). Cells were passaged once they reached 70-80% confluency. All cells, except mesothelial cells, were detached with 1x trypsin-EDTA (Gibco, cat no. 10779413). Mesothelial cells were detached using an enzyme-free cell dissociation buffer (ThermoFisher Scientific, cat no.13151014). Trypsin/cell dissociation buffer activity was deactivated with serum-rich medium. Cells were then pelleted by centrifugation at 200xg for 3 minutes, re-suspended in basal medium, and re-plated in a new T75 flask. P53-mut FT318 cells were re-plated in Primaria^{TM} T75 flasks.

All cell lines were authenticated by short tandem repeat (STR) sequencing. This was done using the FTA Sample Collection Kit for Human Authentication Service 135-XV (American Type Culture Collection, ATCC). All cell lines were also used within 4 passages in culture.

2.3 Long-term cryopreservation and recovery of cells

Cells were detached with trypsin/cell dissociation buffer and centrifuged as described in section 2.2. Next, the cellular pellet was re-suspended in freezing medium, which comprised of 90% FBS (Gibco, cat no. 17256336) and 10% DMSO (Scientific Lab Supplies, cat no. 25-950-CQC). Cells were then transferred into cryogenic storage vials (Corning, Cat.no. 430488), frozen in -80°C overnight and stored in liquid nitrogen (-196°C).

Frozen cells were recovered from liquid nitrogen and thawed at 37°C. Cells were then added to 10mL of culture medium and centrifuged for 3 minutes at 200xg. The cellular pellet was then re-suspended in fresh medium (cell dependent, as described in paragraph 2.2). Cells were then seeded in a T25/T75 tissue-culture treated flask (Corning).

2.4 Dissolving aspirin

180mg of aspirin powder (Merck, cat no. A5376-250G) was dissolved in 250µl DMSO (Scientific Lab Supplies, cat no. 25-950-CQC) to create a concentrated stock solution of 4M. This was then vortexed for 2-3 minutes until the drug was completely dissolved. Intermediate stock solutions of 100mM and 10mM were then made in 2mL

of culture medium (these contained 2.5% DMSO and 0.25% DMSO respectively). Serial dilutions were then carried out in culture medium, in such a way that the final working solution (≤1mM) used for experiments did not contain >1% DMSO (Table 10). The type of medium used was dependent on the cell type. Fresh solutions of aspirin were made before every experiment, to mimic the unstable nature of aspirin *in vivo* (half-life of 15-20 minutes). Given Devall, Drew *et al.*'s recent publication[173], and the peak plasma concentrations of low-medium aspirin being approximately 7.31-40μM, it is essential to adhere to the micromolar range[44]. However, it is difficult to recapitulate the pharmacokinetics of aspirin *in vitro*, and it is therefore not possible to directly compare *in vitro* concentrations to oral dosing *in vivo.*

Table 10. Percentage of DMSO present across different concentrations of aspirin

Aspirin concentration	Percentage of DMSO
4M (stock)	100%
100mM (sub-stock)	2.5
10mM (sub-stock)	0.25
1mM	0.025
100μM	0.0025
10 μM	0.00025

2.5 Drug treatments with celecoxib (COX-2 inhibitor) and PGE_2 receptor antagonists

Primary omental fibroblasts, mesothelial cells and tetra-cultures of HGSOC were also treated with celecoxib (Cat no.PHR1683, Merck) an EP2 receptor antagonist, PF04418948 (Cat no. 4818, Tocris), and an EP4 receptor antagonist, L-161982 (Cat no., 2514, Tocris).

All three drugs were dissolved in DMSO, and vortexed for 2-3 minutes until the drug was completely dissolved. Similar to section 2.4, the final working solution (≤1 μM) did not contain >1% DMSO. This is summarised in Table 11.

Table 11. Stock concentrations for celecoxib, PF-04418948 and L-161982.

Drug	Stock concentration	Mass of drug	Volume of DMSO (ml)
Celecoxib	25mM	1mg	0.1
PF04418948	1mM	1mg	2.44
L-161,982	1mM	1mg	1.53

2.6 Cell growth and viability assays

2.6.1 Cell growth assay

Growth curves were performed to assess how cancer cells, mesothelial cells and fibroblasts responded to aspirin. Cells were seeded on tissue culture treated plates (Corning) 24 hours prior to treatment with aspirin. To assess the short-term effect of aspirin on cell growth (≤72h treatment), 12-well plates were used (Corning). Six well plates (Corning) were used to examine longer-term growth inhibitory effects by aspirin. Cells were then exposed aspirin on a daily basis and incubated in 37°C until endpoint was attained. Cells were then trypsinised and counted using a Vi-CELL XR Viability analyser (Beckman Coulter). Conditioned media was collected and stored in -80°C.

2.6.2 CyQuant Cell Proliferation Assay

A CyQuant Cell Proliferation assay kit (C7026, ThermoFisher Scientific) was used to determine cell proliferation following aspirin treatment. The experiment was carried out following the manufacturer's protocol. In brief, conditioned media was collected, cells were washed once with 1x PBS, and microplates were frozen down in -80°C overnight to ensure efficient lysis. The following day, 200µl of the prepared reagent was added to each well and incubated in the dark for 2-5 minutes at room temperature prior to being transferred into a Nunc™ MicroWell™ 96-Well Optical-Bottom Plates with Polymer Base (Invitrogen, cat no. P8991). A standard curve was prepared, and fluorescence was measured at an excitation/emission wavelength of 480/520nm using a BMG FLUOstar Optima microplate reader (Labtech).

2.6.3 Live/Dead Cell Viability Assay

A live/dead cell viability assay was used to determine the effects of aspirin on cell viability in spheroids of malignant cells. This was done using the Live/Dead Viability/ cytotoxicity kit (Invitrogen, cat no. L3224), following the manufacturer's protocol.

To begin with, a live/dead 'mix' was prepared by diluting reagents 'A' and 'B' in 1x D-PBS (Gibco, cat no. 13492609), as shown in Table 12. Next, gels were washed once in D-PBS for 10 minutes in 37°C. 300µL of the live/dead staining mix was then added to each well. Plates were then incubated in the dark for ≥30 minutes at 37°C. Gels were then carefully transferred to a NUNC-TM glass bottom dish (Thermo Fisher Scientific, cat no. 150680), covered with PBS, and imaged using the Zeiss LSM-710 confocal microscope.

Table 12. Dilutions required for the live/dead mix

Reagent	Dilution in D-PBS
'A' - Calcein AM (live cells)	1 in 2000
'B' – Ethidium homodimer-1 (dead cells)	1 in 1000

2.7 Lipid mediator profiling using mass spectrometry

The prostaglandin profile of malignant and pre-malignant cells was analysed using LC-MS/MS by LC-20aD HPLC and a SIL-20A auto injector (Shimadzu, Kyoto, Japan) paired with a QTrap 6500 (ABSciex). To begin with, cells were cultured in phenol-free medium for 24 hours (DMEM F12, Gibco, cat no.11580546; DMEM, Gibco, cat no. 11330892; RPMI, Gibco, cat no.10363083) in tissue-culture treated 6 well-plates (Corning). A density of 1.5 million cells/well was used, with 2mL of culture medium/well. The following day, cells were exposed to aspirin. At endpoint, 4ml of ice-cold methanol containing 500pg of deuterium-labelled internal standards (d_8-5*S*-HETE, d_4LTB_4, d_5LXA_4, and d_4PGE_2) was added to each well (internal standards were used to facilitate quantification and sample recovery). Samples were then frozen overnight in -80°C and centrifuged the next day for 10 min (2500rpm, 4°C). Supernatants were transferred to a new tube, placed in an automated evaporation

system (TurboVap LV, Biotage), and brought down to a volume of 1ml. Samples were then placed onto an automated extraction system (ExtraHera, Biotage). C18 columns (Biotage) were primed with each solvent (neutral ddH_2O, Methyl formate, 99% spectrophotometric grade (Aldrich), pH 3.5 acidified water, HPLC grade hexane (Fisherbrand), and HPLC grade methanol (Fisherbrand). The solid phase extraction was then run using the method 'CORE C18 SPE MF.' At the end of the extraction, the eluted samples were rinsed twice with methyl formate, twice with methanol, and thoroughly evaporated using TurboVap (Biotage). Samples were then re-suspended in a 1:1 ratio of ddH_2O and methanol to a total volume of 40µl. Samples were then centrifuged (2500rpm, 5 min, 4^oC) for LC-MS/MS automated injections.

To identify and quantify prostaglandin levels, a multiple reaction monitoring method (MRM) developed by Professor Dalli's group was used. Quantification was carried out using the Analyst software (SCIEX) by measuring the area of the peak of the interested analyte. To ensure that the peak was integrated correctly, results were cross-verified against a control containing only methanol with deuterium-labelled internal standards. The recovery for the methods that we used in my experiments was 84.8 ± 0.2 %.

Professor Jesmond Dalli's group carried out the extraction and analysis processes, and provided information on the recovery.

2.8 Spheroids, collagen gels, and multi-cellular models

2.8.1 Spheroids of HT-29 and HCT116 cells

The effects of aspirin on the viability of CRC spheroids were assessed. HT-29 and HCT116 cells were first trypsinised as described previously (section 2.2) and counted using a Vi-CELL XR counter (Beckman Coulter). Thirty thousand cells/ well were then plated in an ultra-low attachment plate (Costar, cat no. 7007). 200µl of medium was used/well. Spheroids were left to remodel for 2-3 days, before being subjected to daily aspirin treatment.

2.8.2 Collagen co-cultures of malignant cells and fibroblasts

To begin with, cells were trypsinised and counted (section 2.2). A 1:1 ratio was used for collagen gels of G164 cells and fibroblasts (100,000 cells of each cell type), and a

1:4 ratio was used for collagen gels of HT-29 CRC cells and fibroblasts. The desired number of cancer cells and fibroblasts were then aliquoted, mixed together in one tube, and centrifuged for 5 minutes at 200xg. The supernatant was removed, and the pellet was re-suspended in 60µl of fresh culture medium, which was then added to the collagen gel 'mix'. For each gel, the 'mix' was made as indicated below (Table 13).

Table 13. Collagen gel 'mix'

Reagent	Volume required / gel
10x DMEM-low glucose (Merck, cat no.D2429-100mL)	5µL
Sodium hydroxide, NaOH (Fisher Scientific, 10475091)	2µL
Rat-tail collagen 1 (Merck, cat no. C867)	33µL

The newly formed collagen gel mix with the cell suspension was left to stabilise on ice for 5 minutes, prior to being pipetted in volumes of 100µl into 96-well plates. Gels were then immediately incubated for 1 hour at 37°C. Once gels were solidified, they were transferred to 24-well plates with two gels per well. Medium was replaced every 2-3 days.

2.8.3 Tetra-culture model of HGSOC

2.8.3.1 Tissue Collection

The Royal London Hospital (RLH) provided patient omental tissue, with full written consent given from patients. Samples were transported from the Royal London hospital to Barts Cancer Institute (BCI), in accordance with the Health and Safety Policy, Human Tissue Authority (HTA) Codes of Practice, The European Agreement Concerning the International Carriage of Dangerous Goods by Road (ADR) and Transport for London Bye-laws (Research Ethic Committee, REC, number: 17/LO/0405).

2.8.3.2 Processing human omentum

First, omental tissue was trypsinised with 10x-trypsin-EDTA (Gibco, cat no. 10779413) diluted in D-PBS (Gibco, cat no. 13492609) in a 1:1 ratio for 20 minutes, 37°C. Trypsin activity was inactivated with DMEM-F12 GlutaMAX™ (ThermoFisher Scientific, cat no.10565018), supplemented with 5% FBS (Gibco, cat no.17256336). The tissue was transferred into a clean petri dish. The remaining contents inside the Falcon tube were centrifuged (200xg, 5 min), and the mesothelial cell pellet was re-suspended in the appropriate basal medium (section 2.2).

The tissue inside the petri dish was carefully minced into small pieces (≥1mm^3) using two scalpels and digested with liberase™-DL (Merck, cat no. 5401160001; 115µL liberase/ 20ml of DMEM-F12 GlutaMAX™ and 5% FBS). The tissue digestion took place for approximately 30 minutes, 37°C at a speed of 65rpm using an Innova 40 Incubator Shaker (New Brunswick). Stiffer tissue was left to digest for a longer period (e.g., 40 minutes). Conversely, smaller, and softer tissues were sometimes digested for a shorter period (<30 minutes).

After digestion was complete, the tissue sample was gently disaggregated with a Pasteur pipette (Greiner Bio-One Ltd, cat no. 612302). The sample was then filtered using a 250µm Pierce™ tissue strainer (Thermo Fisher Scientific, cat no. 87791) and a 1 ml syringe handle in 15ml Falcon tubes (Corning). The filtered products were then transferred into a fresh 50ml tube containing 20ml of DMEM-F12,GlutaMAX™ (ThermoFisher Scientific, cat no.10565018), supplemented with 5% FBS (Gibco, cat no.17256336). This tube was then centrifuged (200xg, 3 minutes) to obtain an adipocyte layer (supernatant), a layer with spent medium, and a stromal vascular fraction (pellet). The adipocyte layer was carefully transferred into a second Falcon tube containing fresh medium and centrifuged for 2 minutes at 200xg. In the meantime, the pellet containing the stromal vascular fraction was re-suspended with fibroblast culture medium (section 2.2) and plated in a T75 flask.

To ensure the removal of residual liberase, the adipocyte layer was carefully transferred into a 15 mL Falcon tube (Corning) containing 3mL of fibroblast basal medium and incubated upright in 37°C for 8 minutes. This process was repeated twice, and the final volume of adipocytes was used to cast gels (2.8.3.3).

2.8.3.3 Casting adipocyte gels

The number of gels casted depended on the volume of adipocytes extracted from human omental tissue. Calculations for casting gels were done as follows in Table 14. The gel mix was prepared on ice to slow down the polymerisation of collagen by NaOH.

Table 14. Calculations required for casting adipocyte gels.

Volume of adipocytes x3 = Total Volume

E.g. 200 µL x3= 600 µL

Reagents	**Volume required**	**Example**
Rat-tail collagen 1 (Merck, cat no. C867)	$\frac{1}{3}^{rd}$ of total volume	200µL
10x DMEM-low glucose (Merck, cat no. D2429-100mL)	$\frac{1}{10}^{th}$ of total volume	60µL
Water, sterile-filtered, BioRegent, suitable for cell culture (Merck, W3500)	Remaining volume	130µL
Sodium hydroxide, NaOH (Fisher Scientific, 10475091)	5 µl for every 100µl of collagen I	10µL
Adipocytes	Dependent on tissue	200µL

100µl aliquots of the gel mix were cast in a tissue culture-treated 96 well plate (Corning) and incubated for 1 hour in 37°C. Next, 200µl of M199 culture medium (Thermo Fisher Scientific, cat no.11150067), supplemented with 10% FBS (Gibco, cat no.17256336), 1% P/S (Merck, cat no. 11074440001) and 1% ITS-1 (Gibco, cat no. 41400045) was added to each well. Gels were then transferred into a 24 well plate containing one mL of basal media (M199 with supplements). Culture medium was replaced every 2-3 days.

2.8.3.4 Building the Tetra-culture model

Adipocyte gels were anchored to the bottom of a tissue culture-treated 96 well plate (Corning) with 1mg/mL rat-tail collagen I solution (Merck, cat no.C867) for 10

minutes. Forty thousand omental fibroblasts were then seeded per gel in 100µL culture medium and incubated at 37°C for at least 2 hours. This was followed by the addition of 200,000-omental mesothelial cells/gel in 100µl mesothelial growth medium. Gels were left for 24 hours in 37°C, 5% CO_2. The following day spent medium was removed and replaced with 40,000 malignant or pre-malignant cells/ gel in a volume of 200µL. 24 hours later, gels were transferred into a 24 well plate using a small spatula/spoon. Culture medium was replaced every 2-3 days until end-point was attained.

2.9 Immunohistochemistry

Gels were fixed in 10% para-formaldehyde (Cellpath Plc, cat no. BAF-0100-25A), embedded in 2% ultra-pure agarose (prepared in dH_2O Thermo Fisher Scientific, cat no. 10264544) and de-hydrated in 70% ethanol (Thermo Fisher Scientific, cat no. 10437341). Gels were then embedded in paraffin and sectioned on the microtome by BCI Pathology Services.

Slides were de-paraffinised in two changes of xylene (Thermo Fisher Scientific, cat no. 10385910) for 5 minutes each and rehydrated in a descending order of 100%, 90% 70% and 50% ethanol for 2 minutes each (Thermo Fisher Scientific, cat no. 10437341), followed by a 3-minute wash in distilled water. To enable antigen-retrieval, slides were incubated in citrate-based antigen retrieval solution (pH6, 1:100, Vector labs, cat no. H-3300) for 30 min at 95°C. Slides were then washed three times in PBS, with 3 minutes per wash. Next, endogenous horseradish peroxidase (HRP) activity was blocked by incubating slides with HRP-blocking buffer for 30 minutes at room temperature. This buffer comprised of 245mL methanol (Thermo Fisher Scientific, cat no. 10675112) and 5mL hydrogen peroxide (Stock concentration, 30% w/v, Thermo Fisher Scientific, cat no. 10687022). Blocking solution was then washed off with one 3-minute wash with PBS- 0.05%tween 20 (PBS-T, Sigma, tween 20 cat no. 10485733), followed by two 3-minute washes in PBS.

An ImmEdge hydrophobic barrier pap pen (Vector labs, cat no H-4000) was used to outline a water-repellent border around the samples to prevent the waste of valuable reagents. For all antibodies except PAX-8, slides were incubated with a

blocking buffer of PBS containing 2.5% goat serum (Sigma, cat no. G9023), and 2.5% bovine serum albumin (BSA, Merck, cat no. A8022) for 45 minutes at room temperature. For PAX-8 staining, additional blocking steps were performed using the avidin-biotin kit (Vector Labs, cat no. SP-2001) following the manufacturer's protocol, before adding blocking buffer to the slides. The appropriate primary antibody was then added (Table 5) to the slides. Slides were then left overnight in a humidified chamber at 4°C.

The next day, slides were washed twice with PBS-T (Merck, tween 20 cat no. 10485733) and once with PBS. For all markers except PAX-8, the ImmPRESS® HRP Goat Anti-Rabbit/Anti-Mouse IgG Polymer Detection Kit (Vector labs, cat nos. MP-7451 and MP-7452 respectively) was added to the slides for 30 minutes at room temperature. For PAX-8 staining**,** a biotinylated goat anti-rabbit IgG secondary antibody (1:200, Vector Labs, BA-1000) was used instead of the ImmPRESS® polymer for 30 minutes at room temperature. All slides were then washed twice in PBS-T (Merck, tween 20 cat no. 10485733) and once in PBS. For PAX-8 staining only, samples were incubated for 30 minutes in ABC Vectastain solution (Vector labs, cat no. PK-6200), followed by two washes in PBS-T (Merck, tween 20 cat no. 10485733) and one wash in PBS.

Next, the 3,3'-diaminobenzidine (DAB) chromogen reagent (DAKO, GC806) was added to the slides to enable the development of a dark brown reaction product. To stop the reaction, slides were rinsed in distilled water. This was followed by a counterstain in 100% Gills haematoxylin (Merk, cat no. MHS16) for one minute. Slides were then dehydrated in one change of 90% ethanol for 3 minutes, two changes of 100% ethanol for 3 minutes each (Thermo Fisher Scientific, cat no. 10437341), and two changes of xylene for 5 minutes each (Thermo Fisher Scientific, cat no. 10385910). Finally, excess xylene was dried, slides were mounted using DPX mountant for histology (Scientific Laboratories Ltd, cat no. 06522), and visualised using a bright-field panoramic digital slide scanner (3DHISTECH).

2.10 Immunofluorescence staining on collagen gels

Collagen gels were first fixed in 70% ethanol (Thermo Fisher Scientific, cat no. 10437341) for 24h. Immunofluorescence on collagen gels were performed on 96 well plates (Corning) due to the size of the gels.

To begin with, gels were washed with three times with D-PBS (Gibco, cat no. 13492609), with 5 minutes/wash. Next, cells were permeabilised with 0.5% triton-X-100 (prepared in dH_2O, Merck, cat no. T8787) for 10 minutes at room temperature. Gels were then washed with 1x-glycine in PBS (Merck, cat no. G8898 1KG) for 3 minutes at room temperature. This was followed by two washes in PBS, with 3 minutes/wash. Wells were then incubated with 5% BSA (Merck- A8022) in PBS for 1 hour at room temperature. Blocking buffer was then replaced with the desired primary antibody (see Table 6 and Table 7) in 1% BSA (Merck- A8022) in PBS overnight in 4°C. Plates were protected from light.

The following day, gels were washed three times with D-PBS, with 3 minutes/wash. This was followed by the addition of the secondary antibody (Table 8) in 1% BSA (Merck- A8022) for 1 hour at room temperature. Gels were washed with D-PBS three times, with 3 minutes/wash. Wells were next stained with DAPI (1:2000 in PBS, Tocris, cat no. 40043) for 15 minutes at room temperature. This was followed by three washes in D-PBS, with 3 minutes/wash. Gels were then carefully transferred to a NUNC™ glass bottom dish (Thermo Fisher Scientific, cat no. 150680), covered with PBS, and imaged using the Zeiss LSM-710 confocal microscope.

2.11 Analysis of the Secretome

2.11.1 Human Cytokine Array

A Proteome Profiler™ Array (RnD systems, cat no. ARY005B) was performed on supernatants of tetra-culture models treated with aspirin. This assay was done according to the manufacturer's protocol. All reagents were provided in the kit, unless stated otherwise. All components in the kit were brought to room temperature and samples were thawed on ice.

Samples were prepared as follows: 200µL of sample was added to 500 µL of array buffer 4 and 800 µL of array buffer 5. Next, 15µL of reconstituted human cytokine

array detection antibody cocktail was added to each sample and incubated at room temperature for at least 1 hour. In the meantime, membranes were carefully placed in the 4-well multi-dish and incubated with 2mL array buffer 4 for 1 hour at room temperature. Array buffer 4 was then aspirated from each well, replaced with the prepared samples and incubated overnight at 4°C on a rocking platform.

The following day, wells were washed 3 times with 1x wash buffer on a rocking platform (3 min/ wash). 2mL of diluted streptavidin-HRP in array buffer 5 was then added to each well and incubated for 30 minutes at room temperature. Wells were then washed 3 times with 1x wash buffer on a rocking platform (3 min/wash).

Finally, membranes were carefully placed inside a plastic sheet protector with 1mL of the prepared 'Chemi Reagent Mix' and developed using the Amersham™ Imager 600 (GE Healthcare Life Sciences).

2.11.2 Enzyme Linked Immunosorbent Assay (ELISA)

ELISAs of Interleukin-6 (IL-6), interleukin-8 (IL-8) and monocyte attractant protein-1 (MCP-1 or CCL2) were done on aspirin-treated cultures. All assays were carried out according to the manufacturer's protocol. Reagents were provided in the kits, unless stated otherwise. All components of the kits were brought to room temperature. Supernatants were thawed on ice and diluted at least 1:2 in standard diluent buffer to ensure that sample concentrations would be within the range of the respective standard curves (IL-6, 0-1690pg/mL; IL-8, 0-1000pg/mL; CCL2, 0-2000pg/mL).

2.11.2.1 Interleukin-6 (IL-6)

An ELISA kit of human IL-6 was provided by ThermoFisher Scientific (cat. no KAC1261). 50µL of 'solution B' was pipetted into the appropriate wells for the standards and 50µL of 'solution A' was pipetted into the appropriate wells for the samples. Next, 100 µL of standards and pre-diluted samples (1:10) were added to the appropriate wells and incubated for 1 hour at room temperature. The solution was then aspirated and washed 3 times with 1x wash buffer. Next, 100µL of anti-IL-6 conjugate was pipetted into all wells. This was followed by the addition of 50 µL of solution A into each well. The plate was then incubated for 1 hour at room temperature, followed by three washes in 1x wash buffer. 200 µL of chromogenic

TMB was then added to each well and incubated for 15 minutes in the dark. Finally, 100 µL of 'stop solution' was added to each well, and absorbance was immediately read at 450nm using a BMG FLUOstar Optima microplate reader (Labtech, not provided by the kit).

2.11.2.2 Interleukin-8 (IL-8)

An ELISA kit of human interleukin-8 (IL-8) was provided by ThermoFisher Scientific (cat. no KHC0081).

50µL of standards and pre-diluted samples (1:2) were added to the appropriate wells. 50µL of human IL-8 biotin conjugate solution was then added into each well. Plates were incubated for 90 minutes at room temperature, followed by four washes using 1x wash buffer. Next, 100µL of 1x Streptavidin-HRP solution was added into each well, except the chromogen blanks. Plates were incubated for 30 minutes at room temperature, followed by four washes in 1x wash buffer. Next, 100 µL of stabilised chromogen (TMB) was added to each well and incubated for 30 minutes at room temperature in the dark. Adding TMB to each well resulted in the solution slowly turning blue. Finally, 100 µL of the stop solution was added to each well. This resulted in the colour of the solution changing from blue to yellow. Absorbance was immediately read at 450nm using a BMG FLUOstar Optima microplate reader (Labtech, not provided by the kit).

2.11.2.3 CCL2 (monocyte chemo-attractant protein-1, MCP-1)

Biotechne provided a human CCL2/MCP-1 quantikine ELISA kit, RnD Systems (cat no. DCP00). 200 µL of standards and pre-diluted samples (1:5) were added to the appropriate well and incubated for 2 hours at room temperature. This was followed by four washes with 1x wash buffer. Next, 200 µL of human MCP-1 conjugate was added to each well and incubated for 1 hour at room temperature. Wells were then washed 4 times with 1x wash buffer and incubated with 200 µL substrate solution for 30 minutes at room temperature in the dark. Finally, 50 µL of 'stop solution' was added to each well, and absorbance was recorded at 450nm using a BMG FLUOstar Optima microplate reader (Labtech).

2.11.3 MesoScale Discovery (MSD®) Multi-Spot Assay System (V-PLEX®)

A custom MSD® was performed on aspirin-treated cultures. This assay was done according to the manufacturer's protocol. All reagents were provided in the kit, unless stated otherwise. Similar to the ELISA assays described above, all components were brought to room temperature. Supernatants were thawed on ice and centrifuged for 3 min at 2000xg, 4°C. All samples were diluted 1:2 for this assay.

First, plates were washed 3 times with 150 µL of wash buffer. Next, 50 µL of standards and pre-diluted samples were added to each well and incubated at room temperature for 2 hours. Plates were then washed 3 times with 150 µL wash buffer. 25 µL of detection antibody cocktail was added to each well and incubated for 2 hours at room temperature. Plates were then washed 3 times with 150 µL wash buffer. Finally, 150 µL of 2x 'Read buffer T' was added to each well. Plates were then analysed on the MESO QuickPlex SQ 120MM MSD instrument.

2.12 Analysis and statistics

GraphPad Prism (Version 8.3.0, La Jolia, California, U.S.A) was used for graphical representation of data and statistical analysis. Error bars represented the standard deviation (SD). An ordinary one-way analysis of variance (ANOVA) test was used to assess statistical significance between untreated controls (DMSO) and samples treated with 10µM, 100µM and 1mM aspirin. A one-way ANOVA test was used as three different aspirin treatment doses were compared against the untreated control group. Results were said to be statistically significant when $p<0.05$.

3 Results - Investigating the short-term and longer-term effects of aspirin on malignant cell growth and viability

3.1 Background

The first aim of my project was to determine the effects of aspirin on the growth of malignant cells and the different components of the tumour microenvironment (TME). I started my PhD by looking at existing RNA-sequencing data to investigate the basal expression of *PTGS1* (COX-1), *PTGS2* (COX-2) and its downstream targets in AOCS-1, G164 and OVCAR3 human HGSOC cell lines. Colleagues in the lab performed the RNA extraction, which was then outsourced to Oxford Genomics Centre and analysed by Dr. Eleni Maniati.

3.2 RNA-sequencing data showing *PTGS1, PTGS2* and downstream target expression in HGSOC cell lines

As shown in Figure 3.1, all three cell lines had measurable transcripts for COX-1 (*PTGS1*). AOCS-1 and G164 expressed *PTGS2* (COX-2), although *PTGS2* levels were higher in G164 compared to AOCS-1. OVCAR3 cells did not have detectable levels of *PTGS2*. In addition, G164 had high levels of three cognate receptors of PGE_2, EP2 (*PTGER2*), EP3 (*PTGER3*), and EP4 (*PTGER4*). EP2-4 receptors are associated with cell proliferation, migration and invasion[31]. On the other hand, mixed results were seen for the AOCS-1 cell line. AOCS-1 expressed low levels of EP2 and EP3, but high levels of EP4. Similarly, OVCAR3 cells expressed EP3 and EP4, but did not express EP2. Overall, AOCS-1 and G164 cells expressed *PTGS2* and *PTGER2-4,* which suggested that the COX-2-PGE_2 pathway was relevant in these two HGSOC cell lines.

I also used PAX-8 as a reference gene, as malignant cells of HGSOC typically express PAX-8. It would be interesting to measure protein levels of these genes.

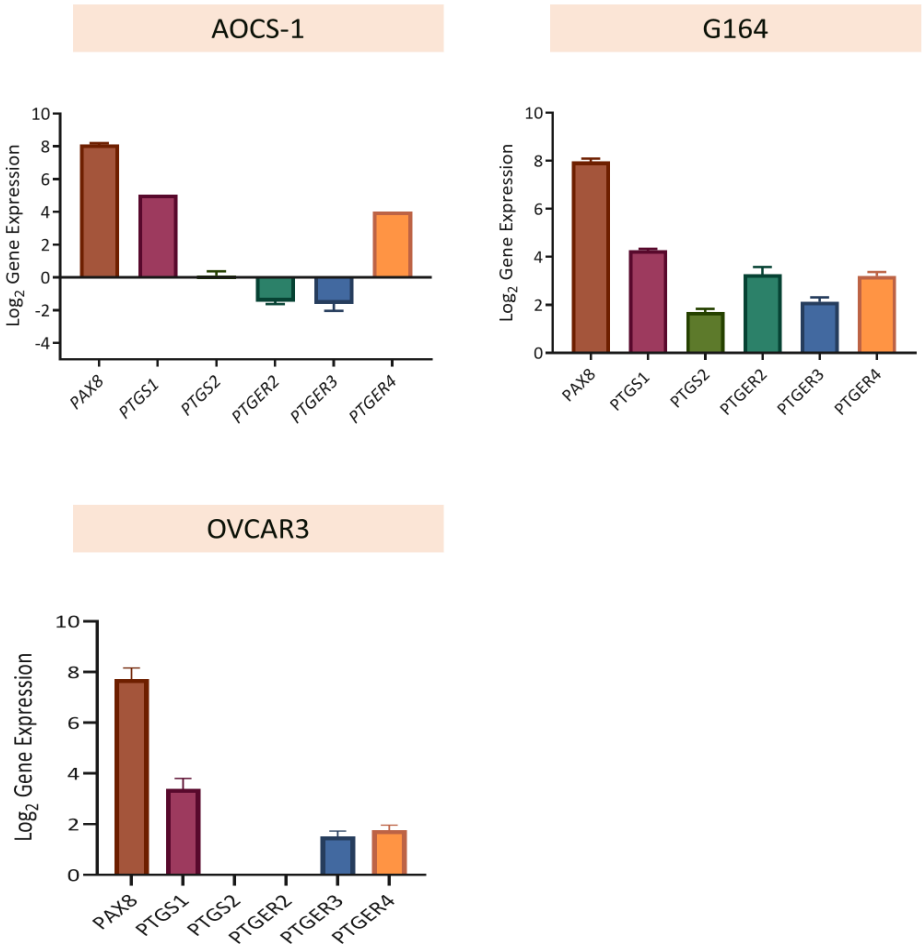


Figure 3.1. RNA-sequencing data of genes involved in regulating the metabolism of arachidonic acid into prostanoids, including *PTGS1* (COX-1) and *PTGS2* (COX-2). RNA sequencing was performed on three HGSOC cell lines: AOCS-1 (ascites), G164 (omental metastases) and OVCAR3 (ascites). I examined the expression of *PTGS-1* (COX-1), *PTGS-2* (COX-2) and PGE_2 receptors *(PTGER 1-4).* AOCS-1 and G164 cells expressed both *PTGS1* and *PTGS2*, whereas OVCAR3 cells only expressed *PTGS1*. PAX-8 was used as a reference gene as HGSOC cells express PAX-8. PTGS-1, prostaglandin endoperoxide synthase-1; PTGS-2, prostaglandin endoperoxide synthase-2; PTGER2, prostaglandin E receptor 2; PTGER3, prostaglandin E receptor 3 ; PTGER4, prostaglandin E receptor 4.

3.3 Short-term effects of low-dose aspirin on the growth of HGSOC cells, primary omental fibroblasts, and primary omental mesothelial cells

After interrogating the RNA-sequencing data, I assessed the effects of aspirin on AOCS-1 and G164 cell lines *in vitro* in 2D monoculture. Since there are little data on the effects of aspirin in ovarian cancer cells, I tested doses and time-points based on publications on aspirin's effects *in vitro* on other cancer types, most notably colorectal cancer (CRC) cell lines [182,183,184]. I used doses ranging from 5μM- 1mM and time-points up to 72 hours.

Treating AOCS-1 and G164 HGSOC cell lines daily with aspirin for 48 and 72 hours had no effect on malignant cell growth, as shown in Figure 3.2 and Figure 3.3. There were also no morphological changes seen in AOCS-1 (Figure 3.2A) and G164 cells (Figure 3.3A). In G164, viable cell counts appeared to stall for the first 48 hours after 50μM and 100μM treatment (Figure 3.3B). However, these differences were not statistically significant.

I then corroborated these results using a CyQuant cell proliferation assay, where I measured DNA concentrations after administering aspirin to cells for 24, 48 and 72 hours. Aspirin did not affect tumour cell proliferation. DNA concentrations of AOCS-1 (Figure 3.2B) and G164 (Figure 3.3B) after aspirin treatment seemed to have marginally decreased relative to untreated controls, but this was not statistically significant. Cells did not display any morphological signs of stress.

Next, I explored the potential effects of aspirin on primary omental mesothelial cells and omental fibroblasts. This is because they are key stromal components in the HGSOC tumour microenvironment that may contribute towards malignant cell invasion and metastasis. Given their importance, they are also crucial components of our tetra-culture model, which I used later in the project (chapter 5). Qualitative observations and quantitative analysis showed that aspirin did not inhibit primary fibroblast or mesothelial cell growth or proliferation (Figure 3.4 and Figure 3.5 respectively). I therefore concluded that low-dose aspirin (5-100μM) had no effect

on the growth of two different HGSOC cell lines, primary omental fibroblasts or mesothelial cells grown in 2D culture.

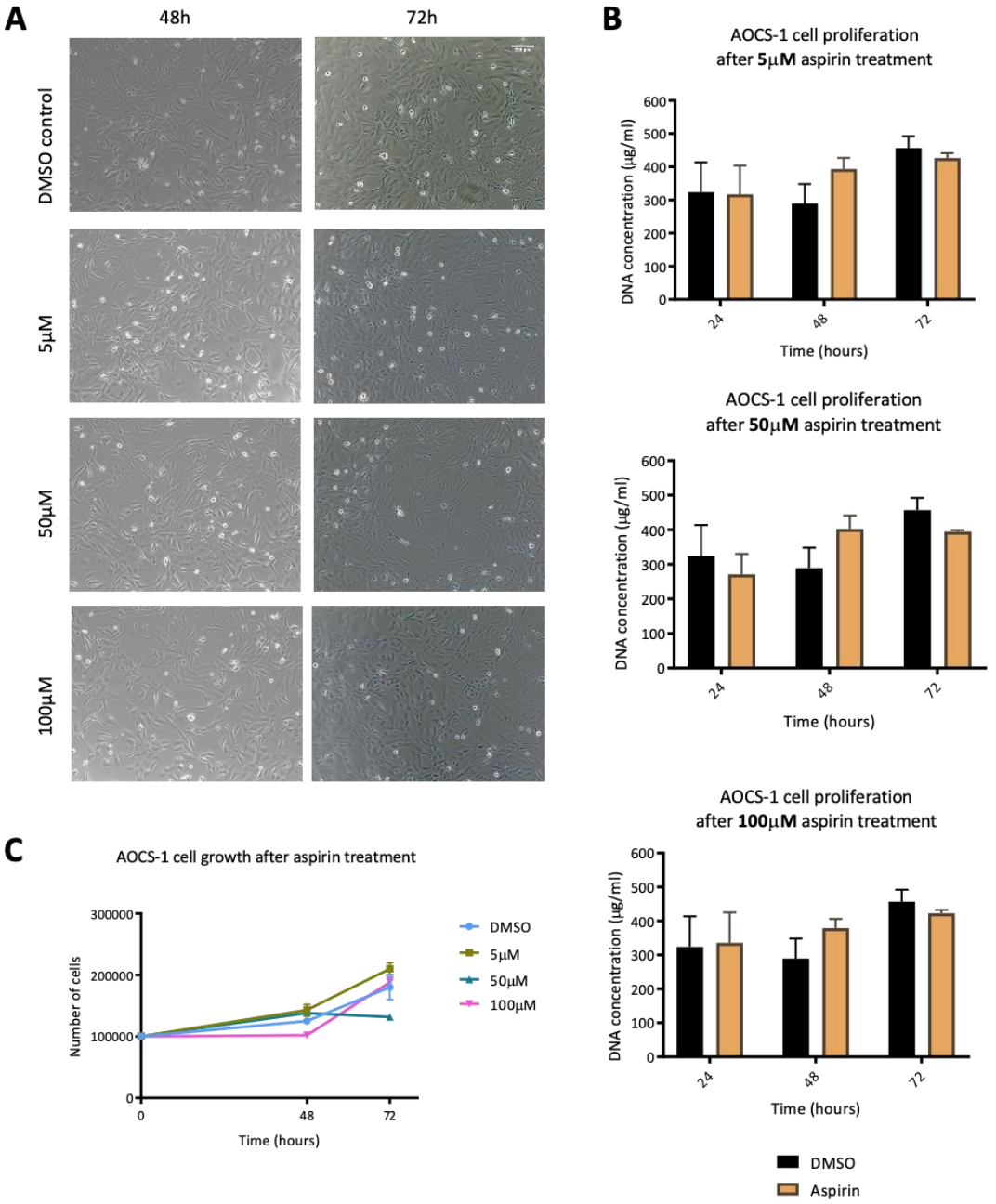


Figure 3.2. Effect of low-dose aspirin treatment on AOCS-1 cell growth and proliferation. Images (10x magnification) were taken after 48h and 72h of aspirin treatment (A), cell growth was measured using a Vi-Cell XR counter (B), and cell proliferation was quantified using a CyQuant cell proliferation assay (C). Overall, aspirin had no effect on AOCS-1 cell growth or proliferation (n=3). Scale bar represents 200µM.

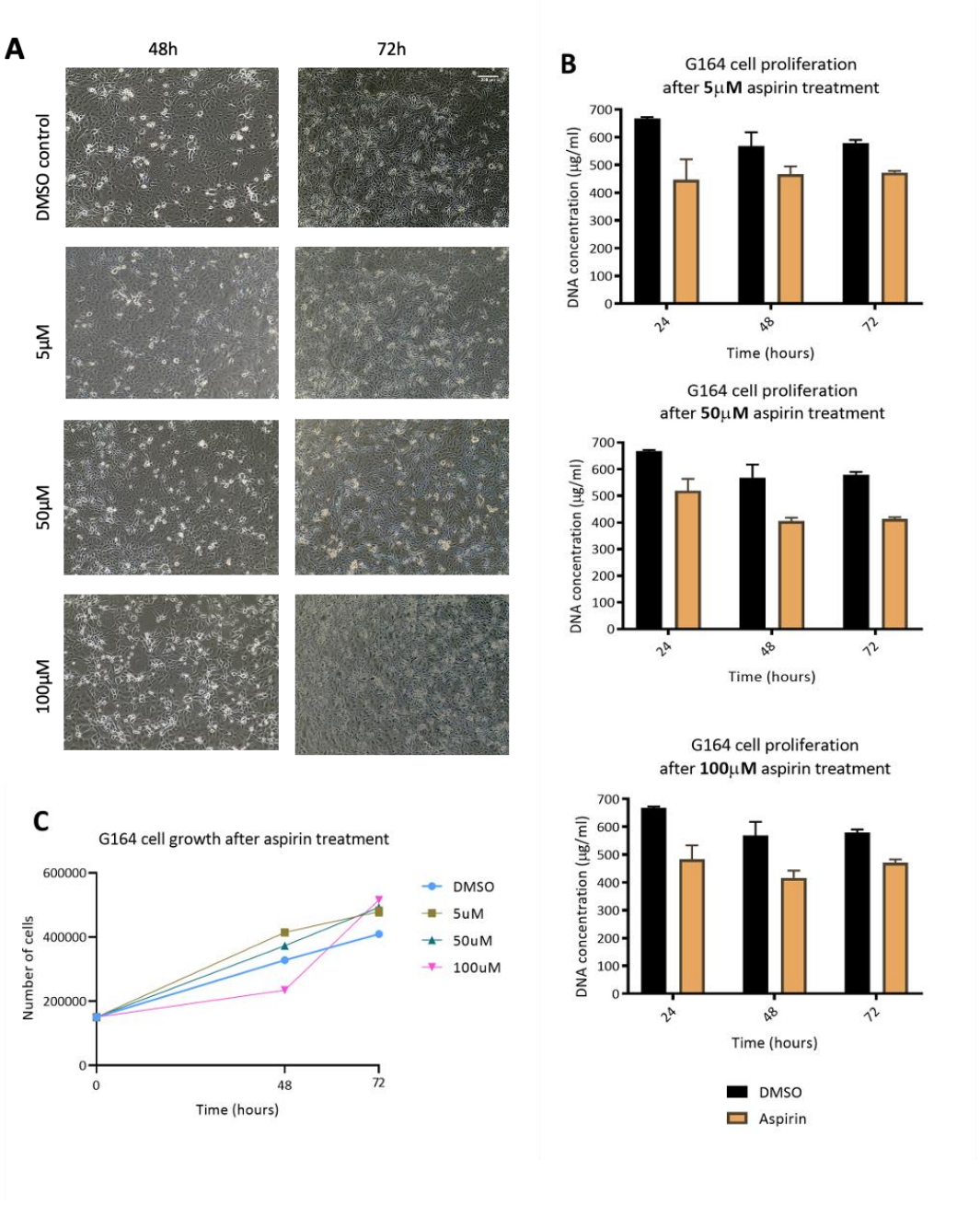


Figure 3.3. Effect of low-dose aspirin treatment on G164 cell growth and proliferation. Images (10x magnification) were taken after 48h and 72h of aspirin treatment (A), cell growth was measured using a Vi-Cell XR counter (B), and cell proliferation was quantified using a CyQuant cell proliferation assay (C). Overall, aspirin had no effect on G164 cell growth or proliferation (n=3). Scale bar represents 200µM.

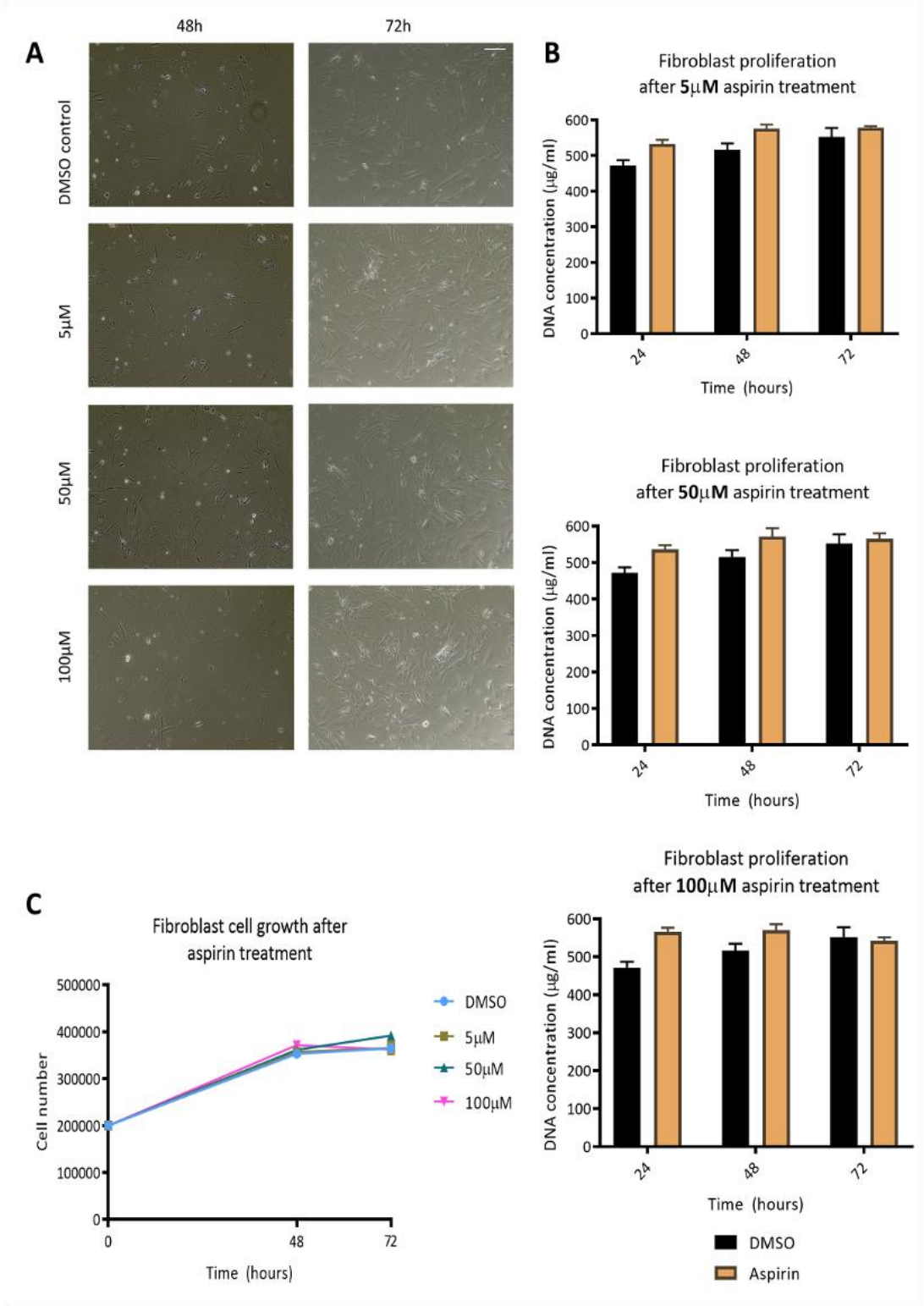


Figure 3.4. Effect of low-dose aspirin treatment on primary fibroblast growth and proliferation. Images (10x magnification) were taken after 48h and 72h of aspirin treatment (A), cell growth was measured using a Vi-Cell XR counter (B), and cell proliferation was quantified using a CyQuant cell proliferation assay (C). Overall, aspirin had no effect on fibroblast cell growth or proliferation (n=3). Scale bar represents 200µM.

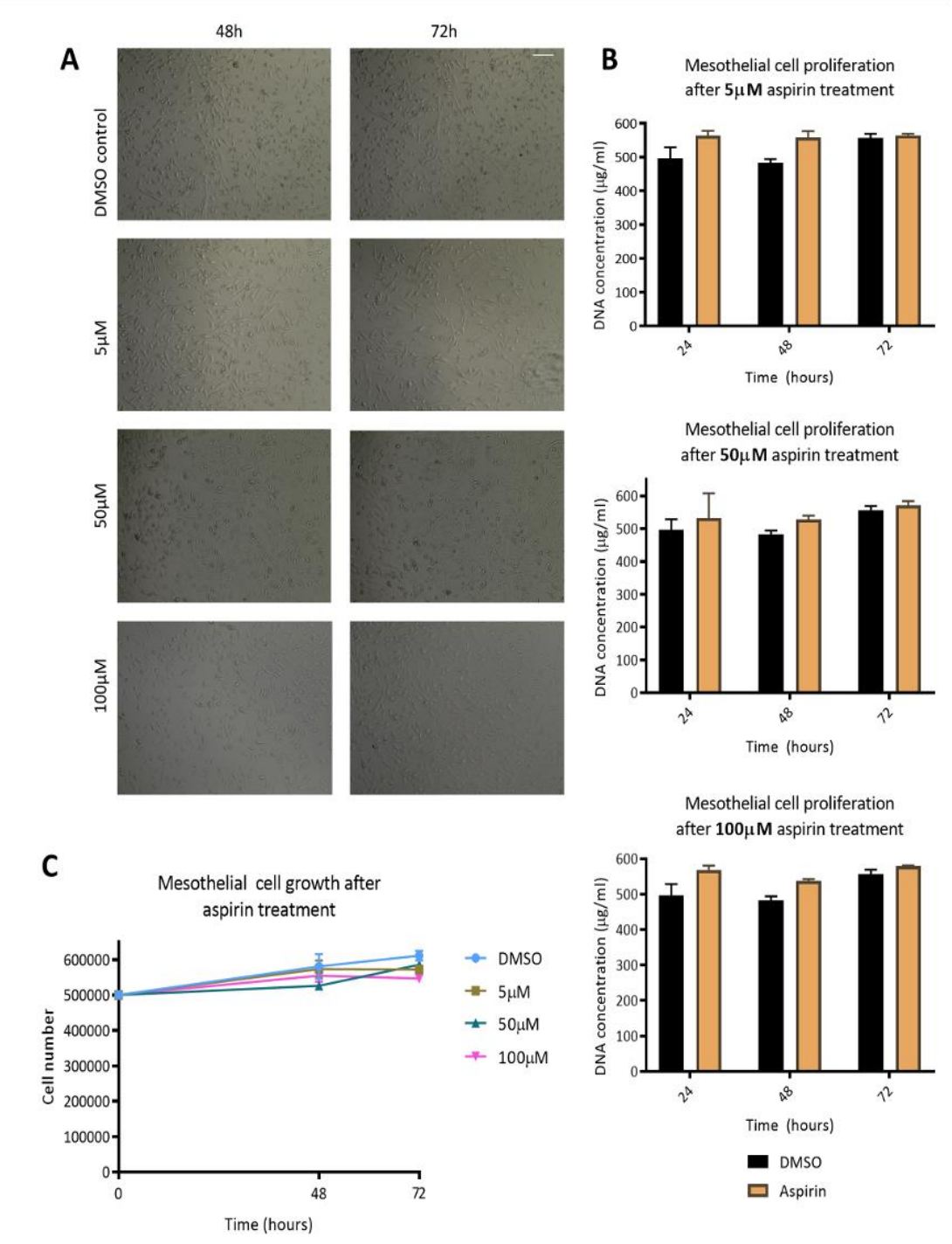


Figure 3.5. Effect of low-dose aspirin treatment on primary mesothelial cell growth and proliferation. Images (10x magnification) were taken after 48h and 72h of aspirin treatment (A), cell growth was measured using a Vi-Cell XR counter (B), and cell proliferation was quantified using a CyQuant cell proliferation assay (C). Overall, aspirin had no effect mesothelial cell growth or proliferation (n=3). Scale bar represents 200μM.

3.4 Short-term effects of low-dose aspirin on colorectal cancer cells

Since low-dose aspirin had no effect on HGSOC cells, primary omental fibroblasts, or mesothelial cells in short term culture, I needed a positive control to confirm aspirin activity in the preparations I had used. There is evidence documenting the effects of daily low-dose aspirin in the prevention of CRC[19,21,185,186,44]. Therefore, I selected the following three cell lines and treated them with aspirin for 48 and 72 hours:

1. HT-29- from grade II colon adenocarcinoma
2. HCT116- from colorectal carcinoma
3. HCT-15- from Dukes' type C, colorectal adenocarcinoma

Figure 3.6 A-C illustrated that short-term aspirin exposure did not inhibit the growth of HT-29, HCT116 and HCT-15 cell lines, despite evidence in literature suggesting otherwise. Moreover, even supra-physiological levels of aspirin (1mM) did not reduce CRC cell line growth.

Overall, short-term aspirin treatment did not reduce the growth of two HGSOC cell lines, three CRC cell lines, primary omental fibroblasts, and primary omental mesothelial cells.

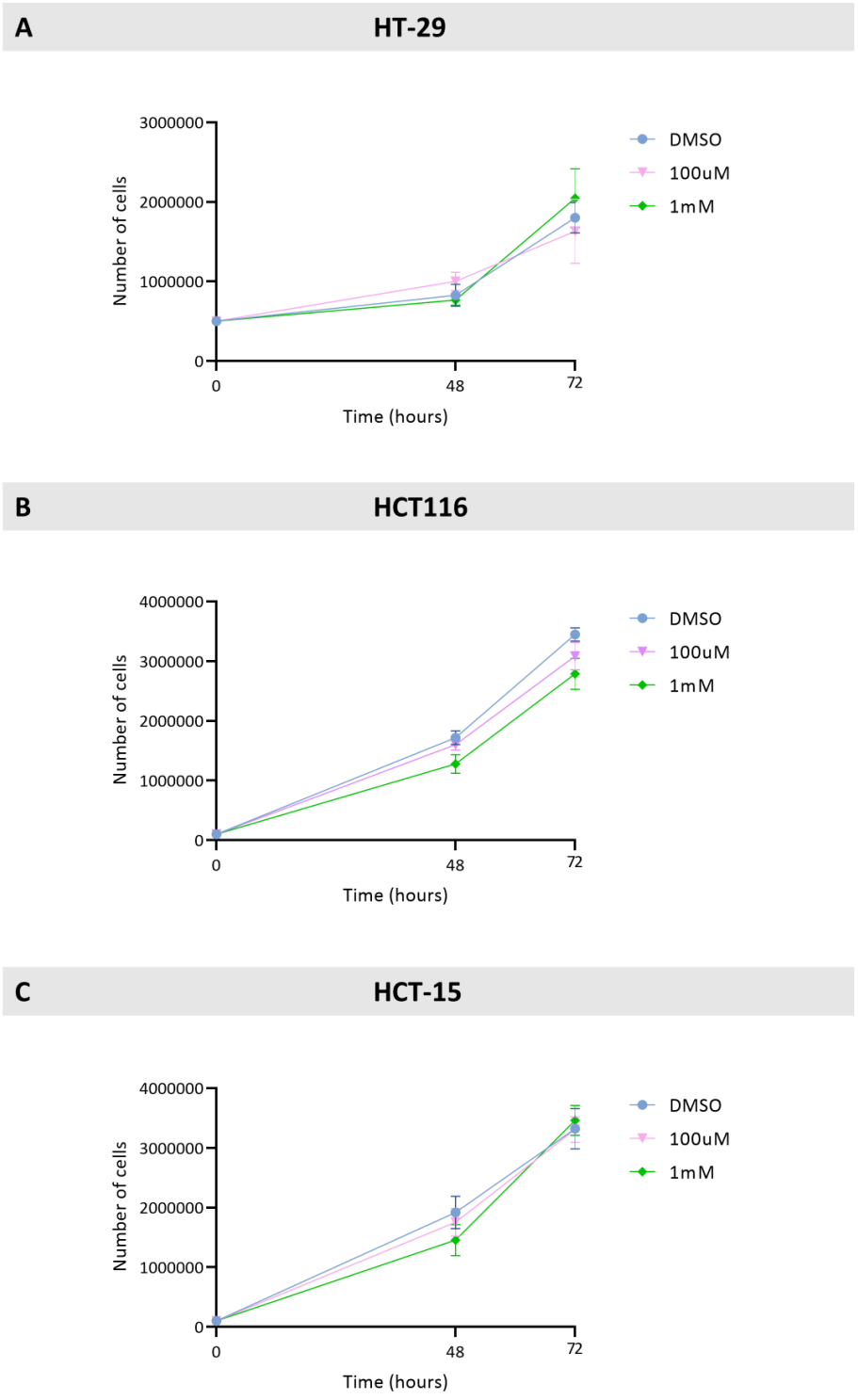


Figure 3.6. Effect of aspirin treatment on HT-29, HCT116 and HCT-15 colorectal cancer cells. Cell growth was measured using a Vi-Cell XR counter after 48h and 72h daily aspirin treatment. (A) Shows HT-29 growth curve following 48h and 72h of aspirin treatment; (B) HCT116 growth curve following 48h and 72h of aspirin treatment; (C) HCT015 growth curve following 48h and 72h of aspirin treatment.

3.5 Longer-term effects of low-dose aspirin on HGSOC and CRC cells

I questioned whether ≤72h aspirin treatment was long enough to see a growth inhibitory effect on malignant cells. Therefore, I explored whether longer-term aspirin exposure would hamper malignant cell growth *in vitro.* Moreover, from a translational standpoint, the therapeutic effects of aspirin in patients are visible only after prolonged administration[19,21]. Consequently, I treated malignant cell lines with 100μM and 1mM aspirin and measured cell growth after 4, 5, 6, and 7 days. I decided to examine lower doses (<100μM) only if there was a visible reduction in malignant cell growth after 100μM and 1mM aspirin treatment.

Longer-term aspirin treatment did not reduce HT-29, HCT116 and HCT-15 cell growth (Figure 3.7). Moreover, 1mM aspirin, a supra-physiological dose, only led to a modest reduction HT-29 and HCT116 growth, although this was not significant. Similarly, a 7-day aspirin treatment did not influence AOCS-1 and G164 cell growth (Figure 3.8). Looking at the data closely, aspirin appeared to stall AOCS-1 cell growth after four, five and 6 days of treatment, but again this was not statistically significant so can be discounted.

Overall, Figure 3.7 and Figure 3.8 indicated that longer-term aspirin administration did not significantly inhibit the growth of CRC and HGSOC cell lines.

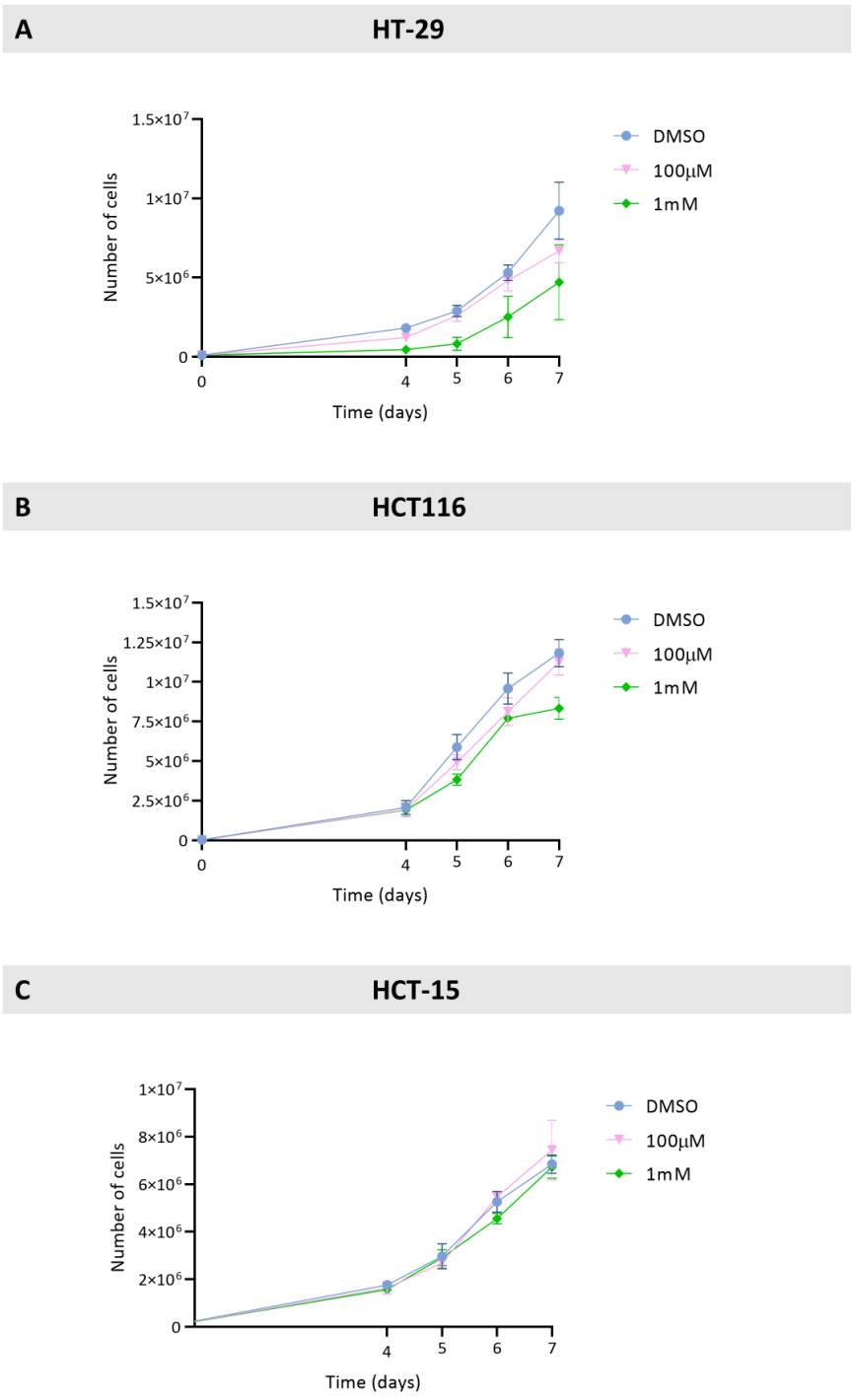


Figure 3.7. Longer-term effects of aspirin treatment on HT-29, HCT116 and HCT-15 colorectal cancer cells. Cell growth was measured using a Vi-Cell XR counter after 4, 5, 6 and 7 days of daily aspirin treatment. (A) Shows HT-29 growth curve following aspirin treatment; (B) HCT116 growth curve following aspirin treatment;(C) HCT-15 growth curve following aspirin treatment.

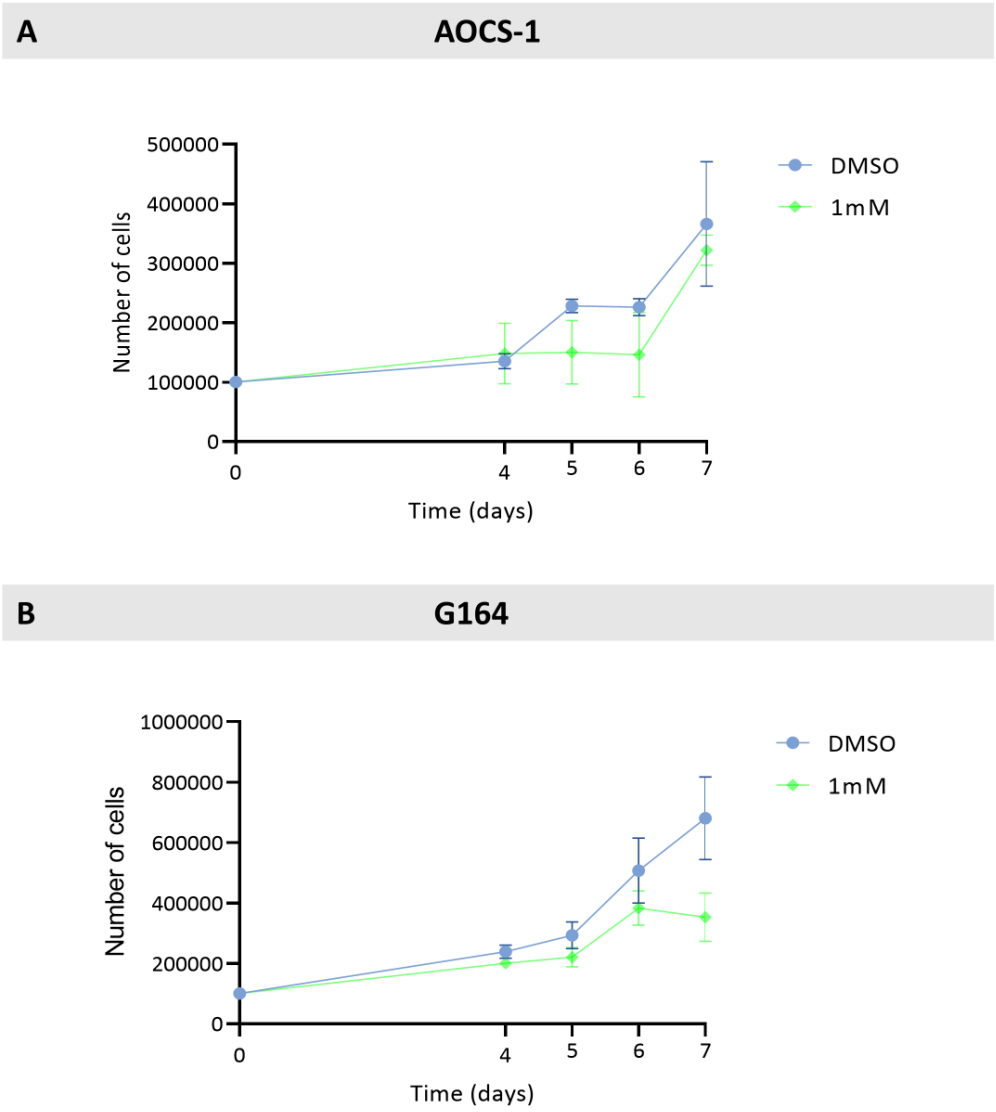


Figure 3.8. **Longer-term effect of aspirin on AOCS-1 and G164 high-grade serous ovarian cancer cells.** AOCS-1 and G164 HGSOC cells were exposed to daily doses of aspirin, and cell growth was measured using a VI-Cell XR counter after 4, 5, 6 and 7 days. 1mM aspirin seems reduce the growth in G164 cells, but this is not significant. Overall, 1mM aspirin did not reduce AOCS and G164 cell growth.

3.6 Effects of aspirin in suspension spheroids of malignant cells

The data acquired so far showed that aspirin treatment (≤7 days) did not decrease malignant cell growth. However, I did this work on 2D monolayers, which is not representative of conditions *in vivo*[171,156,176]. Since I was ultimately keen to study the effects of aspirin in complex 3D multi-cellular models, it made sense to examine the actions of aspirin in simple 3D *in vitro* culture systems.

I first set up spheroids of CRC cells to see if I could obtain a positive control for aspirin treatment. I exposed spheroids of HT-29 and HCT116 cells with aspirin for 7 days and measured cell viability using a live/dead fluorescent stain. As displayed in Figure 3.9, aspirin did not hinder viability of CRC spheroids. This is depicted by an abundance of 'green' cells. At a glance, there seemed to be more dead (red) cells in HT-29 spheroids after 1mM aspirin treatment, but this was not significant.

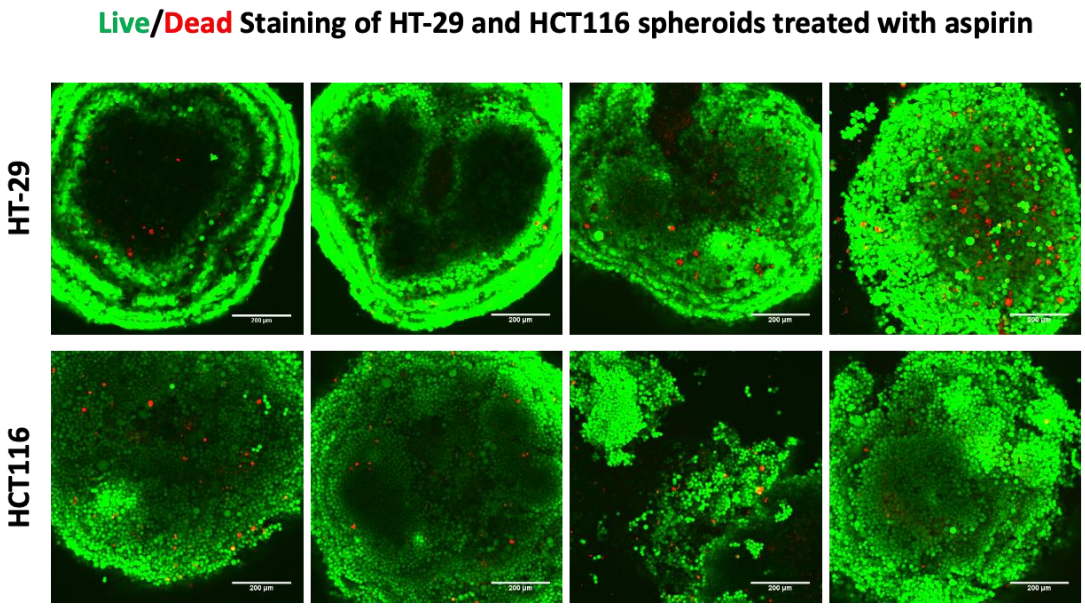


Figure 3.9. Live/Dead stain on CRC spheroids of HT-29 and HCT116 after a 7-day aspirin treatment. Suspension spheroids of HT-29 and HCT116 were subjected to daily aspirin exposure for 7 days. A live/dead staining for cell viability was performed and spheroids were imaged on the LSM-710 confocal microscope at 10-x magnification. 'Green' staining indicates viable cells, and 'red' staining indicates dead cells. Scale bar shows 200µM.

3.7 Effects of aspirin in collagen gels of malignant cells and fibroblasts

Despite the 'negative data' accumulated so far, I proceeded to examine the effects of aspirin in 3D *in vitro* collagen gels of malignant cells and fibroblasts. Three-dimensional (3D) *in vitro* culture systems have shown to be particularly relevant for understanding the TME, as malignant cells act in concert with stromal cell compartments and immune cells to facilitate invasion and metastasis[171,156,176,187]. Three-dimensional models also allow for co-culturing multiple cell types together, as well as the integration of native or bioengineered tumour ECM components, including collagen[158].

I first interrogated the available RNA-sequencing analysis of G164 and fibroblast collagen gels. As depicted in Figure 3.10, there was an increase in *PTGS2* (COX-2) in co-culture gels compared to 2D monolayers of G164 alone. There was also more *PTGER3* (EP3) expressed in collagen gels of G164 and fibroblasts. Furthermore, *PTGER1* (EP1) was present in collagen gels, whereas it was absent in G164 monolayers. There were also slightly elevated levels of *PTGER2* (EP2), *PTGER4* (EP4) and *PTGS1* (COX-1) expression in collagen gels compared to 2D monocultures of G164.

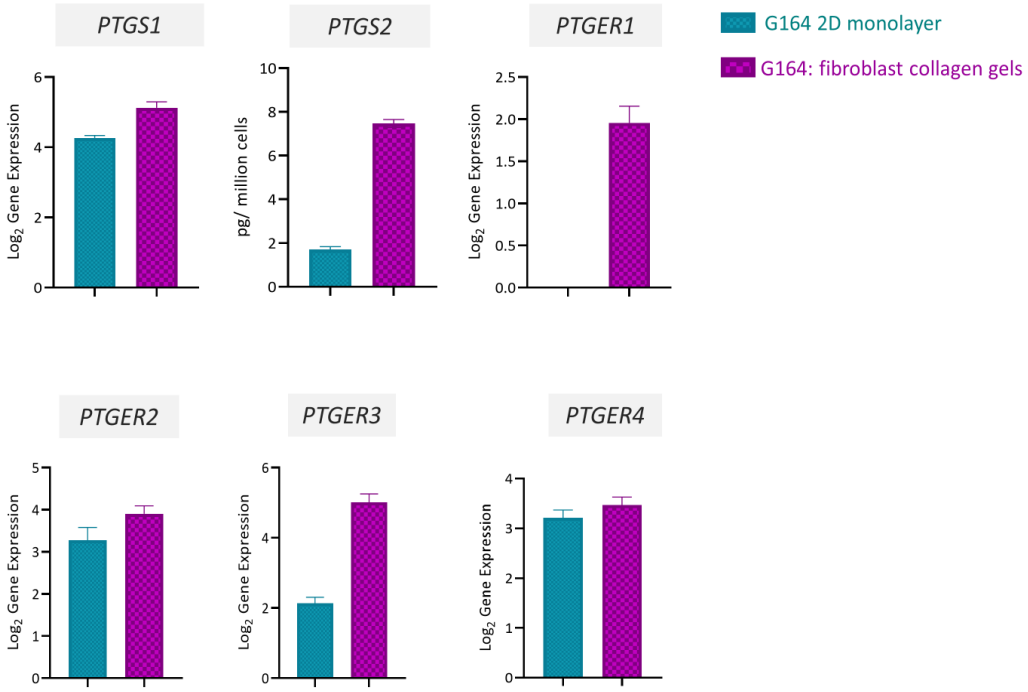


Figure 3.10. RNA-sequencing data of genes involved in regulation of the metabolism of arachidonic acid into prostanoids in collagen co-culture gels of G164 and fibroblasts. I decided to examine RNA-sequencing data of collagen co-culture gels to check for the expression of *PTGS1* (COX-1), *PTGS2* (COX-2) and PGE_2 receptors *(PTGER 1-4).* PTGS-1, prostaglandin endoperoxide synthase-1; PTGS-2, prostaglandin endoperoxide synthase-2; PTGER2, prostaglandin E receptor 2; PTGER3, prostaglandin E receptor 3; PTGER4, prostaglandin E receptor 4. (n=1).

Using 3D collagen co-culture gels of malignant cells and primary omental fibroblasts, I investigated whether aspirin reduced cancer cell proliferation and the number of activated fibroblasts. I cast collagen gels of G164 cells and fibroblasts using a ratio of 1:1, a ratio that was established as optimal by previous lab members. I then treated gels with aspirin and performed immunohistochemical staining on formalin fixed, paraffin embedded sections. As portrayed in figure 3.11, aspirin did not alter cellular proliferation (Ki67), the number of malignant cells (PAX-8) or activated fibroblasts (FAP).

I also tested the anti-proliferative effects of aspirin in CRC collagen gels of HT-29 and fibroblasts. There was no significant effect observed by immunohistochemical staining (Figure 3.12). Additionally, HT-29 cells displaced the fibroblasts by day 7, as they are hyper-proliferative cells. Performing an immunofluorescence stain on these

gels showed that there were much fewer fibroblasts compared to HT-29 cells after 7 days in culture (figure 3.13).

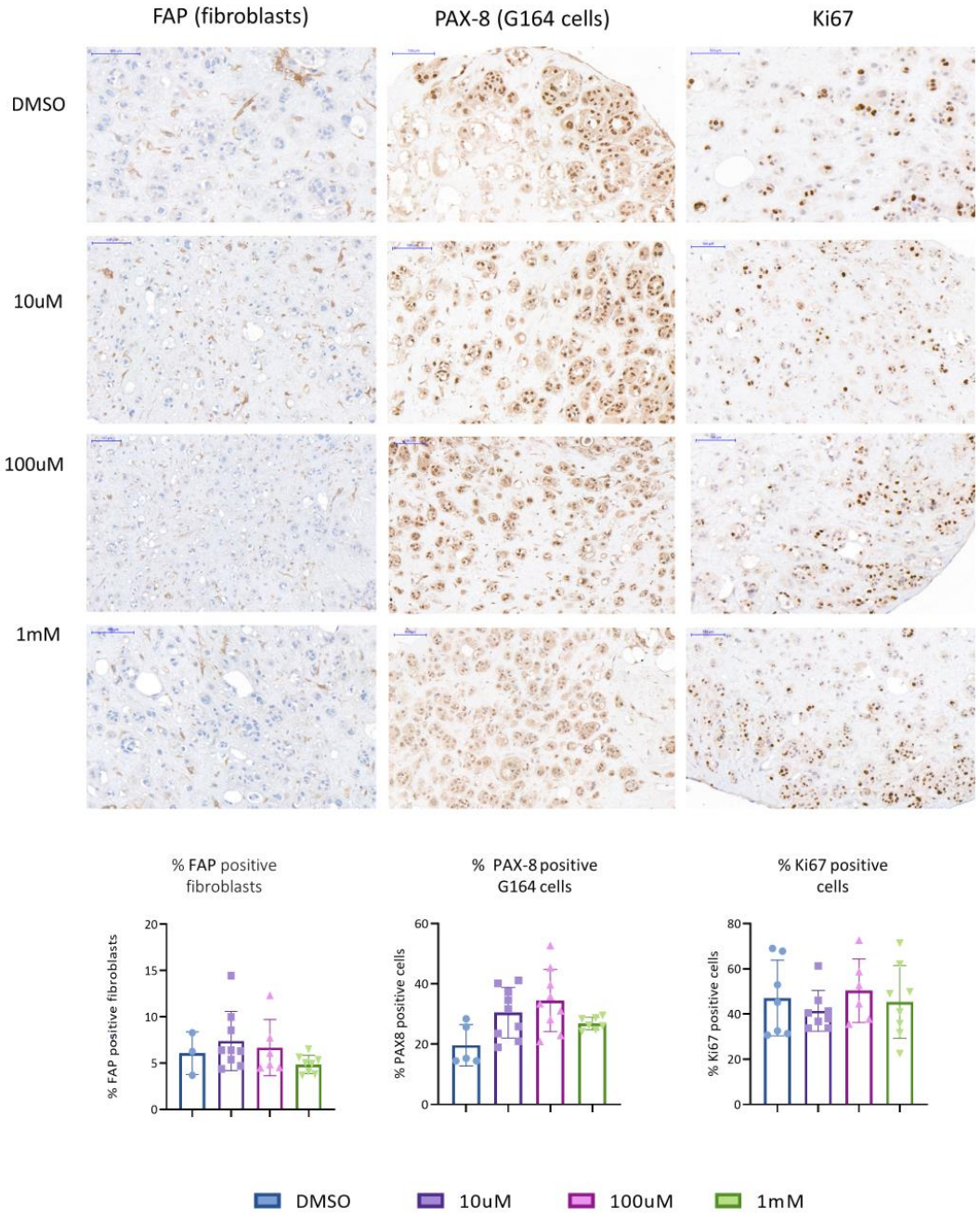


Figure 3.11. Collagen gels of G164 cells and primary fibroblasts treated with aspirin. I treated collagen gels of G164 cells and primary omental fibroblasts with daily aspirin for 7 days. Gels were embedded in 2% agarose, fixed in formalin, and embedded in paraffin. Immunohistochemical staining was performed on the gels. Three slides/condition/marker were stained. Quantification was performed using the QuPath software. FAP, fibroblast activation protein; PAX-8, paired box 8 gene. Scale bar shows 100μM.

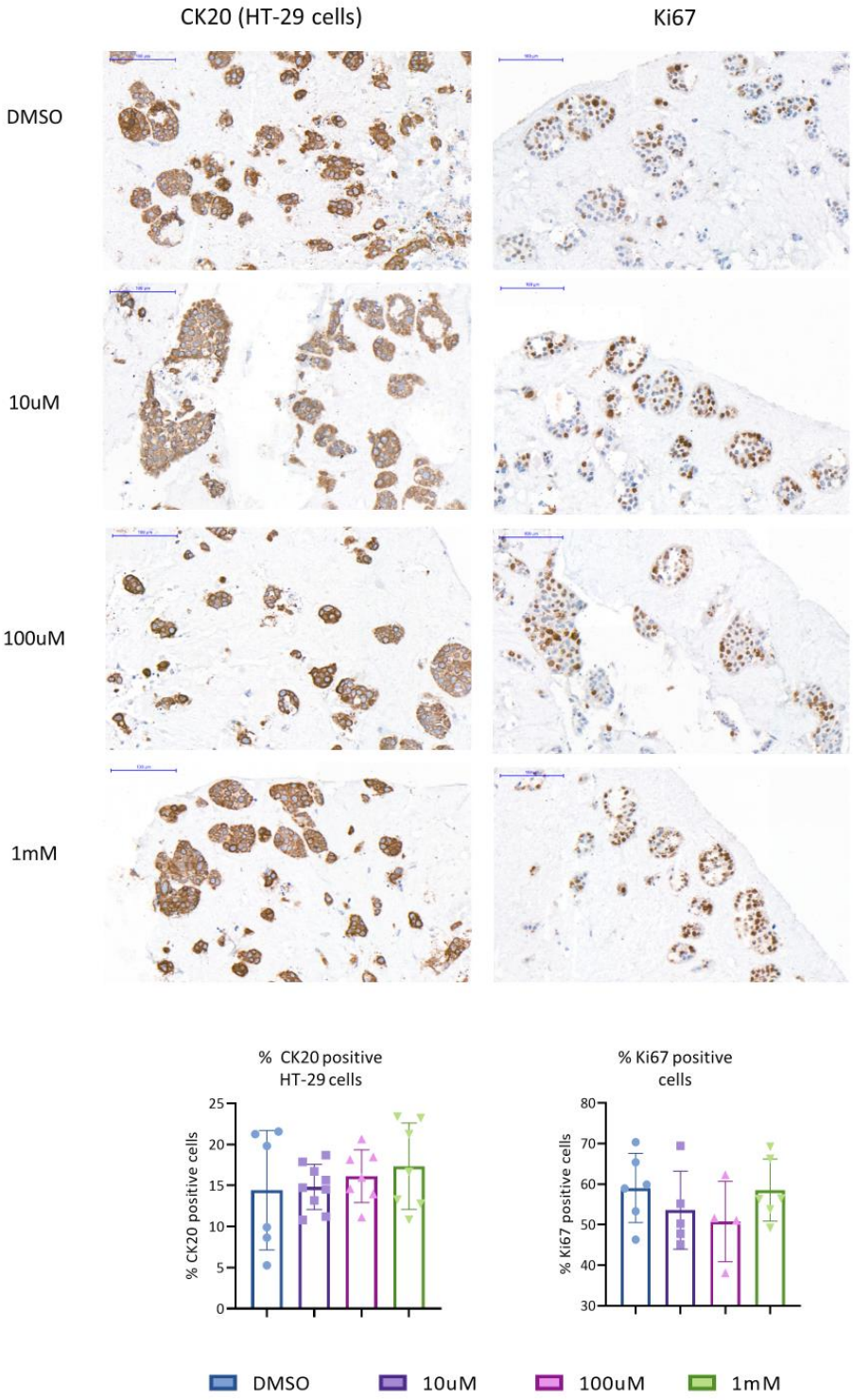


Figure 3.12. Collagen gels of HT-29 cells and primary fibroblasts treated with aspirin. I treated collagen gels of HT-29 and fibroblasts with daily aspirin for 7 days. Gels were embedded in 2% agarose, fixed in formalin and embedded in paraffin. Immunohistochemical staining was performed on the gels. Three slides / condition/marker were stained. Quantification was performed using the QuPath software. FAP, fibroblast activation protein; CK20, cytokeratin 20. Scale bar shows 100μM.

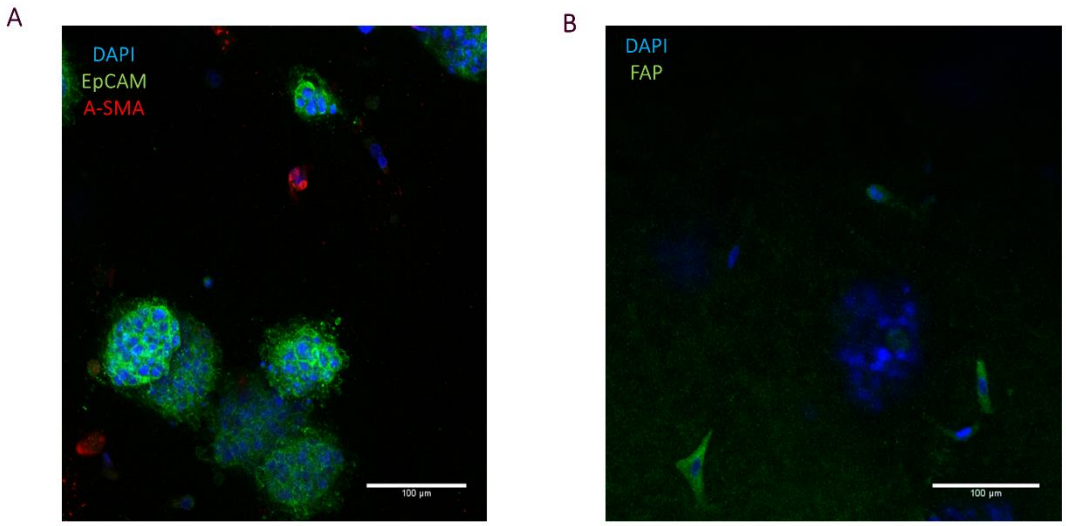


Figure 3.13. Immunofluorescence of collagen gels of HT-29 and fibroblasts after 7 days. I performed immunofluorescence staining on collagen gels of HT-29 and fibroblasts to confirm my IHC results. Immunofluorescent staining confirmed that there were far fewer fibroblasts compared to cancer cells. (A) Shows a staining of EpCAM and a-SMA; (B) is a staining for FAP. EpCAM was used to stain for HT-29, and a-SMA/FAP were used for fibroblasts. EpCAM, Epithelial Cell Adhesion Molecule; A-SMA, alpha smooth muscle actin; FAP, fibroblast activation protein. Scale bar shows 100µM.

3.8 Summary of Results

I have summarised the key findings from this chapter below:

- Short-term aspirin treatment (≤72h) did not inhibit the growth of two HGSOC cell lines (AOCS-1 and G164), three CRC cell lines (HT-29, HCT116, and HCT-15), primary omental fibroblasts and primary omental mesothelial cells.
- Longer-term aspirin exposure (4-7 days) did not inhibit HGSOC and CRC cell growth.
- Aspirin treatment did not reduce viability in spheroids of HT-29 and HCT116 cells.
- Aspirin did not affect malignant cell density, the number of activated fibroblasts and cellular proliferation in collagen co-culture gels of malignant cells (HGSOC and CRC) and fibroblasts.

3.9 Discussion

Aspirin has shown to exhibit promising potential as a cancer-preventative agent in several tumour types, especially colorectal and upper GI malignancies[19,22,186,188]. There is some epidemiological evidence that suggests a potential role of aspirin in preventing ovarian cancer[59,148,149]. Nevertheless, there is no concrete research done *in vitro* that investigates the biological mechanisms by which aspirin may hamper the neoplastic progression of HGSOC.

Based on preliminary RNA-sequencing results, I decided to examine the effects of aspirin on AOCS-1 and G164 HGSOC cell lines. I also tested aspirin on primary omental fibroblasts and mesothelial cells, as they are components of our tetra-culture model[181]. This 3D system aims to replicate the early stages of HGSOC progression in the omentum and understand the vital role played by the TME in promoting tumourigenesis[181]. Ultimately, I was interested to explore the actions of aspirin on the TME in our tetra-culture model, which comprises primary adipocytes, mesothelial cells, fibroblasts and malignant cells[181]. However, before exploring the effects of aspirin in complex multi-cellular models, I decided to study its effects on each cell type in 2D monocultures.

There was no effect elicited by low-dose aspirin (≤100μM) in our HGSOC cell lines, primary omental mesothelial cells, or omental fibroblasts, after 48 and 72 hours of treatment. There is a possibility that the low levels of *PTGS2* expression in the HGSOC cell lines, particularly AOCS-1, limited the response to aspirin. In fact, clinical evidence highlights that CRC patients with tumours that over-expressed COX-2 responded significantly better to aspirin treatment, compared to those that had weak or absent COX-2[189]. Therefore, the strength of COX-2 expression could potentially affect HGSOC cellular response to low-dose aspirin. However, it was unexpected to see that short-term aspirin exposure did not inhibit CRC cell growth, particularly in HT-29 cells. This contradicted publications that researched the effects of aspirin on the HT-29 cell line *in vitro*[190,191] .

These preliminary results led me to investigate the potential longer-term effects of aspirin in CRC and HGSOC cells *in vitro.* This is for two reasons. Firstly, it is more

clinically relevant, as aspirin is usually administered over a prolonged period in patients. Secondly, the current available literature that has investigated the anti-cancer effects of aspirin *in vitro* is limited to the short exposure (≤72 hours). Therefore, I measured malignant cell growth after 4, 5, 6 and 7 days of aspirin treatment. I only used 100µM and 1mM aspirin to begin with before testing lower doses. I was surprised that repeated aspirin exposure did not have an impact on CRC cell growth, especially in HT-29 cells, which is believed to be sensitive to aspirin treatment.[190,191] Similarly, longer-term aspirin administration did not seem to have a growth inhibitory effect in AOCS-1 and G164 HGSOC cells.

I then explored the effects of aspirin in spheroids of malignant cells. Spheroids have shown to be a useful tool to understand how cells proliferate, grow, obtain nutrients, and interact with each other[192]. It also made sense to start with relatively simple 3D model systems before adding aspirin to complex multi-cellular models (≤4 cell types), which I did at a later-stage of my PhD (chapter 5).

I seeded spheroids of HT-29 and HCT116 with the hope of obtaining a positive control. I did not see a reduction in the viability of CRC spheroids after a 7-day aspirin treatment. However, I did not incorporate other cell types of the TME to these spheroids (e.g., fibroblasts). Interestingly, Gilligan *et al* suggested that aspirin may induce a stromal-dependent killing mechanism of malignant cells, as it induced apoptosis of lung cancer cells *in vivo* but had little effect when these cells were treated on their own in 2D *in vitro* cultures[193].

Whilst it was useful to know that aspirin did not elicit any growth inhibitory effects in 2D monocultures, I wanted to explore the actions of aspirin in collagen co-cultures that incorporated components of the TME. RNA-sequencing analysis of collagen gels (G164 and fibroblasts) showed that there was almost a 4-fold increase in *PTGS2* in 3D *in vitro* collagen gels compared to G164 cells alone in 2D. Collagen gels also expressed higher levels of EP1, EP3 and EP4. Given the promising nature of the RNA-sequencing data, I was keen to treat collagen gels of G164 and fibroblasts with aspirin. Aspirin did not reduce cellular proliferation. In addition, there was no difference in malignant cell density (PAX-8) or the number of activated fibroblasts

(FAP) between untreated and treated samples. A similar trend was seen in CRC collagen gels of HT-29 cells and fibroblasts.

To conclude, my findings so far indicated that aspirin had no effect on the growth of two HGSOC cell lines, three CRC cell lines, primary fibroblasts, and primary mesothelial cells in 2D monolayers. Additionally, aspirin did not reduce malignant cell density, the number of activated fibroblasts and overall proliferative index in 3D co-culture gels of cancer cells and fibroblasts.

To move forward, it was critical to confirm that I was preparing aspirin correctly for my experiments. Given aspirin's ability to block prostanoid production, I decided to proceed by measuring prostaglandin levels in malignant cells -pre and -post aspirin treatment. I have described this in the next chapter.

4 Results- Effects of aspirin on the lipid mediator profile in malignant and pre-malignant cells

4.1 Background

As described in the introduction (section 1.2.1), prostaglandins (PG), especially PGE_2, stimulate carcinogenesis[31-4]. PGE_2 can directly promote malignant cell survival and proliferation as well as generate an immunosuppressive TME[31,14]. There is also evidence suggesting that the metabolite of PGE_2, PGE-M, can be used as a biomarker to predict the chemo-preventive response to aspirin[33]. Since aspirin blocks PG production, I questioned whether the PG profile in malignant and pre-malignant cells could be altered following aspirin treatment. To our knowledge, this has not been explored in depth before, especially in HGSOC.

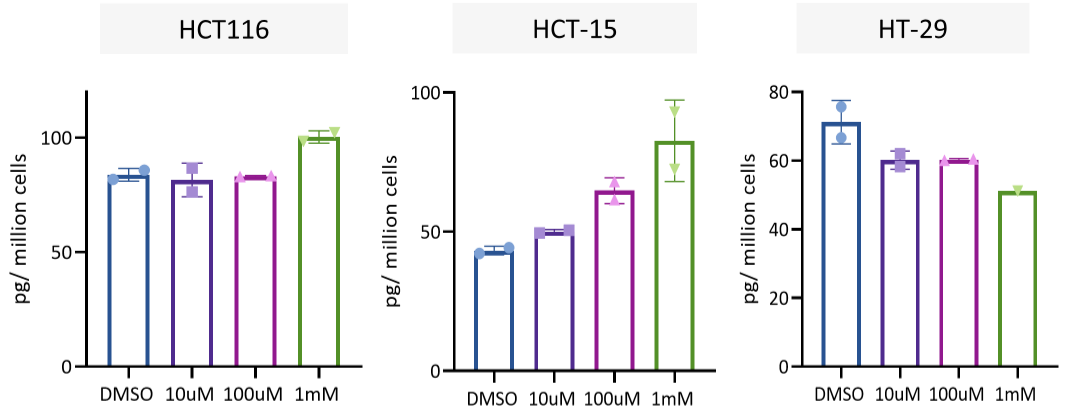
I performed PG profiling analyses on aspirin-treated cells using liquid chromatography-mass spectrometry (LC-MS/MS). I carried out this work in collaboration with Professor Jesmond Dalli's group, based in the Lipid Mediator Unit, William Harvey Research Institute, Queen Mary University of London. I harvested the supernatants and cell pellets in methanol with deuterium labelled internal standards. The Dalli lab then carried out the sample extraction and analysis.

4.2 Effects of ASA on the PG profile in CRC cell lines

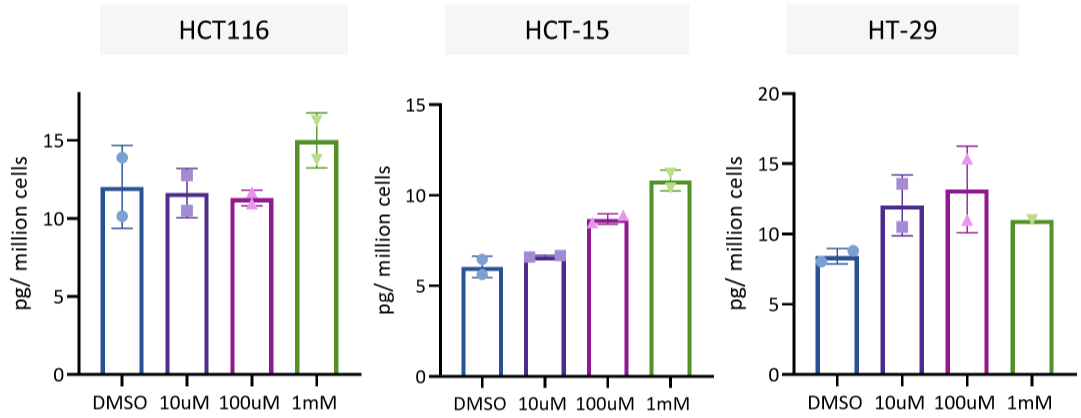
First, I needed to generate a positive control cell line that responded to aspirin. Most of the literature examining the anti-cancer effects of aspirin is focused on CRC[17-188]. Therefore, I treated HT-29, HCT116 and HCT-15 CRC cell lines with aspirin for 7 days and measured PG levels after treatment.

As portrayed in Figure 4.1-A, aspirin did not reduce PG levels in HCT116 and HCT-15 cells. In fact, a slight increase in PGE_2, PGF_{2a}, and TXB_2 was observed in HCT116 cells following 1mM aspirin treatment. A similar trend was seen in HCT-15 cells. Conversely, PG levels in HT-29 cells reduced marginally following aspirin exposure. There was a reduction in PGD_2 (Figure 4.1-C). However, the amount of PGE_2 and TXB_2 did not change following aspirin treatment (Figure 4.1-B and E respectively). Overall, aspirin did not substantially modulate the PG profile in all three CRC cell lines.

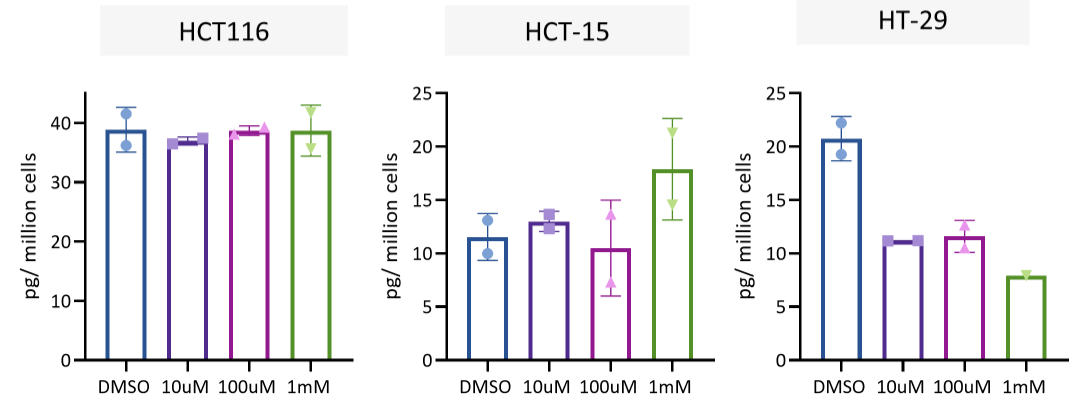
A Total PG levels (PGE_2 + PGD_2 + PGF_{2a})



B PGE_2 only



C PGD_2 only



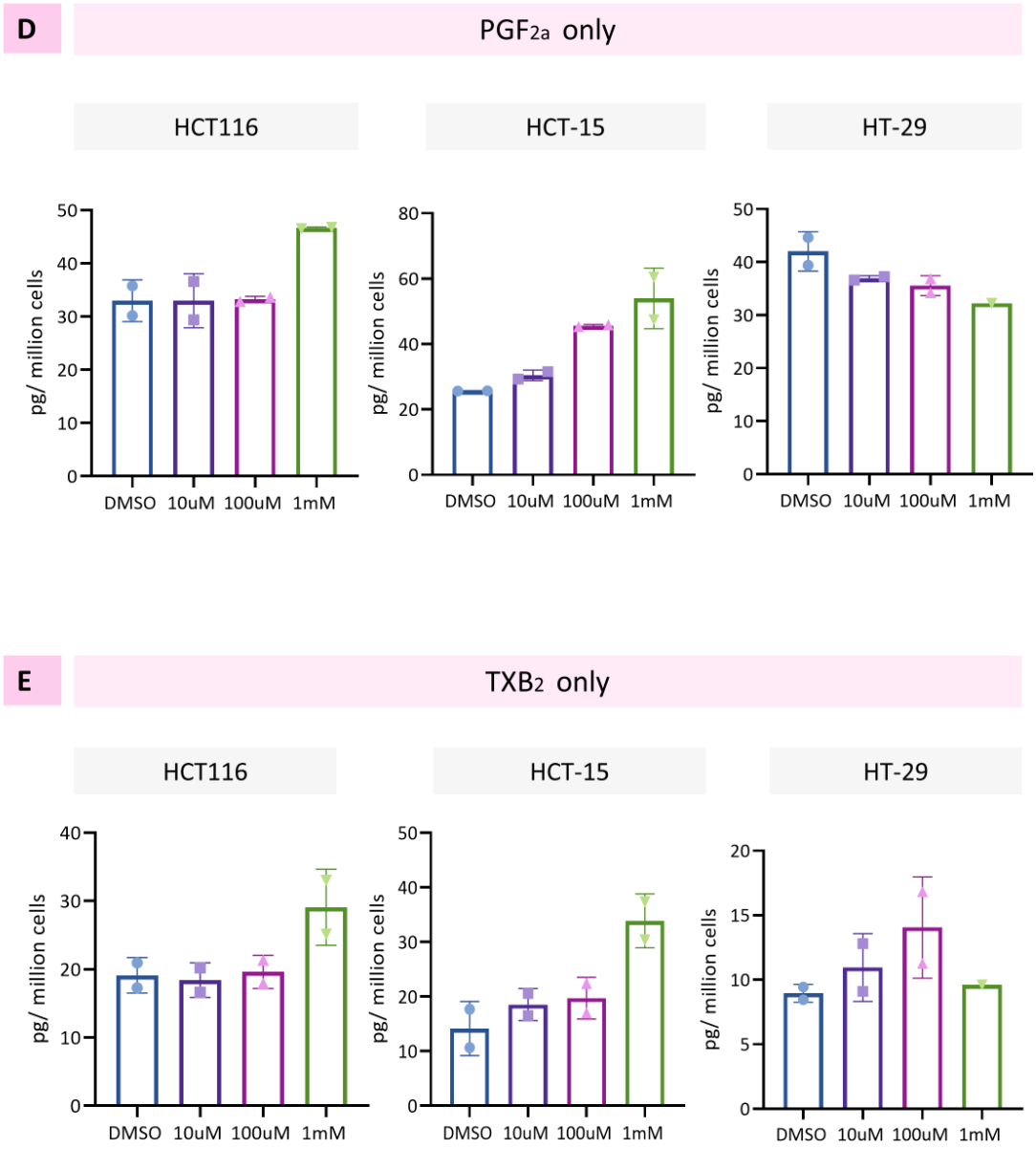


Figure 4.1. Prostaglandin profiling of CRC cells after 7 days of ASA treatment. HCT116, HCT-15 and HT-29 cells were treated with aspirin every day for 7 days. Prostaglandin profiling was performed by mass spectrometry. The total levels of PGs correspond to the sum of PGE_2, PGD_2 and PGF_{2a}. A starting density of 1.5 million cells was used. PGs, prostaglandins; TXB_2, thromboxane B2. N=1. LC-MS/MS is an expensive procedure, (£140/ sample), so I performed my experiments in technical duplicates instead.

4.3 Effects of ASA on the prostaglandin profile in HT-29 cells in a serum-free environment

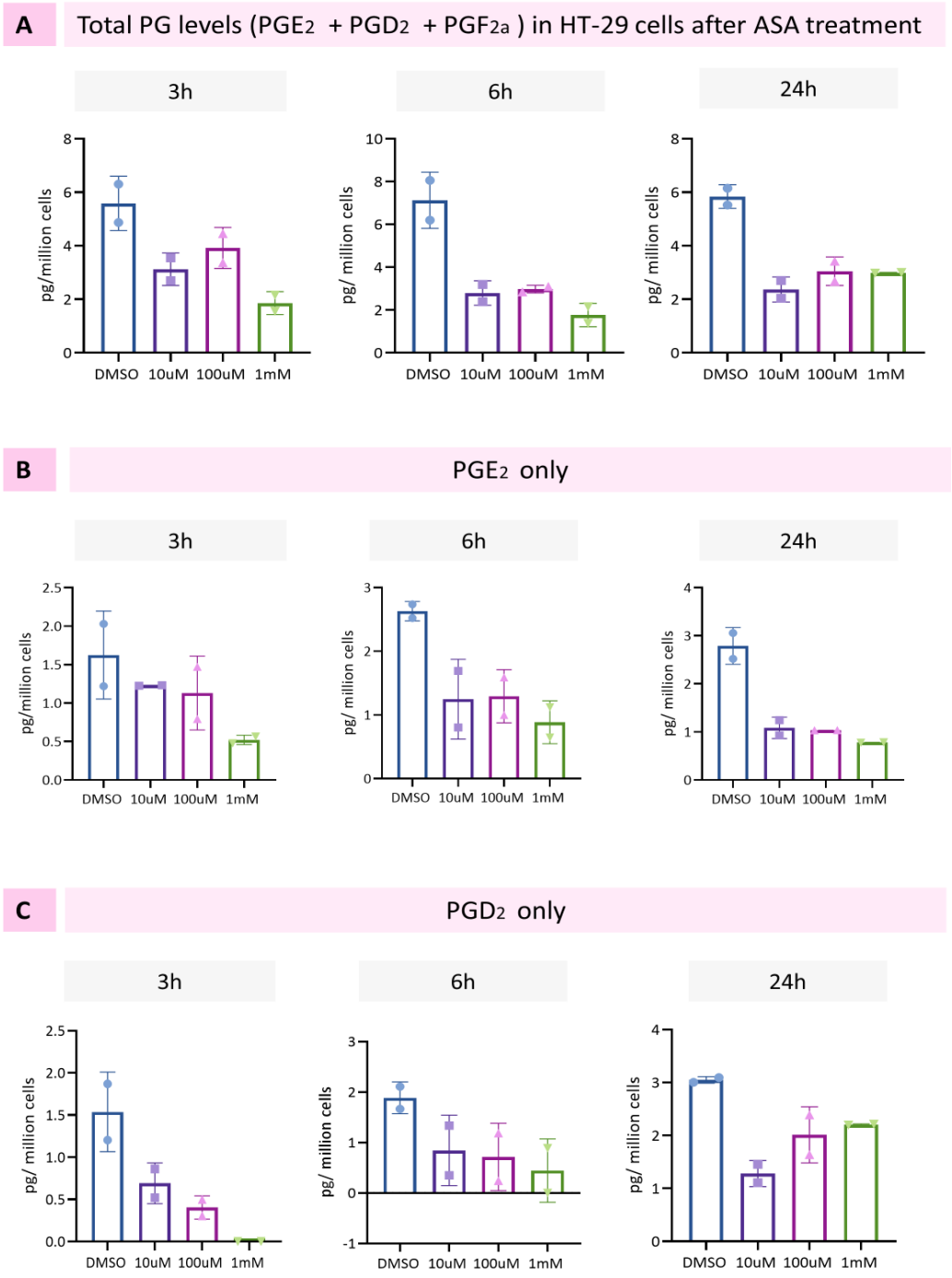
When troubleshooting my experiments in Figure 4.1, I wondered whether the presence of serum in the culture medium obscured the potentially low levels of PGs produced by CRC cells. Therefore, I set up an experiment whereby I treated HT-29 cells with aspirin in serum-free medium. I also reduced the aspirin exposure time to 3, 6, and 24 hours. This is because aspirin has a relatively short half-life of 15-20 minutes, so I postulated that any changes in PG levels would be captured within the first 6 hours of treatment.

As highlighted in Figure 4.2, aspirin seemed to reduce PG levels in HT-29 cells in serum-free medium after 3, 6 and 24h (Figure 4.2-A). In particular, PGE_2 levels decreased after 3 hours of 100µM and 1mM aspirin treatment (Figure 4.2-B). The amounts of PGD_2 and TXB_2 were lower after 3 and 6 hours of aspirin exposure but restored after 24 hours (Figure 4.2-C and E respectively). However, the amount of prostaglandins present did not accumulate over time. Perhaps HT-29 cells stopped synthesising PGs after 3 hours of aspirin treatment. HT-29 cells seemed to respond to aspirin in serum-free conditions, and serum might have masked the potentially low levels of PGs produced by CRC cells in Figure 4.1. Since LC-MS/MS is an expensive procedure (£145/ sample), I could not perform multiple biological replicates of this experiment. I performed my experiments in technical duplicates instead.

Given these findings, I was intrigued to know the basal level of PGs present in serum. Therefore, I performed a lipid mediator analysis on aliquots of FBS and human serum, as I used both types of sera in culture depending on the cell line (2.2). I also used charcoal-stripped serum as a comparison control. This is because charcoal-stripped serum is meant to contain low levels of lipid mediators[194].

As depicted in Figure 4.3, there was a substantial amount of PGs present in FBS and human serum. In particular, human serum had significantly elevated levels of both PGD_2 and PGE_2 compared to both FBS and charcoal-stripped sera. Additionally, PGF_{2a} and TXB_2 levels were significantly higher in FBS, and human sera compared to

charcoal-stripped serum. Overall, the high basal level of PGs present in serum could have interfered with the actions of aspirin in Figure 4.1.



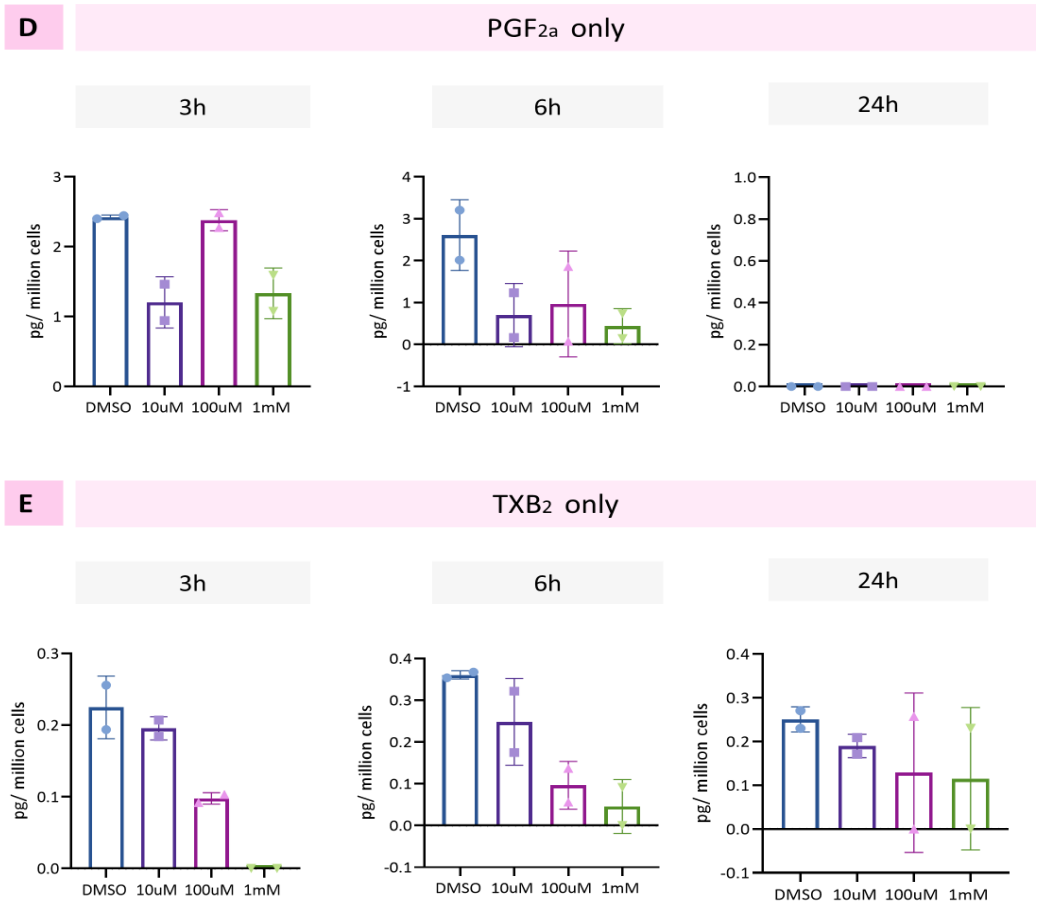


Figure 4.2. Prostaglandin profiling of HT-29 cells after 3, 6, and 24h ASA treatment in serum-free conditions. HT-29 cells were treated with aspirin for 3, 6 and 24h in serum-free conditions. (A) Shows the sum of PGD_2, PGE_2, and PGF_{2a}; (B) shows the levels of PGE_2 only. (C) shows the levels of PGD_2 only; (D) shows the levels of PGF_{2a}, and (E) shows TXB_2 levels. PGE2, prostaglandin E2; PGD2, prostaglandin D2; PGF2a, prostaglandin F2a; TXB2, thromboxane B2. N=1. LC-MS/MS is an expensive procedure, (£140/ sample), so I performed my experiments in technical duplicates instead.

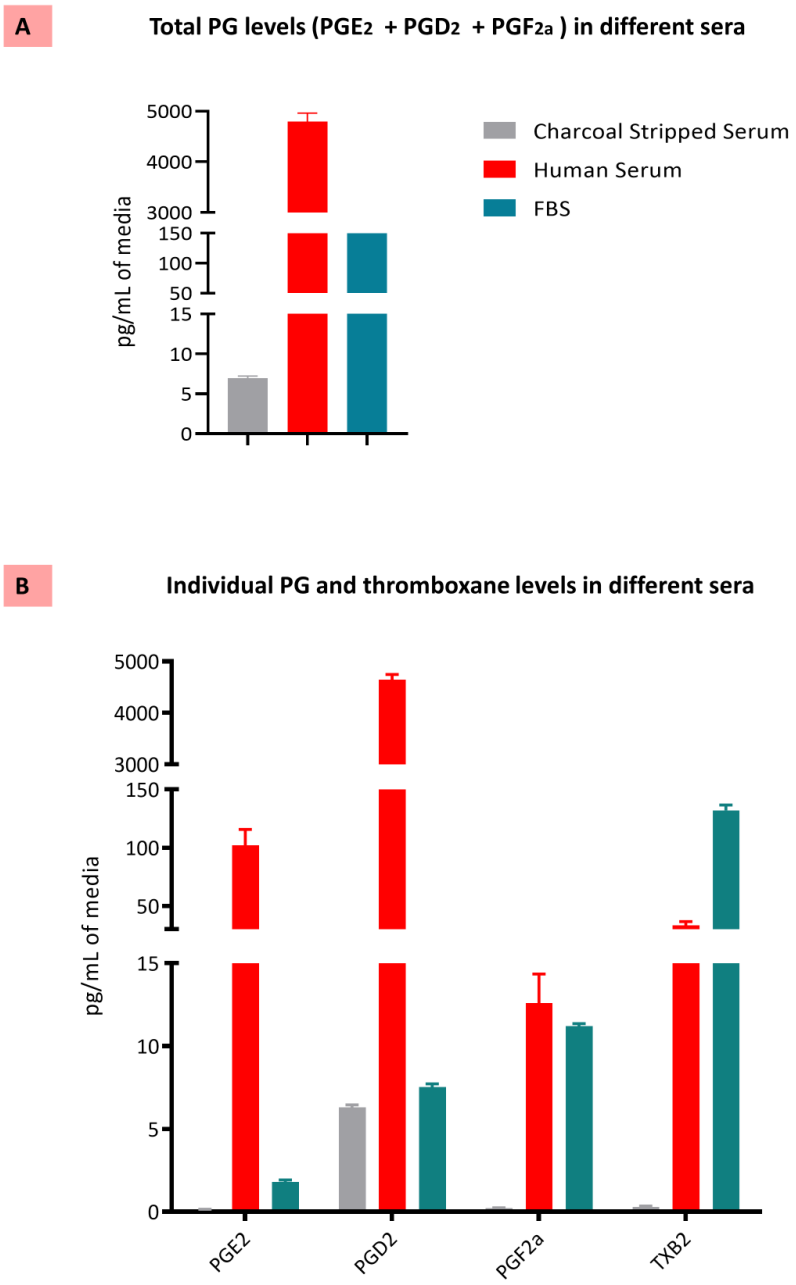


Figure 4.3. Prostaglandin profiling of FBS, human serum and charcoal-stripped serum alone. I performed a prostaglandin profile on FBS and human serum, to confirm that the high levels of PGs present in serum inhibited the actions of aspirin. I used charcoal-stripped serum as a comparison control. (A) Shows the total levels of PGs present in all three types of sera; (B) highlights the individual PG and thromboxane levels present. PGE_2, prostaglandin E2; PGD_2, prostaglandin D2; PGF_{2a}, prostaglandin F2a; TXB_2, thromboxane B2; FBS, foetal bovine serum.

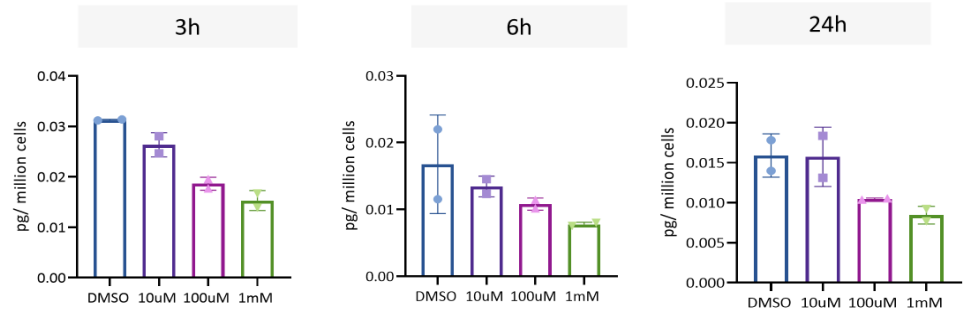
4.4 Effects of ASA on the prostaglandin profile in HGSOC cells in a serum-free environment

Since I established the optimal conditions to carry out a mass spectrometry analysis in the HT-29 positive control cell line, I decided to measure PG levels in aspirin-treated HGSOC cells in serum-free conditions.

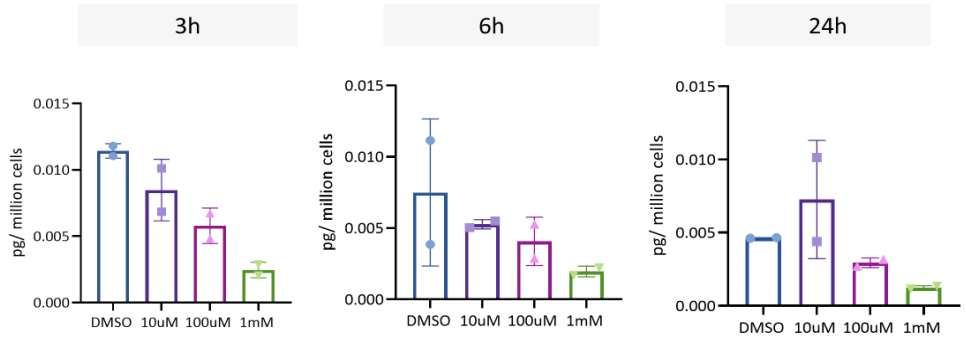
G164 cells responded to aspirin in serum-free conditions in a concentration-dependent manner (Figure 4.4-A). Aspirin decreased PGE_2 secretion after 3 and 6 hours of treatment (Figure 4.4-B). PGD_2 was also reduced following aspirin treatment (Figure 4.4-C). Nevertheless, the amount of PGs present in the untreated control almost halved after 24 hours of serum-free culture, which was a point of concern.

To circumvent this issue, I supplemented G164 cells with 10% charcoal-stripped serum as a potential alternative to serum-free medium. I then treated these cells with aspirin for 3 hours and performed a PG profile. As illustrated in Figure 4.5, there were no differences in PG levels between serum-free conditions and charcoal-stripped serum, suggesting that G164 cells had naturally low amounts of PGs to begin with. In addition, similarly to what observed with the HT-29 cell line, serum might have masked the low levels of PGs present in G164 cells. Overall, G164 cells seemed to respond to aspirin in serum-free conditions.

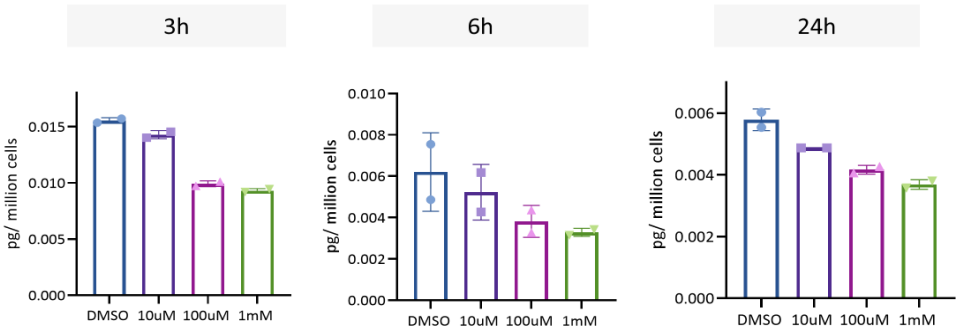
A Total PG levels (PGE_2 + PGD_2 + PGF_{2a}) in G164 cells after ASA treatment



B PGE_2 only



C PGD_2 only



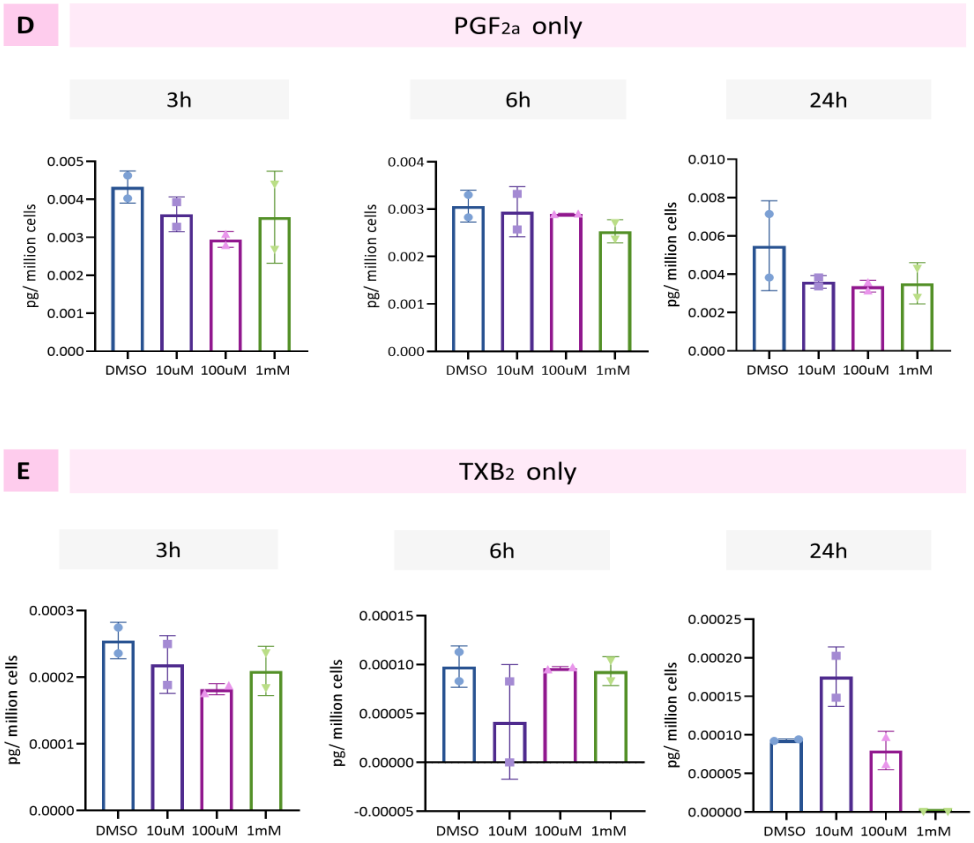
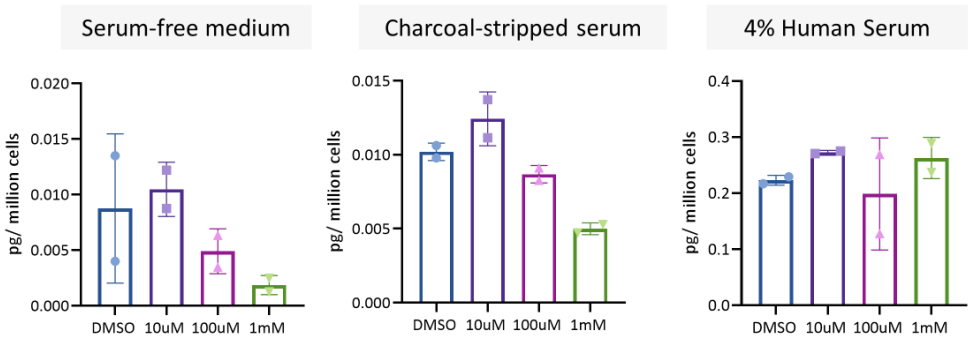
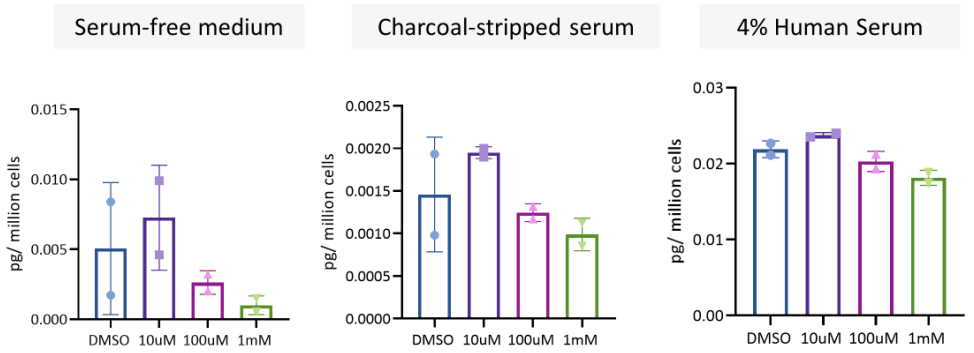


Figure 4.4. **Prostaglandin profiling of G164 cells after 3, 6, and 24h ASA treatment in serum-free conditions**. G164 cells were treated with aspirin for 3, 6 and 24h in serum-free conditions. (A) Shows the sum of PGD_2, PGE_2, and PGF_{2a}. (B) shows the levels of PGE_2 only; (C) shows the levels of PGD_2 only; (D) shows the levels of PGF_{2a} only; (E) shows TXB_2 only. PGE2, prostaglandin E2; PGD2, prostaglandin D2; PGF2a, prostaglandin F2a; TXB2, thromboxane B2.n=1.

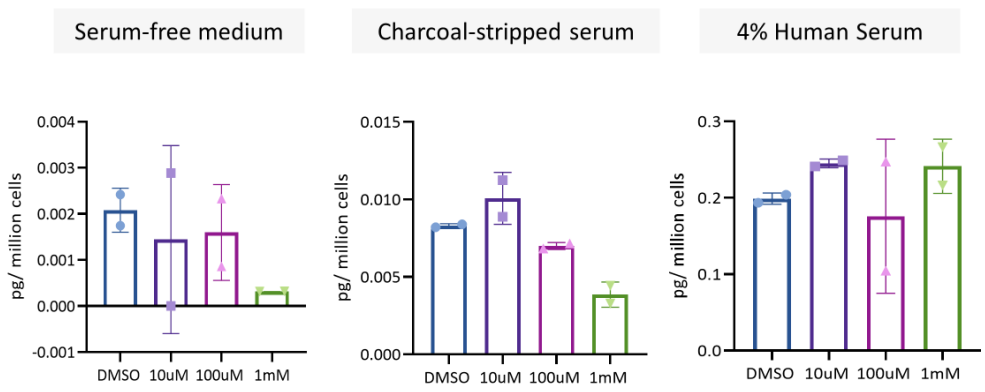
A Total PG levels (PGE_2 + PGD_2 + PGF_{2a}) in G164 cells after ASA treatment



B PGE_2 only



C PGD_2 only



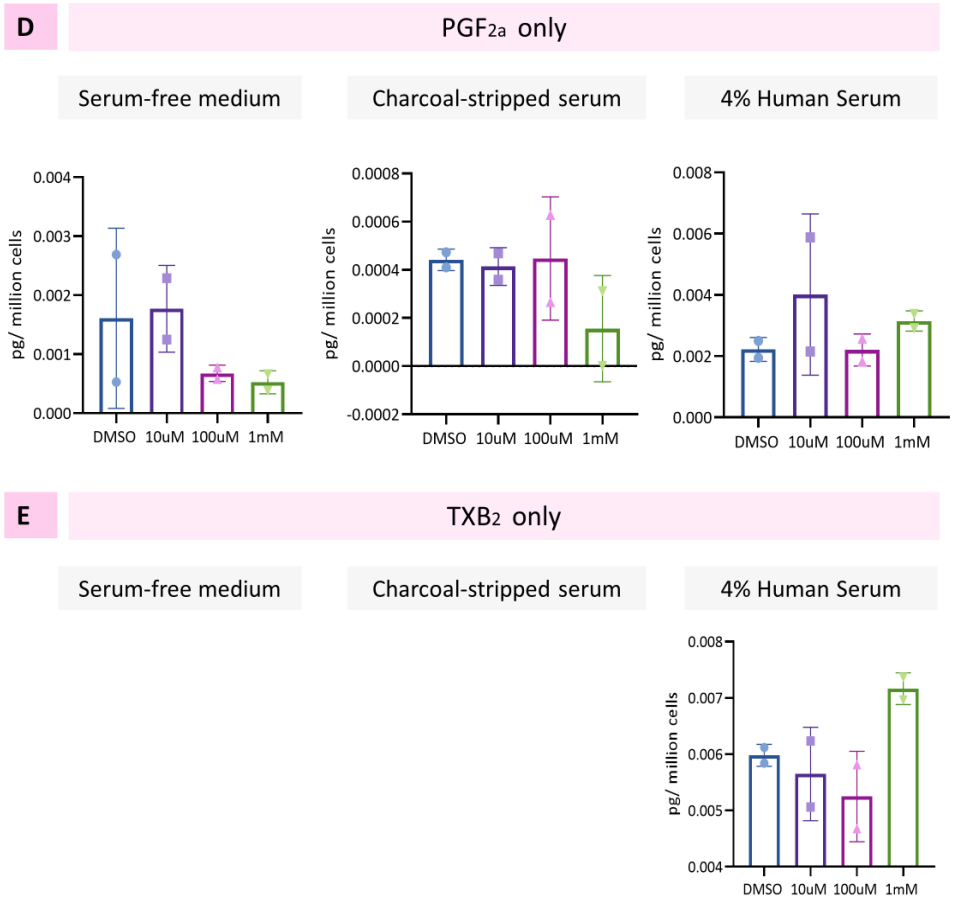
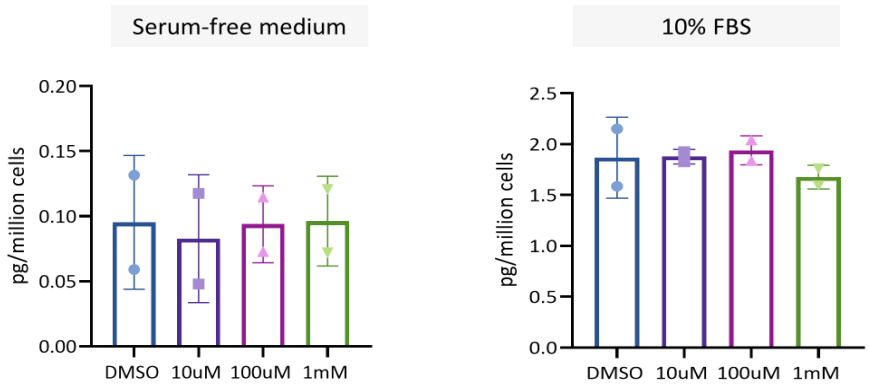


Figure 4.5. Prostaglandin profiling of G164 cells after 3h ASA treatment under different culture conditions. G164 cells were treated with aspirin for 3h in charcoal-stripped serum, serum-free medium, and 4% human serum. This was to confirm whether the PG levels in G164 cells were low, or if it was due to serum-starvation. PGE_2, prostaglandin E2; PGD_2, prostaglandin D2; PGF_{2a}, prostaglandin F2a; TXB_2, thromboxane B2. N=1.

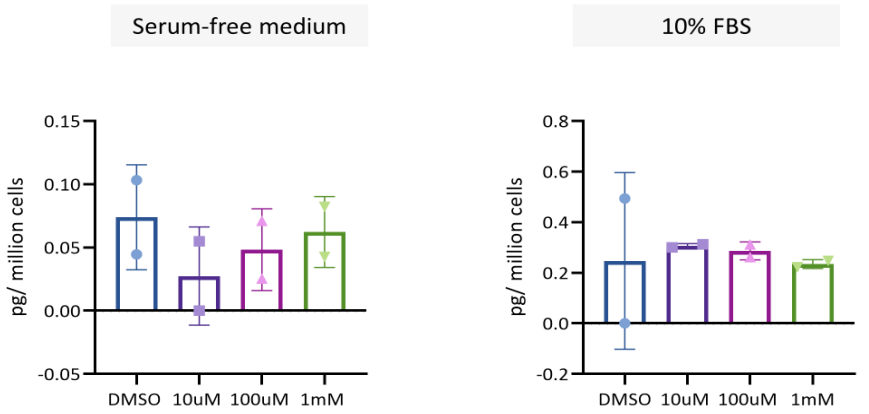
I also treated G33 and AOCS cell lines with aspirin for 3 hours in serum-free medium. As demonstrated in Figure 4.6, G33 cells did not respond to aspirin even in a serum-free environment. In fact, the amount of PGF_{2a} seemed to double after 1mM aspirin treatment (Figure 4.6-D). G33 also had similar basal levels of PGs to G164, although these two omental metastatic cell lines behaved differently when incubated with aspirin.

On the other hand, AOCS-1 cells responded to aspirin in serum-free conditions. A reduction in PGE_2, PGD_2 and PGF_{2a} levels was observed after 100µM and 1mM treatment (Figure 4.7-B-D). There seems to be more variability in the amount of PGs in the untreated control and after 10µM aspirin treatment, but 100µM aspirin seems to inhibit PG production. AOCS-1 cells also had a higher baseline level of PGs compared to G33 and G164. The presence of prostaglandins in serum may have obscured the detection of the low levels of PGs produced by malignant cells.

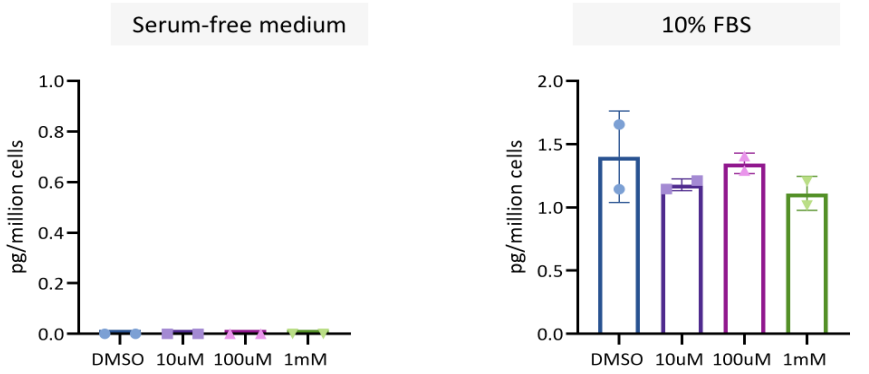
A Total PG levels (PGE_2 + PGD_2 + PGF_{2a})



B PGE_2 only



C PGD_2 only



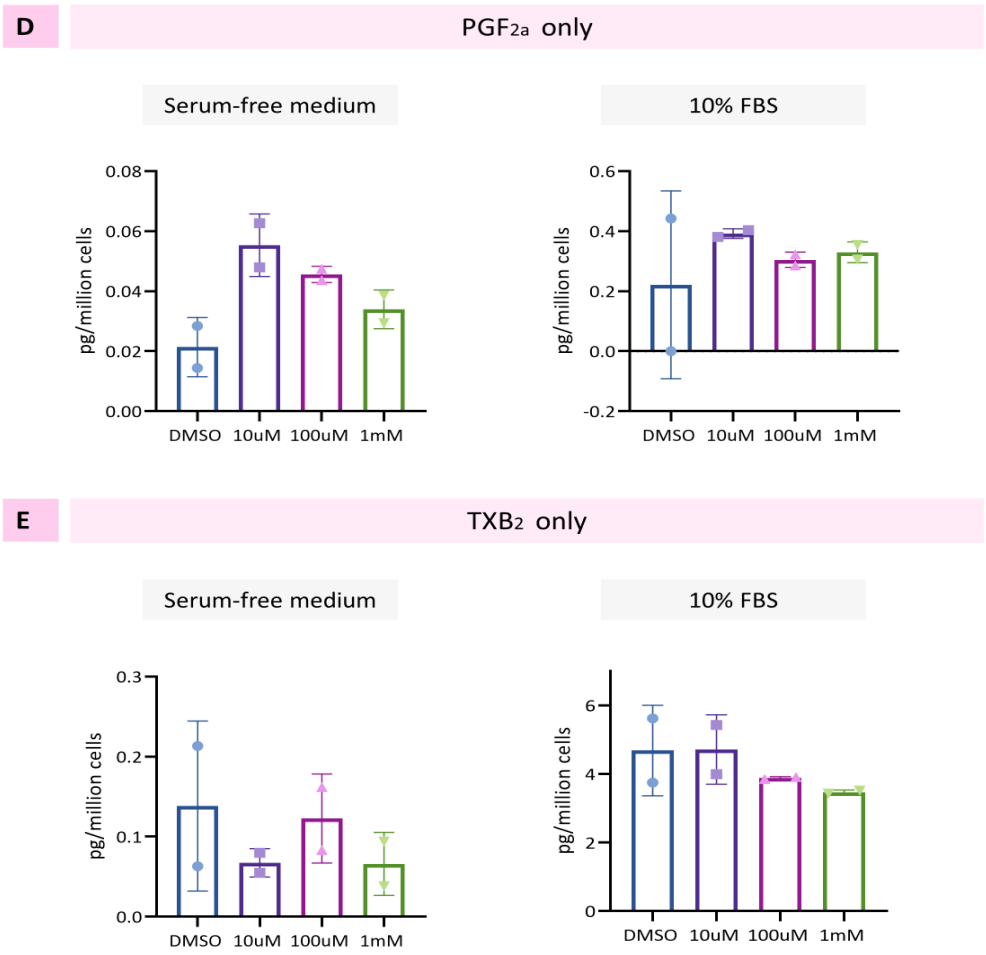
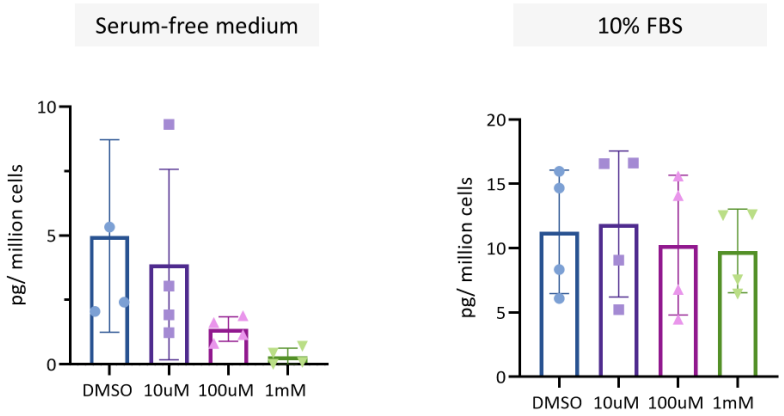
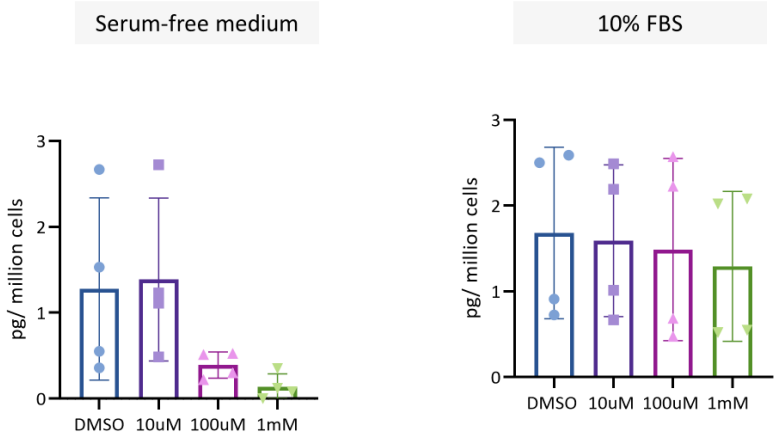


Figure 4.6. Prostaglandin profiling of G33 cells after 3h ASA treatment. G33 cells were treated with aspirin for 3h in serum-free conditions. A corresponding 10% FBS control was used. (A) Shows the sum of PGD_2, PGE_2, and PGF_{2a}. (B) shows the levels of PGE_2 only; (C) shows the levels of PGD_2 only; (D) shows the levels of PGF_{2a} only; (E) shows TXB_2 only. PGE_2, prostaglandin E2; PGD_2, prostaglandin D2; PGF_{2a}, prostaglandin F2a; TXB_2, thromboxane B2. N=1. LC-MS/MS is an expensive procedure, (£140/ sample), so I performed my experiments in technical duplicates instead.

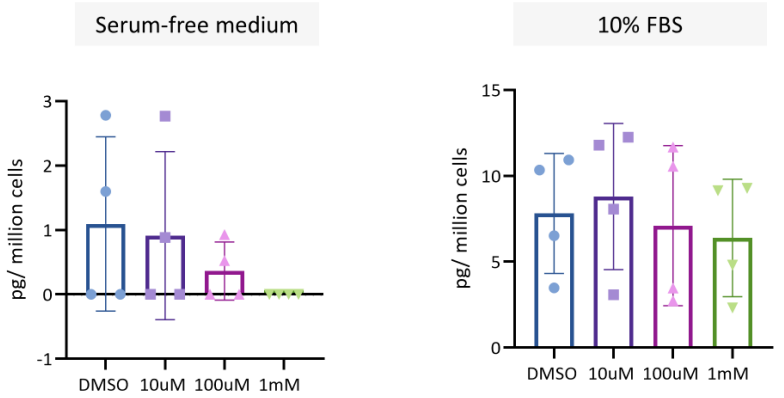
A Total PG levels (PGE_2 + PGD_2 + PGF_{2a})



B PGE_2 only



C PGD_2 only



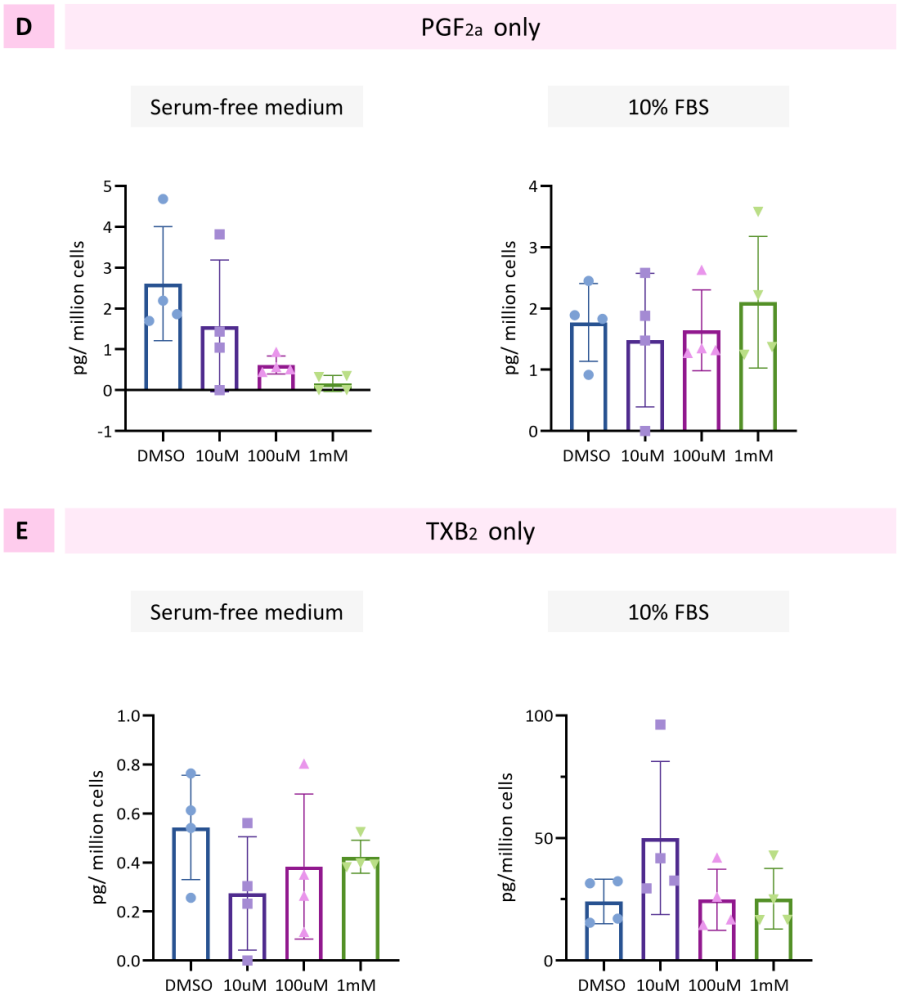


Figure 4.7. **Prostaglandin profiling AOCS-1 cells after 3h ASA treatment**. AOCS-1 cells were treated with aspirin for 3h in serum-free conditions. A corresponding 10% FBS control was used. (A) Shows the sum of PGD_2, PGE_2, and PGF_{2a}. (B) shows the levels of PGE_2 only; (C) shows the levels of PGD_2 only; (D) shows the levels of PGF_{2a} only; (E) shows TXB_2 only. PGE_2, prostaglandin E2; PGD_2, prostaglandin D2; PGF_{2a}, prostaglandin F2a; TXB_2, thromboxane B2. N=2. LC-MS/MS is an expensive procedure, (£140/ sample), so I performed my experiments in technical duplicates instead.

4.5 Effects of ASA on the prostaglandin profile in pre-malignant precursors of HGSOC

Since aspirin has been shown to possess cancer-preventive properties, I wanted to know whether aspirin could modulate PG levels in pre-malignant progenitors of HGSOC. It is now known that most high-grade serous ovarian cancers arise from p53 mutant fallopian tube surface epithelial (FTE) cells[65,151,195]. Many p53-mutant FTE cells transform into lesions called serous tubular intraepithelial carcinomas

(STICs)[65,99]. STICs have shown to resemble HGSOC cells[195-61]. Furthermore, TGFβI production by macrophages generate an immunosuppressive TME in STICs, which is found in patients with advanced HGSOC[99].

I decided to treat p53-mutant (R273C) FT318 FTE cells with aspirin and measure PG levels after treatment. I only used an aspirin dose of 100μM as p53-mutant FT318 cells were slow growing in nature.

As depicted in Figure 4.8, there was a slight reduction in PGE_2 and TXB_2 after 100μM aspirin treatment in serum-free conditions. Conversely, there was a slight increase in PGD_2. P53-mut FT318 cells did not secrete $PGF_{2a.}$

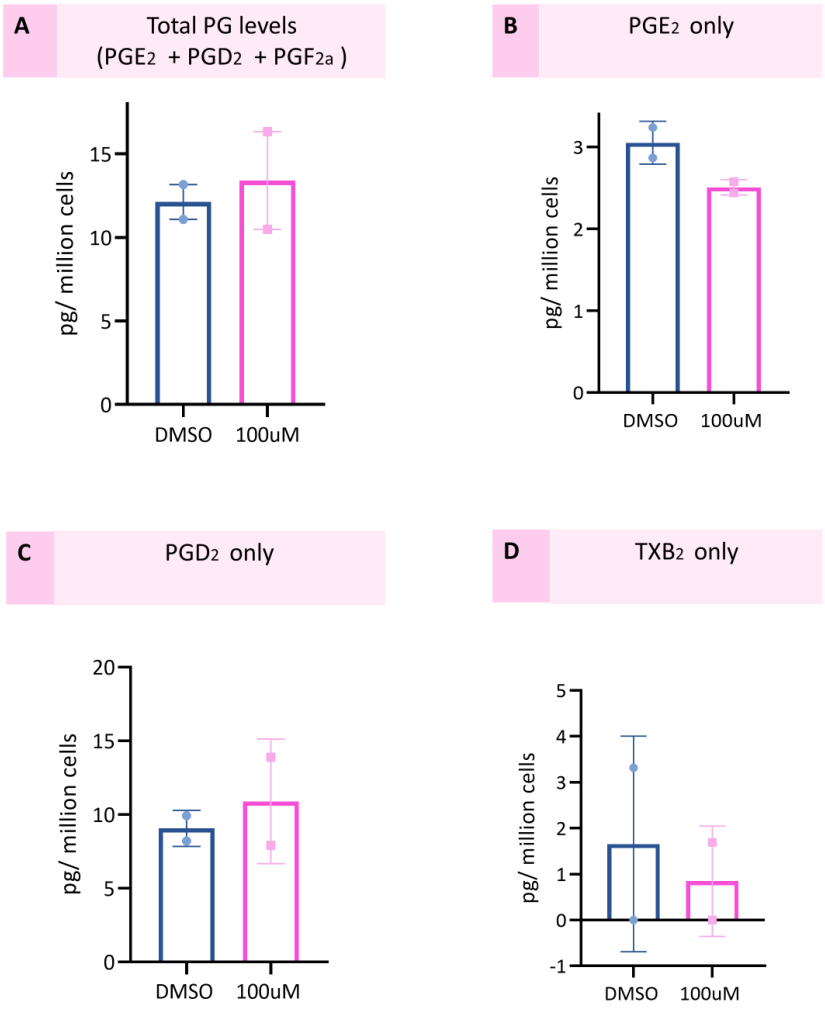


Figure 4.8. Prostaglandin profiling of p53 mutant FT318 cells after 3h ASA treatment in serum-free conditions. P53-mutant FT318 cells were treated with 100µM aspirin for 3h in serum-free conditions. (A) Shows the sum of PGD_2, PGE_2, and PGF_{2a}. (B) shows the levels of PGE2 only; (C) shows the levels of PGD2 only; (D) shows TXB2 only. PGE2, prostaglandin E2; PGD2, prostaglandin D2; PGF2a, prostaglandin F2a; TXB2, thromboxane B2. N=1

4.6 Summary of results

I have summarised the findings so far below in Table 15.

Table 15. Prostaglandin profiles of malignant and pre-malignant cells treated with aspirin.

Cell line	Origin	Potential reduction in PGE_2 in serum-free conditions	Potential reduction in PGD_2 serum-free conditions	Potential reduction in PGF_{2a} serum-free conditions
G33	Omental metastases of HGSOC	No	No	No
G164	Omental metastases of HGSOC	Yes	Yes	No
FT318 p53 mutant R273C	Fallopian Tube Surface Epithelium	Yes	No	-
AOCS-1	Ascites	Yes	Yes	Yes
HT-29	Grade II colon adenocarcinoma	Yes	Yes	Yes

4.7 Effects of ASA on malignant cell growth inhibition in serum-free conditions

It is evident from my mass spectrometry data that serum contained high levels of prostaglandins, which could have obscured the detection of the low levels of PGs produced by HGSOC and CRC cells. As I initially performed my *in vitro* growth curve experiments in serum-rich medium, in chapter 3, I needed to repeat these experiments in serum-free medium. Therefore, I repeated these growth curve experiments in serum-free conditions. I used AOCS-1 and HT-29 for this experiment, as they were the two most responsive malignant cell lines to aspirin treatment in the above experiments.

I first tested two commercially available serum-free media on AOCS-1 and HT-29 cells to ensure that they were viable in culture for up to 72 hours (Table 16). I then measured the growth of AOCS-1 and HT-29 cells after aspirin treatment in serum-free conditions. As exhibited in Figure 4.9, aspirin did not reduce AOCS-1 and HT-29 cell growth even in serum-free conditions. This suggested that cell growth was not the optimal readout when exploring the anti-cancer effects of aspirin on malignant cells.

Table 16. Serum-free media used to culture AOCS-1 and HT-29 cells.

Cell line	**Serum-free medium used**
AOCS-1	OptiMEM Glutamax™
HT-29	PromoCELL- Cancer Cell Line Medium XF

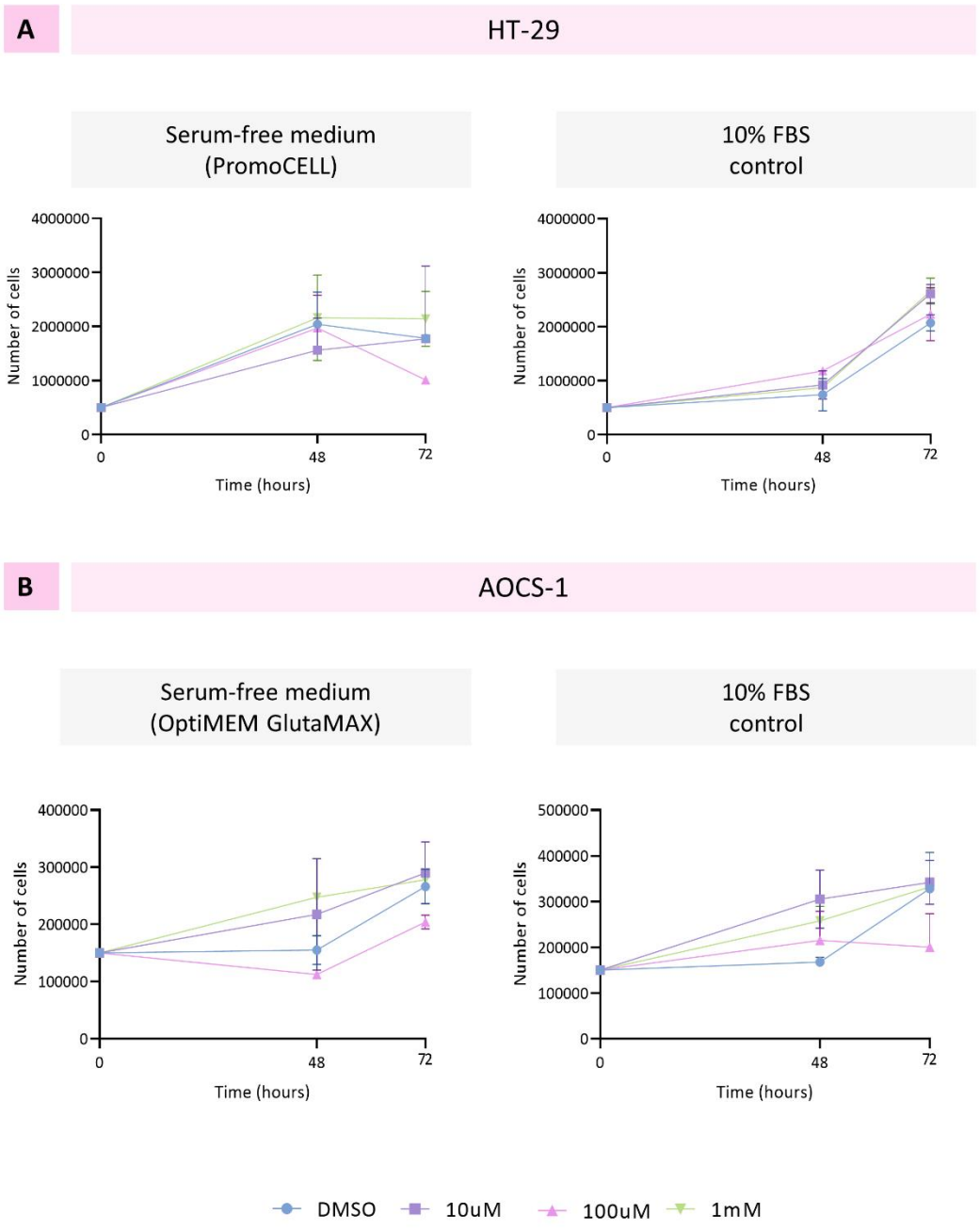


Figure 4.9. **Effects of ASA on malignant cell growth in serum-free conditions**. HT-29 and AOCS-1 cells were treated with aspirin in serum-free medium, and cell growth was measured after 48h and 72h using a Vi-Cell XR counter. (A) Effect of aspirin on HT-29 cell growth in serum-free conditions, using a 10% FBS control; (B) Effect of aspirin on AOCS cell growth in serum-free conditions, using a 10% FBS control. Overall aspirin does not affect malignant cell growth, even in serum-free conditions. N=1

4.8 Discussion and conclusions

In this chapter, I examined the effects of aspirin on the lipid mediator profile in malignant and pre-malignant cells using liquid chromatography-mass spectrometry (LC-MS/MS). I found that aspirin reduced PG production in malignant cells only in a serum-free environment. Aspirin reduced PGE_2, PGD_2 and PGF_{2a} in HT-29 and AOCS-1 cells (Figure 4.2 and Figure 4.7 respectively). Similarly, aspirin reduced PGE_2 and PGD_2 in G164 cells (Figure 4.4) and marginally decreased PGE_2 in p53-mutant FT318 pre-malignant cells (Figure 4.8).

My results highlighted the complexity of performing aspirin treatments *in vitro.* Table 17 shows a list of 31 papers from 1997-2021 which examined the anti-proliferative effects of aspirin *in vitro* on cancer cells. All studies, except for the recent publication in October 2021 by Professor Andrew Chan's group[173], carried out aspirin treatments in serum-rich medium (typically 10% FBS). Since most papers performed aspirin exposures in serum-rich medium, it is not surprising that the concentrations of aspirin used were higher (≥ 1mM). These studies show that higher concentrations of aspirin are usually required when performing *in vitro* treatments in serum-rich medium to account for the presence of proteins (e.g., albumin) and prostanoids present in serum (figure 4.3) [237,238,239]. Whilst I used an aspirin concentration range of 10µM (low), 100µM (high) and 1mM (high) for my experiments, I performed my experiments in medium devoid of serum, which implied that there was more 'free drug' to interact with cells. It is therefore not possible to make a direct comparison between my results and previous literature. Interestingly, Professor Chan's latest paper emphasised the importance of 'limiting *in vitro* doses to those that remain clinically relevant (micromolar range)[173].' However, similar to my experiments, they performed aspirin treatments in serum-free medium, which is probably why a concentration of 50 µM was used.

As highlighted in Table 17, the majority of papers in the literature from 1997-2021 used higher aspirin concentrations. For instance, a paper in 1997 examining the effects of aspirin in the Caco-2 CRC cell line exposed cells to 5mM and 10mM aspirin and observed an apoptotic effect[196]. Given that the peak plasma concentration of

high dose aspirin (1200mg) corresponds to approximately 142µM *in vivo*, perhaps 5mM and 10mM aspirin concentrations are too high. Nevertheless, aspirin is absorbed, distributed and metabolised differently *in vivo* compared to *in vitro* systems, so it is not possible to directly compare concentrations *in vivo* to those *in vitro*.

Another study in 2019 treated SK-N-SHCN human neuroblastoma cells with 2mM aspirin to observe reduction in cell motility and growth[197]. Interestingly, this paper initially included a low concentration of 100µM, but 2mM was chosen as a representative concentration for subsequent assays. This could be because 100µM aspirin treatment in serum-rich medium did not have a pronounced effect on malignant cells. Moreover, Nounu, Aayah *et al.* recently treated colorectal adenoma cell lines with 4mM aspirin to 'identify apparent effects' of the drug[198]. A concentration of 4mM was probably required to compensate for supplementing the cells with 20% FBS. In October 2021, Devall MAM, Drew DA, Dampier CH *et al.* treated organoids of colorectal adenomas with 50µM aspirin in serum-free medium[173]. To our knowledge, this is the first publication exploring the anti-neoplastic effects of aspirin *in vitro* to use medium devoid of FBS. However, as emphasised previously, it is not possible to make a direct comparison between studies that have performed aspirin treatments in serum-rich medium versus those that performed aspirin treatments in a serum-free milieu.

It is equally important to recognise that there are several limitations to performing *in vitro* aspirin treatments using serum-free medium. Firstly, both bovine and human sera contain proteins, such as albumin, which bind to aspirin, and affects its bioavailability[237,238]. Therefore, treating cells with aspirin in a serum-free environment results in a higher concentration of 'unbound' drug, which is not mimetic of conditions *in vivo*. Furthermore, metabolites of arachidonic acid, including prostanoids, can bind to serum proteins, which can affect the detection of prostanoids by LC-MS/MS[239]. On the other hand, the method of administrating cells with aspirin *in vitro* cannot be compared to aspirin dosing *in vivo.* It is ultimately challenging to truly recapitulate the pharmacokinetics of aspirin *in vitro*.

I initially carried out all my aspirin exposures in serum-rich medium. However, repeating the *in vitro* growth curves in serum-free medium showed that aspirin did not inhibit malignant cell growth even in serum free conditions (Figure 4.9). I concluded that, when exploring the anti-cancer effects of aspirin, malignant cell growth in 2D was not one of the affected processes. However, as discussed in chapter 7, there are additional controls that could have been performed in chapter 3 to ensure that I was using the optimal culture systems to investigate the cancer-preventive effects of aspirin.

Ultimately, I deduced the complexity of performing aspirin experiments *in vitro*, and retrospectively identified additional controls that could have been tested at the earlier stages of my PhD. To move forward, I decided to examine the actions of aspirin in multi-cellular models of HGSOC (<4 cell types) that included other cells of the tumour microenvironment (TME), as I wondered if any cancer preventative effects were due to actions on non-malignant cells in the TME. I have described this in the next chapters.

Table 17. List of papers studying the anti-cancer effects of aspirin *in vitro*

Paper	Year	Doses used	Does the Medium contain Serum?	Cancer Type(s)	Cell Lines used	Effects of Aspirin Examined
Ricchi, P et al.	1997	1-10mM	10% FBS	Colorectal Cancer	Caco-2 cells	Aspirin inhibited cell proliferation in colon cancer cell lines
Bellosillo, B et al.	1998	2.5-10mM	10% FBS	B-chronic lymphocytic leukaemia (B-CLL)	B-CLL cells isolated from mononuclear cells	Aspirin decreased cell viability and induced apoptosis
Qiao, L et al	1998	0.4mM, 1mM, 3mM	10% FBS	Colorectal Cancer	HT-29	Aspirin induced apoptosis in HT-29 cells
Castaño, E., Dalmau, M., Barragán, M. *et al.*	1999	2.5-10mM	10% FBS	Colorectal Cancer	HT-29	Aspirin induces cell death in HT-29 cells
Jiang, M C et al.	2001	300uM, 900uM, 2.7mM	10% FBS	Hepatocellular Carcinoma (HCC)	Sk-Hep1	Aspirin inhibited malignant cell invasion
Arango, H A et al.	2001	1-5mM	Charcoal-absorbed FBS	Endometrial Cancer	Ishikawa human endometrial cancer cells	Aspirin inhibited cell growth
Stark, Lesley A et al.	2006	3-5mM	10% FBS	Colorectal Cancer	HT-29	Aspirin induces apoptosis
Dikshit, Priyanka et al.	2006	2.5, 5, 10, 25, 50mM	10% FBS	Neuroblastoma, cervical cancer	mouse Neuro2a, HeLa	Aspirin inhibits cell proliferation and induces apoptosis
Yin, Hongying et al.	2006	1mM- 12.5mM	5% FBS	Colorectal Cancer	HT-29	Aspirin induced apoptosis in HT-29 cells
Yoo, Jinsang, and Yong J Lee.	2007	5mM	10% FBS	Prostate Cancer	LNCaP	Aspirin induced apoptosis
Lu, Meiling et al.	2008	1, 5mM	10% FBS	Breast Cancer	MDA-MB-435, T47D	Aspirin induced apoptosis
Kim, Su-Ryun et al.	2009	5, 8 mM	10% FBS	Glioblastoma	A172	Aspirin induced apoptosis
Pathi, Satya et al.	2012	2.5-10mM	5 and 10% FBS	Colorectal Cancer	RKO, SW480, HT-29, HCT116	Aspirin inhibited colon cancer cell growth
De Luna-Bertos, E et al.	2012	20uM-1mM	10% FBS	Osteosarcoma	MG-63	Aspirin inhibited osteosarcoma cell growth
Claudius, Ann-Katrin et al.	2014	3mM	10% FBS	Colorectal Cancer, Breast Cancer	SW580 , MCF-7	Aspirin inhibited cell proliferation and induces apoptosis
Maity, Gargi et al.	2015	50uM-5mM	10% FBS	Breast Cancer	MCF-7, MDA-MB-231	Aspirin inhibited breast cancer cell growth
Liao, Dan et al.	2015	2.5,5,10,20 mM	10% FBS	Osteosarcoma	U20S, MG63	Aspirin reduced cell viability, migration and invasion of malignant cells
Saha, Shilpi et al.	2016	1-5mM	10% FBS	Breast Cancer	MCF-7, T47D, ZR75-1	Aspirin disrupted chemoresistance via NFKB-IL6 signalling
Dai, Xiaoyang et al.	2017	1mM-20mM	10% FBS	Breast Cancer	MDA-MB-231	Aspirin inhibited angiogenesis in a heparenase-dependent manner
Sun Y, Dai H, Chen S, et al.	2018	0, 0.5, 1, 2, 5mM	10% FBS	Lung Cancer	A549, H1299	Aspirin reduced cell viability/survival in lung cancer cell lines
Chen Z, Li W, Qiu F, et al.	2018	5, 10mM	10% FBS	Colorectal Cancer	P1, P2 (primary CRC cells)	Aspirin enriched apoptosis in colon cancer cells
Zhang, Xiaoqi et al.	2018	0.5, 1, 2, 5, 10mM	10% FBS	Oral Sqamous Cell Carcinoma	TCA8113, CAL27	Aspirin reduced cell proliferation in oral squamous cell carcinoma cells
Jin, Shenghang, and Xianguo Wu	2019	1-10mM	10% FBS	Colorectal Cancer	SW480	Aspirin reduced colon cancer cell migration
Maity, Gargi et al.	2019	1mM, 2.5mM	10% FBS	Breast Cancer	MDA-MB-231, HCC-70	Aspirin blocked breast cancer migration
Pozzoli, Giacomo et al.	2019	2mM (some experiments with 0.1, 0.3m 1mM, 3m	10% FBS	Neuroblastoma	SK-N-SH	Aspirin reduced neuroblastoma cell proliferation
Shi, Tingting et al	2020	2.5-10mM	5 and 10% FBS	Hepatocellular Carcinoma (HCC)	HLE, HLF, Huh-7, PLC/PRF/5, Hep-3B, Li-7	Aspirin suppressed cell proliferation
Zhang, Xiaoyuan et al.	2020	0.5-30mM	10% FBS	Breast Cancer , Lung Cancer	MDA-MB-231, A549	Aspirin reduced tumour growth
Nounu, Aayah et al.	2020	2-4mM	20% FBS	Colorectal Cancer	RG/C2	Identified genes associated with aspirin and CRC risk
Lin, Shengping et al.	2020	0.5, 1mM, 2mM, 4mM	10% FBS	Pancreatic Cancer	PANC-1	Aspirin reduced cell proliferation
Devall MAM, Drew DA, Dampier CH, et al.	**2021**	**50uM**	**NO FBS**	**Colorectal Cancer**	**Organoids of colorectal adenoma**	**Identified genes associated with aspirin chemo-prevention in CRC**

5 Results- Examining the effects of aspirin in multi-cellular *in vitro* models of HGSOC

5.1 Background

This chapter explores the potential effects of aspirin in modulating the TME in multi-cellular models of HGSOC *in vitro*.

My data in chapter 4 demonstrated the importance of carrying out *in vitro* aspirin treatments on malignant cells in serum-free medium, and provided me with useful information required to progress with my project. I next wanted to know whether aspirin could exert its potential cancer-preventive actions by targeting non-malignant cells in the TME. To answer this question, I examined the effects of aspirin in multi-cellular models of HGSOC.

Over the last decade, multi-cellular models have become increasingly popular to study tumour development and progression[156]. Clinically relevant multi-cellular models are inherently more biomimetic than 2D tissue culture plastic, and recapitulate elements of the TME such as hypoxia, invasion, and cell-cell communication[157]. As described in the Introduction (section 1.6.2.2), there are several advantages of using 3D multi-cellular models to answer biological questions. 3D multi-cellular models enable the co-culturing and interaction between malignant and non-malignant cells in the TME, such as stromal cells (e.g. cancer associated fibroblasts, CAFs), and immune cells (e.g. monocytes/macrophages)[156]. It is also possible to incorporate ECM molecules in 3D multi-cellular models, such as collagen I[175,176]. Moreover, 3D multi-cellular models help to overcome some of the barriers associated with patient-derived xenografts (PDXs), including a slow turnaround time, high cost, and a lack of all components of the TME in immunodeficient mice[156,160].

There are different types of 3D *in vitro* multi-cellular models, such as spheroids, collagen I hydrogels, and patient-derived organoids (sections 1.6.2.2.1- 1.6.2.2.3). Since I was interested to study the role of aspirin in preventing early-stage HGSOC development and progression, it was important to use a relevant 3D multi-cellular model to answer my research question. Therefore, I used our disease-specific tetra-culture (four cell types) model to do so, as it recapitulates some elements of early-stage HGSOC progression in the omentum. As described earlier (1.6.2.3), our multi-cellular model is 're-constructed' in a layered approach using primary omental

adipocytes, primary omental fibroblasts, primary omental mesothelial cells, and early-passage HGSOC malignant cells. Furthermore, Malacrida *et al.* recently used our tetra-culture model to demonstrate the role of platelets in stimulating early-stage disease progression, malignant cell invasion into the omentum, and production of cancer-associated ECM molecules[181].

5.2 RNA-sequencing data showing *PTGS1, PTGS2* and downstream targets in tetra-cultures of HGSOC

I started this chapter by interrogating existing RNA-sequencing results to study the basal expression of *PTGS1, PTGS2* and its downstream targets in tetra-cultures of G164 cultured with/without platelets. As described in the Introduction, platelets have been shown to favour tumour progression. Recent work in our group has highlighted the role played by platelets in promoting disease progression by stimulating malignant cell invasion and a cancer-associated ECM in tetra-culture models of HGSOC[181].

As depicted in Figure 5.1, *PTGS1* was elevated in tetra-cultures of G164 compared to 2D monocultures of G164. Furthermore, there was almost a 3-fold increase in *PTGS2* in 3D multi-cellular models compared to G164 cells cultured in 2D monocultures. There was also a significant elevation in *PTGER3* (gene which encodes for the EP3 receptor) in tetra-cultures of G164 +/- platelets. However, there were no significant differences in *PTGER1* (EP1) and *PTGER4* (EP4) between 2D monocultures of G164 and tetra-culture models of G164. Tetra-cultures of G164 did not express the gene which encoded for the EP2 receptor (*PTGER2*).

Overall, tetra-cultures of G164 expressed higher levels of *PTGS2* than 2D monolayers of malignant cells, and also expressed *PTGER1, PTGER3* and *PTGER4,* which collectively suggested that the COX-2-PGE_2 pathway was relevant in our multi-cellular model of HGSOC.

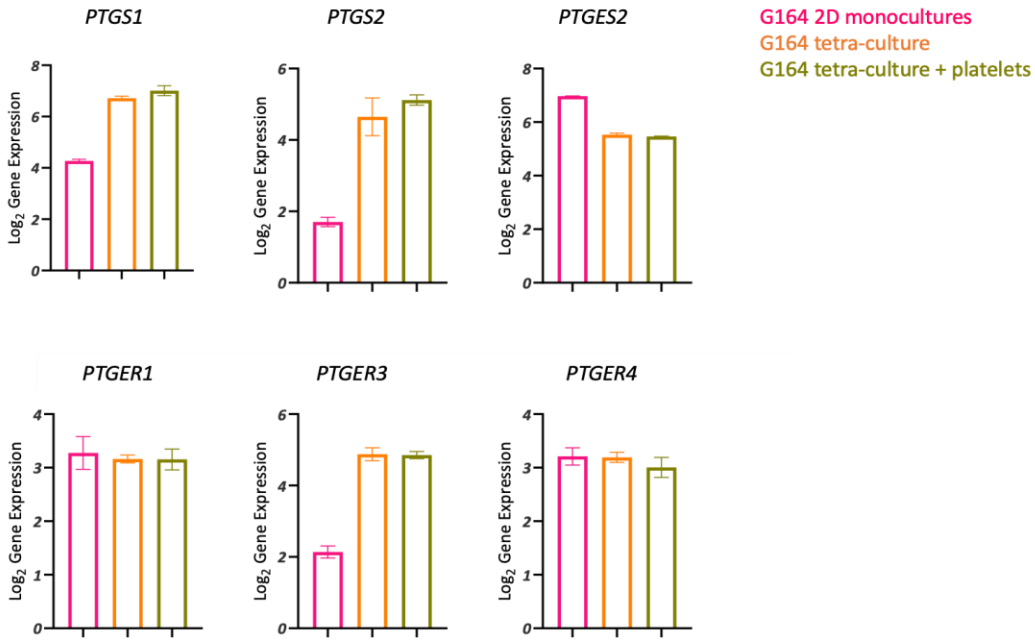


Figure 5.1. RNA-sequencing data of genes involved in regulating the metabolism of arachidonic acid into prostanoids in tetra-culture models of G164. RNA-sequencing was performed in tetra-culture models of G164 cultured with and without platelets. I examined the expression of *PTGS1* (gene that encodes for COX-1), *PTGS2* (gene that encodes for COX-2), and PGE_2 receptors *(PTGER1-4)*. I also incorporated the expression of these genes in 2D monocultures of G164 as an additional reference. PTGS-1, prostaglandin endoperoxide synthase-1; PTGS-2, prostaglandin endoperoxide synthase-2; PTGER2, prostaglandin E receptor 2; PTGER3, prostaglandin E receptor 3 ; PTGER4, prostaglandin E receptor 4.

5.3 Effects of aspirin on the viability in tetra-cultures of HGSOC

After examining the RNA-sequencing data, I proceeded to investigate the effects of aspirin on cell viability in tetra-cultures of AOCS-1, G164 and G33 cells, as well as tetra-cultures of the p53-mutant FT318 pre-malignant cell line. Since I performed all aspirin treatments in serum-free medium, I could not exceed a treatment time-point of 48 hours. At endpoint, I performed an immunohistochemical staining on my gels for cleaved caspase 3, as illustrated in Figure 5.2 .

Aspirin did not reduce cell viability in tetra-cultures of AOCS-1, G164, G33 and p53 mut FT318, as measured by caspase 3 levels. This is particularly evident in tetra-cultures of G33, although it is not too surprising as G33 cells are more aggressive than G164 or AOCS-1 cells. Moreover, tetra-cultures of G164 seemed to display a basal level of stress, which is likely due to serum starvation. Similarly, some cleaved caspase 3 staining was detected in tetra-cultures of AOCS-1 and p53-mutant FT318 cells, but there was no difference between control and treated samples. Overall,

aspirin did not reduce cell viability in tetra-cultures of AOCS-1, G164, G33 and p53 mut FT318, despite being subjected to a challenging, serum-starved environment.

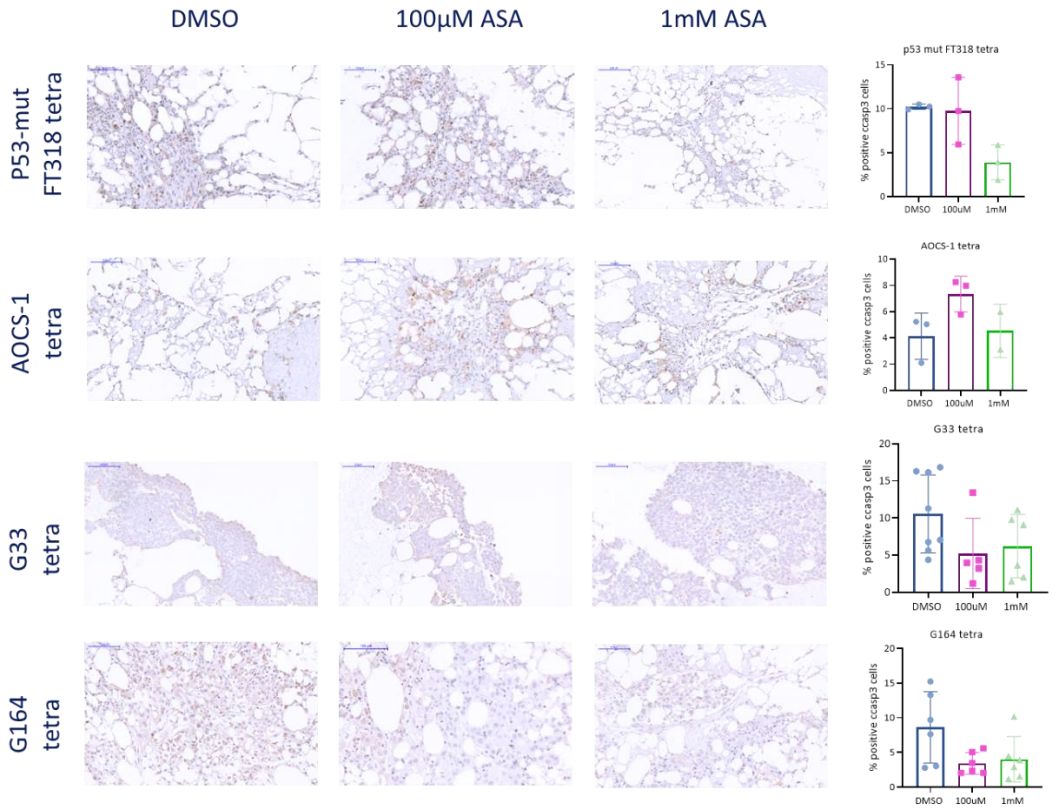


Figure 5.2. Cleaved caspase 3 staining of tetra-cultures treated with aspirin. Tetra-culture models of p53-mut FT318, AOCS-1, G33 and G164 were subjected to aspirin treatment in serum-free medium for 48 hours. Gels were then embedded in 2% agarose, fixed in formalin, and embedded in paraffin. Immunohistochemical staining for cleaved caspase 3 was then performed to assess the viability of the gels. Three slides/condition were stained. Quantification was performed using the QuPath software. Scale bar shows 100µM.

5.4 Effects of aspirin on the secretome in HGSOC *in vitro*

I then asked whether aspirin could modulate other aspects of the TME, such as the secretome. As described in the Introduction (section 1.5.4), there is evidence which points to the crucial role played by cytokines (e.g. IL-6[126,127]), chemokines (e.g. IL-8[135,199,200,102]), and growth factors (e.g. VEGF[145,147]) in generating a pro-inflammatory TME to drive tumour progression. Given aspirin's nature as an anti-inflammatory agent, I questioned whether aspirin could regulate the constitutive production of cytokines in HGSOC models *in vitro*.

5.4.1 Pro-inflammatory cytokine array on tetra-cultures of HGSOC treated with aspirin

First, I carried out a pro-inflammatory 'Proteome Profiler™' array using supernatants of tetra-cultures incubated with aspirin. The aim of this assay was to identify any potential cytokines of interest that may be detected and modulated following aspirin treatment. Since a maximum of only four samples could be used per kit, I performed this experiment using tetra-cultures of AOCS-1 and G164 with and without 100μM aspirin treatment.

IL-6, IL-8, CCL2 and macrophage migration inhibitory factor (MIF) were detected in tetra-cultures of AOCS-1, as displayed in Figure 5.3A. Tetra-cultures of G164 also had detectable levels of the above-mentioned cytokines, with the exception of CCL2 (Figure 5.3B). Interestingly, aspirin appeared to decrease IL-8 secretion in tetra-cultures of AOCS-1. There also seemed to be a potential reduction in IL-6 and CCL2 in tetra-cultures of AOCS-1. Conversely, aspirin seemed to stimulate IL-6 in tetra-cultures of G164.

Overall, this array was a useful start to detect cytokines that may be present in our cultures –pre and –post aspirin treatment. However, a limitation of this assay is its lack of sensitivity and limited number of cytokines available. Therefore, I decided to continue by performing a custom MesoScale Discovery (MSD®) multi-spot assay on aspirin treated HGSOC cultures.

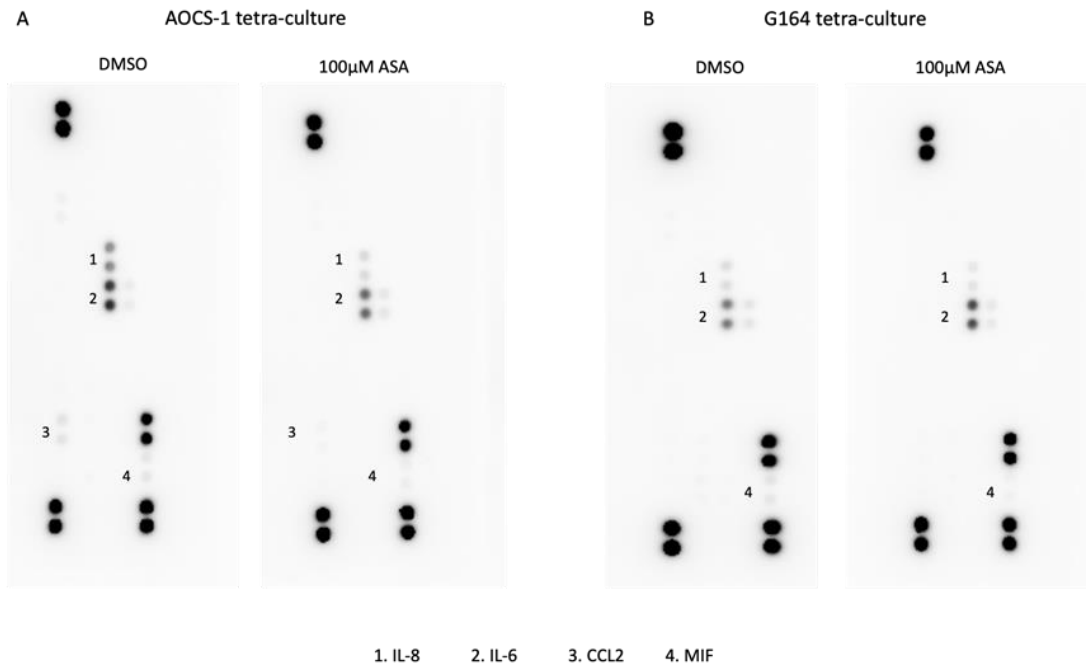


Figure 5.3. Pro-inflammatory cytokine array on aspirin-treated tetra-cultures of AOCS-1 and G164. Pro-inflammatory cytokine array on supernatants of tetra-cultures treated with aspirin (48h, serum-free medium). All membranes were developed at the same time using the Amersham™ Imager 600 (GE Healthcare Life Sciences).

5.4.2 Mesoscale Discovery® multi-spot assay in HGSOC cultures treated with aspirin

I analysed a range of cytokines and chemokines in 2D and 3D HGSOC cultures treated with aspirin, as summarised in Figure 5.4. I also performed additional ELISAs for IL-6, IL-8 and CCL2, as some of my cultures secreted high amounts of these cytokines, and were significantly above the standard curve in the MSD® multi-spot assay.

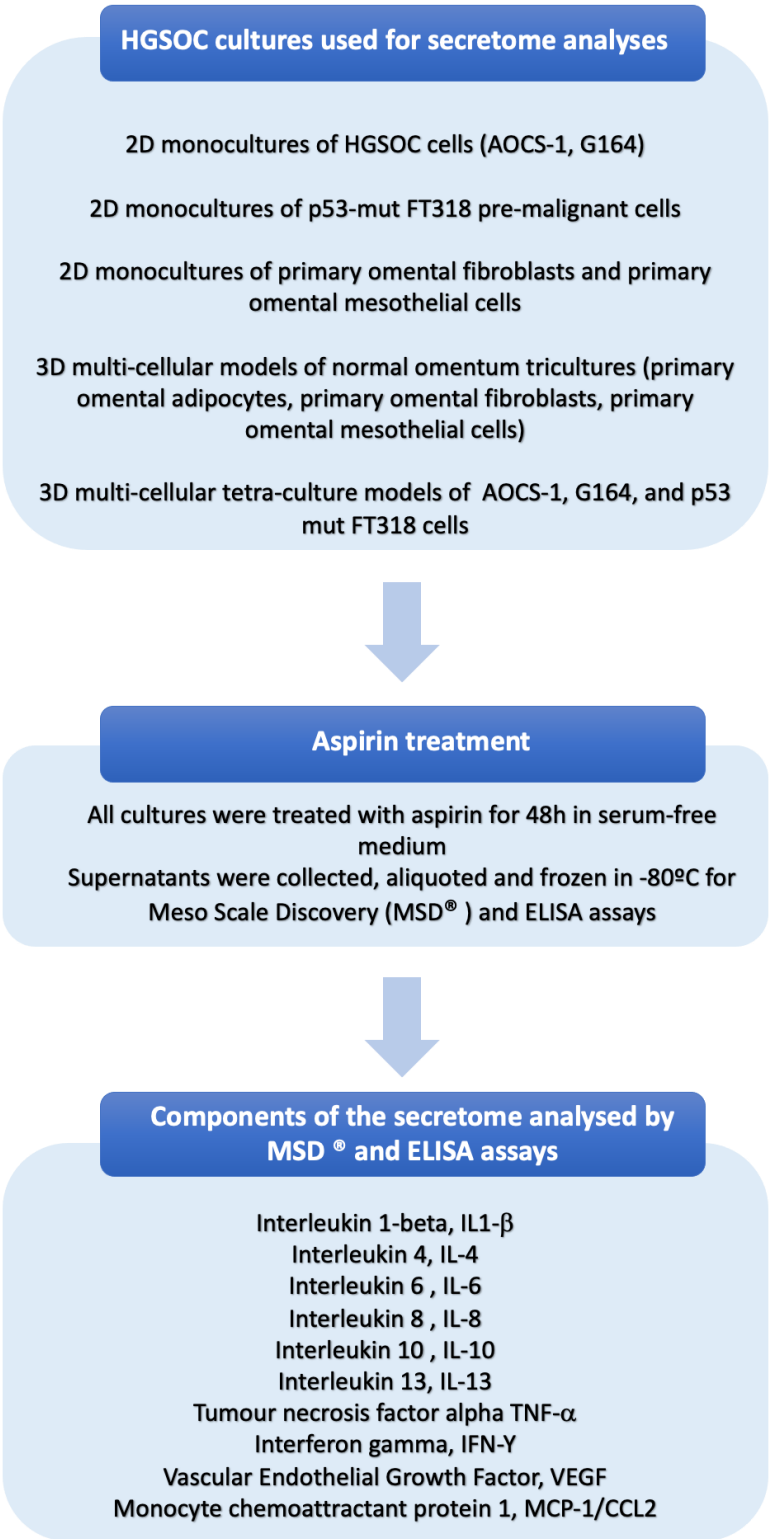


Figure 5.4. Schematic summarising the HGSOC cultures used for cytokine analyses. Meso Scale Discovery (MSD®) multi-spot assay on supernatants of aspirin treated HGSOC cultures. I treated the following cultures with aspirin: 2D monocultures of AOCS-1, G164 and p53 mut-FT318 cells; 2D monocultures of primary omental fibroblasts and primary omental mesothelial cells; normal omental tricultures consisting of adipocytes, primary omental fibroblasts, and mesothelial cells; and tetra-cultures of AOCS-1, G164 and p53 mut-FT318. I had to do an ELISA for IL-6, IL-8 and CCL2 as the concentrations in my samples for these three cytokines were significantly above the MSD standard curve.

5.4.2.1 Interleukin 1 beta, IL1-β secretion in aspirin-treated HGSOC cultures

IL1-β is linked to tumour progression, and has also been shown to induce hypoxia-inducible factor via COX-2 signalling[201].

Aspirin did not significantly modulate the secretion of IL1-β in any of the HGSOC culture conditions, as portrayed in Figure 5.5. At a glance, aspirin seemed to reduce IL1-β in 2D monocultures of AOCS-1 cells, although this cell line secreted relatively small amounts of the cytokine (mean baseline concentration 7.77pg/mL). Conversely, 1mM treatment potentially stimulated IL1-β secretion in p53 mutant FT318 cells. Similarly, aspirin appeared to slightly increase IL1-β release in tricultures of HGSOC. Lastly, the concentration of IL1-β did not seem to fluctuate in monocultures of G164, primary omental fibroblasts and mesothelial cells, and tetra-cultures of HGSOC (AOCS-1, G164, p53-mut FT318). Overall, aspirin did not reduce the secretion of IL1-β in 2D and 3D HGSOC cultures.

5.4.2.2 Interleukin 4, IL-4, secretion in aspirin-treated HGSOC cultures

IL-4 is typically associated with T_H2 T helper T cells, but it is produced by some malignant cells and linked with tumour progression[31]. As depicted in Figure 5.6, IL-4 concentration varied between 2D monocultures and 3D multi-cellular models. Primary omental fibroblasts seemed to be the major contributors of IL-4 secretion (mean baseline IL-4 concentration 98.5pg/mL). This contrasted with monocultures of malignant /pre-malignant cells (AOCS-1, 2.06pg/mL; G164, 7.99pg/mL; p53 mut FT318, 11 pg/mL).

Overall, aspirin seemed to marginally modulate the secretion of IL-4 in some multi-cellular models of HGSOC. As an example, there appeared to be a decrease in IL-4 after 1mM aspirin treatment in tetra-cultures of G164 and p53 mut FT318 (Figure 5.6-H and I). Conversely, aspirin did not regulate IL-4 release in tetra-cultures of AOCS-1 (Figure 5.6-G). Fluctuating levels of IL-4 were seen in normal omental tricultures, as well as monocultures of fibroblasts and mesothelial cells. Furthermore, an unexpected increase in IL-4 secretion after 1mM aspirin treatment was observed in monocultures of p53 mut FT318 cells (Figure 5.6-C). Conversely, 10μM and 100μM aspirin seemed to reduce IL-4 release in monocultures of AOCS-1 and G164,

but these two cell lines secreted little amounts of the cytokine to begin with. Overall, aspirin did not have a clear inhibitory effect on the release of IL-4 in HGSOC cultures.

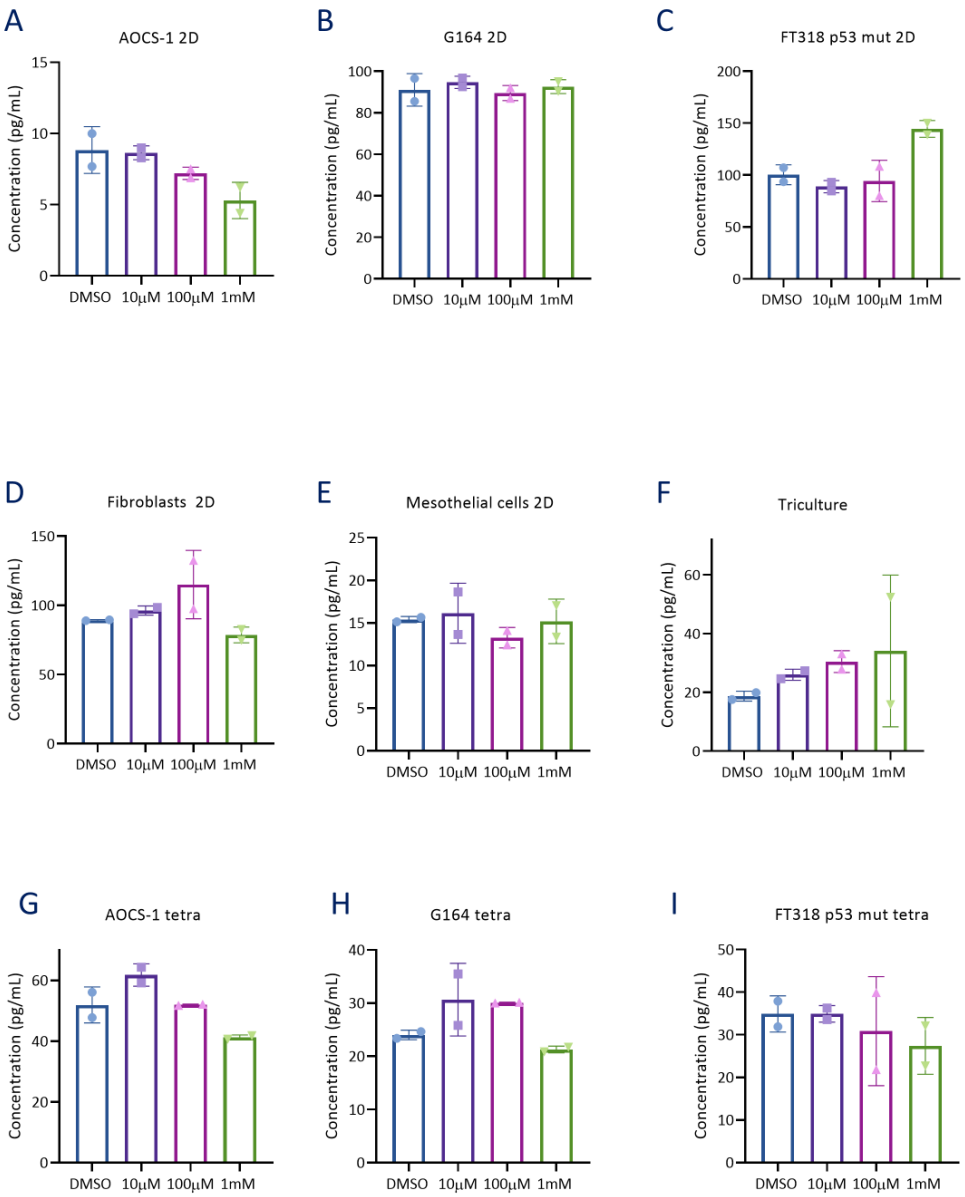


Figure 5.5. Interleukin beta, IL1-β, secretion in aspirin-treated HGSOC cultures *in vitro.* MesoScale Discovery multi-spot assay on the following cell types treated with aspirin for 48h: Malignant cells (AOCS-1, G164), pre-malignant cells (p53 mut FT318), primary omental fibroblasts and mesothelial cells, normal omentum tricultures (adipocytes, fibroblasts, mesothelial cells), and tetra-cultures of AOCS-1, G164 and p53 mut FT318 cells.

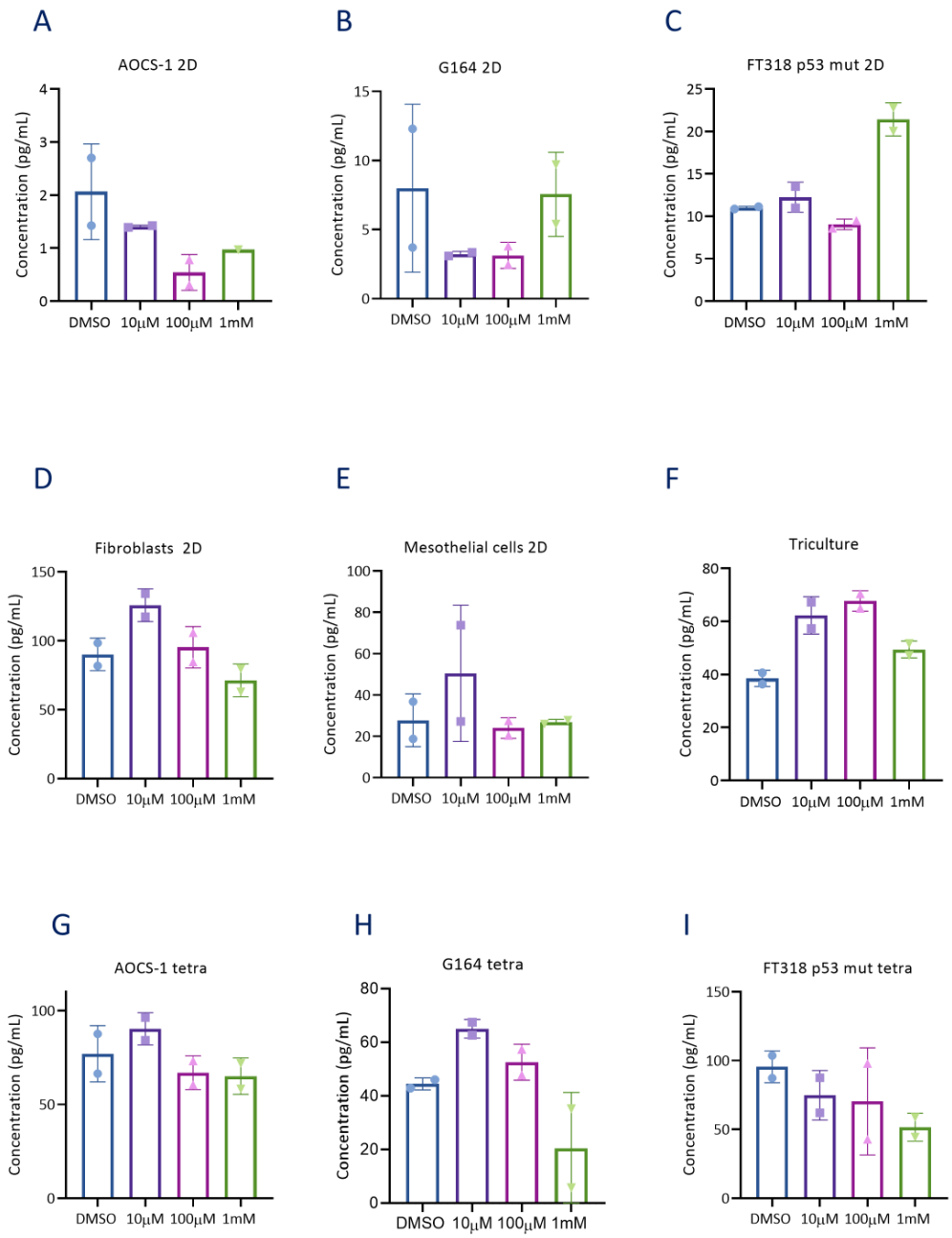


Figure 5.6. Interleukin 4, IL-4 secretion in aspirin-treated HGSOC cultures *in vitro*. Meso Scale Discovery multi-spot assay on the following cell types treated with aspirin for 48h: Malignant cells (AOCS-1, G164), pre-malignant cells (p53 mut FT318), primary omental fibroblasts and mesothelial cells, normal omentum tricultures (adipocytes, fibroblasts, mesothelial cells), and tetra-cultures of AOCS-1, G164 and p53 mut FT318 cells.

5.4.2.3 Interleukin 6, IL-6, secretion in aspirin-treated HGSOC cultures

As detailed in the introduction, IL-6 is a well-established pro-tumourigenic cytokine that has been of interest to our group[126, 127 ,147].

Relatively high levels of IL-6 were seen in HGSOC cultures, as depicted in Figure 5.7. Primary omental fibroblasts and mesothelial cells secreted significantly higher amounts of IL-6 (fibroblasts 2D, 4132 pg/mL); (mesothelial cells 2D, 1380 pg/mL) compared to malignant and pre-malignant monocultures (AOCS-1 2D, 356 pg/mL; G164 2D, 87.2 pg/mL; p53 mut FT318 cells 2D, 538 pg/mL). This may explain why there was a 10-fold increase in IL-6 concentrations in tetra-cultures of HGSOC. Furthermore, tricultures of HGSOC, which contain all stromal components present in our tetra-culture model, also secreted substantial amounts of IL-6 relative to monocultures of malignant and pre-malignant cells.

A potential reduction in IL-6 in monocultures of AOCS-1 was observed following aspirin treatment, although this effect did not appear to be dose-dependent (Figure 5.7-A). On the other hand, there was a notable increase in IL-6 in p53 mut FT318 cells following 1mM aspirin treatment. However, lower doses of aspirin (10μM, 100μM) did not modulate IL-6 concentration in p53 mut FT318 monocultures. Similarly, aspirin did not inhibit IL-6 release from fibroblasts, mesothelial cells, tricultures and tetra-cultures of HGSOC. Overall, aspirin did not seem to elicit an effect on IL-6 in multi-cellular models of HGSOC.

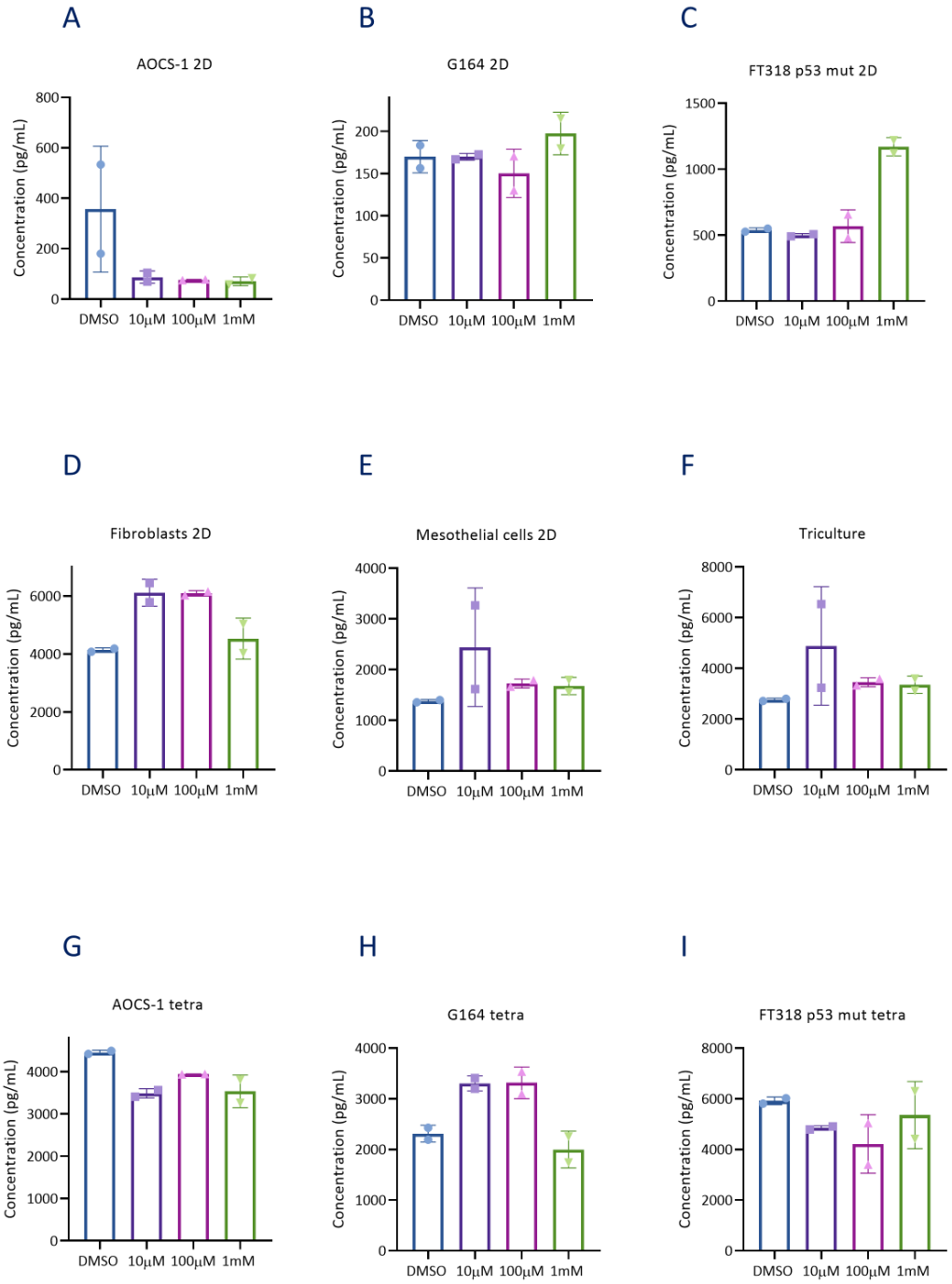


Figure 5.7. Interleukin 6, IL-6 secretion in aspirin-treated HGSOC cultures *in vitro*. Meso Scale Discovery multi-spot assay on the following cell types treated with aspirin for 48h: Malignant cells (AOCS-1, G164), pre-malignant cells (p53 mut FT318), primary omental fibroblasts and mesothelial cells, normal omentum tricultures (adipocytes, fibroblasts, mesothelial cells), and tetra-cultures of AOCS-1, G164 and p53 mut FT318 cells.

5.4.2.4 Interleukin 10, IL-10, secretion in aspirin-treated HGSOC cultures

IL-10 is associated with immunosuppression, downregulating T_H1 responses, and inducing regulatory T cell (T_{Reg}) responses. It is therefore regarded as an important cytokine of the TME. As illustrated in Figure 5.8, there was a relatively low basal level of IL-10 secretion in 2D monocultures of malignant and pre-malignant cells (Figure 5.8 A-C), 2D monocultures of primary omental fibroblasts and primary omental mesothelial cells (Figure 5.8 D-E), normal omental tricultures (Figure 5.8 F), and tetra-cultures of HGSOC (Figure 5.8G-I). There were also no differences in IL-10 concentration between 2D monocultures and 3D multi-cellular models.

The concentration of IL-10 did not significantly change following low dose aspirin treatment (10μM, 100μM) in most HGSOC cultures. There appeared to be a slight dose-dependent reduction of IL-10 following 10μM and 100μM aspirin in monocultures of fibroblasts (Figure 5.8-D). Similarly, 100μM aspirin may have reduced IL-10 levels in monolayers of G164 (Figure 5.8-B). However, IL-10 seemed to be stimulated following high-dose aspirin (1mM) in several HGSOC cultures. This includes monocultures of AOCS-1 (Figure 5.8-A), monocultures of G164 (Figure 5.8-B), normal omental tricultures (Figure 5.8– F), and tetra-cultures of G164. Overall, aspirin did not modulate the release of IL-10 in HGSOC cultures *in vitro.*

5.4.2.5 Interleukin 13, IL-13, secretion in aspirin-treated HGSOC cultures

Similar to IL-4, IL-13 is a cytokine responsible for eliciting T_H2 responses, but can be secreted by some tumour cells too. Whilst the effects of aspirin in reducing IL-13 secretion from malignant cells have not been explored, Perez-G *et al.* showed that aspirin improved allergic response via IL-13-STAT6 signalling[202].

As exhibited in Figure 5.9, IL-13 was secreted in my HGSOC cultures. IL-13 was secreted by 2D cultures of malignant/pre-malignant cells, fibroblasts, and mesothelial cells (Figure 5.9A-E), 3D tricultures (Figure 5.9-F), and tetra-cultures of HGSOC (Figure 5.9 G-I). There were no major differences in the basal concentration of IL-13 between 2D monolayers and multi-cellular models of HGSOC. Moreover, IL-13 levels appeared to be relatively unchanged following aspirin treatment. This was

seen in all cultures of HGSOC. Therefore, aspirin did not affect IL-13 release in 2D and 3D HGSOC cultures *in vitro.*

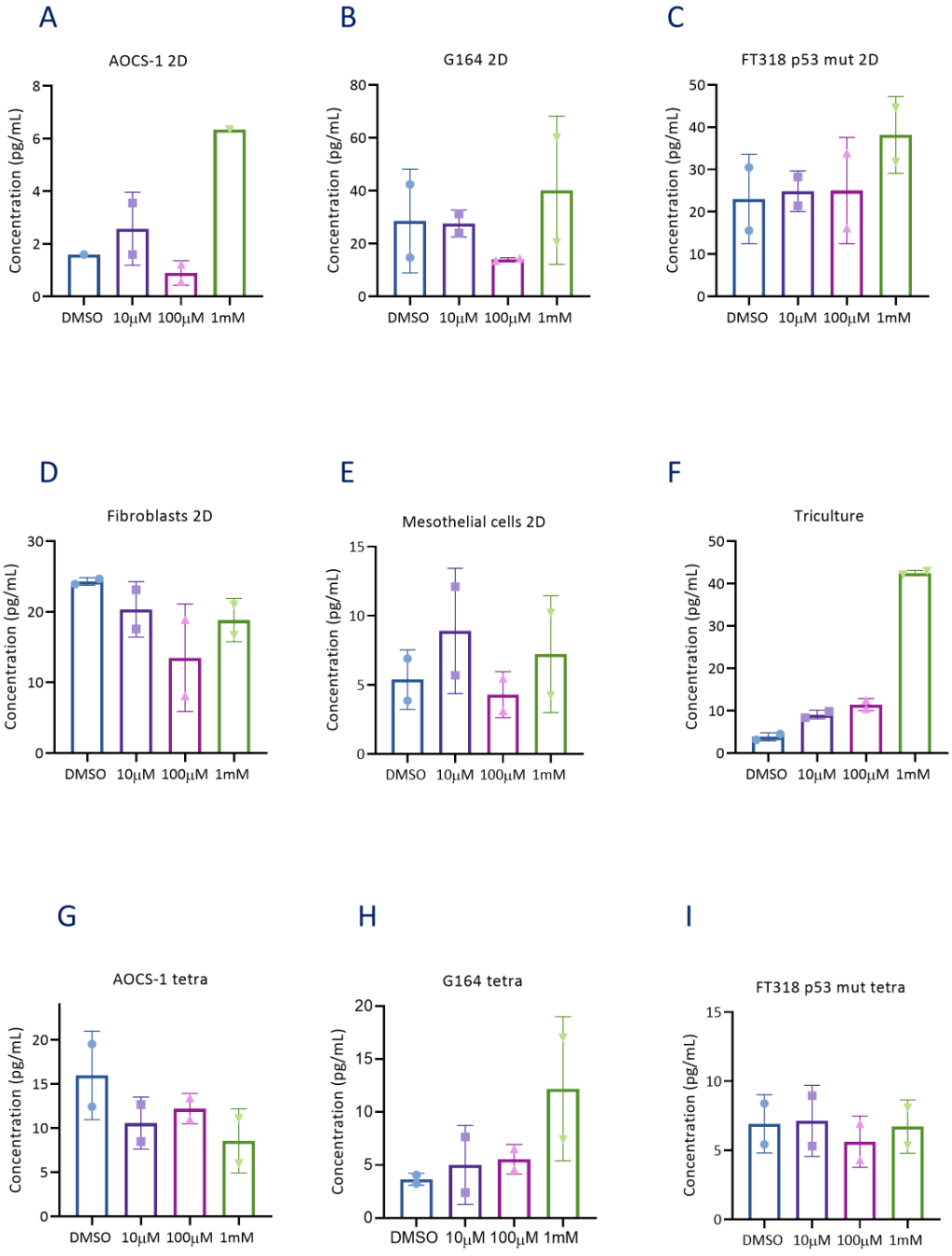


Figure 5.8. Interleukin 10, IL-10 secretion in aspirin-treated HGSOC cultures *in vitro*. Meso Scale Discovery multi-spot assay on the following cell types treated with aspirin for 48h: Malignant cells (AOCS-1, G164), pre-malignant cells (p53 mut FT318),primary omental fibroblasts and mesothelial cells, normal omentum tricultures (adipocytes, fibroblasts, mesothelial cells), and tetra-cultures of AOCS-1, G164 and p53 mut FT318 cells.

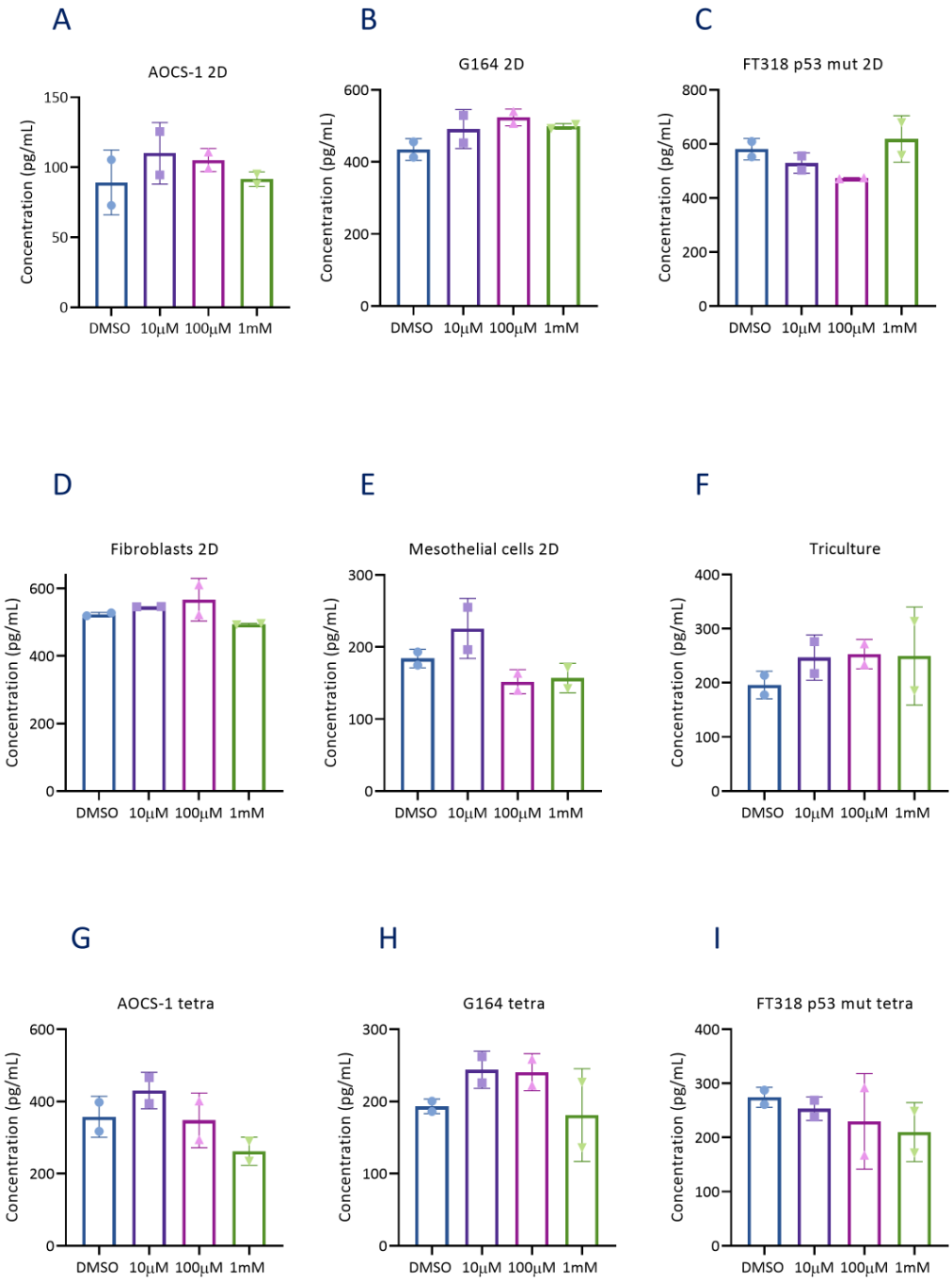


Figure 5.9. Interleukin 13, IL-13 secretion in aspirin-treated HGSOC cultures *in vitro*. Meso Scale Discovery multi-spot assay on the following cell types treated with aspirin for 48h: Malignant cells (AOCS-1, G164), pre-malignant cells (p53 mut FT318), primary omental fibroblasts and mesothelial cells, normal omentum tricultures (adipocytes, fibroblasts, mesothelial cells), and tetra-cultures of AOCS-1, G164 and p53 mut FT318 cells.

5.4.2.6 Tumour necrosis factor alpha, TNFα, secretion in aspirin treated HGSOC cultures

As described in the Introduction (1.5.4.4), TNFα was once thought to be an anti-tumour agent, but it is now known that this cytokine is also known to be pro-tumourigenic in nature. The effects of aspirin in regulating TNFα release are illustrated in Figure 5.10.

Upon initial observation, it appears that aspirin may have potentially modulated the release of TNFα in some HGSOC cultures. For example, 100μM and 1mM aspirin appeared to lower TNFα concentrations in 2D monocultures of primary omental mesothelial cells (Figure 5.10-E). On the contrary, 1mM aspirin may have potentially stimulated the release of this cytokine in monocultures of p53mut FT318 cells (Figure 5.10-C), as well as 3D models of normal omental tricultures (Figure 5.10-F). Similarly, TNFα concentrations increased by 3 times in G164 cells following 10μM, 100μM and 1mM aspirin exposure (Figure 5.10-B). Conversely, aspirin did not substantially fluctuate TNFα release in monocultures of AOCS-1 (Figure 5.10-A), primary omental fibroblasts (Figure 5.10-D), and tetra-cultures of HGSOC (Figure 5.10 G-I). Overall, aspirin did not significantly reduce levels of TNFα in 2D and 3D cultures of HGSOC.

5.4.2.7 Interferon gamma, IFN-Y secretion in aspirin-treated HGSOC cultures

Interferon gamma (IFN-γ) is classically regarded as an anti-tumour cytokine and is secreted by 2D and 3D HGSOC cultures (Figure 5.11). Overall, aspirin did not modulate the release of IFN-γ in 2D monocultures of malignant and pre-malignant cells (Figure 5.11 A-C), 2D monocultures of primary omental fibroblasts and mesothelial cells (Figure 5.11D-E), and tetra-cultures of HGSOC (Figure 5.11G-I). On the contrary, there appeared to be a decrease in IFN-γ following 1mM aspirin treatment in 3D normal omental tricultures (Figure 5.11-F). Overall, aspirin did not significantly regulate IFN- γ release in HGSOC cultures *in vitro.*

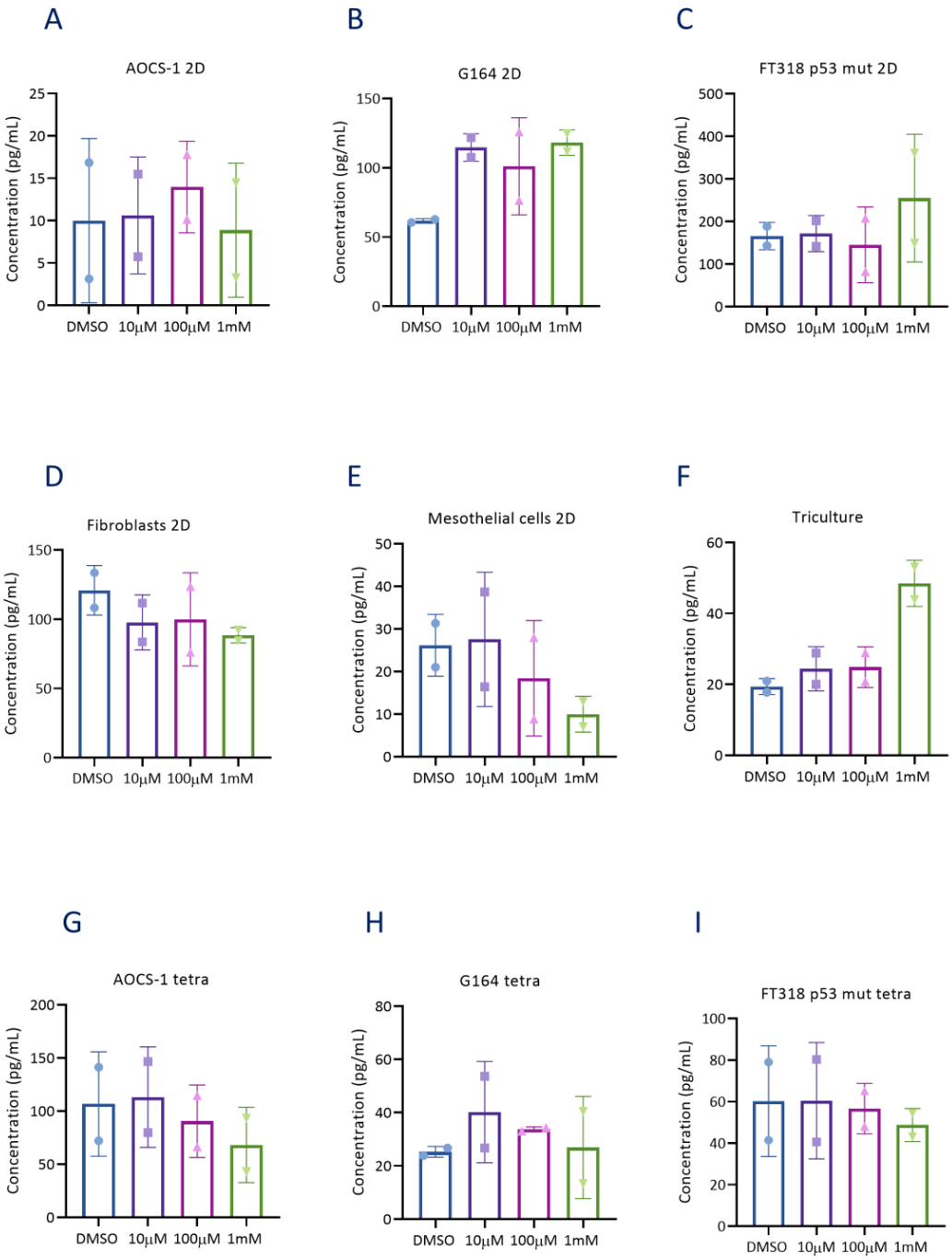


Figure 5.10. Tumour necrosis factor alpha, TNFα secretion in aspirin treated HGSOC cultures *in vitro*. Meso Scale Discovery multi-spot assay on the following cell types treated with aspirin for 48h: Malignant cells (AOCS-1, G164), pre-malignant cells (p53 mut FT318), primary omental fibroblasts and mesothelial cells, normal omentum tricultures (adipocytes, fibroblasts, mesothelial cells), and tetra-cultures of AOCS-1, G164 and p53 mut FT318 cells.

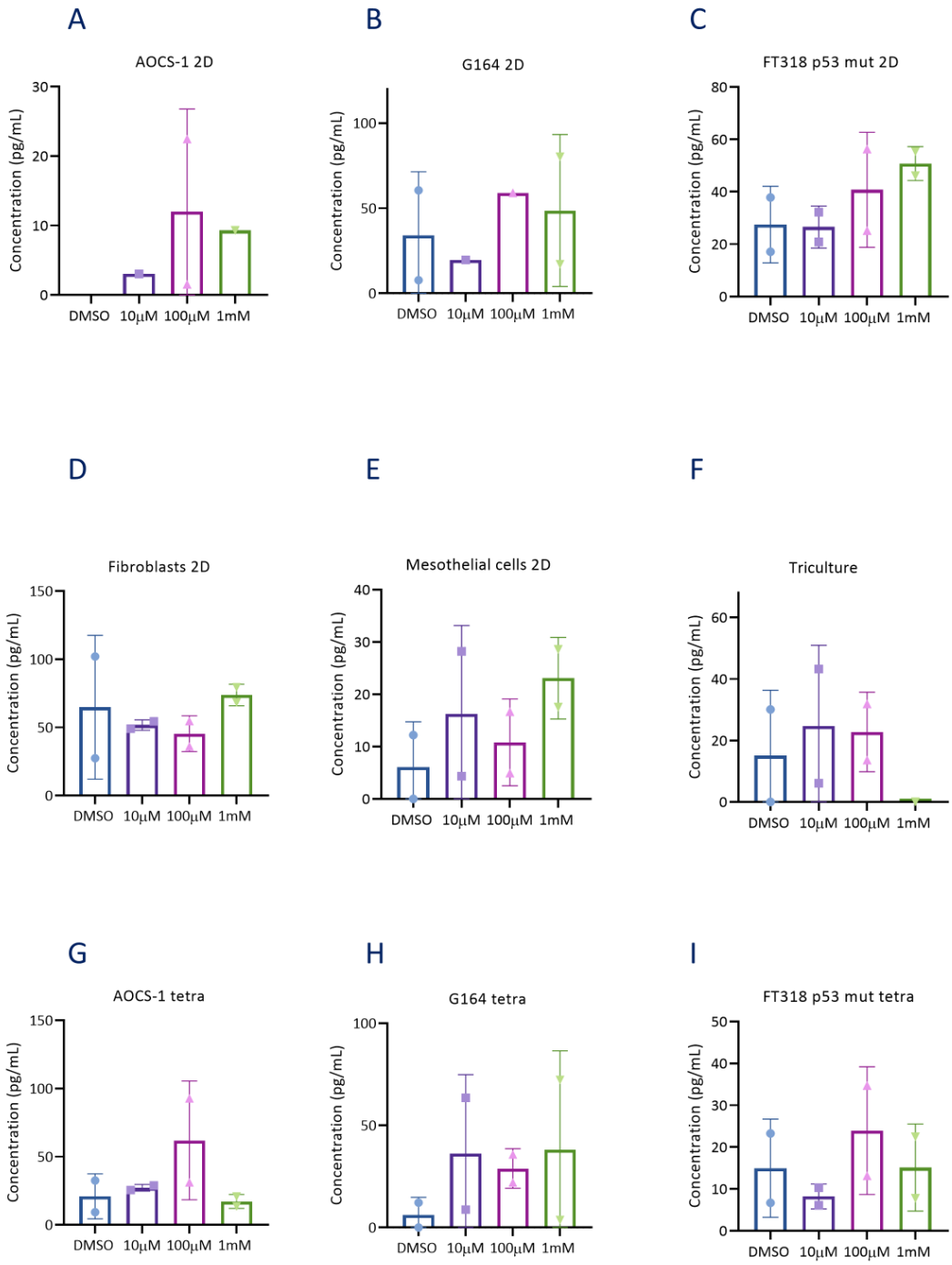


Figure 5.11. Interferon gamma, IFN-γ in aspirin-treated HGSOC cultures *in vitro*. Meso Scale Discovery multi-spot assay on the following cell types treated with aspirin for 48h: Malignant cells (AOCS-1, G164), pre-malignant cells (p53 mut FT318), primary omental fibroblasts and mesothelial cells, normal omentum tricultures (adipocytes, fibroblasts, mesothelial cells), and tetra-cultures of AOCS-1, G164 and p53 mut FT318 cells.

5.4.2.8 Monocyte chemoattractant protein 1 (MCP-1/CCL2) secretion in aspirin-treated HGSOC cultures

CCL2 is a powerful pro-tumourigenic chemoattractant typically linked with the recruitment of monocytes and macrophages to the TME. I have described the role of this chemokine previously (section 1.5.4.3). High concentrations of CCL2 were released from 2D monocultures of AOCS-1, p53 mut FT318, fibroblasts and mesothelial cells (Figure 5.12-A-C). CCL2 was also produced by tetra-cultures of AOCS-1, G164 and p53 mut FT318 cells (Figure 5.12-F-H). G164 cells did not secrete CCL2, which is in accordance with other unpublished results in our group.

Aspirin did not reduce CCL2 secretion in monocultures of AOCS-1 and p53mut FT318 cells as illustrated in Figure 5.12-A and B. Conversely, 10μM and 100μM aspirin reduced the secretion in monocultures of fibroblasts (Figure 5.12C). There also appeared to be a dose-dependent decrease in CCL2 release in tri-cultures of HGSOC (Figure 5.12E). However, mixed results were observed in tetra-cultures of HGSOC. There was a reduction in CCL2 in tetra-cultures of AOCS-1 following 10μM, 100μM and 1mM treatment (Figure 5.12F). Similarly, 10μM and 100μM treatment decreased CCL2 release from tetra-cultures of p53 FT318, but there was a sudden stimulation of the chemokine after 1mM aspirin treatment. On the other hand, aspirin did not seem to modulate the secretion of CCL2 in tetra-cultures of G164 (Figure 5.12G).

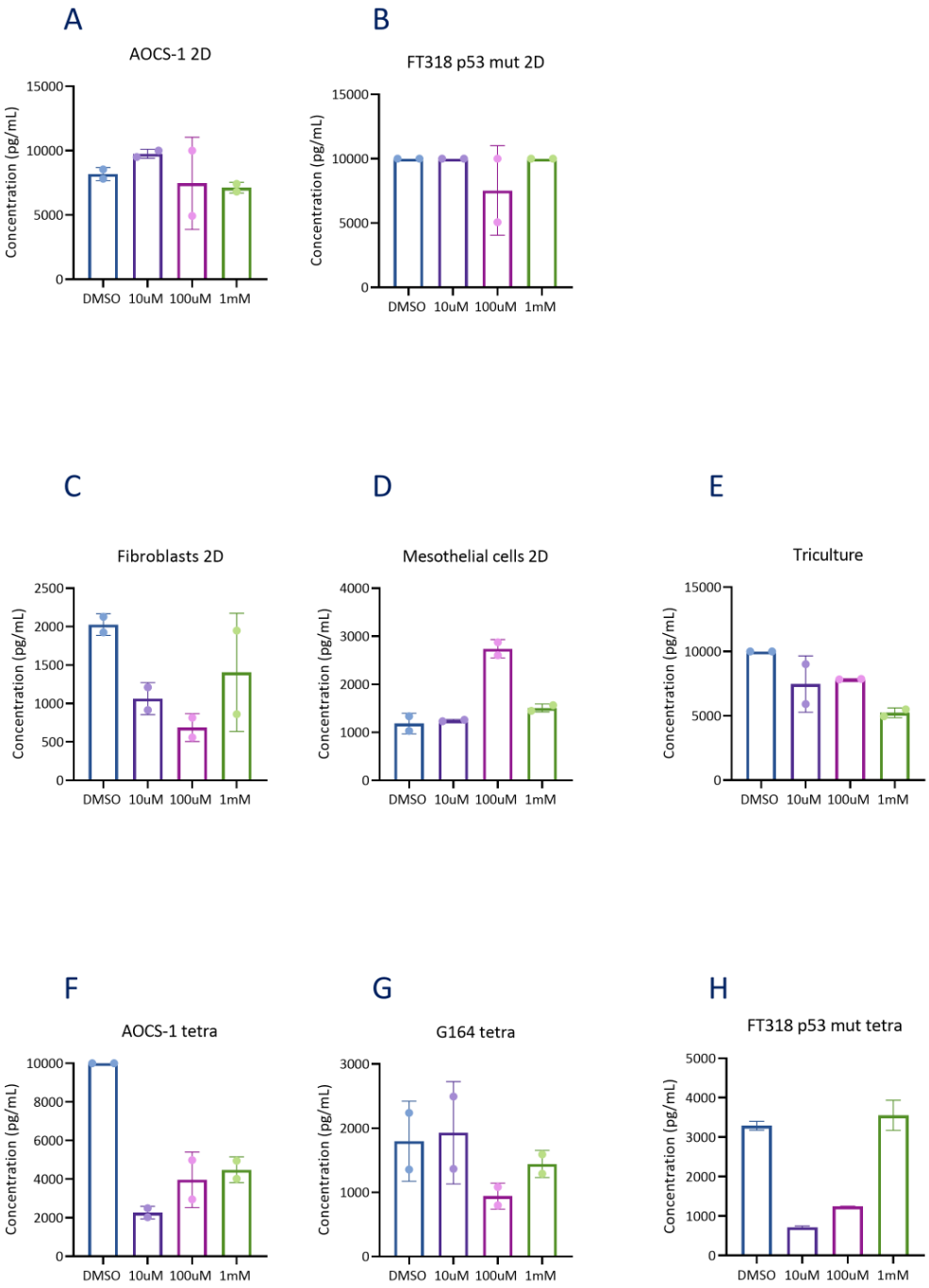


Figure 5.12. CCL2 release in aspirin treated HGSOC cultures in vitro. Meso Scale Discovery multi-spot assay, as well as an ELISA on the following cell types treated with aspirin for 48h: Malignant cells (AOCS-1, G164), pre-malignant cells (p53 mut FT318), primary omental fibroblasts and mesothelial cells, normal omentum tricultures, (adipocytes, fibroblasts, mesothelial cells), and tetra-cultures of AOCS-1, G164 and p53 mut FT318 cells. CCL2 secretion from G164 monocultures could not be detected.

5.4.2.9 Vascular endothelial growth factor (VEGF) release in aspirin treated HGSOC cultures

VEGF is a pro-angiogenic factor dysregulated in a wide range of cancers, including HGSOC. As a result, I was curious to examine whether aspirin would reduce the release of VEGF in 2D and 3D cultures of HGSOC. Overall, aspirin did not reduce the secretion of VEGF in 2D monocultures of HGSOC (Figure 5.13A-E) , although there appeared to be a marginal reduction in VEGF in monocultures of primary omental fibroblasts (Figure 5.13D). Similarly, there was a slight decrease in VEGF release in normal omental tricultures of HGSOC following 1mM aspirin treatment (Figure 5.13F). Furthermore, there may have been a potential reduction in VEGF concentration in tetra-cultures of G164 and p53 mut FT318 cells, but not tetra-cultures of AOCS-1 (Figure 5.13G-I). On the contrary, 1mM aspirin appeared to stimulate VEGF in monocultures of p53 mut FT318 cells (Figure 5.13C), and monocultures of primary omental mesothelial cells (Figure 5.13E). Overall, it is clear that aspirin did not inhibit the secretion of VEGF in 2D monocultures and 3D multi-cellular models of HGSOC.

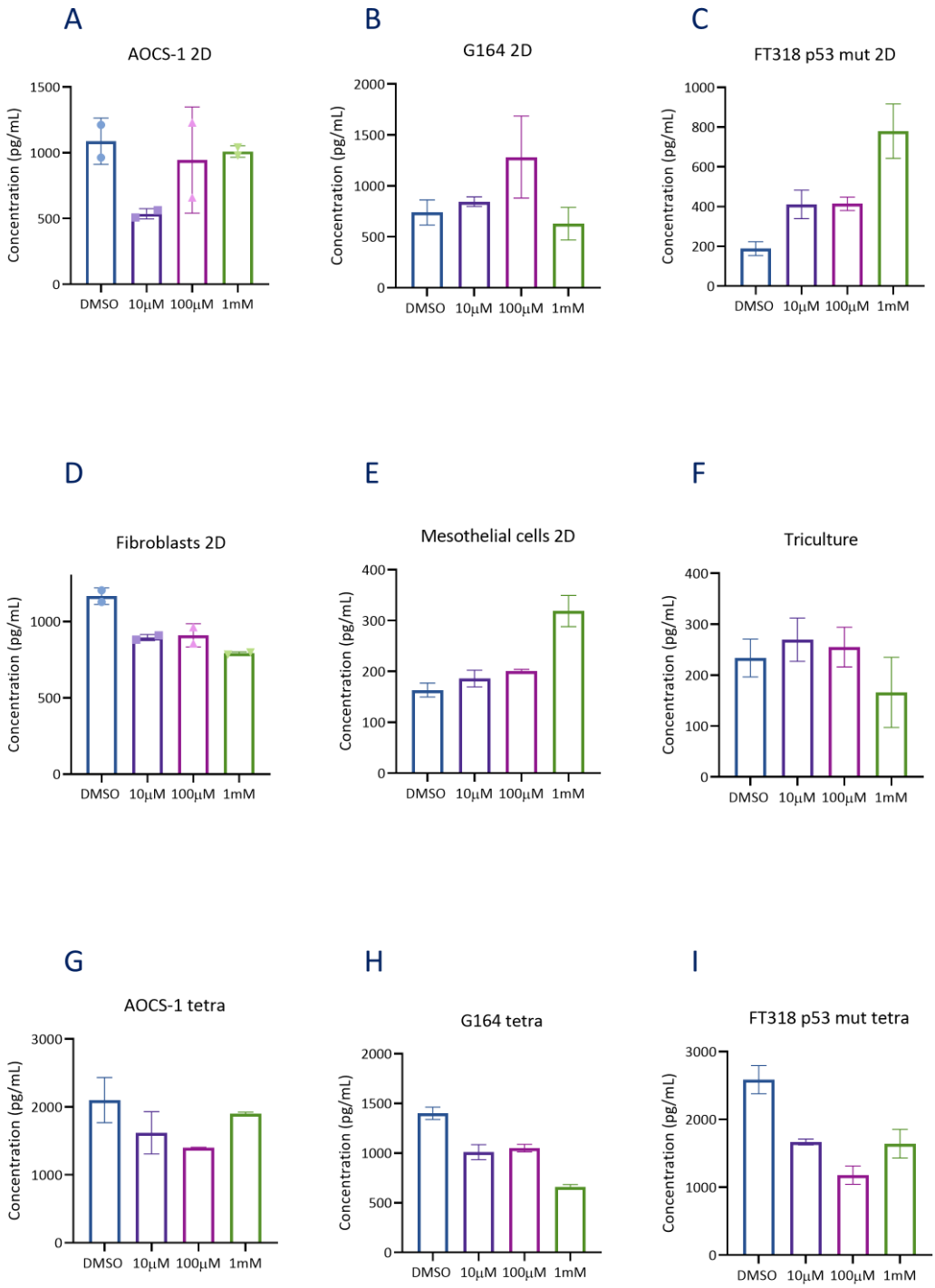


Figure 5.13. Vascular Endothelial Growth Factor (VEGF) release in aspirin-treated HGSOC cultures *in vitro*. Meso Scale Discovery multi-spot assay on the following cell types treated with aspirin for 48h: Malignant cells (AOCS-1, G164), pre-malignant cells (p53 mut FT318),rimary omental fibroblasts and mesothelial cells, normal omentum tricultures (adipocytes, fibroblasts, mesothelial cells), and tetra-cultures of AOCS-1, G164 and p53 mut FT318 cells.

5.4.2.10 Interleukin 8 (IL-8) release in aspirin-treated HGSOC cultures

IL-8 (CXCL8) is a member of the C-X-C chemokine family and is overexpressed in various solid tumours[203]. As described in the introduction (paragraph 1.5.4.2), IL-8 was first identified as a recruiter of neutrophils and other immune cells such as tumour associated macrophages (TAMs), to the cancer microenvironment[102]. IL-8 is now reported to play a crucial role in propagating angiogenesis, tumour growth, invasion, and metastatic spread[138]. High levels of IL-8 confer a poor prognosis, and has recently shown to be involved in resistance to immune-checkpoint inhibitors[132]. Furthermore, there are some studies, which have explored the links between IL-8, COX-2 and PGE_2 in cancer progression *in vitro.* For instance, Yu *et al.* showed that PGE_2 promoted tumour progression in the T84 human colon cancer cell line by inducing IL-8 gene expression[204]. Similarly, Takehara *et al* showed that treating gastric cells with exogenous PGE_2 stimulated IL-8 release[205]. They also confirmed that. PGE_2-mediated IL-8 release was inhibited when gastric cancer cells were treated with PGE_2 receptor (EP2/EP4) antagonists[205].

The effects of aspirin on IL-8 release from HGSOC cultures are shown in Figure 5.14. Aspirin seemed to reduce IL-8 secretion in tetra-culture models of AOCS-1, G164 and p53 mut FT318 (Figure 5.14G-I). There appeared to be a clear dose-dependent reduction of IL-8 in tetra-cultures of G164 and p53 mut FT318. In tetra-cultures of AOCS-1, 10µM aspirin seemed sufficient to reduce IL-8 release (Figure 5.14G). There was also an unexpected increase in IL-8 following 1mM aspirin in tetra-cultures of AOCS-1 (Figure 5.14G).

The effects of aspirin on IL-8 secretion from 2D monocultures were also equally interesting. 10µM and 100µM aspirin seemed to reduce IL-8 secretion from primary omental mesothelial cells (Figure 5.14E). Equally, 100µM aspirin appeared to decrease IL-8 release from primary omental fibroblasts (Figure 5.14D). Conversely, aspirin did not seem to modulate IL-8 release from 2D monocultures of AOCS-1 (Figure 5.14A), G164 (Figure 5.14B) and p53 mut FT318 (Figure 5.14C). Overall, aspirin may have reduced IL-8 release from multi-cellular models of HGSOC, and 2D monocultures of primary omental fibroblasts and mesothelial cells. These results were interesting compared to the 'negative data' with the other cytokines.

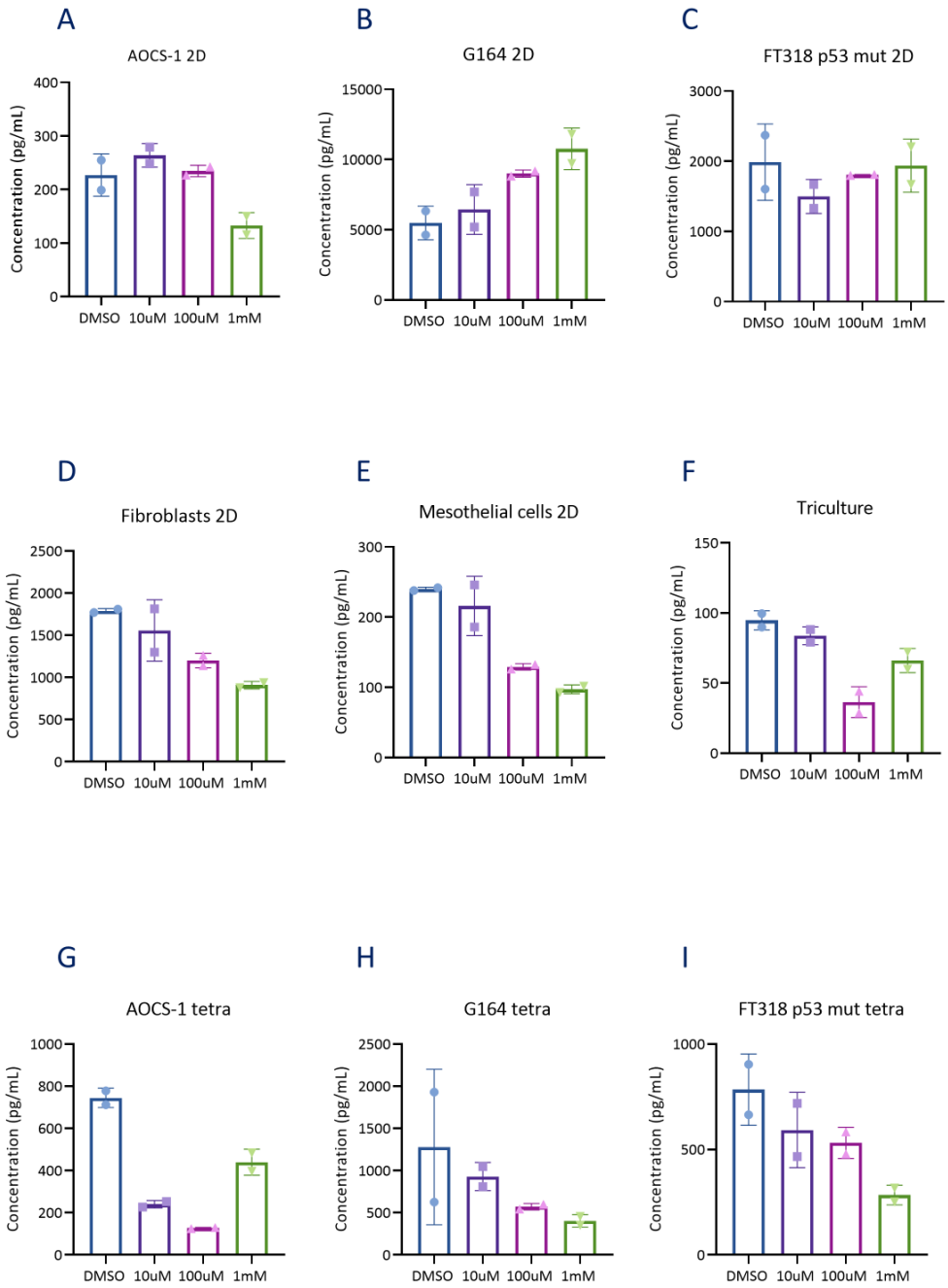


Figure 5.14. Interleukin 8 (IL-8) release in aspirin treated HGSOC cultures *in vitro*. IL-8 ELISA on the following cell types treated with aspirin for 48h in serum-free medium: Malignant cells (AOCS-1, G164), pre-malignant cells (p53 mut FT318), primary omental fibroblasts, and mesothelial cells, normal omentum tricultures (adipocytes, fibroblasts, mesothelial cells), and tetra-cultures of AOCS-1, G164 and p53 mut FT318 cells.

5.4.2.11 Summary of cytokine results

To summarise this section, aspirin did not decrease the secretion of IL-1β, IL-4, IL-6, IL-10, IL-13, TNFα, IFN-γ, CCL2 and VEGF in 2D monocultures of malignant cells (AOCS-1, G164), 2D monocultures of primary omental fibroblasts and mesothelial cells, and 3D multi-cellular models of AOCS-1, G164 and p53 mut FT318 cells. On the contrary, aspirin seemed to reduce IL-8 secretion in 3D multi-cellular models of HGSOC, 2D monocultures of primary omental fibroblasts and mesothelial cells. Aspirin did not reduce IL-8 release from 2D monocultures of AOCS-1, G164 and p53 mut FT318 cells. I have summarised this in Figure 5.15.

Overall, the MSD® multi-spot assay was an exploratory experiment used to identify potential cytokines that may have been affected by aspirin treatment. As the MSD® kit is expensive (approximately £3000), I could only perform technical replicates of two wells/parameter, so it was not possible to perform statistical analyses in this section. However, with the exception of IL-8, aspirin did not modulate the secretion of any other cytokine in the MSD panel. Therefore, it made sense to confirm my findings on aspirin and IL-8 release instead of repeating the MSD. I have shown this in the next section.

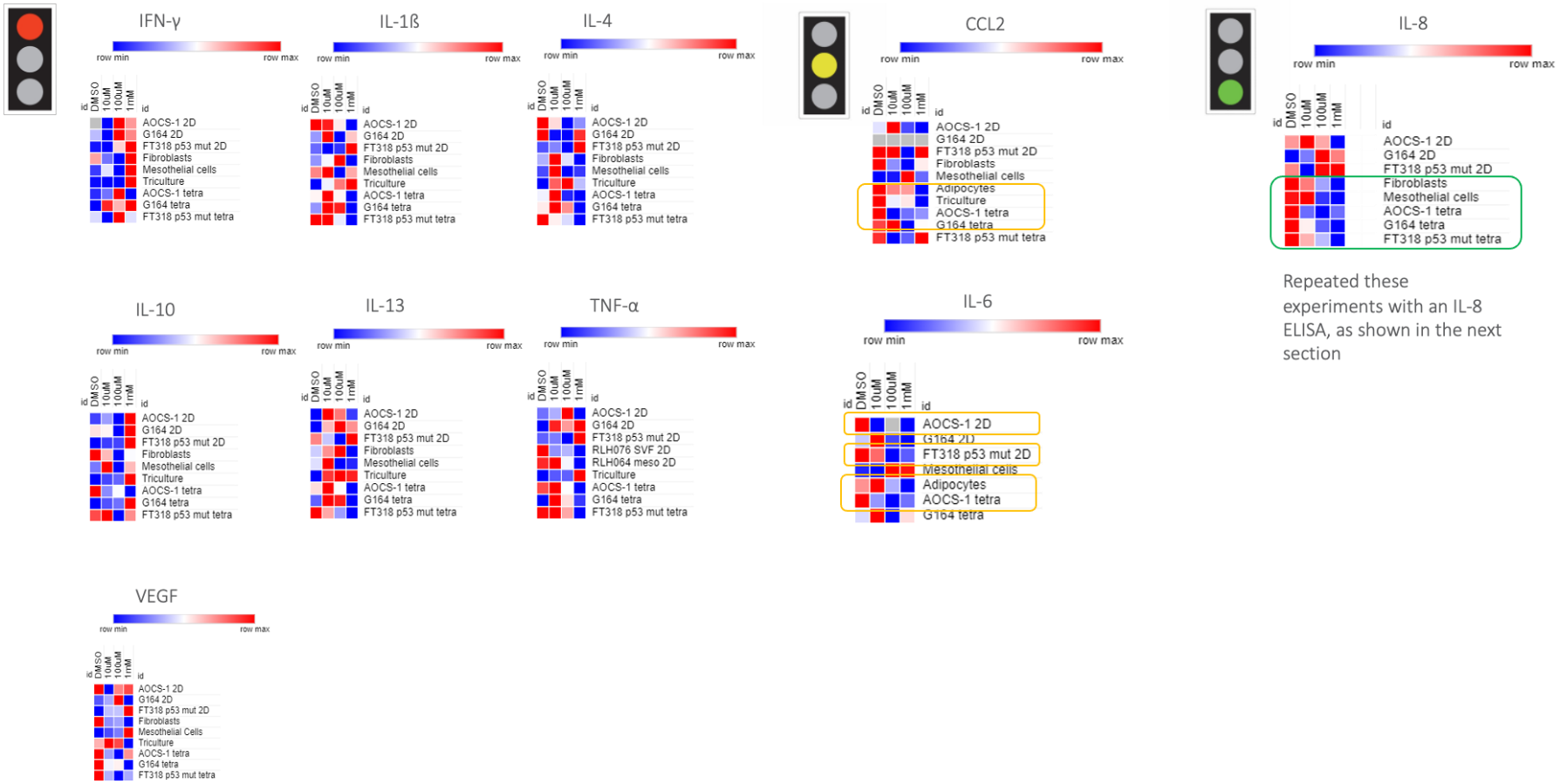


Figure 5.15. Effects of aspirin in modulating cytokine release in HGSOC cultures *in* vitro. This is a summary figure of section 5.4.2. It is evident that aspirin did not inhibit the secretion of IFN-γ, IL-1β, IL-4, IL-10, IL-13, TNF-α and VEGF in 2D and 3D HGSOC cultures *in vitro*. Aspirin may be potentially decreased CCL2, and IL-6 release in some cultures of HGSOC. However, aspirin clearly seemed to reduce the secretion of IL-8 in multi-cellular models of HGSOC, 2D monocultures of primary omental fibroblasts and mesothelial cells, as highlighted in green. Conversely, aspirin did not seem to reduce IL-8 secretion in 2D monocultures of AOCS-1, G164 and p53 mut FT318 cells.

5.4.2.12 Confirming IL-8 reduction in aspirin-treated HGSOC cultures *in vitro*

My data so far suggested that aspirin could inhibit IL-8 release in tetra-cultures of HGSOC, 2D monocultures of primary omental fibroblasts and mesothelial cells, but not 2D monocultures of malignant cells. Therefore, I repeated the above experiments, and measured IL-8 release from aspirin-treated HGSOC cultures using an ELISA.

Figure 5.16 shows the effects of aspirin in inhibiting IL-8 release from 2D and 3D HGSOC cultures. A significant reduction in IL-8 was observed in tetra-cultures of AOCS-1, G164 and p53 mut FT318 (Figure 5.16 G-I). In particular, a clear dose-dependent reduction was witnessed in tetra-cultures of G164 and p53 mut FT318 cells. A similar trend was seen in tetra-cultures of AOCS-1, except 1mM appears to slightly upregulate IL-8 secretion (figure 5.16 G). Moreover, there is a clear dose-dependent reduction of IL-8 in 2D cultures of primary omental fibroblasts and primary omental mesothelial cells (Figure 5.16 D and E). Whilst there was only a slight reduction in IL-8 following 10μM aspirin treatment, 100μM and 1mM aspirin induced a significant decrease in IL-8 release in both cell types. On the contrary, it is interesting to note that aspirin did not inhibit IL-8 secretion from 2D monocultures of HGSOC cell lines (AOCS-1, G164) and the pre-malignant p53-mut FT318 cell line.

In conclusion, aspirin inhibits IL-8 secretion in tetra-cultures of HGSOC, as well as 2D monocultures of fibroblasts and mesothelial cells, but not in 2D monocultures of malignant and pre-malignant cells.

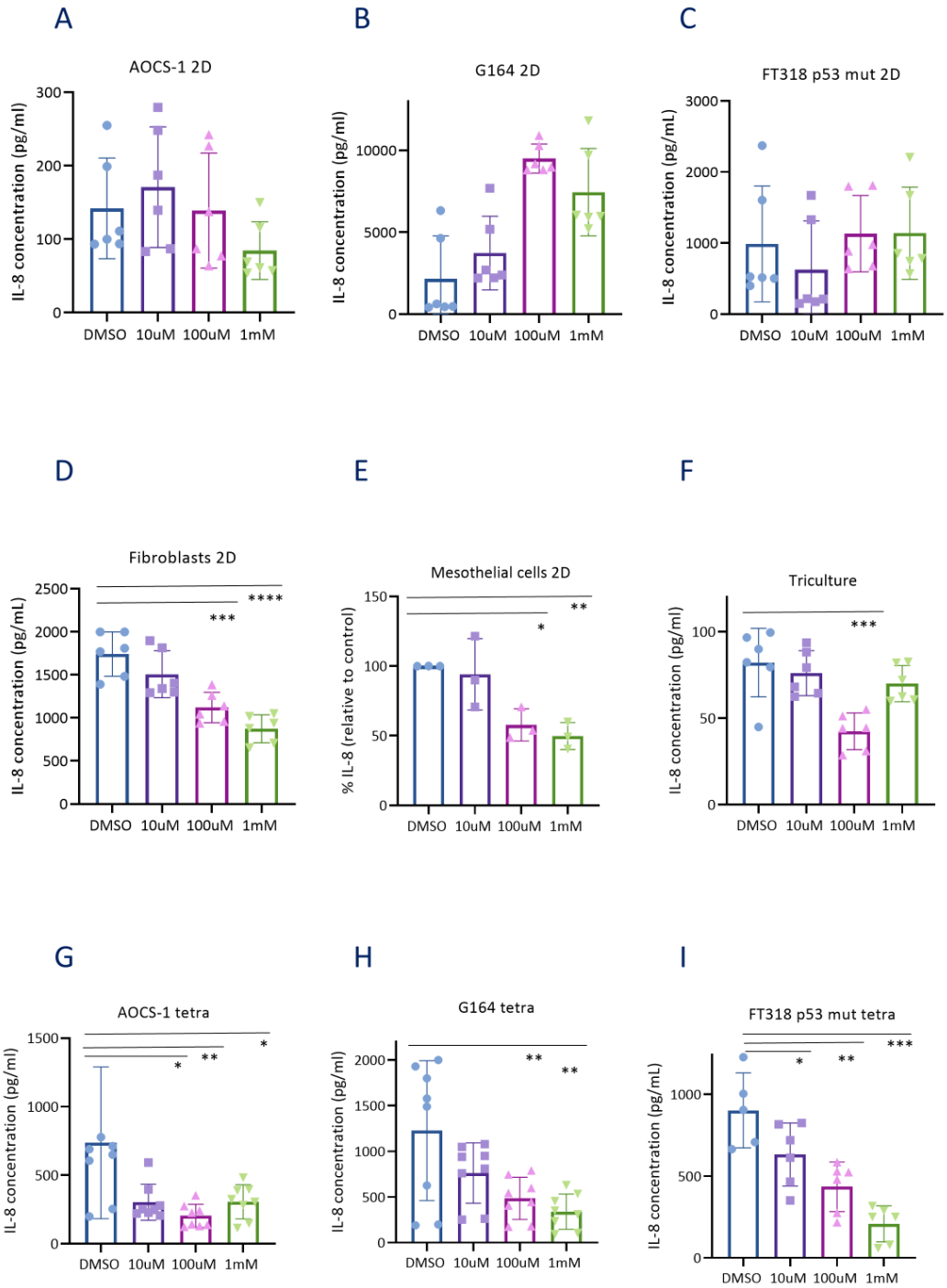


Figure 5.16. **Interleukin 8 (IL-8) release in aspirin treated HGSOC cultures *in vitro*.** IL-8 ELISA on the following cell types treated with aspirin for 48h in serum-free medium: Malignant cells (AOCS-1, G164), pre-malignant cells (p53 mut FT318), primary omental fibroblasts and mesothelial cells, normal omentum tricultures (adipocytes, fibroblasts, mesothelial cells), and tetra-cultures of AOCS-1, G164 and p53 mut FT318 cells. I did an n=3 in all 2D monocultures (A-E), and n=2 patient donors for 3D multi-cellular models (F-I). Statistics were performed using a one-way ANOVA using GraphPad prism. *Indicates p <0.05; ** p < 0.01; *** p<0.001 and ****p<0.0001.

5.5 Summary of results

I have summarised the key findings from this chapter below:

- Aspirin treatment did not reduce cell viability in tetra-cultures of AOCS-1, G164, G33 and p53 mut FT318 cells as measured by caspase 3.
- Aspirin did not reduce the secretion of IL-1β, IL-4, IL-6, IL-10, IL-13, TNFα, IFN-γ, CCL2 or VEGF in 2D monocultures of malignant cells (AOCS-1, G164), 2D monocultures of primary omental fibroblasts and mesothelial cells, and 3D tetra-culture models of AOCS-1, G164 and p53 mut FT318 cells.
- Aspirin reduced the secretion of IL-8 in tetra-cultures of AOCS-1, G164 and p53 mut FT318 and 2D monocultures of primary omental fibroblasts and primary omental mesothelial cells. However, aspirin did not inhibit IL-8 production in 2D monocultures of malignant (AOCS-1, G164) and pre-malignant cells (p53 mut FT318).

5.6 Discussion

The aim of this chapter was to examine the effects of aspirin on the TME in multi-cellular models of HGSOC *in vitro*.

My data show that aspirin inhibits IL-8 release in 3D tetra-culture models of HGSOC, 2D monocultures of primary omental fibroblasts, and 2D monocultures of primary omental mesothelial cells (Figure 5.16 D-H). Conversely, aspirin did not decrease IL-8 secretion from 2D monocultures of malignant and pre-malignant cells of HGSOC (Figure 5.16 A-C). I was intrigued by these results, as IL-8 is a well-established pro-tumourigenic chemokine[23]. Elevated levels of IL-8 are associated with a high tumour burden and confer a poorer prognosis[102,132]. In the 1980s, IL-8 was first discovered to promote the chemotaxis of neutrophils to the tumour site[23]. However, it is now known that IL-8 is central to promoting various processes of tumour progression, including malignant cell survival, invasion, and angiogenesis[131]. IL-8 is also recognised as an 'adipokine', and is secreted by multi-cellular models of HGSOC (unpublished data in our group). IL-8 secretion from multi-cellular models of HGSOC has also been examined by Estermann *et al.* in January 2023[180].

IL-8 binds to its cognate receptor CXCR1 (IL-8RA) or CXCR2 (IL-8RB) to activate a series of G-protein mediated downstream signalling cascades[135]. Luppi *et al.* demonstrated that IL-8 promoted malignant cell proliferation by transactivating EGFR, which led to the downstream activation of the MAPK pathway[137]. They also confirmed that treating malignant cells with inhibitors of EGFR or ERK1/2 abrogated IL-8 induced cell proliferation[137]. This finding goes in line with Venkatakrishnan *et al*[206]. Here, they suggested that IL-8 transactivates EGFR in ovarian cancer cells *in vitro*, which activates the MAPK pathway and increases malignant cell proliferation. Furthermore, they also showed that IL-8 induced a morphological change in malignant cell lines, suggesting that it might play a role in cell motility and migration. Moreover, MacManus *et al.* demonstrated that IL-8 signalling regulates cyclin D1 expression, which promoted malignant cell proliferation in prostate cancer cells[136]. They also showed that IL-8 regulates cell proliferation via PI3K and MAPK pathways, and treating the PC-3 cell line with inhibitors of MEK, PI3K and mTOR significantly reduced cell proliferation. Similarly, Sun *et al.* suggested that IL-8 upregulates integrin αvß3-

mediated malignant cell invasion through the PI3K/Akt pathway in hepatocellular carcinoma (HCC) *in vitro,* and IL-8/CXCR1/2/PI3K/AKT/integrinB3 signalling could provide new insights into tumour progression in HCC[207].

Equally, IL-8 is a well-known pro-angiogenic chemokine[203,138]. Angiogenesis is a key hallmark to cancer progression, as documented by Hannahan and Weinberg[208]. Almost 28 years ago, Streiter *et al.* showed that IL-8 played a role in directly recruiting endothelial cells and promoting angiogenesis[209]. Furthermore, Li *et al.* explored the role of the IL-8-CXCR1/CXCR2 pathway in mediating angiogenesis in human umbilical vascular endothelial cells (HUVEC) *in vitro*[210]. Treating HUVECs with recombinant IL-8 increased cell proliferation and reduced apoptosis. Conversely, inhibiting IL-8 in HUVECs significantly reduced proliferation. Moreover, incubating endothelial cells with IL-8 significantly upregulated MMP2 and MMP9, which promoted invasion and angiogenesis[210]. Similar findings were reported by Heidemann *et al,* where they suggested IL-8 binding to CXCR2 promotes angiogenesis in human intestinal microvascular cells[211]. Furthermore, Li *et al.* also published another paper investigating the autocrine role of IL-8 in regulating angiogenesis in HUVECs[212]. They demonstrated that treating HUVECs with anti-IL8, anti-CXCR1 and anti-CXCR2 antibodies reduced endothelial cell proliferation, MMP2 production and increased apoptosis[212]. Furthermore, anti-IL-8 reduced endothelial cell migration and the formation of capillary tubes.

IL-8 also plays a crucial role in modulating the TME[138]. It is well known that IL-8 preferentially recruits tumour associated macrophages (TAMs) and tumour associated neutrophils (TANs) to the tumour site[102,203]. Chen *et al.* demonstrated that IL-8 secreted by TAMs increased the expression of twist, an epithelial-to-mesenchymal transition (EMT) marker, and promoted motility in pancreatic ductal adenocarcinoma cells *in vitro*[213]. Similarly, Fu *et al.* showed that macrophages secreted IL-8 via the JAK2/STAT3/Snail signalling pathway, and induced EMT in HCC cells[214]. Moreover, Alfaro *et al.* examined the role of IL-8 in recruiting immunosuppressive myeloid cells into the TME[199]. Using peripheral blood samples from advanced cancer patients, they showed that IL-8 increased the formation of neutrophil extracellular traps (NETs) in granulocytic populations. Furthermore, Wu

et al. demonstrated that IL-8 secreted from oesophageal squamous cell carcinoma cells suppressed NKG2D and Nkp30 receptors on NK cells[215]. Consequently, this impaired NK activity, and favoured an immunosuppressive environment. Wu *et al.* also showed that IL-8 inhibited NK cell activity via STAT3, and confirmed that inhibiting STAT3 signalling restored NK cell activity[215]. In addition, Olivera *et al.* studied the pro-tumoural axis of IL-8, IL1-β, and TNF-α, and showed that inhibiting IL1-β or TNF-α reduced IL-8 release in plasma samples of patients with cancer[135].

Recently, there has been emerging evidence to suggest that IL-8 could be used as a biomarker to predict response and resistance to immunotherapy[132]. Schalper *et al.* performed a large-scale retrospective analysis investigating the relationship between IL-8 and response to immune-checkpoint inhibitor (ICI) therapy[216]. They examined four phase III trials with 1344 patients, and demonstrated that high levels of serum IL-8 confers a poorer outcome and response to immunotherapy. They also showed that high levels of *IL-8 g*ene expression positively correlated with an immune microenvironment rich in monocytes and neutrophils, with reduced T cell responses and IFN-Y gene signatures[216]. Similarly, Yuen *et al.* analysed plasma IL-8 and *IL-8* gene expression from 1445 patients with metastatic urothelial carcinoma and metastatic renal cell carcinoma, who were treated with atezolizumab (anti -PDL1 antibody)[200]. Here, they showed that high levels of IL-8 were linked with a reduced response to atezolizumab. On the other hand, low levels of IL-8 in patients with metastatic urothelial carcinoma conferred a better response to anti-PDL1 treatment. Furthermore, single cell RNA sequencing results revealed that elevated levels of *IL8* gene expression were linked to increased neutrophil infiltration, and a decrease in genes associated with antigen presentation[200].

Overall, IL-8 is a powerful, pro-carcinogenic chemokine that can directly influence malignant cell survival and also promote the generation of an immunosuppressive TME, as illustrated in Figure 5.17.

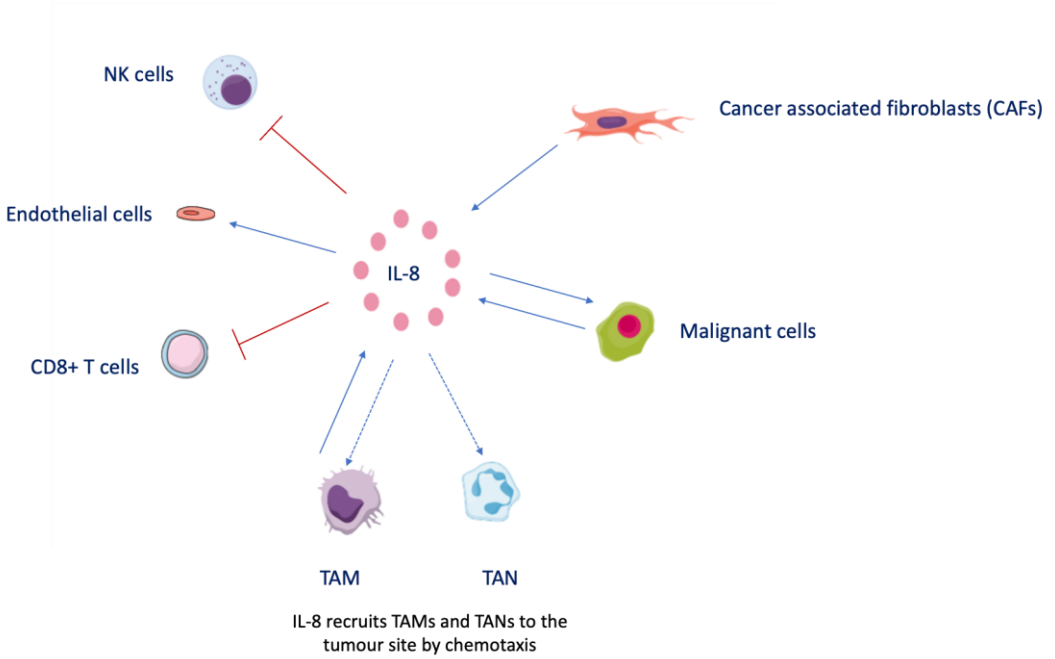


Figure 5.17. Pro-tumourigenic effects of IL-8. This is a simplified figure illustrating some of the pro-tumourigenic effects elicited by IL-8. IL-8 recruits tumour associated macrophages (TAMs) and tumour associated neutrophils (TANs) to the tumour site by chemotaxis. Both TAMs and TANs can directly repress CD8+ T cells and NK cells, resulting in immunosuppression. IL-8 produced by malignant cells and stromal cells, such as CAFs, can activate GCPR-induced signal transduction pathways, such as MAPK and PI3K/AKT cascades. This facilitates malignant cell proliferation, survival, and invasion. IL-8 produced by CAFs can also trigger epithelial-to-mesenchymal transition in malignant cells, which stimulates motility, invasion, and metastatic spread. Furthermore, IL-8 is a pro-angiogenic chemokine, and can directly promote angiogenesis by binding to CXCR1/CXCR2 receptors on endothelial cells. IL-8 can also induce the upregulation of MMP2 in endothelial cells, which results in matrix degradation and physically creates space for vascularisation.

Given the above-mentioned information, it is not surprising that there are some studies which have investigated the effects of aspirin in reducing IL-8 release from malignant cells and co-culture systems *in vitro.* As an example, Johnson *et al.* demonstrated that platelets pre-treated with aspirin inhibited IL-8 secretion from breast cancer cell lines, and consequently mitigated malignant cell invasion[48]. They also showed that treating cancer cell lines with recombinant IL-8 stimulated invasion. Furthermore, Chen *et al.* and Yao *et al.* highlighted that aspirin inhibited IL-8 secretion from co-culture systems of lung cancer cells and macrophages[217,218].

Equally, my results showed that aspirin did not inhibit the secretion of other pro-inflammatory cytokines (Figure 5.5-Figure 5.13), such as IL-6 (Figure 5.7). IL-6 plays a crucial role in propagating tumour progression, and is also extensively investigated in our laboratory[127,126]. I expected to see a reduction in IL-6 in some HGSOC cultures *in vitro*, especially there is some evidence suggesting that aspirin may reduce IL-6 release from malignant cells. For example, Antunes *et al.* examined the effects of

aspirin and celecoxib on cytokine release in cell lines of oral squamous cell carcinoma[219]. Here, they showed that 12 hours of aspirin treatment was enough to reduce IL-6 concentration. However, statistical significance was seen only after 3mM and 6mM aspirin[219]. As discussed in the previous chapter (Chapter 4, Table 17) and as emphasised recently by , Drew *et al.*, it is imperative that *in vitro* aspirin exposures are performed in the micromolar range[173]. Similarly, Kim *et al.* showed that aspirin reduced IL-6 and pSTAT3 expression in A172 glioblastoma cells, but this study was only explored using high doses of 5mM and 8mM aspirin[220]. Furthermore, Hseih and Wang demonstrated that aspirin reduced IL-6 and CCL2 secretion in co-cultures of RAW264.7 murine macrophages and 4T1 murine breast cancer cell lines[221]. Moreover, Brighenti *et al.* highlighted that aspirin reduced IL-6 in breast cancer cells, which resulted in a decreased expression of c-myc, a gene which is linked with malignant cell proliferation and survival[222]. There is also a paper suggesting that aspirin may inhibit IL1-ß signalling in non-small cell lung cancer cell lines[201]. Here, Wang *et al.* suggested that IL1-ß acted through COX-2-dependent-HIF signalling to repress micro-RNA-101 (miR101), which is a micro RNA associated with tumour suppression[201]. They showed that aspirin or celecoxib treatment upregulated IL1-ß induced miR101 expression[201].

In conclusion, it is interesting that aspirin specifically inhibits IL-8 in multi-cellular models of HGSOC, as well as 2D monocultures of primary omental fibroblasts and primary omental mesothelial cells. This effect could potentially contribute to its cancer-preventative actions by reducing IL-8 production by stromal cells in a tumour – or pre-tumour microenvironment. However, the mechanisms by which aspirin inhibits IL-8 in HGSOC are unclear. Therefore, I decided to explore how aspirin may inhibit IL-8 release in HGSOC cultures *in vitro*. I have explained my hypothesis in the next chapter.

6 Exploring the mechanisms by which aspirin inhibits interleukin-8 (IL-8) secretion

6.1 Background

My data in chapter 5 showed that aspirin reduced IL-8 release from 2D monocultures of primary omental fibroblasts, mesothelial cells, and 3D multi-cellular models of HGSOC. To proceed, I wanted to investigate the mechanisms by which aspirin decreases IL-8 secretion from all *in vitro* cultures of HGSOC.

I wondered whether aspirin blocked IL-8 production via PGE_2 signalling. As described in the Introduction (section 1.2.1), PGE_2 is a powerful lipid mediator associated with tumour progression[31,30,14,38]. Out of all prostanoids, PGE_2 is the most abundant prostaglandin found in tumours. PGE_2 binds to one of four G-protein coupled receptors, EP1, EP2, EP3 and EP4, to elicit its downstream effects[31,30,14,38]. The tumour-promoting and immunosuppressive actions of PGE_2 are mainly through the EP2 and EP4 receptors[14,38].

There is some literature which suggests that COX-2-induced PGE_2 stimulates IL-8 production via EP2/EP4 receptors. For instance, a study showed that PGE_2 enhanced IL-8 release via EP4 receptor signalling in human pulmonary microvascular cells[223]. Moreover, PGE_2-EP2/EP4 signalling augments IL-8 production in gastric cancer cells and colonic epithelial cells *in vitro*[224,205].

In view of this information, I questioned whether aspirin inhibits IL-8 via the COX-2-PGE_2-EP2/EP4 pathway in 2D monocultures of primary omental fibroblasts, mesothelial cells, and 3D multi-cellular models of HGSOC. I have illustrated my working hypothesis in Figure 6.1.

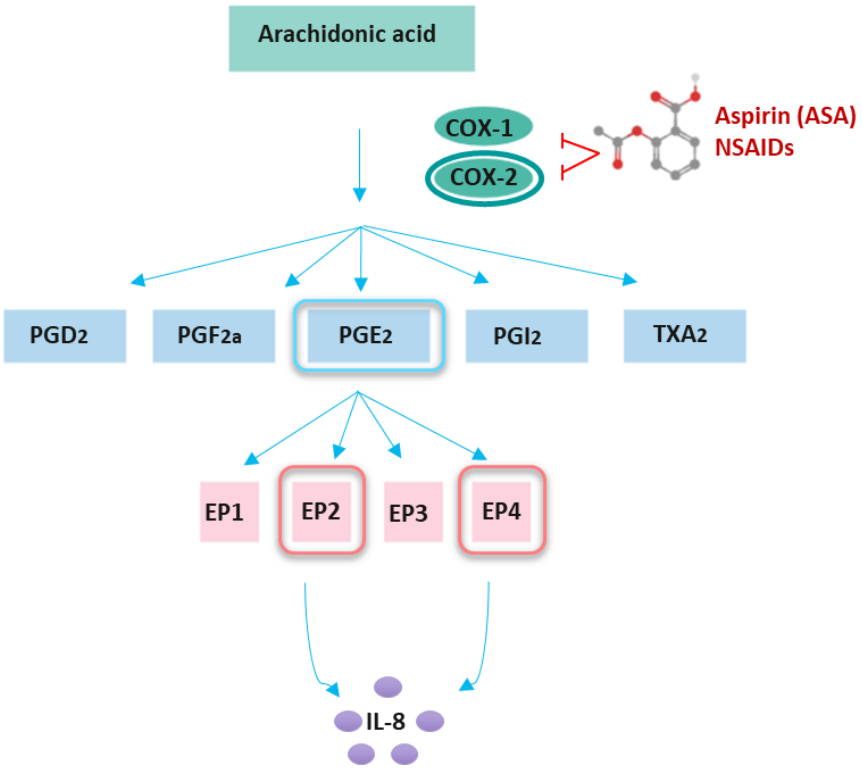


Figure 6.1. Schematic demonstrating one of the mechanisms by which aspirin could inhibit IL-8 secretion in HGSOC *in vitro*. Aspirin blocks the production of prostaglandins (PGs) by irreversibly inhibiting COX-1 and COX-2. Elevated levels of COX-2 and PGE_2 have been implicated in tumour progression. PGE_2 exerts its pro-tumourigenic effects by binding to one of four receptors: EP1, EP2, EP3 and EP4. Furthermore, previous literature suggests that PGE_2 can stimulate IL-8 production by binding to EP2 or EP4 receptors. Therefore, in view of this information, it was hypothesised that aspirin inhibits IL-8 production in HGSOC *in vitro* via the COX-2-PGE_2-EP2/EP4 pathway.

6.2 Effects of aspirin on the prostaglandin profile in primary omental fibroblasts and primary omental mesothelial cells

First, I wanted to know whether aspirin reduced the production of prostaglandins (PGs) in 2D monocultures of primary omental fibroblasts and mesothelial cells. This was done using liquid chromatography-mass spectrometry (LC-MS/MS). As described in the beginning of chapter 4, I performed LC-MS/MS analyses in collaboration with Professor Jesmond Dalli's group. I harvested the supernatants and cell pellets in methanol with deuterium labelled internal standards, and the Dalli lab carried out the sample extraction and analysis.

The effects of aspirin on the PG profile in mesothelial cells is shown in Figure 6.2A. Aspirin appeared to decrease PGE_2 in mesothelial cells, although there was some variability between the two patient donors. Conversely, there was no reduction in

PGD_2 following aspirin treatment. Mesothelial cells did not secrete PGF_{2a}. Aspirin seemed to increase TXB_2 (the more stable form of thromboxane) in mesothelial cells.

The PG profiling data from aspirin-treated fibroblasts is portrayed in Figure 6.2B. At a first glance, primary omental fibroblasts seemed to respond to aspirin treatment. In particular, aspirin reduced PGE_2 by more than 50% in fibroblasts. Aspirin also marginally decreased PGD_2, PGF_{2a} and TXB_2. Furthermore, fibroblasts secreted more PGE_2 (759pg/1,000,000 cells) compared to PGD_2 (49.2pg/1,000,000 cells), PGF_{2a} (0.73pg/1,000,000 cells) and TXB_2 (35.6pg/1,000,000 cells). In addition, fibroblasts produced higher amounts of PGE_2 compared to mesothelial cells, as well as 2D monocultures of malignant and pre-malignant cells (Figure 4.2-Figure 4.8). Overall, aspirin seemed to reduce PG production, especially PGE_2, in primary omental fibroblasts.

Even though COX enzymes catalyse the synthesis of all PGs, not just PGE_2, the production of PGs could be dependent on the cell type, as well as its surrounding microenvironment. For instance, mesothelial cells did not secrete PGF_{2a} to begin with, and fibroblasts secreted an abundance of PGE_2 compared to other PGs. Since both cell types were directly isolated from patients with cancer who had previously undergone chemotherapy, it is possible that the cells came from an environment associated with inflammation, which could partly help explain the high levels of PGE_2 in fibroblasts. Furthermore, the secretion of different PGs and response to aspirin could also be patient dependent.

Overall, my results suggested that IL-8 inhibition by aspirin in primary omental fibroblasts and mesothelial cells was likely to be associated with PGE_2 signalling.

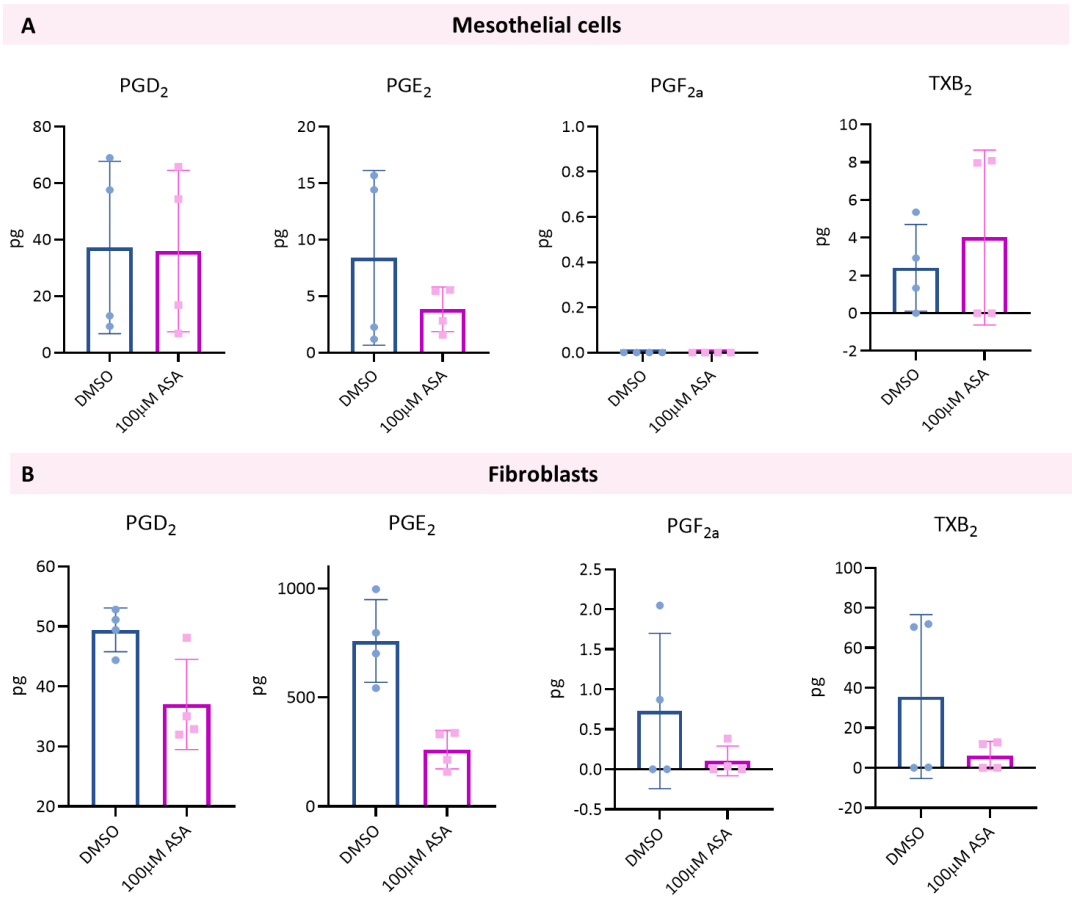


Figure 6.2. **Prostaglandin profiling of primary omental mesothelial cells (A) and primary omental fibroblasts (B) after 3h aspirin treatment.** Mesothelial cells and fibroblasts were treated with aspirin for 3h in serum-free medium. Samples were then harvested in methanol, with deuterium-labelled internal standards. PG profiling was carried out by the Dalli lab. This was done using liquid chromatography-mass spectrometry (LC-MS/MS). This experiment was n=2. Since LC-MS/MS is an expensive procedure (£145/sample), three biological replicates could not be performed. PGE_2, prostaglandin E2; PGD_2, prostaglandin D2; PGF_{2a}, prostaglandin F2a; TXB_2, thromboxane B2.

6.3 Effects of celecoxib, EP2 antagonist and EP4 antagonist on IL-8 release in primary omental fibroblasts and mesothelial cells

I then wondered whether selectively inhibiting COX-2, the EP2 receptor, or the EP4 receptor in HGSOC cultures would reduce IL-8 secretion. Therefore, I decided to treat fibroblasts and mesothelial cells with specific inhibitors of COX-2 (celecoxib), the EP2 receptor (PF 04418948) and the EP4 receptor (L-161,982) for 24 hours. I chose the doses of all three drugs based on previous literature[217,185,225,226]. However, I decided to also incorporate lower doses for my experiments in case the higher doses were too toxic (1µM of celecoxib, and 1nM of EP2/EP4 receptor antagonists). At endpoint,

I collected supernatants for cytokine analyses. I also performed cell counts to ensure that any potential decrease in IL-8 would not be due to a reduction in the number of cells after treatment.

6.3.1 Effects of celecoxib on IL-8 production in primary omental fibroblasts and mesothelial cells

The effects of celecoxib on IL-8 release in primary omental fibroblasts and mesothelial cells are illustrated in Figure 6.3A. Celecoxib significantly inhibited IL-8 secretion in 2D monocultures of primary omental fibroblasts and mesothelial cells. The mean concentration of IL-8 in primary omental fibroblasts was reduced significantly after 10µM and 100µM celecoxib. Similarly, 1µM, 10µM and 100µM celecoxib significantly reduced IL-8 secretion in mesothelial cells. Moreover, as shown in Figure 6.3B, 24h celecoxib treatment did not reduce cell viability. Overall, specifically inhibiting COX-2 with celecoxib substantially reduced IL-8 release in 2D monocultures of primary omental fibroblasts and mesothelial cells. Therefore, these results suggested that aspirin inhibited IL-8 production in fibroblasts and mesothelial cells via COX-2.

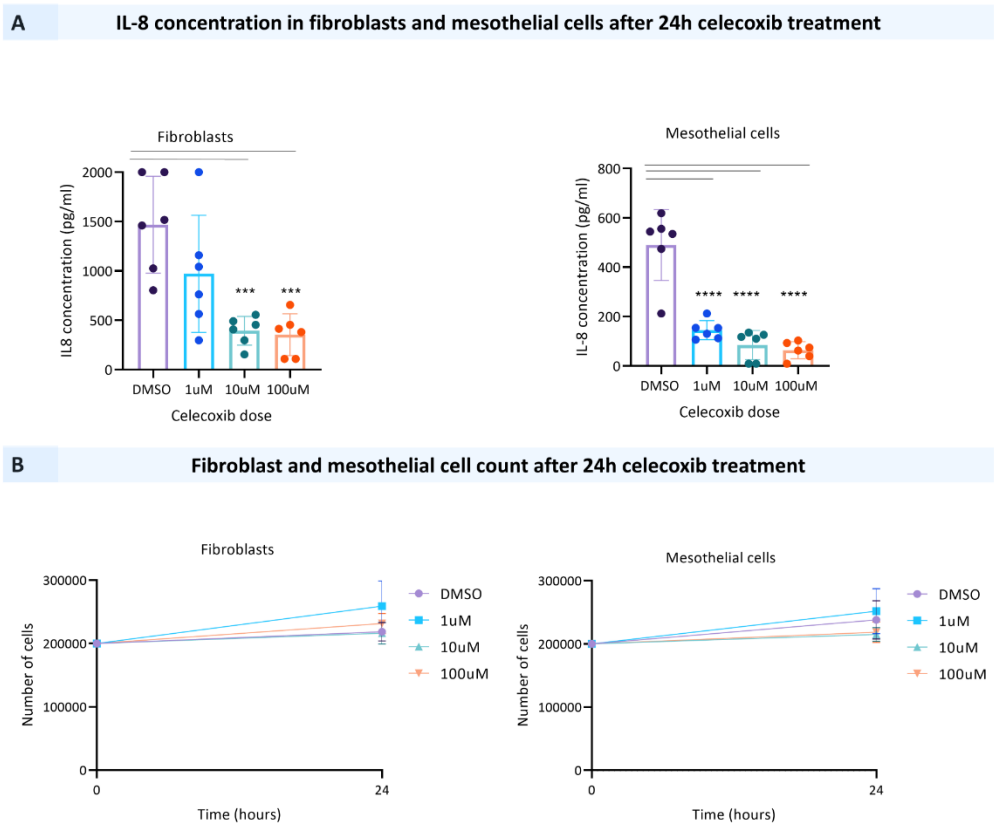


Figure 6.3. Interleukin 8 (IL-8) release in primary omental fibroblasts and mesothelial cells treated with celecoxib. (A) An IL-8 ELISA was performed on primary omental fibroblasts and mesothelial cells treated with celecoxib for 24h in serum-free medium. This experiment was performed using n=3 patient donors. Statistics were performed using a one-way ANOVA using GraphPad prism. *Indicates p>0.05; ** p>0.01; *** p<0.001 and ****p<0.0001. (B) Cell counts were performed after 24h celecoxib treatment to ensure that any changes in IL-8 release was not due to a reduced cell number following treatment.

6.3.2 Effects of PF-04418948 (EP2 receptor antagonist) on IL-8 production in primary omental fibroblasts and mesothelial cells

The effects of PF-04418948 on IL-8 release in primary omental fibroblasts and mesothelial cells are shown in Figure 6.4. PF-04418948 significantly inhibited the secretion of IL-8 in 2D monocultures of primary omental fibroblasts and mesothelial cells. In both cell types, 1nM of PF-04418948 was sufficient to substantially reduce IL-8 release. In fibroblasts, the mean IL-8 concentration was reduced from 1716pg/mL to 436pg/mL after 1nM of PF-04418948 treatment. Similarly, in mesothelial cells, the mean IL-8 concentration was reduced from 374pg/mL to 65.4pg/mL after 1nM treatment. Higher doses of PF-04418948 almost completely inhibited IL-8 release in mesothelial cells (15.06pg/mL after 100nM; 10.72pg/mL

after 200nM). A similar effect was also seen in fibroblasts (197.2pg/mL after 100nM; 210pg/mL after 200nM). Performing a cell count on both cell types after 24h treatment confirmed that PF-04418948 did not reduce cell growth or viability (Figure 6.4-B). Overall, my results so far suggested that IL-8 production in primary omental fibroblasts and mesothelial cells is linked with the COX-2-PGE_2-EP2 signalling pathway.

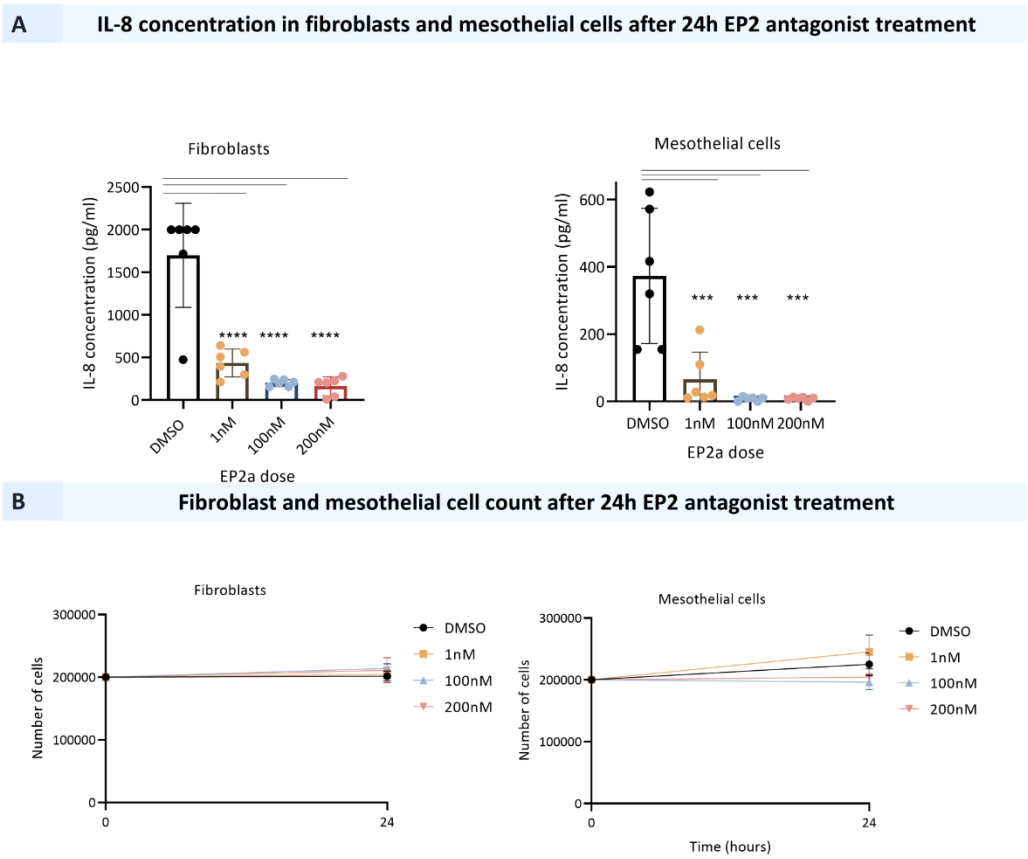


Figure 6.4.Interleukin 8 (IL-8) release in primary omental fibroblasts and mesothelial cells treated with an EP2 receptor antagonist (PF-04418948). (A) An IL-8 ELISA was performed on primary omental fibroblasts and mesothelial cells treated with PF-04418948 for 24h in serum-free medium. This experiment was performed using n=3 patient donors. Statistics were performed using a one-way ANOVA using GraphPad prism. *Indicates p>0.05; ** p>0.01; *** p<0.001 and ****p<0.0001. (B) Cell counts were performed after 24h PF-04418948 treatment to ensure that any changes in IL-8 release was not due to a reduced cell number following treatment.

6.3.3 Effects of L-161,982 (EP4 receptor antagonist) on IL-8 production in primary omental fibroblasts and mesothelial cells

I also treated my cells with L-161,982, an EP4 receptor antagonist. I initially saw that 100nM and 200nM of L-161982 treatment substantially reduced the number of viable fibroblasts. I chose the doses of L-161982 based on previous literature[185]. Therefore, I decided to only use a dose of 1nM to treat primary omental fibroblasts and mesothelial cells. As displayed in Figure 6.5, 1nM of L-161,982 significantly reduced IL-8 production in both cell types. Furthermore, this dose did not reduce cell viability, as demonstrated in Figure 6.5-B.

Overall, my data from Figure 6.3-Figure 6.5 suggested that aspirin inhibited IL-8 secretion from primary omental fibroblasts and mesothelial cells via the PGE_2-EP2/EP4 pathway.

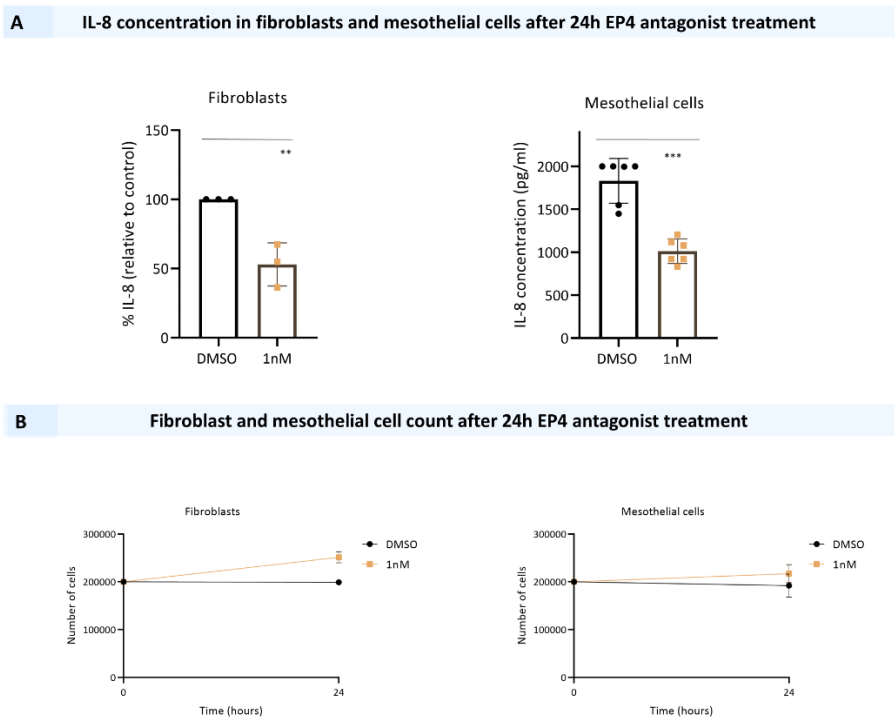


Figure 6.5. Interleukin 8 (IL-8) release in primary omental fibroblasts and mesothelial cells treated with an EP4 receptor antagonist, L-161982. (A) An IL-8 ELISA was performed on primary omental fibroblasts and mesothelial cells treated with L-161982 for 24h in serum-free medium. This experiment was performed using n=3 patient donors. Statistics were performed using a t-test using GraphPad prism. * Indicates p>0.05; ** p>0.01; and *** p<0.001. (B) Cell counts were performed after 24h L-161982 treatment to ensure that any changes in IL-8 release were not due to a reduced cell number following treatment.

6.3.4 Effects of celecoxib, PF-04418948, and L-161982 on IL-8 production in tetra-cultures of HGSOC

Next, I wondered whether celecoxib, PF-04418948 (EP2 receptor antagonist), or L-161,982 (EP4 receptor antagonist) inhibited IL-8 release from multi-cellular models of HGSOC. Therefore, I measured IL-8 levels after treating tetra-cultures of AOCS-1, G164 and p53mut FT318 with 1µM celecoxib, 1nM PF-04418948, or 1nM L-161,982 for 24 hours in serum-free medium. Due to limited tissue supply, I could only perform an n=1 on this experiment.

As depicted in Figure 6.6, celecoxib, PF-04418948, and L-161982 treatment seemed to decrease IL-8 secretion from tetra-cultures of AOCS-1. Celecoxib treatment reduced the mean concentration of IL-8 from 1770pg/mL to 472pg/mL. Similarly, PF-04418948 treatment reduced the mean IL-8 concentration to 878pg/mL. The strongest effect was seen after L-161982 treatment, where the mean IL-8 concentration was 396pg/mL. On the other hand, celecoxib, PF-04418948, and L-161982 treatment did not modulate IL-8 release in tetra-cultures of G164 or p53mut FT318 cells. Perhaps, the doses I used were too low to exert an effect on IL-8 release in multi-cellular models. It is therefore worth repeating this experiment using higher doses of celecoxib, PF-04418948 and L-161982.

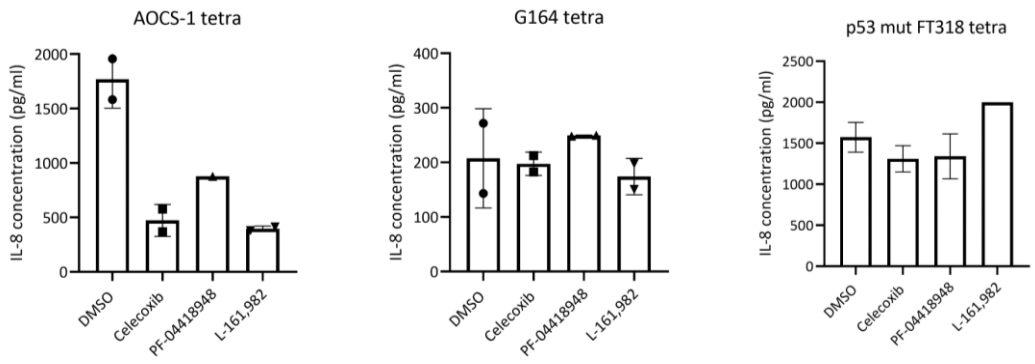


Figure 6.6. Interleukin 8 (IL-8) release in tetra-cultures of HGSOC treated with celecoxib, an EP2 receptor antagonist (PF-04418948) and an EP4 receptor antagonist (L-161982). An IL-8 ELISA was performed on tetra-cultures of AOCS-1, G164 and p53mut FT318 treated with 1µM celecoxib, 1nM PF-04418948 and 1nM L-161982 for 24h in serum-free medium. This experiment was a n=1 due to limited supply. Therefore, statistics could not be performed on this experiment.

6.4 Summary of results

I have summarised the key findings from this chapter below:

- Aspirin reduced PGE_2 in 2D monocultures of primary omental fibroblasts and primary omental mesothelial cells. Aspirin marginally reduced PGD_2, PGF_{2a} and TXB_2 in primary omental fibroblasts, but not in mesothelial cells.
- Celecoxib, the EP2 receptor antagonist (PF-04418948) and the EP4 receptor antagonist (L-161982) significantly reduced IL-8 secretion from 2D monocultures of primary omental fibroblasts and mesothelial cells.
- Celecoxib, PF-04418948 and L-161982 seemed to reduce IL-8 release from tetra-cultures of AOCS-1, but not tetra-cultures of G164 or p53mut FT318.
- Aspirin may reduce IL-8 secretion via the COX-2-PGE_2-EP2/EP4 pathway in 2D monocultures of primary omental fibroblasts and mesothelial cells.

6.5 Discussion

The aim of this chapter was to explore the possible mechanisms by which aspirin inhibits IL-8 secretion from *in vitro* cultures of HGSOC. To our knowledge, this is the first study that has examined how aspirin reduces IL-8 release in HGSOC.

I initially hypothesised that aspirin inhibits IL-8 secretion via the COX-2-PGE_2-EP2/EP4 pathway. Therefore, to confirm my working hypothesis, I first performed a prostaglandin (PG) profile on primary omental fibroblasts and mesothelial cells treated with aspirin. Aspirin reduced PGE_2 in both cell types. Aspirin marginally reduced PGD_2, PGF_{2a} and TXB_2 in fibroblasts, but not mesothelial cells. This suggested that IL-8 inhibition by aspirin in primary omental fibroblasts and mesothelial cells is likely to be mediated by PGE_2. IL-8 has previously been associated with PGE_2[205]. For example, Takehara *et al.* explored the link between COX-2, PGE_2 and IL-8 release in gastric cancer cell lines. They showed that treating gastric cancer cells with exogenous PGE_2 substantially elevated IL-8 levels[205]. Moreover, treating gastric cancer cells with EP2/EP4 receptor antagonists significantly inhibited IL-8 release[205]. Interestingly, IL-8 has also been associated with PGD_2-DP2 signalling[227]. However, my results showed a reduction in PGE_2 in both cell types compared to PGD_2, suggesting that IL-8 inhibition by aspirin in primary omental fibroblasts and mesothelial cells is likely to be via PGE_2.

I then measured IL-8 release after specifically inhibiting primary omental fibroblasts and mesothelial cells with celecoxib (COX-2 inhibitor), PF-04418948 (EP2 receptor antagonist) and L-161982 (EP4 receptor antagonist). Celecoxib significantly inhibited IL-8 secretion from 2D monocultures of primary omental fibroblasts and mesothelial cells. Interestingly, J.W. Chen *et al.* showed that 10μM celecoxib significantly suppressed *IL-8* mRNA expression in co-cultures of lung cancer cells and macrophages, and suggested that NSAIDs may in part exert their cancer-preventive effects by reducing the expression of inducible pro-inflammatory cytokines[217]. In future, an 'add back' experiment with PGE_2 would be interesting to confirm these results.

Similarly, treating primary omental fibroblasts and mesothelial cells with PF-04418948 and L-161982 significantly inhibited IL-8 secretion. Antagonists against the EP receptors are becoming increasingly popular, as the use of these agents block PGE_2-mediated pro-tumourigenic signalling, without risking the side effects associated with prolonged NSAID use[228]. Previously, Prof. Paola Patrignani's group showed that specifically antagonising the EP receptors (especially EP4) inhibited epithelial-to-mesenchymal transition in HT-29 cells *in vitro*[185]. They demonstrated that treating HT-29 with exogenous PGE_2 reduced E-cadherin levels, and increased mesenchymal markers, such as N-cadherin[185]. They then showed that pre-treating HT-29 with EP antagonists, including PF-04418948 and L-161982 upregulated E-cadherin levels[185]. In particular, L-161982 significantly increased E-cadherin levels, suggesting that targeting PGE_2 receptors could inhibit EMT *in vitro*[185]. More recently, in 2022, Prof. Raymond Dubois' group showed that inhibiting the COX-2-PGE_2-EP4 pathway increased CD8+T cell abundance and cytotoxicity, as well as macrophage phagocytosis against cancer cells[229]. Furthermore, drugs that simultaneously target both EP2 and EP4 receptors are being investigated in a clinical setting. One such example is TPST-1495, which is a dual EP2 and EP4 antagonist[230]. A phase 1 study (NCT04344795) is currently evaluating the safety and maximum tolerated dose of TPST-1495 as a monotherapy, or in combination with pembrolizumab, in patients with advanced solid tumours[230]. Overall, these studies showed that specifically blocking EP2/EP4 receptors could inhibit the pro-tumourigenic effects mediated by PGE_2.

Equally, treating multi-cellular models of HGSOC with celecoxib, PF-04418948 and L-161982 generated interesting results. Overall, my data suggested that all three drugs could inhibit IL-8 release in tetra-cultures of AOCS-1, but not tetra-cultures of G164 and p53mut FT318. Perhaps, the doses used were too low to significantly reduce IL-8 release in multi-cellular models of HGSOC. Moreover, it is well known that drug delivery is different in 2D monocultures compared to 3D multi-cellular models[167,171,166]. Ultimately, it is worth confirming my findings using higher doses of all three drugs.

In conclusion, this chapter suggests that aspirin inhibits IL-8 via the COX-2-PGE_2-EP2/EP4 pathway in 2D monocultures of primary omental fibroblasts and mesothelial cells. This effect could, in part, contribute towards its cancer-preventive actions.

7 Overall Discussion and Future Work

7.1 Background

Aspirin is a non-steroidal anti-inflammatory drug. It blocks the production of prostaglandins (PG) by inhibiting COX-1 and COX-2 activity. There is now significant evidence suggesting that aspirin may possess cancer-preventive properties. However, this is most notably documented in colorectal cancer (CRC)[231,16,21,22,56]. Currently, the recommended dose of aspirin is between 75-100mg[22,56]. Furthermore, the CAPP3 trial is exploring the optimal dose of aspirin for people with Lynch syndrome by providing participants with 100, 300 or 600mg of aspirin every day[58].

There is relatively limited evidence examining the actions of aspirin in ovarian cancer. Recently, Hurwitz, Townsend, Jordan *et al.* performed the largest meta-analysis to date on aspirin use and ovarian cancer risk, and demonstrated that aspirin reduces high-grade serous ovarian cancer (HGSOC) risk by 14%[150]. Nevertheless, there is no concrete research investigating the effects of aspirin in HGSOC *in vitro*, even though the pro-inflammatory ovarian TME is important in promoting HGSOC progression. Therefore, the overall aim of my PhD was to study the cancer-preventive effects of aspirin in HGSOC, using *in vitro* models. I did this by:

- Investigating the short-term and longer-term effects of aspirin on the growth and viability in HGSOC cells and stromal components of the TME (chapter 3)
- Examining the effects of aspirin on the lipid mediator profile on malignant cells and stromal components of the TME (chapters 4 and 6)
- Investigating the effects of aspirin in modulating the viability and pro-inflammatory cytokine profile in multi-cellular models of HGSOC (chapter 5)
- Investigating the mechanisms by which aspirin inhibits IL-8 secretion (chapter 6)

In this chapter, I will discuss the extent to which I answered the above aims. I will also address limitations of my project and propose future experiments to be carried out in view of my results.

7.2 Chapter 3- Investigating the short-term and longer-term effects of aspirin on malignant cell growth and viability

The first aim of my PhD was to assess the effects of aspirin on the growth and viability of HGSOC cells and the different components of the TME. I first interrogated existing RNA-sequencing data to confirm that the transcripts for COX-1, COX-2 and its downstream targets were expressed in AOCS-1 and G164 HGSOC cell lines. Both cell lines expressed *PTGS1, PTGS2* and downstream targets associated with PGE_2, suggesting that the COX-2-PGE_2 pathway was relevant in these two cell lines. This goes in line with previous evidence, which highlights the importance of COX-2 in ovarian tumours[232,233,234].

I then investigated the effects of aspirin on malignant cell growth and viability. I also tested the growth inhibitory effects of aspirin in 2D monocultures of primary omental fibroblasts and mesothelial cells, as they are important cell types in our multi-cellular model, which I used in chapter 5. Nevertheless, before examining the effects of aspirin in complex multi-cellular models, I wanted to first examine its actions on each cell type in 2D monocultures. I tested doses and time-points based on previous literature, which has studied the cancer-preventive effects of aspirin *in vitro* (Table 17). The lowest dose I used was 5μM, and the highest dose I used was 1mM. For later experiments (Figure 3.6- Figure 3.8), I decided to use doses of 100μM and 1mM, as I did not see any growth inhibitory effects using lower doses of aspirin. At the time, the then-available literature suggested 1mM was a low dose, as studies used aspirin doses as high as 20mM (Table 17, page 133) [196,235].

In brief, short-term aspirin treatment (≤72h) did not inhibit the growth of two HGSOC cell lines (AOCS-1 and G164), primary omental fibroblasts and mesothelial cells. Similarly, longer-term aspirin treatment (up to 7 days) did not inhibit HGSOC cell growth and viability. Therefore, I examined the growth inhibitory effects of aspirin in CRC cells as a positive control. This is because there is evidence in the literature which has shown that aspirin reduces cell viability in CRC cells[196,191,182]. However, my findings contradicted previous literature, as short-term and longer-term aspirin treatment did not inhibit malignant cell growth. At this point, I did not know that

serum obstructed the potentially low levels of PGs present in HGSOC and CRC cells. However, after discovering this in chapter 4, I confirmed that aspirin did not inhibit malignant cell growth, even in serum-free medium. Overall, it is clear from my data that aspirin did not inhibit malignant cell growth or viability, even when cells were challenged in a serum-free environment.

Nevertheless, there are additional controls I could have performed in this chapter. Firstly, I could have measured protein levels of COX-1 and COX-2 in 2D monocultures of malignant cells, fibroblasts, mesothelial cells, as well as 3D co-culture collagen gels and tetra-cultures of HGSOC. Secondly, it would have been useful to commence my experiments by performing serum growth curves, as this would have optimised the subsequent experiments I did (Figures 3.2-3.8). Thirdly, pre-treating HGSOC cultures *in vitro* with exogenous arachidonic acid would have determined the amount of PGs synthesised in my culture systems, as well as the functionality of aspirin. This could have possibly led to a different culture system being used. Fourthly, I could have tested whether HGSOC cultures *in vitro* were sensitive to exogenous PGE_2 as a pro-proliferative agent. Lastly, it would be worth treating HGSOC cells with a known cytostatic drug (e.g., rapamycin) to determine their sensitivity to anti-proliferative agents.

7.3 Chapter 4- Examining the effects of aspirin on the lipid mediator profile in malignant cells and stromal components of the TME

Given my results in chapter 3, I needed to confirm that I had prepared working solutions of aspirin correctly. Therefore, I performed a prostaglandin (PG) profile on malignant cells treated with aspirin.

This chapter led to one of the most crucial observations of my project. I deduced that aspirin reduced PG levels in some malignant cells (AOCS-1, G164 and HT-29) confirming that I had prepared aspirin solutions correctly for *in vitro* experiments. However, aspirin only reduced PG levels in serum-free medium. However, there was no time-dependent accumulation of PGs (e.g., HT-29 and G164, Figures 4.2 and 4.4). Perhaps, these cells stopped synthesising PGs after 3h treatment. Aspirin did not

reduce PGs in malignant cells cultured in serum-rich medium. Furthermore, performing a lipid mediator profile on aliquots of FBS and human serum validated that both types of sera contained an abundance of PGs, which could have hampered LC-MS/MS analyses from cells. The presence of serum could have masked the low levels of PGs present in HGSOC and CRC cell lines.

I then showed that previous literature from 1997-2021 carried out *in vitro* aspirin treatments in serum-rich medium (Table 17). Therefore, these studies used aspirin concentrations that were higher. For instance, a paper treated colorectal adenoma cell lines with 4mM aspirin, yet supplemented these cells with 20% FBS[198]. As reviewed previously, millimolar doses of aspirin may modulate other proteins independent to COX-1 and COX-2[236,9]. Therefore, it is not possible to know whether aspirin elicited its therapeutic effects in a COX-dependent or a COX-independent manner[236,9]. It was not until October 2021 that Professor Andrew Chan's group subjected organoids of colorectal adenomas with 50µM aspirin in a serum-free environment[173]. In the discussion section of this paper, they emphasised the importance of using *in vitro* doses of aspirin 'to those that remain clinically relevant (micromolar range)[173]'.

However, as discussed in chapter 4, it is not possible to directly compare aspirin treatments conducted in a serum-free environment to those that were performed in serum-rich medium. Serum contains proteins, such as albumin, which reduce the concentration of 'free drug' present, and therefore affect its bioavailability[237,238]. Proteins from serum, such as albumin, can also directly bind to metabolites of arachidonic acid, including prostanoids[239], which can affect their detection by mass spectrometry. Since performing *in vitro* aspirin exposures in serum-free medium ensures that there is more 'free drug' available, my findings cannot be directly compared to the majority of papers that treated cells with aspirin in serum rich medium. Ultimately, it is challenging to accurately recapitulate the pharmacokinetics of aspirin *in vitro*.

Nevertheless, performing the additional controls mentioned in section 7.2 may be able to provide a deeper insight into the functionality of aspirin in HGSOC cultures *in vitro*.

Overall, this chapter highlighted the complexity of using aspirin *in vitro*. Had Devall, Drew *et al.'s* study been published in 2019, I would have started my PhD by performing all aspirin treatments in serum-free medium [173]. This would have taken my project in a different direction, and I would have spent more time investigating findings from chapter 6. Nevertheless, performing experiments in serum-free medium does not recapitulate its bioavailability, but the method of treating cells with aspirin *in vitro* does not mimic oral dosing *in vivo.*

It is also important to recognise that I could not perform n=3 on my experiments. This is because LC-MS/MS is expensive (£145/sample). In future, it would be worth to repeat a lipid mediator profile on AOCS-1 and HT-29 cells, where the strongest effects of aspirin were seen. However, it is clear from my data that aspirin inhibits the production of PGs in some malignant cells of HGSOC and CRC in a serum-free environment, possibly because there is more 'free drug' available. Findings from this chapter influenced future experiments using multi-cellular models of HGSOC.

7.4 Chapter 5- Investigating the effects of aspirin in modulating the viability and pro-inflammatory cytokine profile in multi-cellular models of HGSOC

After having deduced the importance of performing aspirin exposures in serum-free medium, I examined the effects of aspirin in multi-cellular models of HGSOC. I decided to use the tetra-culture model previously developed by Malacrida *et al.*, as it mimics some of the early-stages of HGSOC disease[181]. First, I examined existing RNA-sequencing data of our tetra-culture models. *PTGS2* was significantly elevated in our tetra-culture model compared to 2D monocultures of G164 cells, highlighting the importance of using 3D multi-cellular models to address biological questions. Moreover, downstream targets associated with PGE_2, and its cognate receptors were expressed. Overall, it made sense to study the effects of aspirin using this model.

I showed that aspirin did not reduce cell viability in tetra-culture models of HGSOC, even in serum-free medium. I then wanted to examine the effects of aspirin on the TME. Initially, in view of Malacrida *et al.*'s paper, I was interested to explore whether pre-treating platelets with aspirin could mitigate malignant cell invasion and the production of the 'matrix index' molecules in our tetra-culture model[181]. However, since the tetra-cultures were not viable in serum-free medium for longer than 48h, I could not address this question. Therefore, I decided to examine other components of the TME which could be answered within 48h treatment courses, such as the secretome.

Targeting pro-inflammatory cytokines have been of interest over the last 40 years, as cytokines, such as IL-6 and IL-8, play an important role in generating a pro-tumourigenic TME[123,203,126,127]. Given aspirin's nature as an anti-inflammatory agent, I was keen to examine its potential in modulating the constitutive production of pro-inflammatory cytokines and chemokines from *in vitro* cultures of HGSOC.

This section of my thesis led to the most interesting finding of my PhD. I found that aspirin significantly and specifically inhibited the secretion of IL-8 in 2D monocultures of primary omental fibroblasts, mesothelial cells, and tetra-culture models of AOCS-1, G164 and p53mut FT318 cells. However, aspirin did not inhibit IL-8 secretion from 2D monocultures of AOCS-1, G164 and p53mut FT318 cells. Therefore, I wondered whether aspirin could exert some of its cancer-preventive effects by targeting IL-8 secretion from the stroma. On the contrary, aspirin did not modulate the secretion of other pro-inflammatory cytokines, such as TNF-α and IL-1β. This was surprising, especially in light of recent findings, whereby IL-8, TNF-α and IL-1β were demonstrated to be in a 'pro-tumourigenic loop'[135]. I also thought IL-6 levels might decrease following aspirin treatment, especially because previous papers showed that aspirin inhibited IL-6 release *in vitro*[219,220,221,222]. However, the aspirin doses used in these studies were clinically irrelevant[173].

Ultimately, I was intrigued that aspirin specifically reduced the secretion of IL-8 from *in vitro* multi-cellular HGSOC culture models. IL-8 is a well-documented chemokine known to stimulate a pro-tumourigenic TME[23,102,132,180]. Historically, IL-8 has been

linked with neutrophil and macrophage chemotaxis to the tumour site[131]. However, it is now also associated with angiogenesis, epithelial-to-mesenchymal transition (EMT), malignant cell invasion and migration[206,207,209,218,199]. Furthermore, two recent publications in *Nature* have suggested incorporating IL-8 as a biomarker to predict response to immune checkpoint inhibitors[216,200].

There are some limitations of this chapter, which must be considered for future work. For instance, it is important to test the effects of aspirin on IL-8 release using more patient donors of fibroblasts and mesothelial cells. Whilst I performed an n=3 experiment, factors such as prior chemotherapy and tissue stiffness can change the morphology of the cells (especially fibroblasts) and may affect response to aspirin treatment. In future, it is important to categorise patient donors based on treatment status and obtain at least n=6/7 to confirm my above findings. Furthermore, the tetra-culture model used in this project currently lacks immune cells, such as monocytes/macrophages. This is currently being developed by colleagues in the lab, making it a penta-culture model. In future, I would like to examine the effects of aspirin on IL-8 release in penta-culture models of HGSOC.

Overall, this chapter showed that aspirin inhibits the secretion of IL-8 in multi-cellular models of HGSOC as well as 2D monocultures of fibroblasts and mesothelial cells. This could potentially contribute towards its cancer-preventive effects.

7.5 Chapter 6- Investigating the mechanisms by which aspirin may reduce IL-8 secretion

For the last section of my project, I wanted to explore the mechanisms by which aspirin reduced IL-8 release from *in vitro* cultures of HGSOC. This chapter provided a potential mechanism by which aspirin inhibits IL-8 secretion from 2D monocultures of primary omental fibroblasts, mesothelial cells, and 3D multi-cellular models of HGSOC.

I hypothesised that aspirin inhibits IL-8 via COX-2-PGE_2-EP2/EP4 signalling. This is because PGE_2 is the most abundant prostaglandin implicated in malignancies[31,30,228,35]. PGE_2 can directly exert its effects on malignant cells, or favour the generation of a pro-tumourigenic, immunosuppressive TME[35,37,38,39]. It does so

by typically binding to EP2 and EP4 G-protein coupled receptors[229,223]. Moreover, PGE_2 has also shown to promote IL-8 release *in vitro* via EP2/EP4 signalling in gastric cancer cells[205]. However, I did not disregard the possibility of aspirin inhibiting IL-8 via other prostaglandins, such as PGD_2[227]. Therefore, in the first instance, I performed a lipid mediator profile on aspirin-treated fibroblasts and mesothelial cells. These results showed that aspirin reduced PGE_2 in both cell types. Aspirin marginally reduced PGD_2, PGF_{2a} and TXB_2 in primary omental fibroblasts, but did not modulate the levels of these lipid mediators in mesothelial cells. Consequently, my results suggested that aspirin is likely to inhibit IL-8 release in HGSOC by acting on PGE_2.

I then wanted to see whether specifically inhibiting COX-2, EP2 and EP4 would reduce IL-8 in fibroblasts and mesothelial cells. To do this, I treated cells with a COX-2 specific inhibitor (celecoxib), an EP2 receptor antagonist (PF-04418948) and an EP4 receptor antagonist (L-161982). Overall, this chapter clearly demonstrated that all three drugs significantly reduced IL-8 secretion from primary omental fibroblasts and mesothelial cells. This suggested that IL-8 production is likely to be mediated by COX-2-PGE_2-EP2/EP4 signalling, and aspirin could inhibit IL-8 via this pathway. This also goes in line with previous studies, showing that inhibiting COX-2 or EP2/EP4 receptors inhibited IL-8 *in vitro*[205,217].

I also wanted to know whether treating tetra-culture models of HGSOC with all three drugs would reduce IL-8 release. Due to limited tissue supply, I could only perform an n=1. My data suggested that all three drugs reduced IL-8 in tetra-cultures of AOCS-1, but not tetra-cultures of G164 or p53mut FT318. Perhaps, the doses I used were too low to exert an effect in multi-cellular models of HGSOC. It is also well-known that drug delivery is different when cells are cultured in 3D models compared to 2D monolayers[167,174,166]. Alternatively, the malignant/-pre-malignant cell line incorporated in the model could alter response to the drugs. However, before exploring this possibility, I propose repeating this experiment with higher doses of all three drugs.

I would like to also highlight some additional control experiments required to further validate my findings. Firstly, I would like to measure IL-8 release in fibroblasts and

mesothelial cells treated with exogenous PGE_2. Secondly, it may also be worth treating fibroblasts and mesothelial cells with a dual antagonist of the EP2 and EP4 receptor, such as TPST-1495[230]. Thirdly, a combination treatment of celecoxib and PF-04418948, or celecoxib and L-161982 may further confirm my current data.

Overall, to summarise, this chapter suggested that aspirin inhibits IL-8 in primary omental fibroblasts and mesothelial cells via COX-2-PGE_2-EP2/EP4 signalling, and this could partially contribute towards its cancer-preventive effects. I have illustrated this in Figure 7.1.

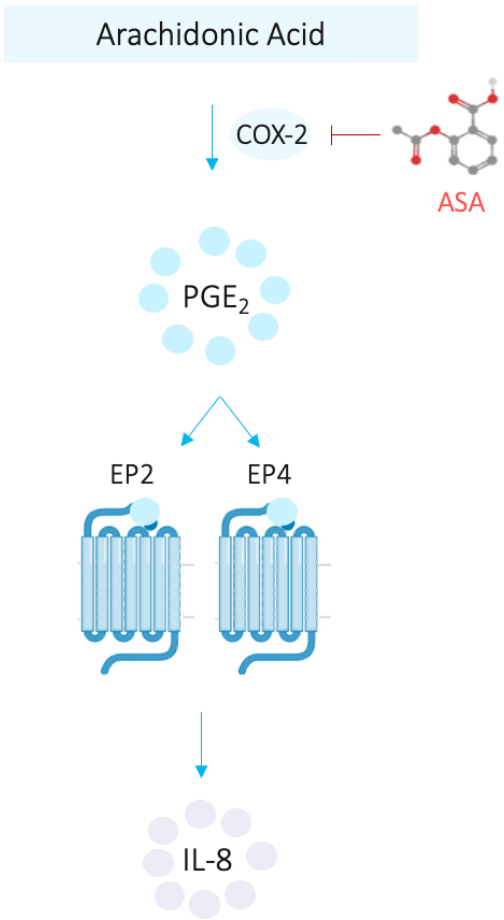


Figure 7.1. Potential mechanisms by which aspirin reduces IL-8 secretion from primary omental fibroblasts and mesothelial cells. Aspirin may reduce IL-8 secretion by inhibiting the COX-2-PGE_2-EP2/EP4 pathway in fibroblasts and mesothelial cells. This effect could partially contribute towards its cancer preventive effects.

7.6 Overall contributions to the field

To our best knowledge, this is the first study which has investigated the effects of aspirin in HGSOC using *in vitro* models. The key findings of my thesis can be summarised as follows:

- Clinically meaningful doses of aspirin do not inhibit malignant cell growth and viability in 2D monocultures and 3D multi-cellular models of HGSOC (chapters 3 and 5).
- It is imperative to perform *in vitro* aspirin treatments in serum-free medium and adhere to the micromolar range. However, it is challenging to truly recapitulate the pharmacokinetics of aspirin *in vitro* (chapter 4).
- Aspirin inhibits the secretion of IL-8, a pro-tumourigenic chemokine, in multi-cellular models of HGSOC, 2D monocultures of primary omental fibroblasts and mesothelial cells, but not 2D monocultures of malignant and pre-malignant cells (chapter 5).
- Aspirin inhibits IL-8 secretion in primary omental fibroblasts and mesothelial cells via the COX-2-PGE_2-EP2/EP4 signalling pathway. This could partially contribute towards its cancer preventive actions (chapter 6).

7.7 Future work

I would now like to pursue my findings from chapter 6 more detail, as I have deduced a possible mechanism by which aspirin inhibits IL-8 release from primary omental fibroblasts and mesothelial cells. I am interested to explore the following questions:

1. **How does aspirin inhibit IL-8 release in multi-cellular models of HGSOC?**

   Before exploring alternative hypotheses, I would like to confirm my initial findings using higher doses of celecoxib, PF-04418948 and L-161982.

2. **Does aspirin reduce IL-8 secretion in plasma samples of patients treated with aspirin?**

   It would be interesting to perform a whole-wide secretome analysis on plasma samples from patients treated with aspirin, to see whether our findings are translatable *in vivo*. Perhaps, aspirin may modulate the secretion of other cytokines too, such as IL-6[240,241,242].

3. **How is IL-8 release regulated when platelets pre-treated with aspirin are incorporated to our tetra-culture model?**

   Previous work shows that platelets promote tumour progression in tetra-culture models of HGSOC[181]. Since aspirin is an anti-platelet drug, I am interested to treat tetra-cultures+ platelets with aspirin, and measure IL-8 release after treatment.

4. **How is IL-8 release regulated in penta-culture models of HGSOC (addition of monocytes/macrophages to our tetra-culture models)?**

   Since immune cells are currently lacking in the multi-cellular model I used, I would like to expose our penta-culture models to aspirin, and measure IL-8 release after treatment.

5. **Can aspirin help improve response to immune-checkpoint inhibitors by reducing IL-8 release?**

   There are two ongoing clinical trials examining the use of aspirin in improving response to immune checkpoint inhibitor therapy. IMpALA, which is a phase II

clinical trial, is examining the use of aspirin to improve response to avelumab in patients with triple negative breast cancer (NCT04188119). Similarly, another study is investigating whether anti-platelet therapy (aspirin /clopidogrel) combined with pembrolizumab (anti-PD-1 immunotherapy) can improve response in patients with recurrent or metastatic squamous cell carcinoma of the head and neck (NCT03245489). It would also be interesting to examine plasma levels of IL-8 in these patients. Furthermore, given that elevated levels of IL-8 confer a poorer response to immune checkpoint inhibitors[132], it would be worth exploring whether aspirin could improve response to immunotherapy in HGSOC.

6. **What are the cancer-preventive effects of aspirin in mouse models of HGSOC?**

I would also like to investigate the cancer-preventive effects of aspirin murine models of HGSOC, which have previously been established by colleagues in our group[161,147]. These models accurately mimic the ovarian TME, and would be a useful system to improve our understanding on the cancer-preventive effects of aspirin in HGSOC. Ultimately, a combination of human 3D *in vitro* models and murine models is required to answer biological research questions.

8 References

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