

Investigating osimertinib resistance in lung cancer using multi-omics and cell biology

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Abstract

Non-small-cell lung cancer (NSCLC) accounts for 80 % of lung cancer cases. Epidermal growth factor receptor mutations (EGFRm) occur in approximately 15 % and 40 % of NSCLC in Western and Asian populations, respectively. Current treatment targets EGFRm with tyrosine kinase inhibitors (TKIs). Resistance to these TKIs however is inevitable. Osimertinib is a 3rd generation EGFR-TKI now used as a first line treatment in the advanced/metastatic setting, exhibiting an average progression free survival of 18.9 months. Dysregulation of metabolism has been suggested to play a role in development of drug resistance. In this thesis, we investigate how aberrant metabolism contributes to the development of osimertinib resistance (OR) using pharmacologically-induced and genetically-modified (GM) resistance models, measured through various -omics analyses; transcriptomics, proteomics, lipidomics and metabolomics. In both pharmacologically-induced and GM OR models, we found alterations in processes relating to metabolism, such as dysregulated sphingolipid metabolism, increased *de novo* lipogenesis, and links to mitochondrial dysfunction, lipid peroxidation and ferroptosis. In pharmacologically-induced models, we identified that OR lines reduce the free pool of ceramides in favour of complex glycosphingolipids. We went on to show that this avoided ceramide-mediated apoptosis via caspase 3 activation. Importantly, when we combined osimertinib with an inhibitor (D-PDMP) of the key enzyme (GCS) responsible for the conversion of ceramide to glycosphingolipids, the pool of free ceramides was increased and glycosphingolipids decreased. This in turn increased activation of caspase-3 and thus restored apoptosis to OR cells. Here, we identified a novel potential therapeutic target for acquired OR, that when inhibited in combination with osimertinib, sensitivity to osimertinib was restored.

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Publications

Dysregulated Metabolism in EGFR-TKI Drug Resistant Non-Small-Cell Lung Cancer: A Systematic Review

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The Mycobacterium lipid transporter MmpL3 is dimeric in detergent solution, SMALPs and reconstituted nanodiscs

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RAS/PI3K pathway mutations sensitise epithelial ovarian cancer cells to a PARP/NAMPT inhibitor combination (*Preprint*)

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Chapter 1 – Introduction

1. Chapter 1 - Introduction

1.1. An introduction to lung cancer

Lung cancer is the second most diagnosed cancer world-wide(1) and accounts for 13 % of new cancer cases. It is currently the third most common cancer in the UK as of 2018(2), and the second most common cancer worldwide as of 2022(3). Global lung cancer mortality in 2020 was 1,796,144 deaths across both genders, with over 2 million new cases of lung cancer detected in the same year(3). The high death rate attributed to lung cancer is most often due to diagnosis occurring when the cancer is in an advanced stage, making an early and accurate diagnosis crucial for long term survival of patients(4). The five year survival of patients diagnosed with stage I lung cancer is over ten times higher than those diagnosed with stage IV, highlighting the importance of early detection(5). Early screening for those in high risk categories, such as smokers and those who work in potentially toxic environments, is key to identifying malignancies in early stages and to identify potential biomarkers to personalise treatment, increasing chances of treatment efficacy and survival(4). There are two major subgroups of lung cancer; small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC)(4). SCLC accounts for a low proportion of total lung cancer cases (15 %)(6) and is characterised by early metastatic ability. Treatment of SCLC is often radiation and chemotherapy with little use for surgical resection due to the fast nature of the cancer's ability to spread via the lymph nodes and blood vessels(6).

1.1.1. Non-small-cell lung cancer (NSCLC)

NSCLC is a lethal and difficult to treat cancer, with 16 % morbidity and 18.4 % mortality(7). NSCLC makes up approximately 85 % of all lung cancer cases, and consists of various histological subtypes, the most common being lung adenocarcinoma (LUAD) comprising 50-60 % of cases and lung squamous cell carcinoma (LUSC), comprising around 20-30 %(8), with the remainder of cases being large cell carcinoma and other subtypes (10-20 %)(9). Within each of these subtypes, there are specific mutations that occur. Epidermal growth factor receptor mutations (EGFRm) are the most common mutation in all lung cancers, accounting for around 50 % in East Asians and 15 % in Caucasians(10). Other common mutations are found within KRAS, MET, HER2, BRAF, ROS1 and RET(9). The prevalence of certain mutations

is dependent on several factors including patient ethnicity, stage of cancer and the therapeutic strategy used for treatment.

There are several stages to NSCLC; Stages 0, I, II, III and IV. As the earliest stage, stage 0 denotes patients who have lesions in the lining layers of the lungs that have not yet invaded deeper tissue(11). Stages I and II are known as early stage NSCLC, and are often treated with surgery followed by adjuvant systemic therapy such as cisplatin chemotherapy(5). Stage III NSCLC, or locally advanced NSCLC (LA-NSCLC), is the most common stage at which patients are diagnosed and is when tumours have metastasized to the lymph nodes or the tumours are large enough to involve local lymph nodes. Treatment at this stage is patient dependent due to the complexity of the tumour burden and symptoms individuals face(12). The goal of treatment in stage III NSCLC is often to prolong survival as opposed to performing curative measures. Here, driving mutations of this stage, such as those mentioned above, will be elucidated to give a more specific form of treatment. Whilst this is a brief overview of the staging of NSCLC, each of these stages can also be divided into further categories, highlighting the complex nature of this disease.

1.1.2. Current treatment landscape for NSCLC

Currently, the treatment regime for NSCLC is surgical resection for early stage, with chemotherapy applied for more advanced patients(13). Historically, stage III NSCLC treatment has involved neoadjuvant chemotherapy and immunotherapy and surgery where applicable(12). Targeted therapeutics or “molecular-targeted therapy”, are recommended for those patients who harbour a specific genetic mutation. The activation of a member of the erythroblastic leukaemia viral oncogene (erbB) family of cell surface receptors, EGFR, via a ligand binding to the extracellular component of the receptor leads to the further activation of downstream signalling pathways involved in key cellular processes such as cell proliferation and survival (**Figure 1.1**). The receptor dimerizes which then activates the tyrosine kinase activity of the intracellular component(13). As a result of this dimerization, amino acid residues within the intracellular region of EGFR are automatically phosphorylated(14). When activated, EGFR transmits cell proliferation, survival and anti-apoptotic signals to the cell nucleus through several downstream pathways such as PI3K/AKT/mTOR, RAS/RAF/MEK/ERK

and JAK/STAT pathways(14)(15). A mutation within EGFR (EGFRm) is one of the more common druggable targets of this disease(16). These drugs are termed EGFR-Tyrosine Kinase inhibitors (EGFR-TKIs).

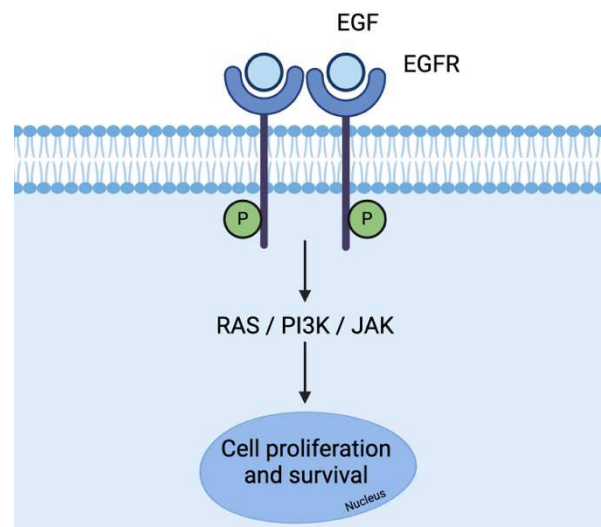


Figure 1.1: EGFR signalling pathway. In most cases of NSCLC, overactivation of EGFR activates downstream signalling pathways such as RAS, PI3K/Akt and JAK/STAT pathways which each play favourable roles in cancer progression, such as cell proliferation, division and survival. These pathways are constitutively overactivated in EGFRm NSCLC. Adapted from Leonetti A et al(15). Created using BioRender.com.

1.1.3. Cell line models for NSCLC

Within the study of NSCLC, LUAD is the most researched histological subtype. Cell lines with the presence of EGFR mutations are often the most represented in research, due to the high proportion of patients with activating EGFR mutations. The most commonly studied cell lines are H1650, H1975, H820, HCC4006, HCC4011, HCC827 and PC9 cells(17). These lines all contain mutations with exons 18-21, such as the common deletion E746-A750 in exon 19 (Ex19del) and the L858R point mutation found within exon 21(17). Additionally, H1975 and H820 contain the T790M mutation in exon 20(17). More uncommon mutations such as C797S in exon 20 can be acquired by these cell lines(17). These additional mutations are what make them resistant to some of the EGFR-TKIs. For example, H1650 contains the Ex19del mutation but can also acquire phosphatase and TENSin homolog (PTEN) deletion or PTEN knockout (-/-)(17). H1975 contains point mutations in exons 20 and 21 but can also acquire p53 mutations(17).

1.1.4. EGFR-TKIs as personalised treatment for EGFRm NSCLC

EGFR-TKIs have been developed to target common mutations within EGFR. They are designed to block signalling to the overactivated downstream pathways which will halt cancer cell survival, proliferation and anti-apoptotic signals. Response to these EGFR-TKIs is however heterogeneous to begin with. It is also understood that less than 5 % of patients with EGFRm have a complete response to EGFR-TKIs, despite the presence of EGFRm being reported to be harboured by virtually all tumour cells(10).

The first-generation EGFR-TKIs include gefitinib and erlotinib(13,14). These are reversible inhibitors that target the Ex19del and L858R point mutation found within EGFR(13,14). Their mechanism of action is to competitively bind to the adenosine triphosphate (ATP) binding site of EGFR, thus inhibiting its activation and disabling downstream signalling pathways that would promote cell proliferation and survival in cancer cells(13,14). The objective response rate (ORR) for patients was between 67-74 % for gefitinib and 64 % for erlotinib(13,14). Progression free survival (PFS) was between 9-11.9 months for gefitinib and 9-13 months for erlotinib(13,14). As its impact on NSCLC patients was vast in improving efficacy and reducing toxicity, it became a first line treatment for NSCLC with the above EGFR mutations in 2015(14,18). However, after around one year, patients gain acquired resistance to these inhibitors, halting any efficacy and PFS. Most commonly, the mutation that develops to render patients resistant is a T790M point mutation within EGFR(13). This mutation occurs in over 50 % of patients and allows resistance to develop due to creating a stronger affinity of the receptor to ATP versus the EGFR-TKI(19).

A second-generation of EGFR-TKIs was then developed to address this acquired resistance to the first-generation drugs. This generation included afatinib, which is an irreversible inhibitor of the original EGFR mutations mentioned above, but is also a pan-Erb inhibitor, meaning not only does it inhibit EGFR (ErbB1), but inhibits HER2 and HER4 (ErbB2 and ErbB4 respectively)(13). The second-generation inhibitor also improved on ORR (70 %) and in the minimum PFS (11-13.6 months). In particular, these effects were observed in patients with the EGFR Ex19del but not in those with the L858R point mutation(14). Efficacy was also observed in some of the rarer EGFR mutations; G719X, L861G and S768I(20). It is therefore true that

both these initial early generations of TKIs improved survival for NSCLC patients with EGFR mutations. Yet acquired resistance occurs in both generations and so a third generation of EGFR-TKIs was developed(13).

The third generation of EGFR-TKIs include osimertinib which was first synthesised by AstraZeneca in 2011. It became a clinical trial candidate the next year and began the AURA phase 1 clinical trial in 2013(21). It is an irreversible inhibitor of both the initial mutations inhibited by the first-generation and second-generation drugs and with T790M mutation which accounts for more than half of the instances of resistance to the early generation therapeutics. It does this whilst selectively ignoring wild type EGFR(13,22). The T790M mutation impacts the binding of EGFR-TKIs to the receptor by creating steric hindrance, which weakens the bond(14). This in turn increases the binding affinity of ATP to the receptor which results in acquired resistance to the EGFR-TKIs(14). To overcome this, the initial third generation TKI, osimertinib, was generated. Osimertinib irreversibly covalently binds to the C797 region of the ATP binding site(22). Its structure contains two main regions of interest; 2-aminopyrimidine which gives osimertinib target specificity for EGFRm and an acrylamide bond, which is the bond responsible for targeting T790M due to alkylating the C797 residue to form the covalent bond (**Figure 1.2**).

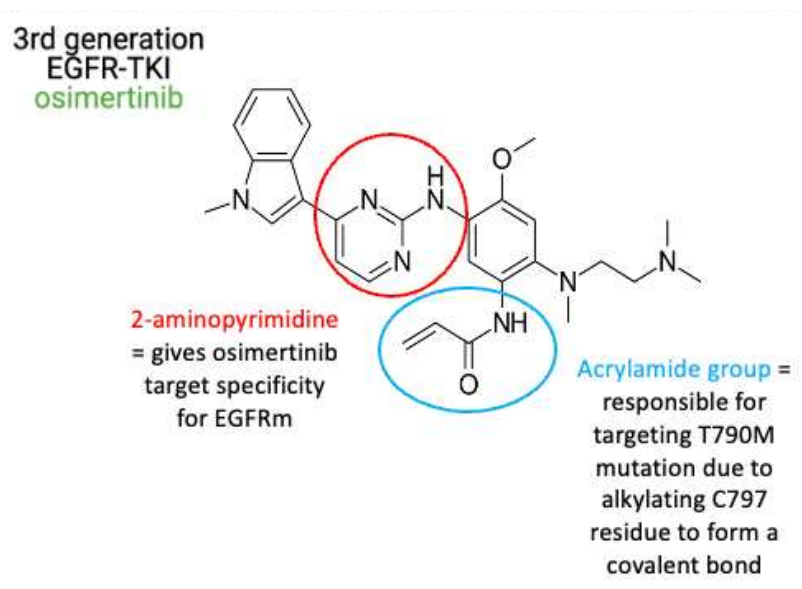


Figure 1.2: The chemical structure of the third-generation EGFR-TKI osimertinib. Osimertinib is unique in two ways; the presence of the 2-aminopyrimidine ring and the acrylamide group. Each of these play a significant role in the efficacy of osimertinib to EGFRm NSCLC.

This inhibitor saw a remarkable increase in ORR (80 %) and PFS (18.9 months)(23). Osimertinib saw a drastic increase in selectivity whilst lowering toxicity. Its ability to cross the blood brain barrier (BBB) allows osimertinib to be more potent. It has also been shown to have efficacy against metastases within the central nervous system (CNS) and most importantly from a drug resistance perspective, can overcome the initial acquired resistance of the first- and second-generation EGFR-TKIs(24). However, unfortunately most patients will also eventually become resistant to osimertinib.

1.2. Drug resistance in NSCLC: common resistance mechanisms

Upon therapeutic treatment, NSCLC cells can have a complete response or show variations of resistance through a partial response or no response at all. Partial response is defined by there being an initial drug-induced response to treatment and therefore decline in tumour growth(25). The development of resistance then leads to no response. The mechanisms of resistance that occur during treatment of NSCLC, and in particular with osimertinib therapy, are complex. There are often several different responses that contribute to drug resistance and disease progression(26). Resistance is multi-faceted and is often the result of a combination of factors both genetic and non-genetic.

1.2.1. General mechanisms behind drug resistance

Tumour heterogeneity and modulation of tumour microenvironment

Tumour heterogeneity can lead to drug resistance through modulation of the tumour microenvironment (TME)(27). Tumour heterogeneity is the measure of diversity between cells of the same type of cancer. This can be because of intrinsic factors such as variations in genetic mutations or extrinsic factors such as hypoxia(27). This leads to treatment not being effective against all cancer cells; those that resist treatment can continue to divide and evade therapy(27).

Increased drug efflux

Drugs may be transported out of the cell and therefore away from the intended target by an increase in the expression of certain efflux pumps and membrane transporters, known as

multidrug resistance proteins (MRP)(28). This is a common resistance mechanism found in chemotherapy. The ATP-binding cassette (ABC) transporter family are the main group of overexpressed transporters involved in drug resistance. For example p-glycoprotein removes intracellular drug content reducing the amount of drug at the target site and enabling disease progression(28). In lung cancer, overexpression of the lung resistance-related protein (LRP) has also been implicated in drug resistance(29).

Induction of DNA repair pathways

In chemotherapy, for example with cisplatin, the primary target is to disrupt cancer cell DNA to trigger apoptosis(29). Resistance then occurs by the cancer cells modulating the expression of DNA-repair enzymes, of which there are many. Repair pathways such as nucleotide excision-repair (NER) and mismatch-repair (MMR) pathways are often modulated in cancer cells and are known to promote resistance to platinum-based chemotherapeutics. Excision repair cross-complementation group 1 (ERCC1) is a critical protein involved in NER as it removes the platinum based adducts in DNA which platinum based chemotherapeutics introduce to disrupt the DNA of cancer cells(29). Therefore, ERCC1 over expression is indicative of drug resistance and has been shown in NSCLC to reduce survival of patients(29).

Altered metabolism of the drug

As cancer drugs are metabolised and broken down into inactive metabolites, the rate of this step can determine the intracellular concentration of the active drug and its bioavailability(30). An increase in expression of enzymes such as members of the cytochrome P450 enzyme family that are responsible for the metabolism and breakdown of a number of substances, can reduce the active drug to its downstream metabolites with no therapeutic effect(31).

Drug tolerance

Drug tolerance occurs when cells reduce response to the drug after consistent exposure, which is different to resistance, where cells fully halt any response to treatment(10). These drug tolerant persisters (DTPs) are often a very small percentage of the total cells targeted, and are able to avoid death from the initial exposure of EGFR-TKIs and persist(10). DTPs are hypothesized to be key in the development of drug resistance, with studies determining that

many resistant cells originate from DTPs. The generation of DTPs occurs by the same mechanisms described here for drug resistance(25).

1.2.2. Genetic alterations in osimertinib resistance

Most commonly, resistance to osimertinib occurs in relation to the expression of additional genetic mutations(23,26,32,33). In this context, they can be classified in two ways; the first is genetic mutations within the EGFR gene, termed here as EGFR-dependent mechanisms of resistance and the second is mechanisms where the genetic mutation occurs within other pathways not directly altering EGFR expression, termed EGFR-independent mechanisms. This phenomenon highlights the clonal evolution of tumour cells and their ability to modulate their genetic expression to avoid the cytotoxic effects of treatment.

1.2.3. EGFR-dependent mechanisms of resistance

Mutations found within EGFR are termed EGFR-dependent activating mutations and are known as on-target mutations(16). The most prevalent of these mutations have already been discussed, and include Ex19del and a point mutation on exon 21 (L858R), therefore in these instances EGFR-TKIs are frontline standard of care therapy for these patients(14). All EGFR mutations within NSCLC fall across exons 18-22, which code for the tyrosine kinase domain. These mutations cause activation of pathways involved in cell proliferation, promotion of cell survival and the continued replication of cancer cells(16). These mutations do this by altering the ligand-receptor binding within EGFR. Whilst osimertinib is specifically used to target the T790M mutation, this mutation has been shown to be lost in patients who received osimertinib, with a gain in KRAS mutations(23). This loss of T790M was also associated with shorter PFS when patients received osimertinib as a second-line treatment(23). The most common EGFR-dependent resistance mechanisms for osimertinib is the C797S point mutation, which disrupts the covalent bond between EGFR and osimertinib, allowing for ATP to competitively bind to this active site once more(34).

1.2.4. EGFR-independent mechanisms of resistance

EGFR-independent mechanisms of resistance often involve mutations within downstream signalling pathways or within molecules belonging to adjacent signalling pathways. Some examples of these are MET, HER2, RAS/RAF, PI3K and PTEN(14).

MET

Mesenchymal-epithelial transition (*MET*) oncogene encodes c-Met, a receptor that when phosphorylated activates downstream pathways such as phosphatidylinositol-3-kinase (PI3K). Its amplification allows for bypassing EGFR inhibition and activating the downstream pathways(34). Around 5-24 % of disease progression following osimertinib treatment can be attributed to *MET* amplifications.

HER2

The human epidermal growth factor 2 receptor (HER2) can be mutated by amplifications, point mutations and upregulation which allow for bypassing of EGFR inhibition. The AURA and FLAURA clinical trials found that HER2 amplification are mutually exclusive to the T790M mutations(23). Point mutations in HER2 account for around 1.5 % of acquired resistance to osimertinib, and the majority of these occur in exon 20(23).

RAS/RAF

Also known as the mitogen-activated protein kinase (MAPK) pathway, RAS/RAF are heavily involved in cell proliferation and growth. There are several genetic alterations to MAPK that trigger resistance to osimertinib(23). Some of these are mutations in NRAS and KRAS discovered by the FLAURA and AURA3 clinical trials. Others were the BRAF^{V600E} mutation(23).

PI3K

Phosphatidylinositol 3-kinase (PI3K) lipid kinase is composed of three subunits; p85 regulatory subunit, p55 regulatory subunit and the p110 catalytic subunit(35). Within the signalling pathway, activation of PI3K occurs normally by recruitment of extracellular factors such as growth factors and hormones to membrane receptors. PI3K catalyses the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate

(PIP3). PIP3 is a secondary messenger that recruits lipid binding domains that can bind to kinases such as Akt and PDK1 which activate downstream pathways that enhance cell proliferation and cell growth(35). One of the major mutations is in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) gene(36). Increase in the copy number of *PIK3CA* and mutations within *PIK3CA* such as H1047R point mutation, are well documented in NSCLC(37). Co-occurring mutations with *PIK3CA* are common and often include mutations within *BRAF*, *HRAS*, *MET* and *HER2*(38).

PTEN

Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) is a tumour suppressor found upstream of the PI3K pathway cascade(39,40). *PTEN* is both a lipid and protein phosphatase that acts by dephosphorylating PIP3, thus halting downstream activation of Akt. It is often mutated in cancer and its inactivation can be caused by numerous mutations that ultimately silence or knockout *PTEN*(41). Loss of function *PTEN* mutations lead to increased tumorigenesis in many differing cancer types(39,40). Its inactivation often correlates with PI3K pathway activation and downstream activation of Akt, highlighting the overlap that *PTEN*, PI3K and Akt signalling have on one another(39,40).

1.3. Dysregulated metabolism in cancer

The dysregulation of cellular metabolism in cancer cells is an acknowledged hallmark of cancer and has been investigated for over 90 years(42). This phenomenon is mostly driven by oncogenes where key steps of metabolism are altered to favour tumorigenesis(43). **Similarly, rewiring of metabolism can play a role in the development of drug resistance.** There are several benefits to aberrant metabolism; to avoid toxic levels of reactive oxygen species (ROS)(44), to avoid apoptosis and other programmed cell death pathways(45) or to increase energy production to fuel continued cell proliferation and promote survival(42). The key cellular dysregulated metabolic pathways include glycolysis, the tricarboxylic acid (TCA) cycle, glutaminolysis as examples of small molecule metabolism, and lipid metabolism via *de novo* lipogenesis (DNL) or lipid peroxidation (**Figure 1.3**). Each of these pathways are linked and often rely upon each other for substrates. In cancer, one example of the dysregulation of glycolysis is The Warburg Effect(42,46).

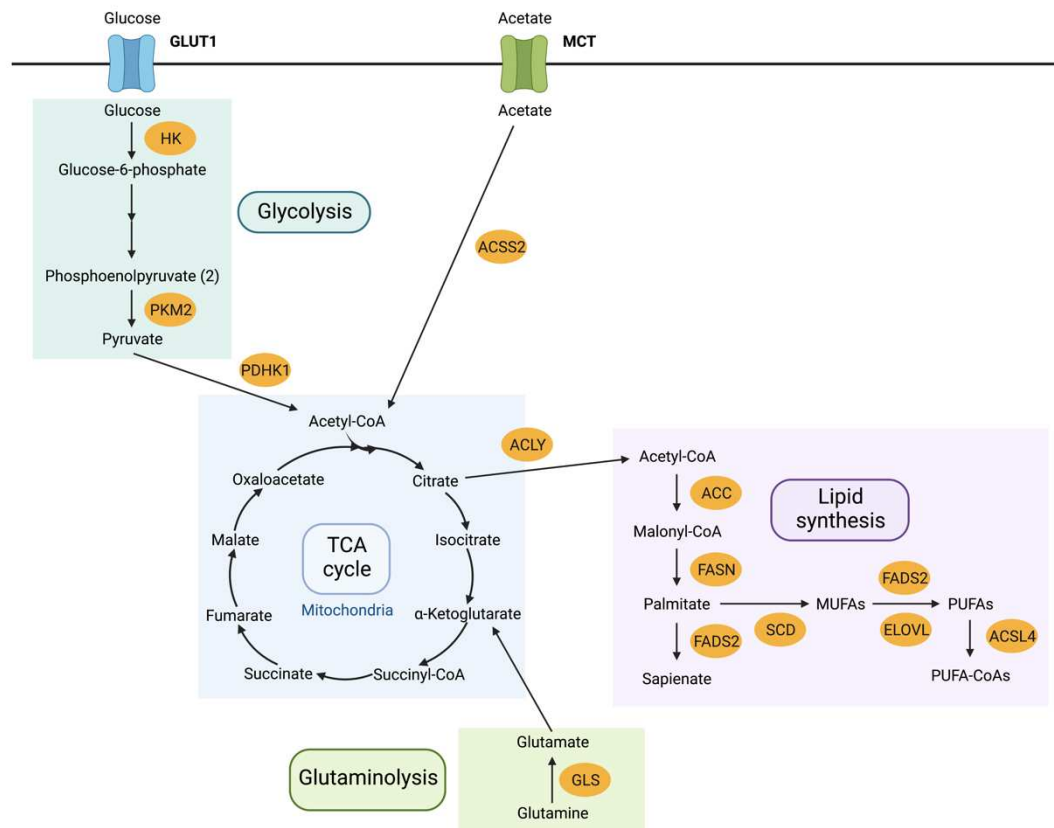


Figure 1.3. An overview of cellular metabolic key pathways; glycolysis, TCA cycle, glutaminolysis and lipid synthesis. Abbreviations: GLUT1 – glucose transporter 1, HK—hexokinase, PKM2—pyruvate kinase M2, PDHK1—pyruvate dehydrogenase kinase 1, MCT – monocarboxylate transporter, ACSS2—acyl-CoA synthetase short chain family member 2, ACLY—ATP citrate lyase, ACC—acetyl-CoA carboxylase, FASN—fatty acid synthase, FADS2—fatty acid desaturase 2, SCD—stearoyl-CoA desaturase 1, ELOVL—elongation of very long chain fatty acids, ACSL4—acyl-CoA synthetase long chain family member 4. Adapted from Koundouros et al(51). Created using BioRender.com.

1.3.1. The Warburg Effect

Glycolysis is a metabolic pathway where glucose is converted to pyruvate through a series of reactions. Glucose is a major energy source for both normal and cancer cells. A hallmark of cancer cells is altered metabolism to support increased cell division, proliferation and survival(47). This is often driven by oncogenes, and these changes distinguish them from healthy normal cells. The Warburg Effect is a well-known phenomenon, whereby cancer cells consume a higher amount of glucose to meet increased energy demands and excrete an increased amount of lactate when compared to normal cells in both normoxic (in the presence of oxygen) and hypoxic (in the absence of oxygen) conditions(42,46).

1.3.2. De novo lipogenesis (DNL)

DNL is the process by which cells synthesise lipids to meet energy requirements of rapidly dividing cells and is known to be upregulated in many cancers(48–50) and linked to poor prognosis and shorter PFS(48). Lipids are synthesised from carbon atoms originating from glucose or amino acids(51). The overview of DNL is summarised in **Figure 1.4**. In hypoxic conditions, cancer cells upregulate acetyl-CoA synthetase 2 (ACSS2), allowing for an increase in acetyl-CoA from acetate and therefore increasing the downstream production of lipids(52–54). The rate limiting step of DNL is catalysed by acetyl-CoA carboxylase (ACC) which converts acetyl-CoA to malonyl-CoA. The main product of *DNL* is palmitate (16:0), a saturated fatty acid (SFA)(51). From here palmitate is desaturated by stearoyl-CoA desaturase (SCD), and elongated by elongation of very long chain fatty acids (ELOVL6) to produce stearate (18:0) and oleate (18:1). Further desaturation and elongation steps follow to create fatty acids with differing chain length and number of double bonds. These then feed into the synthesis of complex lipids, via phosphatidic acid (PA)(51).

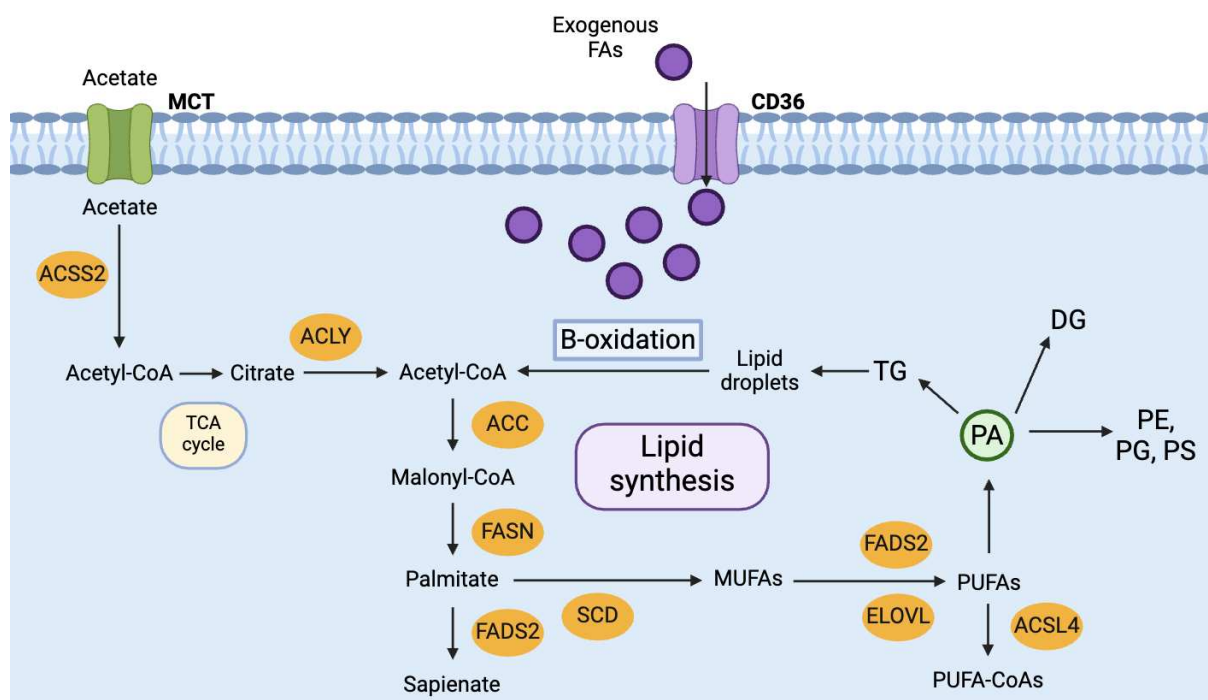


Figure 1.4: An overview of de novo lipogenesis. *De novo lipogenesis stems from acetyl-CoA and ultimately ends in the formation of saturated fatty acids (SFA). These can then be further synthesized into monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs) and complex lipid families(51). Abbreviations: MCT – monocarboxylate transporter, ACLY – ATP citrate lyase, ACC – acetyl-CoA carboxylase, FASN – fatty acid synthase, FADS2 – fatty acid desaturase 2, SCD – stearoyl-CoA desaturase 1, ELOVL – elongation of very long chain fatty acids, ACSL4 – acyl-CoA synthetase long chain family member 4, FA – fatty acid, PA – phosphatidic acid, PE – phosphatidylethanolamine, PG – phosphatidylglycerol, PS – phosphatidylserine, DG – diglyceride/diacylglycerol, TG – triglyceride/triacylglycerol. Adapted from Koundouros et al.(51). Created using BioRender.com.*

Many of the genes involved in DNL (e.g. *FASN*, *ACC* and *SCD*) are regulated by sterol regulatory element binding 1 (*SREBP1*), a frequently upregulated transcription factor in cancer cells(51). Lipid reprogramming in cancer cells can occur from changes at gene level. For example, activation of the PI3K/Akt signalling pathway can lead to an increase in carbon sources available for lipogenesis, activation of *ACLY* and increased acetyl-CoA and fatty acid synthesis(55). As well as utilising DNL to meet increasing energy demands, cancer cells use it as a means of protection against free radicals and as a way of avoiding the effects of chemotherapy(48).

1.3.3. Lipid peroxidation, saturation and ferroptosis

Saturation of membranes can have an impact on the treatment response in cancer (48). The level of saturation, or the number of double bonds, in fatty acyl chains play a key role in the properties of phospholipids. Lipids containing multiple double bonds, such as polyunsaturated fatty acids (PUFA), are susceptible to peroxidation by ROS(48,49,51,56). A main source of ROS comes from the mitochondria as a byproduct of oxidative phosphorylation. The product of lipid peroxidation by ROS are lipid peroxides. Lipid peroxides are able to destabilise the cell membrane through further reaction with the double bonds in PUFAs (44). Lipid peroxides are reduced by glutathione (GSH) peroxidases (e.g. *GPX4*)(57,58). *GPX4* is the master regulator of circulating lipid peroxides, with an accumulation of these triggering ferroptosis(56). Ferroptosis is a programmed form of cell death that relies upon iron metabolism(58). Ferroptosis has three distinctive traits; lipid peroxide accumulation, an increase in iron and a morphologically distinct cell death phenotype(58). *GPX4* requires glutathione (GSH) in order

to reduce the accumulation of lipid peroxides and therefore protect against ferroptosis(58). Nuclear factor erythroid 2-related factor 2 (*NRF2*) is a master regulatory transcription factor of all anti-ferroptotic pathway members. *NRF2* is activated upon degradation of kelch-associated protein 1 (KEAP1) via oxidative stress(58). Once *NRF2* enters the nucleus, it attaches to antioxidant response elements (AREs) which initiates antioxidant and cytoprotective proteins(57).

1.3.4. Accumulation of ceramides to promote apoptosis

Accumulation of ceramides in cells can lead to the activation of apoptosis. Apoptosis is a highly regulated mechanism of programmed cell death, utilised by the cell as a natural response to conditions that cause cell damage, such as DNA damage. Evasion of apoptosis is a hallmark of cancer, since cancer cells look to promote uncontrolled cell division and proliferation of cells that carry mutations(59). Ceramides activate apoptosis via caspases, which play a crucial role in the initiation of apoptosis in cells(45,60). This was first observed in leukemic cells(61), but these mechanisms have since been studied in various cell types(62). Apoptosis activation can occur in an extrinsic or intrinsic way, with the intrinsic pathway relying on mitochondria and mitochondrial outer membrane permeabilization (MOMP)(61). This intrinsic pathway is activated by an extracellular stimuli, such as oxidative stress or chemotherapeutics(63). Following a loss of mitochondrial membrane potential, cytochrome *C* release prompts caspase-9 activation, with subsequent activation of caspase-3(60,62,64). The extrinsic apoptotic pathway occurs through binding of a death ligand such as TNF α and CD95, to its receptor on the plasma membrane which promotes formation of the death-inducing signalling complex (DISC)(65). Several studies report how ceramide accumulation can mediate both the intrinsic and extrinsic apoptosis pathways(45,66,67). **Figure 1.5** shows the intrinsic pathway of ceramide mediated activation of apoptosis in more detail.

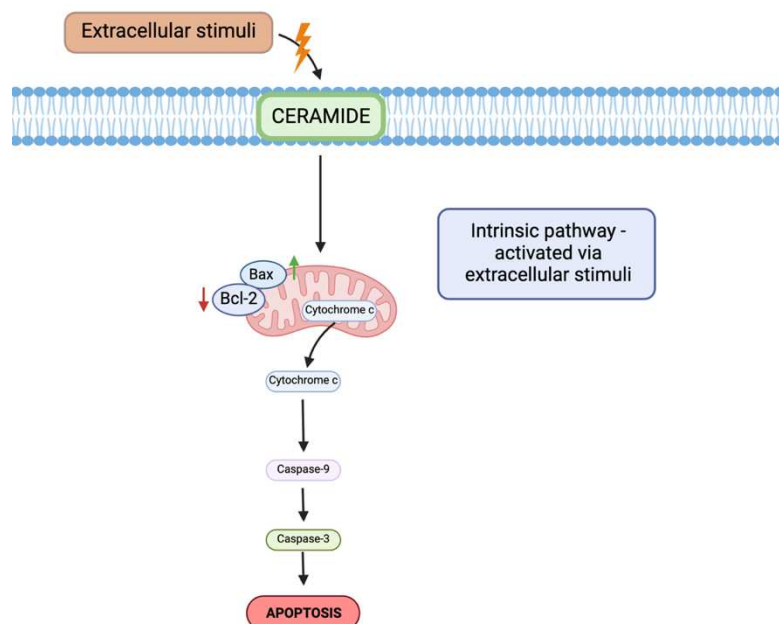


Figure 1.5: Intrinsic pathway of ceramide mediated activation of apoptosis. A schematic of the intrinsic pathway of ceramide mediated caspase activation that is activated via an extracellular stimulus. Adapted from Ogretmen B et al., Sawada M et al., and Park JY et al(61,62,64). Created using BioRender.com.

Previous studies have shown how ceramides can induce MOMP and activate apoptosis intrinsically(63). However, a mechanism of resistance in cancer cells is to resist induction of MOMP, thus stopping the intrinsic apoptosis pathway(63). Modulation of anti-apoptotic or pro-apoptotic proteins can contribute to drug resistance(68). The B cell lymphoma-2 (Bcl-2) family of proteins are a diverse group of proteins that encompass both anti-apoptotic (Bcl-2, Bcl-xL, Bcl-W and MCL1) and pro-apoptotic (Bax, BAD, Bid, Bok, Bik, Bim and PUMA) members(63). Ceramide-induced apoptosis can therefore be triggered by either the downregulation of anti-apoptotic proteins or upregulation of pro-apoptotic proteins(69). One of the proposed mechanisms for apoptosis induced via ceramide accumulation is that this accumulation triggers downregulation of Bcl-2 and may upregulate Bax via a p53-dependent manner(62).

Ceramides are central to sphingolipid metabolism (**Figure 1.6A**). Ceramide accumulation in the cell can occur due to increased *de novo* synthesis, but can also arise from enhanced activity of sphingomyelinases, enzymes responsible for hydrolysing sphingomyelin (SM) to ceramide(45). The salvage pathway is an additional pathway in which ceramide can

accumulate via the recycling of sphingosine and sphingosine-1-phosphate (S1P)(70) and the breakdown of glycosphingolipids (GSL), which occurs in the lysosome.

Sphingolipids are a complex family of bioactive lipids including ceramides, S1P, sphingosine, SM, GSL and phosphosphingolipids (PSL)(71). Sphingolipids are vital structural components within cell membranes and play a signalling role in physiological processes such as apoptosis and cell growth(53). In cancer, sphingolipid levels are dysregulated, disrupting these vital physiological processes(53,72). As a consequence, bioactive sphingolipids are potential biomarkers for cancer progression.

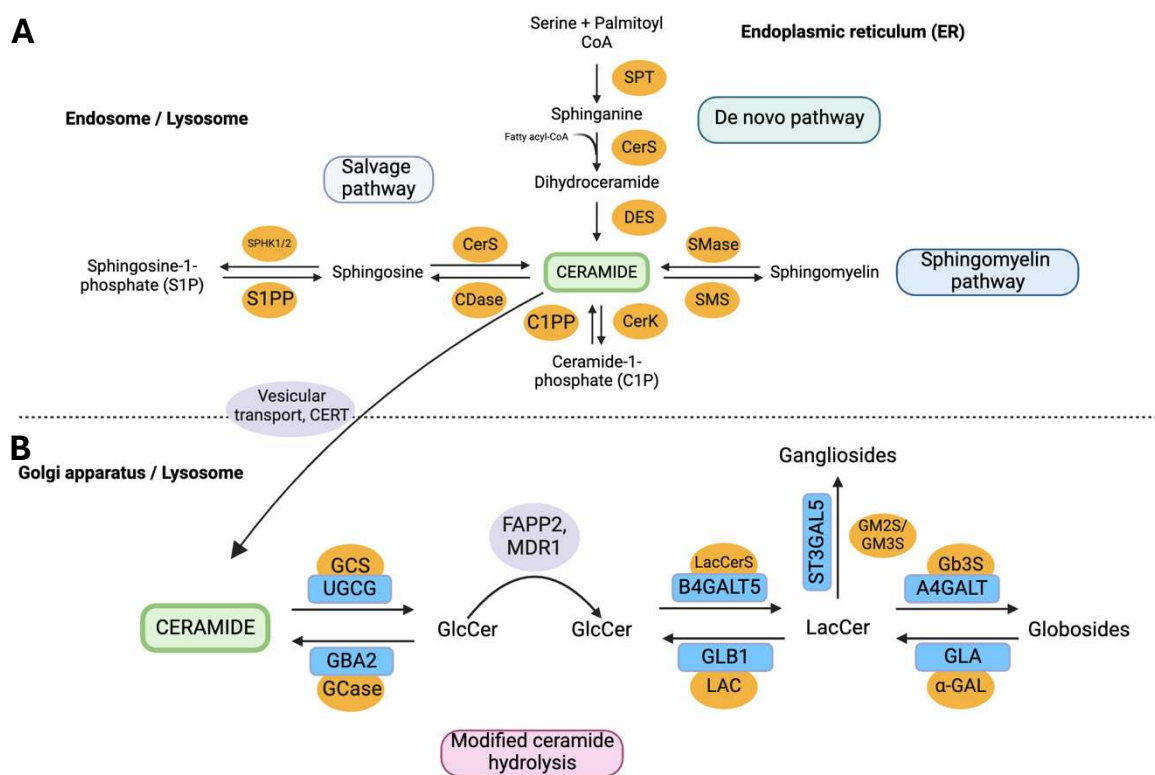


Figure 1.6: Sphingolipid biosynthesis and metabolism. (A) Ceramide biosynthesis pathway showing 3 key pathways; Salvage pathway, De novo pathway and Sphingomyelin pathway. **(B)** Glycosphingolipid biosynthesis pathway (also termed modified ceramide hydrolysis). Enzymes are in orange; transporters are in purple and genes are in blue. Abbreviations: SPT – serine palmitoyltransferase, CerS – ceramide synthase, DES - dihydroceramide desaturase (also known as DEGS), SPHK1/2 – sphingosine kinase, S1PP – sphingosine 1-phosphate phosphatase, CDase – ceramidase, C1PP – ceramide 1-phosphate phosphatase, CerK – ceramide kinase, SMS – sphingomyelin synthase, SMase – sphingomyelinase, CERT – ceramide transfer protein, GCS – glucosylceramide synthase, GCase - β-glucosidase, FAPP2 – 4-phosphate adaptor protein 2, MDR1 – multidrug resistance 1, LacCerS – lactosylceramide synthase, LAC - β-galactosidase, GM2S/GM3S – ganglioside monosialic 2/3 synthase, Gb3S – globo-triaosylceramides synthase, GLA – galactosidase. Adapted from Li RZ et al. and Lin M et al(53,71). Created using BioRender.com.

Ceramides are converted to sphingosine by ceramidase (CDase) and then finally to S1P via sphingosine kinase (SPHK1/2)(70,73). This enzymatic family has been widely studied due to its role in regulating the balance between ceramide, sphingosines and S1P(71). Upregulation of SPHK1 was found to promote cell proliferation and inhibit apoptosis in NSCLC(73). Others have shown S1P promotes cell proliferation and survival, acting in opposition to proapoptotic ceramides(53). S1Ps regulate their biological effects through downstream pathways such as the PI3K/Akt and Ras/ERK1/2 pathways(71). S1P has also been shown to increase EGFR expression, which promotes cancer progression in NSCLC(70). Other utilisation pathways of ceramide include its phosphorylation to ceramide-1-phosphate (C1P) by ceramide kinase (CerK). Finally, in the modified ceramide hydrolysis pathway, ceramides are converted to different bioactive GSLs via several enzymatically driven steps (**Figure 1.6B**).

1.3.5. Glycosphingolipids and glucosylceramide synthase in cancer

GSLs are formed through the addition of glucose or galactose to the 1-hydroxy group of ceramide, in a step called glycosylation(74). Depending on the sugar, simple GSLs can be glucosylceramide (GlcCer) from the addition of glucose, or galactosylceramide (GalCer) from the addition of galactose. More complex GSLs are then synthesized by incorporation of additional moieties, e.g. galactose for lactosylceramides (LacCer); N-acetylgalactosamine for globosides or sialic acid(75) for gangliosides amongst others(74).

Figure 1.6B highlights the glycosylation pathway that occurs in the Golgi apparatus. The first step, in which ceramides are converted into GlcCer, is catalysed by glucosylceramide synthase (GCS) encoded by the uridine-5-diphosphate (UDP)-glucose ceramide glucosyltransferase (*UGCG*) gene(76). It is the only known enzyme responsible for the *de novo* synthesis of GlcCer(77). *UGCG* overexpression has been well documented in the literature as a common occurrence in different cancers(76), and is linked to various cellular processes including multidrug resistance(77), increased glycolysis and increased oxidative phosphorylation and mitochondrial turnover(76). It can also be used as a potential marker of treatment resistance in cancer, since activation of *UGCG* has been shown to promote resistance in breast cancer(78).

Lactosylceramide synthase (LacCerS) is encoded by the beta-1, 4-galactosyltransferase gene (*B4GALT5/B4GALT6*) and is responsible for LacCer synthesis(79). In some cancers, *B4GALT* has been linked to apoptosis, via activation of differing signal transduction pathways, and to multidrug resistance(79). Gangliosides encompass several lipid species which are classified based on the number and position of additional sialic acid groups(75,80). Globosides are synthesised from LacCer by globotriasylceramide synthase (Gb3S), encoded by alpha-1,4-galactosyltransferase (*A4GALT*). Globosides were found to play a potential role in epithelial-to-mesenchymal transition (EMT), which is a feature of tumour invasion and metastasis(81).

1.3.6. Dysregulated metabolism in EGFR-TKI resistant NSCLC

To further explore the potential role of dysregulated metabolism and osimertinib resistance, we conducted a systematic review on “Dysregulated Metabolism in EGFR-TKI Drug Resistant Non-Small-Cell Lung Cancer” (**Chapter 2**). In lung cancer, previous studies have identified metabolite changes within amino acid metabolism, glycolysis, oxidative phosphorylation, gluconeogenesis and fatty acid metabolism to be dysregulated in EGFR-TKI resistant cell lines. For example, GSH and amino acid metabolism were both found to be dysregulated in EGFR-TKI resistant NSCLC, along with aspects of glycolysis and glutamine metabolism (7,82,83)(84). A further change observed in EGFR-TKI resistant cells was within the TCA cycle(84,85), with succinate levels increased in resistant compared to sensitive cells. This implies a loss in succinate dehydrogenase (SDH), which may subsequently activate a hypoxic response in these cells(85). Several studies have also found that upon EGFR inhibition, NSCLC cells re-favour oxidative phosphorylation over anaerobic glycolysis(43,86). This therefore offers a therapeutic window which can be exploited to combat EGFR-TKI resistance. Finally, aberrant lipid metabolism was also linked to EGFR-TKI resistance, through an increase in DNL(49,87–89). These findings are detailed in **Chapter 2**.

1.4. Multi-omics for studying metabolism in cancer

We have summarised some of the major metabolism centred pathways that are dysregulated in cancer in general and in NSCLC, which may play a role in drug resistance. To probe these effectively, the use of -omics technologies can be utilised. A multi-omics approach is a study

which includes more than one of genomics, transcriptomics, proteomics and metabolomics/lipidomics(90,91). This allows for a wider understanding of disease through the combination of information from diverse biological levels(92).

1.4.1. Genomics and transcriptomics

Genomic studies investigate changes in the genome of cancer cells. Classically, cancer is an accumulation of genetic mutations that allow cells to constitutively activate cell division and proliferation(93). These genetic mutations however can be exploited as therapeutic targets, to “correct” the mutation either by silencing the gene or inhibiting the expression of its downstream protein. Integration of genomic information has coincided with clinical outcomes to determine the severity of mutations(93). The study of the genomes provides a personalised outlook on precision medicine and often elucidates mechanisms in which to treat cancer from the source mutation. Changes observed at the genomic level are well documented compared to changes observed within metabolomics(32).

Transcriptomics is the study of the transcriptome, or the set of RNA molecules expressed from a gene(94). This type of study allows us to probe functional elements of the genome(95). Not all changes at gene expression level result in a phenotypic change, therefore studies at protein and metabolite level are also needed.

1.4.2. Proteomics

Proteomics is the study and evaluation of proteins to determine their existence and function within disease. Amino acids are the building blocks of proteins, with molecules containing up to 50 amino acids, linked by amide bonds, termed peptides (**Figure 1.7**). The study of proteins and peptides can provide a more in-depth phenotype of disease when compared to genomics/transcriptomics(96). For example, corresponding protein changes that occur downstream of changes observed in transcriptomics may contain post-translational modifications (PTMs) which influence its activity(97). The protein content of an organism can also be influenced by non-genetic factors, such as the environment.

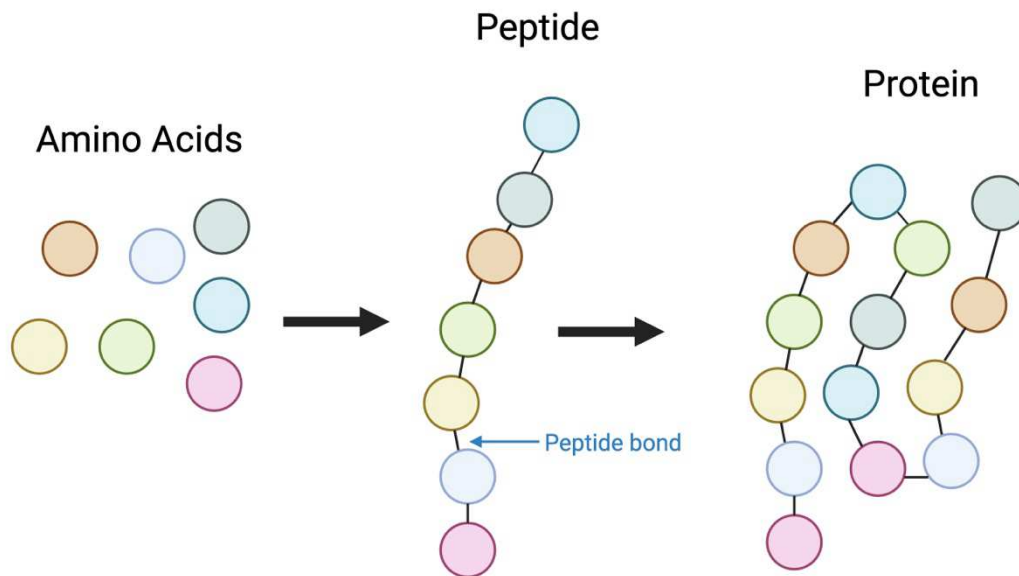


Figure 1.7: The structure of peptides and lipids. Amino acids are the basic building blocks of proteins. When joined together via the peptide bond, they form peptides. Peptides are further combined into more complex structures known as proteins. Created using BioRender.com.

Proteomics analysis can be performed in a “bottom-up” or “top-down” approach. In the bottom-up approach, proteins are digested before analysis by mass spectrometry (MS). These peptides are around 500-3,500 Da and are separated by liquid chromatography (LC) prior to MS evaluation. Bottom-up refers to information being generated from the individually identified peptide fragments that are reconstructed to give information at the protein level(98). Drawbacks to the bottom-up approach are mainly related to inferring a protein structure based on peptide fragments, as peptides may be common across several proteins therefore accurate identification can prove difficult. Enzymatic digestions, such as using trypsin, can alter the peptide which changes the structure prior to analysis(99). A top-down approach utilises intact proteins for analysis and therefore does not involve any digestion step(100). This overcomes some of the difficulties faced with the bottom-up approach, where the peptides detected can only infer the protein match and do not give an exact identification. However chromatographic separation can be more challenging. The most robust analysis of the proteome would benefit from integration of both methods. The data obtained from either method is run through public proteomic databases to determine IDs. These potential IDs can then be investigated in various online libraries, such as UniProt(101), Peptide Atlas(102) or Human Proteome Map(103), which can give support to IDs made and put the proteins into context within signalling pathways, different diseases or patient profiles.

Proteins are important biomarkers of cancer as they can elucidate the stage, severity, genetic mutation and the ability of the cancer to metastasise(96). In NSCLC, an example of proteins that are altered include EGFR whereby its expression or activation can be indicative of disease progression or resistance to treatment(104).

1.4.3. Metabolomics and lipidomics

Metabolomics (also referred to as metabonomics or metabolic profiling) is used to evaluate, measure or quantify specific changes within the metabolome of biological samples, including cells, tissue and biofluids(105). This gives an understanding of the upstream processes occurring which are guided by genes and proteins. Metabolomics measures the small polar metabolites, such as those involved in glycolysis and the TCA cycle, amino acids, sugars and nucleic acids(106,107). Both internal (such as genetics) and external (such as environmental) factors may be responsible for the alteration of metabolite levels(108). When investigating cancer cells for example, metabolomics can be used to determine biomarkers of disease, evaluate disease severity or indicate pathways that may be contributing to drug resistance. Metabolomics studies can also involve measuring metabolic flux. For example by the incorporation of ^{13}C labelled glucose in cell-based studies, we can understand how cancer cells utilise glucose in different metabolic pathways (108).

Lipidomics is a branch of metabolomics and is the study of low-polarity metabolites, including fatty acids and complex lipids(105). Lipids are a large group of biomolecules that share the same base components, fatty acids. Fatty acids or fatty acyl chains are attached to different backbones and head groups, which gives lipids their specificity. There are many diverse families of complex lipids such as phospholipids and sphingolipids. The phospholipid family can be further classified into phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylcholine (PC). Plasmalogens are a type of phospholipid with a vinyl-ether bond or ether bond in the glycerol backbone(109), and are denoted as PC-O, PE-O etc. Other more complex lipid families include triacylglycerides (TGs), diacylglycerides (DGs), and sphingolipids (Cer, SM, GlcCer, GalCer, LacCer, gangliosides and globosides as previously described). **Figure**

1.8A summarises the above lipids along with other complex lipids and their different chemical structures.

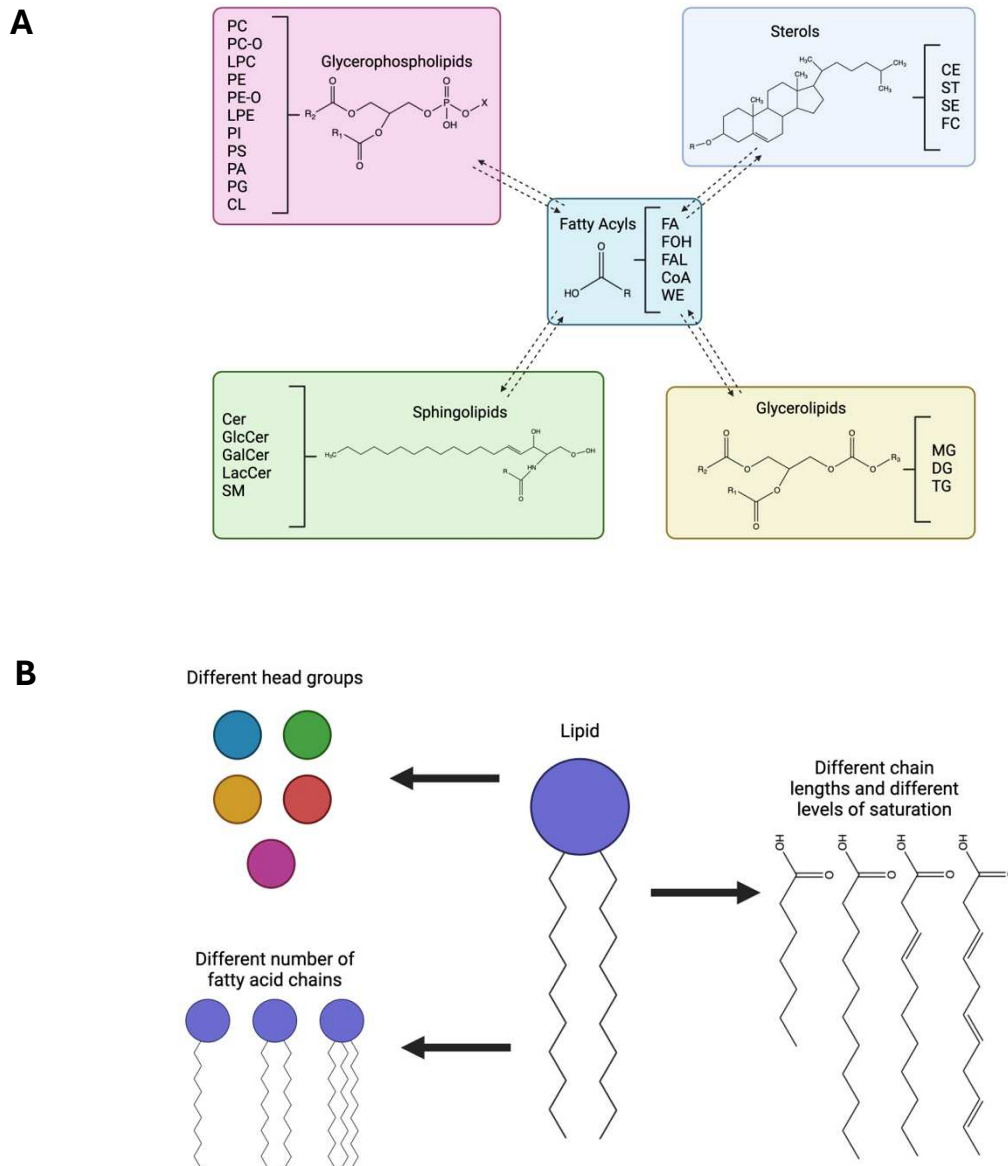


Figure 1.8: Lipid structure and nomenclature. (A) Lipids are composed of fatty acyl chains, and can be synthesized into various complex families of lipids based on specific headgroups as shown. (B) Lipids can be further classified by head groups, the number of fatty acid chains and the saturations of those chains. Created using BioRender.com.

The head group of the lipid denotes the class of lipid, but there are other structural factors that can give lipids distinctive characteristics (summarised in **Figure 1.8B**). For example, lipids can have different numbers of fatty acid chains. Furthermore, these fatty acid chains can vary in length and in saturation. Lipids that contain no carbon-to-carbon double bonds in the fatty acid chains are known to be saturated (SFA), those with one carbon to carbon double bond

are monounsaturated (MUFA), and those with more than one carbon to carbon double bond are polyunsaturated (PUFA). Saturation is an important physicochemical property of lipids and can indicate a variety of functions within the cell, such as the fluidity of the cell membrane(110). As well as important components of membrane structure, lipids can act as secondary messengers in vital signalling pathways. An example of this is PIPs (phosphoinositols) within the PI3K/Akt pathway(51), whereby Akt is activated after binding of PIPs(111).

Metabolomics studies can either be performed in an untargeted or targeted way. With an untargeted approach, the entirety of the detectible metabolome is probed to detect changes in all metabolites, and even unknown molecules. This is often used to discover novel biomolecular changes(112). With targeted analyses, there is a predetermined pool of metabolites to focus on, often generated by a study hypothesis. This may include metabolites that are within specific pathways that can be selectively analysed with more precision and can often be quantified(112,113). Targeted analysis can incorporate measuring the flux of metabolites of interest(114).

1.5. Analytical workflow for conducting metabolomic analysis

1.5.1. Sample collection and preparation

Sample collection and preparation will vary. With all sample types, it is key to quench metabolism of the sample to halt any enzymatic activity, which will give the most accurate representation of metabolism at the point of harvest for analysis. With cell lines, the normal protocol is to use methanol to quench the metabolism of cells, then to scrape and collect the cells from the bottom of plates and from here use an extraction method with suitable solvents to extract the metabolites of interest. Common extraction methods include liquid/liquid extraction (LLE), such as dual phase Folch extraction (115), summarised in **Figure 1.9**.

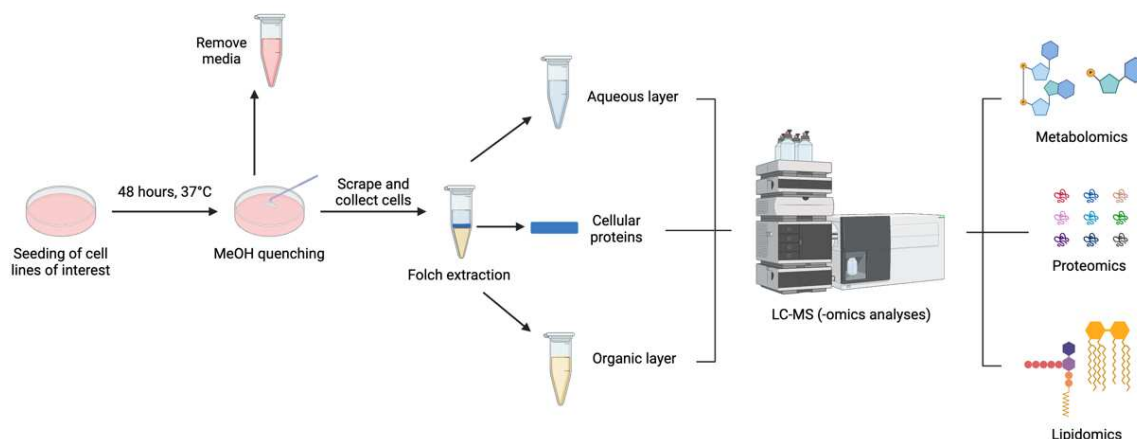


Figure 1.9: Workflow for metabolite extraction and analysis. Cell lines are seeded and after an appropriate time their metabolism is quenched. These are then extracted using several methods, Folch is given here as an example. The aqueous layer can be used for metabolomics, the organic layer for lipidomics and the resultant middle layer for proteomics. Created using BioRender.com.

1.5.2. NMR and GC-MS

There are multiple analytical platforms that can be used to study metabolomics, which mostly fall into nuclear magnetic resonance (NMR) spectroscopy and MS platforms. NMR determines the molecular structure of a compound. It can measure ^1H , ^{13}C , ^{15}N , and ^{31}P to name a few. It does this by applying a magnetic field to the molecule. The nuclei of these atoms spin under the magnetic field after exposure to high frequency pulses that allow them to go with or against the magnetic field. The nuclei are then released from this state and absorb energy. Upon relaxation to the original state, it is this resultant emission of energy detected that can reveal structure information based on the atoms surrounding the specific excited nuclei(116,117). NMR is a less convoluted process than MS as it does not involve the need for any separation technique prior to analysis (such as a chromatographic technique often coupled with MS)(117). The downside to NMR is the lack of sensitivity when compared to MS.

To perform metabolomics studies, the main platform used is MS. MS records the mass/charge (m/z) ratio and intensity of the analyte ion(118). This is often coupled with a separation step before mass analysis, such as liquid or gas chromatography (LC and GC). GC is used to measure smaller metabolites, which includes amino acids and molecules within the TCA cycle(119). The

molecules must be volatile enough to become a gas(119). GC can cover a wide breadth of metabolites with both sensitivity and specificity due to electron ionization which allows for specific fragmentation patterns to be elucidated. This therefore increases reliability on matching the mass profiles with any known libraries. However, as GC is limited to those metabolites which are able to become volatile(120), it cannot be used for every metabolite of interest.

1.5.3. Liquid chromatography mass spectrometry (LC-MS)

LC allows for the separation of metabolites and lipids based on their polarity, weight and other physicochemical properties, to improve the resolvability of the compounds through MS(116). High-performance LC (HPLC) and Ultrahigh-performance LC (UHPLC or UPLC) are branches of LC that differ mainly in the pressures at which they can operate. UHPLC systems perform at a pressure above 6000 psi with HPLC systems able to perform at lower pressures(121).

LC-MS can enable the coverage of a wider range of compounds depending on the stationary phase, column type, composition of mobile phases and any additional modifiers that can alter the separation during LC. Most often than not, there are two mobile phases to drive the separations within the column with one phase being more polar than the other. Two of the common LC methods are reverse phase LC (RPLC) and hydrophilic interaction chromatography (HILIC) (118,122). RPLC separates lower polarity metabolites (e.g. lipids) and uses hydrophobic stationary phase (e.g. C18 columns)(112), whereas HILIC separates highly polar metabolites (e.g. small molecule metabolites)(118,122). To improve on the chromatographic separation, the combination of separation steps can be utilised and is termed multidimensional liquid chromatography (MDLC). This is useful with complex samples where there may be metabolite overlaps that need to be separated (112). The separations methods often run for around 10 to 30 minutes per injection before they can be analysed via MS(122).

1.5.4. Key components of a mass spectrometer

A mass spectrometer involves three major parts as shown in **Figure 1.10**. These include an ion source, a mass analyser and the detector.

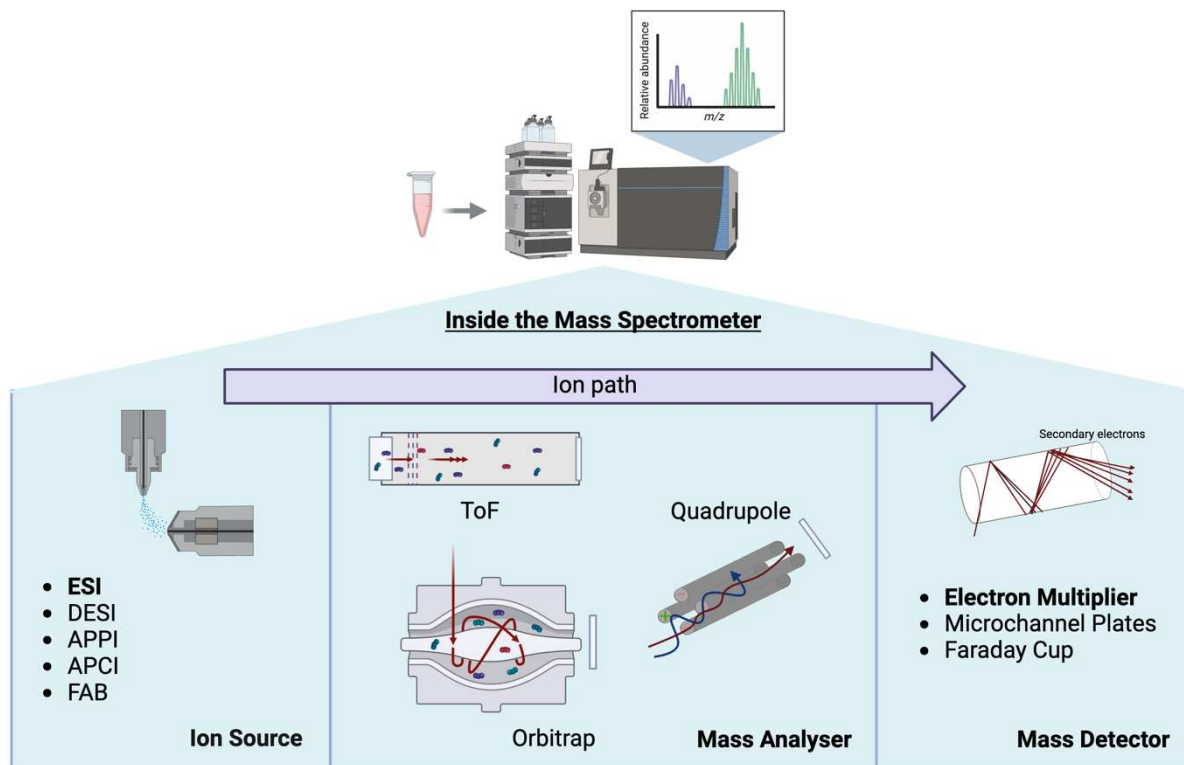


Figure 1.10: Overview of the mass spectrometer. The mass spectrometer is made up of 3 main parts; the ion source (ESI pictured), mass analyser and mass detector (electron multiplier pictured). The ion source produces the ions to then be analysed by the mass analyser which are then passed to the detector to read the signal. The mass spectrometer is under a constant vacuum. Abbreviations: ESI – electrospray ionization, DESI – desorption electrospray ionization, APPI – atmospheric pressure photoionization, APCI – pressure chemical ionization, FAB – fast atom bombardment, ToF – Time of Flight. Adapted from Li C et al., Prabhu GRD et al. and Zubarev RA et al(123-125) Created using BioRender.com.

Ion source

The ion source is responsible for converting the molecules that are separated in LC into ions that can then be measured in the mass analyser. The most common form of ionisation is electrospray ionization (ESI) as it allows both small and large molecules to be ionized(122). ESI is a “soft ionisation” technique, meaning it only creates a small number of larger ions(121). ESI employs a high voltage to the liquid droplets to form gas-phase positively or negatively charged ions(123). The ions formed are most commonly protonated $[M+H]^+$ or deprotonated $[M-H]^-$ in positive and negative ion mode, respectively. However, depending on modifiers in

the mobile phases, they can also include $[M+Na]^+$ and $[M+NH_4]^+$ in positive mode and $[M+Oac]^-$ and $[M+Cl]^-$ in negative(122). Drawbacks to using ESI as an ion source include the negative impact salts can have on the efficiency of ESI, meaning the buffers used in the chromatography are limited. Another common drawback is ion suppression, which is where metabolites co-eluting from the chromatography column compete for ionization which can obscure detection(122,123). Other forms of ionisation include desorption electrospray ionization (DESI), atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI) and fast atom bombardment (FAB)(112,123).

Mass analyser

Mass resolution is the ability of the system to resolve ions and generate peaks(124). Mass analysers mainly differ in the way in which they separate and resolve ions of differing m/z . (125). Low resolution mass analysers such as triple-quadrupole (QqQ) or linear ion trap (LIT) are mostly used in targeted methods and resolve fewer ions as there is less ability to discriminate peaks with similar mass(125,126). Quadrupole mass analysers act as a filter based on the m/z of ions. They consist of four charged cylindrical rods that allow the ions to pass through the centre. The rods are electrically connected to one another with a radio frequency (RF) and direct current (DC) applied. Depending on these parameters, ions can either pass through to the detectors or be filtered out by the rods based on m/z (125). One disadvantage to quadrupoles is that they are limited by the mass range they can operate with and have lower resolution compared to other analysers. LIT mass analysers consist of charged rods with an RF and DC current applied. There is a potential field applied to either end of the charged rods to trap ions within the analyser itself(125). These trapped ions are then selectively ejected to the detector, based on their m/z .

High resolution mass analysers may include time of flight (TOF) or orbital ion trap (Orbitrap) and are often applied to untargeted analysis(112). The higher resolving power, which denotes the ability of the mass analyser to resolve the adjacent peaks as well as main peaks, enables resolution of ions with a smaller degree of difference in mass(127). Mass resolution and resolving power are used interchangeably in most cases. TOF analysers consist of a flight tube in which ions can drift through the tube towards the detector. At the end of the TOF where the ion source is, there is an acceleration grid that allows a group of ions to accelerate from

the ionization source towards the detector(125). TOFs therefore separates ions based on the time it takes them to travel a set distance after passing through the acceleration grid(125). This then allows the m/z to be calculated based on arrival time. TOFs have a wide mass range and are therefore suited for large molecules such as proteins. The orbitrap analyser is comprised of an inner spindle electrode, with two outer electrodes on either side. Ions will spin around the inner electrode based on a voltage potential that is applied, and their motion is dictated by their m/z . The orbitrap uses Fourier transformation to convert the signal produced by ions spinning around the inner electrode(125). Different mass analysers can also be combined to form hybrid instruments, e.g. Q-TOF or LTQ-Orbitrap.

Tandem mass spectrometry (MS/MS or MS²) can be utilised to fragment the precursor ions that are formed after ESI into product ions through various methods such as collision-induced dissociation (CID)(128). In CID, ions are fragmented through the collision with a neutral gas, most commonly nitrogen, helium or argon(128). Other common MS/MS methods include electron-induced dissociation (EID)(129), which is an interaction between the ions and electrons, as opposed to a neutral gas in CID(129), and higher energy collision dissociation (HCD), which is a fragmentation technique specific to Orbitraps(130). The information gathered from fragmented ions allows for another layer of structural information to improve confidence in annotation and is complementary to the exact mass. For example, MS/MS can aid the determination of fatty acyl chain composition and the head group of a complex lipid.

Mass detectors

The detector allows the ions to be displayed as spectral data highlighting the relative intensity or abundance of the ions based on the m/z . There are several ion detectors such as electron multipliers, microchannel plates (MCPs) and faraday cups(126). The electron multiplier detects the ions that have passed through the mass analyser. When the ions meet the detector plate, they release secondary charged particles which can be measured. Electron multipliers have high sensitivity and produce little signal noise(126). MCPs operate in a similar way to electron multipliers, and both contain means to amplify the signal. Faraday cups measure the ion beam current that is released from the mass analyser. The ions hit the surface of the detector plate or dynode and the resultant current produced is recorded. Faraday cups are

less sensitive than both electron multipliers and MCPs as they do not possess an amplification step(126).

1.5.5. Data processing, annotation and statistical analysis

The raw spectral data that is generated from the mass analyser and received by the detector is then displayed as a mass spectrum for m/z values and their intensities, and an ion-chromatogram for retention times in LC/GC-MS. Processing involves peak or feature detection, deisotoping and peak alignment before full annotation can occur. There are many accessible data processing tools that exist to process data, such as MS Dial(131), XCMS(132) and Skyline(133).

Once data has been processed and peaks have been identified, the next step is to annotate or characterise the data. There is great difficulty that comes with accurate and confident annotation, and this is the bottleneck to current metabolomics analyses(122). The structural complexity and diversity of metabolites and lipids makes confident characterisation difficult. The addition of MS/MS data can improve confidence in annotation. There are several MS/MS public libraries that contain nearly 1 million molecular standards across both positive and negative ion mode that can be used as reference(134,135). Yet with these resources it is estimated that only 10-20 % of molecular features from untargeted LC-MS can be annotated based on the mass spectra library matches alone(122). Annotation can be aided by public libraries such as Lipid Maps(136), where you can input a list of possible masses, filter down by the adducts or lipid class you expect and receive outputs of potential IDs. These can then be considered along with the retention time (R_t) of the metabolite, to try to determine a confident annotation.

Before in-depth statistical analysis and any meaningful conclusions or hypotheses can be drawn, the data is normalised. Data normalisation accounts and removes any variation in the data that comes from non-biological sources such as outliers in ion intensity that may skew data, or batch/sample variation(122,137). There are many forms of data normalisation that can be split into sample-based or data-based(122). In sample-based strategies, the use of internal standards or quality control samples (QCs) can be used to normalise the data. Internal standards are known compounds (typically isotopically-labelled) that are spiked into each

sample and therefore can be identified based on the known m/z and retention time (R_t). Examples of data-based normalisation strategies include median run normalisation (MED) and also total ion count (TIC), where the ion intensity of a feature is divided by the sum of all the ions/features found within a sample(137). There are advantages and disadvantages to each normalisation method, for instance TIC can be susceptible to some features with high ion intensity overly influencing the data(137). Data can then be transformed or scaled, for example using log transformations or pareto scaling, to make intensities of metabolites measured more comparable across the dataset. Doing this can enhance statistical reliability.

Pooled QCs, where aliquots of each sample are combined and injected at the beginning, throughout, and end of the sequence, can track any changes such as R_t drift that occur within the run and data acquisition period. QCs can be used for a data quality filtering step, since features with high relative standard deviation (RSD) in repeat QC injections are likely to be false positives. Features are excluded if they fall above a set RSD threshold(138). Other quality controls include repeated injection of blank samples that have been through the exact same treatment as samples, including extraction and analysis procedures. Features are excluded if their signal is not above a multiple (typically x3) of the signal found in the blanks.

1.5.6. Multivariate statistical analyses

Univariate statistical analysis denotes analysing only one dependent variable at a time, such as t-tests or ANOVA to determine significance(139). Multivariate statistical analysis on the other hand accounts for multiple dependent variables to be measured at the same time(139,140). This analysis can investigate the covariance and correlation between variables in sample sets. The most common methods in this thesis are unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA). The PCA model analyses the data without any knowledge of sample set or groups, meaning visualisation of the underlying data structure is performed without bias. PCA measures variation within the data on a linear scale and then combines these for all comparisons, with valuable information garnered from the correlation pattern observed. It is best used as a summary or overview of your data(140). The objective is to preserve the original variance within the data(140). The simplification of the data allows very complex datasets to be

visualised whilst retaining meaningful changes and displaying them as a summary of the overall dataset. This is achieved by projecting data into principal components (PCs)(141). The first PC (PC1) is chosen to explain the maximum variance in the data shown by the distance between each group, and the subsequent PCs then account for less variance. The PCA will give scores, which show a summary of observations, whilst loadings show a summary of the variables(141). PLS-DA is a supervised analysis for classification of groups based on PLS regression. The strategy of PLS-DA involves first dimension reduction where components are constructed, followed by discriminant analysis and predictive model construction(142). The output is the PLS-DA score or X score, which is the original data (X) at a lower dimension, and the predicted class of the matrix which determines groups within the data (Y)(142). PLS measures covariation in a more condensed manner as it constructs components which can be more easily understood(143), however this method can overfit data. There are variations of PLS-DA; Orthogonal PLS-DA (OPLS-DA) and sparse PLS-DA (sPLS-DA). OPLS-DA can help clarifying the variation that is correlated or not as it detects orthogonal variation. It is used to determine the differences between two groups or classes only and is both a regression and prediction model. sPLS-DA provides simpler interpretation of the data by identifying the smallest subset of data that contains the most relevant variables only(144). In short, sPLA-DA focuses on specific feature selection of importance and OPLS-DA focuses on separating predictive variation from irrelevant noise not related to class separation(145).

1.6. Overall hypothesis and project aims

1.6.1. Hypothesis

The hypothesis of this PhD project is that osimertinib resistant (OR) NSCLC cell lines will possess distinct metabolic phenotypes compared to osimertinib sensitive (OS) NSCLC, measurable by multi-omics approaches. Targeting specific metabolic pathways can therefore be an approach to reinstate sensitivity to osimertinib in drug resistant cells, to allow continued benefit from osimertinib therapy.

1.6.2. Aims

Aim 1: Systematic review

Assess the current literature to review whether/how dysregulated metabolism contributes to drug resistant NSCLC in earlier generations of EGFR-TKIs. We sought to address this by conducting a systematic review of the literature (**Chapter 2**).

Aim 2: Multi-omics profiling of pharmacologically-induced (**Chapter 4**) and genetically-modified (**Chapter 5**) models of OR

Using a multi-omics approach we investigated the transcriptome, proteome, lipidome and metabolome of different models of OR. The overall aim was to identify potential metabolic pathways that may be linked to OR.

Aim 3: Targeting metabolism to reinstate osimertinib sensitivity (**Chapter 6**)

The aim of the final chapter was to determine if sphingolipid metabolism (identified as dysregulated in **Chapter 4**) could be used as a therapeutic target to reinstate sensitivity to osimertinib in OR cells. This would highlight a novel pathway that could be used to prolong overall survival for patients with OR NSCLC.

Chapter 2 – Dysregulated Metabolism in EGFR-TKI
Drug Resistant Non-Small-Cell Lung Cancer: A
Systematic Review

2. Chapter 2: Dysregulated Metabolism in EGFR-TKI Drug Resistant Non-Small-Cell Lung Cancer: A Systematic Review

Published in *Metabolites* Journal in July 2022.

Babuta J, Hall Z, Athersuch T. Dysregulated Metabolism in EGFR-TKI Drug Resistant Non-Small-Cell Lung Cancer: A Systematic Review. Metabolites. 2022 Jul 14;12(7):644.

The full published manuscript can be found in **Appendix Section 9.3**.

2.1. Introduction

The purpose of this study was to critically and systematically review the published literature to give insight into the current landscape on investigating TKI resistance in NSCLC, with a focus on the metabolic adaptations of TKI-resistant cell populations. Understanding the differences between resistant and sensitive cells is vital and has the potential to allow interventions that enable the re-sensitisation of resistant cells to treatment, and consequently reinitiate the therapeutic effect of EGFR-TKIs.

The main literature search was performed in April 2021 using relevant keywords in PubMed and Ovid (Medline) and reviewed using the Covidence platform. Of the 1331 potentially relevant literature records retrieved, 27 studies were subsequently selected for comprehensive analysis. Collectively, the literature revealed that NSCLC cell lines resistant to EGFR-TKI treatment possess characteristic metabolic and lipidomic phenotypic signatures that differentiate them from sensitive lines. Further exploration of these reported differences suggests that drug-resistant cell lines are differentially reliant on cellular energy sources and that modulation of relative energy production pathways may lead to the reversal of drug resistance.

2.2. Material and Methods

The review was conducted and written in accordance with the 2020 Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines(146). A literature

search of the entirety of PubMed and Ovid (MedLine) and a search of articles only from Web of Science were conducted in April 2021. The evidence-based PICO (Patient/Population/Problem, Intervention, Comparison and Outcome) model was used to generate relevant search terms to answer the research question “to understand the role of metabolism on the ability for NSCLC cells to acquire resistance to EGFR treatment”. Terms such as “NSCLC”, “metabolomics”, “lipidomics”, “EGFR therapies” and “drug resistance” along with their synonyms were searched (**Table 2.1**).

Table 2.1: The search terms used in the literature search across all databases.

Entry	Keyword
1	NSCLC.mp
2	Non small cell lung cancer.mp
3	Non-small-cell lung cancer.mp
4	1 or 2 or 3
5	Osimertinib.mp
6	Tagrisso.mp
7	Erlotinib.mp
8	Tarceva.mp
9	Gefitinib.mp
10	Iressa.mp
11	3rd line.mp
12	3rd generation.mp
13	Tyrosine kinase inhibitor.mp
14	EGFR therapy.mp
15	EGFR-targeted therapies.mp
16	EGFR-TKI.mp
17	Epidermal growth factor receptor tyrosine kinase inhibitors.mp
18	5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17
19	Epidermal growth factor receptor.mp
20	EGFR.mp
21	T790M.mp
22	EGFRm.mp
23	19 or 20 or 21 or 22
24	metabolomics.mp
25	metabolite.mp

26	metabolome.mp
27	metabolic profiling.mp
28	metabolic biomarker.mp
29	metabolism.mp
30	metabolic profile.mp
31	metabonomics.mp
32	metabolic phenotyping.mp
33	small molecule metabolites.mp
34	lipidomics.mp
35	24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 or 33 or 34
36	drug resistance.mp
37	resistance.mp
38	36 or 37
39	4 and 18 and 23 and 35 and 38

Collectively, 1331 studies were retrieved and input to Covidence, a “primary screening and data extraction tool” for those conducting systematic reviews(147), and after 175 duplicate papers were excluded, papers were then excluded based on account of no full text article being available or the studies were not primary literature reports (e.g. books/reviews/letters) (Table 2.2).

Table 2.2: The inclusion and exclusion criteria used when compiling the literature included in the analysis.

Inclusion Criteria	Exclusion Criteria
English full text articles	Proteomic studies
Metabolites	No full text
Metabolomic techniques (e.g., LC-MS, GC-MS, NMR spectroscopy)	Non-EGFR treatments/pathways
EGFR therapies	Books, reviews, letters, conference abstracts
Original data/results	New treatments to overcome TKI resistance

TKI resistance	RNAseq
Genes/pathways linked to metabolism	Other cancers with EGFR treatment
Metabolic pathways (glycolysis) etc.	

Figure 2.1 depicts the full process of this systematic review based on the PRIMSA guidelines for systematic reviews(148). Finally, 27 studies were selected for deeper analysis. A summary of these papers can be found in the **Appendix Section 9.1, Appendix Table 9.1**.

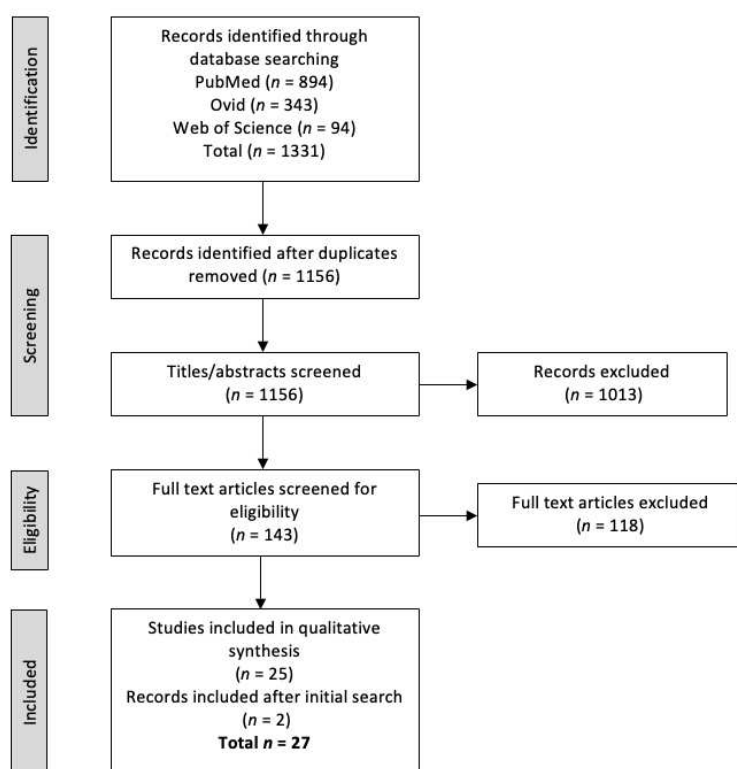


Figure 2.1 PRISMA flow diagram summarising the review process of all papers. Adapted from Moher et al(151).

2.3. Results and Discussion

From the 27 studies selected for comprehensive analysis by review of the full text and all supplementary materials, three main themes were identified: (i) metabolome rewiring, (ii) reversal of drug resistance by metabolic intervention and (iii) alterations in dysregulated lipid metabolism. These are discussed in turn below. **Figure 2.2** is a visual aid representing the

number of papers found within each section, with some papers being discussed in more than one section.

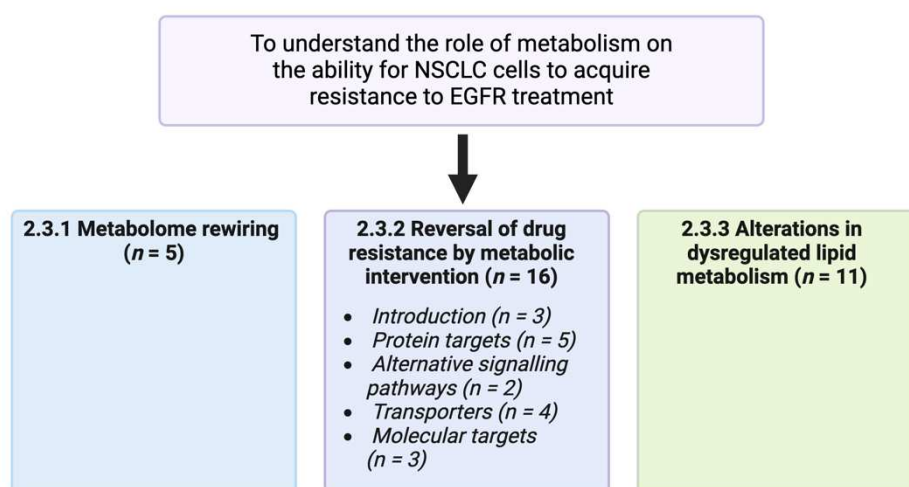


Figure 2.2. Schematic representation of the main themes of this review. With a total of 27 studies selected for deeper analysis, some papers fall into more than one section. Created using BioRender.com.

2.3.1. Metabolome Rewiring

The gold standard for measuring the metabolic phenotype of cells is metabolomics; in this context to compare and contrast the metabolite profiles of EGFR-mutant treatment sensitive and treatment resistant NSCLC cells. Our review of the literature highlighted key multiple metabolic pathways altered during the onset of drug resistance.

Using a ^1H NMR-based metabolomic approach, Li et al., 2016, identified 36 assigned metabolites that differentiated erlotinib-resistant and sensitive cell lines(83). Many of these were involved in GSH metabolism, amino acid and nucleotide synthesis, and choline metabolism—a precursor for phospholipid synthesis(83). Interestingly, there was a notable decrease in the intracellular levels of GSH in resistant cell lines. Ma et al., 2020 took a multi-omics approach, utilising data from metabolomics obtained via LC-MS and proteomics to compare OR and sensitive cells using multivariate statistics(7). Overall, 54 metabolites were found to have different abundances between resistant and sensitive cells, and these were largely related to amino acid and nucleotide metabolism, implicating these pathways in the development of osimertinib resistance(7).

Using capillary electrophoresis-time of flight MS (CE-TOF-MS), Serizawa et al., 2014, measured the metabolic profile of erlotinib-resistant and sensitive cell lines and determined 18 metabolites that significantly contributed to distinguishing sensitive from resistant cell lines(82). These included glucose 6-phosphate and other glycolytic intermediates, which were all lower in resistant cells compared to sensitive cells. Furthermore, metabolites relating to glutamine metabolism were higher in resistant cells(82). This suggests that there was a shift away from glycolysis as the main energy pathway in resistant cells.

Thiagarajan et al., 2016, used transcriptomics and metabolomics, which determined that transforming growth factor beta (TGF- β) contributed to adaptive drug-escape, a phenomenon where cells are able to acquire new methods of resistance, along with altered metabolic bioenergetics, regulated by mitochondrial function(84). Mass spectrometry found a global change in energy metabolism when the cells were in their drug-escape phase, with alterations in both the glycolytic and TCA cycle intermediates(84). There were also significant changes in lipid metabolism, with precursors of fatty acid synthesis elevated in cells after 9 days of erlotinib treatment.

In addition to drug resistance, drug tolerance can also occur(85). Drug tolerance can be defined as a group of cells which survive the initial exposure to TKIs, and are often the pool of cells in which the majority of drug-resistant cells arise from(10). Zhang et al., 2019, report that EGFR^m drug-resistant cells can emerge from drug tolerant cells(85), and therefore imply that these cells should be considered a therapeutic target to prevent resistance. This study used a targeted metabolomics approach via LC-MS/MS, to identify key mediators in the TCA cycle that were altered, for example increased succinate, which suggested loss of function of SDH. SDH is a stabilising agent to the hypoxic inducible factor α (HIF1 α), therefore the study concluded that the alteration of the TCA cycle can activate a hypoxic response, enabling drug tolerance to develop(85).

2.3.2. Reversal of Drug Resistance by Metabolic Intervention

The Warburg effect, as previously stated, describes a switch in energy metabolism where cancer cells adapt to gaining their energy supply from aerobic glycolysis, as opposed to

oxidative phosphorylation(42,43,86). **Figure 2.3** shows the pathways of glycolysis and oxidative phosphorylation. Reversing this altered metabolism is therefore a way to treat cancer cells.

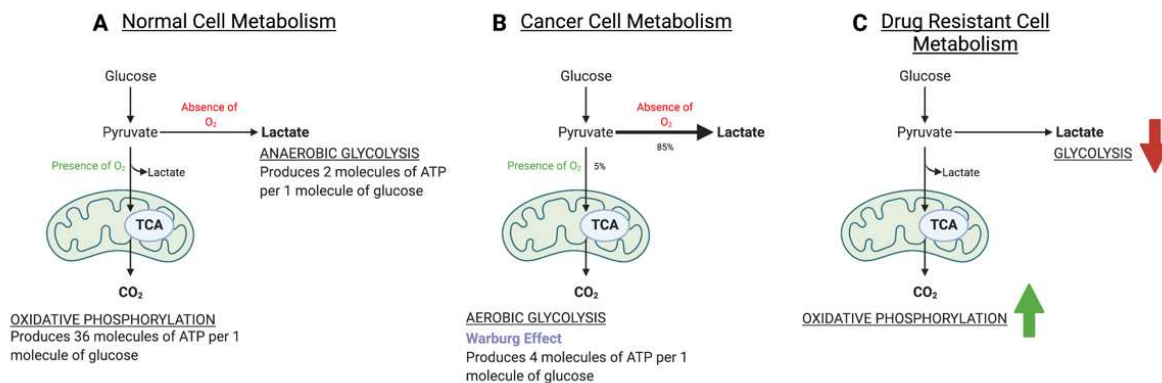


Figure 2.3: Cellular energy metabolism. (A) In normal cells in the presence of oxygen, cells produce around 36 ATP molecules per glucose via glycolysis and the TCA cycle. In the absence of oxygen, cells accumulate lactate and produce only 2 ATP molecules. (B) In cancer cells both in the presence and absence of oxygen only use glycolysis to produce around 4 ATP molecules, whilst producing more lactate than normal cells in the absence of oxygen. (C) Drug-resistant cells favour oxidative phosphorylation to provide energy over aerobic glycolysis. Adapted from Martin MJ et al.(86). Created using BioRender.com.

De Rosa et al., 2015, investigated the effects of EGFR targeting TKIs on glucose metabolism in NSCLC, reversing the Warburg effect(43). They found that efficient inhibition of EGFR signalling restored oxidative phosphorylation as the primary energy source in NSCLC. This inhibition also regulated aerobic glycolysis via downregulation of hexokinase II (HKII); an enzyme that phosphorylates glucose to glucose-6-phosphate in the initiating step of glycolysis(43) and phospho-pyruvate kinase M2 (p-PKM2); which catalyses the formation of pyruvate from phosphoenolpyruvate (PEP), the final step of glycolysis (See **Figure 2.3** for a schematic of the steps in glycolysis)(149). Therefore, it is key to understand the mechanisms behind this shift between both energy pathways to prevent or reverse resistance. Kim et al., 2018, found that the mutation within EGFR itself enhanced glycolysis, which was required for EGFR stability(150). In addition, they determined that depriving resistant cells of glucose inhibited glycolysis, inducing apoptosis.

As a preference for oxidative phosphorylation over glycolysis is a key energy metabolism hallmark of resistant cells, targeting the pathway itself would be one way to revert cells to

favouring glycolysis(86). In a parental EGFRm line, osimertinib suppressed glycolysis and favoured oxidative phosphorylation(86), but does not have this effect in resistant lines. By targeting a component of the electron transport chain (ETC); complex I, with an inhibitor metformin, this study found that when used in conjunction with osimertinib, the development of drug resistance was inhibited in vitro(86).

The metabolomic studies highlighted that key metabolic pathways are altered in drug resistance including the TCA cycle and glycolysis. Taking this further, a second core theme of the papers selected in this systematic review was that dysregulated cellular metabolism in TKI-drug resistance can be reversed by an intervention to reinstate drug sensitivity.

Protein Targets

To look at the dysregulation of energy metabolism in TKI-resistant cells, with the intention to resensitise cells to treatment and reinstate therapeutic effect, targeting proteins implicated in metabolism was an approach used by several authors(1,151–154).

5' adenosine monophosphate-activated protein kinase (AMPK) is a sensory kinase activated by intracellular levels of ATP and AMP(152). Its downstream targets, such as mTOR and acetyl-CoA carboxylase (ACC)(151), when phosphorylated, redirect metabolism towards increased catabolism(155). Metabolic stress through direct activation of AMPK by 2-deoxy-D glucose (2DG), which in turn inhibits glycolysis, enhanced cellular sensitivity and therefore the anti-cancer effect of both afatinib and gefitinib in separate studies(151,152).

Pyruvate dehydrogenase (PDHK1) is a protein complex that when activated can inhibit glycolytic activity(1). To investigate pyruvate metabolism, transcriptomics data from two separate NSCLC cohorts was interrogated and found increased expression of PDHK1. Upon inhibition with dichloroacetate (DCA) in combination with an EGFR inhibitor, metabolism was rewired to favour pyruvate oxidation whilst reducing lactate production, therefore inhibiting glycolysis and increasing the therapeutic effect of the EGFR inhibitor(1).

Targeting glutamine metabolism, a key nutrient for cancer cells, can also be a potential strategy to overcome drug resistance in NSCLC(153). A compound that targeted glutaminase

C (GAC) in combination with erlotinib downregulated both glutamine and glucose metabolism in erlotinib-resistant cells and reinstated sensitivity(153).

Alternative signalling pathways

A further approach to reinstate sensitivity to TKIs and downregulate resistant cells' requirement for glycolysis is by targeting alternative signalling pathways.

Ye et al., 2017, targeted Akt expression in resistant cells in combination with inhibiting glucose metabolism(156). Akt and autophagy were found to be more activated in resistant cells than in the parental line. The study found that an increased uptake of glucose via GLUT1, a major glucose transporter, was a feature of resistant cells, and removing glucose would reverse resistance. However, drug sensitivity did not increase until they combined this with suppressing Akt phosphorylation via an inhibitor MK2206(156).

Cellular metabolism is known to change when cancer cells are under hypoxic conditions, due to the lack of oxygen to the cells. This can result in drug resistance for those drugs which require oxygen to be cytotoxic, or of interest in this review, by the alteration of metabolism(157). In hypoxic conditions, HIFs are activated and can induce a multidrug efflux transporter, p-glycoprotein, and therefore convey drug resistance(7). Ma et al., 2020, found that expression of upstream proteins of HIF1 α were significantly increased in OR cells. Bypass signalling pathways, such as the PI3K/Akt pathway, were also found to be enriched in OR cells(7,156).

Transporters

Transporters play key roles in determining cellular energy fate. An upregulation of a key transporter involved in glycolysis for example, such as GLUT1, would result in more glucose being transported into the cell(156), and if you inhibit this with an inhibitor such as dehydroascorbate (DHA), which is oxidized vitamin C and taken up by GLUT1, glycolysis is inhibited(158). Another study found that GLUT1 expression, and therefore glucose uptake, were more prominent in gefitinib-resistant cells, and once genetic and pharmacological inhibition of GLUT1 was established, resistant cells were re-sensitised to therapy(159). One study found that monocarboxylate transporter 1 (MCT-1), a transporter that secretes lactate

into extracellular space, was upregulated in TKI-resistant cells compared to sensitive cells(160).

Molecular Targets

Bach et al., 2018, investigated bone morphogenetic proteins (BMPs), which belong to the TGF- β family and control many processes including cell differentiation and tumour growth(161). Activation of BMPs has been known to confer resistance to EGFR-TKIs and to test this Bach et al., 2018, used gefitinib-resistant cells to determine the gene expression of these cells compared to parental sensitive cells(161). BMP4 plays a role in energy metabolism through regulation of ACSL4, which is key in lipid metabolism and fatty acid oxidation, as it catalyses the conversion of PUFAs to acyl-CoAs(161). TGF- β was also found to aide adaptive drug-escape through transcriptomics and metabolomics, with cells at this point able to exhibit adaptive metabolic bioenergetics, once again linking back to mitochondrial function(84).

mTORC2 was found to play a role in metabolic reprogramming in erlotinib-resistant cells, as these cells had more metabolic flexibility when compared to sensitive cells, measured via spare respiratory capacity (SRC). SRC is a measure of the extra mitochondrial availability, i.e., the ability for increased ATP production via oxidative phosphorylation, and is an indicator of metabolic reprogramming(162). Knockdown of an mTORC2 component, Rictor, increased SRC in resistant cells. This, therefore, implies that mTORC2 mediated the metabolic reprogramming of resistant cells, as resistant cells before treatment intervention have lower SRC and therefore lower mitochondrial activity(162).

2.3.3. Alterations in Dysregulated Lipid Metabolism

Lipid metabolism is tightly linked to glycolysis and oxidative phosphorylation(51)(167). Lipid metabolism has been found to be dysregulated in TKI-resistant cells compared to TKI-sensitive cells. In particular, DNL is favoured by resistant cells as opposed to utilising endogenous lipids(49). FASN is a key enzyme required for DNL and is frequently upregulated in cancer, whilst *SREBPs* are master transcription factors that regulate lipid and cholesterol metabolism(87–89). Ali et al., 2018 demonstrated a novel link between EGFR signalling and FASN expression in resistant cells. EGFR signalling knockdown decreased levels of both FASN

and its activator SREBP1, demonstrating that blocking lipid synthesis can be of therapeutic benefit to resistant cells(87). This was then confirmed with pharmacological inhibition of FASN using orlistat, which had a cytotoxic effect on gefitinib-resistant cells(87).

Chen et al., 2021, also determined a link between SREBP1 and EGFRm response to osimertinib, which found that the mature form of SREBP1 (mSREBP1) was degraded in the presence of osimertinib, suppressing lipogenesis. However, once these cells acquired resistance to osimertinib, the ability to suppress lipogenesis was lost, and mSREBP1 levels began to increase once more(165). This study used untargeted lipidomic analysis on osimertinib-treated cells compared to vehicle to determine how treatment changed the lipidomic profile of cells. This identified 148 lipid metabolites, with 50 of these including TGs, DGs, Cers, PEs, SMs and PUFA PEs showing a significant decrease in abundance in osimertinib treated cells, highlighting how drug treatment can change the lipidomic phenotype of a cell(165).

Xu et al., 2021, used lipidomics to show that gefitinib treatment altered the ratio of saturated and unsaturated phospholipids in resistant and sensitive cell lines, highlighting that prolonged SREBP1 activation is key for *DNL*, and resistant cells do in fact rely upon this mechanism(166). Another investigation in SREBP1 signalling by Li et al., 2016, found that intervening with MARVELD1, a SREBP binding partner, inhibits lipogenesis and improves therapeutic effects of TKIs(89). As mentioned above, when AMPK is activated, it acts upon ACC, which is vital for the de novo fatty acid biosynthesis pathway. Its activation incurred metabolic stress and inhibited glycolysis, which enhanced TKI therapeutic effect(151,152).

Cholesterol is an essential cell membrane lipid and plays an integral role in maintaining cell function and integrity(167). Cholesterol exists in pockets of the plasma membrane, known as lipid rafts(167). In gefitinib-resistant cells, cholesterol levels within these lipid rafts were higher than in gefitinib-sensitive cells. This knowledge prompted Chen et al., 2018, to determine if there was a link between intracellular cholesterol levels and resistance to EGFR-TKIs(167). They found that cholesterol inhibited binding of gefitinib to EGFR, resulting in resistance. Overall, depletion of cholesterol levels restored drug sensitivity in these resistant cell lines(167). An alternative strategy to target high cholesterol levels in resistant cells is to

target the key mediator of cholesterol uptake; the low-density lipoprotein receptor (LDLR), which is upregulated via EGFR activation through SREBP1(168). Despite this study focusing more on overall therapeutic benefit of TKIs as opposed to resistance, Luo et al., 2021, highlighted another potential way to reduce cholesterol levels.

SCD1 is a key enzyme in lipogenesis, responsible for converting SFAs into MUFAs(169). These can then be incorporated into neutral lipids that are stored in lipid droplets (LDs), which have a high expression in cancer cells(169). Huang et al., 2019, investigated the link between SCD1 mediated lipogenesis and TKI resistance. Intracellular LDs were found to be of a higher proportion in resistant cells, along with expression of SCD1 and its enzymatic product oleic acid. The resistance was overcome by combined pharmacological inhibition of SCD1 with 20(S)-protopanaxatriol and EGFR-TKI(169).

Jung et al., 2015, set out to investigate the phospholipid composition of extracellular vesicles (EVs) that tumour cells secrete(170). Using matrix-assisted laser desorption/ionization (MALDI) MS to look at lipid profiles of resistant and sensitive cells, this study found that there were 67 phospholipids, including PCs, LysoPCs, SMs, PGs, PIs and LysoPIs, that had varying abundances in resistant vs. sensitive cells(170). This result highlights once again that resistant and sensitive cells possess distinct lipidomic phenotypes, suggesting a novel way to reinstate therapeutic effect.

2.4. Conclusions

The investigation into cellular metabolism dysregulation in EGFR-TKI resistant cells compared to those cells that are still sensitive to drug treatment, can elucidate several mechanisms within key pathways that may be targeted to prevent or reverse resistance.

The Warburg effect is the switch seen in cancer cells in which they favour aerobic glycolysis over oxidative phosphorylation, even in oxygenated conditions(171). However, the reverse is true of drug-resistant cell populations(86). More recently, perturbed lipid metabolism has been described in cancer cells and drug-resistant cells. In particular, DNL has been found to be upregulated in drug-resistant cells compared to sensitive cells(165).

This systematic review has highlighted the importance of investigating metabolomic and lipidomic remodelling, and the pathways that are altered allowing resistant cells to dysregulate cellular metabolism. In the papers summarised here, the studies use both metabolomics and lipidomics to investigate the phenotype of resistant cells, to understand the changes observed from gene expression to metabolite abundance.

By elucidating the metabolome and lipidome changes found within resistant cells and comparing these to sensitive cells, specific metabolic pathways can be targeted alongside EGFR-TKI treatment to allow continued therapeutic benefit.

Chapter 3 – Materials and Methods

3. Chapter 3 – Materials and Methods

3.1. Cell Culture

3.1.1. Cell lines

All cell lines were kindly provided by Matthew Martin within the Innovative Medicines team at AstraZeneca Oncology in 2021. Pharmacologically-induced resistant cell lines were generated by chronically dosing PC9 parental cell line (PC9P) with increasing concentrations of osimertinib to a maximum dose of 160 nM, producing 2 osimertinib resistant (OR) clones; PC9R5 and PC9R6, previously described by Eberlein *et al*(172). PC9P was used as the osimertinib sensitive (OS) control. Genetically modified (GM) resistant clones were generated through CRISPR gene editing to produce a PC9 PTEN-knockout line (PC9 PTEN -/-) and a PC9 resistant line which harbours a PIK3CA H1047R point mutation (PC9 PI3KCAm)(173). A PC9 non targeting control (PC9 NTC) was used as the OS control. PC9P, PC9R5, PC9R6 and PC9 PI3KCAm were authenticated in 2024 by Eurofins. PC9 NTC and PC9 PTEN -/- were authenticated in 2019 and 2020 respectively.

3.1.2. Drug preparation

Osimertinib (AZD9291) was provided by AstraZeneca. DL-threo-PDMP (hydrochloride) was purchased from Cambridge Bioscience (CAY10005276). C2-Ceramide (N-Acetyl-D-sphingosine) was purchased from Sigma-Aldrich (A7191). Stocks of all were prepared in DMSO (Sigma-Aldrich, D2650) and aliquots were stored at -20 °C.

3.1.3. Pharmacologically-induced OR models (PC9R5; PC9R6) and PC9 control

All cell lines were grown in RPMI 1640 (Sigma), supplemented with 10 % fetal bovine serum (FBS) (ThermoFisher, 10270106), 2 mM L-glutamine (ThermoFisher, 25030-081) and 100 units/mL penicillin-streptomycin (P/S) (ThermoFisher, 15070-063). The resistant lines (PC9R5 and PC9R6) grew in the same conditions as the parental with an addition of 160 nM osimertinib. Cell lines were incubated at 37 °C with 5 % CO₂ and passaged when they reached 80 % confluency. Cell lines were authenticated using Eurofins and regular mycoplasma testing was performed.

3.1.4. Genetically modified OR models (PC9 PTEN -/-; PC3 PI3KCAm) and PC9 NTC control

All cell lines were grown in RPMI 1640 (Sigma), supplemented with 10 % fetal bovine serum (FBS; ThermoFisher, 10270106), 2 mM L-glutamine (ThermoFisher, 25030-081) and 100 units/mL penicillin-streptomycin (P/S) (ThermoFisher, 15070-063). Cell lines were incubated at 37 °C with 5 % CO₂ and passaged when they reached 80 % confluency. Cell lines were authenticated using Eurofins and regular mycoplasma testing was performed.

3.2. Experimental procedures

3.2.1. Sulphorodamine B (SRB) cell growth assay for osimertinib cytotoxicity and dose response

SRB assay was used to determine cell viability and inhibition of cell growth. Cells (PC9P, PC9R5, PC9R6, PC9 NTC, PC9 PTEN -/-, PC9 PI3KCAm) were seeded in 96-well plates at a density of 20000 cells/well in 200µL of respective media. Cell counts were performed using the Vi-Cell XR Cell Viability Analyzer (Beckman Coulter). Cells lines were incubated at 37 °C with 5 % CO₂ for 24 hours. Spent medium was removed and cells were then incubated in 100uL of media containing 0.0001, 0.001, 0.01, 0.1, 1, and 10 µM osimertinib and vehicle control (0.1 % DMSO) for 72 hours. Six technical replicates were used for each condition. Cells were fixed by addition of 10 % (w/v) trichloroacetic acid (TCA; 25 µL) solution (Sigma-Aldrich, T6399) to each well and left to incubate at 4 °C for 1 hour. The plate was washed using cold water to remove the TCA and incubated at room temperature overnight. Cells were then stained with 100 µL of 0.4 % (w/v) SRB solution (Sigma-Aldrich, 230162) in 1 % (v/v) acetic acid (ThermoFisher, 10005920) for 30 minutes before being aspirated off the cells and the plate was rinsed with 1 % acetic acid (v/v) to remove all unbound dye. Plates were incubated at room temperature overnight. 200 µL of 10 mM Tris Base solution (Sigma-Aldrich, T1699) was added to each well and left on a shaker for 30 minutes to mobilise the bound SRB dye. A second plate identical to the first was set up with 200 µL of 10mM Tris Base solution, and 50 µL of the original plate was added to this second plate and pipetted up and down to dilute the sample 1:5. Absorbance was measured using a CLARIOstar plate reader (BMG LABTECH), with OD set to 545 nm. Cell free wells were used as blanks to remove background noise. Values were

normalised to vehicle control and plotted using GraphPad Prism v10.2.0. Experiments were repeated for $n = 3$.

3.2.2. SRB assay for D-PDMP and C2-Ceramide cytotoxicity and dose response

Cells (PC9P, PC9R5 and PC9R6) were seeded in 96-well plates at a density of 20000 cells/well in 200 μ L of respective media. Cell counts were performed using the Vi-Cell XR Cell Viability Analyzer (Beckman Coulter). Cells lines were incubated at 37 °C with 5 % CO₂ for 24 hours. Spent medium was removed and cells were then incubated in 100 μ L of media containing D-PDMP (10 μ M, 20 μ M, 30 μ M, 40 μ M and 50 μ M) or C2-Ceramide (10 μ M, 20 μ M, 30 μ M, 40 μ M and 50 μ M) as well as vehicle (0.1 % DMSO) for 72 hours. Six technical replicates were used for each condition. Data was acquired using the SRB assay described above (**Section 3.2.1**). Experiments were repeated for $n = 3$. Graphs were generated using GraphPad Prism v10.2.0.

3.2.3. D-PDMP in combination with osimertinib

Cell lines (PC9P, PC9R5 and PC9R6) were seeded in 96-well plates at a density of 20000 cells/well in 200 μ L of respective media. Cells lines were incubated at 37 °C with 5 % CO₂ for 24 hours. PC9P cells were treated with vehicle, osimertinib (1 μ M and 10 μ M) and/or D-PDMP (15 μ M, 35 μ M and 50 μ M) for 72 hours. PC9R5 cells were treated with vehicle, osimertinib (1 μ M, 5 μ M and 10 μ M) and/or D-PDMP (10 μ M, 25 μ M and 50 μ M) for 72 hours. PC9R6 cells were treated with vehicle, osimertinib (1 μ M, 5 μ M and 10 μ M) and/or D-PDMP (10 μ M, 15 μ M and 30 μ M) for 72 hours. Three technical replicates were used per condition. Data was acquired using the SRB assay described above (**Section 3.2.1**). Experiments were repeated for $n = 3$. Graphs were generated using GraphPad Prism v10.2.0.

3.2.4. C2-ceramide in combination with osimertinib

Cell lines (PC9P, PC9R5 and PC9R6) were seeded in 96-well plates at a density of 20000 cells/well in 200 μ L of respective media. Cells lines were incubated at 37 °C with 5 % CO₂ for 24 hours. PC9P cells were treated with vehicle, osimertinib (1 μ M and 10 μ M) and/or C2-

Ceramide (5 μ M, 20 μ M and 50 μ M) for 72 hours. PC9R5 cells were treated with vehicle, osimertinib (0.1 μ M, 1 μ M and 10 μ M) and/or C2-Ceramide (5 μ M, 20 μ M and 50 μ M) for 72 hours. PC9R6 cells were treated with vehicle, osimertinib (0.1 μ M, 1 μ M and 10 μ M) and/or C2-Ceramide (10 μ M, 40 μ M and 50 μ M) for 72 hours. Three technical replicates were used per condition. Data was acquired using the SRB assay described above (**Section 3.2.1**). Experiments were repeated for $n = 3$. Graphs were generated using GraphPad Prism v10.2.0.

3.2.5. Caspase assays (Caspase-Glo® 3/7 Assay)

PC9P, PC9R5 and PC9R6 cells were cultured in 96 well plates, one plate was used for an SRB assay and another white bottomed plate was used for the Caspase-Glo® 3/7 Assay (Promega, G8092). 24 hours after seeding, cells were treated with either vehicle, osimertinib and/or D-PDMP/C2-Ceramide for 72 hours. After treatment, the SRB plates were fixed and processed using the described SRB assay protocol. The plate for the Caspase-Glo® 3/7 Assay were processed following manufacturers guidelines. This protocol was modified slightly to only add 30 μ L of combined caspase reagent to 30 μ L of media within the 96 well plates. Luminescence was measured using a CLARIOstar Plate Reader (BMG Labtech). Caspase activity was background corrected using cell-free wells and normalised to the SRB cell mass data and to VC wells. Graphs were generated using GraphPad Prism v10.2.0.

3.2.6. Dual phase modified Folch extraction for metabolite and lipid extraction

Cells were cultured in 6cm Cornell plates (6 technical replicates) for 48 hours. Media was removed and the cells were washed with 1 mL cold PBS. 1 mL of ice cooled HPLC grade methanol was added and the cells were scraped and collected. A further 500 μ L of HPLC grade methanol was added, scraped and collected. The samples were then dried under nitrogen flow. Folch dual phase extraction was used to then obtain the organic layer for lipidomic analysis and the aqueous layer for metabolomic analysis, both via LC-MS. 100 μ L of a pre-mixed internal standard mix (**Table 3.1**) and 950 μ L of HPLC grade 2:1 chloroform/methanol was added to the dried down samples. The samples were sonicated for 5 minutes. 400 μ L HPLC grade water was added to each sample and then vortexed for 20 seconds, followed by centrifuging the samples for 10 minutes at 13,000 rfc. Both layers were removed separately

using a glass Pasteur pipette and placed into LC-MS vials. Once the layers were removed leaving only the protein pellet, a further 500 μ L 2:1 HPLC grade chloroform/methanol and 200 μ L HPLC grade water was added and the samples were again vortexed for 20 seconds and centrifuged for 10 minutes at 13,000 rfc. The layers were removed as a above and dried down under nitrogen flow before being stored at -80 °C.

Table 3.1: Internal Standard Mix (ISM) used for untargeted lipidomics analysis*.

Standard	Concentration (mg/mL)	Solvent
Fatty acid C12 (FA12) – 12:0-d5 FA	10mg/mL	MeOH
Fatty acid C15 (FA15) – 15:0-d29 FA		MeOH
Fatty acid C17 (FA17) – 17:0-d33 FA		MeOH
Fatty acid C20 (FA20) – 20:0-d39 FA		MeOH
Ceramide (Cer) – C16-d31 Cer		MeOH
Lyso-phosphocholine (LPC) – 14:0-d29 LPC-d13		MeOH
Phosphatidic acid (PA) – 16:0-d31-18:1 PA		MeOH
Phosphatidylcholine (PC) – 16:0-d31- 18:1 PC		MeOH
Phosphatidylethanolamine (PE) – 16:0- d31-18:1 PE		MeOH
Phosphatidylglycerol (PG) – 16:0-d31- 18:1 PG		MeOH
Phosphatidylinositol (PI) – 16:0-d31- 18:1 PI		MeOH
Phosphatidylserine (PS) – 14:0 PS-d54		MeOH
Sphingomyelin (SM) – 16:0-d31 SM		MeOH
Cholesterol Ester (CE) – 18:0-d6 CE		1:1 (CHCl ₃ :MeOH) (v/v)
Triacylglyceride (TG45) – 45:0-d87 TG		1:1 (CHCl ₃ :MeOH) (v/v)
Triacylglyceride (TG48) – 48:0-d93 TG		1:1 (CHCl ₃ :MeOH) (v/v)
Triacylglyceride (TG54) – 54:0-d105 TG		2:1 (CHCl ₃ :MeOH) (v/v)

* All standard stock solution above were then diluted 100-fold in methanol to form the final ²H IS Lipid Mix.

3.2.7. Preparation of organic layers for lipidomics analysis

The organic layers from all cell lines obtained via the earlier mentioned dual phase Folch extraction were reconstituted in 100 µL of 2:1 HPLC grade chloroform/methanol. 20 µL of this was added to a new vial inside a vial insert and 180 µL of HPLC grade 2:1:1 isopropanol (IPA)/acetonitrile (ACN)/water was added to dilute the sample 1:10. Each sample was vortexed for 10 seconds.

3.2.8. Preparation of small molecule aqueous metabolites for metabolomics analysis

The aqueous layers from all cell lines obtained via the earlier mentioned dual phase Folch extraction were reconstituted in 80 µL of a Mobile Phase A (H₂O + 0.2 % Formic Acid (FA) (v/v)). 20 µL of the ISM (components can be found in **Table 3.2**) was also added and each sample was vortexed for 10 seconds before being transferred to a vial insert.

Table 3.2: ISM used for untargeted small molecule metabolite analysis.

Standard	Concentration (µg/ml)	Product Number
Fumaric acid-13C4	10	Sigma (606014)
Lysine HCl, D4	10	Sigma (616192)
L-valine-13C5	20	Sigma (758159)
L-aspartic acid-13C4	20	Sigma (604852)
Succinic acid-13C4	20	Sigma (491983)
Octadecanoate	100	NA
L-glutamic acid-13C5	20	Sigma (604860)
L-isoleucine-13C6,15N	5	Sigma (608092)
L-glutamine-13C5	800	Sigma (604690)
Citric-2,2,4,4,-D4 acid	30	Sigma (485438)
Methionine	200	Sigma (299154)
Phenyl-d5-alanine	10	Sigma (615870-1G)

Myristic-d27	20	Sigma (366889-1G)
Uracil-13C, 15N2	10	Sigma (608459)
Uridine 5,6-D2	10	Santa Cruz Biotechnology (sc-213138)
Nicotinate	5	Supelco (V-034)
Carnitine, C0-d3	10	NA
Carnitine, C2-d3	0.5	NA
Carnitine, C8-d3	0.5	NA
Carnitine, C16-d3	0.5	NA
DCA	2	NA
LCA	0.04	NA
GCA	0.4	NA

3.2.9. Preparation of protein pellets for proteomics analysis

The following sample preparation was performed by Dr Pavel Shilaha and Dr Alex Montoya at the Medical Research Council Laboratory of Medical Sciences London (MRC LMS). Protein pellets were resuspended through addition of 150 μ L of SEPOD surfactant cocktail buffer to the pellet, sonicating at 100 % energy for 2 minutes on a Horn Sonicator before shaking at 1300 rpm on an orbital shaker at 70 °C, as previously described(174). The Pierce BCA Protein Assay Kit (ThermoFisher, 23225) assay(175), was used to determine protein concentration and then was adjusted to 2g/L. 150 μ L of each sample (300 μ g) were reduced and alkylated via addition of equal volume of water containing 20 nM tris(2-carboxyethyl)phosphine (TCEP) and 40 mM chloroacetamide. The samples were then subjected to precipitated assisted capture (PAC)(176) via addition of 1.5 mg of hydroxyl beads (MR-HYX010) and 450 μ L ethanol. The beads were then washed with 500 μ L of 80 % ethanol and digested overnight in 300 μ L of 50 mM ammonium bicarbonate buffer containing 20 ng/ μ L Trypsin and 10 ng/ μ L Lys-C whilst shaking at 1300 rpm in Eppendorf deep well plates. The resulting digest was then transferred to a new plate and the resin was washed with 50 μ L of 1 % trifluoroacetic acid (TFA). The washing solution was combined with the final digest.

3.3. Mass Spectrometry analysis

3.3.1. Lipid analysis via untargeted Reverse Phase method (LC-MS)

The organic layers from all cell lines obtained via dual phase Folch extraction were analysed by LC-MS using an UltiMate 3000 HPLC system coupled to an LTQ-Orbitrap Elite MS system (Thermo Scientific). A 10-minute reverse phase liquid chromatographic method was used to separate lipid species prior to detection by MS in both positive and negative ionisation modes. Separation was achieved using an Acquity UPLC C18 BEH column, 130Å, 1.7 µm, 2.1 mm X 50 mm, 1/pk (Waters Corporation). Mobile phases were prepared with UPLC grade solvents and chemicals (**Table 3.3**): A = 60 % ACN, 40 % H₂O with 10 mM ammonium formate. B = 90 % IPA, 10 % ACN with 10 mM ammonium formate. C = 60 % ACN, 40 % H₂O and 10 mM ammonium acetate. D = 90 % IPA, 10 % ACN and 10 mM ammonium acetate. The mobile phase gradient elution for positive and negative 10-minute reverse phase mode can be found in **Table 3.4**. 5 µL of each sample was injected in positive mode and 10 µL was injected in negative mode, both at a flow rate of 0.5 mL/min at a column temperature of 55 °C. QC pooled samples were used in all experiments and injected every 5 samples to measure quality throughout the run. CID MS/MS fragmentation was performed on the QC sample only. A blank which was taken through all sample preparation steps was also used and injected every 5 samples. The spectra were acquired in both ionization modes in the range of 110-2000 m/z at 60,000 mass resolution. This method has been previously described(177).

Table 3.3: Mobile Phase composition for Untargeted Reverse Phase Method for lipids

Reverse Phase Mobile Phases	Composition
Mobile Phase A (positive)	60 % ACN + 40 % H ₂ O + 10 mM ammonium formate
Mobile Phase B (positive)	90 % IPA + 10 % ACN + 10 mM ammonium formate
Mobile Phase C (negative)	60 % ACN + 40 % H ₂ O + 10 mM ammonium acetate
Mobile Phase D (negative)	90 % IPA + 10 % ACN + 10 mM ammonium acetate

Table 3.4: Gradient elution for Reverse Phase Method for Lipids in positive and negative mode

Time (mins)	Flow rate (mL/min)	Mobile phase A/C (%)	Mobile phase B/D (%)
0	0.5	60	40
0.8		57	43
0.9		50	50
4.8		46	54
4.9		30	70
5.8		19	81
8		1	99
8.5		1	99
8.6		60	40
10		60	40

3.3.2. Processing and analysis of lipidomics data – an untargeted workflow

Data was acquired using Xcalibur (Thermo) and converted from a raw format to mzML via ProteoWizard MS Convert software(178). The mzML files were processed using the XCMS package in R studio (Version R4.2.2.2), using R scripts developed by Nick Bond and modified by Dr Zoe Hall (Imperial College London). All data was blank and QC filtered, whereby features were retained if the average signal was more than 3 times the signal of the blank and less than 20 % RSD for QC repeat injections, before being normalised to TIC and Pareto scaled, which reduces the influence of intense peaks(140). Lipids were annotated based on RT patterns and accurate mass database searches in Lipid Maps® “Bulk” Structure Searches Database (LMSD)(179). Confidently annotated lipids were annotated based on mass, adducts, Rt and MS/MS. Metaboanalyst v6.0 was used for multivariate analysis of the data and generated all statistical figures, except for S-plots which were created using SIMCA v7 (Sartorius). GraphPad Prism v10.2.0 was used to generate any box plots.

3.3.3. Small molecule metabolite analysis via untargeted Luna method (LC-MS)

The aqueous layers from all cell lines obtained via Folch extraction were analysed by LC-MS using an Exion UPLC system coupled to a ZenoTOF 7600 MS system (SCIEX/Danaher, WA, USA). A Luna Omega Polar C18 column, 150 mm × 2.1 mm × 1.6 µm (Phenomenex/Danaher, WA, USA) was used for chromatographic separation in both positive and negative ion mode. Mobile phases were prepared with UPLC grade solvents and chemicals (**Table 3.5**). 5 µL of each sample was injected in both positive and negative modes at a flow rate of 0.45 mL/min at a column temperature of 40 °C for 20 minutes in positive mode and 14 minutes in negative mode. The mobile phase gradient elution for positive and negative modes can be found in **Table 3.6 and 3.7**. QC pooled samples were used in all experiments and injected every 6-8 samples to measure quality throughout the run. A blank which was taken through all sample preparation steps was also used. MS/MS fragmentation was performed on all samples using sequential window acquisition of all theoretical mass spectra (SWATH-MS) mode.

Table 3.5: Mobile Phase composition for untargeted Luna Method for small molecule metabolites

Luna Mobile Phases	Composition
Mobile Phase A (positive)	H ₂ O + 0.2 % FA (v/v)
Mobile Phase B (positive)	ACN + 0.2 % FA (v/v)
Mobile Phase C (negative)	H ₂ O + 0.1 % FA (v/v) + 10 mM ammonium formate
Mobile Phase D (negative)	ACN

Table 3.6: Gradient elution for Luna method in positive mode

Time (mins)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0	0.45	99.8	0.2
2		99.8	0.2
5		98.0	2.0
11		75.0	25.0
13		2.0	98.0
17		2.0	98.0
18		99.8	0.2
20		99.8	0.2

Table 3.7: Gradient elution for Luna method in negative mode

Time (mins)	Flow rate (mL/min)	Mobile phase C (%)	Mobile phase D (%)
0	0.45	95.0	5.0
8		80.0	20.0
10		0.0	100.0
12		0.0	100.0
12.1		95.0	5.0
14		95.0	5.0

3.3.4. Processing and analysis of small molecule metabolite data – a targeted workflow

Data and peaks were acquired using SCIEX OS Software V3.0. An input list of metabolites (generated by Dr Cristina Balcells) that have been detected using the Luna method were searched for in the data. This was then processed and peaks integrated and annotated in MS Dial v5.3.24(131). The integration results were analysed via Targeted Workflow utilising the OptiMSR R package v24_03_15 (designed and optimised by Dr Cristina Balcells). Data analysis included blank filtering, QC filtering, batch effect correction and drift correction. All data was normalised to TIC and Pareto scaled. Metaboanalyst v6.0 was used for multivariate analysis of the data and generated all statistical figures. GraphPad Prism v10.2.0 was used to generate any box plots.

3.3.5. Proteomics analysis via LC-MS

The following analysis was performed by Dr Pavel Shilaha and Dr Alex Montoya at the MRC LMS. The protein digests obtained in **Section 3.2.9** were analysed by LC-MS using an UltiMate 3000 RSLC nano liquid chromatography system coupled to an Exploris 240 MS (Thermo Scientific) via an EASY-Spray source. Electrospray nebulisation was achieved by interfacing to Bruker PepSep emitters (PN: PSFSELF20, 20 μm). Peptides were separated using a 66-minute stepped gradient method in positive mode only using an Acquity CSH C18 1.7 μM beads, 300 μm x 35cm column, where 2 μg of digests resuspended in 5 μL were injected directly into the column at a flow rate of 5 $\mu\text{L}/\text{min}$ for 4 minutes. Mobile phases were prepared with UPLC grade solvents and chemicals (**Table 3.8**). The peptides were then separated using a 66-minute stepped gradient of 0-45 % Mobile Phase B. The data-independent acquisition (DIA) mode was as follows: an initial MS1 scan with a mass range of 410-1650 m/z at 120,000 resolution with an AGC target of 3×10^6 ions for a maximum injection time (IT) of 200 ms. This was followed by an MSX (boxcar) MS1 scan with 10 variable windows covering a range of 410-1650 m/z at 120,000 resolution and 310 DIA scans with variable window width at 30,000 resolution. The ACG target was again set to 3×10^6 ions, with maximum IT set to auto. The normalised collision energy was set to 27 % and the total run acquisition time was 82 minutes.

Table 3.8: Mobile Phase composition for Proteomics analysis

Proteomics Mobile Phases	Composition
Mobile Phase A	95 % H_2O + 5 % DMSO + 0.1 % FA (v/v)
Mobile Phase B	75 % ACN + 20 % H_2O + 5 % DMSO + 0.1 % FA (v/v)

3.3.6. Raw data processing of the proteomic analysis

Raw data was processed using Spectronaut v18.6.231227.55695 (Biognosys). Analysis was carried out in direct DIA as follows:

1. **Pulsar Search.** Library generation and database search was carried out with default settings for a trypsin/p specific digest: “Missed Cleavage Rate” was set to 3, “Variable Modifications” allowed for methionine oxidation, protein N-terminal acetylation, asparagine deamidation and cyclization of glutamine to glutamate. Peptide-spectrum

match (PSM) and protein group FDR were set to 0.01. Searches were carried out against UniProt homo sapiens proteome (UP000005640) 1 gene per protein sequence database(180), which was accessed on 22/01/2024 and yielded 20,596 entries. Searches were also carried out against a universal protein contaminants database(181), which was accessed on 22/01/2024 and yielded 381 entries.

2. **Direct DIA analysis.** A mutated decoy database was employed using protein q-value cut off set to 0.01 at the identification level. The quantification parameters were set to MS2 with the “Proteotypicity” filter set to “Only Proteotypic”. There was no value imputation strategy employed. The protein quantification method was set to MaxLFQ(182).
3. **Data export.** The raw, global median and local normalised protein intensity data generated were then exported for further downstream analysis.

3.3.7. Processing and analysis of the of the proteomic data – an untargeted workflow

A python pipeline, developed at MRC LMS, utilising various visualisation packages such as Plotly(183) and Pandas(184) was used for exploratory and technical analysis. Protein tables were filtered to remove the contaminant proteins discovered via the contaminant database before further analysis. The UniProt ID mapping service(101) was used to retrieve GO ontology terms against a list of protein accession IDs. The Perseus platform v1.6.15.0(185) was used for all further analysis. The data was first log2 transformed prior to additional filtering and statistical analysis. Where multi-group comparisons were performed, data was filtered for proteins that contained ≥ 5 replicate intensities per experimental group (total replicates in each group were 6). 1-way ANOVA of the protein intensities was performed with multiple testing correction and permutation-based FDR set to 0.05, and these were then mean-centred (z-score normalised). Hierarchical Clustering Analysis (HCA) was performed and unique HCA row cluster ID's were assigned which annotated groups of proteins based on their specific protein regulation. These groups of proteins were then mapped to the original protein ID to provide a “Cluster” output column. Perseus gene set enrichment analysis (GSEA)(186) module was used with the following testing included; Fishers exact testing and multiple testing correction with Benjamini-Hochberg and FDR threshold of 0.05, and performed on this “Cluster” output. The resulting enrichment table reflects the relative enrichment of the GO

terms by cluster, against other clusters and background proteins. Tukey's HSD post-hoc testing was carried out on ANOVA significant hits to determine pairs of groups which were defined as having significant differences in means. Where two-way group comparisons, the intensities were filtered as described and Student's t-test analysis was performed with multiple testing correction and permutation-based FDR set to 0.05. The results were visualised as volcano plots. Significant intensities were also z-scored and processed for GO enrichment as described for multi-group comparisons.

3.4. Bioinformatics and other data analyses

3.4.1. Gene Set Enrichment Analysis (GSEA) and Cytoscape

Transcriptomic data was generated by AstraZeneca via the Affymetrix Expression console. RNA sequencing data was generated on 4 unique OR clones, one of which included PC9 AZDR 3, referred to in this thesis as PC9R5. The other 3 clones were generated in the same way as PC9R5(172), with two clones (PC9 AZDR 2 and PC9 AZDR 5) having an alterations in NRAS and the third clone having no additional genetic alterations(172). This data was then input into GSEA v4.3.0 (Broad Institute) for gene set enrichment analysis. The gene set database used, obtained via the Broad Institute Molecular Signatures Database(187), was "c5.bp.v7.1.symbols.gmt". The number of permutations was 1000, the phenotype labels were Sensitive Vs Resistant and the permutation type was phenotype. The Chip platform used was "Human_Gene_Symbol_with_remapping_MSigDB.v2022.1.Hs.chip". The output file was then imported in Cytoscape v3.10.0. The Enrichment Map app in Cytoscape was used to visualise the GSEA data. The FDR q-value cutoff was 0.25 for gene-set filtering (nodes). For the resulting map, WordCloud App was used to group pathways and genes together into GO: Biological Processes. For the resulting enrichment map, the Node cutoff q value was 0.1 and the Edge cutoff was 0.6875.

3.4.2. DAVID

Proteomic data output from Perseus was analysed via the Database for Annotation, Visualization and Integrated Discovery (DAVID)(188,189). Functional annotation and enrichment analysis was performed on proteins that were both statistically significant using

ANOVA and Tukey's post hoc testing in multiple comparisons and using Students t-test in two-way comparisons. Gene ontology biological processes, cellular content and molecular function (DIRECT) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) databases were selected for enrichment analysis. Pathways with FDR < 0.1 were considered significantly enriched.

3.4.3. Synergy analysis

Synergy data analysis was performed on the drug combination data of both D-PDMP and osimertinib, and C2-Ceramide and osimertinib, obtained via SRB assay, to determine if the combinations were synergistic or simply additive. The web application SynergyFinder+(190) was used with the Bliss Independence model of combination analysis chosen. This platform uses statistical synergy models to score each combination. There are four main models used; highest single agent (HSA), Loewe additivity (Loewe), Bliss independence (Bliss) and zero interaction (ZIP)(190). We decided to use the Bliss synergy score model as this model is more appropriate when the drugs work in different pathways and work independently of each other(191). It is important to note that results from calculating synergy using any model should be interpreted with caution, as the complexity of drug interactions and combinations may not be fully reflected. The principle of Bliss scoring is shown below(192);

$$S_{Bliss} = y_c - (y_1(x_1) + y_2(x_2) - y_1(x_1)y_2(x_2))$$

Where;

- x_1 = Drug A at a specific concentration,
- x_2 = Drug B at a specific concentration,
- y_c = combined effect of x_1 and x_2 ,
- $y_1(x_1)$ = single drug effect of Drug A,
- $y_2(x_2)$ = single drug effect of Drug B.

There are several different Bliss score cut-offs reported in the literature, with some papers using the following:

- < -1 is antagonistic, = 0 is additive, > 1 is synergistic(190),

- < 0 is antagonistic, $= 0$ is additive, > 0 is synergistic(190),
- < 5 is antagonistic, -5 to 5 is additive, > 5 is synergistic(192).

The cut-offs for Bliss scoring we used were as follows: < -5 is antagonistic, -5 to 5 is additive and > 5 is synergistic. All graphs were generated using the GraphPad Prism v10.2.0 software.

3.4.4. Multi-omics integration

Metaboanalyst v6.0(193) “Joint Pathway Analysis” was used to perform multi-omics integration of the proteomics, lipidomics and metabolomics data sets. Significant proteins and lipids changes in OR versus OS (p value < 0.05 and FC of > 1.5 for proteins, > 1.5 or 2.0 for lipids depending on the cell line and p value < 0.05 and FC of > 1.2 for metabolites) were selected as input. Lipid IDs were converted to HMDB IDs using the LipidSig 2:0 ID conversion tool(194). In parameter settings, “Metabolic pathways (integrated)” was selected. For algorithm selection, the following settings were applied:

1. Enrichment analysis: Hypergeometric test
2. Topology measure: Degree centrality
3. Integration method: Combine queries

3.4.5. Statistical analyses

Statistical analyses were performed on various different software and programmes including; GraphPad Prism v10.2.0, R Studio v4.0.0, SIMCA v17.0.2.34594, Metaboanalyst v6.0, Perseus v1.6.15.0 and Microsoft Excel. Information on statistical test used can be found in Figure legends, where appropriate. Both multivariate (Metaboanalyst v6.0) and univariate (GraphPad Prism v10.2.0) analyses were used.

For multivariate analyses in Metaboanalyst, TIC normalised data was Pareto scaled. Heatmaps were generated using hierarchical clustering with Euclidean algorithm for distance measure, Ward algorithm for clustering measure and plotted ANOVA values. For proteomic analysis, local regression normalisation was used. For lipidomic and metabolomic analysis, TIC normalisation was used.

For univariate analyses, statistical significance was as follows: *ns* $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P = 0.001$, $****P < 0.0001$ compared with control. For all boxplots, one-way ANOVA followed by Dunnett's multiple correction was used to determine statistical significance of multiple comparisons. Fold change (FC) cut offs were 1.5 for proteomics data, 1.5 or 2.0 for lipidomics data, depending on cell line (this is clarified within specific figure legends) and 1.2 for metabolomics data. For proteomics data, where two-way comparisons were used, student's *t* tests followed by Tukey's HSD post-hoc testing for multiple comparisons was performed.

Chapter 4 – Multi-omics profiling of
pharmacologically-induced models of osimertinib
resistance

4. Chapter 4: Multi-omics profiling of pharmacologically-induced models of osimertinib resistance

4.1. Background

The EGFR-TKIs are notorious for enabling PFS but then ultimately succumbing to drug resistance. Because of this, many groups have studied their resistance mechanisms, often through generation of drug-resistant models. Since resistance can occur in an EGFR-dependent or independent manner, models are generated to take these different mutations into account.

NSCLC pharmacologically-induced models of osimertinib resistance (OR) were generated by AstraZeneca following a dose escalation study whereby PC9 parental (PC9P) cell lines were treated with escalating doses of osimertinib, to a final dose of 160 nM osimertinib (as described in **Chapter 3.1.3**). Two resistant clones (PC9R5 and PC9R6) were generated. Both cell lines contain a deletion within exon 19 at E746-A750 as is present in PC9 cells. Upon generation of PC9R5, there were additional genetic alterations detected within MAPK1/CRKL. This is the only known feature that distinguishes PC9R5 and PC9R6. It could be argued that these cell models present a more accurate account of how drug resistance occurs within patients and therefore is an accurate *in vitro* model to be translated into the clinic.

Our systematic review (**Chapter 2**) revealed that metabolism is altered in a myriad of ways in EGFR-TKI resistant NSCLC, and this occurs across all generations of EGFR-TKIs(105). For instance, many of the identified resistance mechanisms occurred within branches of cell metabolism. This included small molecule metabolism, such as the TCA cycle, and lipid metabolism, such as fatty acid synthesis. However, there is limited data on the non-genetic mechanisms behind acquired osimertinib resistance, since it is one of the most recent generations of TKIs.

In this chapter, we characterise the transcriptome, proteome, lipidome and metabolome of two OR clones (PC9R5 and PC9R6) compared to osimertinib-sensitive control (OS; PC9P), to elucidate potential resistance mechanisms.

4.1.1. Aims

Hypothesis

OR cells have distinct metabolic phenotypes compared to OS. We will characterise this by analysing and comparing their transcriptomes, proteomes, lipidomes and metabolomes.

The aim of this chapter was to determine if pharmacologically-induced models of OR have metabolic differences compared to OS, using multi-omics.

To address this, we have performed:

- Pathway analysis on transcriptomics data, to determine if there are any within metabolism-centric pathways in OR (PC9R5) compared to OS cells.
- Untargeted proteomics to investigate protein-level changes in OR cell lines.
- Untargeted lipidomics analysis to investigate lipid changes in OR cell lines.
- Targeted metabolomics analysis to investigate polar metabolite changes in OR cell lines.

4.2. Characterisation of the pharmacologically-induced models of resistance

To confirm and characterise the models' acquired resistance to osimertinib, an SRB assay (methodology described in **Chapter 3.2.1**) was used to measure the inhibition on cell growth when PC9P (OS), PC9R5 and PC9R6 (OR) were treated with increasing doses of osimertinib (**Figure 4.1**). The OR lines were more resistant to osimertinib at higher doses as expected, with the IC₅₀ values of each of these lines close to 2 µM (1.719 µM for PC9R5 and 1.918 µM for PC9R6), compared to 0.003 µM for OS.

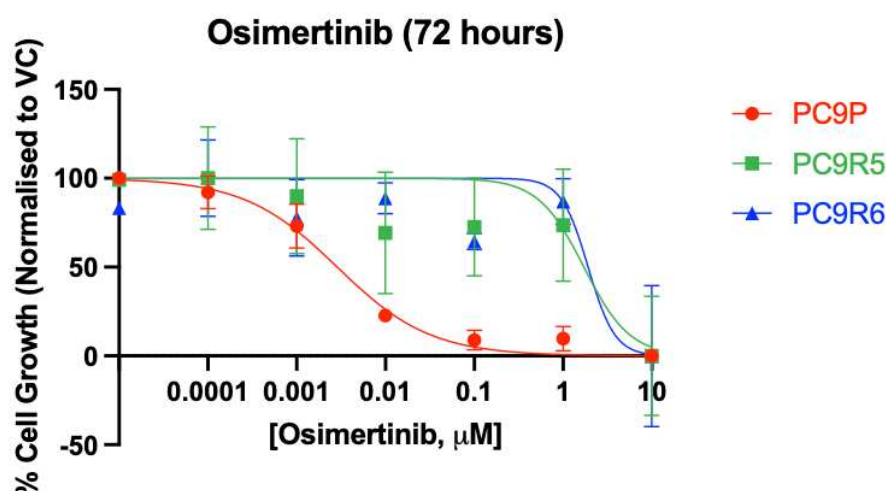


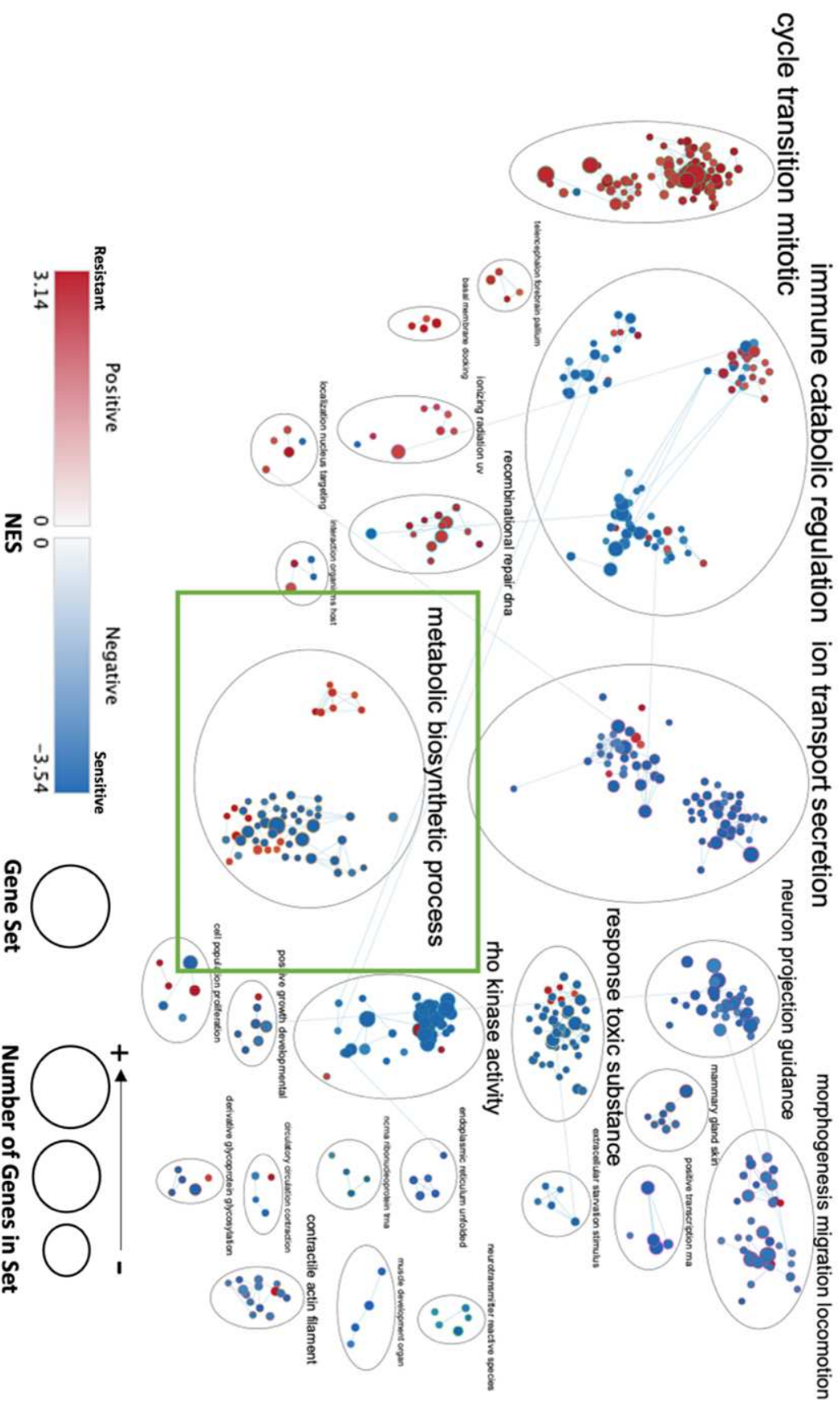
Figure 4.1: Characterisation of pharmacologically-induced models of osimertinib resistance. Pharmacologically-induced resistant lines were treated with 0-10 μM osimertinib for 72 hours before cell growth was measured using the SRB assay. Data expressed as mean $\pm$ SEM (biological replicate, $n = 3$).

4.2.1. Transcriptomics data analysis of OR lines

RNA sequencing was performed by the team at AstraZeneca using the Affymetrix Expression console and data was collected on 4 unique OR clones (including PC9R5, but not PC9R6) and the OS parental line PC9P. Pathway analysis using gene set enrichment analysis (GSEA), and visualised with Cytoscape(195), was performed on all 4 OR clones against OS control. **Figure 4.2A** shows the enriched gene ontologies (GO) which were grouped into word clouds according to common words within each pathway. Interestingly, one of the main word clouds was termed “metabolic biosynthetic process”, which included enriched pathways involved in lipid metabolism (e.g. regulation of lipid biosynthetic process, phospholipid biosynthetic process and fatty acid derivative metabolic process). There were also enriched pathways involved in aqueous/small molecule metabolism (e.g. cellular amino acid metabolic process, small molecule biosynthetic process and glucose metabolic process).

Next, we decided to look in more detail at the enriched pathways in only the PC9R5 cell line compared to OS control, since this was one of the cell lines we were investigating using multi-omics. **Figure 4.2B** shows the significantly enriched pathways in PC9R5 cells compared to PC9P control. Positively enriched pathways were largely related to lipid metabolism like those mentioned above, with negatively enriched pathways including aqueous/small molecule metabolism (e.g. small molecule metabolic process and cellular amino acid

metabolic process). “Lipid metabolic process” itself however was also negatively enriched. Next, we turned to analyse the differential expression of specific genes involved in various aspects of lipid metabolism for PC9R5 compared to control. A list of genes was prepared using the KEGG database (accessed in December 2023 by Laila Evangelista, full list in **Appendix 9.2, Appendix Table 9.2**). Key differentially expressed genes involved in lipid metabolism were identified and grouped according to their role (**Figure 4.2C**). Several genes involved in fatty acid synthesis and lipid remodelling were overexpressed in OR (e.g. *ACLY*, *FASN*, *LPCAT1/3*), with genes involved in other lipid metabolic pathways also showing significant changes in expression. For instance, several genes involved in sphingolipid metabolism were overexpressed. This included *UGCG* which catalyses the conversion of ceramide into glucosylceramide, *B4GALT6* which encodes a lactosylceramide synthase and *CERS5/6* which encodes ceramide synthase(196).



A

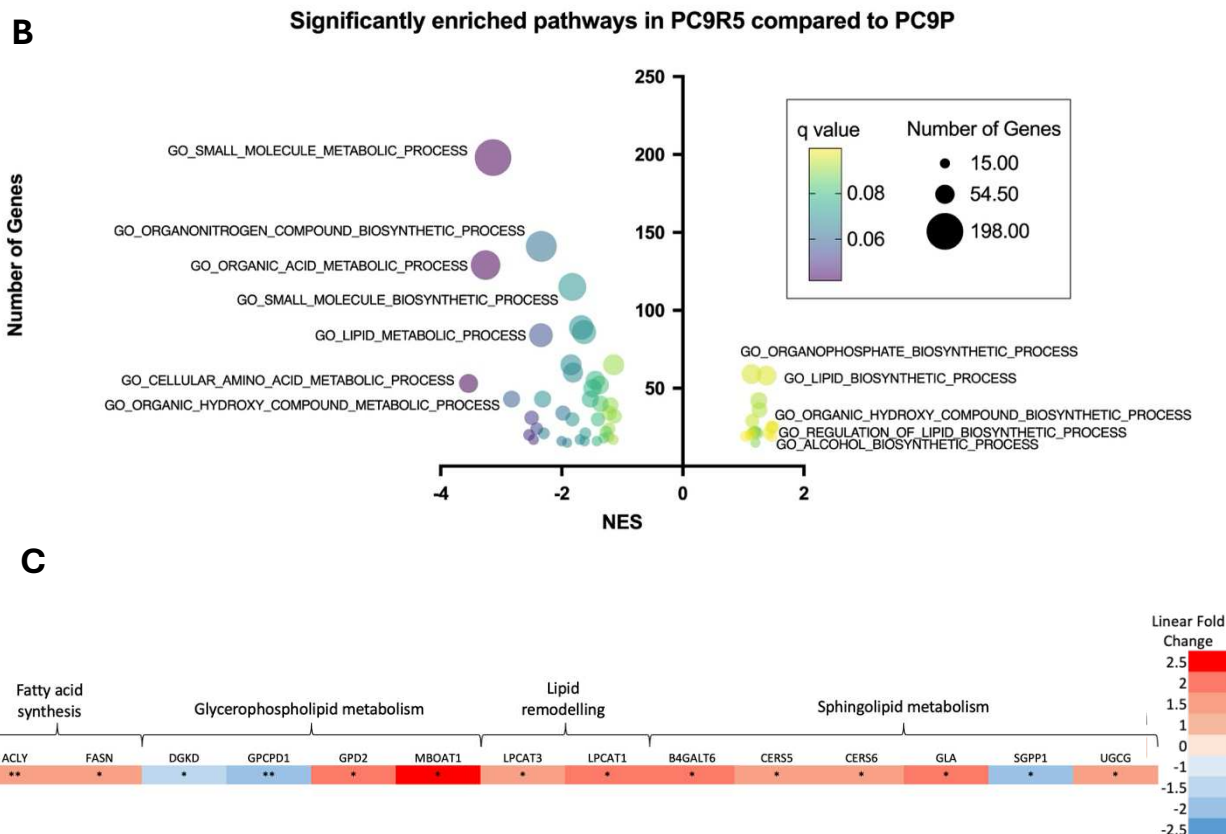
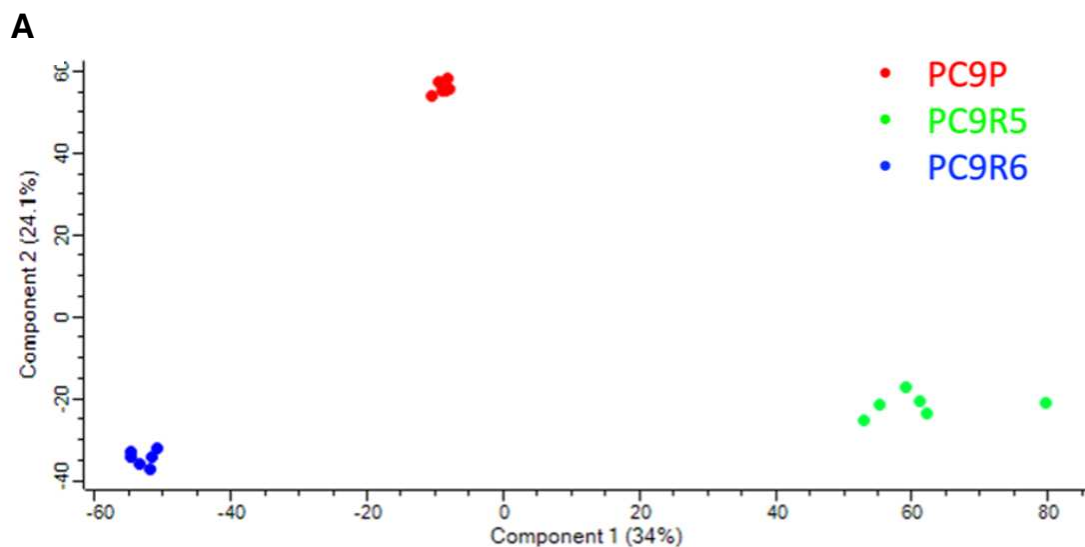


Figure 4.2: Transcriptomic changes between pharmacologically-induced models of OR and OS cells. (A) Enriched gene ontologies (GO) for all 4 resistant clones versus OS generated via GSEA and Cytoscape, which are grouped into word clouds according to common words within each GO pathway. **(B)** Bubble plot of these terms in PC9R5 (OR) versus PC9P (OS), showing which GO's were positively or negatively enriched. The X-axis measures normalised enrichment score (NES), the Y axis measures Number of Genes within each pathway, the colour of the bubbles represents the q-value and the size of the bubble also represents Number of Genes. **(C)** Lipid metabolism genes altered in PC9R5 versus PC9P. Data expressed as linear fold change, and all are significant calculated via ANOVA p-value. A positive linear fold change indicates upregulation in resistant versus sensitive PC9 (OS) cells. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared with control.

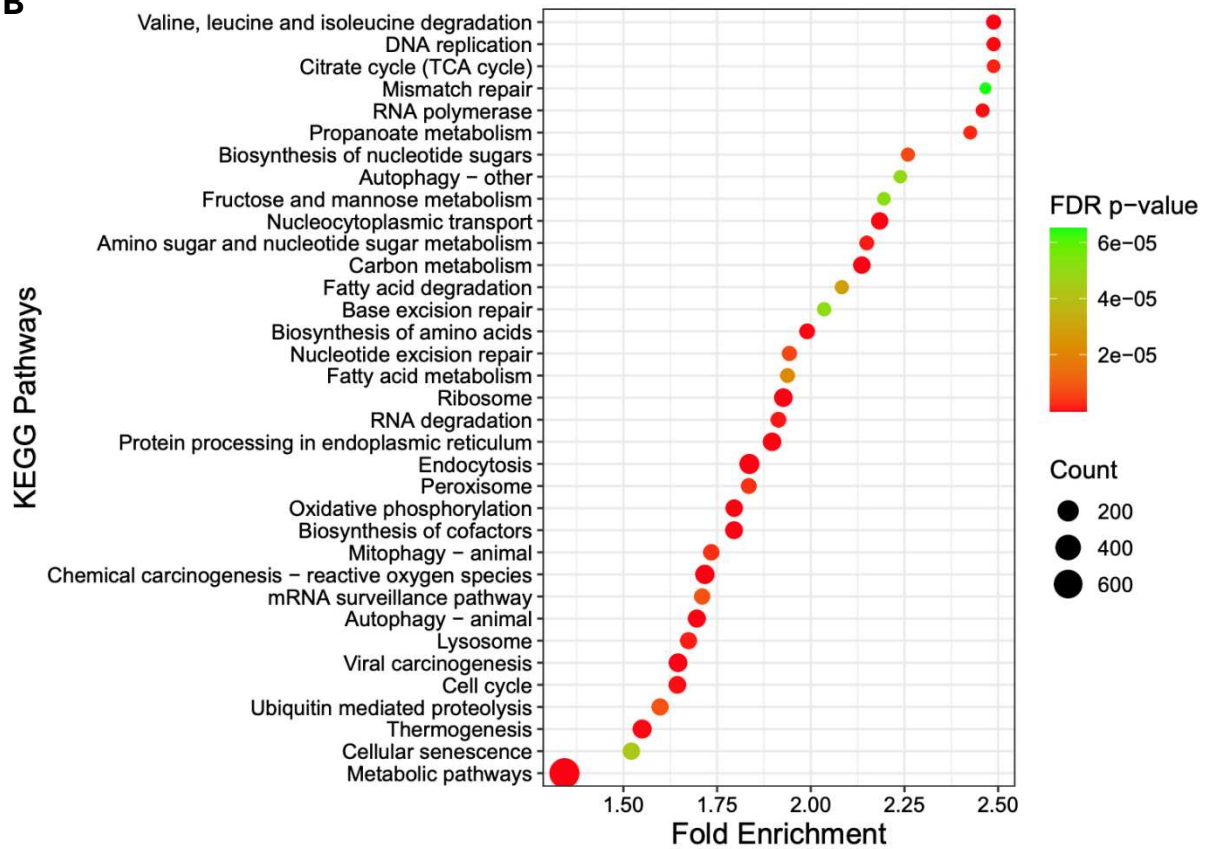
4.3. Untargeted proteomics analysis of OR lines

Untargeted proteomics, lipidomics and metabolomics were all performed on the exact same pharmacologically-resistant samples. Proteomics analysis was performed by Dr Pavel Shliha and Dr Alex Montoya at MRC LMS (see **Chapter 3.2.9** and **3.3.5-3.3.7**). There were 7727 total proteins detected, 6186 of these could be minimally identified by 2 or more peptides and 6112 of these could be minimally quantified by 2 or more peptides.

After processing using Perseus software, there were 5786 proteins that were statistically significant across all comparisons using ANOVA and Tukey's post hoc testing across all groups. Based on these proteins, we transferred these into gene names and performed GSEA using DAVID; an online database for annotation, visualisation and integrated discovery to determine enriched pathways from a gene or protein list (Accessed August 2024)(189). Firstly, PCA of the local normalised protein intensities revealed an obvious distinction between all 3 cell lines in Component 1, whilst OS and OR cell lines were separated in Component 2 (**Figure 4.3A**). We looked at the Top 50 KEGG pathways that were enriched within the significantly changed protein set and removed pathways that were not relevant to human lung cancer (**Figure 4.3B**). Remaining pathways included metabolism-centric pathways such as Citrate cycle (TCA cycle), fatty acid degradation and metabolism, and amino sugar and nucleotide metabolism. We then used GO enrichment to determine the Top 10 GO enriched pathways that were significant in 3 functional areas; Biological processes (BP), cellular components (CC) and molecular function (MF). Many of these enriched pathways were involved in mRNA processing, and others with mitochondrial function and ATP activity (**Figure 4.3C**).



B



C

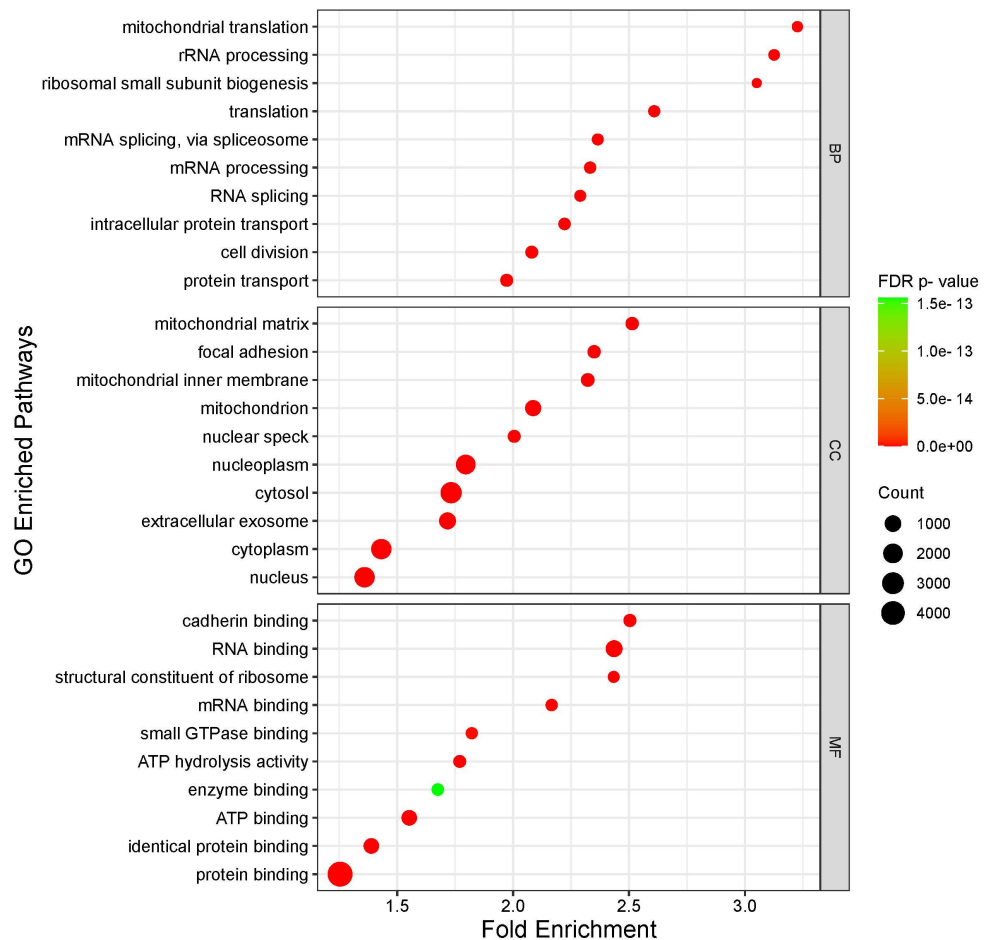


Figure 4.3: Untargeted proteomic analysis of pharmacologically-induced models of OR and OS cells. (A) PCA score plot of proteomic profiles in OR (PC9R5, PC9R6) and OS (PC9P) cells shows distinct proteomic profiles (technical replicate, $n = 6$). (B) KEGG pathways that differentially expressed proteins were enriched in. (C) The Top 10 GO pathways that differentially expressed proteins were enriched in. Biological Process (BP), Cellular Content (CC) and Molecular Function (MF).

We then performed two-way comparisons of each OR cell line compared to OS control. Between PC9R5 and PC9P there were 4099 proteins significantly different based on Student's t test (**Figure 4.4A**). Major underexpressed proteins included members of the aldehyde dehydrogenase families (ALDH1A3 and ALDH3A1) which play roles in lipid metabolism and peroxidation(197), SH3 domain containing kinase binding protein 1 (SH3KBP1) which is involved in apoptosis(198), low density lipoprotein receptor (LDLR) which regulates cholesterol levels(199) and dehydrogenase/reductase 2 (DHRS2) which is involved in lipid metabolism(200). Major overexpressed proteins included solute carrier 25A1 (SLC25A1) which is a mitochondrial carrier and is involved in the movement of citrate across membranes(201), CRK like proto-oncogene (CRKL) which is known to activate alternative signalling pathway RAS(172) and oxidized low density lipoprotein receptor 1 (OLR1) which is involved in cyclic AMP signalling and lipid metabolism(202).

We then compared the proteome of PC9R6 (OR) versus PC9P (OS). 4519 proteins in this comparison were significantly different based on Student's t test and Tukey's HSD post-hoc testing for multiple comparisons (**Figure 4.4B**). Major underexpressed proteins included HPSB1, which has a protective effect against apoptosis(203) and DHRS1. Major overexpressed proteins were ALDH6A1 and ALDH2, whose functions have been described above, ASS1 which catalyses the penultimate step in arginine biosynthesis(204), BDH2 which is a short chain-dehydrogenase/reductase and is involved in NAD binding activity and fatty acid oxidation(205) and GPX3 which protects cells against lipid peroxidation(206).

Differential protein expression

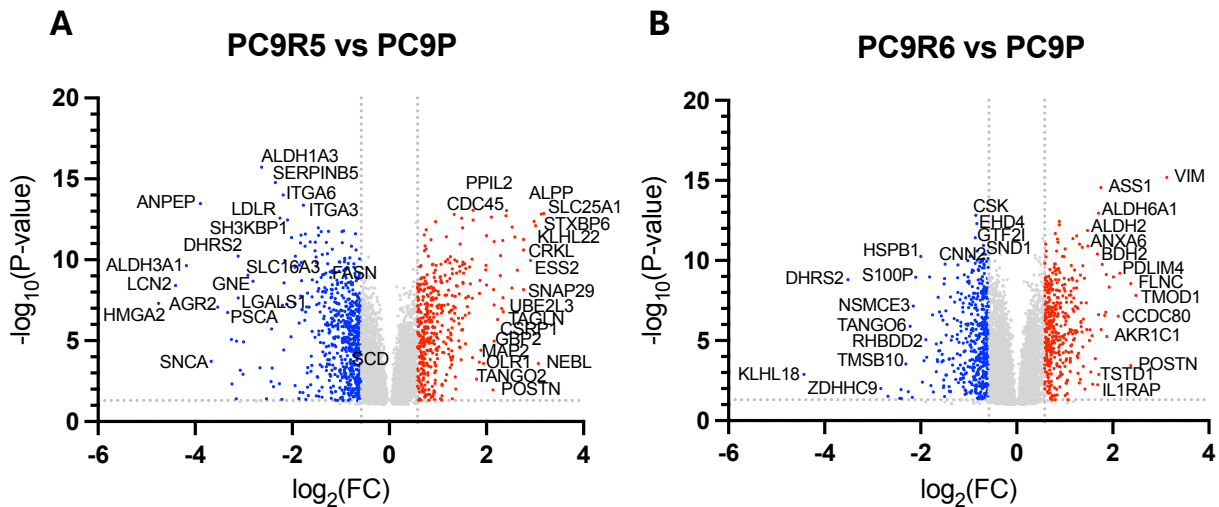


Figure 4.4: A two-way comparison of protein changes in pharmacologically-induced models of OR and OS cells. (A) PC9R5 compared to PC9P and (B) PC9R6 compared to PC9P. Volcano plot showing proteins that are significantly increased (red) and decreased (blue). FC cut-off for significance was 1.5 and FDR adjusted *p*-value cut off was 0.05. Data was normalised via local regression normalisation.

4.3.1. Changes in proteins involved in lipid metabolism

We investigated the proteomics data to determine if proteins involved in lipid metabolism, were also differentially regulated in OR versus OS. Proteins involved in lipid metabolism, according to the KEGG database (accessed in December 2023 by Laila Evangelista), were subset from the full dataset of proteins and analysed separately (full list **Appendix Table 9.2**). Key proteins related to DNL or lipid metabolism were downregulated in each cell line, for example, FASN and SCD in PC9R5 and ELOVL7, which is mostly associated with PUFA lipid metabolism, in PC9R6. On the other hand, SCD5 was upregulated in both OR cell lines. A decrease in FAR1, a rate-limiting protein in plasmalogen synthesis(207), was shown in PC9R5. There was also an increase in lipid remodelling protein LPCAT1 in both OR lines (**Figure 4.5**).

Proteins involved in sphingolipid metabolism also had altered expression. Underexpressed proteins in PC9R5 compared to PC9P sensitive control included PLPP2, KDSR, SPHK2 and UGT8 (**Figure 4.5A**). GLB1 and ST3GAL1 were significantly overexpressed. In PC9R6 versus PC9P sensitive control (**Figure 4.5B**), underexpressed proteins included CERS6 and DEGS1, whilst overexpressed proteins were GBA1 and UGCG. B4GALT5, involved in LacCer synthesis, was significantly increased, however fell just below the chosen fold change cut-off of 1.5.

Lipid metabolism related protein changes

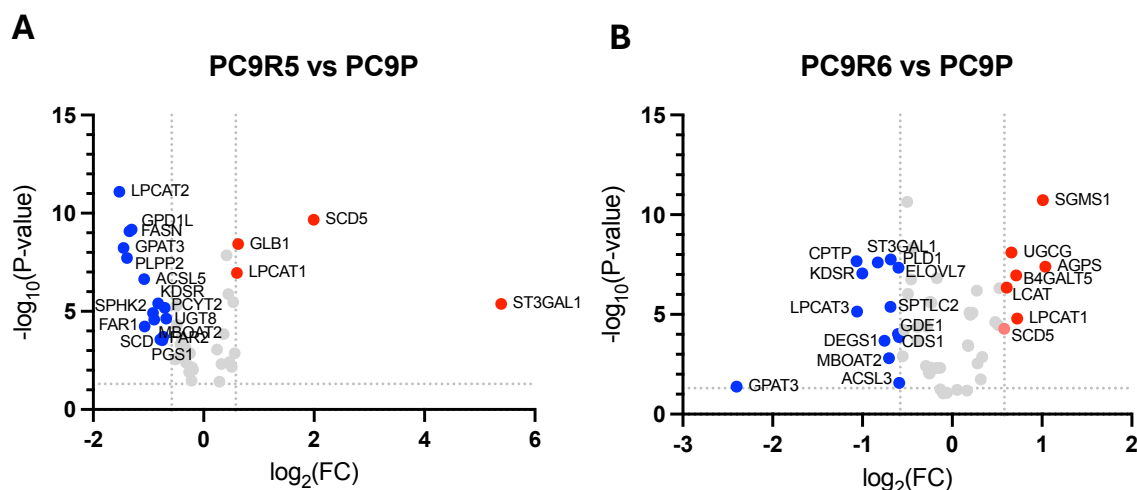


Figure 4.5: Specific changes in proteins involved in all aspects of lipid metabolism in pharmacologically-induced models of OR versus OS cells. (A) PC9R5 compared to PC9P and (B) PC9R6 compared to PC9P. Volcano plot showing proteins that are significantly increased (red) and decreased (blue). FC cut-off for significance was 1.5 and FDR adjusted p-value cut-off was 0.05. Data was normalised via local regression normalisation.

4.4. Untargeted lipidomics analysis of OR lines

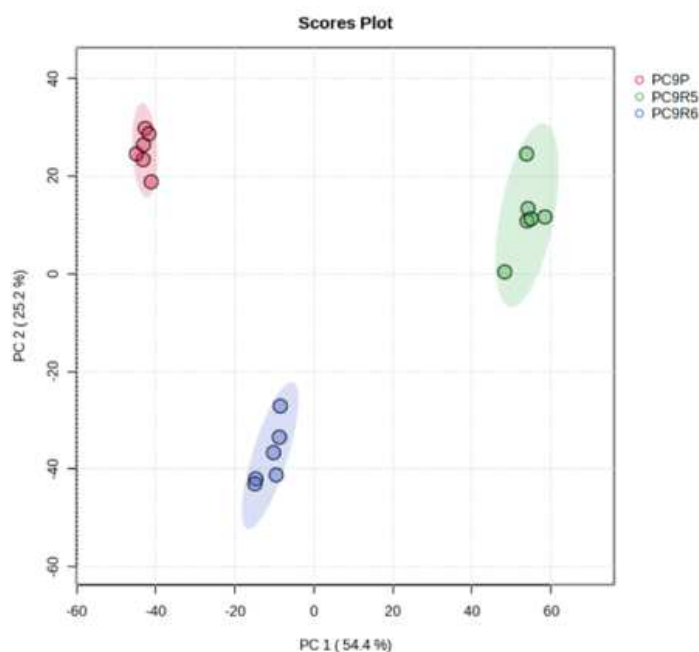
Our systematic review (**Chapter 2**) highlighted lipid metabolism as potentially playing a role in NSCLC drug resistance. This included studies across different EGFR-TKIs and various lipid metabolic pathways. In addition, expression of genes and proteins involved in lipid metabolism had altered abundance in OR cell lines. We therefore elected to perform untargeted lipidomic analysis on the two pharmacologically-resistant NSLSC cell lines and their control, to identify whether (and which) lipid pathways were dysregulated in OR cells. Lipids were extracted and analysed by LC-MS/MS as described in **Chapter 3.2.6 - 3.2.7** and **3.3.1**. Overall, there were 1415 unique IDs identified and retained after quality control filtering in both positive and negative ion modes combined. We confidently annotated 404 of these lipids and tentatively annotated a further 337, with 674 unknown features. Statistical analysis was performed on Metaboanalyst v6.0(193), SIMCA v17.0.2.34594 and GraphPad Prism v10.2.0. Full data processing method is described in **Chapter 3.3.2**.

4.4.1. Multivariate statistical analysis revealed distinct lipid profiles in OR compared to OS

PCA is an unsupervised method that can classify samples based on their variance. PCA of the lipidomics datasets successfully grouped each technical replicate together. In addition, each sample type was grouped separately, highlighting the significant differences in lipid profile for each cell line ($R^2 = 0.98$; **Figure 4.6A**). There was separation in PC1 across all lines, between resistant versus sensitive cell lines and between the two resistant cell lines. In PC2, there was separation of PC9R6 versus other cell lines, however an overlap between PC9P and PC9R5 was observed.

Hierarchical clustering separated the 3 distinct cell lines, recognising lipidomic differences between OR and OS, and between PC9R5 and PC9R6. The Top 50 features, based on ANOVA, that were different across all 3 cell lines included plasmalogens, phospholipids such as PCs and Pls, TGs and sphingolipids (**Figure 4.6B**). Whilst there are some common features clustering together between the two resistant lines, and between PC9R6 and PC9P, overall, each cell line had very different lipid profiles. For this reason, we cannot simply look at “OR versus OS” but must also look at each OR cell line on an individual basis, compared to control.

A



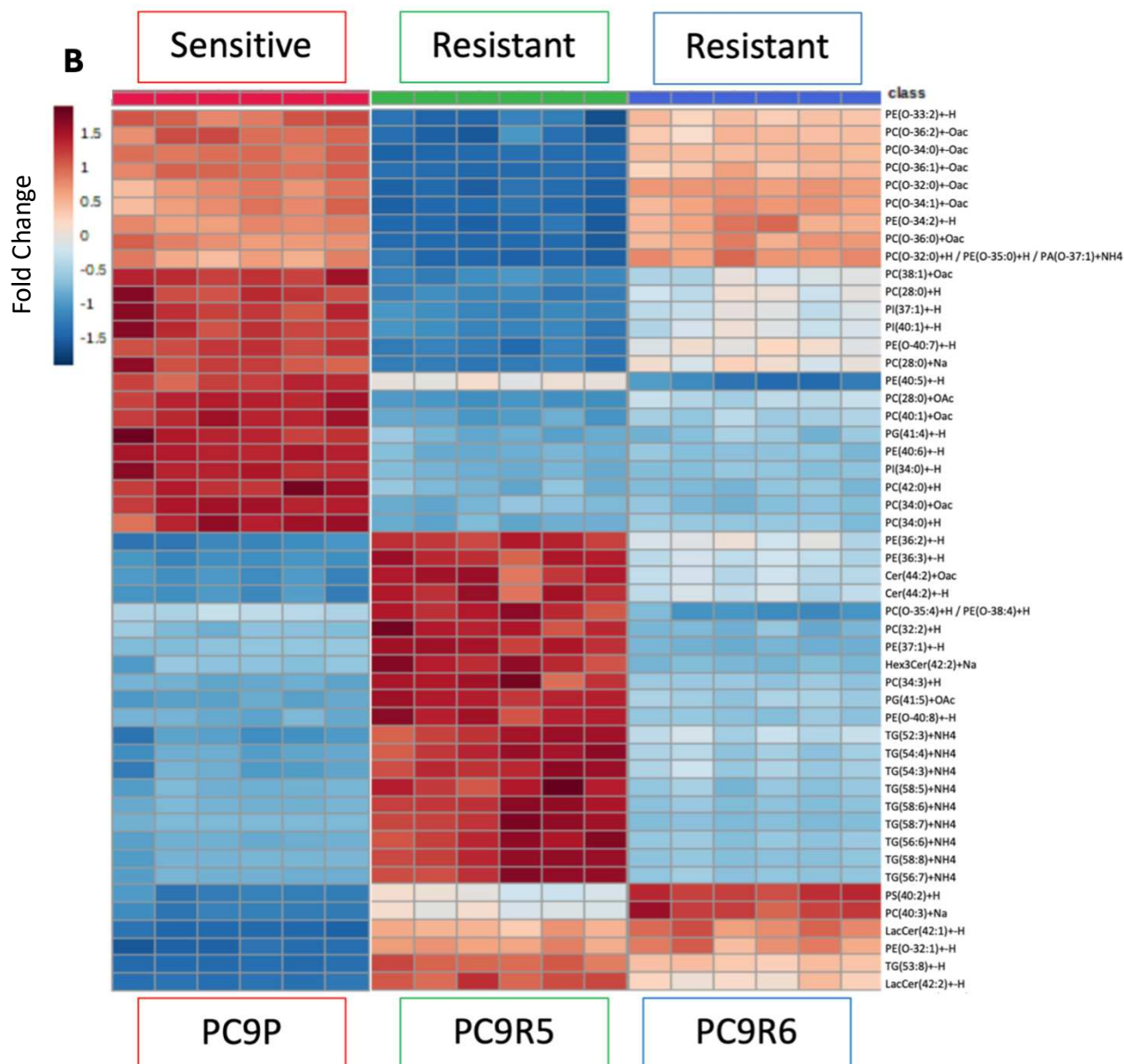


Figure 4.6: Multivariate statistical analysis of untargeted lipidomic profiles of pharmacologically-induced models of OR versus OS cells. A) PCA score plot of lipidomic profiles across the two OR cell lines, PC9R5 and PC9R6, and PC9P sensitive control. (B) Heatmap of the Top 50 features differentiating the 3 cell lines (based on ANOVA). Data normalised to TIC (technical replicate, $n = 6$).

4.4.2. Specific changes in two-way comparisons: PC9R5 versus PC9P

It is clear from the overall data described above that the lipidomic profiles of each resistant cell line are distinct from each other, as well as from PC9P control, as evidenced by some of the key lipid families we analysed. We therefore performed pairwise comparisons of the cell lines.

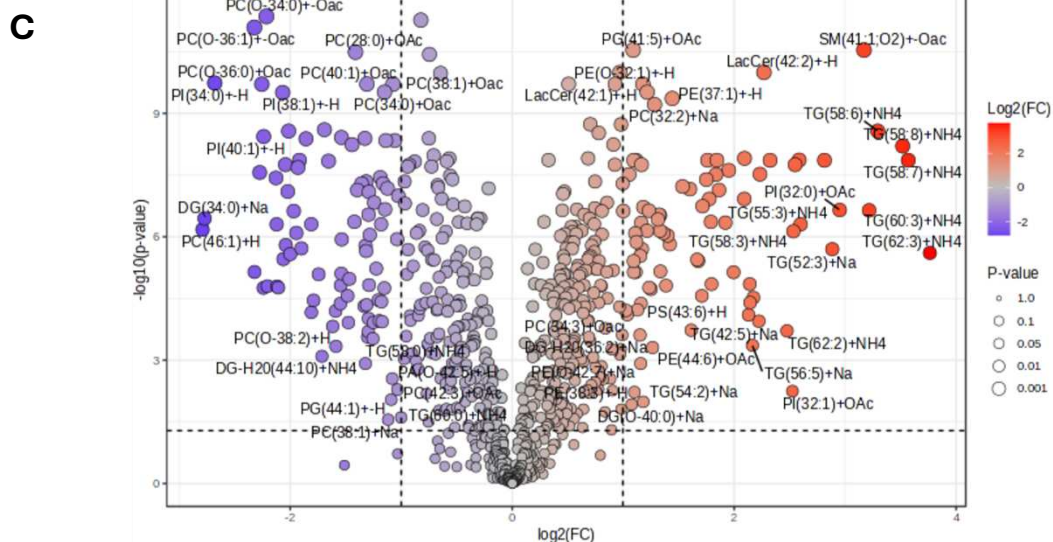
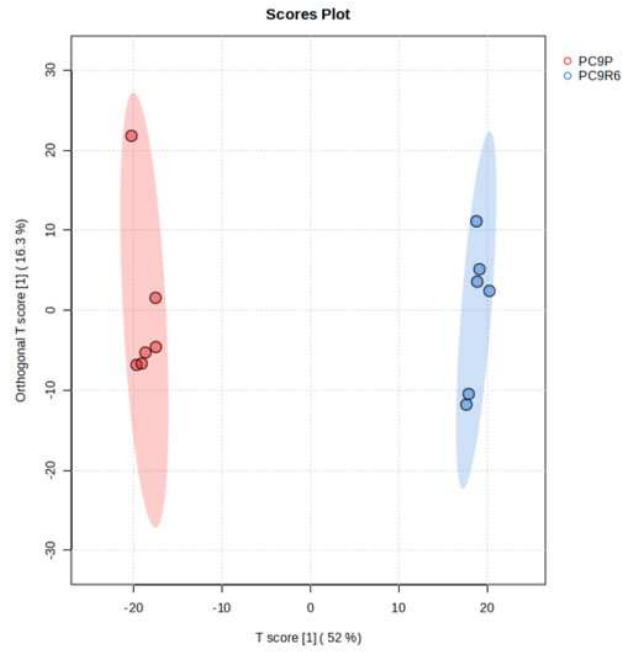


Figure 4.7: A two-way comparison of lipid profiles between PC9R5 compared to PC9P. (A) OPLS-DA scores plot of PC9R5 and PC9P. (B) S-plot showing lipids increased (green) and decreased (red) in PC9R5 compared to PC9P. Highlighted data points, were annotated to show representative lipids that are changing. (C) Volcano plot showing lipids that are significantly increased (red) or decreased (blue) in PC9R5 compared to PC9P. FC cut-off for significance was 2.0 and FDR adjusted p-value cut-off was 0.05. Data was normalised to TIC (technical replicate, $n = 6$).

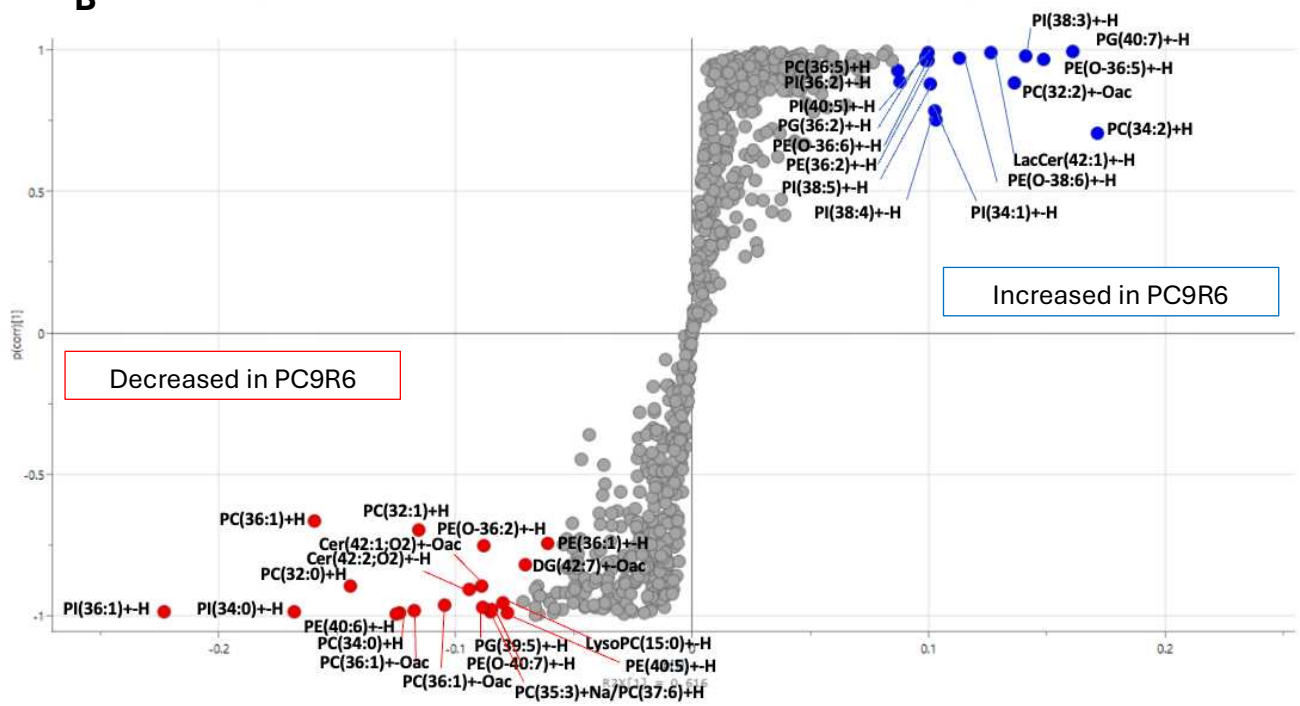
4.4.3. Specific changes in two-way comparisons: PC9R6 versus PC9P

We again used OPLS-DA to compare PC9R6 with PC9P (**Figure 4.8A**), confirming the distinct lipid profiles of the two cell lines ($R^2X = 0.52$, $R^2Y = 0.99$, $Q^2 = 0.97$, $p \text{ value} = 5.60\text{e-}08$). The S-plot (**Figure 4.8B**) revealed increased MUFA and PUFA-containing phospholipids and LacCers in PC9R6 compared to PC9P. Decreased lipids included ceramides and a mixture of phospholipids and plasmalogens. Lastly, the volcano plot (**Figure 4.8C**) analysis highlighted increased PUFA TGs and complex GSLs (GlcCers and LacCers). Lipids that were significantly decreased in this analysis came from a mixture of many different lipid classes.

A



B



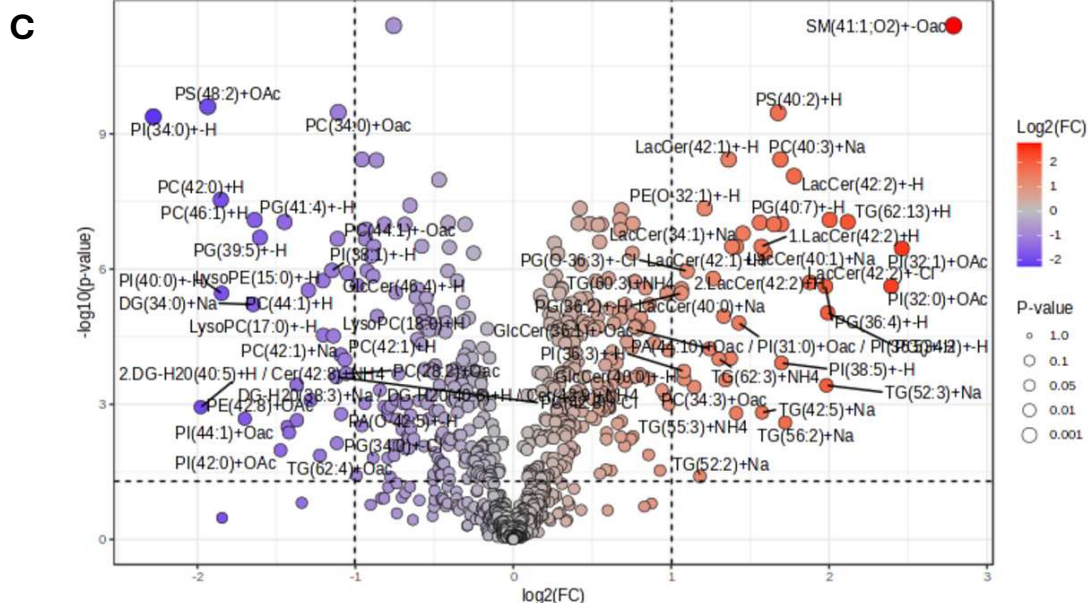


Figure 4.8: A two-way comparison of lipid profiles between PC9R6 compared to PC9P. (A) OPLS-DA scores plot of PC9R6 and PC9P. (B) S-plot showing lipids increased (blue) and decreased (red) in PC9R6 compared to PC9P. Highlighted data points, were annotated to show representative lipids that are changing. (C) Volcano plot showing lipids that are significantly increased (red) and decreased (blue) in PC9R6 compared to PC9P. FC cut-off for significance was 2.0 and FDR adjusted p-value cut-off was 0.05. Data was normalised to TIC (technical replicate, $n = 6$).

4.4.4. Key families of lipids that are altered in OR compared to OS

We plotted the total intensity of the key families of lipids that were found to be important in driving the differences between the cell line groups. **Figure 4.9A** shows the total abundance of TGs in each cell line. TGs overall were increased in both OR lines compared to OS. We then compared the profiles of PUFA versus non-PUFA TGs, to see if fatty acyl chain saturation was altered in resistant lines (**Figure 4.9B**). There was a significant increase in PUFA/Non-PUFA TGs in PC9R5 compared to PC9P and PC9R6. Next, we investigated the levels of plasmalogens, particularly PC and PE plasmalogens as they were the most abundant in our data (**Figure 4.9C**). There was a significant decrease in total plasmalogens in PC9R5 compared to PC9P and PC9R6.

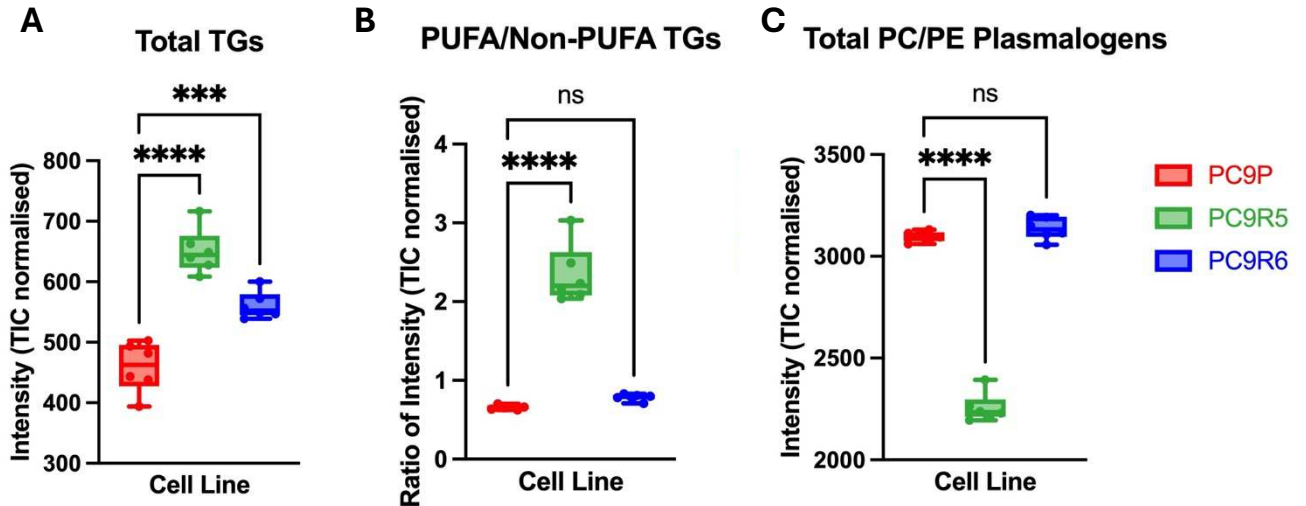


Figure 4.9: Specific lipid family profiles in pharmacologically-induced models of OR versus OS cells. (A) Total TGs in OR (PC9R5 and PC9R6) and OS (PC9P) cells. **(B)** TG saturation, with the ratio of PUFA/Non-PUFA TGs in OR and OS cell lines. **(C)** Total PC and PE plasmalogens in OR and OS cell lines. Lipidomic data was normalised to TIC. Statistical differences in box plots determined by one-way ANOVA with Dunnett's correction multiple comparisons. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, **** $P < 0.0001$ compared with control (technical replicate, $n = 6$).

4.5. Dysregulation of sphingolipid metabolism evidenced by multi-omics analyses

Finally, we investigated the sphingolipid family which revealed dysregulation in sphingolipid metabolism. Specifically, we found there to be a decrease in total ceramides and an increase in GSLs in OR cells compared to OS controls (**Figure 4.10A**). To understand which specific sphingolipid pathways were altered, we looked at the ratio of ceramide relative to complex GSLs, and to SM. SM can be converted to ceramide via sphingomyelinase (SMase) in the sphingomyelin pathway(71). The ratio of total ceramides to SM was decreased in OR cells (**Figure 4.10B**). Similarly, the ratio of ceramide to complex GSLs was decreased in OR cells compared to OS (**Figure 4.10C**). Next, we then looked at the abundance of specific sphingolipid classes relative to the total sphingolipid composition (**Figure 4.10D**). As expected, the % Ceramide was lower in OR cells compared to OS. There was a small significant decrease in % GlcCer in PC9R5 compared to PC9P, however there was no significant difference when PC9R6 was compared to PC9P. A dramatic increase in % LacCer was observed in both OR lines compared to the OS control. The % Globosides was significantly increased in PC9R5 compared to PC9P, however there was no significant

difference between PC9R6 and PC9P. Overall, there are clear alterations to sphingolipid metabolism in OR versus OS cells, in ceramides and different GSL species present in the lipidome of each cell line.

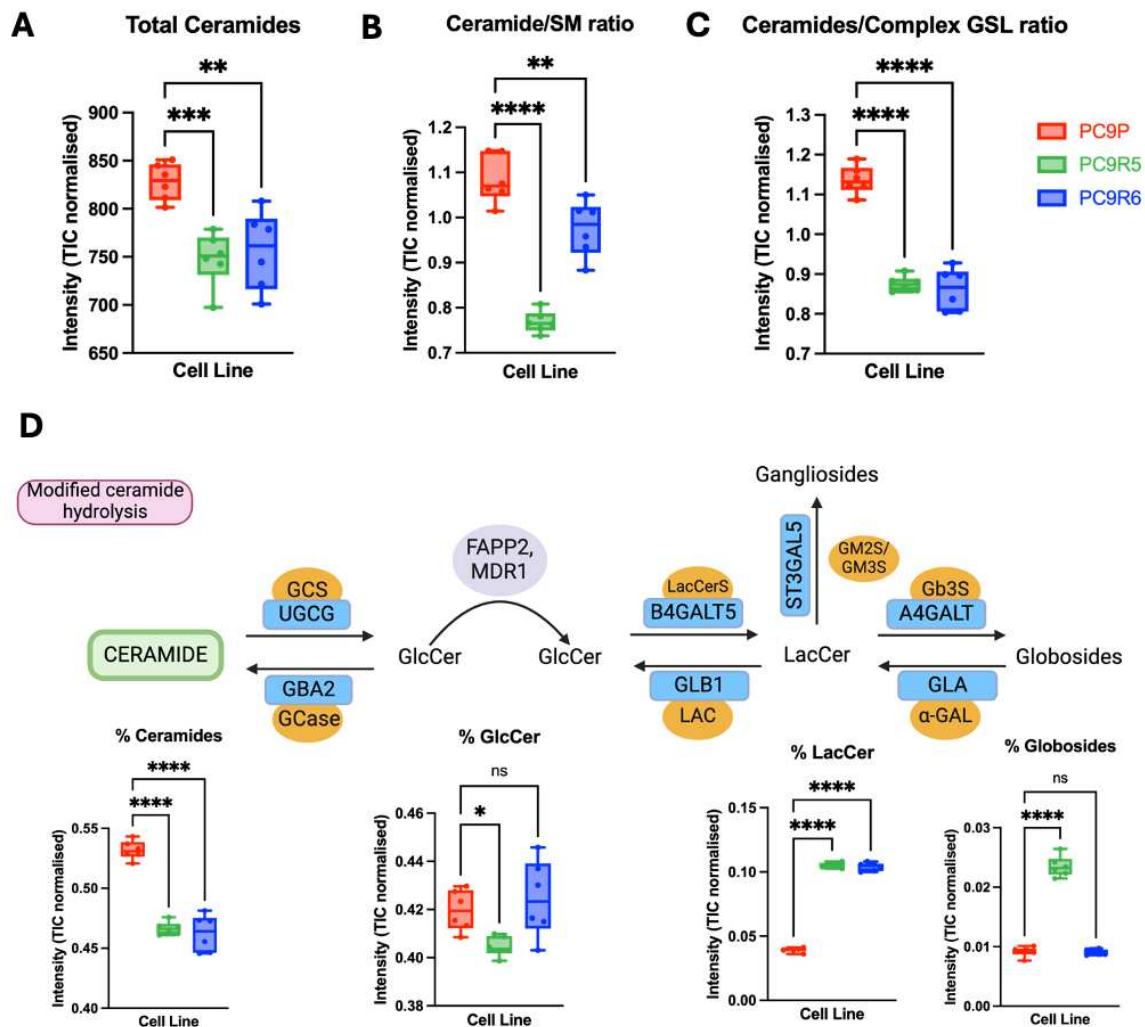


Figure 4.10: Sphingolipid profiles of pharmacologically-induced resistant models. (A) Total ceramide levels in OR and OS cells. (B) Ceramide to SM ratio in OR and OS cells. (C) Ceramide to GSL ratio in OR and OS cells. (D) GSL biosynthesis pathway starting from free ceramide and box plots showing the relative amount of ceramides, GlcCers, LacCers and globosides as a percentage of total SL composition. Genes are in blue, enzymes are in orange and transporters in lilac. Lipidomic data was normalised to TIC. Statistical differences in box plots determined by one-way ANOVA with Dunnett's correction multiple comparisons. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, * $P = 0.001$, **** $P < 0.0001$ compared with control (technical replicate, $n = 6$).. Created using BioRender.com.**

In summary, both the lipidomics and proteomics analyses revealed altered sphingolipid metabolism in the OR cell lines compared to OS control. A reduction in relative ceramide

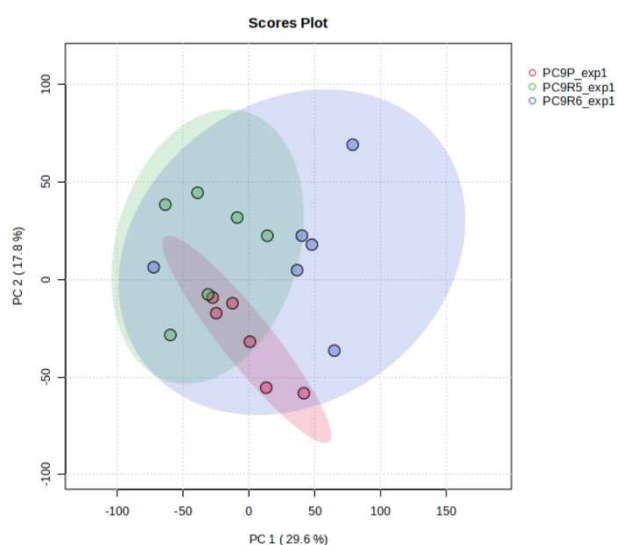
levels in OR cells were observed compared to OS, which could play a role in reducing apoptosis in OR cells.

4.6. Targeted metabolomics analysis of OR lines

Targeted metabolomics analysis was performed on the exact same pharmacologically-resistant cell line sample set and their controls as for the proteomics and lipidomics study. Details of the methodology and instrumentation used can be found in **Chapter 3.3.3 - 3.3.4**. After alignment and processing was completed using MS Dial and OptiMSR package in R Studio, 28 confidently annotated metabolites were identified that passed QC checks.

Multivariate statistical analysis of the targeted metabolomic data revealed some metabolite differences in OR versus OS cell lines, however this was not as clear as we hypothesised. OR and OS cells were not clearly differentiated based on their metabolic profiles by PCA ($R^2 = 0.43$; **Figure 4.11A**). Volcano plots between OR and OS revealed that between PC9R5 and PC9P (**Figure 4.11B**), significantly decreased metabolites included L-glutamic acid, a derivative of aspartic acid (N-Acetyl-L-aspartic acid; NAA), malate and uridine 5'-monophosphate in OR. In PC9R6 versus OS, decreased metabolites were L-glutamic acid only, and increased was 3-Methyl-2-oxovaleric acid, reduced glutathione (GSH) and 2'-Deoxyuridine 5'-monophosphate (**Figure 4.11C**).

A



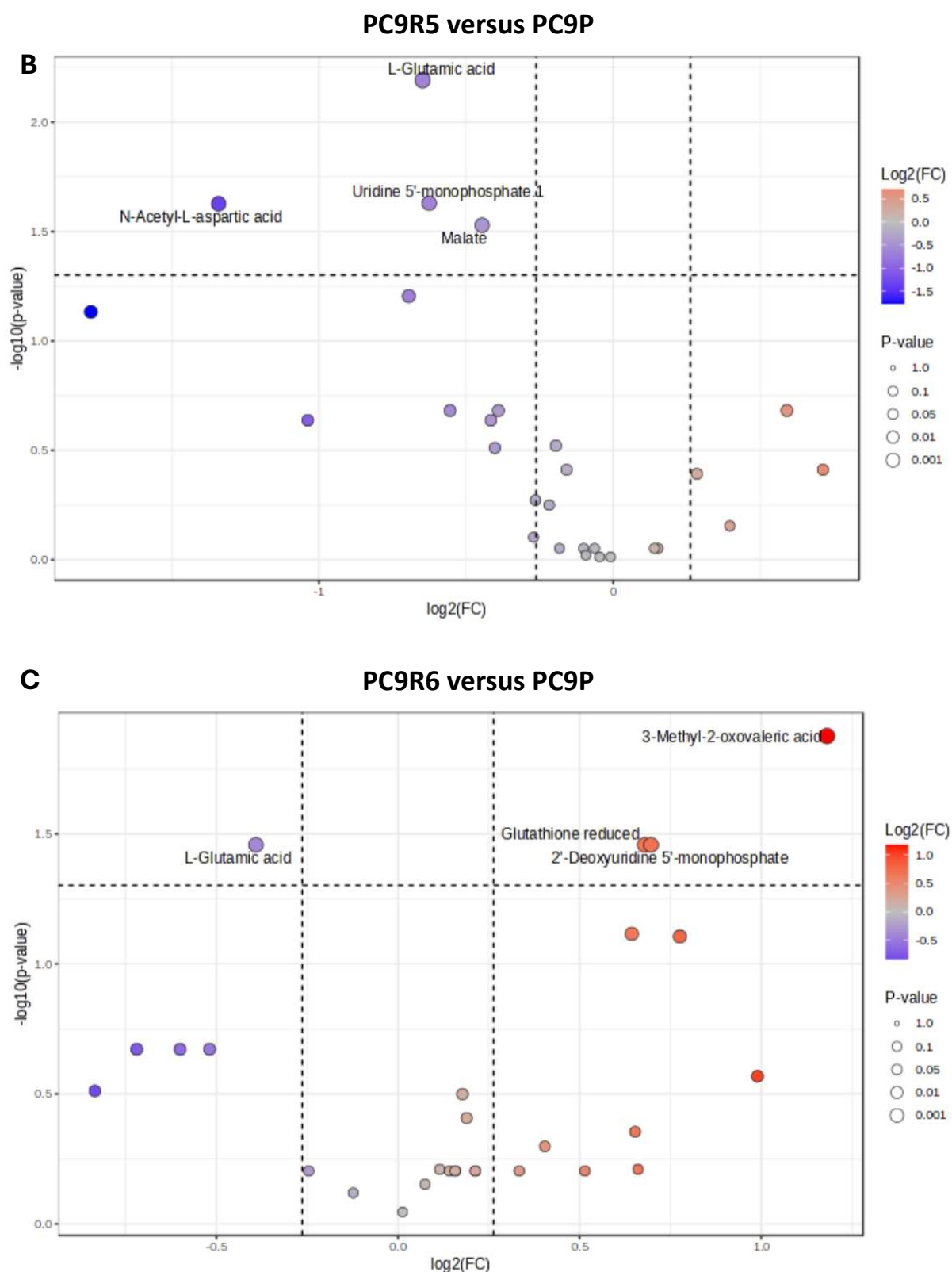


Figure 4.11: An overview of targeted metabolomics of pharmacologically-induced models of OR versus OS. (A) PCA scores plot of PC9P, PC9R5 and PC9R6. (B) Volcano plot showing lipids that are significantly increased (red) and decreased (blue) in PC9R5 compared to PC9P. (C) Volcano plot showing lipids that are significantly increased (red) and decreased (blue) in PC9R6 compared to PC9P. FC cut-off for significance was 1.2 and FDR adjusted p -value cut-off was 0.05. Data was normalised to TIC. ** $P < 0.0001$ compared with control (technical replicate, $n = 6$).**

Taking a closer look at some of the metabolites of interest revealed changes in amino acids, GSH, and TCA cycle (**Figure 4.12A**). Glutamic acid, the product of glutamine is a precursor to GSH(209). There was a reduction of glutamic acid in both OR cell lines compared to OS (**Figure 4.12B**), and an increase in GSH (which is significant in PC9R6; **Figure 4.12C**). This may indicate that cancer cells are utilising glutamic acid to produce more GSH, an important ROS scavenger(209). High homocysteine levels have an association with oxidative stress and cancer progression(210) and are also a substrate for GSH production; homocysteine was increased in PC9R6 compared to OS (**Figure 4.12D**). Malate is an intermediate of the TCA cycle, and was significantly decreased in PC9R5 versus OS (**Figure 4.12E**). N-acetyl-L-aspartic acid (NAA), a derivative of aspartic acid was decreased in OR compared to OS, reaching significance in PC9R5 only(211) (**Figure 4.12F**).

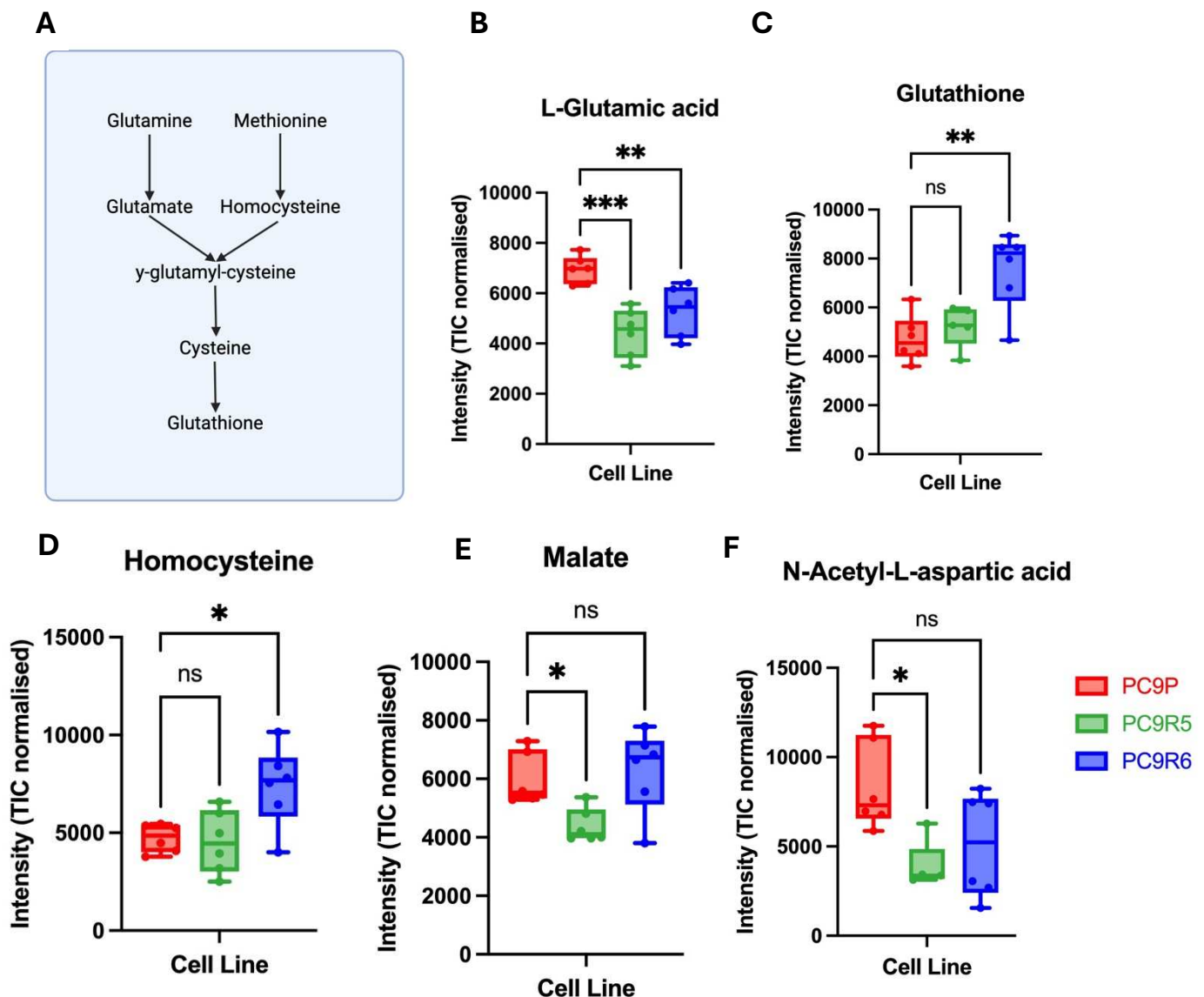


Figure 4.12: Specific metabolite changes in pharmacologically-induced models of OR versus OS cells. (A) A simplified overview of glutathione synthesis involving key metabolites. (B) L-Glutamic acid levels in OR versus OS. (C) Glutathion (GSH) levels in OR versus OS. (D) Homocysteine levels in OR versus OS. (E) Malate levels in OR versus OS. (F) N-Acetyl-L-aspartic acid (NAA) levels in OR versus OS. Data was normalised to TIC. Statistical differences in box plots determined by one-way ANOVA with Dunnett's correction multiple comparisons. *ns* $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P = 0.001$, $****P < 0.0001$ compared with control (technical replicate, $n = 6$). Adapted from Franco R et al(214). Parts of figure created using BioRender.com.

4.7. Multi-omics integration of all -omics analyses in OR lines

Multi-omics integration was performed on the proteomics, lipidomics and metabolomics datasets for significantly changed proteins (p value < 0.05 , $FC > 1.5$), lipids (p value < 0.05 , $FC > 2.0$) and metabolites (p value < 0.05 , $FC > 1.2$) in OR versus OS. Details on the joint pathway analysis can be found in **Chapter 3.4.4**.

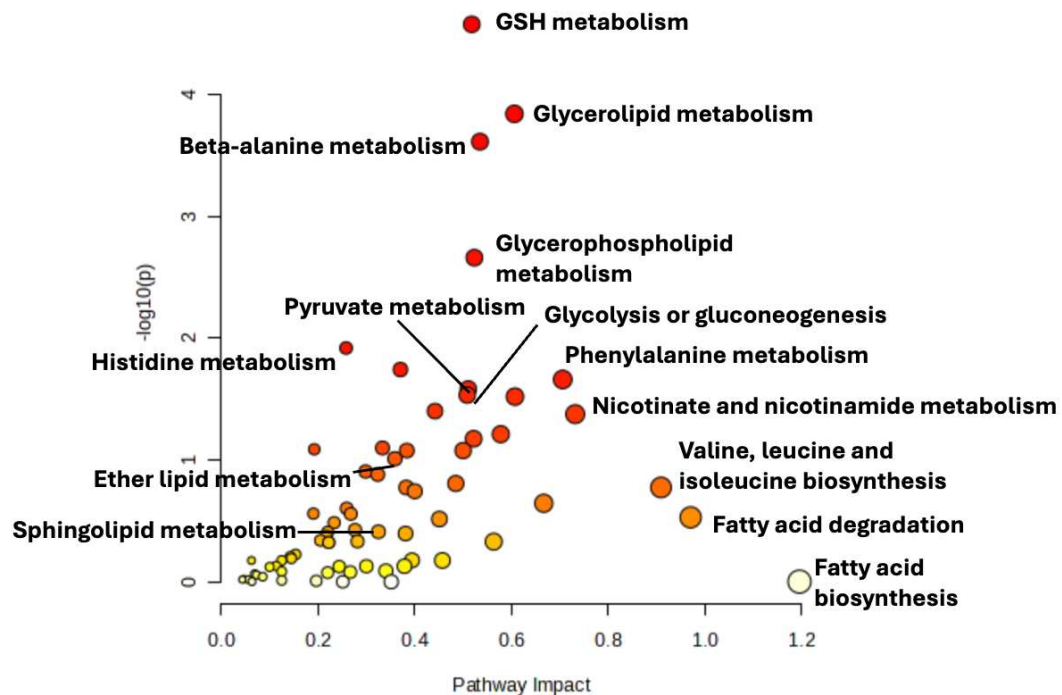
Significantly enriched pathways in PC9R5 included GSH metabolism ($p_{adj} = 0.002$), glycerolipid metabolism ($p_{adj} = 0.006$) and glycerophospholipid metabolism ($p_{adj} = 0.05$).

In PC9R6, many of the same pathways identified in PC9R5 were also enriched, highlighting the potential overlap of resistance mechanisms between the 2 cell lines (**Figure 4.13B**). However, the only statistically significant pathway was glycerophospholipid metabolism ($p_{adj} = 0.007$).

A

PC9R5

Overview of Pathway Analysis



B

PC9R6

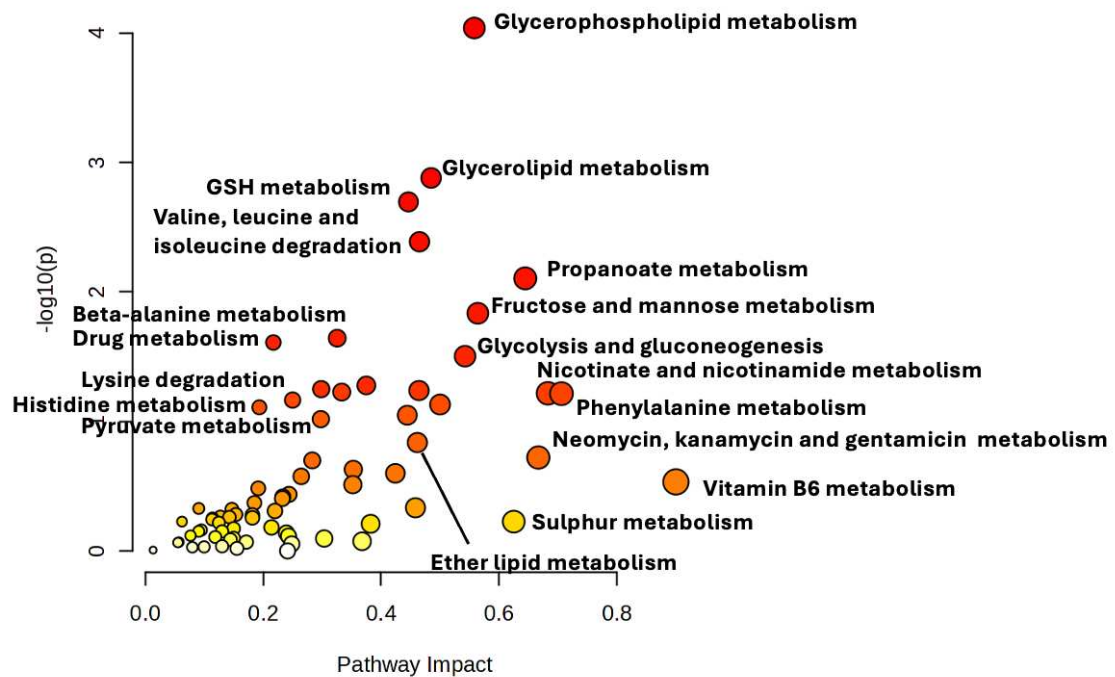


Figure 4.13: Multi-omics joint pathway analysis in (A) PC9R5 and (B) PC9R6. The significantly altered proteins (p value < 0.05 , $FC > 1.5$), lipids (p value < 0.05 , $FC > 2.0$) and metabolites (p value < 0.05 , $FC > 1.2$) in each cell line were used as input to determine any common pathways that may be enriched in OR versus OS on proteomic, lipidomic and metabolomic level.

4.8. Discussion

Many of the genetic mutations occurring in NSCLC may also lead to dysregulated metabolism, including in pathways such as glycolysis, gluconeogenesis, the TCA cycle and oxidative phosphorylation(47). Genetic mutations leading to osimertinib resistance mechanisms have also been reported, and are mainly found in genes involved in cell proliferation or other known proto-oncogenes(23). In this chapter, we explored metabolic rewiring as a potential contributor to drug resistance. Using multi-omics, we established that pharmacologically-induced OR cell lines have altered metabolic phenotypes compared to sensitive control.

Dysregulation of lipid metabolism: DNL and lipid peroxidation

Analysis of transcriptomics data revealed enrichment of cellular metabolic processes such as lipid biosynthesis and fatty acid synthesis, which have also been noted previously in other EGFR-TKI resistant NSCLC(52,212)(49). Here, we found *ACLY*, *FASN* and *SCD* genes to be significantly overexpressed in PC9R5 (OR) compared to OS. *FASN* is a key enzyme in DNL, which is often favoured in cancer cells over the use of exogenous lipids(105). However, we found *FASN* was decreased at protein level in PC9R5 and not significantly changed in PC9R6. On the other hand, the proteomics revealed an increase in *SCD5* in both OR lines compared to OS. Lipidomics analysis revealed an increase in total TG in both OR cell lines compared to control. Our multi-omics analyses highlighted pathways such as glycerolipid and glycerophospholipid metabolism as enriched in PC9R5 versus OS. Taken together this may be interpreted as evidence to support increased DNL in OR cell lines.

The main product of DNL is palmitate, a saturated fatty acid. Increased *DNL* in cancer cells may therefore increase membrane lipid saturation to mitigate oxidative stress caused by common cancer treatments, since PUFA-containing lipids are more susceptible to lipid peroxidation and ultimately ferroptosis(48,49). Increased lipid saturation may also alter uptake of chemotherapeutic agents by decreasing membrane fluidity(48). Therefore, we might expect cells more susceptible to osimertinib to have higher PUFA-containing lipids. However, we found the opposite with an increase in the ratio of PUFA/Non-PUFA TGs in PC9R5 (but not PC9R6) compared to PC9P. In addition, phospholipids (e.g. PCs and PIs)

increased in OR were mainly MUFA- or PUFA-containing, with SFA-phospholipids higher in OS.

On the other hand, several of the top significantly changed proteins in the untargeted proteomics analysis pointed to modulation within lipid peroxidation. ALDH1A3 and ALDH3A1 were underexpressed in PC9R5 compared to OS, whilst ALDH6A1 and ALDH2 were both overexpressed in PC9R6. These proteins are all involved in lipid peroxidation(197) and increased expression in NSCLC is linked to worse overall survival(197). Increased ALDH proteins accompany an increase in oxidative damage to lipids(213). Of interest, when ALDH2 expression was inhibited, LUAD cells were more sensitive to platinum-based chemotherapy. This same study also linked ALDH2 expression to regulation of ferroptosis, a programmed form of cell death reliant upon iron and lipid peroxides(214). Furthermore, GPX3 which has a protective effect against lipid peroxidation (206,215) was overexpressed in PC9R6, supporting an avoidance of ferroptosis as a potential resistance mechanism in this cell line. It is interesting to note that this mechanism may not be active within PC9R5, given that the ALDH proteins were underexpressed in this line compared to OS (and increased PUFA-containing lipids), suggesting that different mechanisms may contribute to OR in each cell line. Yet, it is interesting to note that there was a significant decrease (however this did not pass our FC cut-off) in expression of KEAP1 in PC9R5 versus PC9P, which has been shown to drive LUAD progression via increased NRF2 and antioxidant activity and avoidance of ferroptosis(216). This reduction in KEAP1 could be due to its degradation which then triggers the antioxidant activity(57).

Finally, the metabolomics analysis revealed aberrations within GSH metabolism, which was also identified as one of the most significantly enriched pathways in our multi-omics analysis in PC9R5. We observed a reduction in glutamic acid in both OR lines and an increase in GSH in PC9R6. We hypothesise this could be due to increased utilisation of glutamic acid to be converted into GSH(209). GSH metabolism has been well documented as dysregulated in NSCLC and drug resistance(82,83,217,218). Li *et al.*, found that GSH levels were reduced in erlotinib-resistant NSCLC lines compared to erlotinib-sensitive cells. This reduction was dependent on the T790M mutation as there is a lack of GSH synthesis enzyme expression when this mutation is present(83). Historically, drug resistance was associated with

increased GSH levels as an alternative energy provider(219), highlighting the modulation of different metabolic pathways within cancer cells to meet their everchanging needs(47,82,217,218). Increased GSH levels can also be linked to oxidative stress and ferroptosis(44,220). Reduced GSH is converted to the oxidized form (GSSG) by glutathione peroxidase under oxidative stress conditions(221). GSH has a protective role against oxidative stress, and when GSH levels are depleted, cells are more susceptible to oxidative stress. This then can decrease GPX4, a modulator of ferroptosis, thus activating this programmed death pathway(220).

Plasmalogens

The untargeted lipidomics analyses revealed decreased levels of individual plasmalogen species in OR versus OS. In addition, there was a significant decrease in total plasmalogens in PC9R5, but not in PC9R6, compared to PC9P control. Similarly, FAR1, a protein involved in the synthesis of plasmalogens, was significantly decreased in PC9R5 compared to OS control. Interestingly, the literature indicates that a reduction in FAR1 would increase plasmalogen levels(207), which is the opposite of what we see in the lipidomics. Plasmalogens are another family of lipids frequently increased in cancer cells(222), however, they have not been investigated as widely as some of the other lipid families discussed. Other than being linked to metastasis in several cancers, to our knowledge, plasmalogens have not been linked to NSCLC or in EGFR-TKI resistance. One possibility is that plasmalogens mitigate oxidative stress by reacting with ROS(223), however this would not account for a decrease in their abundance in OR, as found in PC9R5. One potential reason for this could be that PC9R5 has used up plasmalogens in reactions with ROS as a mechanism of mitigating oxidative stress. This could be instead of reducing PUFA content as a way to mitigate oxidative stress, which we observe in PC9R6.

Dysregulation of the TCA cycle and small molecule metabolism

The TCA cycle and its dysregulation in cancer is well known and has been investigated in drug-resistant NSCLC and summarised in Babuta *et al.*, 2022(105). Studies have found key TCA-intermediates such as succinate to be increased, which can lead to a hypoxic response and the development of drug tolerance(10). Others showed that the Warburg effect, where cancer cells switch to aerobic glycolysis over oxidative phosphorylation, was observed in OR

cells(224). Unfortunately, whilst the multi-omics revealed several enriched small molecule metabolic pathways, they did not retain significance after correction for false discovery. Our untargeted proteomics analysis revealed that enriched pathways in OR and OS included the TCA cycle, fatty acid degradation and metabolism, as well as amino sugar and nucleotide metabolism. Malate is an intermediate of the TCA cycle and had decreased abundance in PC9R5 versus OS. The Warburg effect dictates that cancer cells switch from oxidative phosphorylation pathways such as the TCA cycle for energy, and primarily utilise anaerobic glycolysis(42,46). Therefore, a decrease in malate may support this shift. An analogue of aspartate, N-acetyl-aspartic acid (NAA) was reduced in OR versus OS. NAA is associated with worse overall survival and tumour progression in cancer(225). Aspartate in cancer is known to be limiting for cell growth and is a major source for the biosynthesis of nucleotides(226). Aspartate also is used in the synthesis of asparagine, and this may explain the reduction we observe here, as we hypothesise it is being utilised in asparagine synthesis. Asparagine is consumed for the production of other complex energy sources such as lipids and thus its increased production would be favourable in OR cells(227).

Overall, there were limited significantly altered metabolites, fewer than we expected from both literature and our own proteomics analysis; this could be due to their inherently dynamic nature, with levels fluctuating in response to exogenous stimuli. Metabolites are often utilised in multiple pathways at a time and therefore metabolic flux may have been beneficial in this analysis to determine how and where they may be used up in the cell. Alternatively, metabolite stability could potentially be more prone to experimental/analytical factors.

Dysregulated sphingolipid metabolism

Transcriptomics analysis revealed that the expression of genes involved in sphingolipid metabolism were altered. *CERS5* and *CERS6*, involved in ceramide synthesis, and *UGCG*, involved in formation of GSLs, were significantly overexpressed in PC9R5 compared to OS. Dysregulated sphingolipid metabolism has been investigated in NSCLC but only very recently in one study(212) has an overexpression of *UGCG* and the GCS enzyme been reported in OR NSCLC(33–35).

Untargeted lipidomics revealed changes in ceramide levels in both OR lines versus OS. Modulation of ceramide levels has been well documented as a way for cancer cells to maintain cell proliferation and survival(228,229), with cancer cells reducing ceramide to avoid apoptosis(64,229,230). Here, we observed that total ceramide levels were decreased, with complex GSLs, particularly LacCer, increased in both OR lines compared to OS. A very recent study which independently conducted similar experiments found that there were increased levels of nearly all complex GSL species investigated in OR compared to OS cells(212).

To further investigate sphingolipid metabolism, we explored the proteomics data. This revealed that proteins pertaining to sphingolipid metabolism were altered in OR cells compared to OS. When comparing PC9R5 to PC9P sensitive control, underexpressed proteins included PLPP2, KDSR, SPHK2 and UGT8. PLPP2 belongs to the phospholipid phosphatase family which catalyses dephosphorylation of sphingolipids such as S1P and C1P(231). KDSR (3-ketodihydrosphingosine reductase) is involved in the *de novo* synthesis of sphingolipids in mammalian cells(232). KDSR reduces KDS into dihydrosphingosine (DHS), a precursor to ceramide via CerS. Downregulation of KDSR would therefore lead to a decrease in DHS, and ultimately ceramide. SPHK2 is one of two sphingosine kinase isoforms that act by phosphorylating sphingosine to produce S1P(233). Downregulation of SPHK2 would lead to a decrease in sphingosine, and therefore a decreased availability of a precursor to ceramide synthesis within the salvage pathway of sphingolipid metabolism. Finally, UGT8 (ceramide or UDP-galactosyltransferase) is commonly dysregulated in cancer cells and has been found to be overexpressed in breast cancer cells(53). Its role in sphingolipid metabolism is glycosylation of ceramide to GalCer(234). One significantly overexpressed protein in PC9R5 cells compared to PC9P was β -galactosidase (GLB1) which catalyses the breakdown of GM1 gangliosides(235). Another was ST3GAL1, the protein which converts LacCer to gangliosides. It has also been associated with drug resistance in ovarian cancer, with an increase in its expression associated with worse overall survival(236). An increase in both of these proteins suggests increased production of GSLs.

When comparing PC9R6 and PC9P sensitive control there were two significantly underexpressed proteins and two significantly overexpressed proteins in this subset of

sphingolipid-related proteins. CERS6 and DEGS1 (DES), both involved in synthesis of ceramide were underexpressed in PC9R6 compared to PC9P. Overexpressed proteins in PC9R6 compared to PC9P were GBA1 and UGCG. GBA1 (or GCase) is responsible for the cleavage of the β -glucosidic linkage in GlcCers, to produce ceramide(237). UGCG catalyses the formation of GlcCer from ceramides, and therefore works in opposition to GBA1(76). An upregulation of both enzymes could indicate increased metabolic flux within the GSL pathway. This infers that there are competing factors that are driving dysregulation of sphingolipid metabolism in PC9R6 cells. One protein we found to be of interest however fell just short of our significance parameters was B4GALT5, the enzyme responsible for the synthesis of LacCer from GlcCer. This was increased in PC9R6 versus PC9P.

We explore the role of sphingolipid metabolism in osimertinib resistance in more detail in **Chapter 6**.

Conclusion

This chapter has revealed key differences in pharmacologically-induced OR cells versus OS at the transcriptomic, proteomic and lipidomic levels. These included alterations in *DNL*, lipid peroxidation and small molecule metabolism. Our most interesting discovery lay within dysregulated sphingolipid metabolism, which was evident at transcriptome, proteome and lipidome levels, and was found in both OR cell lines. This will be further investigated in **Chapter 6**. Many of the key lipids, genes and proteins identified in this chapter have been previously investigated in the literature as potential mechanisms or biomarkers of resistance with other EGFR-TKIs (but not osimertinib), implying that the mechanisms of resistance may be conserved and predictable for future generations of TKIs.

Chapter 5 – Multi-omics profiling of genetically modified models of osimertinib resistance

5. Chapter 5: Multi-omics profiling of genetically-modified models of osimertinib

5.1. Background

In the previous chapter, we investigated the metabolism-centred changes in acquired OR using models that were treated with escalating doses of osimertinib. An alternative approach to induce OR is by genetic alterations, where mutations in alternative or downstream signalling pathways to EGFR allow for drug resistance to occur. The advantage of using a genetic model of resistance is we can more clearly distinguish cause and effect, compared to a pharmacologically-induced model.

Genetically modified (GM) resistant cell lines were generated at AstraZeneca. These were a PTEN CRISPR knockout and a CRISPR gene edited PC9 line harbouring a PIK3CA point mutation at H1047R. The PIK3CA gene encodes for the p110 subunit of PI3K, and therefore the mutation is within this subunit(238). These cells were then cultured in 160 nM osimertinib(173). Both PTEN and PI3K act within the same pathway (as summarised in **Figure 5.1**), whereby through extracellular activation of RAS, PI3K catalyses the production of a lipid secondary messenger PIP3 to the cell membrane, which in turn activates Akt and inhibits BAD, promoting survival(35). As a tumour suppressor, PTEN is both a lipid and protein phosphatase with the ability to dephosphorylate and therefore deactivate PIP3, which stops downstream activation of Akt(40). When PTEN is knocked out, Akt is activated (**Figure 5.1**).

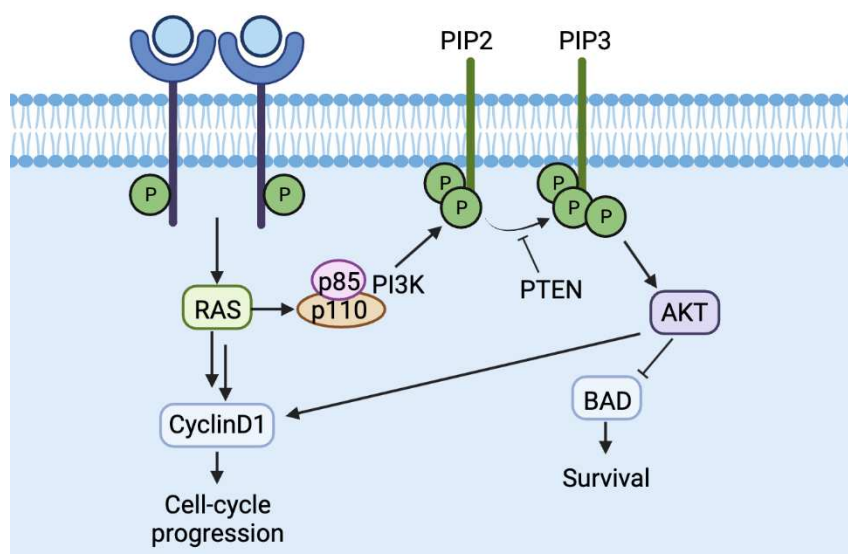


Figure 5.1: An overview of PI3K/Akt signalling pathway. *PI3K is activated by RAS and upon this activation, the phosphorylation of PIP2 which follows on to phosphorylation of PIP3 allows for various signalling proteins such as Akt to localise to the cell membrane and activate cell survival pathways (such as BAD). PTEN functions by inhibiting the phosphorylation of PIP3 and therefore inhibiting further downstream effects. Adapted from Yang J et al(35). Created using BioRender.com.*

In the literature, PIK3CA mutants are found within advanced NSCLC and are responsible for lower overall survival and reduced PFS. Following PIP3 generation and Akt activation, several downstream pathways such as mTOR are activated in the cascade(36). Activation of this complex pathway has been linked to aberrant lipid metabolism, with increased expression of key lipid metabolism proteins such as SREBP1 and FASN(239). PTEN knockout or loss of PTEN function through mutation was expressed in 42.4 % of NSCLC and is linked to a shorter PFS and more severe disease(41,240) and has links to EGFR-TKI resistance(241). In metabolomics studies in the literature, which investigate PTEN knockout models of prostate cancer, metabolites relating to glycolysis, glutaminolysis, DNL, and fatty acid β -oxidation were found to have increased, highlighting the extensive metabolic effects that knockout of this gene can have(242).

5.1.1. Chapter Aims

Hypothesis

Since both genetic modifications of PC9 PTEN $-/-$ and PC9 PIK3CAm cell lines act within the same pathway and influence the same proteins downstream, we hypothesised that these two cell lines would possess similar metabolic phenotypes (proteome, lipidome and metabolome) to each other and different to OS control (PC9 NTC).

The aim of this chapter was to determine if GM models of OR have metabolic differences compared to OS, using multi-omics.

To address this, we have performed:

- Untargeted proteomics to investigate protein-level changes.
- Untargeted lipidomics analysis to investigate lipid profile changes.
- Targeted metabolomics analysis to investigate polar metabolite changes.

5.2. Characterisation of the genetically modified (GM) models of resistance

To confirm and characterise the GM cell lines' resistance to osimertinib, an SRB assay (methodology described in **Chapter 3.2.1**) was used to measure the inhibition of osimertinib on cell growth when PC9 NTC (OS), PC9 PTEN $-/-$ (OR) and PC9 PI3KCA H1047R mutant (known as PC9 PIK3CAm from here on) (OR) are treated with increasing doses of osimertinib (**Figure 5.2**). Information on the generation of these cell lines can be found in **Chapter 3.1.4**. The OR lines were more resistant to osimertinib at higher doses as expected, with the IC_{50} values of each of these lines around 0.06 μ M (0.059 μ M and 0.065 μ M) compared to OS (0.004 μ M).

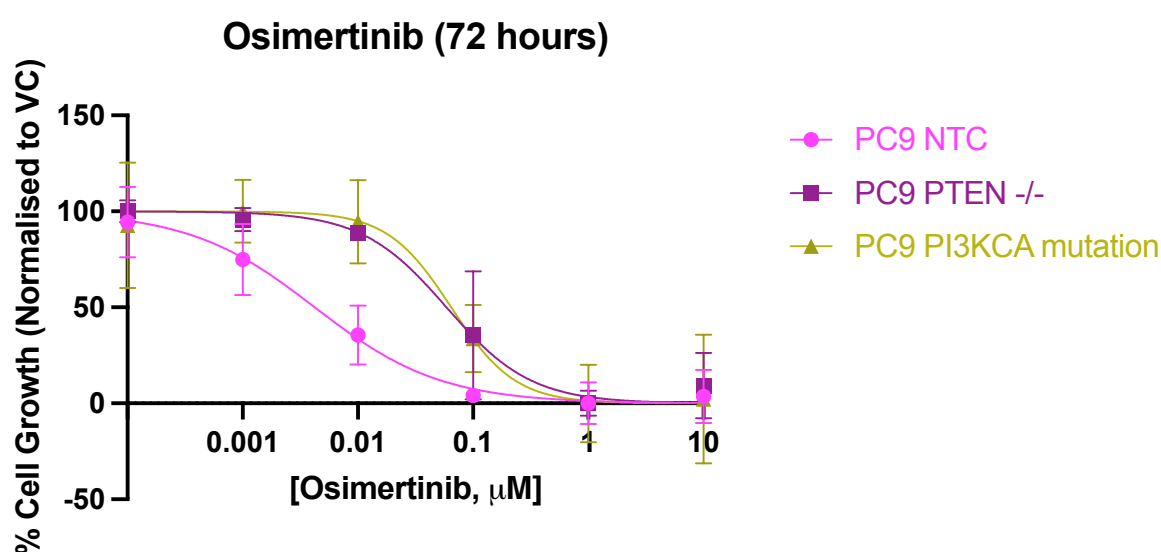


Figure 5.2: Characterisation of GM models of osimertinib resistance. GM lines were treated with 0-10 μ M osimertinib for 72 hours before cell growth was measured using the SRB assay. Data expressed as mean $\pm$ SEM (biological replicate, $n = 3$).

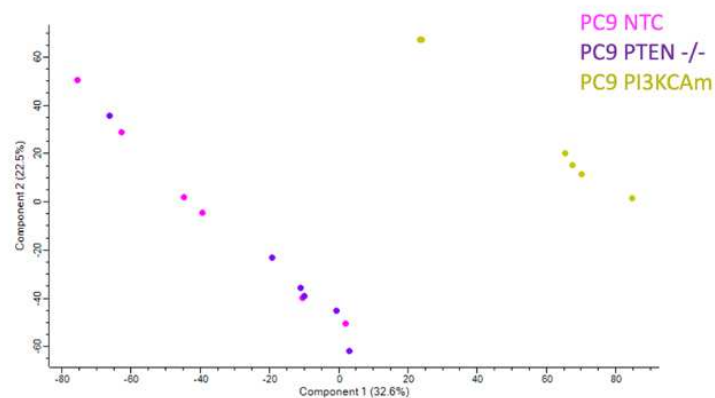
5.2.1. Untargeted proteomics analysis of GM models of resistance

Proteomic analysis was performed by Dr Pavel Shliha and Dr Alex Montoya at MRC LMS. Full analysis information can be found **Chapter 3.2.9** and **3.3.5-3.3.7**. There were 7536 total proteins detected, 6471 of these could be minimally identified by 2 or more peptides and 6397 of these could be minimally quantified by 2 or more peptides.

After processing using Perseus software, there were 4556 proteins that were statistically significant using ANOVA and Tukey's post hoc testing across all 3 groups. We first carried out PCA (**Figure 5.3A**) which revealed that PC9 PTEN $-/-$ samples clusters together with PC9 NTC

highlighting a similar overall proteomic profile, whereas PC9 PIK3CAm was well separated in the first principal component relative to both PC9 PTEN $-/-$ and PC9 NTC. The significantly altered proteins were translated into gene names and gene set enrichment analysis (GSEA) was performed using DAVID(189). The Top 50 KEGG pathways that were enriched across all cell lines included amino and nucleotide sugar metabolism (including biosynthesis of nucleotide sugars and biosynthesis of amino acids), ferroptosis, fatty acid metabolism and glycolysis (**Figure 5.3B**). Pathways that were not relevant to human lung cancer were removed from the plot.

A



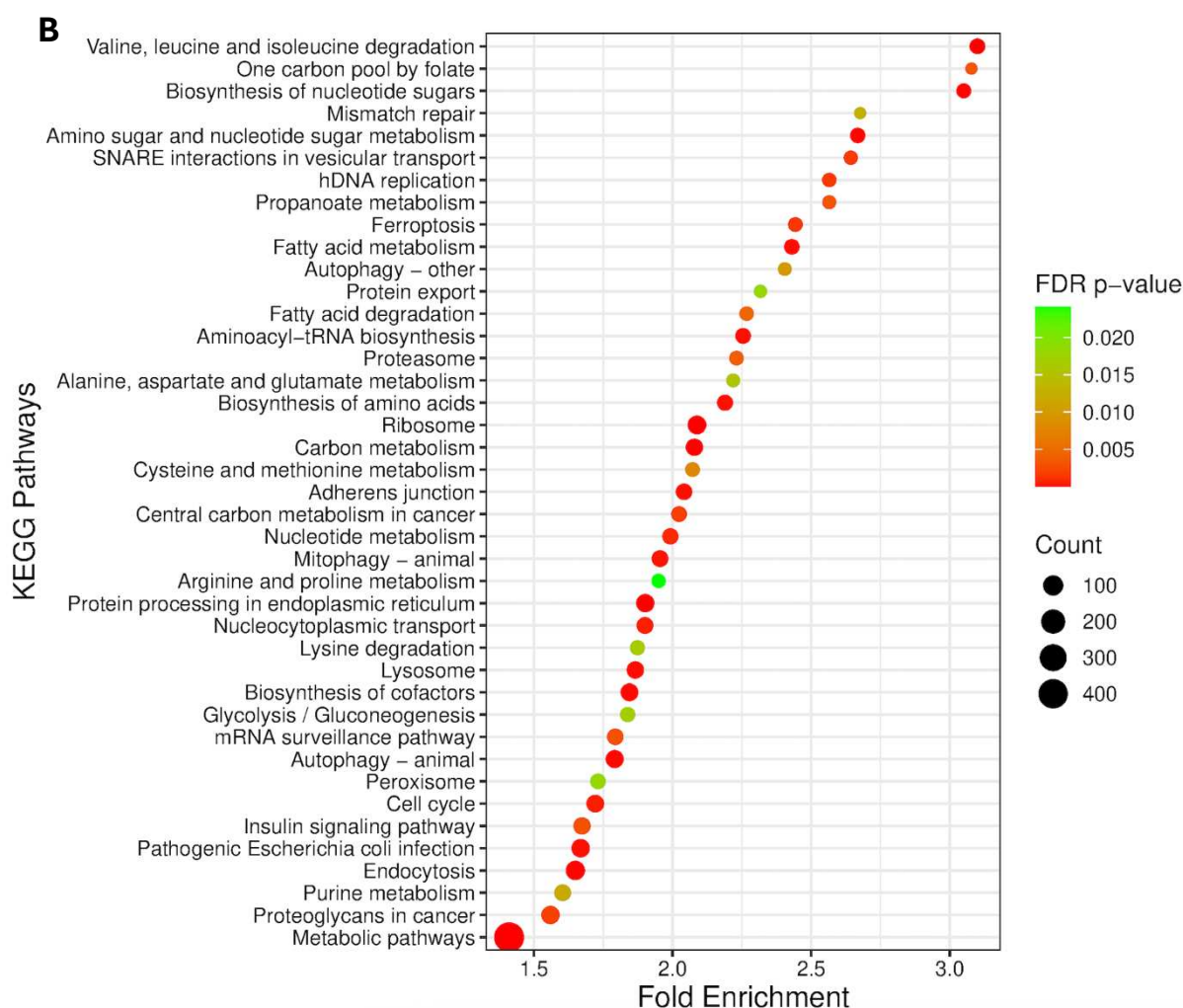


Figure 5.3: Untargeted proteomic analysis of OR versus OS highlighting differentially expressed KEGG pathways. (A) PCA score plot of proteomic profiles in OR versus OS cells shows distinct proteomic profiles (technical replicate, $n = 6$). (B) KEGG pathways that differentially expressed proteins were enriched in. (C) The Top 10 GO pathways that differentially expressed proteins were enriched in. Biological Process (BP), Cellular Content (CC) and Molecular Function (MF).

We then performed two-way comparisons on each OR and OS cell line to determine which proteins were significantly altered. **Figure 5.4A** shows specific proteins which were either over or underexpressed in PC9 PTEN $-/-$ versus PC9 NTC. 1624 proteins in this comparison were significant based on Student's t test and Tukey's HSD post-hoc testing for multiple comparisons. Major underexpressed proteins included ALDH3A1 (involved in lipid peroxidation), programmed cell death 4 (PDCD4) which is a known tumour suppressor and

documented to be reduced in lung cancer(243), alpha-ketoglutarate-dependent dioxygenase ALKB Homolog 7 (ALKBH7), a protein involved in a cells response to DNA damage and a change in MOMP which can lead to necrotic cell death(244), succinate dehydrogenase complex subunit D (SDHD) which is responsible for the oxidation of succinate(245) and solute carrier family members SLC66A2 and SLC35A3, with the second being a UDP-N-Acetylglucosamine transporter(246). Major overexpressed proteins included asparagine synthetase (ASNS), ALDH2 (protects against ferroptosis), HSPB1, and peroxisomal trans-2-enoyl-CoA reductase (PECR) which may promote proliferation(247).

We next compared the proteomic phenotype of PC9 PIK3CA^m versus PC9 NTC (**Figure 5.4B**). 3538 proteins in this comparison were significant based on Student's t test and Tukey's HSD post-hoc testing for multiple comparisons. Underexpressed proteins included ALDH3A1, ASS1 (arginine synthetase), UDP-glucose 6-dehydrogenase (UGDH) which converts UDP-glucose to UDP-glucuronate and is part of the glycosaminoglycan biosynthesis pathway(248), caspase-14 (CASP14) which triggers apoptosis and is a marker of triple negative breast cancer(249), SLC16A3, also known as monocarboxylate transporter 4 (MCT4), which transports lactic acid and pyruvate across plasma membranes(250), two solute transporters (SLC37A4 and SLC38A10) with the first regulating the transport of glucose-6-phosphate(251) and the second regulating transmembrane transport of amino acids(252), AKT1 which is involved in PI3K signalling, UDP-glucose glycoprotein glucosyltransferase (UGGT2) a protein that re-glycosylates unfolded glycoproteins and TLC domain containing 1 (TLCD1) which is involved in phospholipid homeostasis and lipid distribution regulation within membranes(253). Major overexpressed proteins were thyroid hormone receptor interactor 13 (TRIP13), which is overexpressed in gefitinib resistant NSCLC(254), SH3KBP1 which has been found to promote EGFR signalling(255), SLC1A3 which was found to be increased in OR NSCLC by Ochi *et al.*(256), adiponectin receptor 2 (ADIPOR2), a protein also found to be overexpressed in NSCLC(257) and ASNS and HSPB1 which have both already been described.

Differential protein expression

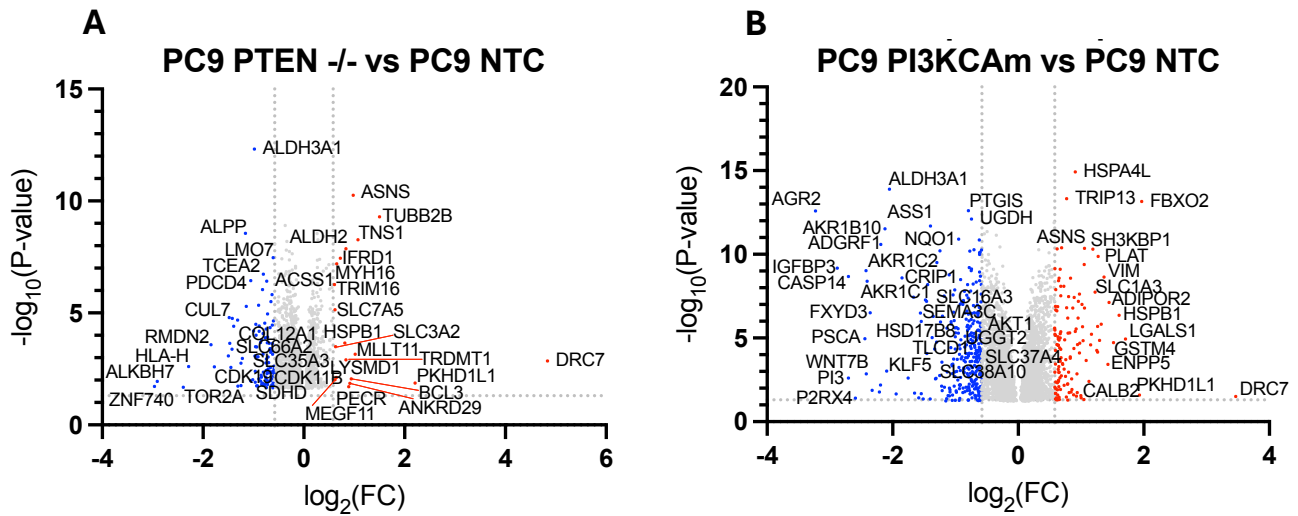


Figure 5.4: A two-way comparison of protein changes in GM models of OR versus OS. (A) PC9 PTEN $-/-$ compared to PC9 NTC and (B) PC9 PI3KCam compared to PC9 NTC. Volcano plot showing proteins that are significantly increased (red) and decreased in (blue). FC cut-off for significance was 1.5 and FDR adjusted p -value cut-off was 0.05. Data was normalised via local regression normalisation.

5.2.2. Changes in proteins involved in lipid metabolism

After dysregulation of various aspects of lipid metabolism were revealed in the pharmacologically-induced cell line (**Chapter 4**), we investigated the proteomics dataset to determine if these proteins were also differentially regulated in GM lines. As with our other data, there were not many significant differences observed in PC9 PTEN $-/-$ compared to OS, except for under expression of PPIPSK1 (**Figure 5.5A**). In PC9 PIK3CAm versus OS, PLPP3 was underexpressed and ACSL4 and SCD were overexpressed (**Figure 5.5B**).

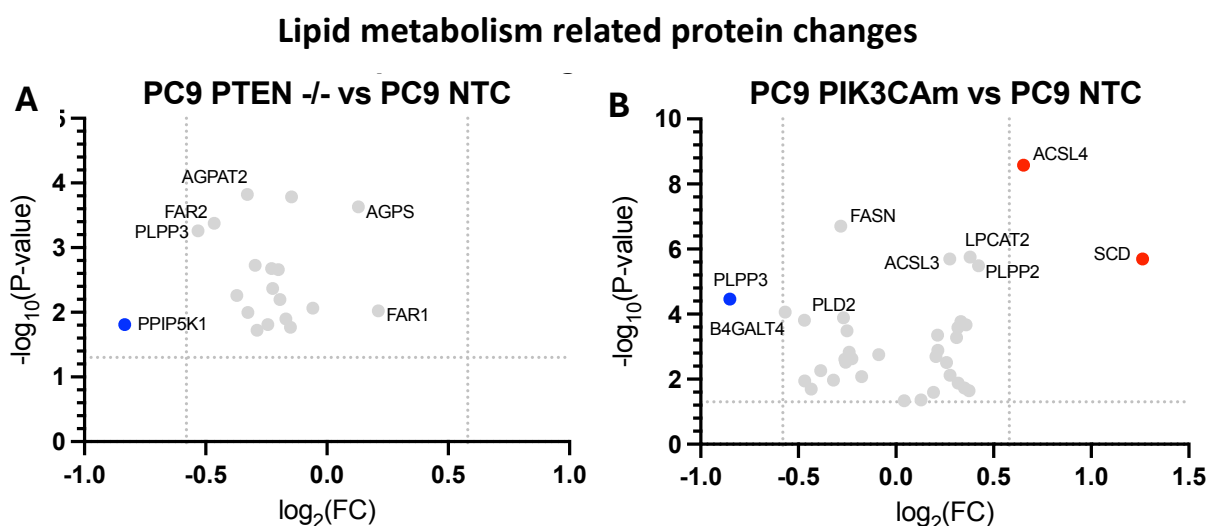


Figure 5.5: Specific changes in proteins involved in all aspects of lipid metabolism in GM models of OR versus OS. (A) PC9 PTEN $-/-$ compared to PC9 NTC and (B) PC9 PI3KCAm compared to PC9 NTC. Volcano plot showing proteins that are significantly increased (red) and decreased in (blue). FC cut-off for significance was 1.5 and FDR adjusted p-value cut-off was 0.05. Data was normalised via local regression normalisation.

5.3. Untargeted lipidomic analysis of GM models of resistance

We then used untargeted lipidomic analysis to investigate the lipid profiles of the GM resistant lines. This was performed on the exact same GM resistant cell line sample set as the proteomics. Lipids were extracted and analysed by LC-MS/MS as described in **Chapter 3.2.6 - 3.2.7** and **3.3.1**. Overall, there were 1054 unique IDs identified and retained after QC filtering in both positive and negative ion modes combined. We confidently annotated 552 of these lipids and tentatively annotated a further 111, with 390 unknown features. Statistical analysis was performed using Metaboanalyst v6.0, SIMCA v17.0.2.34594 and GraphPad Prism v10.2.0. Full data processing method is described in **Chapter 3.3.2**.

5.3.1. Multivariate statistical analysis revealed distinct lipid profiles between PC9 PIK3CAm and PC9 NTC

PCA on the lipid profiles of the three groups revealed PC9 PIK3CAm was separated from the other two cell lines on the first principal component ($R^2 = 0.79$; **Figure 5.6A**). Hierarchical clustering was performed and distinguished PC9 PI3KCAm from both PC9 PTEN $-/-$ and OS, however highlighted the closer similarity of the lipid profiles of PC9 PTEN $-/-$ and OS. The Top 50 features, based on ANOVA, that appear to be driving the differences across the cell lines

included sphingolipids such as ceramides and complex GSLs, plasmalogens and phospholipids (Figure 5.6B).

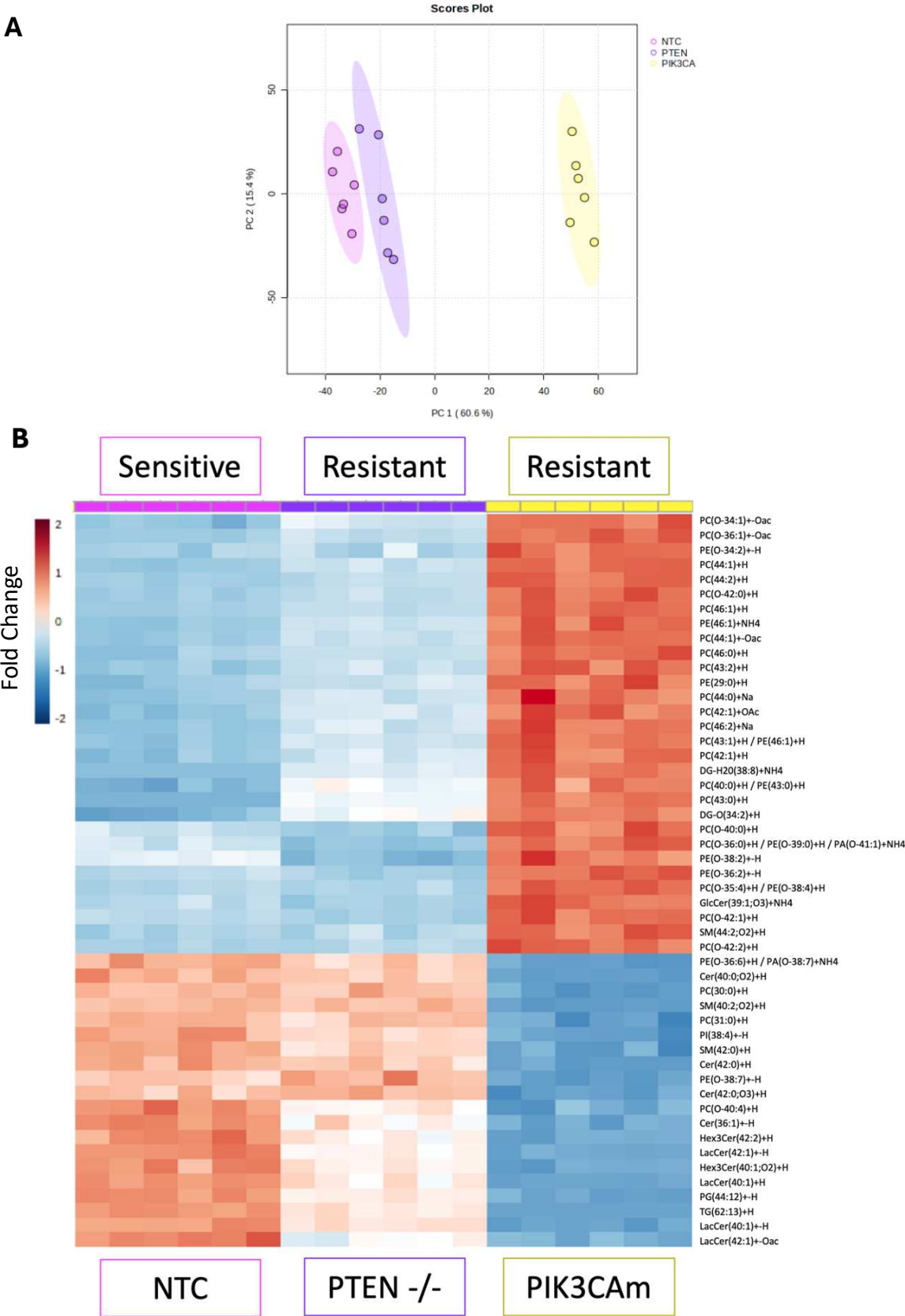
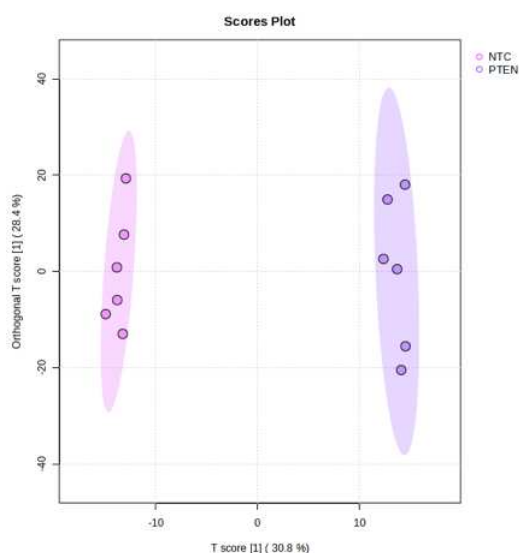


Figure 5.6: Multivariate statistical analysis of untargeted lipidomic profiles of GM models of OR versus OS cells. (A) PCA score plot of lipidomic profiles in OR versus OS cells shows distinct lipidomic profiles. (B) Heatmap of the Top 50 features comparing OR with OS. Data normalised to TIC (technical replicate, n = 6).

5.3.2. Specific changes in two-way comparisons: PC9 PTEN -/- versus PC9 NTC

An OPLS-DA model significantly discriminated between PC9 PTEN -/- and PC9 NTC ($R^2X = 0.31$, $R^2Y = 0.98$, $Q^2 = 0.93$, p-value = 0.0003; **Figure 5.7A**). The associated S-plot (**Figure 5.7B**) revealed increased phospholipids (mainly PI) and ceramides and decreased phospholipids (across many classes) in PC9 PTEN -/- compared to control. Finally, we used a volcano plot, which identifies key significantly changed lipids based on univariate linear fold changes (FC) (**Figure 5.7C**). Only 6 lipids were significantly increased in PC9 PTEN -/- compared to OS using a fold change cut-off of 1.5 these were DGs and PCs. Only one lipid was significantly decreased.

A



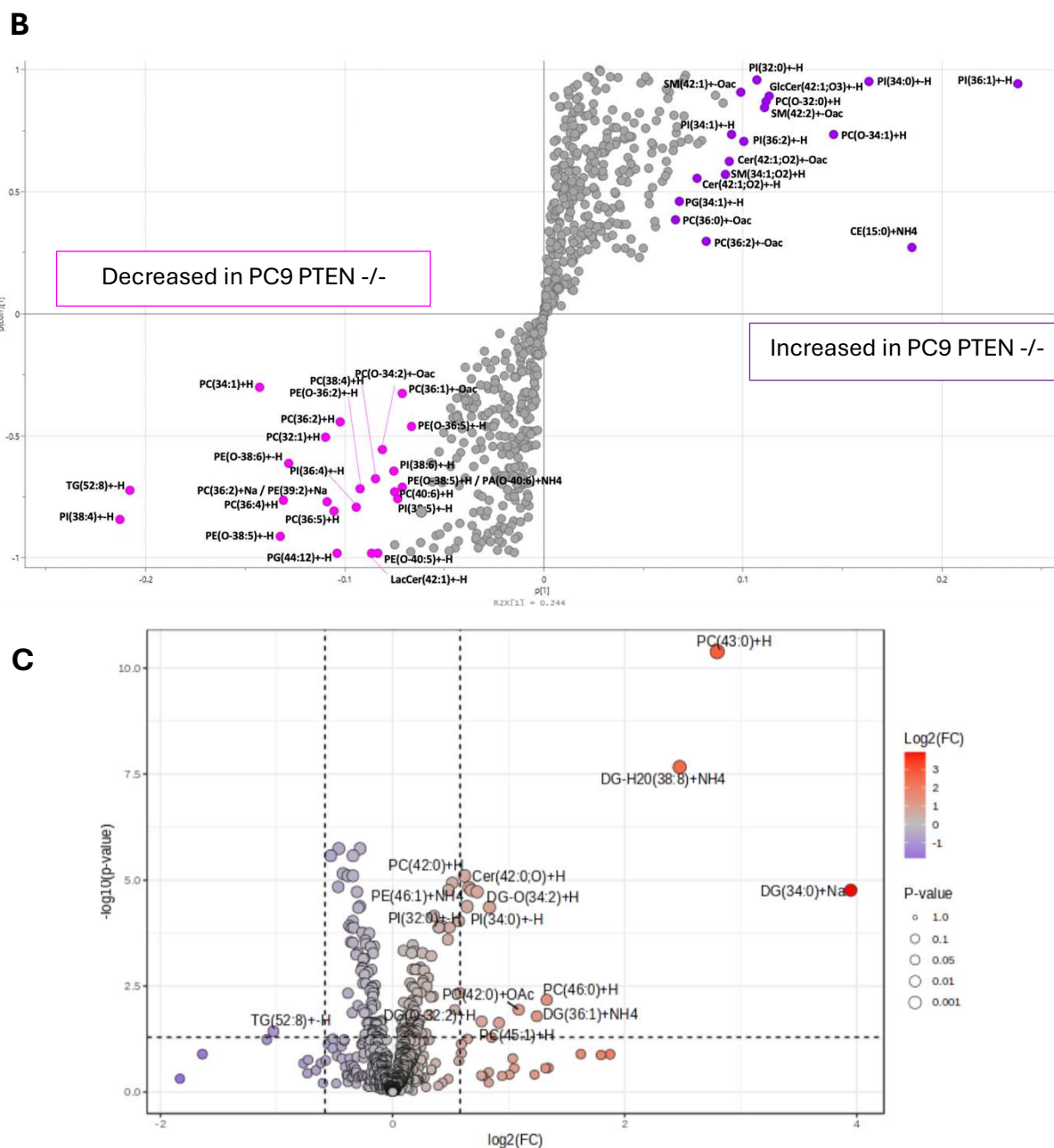


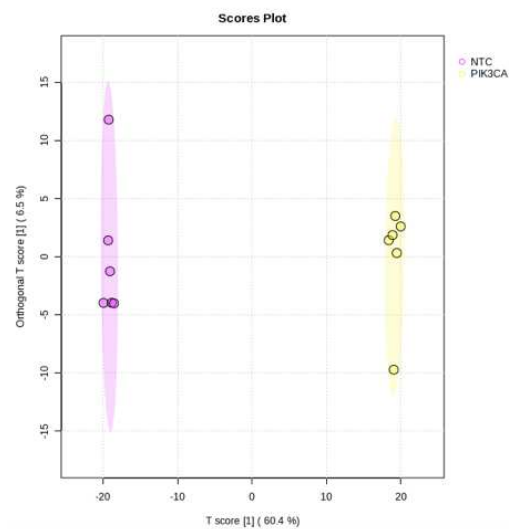
Figure 5.7: A two-way comparison of specific lipid changes between PC9 PTEN $-/-$ compared to PC9 NTC. (A) OPLS-DA scores plot of PC9 PTEN $-/-$ versus PC9 NTC. (B) S-plot showing lipids increased (purple) and decreased (pink) in PC9 PTEN $-/-$ versus PC9 NTC. Highlighted data points were annotated to show representative lipids that are changing. (C) Volcano plot showing lipids that are significantly increased in PC9 PTEN $-/-$ (in red) and decreased in PC9 PTEN $-/-$ (in blue). FC cut-off for significance was 1.5 and FDR adjusted p-value cut-off was 0.05. Data was normalised to TIC (technical replicate, $n = 6$).

5.3.3. Specific changes in two-way comparisons: PC9 PIK3Cam versus PC9 NTC

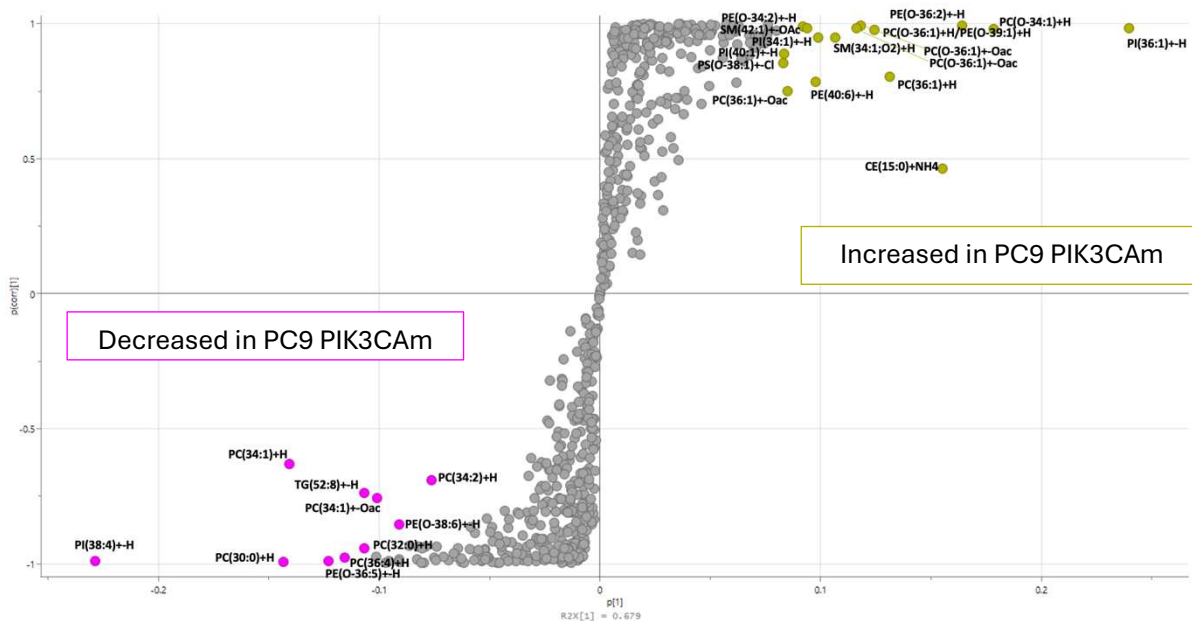
OPLS-DA also showed the PC9 PIK3Cam and PC9 NTC lipid profiles to be significantly distinct ($R^2X = 0.60$, $R^2Y = 0.997$, $Q^2 = 0.99$, p value = $1.8e-10$; **Figure 5.8A**). The associated S-plot

(Figure 5.8B) revealed modulation of plasmalogens and phospholipids in the OR line compared to OS. Finally, the volcano plot analysis (Figure 5.8C) showed lipids species from many different classes were significantly changed in OR compared to OS with a fold change cut off 2.0. Lipids that were significantly increased are shown in red and included a mixture of different lipid families, with DG and PC phospholipids (especially MUFA-containing) being the most significantly increased. Decreased lipids shown in blue included some ceramides and LacCers but also complex GSL species such as some globo/gangliosides (denoted as Hex3-HexNAc-Cer).

A



B



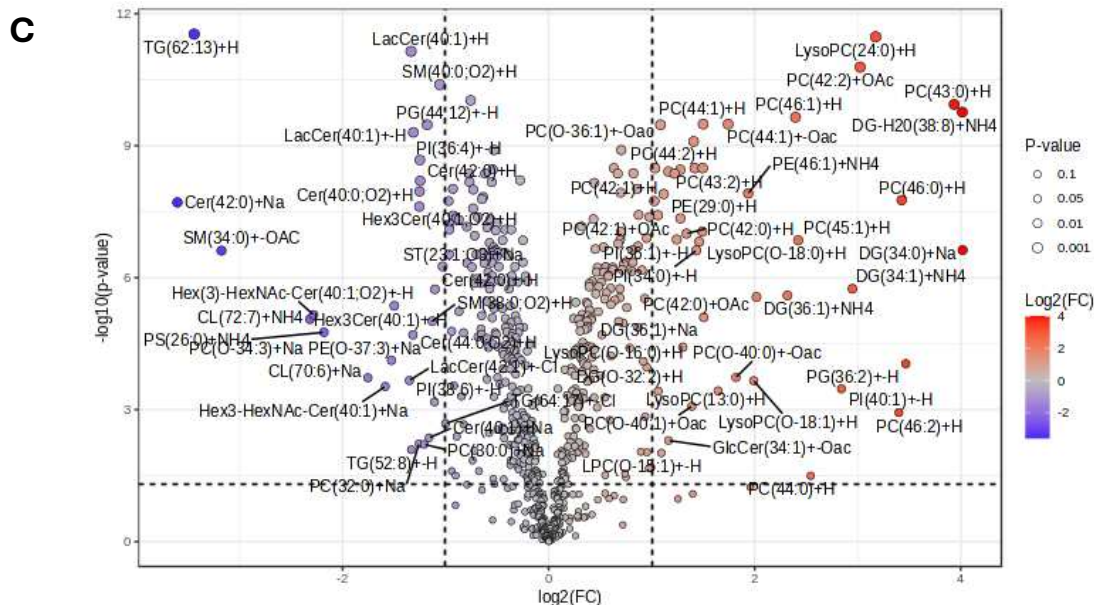


Figure 5.8: A two-way comparison of specific lipid changes between PC9 PIK3CAm compared to PC9 NTC. (A) OPLS-DA scores plot of PC9 PIK3CAm versus PC9 NTC. (B) S-plot showing lipids increased (yellow) and decreased (pink) in PC9 PIK3CAm compared to PC9 NTC. Highlighted data points were annotated to show representative lipids that are changing. (C) Volcano plot showing lipids that are significantly increased in PC9 PIK3CAm (in red) and decreased in PC9 PIK3CAm (in blue). FC cut-off for significance was 2.0 and FDR adjusted p-value cut-off was 0.05. Data was normalised to TIC (technical replicate, n = 6).

5.3.4. Key families of lipids that are altered in OR and OS

We investigated different lipid families highlighted in the multivariate/univariate analyses. There was a significant decrease in total PCs and increased total plasmalogens in PC9 PIK3CAM compared to OS, with no significant difference between PC9 PTEN $-/-$ and OS (**Figure 5.9A**). In addition, we looked at the saturation of fatty acyl chains in different lipid classes and noted that PC9 PIK3CAM (but not PC9 PTEN $-/-$) had significantly reduced ratio of PUFA/Non-PUFA PCs compared to OS, suggesting increased fatty acyl saturation (**Figure 5.9B**). There was a significant increase in PC/PE plasmalogens in PC9 PIK3CAM versus OS (**Figure 5.9C**). We found there to be significantly increased total DGs in both OR lines compared to OS, in line with the expected link between DG and PI3K signalling(258) (**Figure 5.9D**). We then looked at ceramide and sphingolipid classes as a proportion of total sphingolipids. In contrast to the pharmacological models, % ceramide was not significantly changed in OR (**Figure 5.9E**). We next looked at the content of different sphingolipids. There was a significant increase in % GlcCer in both OR lines compared to OS (**Figure 5.9F**), with significantly decreased % LacCers

(Figure 5.9G), in contrast to what was observed in the pharmacologically-induced resistant cell lines.

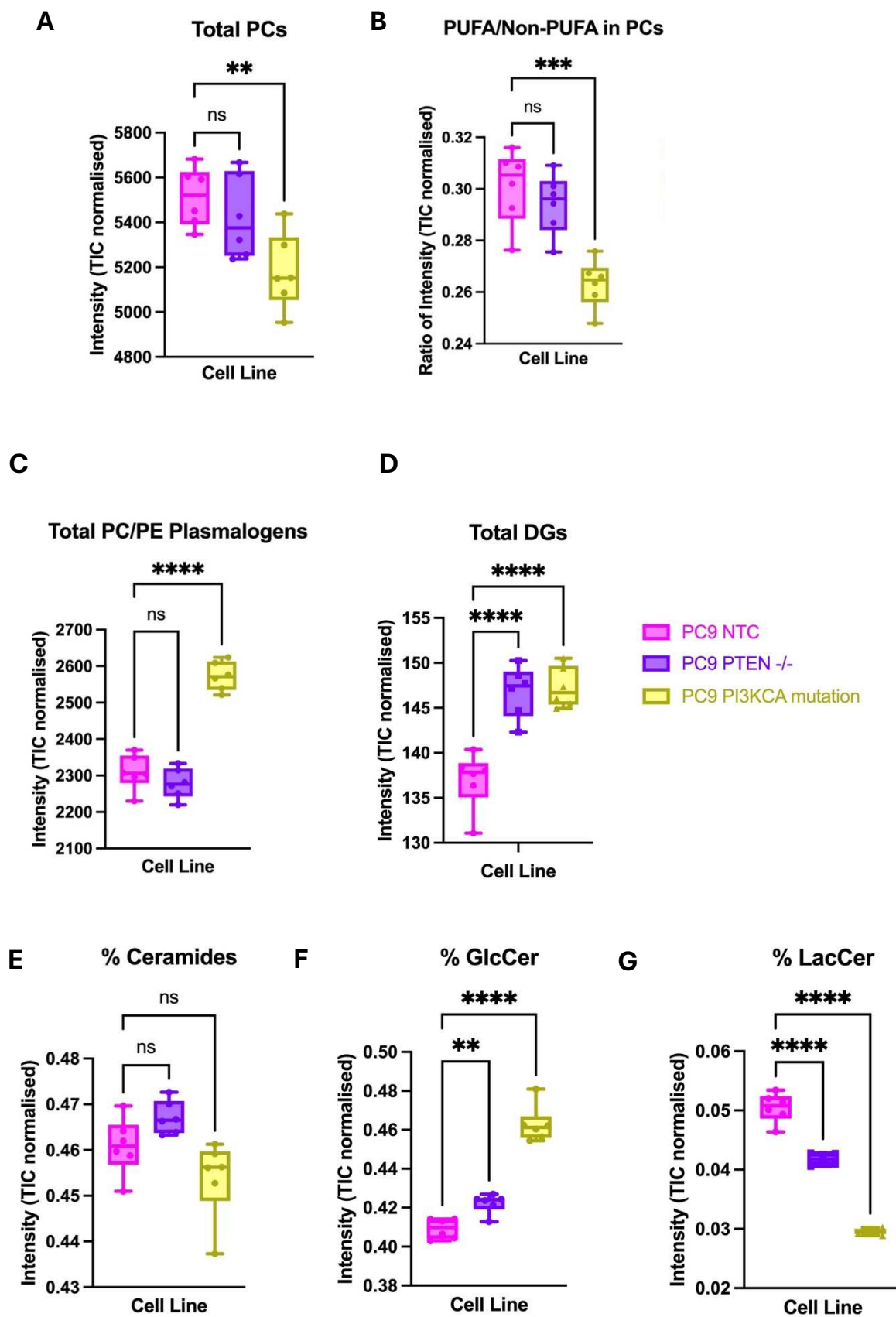
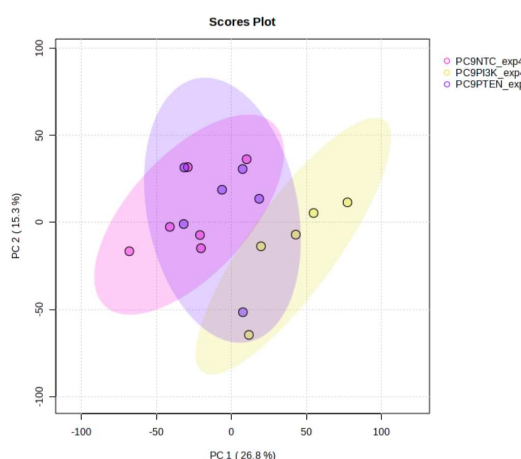


Figure 5.9: Specific lipid family profiles in GM models of OR versus OS. (A) Total PCs in OR and OS cells. (B) PC saturation, with the ratio of PUFA/Non-PUFA in PCs in OR and OS cells. (C) Total PC/PE plasmalogens in OR and OS cells. (D) Total DGs in OR and OS cells. (E) Relative amount of % Ceramides in OR and OS cells. (F) Relative amount of % GlcCer in OR and OS cells. (G) Relative amount of % LacCer in OR and OS cells. Lipidomic data was normalised to TIC. Statistical differences in box plots determined by one-way ANOVA with Dunnett's correction multiple comparisons. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, **** $P < 0.0001$ compared with control (technical replicate, $n = 6$).

5.4. Targeted metabolomic analysis of GM models of resistance

Targeted metabolomics analysis was performed on the exact same GM cell line sample set and their controls as for the proteomics and lipidomics study. Details of the methodology and instrumentation used can be found in **Chapter 3.3.3-3.3.4**. After alignment and processing was completed using MS Dial and OptiMSR package in R Studio, 29 confidently annotated metabolites were identified that passed QC checks. Multivariate statistical analysis of the targeted metabolomic data revealed subtle differences in OR versus OS cell lines, however this was not as clear the proteomic or lipidomic profiles. OR and OS cells were not completely differentiated based on their metabolic profiles by PCA ($R^2 = 0.43069$; **Figure 5.10A**). There were only statistically significant metabolites altered in the volcano plot of PC9 PIK3CA^{mut} versus OS (**Figure 5.10B**), and none observed in PC9 PTEN^{-/-} versus OS based on a fold change cut off of 1.2.

A



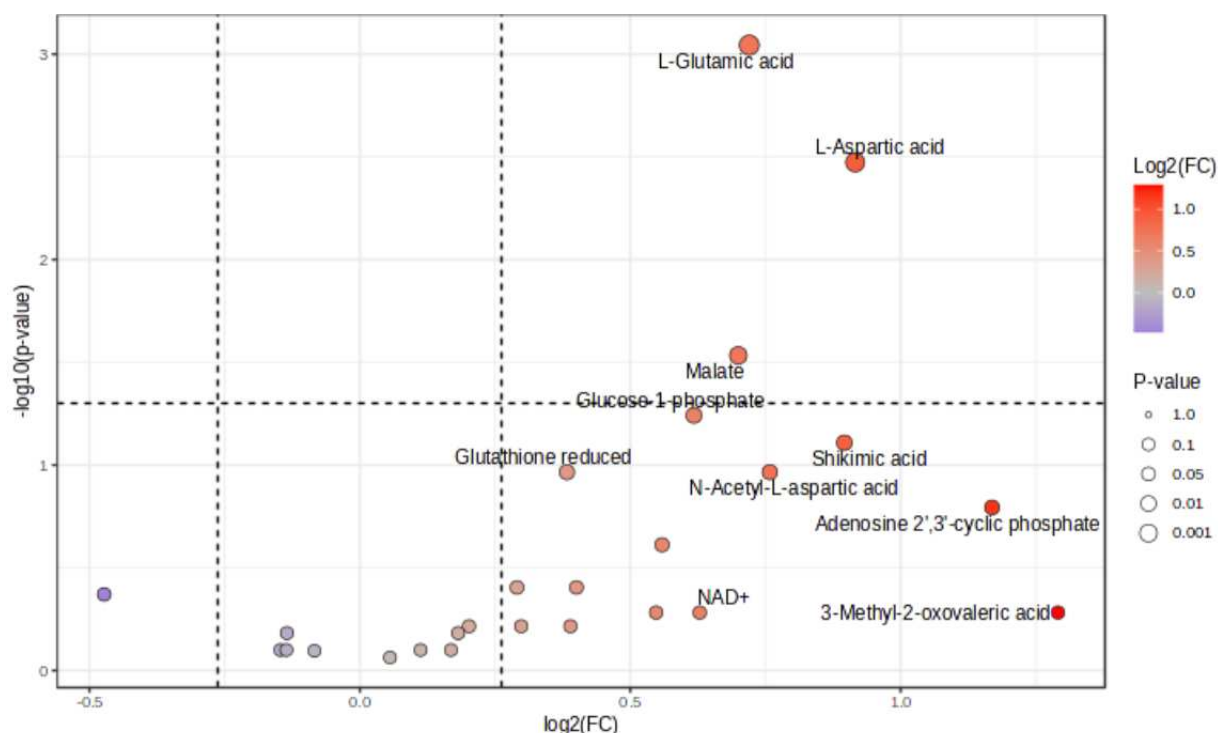
B

Figure 5.10: An overview of targeted metabolomics of GM models of OR versus OS cells. (A) PCA scores plot of PC9 NTC (pink), PC9 PTEN -/- (yellow) and PC9 PIK3CAm (purple). (B) Volcano plot showing lipids that are significantly increased in PC9 PIK3CAm (in red). FC cut-off for significance was 1.2 and FDR adjusted p-value cut-off was 0.05. Data was normalised to TIC. ** $P < 0.0001$ compared with control (technical replicate, $n = 6$).**

We plotted individually the altered metabolites in PC9 PIK3CAm which included glucose-1-phosphate (**Figure 5.11A**) and malate (**Figure 5.11B**), intermediates of glycolysis and the TCA cycle, respectively. The Warburg effect describes cancer cells increasing the use of glycolysis to meet energy demands(259), but in OR cells it has been shown that they re-utilise the TCA cycle to meet energy demands(43,86). GSH was increased in PC9 PIK3CAm compared to control (**Figure 5.11C**), along with amino acids glutamic acid (**Figure 5.11E**) and aspartic acid (**Figure 5.11F**) and aspartic acid derivative N-acetyl-L-aspartic acid (NAA) (**Figure 5.11F**).

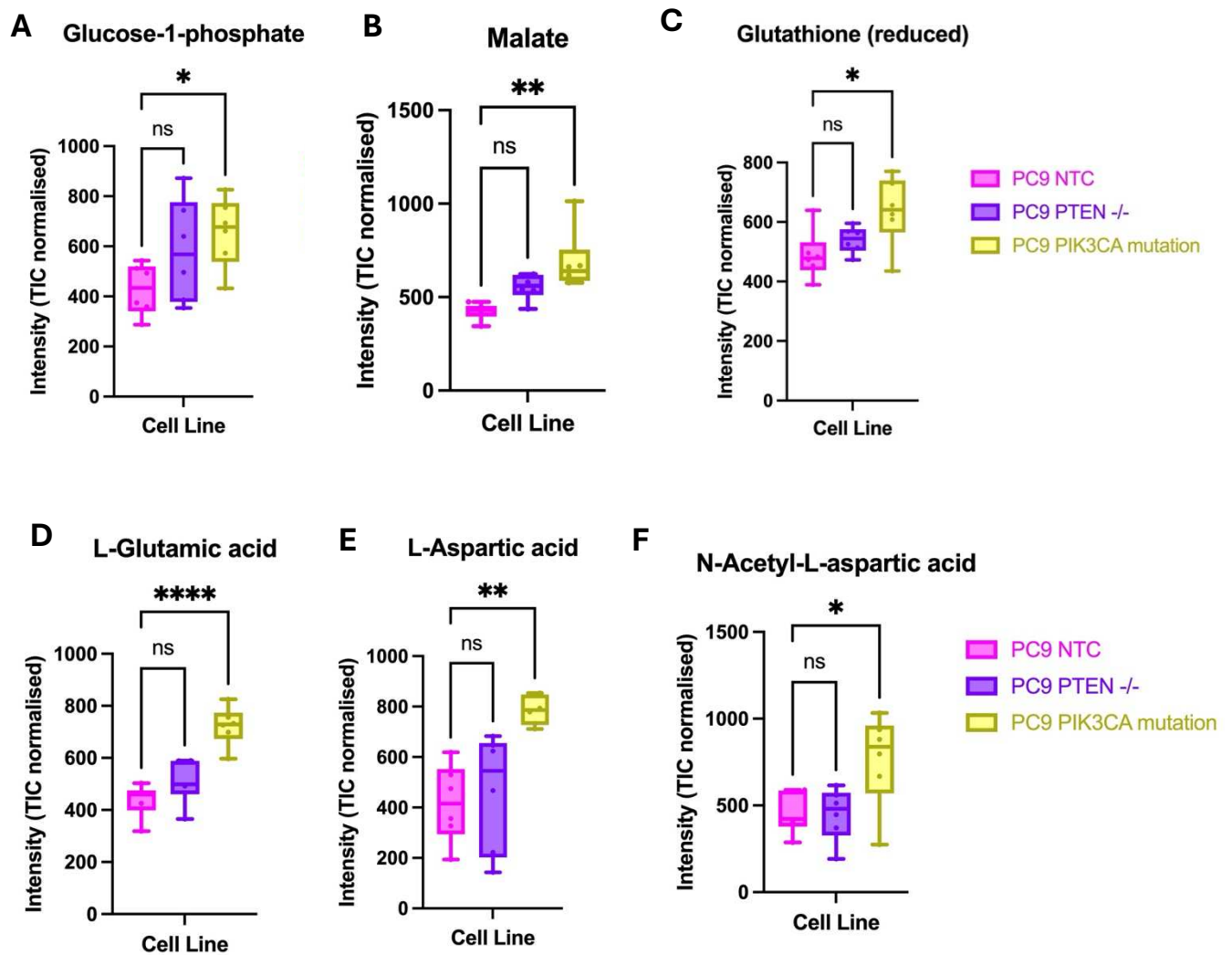


Figure 5.11: Specific metabolite changes in GM models of OR versus OS cells. (A) *Glucose-1-phosphate* levels in OR versus OS. (B) *Malate* levels in OR versus OS. (C) *Reduced glutathione* levels in OR versus OS. (D) *L-glutamic acid* levels in OR versus OS. (E) *L-aspartic acid* levels in OR versus OS. (F) *N-acetyl-L-aspartic acid* levels in OR versus OS. Data was normalised to TIC. Statistical differences in box plots determined by one-way ANOVA with Dunnett's correction multiple comparisons. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, **** $P < 0.0001$ compared with control (technical replicate, $n = 6$).

5.5. Multi-omics integration in OR lines

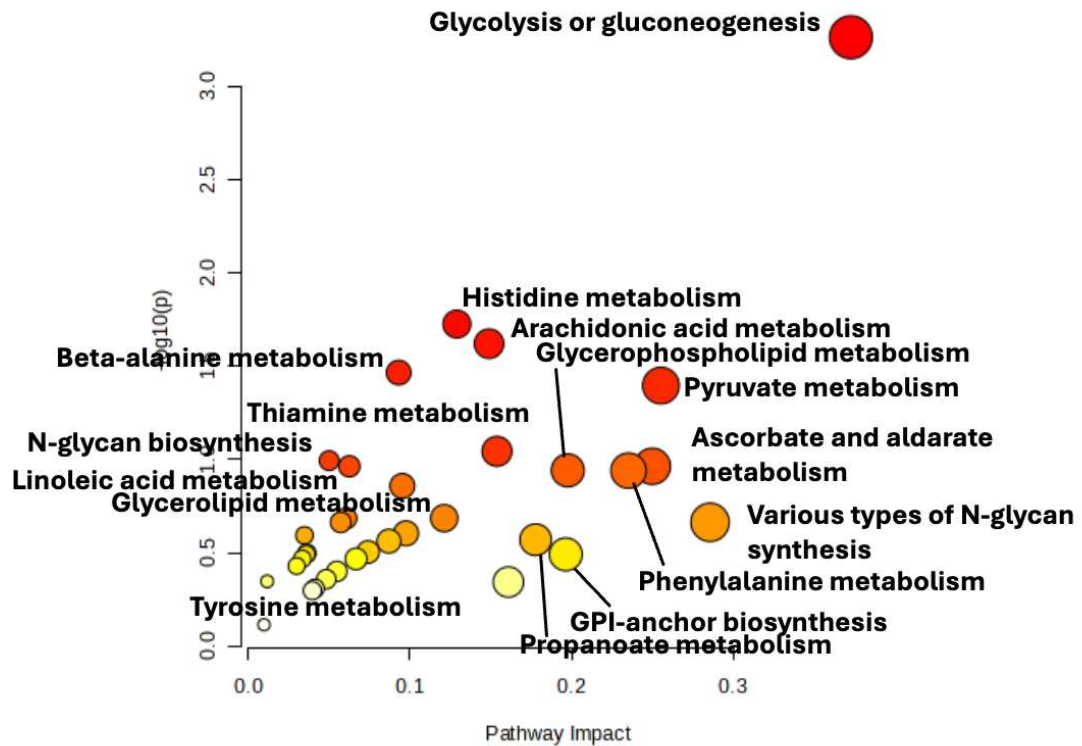
Multi-omics integration was performed on the significantly changed proteins (p value < 0.05 , $FC > 1.5$), lipids (p value < 0.05 , $FC > 1.5$ or 2.0) and metabolites in OR versus OS (p value < 0.05 , $FC > 1.2$). Details on the joint pathway analysis can be found in **Chapter 3.4.4**.

In PC9 PTEN $-/-$, the only significantly enriched pathway was glycolysis or gluconeogenesis ($p_{adj} = 0.04$) (**Figure 5.12A**). Whilst enriched pathways in PC9 PIK3CAm included lipid and small molecule-related metabolism (**Figure 5.12B** none were statistically significant).

A

PC9 PTEN $-/-$

Overview of Pathway Analysis



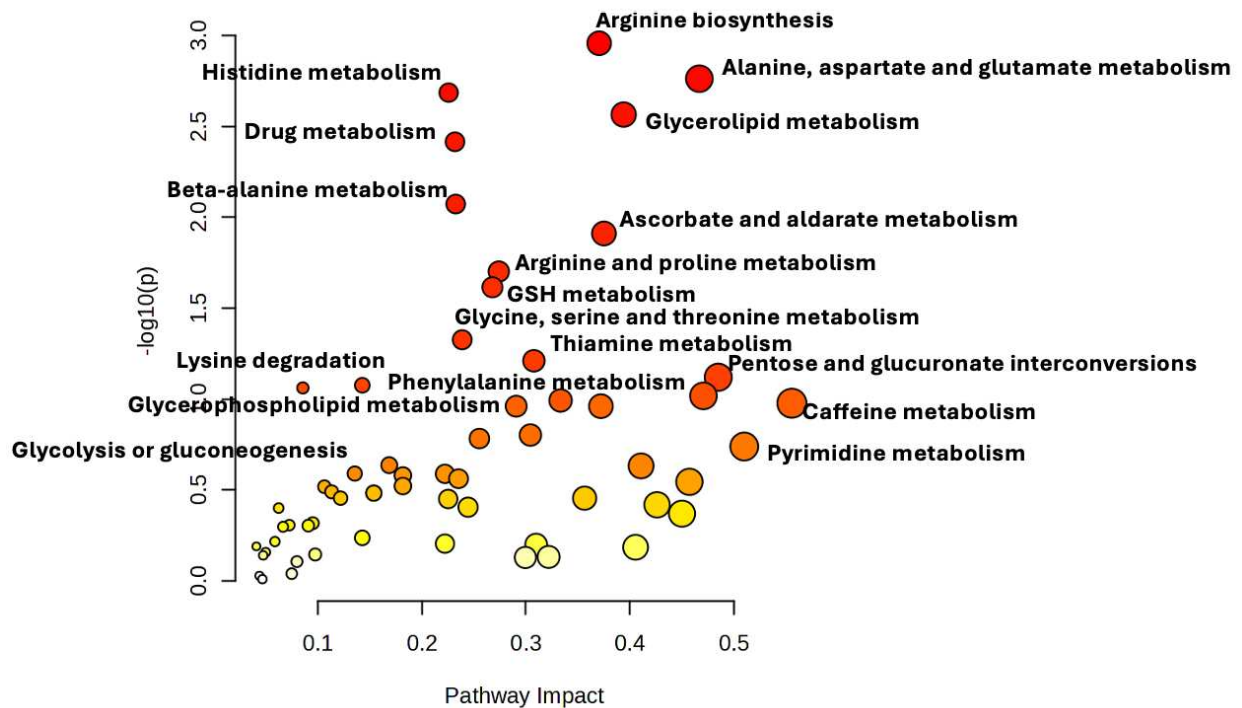
B**PC9 PIK3CAm**

Figure 5.12: Multi-omics joint pathway analysis in (A) PC9 PTEN $-/-$ and (B) PC9 PIK3CAm. The significantly altered proteins (p value < 0.05, FC > 1.5) and lipids (p value < 0.05, FC > 1.5 or 2.0) in each cell line were used as input to determine any common pathways that may be enriched in OR versus OS on both the proteomic and lipidomic level.

5.6. Discussion

This chapter examines two genetically-induced cell models of OR. The genetic modifications are on distinct genes but within the same super pathway and therefore we would have expected similar effects on downstream pathways. However, our analysis revealed that there were striking differences between the two variants in their proteome, lipidome and metabolome. Nevertheless, both OR cell lines could be differentiated from the OS control based on their metabolic phenotypes, particularly with respect to lipid metabolism.

Dysregulation of lipid metabolism, lipid peroxidation and ferroptosis

Proteomics analysis revealed protein-level changes pertaining to lipid metabolism, through identifying enriched pathways such as fatty acid metabolism and ferroptosis. The PC9 PIK3CAm cell line showed TLCD1 to be underexpressed. TLCD1 regulates membrane fluidity through PUFA phospholipid synthesis and has a protective effect when knocked

down(253,260). A decrease in this protein would therefore impair the membrane structure in cells and limit production of PUFA containing phospholipids(260). We hypothesised that OR cells may reduce the PUFA-content of phospholipids to avoid lipid peroxidation, and this could contribute to drug resistance in the PIK3CAm cell line. Supporting this, our lipidomics analysis confirmed a decrease in PUFA/Non-PUFA PC ratio for PC9 PIK3CAm cell line compared to control (but not PC9 PTEN -/-). In addition, plasmalogens were increased in PC9 PIK3CAm compared to both PC9 PTEN -/- and the sensitive control. Increased plasmalogen levels have been shown to enhance activation of the PI3K pathway and reduce activation of caspase-9 and therefore apoptosis(261). Plasmalogens are also thought to act as scavengers of ROS, and therefore increased levels could mitigate against oxidative stress in OR cells(223,262), however this mechanism is not fully understood(263). There was a significant increase in DGs in both OR lines revealed by the lipidomics. DGs can be used to generate PAs within lipid metabolism by the expression of DG kinases (DGKs). These enzymes are often highly expressed in cancer cells, and the PA produced may be involved in pathways which promote cell proliferation and avoidance of apoptosis(264). DGKs are also linked to PI3K signalling as they may regulate downstream signalling of Akt(258). DGs can behave as secondary messengers in the same way that PIPs do in the PI3K/Akt pathway. In the formation of PIP3, DGs are often also produced(258). Therefore, an increase in DGs would align with an increase in PI3K signalling as is the case in these OR lines.

We probed the proteomics for specific changes in proteins involved in all aspects of lipid metabolism using a predefined list of proteins (accessed in December 2023 by Laila Evangelista, full list in **Appendix 9.2, Appendix Table 9.2**) In PC9 PTEN -/- versus OS, the only significantly changed protein was PPIP5K1 which catalyses production of diphosphoinositol polyphosphates(265). In PC9 PIK3CAm, SCD was overexpressed which would account for the increased level of MUFA-containing PCs observed and points to increased DNL in OR, as hypothesised(51,266).

In our targeted metabolomics analyses, we found increased glutamic acid and GSH in PC9 PIK3CAm compared to control. As discussed previously, glutamic acid and GSH are key metabolites in glutathione synthesis. GSH has a protective role against oxidative stress and is

dysregulated in cancer(83,217,219,220) and has been linked to drug resistance in previous studies(83,220,267).

Whilst the changes in plasmalogens, PUFA-PC and GSH were not found in PC9 PTEN^{-/-} model of OR, we did note that key proteins involved in lipid peroxidation and ferroptosis were significantly altered in both GM models (as also noted in **Chapter 4** for the pharmacologically-induced models). For example, ALD2 which inhibits ferroptosis was overexpressed in PC9 PTEN^{-/-} versus PC9 NTC. The PI3K/Akt pathway is a regulator of ferroptosis, via NRF2 and GPX4(57). By inhibiting the PI3K/Akt pathway, cancer cells may be resensitised to ferroptosis, and this could potentially overcome drug resistance. The proteomics did reveal a significant reduction in KEAP1 in both OR models compared to OS however this did not meet our FC cut off. However, the reduction in KEAP1 may be due to its degradation which activates NRF2 and downstream, can inhibit ferroptosis.

Taken together, our findings in the proteome, lipidome and small molecule metabolism all support OR cells being more resistant to oxidative stress and/or ferroptosis and this could be a contributory factor in their resistance to osimertinib.

Dysregulated sphingolipid metabolism

In the pharmacologically-induced models of OR, we found decreased free ceramide whilst complex GSLs such as LacCer were increased in OR compared to OS (**Chapter 4**). In contrast, in the GM models, free ceramide was unchanged, whilst GlcCer and LacCer were both significantly changed (increased and decreased, respectively) in both OR cell lines compared to OS control. Modulation of the PI3K pathway is expected to regulate ceramide biosynthesis and glycosylation via regulation of transport of ceramide from the endoplasmic reticulum (ER) to the golgi apparatus, where the biosynthesis of GSLs occurs(268). Another example of this crosstalk is in accumulation of GlcCer, activating Akt signalling, which is downstream to both PI3K and PTEN(78). Despite the observed differences, both pharmacological models and genetic models of OR seem to have increased flux through the ceramide salvage pathway, with increased synthesis of glycosphingolipid families, via GCS. However in the GM models, this was not supported by the proteomics data, as noted in the pharmacologically-induced models.

Changes in small molecule metabolism: glycolysis, the TCA cycle and amino acid metabolism

Enriched pathways in the proteomics analysis included amino and nucleotide sugar metabolism and aspartate and glutamate metabolism. In addition, targeted metabolomics revealed an increase in glycolytic intermediates, such as glucose-1-phosphate, in PC9 PIK3CAm compared to control. Key metabolic transporters, such as SDHD and MCT4, were dysregulated in OR. SDHD is a key enzyme involved in the TCA cycle and electron transport chain and was underexpressed in PC9 PTEN -/- compared to OS. Its responsible for oxidation of succinate(245). The transporter MCT4, which transports lactic acid and pyruvate across the plasma membrane, was also underexpressed in OR. MCT4 is a biomarker for poor survival in NSCLC when it is found in high levels(269). One study did show that EGFR-mutated NSCLC lowered lactate consumption; as such decreased MCT4 would be expected to reduce lactate levels in the cell(270). Some drug resistant cancers reduce lactate consumption and glycolysis in order to increase energy gain via oxidative phosphorylation(86,105). Malate, an intermediate of the TCA cycle, was increased in PC9 PIK3CAm compared to OS. Taken together, this could suggest an increase in use of oxidative phosphorylation. However, without studies to monitor metabolic flux via isotope labelling as previously described, a conclusive statement as to the energy pathways utilised by OR cannot be confidently determined.

Protein changes in PC9 PIK3CAm to PC9 NTC also included decreased ASS1, the enzyme that catalyses the final step in arginine biosynthesis(204), whilst in both GM-OR cell lines, ASNS which synthesises asparagine was overexpressed. The metabolomics revealed increased aspartic acid and its derivative in PC9 PI3KCAm compared to OS, in contrast to the decrease observed in the pharmacologically-induced OR cell lines. SLC1A3 is an aspartate/glutamate transporter, and was overexpressed in PC9 PI3KCAm(226). This may account for the increased levels of aspartic and glutamic acid measured in PI3KCAm in the metabolomics study. Cancer cells with underexpressed ASS1 are more reliant upon extracellular arginine(204). One study showed that prolonged deprivation of arginine induced mTOR1 activation via PI3K/Akt(271). In NSCLC, an overexpression of ASNS has been shown to protect cancer cells against apoptosis and has been linked to drug resistance in some cancers(272,273). Interestingly, SLC1A3 has been suggested as a biomarker for OR in NSCLC by Ochi *et al.*, and when silenced increased sensitivity to osimertinib(256). One study found that increased aspartate levels rendered

cancer cells resistant to inhibition of the electron transport chain (ETC)(226). Therefore, this increase in aspartic acid may be a resistance mechanism linked to mitochondrial respiration.

In summary, dysregulation of key metabolic transporters and amino acid metabolism may contribute to OR in NSCLC. The alterations in metabolites involved with the TCA cycle/mitochondrial respiration may also play a role.

Conclusions

This chapter has highlighted that the genetic mutations rendering NSCLC resistant to osimertinib can yield different metabolic phenotypes, despite the mutations acting within the same pathway. Whilst PC9 PTEN -/- cell line had a partial overlap in proteomic profile to OR and a similar metabolic profile to the OS control, it remains resistant to osimertinib, suggesting that at minimum small molecule metabolism may not play a major role in resistance within this cell line. On the other hand, PC9 PTEN -/- showed distinct lipidomic profiles to OS, albeit not as pronounced as PC9 PIK3CAm. PC9 PIK3CAm had a significantly different proteomic, lipidomic and metabolomic profile when compared to OS and PC9 PTEN -/-. PI3K signalling is limited by Akt and mTOR dependent feedback loops and this could explain why both OR cell lines have different proteomes, lipidomes and metabolomes despite their similar mutated pathways and measurable drug resistance(274). Lipid peroxidation, saturation of lipids and ferroptosis were identified across various -omics platforms to be in some way dysregulated or enriched in these cell lines. Sphingolipid metabolism, particularly formation of GSLs, was also dysregulated as noted in **Chapter 4**.

**Chapter 6 – Sphingolipid metabolism as a therapeutic
target to reinstate osimertinib sensitivity**

6. Chapter 6: Sphingolipid metabolism as a therapeutic target to reinstate osimertinib sensitivity

6.1. Background

Our investigation into resistance mechanisms pertaining to metabolism in the pharmacologically-induced cell lines revealed through transcriptomics, proteomics and lipidomics, a disruption within sphingolipid metabolism. Dysregulation of sphingolipid metabolism has been reported in the literature in different cancers, however upon the investigations made within this chapter, this phenomenon had not yet been investigated in OR-NSCLC.

Ceramide is central to sphingolipid metabolism and is converted to GlcCer by GCS, encoded by *UGCG*. This is the first step in GSL synthesis that goes on to yield LacCer, GalCer, globosides and gangliosides. *UGCG* overexpression is well documented in the literature to occur in cancer and has links to drug resistance(76–78). The production of complex GSLs, and therefore an overexpression of the enzymes that synthesise them, have also been linked to apoptosis and drug resistance in cancer(79).

The accumulation of ceramides can activate apoptosis via caspase-3 activation. We showed in **Chapter 4** that OR cell lines had a reduced pool of ceramides compared to OS. Instead, they had an increased supply of GSLs, such as LacCer, when compared to OS. Therefore, we investigated whether this reduction in ceramide could avoid apoptosis in OR cells and contribute to drug resistance. This could point to a potentially novel resistance mechanism that can be targeted by inhibiting GSL synthesis (and thereby increasing ceramide), in conjunction with osimertinib, to reinstate therapeutic effect.

Over the last few years, targeting sphingolipid metabolism in cancer has gained increased attention. The enzymes involved in key steps of sphingolipid metabolism are often dysregulated in tumour cells making them potential targets for cancer treatment(72). Several pharmacological inhibitors have been developed including: fenretinide, a DES inhibitor in Phase III clinical trials for breast cancer; safinol which inhibits SPHK1 and is in Phase I clinical

trials for metastatic solid tumours; and opaganib which selectively inhibits SPHK2 and DES1 and is in Phase II clinical trials for multiple cancer types such as prostate cancer (72). Ceramide nanoliposomes are also in Phase I and II clinical trials for multiple cancer types including solid tumours and acute myeloid leukaemia, as a way to induce ceramide accumulation in cells(72). Sphingolipid enzymes are known to be therapeutic targets in diseases other than cancer. For instance, miglustat which is an inhibitor of GCS and used to treat Gaucher disease type 1(275). D-PDMP, the GCS inhibitor used in this project, has not itself been used in a therapeutic setting to date. However, these sphingolipid pathway inhibitors have not yet been utilised to combat chemotherapy resistance(276). There is therefore an argument to be made that these inhibitors could be repurposed to target osimertinib drug resistance in NSCLC.

6.1.1. Chapter Aims

Hypothesis

Inhibiting GCS will increase levels of ceramides, which will in turn increase apoptotic cell death in OR cells and resensitise them to osimertinib.

The aim of this chapter is to determine if increasing ceramide levels by inhibiting the formation of complex GSLs will reinstate sensitivity to osimertinib in pharmacologically-induced OR cells. To address this, we will;

- Quantify changes in ceramides and complex GSLs when GCS is inhibited by D-PDMP, in combination with osimertinib
- Measure apoptotic death via caspase-3 activation in OR and OS cells, when GCS is inhibited by D-PDMP, with and without osimertinib.
- Investigate effects on apoptosis when treating OR and OS cells with C2-ceramide, with and without osimertinib.
- Determine if the effects of combining osimertinib with either D-PDMP or C2-ceramide are synergistic.

6.2. Inhibiting glucosylceramide synthase in OR cells

6.2.1. D-PDMP toxicity

To determine the maximum non-toxic dose of D-PDMP, we used an SRB assay to treat each cell line with increasing doses of D-PDMP from 10 μ M to 50 μ M for 72 hours to assess cell growth (**Figure 6.1**). The IC₅₀ values calculated in this assay were then used to define the optimum concentration to use in the following experiments. The data highlights that PC9P was most resistant to D-PDMP, whilst PC9R6 was most sensitive. This correlates with the observed changes we saw in lipid profiles, with OR lines having reduced ceramide and increased GSLs, suggesting an increase in expression of GCS to mediate these lipid changes.

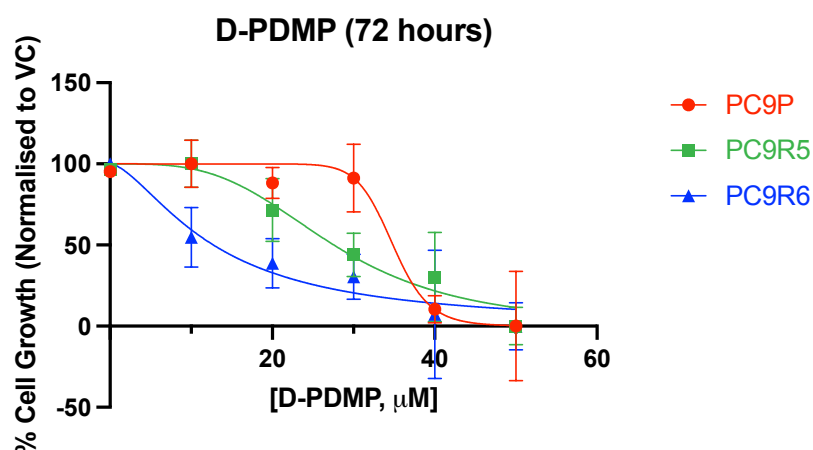


Figure 6.1: Dose response curves of D-PDMP treatment in PC9P, PC9R5 and PC9R6 cells. Cell lines were treated with D-PDMP for 72 hours before cell growth was measured using the SRB assay. Data expressed as mean $\pm$ SEM (biological replicate, $n = 3$).

6.2.2. D-PDMP in combination with osimertinib reduces cell growth and activates apoptosis in OR cells

First, we assessed the combination of D-PDMP and osimertinib and its effect on cell growth, to determine if D-PDMP resensitised the resistant cell lines to osimertinib. For each cell line, we treated with a range of osimertinib doses (0.01-10 μ M) to encapsulate both the therapeutic dose of osimertinib and a high dose of osimertinib, and two doses of D-PDMP (**Figure 6.2**) – the cell-line specific IC₅₀ (as determined above) and one higher dose (HD). In PC9P cell line, increasing doses of D-PDMP in the absence of osimertinib resulted in a significant reduction in growth (**Figure 6.2A**). In the presence of osimertinib, there was a

dramatic decrease in cell growth, as expected since PC9P is osimertinib-sensitive. There was no further significant effect on cell growth with the addition of D-PDMP in the presence of osimertinib. In contrast, for PC9R5 cell line (**Figure 6.2B**) there was a significant combination effect on inhibiting cell growth for D-PDMP HD co-treatment at 1 μ M osimertinib, when compared to osimertinib alone. For PC9R6 (**Figure 6.2C**), co-treatment with 10 μ M osimertinib and either dose of D-PDMP had a significant combination effect on cell growth.

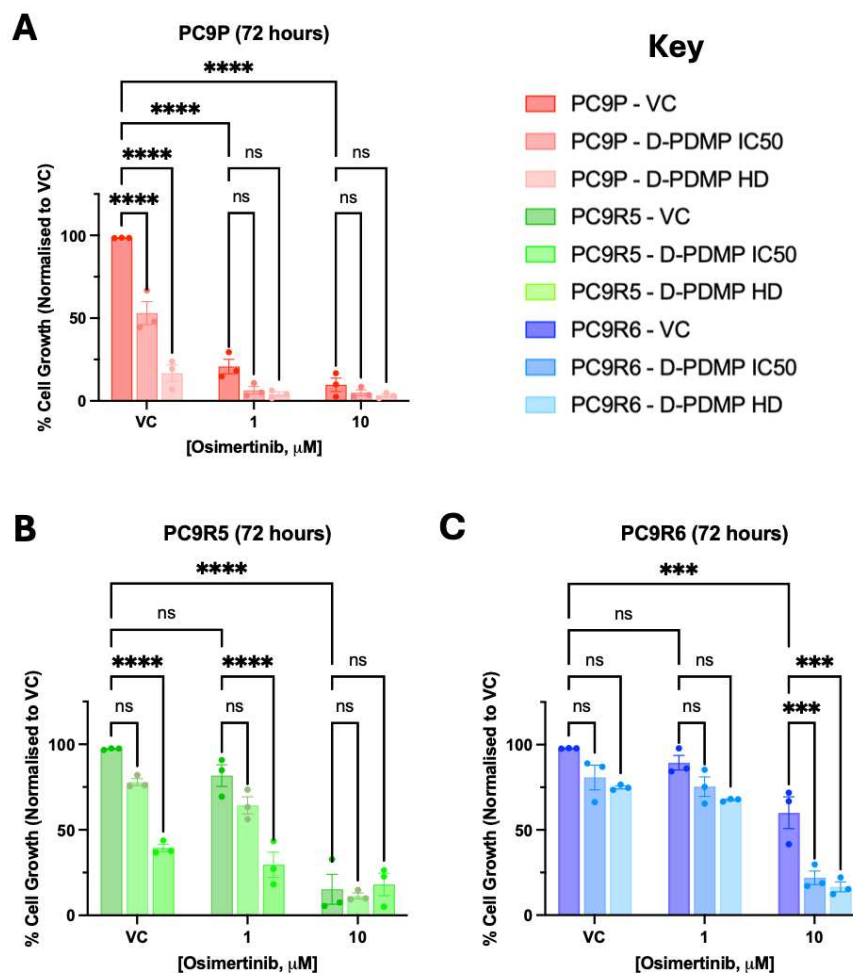


Figure 6.2: D-PDMP treatment in combination with osimertinib. Percentage (%) change in growth, measured via an SRB assay, after cells were treated with various combinations of D-PDMP and osimertinib for 72 hours. (A) PC9P, (B) PC9R5 and (C) PC9R6 cells were treated with DMSO VC, osimertinib (1 and 10 μ M) monotherapies, D-PDMP (IC₅₀ and HD) monotherapies and combinations of both drugs for 72 hours. D-PDMP IC₅₀/HD were 35 μ M/50 μ M, 25 μ M/50 μ M and 15 μ M/30 μ M for PC9P, PC9R5 and PC9R6 cell lines respectively; see also **Table 6.1**. Statistical differences in box plots determined by two-way ANOVA with Dunnett's correction multiple comparisons. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, **** $P < 0.0001$ compared with control/monotherapy. Data expressed as mean $\pm$ SEM (biological replicate, $n = 3$).

Next, we used the Caspase-Glo 3/7 assay to determine if D-PDMP treatment (either as a monotherapy or in combination with osimertinib) increased caspase activity (**Table 6.1**). Each cell line showed differing caspase activation to the treatments, with PC9P having the most caspase activation and PC9R6 having the least caspase activation, evidenced by the differing Y axes scales in **Figure 6.3**. In PC9P (**Figure 6.3A**) there was a highly significant combination benefit on caspase activity for D-PDMP + osimertinib compared to either drug alone. This was found for both the D-PDMP IC₅₀ and HD treatment groups. In PC9R5, there was a highly significant combination benefit on caspase activity for D-PDMP + osimertinib compared to either monotherapies in the D-PDMP HD group only (**Figure 6.3B**). Finally, in PC9R6 there was a significant combination effect on caspase activity in both the IC₅₀ and HD D-PDMP combination groups compared to osimertinib monotherapy, but not the D-PDMP monotherapy (**Figure 6.3C**). As expected, PC9P cell line has the highest overall caspase activity, which suggests D-PDMP treatment increased apoptosis more in the OS cell line. PC9R6 is the OR cell line that is most resistant to osimertinib and had the lowest overall caspase activity, suggesting it was the least susceptible to apoptosis.

In summary, inhibiting GCS with D-PDMP in combination with osimertinib, when compared to the effects of osimertinib monotherapy, reduced cell growth and increased apoptosis, evidenced by an increase in caspase activity.

Table 6.1: Conditions for D-PDMP and osimertinib combination treatment on pharmacologically resistant models of OR and OS control for lipidomics and caspase experiments.

IC₅₀ values determined from previous monotherapy dose response curves generated via SRB assays.

Cell line	VC	Osimertinib IC ₅₀ [#] (μM)	D-PDMP IC ₅₀ + Osimertinib IC ₅₀ (μM)		D-PDMP high dose (HD) + Osimertinib IC ₅₀ (μM)	
			OSI	D-PDMP	OSI	D-PDMP
PC9P (OS)	0.1 % DMSO	0.003	0.003	35	0.003	50
PC9R5 (OR)	0.1 % DMSO	1.72	1.72	25	1.72	50
PC9R6 (OR)	0.1 % DMSO	1.92	1.92	15	1.92	30

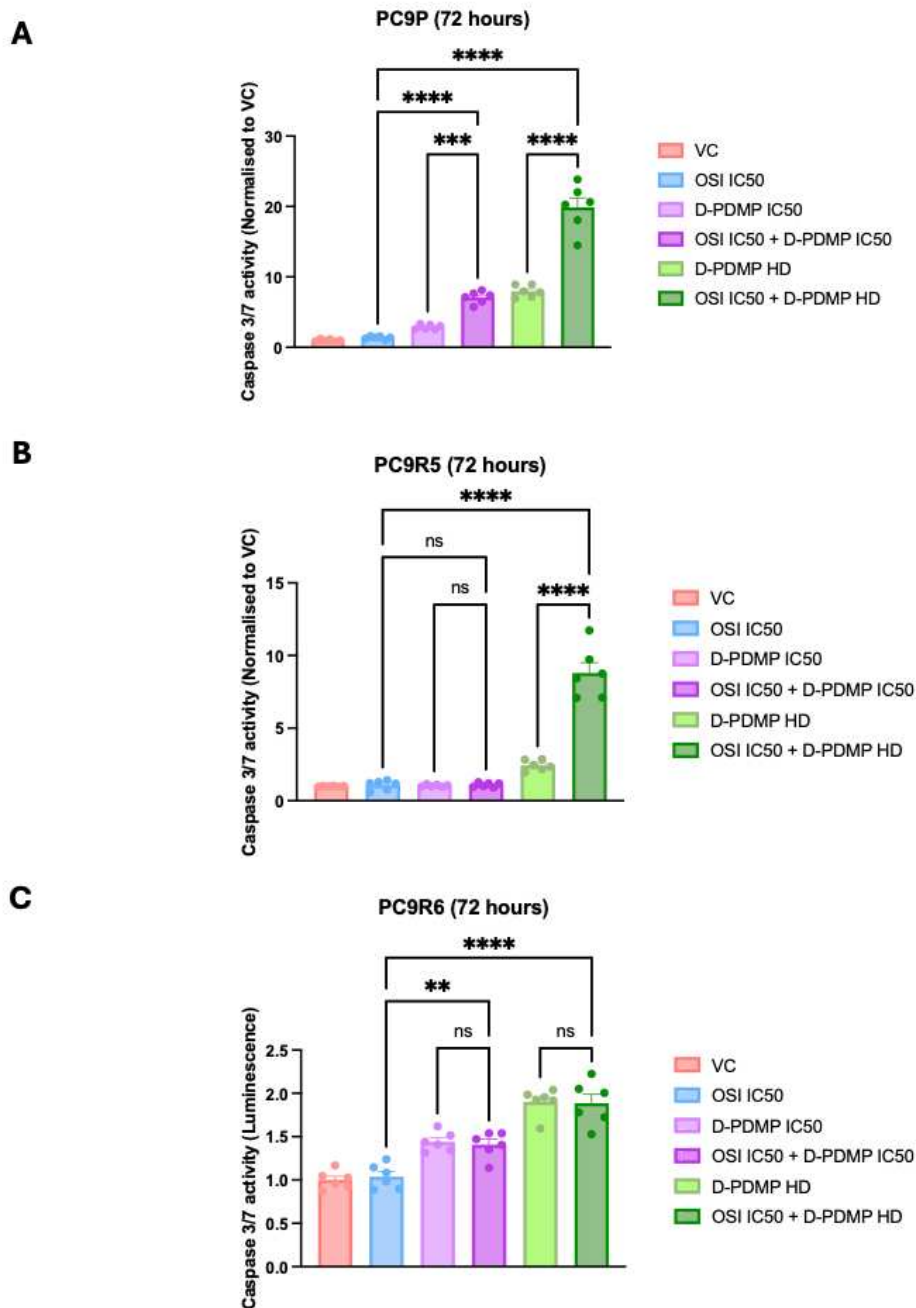


Figure 6.3: Combination treatment increases caspase 3/7 activity in OR and OS cells. Cell lines (A) PC9P, (B) PC9R5 and (C) PC9R6 were treated with either DMSO VC, osimertinib IC₅₀ monotherapy, D-PDMP IC₅₀ monotherapy, osimertinib IC₅₀ and D-PDMP IC₅₀ combination, D-PDMP HD monotherapy, and osimertinib IC₅₀ and D-PDMP HD combination. Caspase activity was measured using the Caspase 3/7 Glo assay. Osimertinib IC₅₀ was determined for each cell line separately and was 0.003 μ M, 1.72 μ M and 1.92 μ M for PC9P, PC9R5 and PC9R6 cell lines respectively. D-PDMP IC₅₀/HD were 35 μ M/50 μ M, 25 μ M/50 μ M and 15 μ M/30 μ M for PC9P, PC9R5 and PC9R6 cell lines respectively; see also **Table 6.1**. Statistical differences in box plots determined by one-way ANOVA with Dunnett's correction multiple comparisons. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, **** $P < 0.0001$ compared with control. Data expressed as mean $\pm$ SEM (technical replicate, $n = 6$). Representative plot from 3 individual experiments.

6.2.3. D-PDMP and osimertinib combination shows synergy in OR cells

To determine if the effects on cell growth observed with the combination of D-PDMP and osimertinib in PC9R5 is synergistic or additive, the Bliss synergy score was used (see Chapter 3.4.3). The cut-offs for Bliss scoring are as follows: < -5 is antagonistic, -5 to 5 is additive and > 5 is synergistic(190,277). There was a reduction in cell growth with increasing doses of D-PDMP and osimertinib, individually and in combination (**Figure 6.4A**). However, increasing doses of D-PDMP did not have a significant effect on cell growth at 10 μ M osimertinib. There was a synergistic effect across several dose combinations (**Figure 6.4B**). There is significance in the drug combination compared to both osimertinib and D-PDMP monotherapies at 25 μ M D-PDMP and 5 μ M osimertinib (**Figure 6.4C**).

In PC9R6, there was a significant reduction in cell growth with increasing doses D-PDMP at all doses of osimertinib (**Figure 6.4D**). The Bliss synergy score shows a significant synergy profile in the combination, which increases in a dose dependent manner (**Figure 6.4E**). The combination therapy is only significant compared to D-PDMP monotherapy at 15 μ M and 30 μ M (**Figure 6.4F**). It is therefore clear that in these OR cell lines, D-PDMP and osimertinib combination shows a synergistic benefit.

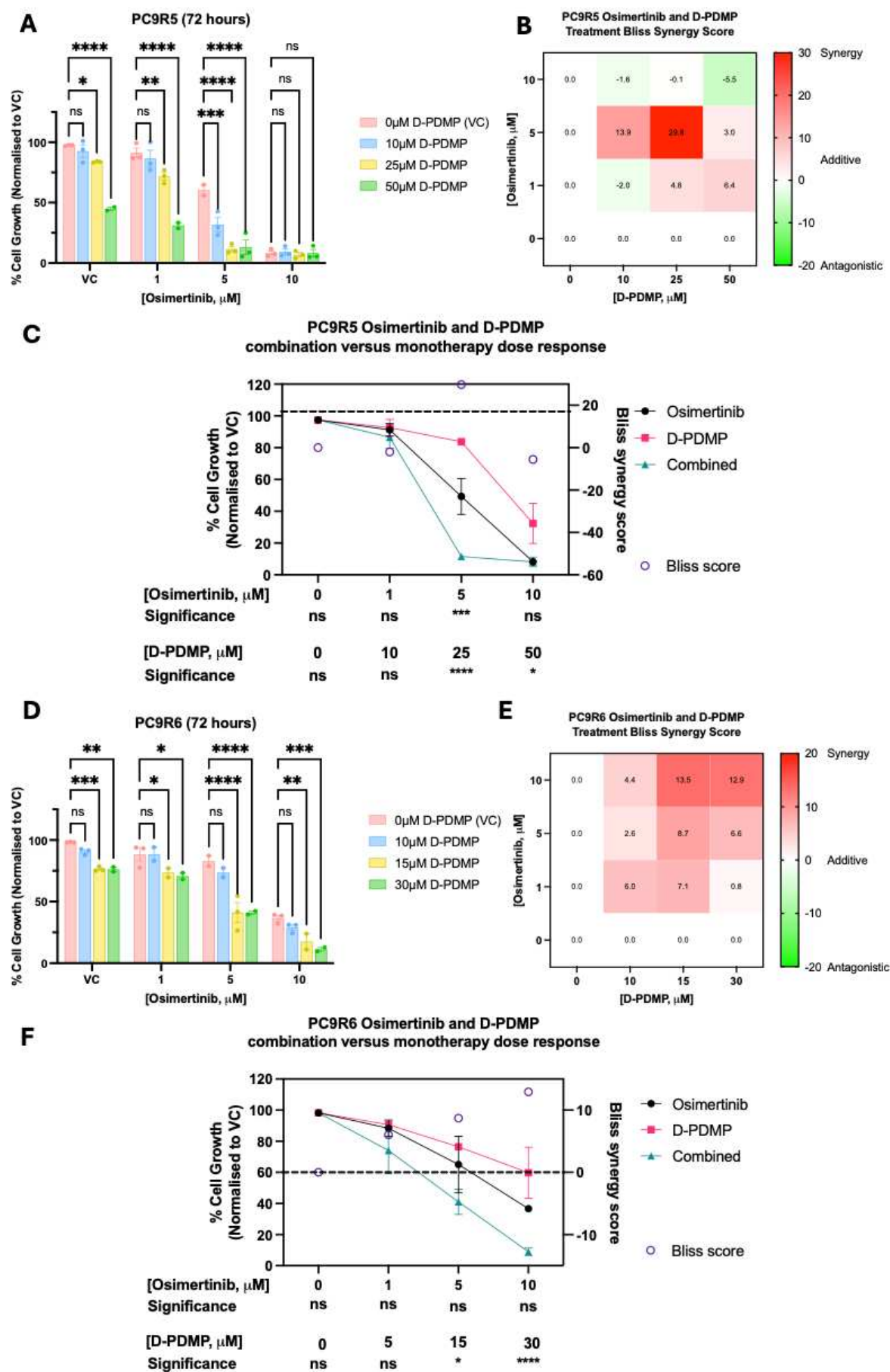


Figure 6.4: Synergy study of D-PDMP in combination with osimertinib in pharmacologically-induced models of OR. **A-C** show data for PC9R5 cells; **D-F** show data for PC9R6 cells. (**A** and **D**) Cells were treated with a combination of D-PDMP and osimertinib for 72 hours before cell growth was measured using the SRB assay. (**B** and **E**) Synergy data using the Bliss scoring system at different combinations of D-PDMP and osimertinib. (**C** and **F**) Monotherapies and combination of D-PDMP and osimertinib effects on cell growth (left y-axis) with the Bliss synergy score (right y-axis) for each dose combination (x-axis). The dashed line indicates 0 on the Right y-axis / Synergy score. Statistical differences in box plots determined by two-way ANOVA with Dunnett's correction multiple comparisons. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, **** $P < 0.0001$ compared with VC of D-PDMP group. Data expressed as mean $\pm$ SEM (biological replicate, $n = 3$). Some outlier data points were excluded. These points represent $n = 1$ biological replicate and were excluded due to experimental variation.

6.3. Combination of osimertinib and D-PDMP alters ceramide and glycosphingolipid levels in OR cells

To investigate if inhibition of GCS by D-PDMP in combination with osimertinib altered the levels of ceramides and complex GSLs, cells were treated with the conditions described in **Table 6.1** for 72 hours. After treatment the cell pellet was collected, and lipids were extracted using the dual phase Folch method.

Untargeted lipidomics analysis, with post data acquisition and processing focused on sphingolipids, was performed under the same conditions as previously described in **Chapter 4**. In PC9P OS cells, there was a significant increase in ceramide to complex GSLs ratio compared to VC in the osimertinib/D-PDMP HD combination, but a significant decrease at the osimertinib/D-PDMP IC₅₀ dose combination (**Figure 6.5A**). As expected, the ratio of ceramide to complex GSLs was significantly increased in PC9R5 and PC9R6 cell lines, at both doses of D-PDMP, when in combination with osimertinib (**Figure 6.5B and C**).

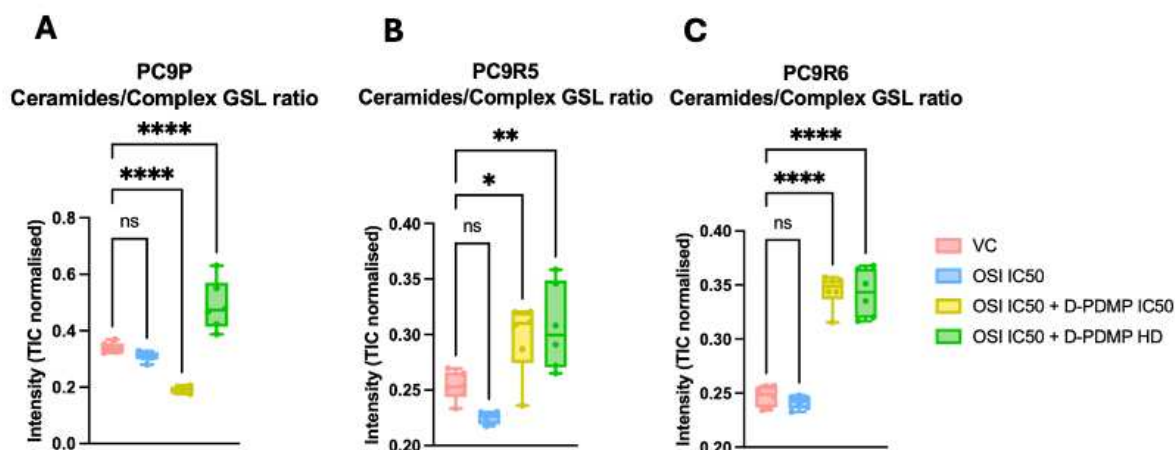
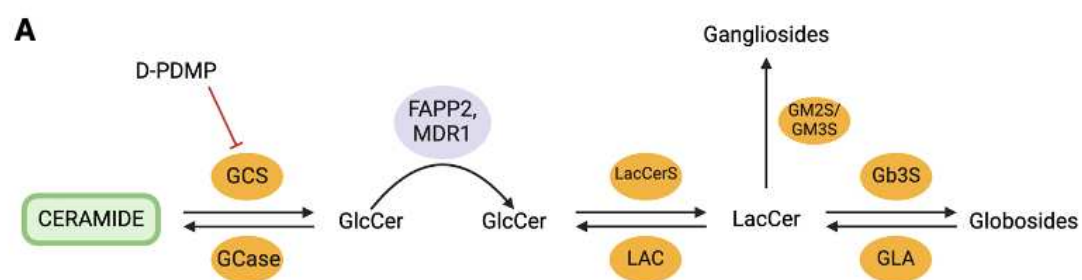


Figure 6.5: Ratio of free ceramides to complex GSLs in pharmacologically-induced models of OR (A) PC9P cells, (B) PC9R5 cells and (C) PC9R6 cells. Lipidomics data was normalised to TIC. Statistical differences in box plots determined by one-way ANOVA with Dunnett's correction multiple comparisons. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, **** $P < 0.0001$ compared with control. Data expressed as mean $\pm$ SEM (technical replicate, $n = 6$).

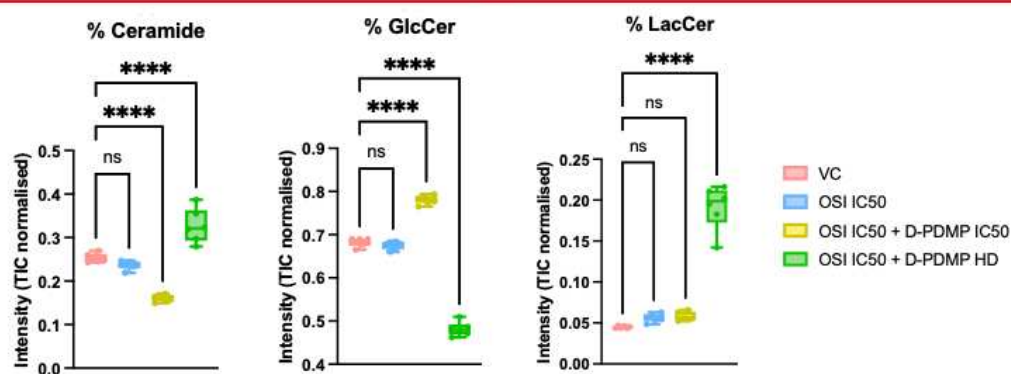
To then determine if the levels of specific GSLs, such as GlcCer and LacCer, have been reduced due to inhibition of GCS, we looked at the proportion of these relative to total sphingolipid content in each cell line. All treatment groups were compared for statistical significance against VC. **Figure 6.6A** highlights the biosynthesis pathway of GSLs from ceramide and where D-PDMP acts within this pathway.

Overall, the lipidomics analysis revealed that GCS inhibition with D-PDMP in combination with osimertinib significantly increased % Ceramides in OR cells at both doses of D-PDMP compared to VC and osimertinib monotherapy (**Figure 6.6B**). Unexpectedly, there was an increase in % GlcCer in OR cells at D-PDMP IC₅₀ combination group (and in D-PDMP HD combination group for PC9R6 cell line). However, % LacCer was found to decrease in D-PDMP IC₅₀ combination group for both OR cell lines, as hypothesised. It appears that in PC9R6, both the D-PDMP IC₅₀ and HD combination groups altered sphingolipid levels similarly, thus showing no benefit in increasing the dose of D-PDMP past the IC₅₀ dose here. In all cell lines, there was no significant change to % Ceramides, % GlcCer or % LacCer in OSI IC₅₀ monotherapy compared to VC, therefore all alterations to sphingolipid metabolism can be attributed to GCS inhibition with D-PDMP (**Figure 6.6B**).

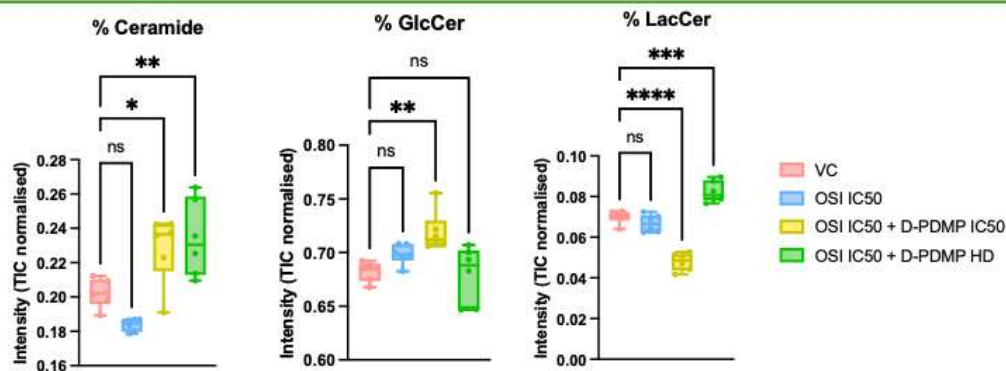


B

PC9P (OS)



PC9R5 (OR)



PC9R6 (OR)

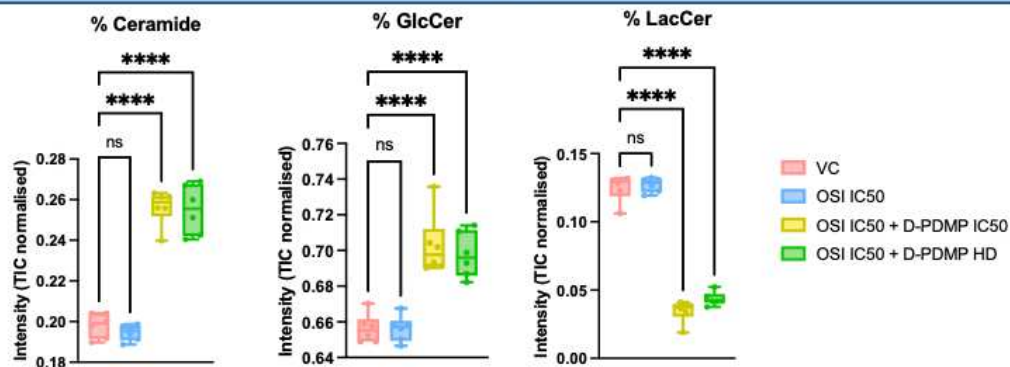


Figure 6.6: % Ceramide, % GlcCer and % LacCer levels in pharmacologically induced resistant models. (A) Summary of GSL biosynthesis pathway with inhibition of GCS by D-PDMP. **(B)** Relative levels of ceramide, GlcCer and LacCer in cells when treated with DMSO VC, OSI IC_{50} and combinations of osimertinib (OSI) and D-PDMP IC_{50} and HD. OSI IC_{50} was determined for each cell line separately and was 0.003 μ M, 1.72 μ M and 1.92 μ M for PC9P, PC9R5 and PC9R6 cell lines respectively. D-PDMP IC_{50} /HD were 35 μ M/50 μ M, 25 μ M/50 μ M and 15 μ M/30 μ M for PC9P, PC9R5 and PC9R6 cell lines respectively; see also **Table 6.1**. Lipidomics data was normalised to TIC. Statistical differences in box plots determined by one-way ANOVA with Dunnett's correction multiple comparisons. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, **** $P < 0.0001$ compared with control. Data expressed as mean $\pm$ SEM (technical replicate, $n = 6$). Parts of figure created using BioRender.com.

6.4. Treatment of OR cell lines with C2-ceramide

6.4.1. C2-ceramide toxicity in OR cells

Lastly, to confirm our D-PDMP inhibitor experiment results, we increased ceramide levels directly. We treated the OR and OS cell lines with C2-ceramide to determine if we can trigger apoptosis and resensitise the cell lines to osimertinib. We used an SRB assay to determine the maximum non-toxic dose of C2-ceramide in all cell lines and used this data to determine IC_{50} values. Doses of C2-ceramide that were used were between 10 μ M and 50 μ M and treatment was for 72 hours (**Figure 6.7**). PC9R6 was most resistant to C2-ceramide, which highlights the link between ceramide and apoptosis and that PC9R6 appears least susceptible to apoptosis via caspase activation.

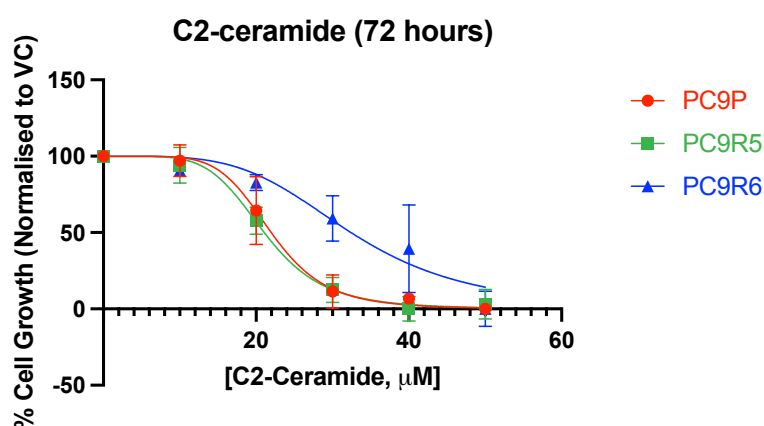


Figure 6.7: Dose response curves of C2-ceramide treatment in PC9P, PC9R5 and PC9R6 cells. Cell lines were treated with C2-ceramide for 72 hours before cell growth was measured using the SRB assay. Data expressed as mean $\pm$ SEM (biological replicate, $n = 3$).

6.4.2. C2-ceramide in combination with osimertinib reduces cell growth and activates apoptosis

We assessed the combination of C2-ceramide and osimertinib and its effects on cell growth, to determine if C2-ceramide resensitised the OR lines to osimertinib. For each cell line, we treated with osimertinib (range 0.01-10 μ M) and two doses of C2-ceramide (**Figure 6.8**) – the cell line specific IC₅₀ values (as determined above) and one higher dose (HD). The combination benefit in PC9P is shown in **Figure 6.8A**, where increasing doses of C2-ceramide had a significant reduction in growth. The co-treatment of osimertinib with C2-ceramide however had no further significant reduction in growth, likely due to osimertinib alone substantially reducing cell growth in these OS cells. In PC9R5 (OR) (**Figure 6.8B**), there was a significant combination benefit and reduction in growth at both doses of C2-ceramide when combined with 1 μ M osimertinib when compared to osimertinib alone, but no significant reduction in growth at 10 μ M osimertinib (similar to OS cells, at 10 μ M osimertinib growth was already substantially reduced). **Figure 6.8C** shows the combination benefit in PC9R6 (OR), where there is a very significant reduction in cell growth at all combination doses.

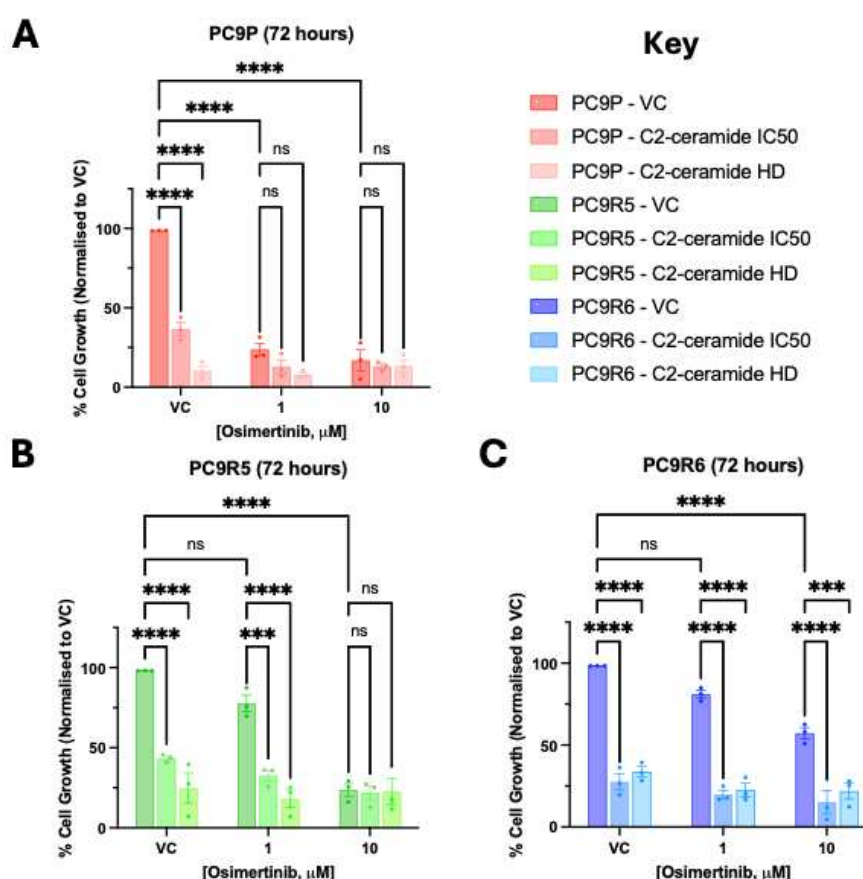


Figure 6.8: C2-ceramide treatment in combination with osimertinib. Percentage (%) change in growth, measured via an SRB assay, after cells were treated with various combinations of C2-ceramide and osimertinib for 72 hours. (A) PC9P, (B) PC9R5 and (C) PC9R6 cells were treated with DMSO VC, osimertinib (1 and 10 μ M) monotherapies, C2-ceramide (IC_{50} and HD) monotherapies and combinations of both drugs for 72 hours. C2-ceramide IC_{50} /HD were 20 μ M/50 μ M, 20 μ M/50 μ M and 40 μ M/50 μ M for PC9P, PC9R5 and PC9R6 cell lines respectively; see also **Table 6.2**. Statistical differences in box plots determined by two-way ANOVA with Dunnett's correction multiple comparisons. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, **** $P < 0.0001$ compared with control/monotherapy. Data expressed as mean $\pm$ SEM (biological replicate, $n = 3$).

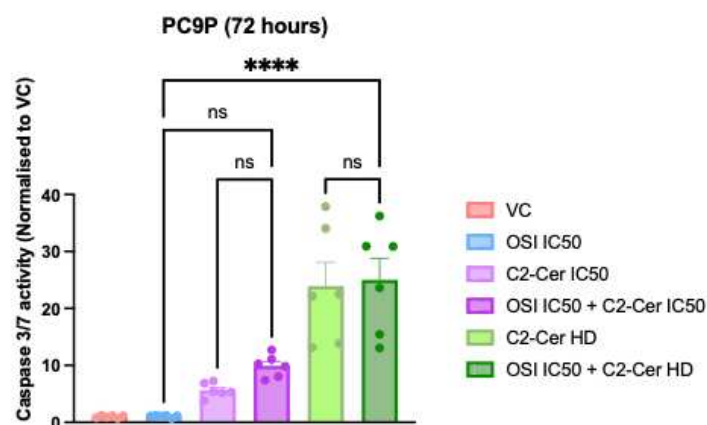
To investigate if C2-ceramide (either as a monotherapy or in combination with osimertinib) increased caspase activation, we used the Caspase-Glo 3/7 assay. The conditions used can be found in **Table 6.2**. In PC9P (**Figure 6.9A**), we saw a significant increase in caspase activation in the C2-ceramide HD combination group compared to osimertinib alone. There was no significant change in caspase activity between either combination group and the corresponding monotherapy treatment of C2-ceramide. In PC9R5 (**Figure 6.9B**), there was a significant increase in caspase activity in the C2-ceramide HD combination group compared to osimertinib alone. There was also a significant increase in caspase activity in the C2-ceramide IC_{50} combination group compared to osimertinib alone. However, as observed with PC9P, there was no significant change in either combination group compared to C2-ceramide as a monotherapy treatment. Finally, in PC9R6 (**Figure 6.9C**), we saw a highly significant increase in both combination groups compared to osimertinib alone. This increase was not observed when we compared the combination groups to C2-ceramide monotherapy treatment however. As expected, PC9P was the cell line which had the overall highest caspase activation when normalised to VC, and PC9R6 was the lowest. This therefore suggests that PC9P was more sensitive to C2-ceramide treatment and PC9R6 was more resistant, similar to the observations with osimertinib monotherapy.

Table 6.2: Treatment conditions for C2-ceramide and osimertinib combination treatment on pharmacologically resistant models of OR versus OS for caspase experiments.

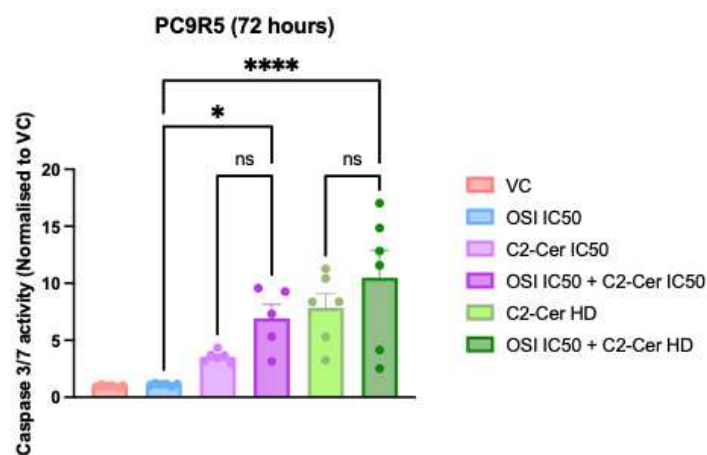
IC₅₀ values determined from previous monotherapy dose response curves generated via SRB assays.

Cell line	VC	Osimertinib IC ₅₀ (μM) #	C2-ceramide IC ₅₀ + Osimertinib IC ₅₀ (μM)		C2-ceramide high dose (HD) + Osimertinib IC ₅₀ (μM)	
			OSI	C2-ceramide	OSI	C2-ceramide
PC9P (OS)	0.1 % DMSO	0.003	0.003	20	0.003	50
PC9R5 (OR)	0.1 % DMSO	1.72	1.72	20	1.72	50
PC9R6 (OR)	0.1 % DMSO	1.92	1.92	40	1.92	50

A



B



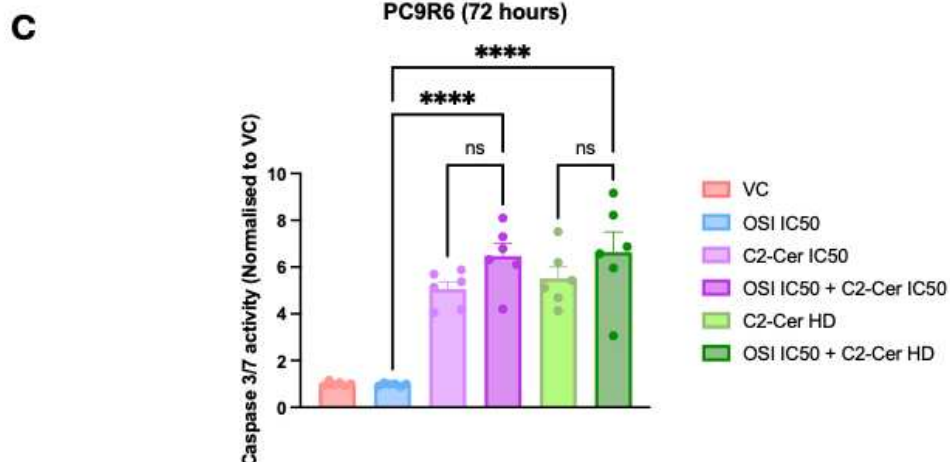


Figure 6.9: Combination treatment increases caspase 3/7 activity in OR and OS cells. Cell lines (A) PC9P, B) PC9R5 and (C) PC9R6 were treated with either DMSO VC, osimertinib IC₅₀ monotherapy, C2-ceramide IC₅₀ monotherapy, osimertinib IC₅₀ and C2-ceramide IC₅₀ combination, C2-ceramide HD monotherapy, and osimertinib IC₅₀ and C2-ceramide HD combination. Caspase activity was measured using the Caspase 3/7 Glo assay. Osimertinib IC₅₀ was determined for each cell line separately and was 0.003 μ M, 1.72 μ M and 1.92 μ M for PC9P, PC9R5 and PC9R6 cell lines respectively. C2-ceramide IC₅₀/HD were 20 μ M/50 μ M, 20 μ M/50 μ M and 40 μ M/50 μ M for PC9P, PC9R5 and PC9R6 cell lines respectively; see also Table 6.2. Statistical differences in box plots determined by one-way ANOVA with Dunnett's correction multiple comparisons. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$, **** $P < 0.0001$ compared with control. Data expressed as mean $\pm$ SEM (technical replicate, $n = 6$). Representative plot from 3 individual experiments.

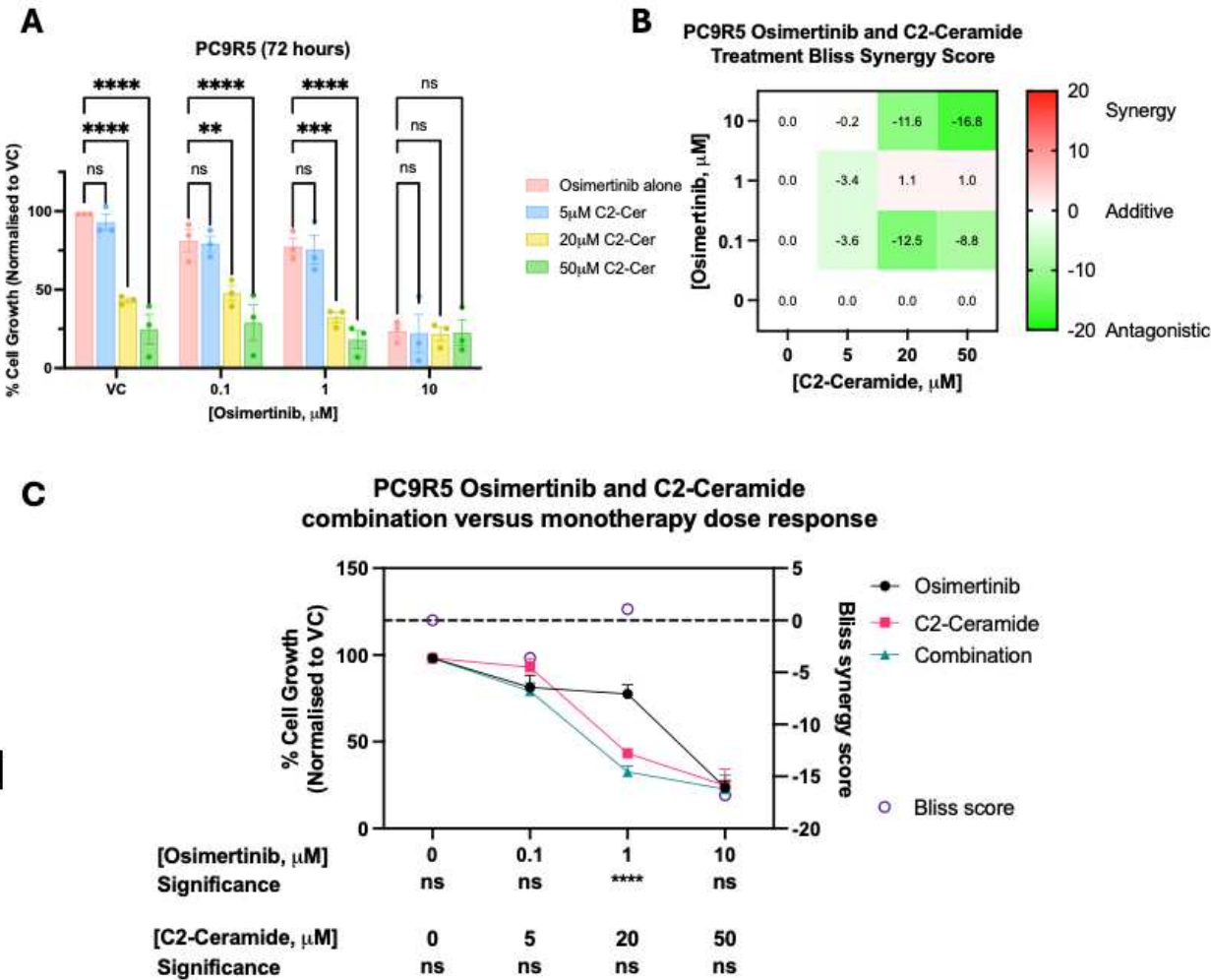
In summary, treating OR cells with C2-ceramide in combination with osimertinib, when compared to the effects of osimertinib monotherapy, reduced cell growth and increased apoptosis, evidenced by an increase in caspase activity.

6.4.3. C2-ceramide and osimertinib combination shows some synergistic benefit in pharmacologically resistant cells

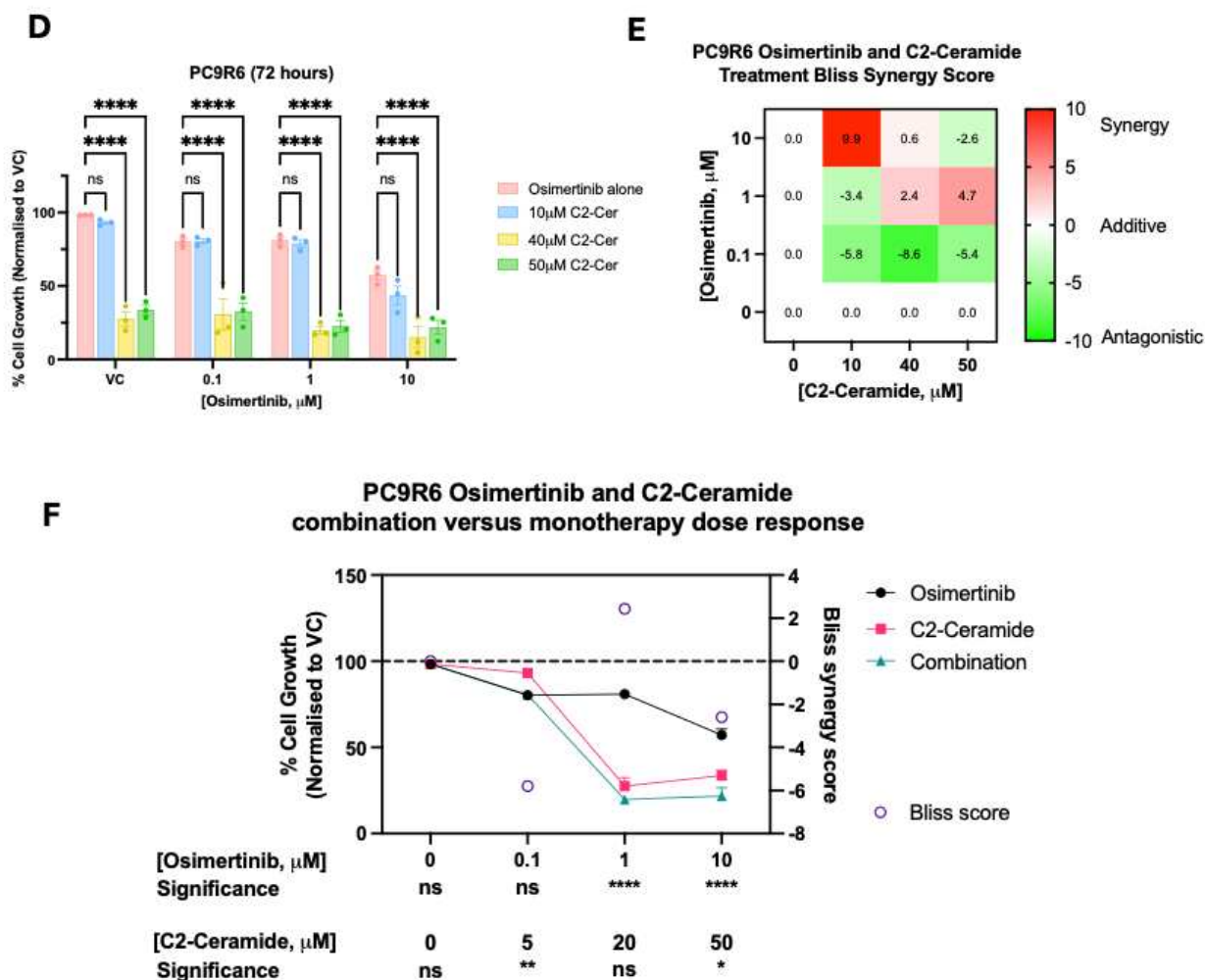
To determine if the above effects on cell growth observed with the combination of C2-ceramide and osimertinib in PC9R5 is synergistic or additive, the Bliss synergy score was used as previously described. There was a reduction in cell growth as both osimertinib and C2-ceramide doses are increased to a max of 10 μ M osimertinib, where the increase of C2-

ceramide has no additional effect on cell growth (**Figure 6.10A**). There was no synergy observed at any combination of doses, with some additive effects observed at low doses of C2-ceramide and antagonistic effects at higher doses of C2-ceramide (**Figure 6.10B**). There was only one dose combination (1 μ M osimertinib and 20 μ M C2-ceramide) found to have a significant change in cell growth compared to osimertinib monotherapy (**Figure 6.10C**), however the Bliss score indicated this to be an additive effect.

In PC9R6, there was a reduction in cell growth at increasing doses of both osimertinib and C2-ceramide (**Figure 6.10D**). The reduction in growth is observed with increased doses of C2-ceramide from 10 μ M to 40 μ M, however at all doses of osimertinib there is a slight increase in cell growth from 40 μ M to 50 μ M C2-ceramide. The Bliss score suggests synergy at 10 μ M osimertinib and 10 μ M C2-ceramide, but mostly shows antagonistic effects at several combination doses. There are some additive effects close to synergy at 1 μ M osimertinib an



50 μ M C2-ceramide (**Figure 6.10E**). There is significance in the combination compared to osimertinib monotherapy at both 1 μ M and 10 μ M osimertinib (**Figure 6.10F**).



In summary, there was a reduction in cell growth with the combination of C2-ceramide and osimertinib, when compared to osimertinib alone. The caspase assay revealed the highest activation of caspase occurred in the combination groups compared to osimertinib alone, however there was no significant difference between the combination groups and C2-ceramide monotherapy in any cell line based on Bliss synergy. This may be due to C2-ceramide directly affecting apoptosis as a monotherapy, therefore the need for another drug (osimertinib) to increase cytotoxicity is redundant.

6.5. Discussion

Studies have shown that dysregulated sphingolipid metabolism is a common mechanism of cancer progression and there is potential for these pathways to be therapeutic targets(72,278). Several studies have reported lower ceramide levels in cancers compared to normal tissue, such as in hepatocellular carcinoma(279) and head and neck squamous cell carcinoma(280). However, Separovic *et al.*, 2017 found increased levels of ceramides in colorectal cancer (CRC) compared to a control group(281). These studies highlight the modularity of sphingolipid metabolism, and the potential for ceramides to be a biomarker of progression for various different cancer types(281). In **Chapter 4**, untargeted lipidomics and proteomics revealed dysregulation within sphingolipid and ceramide metabolism.

In this chapter, we have further demonstrated that sphingolipid and ceramide metabolism may play a role in osimertinib drug resistance in NSCLC.

Inhibition of GCS with D-PDMP

We investigated if the use of GCS inhibitor D-PDMP would reduce the production of GSLs and increase ceramide levels in OR cells. We hypothesised that this accumulation of ceramide would then trigger apoptosis when an extrinsic damaging agent, such as osimertinib, is introduced and thereby reinstating osimertinib drug-sensitivity. We first determined the toxicity of the inhibitor and calculated an IC_{50} for each cell line to use in further experiments. The combination of D-PDMP and osimertinib revealed a decrease in cell growth in all cell lines at increasing doses of both compounds. These effects were found to be synergistic in various combinations of doses in OR cells. Synergy appeared to be dose dependent in PC9R6 cells. In

PC9R5, at the highest doses of both compounds, the interaction appeared to be antagonistic. We suggest this is because high doses of osimertinib in monotherapy reduces cell growth by 90 % in this cell line, and therefore the combination response is less than would be anticipated by the synergy model(277). Increasing doses of D-PDMP have been shown previously in both OR and OS cells to inhibit cell proliferation measured via MTT assay and to inhibit colony formation(212). The Caspase-Glo 3/7 assay revealed that D-PDMP HD had the most significant effect on caspase activation in all cell lines, both as a monotherapy and in combination with osimertinib. Similar findings have been observed in other lung cancer cell lines, such as in A549 cells (human lung adenocarcinoma) where D-PDMP treatment was found to increase ceramide accumulation which ultimately led to caspase-3 activation and apoptosis(45).

Next, we investigated the relative proportion of ceramides and GSLs in OR cell lines when treated with osimertinib monotherapy and a combination of D-PDMP and osimertinib at varying doses unique to each cell line. In both OR lines, both combination treatment groups showed an increase in ceramides compared to GSLs. This increase was significant in PC9R5 (D-PDMP HD combination group), and PC9R6 (both doses of D-PDMP). PC9P cells showed a decrease in ceramide compared to GSLs when treated with the D-PDMP IC₅₀ combination group. This is the opposite of what we would expect to see with this inhibitor, however the monotherapy toxicity profile of D-PDMP showed that PC9P cells are more resistant to D-PDMP. This may be due to PC9P cells already having increased ceramide levels prior to D-PDMP treatment (due to its osimertinib sensitivity). In both PC9P and PC9R5 cells, in the D-PDMP HD combination groups, % GlcCer was also decreased. GlcCer is synthesised from ceramides via GCS and is a precursor to LacCer. Therefore, GlcCer levels may be in constant flux due to being converted into other GSLs, for example LacCer which was increased in both OR cell lines.

It is also interesting to note the differences between PC9R5 and PC9R6. Whilst PC9R6 exhibits the expected changes in sphingolipid profile on GSC inhibition, PC9R5 shares some similarities with PC9P control. One possible reason PC9R5 is dissimilar to PC9R6 with respect to sphingolipid metabolism is because there may be different (or additional) resistance mechanisms driving OR in PC9R5. One study published earlier this year investigated the levels of common GSLs in OR cells of PC9 origin compared to OS cells when treated with D-PDMP(212). The OR cells this study generated had different mutations to the cell lines used in

this project. Interestingly, when investigating the fold change of common GSL families in OR cells compared to OS upon treatment of D-PDMP, these differing mutations yielded different results. For example, in PC9^{BRAF^{G469A}} there was a significant increase in GlcCer and LacCer species, however in PC9 T790M^{CIA} the increase was found only in LacCer and there was a decrease in globosides(212). This paper therefore demonstrates that despite all OR cell lines having increased GSLs, the level of specific families of GSLs varies depending on the specific cell line mutation. In this project, PC9R5 and PC9R6 differ based on a genetic alteration observed in PC9R5 and not PC9R6, which is a MAPK1/CRKL gain. This could explain the variation in GSL species observed upon inhibition with D-PDMP.

C2-ceramide treatment

In addition to inhibiting GCS to increase ceramide levels in OR cells, we also separately treated all cell lines with C2-ceramide, a non-physiological ceramide analogue, to mimic the effects of intracellular ceramide-mediated cell death(282). In HNSCC, C2-ceramide treatment showed dose-dependent cytotoxicity and activation of apoptosis(230). In this project, the treatment combination of C2-ceramide HD and osimertinib caused significant increase in caspase activation across all cell lines when compared to osimertinib alone. However, there was no significant increase in caspase in any line between the combination groups and C2-ceramide alone, implying that the apoptosis activation is not from a combination effect, but from C2-ceramide treatment alone. Synergy analysis confirmed this; across all lines, there was little synergy observed between C2-ceramide and osimertinib and therefore no combination benefit.

Conclusion

In summary, the results from this chapter indicate that inhibition of GCS in pharmacologically-induced models of osimertinib resistance resensitises these OR lines to osimertinib in a combination treatment. These effects are synergistic; we suggest the mechanism of action is due to increased ceramide levels upon treatment, which lead to caspase-3 activation and increased apoptosis. These findings, which are in line with other studies in varying cancer types as described above, could indicate a potential novel combination treatment for patients carrying mutations which render them resistant to osimertinib. Through combination

therapies which target identified resistance mechanisms such as GSL synthesis, this treatment strategy could potentially reinstate the therapeutic effects of osimertinib.

Chapter 7 – Final discussion, future directions and conclusions

7. Chapter 7: Final discussion, future directions and conclusions

The key difficulty with treatment of EGFRm NSCLC is the development of acquired resistance, with EGFR-TKIs losing therapeutic effect after around 1 year of PFS(23). There are several generations of EGFR-TKIs, each to combat differing mutations within EGFR and to overcome resistance acquired to previous generations. Treatment with osimertinib, a 3rd generation TKI developed by AstraZeneca, similarly results in cancer cells developing mutations that render them resistant (23,283,284).

In this thesis, we have explored the link between dysregulated metabolism and osimertinib resistance, and importantly whether we can reinstate osimertinib sensitivity by targeting metabolism. In **Chapter 2**, we assessed the literature in the form of a systematic review to determine whether studies in other TKIs could shed light on the role of metabolism in drug resistance. Our analysis revealed that drug-resistant cells reversed the Warburg effect, a common hallmark in cancer cells, by increasing the use of oxidative phosphorylation for energy production. It also highlighted an increase in glutathione metabolism to regulate oxidative stress. Interestingly, dysregulated lipid metabolism, particularly increased *DNL*, was also linked to resistance in other generations of TKIs.

Next, we turned to look at cellular models of osimertinib resistance using multi-omics (transcriptomics, proteomics, metabolomics, lipidomics). This included pharmacologically-induced (**Chapter 4**) and genetically-modified (GM) models (**Chapter 5**). Amongst other changes, we discovered dysregulated sphingolipid metabolism, particularly in the pharmacological models, which could contribute to resistance mechanisms through suppression of apoptotic pathways in OR cells. We also identified changes to phospholipid saturation, plasmalogens and GSH in the GM models that may contribute to resistance through a reduction in oxidative stress and ferroptosis, which was also supported by changes in protein abundance.

Finally, in **Chapter 6**, we targeted sphingolipid metabolism by inhibiting the formation of glycosphingolipids via GCS, using D-PDMP. This resulted in partial recovery of osimertinib sensitivity in pharmacologically-induced OR cells, through an increase in free ceramide and

apoptosis as evidenced by increased caspase-3 activity. Furthermore, we established that D-PDMP acted synergistically with osimertinib. Overall, this study has revealed different metabolic changes that may be worth exploring further as targets for co-therapies with osimertinib to mitigate acquired resistance.

7.1. Pharmacologically-induced models of osimertinib resistance

We explored two pharmacologically-induced models of OR: PC9R5 and PC9R6. These cell lines closely mimic the response that patients would exhibit to continued and increased administration of osimertinib. Interestingly, whilst these two cell lines were both phenotypically distinct from the sensitive control, they were also distinct from each other at metabolomic/lipidomic and proteomic levels. One possible reason these two OR cell lines have different metabolite/lipid levels and proteomic profiles may be due to the additional genetic mutation in *MAPK1/CRKL* which was gained by PC9R5 when the cell line was being produced. MAPK1, also known as ERK2, has been investigated in EGFR-TKI resistant NSCLC previously(285). Aberrant activation of MAPK1 enabled resistance to EGFR-TKI WZ4002, and upon inhibition, sensitivity was restored(286). Increased MAPK1 expression was also found in erlotinib-resistant EGFRm NSCLC. However, it should be noted that the dose response to osimertinib and calculated IC₅₀ was almost identical for the two cell lines, despite the additional mutation in MAPK1. This suggests that the metabolic differences observed between the two OR lines may be unrelated to drug resistance.

In contrast, there were also shared metabolic pathways altered in PC9R5/6 compared to sensitive control. Aberrant lipid metabolism, with one pathway being *DNL* was suggested to be enhanced in OR through transcriptomics, proteomics and some findings within the lipidomics datasets. In addition, proteomics data suggested altered lipid peroxidation. Both increased DNL and avoidance of lipid peroxidation (and ultimately ferroptosis) could act as contributory mechanisms of drug resistance. We explored the lipidomics data for changes to fatty acyl chain saturation in different lipid classes. An increase in SFA- and MUFA-containing phospholipids could support an increase in DNL, and reduction in lipid peroxidation (which mainly involves PUFA). However this was not observed, although total TAGs were increased in both OR lines. We did observe a reduction in PUFA-TGs in PC9R6 compared to control, which

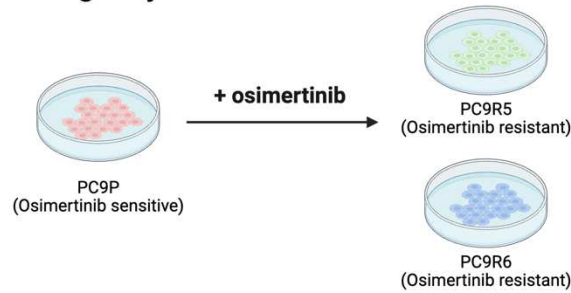
would indicate that this is a potential mechanism to mitigate oxidative stress in this cell line. Whilst this was not observed in PC9R5, we did observe a reduction in plasmalogens, which may be reacting with ROS as another mechanism to mitigate oxidative stress. On the other hand, we found increased GSH in PC9R6 compared to control, which would offer a protective effect against oxidative damage.

Our most interesting finding was within sphingolipid metabolism (**Figure 7.1**). The transcriptomics, proteomics and lipidomics analyses all point towards dysregulated sphingolipid metabolism. In particular, increased gene expression of *UGCG* (coding for GCS, which converts ceramide to GlcCer) was observed in transcriptomics dataset of PC9R5 compared to control. This was supported by upregulated GCS at protein level (PC9R6 compared to control). Finally, lipidomics analysis revealed a decrease in ceramide and increase in complex GSLs (e.g LacCer) in both OR cell lines compared to OS control.

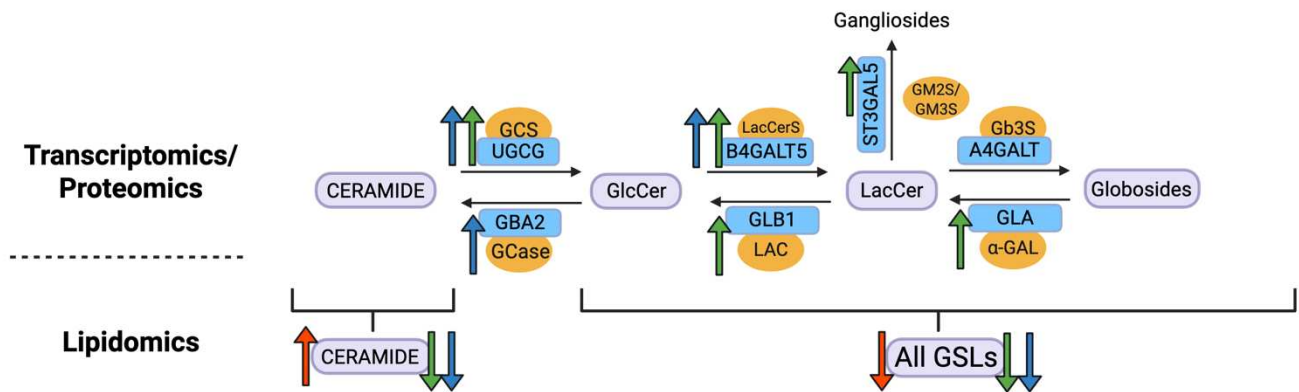
It is worth noting also that when the proteomics, lipidomics and metabolomics data were combined in a multi-omics analysis, many of the enriched pathways were not statistically significant. One reason could be that we didn't have sufficient coverage of metabolites/lipids/proteins across the different pathways.

We further investigated dysregulation of sphingolipid metabolism in **Chapter 6**, where we discovered that both pharmacologically-induced OR cell lines had a reduction of caspase-3 induced apoptosis, a phenomenon also observed in chemotherapy-resistant NSCLC(29). When GCS was inhibited by D-PDMP, ceramide levels were increased and GSL levels decreased. This inhibitor, when combined with osimertinib, also increased activation of apoptosis and increased osimertinib sensitivity in the OR lines. We therefore suggest sphingolipid metabolism via GSL synthesis as a potential therapeutic target for reinstating osimertinib sensitivity and prolonging drug efficacy (**Figure 7.1**).

Pharmacologically-induced models of osimertinib resistance



Multi-omics reveals dysregulated sphingolipid metabolism



Inhibition of GCS by D-PDMP reverses dysregulated sphingolipid metabolism, induces apoptosis and reinstates osimertinib sensitivity

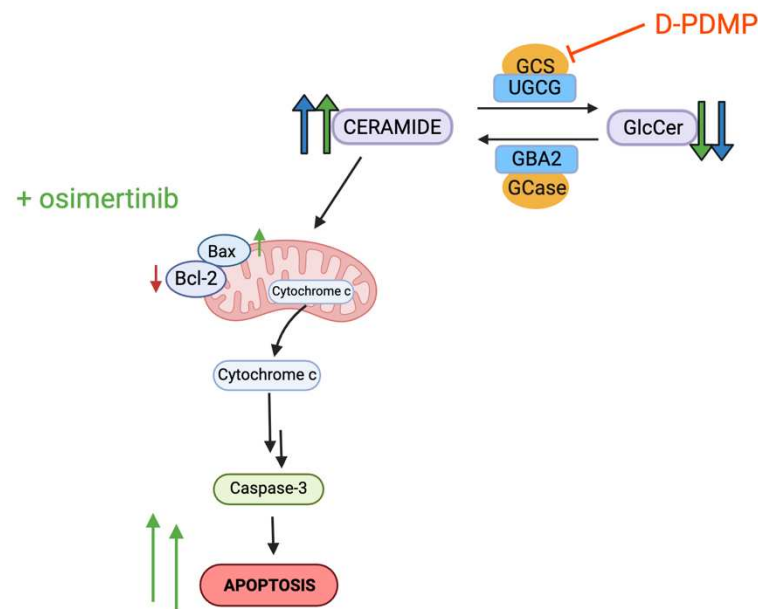


Figure 7.1: A summary of the main findings from the pharmacologically-induced models of resistance.

Through multi-omics, dysregulated sphingolipid metabolism was identified to be a key pathway altered in OR versus OS, which may be contributing or causing OR. We probed this by inhibiting a key enzyme in the pathway to increase ceramides and promote apoptosis. This inhibitor in combination with osimertinib reversed OR.

7.2. Genetically modified (GM) models of osimertinib resistance

The proteomics investigation into the GM OR lines identified *DNL*, lipid peroxidation and ferroptosis as enriched pathways relative to sensitive control. These may contribute to drug resistance, as already discussed in the above section, however it is also possible that these changes simply arise as a consequence of the mutation.

Whilst the mutations of each cell line are on different proteins, they act within the same pathway. PTEN regulates activation of PI3K and when inhibited or silenced allows constitutive activation of PI3K. On the other hand, PIK3CA mutation gives rise to overactivation of PI3K and its downstream components. However, despite acting in a similar way, we observed that PC9 PTEN ^{-/-} and PC9 PI3KCAm cells had highly distinct proteomic and metabolomics/lipidomic profiles. The two OR cell lines differed from PC9 NTC sensitive control to different extents, with PC9 PTEN ^{-/-} more similar to control than PC9 PI3KCAm. One explanation for this may be found in PIP3 activity. The activation of PI3K and the loss of PTEN as described above have the same effects on the downstream pathway, and therefore the secondary messenger PIP3. PIP3 then activates Akt signalling. There have been reports however that activation of PIP3 is not proportional to the activation of Akt, and that other factors may compete with Akt activation(40). This was evidenced by one study which found that PIK3CA activating mutations and PTEN inactivation or knockdown had differential expression of Akt phosphorylation(285). PTEN inactivation was found to increase Akt phosphorylation more than PIK3CA activating mutations, indicating that the effects we observed in PC9 PI3KCAm may be influenced by other downstream factors aside from Akt. It is also worth noting that PTEN knockout models do not exhibit any associated benefit when PI3K inhibitors are used, suggesting that there are other important molecular targets involved. Genetic models may not capture the true nature of acquired drug resistance in the same way the pharmacologically-induced models do, and therefore may not relate as closely to how resistance occurs within patients.

Whilst sphingolipid metabolism was also shown to be dysregulated within these cell lines, the avoidance of lipid peroxidation and ultimately ferroptosis was identified as a potential resistance mechanism contributing to the resistant profile of the GM OR cell lines. Proteomics

data revealed modulation of ALDH family of proteins and in solute transporters responsible for shuttling nutrients to important metabolic pathways such as GSH metabolism and the TCA cycle. Pathways enriched in the proteomics data included “Ferroptosis” and “Fatty acid metabolism” pathways. To support this, we found a reduction in PUFA-containing PCs and an increase in plasmalogens and GSH in PC9 PIK3Cam compared to OS control (**Figure 7.2**).

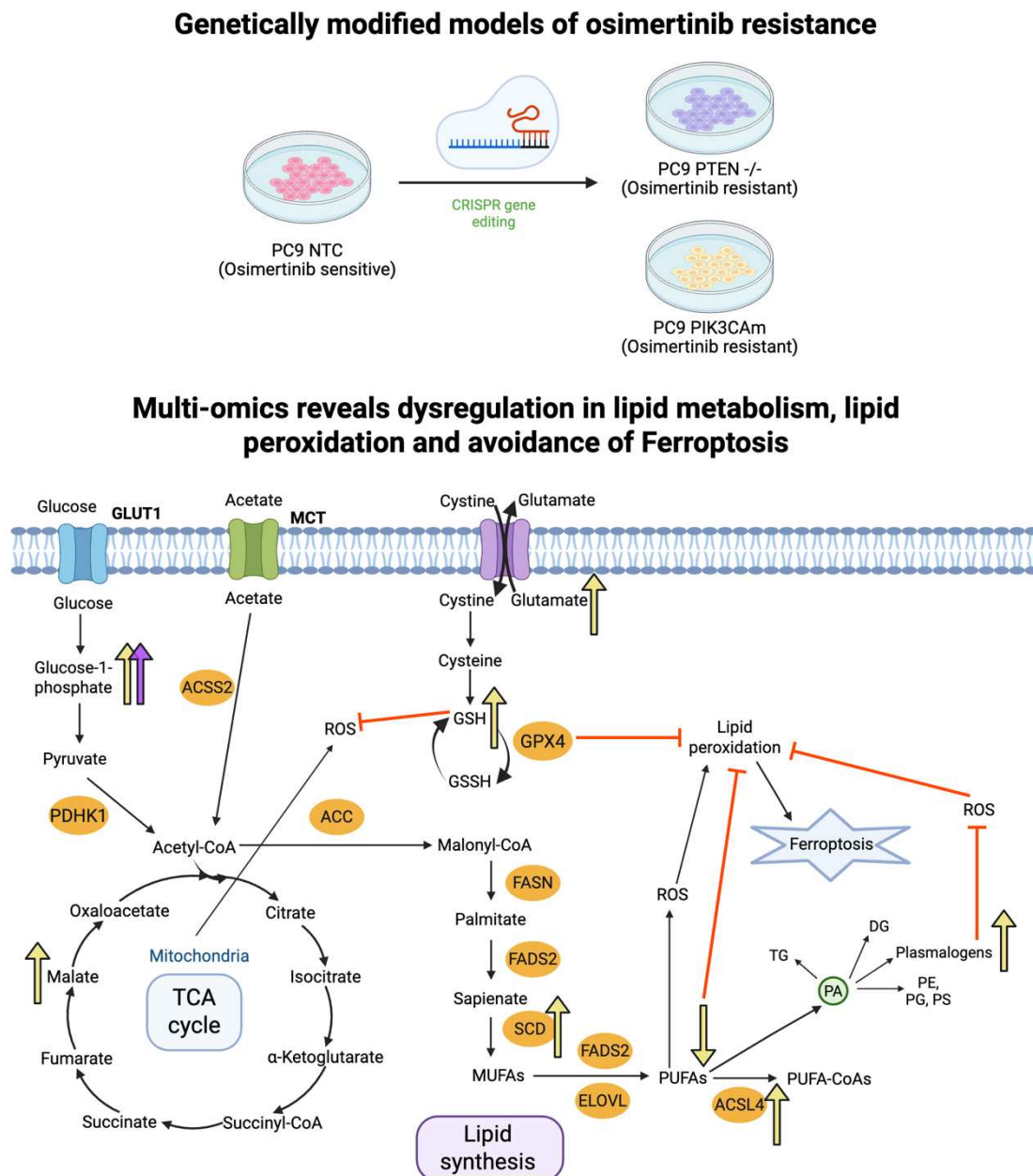


Figure 7.2: A summary of the main findings from the genetic models of resistance. Through multi-omics, alterations within lipid metabolism and modulation of lipid peroxidation was identified, as a potential resistance mechanism by inhibiting ferroptosis in PC9 PIK3Cam compared to PC9 NTC.

7.3. A brief comparison of pharmacologically-induced and GM cell lines

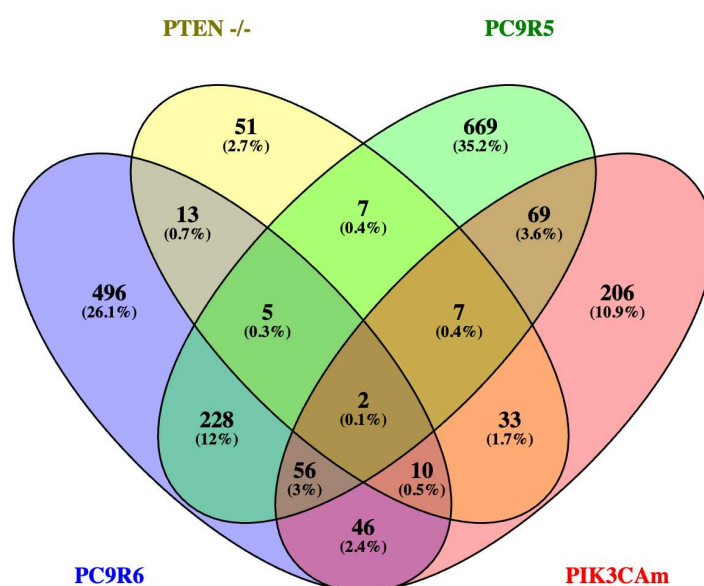
This thesis has investigated resistance which was generated in different ways; through pharmacological dose escalation and through genetic modifications. We therefore decided to compare only the statistically significant proteins and lipids (p value < 0.05 and FC > 1.5 or 2.0 cell line depending) that were found to be altered in the pharmacologically-induced cell lines versus the GM cell lines, to determine if any key changes were conserved across all manners of resistance.

In the proteomics, there were 228 significantly changed proteins common to both pharmacologically-induced lines but only 33 significantly changed proteins common to both GM cell lines (**Figure 7.3A**). Out of the pharmacological lines, PC9R6 had more significantly changed proteins in common with PC9 PTEN -/- (13 proteins) than PC9R5 (7 proteins), whilst PC9R5 had 69 significantly changed proteins in common with PC9 PIK3CAm. PC9 PIK3CAm had more significantly changed proteins in common with the pharmacologically-induced cell lines compared to PC9 PTEN -/-, which is to be expected due to PC9 PTEN -/- having less in common with an OR phenotype than all other OR cell lines. There were only 2 significantly changed proteins that were changing in both pharmacologically-induced and GM lines, and these were ALDH3A1 and ALDH2. Both proteins are involved in lipid peroxidation, suggesting that ferroptosis may be a common resistance mechanism.

In the lipidomics, there were 41 statistically significant lipids common to both pharmacologically-induced lines but only 10 statistically significant lipids common to both GM cell lines (**Figure 7.3B**). PC9 PTEN -/- had no statistically significant lipids in common with either pharmacologically-induced cell line, and PC9 PIK3CAm only had 5 statistically significant lipids in common with PC9R5 (and none with PC9R6). There were only 3 statistically significant lipids that were changing in both pharmacologically-induced and GM lines; PI(34:0)+-H, PC(42:0)+H and DG(34:0)+Na. Whilst these do not fit with any of the pathways discussed, it is interesting to note they all contain saturated fatty acids.

A

Proteomics



B

Lipidomics

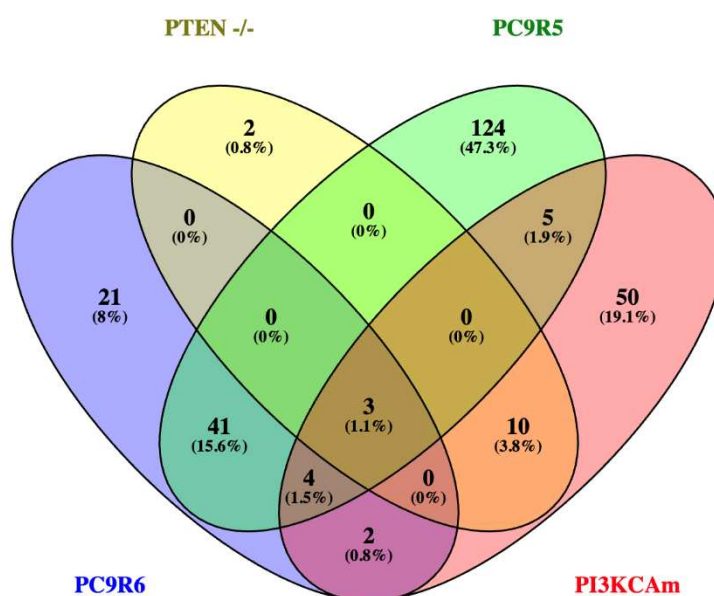


Figure 7.3: Comparison between pharmacologically-induced cell lines versus GM cell lines, with respect to A) Proteomics and B) Lipidomics. The inputs were taken from the statistically relevant proteins and lipids that were plotted in the volcano plots in Chapters 4 and 5.

7.4. Overall limitations and future research

In this study, our aim was to determine which mechanisms cell lines utilise to become resistant to osimertinib treatment or further their resistance to treatment, with respect to metabolism. In the pharmacologically-induced lines, the driving mutations which cause resistance are unknown. We hypothesised that metabolic differences observed between OR and OS could contribute to drug resistance. However, it is difficult to distinguish between metabolic changes that are responsible for the occurrence of resistance, and those which are incidental to another causative mechanism not involved in resistance.

Drug resistance is most often caused by a combination of factors, such as those in EGFR-TKI resistant cells. Several generations of TKIs are required to overcome the resistance mechanisms of the previous generation of therapy. As there is then acquired resistance developing to this new generation of treatment, multiple resistance mechanisms stack up and further limit treatment options.

A second complication that arises when investigating drug resistance is that additional mechanisms of resistance can occur downstream of the initial resistance pathway. For example, with the GM modified cell lines, the original mechanism of resistance is predetermined (the driving genetic mutation in PI3K/Akt pathway). The mutation allows for constitutive activation of the PI3K/Akt pathway, which can contribute to uncontrolled cell proliferation and increased survival mainly, but also dysregulate metabolism in cancer cells(35). However, as we have investigated within the project, there may be additional mechanisms contributing to resistance that occur downstream, such as avoidance of ferroptosis. On the other hand, dysregulated metabolic pathways that we have determined here may simply exist as a consequence of overactivation of the PI3K/Akt pathway, without actually conveying drug resistance. Ultimately, we would need to perform validation experiments to confirm, for example inducing ferroptosis by modulating lipid saturation and assessing the effect on osimertinib sensitivity.

The targeted metabolomics analysis proved to be the weakest “omics” experiment for distinguishing the OR and OS groups based on their phenotypic profiles, in contrast to the

proteomics and lipidomics datasets. There may be limitations with the processing of the data, or the libraries used for annotations, or simply experimental limitations when extracting or preserving the metabolite fractions. The wider literature, our systematic review, and our own proteomics data provided strong evidence to expect significant dysregulation within small molecule metabolism, such as within the TCA cycle and glycolysis. Whilst there were some metabolites of interest that we identified to be altered in OR versus OS (such as GSH and glutamine), there were limited changes in key intermediates of the TCA cycle and glycolysis that were significantly altered. Only malate and glucose-1-phosphate were increased in PC9 PIK3CAm compared to control. This may indicate increased reliance upon both glycolysis and the TCA cycle, however this would need to be confirmed by further experiments such as stable isotope labelling to track metabolic flux.

There were several findings in this thesis that we would have liked to explore further. DNL was identified in some OR cell lines (at transcriptomic or proteomic level, further highlighted in the lipidome). Performing Western blot on key proteins in this pathway, such as FASN, ACLY, and SCD which had altered expression at transcriptome and/or proteome would have been useful to measure DNL more directly. Furthermore, we could have inhibited lipid synthesis and measured changes in lipidomic output, such as the lipid saturation in different classes and the effect on osimertinib sensitivity. We could also use ^{13}C labelling to label glucose and track its utilisation within these cells on a GC-MS. Of interest, the cell lines with more evidence of DNL at the lipidomic/proteomic level were PC9R6 and PC9 PIK3CAm, both of which were more resistant to osimertinib than the other pharmacologically-induced and GM line.

Lipid peroxidation and ferroptosis are other pathways we would have wanted to interrogate further. Investigating GPX3/4 and *NRF2* signalling by first measuring the expression of these in OR compared to OS, and then modulating these pathways *in vitro*, could confirm whether they contribute to resistance. One way to trigger ferroptosis could be to inhibit the formation of GSH itself e.g. through inhibition of SLC7A11, which regulates GSH synthesis(287). SPTBN2 binds to SLC7A11 to allow it to transport GSH throughout the cell membrane and can be inhibited by Abrine. Upon inhibition and when given as a co-treatment with cisplatin, ferroptosis was triggered and sensitivity to cisplatin was restored in NSCLC(287). Cisplatin, levobupivacaine and orlistat are examples of drugs which can induce ferroptosis(288).

Further investigation into small molecule metabolism would be beneficial to this project. This would allow us to focus on the pathways in which we hypothesise there to be changes, as this was not entirely captured in our data despite being evidenced by the wider literature. As our metabolomic data was acquired using an untargeted workflow but processed in a targeted way, we would like to re-obtain our data using a targeted selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) method on a triple quadrupole instrument(289). This allows for higher sensitivity and selectivity in detection of metabolites and is overall an easier workflow compared to untargeted data analysis. NMR could also be used to measure the consumption of different metabolites, such as glucose, pyruvate, lactate and GSH across different cell lines through the analysis of cell media. This indicates the overall usage of specific metabolites in OR versus OS and therefore which pathways they may be reliant upon for energy demands.

One specific finding which was also of interest and which could warrant further investigation was the differential expression of ASS1. This was overexpressed in PC9R6 compared to OS and underexpressed in PC9 PTEN -/- compared to OS. ASS1 is the enzyme responsible for arginine biosynthesis. Unfortunately, we were not able to measure arginine levels directly to confirm due to it not passing QC filtering checks in the metabolomics. If this mechanism is contributing to OR, a potential approach to reinstate drug sensitivity could be arginine deprivation, which has been shown to reduce oxidative phosphorylation and therefore induce mitochondrial dysfunction and ultimately cell death(290,291).

Finally, plasmalogens were consistently shown to be significantly altered in OR versus OS. Plasmalogens are under researched in the context of cancer and drug resistance. As our findings are novel within this setting, plasmalogens would be an interesting lipid class to further investigate. There is some evidence that plasmalogens may modulate the fluidity of the cell membrane, which could link to membrane lipid modifications already observed in NSCLC and within PI3K signalling(292). In addition, plasmalogens are thought to mitigate oxidative stress by reacting with ROS(223).

7.5. Relation to the clinic and final conclusions

Acquired resistance is a known difficulty faced by the clinic when treating NSCLC patients(172,293,294). The research provided by this project gives a novel insight into osimertinib acquired resistance with respect to dysregulated metabolism. PIK3CA NSCLC has been observed in around 5-8 % of NSCLC cases and is a known driver in acquired resistance to EGFR-TKIs(33). PIK3CA are active in numerous solid tumour cancers such as breast cancer, and therefore these are widely studied. In lung cancer, some studies report a correlation between PIK3CA and poor survival in patients(295), however others observe no negative impact on survival after first line treatment with chemotherapy or surgery(33). PTEN knockout or loss of PTEN function through mutation was expressed in 42.4 % of NSCLC and is linked to a shorter PFS and more severe disease(41,240) and has links to EGFR-TKI resistance(241). Therefore, the two GM OR-models selected to study in this thesis have key clinical relevance. In addition, the use of pharmacologically-induced models of OR, generated by dose escalation, may shed light on how resistance might develop in a therapeutic setting. Overall, the use of a combination of models will provide greater insights into possible drug resistance mechanism acting in patients.

In general, combination therapies as a treatment regime in cancer is widely utilised. Often this includes combinations of different chemotherapy, or chemotherapy and radiotherapy. It can also include chemotherapy, such as cisplatin, with small molecule inhibitors. For example, ceramide levels have been inhibited using genetic and small molecule inhibitors in combination with cisplatin in cisplatin-resistant cell lines to reinstate sensitivity(296). Sphingolipid metabolism has also been targeted by inhibiting *UGCG* (genetically and pharmacologically) in combination with chemotherapeutics to combat drug resistance(297).

Combination therapies to combat EGFR-TKI resistance in particular are well documented and used in numerous settings(298,299). In addition, metabolism has been investigated in NSCLC, with studies targeting important transporters (e.g. GLUTs, SLC family members, MCTs) or crucial enzymes (e.g. HKs, PDK, GCS) common. However, a combination of an EGFR-TKI with a therapy specifically aimed at targeting metabolism is not yet used in the clinic. This has been explored *in vitro*: one study targeted PDK in combination with EGFR-TKI to successfully reverse

resistance(300), and another very recent study, like us, targeted GSL biosynthesis via GCS inhibition(212). These studies were performed *in vitro* using monocultured cell lines, which do not give an accurate representation of how pharmacological inhibitors would behave in patients. Therefore, to progress this research and the conclusions we found here, the next step could be to explore in 3D cultures such as spheroids or by performing an *in vivo* study. One complexity to administering combination therapies over monotherapies is treatment-induced toxicity, which is increasingly likely when administering more than one drug(301). This often means that more care and attention is required to develop a treatment regime, usually meaning patients do not take both treatments at once but in a staggered approach. As our data was generated by continuously dosing both drugs at once, this is not reflective of real-world drug treatment regimes, that would be used with patients.

In conclusion, this project explores how dysregulated metabolism may contribute to resistance mechanisms in osimertinib-resistant NSCLC. In pharmacologically-resistant cell lines, dysregulated sphingolipid metabolism in the form of reducing ceramide levels in favour of complex GSLs, to avoid ceramide mediate caspase-3 activation and apoptosis, was determined as a potential resistance mechanism. We validated this by inhibiting GCS in conjunction with osimertinib therapy, which reduced ceramide levels, increased caspase-3 apoptosis, and resensitised PC9R5 and PC9R6 to osimertinib. This finding has simultaneously been confirmed by another independent research group in similar cell lines (212). The GM cell lines, PC9 PTEN -/- and PC9 PI3KCAm, exhibit different metabolic phenotypes to each other and to sensitive control. Increased DNL and avoidance of lipid peroxidation/ferroptosis appear to be key modulated pathways in PC9 PI3KCAm and may contribute to resistance. We have determined several avenues that warrant further exploration in future studies and suggest a potential novel therapeutic strategy to combat osimertinib resistance in patients.

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Appendix

9. Appendix

9.1. A summary of all papers used in the literature analysis conducted in the systematic review (Chapter 2).

Appendix Table 9.1: A summary of all 27 papers included in the analysis of this systematic review.

Study	Drug Treatment	Cell Lines	Methods	Statistical Analysis	Summary of Findings
Dyrstad et al., 2021	Erlotinib	HCC827, H1975 (erlotinib resistant and sensitive)	1) Seahorse XF assay to measure OCR 2) CO ₂ trapping	ANOVA and unpaired two-tailed t-test and $p < 0.05$ considered statistically significant	Targeted glycolysis to prevent TKI resistance. Inhibited PDHK activity as this is increased in resistant cells. Redirecting metabolism to restore therapeutic effect of EGFR-TKI.
Li H et al., 2016	Erlotinib	PC9, H3255, H1975	1) Metabolomics: ¹ H-NMR 2) GSH colorimetric assay	OPLS-DA	Erlotinib resistant cells were re-sensitised to TKI when GSH was increased. 36 differential metabolites, 14 involved in GSH, amino acids, nucleotides and choline metabolism correlated with resistance.
Ma et al., 2020	Osimertinib	H1975 (osimertinib resistant and sensitive)	1) Metabolomics and proteomics: LC-MS – Linear ion trap-Orbitrap	PCA, OPLS-DA, two-tailed t-test and $p < 0.05$ considered statistically significant	54 differential metabolites found all related to amino acid metabolism. Increased reliance on oxidative phosphorylation, and decreased glycolysis in resistant cells. Increase in PI3k/Akt signaling and HIF1 α in resistance cells and a decrease in arginine/proline metabolism.

DeRosa et al., 2015	Erlotinib	H1975, HCC827, H1993	1) F-FDG glucose uptake and consumption 2) Lactate secretion 3) Intracellular ATP levels 4) Seahorse XF assay to measure OCR and ECAR	Unpaired and paired t-test and $p < 0.05$ considered statistically significant	EGFR-TKIs reactivated oxidative phosphorylation, this shift was mediated by PI3K/Akt and RAS/MAPK.
Martin et al., 2016	Osimertinib	PC9, H1975 (osimertinib resistant and sensitive)	1) Lactate assay 2) Seahorse XF assay to measure OCR	Two-tailed t-test and $p < 0.05$ considered statistically significant	Osimertinib suppressed glycolysis in sensitive lines. oxidative phosphorylation inhibitor delayed or prevented drug resistance. Osimertinib treatment induced oxidative phosphorylation.
Kim et al., 2018	Gefitinib Erlotinib	A549, H1299, HCC827, H1975, PC9 (PC9 gefitinib and erlotinib resistant and sensitive)	1) Metabolomics: LC-MS 2) Glucose consumption/Lactate production assay 3) Seahorse XF assay to measure OCR and ECAR 4) ROS quantification 5) Intracellular ATP quantification	t-test and p-value < 0.05 considered statistically significant	EGFR-TKI can overcome resistance via inhibition of glucose metabolism or JNK activation.
Xu et al., 2021	Gefitinib	H1975, PC9 (gefitinib resistant and sensitive)	1) Lipidomics: ESI-MS/MS on AB SCIEX Triple TOFTM 5600+	Two-tailed t-test and $p < 0.05$ considered statistically significant	Gefitinib induced downregulation of SREBP1 in sensitive cells only. Gefitinib altered the ration of saturated and unsaturated phospholipids in

			2) Intracellular measurements of fatty acid, cholesterol and triglyceride levels		sensitive vs resistant cells. Inhibition of SREBP1 resensitised cells to gefitinib.
Chen et al., 2021	Osimertinib	PC9, HCC827	1) Lipidomics: LC-MS	Two-sided unpaired Student's t-test and $p < 0.05$ considered statistically significant	Lipogenesis was suppressed by osimertinib treatment as it degraded mSREBP1. Osimertinib treated cells showed a decrease in abundance of 50 lipid metabolites, highlighting that drug treatment can change the lipid profile of cells.
Serizawa et al., 2014	Erlotinib	PC9 (erlotinib resistant and sensitive)	1) Metabolomics: CE-TOF-MS	Welch's t-test and $p\text{-value} < 0.01$ considered statistically significant OPLS-DA	Glutamine metabolism was enhanced in erlotinib resistant cells compared to glycolysis. Resistance occurred via constitutive activation of CRKL/PI3K/Akt. 5 genes involved in glutamine/glutathione synthesis had significant gains in copy number in resistant cells. 18/24 metabolites contributed to erlotinib resistance.
Thiagarajan et al., 2016	Erlotinib	HCC827, H1975	1) Affymetrix Array 2) Glutamine deprivation 3) Metabolomics	PCA	TGF β 2 upregulation increased EMT-ness and remarkable adaptive transcriptome-metabolome bioenergetic alterations correlated with early-onset drug escape of EGFRm NSCLC. Global reduction in glucose metabolism, TCA cycle activity and reduction of synthesis in fatty acids and membrane lipids.

Zhang et al., 2019	Osimertinib Gefitinib	H358, H460, A549, H1299, H69, H1650, H1975, HCC827 (osimertinib and gefitinib resistant and sensitive), PC9 (osimertinib and gefitinib resistant and sensitive), H3255	1) Metabolomics: HPLC-MS/QTRAP Triple- Quadrupole	t-test and p-value < 0.05 considered statistically significant	Increased levels of miR-147b created a drug tolerance for EGFR inhibition. This disrupted the TCA cycle and activation of hypoxic responses irrespective of oxygen levels.
Chen et al., 2017	Gefitinib	H1975, H820, H1299	1) Glycolysis stress test 2) Cholesterol staining/quantity assay	One-way ANOVA and Dunnett's test, *p < 0.05, **p < 0.01, ***p < 0.001 considered statistically significant	AMPK regulated metabolic homeostasis through inducing metabolic changes to balance energy consumption and production intracellularly. AMPK inhibitor shows cytotoxicity towards both gefitinib sensitive and resistant cells.
Kim et al., 2013	Afatinib	H1975, PC9 (afatinib resistant and sensitive)	1) Lactate production assay 2) Orimetric assay for intracellular ATP quantification	t-test and p-value < 0.05 considered statistically significant	Inhibiting glycolysis by 2DG improves TKI efficacy in all cell lines used. Downregulation of MCL1 via alteration of AMPK/mTOR signaling improved afatinib efficacy via inhibition of glycolysis.
Xie et al., 2016	Erlotinib	HCC827 (erlotinib	1) Metabolomics: ¹ H-NMR spectroscopy	PCA	Inhibited glutaminase which inhibited cell proliferation in resistant cells and in combination

		resistant and sensitive)	2) Glutaminase activity assay	One way ANOVA with p-value < 0.05 considered statistically significant	downregulated glutamine and glycolysis metabolism, implying that inhibition with compound 968 and TKI inhibited whole cellular metabolism.
Yang et al., 2018	Erlotinib	H1975, H1650	1) Seahorse XF assay to measure OCR 2) Detection of intracellular ATP 3) Metabolomics: LC-MS	ANOVA, t-test and p-value < 0.05 considered statistically significant	PKD1 regulates glycolysis. Inhibiting this enhanced TKI effect in hypoxic conditions and improved oxidative phosphorylation. This increased the anti-cancer efficacy of erlotinib.
Ye et al., 2017	Erlotinib	HCC827 (erlotinib resistant and sensitive)	1) Seahorse XF assay to measure OCR and ECAR 2) ROS, GSH and GSSG measurement 3) Metabolomics: UPLC-TOF for metabolites 4) Lactic acid and glucose measurements	ANOVA and p-value < 0.05 considered statistically significant	Mitochondrial dysfunction plays a role in the erlotinib resistance as mitochondria rely on glycolysis. Blocking autophagy/AKT and causing glucose deprivation re-sensitised the cells to TKI.
Ye et al., 2019	Erlotinib	HCC827 (erlotinib resistant and sensitive)	1) Seahorse XF assay to measure OCR and ECAR 2) Metabolomics: UPLC-TOF	ANOVA with p-value < 0.05 considered statistically significant	Compared the sensitive and resistant cell lines. Erlotinib resistant cells switched to higher glycolysis as opposed to oxidative phosphorylation like the other findings. Used Vitamin C to inhibit this glycolysis and prevent drug resistance.

Suzuki et al., 2018	Gefitinib Erlotinib	A549, H1299, PC9, HCC827	1) Glucose uptake assay	Two-tailed t-test (with Bonferroni correction) and $p < 0.05$ considered statistically significant	GLUT1 mediated glucose metabolism led to gefitinib resistance. Targeting GLUT1 overexpression returned sensitivity to gefitinib.
Huang et al., 2020	Gefitinib	PE089 (gefitinib resistant and sensitive), PF001 (gefitinib sensitive), PF002 (gefitinib resistant)	1) Mitochondrial extraction 2) Seahorse XF assay to measure OCR and mitochondrial complex activity	t-test and p -value < 0.05 considered statistically significant	Mitochondrial translocation allowed for EGFR-TKI resistant cells to switch from glycolysis to oxidative phosphorylation. EGFR translocation to the mitochondria shifted metabolism and increased MCT1, LDHB and PDHA1. The resistant cells responded to an MCT1 inhibitor.
Bach et al., 2018	Erlotinib Gefitinib	H1993 (gefitinib/erlotini b resistance)	1) Affymetrix Array 2) Metabolomics: 1H-NMR spectroscopy	t-test and $*p < 0.05$, $**p < 0.01$, $***p$ < 0.001 considered statistically significant	Association between gefitinib resistant NSCLC cells and BMP4 overexpression. BMP4 interacts with lipid metabolism via ACSL4 and p53 signaling pathway.
Chiang et al., 2018	Erlotinib	PC9, H3255 (erlotinib resistant and sensitive) HCC4011 (MET amplified)	1) Agilent Seahorse XF metabolic assay	Two-tailed t-test and $*p < 0.05$, $**p <$ 0.01 , $***p < 0.001$ considered statistically significant	mTORC2 metabolically reprogrammed erlotinib resistant cells therefore mTOR plays a role in glucose metabolism regulation.
Ali et al., 2018	Gefitinib	EGFRm – PC9, HCC4006,	1) Free fatty acid measurement		EGFR signaling relies upon EGFR palmitoylation. FASN regulation by EGFRm mediated TKI

		H1650, H820 and H1975 EGFRwt – H1703 Immortalized non-transformed lung NL20		t-test and $p < 0.05$ considered statistically significant	resistance. Targeting FASN with Orlistat halts EGFRm signaling. Fatty acid metabolic pathway is upregulated in EGFRm NSCLC cells with acquired resistance to gefitinib. Knockdown of EGFR signaling decreased levels of both FASN and its activator SREBP1, implying that blocking the fatty acid metabolism can therapeutically benefit resistant cells.
Li et al., 2016	Gefitinib	PC9, A549	1) Lipidomics: GC-MS 2) Triglyceride and cholesterol assay	t-test and $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ considered statistically significant	Gefitinib sensitivity was enhanced when knockdown of MARVELD1 occurred (usually binds to SREBP1 to stimulate lipid metabolism). Reduced proportion of unsaturated fatty acids (by SREBP) in cell membrane resulted in decreased cell membrane fluidity.
Chen et al., 2018	Gefitinib	A549, H1975, H1650, PC9 (gefitinib resistant and sensitive)	1) Lipid raft isolation 2) Amplex red cholesterol assay	Two-tailed t-test and $p < 0.05$ considered statistically significant	Cholesterol levels were higher in gefitinib resistant cells compared to sensitive cells. Lovastatin reduced cholesterol levels to inhibit tumor growth in gefitinib resistant cells.
Luo et al., 2021	Gefitinib Osimertinib	H1993, A549, PC9, HCC827 and H1975	1) Immunoblotting 2) MTT 3) siRNA	Two-tailed t-test and $p < 0.05$ considered statistically significant	EGFR signaling upregulated LDLR expression via SREBP1. EGFRm count on lipids for survival. Combination of a statin and TKI could improve treatment.

Huang et al., 2019	Gefitinib	HCC827, PC9, H1975 (gefitinib resistant and sensitive)	1) BODIPY 493/503 staining for LD 2) Treatment with OA to simulate high lipid environment	t-test followed by one-way ANOVA and Tukey's post-test. p-value < 0.05 considered statistically significant	Main link between lipid metabolic reprogramming and TKI resistance. Hypothesized that LD metabolized into small molecule phospholipids and fatty acids to maintain the survival of resistant cells.
Jung et al., 2015	Gefitinib	PC9 (gefitinib resistant and sensitive)	1) Lipidomics: Lipid MALDI-MS and Triple Quadrupole LC-MS	PCA	35 differentially expressed lipids between resistance and sensitive cells. EV's contain lipid signatures including phospholipid changes that related to drug resistance (67 differentially expressed phospholipids identified).

Abbreviations: AMPK; AMP-activated protein kinase, BMP4; bone morphogenic protein 4, CE-TOF-MS; capillary electrophoresis-time of flight-MS, ECAR; extracellular acidification rate, EGFR-TKI; epidermal growth factor receptor-tyrosine kinase inhibitor, ESI; electron spray ionisation, FASN; fatty acid synthase, F-FDG; fluorodeoxyglucose, GSH; glutathione, HPLC-MS; high performance liquid chromatography MS, LC-MS; liquid chromatography-mass spectrometry, LDLR; low density lipoprotein receptor, LD; lipid droplet, MTT; 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide, NMR; nuclear magnetic resonance, OCR; oxygen consumption rate, PDHK; pyruvate dehydrogenase kinase, ROS; reactive oxygen species, SREBP1; sterol regulatory binding protein 1, mSREBP1; matureSREBP1.

9.2. The full list of lipid metabolism genes/proteins used in multiple chapters when discussing key lipid proteins involved in DNL and sphingolipid metabolism

Appendix Table 9.2: *A list of lipid metabolism genes with their full name and specific process in which they act.*

Full Name	Gene	Process
Acetyl-CoA Carboxylase Alpha	ACACA	Fatty acid synthesis
ATP Citrate Lyase	ACLY	Fatty acid synthesis
ELOVL Fatty Acid Elongase 6	ELOVL6	Fatty acid synthesis
Fatty Acid Synthase	FASN	Fatty acid synthesis
Stearoyl-CoA Desaturase	SCD	Fatty acid synthesis
Acetylcholinesterase	ACHE	Glycerophospholipid metabolism
1-acylglycerol-3-phosphate O-acyltransferase1	AGPAT1	Glycerophospholipid metabolism
1-acylglycerol-3-phosphate O-acyltransferase2	AGPAT2	Glycerophospholipid metabolism
1-acylglycerol-3-phosphate O-acyltransferase3	AGPAT3	Glycerophospholipid metabolism
Alkyldihydroxyacetonephosphate synthase, peroxisomal	AGPS	Glycerophospholipid metabolism
acyl/alkyldihydroxyacetone	AYR1	Glycerophospholipid metabolism
CDP-diacylglycerol--inositol 3-phosphatidyltransferase	CDIPT	Glycerophospholipid metabolism
Phosphatidate cytidyltransferase1	CDS1	Glycerophospholipid metabolism
Phosphatidate cytidyltransferase2	CDS2	Glycerophospholipid metabolism
Choline O-acetyltransferase	CHAT	Glycerophospholipid metabolism

Cardiolipin synthase (CMP-forming)	<i>CRLS1</i>	Glycerophospholipid metabolism
Diacylglycerol O-acyltransferase	<i>DGAT</i>	Glycerophospholipid metabolism
Diacylglycerol Kinase Alpha	<i>DGKA</i>	Glycerophospholipid metabolism
Diacylglycerol Kinase Beta	<i>DGKB</i>	Glycerophospholipid metabolism
Diacylglycerol Kinase Delta	<i>DGKD</i>	Glycerophospholipid metabolism
Diacylglycerol Kinase Epsilon	<i>DGKE</i>	Glycerophospholipid metabolism
Diacylglycerol Kinase Gamma	<i>DGKG</i>	Glycerophospholipid metabolism
Diacylglycerol Kinase Eta	<i>DGKH</i>	Glycerophospholipid metabolism
Diacylglycerol Kinase Iota	<i>DGKI</i>	Glycerophospholipid metabolism
Diacylglycerol Kinase Kappa	<i>DGKK</i>	Glycerophospholipid metabolism
Diacylglycerol Kinase Zeta	<i>DGKZ</i>	Glycerophospholipid metabolism
Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	<i>ENPP2</i>	Glycerophospholipid metabolism
Glycerophosphocholine cholinephosphodiesterase	<i>ENPP6</i>	Glycerophospholipid metabolism
Ethanolamine kinase	<i>ETNK2</i>	Glycerophospholipid metabolism
Fatty acyl-CoA reductase 1	<i>FAR1</i>	Glycerophospholipid metabolism
Fatty acyl-CoA reductase 2	<i>FAR2</i>	Glycerophospholipid metabolism
Glycerophosphodiester phosphodiesterase	<i>GDE1</i>	Glycerophospholipid metabolism
Lysophospholipase D GDPD1	<i>GDPD1</i>	Glycerophospholipid metabolism
Dihydroxyacetone phosphate acyltransferase	<i>GNPAT</i>	Glycerophospholipid metabolism
Glycerol-3-phosphate acyltransferase1	<i>GPAT</i>	Glycerophospholipid metabolism

Glycerol-3-phosphate acyltransferase2	<i>GPAT2</i>	Glycerophospholipid metabolism
Glycerol-3-phosphate acyltransferase3	<i>GPAT3</i>	Glycerophospholipid metabolism
Glycerol-3-phosphate acyltransferase4	<i>GPAT4</i>	Glycerophospholipid metabolism
Glycerophosphodiester phosphodiesterase	<i>GPCPD1</i>	Glycerophospholipid metabolism
Glycerol-3-phosphate dehydrogenase	<i>GPD1L</i>	Glycerophospholipid metabolism
Glycerol-3-phosphate dehydrogenase 2	<i>GPD2</i>	Glycerophospholipid metabolism
Phosphatidylcholine-sterol acyltransferase	<i>LCAT</i>	Glycerophospholipid metabolism
Lysocardiolipin acyltransferase 1	<i>LCLAT1</i>	Glycerophospholipid metabolism
Lysophosphatidylglycerol Acyltransferase 1	<i>LPGAT1</i>	Glycerophospholipid metabolism
Acyl-protein thioesterase 1	<i>LYPLA1</i>	Glycerophospholipid metabolism
Lysophospholipid acyltransferase 1	<i>MBOAT1</i>	Glycerophospholipid metabolism
Lysophospholipid acyltransferase 2	<i>MBOAT2</i>	Glycerophospholipid metabolism
Lysophospholipid acyltransferase 7	<i>MBOAT7</i>	Glycerophospholipid metabolism
Ethanolamine-phosphate cytidyltransferase 2	<i>PCYT2</i>	Glycerophospholipid metabolism
Phosphatidylethanolamine N-methyltransferase	<i>PEMT</i>	Glycerophospholipid metabolism
Phosphatidylglycerophosphate Synthase 1	<i>PGS1</i>	Glycerophospholipid metabolism
Phosphoethanolamine/phosphocholine phosphatase 1	<i>PHOSPHO1</i>	Glycerophospholipid metabolism
Phosphatidylinositol-4-phosphate 5-kinase type-1 alpha	<i>PIP5K1A</i>	Glycerophospholipid metabolism
Phosphatidylserine decarboxylase proenzyme, mitochondrial	<i>PISD</i>	Glycerophospholipid metabolism

Phospholipase A1 member A	<i>PLA1A</i>	Glycerophospholipid metabolism
Phospholipase A2	<i>PLA2G1B</i>	Glycerophospholipid metabolism
Phospholipase A1	<i>PLAAT3</i>	Glycerophospholipid metabolism
Phospholipase C	<i>PLC</i>	Glycerophospholipid metabolism
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1	<i>PLCB1</i>	Glycerophospholipid metabolism
Phospholipase D1	<i>PLD1</i>	Glycerophospholipid metabolism
Phospholipase D2	<i>PLD2</i>	Glycerophospholipid metabolism
Phospholipid Phosphatase 4	<i>PLPP4</i>	Glycerophospholipid metabolism
Phospholipid Phosphatase 5	<i>PLPP5</i>	Glycerophospholipid metabolism
Phosphatidylserine synthase 1	<i>PTDSS1</i>	Glycerophospholipid metabolism
Phosphatidylserine synthase 2	<i>PTDSS2</i>	Glycerophospholipid metabolism
Ethanolaminephosphotransferase 1	<i>SELENOI</i>	Glycerophospholipid metabolism
Lysoplasmalogenase	<i>TMEM86B</i>	Glycerophospholipid metabolism
Choline kinase alpha	<i>CHKA</i>	Glycerophospholipid metabolism, PC synthesis
Cholinephosphotransferase 1	<i>CHPT1</i>	Glycerophospholipid metabolism, PC synthesis
Choline-phosphate cytidyltransferase A	<i>PCYT1A</i>	Glycerophospholipid metabolism, PC synthesis
ELOVL Fatty Acid Elongase 1	<i>ELOVL1</i>	Lipid remodelling
ELOVL Fatty Acid Elongase 3	<i>ELOVL3</i>	Lipid remodelling
ELOVL Fatty Acid Elongase 7	<i>ELOVL7</i>	Lipid remodelling
Lysophosphatidylcholine acyltransferase 3	<i>LPCAT3</i>	Lipid remodelling
Lysophosphatidylcholine acyltransferase 1	<i>LPCAT1</i>	Lipid remodelling, Glycerophospholipid metabolism

Lysophosphatidylcholine acyltransferase 2	<i>LPCAT2</i>	Lipid remodelling, Glycerophospholipid metabolism
Lysophosphatidylcholine acyltransferase 4	<i>LPCAT4</i>	Lipid remodelling, Glycerophospholipid metabolism
Alkaline Ceramidase 3	<i>ACER3</i>	Sphingolipid metabolism
Long chain fatty acid CoA ligase	<i>ACSL</i>	Sphingolipid metabolism
Arylsulfatase A	<i>ARSA</i>	Sphingolipid metabolism
Acid ceramidase	<i>ASAH1</i>	Sphingolipid metabolism
Galactosyltransferase6	<i>B4GALT6</i>	Sphingolipid metabolism* also B4GALT5
Choline/Ethanolamine Phosphotransferase 1	<i>CEPT1</i>	Sphingolipid metabolism
Ceramide kinase	<i>CERK</i>	Sphingolipid metabolism
Ceramide synthase	<i>CERS</i>	Sphingolipid metabolism
Ceramide synthase 2	<i>CERS2</i>	Sphingolipid metabolism
Ceramide synthase 4	<i>CERS4</i>	Sphingolipid metabolism
Ceramide synthase 5	<i>CERS5</i>	Sphingolipid metabolism
Ceramide synthase 6	<i>CERS6</i>	Sphingolipid metabolism
Ceramide-1-Phosphate Transfer Protein	<i>CPTP</i>	Sphingolipid metabolism
Sphingolipid 4-desaturase	<i>DEGS1</i>	Sphingolipid metabolism
Sphingolipid 4-desaturase	<i>DEGS2</i>	Sphingolipid metabolism
Galactosylceramidesulfotransferase	<i>GAL3ST1</i>	Sphingolipid metabolism* also GST
Galactosylceramidase	<i>GALC</i>	Sphingolipid metabolism
Glucosylceramidase	<i>GBA</i>	Sphingolipid metabolism
Glucosylceramidase Beta 2	<i>GBA2</i>	Sphingolipid metabolism
Alpha-galactosidase A	<i>GLA</i>	Sphingolipid metabolism

Betagalactosidase	<i>GLB1</i>	Sphingolipid metabolism
3-ketodihydrosphingosine reductase	<i>KDSR</i>	Sphingolipid metabolism
Sialidase-3	<i>NEU3</i>	Sphingolipid metabolism
Shingomyelin synthase 1	<i>SGMS1</i>	Sphingolipid metabolism
Shingomyelin synthase 2	<i>SGMS2</i>	Sphingolipid metabolism
Sphingosine-1-phosphate lyase	<i>SGPL1</i>	Sphingolipid metabolism
Sphingosine-1-phosphate phosphatase 1	<i>SGPP1</i>	Sphingolipid metabolism
Sphingosine-1-phosphate phosphatase 2	<i>SGPP2</i>	Sphingolipid metabolism
Sphingomyelin phosphodiesterase 1	<i>SMPD1</i>	Sphingolipid metabolism
Sphingomyelin phosphodiesterase 2	<i>SMPD2</i>	Sphingolipid metabolism
Sphingomyelin phosphodiesterase 3	<i>SMPD3</i>	Sphingolipid metabolism
Sphingomyelin phosphodiesterase 4	<i>SMPD4</i>	Sphingolipid metabolism
Sphingosine kinase 1	<i>SPHK1</i>	Sphingolipid metabolism
Sphingosine kinase 2	<i>SPHK2</i>	Sphingolipid metabolism
Serine palmitoyltransferase 1	<i>SPTLC1</i>	Sphingolipid metabolism
Serine palmitoyltransferase 2	<i>SPTLC2</i>	Sphingolipid metabolism
Serine palmitoyltransferase 3	<i>SPTLC3</i>	Sphingolipid metabolism
Ceramide glucosyltransferase	<i>UGCG</i>	Sphingolipid metabolism
2-hydroxyacylsphingosine 1-beta-galactosyltransferase	<i>UGT8</i>	Sphingolipid metabolism
Phospholipid Phosphatase 1	<i>PLPP1</i>	Sphingolipid metabolism, Glycerophospholipid metabolism

Phospholipid Phosphatase 2	<i>PLPP2</i>	Sphingolipid metabolism, Glycerophospholipid metabolism
Phospholipid Phosphatase 3	<i>PLPP3</i>	Sphingolipid metabolism, Glycerophospholipid metabolism

9.3. A copy of the published systematic review discussed in Chapter 2

Systematic Review

Dysregulated Metabolism in EGFR-TKI Drug Resistant Non-Small-Cell Lung Cancer: A Systematic Review

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Abstract: Drug resistance is a common barrier to continued effective treatment in cancer. In non-small-cell lung cancer (NSCLC), tyrosine kinase inhibitors that target the epidermal growth factor receptor (EGFR-TKIs) exhibit good efficacy in cancer treatment until acquired resistance occurs. It has been observed that drug resistance is accompanied by numerous molecular-level changes, including significant shifts in cellular metabolism. The purpose of this study was to critically and systematically review the published literature with respect to how metabolism differs in drug-resistant compared to drug-sensitive NSCLC. Understanding the differences between resistant and sensitive cells is vital and has the potential to allow interventions that enable the re-sensitisation of resistant cells to treatment, and consequently reinitiate the therapeutic effect of EGFR-TKIs. The main literature search was performed using relevant keywords in PubMed and Ovid (Medline) and reviewed using the Covidence platform. Of the 1331 potentially relevant literature records retrieved, 27 studies were subsequently selected for comprehensive analysis. Collectively, the literature revealed that NSCLC cell lines resistant to EGFR-TKI treatment possess characteristic metabolic and lipidomic phenotypic signatures that differentiate them from sensitive lines. Further exploration of these reported differences suggests that drug-resistant cell lines are differentially reliant on cellular energy sources and that modulation of relative energy production pathways may lead to the reversal of drug resistance.

Keywords: NSCLC; EGFR-TKI; metabolomics; lipidomics; drug resistance; metabolism; metabolites



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1. Introduction

Lung cancer is the second most diagnosed cancer world-wide [1], with NSCLC accounting for 80% of lung cancer cases [2,3]. NSCLC is a lethal and difficult to treat cancer, with 16% morbidity and 18.4% mortality [4]. One of the most common driving mutations in NSCLC patients is found in the epidermal growth factor receptor (EGFR). Such cancers respond well to EGFR-tyrosine kinase inhibitors (EGFR-TKIs) [3], which target the ATP binding site of the TK domain of EGFR, blocking downstream signalling pathways such as Ras/MAPK and PI3K/Akt [3]. These signalling pathways are heavily involved in cell proliferation, and when constitutively activated in NSCLC patients, can allow for increased proliferation whilst simultaneously evading apoptosis [5].

Targeted therapies that include EGFR-TKIs are currently an effective standard of care treatment, with the first-generation TKIs-erlotinib and gefitinib-giving patients up to one year of progression-free survival (PFS) [6] before acquired resistance occurs. Resistance can arise in both an EGFR-dependent and/or an EGFR-independent manner. The most common form of EGFR-dependent acquired resistance arises as a point mutation within EGFR (EGFR_M), at T790M residue occurring in around 60% of cases [6]. EGFR-independent resistant mechanisms occur as a consequence of mutations in other oncogenes such as c-MET [1] or by mutations downstream of EGFR that allow for the pathway to be constitutively activated once more, promoting tumorigenesis. It is because of this arising

resistance to therapies that further generations of TKIs have been developed, including third-generation drug osimertinib (AZD9291). Osimertinib selectively targets both the initial EGFRm and T790M mutations whilst selecting against EGFR wild type (EGFRwt). However, similar to previous generations of TKIs, resistance to the newer generation TKIs is inevitable. Understanding the process of drug resistance acquisition for TKIs offers an opportunity to identify new adjuvant therapeutic targets that prevent or reverse drug resistance and reinstate the desired therapeutic effect.

The dysregulation of cellular metabolism has been acknowledged as a hallmark of cancer for over 90 years [7]. Driven by oncogenes, this phenomenon allows key steps of metabolism to be altered to favour tumorigenesis [8]. A specific example of this is the ability of cancer cells to switch their energy metabolism to favour aerobic glycolysis over oxidative phosphorylation, the key metabolic process for ATP production in healthy cells, via the mitochondria [8]. This results in an increased production of lactate from glucose even in the presence of oxygen, and is known as the Warburg effect [7–9]. A shift from oxidative phosphorylation can be attributed to oncogenic signalling from pathways downstream of EGFR [8]. In NSCLC specifically, EGFR signalling enhances glycolysis [10]. This phenomenon of creating additional ATP energy also promotes glucose utilisation in anabolic processes for synthesis of other vital compounds such as DNA, proteins and lipids—key building blocks required for cancer cells to proliferate [1,7,9,11]. Medes et al. discovered that tumour cells synthesize lipids from glucose or acetate, and rely upon de novo lipogenesis as the primary form of fatty acid generation [12] to allow for cancer cells' high demand for lipids (Figure 1).

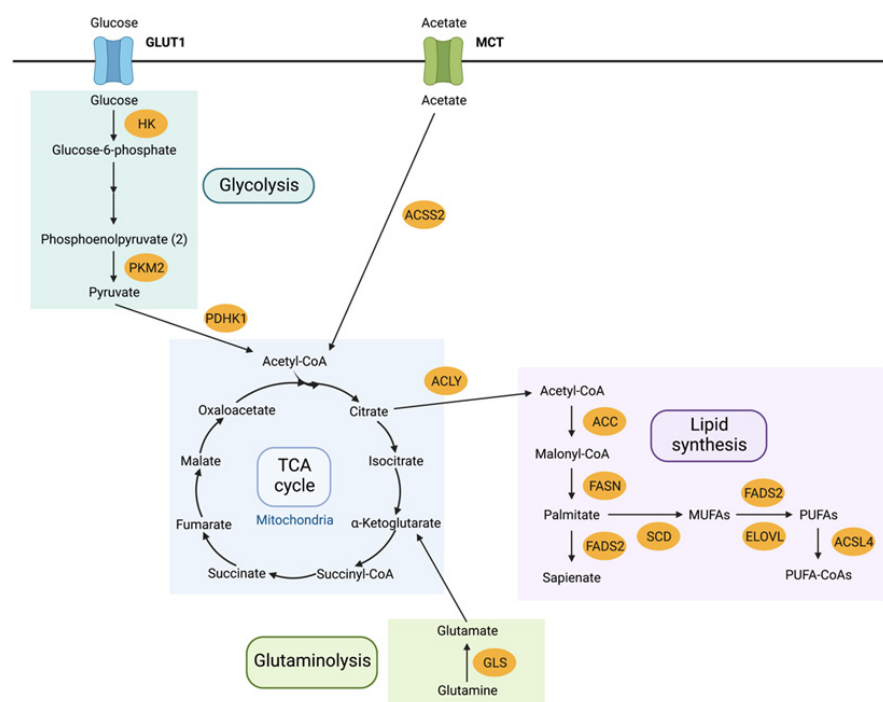


Figure 1. An overview of cellular metabolic key pathways; glycolysis, TCA cycle, glutaminolysis and lipid synthesis. Adapted from Koundouros et al., 2019 [13]. Abbreviations: GLUT1 – glucose transporter 1, HK—hexokinase, PKM2—pyruvate kinase M2, PDHK1—pyruvate dehydrogenase kinase 1, MCT – monocarboxylate transporter, ACSS2—acyl-CoA synthetase short chain family member 2, ACLY—ATP citrate lyase, ACC—acetyl-CoA carboxylase, FASN—fatty acid synthase, FADS2—fatty acid desaturase 2, SCD—stearoyl-CoA desaturase 1, ELOVL—elongation of very long chain fatty acids, ACSL4—acyl-CoA synthetase long chain family member 4.

A multi-omics approach including genomics, proteomics and metabolomics allows for a wider comprehension of disease by combining information from diverse biological

levels [14]. While changes at the genomic level are well documented [15], the full spectrum of metabolic contributions to drug resistance in cancer are less well defined; this review will focus on studies which measure the metabolic phenotype, including but not limited to metabolomics and lipidomics. In lung cancer, previous studies identified metabolites involved in amino acid metabolism, glycolysis, oxidative phosphorylation, gluconeogenesis and fatty acid metabolism to be dysregulated in drug-resistant cells [16].

In its broadest sense, metabolomics (commonly referred to as metabonomics, metabolic profiling, or metabolic phenotyping) is the study of measuring the levels of metabolites in biological samples such as cells, tissues and biofluids [16], allowing the metabolic state of the cell to be determined. Metabolites of interest are often intermediates of key pathways such as glycolysis, and their levels within cancer cells or in drug-resistant vs. drug-sensitive cell lines can give an indication of which metabolic pathway has been rewired [17]. Metabolomics is largely studied using liquid chromatography–mass spectrometry (LC-MS), gas chromatography–mass spectrometry (GC-MS) and proton nuclear magnetic resonance (^1H -NMR) spectroscopy [18]. LC-MS is most frequently used due to its sensitivity and high throughput [16].

Lipidomics is the study of all the non-polar metabolites within a sample to obtain the lipidomic phenotype or lipid profile. Cancer cells heavily rely on *de novo* lipogenesis for energy production and membrane biosynthesis to maintain the rapid proliferation of cells [19–21]. In drug-resistant cancer cells, lipid metabolism, with emphasis on *de novo* lipogenesis, is found to be even more active [19,21]. Lipid storage and uptake of endogenous lipids is also known to increase in resistant cells [20]. *De novo* lipogenesis primarily occurs through the sterol regulator element binding (SREBP1) transcription factor, which activates fatty acid synthase (FASN), acetyl CoA carboxylase (ACC) and stearoyl CoA desaturase (SCD). These all play a role in fatty acid synthesis, including the synthesis of more complex lipids such as phospholipids [19,20]. Phospholipids, triglycerides and sphingolipids are examples of complex lipid species in which fatty acids are bound to a backbone and head group [13,19]. They can then be synthesised into other complex lipids, such as triacylglycerides (TAGs), diacylglycerides (DAGs), phosphoglycerides such as phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) [13].

Untargeted-omics studies provide a means to generate metabolomic or lipidomic profiles that are representative of the small molecule composition of samples obtained from biological systems with minimal *a priori* selection of analytes, but often with a substantial requirement for annotation despite providing an efficient route to the generation of new hypothesis. By contrast, targeted-omics studies (often incorporating isotope labelling to measure metabolite flux) can be used to measure a specifically predetermined selection of small molecule metabolites, and are often a direct route to interrogating pathways of interest [22]. Multivariate statistics, such as principal component analysis (PCA), an unsupervised statistical method, or a supervised orthogonal partial least squares discriminant analysis (OPLS-DA), can then be used to identify distinctions within groups such as between an experimental and control group, or in the theme of this review, when comparing the drug-resistant and sensitive lines [23].

The aim of this systematic review was to give insight into the current landscape on investigating TKI resistance in NSCLC, with a focus on the metabolic adaptations of TKI-resistant cell populations.

2. Materials and Methods

The review was conducted and written in accordance with the 2020 Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines [24]. A literature search of the entirety of PubMed and Ovid (MedLine) and a search of articles only from Web of Science were conducted. The evidence-based PICO (Patient/Population/Problem, Intervention, Comparison and Outcome) model was used to generate relevant search terms to answer the research question “to understand the role of metabolism on the abil-

ity for NSCLC cells to acquire resistance to EGFR treatment". Terms such as "NSCLC", "metabolomics", "lipidomics", "EGFR therapies" and "drug resistance" along with their synonyms were searched (see Supplementary Materials Table S1). Collectively, 1331 studies were retrieved and input to Covidence, a "primary screening and data extraction tool" for those conducting systematic reviews [25], and after 175 duplicate papers were excluded, papers were then excluded based on account of no full text article being available or the studies were not primary literature reports (e.g., books/reviews/letters). Full inclusion and exclusion criteria can be found in the Supplementary Materials (Table S2). Figure 2 depicts the full process of this systematic review based on the PRISMA guidelines for systematic reviews [26]. Finally, 27 studies were selected for deeper analysis. A summary of these papers can be found in the Supplementary Materials (Table S3).

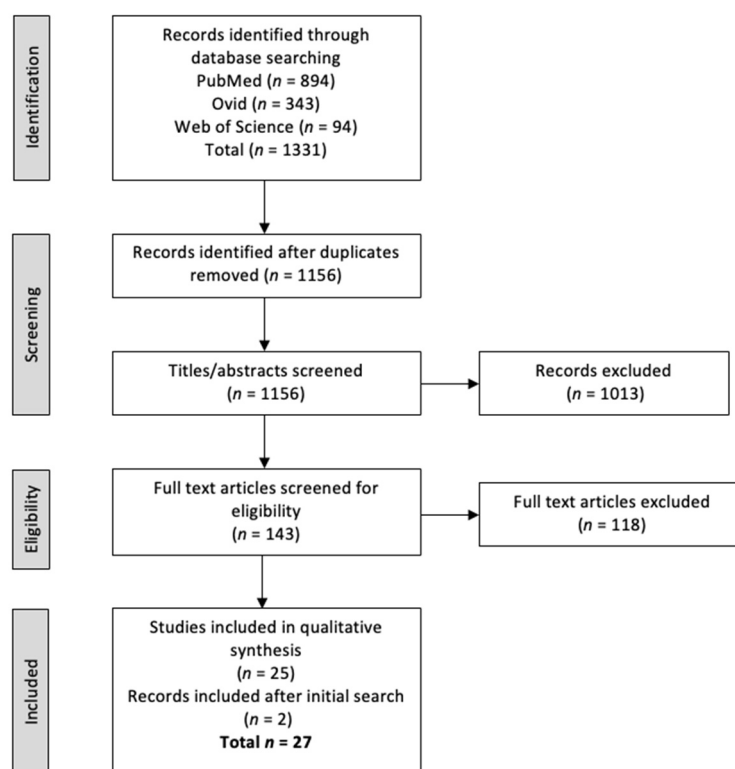


Figure 2. PRISMA flow diagram summarising the review process of all papers. Adapted from Moher et al., 2009 [26].

3. Results and Discussion

From the 27 studies selected for comprehensive analysis by review of the full text and all supplementary materials, three main themes were identified: (i) metabolome rewiring, (ii) reversal of drug resistance by metabolic intervention and (iii) alterations in dysregulated lipid metabolism. These are discussed in turn below. Figure 3 is a visual aid representing the number of papers found within each section, with some papers being discussed in more than one section.

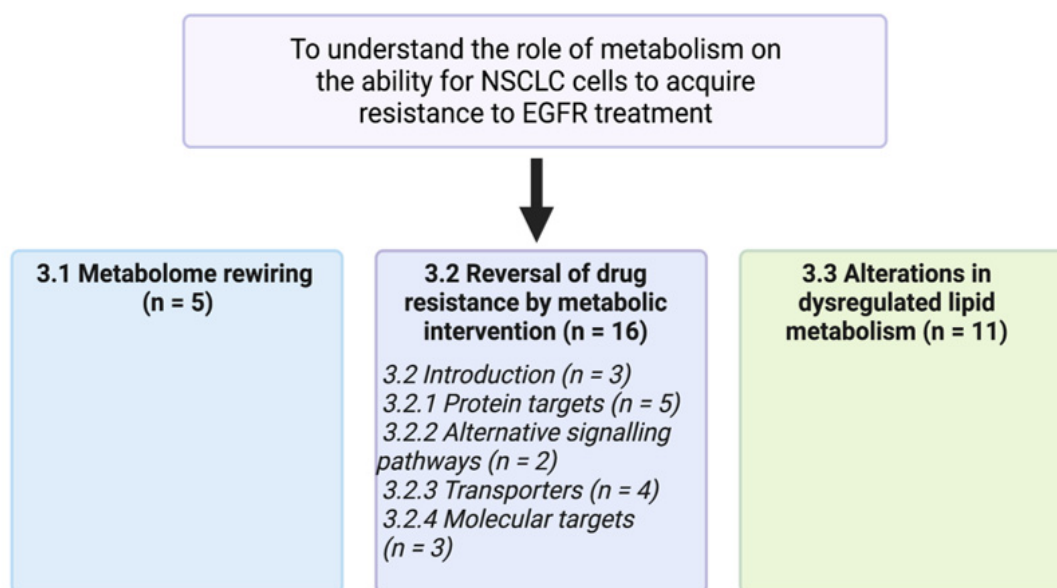


Figure 3. Schematic representation of the main themes of this review. With a total of 27 studies selected for deeper analysis, some papers fall into more than one section.

3.1. Metabolome Rewiring

The gold standard for measuring the metabolic phenotype of cells is metabolomics; in this context to compare and contrast the metabolite profiles of EGFR-mutant treatment sensitive and treatment resistant NSCLC cells. Our review of the literature highlighted key multiple metabolic pathways altered during the onset of drug resistance.

Using a ^1H NMR-based metabolomic approach, Li et al., 2016, identified 36 assigned metabolites that differentiated erlotinib-resistant and sensitive cell lines [3]. Many of these were involved in glutathione (GSH) metabolism, amino acid and nucleotide synthesis, and choline metabolism—a precursor for phospholipid synthesis [3]. Interestingly, there was a notable decrease in the intracellular levels of GSH in resistant cell lines. Ma et al., 2020 took a multi-omics approach, utilising data from metabolomics obtained via LC-MS and proteomics to compare osimertinib-resistant and sensitive cells using multivariate statistics [4]. Overall, 54 metabolites were found to have different abundances between resistant and sensitive cells, and these were largely related to amino acid and nucleotide metabolism, implicating these pathways in the development of osimertinib resistance [4].

Using capillary electrophoresis-time of flight MS (CE-TOF-MS), Serizawa et al., 2014, measured the metabolic profile of erlotinib-resistant and sensitive cell lines and determined 18 metabolites that significantly contributed to distinguishing sensitive from resistant cell lines [27]. These included glucose 6-phosphate and other glycolytic intermediates, which were all lower in resistant cells compared to sensitive cells. Furthermore, metabolites relating to glutamine metabolism were higher in resistant cells [27]. This suggests that there was a shift away from glycolysis as the main energy pathway in resistant cells.

Thiagarajan et al., 2016, used transcriptomics and metabolomics, which determined that transforming growth factor beta (TGF- β) contributed to adaptive drug-escape, a phenomenon where cells are able to acquire new methods of resistance, along with altered metabolic bioenergetics, regulated by mitochondrial function [28]. Mass spectrometry found a global change in energy metabolism when the cells were in their drug-escape phase, with alterations in both the glycolytic and TCA cycle intermediates [28]. There were also significant changes in lipid metabolism, with precursors of fatty acid synthesis elevated in cells after 9 days of erlotinib treatment.

In addition to drug resistance, drug tolerance can also occur [29]. Drug tolerance can be defined as a group of cells which survive the initial exposure to TKIs, and are often the pool of cells in which the majority of drug-resistant cells arise from [30]. Zhang et al.,

2019, report that EGFRm drug-resistant cells can emerge from drug tolerant cells [29], and therefore imply that these cells should be considered a therapeutic target to prevent resistance. This study used a targeted metabolomics approach via LC-MS/MS, to identify key mediators in the TCA cycle that were altered, for example increased succinate, which suggested loss of function of succinate dehydrogenase (SDH). SDH is a stabilising agent to the hypoxic inducible factor α (HIF1 α), therefore the study concluded that the alteration of the TCA cycle can activate a hypoxic response, enabling drug tolerance to develop [29].

3.2. Reversal of Drug Resistance by Metabolic Intervention

The Warburg effect, as stated, describes a switch in energy metabolism where cancer cells adapt to gaining their energy supply from aerobic glycolysis, as opposed to oxidative phosphorylation [7–9]. Figure 4 shows the pathways of glycolysis and oxidative phosphorylation. Reversing this altered metabolism is, therefore, a way to treat cancer cells.

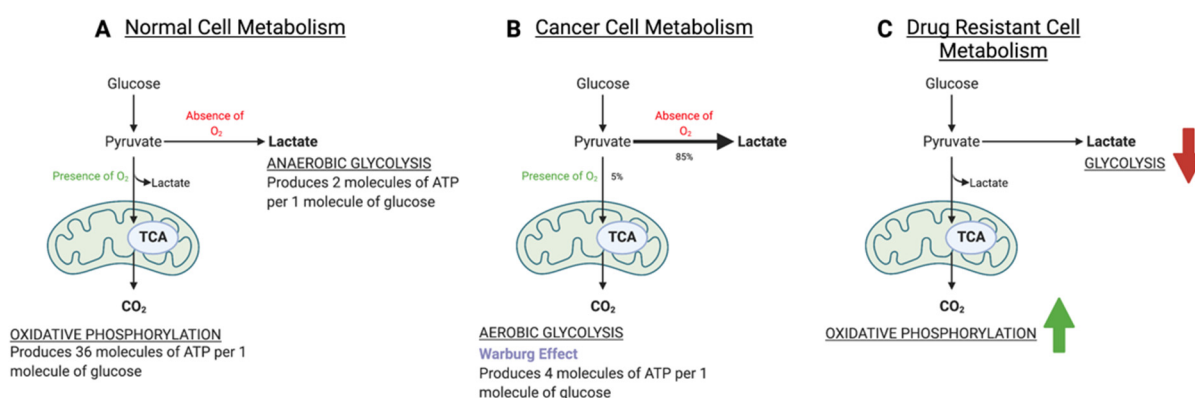


Figure 4. Cellular energy metabolism in (A) normal cells in the presence of oxygen, cells produce around 36 ATP molecules per glucose via glycolysis and the TCA cycle. In the absence of oxygen, cells accumulate lactate and produce only 2 ATP molecules. (B) Cancer cells both in the presence and absence of oxygen only use glycolysis to produce around 4 ATP molecules, whilst producing more lactate than normal cells in the absence of oxygen. (C) Drug-resistant cells favour oxidative phosphorylation to provide energy over aerobic glycolysis.

De Rosa et al., 2015, investigated the effects of EGFR targeting TKIs on glucose metabolism in NSCLC, reversing the Warburg effect [8]. They found that efficient inhibition of EGFR signalling restored oxidative phosphorylation as the primary energy source in NSCLC. This inhibition also regulated aerobic glycolysis via downregulation of hexokinase II (HKII); an enzyme that phosphorylates glucose to glucose-6-phosphate in the initiating step of glycolysis [8] and phospho-pyruvate kinase M2 (p-PKM2); which catalyses the formation of pyruvate from phosphoenolpyruvate (PEP), the final step of glycolysis (See Figure 1 for a schematic of the steps in glycolysis) [31]. Therefore, it is key to understand the mechanisms behind this shift between both energy pathways to prevent or reverse resistance. Kim et al., 2018, found that the mutation within EGFR itself enhanced glycolysis, which was required for EGFR stability [11]. In addition, they determined that depriving resistant cells of glucose inhibited glycolysis, inducing apoptosis.

As a preference for oxidative phosphorylation over glycolysis is a key energy metabolism hallmark of resistant cells, targeting the pathway itself would be one way to revert cells to favouring glycolysis [9]. In a parental EGFRm line, osimertinib suppressed glycolysis and favoured oxidative phosphorylation [9], but does not have this effect in resistant lines. By targeting a component of the electron transport chain (ETC); complex I, with an inhibitor metformin, this study found that when used in conjunction with osimertinib, the development of drug resistance was inhibited in vitro [9].

The metabolomic studies in Section 3.1 highlighted that key metabolic pathways are altered in drug resistance including the TCA cycle and glycolysis, with the Warburg effect

being an important cancer hallmark. Taking this further, a second core theme of the papers selected in this systematic review was that dysregulated cellular metabolism in TKI-drug resistance can be reversed by an intervention to reinstate drug sensitivity.

3.2.1. Protein Targets

To look at the dysregulation of energy metabolism in TKI-resistant cells, with the intention to re-sensitise cells to treatment and reinstate therapeutic effect, targeting proteins implicated in metabolism was an approach used by several authors [1,32–35].

5' adenosine monophosphate-activated protein kinase (AMPK) is a sensory kinase activated by intracellular levels of ATP and AMP [33]. Its downstream targets, such as mTOR and acetyl-CoA carboxylase (ACC) [32], when phosphorylated, redirect metabolism towards increased catabolism [36]. Metabolic stress through direct activation of AMPK by 2-deoxy-D glucose (2DG), which in turn inhibits glycolysis, enhanced cellular sensitivity and therefore the anti-cancer effect of both afatinib and gefitinib in separate studies [32,33].

Pyruvate dehydrogenase (PDHK1) is a protein complex that when activated can inhibit glycolytic activity [1]. To investigate pyruvate metabolism, transcriptomics data from two separate NSCLC cohorts was interrogated and found increased expression of PDHK1. Upon inhibition with dichloroacetate (DCA) in combination with an EGFR inhibitor, metabolism was rewired to favour pyruvate oxidation whilst reducing lactate production, therefore inhibiting glycolysis and increasing the therapeutic effect of the EGFR inhibitor [1].

Targeting glutamine metabolism, a key nutrient for cancer cells, can also be a potential strategy to overcome drug resistance in NSCLC [34]. A compound that targeted glutaminase C (GAC) in combination with erlotinib downregulated both glutamine and glucose metabolism in erlotinib-resistant cells [34].

3.2.2. Alternative Signalling Pathways

A further approach to reinstate sensitivity to TKIs and downregulate resistant cells' requirement for glycolysis is by targeting alternative signalling pathways.

Ye et al., 2017, targeted AKT expression in resistant cells in combination with inhibiting glucose metabolism [37]. AKT and autophagy were found to be more activated in resistant cells than in the parental line. The study found that an increased uptake of glucose via GLUT1, a major glucose transporter, was a feature of resistant cells, and removing glucose would reverse resistance. However, drug sensitivity did not increase until they combined this with suppressing AKT phosphorylation via an inhibitor MK2206 [37].

Cellular metabolism is known to change when cancer cells are under hypoxic conditions, due to the lack of oxygen to the cells. This can result in drug resistance for those drugs which require oxygen to be cytotoxic, or of interest in this review, by the alteration of metabolism [38]. In hypoxic conditions, HIFs are activated and can induce a multidrug efflux transporter, p-glycoprotein, and therefore convey drug resistance [4]. Ma et al., 2020, found that expression of upstream proteins of HIF1 α were significantly increased in osimertinib-resistant cells. Bypass signalling pathways, such as the PI3K/Akt pathway, were also found to be enriched in osimertinib-resistant cells [4,37].

3.2.3. Transporters

Transporters play key roles in determining cellular energy fate. An upregulation of a key transporter involved in glycolysis for example, such as GLUT1, would result in more glucose being transported into the cell [37], and if you inhibit this with an inhibitor such as dehydroascorbate (DHA), which is oxidized vitamin C and taken up by GLUT1, glycolysis is inhibited [39]. Another study found that GLUT1 expression, and therefore glucose uptake, were more prominent in gefitinib-resistant cells, and once genetic and pharmacological inhibition of GLUT1 was established, resistant cells were re-sensitised to therapy [40]. One study found that monocarboxylate transporter 1 (MCT-1), a transporter that secretes lactate into extracellular space, was upregulated in TKI-resistant cells compared to sensitive cells [41].

3.2.4. Molecular Targets

Bach et al., 2018, investigated bone morphogenetic proteins (BMPs), which belong to the transforming growth factor β (TGF- β) family and control many processes including cell differentiation and tumour growth [42]. Activation of BMPs has been known to confer resistance to EGFR-TKIs and to test this Bach et al., 2018, used gefitinib-resistant cells to determine the gene expression of these cells compared to parental sensitive cells [42]. BMP4 plays a role in energy metabolism through regulation of acyl-CoA synthetase long chain family member number 4 (ACSL4), which is key in lipid metabolism and fatty acid oxidation, as it catalyses the conversion of polyunsaturated fatty acids (PUFAs) to acyl-CoAs [42]. TGF- β was also found to aid adaptive drug-escape through transcriptomics and metabolomics, with cells at this point able to exhibit adaptive metabolic bioenergetics, once again linking back to mitochondrial function [28].

mTORC2 was found to play a role in metabolic reprogramming in erlotinib-resistant cells, as these cells had more metabolic flexibility when compared to sensitive cells, measured via spare respiratory capacity (SRC). SRC is a measure of the extra mitochondrial availability, i.e., the ability for increased ATP production via oxidative phosphorylation, and is an indicator of metabolic reprogramming [43]. Knockdown of an mTORC2 component, Rictor, increased SRC in resistant cells. This, therefore, implies that mTORC2 mediated the metabolic reprogramming of resistant cells, as resistant cells before treatment intervention have lower SRC and therefore lower mitochondrial activity [43].

3.3. Alterations in Dysregulated Lipid Metabolism

Lipid metabolism is tightly linked to glycolysis and oxidative phosphorylation, see Figure 1 [13,44]. Lipid metabolism has been found to be dysregulated in TKI-resistant cells compared to TKI-sensitive cells. In particular, de novo lipogenesis is favoured by resistant cells as opposed to utilising endogenous lipids [19]. FASN is a key enzyme required for de novo lipogenesis and is frequently upregulated in cancer, whilst SREBPs are master transcription factors that regulate lipid and cholesterol metabolism [45–47]. Ali et al., 2018 demonstrated a novel link between EGFR signalling and FASN expression in resistant cells. EGFR signalling knockdown decreased levels of both FASN and its activator SREBP1, demonstrating that blocking lipid synthesis can be of therapeutic benefit to resistant cells [45]. This was then confirmed with pharmacological inhibition of FASN using orlistat, which had a cytotoxic effect on gefitinib-resistant cells [45].

Chen et al., 2021, also determined a link between SREBP1 and EGFRm response to osimertinib, which found that the mature form of SREBP1 (mSREBP1) was degraded in the presence of osimertinib, suppressing lipogenesis. However, once these cells acquired resistance to osimertinib, the ability to suppress lipogenesis was lost, and mSREBP1 levels began to increase once more [20]. This study used untargeted lipidomic analysis on osimertinib-treated cells compared to vehicle to determine how treatment changed the lipidomic profile of cells. This identified 148 lipid metabolites, with 50 of these including TAGs, DAGs, ceramides (CERs), PEs, sphingomyelins (SMs) and PUFA PEs showing a significant decrease in abundance in osimertinib treated cells, highlighting how drug treatment can change the lipidomic phenotype of a cell [20].

Xu et al., 2021, used lipidomics to show that gefitinib treatment altered the ratio of saturated and unsaturated phospholipids in resistant and sensitive cell lines, highlighting that prolonged SREBP1 activation is key for de novo lipogenesis, and resistant cells do in fact rely upon this mechanism [19]. Another investigation in SREBP1 signalling by Li et al., 2016, found that intervening with MARVELD1, a SREBP binding partner, inhibits lipogenesis and improves therapeutic effects of TKIs [47]. As mentioned above, when AMPK is activated, it acts upon ACC, which is vital for the de novo fatty acid biosynthesis pathway. Its activation incurred metabolic stress and inhibited glycolysis, which enhanced TKI therapeutic effect [32,33].

Cholesterol is an essential cell membrane lipid and plays an integral role in maintaining cell function and integrity [48]. Cholesterol exists in pockets of the plasma membrane,

known as lipid rafts [48]. In gefitinib-resistant cells, cholesterol levels within these lipid rafts were higher than in gefitinib-sensitive cells. This knowledge prompted Chen et al., 2018, to determine if there was a link between intracellular cholesterol levels and resistance to EGFR-TKIs [48]. They found that cholesterol inhibited binding of gefitinib to EGFR, resulting in resistance. Overall, depletion of cholesterol levels restored drug sensitivity in these resistant cell lines [48]. An alternative strategy to target high cholesterol levels in resistant cells is to target the key mediator of cholesterol uptake; the low-density lipoprotein receptor (LDLR), which is upregulated via EGFR activation through SREBP1 [49]. Despite this study focusing more on overall therapeutic benefit of TKIs as opposed to resistance, Luo et al., 2021, highlighted another potential way to reduce cholesterol levels.

SCD1 is a further key enzyme in lipogenesis, responsible for synthesizing saturated fatty acids (SFAs) into monounsaturated fatty acids (MUFAs) [50]. These can then be incorporated into neutral lipids that are stored in lipid droplets (LDs), which have a high expression in cancer cells [50]. Therefore, Huang et al., 2019, investigated the link between SCD1 mediated lipogenesis and TKI resistance. Intracellular LDs were found to be of a higher proportion in resistant cells, along with expression of SCD1 and its enzymatic product oleic acid. The resistance was overcome by combined pharmacological inhibition of SCD1 with 20(S)-protopanaxatriol and EGFR-TKI [50].

Jung et al., 2015, set out to investigate the phospholipid composition of extracellular vesicles (EVs) that tumour cells secrete [51]. Using matrix-assisted laser desorption/ionization (MALDI) MS to look at lipid profiles of resistant and sensitive cells, this study found that there were 67 phospholipids, including PCs, LysoPCs, SMs, PGs, PIs and LysoPIs, that had varying abundances in resistant vs. sensitive cells [51]. This result highlights once again that resistant and sensitive cells possess unique lipidomic phenotypes, suggesting a novel way to reinstate therapeutic effect.

4. Conclusions

The investigation into cellular metabolism dysregulation in EGFR-TKI resistant cells compared to those cells that are still sensitive to drug treatment, can elucidate several mechanisms within key pathways that may be targeted to prevent or reverse resistance.

The Warburg effect is a well characterised hallmark of cancer and alludes to the switch seen in cancer cells in which they favour aerobic glycolysis over oxidative phosphorylation, even in oxygenated conditions [52]. However, the reverse is true of drug-resistant cell populations [9]. More recently, perturbed lipid metabolism has been described in cancer cells and drug-resistant cells. In particular, de novo lipogenesis has been found to be upregulated in drug-resistant cells compared to sensitive cells [20].

This systematic review has highlighted the importance of investigating metabolomic and lipidomic remodelling, and the pathways that are altered allowing resistant cells to dysregulate cellular metabolism. In the papers summarised here, the studies use both metabolomics and lipidomics to investigate the phenotype of resistant cells, to understand the changes observed from gene expression to metabolite abundance.

By elucidating the metabolome and lipidome changes found within resistant cells and comparing these to sensitive cells, specific metabolic pathways can be targeted alongside EGFR-TKI treatment to allow continued therapeutic benefit.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/metabo12070644/s1>, Table S1: Search terms used in the literature search across all databases; Table S2: Inclusion and exclusion criteria used for literature selection; Table S3: A brief summary of all 27 papers included in the full analysis of this systematic review.

Author Contributions: J.B. designed and coordinated this systematic review and performed the literature search, with Z.H. and T.A. reviewing the included articles and revising any conflicts. J.B. interpreted the study results and wrote the manuscript. Z.H. and T.A. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data set (27 papers) analysed in this study is available upon request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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