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# Investigating the possible mechanisms and gender effects of Omega-3 fatty acids on neuroblastoma, breast and prostate cancers

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## II Abbreviations

| | |
|---|---|
| AA | Arachidonic acid |
| AACOCF3 | Arachidonoyl trifluoromethyl ketone |
| ADT | Androgen deprivation therapy |
| ALA | Alpha-linolenic acid |
| AR | Androgen receptor |
| ATPR | 4-amino-2-tri- fluoromethyl- phenyl ester |
| Bak | Pro-apoptotic protein |
| Bax | Bcl2 like protein 4 |
| Bcl-2 | B cell lymphoma 2 |
| BEL | Bromoenol lactone |
| COX | Cyclooxygenase |
| cPLA2 | Cytosolic calcium dependant phospholipase A2 |
| CREB | cAMP response element binding protein |
| CNS | Central nervous system |
| Cyt c | Cytochrome c |
| DGLA | Dihomo-γ-linolenic acid |
| DHA | Docosahexaenoic acid |
| DISC | Death inducing signal complex |
| DMEM | Dulbecco modified eagle medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DR | Death receptor |
| EMEM | Eagle's minimum essential medium |
| EPA | Eicosapentaenoic acid |

| | |
|---|---|
| ER | Oestrogen receptor |
| ERK | Extracellular receptor kinase |
| FASN | Fatty acid synthase |
| FBS | Foetal bovine serum |
| FCS | Foetal calf serum |
| GPR40 | G-protein coupled receptor 40 |
| iPLA2 | Calcium-independent phospholipase A2 |
| LA | Linoleic acid |
| LOX | Lipoxygenase |
| MAPK | Mitogen-activated protein kinase |
| MTT | 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide |
| NEAA | Non-essential amino acids |
| NR | Nuclear receptor |
| P13K-AKT | Phosphatidylinositol 3-kinase-AKT |
| PACOCF3 | Palmityl trifluoromethyl ketone |
| PBS | Phosphate buffered saline |
| PDK | Phosphoinositide-dependent ketone |
| PLA2 | Phospholipase A2 |
| PlsEtn | Plasmenylethanolamine |
| PPARγ | Peroxisome proliferator activated receptor gamma |
| PR | Progesterone receptor |
| PSA | Prostate-specific antigen |
| PtdEn | Phosphatidylethanolamine |
| PtdSer | Phosphatidylserine |
| PUFA | Poly-unsaturated fatty acid |

| | |
|---|---|
| ROS | Reactive oxygen species |
| RxR | Retinoid-X-Receptor |
| SDC-1 | Syndecan-1 |
| SERDs | Selective oestrogen receptor down regulators |
| SERMs | Selective oestrogen receptor modulators |
| sPLA2 | Secreted calcium-independent phospholipase A2 |
| TNF | Tumour necrosis family |
| TUNEL | Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling |
| UK | United Kingdom |

# 1 Abstract

Cancer is a worldwide disease characterised by uncontrollable cell division and tumour growth making it a leading cause of death worldwide. The use of dietary products such a poly-unsaturated fatty acids (PUFAs) to reduce cancer growth is a current area of interest in research. Docosahexaenoic acid (DHA), Alpha-linolenic acid (ALA) and Arachidonic acid (AA) are examples of omega-3 and omega-6 fatty acids found to play a role in carcinogenesis, for example DHA has previously been shown to induce apoptosis in cancer cells without influencing healthy cells. In neuroblastoma cancer development has been delayed by DHA treatment as a result of *in vitro* mechanisms involving intracellular peroxidation. A further protective effect of omega-3 fatty acids was found in breast cancer patients following high fish consumption and in prostate cancer development the role of omega-3 fatty acids have been highlighted in animal studies, though the later remains a conflicted area of research. Both prostate and breast cancers are gender specific and influenced by hormones, therefore the success of treatments in both these cancers may elicit gender differences. For this reason, neuroblastoma, breast and prostate cancer cell lines have been investigated in the current research to see if PUFAs or gender effects play a role in cancer development. The supplementation of DHA, ALA and AA were investigated across the three cancer cell lines (SH-SY5Y neuroblastoma, MCF-7 breast, PC-3 prostate) using a dose range of 0-1000μM to produce dose-response curves displaying the effects on cell viability. A concentration of 800μM was found to produce an optimal reduction in cell viability across the three cell lines. DHA was found to decrease cell viability of all three cells lines following 24hr and 48hr treatment periods. In this study antagonists of calcium-independent phospholipase A2 (iPLA2), G-protein coupled receptor 40 (GPR40), retinoid-X-receptor (RxR) and nuclear receptors (NR) (bexarotene, tamoxifen, mifepristone, nilutamide) were investigated as pharmacological targets to see if these specific receptors were involved in PUFA effects on cancer cell viability. The

antagonists investigated had differing effects on the different cell lines, with certain antagonists eliciting significant cell viability differences in select cell lines. iPLA2 antagonists acting on the SH-SY5Y and MCF-7 lines whereas RxR induced changes in cell viability across all the cell lines. In conclusion, this research has shown IPLA2, GRP40, RxR and nuclear receptors all have an involvement across the three cancer cell lines (SH-SY5Y, MCF-7, PC-3) by significantly affecting cell viability. The prostate cancer cell line did not respond as well to PUFA treatment when compared to the other two cell lines suggesting there may be gender differences, which is in line with previous research displaying no consistent evidence. This research supports the role of PUFAs in prevention and causation of neuroblastoma, breast and prostate cancer.

# 2 Introduction

## 2.1 What is cancer?

Cancer is a major worldwide disease with 359, 960 new cases in the United Kingdom (UK) in 2015; with a 50% survival chance in England and wales, of which 38% of cases were preventable (Researchuk., 2018). The disease is characterised by uncontrollable cell division which leads to tumour growth made up of an abnormal mass of tissue (D'Eliseo and Velotti, 2016). It is the second most common cause of death following cardiovascular disease with more males (155,019) being diagnosed with cancer than females (148,116) (Ons.gov.uk. 2018). Breast cancer accounts for 15.2% of cancer registrations and prostate cancer accounts for 13.4%, this makes these gender specific cancers leading types (Ons.gov.uk. 2018). Though these cancers arise from different organs there are major similarities that may suggest why the two most leading cancers are gender specific as both the breast and the prostate use gonadal steroids and tumours that develop here are hormone-dependant (Risbridger et al., 2010).

Neuroblastoma is a tumour of the sympathetic nervous system, most often occurring in the abdomen (ChildrenwithcancerUK, 2019). Differentiation of neuroblastoma cells is disrupted by a mechanism dependant on amplification of the MYCN proto-oncogene; increased levels of this gene leads to repression of the genes involved in terminal differentiation in the sympathetic nervous system (Szemes, 2018). Other signalling pathways seen to be affected in neuroblastoma are; dysregulated kinase pathways, increase AKT signalling and mutations in the Ras- mitogen-activated protein kinase (MAPK) pathway (Eleveld, 2015). Treatment so far includes surgery and chemotherapy (Arendonk, 2019) therefore mechanisms need to be clarified in order to understand neuroblastoma tumorigenicity (Yao, 2017) and to develop better treatments for this cancer. In this study the neuroblastoma cell line provides a cancer of female origin with no gender influence as it effects both males and females fairly equally. Whilst

breast and prostate cancer are gender specific, therefore comparison across the three would highlight any gender effects.

Human cell division, differentiation and death are highly regulated events. When disrupted these processes no longer work optimally and the delicate balance is disrupted which leads to the avoidance of death by cancer cells causing an accumulated malignant tumour (Fig 1). When apoptosis becomes defective carcinogenesis may be promoted as well as cell resistance to cancer treatment.

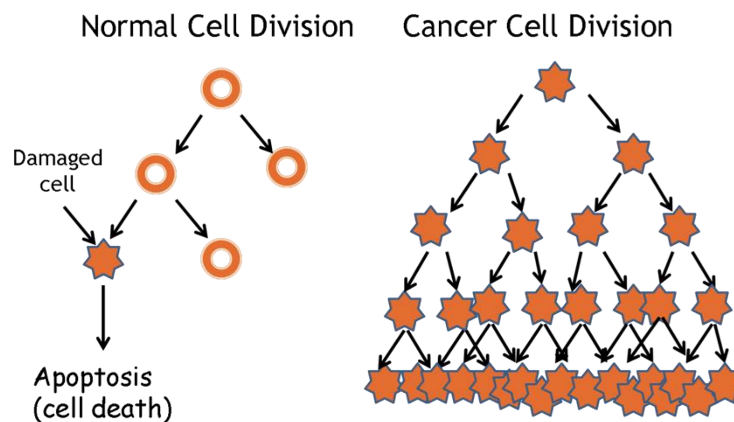


**Figure 1: Demonstration of cancer pathogenesis;** normal cell division results in apoptosis but cancer cell division avoids the cell death mechanism.

In healthy cells the apoptotic (programmed cell death) mechanism is vital for maintaining a balance between survival and death of cells (Fulda., 2010), the signals involved allow genomic integrity to be maintained through the removal of unnecessary, damaged and aged cells (Fulda., 2010). Therefore, when this mechanism becomes defective carcinogenesis may be promoted as well as cell resistance to cancer treatment.

The triggering of apoptosis is a result of caspase activity leading to the cleavage of key cellular components (Perez-Garijo., 2018), consequently causing cellular dysfunction and death. Cell shrinkage, membrane blebbing and nuclear fragmentation occur in cells undergoing apoptosis.

This mechanism permits the removal of unhealthy, dying cells from the tissue via macrophage activity (Gregory., 2004). When this process is interrupted it can result in carcinogenesis with the ability of cells to avoid death signals causing continuous growth (Fig 1) (Plati., 2011). Specifically, by the hijacking of normal cellular growth pathways survival and death signals are avoided by cancer cells (Hanahan and Weinberg., 2011). The avoidance of apoptosis then becomes a hallmark of cancer cells; Bcl-2 is an anti-apoptotic protein regulated by the p53 tumour suppressor gene and is expressed in malignant cells (Fig 2) (Jacobson., 1993).

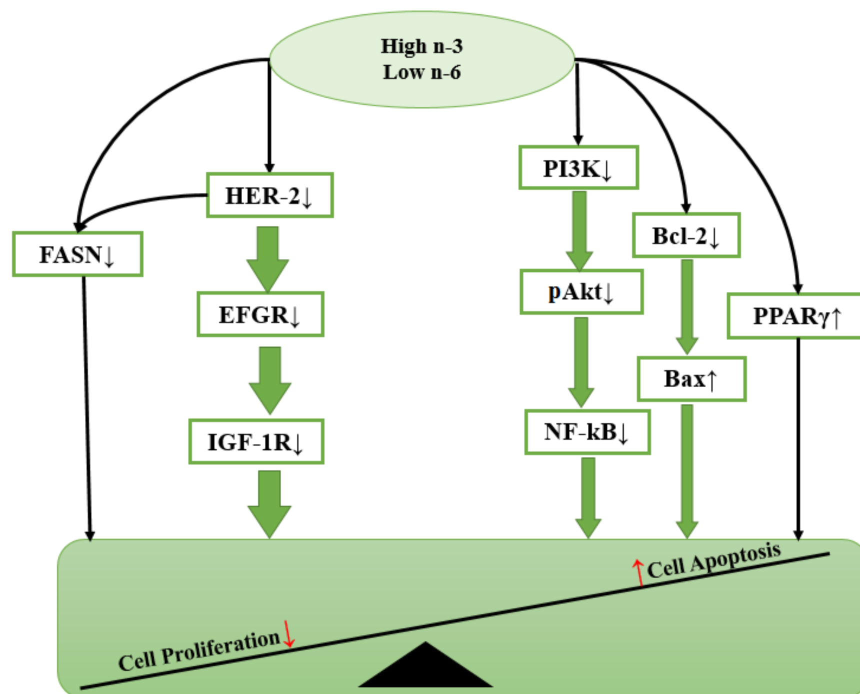


**Figure 2: Hypothetical scheme showing omega-3 PUFAs modulating intracellular signalling molecules**, decreasing cell proliferation and increasing apoptosis following high omega-3 (n-3) and low omega-6 (n-6) levels. Reduced expression of growth factors; human epidermal growth factor receptor 2 (Her2), epidermal growth factor receptor (EGFR) and insulin-like growth factor (IGF-1R). Decreasing levels of fatty acid synthase (FAS) or activating peroxisome proliferator-activated receptor gamma (PPARγ) to inhibit cell proliferation. Blocking phosphatidylinositol 3-kinase and downregulating Protein kinase B (P13K/Akt),

inhibiting Nuclear Factor kappa light-chain-enhancer of activated B cells (NF-κB) and lowering Bcl-2 like protein 4 (Bcl-2/BAX) to promote apoptosis.

There are a variety of mechanisms that cancer cells adopt to avoid apoptosis such as defects in signalling of the intrinsic and extrinsic pathways (Fig 2) (Liu and Ma, 2014). Intrinsically, apoptosis relies on mitochondrial processes which respond to death signals such as deoxyribonucleic acid (DNA) damage (Liu and Ma, 2014). This is modulated by the Bcl-2 protein family. Bcl-2 along with BH-3 proteins regulate Bax and Bak proapoptotic proteins that are key regulators of the internal signals deciding a cells fate (Fig 3). While Bcl-2 proteins and BH-3 proteins regulate Bax/Bak, these suppression and pro-survival proteins are not enough to kill a cell without Bax/Bak (Liu and Ma, 2014).

A key upstream molecular event for apoptotic intrinsic signalling is the release of Cytochrome c (Cyt c) and Bax translocation (Kim., 2006). Although it is not clear how Bax translocates to mitochondria, Bak is constantly residing on the mitochondria resulting in membrane permeabilization and Cyt c release. In the cytosol, Cyt c undergoes various interactions ultimately activating procaspase 3 and 7 (Ott., 2002) which in turn cleave several proteins leading to morphological and biochemical changes resembling apoptosis (Fig 3).

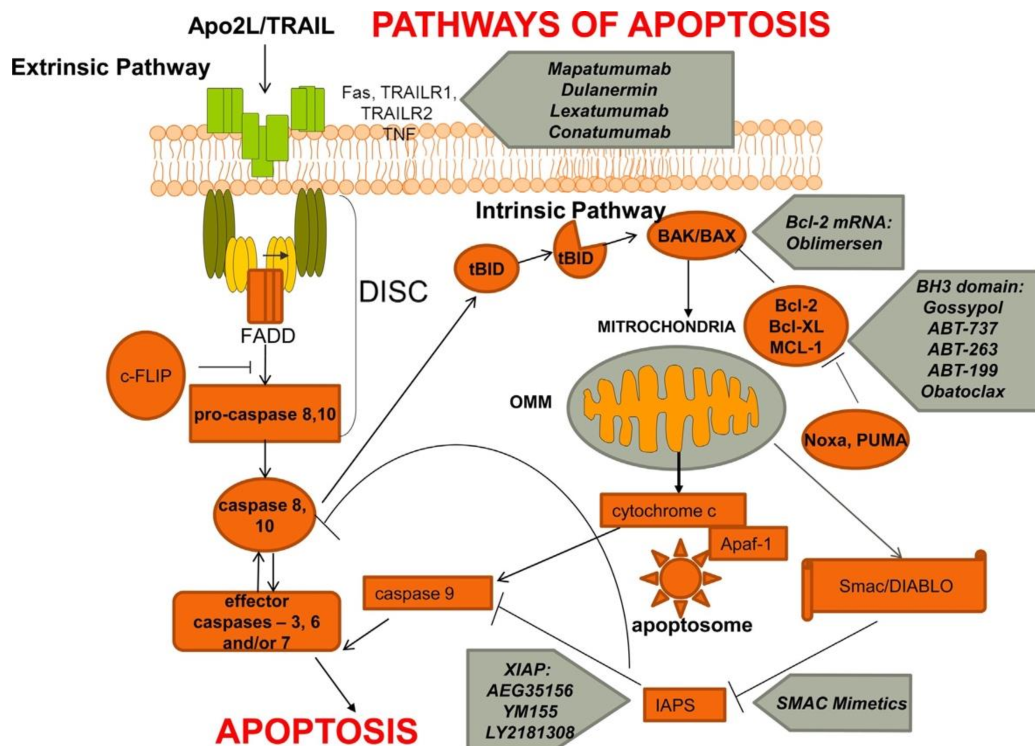


**Figure 3:** Displaying the extrinsic and intrinsic pathway of the apoptosis mechanisms (Khan et al., 2014). Apo2L/TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) binds to tumour necrosis factor (TNF) or TRAIL receptors (TRAILR1, TRAILR2). This recruits Fas associated death domain protein (FADD) and procaspases producing the death inducing signalling complex (DISC). BID and its truncated form BID (tBID) are activated and BAK and BAX form pores in the outer mitochondrial membrane (OMM). Bcl-2 related gene long isoform (Bcl-XL), myeloid cell leukemia 1 (MCL-1), second mitochondrial-derived activator of caspases/ direct inhibitors of apoptosis proteins-binding protein with low pI (Smac-DIABLO) and p53 up regulated modulator of apoptosis (PUMA).

The extrinsic pathway involves the activation of death receptors (DR); e.g. TRAILR1,R2 which are members of the tumour necrosis factor receptor superfamily (TNF). Ligands binding to the DR cause clustering of the receptors and an increase in the apoptotic response as well as recruiting caspases 8 and 10 to form a death-inducing signal complex (DISC) (Sun., 2008) (Fig 3).

These extrinsic and intrinsic pathways involved in apoptosis are highly-regulated events and are critical for proliferation, differentiation and removal of harmful cells (Liu and Ma, 2014). As cancer cells can avoid apoptosis the suppression of apoptosis is thought to play a central role in the development of many cancers. The ability of cancer cells to conduct themselves in such a way is due to either; gene faults, mutations or resistance. These errors thereby allow the cancer cells to override apoptotic signals, which would usually tell them to self-destruct when they become dangerous to the human body (D'Eliseo and Velotti., 2016).

## 2.2 Gender specific cancer and nuclear receptors

Most forms of cancer display fairly equitable distribution across both male and female genders. Breast cancer accounts for approximately 15.2% of all new cancer diagnosis in the UK, with 55,000 new diagnoses of breast cancer each year (statistics, 2018). Whilst 0.6% of all new diagnoses of breast cancer are from male patients, breast cancer is well regarded to constitute a female specific form of cancer. Prostate cancer accounts for approximately 13.4% of all new cancer diagnosis in the UK, with 47,000 new diagnoses of prostate cancer each year (statistics, 2018), and it is well regarded to constitute a male specific form of cancer. Neuroblastoma accounts for 100 new diagnoses of childhood cancer and is a gender-neutral cancer with only a slightly higher incidence rate in males versus females (6:5).

The pathological signalling mechanisms involved in cancer are highly complex and differ across breast, prostate and neuroblastoma cancers. Specifically, breast cancer can be divided into three types; hormone receptor positive, human epidermal growth factor-2 overexpressing and triple negative breast cancer (Nagini, 2017). Current treatment for hormone receptor positive breast cancer includes selective oestrogen receptor modulators (SERMs) or selective oestrogen receptor down regulators (SERDs), aromatase inhibitors and targeting of the oestrogen receptor (ER) with drugs like tamoxifen (Nagini, 2017), to name a few. Despite

these treatments understanding of the mechanisms of nuclear receptor signalling in breast cancer has been limited to two nuclear receptors; ER and progesterone receptor (PR) (Hilton, 2018).

Clinical data has expanded the knowledge on breast cancer development to show oestrogen is critical in hormone receptor positive breast cancer proliferation and progression (Capper, 2016). Specifically, unequal cell proliferation rates are a result of ER signalling with pro-survival signals overtaking pro-death signals (Tyson, 2011). Apoptosis inhibition in breast cancer has previously been shown to be a result of Bcl-2 upregulation by oestrogen, thereby highlighting the nuclear receptors role in preventing cancer cell death (Wang, 1995).

Interestingly, progesterone can reverse tamoxifen's anti-tumorigenic effects through a PR-mediated mechanism (Robinson, 1987). Research has shown increased breast cancer risk in women may be due to progesterone's ability to stimulate proliferation and inhibit apoptosis, as well as to stimulate progenitor cells in the breast (Hilton, 2018). This is supported by the ability of anti-progestins, such as mifepristone, to promote apoptosis (Engman, 2008) and their anti-tumorigenic effect in a patient-derived breast tumour xenograft model (Esber, 2016).

The androgen receptor, glucocorticoid receptor and epidermal growth factor receptors are a few examples now being studied as potential molecular targets for breast cancer treatment (Nagini, 2017; Hilton, 2018). The glucocorticoid receptor has been previously implicated in pathological and physiological processes such as cell survival, differentiation and immune suppression (Zhou, 2005) highlighting its possible relevance in breast cancer signalling. Identifying these mechanisms is a way forward to develop mechanism-based drugs for treatment and to develop a pathophysiological understanding of breast cancer (Nagini, 2017).

Prostate cancer hormone responsiveness has also been recognised following research into the androgen receptor (AR) axis (Pelekanou, 2016). The AR is a member of the steroid receptor family with the primary agonist being testosterone, once the AR is translocated in the nucleus an interaction occurs with androgen-responsive elements leading to cell differentiation, growth and prostate-specific antigen (PSA) secretion (Querol Cano, 2013).

Previous research has shown androgens are crucial to normal functioning, differentiation and development of the prostate (Yedjou, 2019) because androgens stimulate prostate cancer cells to grow (cancer, 2019). Lowering androgen levels can prevent prostate cancer progression and currently, androgen deprivation therapy (ADT) is a hormonal therapy used for prostate cancer treatment (cancer, 2019). Nevertheless, hormone therapy alone cannot cure prostate cancer. In addition, AR antagonists, such as nilutamide, bicalutamide and more recently enzalutamide make androgen action blockage possible (Pelekanou, 2016) and highlight the AR to be a key target for the management of prostate cancer and future research.

## 2.3 Fatty acids and their signalling pathways

PUFAs are fatty acids with more than one double bond in their backbone that are commonly found in fish and plant food sources; they affect a wide variety of physiological processes and have been shown to display preventative characteristics across a range of pathologies (Ander, 2003).

DHA (22:6, w3) and ALA (18:3, Ω3) are omega-3 PUFA's and are considered essential fatty acids due to their inability to be synthesised *de novo*, requiring consumption from dietary sources (Fig 4). DHA is obtained directly via consumption of fish while ALA is obtained directly via consumption of flax and seed food sources. AA (20:4 Ω6) is an omega-6 PUFA;

also considered an essential fatty acid, and obtained directly via consumption of meat, or indirectly through the metabolism of linoleic acid (LA, 18:2 Ω6). Both Omega-3 and Omega-6 PUFA's are ubiquitously enriched across all mammalian cellular membranes, (Scott and Bazan, 1989).

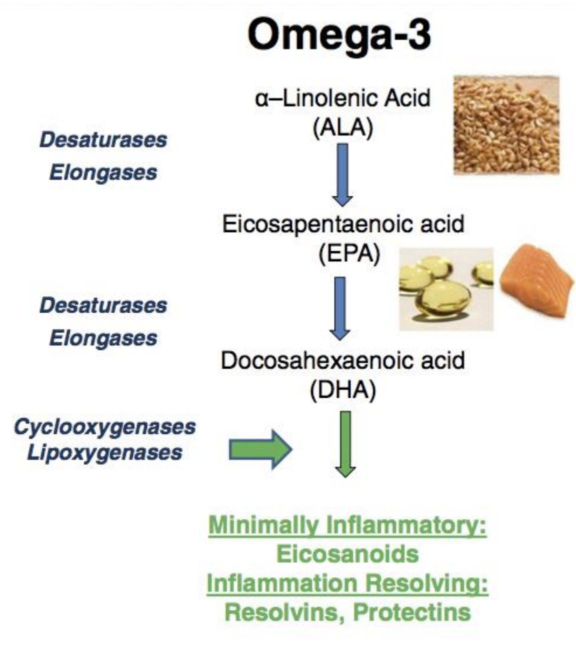


**Figure 4: The general metabolic pathway for omega-3;** conversion from α-Linolenic acid (ALA) to eicosapentaenoic acid (EPA) to Docosahexaenoic acid (DHA).

DHA is predominantly found on the sn-2 position of membrane phospholipids; phosphatidylethanolamine (PtdEtn), plasmenylethanolamine (PlsEtn) and phosphatidylserine (PtdSer). DHA is readily cleaved from the neuronal membrane via diacylation (Farooqui *et al.*, 2000), where approximately 2-8% of the total cerebral DHA content is deacylated and replaced on a daily basis (Rapoport *et al.*, 2001) and as DHA cannot be made by the human body it needs to be obtained from the diet.

Phospholipase A2 (PLA2) is a diverse family of enzymes ubiquitously expressed across most mammalian cells, along with being a major constituent of vertebrate and invertebrate venoms (Scott *et al.*, 1990). The major function of PLA2 is to catalyse the cleavage of fatty acids from the sn-2 position of membrane phospholipids, leading to an increase in intracellular free fatty acid levels. A range of distinct sub-types of PLA2 have been identified, and classified into three main subgroups based on their modality, and calcium regulation: secreted calcium-dependent PLA2 (sPLA2); cytosolic calcium-dependent (cPLA2); and iPLA2. sPLA2 is located within a predominantly extracellular location, has a calcium regulated activity, and is considered to play a predominantly inflammatory role through the AA mediated prostaglandin and leukotriene signalling pathways (Murakami et al., 1998). cPLA2 and iPLA2 are located within a predominantly intracellular location, and are considered responsible for the majority of signalling based PLA2 activity (Exton, 1994). cPLA2 is considered activity-dependent, via calcium dependent mechanisms, where increases in intracellular calcium levels leads to almost selective cleavage of AA from the phospholipid membrane (Murakami et al., 1998). iPLA2 is considered to mediate the majority of basal PLA2 activity, and has been shown to favour DHA selective cleavage, over AA, from the phospholipid membrane (Murakami and Kudo, 2002).

DHA's chemical and signalling pathways are thought to be beneficial towards the treatment of cancer through inducing cell cycle arrest and apoptosis in cancer cells (Siddiqui, 2004). DHA and AA can be used as substrates by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes; COX-1 and COX-2 are responsible for AA-derived prostaglandin E2 while 5-LOX and 12-LOX generate leukotrienes which have chemotactic effects on inflammatory cells (Fabian, 2015). This therefore can be linked to the reduction of breast cancer through the reduction of proinflammatory eicosanoids as well as an increase in inflammatory resolving derivatives.

Additionally, DHA reduces translocation of Nuclear factor-kB via its effects on peroxisome proliferator-activated receptor gamma and GRP120 receptors (Fabian, 2015).

Due to faults in apoptotic pathways DHA can induce apoptosis in cancer cells. This results in cell death as a result of signalling via extracellular receptor kinase (ERK), P13K and JAK/STAT pathways (Hanahan and Weinberg, 2011). In relation to these pathways omega-3 has been found to induce cytotoxicity in different types of tumour cells and support has been found during *in vivo* studies that omega 3 PUFAs act on apoptotic mechanisms (D'Eliseo and Velotti, 2016); omega-3 can be linked to the reduction of cancer and is promising for future treatment (Siddqui, 2011). Further, animal models have shown the suppression of tumours during DHA treatment (Murray, 2015) and DHA in combination with other therapies and/ or alone can cause cytotoxicity via the induction of apoptosis in cancer cells resulting in cell death and reduction in tumour growth (D'Eliseo and Velotti, 2016).

ALA is an omega-3 fatty acid and a precursor of both EPA and DHA. Studies have investigated whether or not the conversion of ALA is beneficial in terms of increasing DHA levels throughout the body for therapeutic use (Gerster, 1998). ALA has 18 carbon atoms and three methylene-interrupted double bonds (Stark, 2016) (Fig 5), this omega-3 fatty acid is essential but often overlooked as DHA is the vital omega-3 involved in brain development (Stark, 2016). In the past, studies have shown low levels of ALA can cause fatty liver, loss of elastic tissue and in monkeys, severe behaviour pathology (Fiennes, 1973). Also, the addition of ALA to diets causes an increase in brain weight and higher concentrations of DHA in the brains of pregnant rats (Alemida, 2011). Therefore, ALA may play a more important role than previously thought. However, it was found men with a low risk of prostate cancer had low levels of ALA in their prostate therefore it was suggested ALA may cause damage to the prostate. (Christensen, 2006).

In addition, omega-6 PUFAs are precursors to eicosanoids (lipid mediator signalling molecules) which are pro-inflammatory (Patterson, 2012). Specifically, Arachidonic acid (AA) is a polyunsaturated omega-6 fatty acid with 20 carbon atoms and four double bonds (Martin, 2016), this fatty acid is very abundant in human tissue (Fig 5).

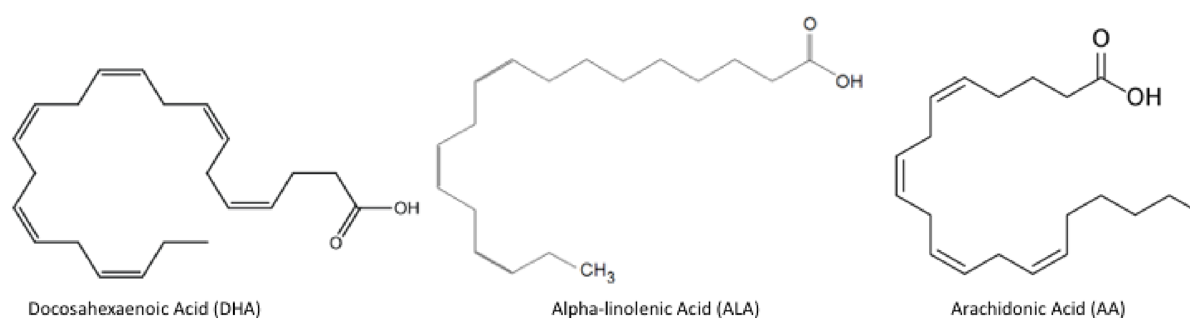


**Figure 5:** Omega-3 and Omega-6 structures; Docosahexaenoic acid (DHA), Alpha-linolenic acid (ALA) and Arachidonic acid (AA)).

The n-6 fatty acid pathway involves AA, synthesised from the desaturation of dihomo-γ-linolenic acid (DGLA), being elongated into its eicosanoids by COX and LOX enzymes (Patterson, 2012). These enzymes can convert AA to prostaglandins that are active, short-lived hormones (eicosanoids) which are involved in many pathological processes (Patterson, 2012).

Over the last few decades the dietary intake ratio of omega-6 to omega-3 fatty acids have drastically changed to ~15:1 compared to an optimal ~4:1, with a higher intake of omega-6 fatty acids associated with increased inflammatory diseases e.g. cardiovascular disease, rheumatoid arthritis and Alzheimer’s disease (Patterson, 2012). This suggests omega-6 fatty acids may play a role in particular diseases.

## 2.4 The interaction between PUFAs and cancer

Cancer cells undergo division and proliferation uncontrollably using certain nutrients along the way as building blocks such as sugars and lipids (Zarate, 2017). For many years the importance of lipids in regard to cancer was dismissed but emerging research displays the need, in particular for fatty acids, for the building of new cell membranes (Zarate, 2017). Research has shown that metabolism especially lipid metabolism is altered in cancer cells to allow them to proliferate uncontrollably (Beloribi-Djefaflia, 2016).

Fatty acid synthase (FASN) is overexpressed in both breast and prostate cancer (Santos, 2012) and FASN is affected by the phosphatidylinositol 3-kinase-AKT (P13K-AKT) and MAPK signalling pathways. Subsequently, these pathways are affected by overexpression of lipid metabolism transcription factors and signal regulated genes in cancer cells (Baenke, 2013). Also, membrane physiology and plasticity are affected in cancer cells as a result of lipid composition changes in either saturated or unsaturated fatty acids (Zarate, 2017); but certain PUFAs have been shown to have differing effects on cancer. Specifically, research has shown omega-6 (AA) levels to be increased in cancer while omega-3 (DHA) levels are reduced (Zarate, 2017). This suggests omega-3 fatty acids to have anti-cancer properties as increased intake of omega-3 has been associated with a reduced risk of breast and prostate cancer (Zarate, 2017).

## 2.5 DHA mechanisms in cancer

DHA has undergone extensive research into its involvement in cancer cells and its mechanisms of action are thought to be; apoptosis, oxidative stress, potentiation of cytostatic drugs and the inhibition of COX-2 (Gleissman, 2010). Previous research has shown that DHA induces dose-dependent apoptosis in cancer cells via the intrinsic and extrinsic pathways (Serini, 2009). The

intrinsic pathway, also known as the mitochondrial pathway, is activated in response to cellular stress signals. Part of this pathway involves upregulation of BID and PUMA pro-apoptotic proteins (Khan, 2014). Once the pathway is activated BAK and BAX form pores on the outer mitochondrial membranes leading to permeabilization. Cytochrome c is released and caspases are activated (Khan, 2014). The expression of proteins from the Bcl-2 family are modified which increases the Bak and Bcl-xS pro-apoptotic proteins (Yamagami, 2009), inducing apoptosis in cancer cells. The extrinsic pathway involves ligands binding and activating death receptors. This leads to trimerization and recruitment of receptors to form clusters, which amplify the apoptotic response. DISC is formed and initiator caspases are activated ready for the process of apoptosis to begin (Khan, 2014).

Also, DHA has been shown to increase the effectiveness of cytostatic drugs including; doxorubicin, irinotecan and vincristine though the mechanisms responsible are not yet known (Biondo, 2008). One way is thought to be DHA's action on membrane-associated signal transduction e.g. decreasing P13K-AKT, Her-2/neu signalling and changing lipid raft composition. Another is said to be the stimulation of oxygen free radicals through peroxidation by drug potentiation (Biondo, 2008). Additionally, as DHA is incorporated into cell membranes AA is lost which reduces the amount of AA derived eicosanoids which are said to drive tumour growth (Gleissman, 2010). As a result, the combined treatment of DHA and a COX-2 inhibitor in neuroblastoma cells has shown an induction of cytotoxicity through the blocking of COX-2 and modulation of NFk-B activity (Narayanan, 2005). Therefore, the mechanisms of DHA are beginning to be elucidated on a physiological and molecular basis (Gleissman, 2010).

The incorporation of DHA into membranes in the central nervous system is affected by its synthesis from its precursors (Langelier, 2005); this uptake in nerve cell membranes is critical at early stages of development for cerebral and retinal functions. The mechanisms of action of omega-3 and omega-6 fatty acids in neuroblastoma tumours has previously been investigated *in vivo* (Barnes, 2011) and display the critical need for omega-3 within the body. In neuroblastoma tumour models it has been shown that omega-3 anti-tumour effects may be a result of induced apoptosis or proliferation (Barnes, 2011) suggesting PUFAs to have anti-carcinogenic effects.

Omega-3 fatty acids and breast cancer have been previously studied and it has been shown that a high intake of omega-3 reduces the risk of breast cancer. The likely mechanisms involved are thought to work via reduction in pro-inflammatory lipid derivatives, inhibition of NF-kB-induced cytokine production and a decrease in growth factor signalling. DHA is also seen to prevent some of the common problems that occur after breast cancer diagnosis (Fabian., 2015); including cognitive dysfunction and chemo-therapy induced neuropathy. Advantageously, DHA treatment may overcome the loss of muscle mass and weight gain seen often following cancer treatment (Fabian., 2015).

Also, it was found that DHA could strongly inhibit the cell growth of MCF-7 human breast cancer cells and it reduced b-catenin expression in 4T1 mouse breast cells, highlighting anticancer properties of fatty acids (Xue, 2014). Along with this DHA also down regulated specific genes including c-myc and cyclinD1. The main findings came from feeding the mice 5% supplementation of fish oil; after 30 days this significantly reduced breast cancer growth in the mice (Xue, 2014). Apoptosis was induced in the cancer cells signifying one of many modes of action that DHA can take to produce anticancer effects.

Additionally, Rahman et al (2013) treated MDA-MB-231 human breast cancer cell lines with DHA and EPA. They found DHA to inhibit the proliferation and invasion of the breast cancer in *in vitro* culture. These finding were followed by a study using doxorubicin, a chemotherapy agent used to treat cancer, on the MCF-7dox human breast cancer cell line which is resistant to the treatment of doxorubicin. Following treatment with DHA, the resistance to doxorubicin was overcome and cell proliferation was reduced (Rahman., 2013). This strongly shows DHA in conjunction with current therapies can enhance therapeutic mechanisms and reduce breast cancer. Further support comes from a study by Sun (2008) who studied human breast cancer cells and found omega-3 to inhibit growth through the induction of apoptosis and increase of the protein syndecan-1 (SDC-1). Strongly suggesting omega-3 fatty acids to mechanistically work within cancer cells to reduce the growth.

Support that DHA can be used in prevention of breast cancer comes from a study by Skibinski et al (2016). Following formulation of a DHA acid stable liposome and its comparison with free DHA in human breast cancer cells, a novel biologically active stable liposomal DHA formulation was created for breast cancer prevention (Skibinski et al., 2016).

Furthermore, Liu and Ma (2014) reviewed ALA, eicosapentaenoic acid (EPA) and DHA consumed individually and as a mixture to investigate the effect on breast cancer cells and highlighted findings from both mouse models and cell culture studies stating that omega-3 fatty acids have promising anticancer effects. Also, Barascu et al (2006) found decreased cell growth of MCF-7 cells following treatment with DHA as well as an increased number of apoptotic cells that were seen to follow a concentration- dependant manner. Particularly, DHA was seen to lengthen the G2/M phase of the cell cycle (Barascu et al., 2006) which is supported by Kachhap et al (2001) who also found DHA obstructed the G2/M phase.

Yee et al (2005) used transgenic mouse models and examined mammary tumour development following being fed fish oil. They found tumour incidence to be greatly reduced as a result of COX-2 downregulation which reduced cell proliferation. This is supported by MacLennan et al (2013) who showed lifelong dietary supplementation using n-3 PUFAs alleviates tumour development therefore providing evidence of n-3 PUFA protective effects. Supplementation with DHA increases DHA serum levels as found through clinical trials, this in combination with research showing DHA can reduce cancer growth suggests dietary intake of DHA may be beneficial for cancer patients (Yee et al, 2010).

Whilst studies show direct effects of DHA on growth inhibition of breast cancer cell lines there are few displaying this effect of ALA (Liu and Ma., 2014). These combined findings show n-3 PUFAs, particularly DHA, are promising anticancer agents in breast cancer that need further research (Liu and Ma., 2014). Bringing together findings from transgenic mouse models and cell culture studies, connections can be seen to link the dysregulation of apoptosis in cancer cells to agents including DHA/ALA. Novel mechanisms do arise including; inhibition of the phosphorylation of AKT and the decreased expression of Bcl- 2 (Schley et al., 2005). Therefore, the common train of thought now is that omega-3 PUFAs can inhibit the proliferation of breast cancer cells by inducing apoptotic cell death and reducing tumour cell proliferation (Liu and Ma., 2014).

In western countries, prostate cancer is the most common type of cancer amongst men (Hu., 2015) though the initial cause is not yet known it is thought dietary intake can have an influence. As shown by several studies (Olivo., 2005; Chen., 2014 and Apte., 2015) PUFAs can promote or inhibit several types of tumour. Prostate cancer cells highlight how DHA can induce apoptotic cell death which occurs via the syndecan-1 dependant mechanism (O'Flaherty., 2013). Syndecan 1 is a protein encoded for by the SDC-1 gene, this protein is involved in

growth factor signalling and cell-to-cell interactions (Sun., 2008). A study by O'Flaherty (2013) considered the metabolism of DHA via LOX and COX lipoxygenases; these are enzymes used in the metabolism of fatty acids. It was found that the effect of DHA on apoptotic mechanisms was blocked by pan-LOX/ 15-LOX inhibitors suggesting the need of oxygenase enzymes for DHA to exert an effect in prostate cancer cells. Furthermore, by silencing 15-LOX-1 during DHA metabolism, DHA's effects on the phosphoinositide-dependent kinase-1 (PDK)/AKT signalling pathway were evaded (O'Flaherty., 2013). This demonstrates for apoptosis to occur in prostate cancer cells, 15-LOX-1 metabolism of DHA is needed to activate the SDC-1 signalling pathway. It was also found in knockout COX-1 and 15-LOX-2 mice, DHA induced apoptosis still occurred. This provides high specificity around 15-LOX-1 being central to DHA metabolism and may be utilised for therapy regarding prostate cancer (O'Flaherty., 2013) though other receptors and pathways are likely to be involved and need further investigation.

Edwards (2008) using breast and prostate cancer cell lines, showed DHA works via peroxisome proliferator-activated receptor gamma (PPARγ) receptors to upregulate SDC-1 which subsequently induce apoptosis. Inhibition of SDC-1 or PPARγ caused a reduction in apoptotic cell death (O'flaherty, 2012). Additionally, Sun (2008) studied prostate epithelial cells and the regulation of syndecan-1 by omega-3. It was found in the prostate gland of animals fed on an omega-3 enriched diet, syndecan-1 mRNA levels were higher than in mice fed on non-enriched diets. In the PC-3 human prostate cell line treated with omega-3, the protein syndecan-1 was increased. In addition, this was mimicked by PPARγ agonists and inhibited by PPARγ antagonists. This shows omega-3 up-regulates syndecan-1 which specifically occurs via the PPARγ pathway (Sun., 2008). Therefore, this suggests the pathway plays a role in the ability of omega-3 to act as a chemo preventive agent and highlights the complexity behind fatty acid anticancer mechanisms.

The underlying mechanisms remain to be clarified; Hu (2015) found DHA does inhibit hormone dependant growth of LNCaP prostate cancer cells via reduced protein expression and repression of specific genes (Hu., 2015). This thereby provides a novel mechanism where DHA exerts an inhibitory effect on growth of prostate cancer cells (Hu., 2015).

## 2.6 Why is this topic important?

The contribution of gender specific nuclear hormone receptors in relation to human breast and prostate cancer is highly significant and could help future research and treatment of patients with these gender specific diseases. Though the diagnosis and treatment of cancer continues to progress, the use of PUFAs may reduce or avoid chemo- and radio-therapy associated detrimental side-effects. An approach addressing this is the use of PUFA dietary supplementation as it has been shown to influence cancer cells via induction of cancer cell apoptosis (D'Eliseo and Velotti, 2016); as mechanisms are not yet completely understood there is a need for specific pathways to be targeted to either rule-out or rule-in the role they play in cancer which could then be utilised for improved therapeutics.

Even though it has encouragingly been shown that PUFAs can sensitise tumours in favour of current therapies such as chemo- and radio-therapy; creating a better outcome for the treatment of certain cancer tumours may be achievable (D'Eliseo and Velotti, 2016). A major gap in current research is PUFA actions on the RxR and gender specific nuclear receptors in hormone related cancers such as breast and prostate cancer as well as non-gender specific cancers e.g. neuroblastoma. Therefore, to research DHA, ALA and AA's ability to induce apoptosis in cancer and the possible link to gender specific nuclear hormone receptors would be one of the first studies designed to target specific receptors and pathways involved in this process.

Additionally, a major finding has been that DHA is toxic to cancer cells with no effect on healthy cells (Merendino, 2013), this provides a huge advantage should DHA be proved suitable as a cancer therapy. This will also provide mechanistic understanding of DHA's involvement in apoptosis and gender specific nuclear hormone receptors which is not yet known. Though mechanisms of action have been proposed, including DHA combining with the cell membrane to disrupt signalling, DHA increasing oxidative stress, DHA modulating eicosanoid metabolites and DHA's ability to bind to nuclear hormone receptors consequently changing gene expression (D'Eliseo and Velotti, 2016). Refining these proposed routes would provide the knowledge and evidence needed to show DHA as a possible cancer therapy alone or in conjunction with chemo- and radio-therapies.

A purpose for the current research therefore is to fill this gap to progress with cancer treatment and perhaps find a safer way to induce apoptosis in cancer cells without harming present healthy cells needed for bodily functions. This would further the findings of Siddqui (2011) who successfully showed PUFAs to be a safer treatment of cancer resulting in less side effects than that of current anticancer therapies being used on the market. Therefore, the current study proposes to add to the body of evidence that PUFAs (DHA, ALA and AA) may be promising treatments for neuroblastoma, breast and prostate cancers. Through the use of antagonists on different cell lines it will be investigated where these fatty acids act to see if support for studies that specifically show DHA to leave healthy cells unaffected by supplementation can be found (Colas, 2004) and whether gender effects arise across the gender specific cancers (breast and prostate).

## 2.7 Current problems and trends in the field

D'Eliseo and Velotti (2016) like many other studies show cytotoxic therapies to have major limitations including failure to induce apoptosis in cancer cells or cause relapse. This is because

the cancer cells develop resistance to the therapy, resulting in resistance to apoptosis. A problem in this area of research is the limited amount of studies linking omega-3 PUFAs supplementation to a decrease in tumour size, therefore any conclusions made are also of limited validity (D'Eliseo and Velotti, 2016). One study demonstrating an association between fatty acids and tumour size was conducted by (Bougnoux, 2009) on breast cancer using an open-label, open arm phase II study. A low n of 25 patients was used and the efficacy of adding DHA as an oral supplement to reactive oxygen species (ROS) generating chemotherapy was investigated. This study found higher plasma DHA concentrations were associated with an increased median time of cancer progression (Bougnoux, 2009). This highlights DHA's ability to reduce or slow cancer growth which is promising for future treatments.

Also, a study by Khankari (2015) also investigated breast cancer and DHA. Following background knowledge that omega-3 induces a cytotoxic environment by reducing the number of inflammatory eicosanoids that are produced by omega 6 metabolism, apoptosis is induced. This provides a platform to continue research into PUFAs as studies are limited regarding whether DHA benefits cancer patients by reducing cancer cell viability after breast cancer diagnosis (Khankari, 2015).

Nevertheless, in conflict is a study conducted by (Lovegrove, 2014) who found that DHA, specifically in relation to prostate cancer, does not reduce prostate cancer aggressiveness or incidence. Therefore, further research is needed to answer questions such as why this is the case and why conflicting research still occurs. A commonly used therapy for prostate cancer is known as androgen ablation which causes tumour regression, however relapse usually occurs following this treatment. This may be because there is no evidence to show an increase in the apoptotic process following androgen ablation, therefore resistance can arise. From this study,

it is suggested promotion of apoptosis by other methods, such as PUFA supplementation, would see regression of the cancer (Bruckheimer, 2000) warranting further research.

Also, there are currently few studies that address PPARγ and RxR receptors as a combined therapy for breast cancer prevention and treatment (Crowe, 2004) as studies lack focus on their expression in human breast cancer (Ditsch, 2012). PUFA ability to reduce cancer risk is also far from conclusive as *in vitro* studies and human studies to date give highly variable results possibly reflecting the methods used to assess omega-3 including; time of exposure and dose/ response endpoint (Fabian, 2015).

A trend in both animal and cell culture studies is that fatty acids have been shown to consistently inhibit progression of mammary carcinogenesis (Stoll, 2002). However, clinically this effect is inconsistent. The activation of PPARγs by fatty acids causes growth inhibition in human breast cancer cells in culture, this effect is then enhanced by ligands of the RxR receptor (Stoll, 2002). The current study may provide the additional evidence needed to then progress with more advanced clinical trials.

Another trend is that RxR receptors are well documented but limited in relation with PPARγ therefore the current study investigating RxR and gender receptor research in conjunction with PUFA treatment in neuroblastoma, breast and prostate cancer cell lines would be a new innovative way of inducing reduced cell viability. There isn't much research concerning several of the gender specific receptors including; oestrogen, progesterone and androgen. Therefore, the current study would fill this gap in an attempt to identify specific mechanisms and receptors involved in DHA, ALA and AA's action to reduce cell viability in neuroblastoma, breast and prostate cancer. There has been a lot of progress with oestrogen as a target for treatment though further research would improve knowledge in this area.

## 2.8 The aims of this present study

The aims of this present study are:

1. Investigate the ability of three omega-3 fatty acids including DHA, ALA and AA to reduce cancer cell viability in neuroblastoma, breast and prostate cancer cell lines.
2. Identify if omega-3 fatty acids work at specific receptors (iPLA2, GPR40, RxR) to elicit said effect on cancer cell viability. in three cancer cell lines in response to DHA, ALA and AA treatment.
3. Characterise if nuclear hormone receptors are utilised by omega-3 fatty acids to affect cancer cell viability in the three cancer cell lines in response to DHA, ALA and AA treatment.

# 3 General Methods

Cell Culture

The human, female origin, neuroblastoma cell line SH-SY5Y was obtained from the ECACC (ECACC, UK). *Homo sapiens*, human SH-SY5Y cells obtained from neuroblastoma bone marrow of epithelial morphology. SH-SY5Y cells had a doubling time of 24-48hrs. Cells were cultured in maintenance media containing 87% Dulbecco modified eagle medium (DMEM):F12 (Lonza, UK), 10% Foetal Bovine Serum (FBS; Lonza, UK), 1% Glutamax™ (Life Technologies, UK), 1% Non-Essential Amino Acids (NEAA; Life Technologies, UK), 1% Penicillin/Streptomycin (Sigma-Aldrich, UK).

The *Homo sapien*, human, female origin, breast cancer cell line MCF-7 obtained from the mammary gland of the breast, derived from the metastatic site of adenocarcinoma. Supplied as a gift from Prof Gwyn Williams at Keele University. MCF-7 cells had a doubling time of 24hrs. Cells were cultured in maintenance media containing 87% Eagle's minimum essential medium

(EMEM) (Lonza, UK), 10% Foetal Bovine Serum (FBS; Lonza, UK), 1% human recombinant insulin (Life Technologies, UK).

The *Homo sapien*, human, male origin, prostate cancer cell line PC-3 obtained from the prostate; derived from bone metastatic site of grave IV adenocarcinoma. Supplied as a gift from Prof Gwyn Williams at Keele University. PC-3 cells had a doubling time of 24hrs. Cells were cultured in maintenance media containing 87% RPMI (Lonza, UK), 10% Foetal Calf Serum (FCS; Lonza, UK), 1% Glutamax (Life Technologies, UK), 1% Non-Essential Amino Acids (NEAA; Life Technologies, UK), 1% Penicillin/Streptomycin (Sigma-Aldrich, UK).

In all cases cells were grown in tissue culture treated T75 flasks (Starstedt, UK) at 37°C and 5% $CO_2$. For all experiments, cells were seeded into tissue culture treated 96-well plates, at a density of $1x10^4$ cells/ml (Starstedt, UK). Preliminary experiments assessed the best time point to treat cells across 24hr and 48hr periods of fatty acid treatment; a 24hr time period was selected. Exponential growth phase was not identified as cells were taken at 70% confluency commonly accepted to represent log growth phase.

## 3.1 Pharmacology

DHA (Tocris, UK), AA (Tocris, UK) and ALA (Sigma-Aldritch, UK) were dissolved in 100% ethanol at a stock solution of 10mM, bubbled with nitrogen, and stored at -20°C. Working concentrations of all PUFA's were made fresh on each day of experimentation.

Bromoenol lactone (BEL: Sigma-Aldrich, UK), GW1100 (Cambridge Bioscience, UK), Arachidonoyl trifluoromethyl ketone ($AACOCF_3$: Tocris, UK), Palmityl trifluoromethyl ketone ($PACOCF_3$: Tocris, UK), DC260126 (Tocris, UK), PA456 (Tocris, UK), HX531

(Tocris, UK), bexarotene (Tocris, UK), mifepristone (Tocris, UK), tamoxifen (Tocris, UK), nilutamide (Tocris, UK) were dissolved in dimethyl sulfoxide (DMSO) at a stock solution of 10mM and stored at -20$^{o}$C.

### Dosage of inhibitors

Preliminary experiments were conducted to assess the best concentration of each inhibitor to use. Cell viability was assessed in each cell line when treated with the different antagonists alone for 24 h. The SH-SY5Y cell line was treated with 0.01μM, 0.1μM and 1μM DC260126, AACOCF3 and BEL. The MCF-7 breast cancer cell line was treated with 0.1μM, 1μM and 10μM DC260126 and BEL. While the PC-3 prostate cell line was treated with 1μM and 10μM DC260126 and AACOCF3 antagonists. Antagonist concentrations determined in preliminary experiments (see appendix I, II, III).

### Control conditions

Controls were used in every experiment to ensure the effects seen were due to the fatty acids alone and not influenced by the vehicle. The vehicle used to make a stock solution for each fatty acid was ethanol, therefore ethanol was used as the control instead of DMSO.

### MTT assay

Cells were plated in triplicate for each condition tissue culture treated 96-well plates (Starstedt, UK). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT: Tocris, UK) was dissolved in sterile phosphate buffered saline (PBS) at a concentration of 5mg/ml. 20µl of MTT stock solution was added to each well, and incubated at 37$^{o}$C for 3 hours. Media was then removed from each well and 100µl of DMSO was added to every well. Plates were transferred to the orbital shaker for 15mins before being read on a plate reader at 565nm.

### Other methods considered

Trypan blue exclusion is a method that could have been used to assess the viability of the cells. The cells are visually examined to see whether or not they have taken up or excluded the dye; dead cells take up the dye due to their ruptured membrane. These cells can then be counted on a haemocytometer to determine how many viable cells are in a given suspension of cells.

Flow cytometry is another method that could be used to measure the physical and chemical characteristics of the cells. The sample of cells is suspended in fluid and injected into the flow cytometer instrument. This can be used determine cell viability and is a rapid and reliable technique.

These techniques were not included in the current work due to a lack of time, however, they would work well to confirm cell viability in the cancer cell lines following antagonist treatment.

## 3.2 Statistics

All data were normalised as viability measured as a percentage of control or vehicle treatments. In all cases, statistical significance was assessed via Two-way ANOVA and post-hoc Tukey tests in GraphPad Prism 8. A two-way ANOVA was used to compare the mean differences between the two treatments of omega-3 fatty acids and the antagonists to see if there is any interaction between these independent variables on the cell viability (dependant variable). This analysis was followed by a post hoc tukey test to show exactly where the differences lie within the samples. Specifically, identifying which means within a set of means differ from the rest.

# 4 Results

## 4.1 *Does DHA induce an effect on cell viability across the three cell lines (SH-SY5Y, MCF-7, PC-3) following 24hr and 48hr treatment?*

SH-SY5Y neuroblastoma cancer cells were subjected to DHA treatment for a period of 24hours (Fig 6A) and 48hours (Fig 6B). MCF-7 breast cancer cells were subjected to DHA treatment for a period of 24hours (Fig 6A) and 48hours (Fig 6B). PC-3 prostate cancer cells were subjected to DHA treatment for a period of 24hours (Fig 6A) and 48hours (Fig 6B). There was no significance across PC-3 cell viability.

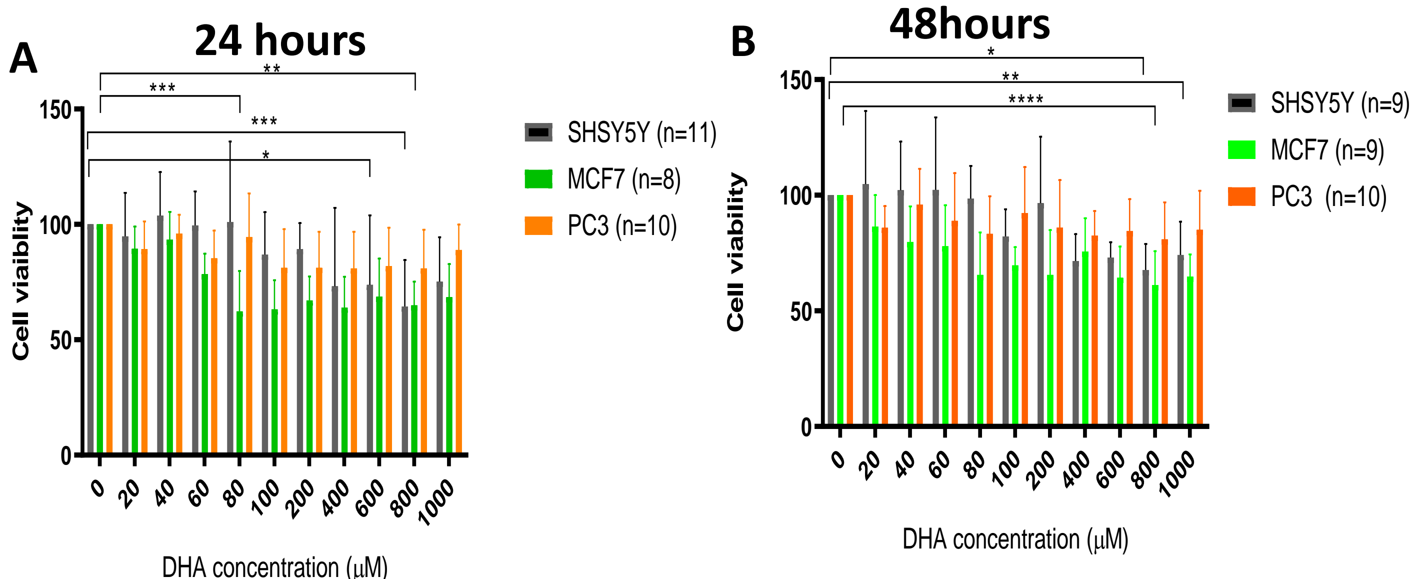


**Figure 6: Cell viability of SH-SY5Y, MCF-7 and PC-3 cells following 24hr and 48hr DHA treatment.** (A) Bar chart generated from cells treated with 24hr 0-1000µM DHA, (B) Bar chart generated from cells treated with 48hr 0-1000µM DHA. Cell viability expressed as a percentage of controls.

A significant reduction was seen across SH-SY5Y and MCF-7 cell lines following 24hr DHA treatment $F_{(2,286)}=17.64$ $P<0.001$ and a significant difference was found across the DHA concentrations for a 24hr time period $F_{(10,286)}=1.829$ $P<0.001$ (Fig 6A). A significant reduction of cell viability was also seen across SH-SY5Y and MCF-7 cells following 48hr DHA treatment $F_{(10,275)}= 9.05$ $P<0.001$ and a significant difference was found across the DHA concentrations following 48hr treatment $F_{(2,275)}=25.63$ $P<0.001$ (Fig 6B). Also, a significant interaction was

found following 24-hour treatment $F_{(20,286)}=1.829$ $P<0.01$ and after 48hour DHA treatment $F_{(20,275)}=2.13$ $P<0.01$.

Specifically, a significant reduction in SH-SY5Y cell viability was seen between 0µm and 800µm 24hr DHA treatment (P<0.001) (Fig 6A) and a significant cell viability reduction was seen between 0µm and 600µm 24hr DHA (P<0.05) in SH-SY5Y cells (Fig 6A). A significant reduction following 48hr DHA treatment between 0µm and 800µm was found (P<0.05) (Fig 6B). Comparison across 0µm and 100µm after 48hr DHA treatment also found a significant reduction in cell viability (P<0.01) (Fig 6B).

A significant reduction in MCF-7 cell viability was seen across 0µm and 800µm after 24hr DHA treatment (P<0.01) (Fig 6A) A significant reduction was also seen at 0µm to 80µm DHA 24hr (P<0.001) in MCF-7cells (Fig 6A). Following 48hr DHA treatment a significant cell viability was also found when comparing 0µm to 800µm DHA (P<0.0001) (Fig 6 B).

### *4.2 Does ALA induce an effect on cell viability across the three cell lines (SH-SY5Y, MCF-7, PC-3) following 24hr and 48hr treatment?*

SH-SY5Y cells were subjected to ALA treatment for a period of 24hours (Fig 7A) and 48hours (Fig 7B). SH-SY5Y cells subjected to ALA treatment for 24hours , a significant reduction was seen at 0µm to 400µm ALA 24hr (P<0.05) in SH-SY5Y cells (Fig 7A). SH-SY5Y after 48hrs treatment with ALA displayed a significant reduction in cell viability between 0µm and 600 µm (P<0.05). MCF-7 cells were subjected to ALA treatment for a period of 24hours (Fig 7A) and 48hours (Fig 7B). MCF-7 cells subjected to ALA treatment for 24hours displayed a significant reduction in cell viability between 0µm and 800µm (P<0.05) (Fig 7A) and 48hr displayed a significant difference between 0µm and 80µm ALA treatment (P<0.05) (Fig 7B).

PC-3 cells were subjected to ALA treatment for a period of 24hours (Fig 7A) and 48hours (Fig7B). PC-3 subjected to 24hr treatment with ALA displayed a significant difference between 0µm and 1000µm ($P<0.05$) (Fig7A). While PC-3 cells subjected to 48hr treatment with ALA displayed a significant difference in cell viability between 20µm and 600µm (Fig7B).

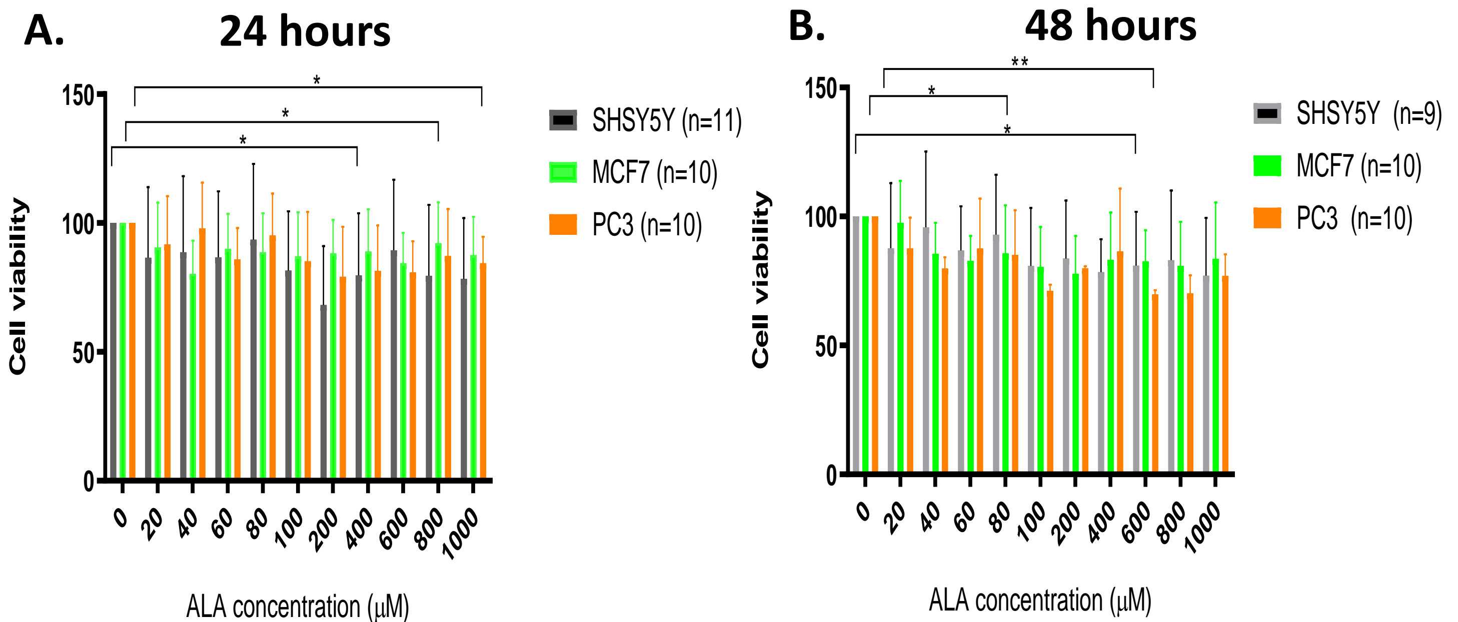


**Figure 7: Cell viability of SH-SY5Y, MCF-7 and PC-3 cells following 24hr and 48hr ALA treatment.** (A) Bar chart generated from cells treated with 24hr 0-1000µM ALA, (B) Bar chart generated from cells treated with 48hr 0-1000µM ALA. Cell viability expressed as a percentage of control.

### *4.3 Does AA induce an effect on cell viability across the three cell lines (SH-SY5Y, MCF-7, PC-3) following 24hr and 48hr treatment?*

SH-SY5Y cells were subjected to AA treatment for a period of 24hours (Fig 8A) and 48hours (Fig 8B). No significance was found. MCF-7 cells were subjected to AA treatment for a period of 24hours (Fig 8A) and 48hours (Fig 8B). PC-3 cells were subjected to AA treatment for a period of 24hours (Fig 8A) and 48hours (Fig 8B).

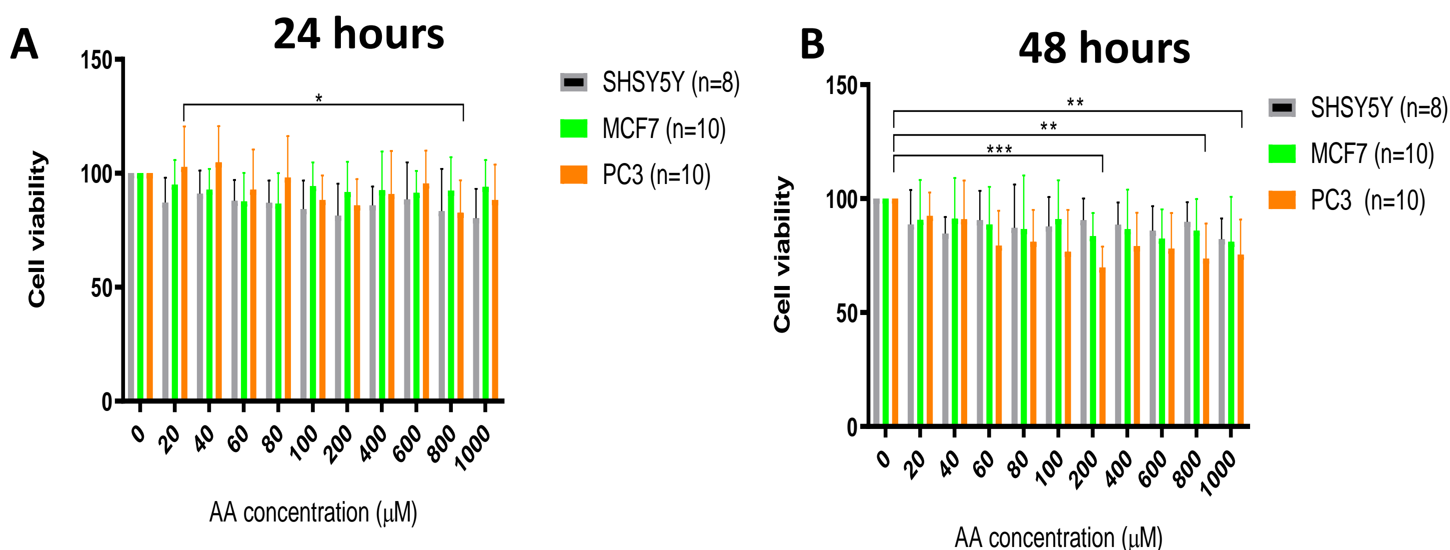


**Figure 8: Cell viability of SH-SY5Y, MCF-7 and PC-3 cells following 24hr and 48hr AA treatment.** (A) Bar chart generated from cells treated with 24hr 0-1000µM AA, (B) Bar chart generated from cells treated with 48hr 0-1000µM AA. Cell viability expressed as a percentage of control.

A significant reduction was seen across the PC-3 cell line following 24hr AA treatment $F_{(2,275)}=7.041$ $P<0.001$ and a significant difference was found across AA concentrations $F_{(10,275)}=3.058$ $P<0.001$ (Fig 8B). A significant reduction was also seen across the cell lines following 48hr AA treatment $F_{(2,286)}=8.506$ $P<0.01$ and a significant reduction was seen across AA concentrations $F_{(10,286)}=4.569$ $P<0.001$ (Fig 8D).

A significant reduction in PC-3 cell viability was seen following 24hr AA treatment from 20µm to 800µm (P<0.05) (Fig 8A). Following 48hr AA treatment of PC-3 cells 0µm compared to 200µm AA also resulted in a significant reduction in cell viability (P<0.001) (Fig 8B) and 0µm to 800µm produced a significant reduction (P<0.01), similarly 0µm compared to 1000µm produced a significant effect (P<0.01) (Fig 8B).

## 4.4 Do IPLA2 inhibitors induce an effect on cell viability following 24hr treatment with one of three fatty acids (DHA, ALA, AA) across three cell lines (SH-SY5Y, MCF-7, PC-3)?

A significant difference was found in the SH-SY5Y cell line across the three fatty acids $F_{(3,144)}=46.32$ $P<0.001$. Treatment with iPLA2 antagonists also produced a significant difference in cell viability $F_{(3,144)}=7.318$ $P<0.001$. A significant interaction effect was not seen across the fatty acids and iPLA2 inhibitors $F_{(9,144)}=2.388$ $P<0.1$ (Fig 9A). A significant difference was found in the MCF-7 cell line across the three fatty acids $F_{(3,144)}=16.42$ $P<0.001$. Treatment with iPLA2 antagonists also produced a significant difference in MCF-7 cell viability $F_{(3,144)}=5.398$ $P<0.01$. A significant interaction effect was not seen across the fatty acids and iPLA2 inhibitors $F_{(9,144)}=2.519$ $P<0.1$(Fig 9B). Also, a significant difference was seen across the three fatty acids in the PC-3 cell line $F_{(3,144)}=19$ $P<0.001$ but no significant difference was seen across the iPLA2 inhibitors $F_{(3,144)}=2.778$ $P<0.1$ (Fig 9B).

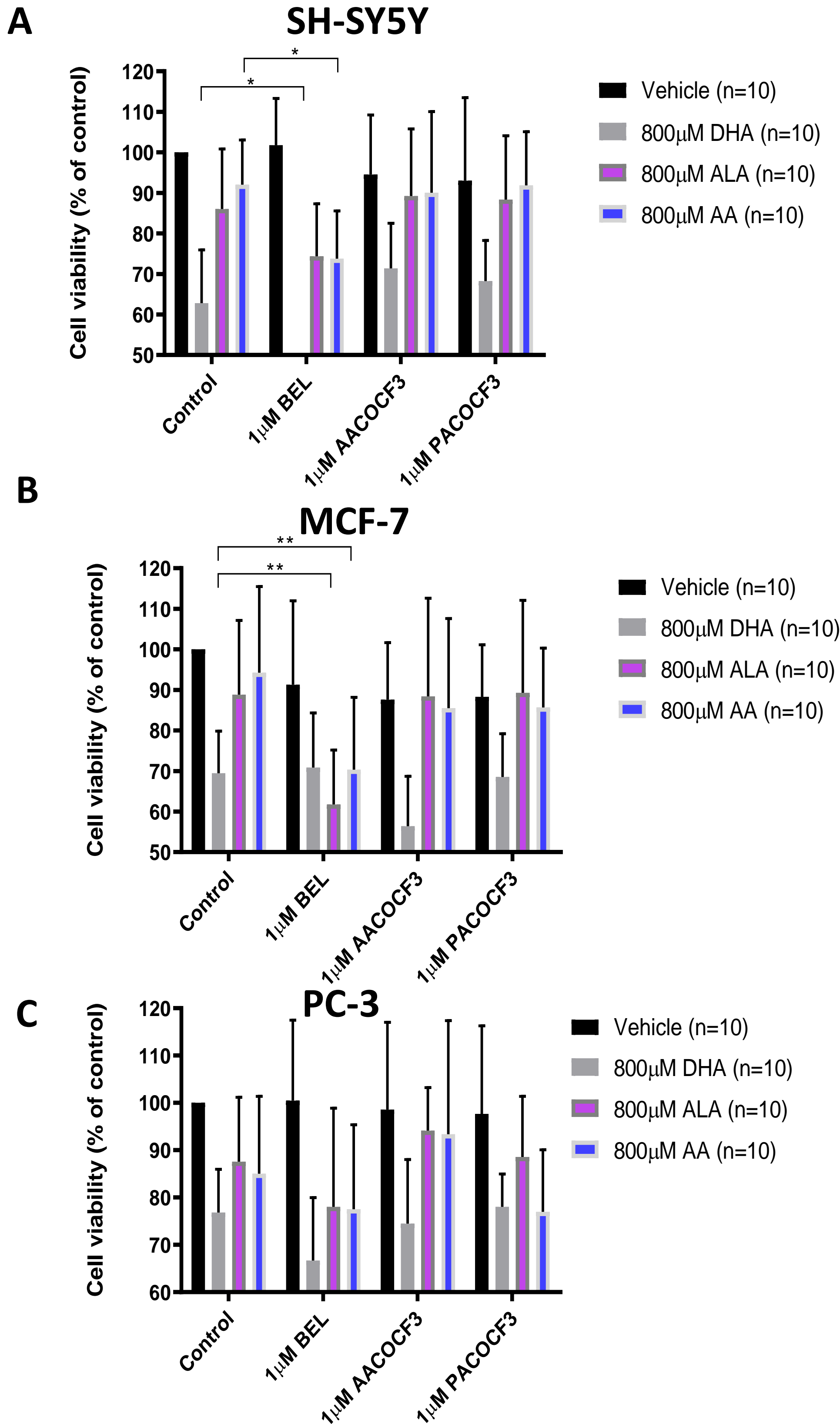


**Figure 9: Cell viability of SH-SY5Y, MCF-7 and PC-3 cells following 24hr fatty acid treatment in combination with iPLA2 antagonists** (A) Bar chart generated from SH-SY5Y cells treated with 800µM DHA, ALA or AA in combination with either BEL, AACOCF3 or PACOCF3, (B) Bar chart generated from MCF-7 cells treated with 800µM DHA, ALA or AA in combination with either BEL, AACOCF3 or PACOCF3, (C) Bar chart generated from PC-3 cells treated with 800µM DHA, ALA or AA in combination with either BEL, AACOCF3 or PACOCF3. Cell viability expressed as percentage of control.

Following 800µm DHA treatment a significant difference was found between the control and 1µm BEL iPLA2 antagonist treatment ($P<0.05$) in SH-SY5Y cells (Fig 9A). Comparison across iPLA2 antagonists displayed a significant difference between BEL and AACOCF3 ($P<0.01$) as well as PACOCF3 ($P<0.01$). Following 800µm AA treatment in SH-SY5Y cells a significant difference was also seen between control and 1µm BEL ($P<0.05$).

Following 800µm DHA treatment in MCF-7 cells a significant difference was found between control and 1µm BEL treatment ($P<0.01$) as well as across BEL treatment and PACOCF3 ($P<0.01$). Similarly, following 24hr 800µm AA treatment of MCF-7 control against BEL treatment displayed a significant difference in cell viability ($P<0.01$) (Fig 9B).

### *4.5 Do GPR40 inhibitors induce an effect on cell viability following 24hr treatment with one of three fatty acids (DHA, ALA, AA) across three cell lines (SH-SY5Y, MCF-7, PC-3)?*

A significant difference was found across the three types of fatty acids in SH-SY5Y cell viability $F_{(3,108)}=30.78$ $P<0.001$. An interaction was also found across the fatty acids and GPR40 antagonists $F_{(6,108)}=8.496$ $P<0.001$ (Fig 10A). The MCF-7 cell lines also a significant fatty acid affect $F_{(3,108)}=8{,}258$ $P<0.001$ and an interaction effect $F_{(6,108)}=3.319$ $P<0.01$. PC-3 cell viability displayed a significant difference across the three fatty acids treatments $F_{(3,108)}=9.848$ $P<0.001$ (Fig 10B).

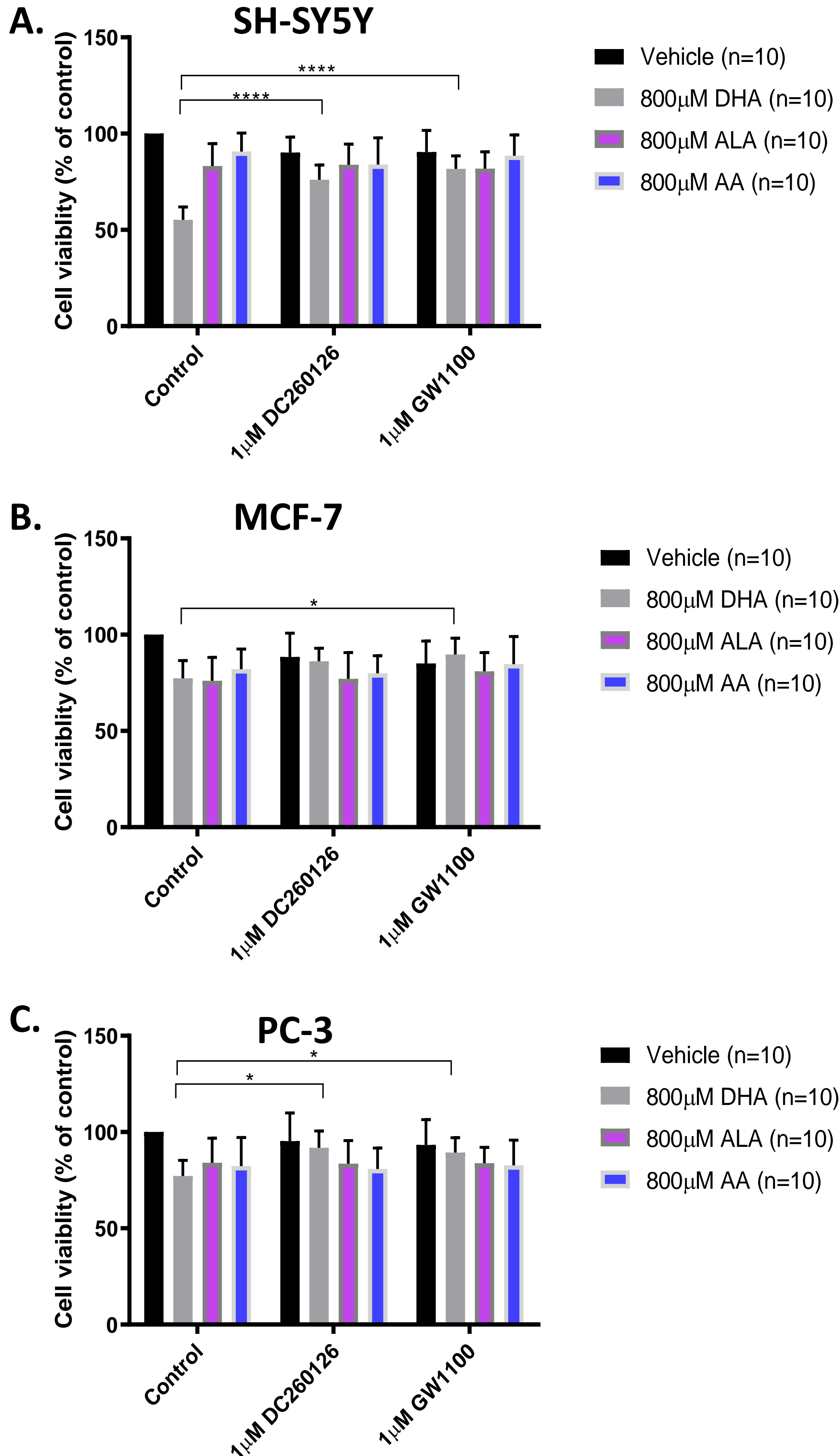


**Figure 10: Cell viability of SH-SY5Y, MCF-7 and PC-3 cells following 24hr fatty acid treatment in combination with GPR40 antagonists** (A) Bar chart generated from SH-SY5Y cells treated with 800µM DHA, ALA or AA in combination with either DC260126 or GW1100, (B) Bar chart generated from MCF-7 cells treated with 800µM DHA, ALA or AA in combination with either DC260126 or GW1100, (C) Bar chart generated from PC-3 cells treated with 800µM DHA, ALA or AA in combination with either DC260126 or GW1100. Cell viability expressed as percentage of control.

Following 800μm DHA treatment in SH-SY5Y cells results display a significant difference between control and 1μm DC260126 GPR40 antagonist treatment ($P<0.001$). The GPR40 antagonist GW1100 also displayed a significant difference in cell viability to controls ($P<0.001$) (Fig 10A).

Treatment of the MCF-7 cell line found significant difference following 800μm DHA treatment, control was significantly different to GW1100 treated cells ($P<0.05$) (Fig 10B). Similarly, in the PC-3 cell line antagonist treatment using DC260126 was significantly different to DHA control treatment cell viability ($P<0.015$ as well as GW1100 treatment ($P<0.5$) (Fig 10C).

### *4.6 Do RxR inhibitors induce an effect on cell viability following 24hr treatment with one of three fatty acids (DHA, ALA, AA) across three cell lines (SH-SY5Y, MCF-7, PC-3)?*

A significant difference was found across the three cell lines $F_{(2,108)}=4.112$ $P<0.01$ as well as a significance difference in cell viability across the RxR inhibitors $F_{(3,108)}=7.709$ $P<0.01$. The interaction of the two was found to be significant $F_{(6,108)}=12.12$ $P<0.01$. The three fatty acids also displayed a significant affect in the MCF-7 cell line on cell viability $F_{(3,108)}=4.478$ $P<0.01$ in addition to a significant interaction affect $F_{(6,108)}=3.842$ $P<0.01$ (Fig 11B). The PC-3 cell line showed a significant difference in cell viability across the three fatty acids $F_{(3,108)}=6.29$ $P<0.001$ and the interaction effect was significant $F_{(6,108)}=4.188$ $P<0.001$ (Fig 11C).

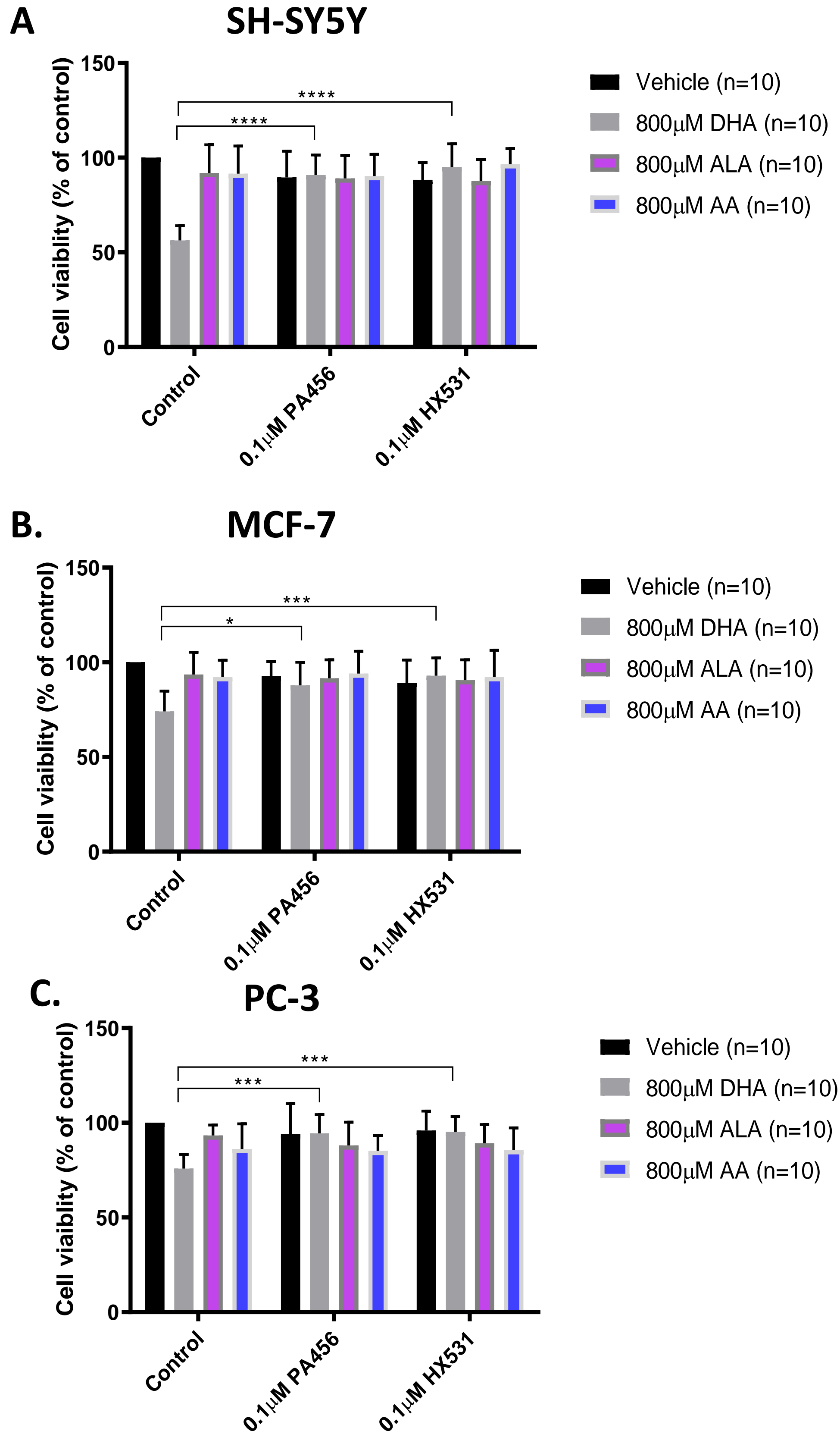


**Figure 11: Cell viability of SH-SY5Y, MCF-7 and PC-3 cells following 24hr fatty acid treatment in combination with RXR antagonists** (A) Bar chart generated from SH-SY5Y cells treated with 800µM DHA, ALA or AA in combination with either PA456 or HX531, (B) Bar chart generated from MCF-7 cells treated with 800µM DHA, ALA or AA in combination with either PA456 or HX531, (C) Bar chart generated from PC-3 cells treated with 800µM DHA, ALA or AA in combination with either PA456 or HX531. Cell viability expressed as percentage of control.

Following 800µm DHA treatment of SH-SY5Y cells the control cell viability was significantly different to the treatment with either 1µm PA456 ($P<0.001$) or 1µm HX531 RxR antagonists ($P<0.001$) (Fig 11A). Similarly, MCF-7 cell treated with 800 DHA control cell viability was significantly different to both RxR antagonists; PA456 ($P<0.05$) and HX531 ($P<0.001$) (Fig 11B). The RxR antagonists also influence the PC-3 cell line viability displaying a significant difference from the control 800µm DHA cell viability to the 1µm PA456 ($P<0.001$) and HX531 ($P<0.001$) cell viability (Fig 11C).

### *4.7 Do nuclear hormone receptor inhibitors induce an effect on cell viability following 24hr treatment with one of three fatty acids (DHA, ALA, AA) across three cell lines (SH-SY5Y, MCF-7, PC-3)?*

In the SH-SY5Y cell line a significant difference in cell viability was found across all three fatty acids $F_{(3,180)}=36.94$ $P<0.001$ and the nuclear receptor antagonists $F_{(4,180)}=83.77$ $P<0.001$. The interaction effect across the SH-SY5Y cell line was also significant $F_{(12,180)}=6.712$ $P<0.001$ (Fig 12A). Also, the MCF-7 line displayed significant cell viability reduction following treatment from all three fatty acids $F_{(3,180)}=56.02$ $P<0.01$ and the nuclear receptor antagonists $F_{(4,180)}=141.2$ $P<0.001$; an interaction effect was also found $F_{(12,180)}=13.62$ $P<0.001$ (Fig 12B). Furthermore, the PC-3 cell line similarly displayed a significant effect of the three fatty acids on cell viability $F_{(3,180)}=9.685$ $P<0.001$ as well as the nuclear receptor antagonists $F_{(4,180)}=97.75$ $P<0.001$. Also, the interaction affect across the fatty acids and the nuclear receptor antagonists was found to be significant in the PC-3 cell line $F_{(12,180)}=3.423$ $P=0.01$ (Fig 12C).

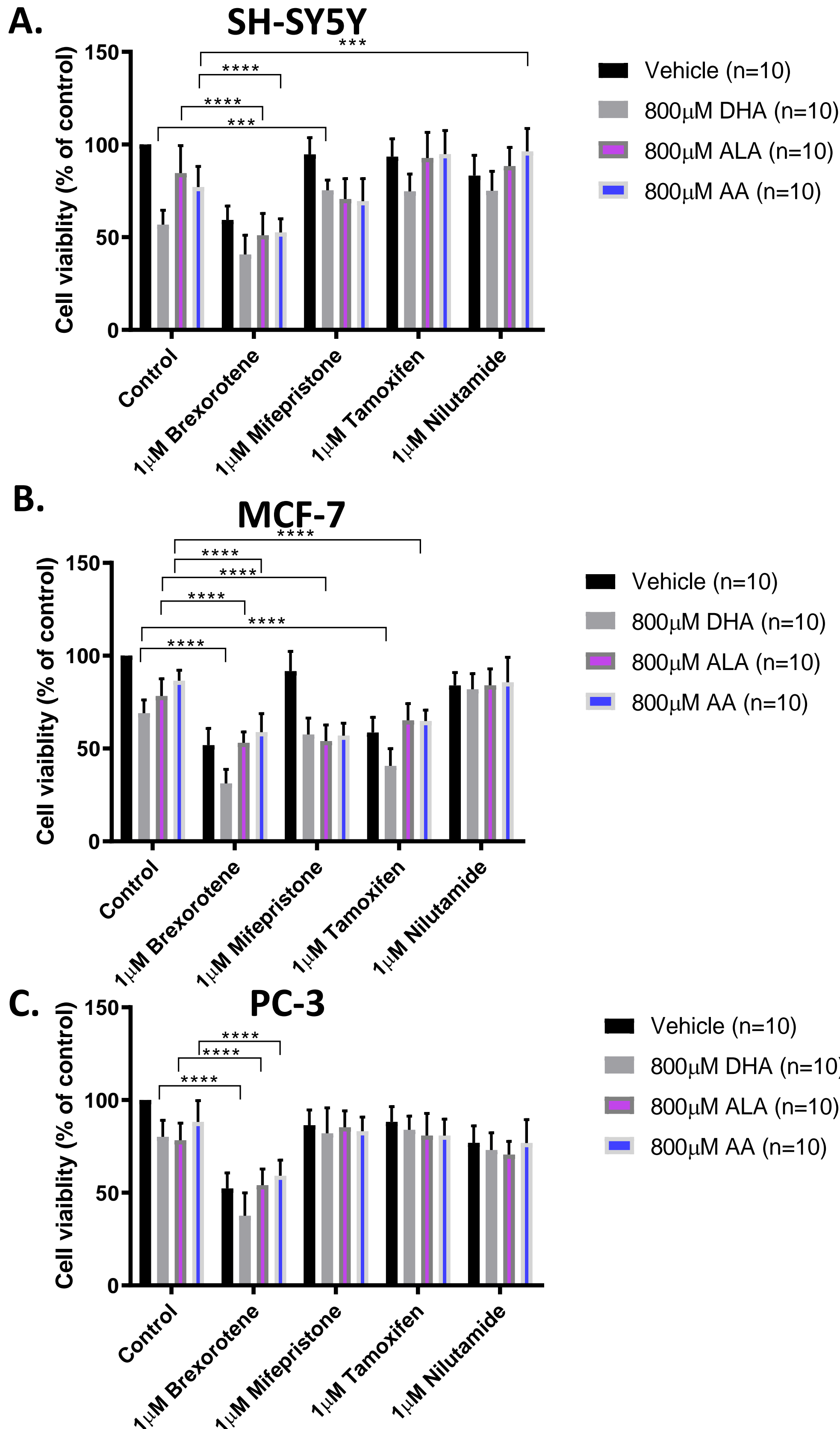


**Figure 12: Cell viability of SH-SY5Y, MCF-7 and PC-3 cells following 24hr fatty acid treatment in combination with hormone receptor antagonists** (A) Bar chart generated from SH-SY5Y cells treated with 800µM DHA, ALA or AA in combination with either Brexarotene, Mifepristone, Tamoxifen or Nilutamide, (B) Bar chart generated from MCF-7 cells treated with 800µM DHA, ALA or AA in combination with either Brexarotene, Mifepristone, Tamoxifen or Nilutamide, (C) Bar chart generated from PC-3 cells treated with 800µM DHA, ALA or AA in combination with either Brexarotene, Mifepristone, Tamoxifen or Nilutamide. Cell viability expressed as percentage of control.

Following treatment of the SH-SY5Y cell line with 800μm DHA a significant effect was found between control cell viability and mifepristone treated cells. Mifepristone increased cell viability compared to controls (P<0.001) (Fig 12A). Brexarotene further reduced cell viability when compared to ALA control (P<0.01) (Fig 12A). Brexarotene and Nilutamide significantly changed cell viability compared to AA control treatment of SH-SY5Y cells (P<0.001) (Fig 12A).

The MCF-7 cell line also displayed significant cell viability reduction following 800μm DHA treatment and there was a significant difference across the controls compared to bexarotene (P<0.001) and tamoxifen (P<0.001) antagonist treatment (Fig 12B). Also following treatment with 800μm ALA there was a significant difference across control and bexarotene (P<0.001) cell viability as well as mifepristone (P<0.001) treatment on cell viability. Significance was also found following 800μm AA treatment when comparing control cell viability to brexarotene (P<0.001) and nilutamide (P<0.001) (Fig 12B).

The PC-3 cell line displayed a significant cell viability reduction following 800μm DHA, ALA and AA treatment. Following DHA treatment there was a significant difference between control cell viability and 1μm bexarotene antagonist treated cells (P<0.001) (Fig 12C). ALA treatment also found significance across one nuclear receptor antagonists; when compared to controls treated with 800μm ALA a significant difference was found in cell viability reduction compared to 1μm bexarotene (P<0.001) (Fig 12C). Also, PC-3 cell viability following 800μm AA fatty acid treatment in combination with nuclear receptor antagonists displayed significance across comparison of control cell viability to bexarotene cell viability (P<0.001) (Fig 12C).

# 5 Discussion

## 5.1 Cell effects

From this research differences arise across the three cancer cell types in response to fatty acid treatment as well as antagonist treatment of certain receptors. Across three cancer cell lines; neuroblastoma, breast and prostate cancer, this research has shown DHA, ALA and AA to have anti-cancer properties through the reduction of cancer cell viability. This is in line with research showing DHA's anticancer activity to be a result of apoptosis in human cancer cells with no effect on healthy cells (Song, 2016).

Cell viability was reduced across all three cell lines following DHA treatment. Specifically, following 24hr DHA treatment the MCF-7 breast cancer cell line produced the greatest response as cell viability was reduced the most. Prostate cancer cells and the neuroblastoma cells also responded well to 24hr DHA treatment with significant reductions seen at higher concentrations of the fatty acid. To compliment this data the same trend was seen following 24hr DHA treatment, that being the MCF-7 cell line [producing the greatest reduction in cell viability compared to SH-SY5Y cells and PC-3 cells. This may suggest the role of a gender effect on the ability of DHA to reduce cancer cell viability, in favour of female origin cells.

Cell viability was also reduced across all three cell lines following ALA fatty acid treatment for both 24hrs and 48hrs. Specifically, the greatest reduction in cell viability was seen in the SH-SY5Y neuroblastoma cells following ALA treatment. Both the SH-SY5Y cells line and PC-3 cell line viability was nicely decreased as ALA fatty acid concentration increased, whereas MCF-7 cell viability was reduced at lower concentrations of ALA fatty acid but as the concentration of ALA was above 200µM the fatty acid had a reduced effect on MCF-7 cell

viability. This may suggest ALA produces a greater reduction in the gender neutral and the male cancer cell line and therefore a possible gender difference in the fatty acids mode of action. Furthermore, omega-6 fatty acid AA produced similar effects across all three cells lines with only minimal cell viability reduction seen in the SH-SY5Y, MCF-7 and PC-3 cell lines. Interestingly, significant cell viability reduction was mostly seen with in the PC-3 cell line following treatment with AA. Following 48hr treatment with AA cell viability was reduced in the PC-3 cell line as concentration of the fatty acid increased. Though cell viability of all three cell lines was slightly reduced compared to controls, the main effect seen was within the male prostate cancer PC-3 cell line, this would suggest omega-6 fatty acid AA has a greater ability to reduce male cancer cell viability when compared to a gender neutral and female cancer cell lines.

## 5.2 Omega-fatty acid effects

Of the three fatty acids DHA, ALA and AA; DHA an omega-3 fatty acid is the most widely studied. Previous research has shown DHA treatment does not impact healthy cells making DHA an appealing candidate for cancer treatment out of the other fatty acids as well as other cancer treatments currently clinically used (D'Eliseo and Velotti, 2016). Specifically, treatment of neuroblastoma cells with DHA has been shown to result in depolarisation of the mitochondrial membrane (Lindskog, 2006); this may be key to DHA's ability to induce cytotoxicity. This highlights an advantage to the use of DHA for cancer therapy either alone or in combination with other treatments as healthy cells will remain unaffected. However, some studies have shown anti- proliferative effects on healthy cells (Yusufi., 2003).

DHA has also been shown to enhance the activity of current anticancer drugs by both survival pathways being supressed and drug uptake being increased (Song, 2016). A possible therapeutic approach could be the use of omega-3 fatty acids as an alternative or combination

treatment but further work is required before clinical studies are conducted. The effects of DHA, ALA and AA were investigated across the three cell lines. This is in line with previous research as neuroblastoma cell survival has been shown to decrease in a dose-dependent manner following treatment with DHA (Lindskog, 2006). In the current study DHA treatment reduced cell viability after both 24hr and 48hr treatment, this omega-3 fatty acid produced the greatest reduction in SH-SY5Y cell viability when concentrations of DHA were above 100µM. DHA reduced cell viability optimally at 800µM. This concentration was then chosen for further experimentation using antagonists of the GRP40, iPLA2, RxRRxR and nuclear receptors.

The previous findings of Lindskog (2006) were time dependant; cell viability was reduced with longer treatment. This is different to the current study which found that 24hour treatment had a greater impact on viability compared to 48 hours. Nevertheless, Lindskog (2006) did not specifically focus on the SH-SY5Y neuroblastoma cell line, their study compromised SH-SY5Y cells in addition to SK-N-DZ and IMR-32 cell lines. Therefore, time periods may differ as a result of the different cell lines used.

The SH-SY5Y cells line is commonly used as the cell line provides a representation of neuroplastic cells which in many studies are compared to healthy cells to identify therapeutics for neuroblastoma cancer (Das, 2016). Also, it has been found following treatment of SH-SY5Y cells and primary astrocytes, as a healthy control cell, that 100µM DHA for 24hrs results in significant cell viability loss in the cancer cells but not in the primary astrocytes (Das, 2016), as determined by MTT assay. This supports the current study as DHA was also found to reduce cell viability in SH-SY5Y cells; 800µM DHA, ALA and AA treatment for a 24 h time period was the optimum concentration for reduced cell viability.

We have shown cell viability of a neuroblastoma cell line is reduced following omega-3 fatty acids DHA, ALA and AA administration. With the greatest reduction of cell viability seen following DHA treatment. Other studies have developed this further by showing this may be due to apoptotic processes for example cell loss was shown to be due to apoptotic processes confirmed by Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling (TUNEL) staining and caspase-3 activation (Das, 2016) in a previous study. From this, six proteins (annexin A2, calumenin, pyruvate kinase M2 isoform, 14-3-3x, ket0-reductase-1B8 and glutathione-S- transferase P1 subunit) were identified in SH-SY5Y cells upon DHA exposure unlike in the astrocytes; which suggests the involvement of these proteins in apoptosis of these cells (Das, 2016).

Furthermore, the effects of fatty acids on MCF-7 cell viability were investigated via MTT assay as previous research has found omega-3 fatty acids may affect cell proliferation and/ or apoptosis mechanisms in breast cancer. The current study found DHA, ALA and AA decreased MCF-7 cell viability after 24 h and 48 h treatment, specifically 800μM DHA and ALA were chosen as optimum concentrations for further experimentation with antagonists as the greatest reduction in cell viability was seen at 800μM. Treatment of MCF-7 cells with ALA and AA displayed the greatest reduction in cell viability after a 48hr treatment period. These findings are consistent with other research; LeMay- Nedjelski (2018) found ALA, at a concentration of 100μM, reduced MCF-7 cell viability after 48h and 96h treatment. It was suggested to be due to a reduction in cell viability rather than the induction of apoptosis.

LeMay- Nedjelski (2018) also found DHA at a high concentration can reduce cell viability and it has been suggested DHA may work through increasing the production of reactive oxygen species in breast cancer cells (Kang, 2010). This may be due to DHA's structural properties

including the double bonds and large molecular size, consequently leading to cell death of breast cancer cells via apoptosis (Kang, 2010). Previous research into the effects of DHA have been shown to involve Bcl-2 and the induction of apoptosis (Mason-Ennis, 2016). It has also been shown that DHA displays the most bioactive capacity of the omega-3 fatty acids meaning DHA can have the most significant impact on viability (Roy, 2017). This was also found in the current study, DHA compared to ALA and AA displayed the greatest impact on cell viability in both the SH-SY5Y cell line and the MCF-7 cell line. Therefore, it is a suitable fatty acid to investigate for cancer therapy. Therefore, fatty acids including DHA are shown to work at the molecular level in cancer cells and can impact cellular growth of breast cancer cells (Chamras, 2002) via mechanisms other than apoptosis. This proposes induction of differentiation associated with lipids may be the main pathway by which DHA works to inhibit breast cancer cell growth (Chamras, 2002).

This is supported by a DHA induced dose-related inhibition of breast cancer cell growth (Rose, 1990) using a range of DHA concentrations from 0.5-2.5µg/ml, whereas EPA was less effective. The MDA-MB-231 breast cancer cell line was also inhibited by DHA which is thought to be via inhibition of one or both eicosanoid classes (Rose, 1990). Also, a study conducted to treat breast cancer using DHA or EPA, found DHA is more potent at inhibiting cell proliferation and invasion than EPA (Rahman, 2013). This is comparable to the current research that found DHA was more potent at inducing reduced cancer cell viability compared to ALA. Specifically, in MCF-7 dox cells DHA significantly decreased proliferation, doxorubicin did not affect cell proliferation but DHA and doxorubicin in combination did dramatically inhibit proliferation (Rahman, 2013). This presents strong evidence to suggest DHA reduces MCF-7 cell proliferation, therefore similar research with DHA treatment may find a reduction cell viability.

Furthermore, it is known cancer cells can resist chemotherapy because of increased P-glycoprotein expression. PUFAs, alone or in combination with doxorubicin, could decrease the expression of P-gp in breast cancer cells (Rahman, 2013). Therefore, it is suggested that PUFAs can be used as an adjuvant to increase the effectiveness of other drugs and when membrane lipids are enhanced with DHA it has been found that the tumour cells are more sensitive to chemotherapy (Rahman, 2013).

The current research shows DHA, ALA and AA can reduce MCF-7 cancer cell viability which could warrant the use of fatty acids as modulators for tumour cell chemo-sensitivity (Menendez, 2005). This ability to increase cell sensitivity to chemotherapy drugs may be a result of increased membrane phospholipid DHA following DHA supplementation in combination with doxorubicin (Maheo, 2005). Therefore, the effect of DHA on chemotherapy drug toxicity is a result of oxidative stress due to doxorubicin. This is a novel, combinational therapy approach for cancer treatment because omega-3 fatty acids such as DHA and ALA are physiological molecules found in food and are non-toxic in vivo (Maheo, 2005). From this and the current findings that DHA and ALA reduce cell viability of MCF-7 cells, further research should look into combinational therapies for breast cancer.

In support, Colas (2004) has shown DHA can improve chemotherapy outcome by sensitising tumours, and Colas (2006) has also found that DHA can make resistant malignant mammary tumours sensitive to the effect of chemo and radio-therapy (Colas, 2006).

Omega-3 fatty acids are thought to play a role in prostate cancer yet the mechanism underlying the effects are not yet known (Liu, 2014). DHA has been shown to suppress growth of prostate cell lines in culture (Wang, 2012). The PC-3 cell line is regularly used to investigate prostate

cancer; PC-3 cells are androgen- independent prostate cancer cells and from studies using this cell line, DHA has been shown to decrease PC-3 cell proliferation, invasion and migration (Oono, 2017). Previously it has been demonstrated that DHA may cause these effects via influence over growth factor signalling (Liu, 2014). All three fatty acids used in the current study reduced prostate cancer cell viability, with the greatest reduction of cell viability produced by DHA treatment. Interestingly, comparison across all three cell lines displayed AA produced the greatest reduction of prostate cancer cell viability.

Prostaglandin E2 is seen to be reduced following treatment with omega-3 fatty acids (Kobayashi, 2006) leading to decreased tumour growth. Expression of the transmembrane heparan sulphate proteoglycan (SDC-1) is decreased in cancer cells and following treatment with DHA, it's expression is increased, and cell growth is inhibited. The ability of DHA treatment to restore SDC-1 in prostate cancer cells may restore a homeostatic mechanism which can slow the progression of prostate cancer (Hu, 2010). The current study's finding that DHA, ALA and AA have reduced PC-3 cell viability are consistent with these previous publications. Other studies have found that DHA may act via prostaglandin and proteoglycans. Additionally, it has also been found in vitro DHA reduced prostate cancer growth when combined with the celecoxib non-steroidal anti-inflammatory drug (Calviello, 2004).

Interestingly, the use of dietary supplements such as omega-3 have been shown to have no effect on prostate cancer; long chain n-3 fatty acids were not significantly seen to reduce or increase prostate cancer risk (Kristal, 2010). Though, the current study did see a reduction in PC-3 cell viability following omega-3 fatty acid treatment; the prostate cell line, out of the three cell lines, was the most affected by omega-6 fatty acids treatment. This may suggest alternate modes of action across omega-3 fatty acids and omega-6 fatty acids. Advanced prostate

tumours tend to develop resistance to therapeutic techniques that work via the induction of apoptosis (Hu, 2010); this may hinder any form of treatment using DHA and needs to be investigated further.

Importantly, using the DU145 prostate cancer cell line it was found that proliferation between control and DHA treated cells did not differ (Liu, 2014). Although, this contradicts the current study as it has been reported that DHA can induce a dose-dependent decrease of prostate cell viability. Therefore, it is still not known if or how omega-3/6 fatty acids work to affect cancer cell viability (Liu, 2014) and mechanisms underlying the effect in PC-3 cells need further elucidating (Oono, 2017). These differences between cell lines and fatty acids illustrate that care has to be taken when extrapolating from cell lines to human tumours.

## 5.3 Receptors involved/ mechanisms

Following treatment of the cell lines with the omega fatty acids, the current study went on to investigate whether receptors and different mechanisms were involved to elicit the different effects seen. The GPR40, iPLA2, RxR and hormone receptors were studied.

DHA is a physiological agonist of the GPR40 receptor, also known as free fatty acid receptor 1, therefore the high levels found in the central nervous system (CNS) are seen to activate this receptor (Contreras, 2000). In 2012, the "PUFA-GPR40-CREB signalling" hypothesis was put forward suggesting subsequent activation of cAMP response element binding protein (CREB) following GPR40 activation (Yamashima, 2012); though this is limited by the lack of receptors found in the rodent brain, it provides a link whereby DHA may be mechanistically working on the cancer cells.

Furthermore, a study by Zamarbide (2014) aimed to investigate if there is an existing link between GPR40 and CREB activation as suggested by the above hypothesis, using the SH-

SY5Y cell line. It was found that there is a direct link between the receptor and CREB phosphorylation in the neuroblastoma cell line (Zamarbide, 2014). This suggests if DHA and ALA works to activate the GPR40 receptor, activation of CREB phosphorylation can be a result of its administration. This may explain the results showing cell viability to significantly change following GPR40 antagonist treatment. This is supported by Das (2016) stating it is known that DHA works via the GPR40 receptor and shows distribution of these receptor in different cells will affect the signal induction. Therefore, the current research builds the body of knowledge in this area displaying DHA's effects on neuroblastoma cells are impacted by GPR40 antagonists.

When SH-SY5Y cells were treated with GPR40 antagonists cell viability was almost restored following fatty acid treatment. This may suggest utilisation of the GPR40 receptor by the fatty acids to reduce cancer cell viability. Therefore, this specific receptor can be said to play a large role in the effect of fatty acids treatment and can be considered as a potential target to explain the differing effects of treatment. The current study assessed the involvement of the GPR40 receptor in DHA, ALA and AA mechanisms and its ability to reduce SH-SY5Y cell viability. Following 24 h treatment using ALA on SH-SY5Y cells, cell viability was reduced; in comparison treatment with 1µM DC260126 and GW1100 both GPR40 receptor antagonist a significant affect was found. This suggests blocking the GPR40 receptor did affect DHA, ALA and AA treatment on SH-SY5Y cells, therefore DHA, ALA and AA may mechanistically work at these receptors.

Additionally, the iPLA2 receptor plays a role in the external signalling pathway and was investigated in the second lot of experiments. iPLA2-dependant pathways are important and thought to be involved in cell proliferation, differentiation and motility (Kispert, 2017). These

processes are dysregulated in cancer and iPLA2 may play an important role in the ability of DHA, AA and ALA to reduce SH- SY5Y cell viability, therefore this was investigated in the current research using iPLA2 antagonists arachidonoyl trifluoromethyl ketone (AACOCF3) and BEL (Ackermann, 1995) which have previously been seen to inhibit the receptor. In the current study following reduced cell viability from DHA and ALA treatment, BEL and AACOCF3 produced a significant difference in cell viability suggesting iPLA2 receptors may also play a role in omega-3 fatty acid affects. BEL reduced cell viability further when compared to DHA alone whereas AACOCF3 increased cell viability compared to DHA treatment alone. It has been shown that the iPLA2 does have an important impact on cancer as inhibition of the receptor can prevent tumour progression (Kispert, 2017); this requires further investigation as it may be the case that omega-3 fatty acids work elsewhere mechanistically, such as at hormone receptors, RxR's or GPR40 receptors, even though the iPLA2 receptor has been shown previously to be involved.

Additionally, the involvement of the GPR40 and iPLA2 receptors were investigated in the MCF-7 cell line. It was found 1µM GW1100 significantly affected the cell viability of MCF-7 cells. As previously stated 800µM DHA reduced cell viability when compared to controls and the addition of 1µM GW1100 antagonised the GPR40 receptor recovering the cell viability and reversing DHA's effect. This shows that the breast cancer cell line MCF-7 contains the GPR40 receptor and the antagonist GW1100 works at the GPR40 receptor, subsequently affecting cancer cell proliferation. Furthermore, in combination with 800µM DHA significant change in cell viability was elicited by 1µM GW1100. To confirm the presence of the GPR40 receptor additional techniques could be used such as immunohistochemistry or western blotting.

The current study also showed the effects of DHA on breast cancer cell viability were reduced by the action of BEL as an antagonist working at the iPLA2 receptor. The results are consistent with a study that investigated whether iPLA2b can mediate nicotine induced breast cancer cell proliferation and if BEL can attenuate this (Calderon, 2015). Via MTT assays it was shown BEL did attenuate breast cancer cell proliferation and migration. After 24 h 800µM DHA, ALA and AA treatment MCF-7 cell viability was reduced but when combined with 1µM BEL cell viability was not recovered. The results go against expectation showing that BEL, an antagonist of the iPLA2 receptor reduced cancer cell viability further, therefore DHA, ALA and AA may not utilise the iPLA2 receptor.

In support, genetically suppressed iPLA2b knockout mice did not show a reduction in breast cancer tumour size (McHowatt., 2011), therefore inhibition of this receptor alone in the tumour's host is not necessarily enough to reduce cell growth (Calderon, 2015). This suggests the need for direct inhibition of the iPLA2b receptor in breast cancer cells to attenuate the tumour development.

Nevertheless, BEL is shown to be an effective treatment for early-stage breast cancer as it is shown to interfere with cellular proliferation, migration and metastasis (Calderon, 2015). Several studies show the antagonist has also been shown to be effective in long-term less invasive breast cancer, though low doses do show toxicity tolerance in the cancer cells (Kispert, 2015) (Calderon, 2015). Therefore, external signalling has been shown to play a role in the ability of DHA to reduce MCF-7 cell viability, but mechanisms are not yet specifically known and require further research.

In addition, previous research has investigated the GPR40 receptors in prostate cancer. It is known to specifically bind omega-3 fatty acids as agonist ligands and a study considered the effects of these PUFAs on prostate cancer cells (Liu, 2014). This proposes a mechanism by which omega-3 fatty acids suppress cancer cell proliferation (Liu, 2014). Liu and Ma (2014) also investigated whether omega-3 has inhibitory effects on prostate cancer growth factor signalling and was found to be the case. They showed that omega-3 fatty acids can inhibit the growth and survival of prostate cancers (Liu, 2014) and that the GPR40 receptor antagonists, DC260126 and GW1100, prevented these effects.

The current study investigated the GPR40 receptor antagonist, DC260126. Following 24 h DHA and 1μM DC260126 and 1μM GW1100 treatment it was found that cell viability was significantly affected; GRP40 antagonist treatment significantly reduced the impact of DHA on PC-3 cell viability. This supports Liu and Ma (2014) suggesting DHA works via external GPR40 receptors to consequently induce prostate cancer cell death. Though not significant, the antagonist of the iPLA2 receptor, AACOCF3, did slightly restore PC-3 cancer cell viability following ALA and AA treatment. Further work is needed to confirm if these receptors are actively used by fatty acids in prostate cell to reduce the cell viability.

It is thought RxR receptors may play a role in the ability of PUFAs to reduce cell viability in SH-SY5Y cells. Previous research has shown that retinoids have a chemo-preventative effect (Kanayasu-Toyoda, 2005) by regulating the transcription of certain genes through activating retinoid X receptors (RxR). On the other hand, RxR agonists have previously been shown to induce apoptosis in neuroblastoma cancer cells, thereby suggesting RxR receptor involvement in the disease (Ferreira, 2013).

The current study investigated whether antagonists working at the RxR could prevent DHA, ALA and AA from reducing SH-SY5Y neuroblastoma cell viability. Antagonists Hx531 and PA452 were used to treat the neuroblastoma cell line in combination with DHA, ALA and AA. Hx531 is an antagonist of the RxR which inhibits both the RxR homodimer and heterodimer, subsequently it is thought to work at the nuclear receptors to modulate gene expression (Kanayasu-Toyoda, 2005). The exact mechanism is not yet known though Hx531 has been shown to inhibit 9-cis retinoic acid-induced neutrophilic differentiation in HL-60 cells (Kanayasu-Toyoda, 2005).

It was found that both RxR antagonists did induce significant effects on the SH-SY5Y cell line. Following reduced cell viability due to 800μM DHA treatment, the addition of HX531 significantly increased cell viability; the bar chart displays an increase in cell viability when compared to DHA treatment alone. This suggests DHA must work at these receptors and the pathways involved with the RxR receptor. The RxR antagonist PA456 also significantly increased cell viability after 800μM DHA treatment. Therefore, DHA may act at the RxR receptor to bring about decreased cell viability in the SH-SY5Y neuroblastoma cell line this is supported by (Tanaka, 2009) who suggested RxR-selective antagonist may be a safe way to treat cancer.

Additionally, RxR's may play an important role in breast cancer tumour biology. Activation of the RxR receptor has been previously shown to induce apoptosis, thereby reducing cell growth in breast cancer cells (Elstner, 2002). Research is limited in this area, nevertheless it has been shown RxR receptor positivity in breast cancer can be used to predict prognosis (Heublein, 2017). Tributyltin chloride and triphenyltin chloride are agonists at the RxR receptor and have previously been studied to identify their anti-cancer affects (Hunakova,

2016). These agonists of the RxR receptor were also found to slow down the migration of the breast cancer cells (Hunakova, 2016) in vitro. This suggests agonising the RxR receptor does play a role in cancer growth.

Wang (2014) used a synthetic retinoid 4-amino-2-tri- fluoromethyl- phenyl ester (ATPR) to investigate the possible involvement of RxRs in breast cancer. They found RxR receptor protein expression was reduced after ATPR treatment and after 48 h treatment cell numbers were clearly reduced. This suggests apoptosis was induced in breast cancer cells by an ATPR highlighting a mechanism involving binding to the RxR and subsequent induction of ER stress and activation the MAPK pathway (Wang, 2014). The majority of studies in the literature use RxR agonists to decrease cancer cell growth (Kim, 2015), Specifically, UAB30 and Targretin (bexarotene) which are RxR selective agonists have been shown to prevent certain types of breast cancers including oestrogen receptor positive and negative breast cancer (Kim, 2015). The current study developed the previous research by using RxR antagonists to inhibit DHA induced cell death. Further supporting the role of RxR's in breast cancer by showing that omega-3 fatty acids (DHA, ALA) may have a role in reducing breast cancer growth via the RxR receptor.

Furthermore, retinoids play a role in cell differentiation, growth and death (Kim, 2015) and an RxR agonist Triphenyltin has been shown to induce apoptosis in P-12 prostate cancer cells (Viviani, 1995). The current study investigated whether antagonists of the RxR affects DHA, ALA and AA mechanisms in prostate cancer cell death to determine if omega-3 fatty acids exert their effects through this receptor.

Treatment with DHA and RXR antagonists (HX531 and PA456) for 24h was investigated. HX531 and PA456 recovered cell viability compared to DHA treatment but did not significantly recover viability compared to ALA and AA treatment. The 24 h treatment did suggest DHAs use of the RXR receptor as part of its mechanism for inducing PC-3 cell viability reduction. However, because of the variability of the findings, further research is needed to confirm whether or not external cell signalling via RXRs is involved in DHAs effects on PC-3 cell viability.

In addition, various hormone receptors were next investigated to see if there was a change in cell viability following DHA, ALA and AA treatment on the neuroblastoma cell line. Oestrogen and Androgen hormones and their receptors are thought to play a role in cancer and it has been suggested, if DHA works at these receptors, that tumour progression could be reduced (Omoto and Iwase., 2015). In the current study this was investigated in SH-SY5Y neuroblastoma cells via 24 h treatment with the fatty acids in addition to treatment with bexarotene, mifepristone, tamoxifen and nilutamide.

Oestrogen regulates growth and differentiation of cells and can be dysregulated in cancer (Tocris., 2018). Tamoxifen is an oestrogen antagonist and blocks the hormone oestrogen from binding, 24 h tamoxifen treatment was compared to 24 h 800μM DHA treatment in terms of cell viability. All nuclear receptor treatment did significantly affect cell viability in the neuroblastoma cell lines. This supports a study suggesting tamoxifen is anti-carcinogenic as cell viability was significantly increase after 24 h and treatment (Tocris., 2018) even though this cell line is gender neutral. This suggests an interaction between DHA and oestrogen receptors.

Additionally, nilutamide is an androgen antagonist and was investigated in combination with DHA, ALA and AA for a 24 h time period. 800µM DHA reduced cell viability in SH- SY5Y cells and the addition of the androgen antagonist nilutamide (1µM) did increase cell viability following this treatment, therefore this suggest DHA may work at the androgen receptor to bring about cell death in SH-SY5Y cells. Use of mifepristone, a glucocorticoid antagonist also significantly restored the cell viability following previous DHA treatment. This is evidence to suggest omega-3 fatty acids may work via hormone receptors to elicit an effect on cancer cell viability. Also, following AA treatment, nilutamide prevent cell viability reductions, again suggesting omega-6 fatty acids may work via hormone receptors or mechanisms to influence cancer cell growth.

Furthermore, breast cancer is a hormone-dependant cancer as growth and development is related to the oestrogen hormone (Omoto and Iwase., 2015). The current study, therefore looked into several nuclear hormone receptor antagonists and their effect on omega-3 and omega-6 fatty acid mechanisms. The oestrogen antagonist tamoxifen was investigated in the current study; tamoxifen did not prevent the reduction in cell viability seen with 800µM DHA, ALA and AA. This suggests DHA may utilise other receptors to induce cell death as cell viability was not recovered following treatment with an oestrogen antagonist. These tamoxifen results do not support the idea that DHA acts at the oestrogen receptor as antagonists acting here did not see an increase in cell viability.

Additionally, mifepristone is a progesterone antagonist and was also investigated in MCF-7 cells in combination with 24 h DHA, ALA and AA treatment; 800µM ALA in combination with 1µM mifepristone did not see recovered cell viability. This suggests other receptors could play a role in the ability of omega-3 PUFAs to affect cancer cell proliferation. Specifically, the

antagonist nilutamide significantly recovered cell viability of the MCF-7 cells compared to controls suggesting DHA, ALA and AA may work via the androgen hormone receptor.
Moreover, hormone receptors were found to produce a slight affect in all three fatty acids on PC-3 cells; mifepristone increased cell viability of cells following ALA treatment and tamoxifen produced a slight increase of cell viability in PC-3 cells following DHA treatment.
Critique of whether aims were achieved

## 5.4 Critique

This research involves considerable work towards the three main aims presented at the start of the study. All three fatty acids (DHA, ALA, AA) were successfully used as treatment on the three cancer cell lines (SH-SY5Y, ,CF-7, PC-3) and MTT assays were conducted to assess cell viability before and after treatment. This had given insight into which fatty acids reduce cancer cell viability. Work has been conducted towards identifying if these fatty acids work at specific receptors or via specific mechanisms via the use of antagonists. Further work could be conducted to look at the most specific antagonists in more detail. Finally, hormone receptors were also investigated in this study to see if the fatty acids utilised them in their ability to reduce cancer cell viability.

One of the aims of this thesis has be to demonstrate that omega-3 fatty acids are able to exert effects on cancer cells that reduce cell viability, therefore positioning them in line for a combinational approach with chemo- and radio-therapy already clinically used. Though I have clearly demonstrated that omega-3 fatty acids do reduce cell viability in neuroblastoma, breast and prostate cancers this observation provokes more questions that future research should address, for example whether apoptosis is involved across all DHA, ALA and AA fatty acids. Also, whether the effects seen on cancer cells could be used in treatments as a non-harmful

therapy to healthy cells, as this provides an advantage over the chemo- and radio-therapies already being used.

If this study was to be conducted differently, said approach could be changed to allow for more techniques to be conducted as a way of confirming the reduction in cell viability and whether this involved apoptotic processes. To better the approach to this study I would suggest to conduct less MTT assays, only those necessary, to then allow confirmation techniques such as trypan blue cell counts or flow cytometry to be conducted. This would retain data on all cell types and fatty acids but would add detail as to why the effects in cell viability are being seen following certain treatments.

## 5.5 Future work

The current research adds to the body of evidence within the cancer field demonstrating that fatty acids may be a potential way to treat different types of cancers. It answers questions such as; which of the fatty acids best reduces cancer cell viability and where there fatty acids may be working to elicit said effect. This research contributes and open doors for the field of cancer biology by starting to look more specifically into why these omega-3 and omega-6 fatty acids effects are being seen across cancers.

Cancer is a worldwide disease (D'Eliseo and Velotti, 2016) with the leading therapies being chemo- and radio-therapy. These therapies induce death of cancer cells as well as healthy cells, therefore it is thought that these therapies could be optimised to reduce healthy cell loss. In an experiment looking at the effects of EPA on PC-3 prostate cells it was found that interference with the voltage gated sodium channels may play a role in reduction of cancer cell growth (Nakajima, 2009). This was not looked at in the current study and may be of interest for further investigation. Clarification is also required as to whether these specific receptor subtypes are

involved in omega-3 PUFAs ability to impact cell viability in PC-3 cells. Differences across cell lines were seen and may be due to limitations in in vivo and in vitro models, therefore models with xenografts would provide greater insights in future research.

The consensus now is that the omega-3/omega-6 ratio is critical to health (Zarate, 2017) as omega-3s are seen to reduce cancer while omega-6s are seen to promote cancer. The anti-inflammatory effects of omega-3 PUFAs are thought to play a huge role in the ability to reduce cancer proliferation (Zarate, 2017). Therefore, the omega-3/ omega-6 ratio should also warrant further research to answer these emerging questions. The current research touches on possible differences between omega-3 and omega-6 fatty acids and warrants further investigation into their effects on cancer cell viability.

Specifically, the leading gender specific cancers, breast and prostate, would benefit from therapies either alone or in combination with those currently used clinically; as statistically shown breast cancer is the leading cancer in women with roughly 1.8 million new cases arising in 2013 (LeMay-Nedjelski et al., 2018).

This work is significant and useful for further research to detail exactly where omega-3 fatty acids are acting to elicit reduced cancer growth. With cancer being a leading world-wide disease research into the area of possible new therapeutics is critical and the work presented here is extending the current understanding of how omega-3 fatty acids could help prevent cancer.
Traditionally it is thought that omega-3 fatty acids act as antioxidants removing unwanted compounds from the cellular environment to reduce the risks of cancer development. The more contemporary outlook is that omega-3 fatty acids reduce cancer cell viability and therefore have anticancer properties. The research here begins to show that omega-3 fatty acids may

specifically work via several different receptors and pathways to reduce cancer cell viability including; external pathway signalling via GPR40 and iPLA2 receptors, RxRs and nuclear receptors. The results begin to elaborate on whether a gender effect is seen across the three specific cancers and whether nuclear receptor involvement is key for omega-3 fatty acids to implement such an effect.

## 5.6 Conclusion

To conclude across all three cells lines it was found that DHA, ALA and AA were able to reduce cell viability to differing extents. Specifically, of the fatty acids, DHA reduced cell viability the most across all three cancer cell lines. Investigations into whether these fatty acids work at specific receptors or via specific mechanisms were conducted through the use of receptor antagonists. Across the three cell lines it was found that DHA may work at the GPR40 receptor to reduce cell viability. Also, DHA may work at RxR receptors as cell viability was restored following RxR antagonist treatment across the three cancer cell lines following DHA treatment. Finally, hormone antagonist effects differed across the three cell lines suggesting the fatty acids may act at different hormone receptors dependant on the type of cancer.

In this thesis I have sought to address whether DHA, ALA and AA can reduce cell viability in neuroblastoma, breast and prostate cancer cell lines. This research is important for the development of less harmful therapies, and the driving force behind research into cancer therapies is its clinical relevance and possible application for further development.

We know DHA specifically can act on cancer cells, but it is not known by what mechanism these fatty acids work, therefore this thesis has shown more work into this area detailing the possibility for the fatty acids to work at receptors such as the GPR40, IPLA2 and RXRs as well as nuclear hormone receptors. This has begun to highlight possible gender effects across the

different types of cancer as well as the different fatty acids used in this research. In my studies of nuclear hormone receptors using bexarotene, mifepristone, nilutamide and tamoxifen gender effects have been looked into and it has been demonstrated that there may be a gender effect as prostate cancer cells responded less than the other two cancer cells lines to omega-3 fatty acid treatment but more of an effect was elicited from omega-6 fatty acid treatment on the PC-3 cell line. This is really critical for the further understanding of PUFA's effects on cancer and the mechanisms behind it.

Overall, of the three fatty acids DHA reduced cancer cell viability of neuroblastoma, breast and prostate cancer cell lines the most when compared to ALA and AA fatty acid treatment. Also, experiments into receptors highlights DHAs possible use of the GPR40 receptors and RxR receptors to elicit a reduction in cell viability across SH-SY5Y, MCF-7 and PC-3 cancer cell lines.

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Appendix:

I:

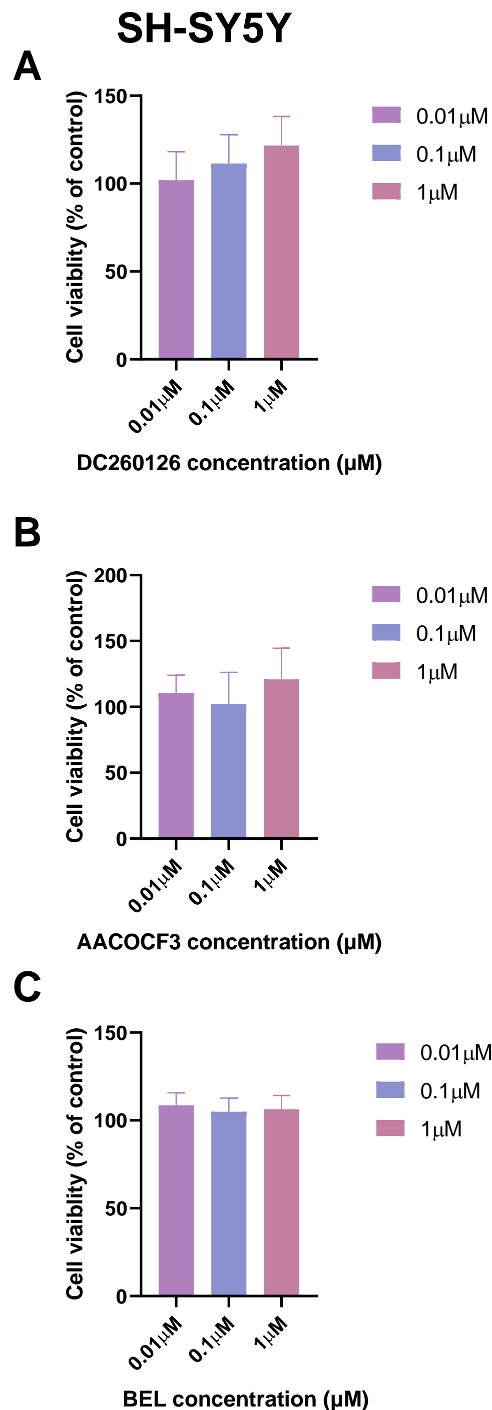


**Figure I: Cell viability of SH-SY5Y cells following GPR40 (DC260126) and iPLA2 (AACOCF3, BEL) antagonist treatment for 24hrs.** A) bar chart showing cell treatment with 0.01µM, 0.1µM, 1µM DC260126, B) bar chart showing cell treatment with 0.01µM, 0.1µM, 1µM AACOCF3, C) bar chart showing cell treatment with 0.01µM, 0.1µM, 1µM BEL.

II:

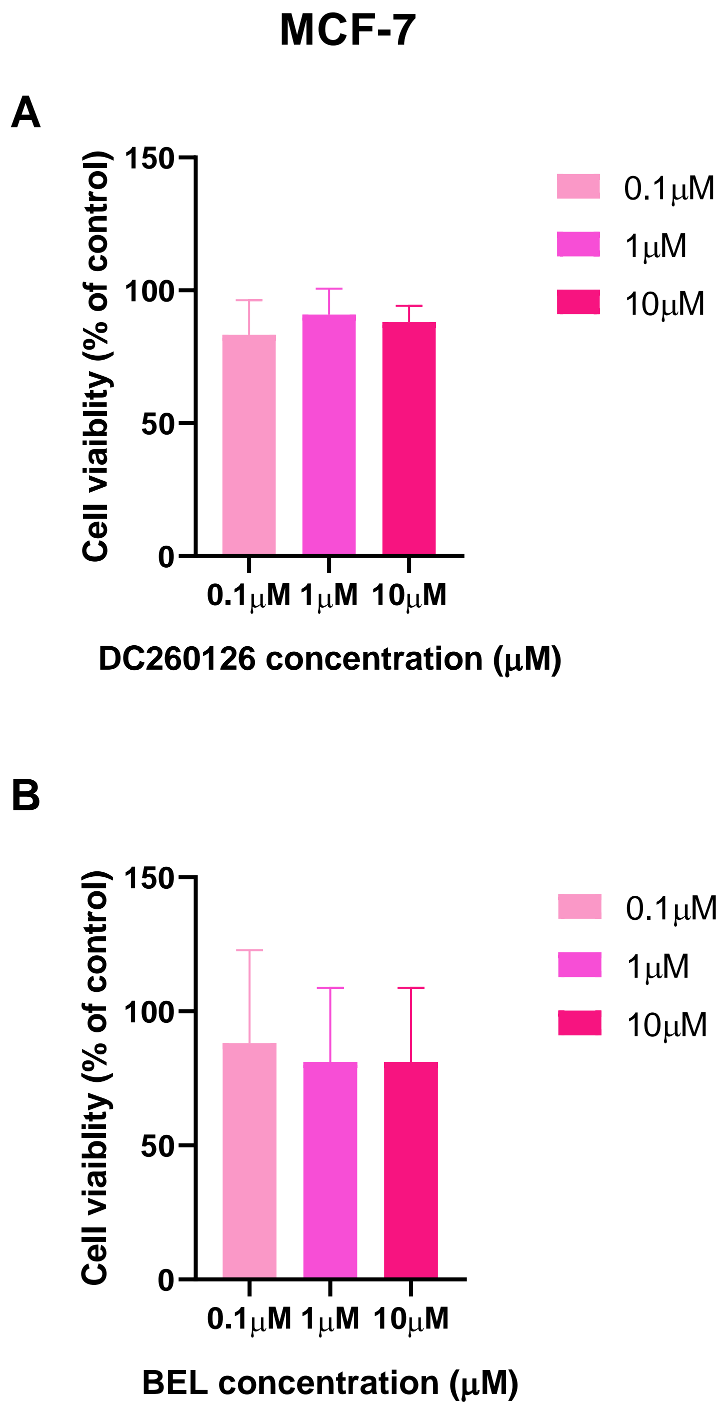


**Figure II: Cell viability of MCF-7 cells following GPR40 (DC260126) and iPLA2 (BEL) antagonist treatment for 24hrs.** A) bar chart showing cell treatment with 0.1μM, 1μM, 10μM DC260126, B) bar chart showing cell treatment with 0.1μM, 1μM, 10μM BEL.

III:

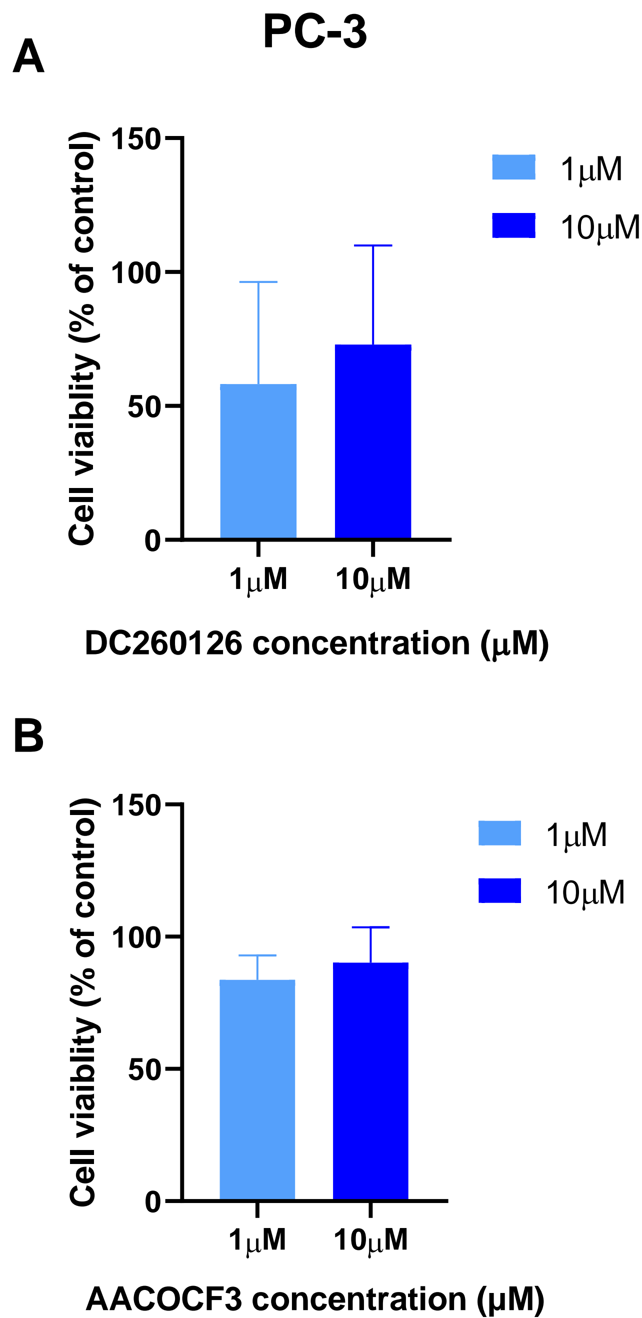


**Figure III: Cell viability of PC-3 cells following GPR40 (DC260126) and iPLA2 (AACOCF3) antagonist treatment for 24hrs.** A) bar chart showing cell treatment with 1μM and10μM DC260126, B) bar chart showing cell treatment with 1μM and 10μM AACOCF3.