Multifactorial Impacts on Early Breast Carcinogenesis- Assessing the Combined Effects of Mixtures of Endocrine Disrupting Chemicals and Fatty Acids.

A Thesis Submitted for the Degree of Doctor of Philosophy

by

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Declaration

I hereby declare that I carried out all the research presented in this thesis, except where clearly stated otherwise within the text. This original work has not been submitted anywhere for any other academic degree.

Gideon Kweku Enimah

I dedicate this thesis to my entire family

Abstract

The percentage of breast cancer attributed to genetic factors is estimated to be between 5-10%, suggesting that a large proportion of breast cancer cases can be attributed to external factors. There has been extensive research on external factors such as obesity, alcohol, smoking, physical activity, diet and environmental chemicals and their role in breast cancer development. However, the evidence of environmental chemicals such as Endocrine Disrupting Chemicals (EDCS) on breast cancer initiation has been inconclusive because they have been studied as single compounds at concentrations not reflective of tissue concentrations. This phenomenon is also applicable to other risk factors, such as diet, suggesting that several multiple risk factors interact to initiate breast carcinogenesis.

Using a non-tumorigenic cell line, MCF-12A, in// a 3D model that recapitulates human mammary structure, mixtures of thirteen EDCs and four fatty acids were tested at tissue concentrations for their effect on breast carcinogenesis. The results showed that the mixtures disrupted acini formation and affected genes and pathways involved in carcinogenesis, such as cell proliferation, migration and apoptosis. There were increases in the sizes of acini and decreases in circularity, which are markers of neoplastic transformation. Changes to gene expression and pathways involved in breast cancer were also found when BRCA1-silenced MCF-12A cells were exposed to mixtures of EDCs and fatty acids, suggesting a possible increase in absolute risk for people with a familial mutation in the BRCA1 gene. The ability of 3D models to appropriately model processes involved in normal cellular functions such as proliferation and glycolysis was also demonstrated through a meta-analysis of published data.

Overall, we show that exposure to mixtures of EDC and fatty acids can increase induce genes and pathways that can lead to breast carcinogenesis. We also demonstrated the ability of EDCs to induce the expression of carcinogenic genes in BRCA1 silenced cells, suggesting a possible increase in breast cancer risk.

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Abbreviations

℃	Degree Centigrade
∆Ct	Delta
2D	Two dimensional
3-BC	3- Benzylidene Camphor
3D	Three dimensional
ACTB	Beta-Actin
AF1	Transcriptional Activation Function 1
AHTN	6-acetyl-1,1,2,4,4,7-hexamethyltetraline
AICR	American Institute for Cancer Research
AKR1C1	Aldo-Keto Reductase 1C1
ALDH1A3	Aldehyde dehydrogenase 1 family member A3
ALDH1	Aldehyde dehydrogenase 1
ANOVA	Analysis of Variance
AR	Androgen Receptor
ARMC6	Armadillo Repeat Containing 6
ATM	Ataxia-telangiectasia mutated
BAK	BCL2 Antagonist/Killer 1
BARD1	BRCA1 Associated RING Domain 1
BASC	BRCA1-associated genome surveillance complex
BAX	BCL2 Associated X
Bcl-2	B-cell lymphoma-2
BDE 100	Brominated diphenyl Ether 100
bFGF	Fibroblast growth factor 2
BID	BH3 Interacting Domain Death Agonist
BIM	BCL-2 interacting mediator of cell death
BMI	Body Mass Index
BP-3	Benzophenone-3
BPA	Bisphenol A
BPF	Bisphenol F
BPS	Bisphenol S
BRCA 1/2	Breast Cancer Susceptibility Genes 1/2
BRCT	BRCA1 C-terminus
BRIP1	BRCA1 Interacting protein C-terminal helicase 1
BuP	Butylparaben
CA	Concentration Addition
CA12	Carbonic Anhydrase 12

CCND1	Cyclin D1
CD36	Cluster of Differentiation 36
CD44	Cluster of Differentiation 42
CDC42	Cell Division cycle 42
CDH1	Cadherin-1
CDH13	Cadherin-13
CDKS	Cyclin Dependent Kinases
cDNA	Complementary DNA
CHEK2	Checkpoint kinase 2
CHFR	Checkpoint with Forkhead and Ring finger domains
CHLs	Chlordane compounds
COL7A1	Collagen type VII alpha 1 chain
COPG2	Coatomer subunit gamma-2
CtIP	C-terminus binding protein
DAPI	4', 6-diamidino-2-phenylindole
DBD	DNA Binding Domain
DCE	1,2-dicloroethane
DDT	Dichloro-diphenyl-trichloroethane
DEGs	Differentially Expressed Genes
DEPP1	Decidual protein induced by Progesterone 1
DMBA	7, 12-dimethylbenz[a]anthracene
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
E1	Estrone
E2	estradiol
E3	estriol
ECM	Extracellular Matrix
EDC	Endocrine Disrupting Chemicals
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Agency
EGF	Epidermal Growth Factor
EIF2AK3	Eukaryotic Translation initiation factor 2-alpha kinase 3
EMT	Epithelial-Mesenchymal transition
EPIC	European Prospective Investigation into Cancer and Nutrition
ERE	Estrogen responsive element
ERK1/2	Extracellular Signal-regulated kinase 1/2
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ESR1	Estrogen receptor 1
ESR2	estrogen receptor 2
EtOH	Ethanol

EtP	Ethylparaben
FABpm	Plasma membrane fatty acid binding protein
FAK	Focal Adhesion Kinase
FASN	Fatty Acid Synthase
FATPs	Fatty Acids Transport Protein Family
FDR	False Discovery Rate
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFR	Growth Factor Reduced
GLOBOCAN	Global Cancer Observatory
GO	Gene Ontology
GPER	G protein-couples receptor 1
GPNMB	Glycoprotein non-metastatic B
GR	Glucocorticoid Receptor
GSTP1	Glutathione S-transferase P1
GWS	Genome-Wide Studies
HBSS	Hanks' Balanced Salt Solution
HCB	Hexachlorobenzene
HER2	Human epidermal growth factor 2
HHCB	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[g]benzopyran
IA	Independent Action
IF	immunofluorescence
IGSF4	Immunoglobulin superfamily member 4
JAK	Janus Kinase
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KLF1	Krüppel-like factor 1
KLK10	Kallikrein-related peptidase 10
KLK7	Kallikrein-related peptidase 7
KRAS	Kirsten rat sarcoma virus
LBD	Ligand Binding Domain
LIPE	hormone Sensitive Lipase
LogFC	Log2(fold change) over control
MAL2	T-cell differentiation protein 2
MAPK	Mitogen-activated protein kinases
MAPK15	Mitogen-activated protein kinases 15
MAPK9	Mitogen-activated protein kinases 9
MCM3AP	Minichromosome maintenance protein 3
MeP	Methylparaben
mRNA	messenger RNA
mTOR	Mechanistic target of rapamycin
MUFAs	Monosaturated Fatty Acids
NUF2	NDC80 kinetochore complex component

p,p'-DDE	Dichlorodiphenyldichloroethylene
PALB2	Partner and Localizer of BRCA2
PBDE	Polybrominated diphenyl Ethers
PBS	Phosphate Buffered Saline
PCBs	Polychlorinated biphenyls
PCNA	Polybrominated diphenyl Ethers
PG	Prostaglandins
PI3K	Polychlorinated biphenyls
PKDCC	Protein Kinase Domain Containing Cytoplasmic
PPAR	Prostaglandins
PR	Progesterone receptor
PrP	Propylparaben
PTEN	Peroxisome Proliferation Activated Receptor
PTPRN2	Progesterone receptor
PUFAs	Propylparaben
qPCR	Phosphatase and tensin homolog
RB	Polysaturated Fatty Acids
rBM	quantitative Polymerase Chain Reaction
RFU	Relative fluorescence unit
RING	Retinoblastoma protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
SAPCD2	Suppressor APC domain containing 2
siRNA	small interfering RNA
SMCO4	Single-Pass Membrane Protein with Coiled-coil Domains 4
SML	Specific Migration Limit
SNP	Single-Nucleotide Polymorphism
SR	Serotonin Receptor
Src	Tyrosine-protein kinase
SREBP-1c	Sterol Regulatory Element-Binding Protein-1c
STK11	Serotonin Receptor
TCE	Trichloroethylene
TDI	Tolerable Daily Intake
TDLU	Terminal ductal lobuaveolar units
TFF1	Trefoil factor1
TIMP3	TIMP Metallopeptidase Inhibitor 3
TMSB4XP6	thymosin beta 4, X-linked pseudogene 6
TNFα	Tumour necrosis factor alpha
TP53	Tumour Protein p53
TR	Thyroid Receptor
TRAIL	TNF-related apoptosis-inducing ligand

TREM1	Triggering receptor expressed on myeloid cell 1
UMAP	Uniform Manifold Approximation and Projection for Dimension Reduction
WCRF	World Cancer Research Fund
WHO	World Health Organization
YES	The Yeast Estrogen Screen

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Finally, I ascribe all glory and gratitude unto Him who is able to do exceedingly abundantly above all we can think or ask, Jesus Christ, the Son of the living God.

Chapter One: Multifactorial Impact on Early Breast Carcinogenesis

1 Multifactorial Impacts on Early Breast Carcinogenesis

1.1 Development of Human Breast

The breast is a bilateral organ that undergoes morphological and functional changes throughout female life (Russo and Russo, 2004). The mammary gland develops through a series of processes linked to sexual and reproductive development, from embryonic to prepubertal and pubertal stages, pregnancy to lactation and involution (Hennighausen and Robinson, 2001). The majority of the differentiation of the mammary glands occurs after birth, initiated by hormones and growth factors (Kuperwasser *et al.*, 2004).

Non-functional mammary rudimentary structures and cell lineages of the mammary glands are formed in the embryonic stage (Capuco and Ellis, 2013). These rudimentary mammary glands occur on day 10 of embryogenesis and emerge as five pairs of placodes. Primary mammary mesenchyme forms by day 15, further dividing into ductal structures and the fat pad (Visvader and Lindeman, 2003). The embryonic stage of mammary gland development is regulated by signalling between the epithelium and mesenchyme (Robinson, 2007). At puberty, under the control of estrogen, the epithelium develops into a branching bilayered ductal structure consisting of outer myoepithelial cells and luminal cells, which can be further divided into ductal luminal cells lining the ducts and alveolar luminal cells, which produce milk. The primary ducts that spread into the nipple develop into other subsidiary ducts: segmental and subsegmental ducts. The subsegmental ducts form the terminal duct formation, which further subdivides terminal ductules or acini. The acini come together to form one terminal duct with the surrounding intralobular stroma, called a terminal lobular duct unit (TDLU) (Figure 1.1). The terminal duct lobular unit is the functional unit of the breast (Javed and Lteif, 2013; Rosenfield, Cooke and Radovick, 2008).

The second phase of post-natal mammary gland development occurs during pregnancy, as the mammary gland undergoes various changes in preparation for lactation. There is an extensive increase in the secondary and tertiary ductal bifurcation and maturation of the alveolar buds into separate alveoli, which become the milk-producing lobules during lactation. The mammary gland does not undergo any morphological changes during lactation. However, the lumen becomes bigger and filled with granular, basophilic material mixed with fat. Milk is produced and released into the acini and ductal system by the alveolar cells and stored for 48 hours. Post lactation, the acini structure collapses and the lobular connective tissues regress (Russo and Russo, 2004)

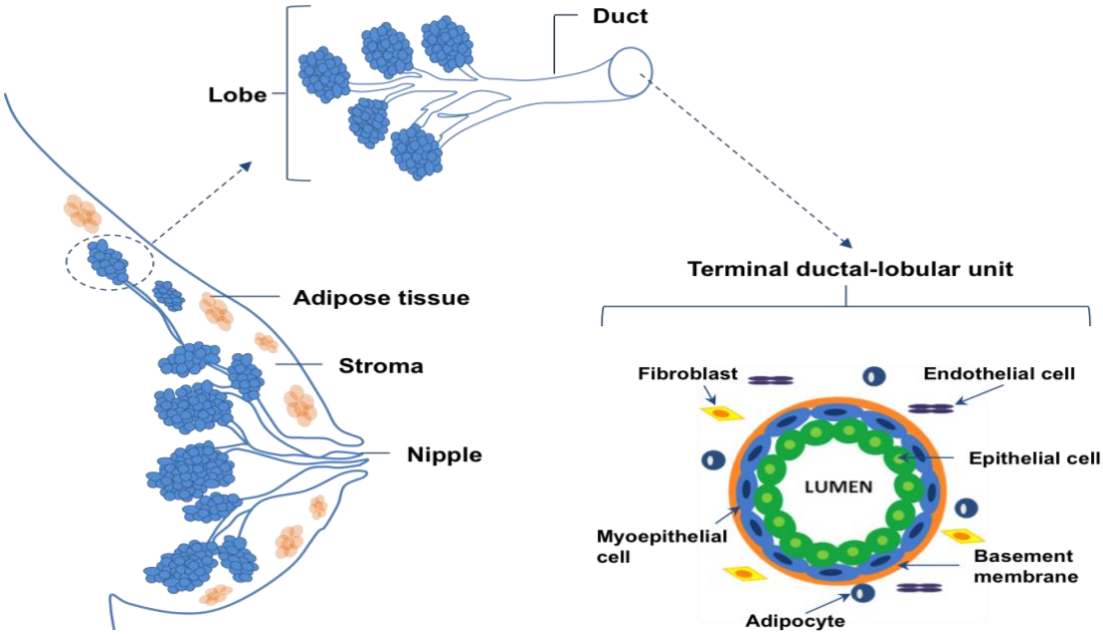


Figure 1.1 Schematic representation of mammary gland morphology. *Structure of the female breast, focusing on the terminal ductal-lobular unit (TDLU). TDLUs consist of acini structures. A cross-section of the acini shows myoepithelial cells surrounding epithelial cells, forming a hollow lumen (Maria Riverso, 2013).*

1.2 Breast Cancer Epidemiology

According to GLOBOCAN 2020, breast cancer accounted for 47.8 new diagnoses per 100,000 women, making it the most common cancer. This translated to 2,261,419 new breast cancer cases and 684,996 deaths globally (Sung *et al.*, 2021) (Figure 1.2). In the UK, there are 55,900 new breast cancer cases every year, which makes it the most common cancer in the country, representing 15% of all new cancer cases based on data collected between 2017 and 2019. It is projected that, in the UK, there will be a 1% increase in breast cancer incidence between 2023-2025 and 2038-2040 (Cancer Research UK). 76% of all women diagnosed with breast cancer in England survive the condition for ten years or more. However, there are 11,499 breast cancer deaths in the UK annually (Cancer Research UK).

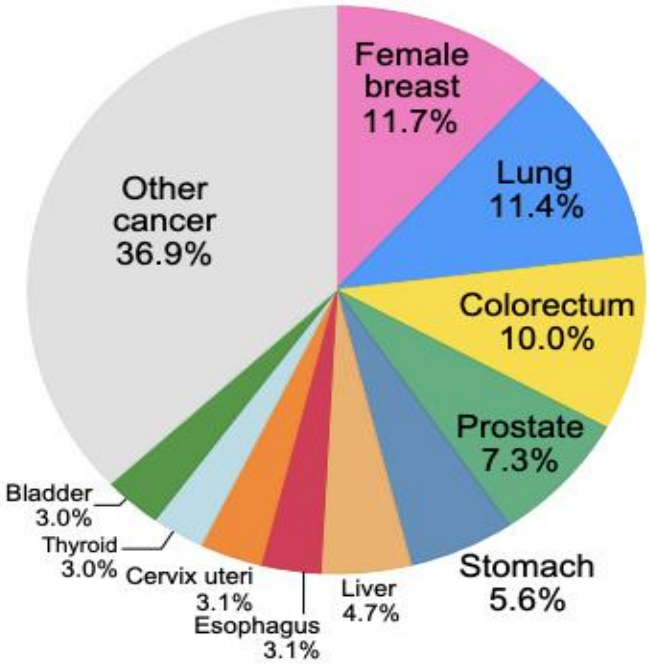


Figure 1.2. Pie Chart showing cancer incidence around the world. *Global distribution of different cancers in both males and females according to GLOBACAN 2020. Female breast cancer was the most prevalent cancer, with 11.7% (Sung et al., 2021)*

Over the last 30 years, breast cancer mortality has been declining due to the significant improvement in early screening and detection, improved treatment and increased knowledge of the biological mechanism underlying the disease (DeSantis *et al.*, 2015; Gonzalez, Rae and Colacino, 2019). Early screening (e.g., frequent mammograms, magnetic resonance imaging) and other preventive factors are available to reduce the incidence and mortality of breast cancer. Age-based screening has been shown to reduce breast cancer mortality by 15%-40%, but there are concerns about possible false positives, over-diagnosis and psychological harms (Khan *et al.*, 2021b). Pashayan and colleagues argued that risk stratification is a cost-effective way of screening the population to reduce overdiagnosis and improve the benefits of screening (Pashayan *et al.*, 2018).

1.3 Breast Cancer Subtypes

Breast cancer is a heterogeneous disease that can be grouped into subtypes based on their clinical, histological and molecular characteristics (Russnes *et al.*, 2017). Immunohistochemistry and gene expression analysis are two primary techniques for classifying breast cancers (Perou *et al.*, 2000; Nielsen *et al.*, 2004). Gene expression profiling of breast cancer cells divides them into four subtypes (Table 1.1): luminal A, luminal B, basal-like, and HER-2 enriched (Perou *et al.*, 2000; Sørlie *et al.*, 2001). Luminal A subtype is both estrogen receptor (ER) and progesterone receptor (PR) positive and HER2 negative with a low Ki-67 expression. Luminal B is also positive for ER and PR expression but can either be HER2 negative or positive, with a high Ki-67 expression suggesting high proliferation. HER2 enriched subtype has HER2 overexpressed but negative for both ER and PR. Basal-like is ER, PR and HER2 negative, and therefore triple negative (Goldhirsch *et al.*, 2013; Prat *et al.*, 2012). In 2007, another subtype, claudin-low breast cancer, was classified using DNA

microarray from 13 murine models. This type was typified by reduced tight junction protein claudin 3, 4 and 7 expression and E-cadherin. Claudin-low, however, is a phenotype of basal breast cancer rather than a distinct subtype of breast cancer. It is ER, PR and HER2 negative and, therefore, has a poor prognosis (Herschkowitz *et al.*, 2007; Prat *et al.*, 2010; Fougner *et al.*, 2020).

Based on cell morphology, growth and architecture, breast cancer has been grouped into 21 histological classes by the World Health Organisation (Lakhani *et al.*, 2012). The most common histological forms of breast cancer are the invasive ductal carcinoma of no special type (IDC) (75-80%) and the invasive carcinoma, classical type (10-75%). Breast cancer of special types accounts for 25% of all breast cancer cases and has distinct biological markers that are useful for prognosis and treatment (Jenkins *et al.*, 2021; Weigelt, Geyer and Reis-Filho, 2010). Other less common histological types include mucinous, cribriform, micropapillary, papillary, tubular, medullary, metaplastic, and apocrine carcinomas (Lakhani *et al.*, 2012). Clinically, breast cancer therapies are guided by the presence of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER2) (Houghton and Hankinson, 2021).

Table 1.1 Intrinsic molecular subtypes of breast Cancer (Perou *et al.*, 2000; Sørlie *et al.*, 2001)

Molecular subtype	**Receptor status**	**Clinical Prognosis**
Luminal A	$ER^{`+}$, PR^{-}, $HER2^{-}$	Good
Luminal B	ER^{+}, $PR^{+/-}$, $HER2^{+/-}$	Good (worse than luminal A)
HER2 enriched	$HER2^{+}$, ER^{-}, PR^{-}	Intermediate
Basal-like	$HER2^{-}$, PR^{-}, ER^{-}	Poor. Threefold risk early distant recurrence within 5 years. 3% risk of late recurrence after 5 years (Zagami and Carey, 2022). 5 year overall survival of metastatic TNBC is 11% (Kesireddy *et al.*, 2024)

1.4 Breast Cancer Initiation and Progression

Cells acquire certain traits that influence cancer initiation and progression. These traits or hallmarks, include tissue invasion and metastasis, circumvented apoptosis, sustained indefinite replication, evasion of anti-metastatic signalling, induced angiogenesis, self-sufficiency in growth signals, altered cellular energy metabolism and avoidance of immune destruction (Hanahan and Weinberg, 2000; Hanahan and

Weinberg, 2011). Douglas Hanahan has enumerated additional hallmarks of cancer. These include unlocking phenotypic plasticity, epigenetic reprogramming, and polymorphic microbiome (Hanahan, 2022).

Breast cancer is primarily initiated in the epithelial cells lining ducts and milk lobules (Hinck and Näthke, 2014). Genetic and epigenetic changes drive the transitions in the morphology of cells in the progression and development of cancers (Rivenbark, O'Connor and Coleman, 2013). Laser microdissection and microarrays of 14 patient-matched normal epithelium, normal stroma, tumour epithelium and tumour-associated stroma specimens showed that the tumour microenvironment is involved in the transition of cells into cancer states (Ma *et al.*, 2009). Different linear models have been proposed to explain breast cancer initiation, transformation, and progression (Bombonati and Sgroi, 2011). One of the most accepted models of breast carcinogenesis proposes that it arises from stem cells in TDLUs, which progress into atypical hyperplasia (atypical ductal hyperplasia, ADH, and atypical lobular hyperplasia, ALH). ADH and ALH are premalignant but possess the ability to be malignant over time (Allred, Mohsin and Fuqua, 2001). ALH/ADH increases the risk of developing breast cancer by 5-fold, and this risk can vary depending on menopausal status (DI, 2003). ALH and ADH transition to in situ ductal carcinoma (DCIS), which is a proliferation of neoplastic luminal cells that are restricted in the ductolobular system of the breast (Figure 1.3). Though DCIS are pre or non-invasive, they are generally considered as the early stages of breast cancer (Stage 0) and make up an estimated 25% of all breast cancer diagnoses (Allred, Mohsin and Fuqua, 2001; van Seijen *et al.*, 2019). DCIS progresses into invasive breast cancer by breaking down the basement membrane of the ducts and invading the stroma either by independent evolution or direct evolution (Grimm *et al.*, 2022).

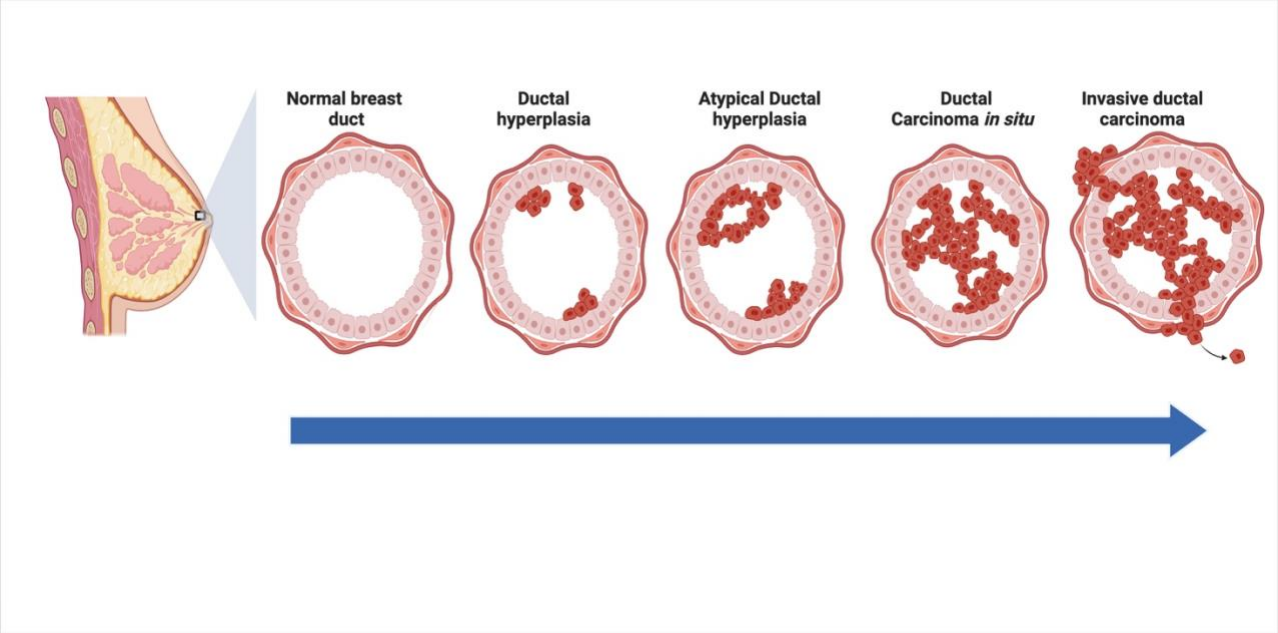


Figure 1.3 Breast cancer progression. *Breast cancer arises from the cells lining the lumen of ducts. The first stage is ductal hyperplasia, which includes non-malignant lesions that resemble normal TDLU. The next step is atypical hyperplasia (ADH), which is pre-invasive but shows proliferation and changes in morphology. ADH transitions into DCIS, where proliferated neoplastic cells are restricted to the lumen. This is generally considered a stage of breast cancer. DCIS become invasive breast cancer by breaking the basement membrane into the surrounding stroma. Image created with Biorender software (https://biorender.com/).*

1.4 Breast Cancer Risk Factors

The aetiology of breast cancer is complex and multifactorial (McPherson, Steel and Dixon, 2000; Hiatt *et al.*, 2014). In the UK, 1 in 7 females born after 1960 will be diagnosed with breast cancer in their lifetime; however, it is argued that 23% of breast cancer cases could be prevented (Cancer Research UK). Masala and colleagues showed that 30% of postmenopausal breast cancer can be prevented through

increased leisure time, physical activity, consumption of one drink per day and keeping a BMI below 25.

Only 5-10% of breast cancers are due to hereditary factors (Economopoulou, Dimitriadis and Psyrri, 2015). In adults over 30 years old, the International Agency for Research on Cancer (IARC) estimates that 36.8% of breast cancer incidence can be linked to environmental and lifestyle factors (Eve *et al.*, 2020). Risk factors can be grouped generally into genetic factors/non-modifiable and lifestyle/modifiable factors. These factors can interact to increase the risk of breast cancer for women and men.

1.4.1 Genetic Risk Factors

The link between familial history and breast cancer is conclusively established (Wendt and Margolin, 2019). It has been established that an estimated 5-10% of all breast cancers are due to mutations in one or two of the major cancer susceptibility genes (Yedjou *et al.*, 2019; Clamp, Danson and Clemons, 2003).

Penetrance of a disease-causing mutation in a cancer gene refers to the relative risk that the mutation will initiate the specific cancer and show clinical symptoms (Economopoulou, Dimitriadis and Psyrri, 2015; Lynch, Venne and Berse, 2015). Breast cancer susceptibility genes can be grouped into high penetrance, intermediate penetrance, and low penetrance (Table 1.2). *BRCA1, BRCA2, TP53, PTEN, STK11,* and *CDH1* are in the high penetrance category, while the intermediate genes are *CHEK2, ATM, BRIP1,* and *PALB2* (Turnbull and Rahman, 2008). BReast CAncer gene 1/2 (*BRCA1 and BRCA2*) are tumour suppressor genes involved in the repair of DNA double-strand break repair and regulating transcription of estrogen and progesterone receptors (Shiovitz and Korde, 2015; Bogdanova, Helbig and Dörk, 2013). Deleterious germline mutations in BRCA1 and BRCA2 contribute 50%-85% of the overall lifetime risk of a woman developing breast cancer (Hoskins *et al.*, 2008). However, *BRCA1*

and *BRCA2* mutations account for approximately 20% of all hereditary breast cancer and 5-10% of all breast cancers in the general population (Balmana *et al.*, 2011). Genome-wide studies (GWS) have identified alleles that can confer breast cancer risk in a polygenic manner. These form low-penetrance alleles (Table 2) consisting of single-nucleotide polymorphisms (SNP) located in gene and intergenic areas (Jia *et al.*, 2023). These have been used to detect genetic variability across the genome and have been shown to increase breast cancer risk (Shiovitz and Korde, 2015; Lilyquist *et al.*, 2018). Eight low-penetrance alleles have been identified: 10q26, 16q, 12q35, 8q24, 5p12, 11p15, 5q11 and 2q33 (Turnbull and Rahman, 2008; Lilyquist *et al.*, 2018; Ellsworth *et al.*, 2018; Han *et al.*, 2017; Ellsworth, Turner and Ellsworth, 2019). The overall breast cancer risk due to the inheritance of low penetrance genes remains inconclusive. While it has been suggested that low-penetrance genes confer a 50% increase in breast cancer risk (Houlston and Peto, 2004), other studies have indicated that they do not significantly contribute to the overall genetic risk of breast cancer (Shiovitz and Korde, 2015)

Table 1.2 Penetrance of known genes in breast cancer (Turnbull and Rahman, 2008; Han *et al.*, 2017; Ellsworth, Turner and Ellsworth, 2019; Jia *et al.*, 2023).

Category	Gene/Locus	Relative Risk	Candidate Genes	Associated SNPs
High penetrance	*BRCA1*	>10		rs8176085
	BRCA2	>10		rs28897700
	TP53	>10		rs1042522
	PTEN	2-10		rs1903858
	STK11	2-10		rs2075606
	CDH1	2-10		rs16260
Intermediate Penetrance	*ATM 2–3*	2-3		rs1801516
	CHEK2	2-3		rs17879961
	BRIP1	2-3		rs137852986
	PALB2	2-4		rs180177102
Low Penetrance	10q26	1.08–1.26	FGFR2	rs2981582
	16q12	1.07–1.13	TOX3	rs3803662
	2q35	1.08-1.26	IGFBPS	rs13387042
	8q24	1.08-1.26	MYC	rs13281615
	5p15	1.25	TERT/CLPTM1L	rs10069690
	11p15	1.07-1.13	LSP1	rs3817198
	5q11	1.07-1.13	MAP3K1	rs889312
	2q33	1.13	CASP8	rs1045485
	19p13.1	1.25	ANKL1, BABAM1	rs8170
	1q32.1	1.16	MDM4	rs4245739

1.4.1.3 Mammographic Density

Mammographic density is an important and established breast cancer risk factor (Vachon *et al.*, 2007; Sieh *et al.*, 2020). Women with an estimated breast density of 75% are four to six times more likely to develop breast cancer than those with low mammographic density (Boyd *et al.*, 2011). Bodewes and colleagues found that

increased mammographic density is associated with a two-fold increase in breast cancer risk (Bodewes *et al.*, 2022). A nested case-control cohort study from the Joanne Knight Breast Health Cohort of 10 481 cancer-free women between 2008 to 2020. The study showed that the rate of change in breast density was linked to the risk of subsequent breast cancer (Jiang *et al.*, 2023).

1.4.2 Lifestyle Risk Factors

1.4.2.1 Alcohol Consumption

Alcohol intake at low to moderate levels is a significant and well-known breast cancer risk, contributing to a 7-10% increase in breast cancer risk (Zeinomar *et al.*, 2019). A study of 242918 postmenopausal women from EPIC found that the risk associated with alcohol consumption increased in a dose-dependent manner. They found that alcohol consumption greater than 20g per day was associated with greater breast cancer risk (McKenzie *et al.*, 2015). This finding was also corroborated in another cross-sectional study of the relationship between breast cancer risk factors and circulating sex hormones in 6000 postmenopausal women, which showed that women who took more than 20g of alcohol per day had higher levels of sex hormones than non-drinkers, which positively linked to increased breast cancer risk (Hormones and Group, 2011). A prospective study of 105986 women followed between 1980 and 2008 showed that consumption of alcohol as low as 5 g to 9.9 g per day (the equivalent of 3 drinks per week) increased breast cancer risk (Relative risk, 1.15, 95% CI, 1.06-1.25) (Chen *et al.*, 2011).

Several mechanisms have been proposed to explain the role of alcohol in breast carcinogenesis. These include its role in estrogen metabolism and response, the mutagenicity of acetaldehyde (a major metabolite of alcohol), oxidative damage and impact on one-carbon metabolic pathways resulting in adverse effects in cellular

response, proliferation, and DNA damage (Dumitrescu and Shields, 2005; Wright, McManaman and Repine, 1999).

1.4.2.2 Obesity

There is a positive link between body weight and breast cancer risk (Lofterød *et al.*, 2020). Obesity is an established risk factor for breast cancer and contributes 6%-19% to the overall risk burden (Carmichael, 2006). Obesity leads to poor long-term survival after breast cancer treatment (Iwase *et al.*, 2021). A meta-analysis of 82 follow-up studies, including 213,075 breast cancer survivors (23,182 from breast cancer), assessed the impact of body mass index (BMI) and breast cancer. The study reported that obesity was positively associated with reduced breast cancer survival and a higher risk of mortality (Chan *et al.*, 2014). Furthermore, an elevated BMI leads to a decrease in pathological complete response rate and disease-free survival rate after adjuvant chemotherapy (Fontanella *et al.*, 2015). In postmenopausal women, the risk ratio for breast cancer incidence increases by 1.12 per 5kg/m^2 increase in BMI (Renehan *et al.*, 2008).

1.4.2.3 Diet

The evidence of a link between diet and breast cancer remains inconclusive (Turati *et al.*, 2022). Diets contain different food classes that can act together, and this combined effect may be more important than that of any single food or nutrient (Torres *et al.*, 2023). Nutritional epidemiologists have argued that to determine a causal link between diet and cancer, the focus should shift from a single nutrient to investigating holistic eating patterns (Mourouti and Panagiotakos, 2016).

The third expert report of the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR), released in 2018, proposed dietary actions to

prevent cancer. These include reducing the consumption of fast food and highly processed foods rich in fat and starch, increasing the consumption of vegetables, whole grains, and fruits, consuming less red meat, and reducing the consumption of sugar-sweetened drinks (International, 2018). Adherence to these expert recommendations has been shown to reduce breast cancer risk (Turati *et al.*, 2020). A study has shown that red meat and processed meat increase breast cancer risk by 6% and 9%, respectively (Farvid *et al.*, 2018). Interestingly, a UK Biobank study of 262,195 women showed that processed meat increased the risk of postmenopausal breast cancer but not pre-menopausal breast cancer. However, this study did not show a causal link between red meat and breast cancer (Anderson *et al.*, 2018). Adherence to Mediterranean diets has been shown to lower breast cancer risk (Mourouti and Panagiotakos, 2016; Torres *et al.*, 2023). A population-based case-control study among 3024 women between the ages of 25-74 years in Ontario, Canada, showed that an increase in the consumption of phytoestrogens during the adolescent age had been associated with lower breast cancer risk (Thanos *et al.*, 2006). A study of food intake by 272,098 women in the European Prospective Investigation into Cancer and Nutrition (EPIC) study showed that intake of dietary fibre and fruits is associated with lower breast cancer risk (Heath *et al.*, 2020). Epidemiological studies have shown that high dietary fat, low-quality food and high alcohol intake in preadolescence and adolescence increased breast cancer risk, while intake of soy decreased risk (Mahabir, 2013). Ambrosone and colleagues showed that using antioxidants (Vitamin A, C and E; carotenoids, coenzyme Q10) before and during breast cancer treatment increases the risk of recurrence and likely death (Ambrosone *et al.*, 2020). A systematic study by Wiggs and colleagues of published cohort, cross-sectional and interventional scholarly studies focusing on the link between diet and /or exercise and

breast cancer risk showed that diet and exercise interventions reduced endogenous estrogen, which can consequently reduce breast cancer risk (Wiggs *et al.*, 2021).

1.4.2.4 Physical Activity

There is an inverse relationship between physical activity and breast cancer risk, particularly among postmenopausal women. The putative mechanisms for the decrease in breast cancer incidence due to physical activity are the reduction of obesity, reduction in insulin and insulin-like growth factors, and a decrease in lifetime exposure to steroid sex hormones (Monninkhof *et al.*, 2007). A prospective case-control study of 47456 premenopausal and 126704 postmenopausal women in the UK Biobank followed between 2006-2014 showed that increased physical exercises reduced breast cancer risk (Guo *et al.*, 2020b). A study has shown that 7 hours or more per week of physical activity leads to a 20% reduction in breast cancer (Colditz and Hankinson, 2005). Although physical activity is an established risk factor for breast cancer, the exact percentage and mechanism of risk reduction have not been conclusively determined.

1.4.2.5 Smoking

The impact of smoking on breast cancer risk has been extensively studied with inconclusive results. Although breast cancer is not a typical tobacco-related disease, many studies have established a link between smoking and breast cancer risk (Catsburg, Miller and Rohan, 2015). A systematic analysis of observational studies by Macacu and colleagues reported a moderate increase in breast cancer for both passive and active smokers (Macacu *et al.*, 2015). This is supported by results in the European Prospective Investigation Into Cancer and Nutrition (EPIC), which examined breast cancer incidence in 322,988 women. The study reported an increase in breast

cancer risk for both active and passive smokers (Dossus *et al.*, 2014). A hospital-based case-control study involving 877 breast cases and 890 controls in China also reported a positive association between passive smoking and increased breast cancer risk, especially among postmenopausal women. A study of 4,402 breast cancer in the Norwegian Breast Cancer Screening program during 2006-2004 showed an association between smoking and the incidence of luminal A-like breast cancer subtype (Ellingjord-Dale *et al.*, 2017). The Generation Study, which studied a cohort of 102,927 women in the United Kingdom, showed a causal link between smoking and a moderate increase in breast cancer risk. However, some earlier studies reported a lack of association between smoking and breast cancer risk. A population-based case-control study of 1273 participants did not establish a causal link between smoking and all-cause and breast cancer-specific mortality (Sagiv *et al.*, 2007). A cohort study of 34,401 Japanese women followed over 10 years did not show an increased risk in breast cancer (Lin *et al.*, 2008).

1.4.2.7 Exposure to Environmental Chemicals

Animal studies have shown that ubiquitous environmental chemicals, such as dioxins, polychlorinated biphenyls, and organochlorides (Koual *et al.*, 2020), can induce tumours in the mammary gland by the activation of hormonal pathways, DNA damage or and increasing breast cancer susceptibility by altering mammary morphogenesis (Brody *et al.*, 2007). A case-control study of 170 women (75 cases and 95 controls) selected from AN Medical Centre in Alaska between 1999 to 2002 concluded that exposure to environmental compounds (phthalates and their metabolites) could be associated with breast cancer (Holmes *et al.*, 2014). However, many epidemiological studies have found weak or no links to breast carcinogenesis (Brody and Rudel, 2003).

To fully understand the risk posed by environmental chemicals to breast cancer, it is imperative to consider that women are exposed to a cocktail of chemicals that interact with each other and also interact with other lifestyle and genetic risk factors.

1.5 Hormonal Carcinogenesis

The breast is an endocrine-sensitive organ, and its development from puberty to lactation is influenced by various hormonal signalling, chiefly the hypothalamic-pituitary-gonadal axis (Macon and Fenton, 2013; Darbre and Williams, 2022). Estrogen is a major hormone that acts on breast tissues and plays a critical role in physiological processes such as cell growth, reproduction, differentiation and development (Halada *et al.*, 2022; Jia, Dahlman-Wright and Gustafsson, 2015). An imbalance in estrogen has been implicated in the initiation and progression of cancers such as breast cancer (Satpathi *et al.*, 2023). One of the main risk factors of hormonal cancers such as breast cancer is exposure to endogenous and exogenous estrogens (Kim and Lee, 2017). Carcinogenesis is described as “hormonal” when the events leading to the initiation of the cancer are potentiated or accelerated by natural or synthetic hormones (Banerjee, 2011). The action of estrogen and its metabolites has been demonstrated through two different but complementary pathways (Figure 1.4). Estrogen activation via ERs occurs through genomic and non-genomic mechanisms. In the genomic pathway (also classical mechanisms or direct genomic), estrogen binds to ERs in the cytoplasm, and ERs undergo conformational changes, inducing receptor dimerisation. The estrogen-ER complex is then translocated to the nucleus, where it interacts with chromatin at the estrogen receptor element (ERE) sequences and promoter regions of target genes (Fuentes and Silveyra, 2019). This estrogen

signalling pathway is slow and sustained; its biological effects show after hours of activation (Silva, Kabil and Kortenkamp, 2010).

The non-genomic pathway (also called indirect genomic) of estrogen signalling is via the activation of membrane-bound ERs, including their splice variants and G protein-coupled receptor 1 (*GPER1*) (Thiebaut *et al.*, 2021). Activation of GPER1 leads to the release of estrogen growth factor (EGF) and its related ligands, which induces a cascade of intracellular events, such as rapid phosphorylation of mitogen-activated protein kinase (MAPK ERK ½), elevated concentration activation of phosphoinositide 3 kinase(PI3K), phospholipase C (PLC) and intracellular aggregation of calcium (Pupo, Maggiolini and Musti, 2016). As compared to the genomic pathway, non-genomic actions of estrogen occur rapidly (Silva, Kabil and Kortenkamp, 2010). Genomic and non-genomic estrogenic pathways play vital roles in breast cancer development, progression and survival (Bhardwaj *et al.*, 2019).

In MCF7 breast cancer cell lines, estrogen interacts with signalling molecules such as PI3K and, consequently, downstream proteins such as AKT (Lee *et al.*, 2005). Cotrim and colleagues demonstrated the interaction between estrogen and Mitogen-activated protein kinase (MAPK ERK 1/2) in MCF-7 cell lines (Cotrim *et al.*, 2013). The interaction of estrogens and signalling molecules (PI3K and MAPK) is vital for regulating proliferation, differentiation, apoptosis, and stress responses (Guo *et al.*, 2020c; Khatpe *et al.*, 2021).

There is a positive association between exposure to endogenous estrogen and its metabolites and overall breast cancer risk in women (Moore *et al.*, 2016). A retrospective cohort study of 620 women with ER-positive and HER2-negative breast cancer in Israel showed that endogenous estrogen impacts breast cancer risk, albeit small when compared with the impact of early menopause and multiparity (Korzets *et*

al., 2021). Early menarche and late menopause are a significant breast cancer risk factor chiefly because they extend the exposure time to endogenous estrogens, which is attributable to the increased number of ovarian and allied endocrine processes (Cancer, 2012). Breast cancer risk increases by a factor of 1.050 for every year younger at menarche and 1.029 for every year older at menopause(Cancer, 2012). Conversely, It has been established that with every year delay in menarche after the age of 12, premenopausal and postmenopausal breast cancer risk is reduced by 7% and 3%, respectively (Ozsoy *et al.*, 2017).

Studies have shown that pregnancy and childbirth have a dual effect on breast cancer risk. Pregnancy before the age of 20 years has been found to confer 50% protection against developing breast cancer. For women over 25 years, there is a transient increase in the risk of breast cancer immediately after childbirth, which is more pronounced in women older than 30 years. Women who have their first full-term pregnancy between 30 and 34 years have little protection and have an increased risk of developing breast cancer from 35 years old (Meier-Abt and Bentires-Alj, 2014).

A cohort study of women between 20 and 75 years in Norway showed that first and subsequent pregnancies have protective effects. However, there was an increase in breast cancer when the first pregnancy was after 30 years or older (Albrektsen *et al.*, 2005). A Danish cohort study consisting of over 2 million women with over 3 million births showed that early pregnancy was associated with reduced breast cancer risk (Husby *et al.*, 2018). However, a study by The International Premenopausal Breast Cancer Collaborative Group using pooled individual-level data from 15 prospective studies involving 18,826 breast cancer cases showed that parous women have an increased risk of developing breast cancer for more than 20 years after childbirth as

compared to childless women, especially in women with a family history of breast cancer, older at first birth and had multiple births (Nichols *et al.*, 2019).

Callihan and colleagues conducted a retrospective cohort study of women aged≥ Forty-five who were diagnosed with breast cancer. The breast cancer cases were categorised between giving birth and diagnosis. They reported that women with a breast cancer diagnosis within five years of giving birth have a 2.8 times higher risk of metastasis and 2.7 times higher mortality as compared to nulliparous women (Callihan *et al.*, 2013). A population-based cohort study examining the impact of a secular trend in parity on breast cancer incidence among women aged between 25 and 28 years from 1935 to 2015 in Connecticut showed that the increased trend in breast cancer incidence did not correlate with changes in parity over the period (Lima *et al.*, 2020). Two nested case-control studies (QResearch or Clinical Practice Research Datalink) in the UK assessed the impact of hormone replacement therapy (HRT) on breast cancer risk. 98611 women (50-79 years old) with a primary diagnosis of breast cancer between 1998 and 2018 showed that combined HRT increased breast cancer risk and suggested that doctors assist women in choosing the appropriate HRT formulation and regimen (Vinogradova, Coupland and Hippisley-Cox, 2020). A prospective follow-up study of 108647 women with a mean age of 65 years showed that the use of menopausal hormonal therapy (MHT) increased breast cancer risk (Cancer, 2019). Consequently, the positive association between estrogenic compounds and their metabolites on breast carcinogenesis has made it imperative to assess whether exposure to estrogen-mimicking endocrine-disrupting chemicals (EDCs) can increase breast cancer risk through similar pathways and mechanisms.

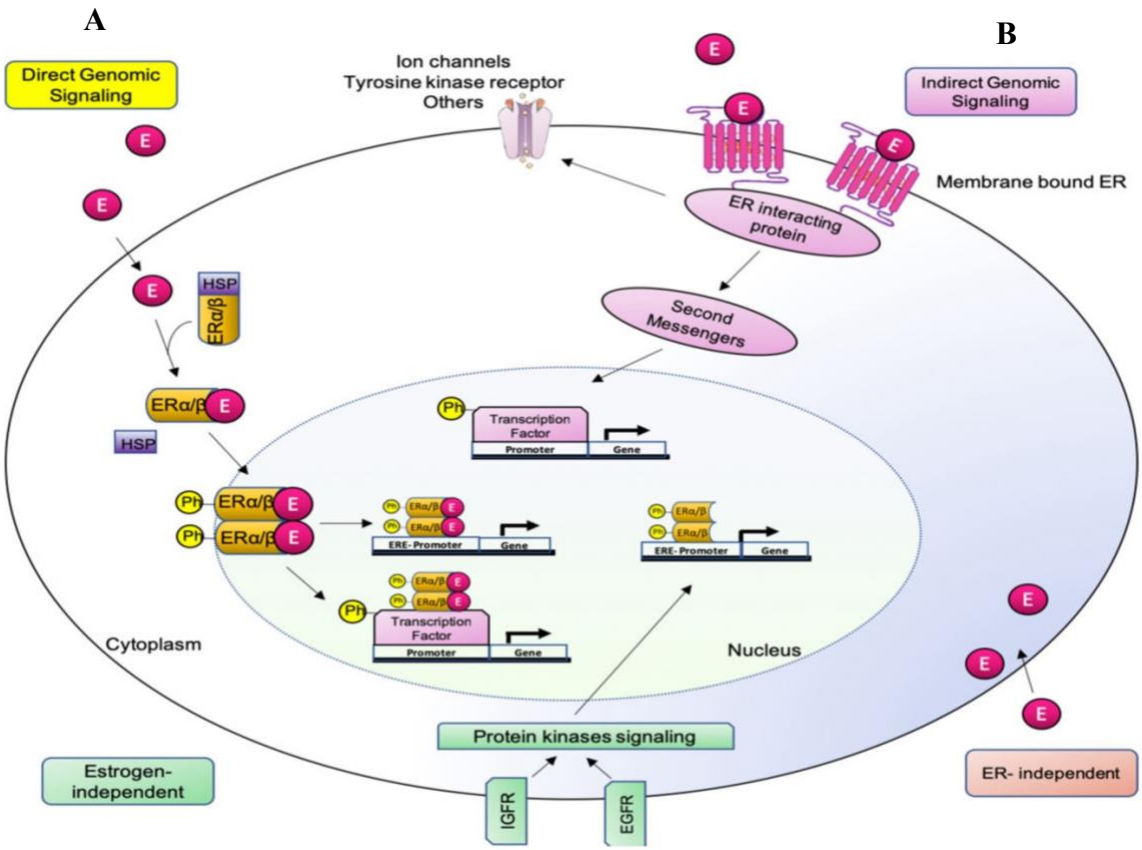


Figure 1.4 Genomic and non-genomic pathways of estrogen signalling

Estrogen-mediated signalling via ERs occurs via two pathways. ***A****) Genomic (Direct Genomic) pathway: estrogen binds to ERs in the cytoplasm and undergoes conformational changes. The estrogen-ER complex is then translocated to the nucleus, where it interacts with chromatin at the estrogen receptor element (ERE) sequences and produces transcriptional changes in the nucleus.* ***B****) Non-genomic (indirect genomic) signalling pathway: estrogens bind to membrane-bound ERs such as GPER1 in the cytoplasm, which activates second messengers, which in turn activate transcription factors. Adapted from (Fuentes and Silveyra, 2019)*

1.6 Endocrine Disruptors and Breast Carcinogenesis

Endocrine disruptors are exogenous compounds or mixtures of compounds that change the function(s) of the endocrine systems, resulting in deleterious health effects for an intact organism, its offspring or subpopulations (World Health Organization (WHO), 2002). These chemicals alter hormonal synthesis, release, transport, metabolism, excretion, binding and effect in the body (Soto and Sonnenschein, 2010). Mechanistically, they can act by interaction with hormone receptor protein complexes or proteins involved in hormone synthesis and delivery (Bergman *et al.*, 2013). They exert their influence via the activation of estrogen receptors (ERs) (Table 1.3), androgen receptors (AR), progesterone receptors (PR), thyroid receptors (TR), serotonin receptors (SR), dopamine receptors, aryl hydrocarbon receptors (AhR), glucocorticoid receptors (GR), peroxisome proliferation activated receptors (PPAR), enzymatic and metabolic pathways (Diamanti-Kandarakis *et al.*, 2009; Giulivo *et al.*, 2016). A diverse range of natural and artificial substances have been known to cause endocrine disruption; these include Bisphenol A (plastic, metal food cans), phthalate (toys, medical devices), parabens (cosmetics, pharmaceuticals), polybrominated biphenyls (flame retardants), and DDT and its metabolites (pesticides) (Table 1.3) (Monneret, 2017). The routes of human exposure to endocrine disruptors are dermal (e.g. body creams), inhalation (polybrominated diphenyl ethers), oral (food contaminants), placental, storage in adipose tissues and exposure to offspring through feeding (e.g. breast milk) (Sharpe and Irvine, 2004).

Table 1.3 Selected environmental chemicals with endocrine-disrupting activity, their use(s) and sources of human exposures (Darbre, 2019; Darbre and Williams, 2022)

Compound	Use	Source of Human Exposure
Polybrominated diphenyl ethers (PBDEs)	Flame retardants	Workplace/ domestic environment/ inhalation
Bisphenol A	Plastics, epoxy resin	Storage of food and beverages (diet), domestic environment
Phthalate esters	Plastics	Domestic consumer products
Parabens	Preservative	Personal care products, food, pharmaceuticals
Benzophenones	UV-filters	Personal care prod
DDT (and metabolites)	Pesticides	Animal fat (diet), restricted uses allow for inhalation and dermal exposures
Phytoestrogens	Natural component of plants	Plant materials in food, nutraceuticals, personal care products
Synthetic estrogens	Pharmaceuticals, cosmeceuticals	Contraceptive pill, hormone replacement therapy, personal care products
Alkyl phenols	Detergents	Workplace/domestic environment, personal care products

1.6.1 Bisphenol A

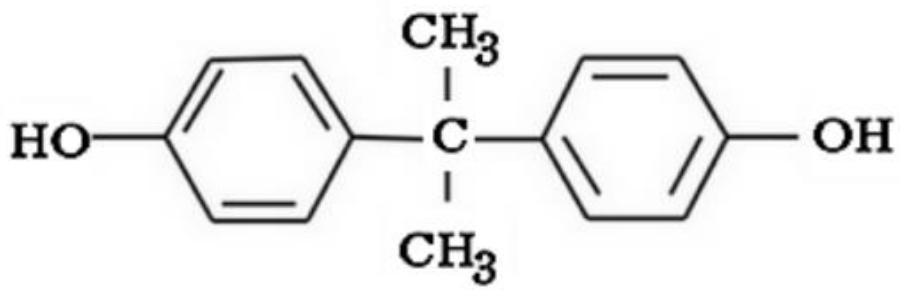


Figure 1.5. Structure of Bisphenol A

Bisphenol A (BPA, 2,2-bis(4-hydroxyphenol) propane is a plasticiser and a monomer used to produce plastics. It is one of the most widely produced chemicals globally, with an estimated 8 million tons produced annually (Vasiljevic and Harner, 2021). It is estimated that the global production of BPA will reach 9.5 million tons/year by 2028, with a market value of USD 26.67 billion (Loganathan *et al.*, 2023). However, the production of BPA has decreased because of the availability of alternatives such as BPS and BPF (Chi *et al.*, 2024). Alternatives to BPA, including bisphenol B (BPB), bisphenol S (BPS), bisphenol F (BPF), bisphenol P (BPP), and bisphenol AF (BPAF), are used commercially to produce BPA-free products (Garrison *et al.*, 2021). Other alternatives commonly regulated by regulatory agencies include bisphenol E (BPE), bisphenol Z (BPZ), bisphenol C (BPC), and bisphenol M (Mhaouty-Kodja *et al.*, 2024; den Braver-Sewradj, van Spronsen and Hessel, 2020). However, it has been shown that some of these BPA alternatives have deleterious effects. For example, BPA analogues (BPAF, BPF and BPS) showed estrogenic and developmental effects in a

zebrafish embryo. It was also shown that BPAF showed greater lethality, developmental toxicity and estrogenicity than BPA (Mu *et al.*, 2018). BPS and BPF have also been shown to interfere with the normal function of the thyroid hormone signalling in both in vitro and in vivo studies (Zhang *et al.*, 2018). Bjornsdotter and colleagues showed BPA alternatives found in paper products in the Netherlands, Spain, Sweden and Norway, including BPS, Pergafast 201, 4-hydroxyphenyl 4-isopropoxyphenyl sulfone (D-8), diphenyl sulphone(D-90), 4,4'-sulfonylbis(2-allylphenol) (TGSA) and 4-(4-prop-2-enoxyphenyl) sulfonyl (BPS-MAE). Analysis showed that BPS showed a lower but significant estrogenic effect compared to BPA. However, Pergafast 201, D-8, D-90, and TGSA showed little or no estrogenic effect compared to BPA but showed developmental effects (Björnsdotter *et al.*, 2017). A comparison of BPA to BPA analogues, such as BPS, BPF, BPAP, BPF, BPZ and BPB, using MCF-7 cell lines, showed that BBP and BPZ had greater estrogenicity than BPA, suggesting that BPA alternatives are not necessarily less estrogenic and have to be used with caution (Mesnage *et al.*, 2017). Overall, some alternatives to BPA have shown effects such as estrogenicity comparable to BPA. However, most of the BPA alternatives have weaker estrogenic effects than BPA.

BPA was originally synthesised as an artificial estrogen in 1890 and was shown in the 1930s to have the potency of estrone in affecting the female reproductive system in rats (Rochester, 2013). It is widely used in the production of polycarbonate (60% global production), epoxy resins (30% of production) and other applications (5%) (Darbre and Williams, 2022; Hahladakis, Iacovidou and Gerassimidou, 2023). Polycarbonate plastics manufacture plates, mugs, cookware and water tanks. The epoxy resins are used to coat the inner surfaces of food cans, drinking water reservoirs, and supply tubing. Other uses of BPA include manufacturing printing ink,

flame retardants, toys and pacifiers with polycarbonate shields, and thermal paper products used for cash receipts and recorders (EFSA Panel on Food Contact Materials *et al.*, 2023).

Humans are exposed to BPA through leaching from products, inhalation of dust from the environment, ingestion and dermal exposures (Prueitt *et al.*, 2023). BPA has been detected in human fluids and tissues such as amniotic fluid (Zhang *et al.*, 2020), breast milk (Altamirano *et al.*, 2015), follicular fluids (Vállez-Gomis *et al.*, 2024), urine (Zhang *et al.*, 2024b), sperm (Santiago *et al.*, 2024), urine (Jamka *et al.*, 2024; Jäger *et al.*, 2023), cord and placental tissues (McCabe *et al.*, 2023).

In the EU, the specific migration limit (SML), which is the maximum amount of chemicals allowed to leach from packaging into food, of BPA is 0.05mg/kg of food (Agriculture and Unit, 2021). The European Food and Safety Agency (EFSA) set a tolerable daily intake (TDI) of 0.2ng BPA/kg/bodyweight/day in 2023 (EFSA Panel on Food Contact Materials *et al.*, 2023). In 2017, Huang and colleagues estimated the global daily intake of BPA using urinary data from 2000-2016 from 30 countries, including the UK. They reported an estimated daily BPA intake of 30.8 ng/kg bw/day for adults and 60.1.ng/kg bw/day for children. They estimated the daily intake in the United Kingdom to be 34.89 ng/kg bw/day using urinary data from 2210 people (Huang *et al.*, 2017). This suggests that the concentration of BPA is still higher in people than the TDI set by EFSA.

1.6.2 Bisphenol A and Breast Cancer

In the body, BPA is metabolized into hepatic BPA-glucuronide, which enables easy urinary elimination, but unconjugated active BPA is responsible for its toxic effects (Vom Saal *et al.*, 2007). BPA binds to both subtypes of the estrogen receptor (ERα

and ERβ), showing a ten-fold affinity for ERβ. However, the affinity of BPA for estrogen receptors is 1000-2000 weaker than estradiol (Delfosse *et al.*, 2012). Studies have shown that even at low concentrations, BPA can activate other pathways and produce molecular effects (Leung *et al.*, 2020; Tonini *et al.*, 2020). BPA has been shown to increase the proliferation of breast cancer cells. Deng and Colleagues showed that BPA increases the proliferation and migration of MCF-7 cell lines (Deng *et al.*, 2021). BPA stimulated the proliferation and progression of the cell cycle in MCF-7 breast cancer cells in a nonmonotonic manner. There was also the upregulation of *ERα*, *pS2*, and *Bcl-2* mRNA (Wang *et al.*, 2020). BPA causes migration, invasion and progression in MDA-MB-231 breast cancer cell lines through the activation of *GPER, FAK, Src* and ERK2-dependent pathways (Castillo Sanchez, Gomez and Perez Salazar, 2016). Similarly, low-dose BPA was able to induce the proliferation of breast cancer cells by activating the ERK1/2/ERRγ signalling (Song *et al.*, 2015). BPA exposure contributes to the poor prognosis of breast cancer through the obesity-inflammation-aromatase axis and the progression of triple-negative breast cancers (Engin and Engin, 2021; Zhang *et al.*, 2016). Low BPA also reduces the efficacy of chemotherapeutic agents in ERα positive and negative breast cancers (LaPensee *et al.*, 2009). BPA has been shown to distort fetal mammary structure formation at very low and environmentally relevant concentrations (Vandenberg *et al.*, 2007; Wadia *et al.*, 2013).

The effect of BPA on breast cancer risk has been tested in many *in vivo* studies. Wormsbaecher and colleagues exposed mice in utero to BPA, which altered the stroma surrounding the mammary gland. Significant transcriptional deregulation of genes involved in cancer after 25μg kg/body weight, including collagen genes, resulted in increased collagen deposition in the mammary glands and stiff extracellular matrix (Wormsbaecher *et al.*, 2020). A mice xenograft exposed to 2.5 μg/L of BPA

through drinking water for 70 days resulted in an estimated 2-fold promotion in the primary tumour growth rate, lymph node metastasis and upregulation of CD206+ polarization of macrophages, suggesting that BPA can accelerate the initiation of invasive breast cancer (Kim *et al.*, 2019). To examine the impact of BPA on fetal programming for mammary tumour susceptibility, fetal mice were treated with 0, 25 and 250 μg/kg BPA via oral gavage. They were subsequently treated with the carcinogen 7,12-dimethylben[a]anthracene (DMBA). The results showed an increase in susceptibility to breast cancer in both low and high-dose BPA compared to controls, suggesting the ability of BPA to increase breast tumorigenesis through molecular alteration of fetal gland programming (Weber Lozada and Keri, 2011). Jenkins and colleagues examined the effect of chronic exposure to BPA on adult breast mammary carcinogenesis. Transgenic mice were fed with different concentrations of BPA (0, 2.5, 25, 250 and 2500 μg/L) via drinking water for 56 to 122 or 252 days. The results showed that BPA significantly increased mammary tumorigenesis and metastasis in transgenic mice in a nonmonotonic manner (Jenkins *et al.*, 2011).

Epidemiological studies on the impact of BPA on breast cancer risk have been mixed. In the Wisconsin Breast Density Study involving 264 postmenopausal women, BPA was found to be positively associated with increased breast density, which is a breast cancer risk (Sprague *et al.*, 2013). Increased breast density was associated with both low and high urinary BPA concentrations of 200 adolescent girls in the Growth and Obesity Cohort Study in Chile (Binder *et al.*, 2018). A prospective cohort study of the urinary BPA from 8,035 participants in the USA showed that a BPA concentration of less than 1.99 ng/mL was associated with a higher risk of cancer mortality (Yuan *et al.*, 2024). However, a meta-analysis of 9 studies, comprising 7820 breast cancer cases and controls, showed no correlation between breast cancer and BPA (Liu *et al.*,

2021). A Multiethnic Cohort Study of 1,032 postmenopausal women with breast cancer and 1030 matched controls found no correlation between BPA and breast cancer risk (Wu *et al.*, 2021)

1.6.3 *p, p*'-DDE

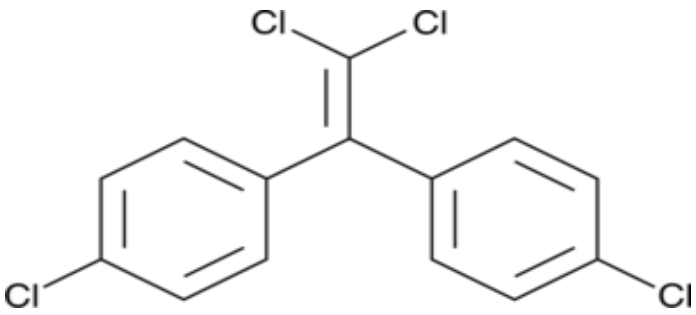


Figure 1.6 Chemical structure of p,p'-DDE

p,p' DDE (1 dichloro 2,2 bis (p chlorophenyl) ethylene) is one of the major metabolites of the organochlorine pesticide DDT (1,1,1 trichloro-2,2 bis(p chlorophenyl) ethane) (Koureas *et al.*, 2019). It is an endocrine disruptor in both humans and wildlife (Burgos-Aceves *et al.*, 2021). Like its parent compound, *p,p'*-DDE is lipophilic, stable, persistent, and bioaccumulated in the environment and human adipose tissues (Mirmigkou and de Boer, 2016). It is not easily metabolised and has a half-life of 10 years in human plasma (Wong *et al.*, 2015; Rogan and Chen, 2005). It is more detected in the human body than DDT (Yipei *et al.*, 2022). Humans are exposed to *p,p'* DDE primarily through diet due to its bioaccumulation in the food chain (Kezios *et al.*, 2013). *p,p*'- DDE is easily absorbed by the gastrointestinal tract and the highest concentrations in the body are found in the adipose tissue (Kirman *et al.*, 2011). It can also be passed on from the placenta and breast milk to the foetus and babies (Rogan

and Chen, 2005). An analysis of the breast tissue of 51 women with different breast health at the Walter Reed National Military Medical Center showed that *p,p'*-DDE was the most abundant organochloride (Ellsworth *et al.*, 2018). A prospective study of 93,000 daughters in the Child Health and Development Studies (CHDS) showed that maternal exposure to o,p'-DDT increased the risk of breast cancer for the daughter (odds ratio 3.7, 95 CI 1.5-9.0) by the age of 52 years (Cohn *et al.*, 2015). Another prospective study of 309 daughters from CHDS showed that in utero exposure to o,p'-DDT increased the breast density of the daughter by 6% (95 CI, 1.5-10.8, p-value= 0.01) (Krigbaum *et al.*, 2020).

A complex mixture of organochloride (OCs) containing *p,p'*-DDE caused the proliferation of MCF-7 breast cancer cell lines due to its estrogenic activity (Aube, Larochelle and Ayotte, 2011). *p,p'*- DDE was able to cause proliferation in CAMA-1 and MCF7 cell lines in a dose-dependent manner (Aubé, Larochelle and Ayotte, 2008). Prepubertal mice were exposed to *p,p*'-DDE at human-relevant concentrations, which accelerated the initiation of mammary tumours (Johnson *et al.*, 2012). A mixture of organochlorides, including *p,p'*-DDE, was able to induce mammary lesions but did not cause obvious tumours in neonatal rats (Desaulniers *et al.*, 2001).

Kloas and Hoffman demonstrated in *Xenopus laevis* that *p,p'*-DDE has both estrogenic and anti-androgenic effects. They did this by exposing male Xenopus laevis to *p,p'*-DDE and analysing their mate-calling behaviour. They reported an alteration and disruption in the mate-calling when exposed to p,p'-DDE, which resulted in reduced reproduction (Hoffmann and Kloas, 2016). *In vitro* studies showed that *p,p'*-DDE increased proliferation in breast cancer cell lines CAMA-1 and MCF7 in a dose-dependent manner (Aubé, Larochelle and Ayotte, 2008).

The causal link between organochlorine pesticides such as *p,p'*-DDE and breast cancer has been inconclusive. Some epidemiological studies have shown a causal link between DDT and its analogues, such as *p,p'*-DDE and breast cancer (Høyer *et al.*, 2000; Romieu *et al.*, 2000; Arrebola *et al.*, 2015). This was, however, not supported in other epidemiological studies that found no link between organochlorine compounds and breast cancer (Itoh *et al.*, 2009; López-Cervantes *et al.*, 2004; Iwasaki *et al.*, 2008). A meta-analysis of studies published between 2004-2012 on DDE exposure and breast cancer did not show a positive correlation (Ingber *et al.*, 2013). Another meta-analysis of 35 studies, hospital-based case-control, population-based case-control, and nested case-control, showed that exposure to *p 'p*-DDE did not elevate breast cancer risk in humans (Park *et al.*, 2014).

A case-control study (72 women with breast cancer and 78 controls) to examine the effect of residual DDT in agricultural soils revealed DDT metabolite, *p,p'*-DDE, was found to increase breast cancer risk in many agrarian regions in China (Tang *et al.*, 2014). In the Carolina Breast Cancer Study Phase I, 456 white and 292 black women had their blood samples measured for DDE/DDT 4.1 months after diagnosis. It showed that *p,p'*-DDE exposure negatively impacted breast cancer survival (Parada Jr *et al.*, 2019). A study of 100 women (50 breast cancer cases and 50 controls) at the Addis Ababa University Oncology on the impact of organochlorides on breast cancer risk in 2020 showed that although DDT was associated with increased breast cancer risk, its metabolites, such as p,p'-DDE, were not among Ethiopian women (Mekonen *et al.*, 2021).

1.6.4 Parabens

Parabens are a class of para-hydroxybenzoic esters with alkyl groups of varying length (methyl to butyl or benzyl), which are widely used as preservatives in cosmetics, shampoos, skin care products, toothpaste, fragrances, pharmaceuticals, food and beverages (Jonkers *et al.*, 2009). Parabens are absorbed orally, subcutaneously and orally (Aubert, Ameller and Legrand, 2012). Parabens have been detected in human breast milk (Ye *et al.*, 2008). They have also been detected in normal breast tissues (0.15 to 0.31 ng/mg), with higher levels detected in cancerous tissues (1.01 ng/mg) (Alampanos *et al.*, 2020). Parabens have been extracted from human breast tumours using high-pressure liquid chromatography followed by tandem mass spectrometry, indicating that parabens can be found intact in breast tissues (Darbre *et al.*, 2004). Methyl paraben, propyl paraben, ethyl paraben, butyl paraben and isobutyl paraben have been detected in urine samples of women, men and children in Spain, Canada, and the United States (Casas *et al.*, 2011; Ye *et al.*, 2006; Genuis, Birkholz and Curtis, 2013).

Paraben compounds have shown estrogenic activity in *in silico* and *in vitro* approaches (Wei, Cheng and Sang, 2022). However, Routledge and colleagues demonstrated that the estrogenic effect of parabens is weaker than that of 17β-estradiol (Routledge *et al.*, 1998). This estrogenic activity has been demonstrated in in vitro studies using ZR-75-1 and MCF7 breast cancer cell lines (Okubo *et al.*, 2001; Darbre *et al.*, 2003). The estrogenicity of parabens has also been demonstrated in *in vivo* experiments in immature mice. After mice were exposed to three daily doses of 1.2 or 12 mg of isobutylparaben subcutaneously, the uterine weight increased (Darbre *et al.*, 2002). Parabens bind to estrogen receptors (ERα and ERβ) in both breast cancer cell lines

(MCF-7) and non-cancerous cell lines (MCF-10A), suggesting their roles in contributing to breast cancer risk (Wróbel and Gregoraszczuk, 2014).

Paraben induced oncogene expression and caused the proliferation of the HER2-positive human BT-474 breast cancer cell line via estrogen receptor activation (ERα) (Pan *et al.*, 2016). Parabens measured at human breast tissue concentrations, acting individually and as a mixture, induced proliferation in MCF7 cells in vitro, suggesting that to assess the effect of parabens, they must be tested as a mixture and not individually (Charles and Darbre, 2013). Long-term exposure to paraben increases proliferative, migratory, and invasive activity in human breast cancer cells (MCF7, T47-D, and ZR-75-1) (Khanna, Dash and Darbre, 2014). Parabens are chemicals of concern, and their exposure impacts breast cancer risk because of their ubiquitous presence in human breast tissues, their ability to induce proliferative, invasive, and migratory activity in breast cancer cell lines, and the suppression of breast cancer cell proliferation (Darbre and Harvey, 2014).

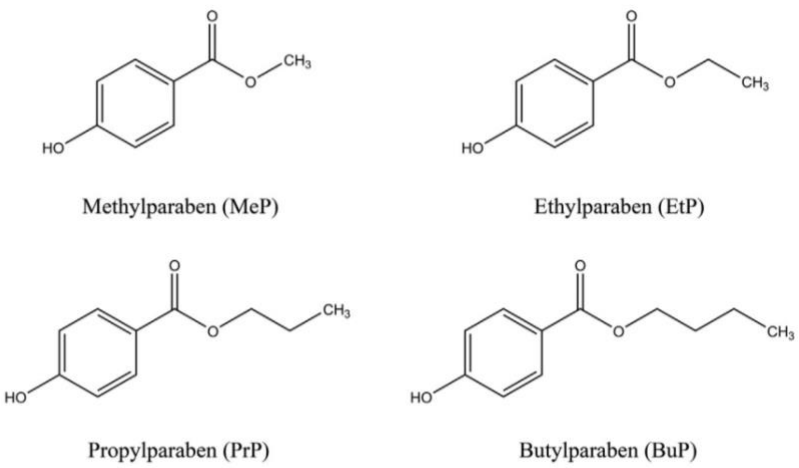


Figure 1.7. *Chemical Structure of Methylparaben (MeP), Ethylparaben (EtP), Propylparaben (PrP) and Butylparaben (BuP).*

1.6.5 Musks

Synthetic polycyclic musks are a class of aromatic, lipophilic compounds consisting of a benzene ring. Synthetic musks include classes of chemicals such as nitro-musks, polycyclic musks and macrocyclic musks (Sumner *et al.*, 2010). Of these classes, polycyclic musks are widely used because of their excellent aroma, low cost, affinity to fabrics and stability under light and alkaline conditions (Li *et al.*, 2020b). Synthetic musks are widely used as fragrances in soaps, perfumes, cosmetics, air fresheners, detergents and shampoos (Gooding *et al.*, 2006).

Twelve synthetic musks are commercially used in personal care products (Pinkas, Gonçalves and Aschner, 2017). The most widely used polycyclic musks (95% of EU market and 90% of US market) are galaxolide (HHCB,1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran)and tonalide (AHTN, 7-acetyl-1,1,3,4,4,6 hexamethyl tetrahydronaphthalene) (Santiago-Morales *et al.*, 2012). Globally, 4500 tons of galaxolide and tonalide are produced annually (Ayuk-Takem *et al.*, 2014). They are commonly detected in surface water and have been found in wastewater plant (WWTP) influents and effluents in the USA, Germany, Spain, and the Western Balkans (Lange, Kuch and Metzger, 2015). In the UK, Galaxolide (987-2098 ng/L) and Tonalide (55-159 ng/L) were detected in effluents at the Tamar and Plym Estuaries (Sumner *et al.*, 2010).

They are lipophilic with a high octanol/water partition coefficient (K_{ow}) and, therefore, can bioaccumulate in human fat, blood, breast milk, and aquatic organisms and adhere to sediments (Lange, Kuch and Metzger, 2015; Ehiguese *et al.*, 2019). Using the E-screen assay, Bitsch and colleagues showed that Tonalide increases cell proliferation in MCF-7 breast cancer cell line. However, the estrogenic effect was

significantly lower than 17β-estradiol (Bitsch *et al.*, 2002). On the other hand, galaxolide showed antiestrogenic activity by inhibiting the activity of 17β-estradiol in a yeast estrogenicity assay (YES) (Simmons *et al.*, 2010). An *in vitro* reporter assay showed that tonalide and galaxolide showed both estrogenic and antiestrogenic activity depending on the cell line and the subtype of the estrogen receptor that was targeted (Schreurs *et al.*, 2002). Another reporter gene assay showed that tonalide and galaxolide had antiestrogenic, antiandrogenic and antiprogestogen activity (Van Der Burg *et al.*, 2008). Cavanagh and colleagues also confirmed the estrogenicity of galaxolide (Cavanagh *et al.*, 2018). Sub-lethal level tonalide and galaxolide have been shown to accelerate cell metastasis of glioblastoma spheroids (Doğanlar *et al.*, 2021).

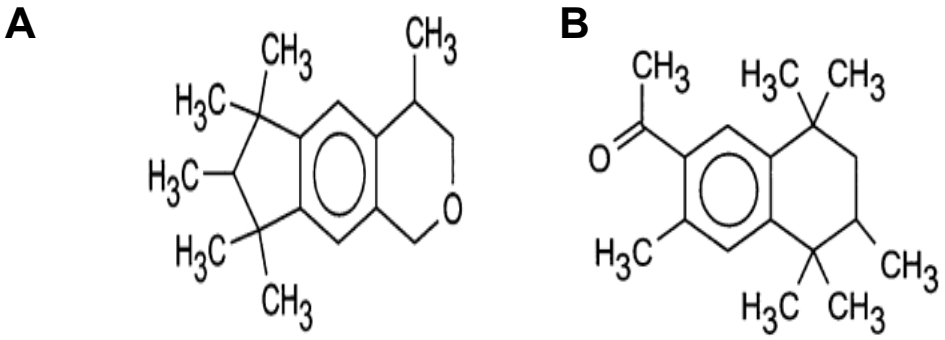


Figure 1.8. *Chemical Structure of musks: (A) Galaxolide and (B) Tonalide*

1.6.6 3-Benzylidin Camphor

3-benzylidene camphor (3-BC) is a UV filter used in sunscreens and cosmetic products (Søeborg *et al.*, 2006; Kunz, Gries and Fent, 2006). A recombinant yeast assay has shown that 3-BC has estrogenic activity by activating the human ERα (Kunz and Fent, 2006). It induces the proliferation of MCF-7 cell line and has been shown in the rat uterotrophic assay to be strongly selective towards ERβ (Schlumpf *et al.*, 2004).

The Yeast Estrogen Screen (YES) was used to test for the estrogenicity of 3-BC. It showed that at an EC_{50} value of 44.2μM, 3-BC activated human estrogen α (Schmitt *et al.*, 2008).

Due to their lipophilic nature, they can be absorbed and accumulate in the skin after prolonged topical application.3-BC was detected in rat adipose tissues, muscle, brain, liver, and plasma following dermal application of the chemical, indicating passage through the skin (Søeborg *et al.*, 2006; Paul, 2019).

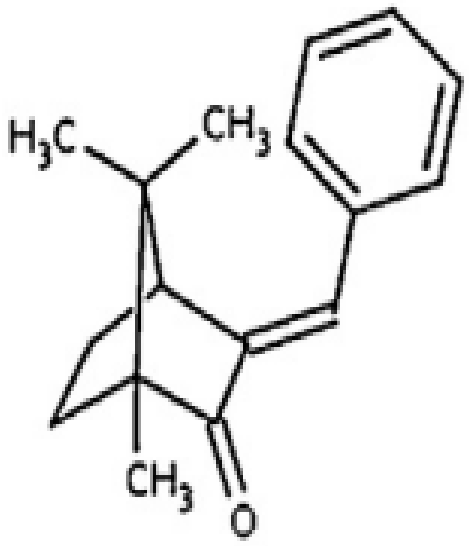


Figure 1.9. *Chemical Structure 3-Benzylidin Camphor*

1.6.7 Benzophenones

Benzophenones(BPs) are a class of aromatic ketones consisting of two main rings linked by a carbonyl group (Mao, He and Gin, 2019). BPs are able to absorb ultraviolet-A (UV-A, 315-400nm) and ultraviolet-B (UV-B, 280-315nm) radiations (Kerdivel *et al.*, 2013). They are used extensively to manufacture sunscreens, food packaging, clothing, optical lenses, agricultural chemicals, shampoos, toothpaste, deodorants, pharmaceuticals and cosmetics (Coutinho, Vianna and Marques, 2022; Fent, Zenker

and Rapp, 2010). Structurally, BPs consist of two benzene rings connected by a carbonyl group with different substitutions, yielding twelve derivates used in commercial products (BP1-BP12) (Park *et al.*, 2013). Benzophenone derivatives are absorbed through the skin and bioaccumulated in humans and wildlife due to their lipophilicity (Suzuki *et al.*, 2005; Kerdivel *et al.*, 2013). In Europe, most sunscreen products contain about 10% benzophenone (Guo *et al.*, 2020a). They are mostly released into the environment by bathing in water bodies, washing personal hygiene into wastewater treatment plants (WWTPs), and leaching from plastics and coatings (Carstensen *et al.*, 2022).

Benzophenone-3 (BP-3, 2-hydroxy-4-methoxybenzophenone, oxybenzone) was synthesised in 1906 and approved by the United States Food and Drug Administration for use in the 1980s. It is one of the widely used derivatives of benzophenones in the manufacture of sunscreens and cosmetics as a UV filter. It is also approved to be used in building materials, furniture, toys, and food plastic containers (Mustieles *et al.*, 2023).Benzophenone-2(BP-2,2',2',4,4'-tetrahydroxybenzophenone) is a benzophenone derivate used in cosmetics, acrylic adhesives, plastics, and coatings (Downs *et al.*, 2014). In the EU and America, BP-2 has been banned in sunscreens due to the high concentration normally used in the formulations (de Sousa *et al.*, 2016). BP-3 showed an estrogenic effect in MCF-7 breast cancer cells by increasing cell proliferation (Schlumpf *et al.*, 2001). Long-term exposure to BP-3 increases migration and invasion of MCF-7 and MDA-MB-231 breast cancer cell lines through estrogen-dependent and estrogen-independent mechanisms (Alamer and Darbre, 2018). *In vivo,* estrogenicity of BP-3 has been reported in uterotrophic assays, where immature rats fed with 1525 mg/kg bw/day exhibited a 25% increase in uterine weight (Schlumpf *et al.*, 2001). BP-3 elicited epithelial mammary tumorigenesis in mice that had been

fed a lifelong high-fat diet but was protective in mice fed a lifelong low-fat diet (Kariagina *et al.*, 2020).

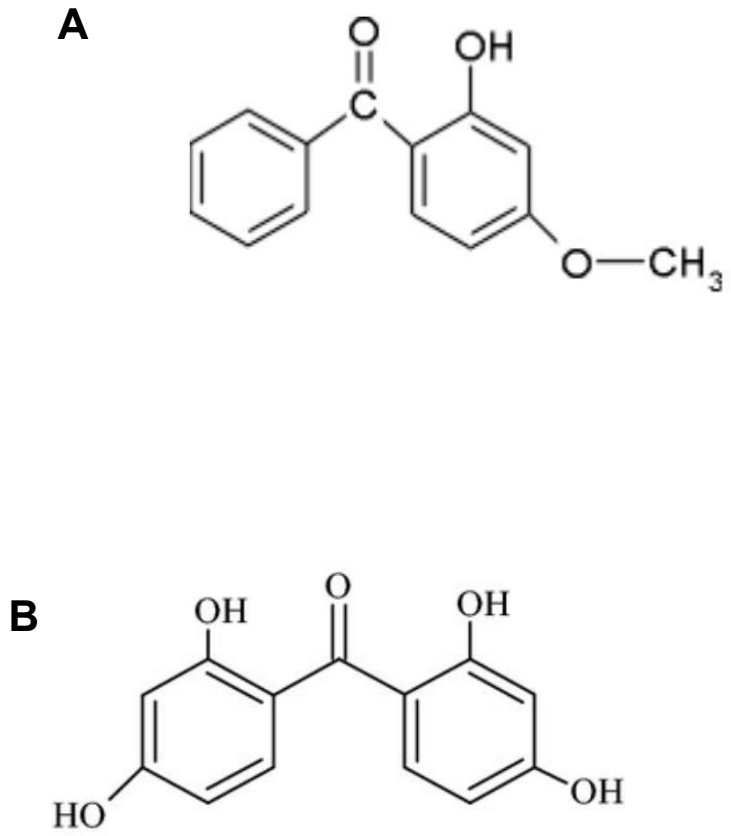


Figure 1.10 *Structure of (A) Benzophenone-3 and (B) Benzophenone-2*

1.6.8 BDE 100

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants (BFRs) that are added to resins and polymers to reduce the risk of flammability (Stapleton *et al.*, 2008). The commercially produced PBDEs are pentabromodiphenyl ethers (Penta-BDE), octabromodipehenyl ethers (Octa-BDE), and decabromodiphenyl ethers (Deca-BDE) (Linares, Bellés and Domingo, 2015). The use of PBDEs has been phased out in many countries, as they have been found in human tissues worldwide,

suggesting ongoing exposure (Kanaya *et al.*, 2019). Humans are exposed to PBDEs through oral ingestions through diet and dust, atmospheric inhalation, and dermal contact. The PBDE and its congeners have been detected in human serum, breast milk and fat tissues (Wu *et al.*, 2020). The estrogenicity of PBDEs has been shown in a luciferase reporter gene assay using a T47D breast cancer cell line. BDE-100 showed the most potent estrogenic activity of the 11 PBDEs tested (Meerts *et al.*, 2001). PBDE showed estrogenic activity by increasing proliferation in the MCF-7 breast cancer cell line (Mercado-Feliciano and Bigsby, 2008).

A case-control study of 209 breast cancer cases and 165 controls in Shantou, China, between January 2014 and May 2016 showed that BDE-100 was positively associated with breast cancer risk (He *et al.*, 2018).

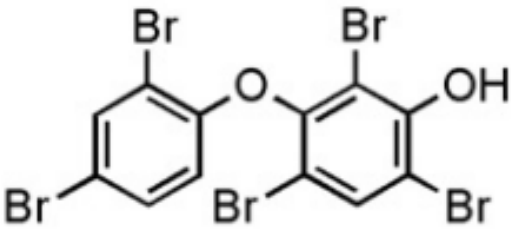


Figure 1.11 Structure of BDE-100

1.7 Fatty Acids and Breast Carcinogenesis

1.7.1 Fatty Acids

Fatty acids are a diverse group of molecules that are important cellular building blocks and act as energy sources via β-oxidation, membrane building blocks and cytokine synthesis (Falomir-Lockhart *et al.*, 2019). Structurally, they are hydrophobic molecules made up of a carboxylic group and a chain of carbon and hydrogen atoms. They can be grouped in four different ways depending on the presence and number of double bonds between the carbon atoms, the number of carbon atoms, the position of the double bonds and the orientation of the double bond. Based on the type of bonds, fatty acids are divided into saturated (no double bond) and unsaturated (presence of double bonds). Unsaturated fatty acids are divided based on the number of double bonds: single double bonds (*monounsaturated*, MUFAs) and multiple double bonds (*polyunsaturated*, PUFAs) (Maltsev and Maltseva, 2021). They can also be classified based on the number of carbon atoms in the structure. Based on the carbon number, saturated fatty acids are divided into short chain (3-7 carbon atoms), medium chain (8-13), long chain (14-20 carbon atoms) and very long (>21 carbon atoms). Unsaturated fatty acid can be divided into three groups based on carbon length: short-chain (<19 carbon atoms), long-chain (20-24 carbon atoms) and very long chain (>25 carbon atoms. The orientation of the carbon atoms in relation to the double bond gives rise to *cis* or *trans* fatty acid (Kalish, Fallon and Puder, 2012). Fatty acids can alter cellular processes such as metabolism, gene expression, hormone response, and the production of bioactive lipid mediators. These perturbations can affect physiological activities and health and cause disease states (Calder, 2015).

Generally, fatty acids can be synthesised *de novo* from sugars such as glucose, sucrose and their derivates (Zhai *et al.*, 2021). Cells can also obtain fatty acids from

blood circulation through transporter-mediated uptake or passive permeation. This uptake is facilitated by transporter proteins such as CD36, also called fatty acid translocase (FAT), fatty acid transport protein family (FATPs), and plasma membrane fatty acid-binding protein (FABpm)(Koundouros and Poulogiannis, 2020). Healthy cells use circulation fatty acids; however, cells synthesise fatty acids when they become malignant (Vander Heiden, Cantley and Thompson, 2009; Ray and Roy, 2018). Fatty acids are a major metabolic substrate to meet the high energy demand needed to sustain the rapid proliferation of cancer cells (Hanahan and Weinberg, 2011).

Increased lipogenesis is one of the important metabolic characteristics of cancer cells. Depending on the cancer type, cancer cells can synthesise 95% of saturated and monosaturated fatty acids *de novo* (Zaidi *et al.*, 2013). Elevated levels of lipogenic enzymes such as FASN and SREBP mark increased fatty acid synthesis in cancer cells (Williams *et al.*, 2013; Flaveny *et al.*, 2015). Inhibition of genes involved in the *de novo* synthesis of fatty acids has become important in developing effective therapy for different types of cancers (Danhier *et al.*, 2017).

A causal link between dietary fatty acids and breast cancer risk has not been conclusively established (Matta *et al.*, 2021). A case-control study to assess the association between saturated fatty acids and breast cancer of 1661 breast cancer cases and 1674 controls in China revealed that all subgroups of fatty acids (medium chain, long chain and very long-chain) were inverse to the odds of developing breast cancer (Jiang *et al.*, 2024). A hospital-based case-control study including 1589 breast cancer cases and 1621 controls showed that higher intake of polyunsaturated fatty acid (n-3 PUFA) and its subtypes resulted in lower breast cancer risk (Zhang *et al.*, 2022). A multicentre case-control in Spain (EpiGEICAM study) conducted a food and

lifestyle audit via a questionnaire of 1017 breast cancer cases and controls. The study showed that women with higher serum concentrations of stearic acid, linoleic acid, and arachidonic acid had lower breast cancer risk. In contrast, women with higher levels of serum palmitoleic acid, palmitelaidic acid and elaidic acid showed a higher risk of breast cancer (Lope *et al.*, 2020). Another case-control study recruited 473 women with breast cancer and 501 healthy individual women as controls. Dietary intakes of saturated fatty acids were validated by a food frequency questionnaire. The results established women who consumed a high intake of polyunsaturated fatty acid were 1.5 times more likely to develop breast cancer as compared to controls. There was a positive association between saturated fatty acids and breast cancer risk among postmenopausal women (Mozafarinia *et al.*, 2021). Lipid synthesis and alteration in lipid-induced signalling are some of the ways fatty acids contribute to breast cancer initiation and progression (Baumann, Sevinsky and Conklin, 2013).

The European Prospective Investigation into Cancer and Nutrition (EPIC) study used questionnaires to investigate the dietary fatty acid intake of 60 individuals. An analysis of the plasma fatty acids showed the presence of different fatty acids (Huybrechts *et al.*, 2023). The fatty acids chosen for this project were among the most frequently consumed in Europe.

1.7.1.1 Palmitic Acid

Palmitic Acid (16:0, PA) is the most common saturated fatty acid in the human body. It is supplied in diet or synthesised endogenously from other fatty acids, carbohydrates, and amino acids. Palmitic acid accounts for 20-30% of total fatty acids in membrane phospholipids and adipose triacylglycerols (TAG) (Carta *et al.*, 2017). It is primarily found in palm oil (44% of all fatty acids in palm oil). It is also found in meat

and dairy products (50-60%), cocoa butter (26%), olive oil (8-20%) and breast milk (20-30%) (Innis, 2016). The concentration of palmitic acid in breast milk is 20-30% of total fat. This concentration is unaffected by dietary changes, made possible by endogenous biosynthesis of palmitic acid by de novo lipogenesis that counterbalances dietary intake (Carta *et al.*, 2017). This tight homeostatic regulation of palmitic acid is essential due to its importance in physiological processes such that disruption causes diseases such as atherosclerosis, cancers and neurodegenerative conditions (Innis, 2016; Carta *et al.*, 2017). A causal link between palmitic acid and breast cancer has been inconclusive so far. A meta-analysis of 3 cohort studies and 7 case-control studies comprising 2,031 cases and 2,334 controls was conducted to establish the relationship between breast cancer and fatty acid biomarkers. In the cohort studies, palmitic acid was associated with increased breast cancer risk (Saadatian-Elahi *et al.*, 2004). The dietary intake of specific fatty acids among 722 postmenopausal women in the VITAL (VITamins And Lifestyle) cohort study. The study used a semi-quantitative Food Frequency Questionnaire (FFQ) to estimate the frequency and portion sizes of 120 foods and beverages consumed in the previous year. The study reported a strong association between fatty acids such as palmitic acid 1.68 (95% CI 1.13-2.50, p-trend =0.02) and breast cancer (Sczaniecka *et al.*, 2012). However, a study carried out in Northern Italy found no link positive link between breast cancer incidence and palmitic acid (Pala *et al.*, 2001).

1.7.1.2 Stearic Acid

Stearic acid (IUPAC: octadecanoic acid) was purified and identified in 1823 by Michel-Eugene Chevreul (Patti *et al.*, 2021). It is an 18-carbon (C18) saturated fatty acid and one of the major SFA in Western diets alongside palmitic acid (Loften *et al.*, 2014; van

Rooijen and Mensink, 2020). Foods rich in stearic acid include vegetable fat, beef, chocolate and dairy (Shen *et al.*, 2014; Loften *et al.*, 2014). It is used extensively in the cosmetic, pharmaceutical, and plastics industries (Patti *et al.*, 2021). Stearic acid has been shown to selectively induce apoptosis in cancerous cells in a time and dose-dependent manner (Evans *et al.*, 2009a). It has also shown anti-metastatic properties to breast cancer by reducing the size of tumours by 50% in athymic nude mice (Evans *et al.*, 2009b). Exposure of human breast cancer cell lines (MDA-MB-361, MCF-7 and MDA-MB-231) to esters of stearic acid showed an inhibition of cell proliferation, suggesting an anti-cancer effect of stearic acid (Khan *et al.*, 2013). Some evidence suggests a protective effect of stearic acid. For example, it has been shown that Stearic acid inhibited epidermal growth factor (EGF) (Wickramasinghe *et al.*, 1996) and prevented breast cancer neoplastic progression at G_1 and G_2 phases at physiological conditions (Li *et al.*, 2011). Conversely, a cohort study of 722 women postmenopausal women (50-76yrs) showed an increase in breast cancer risk due to intake of saturated fatty acids such as stearic acid 1.65 (95 CI: 1.12-2.43, P-trend = 0.02) (Sczaniecka *et al.*, 2012).

1.7.1.3 Elaidic Acid

Trans fatty acids are a class of unsaturated fatty acids having at least 1 unconjugated double bond in their *trans* configuration (Chajès *et al.*, 2011). TFAs are grouped into two depending on their sources: industrial TFA (iTFA) produced from the partial hydrogenation of vegetable oils and ruminant TFA (rTFA) from the bacterial hydrogenation in the rumen and mostly found in milk and meat of ruminants (Kuhnt *et al.*, 2011; Kuhnt, Degen and Jahreis, 2016). Industrial fatty acids (iTFA) constitute an estimated 9% of total energy consumption (Krogager *et al.*, 2015). Elaidic acid (trans-

octadecanoic acid, C18:1 trans9; t9 and C18:1 t10) is the major industrial trans fatty acid (iTFA). It is a semi-solid monosaturated fatty acid with a double at position 9 in the trans configuration and has a melting point between 44.5-45°C (Kuhnt, Degen and Jahreis, 2016; Kwon, 2016; Vendel Nielsen *et al.*, 2013). It is an exogenous fatty acid and is supplied through dietary sources such as margarine, cakes and other industry-processed foods (Michels *et al.*, 2021).

A systematic review of 46 articles showed that dietary TFAs, including elaidic acid, showed a positive association with colorectal cancer and prostate cancer but not breast cancer (Michels *et al.*, 2021). On the other hand, breast cancer risk in 318,607 women aged 35 years and older in the European Prospective Investigation into Cancer and Nutrition (EPIC) enrolled between 1992-2000 showed that increased elaidic acid consumption led to an elevated breast cancer risk (1.14 95% CI: 1.06-1.23 p trend = 0.001) (Matta *et al.*, 2021).

1.7.1.4 Linoleic Acid

Linoleic acid (LA, 18:2n-6) is the major n-6 polyunsaturated fatty acid (PUFA) that is found in Western diets, accounting for 7% of daily calorie intake (Taha, 2020). It is an essential fatty acid supplied through dietary sources such as vegetable oils, nuts, cereals, legumes, meats, eggs and milk (Marangoni *et al.*, 2020). It is a structural component of membrane phospholipids that keeps membrane fluidity and water permeability of the epidermis (Whelan and Fritsche, 2013). Linoleic acid and its metabolites have been implicated in various metabolic disorders and cancers (Vangaveti *et al.*, 2016). The role of linoleic acid in breast cancer has been inconclusive.

Linoleic acid increased the growth of T47D cells by activating the estrogen receptor (ERα), G13α G protein, and p38 MAP kinase gene (Serna-Marquez *et al.*, 2017). In MDA-MB-231 cells, linoleic acid promoted migration and invasion of breast cancer cells by activating the EGFR-/PI3K-/Akt pathway (Serna-Marquez *et al.*, 2017).

Linoleic was shown to promote mammary tumours due to its role in tumour prostaglandins (PG), but this effect has not been mechanistically elucidated (Cohen *et al.*, 1986). A combined meta-analysis and review of in vivo experiments, case-control, prospective, and ecological human trials showed that high intake of linoleic acid did not have a significant increase in breast cancer risk (Zock and Katan, 1998). A review of animal and epidemiologic studies showed that linoleic acid is involved in mammary cancers, possibly because it causes oxidative DNA damage, increased cell proliferation, and elevated levels of free estrogen (Bartsch, Nair and Owen, 1999). However, a meta-analysis of eight prospective cohort studies and four nested control case-control studies showed that dietary linoleic acid reduced breast cancer risk, although the reduction was not statistically significant (Zhou *et al.*, 2016).

1.4 List of Fatty Acids and References

Fatty Acid	References
Palmitic Acid	(Sczaniecka *et al.*, 2012), (Pala *et al.*, 2001; Carta *et al.*, 2017; Innis, 2016)
Linoleic Acid	(Serna-Marquez *et al.*, 2017), (Cohen *et al.*, 1986)
Stearic Acid	(Sczaniecka *et al.*, 2012), (Wickramasinghe *et al.*, 1996), (Khan *et al.*, 2013), (Zhou *et al.*, 2016; Matta *et al.*, 2021)
Elaidic Acid	(Matta *et al.*, 2021), (Michels *et al.*, 2021), (Kuhnt, Degen and Jahreis, 2016)

1.8 Mixture Effect of Chemicals

Human exposure to environmental toxicants does not occur as a single chemical exposure. Generally, humans are exposed to low doses of mixtures of chemicals, and the exposures may be episodic, chronic, sub-chronic or acute (Teuschler and Hertzberg, 1995). Elucidating and quantifying the potential deleterious effect of chemical mixtures has been an age-long challenge in pharmacology and toxicology (Liu and Sayes, 2024). Despite the ubiquitous nature of chemical mixtures in the environment, regulatory bodies have historically focused on a single chemical risk assessment approach in their legislation (Backhaus and Faust, 2012). However, over the last few decades, legislation and frameworks have been developed to assess the potential effect of chemical mixtures (Lasch *et al.*, 2020), seeing as *in vitro* and *in vivo* studies have shown that endocrine-disrupting compounds (EDCs) can act in combination at low and ineffective levels to elicit significant and detectable effects (Silva, Rajapakse and Kortenkamp, 2002; Chen *et al.*, 2015; Benoit *et al.*, 2022; Howdeshell *et al.*, 2008).

Evidence shows that humans are exposed to a cocktail of compounds in breast tissues. Earlier studies by Aronson and colleagues detected the presence of a mixture of organochlorides and polychlorinated biphenyls in breast tissues. They found these chemicals after analysing 217 breast cancer cases and 213 benign cases (Aronson *et al.*, 2000). An analysis of thirty-nine food samples and five mammary glands collected in Shanghai, China, from 2000-2001 showed the presence of low concentrations of environmental chemicals such as *o,p'*-DDT, chlordane compounds (CHLs), hexachlorobenzene (HCB), and polychlorinated biphenyls (PCBs) (Nakata *et al.*, 2002). Another study analysing maternal serum, urine and amniotic fluids from 200 pregnant women recruited between 2001-2004 in Denmark showed the presence of

parabens (methylparaben and ethylparaben), phenols (2,4-chlorophenol, 2,5-chlorophenol, 2-polyphenols), and phthalate metabolites (mono-(2-carboxymethyl-hexyl)phthalate and mono-carboxy-*iso*-octyl phthalate at very low concentrations (Bräuner *et al.*, 2022). Furthermore, Wang and colleagues analyzed 20 adipose tissues sourced from New York City, USA, for the presence of environmental chemicals. They reported the presence of BPA, BP-3, and parabens (Wang, Asimakopoulos and Kannan, 2015). The presence of these mixtures of chemicals in the human body at low concentrations ultimately increases an individual's 'estrogenic load' and could consequently increase breast cancer risk (Kortenkamp, 2008).

1.8.1 Models For Measuring Mixture Effects

Two models have been developed to predict the expected effect of a mixture of chemicals: concentration addition (CA) and independent action (IA) (Lasch *et al.*, 2020). Concentration addition (also known as dose or response addition) was developed by German scientist Loewe in 1926 (Loewe, 1926). It is the concept generally used in risk assessment of mixture toxicity and is applied in mixtures where individual chemicals have similar mechanisms of action. This concept assumes that chemicals behave as dilutions of each other and, therefore, allow for a mixture effect even if the individual components are present at a no-effect level. The contribution of each chemical to the overall toxicity of the mixture is directly proportional to their respective concentrations (Kortenkamp, Backhaus and Faust, 2009). Independent action (IA) was introduced by Bliss (Bliss, 1939) and is generally applicable to mixtures where the individual components have different mechanisms of action. It assumes that the chemicals in the mixture affect the same target tissues, cells, and molecular receptors (Backhaus and Faust, 2012). However, it is seldom used in risk assessment

of mixture toxicity because strictly independent events rarely occur in biological organisations (Kortenkamp, Backhaus and Faust, 2009).

In a chemical mixture, the individual chemicals interact with each other, and elucidating this interaction allows for the determination of each chemical's contribution to the mixture effect. Chemicals in a mixture interact in three main ways: additive, synergistic, or antagonistic. A mixture effect is said to be additive, where individual compounds do not reduce or increase the effect of each other. The chemicals in additive mixtures do not interact with each other but produce the same effects as they would if acting independently. The components in the mixture act as a dilution of each other, and the overall mixture effect can be derived by adding the individual concentration of chemicals to the mixture. Additivity normally indicates the absence of synergism or antagonism (Olszowy-Tomczyk, 2020).

A mixture effect is said to be antagonistic when chemicals in a mixture produce an effect less than the predicted additive effect (Meek *et al.*, 2011). For example, a mixture of inorganic arsenic (As), 1,2 dichloroethane (DCE) and trichloroethylene (TCE) was demonstrated to have an antagonistic effect on preneoplastic hepatic lesions, bronchoalveolar hyperplasia and adenoma formation in male Fisher 344 rats (Pott, Benjamin and Yang, 1998). Another study has demonstrated that the toxic effect of the heavy metal cadmium (anaemia and nephrogenic hypertension) is considerably reduced when mixed with calcium, zinc and selenium (Yu, Tsunoda and Tsunoda, 2011). Ramirez-Acosta and colleagues also demonstrated the antagonistic effect of selenium on Cadmium. Hepatic cells (HepG2) were exposed to different concentrations of cadmium (5 μM, 15μM and 25μM) and selenium (100 μM) alone and in combination. They reported that co-exposure to Selenium and cadmium increased hepatic cell viability and diminished cadmium accumulation (Ramírez-Acosta *et al.*,

2022). Conversely, a mixture effect is said to be synergistic if the effect is greater than the predicted effect of the individual chemicals in the mixture (Kortenkamp and Altenburger, 1999; Cedergreen, 2014). Synergistic mixtures have posed a challenge to regulatory agencies for many years. However, a review of existing literature on mixtures of pesticides, metal ions and antifouling agents showed that synergism was present in between 7-26% of the studies (Cedergreen, 2014). Recently, Martin and colleagues conducted a quantitative reappraisal of mixture studies between 2007 and 2017. Out of the 388 studies reanalysed, 20% of the mixture studies were synergistic (Martin *et al.*, 2021). The search for synergistic interactions between chemicals has been of great interest among toxicologists and chemical regulators. Jatkowska and colleagues reported a synergistic relationship between BPA, Bisphenol A Diglycidyl Ether (BADGE), and Bisphenol A Diglycidyl Ether Dichlorohydrin (BADGE.2HCL). The authors further suggested that this synergism was confirmed in both Concentration Addition (CA) and Independent Action (AI) models. They further confirmed that the synergistic effect is due to the fact that these chemicals have similar mechanisms of action (Jatkowska *et al.*, 2021). However, the conclusions of one of the seminal papers on the synergistic effect of estrogenic pesticides by Arnold and colleagues (Arnold *et al.*, 1996) could not be replicated by other researchers, such as Ashby and his team (Ashby *et al.*, 1997). Consequently, Silva argued that scientists are at risk of discarding the additive effect of exposure to cocktails of EDCs because of their emphasis on synergistic interactions (Silva, 2003).

1.9 Multifactorial effects on breast cancer

The extent to which mixtures of endocrine disruptors interact with other breast cancer risk factors such as diet, genetic mutation, mammographic density, and obesity are

uncertain. For example, Rose and Vona-Davis studied the interaction between menopausal status and obesity on breast cancer risk. Their review of epidemiological studies revealed that the menopausal status of women modifies the effect of obesity on breast cancer in an age-dependent manner. Overall, their study revealed that weight reduction resulted in risk reduction in postmenopausal breast cancer (Rose and Vona-Davis, 2010). Another study examined the association and interactions between mammographic density and other risk factors. 491 breast cancer cases and 982 controls were used in this cohort in Singapore. They found that mammographic density increased the breast cancer risk of women with high BMI and women on long-term use of oral contraceptives (Wong *et al.*, 2011).

Regarding genetic risk factors, the interactions between 12 single nucleotide polymorphisms (SNPs), reproductive history, and body mass index have been studied. The study involved data from 26,349 invasive breast cancer cases and 32,208 controls from 21 case-control studies. The results from this study showed that the risk associated with individual alleles was not modified by age at menarche, age at first birth or BMI. However, the study established the combined effect of susceptibility alleles and other breast cancer risk factors (Milne *et al.*, 2010). Travis et al. showed that the interactions between 12 SNPs and other risk factors after analysis of 7610 breast cancer cases and 10196 controls from the Million Women study showed no statistical significance of gene-environment interaction (Travis *et al.*, 2010). The result by Travis et al. was also seen in another study by Campa et al., where the hypotheses of interaction between SNPs and established breast cancer risk factors were not supported after analysing data from 8576 breast cancer cases and 11892 control cases in the Breast and Prostate Cancer Cohort (Campa *et al.*, 2011).

The probability that a woman will develop breast cancer in their lifetime can be calculated using various breast cancer risk models. These models are useful for counselling, identifying women who are 'high risk' for intervention and the allocation of limited prevention resources (Gail, 2015). The possibility of risk factors interacting and influencing each other must be considered in designing these risk models to produce a comprehensive risk profile. A lack of relevant risk models is likely to result in miscommunicating the probability of individuals developing breast cancer and misapplication of preventative measures. It is, therefore, imperative that a multifactorial approach that considers the contribution of all the risk factors is used in determining an individual's risk.

1.10 In vitro Models for Studying Breast Cancer

1.10.1 Two-dimensional Models

Since the first mammalian cell culture in the early 20th century, the culture of immortalised and primary cells has been used to study a wide range of biological properties and functions, including cell migration, differentiation, growth, and *in vivo* systems (Duval *et al.*, 2017). In a 2D culture, cells are normally grown as a monolayer on flat surfaces of flasks or petri dishes. These are supplemented with synthetic media, providing nutrition, and incubated at 37°C and 5% carbon dioxide (CO_2) (Breslin and O'Driscoll, 2013). Two-dimensional cell cultures are very reproducible, results are easy to interpret, have high performance and are relatively inexpensive to set up (Brancato *et al.*, 2020; Kapałczyńska *et al.*, 2018).

However, two-dimensional (2D) cultures have many limitations: it does not sufficiently recapitulate the extracellular matrix (ECM), leading to a lack of tumour matrix and cell-cell interaction, which is present in tumours *in vivo* (Redmond *et al.*, 2021; Nath and

Devi, 2016). Culturing cells on stiff plastic surfaces of flasks and Petri dishes introduces significant changes in cell behaviour, such as gene expression, morphology, drug sensitivity, proliferation and differentiation, when compared to *in vivo* conditions (Brancato *et al.*, 2020; Egger and Nebel, 2021). These limitations led to the development of 3-dimensional (3D) cell cultures to provide a physiologically relevant platform to recapitulate *in vivo* conditions.

1.10.2 Three-Dimensional Models

Hamburger and Salmon first used 3D cell culture in the 1970s to culture human cells on soft agar (Hickman *et al.*, 2014). Its popularity in biomedical research has grown significantly in recent years as it overcomes many of the deficiencies of the traditional 2D method and represents the heterogenicity and complexity of in vivo conditions (Angeloni *et al.*, 2017; Bassi *et al.*, 2020). It has been an effective model in the drug development process by enhancing the selection of promising drug molecules in the pre-clinical stage before clinical trials (Herrmann and Jayne, 2019).

Specifically, 3D systems have proven to be an important model for studying carcinogenesis and tumorigenesis with a greater degree of biological and molecular replication of in vivo conditions than 2D cell culture models (Pradhan *et al.*, 2017; Jain *et al.*, 2020). The model better replicates the complexity of the tumour microenvironment and aids in the mechanistic study of cellular interactions, signalling and programming (Thoma *et al.*, 2014). Different cell types can be co-cultured in a 3D model, which encourages intracellular and cell-matrix interactions as they occur in tumours *in vivo* (Nath and Devi, 2016). The model has been used to replicate the development of acini of the breast and its relationship with the ECM. This has provided a platform to study breast epithelial carcinogenesis and drug development (Breslin and O'Driscoll, 2013; Marchese and Silva, 2012; Debnath, Muthuswamy and Brugge,

2003; Magdaleno *et al.*, 2021; Malhão *et al.*, 2022). Imamura and colleagues studied the differences in the sensitivity of breast cancer cell lines to chemotherapeutic agents (paclitaxel and doxorubicin) in 2D and 3D. The cell lines (BT-549, BT-474, T-47D) formed dense multicellular spheroids (MCS) in 3D cultures akin to *in vivo* acini structures and showed greater resistance to the chemotherapeutic agent in 3D cultures than in 2D cultures. However, other breast cancer cell lines, MCF-7, HCC-1954 and MDA-MB-231, formed loose MCS and showed similar sensitivity in both 2D and 3D cell cultures, suggesting that 3D cultures forming dense MCSs are more an appropriate system to study resistance than 2D (Imamura *et al.*, 2015).

Generally, three models can be grouped based on their use or lack of scaffold (Foglietta *et al.*, 2020). Scaffold-free cancer models include the hanging drop method, ultra-low attachment, magnetic levitation, and microfluidic systems. Scaffold-based systems employ natural and synthetic hydrogels, such as Matrigel, collagen, fibrin, gelatine, chitosan, nano fibrillar cellulose alginate, polyethene glycol (PEG), and polyesters (lactic-co-glycolic acid).

1.11 Aims and Objectives

The increasing incidence of breast cancer is not only attributable to genetic factors. Environmental chemicals, especially endocrine-disrupting chemicals (EDCs), are known to initiate and promote cancer progression (Knower *et al.*, 2014). The effects of these estrogenic chemicals have been seen in high doses in laboratory assays, which are unrepresentative of real-life situations (Silva, Rajapakse and Kortenkamp, 2002). However, humans are exposed to a myriad of chemicals at low concentrations that could act together to increase cancer risk. Lifestyle factors such as physical exercise, diet, consumption of alcohol, and use of contraceptive pills can impact the

overall risk of developing breast cancer. Most toxicological and epidemiological studies have investigated the effect of single chemicals without incorporating the potential effect of other risk factors such as high-fat diet and genetic mutation.

The main aim of this project is to study the impact of mixtures of endocrine disruptors and fatty acids, both independently and in combination, and their ability to induce alterations in the morphology and genetic profiles of human epithelial breast cells and if these alterations can be associated with breast cancer risk. To achieve this aim, we will address these objectives:

1. Investigate the effect of a mixture of EDCs or fatty acids on early breast carcinogenesis using a human breast epithelial cell line (MCF12A) in a 3D model.
2. Study the combined impact of a mixture of EDCs and fatty acids on breast carcinogenesis utilising MCF12A cell lines in a 3D model.
3. Investigate the effect of mixtures of EDCs and fatty acids on breast carcinogenesis in the presence of BRCA1 mutation.
4. To investigate the differences in genetic profiles and signalling pathways of breast cell line (MCF12A) when cultured in 3D compared to 2D monolayer models.

Chapter Two: Validation of the presence of estrogen receptor and fatty acid uptake of MCF-12A non-tumorigenic cell line

2 Validation of the presence of estrogen receptor and fatty acid uptake of MCF-12A non-tumorigenic cell line

2.1 Introduction

As discussed in Chapter One, hormones such as estrogen influence the development of the human breast from embryonic to full maturation (Macias and Hinck, 2012). In this thesis, we aim to study the effect of mixtures of EDCs and diet (fatty acids) in an *in vitro* model representative of the normal human breast. The model chosen is a non-tumorigenic breast epithelial cell line (MCF-12A), which recapitulates the normal epithelium of the human breast.

This chapter aims to optimise the MCF-12A cell line's estrogen receptor status and ability to uptake the fatty acid mixture. The estrogen receptor status of the MCF-12A cell line will be assessed by exposing the cells to BPA and propylparaben. This is essential to determine whether the cell line expresses a functional estrogen and other downstream receptors regulated by the estrogen receptor. This chapter also examines whether fatty acids can enter and affect MCF-12A cells. This was done to ascertain the uptake of the fatty acid mixture by the cells. We assessed the effect of the mixture of fatty acids on genes involved in fatty acids synthesis and metabolism and implicated in breast cancer.

2.1.1 MCF12A Cell line

Cell lines have been used extensively, especially as *in vitro* models, to study cancers and have contributed to elucidating molecular cancer biology. They are beneficial because they are easy to handle, have indefinite self-replicating ability, have unlimited supply, show a high degree of homogeneity and are easy to store as frozen stocks that can replace contaminated cultures (Burdall *et al.*, 2003; Vincent, Findlay and

Postovit, 2015). However, cell lines may not fully recapitulate the cellular heterogenicity of primary organs, as they are not cultured in normal physiological conditions. Continuous passage of cell lines over a period of time can introduce genotypic and phenotypic alterations, which can cause heterogenicity in cultures thereby producing different results. There is also the possibility of contamination with other plasma and mycoplasma (Kaur and Dufour, 2012; Puschhof, Pleguezuelos-Manzano and Clevers, 2021; Bahia *et al.*, 2002).

MCF-12A is a human mammary non-tumorigenic cell line established at Michigan Cancer Foundation and was derived from the reduction mammoplasty of a postmenopausal 63-year nulliparous woman (Paine *et al.*, 1992). Previous work from the Silva group has shown that MCF-12A cells, when cultured in a 3D system using Matrigel, formed acini-like structures which were disrupted when treated with 17β-estradiol, BPA and propylparaben (Marchese and Silva, 2012). Gelfand et al. used the MCF-12A cell lines to determine the effect of long-term exposure to ethanol on breast cancer initiation. MCF-12A was exposed to concentrations (1mM, 2.5mM and 25mM) of ethanol and acetaldehyde (2.5mM and 1.0 mM) between 1-4 weeks. Results showed that ethanol and acetaldehyde upregulated metallothionein family genes, alcohol metabolic genes and oncogenic transformation in the EMT, such as anchorage-independence in the normal epithelial cells (Gelfand *et al.*, 2016). It has also been used to study the anti-cancer properties of organic isothiocyanates in cancer treatment by measuring the cell growth after exposure of MCF-12A cells to dietary isothiocyanate compounds. Organic Isothiocyanates inhibited cell growth, suggesting their potential use in chemotherapy (Tseng, Ramsay and Morris, 2004). To confirm epidemiological studies on the risk of cigarette smoke on breast cancer, MCF-12A cells were exposed to cigarette smoke containing 253±22μg/ml of nicotine. Their work

showed that exposure of MCF-12A cells to cigarette smoke increased colony formation by 4 to 5fold and resulted in a significant elevation in migration after 18 days of treatment compared to the untreated (Di Cello *et al.*, 2013).

2.2 Validation of the presence of the estrogen receptors in the MCF-12A cell line.

The estrogen receptor status of MCF-12A and its response to estrogen have been sources of controversy. Some studies have reported that MCF-12As are ER-negative and not responsive to estrogen (Paine *et al.*, 1992; Subik *et al.*, 2010; Sweeney, Sonnenschein and Soto, 2018), while others have reported that the cell line is ER-positive and responsive to estrogen (Engel *et al.*, 2011; Gelfand *et al.*, 2016; Marchese and Silva, 2012).

MCF-12A is the cell line to be used in this project due to its established status as an ERα, ERβ, and GPER1 competent cell line, which allows for the study of the role these receptors play in breast morphogenesis, the impact of EDC on the formation and disruption of the mammary gland and, consequently, on carcinogenesis (Marchese and Silva, 2012). It is important to establish the receptor status of the batches of cell lines to be used and their responsiveness to EDCs. This is because there are reported variations in results obtained from different batches of the same cell line. Cell lines are extremely susceptible to genomic, proteomic and phenotypic alteration based on changes in experimental conditions and growth media (Freedman *et al.*, 2015; Payne, Scholze and Kortenkamp, 2001).

2.2.1 Genes of Interest

In answering the aims of this chapter, we set out to classify the cell line to ensure that it would respond to estrogens and pathways involved in hormonal carcinogenesis.

Using the mechanism of action of the test compound, we selected genes that are directly or indirectly implicated in breast carcinogenesis. The selected genes are also known to be dysregulated by estrogen and estrogen-mimicking compounds and affect other downstream pathways.

2.2.2 Estrogen Receptors (ERα / ERβ)

Estrogens are 18-carbon (C18) steroidal molecules consisting of a benzene ring, a phenolic hydroxyl at C3 and a hydroxyl group (17β-estradiol) or a ketone group (estrone) at C17. The major estrogens in the body are estradiol, estrone and estriol (Samavat and Kurzer, 2015). Endogenous estrogen is produced mainly in the gonads but also synthesised in other organs such as the brain (Bean, Ianov and Foster, 2014). The functions of estrogen are mainly mediated by the activation of ERα and ERβ (Haldosén, Zhao and Dahlman-Wright, 2014). Estrogens play an active role in the proliferation of both normal and neoplastic mammary epithelium (Russo and Russo, 2006). Deregulation of estrogenic signalling can result in transcriptional activities that can lead to the development of breast cancer (Aquino *et al.*, 2012). Approximately 70-75% of breast cancers express the estrogen receptor (Gil, 2014) , and therefore, inhibition of estrogenic activity is an effective therapy for breast cancer (Yue *et al.*, 2013).

Estrogen receptors (ER) are members of the nuclear receptor superfamily, comprising two subtypes: Estrogen receptor α (ERα) and Estrogen receptor β (ERβ), which are encoded by the genes *ERS1* and *ESR2*, respectively (Kovats, 2015). ERα was identified in the 1960s and cloned in 1986, while ERβ was discovered and cloned in 1996 (Khalid and Krum, 2016). Estrogen receptors play an essential role in reproduction and physiological functions in different organs (Hamilton, Arao and

Korach, 2014). ERα is highly expressed in tissues of the breast, uterus, ovary, kidney, bone, and liver, while ERβ is mainly found in the central nervous system, cardiovascular system, lungs, testis, prostate, colon, kidney, ovary and the immune system (Yakimchuk, Jondal and Okret, 2013).

ERα and ERβ genes (*ESR1, ESR2*) are located on chromosomes 6q25.1 and 14q22-24, respectively and share 96% DNA-binding and 58% ligand-binding homology (Speirs *et al.*, 2002). The full-length size of ERα is 595 amino acid (67kDa), and that of ERβ is 530 amino (59kDa) respectively (Fuentes and Silveyra, 2019). Structurally (Figure 2.1), estrogen receptors have six distinct regions, designated A through F. An amino-terminal domain (A/B domain), a DNA binding domain (DBD, C-domain), a hinge domain (D-domain), a ligand binding domain (LBD, E- domain), and a carboxyl-terminal domain (F-domain) (Hamilton, Arao and Korach, 2014). The A/B domain is the largest and houses the transcriptional activation function (AF1), which interacts with transcription factors to increase the rate of RNA transcription in a cell- and promoter-dependent manner (Hewitt and Korach, 2018).

The DNA binding domain (C-domain) is the most conserved region of estrogen receptors among species and the place for DNA recognition and receptor dimerisation. The D-domain is a hinge between the DNA binding domain (DBD) and the ligand binding domain (LBD) and confers nuclear receptor localisation signals and the region for interaction between nuclear receptor co-repressors (Nelson and Habibi, 2013). The LBD (E-domain) houses the activation function 2 (AF-2), which is vital for ligand-dependent transcriptional regulation. The configuration of the helix at the core of the AF-2 in the LBD is determined by either an agonist or antagonist (Hamilton, Arao and Korach, 2014). The F domain is the end of the AF2 and plays a critical role in the

activity of ERα and ERβ. The differences in the F domain between ERs allow for selective transcriptional activities of specific target genes (Lee, Kim and Choi, 2012).

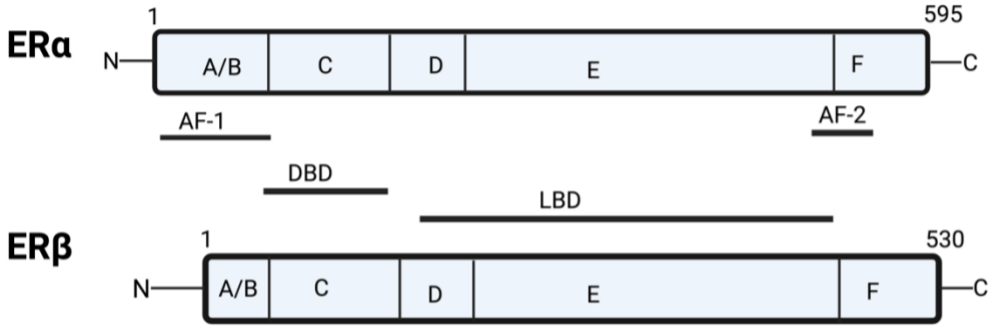


Figure 2.1 Structure and functional domains of estrogen receptor α and β (ERα and ERβ). *Schematic representation of ERα and ERβ highlighting the functional domains: the ligand-independent activation function (AF-1), ligand-dependent activation function (AF-2), DNA binding domain (DBD) and ligand-binding domain (LBD). Adapted from (Haldosén, Zhao and Dahlman-Wright, 2014).*

2.2.3 G protein-protein estrogen receptor 30 (GPER)

G protein-coupled estrogen receptor (*GPER1*), formally known as G protein-coupled receptor 30 (*GPR30*), is a seven transmembrane domain protein that binds to 17β estradiol but is structurally different from estrogen receptor α and β (ERα and ERβ) (Ariazi *et al.*, 2010).

it is widely expressed in reproductive organs, pancreas, liver, heart, arteries, breast, lung, neural tissues and leucocytes (Feldman and Limbird, 2017) In humans, *GPER* is located on chromosome 7p22.3 and is composed of three exons (MizukaMi, 2010). The exact function of *GPER1* in disease states is still a source of debate. *GPER1* activation regulates cellular functions such as growth, differentiation, and proliferation (Arias-Pulido *et al.*, 2010; Weißenborn *et al.*, 2014a). *GPER1* is highly expressed in

50% of breast cancer patients and is a marker of good clinical outcomes for patients (Weißenborn *et al.*, 2014a). It has been demonstrated in MCF-7, SkBr3, MDA-MB-231 and MDA-MB-468 breast cancer cell lines that *GPER1* is a potential tumour suppressor gene (Weißenborn *et al.*, 2014a; Weißenborn *et al.*, 2014b). Mechanistically, the antiproliferative actions of *GPER1* are achieved through induction of cell cycle arrest and epigenetic regulation through hypermethylation of promoter regions (Weißenborn *et al.*, 2014b; Weißenborn *et al.*, 2014a). *GPER's* inhibition in triple-negative breast cancer cell lines (HCC1806, HCC70 and MDA-MB-231) by estriol led to a decrease in cell proliferation and signalling activities mediated by 17β-estradiol suggesting that *GPER1's* inhibition can be a promising treatment option for TNBC treatment (Girgert, Emons and Gründker, 2014). Another study using breast cancer SkBr3 and MDA-MB-231 and an MDA-MB-231 tumour xenograft showed that activation of *GPER1* inhibits ER-negative breast cancer both *in vivo* and *in vitro* (Wei *et al.*, 2014).

It has been demonstrated in SkBr3, an ER-negative breast cancer cell line, that activation of Erk1 and Erk2 by 17β-estradiol is mediated by *GPER1* (Filardo *et al.*, 2000). In the treatment of hormone-dependent breast cancer, *GPER1* has been shown to contribute to tamoxifen resistance by disrupting EGFR signalling and stopping the inhibition of MAP kinases (Mo *et al.*, 2013).

2.2.4 Breast cancer susceptibility gene 1

Breast cancer susceptibility gene 1 (*BRCA1*) is a 220-kDa tumour suppressor gene which was mapped in 1990 and cloned in 1994. It has 24 exons, which encodes a nuclear protein of 1863 amino acids and is located on chromosome 17q21 (Rakha *et al.*, 2008; Caestecker and Van de Walle, 2013). It plays an important role in controlling

DNA damage response and centrosome replication that ensures the survival of both normal and malignant breast cells (Barcellos-Hoff and Kleinberg, 2013; Armstrong *et al.*, 2019). It is also involved in DNA strand break repair and nucleotide excision repair (Zhang and Powell, 2005). BRCA1 affects and interacts with other regulatory proteins involved in DNA repair (*ATM, CHK2, BRCA2, RAD51*, *RAD50/MRE11/NBS1, BASC, PCNA, H2AX*), transcription (*HDAC2, E2F, CBP/p300, SWI/SNF* complex, CtIP, p53) and cell cycle (*RB, CDK2, p21, p27, BARD1*) (Yoshida and Miki, 2004).

Deleterious Germline mutations in BRCA1 predispose a woman to a greater risk of developing breast cancer and account for 3% of all breast cancers (Elstrodt *et al.*, 2006; Fraser *et al.*, 2003). BRCA1 mutation in women confers more than 80% lifetime risk of developing breast cancer. By the age of 70, BRCA1 mutation confers between 45-85% risk of developing breast cancer (Thompson and Easton, 2002; King, Marks and Mandell, 2003). Over 80% of breast cancer arising from inherited BRCA1 mutation is triple-negative breast cancer (TNBC), while 20% of them are ER-positive, present predominantly in sporadic breast cancers (Lips *et al.*, 2013; Foulkes *et al.*, 2004).

BRCA1 protein interacts and inhibits the transcriptional activities of ligand-activated ERα via the estrogen receptor elements (ERE) in breast cancer cell lines (T47D) (Fan *et al.*, 1999). This is achieved through the direct interaction of BRCA1 and ERα protein and down-regulation of nuclear receptor coactivator, p300 (Rosen, Fan and Isaacs, 2005). The interaction between ER and *BRCA1* is essential to maintain the quality of replicated genome DNA when cells undergo proliferation under the mitogenic signalling of estrogen.BRCA1 mutation leads to genomic instability and accumulation of genomic mutation, which can result in cancerous transformation of breast epithelial cells (Wang and Di, 2014). A study of *BRCA1* mutated mice showed that loss of *BRCA1* activity leads to proliferation and tumour development in mammary epithelial

cells in response to exogenous estrogen signalling (Jones *et al.*, 2008). *BRCA1* mutation increased the proliferation of cells, leading to mammary tumorigenesis in both *in vitro* and *in vivo* under extracellular estrogen signalling (Li *et al.*, 2007).

It has been demonstrated in breast cancer cell lines (MCF-7, MDA-MB-231) that endocrine disruptors (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and polychlorinated biphenyls: PCB #138, PCB #153, PCB #180) alter the expression of BRCA1 gene (Rattenborg, Gjermandsen and Bonefeld-Jørgensen, 2002). Silva et al. demonstrated that 17β-estradiol, B-hexachlorocyclohexane, and *o, p'*-DDT at very low concentrations expressed the *BRCA1* gene in the MCF-7 breast cancer cell line (Silva, Kabil and Kortenkamp, 2010).

2.2.5 Cyclin D1

A family of cyclins and their corresponding cyclin-dependent kinases (CDKS) (Gao, Leone and Wang, 2020) regulate the mammalian cell cycle. Four main cyclins are involved in the different phases of the cell cycle: cyclins A, B, C, D and E. The cyclin D family (cyclin D1, D2 and D3) plays regulatory roles in cell proliferation and core cell cycle machinery (Pawlonka, Rak and Ambroziak, 2021).

Cyclin D1 is a member of the cyclin protein family (Ahlin *et al.*, 2017). Cyclin D1 protein is encoded by the *CCND1* gene, located on chromosome 11q13 (Ramos-Garcia *et al.*, 2017). It regulates G_1/S-phase transition by binding and activating cyclin-dependent kinases (*CDK4* and *CDK6*). Additionally, because of its role in the cell cycle, cyclin D1 regulates key stages in cancer initiation and progression, such as DNA damage response, chromosome duplication and stability, senescence, autophagy, mitochondrial respiration, migration, metabolism and immune surveillance (Tchakarska and Sola, 2020). Cyclin D1 is dysregulated in many human solid cancers,

including ER-positive breast cancers. Tumours with elevated levels of cyclin D1 exhibit unchecked proliferation in response to cell cycle malfunction at the checkpoint of the G1 phase (Tchakarska and Sola, 2020; Ewen and Lamb, 2004; Ahlin *et al.*, 2017). Cyclin D1 is expressed in an estimated 50% of all breast cancer and is a maker of poor clinical outcomes (Wang *et al.*, 2018; Lundgren *et al.*, 2012). It is associated with resistance to endocrine therapy such as tamoxifen in breast cancer cell lines (Ahlin *et al.*, 2017; Shi *et al.*, 2020).

The expression of cyclin D1 is directly regulated by the estrogen receptor (Hu *et al.*, 2020b; Inoue and Fry, 2015). Even in the absence of estrogen, cyclin D1 can bind directly to the hormone-binding domain of ERα to mediate gene transcription (Montalto and De Amicis, 2020). It has been shown that the activation of cyclin D1 in the ZR-75 breast cancer cell line by 17β-estradiol is regulated by ERα (Castro-Rivera, Samudio and Safe, 2001). Lin and colleagues showed that bisphenol S (BPS) promotes cell cycle progression and cell proliferation by activating the ERα-cyclin D1 complex (Lin *et al.*, 2019).

2.2.6 Trefoil family factor 1 (TFF1)

Trefoil factors are small (7-12kDa) and stable peptide molecules that are secreted by epithelial mucus-secreting cells in the gastrointestinal tract. They derived their name due to the presence of a common three-loop leaf-like structure, which makes trefoil factors extremely stable to proteolytic digestion, acid, and thermal degradation (Ishibashi *et al.*, 2017). Trefoil factors promote repair and restoration of the epithelial layer after injury to the mucosal lining of the GI tract by increasing cell migration (Perera *et al.*, 2015).

TFF1, also known as pS2, is a cysteine-rich member of the family that was identified in the MCF-7 cell line (Masiakowski *et al.*, 1982). The promoter region contains an

estrogen response element (ERE) and a complex enhancer region that is sensitive to epidermal growth factors (EGF), tumour promoter 12-tetradeconylphorbol (TPA), and proto-oncoproteins c-Has-ras and c-jun (Ribieras, Tomasetto and Rio, 1998).

The presence of the ERE in TFF1 allows the gene to be regulated downstream by estrogen receptors. Up-regulation of TFF1 is a good marker for estrogen receptor functions (Kang, Eyun and Park, 2021; Prest, May and Westley, 2002).

TFF1 is expressed in estrogen-positive breast cancer cell lines, and estrogen increases the concentration of TFF1 mRNA up to 100-fold (May *et al.*, 2004). Apart from estrogen, TFF1 expression can be induced by epidermal growth factors (EGF), 12-O-tetradecanoylphorbol acetate (TPA), proto-oncogenes c-Ha-ras and c-jun by activating estrogen-response element and a TPA-response element (TRE). It has also been shown in MCF-7 cell lines that TPA and EGF can act synergistically with estrogen to upregulate TFF1 (Markićević *et al.*, 2014; Beck, Fegert and Gott, 1997).

TFF1 is involved in estrogen-promoted resistance to apoptosis caused by doxorubicin in the MCF-7 breast cancer cell line, suggesting that the gene can be targeted for enhancing sensitivity to therapeutic agents in breast cancer treatment (Pelden *et al.*, 2013). However, TFF1 has also been shown not to possess tumorigenic properties in breast cancer cell lines and intratumoral TFF1 levels are associated with better clinical outcomes in patients with node-negative tumours (Buache *et al.*, 2011; Corte *et al.*, 2006).

2.3 Validation of fatty acid Uptake by the MCF-12A Cell line

As discussed in Chapter One, studies have shown the potential implication of a high-fat diet on breast cancer initiation and progression. Fatty acid uptake by cells occurs either through membrane diffusion or facilitated transport by membrane proteins. These transport mechanisms require the movement of fatty acids (FA) from an

aqueous solution through the lipid bilayer into the cells (Alsabeeh *et al.*, 2018). Due to their poor solubility, fatty acid uptake into cells is usually facilitated by complexing with albumin, such as bovine serum albumin (BSA) (Alsabeeh *et al.*, 2018; Oliveira *et al.*, 2015). However, an issue arises with mixtures of fatty acids as constituent FAs have different binding affinities to BSA, such that to achieve a uniform binding ratio, the concentrations of the mixture components have to be adjusted (Huber *et al.*, 2006). We, therefore, decided to test our mixture of fatty acids without conjugating to BSA to ascertain if they cross the cell membrane. Some studies have also exposed cells to fatty acids without conjugating with BSA. This thesis will use a mixture of four fatty acids (Elaidic acid, stearic acid, linoleic acid, and palmitic acid), all reviewed in Chapter One. Therefore, we must determine the uptake of the fatty acid mixture by the MCF-12A cell line to ensure that they exert the intracellular effect we aim to study.

2.3.1 Genes Of Interest

Genes were chosen based on their known involvement in fatty acid metabolism and breast carcinogenesis, as found in the literature. More importantly, they were selected as their expression is known to be mediated by the direct, intracellular effect of fatty acids. This way, their expression regulation can be used as an indicator of the uptake of FA by the cells in this study.

2.3.2 Aldo-keto reductase 1C1 (AKR1C1)

*AKR1C1*s (also known as 20α-hydroxysteroids dehydrogenase) are NADPH-dependent oxidoreductases that play essential roles in the metabolism of progesterone, steroids, fats, hormones, glucocorticoids, drugs, polycyclic aromatic hydrocarbons, and prostaglandins (Chu *et al.*, 2022; Ebert *et al.*, 2011). *AKR1C1* is

overexpressed in a myriad of cancers, such as lung, gastric and cervical cancers. Upregulation of *AKR1C1* promotes cancer initiation progression and resistance to anticancer therapy (Hong *et al.*, 2018; Penning *et al.*, 2021; Bortolozzi *et al.*, 2018). Research has shown that long-chain fatty acids are inhibitors of *AKR1C1* (Hara *et al.*, 2017).

AKR1C1 is highly expressed in human breast tissue (Rižner and Penning, 2014) and is upregulated in breast cancer (Zhang *et al.*, 2005). It is an independent prognostic marker for breast cancer (Wenners *et al.*, 2016). Other studies have shown that *AKR1C1* was reduced in cancerous compared to normal breast cells (Lewis, Wiebe and Heathcote, 2004). It is a ferroptosis-related gene connected with the immune microenvironment and can, therefore, influence the progression and prognosis of breast cancer (Zhang *et al.*, 2021b).

2.3.3 Cluster of Differentiation 36 (CD36)

CD36 is an 88kD transmembrane membrane glycoprotein encoded by the *CD36* gene. It has other names, such as Fatty Acid translocase (FAT), scavenger receptor class B type 2 (SR-B2), GP88, and platelet GPIV. It belongs to the scavenger receptor and is found in different tissues and cells such as dendritic cells, microvascular cells, endothelial cells, retinal epithelial cells, monocytes, adipocytes, platelets, enterocytes, and podocytes, Kupffer cells, taste receptor cells, skeletal muscle cells, mammary epithelial cells, and erythrocytes (Yang *et al.*, 2017; Tanase *et al.*, 2020). It plays an important role in the metabolism and uptake of fatty acids and oxidised low-density lipoprotein, immunological response, inflammation, molecular adhesion and apoptosis (Wang and Li, 2019; Hao *et al.*, 2020). High-fat diets increase the expression of *CD36* (Ramos-Jiménez *et al.*, 2022).

It has been implicated in the proliferation and progression of breast cancer, ovarian cancer, pancreatic cancer, and glioblastoma (Drury *et al.*, 2022). In breast cancer, *CD36* has been shown to promote aggressive cancer and also inhibit breast cancer progression (Feng, Bang and Kurokawa, 2020). While deregulation of *CD36* has been linked to aggressive breast cancer (DeFilippis *et al.*, 2012), a lysophosphatidic acid/protein kinase D1 (LPA/PKD-1)-CD36 signalling plays a vital role in promoting breast cancer by promoting microvascular remodelling in chronic diet-induced obesity (Dong *et al.*, 2017).

2.3.4 Sterol Regulatory Element-Binding Prottein-1c (SREBP-1c)

Sterol regulatory element binding proteins (SREBP) are a family of basic helix-loop-helix leucine zipper membrane-bound transcription factors vital in the induction of genes involved in lipid metabolism, including THRSP and FASN (Shao and Espenshade, 2012; Song *et al.*, 2012). There are three mammalian isoforms of *SREBPs: SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and SREBP-1c* originate from the same gene, and SREBP-2 originates from a different gene (Goldstein, DeBose-Boyd and Brown, 2006). *SREBP-1* mainly regulate the lipogenic pathway, whereas SREBP-2 controls cholesterol biosynthesis (Sethi, Shanmugam and Kumar, 2017). *SREBP-1c* is the most dominant isoform in many animal tissues, and it is highly expressed in adipose tissues and liver (Deng *et al.*, 2014).

SREBPs combine multiple cell signals to regulate lipogenesis and other pathways involved in type 2 diabetes, malignancies, immune response, neuroprotection, and autophagy (Shao and Espenshade, 2012). *SREBPs* are essential regulators of lipogenesis and, therefore, potential targets for chemotherapy agents since cancer cells require a constant supply of lipids to sustain tumour growth (Shao and

Espenshade, 2012). High expression of *SREBP-1* is a marker for poor prognosis in patients with breast cancer (Bao *et al.*, 2016). Expression of *SREBP-1c* has been assigned as a phenotype in breast cancer cells (Nieva *et al.*, 2012). Unsaturated fatty acids have been found to down-regulate SREBP-1c (Hannah *et al.*, 2001; Takeuchi *et al.*, 2010).

2.3.5 Hormone Sensitive Lipase (LIPE)

Hormone-sensitive lipase (*LIPE*, previously known as HSL) is an intracellular neural lipase that catalyses fatty acid collection from diacylglycerols, monoacylglycerols, and cholesteryl esters into fatty acids. It is one of the main enzymes that mediate hormone-induced lipolysis. They are highly expressed in adipose and steroidogenic tissues, muscles, and macrophages (Goszczynski *et al.*, 2014; Kraemer and Shen, 2002). The *LIPE* gene encodes Human hormone-sensitive lipase found on chromosome 19 in the q13.2 region (Recazens, Mouisel and Langin, 2021). It was found to be upregulated in susceptible breast epithelium (Marino *et al.*, 2020a). Up-regulation of *LIPE* is a marker for obesity and lipodystrophy (Lampidonis *et al.*, 2011). Conjugated linoleic acids (CLA) down-regulated *LIPE* (Veshkini *et al.*, 2023).

2.3.6 Fatty Acid Synthase (FASN)

Fatty Acid Synthase (*FASN*) is a ~278kDa lipogenic polypeptide that plays a vital role in de novo fatty acid synthesis. There are two kinds of *FASN* in mammals: type I cytosolic *FASN* and type II mitochondrial *FASN*, with type I being commonly called *FASN*. *FASN* catalyses the biosynthesis of fatty acids from dietary carbohydrates in a reaction that uses acetyl-CoA as a primer, malonyl-CoA as a C2 donor and NADPH as a reducing agent (Wu *et al.*, 2014). In normal physiological states, *FASN* is

regulated by environmental, hormonal, and nutritional signalling (Khan *et al.*, 2014; Abramson, 2011). *FASN* is upregulated in a myriad of cancers such as breast, prostate, and colon (Bessadóttir *et al.*, 2014). The overexpression of *FASN* is observed in later stages of cancers with poor prognosis, suggesting that *FASN* confers survival advantages to cancer cells (Ligorio *et al.*, 2021). It is highly expressed in HER2+ breast cancer, plays a vital role in the interaction between HER2 and signalling proteins at lipid raft domains, and regulates HER2 gene transcript and protein expression (Corominas-Faja *et al.*, 2017; Menendez *et al.*, 2021).

The level of expression of *FASN* in breast cancer is directly proportional to tumour progression and aggressiveness. Therefore, an upregulation of *FASN* is a marker for poor prognosis (Alwarawrah *et al.*, 2016). Breast cancer that shows high expression of *FASN* were four times more likely to recur and proliferate than those with low expression of *FASN* (Wang *et al.*, 2021a). *FASN* expression has been associated with resistance to drugs and radiation through DNA damage, up-regulating PARP-1, overproduction of palmitic acid, and inhibition of TNF-alpha and ceremide production (Wu *et al.*, 2016; Liu *et al.*, 2013; Liu, Liu and Zhang, 2008).

Disrupting cellular metabolism has become one of the focal mechanisms to bypass drug resistance during breast cancer treatment, and inhibition of *FASN* has become a bonafide target in breast cancer. Disruption of *FASN* and downstream ER resulted in suppression of cell growth. Functional disruption in *FASN* leads to decreased proliferation and migration of breast cancer cells (Gonzalez-Salinas *et al.*, 2020).

2.3.7 Chapter Scope

This chapter aims to ascertain the suitability of the MCF-12A cell line for the project by evaluating whether these cells express functional estrogen receptors and if they respond to the effect of fatty acids. The overarching aim was achieved by asking the following questions:

1. Are *ESR1, ESR2*, and *GPER1* receptors expressed in MCF12A cells under control conditions? Is the expression of these receptors regulated by estrogenic-mimicking compounds like Bisphenol A (BPA)?
2. Do estrogen-mimicking compounds such as BPA regulate the expression of estrogen receptor responsive genes such as cyclin *D1, BRCA1,* and *TFF1*?
3. Is there a regulation of fatty-acid responsive genes (*CD36, LIPE, SREBP1c*, *FASN,* and *AKR1C1*) when MCF-12A cells are exposed to a mixture of fatty acids?

2.4 Methodology

2.4.1 Routine cell culture

Unless otherwise stated, all reagents were procured from Sigma-Aldrich (Dorset, UK). MCF12A cells were purchased from the America Type Culture Collection and grown in monolayer T75 canter-neck tissue culture flasks. MCF-12A cells were cultured in Dulbecco's Modified Eagle Medium (DMEM: F12, Invitrogen, Paisley, UK), supplemented with 5% horse serum (Invitrogen, UK), 0.02% epidermal growth factor, 0.01% cholera toxin, 0.1% insulin, 0.05% hydrocortisone and 1% Pen-Strep. The cell culture medium was replaced every three days, and cells were kept in a humidified incubator at 37°C and 5% $C0_2$. When cells were 70% confluent, they were passaged with 0.25% trypsin-ethylene diamine tetraacetic acid (trypsin-EDTA).

2.4.2 Chemical exposure (BPA)

BPA was purchased from Fisher Scientific. The stock of BPA was prepared in 100% HPLC-grade ethanol at 1×10^{-3}M and stored in critically clean glass bottles at -20°C. Two concentrations of BPA were used: 1×10^{-7}M and 1×10^{-5}M, which were chosen based on work done in the Silva lab, which showed their ability to induce estrogen receptor expression in MCF-12A (Marchese and Silva, 2012). Cultures of MCF-12A cells were exposed to BPA for 24 hours and 72 hours, after which the experiment was stopped, and RNA was extracted. In all the treatments, the final concentration of solvent did not exceed 0.5% to avoid ethanol toxicity(Marchese and Silva, 2012). Three independent experiments were carried out.

2.4.3 Fatty Acid Mixture

The study used mean plasma fatty acid concentrations of palmitic acid, elaidic acid, stearic acid and linoleic acid as reported by (Abdelmagid *et al.*, 2015; Staff, 2015). A mixture of the fatty acids was prepared where each of them was present in their tissue concentration, as shown in Table 2.5. The rationale for selecting these particular fatty acids was presented in Chapter One.

Table 2.1 List of fatty acids, their mean plasma concentration (Abdelmagid *et al.*, 2015) and their percentage in the mixture solution

Fatty Acids	**Mean Plasma Concentration (M)** (Abdelmagid *et al.*, 2015)	**Proportion of Fatty Acid in Mixture (%)**
Elaidic Acid	4.40×10^{-5}	0.6
Linoleic Acid	2.63×10^{-3}	48.66
Palmitic Acid	2.1747×10^{-3}	40.32
Stearic Acid	5.62×10^{-4}	10.42
Total Mixture Concentration	5.39×10^{-3}	

2.4.4 Chemical Exposure (Fatty Acids)

The fatty acid mixture was prepared as a 5.4 $\times10^{-3}$M stock solution. A working concentration of 5.4×10^{-5} was prepared in critically clean glassware and stored at -20°C. MCF-12A cultures were treated with the fatty acid mixture. RNA was extracted

24 hours after chemical exposure. In all cases, the final solvent concentration did not exceed 0.5% to prevent ethanol toxicity. A total of three independent experiments were conducted.

2.4.5 RNA Extraction

After 24 hours and 72 hours exposure, the media was aspirated, and the cells were washed with Hank's Balanced Solution (HBSS) to remove dead cells. Trypsin-EDTA was then added, and cells were incubated for 5 mins at 37°C and 5% CO_2. When the cells were detached from the flask, they were suspended in full DMEM: F12 medium and centrifuged at 1000 rpm for 5 minutes. The media was removed, and the pellet was used for RNA extraction. The RNA was isolated using the RNAeasy Mini Kit (Qiagen, Manchester, UK) in accordance with the manufacturer's instructions. RNA samples were analysed for purity using 260/280 and 260/230 ratios with the Nanodrop One (Thermo Scientific, Loughborough, UK). The acceptable range of purity was 1.7-2.1.

2.4.6 Quantitative Real-time PCR (qPCR)

qPCR is a powerful and widely used molecular biology technique used for the precise amplification and quantification of specific DNA molecules in biological and environmental samples in real time (Taylor *et al.*, 2019; Pabinger *et al.*, 2014). Gene amplification is measured after every cycle, thereby allowing for the determination of the concentration of nucleic acid in each sample. Minute changes in fold changes in gene expression are detectable, which may be significant when analysing samples treated with low doses of EDCs.

We used the two-step method of gene amplification: reverse transcription of extracted mRNA into cDNA and subsequent qPCR. mRNA was reverse transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems). This allows single-strand mRNA to make double-strand complementary DNA molecules needed for gene amplification. A master mix was prepared using the kit's components according to the quantities in Table 2.1. The kit components were thawed on ice and the master mix was prepared on ice for the number of samples to be analysed.

Table 2.2 Reagents volumes for reverse transcription for one reaction using the High-capacity cDNA reverse transcription kit

Components	Volume/Reaction (μl)
10x RT Buffer	2.0
25x dNTP mix (100mM)	0.8
10X RT Random Primers	2.0
Multiscribe^{TM} Reverse Transcriptase	1.0
Nuclease-free water	4.2
Total for 1 reaction	**10**

mRNA was removed from the -80 °C and kept on ice. 10μl of the mRNA of each sample was added to 10μl of the master mix in Eppendorf tubes and spun down for 5 seconds to remove air bubbles. The reaction tubes were then loaded into a programmed Thermocycler for the reverse transcription (Table 2.3). cDNA was used immediately for quantitative PCR or stored in the short term in the refrigerator (2-6°C) and at -20°C for long-term storage.

Table 2.3 The condition of the thermocycler, showing the temperature and time for each step of cDNA synthesis.

	Step1	**Step2**	**Step3**	**Step4**
Temperature (°C)	25	37	85	4
Time (mins)	10 mins	120 mins	5 mins	∞

The next step was qPCR, which was done using iTaq SYBR Green PCR Master Mix (Bio-Rad Laboratories, Inc) in a Bio-Rad CFX96 system programmed to specific conditions. SYBR Green binds to the cDNA, which enables the detection of levels of expression of target genes. Bound SYBR Green emits a fluorescent signal, while unbound SYBR Green does not. The level of signal emitted by the SYBR Green is proportional to the amplification of the target gene. The intensity of the signal increases because the amount of PCR products also increases. A triplicate of the PCR reaction was performed in a Semi-Skirted 96-well PCR plate (Bio-Rad Laboratories, Inc). Each reaction is comprised of Master Mix and cDNA products. The Master mix consists of iTaq Universal SYBR Green Supermix, Primers and nuclease-free water (Table 2.4).

Table 2.4 Volume of components of master mix for one gene in qPCR experiment using iTaq SYBR Green PCR Master Mix

Reagent	Volume/Reaction (μl)
iTaq Universal SYBR Green Supermix	10
Forward Primer	0.5
Reverse Primer	0.5
Nuclease-free water	8
Total for 1 Master mix	**19**

Master mixes were made for all the target and housekeeping genes. 19μl of the master mixes, and 1μl of cDNA product was added to each well of the plate. The plate was sealed with Microseal 'B' PCR Plate Sealing Film (Bio-Rad Laboratories, Inc) and centrifuged for 2 mins at 1000 rpm to ensure all the content was at the bottom of the well. The plate is then put in Bio-Rad CFX96, which has already been programmed according to the following conditions (Table 2.5).

Table 2.5 Rea-time PCR program, showing steps, temperature, and number of cycles for each step.

Step	Temperature (°C)	Time (sec)	Cycle
Activation	95	30	1
Denaturation	95	5	38
Amplification	60	30	
Melt curve analysis	60	Increment of 5	

2.4.7 Primers for the Presence of Estrogen Receptor

The primers pairs used for this study were tested and optimised in a previous study in the Silva Lab(Marchese and Silva, 2012). The primers were purchased from Sigma-Aldrich. The primers were supplied as dry power, they were centrifuged, and then suspended in volumes of nuclease-free water according to the manufacturer's instructions. The table below lists all the primers used (Table 2.6).

Table 2.6 Gene primer sequences. Forward and reverse sequences of genes used in the real-time PCR

Gene Name	Primer Sequence (5'-> 3')	
ACTB	Forward	TCAGCAAGCAGGAGTATG
	Reverse	GTCAAGAAAGGGTGTAACG
GAPDH	Forward	TCTCTGCTCCTCCTGTTC
	Reserve	GCCCAATACGACCAAATCC
ESR1	Forward	GCCCTCCCTCCCTGAAC
	Reserve	TCAACTACCATTTACCCTCATC
ESR2	Forward	CCTCCCAGCAGCAATCC
	Reverse	CCAGCAGCAGGTCATACAC
GPR30	Forward	GTTCCTCTCGTGCCTCTAC
	Reserve	ACCGCCAGGTTGATGAAG
BRCA1	Forward	ACATACCATCTTCAACCTCTG
	Reserve	CGATGGTATTAGGATAGAAG
CCND1	Forward	TGGAATGGTTTGGGAATAT
	Reserve	CCTGGCAATGTGAGA
TFF1	Forward	CCGTGAAAGACAGAATTG
	Reverse	CGATGGTATTAGGATAGAAG

2.4.8 Primers for the Uptake of Fatty Acids

Primers were designed using Harvard Primer Bank. They were ordered from Sigma Aldrich as dry powder and dissolved using nuclease-free water (Table 2.7).

Table 2.7 Gene Primer Sequence. Forward and reverse primer sequences used in the real-time PCR

Genes		Primer Sequence (5'-> 3')
CD36	Forward	GGCTGTGACCGGAACTGTG
	Reverse	AGGTCTCCAACTGGCATTAGAA
LIPE	Forward	TCAGTGTCTAGGTCAGACTGG
	Reverse	AGGCTTCTGTTGGGTATTGGA
SREBP1c	Forward	GCGCCTTGACAGGTGAAGTC
	Reverse	GCCAGGAAGTCACTGTCTTG
AKR1C1	Forward	TCCAGTGTCTGTAAAGCCAGG
	Reverse	CCAGCAGTTTTCTCTGGTTGAA
FASN	Reverse	AGATTGTGTGATGAAGGACATGG
	Forward	TGTTGCTGGTGAGTGTGCATT

2.4.9 Relative Gene Expression Statistical Analysis

Many methods are available for the relative quantification of gene expression. These include the Pfaffl model, $2^{-\Delta\Delta ct}$ or comparative Ct method and the qBase software. The principle that underpins the various approaches is the establishment of a threshold at which the fluorescence of PCR products goes above the background fluorescence. The number of cycles required to reach this threshold, depending on the number of templates in a sample, is called the Ct value. Lower ct values correspond to higher

amounts of templates (Regier and Frey, 2010). This analysis was done for the experiments using the following calculations:

$$\Delta Ct = Ct\ (target\ gene) - Ct\ (reference\ gene)$$

$$\Delta\Delta Ct = \Delta Ct\ (treatment\ group) - mean\ \Delta Ct\ (Control\ group) \quad \textbf{(Equation 1)}$$

$$Fold\ Change = 2^{-\Delta\Delta Ct}$$

The results are reported as log2(fold change), indicated as Log (Fold Change), with control values normalised to 0. A LogFC-value approaching 0 indicates that there is no change in gene expression between the control and treated samples. Negative values represent a decrease in gene expression, while positive values denote an increase. Log (Fold Change) was applied to the values to ensure normal distribution and easy visualisation of the fold changes. Normal data distribution was confirmed, and a parametric test was required. A one-way Analysis of Variance (ANOVA) was done to ascertain the significance of changes in gene expression between treated and control samples. Bonferroni, a multiple comparison test, was the post-hoc test used to determine the specific treatment that caused a significant differential gene expression. All statistical analysis and visualisation were done in GraphPad Prism (Version 10.2.3).

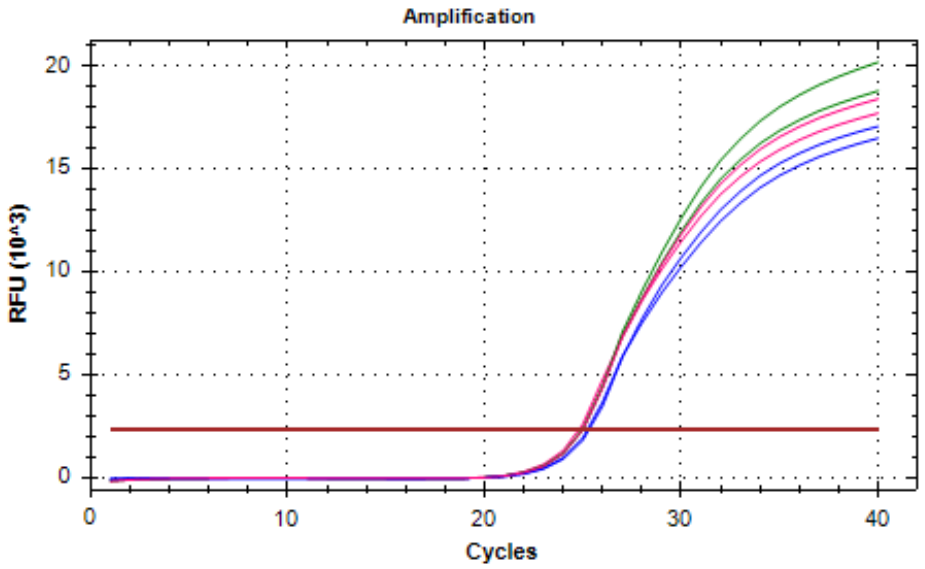
2.5 RESULTS

2.5.1 MCF-12A cell line expressed ESR1, ESR2 and GPER1

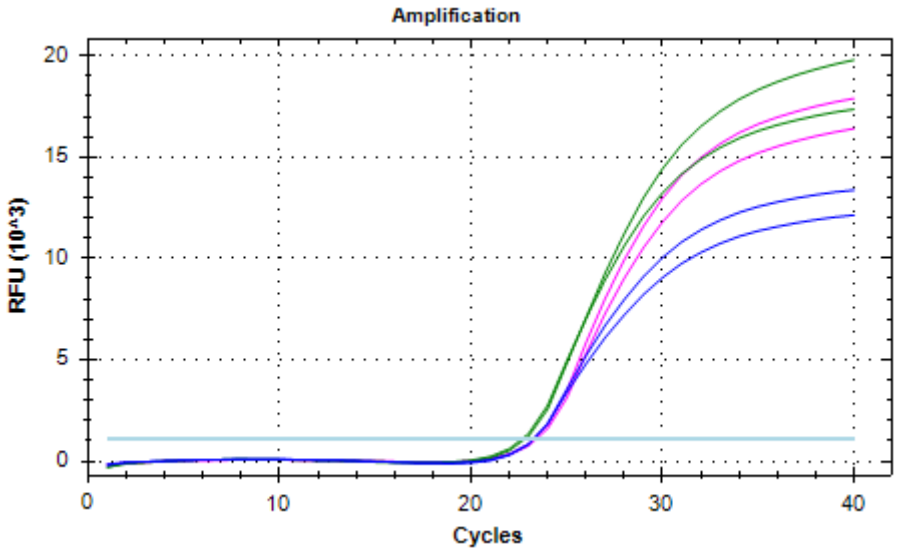
As discussed above, the estrogen receptor status of MCF-12A has been conflicting(Sweeney, Sonnenschein and Soto, 2018; Marchese and Silva, 2012; Soule *et al.*, 1990). The aim of the thesis is to examine the effect of mixture of EDCs on normal mammary epithelium which exert their effect by binding to and activating both the ER and GPER. We therefore tested for the genetic expression of ESR1, ESR2 and GPER1 of the passages of cells to be used in the experiments.

Analysis of real-time PCR data confirmed that ESR1, ESR2 and GPER1 were expressed in both control samples. Exposure to two concentrations of BPA (1×10^{-7} M and 1×10^{-5} M) for 24 hours also resulted in the expression of the genes. The higher Ct values in the treated samples suggest a decrease in gene expression (Figure 2.3)

A



B



C

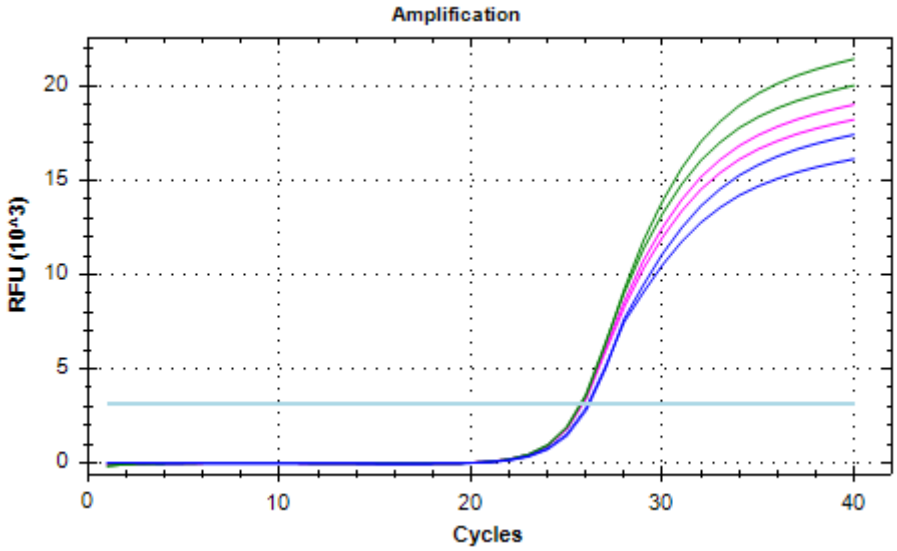
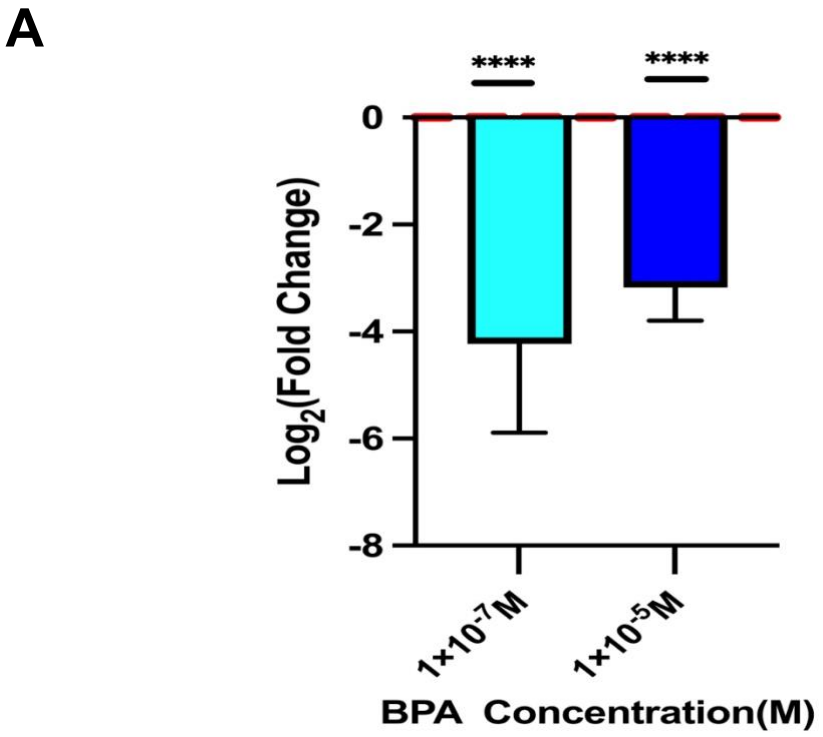


Figure 2.2 Amplification of curves for the expression of ER in MCF-12A cell line. *Amplification done in replicate, depicting (A) presence of ESR1 in the presence of 0.05% pure ethanol (green), 1×10^{-7}M ethanol (pink), and 1×10^{-5} M (blue). ESR2 is also expressed in the presence of the same controls and treatments, the same as (C) GPER1. Amplification curves representative of three independent experiments.*

2.5.2 BPA exposure significantly down-regulated *ESR1, ESR2* and *GPER1*

To confirm the estrogen receptor competence of the MCF-12A cells, they were exposed to two concentrations of BPA. Real-time PCR was carried out to assess the changes in gene expression. ACTB was used as the reference gene to normalise the real-time PCR data. There was a significant down-regulation of *ESR1* and *GPER1* in both concentrations of BPA. There was significant down-regulation of ESR2 only in 1×10^{-5} M.



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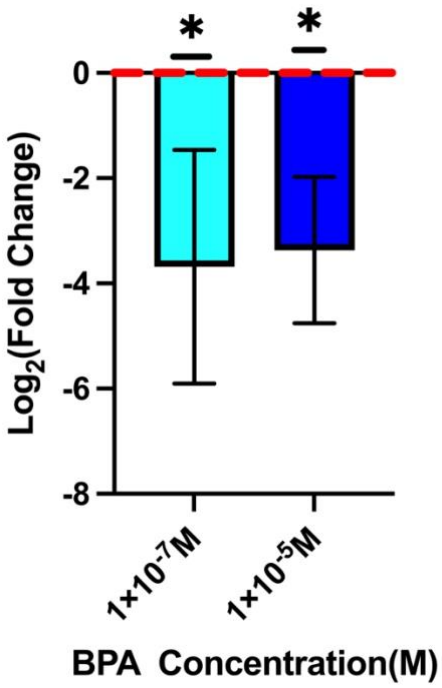
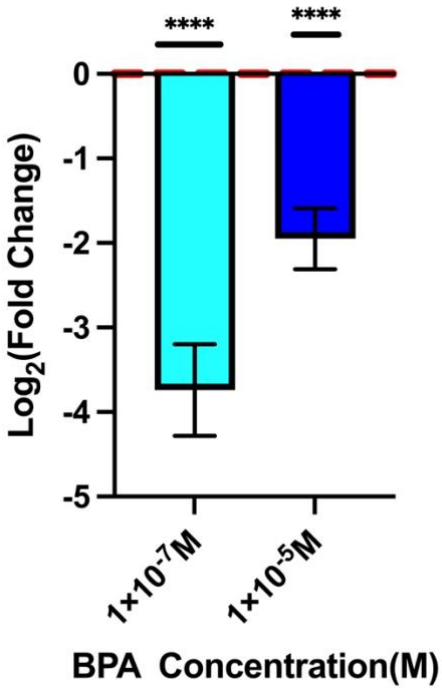

*
*
0
-2
-4
-6
-8
Log2(Fold Change)
1×10-7M
1×10-5M
BPA Concentration(M)

C


****
****
0
-1
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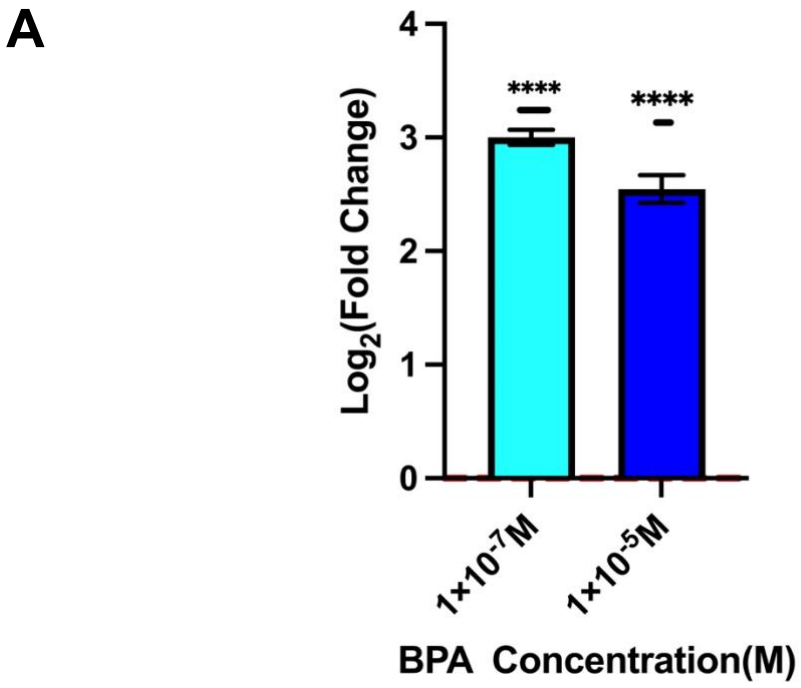
-3
-4
-5
Log2(Fold Change)
1×10-7M
1×10-5M
BPA Concentration(M)


1×10-7M
1×10-5M

Figure 2.3 Bar graphs showing BPA-induced gene expression denoted by log2(fold change) in (A) *ESR1, (B) ESR2* and (C) *GPER1*. *MCF-12A cells were treated in parallel for 24 hours with 0.5% (solvent control) and BPA at 1e-7M and 1E-5M concentrations. Gene expression levels were determined by qPCR. Expression profiles were determined by comparing treated samples to controls. A decrease in gene expression was observed in ESR1 (A), ESR2 (B) and GPER1 (C). Error bars represent the standard error of the mean (SEM). Significance is denoted by ****<0.0001, *0.03 as determined by ordinary one-way ANOVA*

2.5.3 BPA exposure induced significant changes in gene expression in *CCND1*, *BRCA1 and TFF1*

We also assessed the effect of BPA on the regulation of downstream estrogen-responsive genes. We tested CCND1, BRCA1, and TFF1, which are directly regulated by estrogen. CCND1 and TFF1 were up-regulated, while BRCA1 was down-regulated after 24 hours of exposure to both concentrations of BPA.



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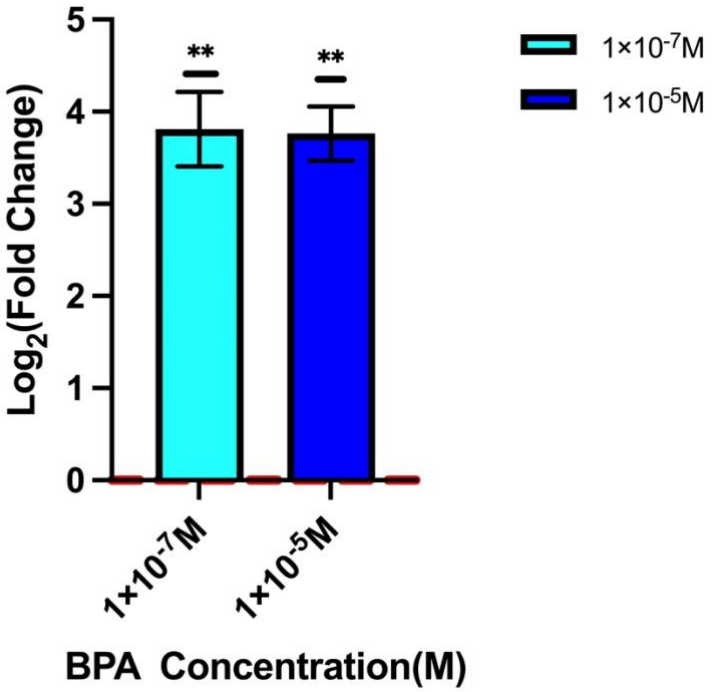
5
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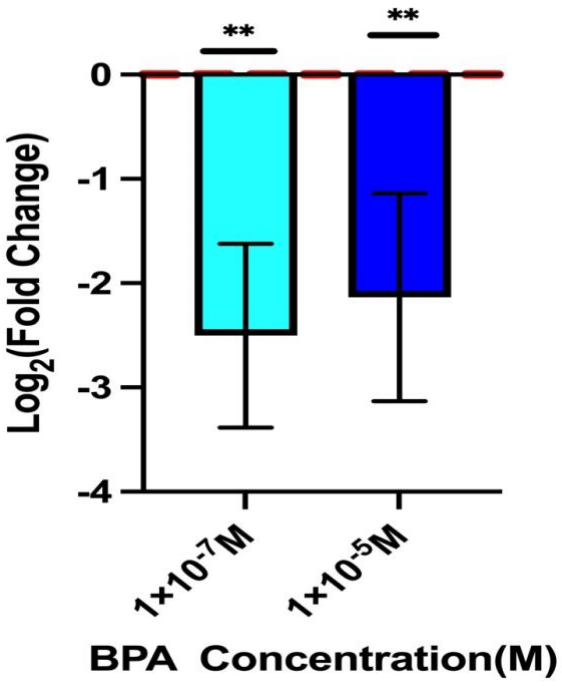
1×10-7M
1×10-5M
Log2(Fold Change)
BPA Concentration(M)

C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-4
1×10-7M
1×10-5M
Log2(Fold Change)
BPA Concentration(M)

Figure 2.4 Bar graphs showing BPA-induced gene expression denoted by log2(fold change) in (A) *CCND1, (B) TFF1* and (C) *BRCA1*. *MCF-12A cells were treated in parallel for 24 hours with 0.5% (solvent control) and BPA at 1e-7M and 1E-5M concentrations. Gene expression levels were determined by qPCR. Expression profiles were determined by comparing treated samples to controls. An increase in gene expression was observed in CCND1 (A) and TFF1 (B), and a decrease was observed in BRCA1 (C). Error bars represent the standard error of the mean (SEM). Significance is denoted by ****<0.0001, *0.03 as determined by ordinary one-way ANOVA.*

2.5.4 Fatty Acid resulted in significant up-regulation of FASN, LIPE, SREBP-1c, AKR1C1 and CD36

We examined whether our mixture of fatty acids crosses the cell membrane into the MCF-12A cells. To access that, we tested for the expression of FASN, LIPE, SREBP-1c, AKR1C1 and CD36. As discussed earlier, these genes are linked to various cellular activities when activated by fatty acids. Real-time PCR analysis showed an up-regulation of these genes after 24 hours of exposure to the fatty acids mixture.

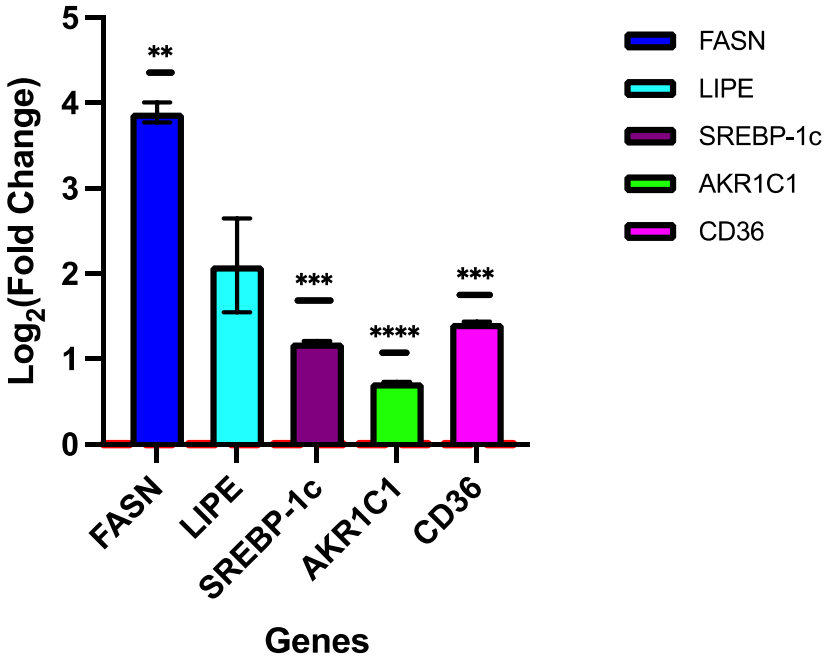


Figure 2.5 Bar plots showing the expression fold changes in *FASN*, *LIPE*, *SREBP-1c*, *AKR1c1* and *CD36*. *MCF-12A cells were treated in parallel for 24 hours with 0.5% (solvent control), and a mixture of fatty acids at concentrations of 5.39×10^{-3} M. Gene expression levels were determined by qPCR. Expression profiles were determined by comparing treated samples to controls. An increase in gene expression was observed in FASN, LIPE, SREBP-1c, AKR1C1, and CD36. Error bars represent the standard error of the mean (SEM). Significance is denoted by ****<0.0001, *0.03 as determined by ordinary one-way ANOVA.*

2.6 Discussion

MCF-12A cells have been published as both ER-positive (Engel *et al.*, 2019; Marchese and Silva, 2012; Gelfand *et al.*, 2016) and ER-negative (Subik *et al.*, 2010; Sweeney, Sonnenschein and Soto, 2018). It has also been reported that passaging cells over a period can introduce phenotypic and genotypic alterations (Puschhof, Pleguezuelos-Manzano and Clevers, 2021). Therefore, it was necessary to establish the ER and *GPER1* status of the batch of cells for this project. Our results showed that the cells used here expressed measurable constitutive levels of *ESR1* and *ESR2*. At the tested BPA concentrations of 1×10^{-7} M and 1×10^{-5} M, ESR1 and ESR2 were down-regulated after 24 hours of exposure. This result is similar to what was reported by Stephanie Marchese, who showed that *ESR1* and *ESR2* were down-regulated after exposure to 1×10^{-9} M E2 (Marchese, 2013). Furthermore, significant down-regulation of ESR1 and ESR2 after exposure to BPA has been established by Maund, who also had to establish the estrogen competence of MCF-12A cell lines (Maund, 2018). Down-regulation of *ESR1* was also reported at the gene and protein level when MCF-7 cell lines were exposed to 1×10^{-9} M of E2 after 15 minutes (Jensen *et al.*, 1999). Mechanistically, we propose that the down-regulation of *ESR1* in our experiments is caused by a cessation in further transcription due to changes in the negative feedback loop, as reported in a study by Castellano and colleagues(Castellano *et al.*, 2009).

The expression of *GPER1* was significantly reduced after 24 hours of exposure to 1×10^{-7} M and 1×10^{-5} M BPA. Earlier research has shown that GPER1 was down-regulated in MCF-7 cells after exposure to 1×10^{-9} M of 4-methyl-2,4-bis(hydroxyphenyl)pent-1-ene (MBP). MBP is a metabolite of BPA down-regulated *GPER1* gene expression after 24 hours of exposure (Hirao-Suzuki *et al.*, 2021).

We also wanted to determine how the MCF12A cell lines expressed other genes that have been found in the literature to be regulated by estrogens. This was done further to establish the cell line's appropriateness for the project, as we needed to ensure, not only that the receptors were present and responsive to estrogens but also functional and capable of regulating downstream target genes. We showed that *CCND1* was significantly up-regulated after 24 hours of BPA. The up-regulation of cell-cycle regulators has been shown in other studies. It has been demonstrated in MCF-7 breast cancer cells that 1×10^{-9} M of BPA up-regulated the expression of *CCND1* after 24 hours of exposure (Mlynarčíková, Macho and Ficková, 2013). Pfeifer and colleagues showed that 10nM BPA significantly increased *CCND1* expression in MCF-7 cell lines (Pfeifer, Chung and Hu, 2015). BPA alternatives such as Bisphenol S (BPS) have also been shown to increase the expression of *CCND1* after 24 hours of exposure (10μM BPS) (Lin *et al.*, 2019).

We also demonstrated the presence of the TFF1 gene in the MCF12A cell line. We showed that both concentrations of BPA up-regulated *TFF1* after 24 hours. TFF1 has an imperfect palindromic estrogen-responsive element in its promoter region and is regulated by the estrogen receptor (Marchese and Silva, 2012). Marchese and Silva demonstrated that 1nM E2 up-regulated the expression TFF1 in MCF12A cell lines after 24 hours of exposure(Marchese and Silva, 2012). Sengupta and colleagues also demonstrated that E2 (10nM) and BPA (1×10^{-5} M) upregulated TFF1 expression in MCF-7 cell lines (Sengupta *et al.*, 2013). The upregulation of TFF1 in our experiments further confirms the presence of a functional estrogen receptor in the batch of MCF12 to be used for the project.

Finally, we demonstrated that both concentrations of BPA significantly down-regulated the expression of BRCA1. Down-regulation of BRCA1 by BPA was demonstrated in

the breast cancer cell line ERα-HA. Singleton and colleagues demonstrated that BRCA1 was down-regulated in the ERα-HA breast cancer cell line after 3 hours of BPA treatment (1×10^{-6} M) (Singleton *et al.*, 2006).

As stated earlier, the ER status of MCF12A has been controversial. Overall, the first part of this chapter aimed to establish the estrogen competence of the batch of the MCF-12A cell to be used. The data analysis clearly shows the presence of the *ESR1, ESR2,* and *GPER1* genes. Other genes downstream of estrogenic action, such as *CCND1, TFF1,* and *BRCA1,* were also found to be activated to varying degrees.

The next section of this chapter was to ascertain whether the fatty acid mixture could cross into the cells and cause changes, such as an alteration in gene expression. The genes we chose were those affected by fatty acids and implicated in various cancers, such as breast cancer. Our data suggested that after exposing MCF-12A cells to 5.39 $\times 10^{-5}$ M fatty acid mixture, *FASN, LIPE, SREBP-1c, AKR1C1*, and *CD36* were up-regulated. *FASN* (p = 0.0072) plays a vital role in the *de novo* synthesis of fatty acids. In normal cells, the expression of FASN is very low but up-regulated in breast cancer cell lines such as MCF-7 and MBD (Xu *et al.*, 2021; Li, Tian and Ma, 2014). The up-regulation of *FASN* in MCF-12A by the fatty acid mixture could be attributed to the fact that *FASN* is up-regulated in hormone-sensitive cells (Kalkhoven *et al.*, 1994; Kusakabe *et al.*, 2000). It has also been demonstrated in MCF-7 and BT-474 cells that *FASN* regulates Estrogen receptor-α signalling (Menendez *et al.*, 2021). MCF-12A cells, being an estrogen-competent cell line, could therefore express *FASN*.

SREBP-1c (p= 0.0004) was also significantly up-regulated after MCF-12A cells were exposed to our mixture of fatty acids. *SREBP-1c* up-regulation has also been shown to regulate in the activation of *FASN* transcription (Zhao *et al.*, 2016). The mixture of fatty acids up-regulated LIPE; however, this was not significant compared to the

controls. Up-regulation of *LIPE* has been associated with increased lipid metabolism in breast cancer cells (Marino *et al.*, 2020b). The expression of *AKR1C1* ($p < 0.0001$) was significant but lower than all the genes tested. The lower expression of AKR1C1 could be attributed to an inhibition by the fatty acids. It has been demonstrated that long-chain fatty acids such as oleic, linoleic, eicosapentaenoic acid, palmitoleic acid and docosapentaenoic acid inhibited *AKR1C1* (Hara *et al.*, 2017).

The mixture of fatty acids up-regulated CD36 ($p = 0.004$). Vallvé and colleagues showed that unsaturated fatty acids such as linoleic and arachidonic acids up-regulated CD36 in THP-1 macrophages after 24 hours of exposure (Vallvé *et al.*, 2002). An up-regulation of CD36 signifies fatty acid uptake, as elucidated by Ehehalt and colleagues (Ehehalt *et al.*, 2008). It has also been demonstrated that *CD36* facilitated the uptake of palmitic acid into cells during matrix detachment and tumour progression (Terry *et al.*, 2023).

Overall, we have shown that our MCF-12A cells are estrogen-competent and, therefore, will be an appropriate model for studying the effect of EDCs on breast cancer initiation and progression. We also demonstrated for the first time that a mixture of fatty acids present in their tissue concentrations can affect lipogenic genes in MCF-12A cells after 24 hours of exposure.

Chapter Three: Effect of Mixture of Endocrine Disruptors and Fatty Acids on MCF-12A in 3-Dimensional Models

3 Effect of Mixture of Endocrine Disruptors and Fatty Acids on MCF-12 in a 3-Dimensional Models

3.1 Introduction

As explained in Chapter One, endocrine disruptors have been implicated in breast cancer initiation and progression. For example, low-dose exposure of breast cancer cell lines to phenolic endocrine disruptors (*p,p'*-DDT, methoxychlor, benzophenone-2, bisphenol A, bisphenol S, 4-phenyl phenol, and n-butylparaben) at environmentally relevant concentrations resulted in an up-regulation of aromatase activity, increased estradiol biosynthesis and estrogen-regulated breast cancer proliferation (Williams and Darbre, 2019). BPA induces the expression of breast cancer oncogenes such as HOXB9 and causes epigenetic methylation in tumour suppressor genes such as TIMP3, CHFR, IGSF4, CDH13, and GSTP1 both *in vivo* and *in vitro* (Deb *et al.*, 2016; Nair *et al.*, 2020; Khan *et al.*, 2021a). Epidemiological studies on the effect of EDCs on breast cancer progression have not been conclusive. A population-based case-control study of 575 postmenopausal women in Poland revealed that urinary Bisphenol A glucuronide did not correlate to breast cancer risk (Trabert *et al.*, 2014). Morgan and colleagues also conducted a cross-sectional study on samples collected from women (20 years and older) between 1994-2004. They reported that there was no significant association between phthalates and BPA on breast cancer risk (Morgan *et al.*, 2017). However, there have been studies that have reported that EDCs, such as PCBs, increase breast cancer risk (Huang *et al.*, 2019; Morgan *et al.*, 2017; Rusiecki *et al.*, 2020). It has been argued that the lack of causality in these epidemiological studies was due to the focus on individual compounds present in low concentrations in tissues and many confounding factors such as diet, occupation and exercise.

Furthermore, the effects of these EDCs have been assessed in comparison to endogenous hormones E1 (estrone, E2 (estradiol) and E3 (estriol), which have significantly more potent estrogenic effects than EDCs. Therefore, when assessing the impact of EDCs individually, the common conclusion is that they do not affect breast cancer risk as the tissue concentrations are not high enough (Kortenkamp, 2006). However, this assumption fails to consider the fact that humans are exposed to low levels of a cocktail of chemicals that can act together to increase a person's internal estrogenic burden and elevate breast cancer risk (Kortenkamp, 2006; Gaudriault *et al.*, 2017). Due to the ubiquitous nature of EDCs and the multiple routes of exposure, there is a compelling argument to assess their toxicity as a mixture (Lazarevic *et al.*, 2019; Stordal *et al.*, 2024).

In Chapter One, we discussed that the effect of a high-fat diet on breast cancer initiation and progression is inconclusive (Zhou *et al.*, 2016; Yang *et al.*, 2014). While some studies have established a causal link between saturated fatty acid and breast cancer risk, it is still contentious, and the mechanisms behind the effects have not been elucidated. However, it could be argued that the association between diet and cancer development follows the scenario of EDCs. While fatty acids may not significantly impact breast cancer risk on their own, it is plausible they can add to the overall cancer risk when combined with a high body burden of mixtures of endocrine disruptors.

3.1.1 Three-Dimensional Breast Cancer Cultures

As discussed in Chapter One, three-dimensional (3D) cultures are highly used in biomedical research because of their ability to replicate the complexity of tissue morphology and architecture in homeostasis and disease states (Roberts, Peyman

and Speirs, 2019). 3D cultures have been developed using suspension-triggered spheroids, synthetic scaffolds, natural and synthetic hydrogels, and more complex bioprinting matrices. As discussed in Chapters One and Five, 3D cultures have been used to study the proliferation and invasiveness of breast cancer cells (Pal *et al.*, 2020; Lee *et al.*, 2022), angiogenesis in breast cancer progression (Correa de Sampaio *et al.*, 2012), and breast cancer invasiveness and metastasis (Katz *et al.*, 2011).

The effects of EDCs on breast cell lines have been studied on 3D models. For example, Atlas and Dimitrova studied the impact of Bisphenol S (BPS) and Bisphenol A on breast cell line MCF-12A cultured on Matrigel. They reported that MCF-12A cell lines formed structured acini that were disrupted on Matrigel in an equipotent manner and caused luminal clearing. They concluded that environmental pollutants affect mammary development and could impact breast cancer risk (Atlas and Dimitrova, 2019). Winkler and colleagues exposed primary human non-malignant breast organoids to EDCs (Bisphenol S and Bisphenol F). They reported that the EDCs produced protumorigenic effects at both the morphology and proteomic levels (Winkler *et al.*, 2022). Earlier work by Marchese and Silva also demonstrated the effect of BPA, propylparaben and E2 on MCF-12A cells cultured on Matrigel. They reported disruptions in organisation and size of acini suggestive neoplastic activity (Marchese and Silva, 2012). While the number of studies using a 3D system to study the effects of chemicals on the breast is relatively low, the work described above indicates that 3D cultures present an appropriate and useful method to study the impact of mixtures of endocrine disruptors and fatty acids on early breast carcinogenesis (Figure 3.1).

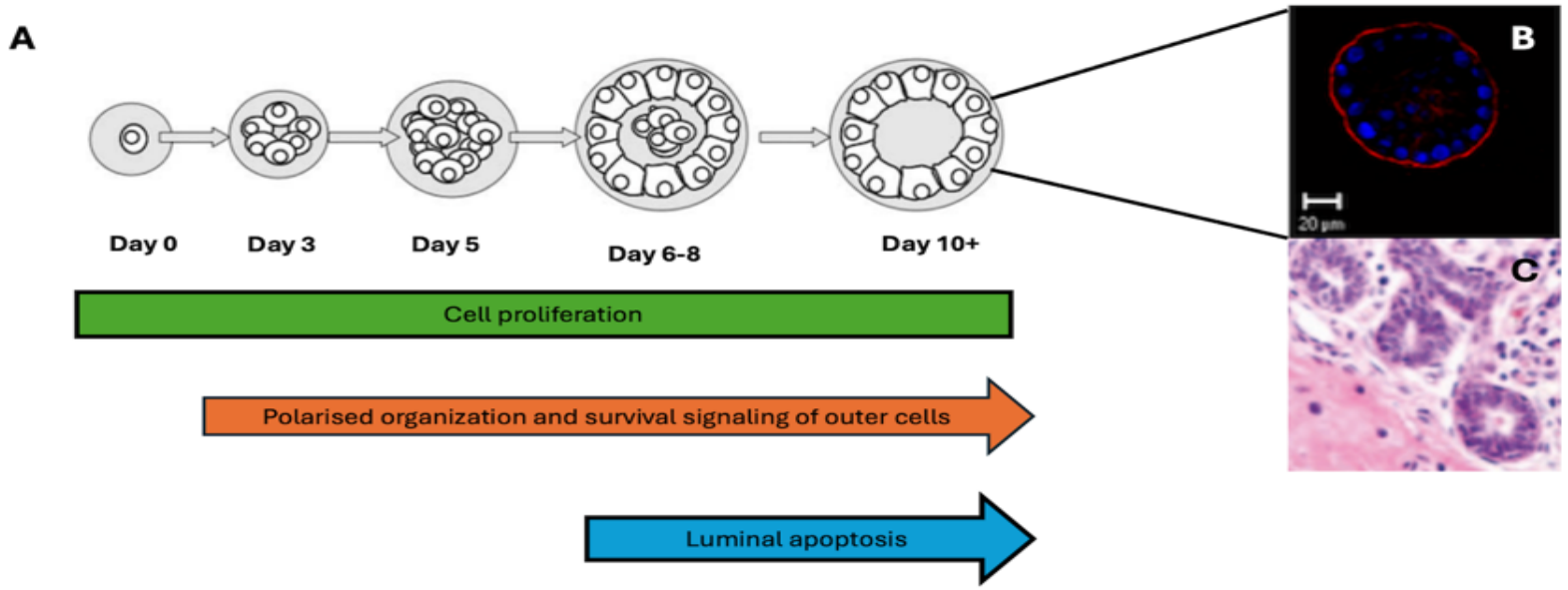


Figure 3.1 Mammary epithelial cells organise into acini-like structures when grown on Matrigel. (***A***) *A schematic representation of the sequence of development of acini when mammary epithelial cells are cultured on Matrigel, including proliferation, polarisation and apoptosis. Cells begin to proliferate and begin to form acini. On days 5-8, there is a visible inner and outer layer; the outer layer interacts with the ECM and remains deformed throughout the development of the acini. Apoptosis and lumen formation occur from day 8 and fully formed from 10 (**B**) Confocal imaging of MCF12A grown on Matrigel showed that by day 10 normal epithelial cells develop into acini-like structure with a hollow lumen. (C) In vitro acini structures recapitulate in vivo acini structure (Marchese and Silva, 2012; Debnath, Muthuswamy and Brugge, 2003; Debnath and Brugge, 2005)*.

3.1.2 Cell Proliferation

The cell cycle is a sequence of events which results in the doubling of cellular components and the segregation of daughter cells. The cell cycle is divided into four phases in eukaryotic cells: gap 1 (G1), DNA synthesis (S), gap 2 (G2) and mitosis (M) (Figure 3.1) (Liu *et al.*, 2019b). These phases contain checkpoints that ensure an error-free replication and segregation of chromosomes into daughter cells. The checkpoints also prevent genomic instability, which can induce and progress tumourigenesis (Thu *et al.*, 2018). The majority of cells in the human body are in a non-proliferative state (Matthews, Bertoli and de Bruin, 2022). The decision to enter the cell cycle is determined by triggers such as nutrients and growth factors like human growth hormone (HGH) (Øvrebø, Ma and Edgar, 2022). In breast cells, progesterone and estrogen are significant triggers of proliferation (Hilton, Clarke and Graham, 2018). During the G1 phase, the cell grows, increases in size, and enters the S phase after reaching a certain size. DNA synthesis and duplication occur in the S phase (Wang, 2021). Optimal entry into the S phase is essential for development, tissue repair and immune defences. However, a dysregulated entry leads to replication stress, DNA damage and oncogenesis (Hume, Dianov and Ramadan, 2020). In the G2 phase, the cell checks for DNA replication completion and enters mitosis. In the M phase, mitosis occurs where chromosomes are segregated, and cell division is completed. A successful cell cycle will result in the two daughter cells receiving an equal number of chromosomes from the parents. The whole cell cycle process occurs between 10-30 hours in most mammalian cells (Wang, 2021). Cells can leave the cell cycle at G1, return to G0, and become quiescent (Johnson and Walker, 1999).

This process is regulated by cyclin-dependent kinases (CDKs) (Barnum and O'Connell, 2014). Though numerous CDKs and cyclins exist in the loci in human cells,

only a few cyclin-CDK complexes are directly involved in regulating the cell cycle. These include CDK2, CDK4, CDK6, CDK1 and 10 cyclins in four different classes (A, B, D and E-type cyclins) (Figure 3.2). Tumour-associated dysregulation of CDK-cyclin leads to continued proliferation or unscheduled re-entry into the cell cycle, which characterises most human tumours (Malumbres and Barbacid, 2009). The activity of CDKs is controlled primarily by the concentration of cyclins. While the concentration of CDKs remains constant, the concentrations of cyclins fluctuate due to transcriptional regulation and controlled degradation by ubiquitin proteolytic pathways (as reviewed in (Rais *et al.*, 2023).

There are regulatory proteins that can arrest the cell cycle, commonly referred to as negative regulators. The most widely studied negative regulators include retinoblastoma protein (Rb), p53 and p21, as reviewed in (Engeland, 2022). They function by mediating the transcriptional dysregulation of many cell cycle genes (Kurimchak and Graña, 2015). Retinoblastoma protein (Rb protein) is involved in suppressing G1 phase transition. In early G1 phase, pRB is unphosphorylated but becomes inactivated by phosphorylation in the mid/late G1 phase by cyclins (D/E) and cyclin-dependent kinases (Palaiologos *et al.*, 2019). Phosphorylation of pRb leads to the accumulation of E2F transcription factors. E2F transcriptional factors target genes needed to synthesise proteins required for progression from G1 to S-phase (Bretones, Delgado and León, 2015; Otto and Sicinski, 2017). Dephosphorylated pRb leads to heterodimerisation with E2F and inhibition of activity (Sheldon, 2017). p53 is a well-known cell cycle regulator that induces transcription of the gene encoding p21 and inhibits CDK-mediated phosphorylation of retinoblastoma protein (Kuganesan *et al.*, 2021).

Various cell cycle regulators have been shown to mediate mammary epithelial cell proliferation. For example, cyclin-dependent kinase inhibitors (CKDKI), $p27^{kip1}$ and $p21^{cip1}$, were found to be involved in the regulating acini formation of MCF10A cell lines in a cultured on Matrigel. While $p21^{cip1}$ was highly expressed in the early stages of proliferation and acini formation, $p27^{kip1}$ increased when proliferation decreased, and acini reached full formation. There was an increase in the proliferation marker Ki67 in the early stages of acini formation (Coppock *et al.*, 2007). Overexpression of cyclin D1 has also been found to disrupt acini formation in the MCF10A cell line cultured on Matrigel (Debnath, Muthuswamy and Brugge, 2003). Cyclin D1 was also found to mediate the proliferative effect of helix-loop-helix (HLH) protein Id1 in MCF12A cell lines cultured in Matrigel, further demonstrating the suitability of 3D in vitro assays to study early breast carcinogenesis (Caldon *et al.*, 2008).

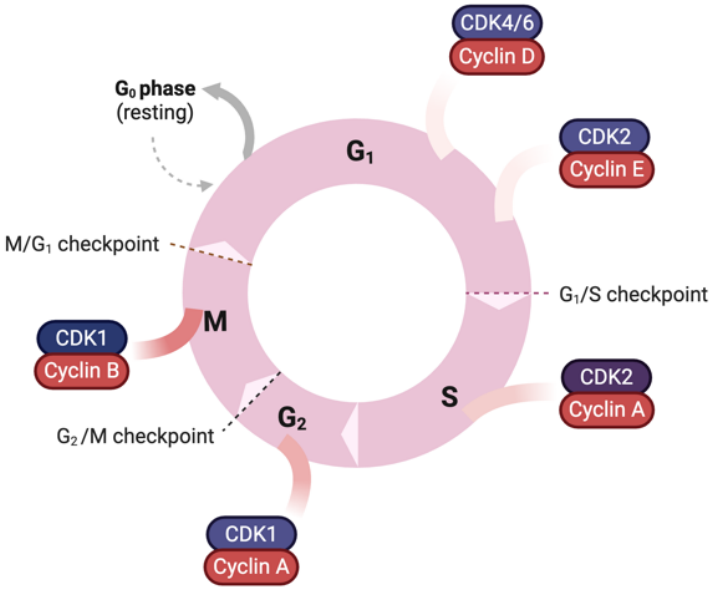


Figure 3.2 Mammalian cell cycle regulated by Cdk/cyclin complexes. *Under the influence of mitogenic signals, normal eukaryotic cells leave quiescence and irreversibly enter successive stages of the cell cycle: G1, S, G2 and M phases. These stages are regulated by complexes of cdk /cyclin (Tchakarska and Sola, 2020). Image created with Biorender software* (https://biorender.com/).

3.1.3 Luminal filling

The ducts of human breasts consist mainly of two cellular elements in a bilayer structure: luminal epithelial cells, which surround the central ductal cavity and myoepithelial cells, found between the basement membrane and luminal epithelial cells (Carter *et al.*, 2017). In adult breasts, these ducts carry nutrient-rich milk to offspring during lactation (Shore *et al.*, 2016). The lumen in the ducts is formed by the apoptosis of the inner cells in newly branched epithelial chords (Mailleux, Overholtzer and Brugge, 2008). Autophagy is also involved in the formation of lumen in the mammary gland (Wärri *et al.*, 2018). The loss of luminal space during tumorigenesis is commonly called luminal filling and is a characteristic of glandular epithelial tumours (Halaoui *et al.*, 2017; Debnath and Brugge, 2005). Previous studies have reported that luminal filling is enhanced by the co-expression of oncogenes that increase proliferation and those that inhibit apoptosis or the upregulation of HER2 by heterodimerisation (Debnath *et al.*, 2002). Using MCF10A cell line cultured on Matrigel, it has been demonstrated that caspase-mediated apoptosis and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated autophagy are both needed for luminal clearance (Mills *et al.*, 2004). As stated earlier, because luminal filling is a hallmark of early carcinogenesis, it is, therefore, necessary to study how extrinsic factors such as mixtures of endocrine disruptors and fatty acid diet affect the mechanisms involved to provide an insight into breast cancer risk.

Apoptosis, also called programmed cell death, is a fundamental process in morphogenesis and can be triggered by intrinsic or external stimuli. The extrinsic apoptosis pathway is mediated by activating receptors such as Fas receptors, DR4/DR5, tumour necrosis factor receptors (TNF-R), and TNF-related apoptosis-inducing ligand receptors (TRAIL-R). These receptors interact with adaptor proteins

such as Fas-associated proteins with death domain (FAAD), tumour necrosis factor receptor type1 associated death domain protein (TRADD) and caspase-8 (as reviewed in (Kashyap, Garg and Goel, 2021). Intrinsic or mitochondrial apoptosis pathway is triggered by DNA damage or oxidative stress. Proapoptotic members of the BCL-2 family found in the mitochondrial outer membrane instigate mitochondrial outer membrane permeabilization (MOMP). The proapoptotic members of BCL-2 include BAX, BAK, BIM, BID, and PUMA. This leads to the release of cytochrome C from the mitochondria into the cytoplasm. The release of Cytochrome C activates caspase 9, followed by the activation of executioner caspase 3 (Carneiro and El-Deiry, 2020; Kale, Osterlund and Andrews, 2018). Caspase 3 induces the degradation of chromosomal DNA and causes chromatin condensation, cytoskeletal reorientation, and cell disintegration (Cavalcante *et al.*, 2019). It has been demonstrated in 3D cultures that apoptosis can be seen by the presence of caspase 3 in the inner cells around day 8, initiating the hollowing of the acini lumen (Figure 3.3)

Apart from apoptosis, autophagy is involved in luminal clearing and lumen maintenance (Mills *et al.*, 2004). It is a conserved physiological process by which cells degrade their components by lysosomal action (Ichimiya *et al.*, 2020). There are three autophagic pathways: chaperone-mediated autophagy, microautophagy, and macroautophagy. Macroautophagy is the major and well-studied form of autophagy and is herein referred to as autophagy (Schmeisser, Bekisz and Zoon, 2014; Mizushima and Komatsu, 2011). It is induced by external and internal cellular stressors, such as starvation and oxidative stress, and inhibitors of TOR, such as rapamycin (Li, He and Ma, 2020; Tian *et al.*, 2020). Mechanistically, autophagy involves the formation of double-membrane vesicles called autophagosomes. These vesicles then fuse with lysosomes, which are degraded acidic lysosomal hydrolases

and the product is recycled (Russo and Russo, 2018). Autophagy has been demonstrated in 3D mammary cultures in the centrally located cell of developing acini. It was observed by the overexpression of Bcl-2 (Debnath *et al.*, 2002; Fung *et al.*, 2008).

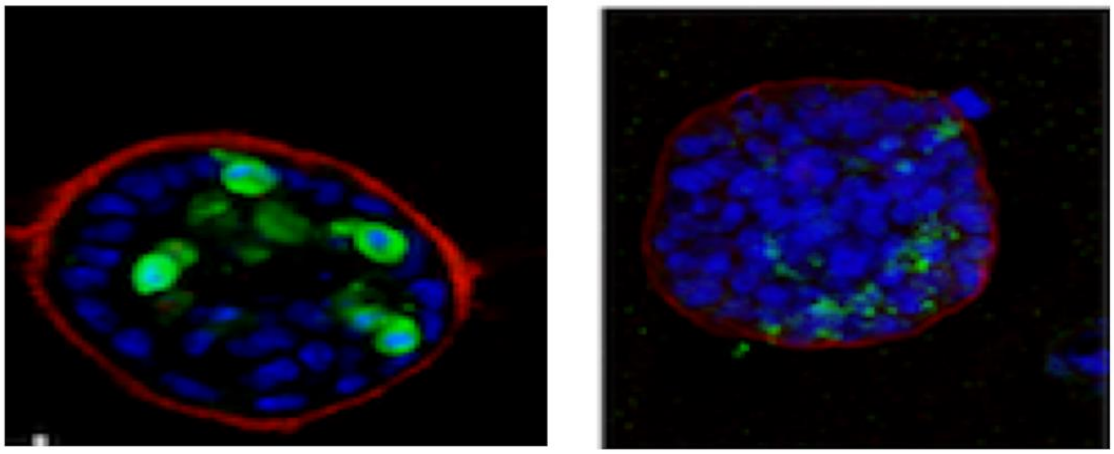


Figure 3.3 Evidence of hollow formation and apoptosis in 3D cultured MCF-10A cells. *Immunofluorescent images demonstrating the formation of lumen formation and apoptosis by day 12. Images adapted from* (Marchese and Silva, 2012).

3.1.4 Loss of polarity

Normal epithelial cells demonstrate apical polarity, which separates the plasma membrane into distinct compartments and is essential for cellular homeostasis, tissue function, and cancer suppression. Polarized cells have an apical and basolateral membrane separated by tight junctions (Catterall, Lelarge and McCaffrey, 2020). Polarity protein complexes mediate the maintenance of cell polarity within the epithelial tissues and the asymmetric segregation of cellular proteins (Atashrazm and Ellis, 2021). Three conserved polarity protein complexes: Par (Par3, Par6 and aPKC), Crumbs (PATJ, Pals1, crumbs) and scribble (lgl, Dlg, scribble) are involved in the maintenance of cellular polarity. Par and Crumbs complexes form the apical polarity domain, while the scribble complex is involved in basolateral polarity (Grifone, 2020).

In acini architecture, the presence of cell-cell tight junctions and hemidesmosomes at the junction of the basement membrane indicates polarity. Molecular markers of apical-basal polarity, α6/β6 integrin indicating hemidesmosomes and ZO-1 indicating tight junctions have been found in 3D mammary cultures (Vidi, Bissell and Lelièvre, 2013).

Breast luminal epithelial cells exhibit apical-basal polarity and the inability to maintain the organizational structure due to dysregulation of polarity protein complexes is associated with hyperplasia and cancers (Rejon, Al-Masri and McCaffrey, 2016). Loss of polarity is a common hallmark of invasive breast cancer, irrespective of the type, grade, ER or HER2 status (Fomicheva, Tross and Macara, 2020). In 3D cultures, loss of apical polarity is shown by the disorganisation of the acini structures (Vidi, Bissell and Lelièvre, 2013).

3.1.5 Chapter Scope

As stated earlier, breast cancer is a multifactorial disease, and risk factor interaction contributes to overall lifetime risk. So far, there has not been any research that has looked at the effect of combinations of different types of external factors, such as EDCs and fatty acids on breast cancer risk. Within this chapter, we aimed to assess whether a mixture of twelve endocrine disruptors representative of real exposures and four fatty acids representative of real diet patterns could cause effects that indicate an increase in breast cancer risk, both as individual mixtures and in combination. Utilising a 3D Matrigel culture that recapitulates the human breast in a better way than traditional 2D assays, we addressed the aims by investigating the following questions:

1. Do mixtures of endocrine disruptors and fatty acids significantly affect the morphology of acini indicative of early breast carcinogenesis?

2. Do mixtures of endocrine disruptors and fatty acids act in combination to affect the morphology of acini, indicating early breast carcinogenesis?
3. Does the exposure to mixtures of endocrine disruptors and fatty acids, both individually and in combination, significantly alter genes associated with early breast carcinogenesis, indicating a possible increase in the risk of breast cancer?

3.2 Methodology

3.2.1 Chemical Handling

All the EDCs in the mixture were of analytical standard (> 95% purity). All the chemicals were purchased from Sigma Aldrich except the parabens (propyl-butyl paraben), which were purchased from Fisher Scientific. Stock concentrations were prepared for all chemicals. These were subsequently diluted and combined according to their average serum concentration concentrations, (Kortenkamp, Scholze and Ermler, 2014) as shown in Table (3.2). In determining the chemicals for our mixtures, we chose ubiquitous chemicals that have been tested for their endocrine-disrupting activity. Kortenkamp and colleagues determined the average concentration of these chemicals in human serum from an average exposure scenario in Europe (Kortenkamp, Scholze and Ermler, 2014). In preparing the fatty acid mixtures, we chose fatty acids that were among those most consumed in Europe, according to the EPIC study (Huybrechts *et al.*, 2023). We used the total plasma concentration reported by (Abdelmagid *et al.*, 2015). The concentrations of fatty acids used in the mixture were found by calculating the average between the reported maximum and minimum concentrations.

The mixture of fatty acids was prepared, as shown in (Table 3.1). Stock concentrations were stored at -20°C for long-term storage.

3.2.2 Routine Cell Culture

All reagents were obtained from Sigma Aldrich unless otherwise stated. MCF-12A cells were obtained from the American Type Collection and cultured in T75 cm^2 canter-neck tissue culture flasks in a monolayer. Cells were supplied with Dulbecco's Modified Eagle Medium (DMEM: F12) supplemented with 5% horse serum, 0.02% epidermal growth factor, 0.01% cholera toxins, 0.1% insulin, 0.05% hydrocortisone and 1% pen/strep. Cultures were maintained in 37°C and 5% CO_2 humidified incubator, and the medium was replaced every three days.

Table 3.1 List of fatty acids, their mean plasma concentration, and their percentage in the mixture solution (Abdelmagid *et al.*, 2015)

Fatty Acids	**Mean Plasma Concentration (M)**	**Proportion of Fatty Acid in Mixture (%)**
Elaidic Acid	4.40×10^{-5}	0.6
Linoleic Acid	2.63×10^{-3}	48.66
Palmitic Acid	2.1747×10^{-3}	40.32
Stearic Acid	5.62×10^{-4}	10.42
Total Mixture Concentration	5.39×10^{-3}	

Table 3.2 Endocrine disruptors, their tissue (serum) concentrations and percentage proportion in the mixture used in the mixture (Kortenkamp, Scholze and Ermler, 2014)

Chemical	**Tissue Concentration (M)**	**Proportion of Chemical in Mixture (%)**
BDE 100	8.12×10^{-13}	0.124125341
Propylparaben	2.34×10^{-11}	0.816893268
Methylparaben	1.54×10^{-10}	0.117229488
Ethylparaben	2.21×10^{-11}	0.130490743
Butylparaben	2.46×10^{-11}	0.938896808
Tonalide	1.77×10^{-10}	0.938896808
Bisphenol A	8.76×10^{-9}	46.46743524
3-benzyliden Camphor	4.81×10^{-10}	2.551465
Galaxolide	7.10×10^{-10}	3.766196
Benzopehenone-2	7.50×-09	39.78376
Benzophenone-3	8.09×10^{-10}	1.007855
p p-DDE	1.90×10^{-10}	4.291342
Total Tissue Concentration	1.89×10^{-8}	100%

3.2.3 Three-dimensional Matrigel Cultures

MCF12A cells were cultured on a Growth Factor Reduced (GFR) Matrigel bed per established protocols (Marchese and Silva, 2012; Debnath, Muthuswamy and Brugge, 2003). 8-well chamber slides (Merck Life Science, Gillingham, UK) were coated with 100μl 100% growth factor reduced (GFR) Matrigel (Fisher Scientific, Loughborough, UK) and allowed to polymerise at 37°C and 5% CO_2 for 15 minutes in an incubator. 70% confluent cells were trypsinised, and a single cell suspension was prepared in a growth medium (2×10^4 cells/ml). Cell suspension was then mixed in a 1:1 ratio to assay medium (supplemented DMEM F-12 + 4% GFR Matrigel). Chemical treatments dissolved in molecular grade EtOH (100%) were dissolved in the cell suspension solution. The final concentration of EtOH did not exceed 0.5%. Negative control samples were exposed to 0.5% EtOH. 400μl (500 cellss) per well cell suspension solution was overlayed on the polymerised Matrigel bed. (Figure 3.4). Cultures were maintained in an incubator at 37°C and 5% CO_2. The growth medium was replenished every four days, containing 2% Matrigel and the test chemical at the concentration mentioned above. Cultures were incubated for 16 days and 10 days before being used for morphological and RNA sequencing analysis, respectively. At 16 days, Marchese and Silva showed that the non-malignant MCF-12A cells form growth-arrested acini, and there were apparent differences between the control and treated samples(Marchese and Silva, 2012). We settled on 10 days for the RNAseq because we observed well-formed acini that were starting to hollow out. The setup is described schematically, as shown in Figure 3.4.

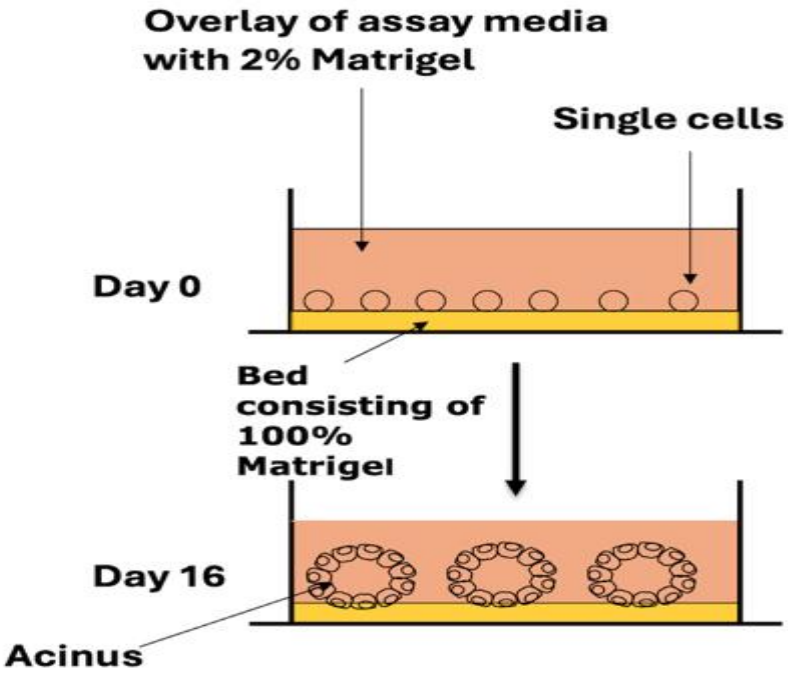


Figure 3.4 Schematic representation of MCF-12A three-dimensional assay. *A polymerised 100% GFR Matrigel was used as the scaffold on which MCF-12A suspended in the growth medium is overlayed. After 16 days of incubation, single cells organise into acini-like spheroids akin to the structures9 seen in the human breast.*

3.2.4 Immunocytochemistry

Following incubation for 16 days, the medium was removed, and the Matrigel bed was washed twice with PBS. The slides were then fixed with freshly cold methanol and acetone solution (1:1) for 12 minutes at -20°C. Cells were then permeabilised to allow antibody passage across the plasma membrane using 0.5% Triton-X in PBS for 10 minutes at 4°C. The slides were then washed three times with 100mM glycine solution for 10 minutes at room temperature on an orbital rocker to reduce cross-linking produced by fixation process. Slides were then incubated with the primary blocking

solution (Immunofluorescence buffer (IF buffer) and 10% fetal bovine serum) for 1 hour on an orbital rocker at room temperature to prevent unspecific antibody binding. IF buffer contained Bovine Serum Albumin (BSA), PBS, Triton and Tween. The primary block was then aspirated, and slides were incubated with the secondary block (900 μl Immunofluorescence buffer + 15.4 μl goat anti-mouse F(ab')2 fragment+ 100μl fetal bovine serum) for 40 minutes at room temperature on an orbital rocker at 4°C to reduce unspecific binding of antibody the Fc receptor and reduce background staining. Primary antibodies were centrifuged (5000 rpm for 5 minutes) to break down aggregates and prepared in freshly prepared second block solution according to the concentration in Table (3.3). Slides were incubated with primary antibodies overnight at 4 degrees on an orbital shaker.

Table 3.3 Primary antibodies used for immunochemistry to investigate apoptosis and the presence of basement membrane in MCF-12A cells cultured on Matrigel

Primary Antibody	Species	Source	Purpose	Dilution
Cleaved caspases-3	Rabbit	Cell Signalling	Apoptotic marker	1:200
Laminin	Mouse	Abcam	Basement Membrane	1:200

After incubation, slides were washed thrice (20 minutes for each wash) with IF buffer at room temperature on an orbital. Secondary antibodies (Table 3.4) were centrifuged to break aggregates and diluted in freshly prepared secondary buffer (IF + 10% fetal bovine serum). Samples were incubated with the secondary antibodies for 50 minutes at room temperature on an orbital rocker.

Table 3.4 Secondary antibodies used for immunocytochemistry to investigate apoptosis and the presence basement membrane in MCF-12A cells cultured on Matrigel.

Secondary Antibody	Species	Source	Dilution
Alexa Fluor ® 488 anti-rabbit IgG	Goat	Molecular Probes	1:200
Alexa Fluor ® 555 anti-mouse IgG	Goat	Molecular Probes	1:200

After the incubation with the secondary antibody, the samples were washed three times (20 minutes per wash) with immunofluorescence buffer (IF buffer) on an orbital shaker. They were allowed to dry in the dark and counterstained with DAPI (4' 6-diamidino-2-phenylindole) in mounting media. The slides were kept in the dark overnight to dry. Image acquisition was done with a Nikon Spinning Disk Confocal Microscope.

3.2.5 Morphological Analysis

Light microscope images of the cultures were taken every 2 days for a period of 16 days. At the end of the 16 days of culture, immunochemistry was performed, and acini were imaged using a Nikon Spinning Disk Confocal Microscope.

To measure the disruption of acini of the light microscope images, a minimum of 10 acini were randomly selected from each duplicate and were analysed for each treatment for each independent experiment. The area and circularity were the parameters chosen to quantify disruption. These two parameters have been used in previous studies to analyse the morphology of acini (Marchese and Silva, 2012; Carey, Martin and Reinhart-King, 2017; Corda *et al.*, 2017). Circularity was calculated using the following method:

$$Circularity = \frac{4\pi\ acini\ area}{acini\ perimeter^2}$$ **(Equation 2)**

A perfect circle has a circularity value of 1, representing a perfect and undisrupted circle. As the value approaches 0, the shape is irregular and disrupted. The area and circularity of the acini were measured using ImageJ (www.imagej.net) (Collins, 2007; Schindelin *et al.*, 2015), and GraphPad Prism (version 10.4.1) was used for the statistical analysis. Two-way ANOVA followed by Bonferroni correction was used to determine the statistical significance.

3.2.6 RNA Extraction for RNA Sequencing

To isolate acini for RNA sequencing at the end of day 10, the assay medium was removed from the wells and washed three times with warm PBS. 200μl of Cell Recovery Solution (Corning, New York, USA) was added to the wells, and the Matrigel/Cell Recovery solution was aspirated into an Eppendorf tube. An additional 200μl of Cell Recovery Solution was used to rinse the well to remove any remaining acini. The Matrigel/Recovery solution was then placed on ice for 1 hour. After incubation, the solution was centrifuged at 1000 rpm for 5 minutes; the supernatant was removed. The remaining pellet was washed twice with PBS. RNA was isolated using the Qiagen RNeasy® Mini Kit, as described in Chapter Two. Samples were treated in triplicate

3.2.7 RNA Sequencing (RNA-seq)

Sample sequencing was done with Illumina sequencing PE150, where the average reads of each replicate for the three experiments were taken (Table 3.5)

Table 3.5 Samples and reads obtained after RNA sequencing. RNA-sequencing was carried out using Illumina Sequencing PE150

Samples	Reads
Control	242701622
EDC Mixture	304706342
Fatty Acid Mixture	248556458
EDC Mixture + Fatty Acid Mixture	316715988

3.2.8 RNA-Seq Data Processing

The pipeline for processing the RNA-seq data was designed as previously described (Zahra *et al.*, 2022). Briefly, the data was aligned to the reference human genome GRCH38 using TopHat2 (v2.1.1) via the ultra-high throughput short-read aligner Bowtie (v2.2.6). Subsequently, the experimental replicates of the three experiments were merged using Samtools (v.0.1.19), where a minimum of 30 high-quality reads were selected. Cufflinks (v.2.2.1) was used to assemble each sample's transcripts and expression quantification. Finally, Cuffdiff was used to obtain the differential expression between the various experiments and treatments. Omics Playground (v3.4.4) (Akhmedov *et al.*, 2020), an online analytics and visualisation platform, was used to analyse and produce graphs for the RNAseq results. The platform was also used to identify DEGs, gene signatures, functional annotation, and biomarkers. FunRich (Pathan *et al.*, 2017) was utilised for gene enrichment to analyse parameters such as biological processes, molecular function and biological pathways.

3.3 RESULTS

3.3.1 Exposure of MCF-12A cells to mixtures of EDCs and fatty acids individually and in combination induced morphological changes of acini.

To understand the effect of endocrine disruptors and fatty acids on early breast carcinogenesis, we tested the mixtures of these individually and in combination. Earlier work has revealed that MCF12A cells cultured on Matrigel form acini with a single layer of cells and a hollow lumen and deposition of a basement membrane (Marchese and Silva, 2012). In this project, throughout the 16 days of incubation, the negative control (0.5% EtOH) formed a single layer of epithelial cells surrounding a slightly formed hollow lumen. When the cells were exposed to EDCs mixture, FA mixture and EDC+FA mixture, we observed a significant increase in the area and a decrease in the circularity in the acini, suggesting an increase in proliferation (Figure 3.5).

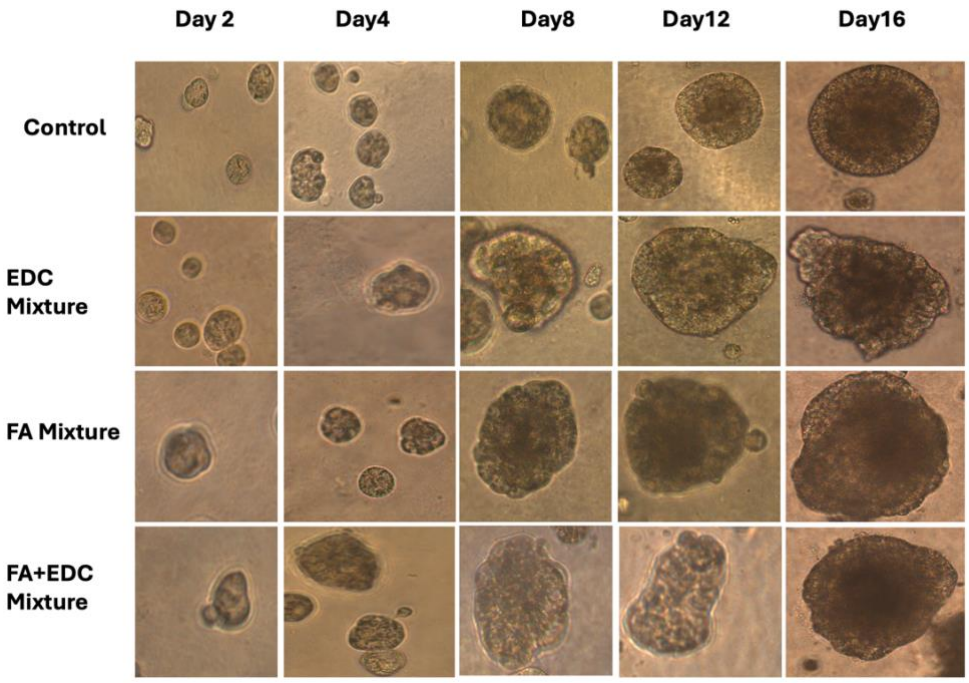


Figure 3.5 Light microscope images of 3D cultures of mammary epithelial cells MCF-12A grown on a Matrigel scaffold. *Structures show a time course of acini growth after treatment with mixtures of EDCs, fatty acids and the two mixtures combined.*

After fixing the acini on day 16, we took confocal images of the acini resulting from the various treatments: solvent control, EDC mixture, FA mixture, and EDC+FA mixture. Staining the acini with an antibody against Laminin V (yellow) clearly showed the presence of a basement membrane. However, antibodies against Caspase-3 did not identify any apoptotic cells. Counterstaining with Alexa Fluor 488 and 588 (blue) clearly showed cell nuclei that are well-organised cells into acini structures. Figure 3.6 is a representative image of several images taken of the acini. Overall, the controls formed well-rounded acini, while the treated samples were deformed and larger at the end of the 16 days of incubation.

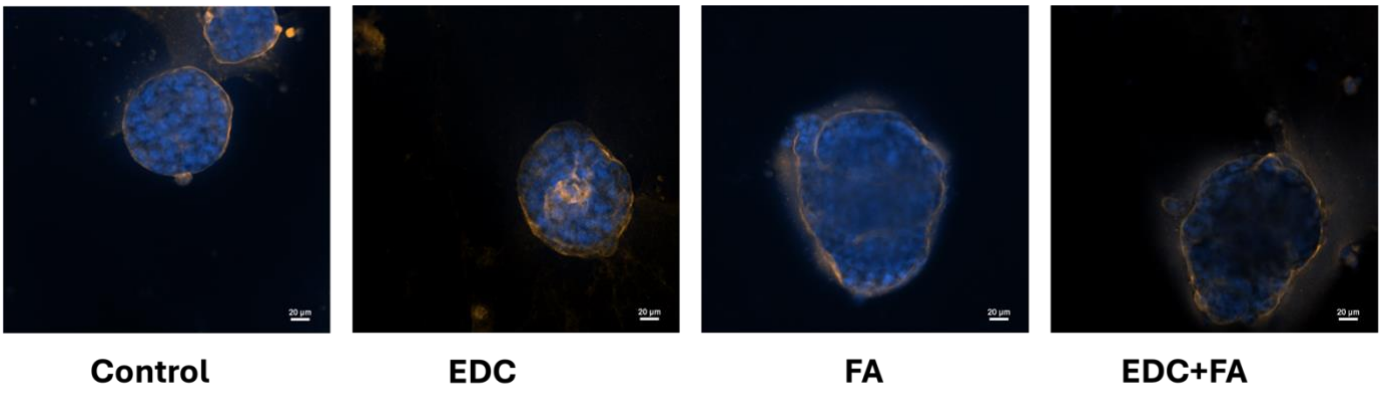


Figure 3.6 Confocal images of 3D cultures of non-tumourigenic epithelial breast MCF12A cells grown on Matrigel for 16 days. *Cells were treated with 0.5% (Control), EDC mixture, FA mixture and the two mixtures combined. Acini were stained with antibodies against Laminin V (yellow) to stain the basement membrane, caspase-3 (green) to identify apoptotic cells and counterstained with Alexa Fluor 488® and 588 to visualise the nuclei. Confocal images are representative of 3 independent experiments.*

The area of the acini increased as the time points measured increased. A slight increase in area could be seen between the negative controls and the treatments on day 2 (Figure 3.7). However, from day 4, a significant increase could be seen in the area between the controls and the treatments (Control_vs_EDC = 32516 ± 1284.82 μm^2, $p<0.0001$, Control_vs_FA = 33050 ± 13221.48 μm^2, $p<0.0001$, Control_vs_FA+EDC = 49823.47 ± 25081.88 μm^2, $p< 0.0001$) (Figure 3.4). There was also a significant difference between the samples treated with EDC mixture, FA mixture and FA+EDC mixture on day 2 (EDC_vs_FA+EDC 16521.56± 2614.19μm^2, $p>0.0001$, FA_vs_FA+EDC 16521± 2835.33μm^2, $p<0.0001$), day 4 (EDC_vs_FA+EDC 49823.46 ± 12238.06μm^2, $p<0.0001$, FA_vs_FAEDC 49823.46± 11860.39μm^2,$p<0.0001$) and day 8 (EDC_vs_FA+EDC 176342±35165.72μm^2, $p>0.0001$, FA_vs_FA+EDC 176342 ± 33953.07μm^2, $p<0.0001$) (Figure 3.7). However, on day 12 and day 16 of the cultures, there was no significant difference between the samples treated with the individual mixtures (EDCs and FA) and those treated with the combined mixture (EDC+FA). The results showed that proliferation did not increase significantly in the controls, and cells continued to proliferate at the expected rate throughout the incubation period. However, the increase in area and deformation of the acini suggests an increased rate of uncontrolled proliferation.

The circularity of the acini of the control samples was closer to 1, indicating a normal acini formation without disruption when compared to the treated samples. As the incubation days increased, the circularity of the treated samples significantly decreased compared to the controls, suggesting increased disruption by the treatments. On day 2, there was a significant difference in loss of circularity between the samples treated with the EDC mixture and those treated with the FA+EDC mixture (0.8826±0.019, $p>0.0001$) (Figure 3.8). However, from day 4 to day 16, the circularity

of samples treated with the combined mixture was lower than that of samples treated with the single mixtures. However, the decrease was not significant between the treatments, meaning the FA+EDC mixture did not increase disruption of the acini.

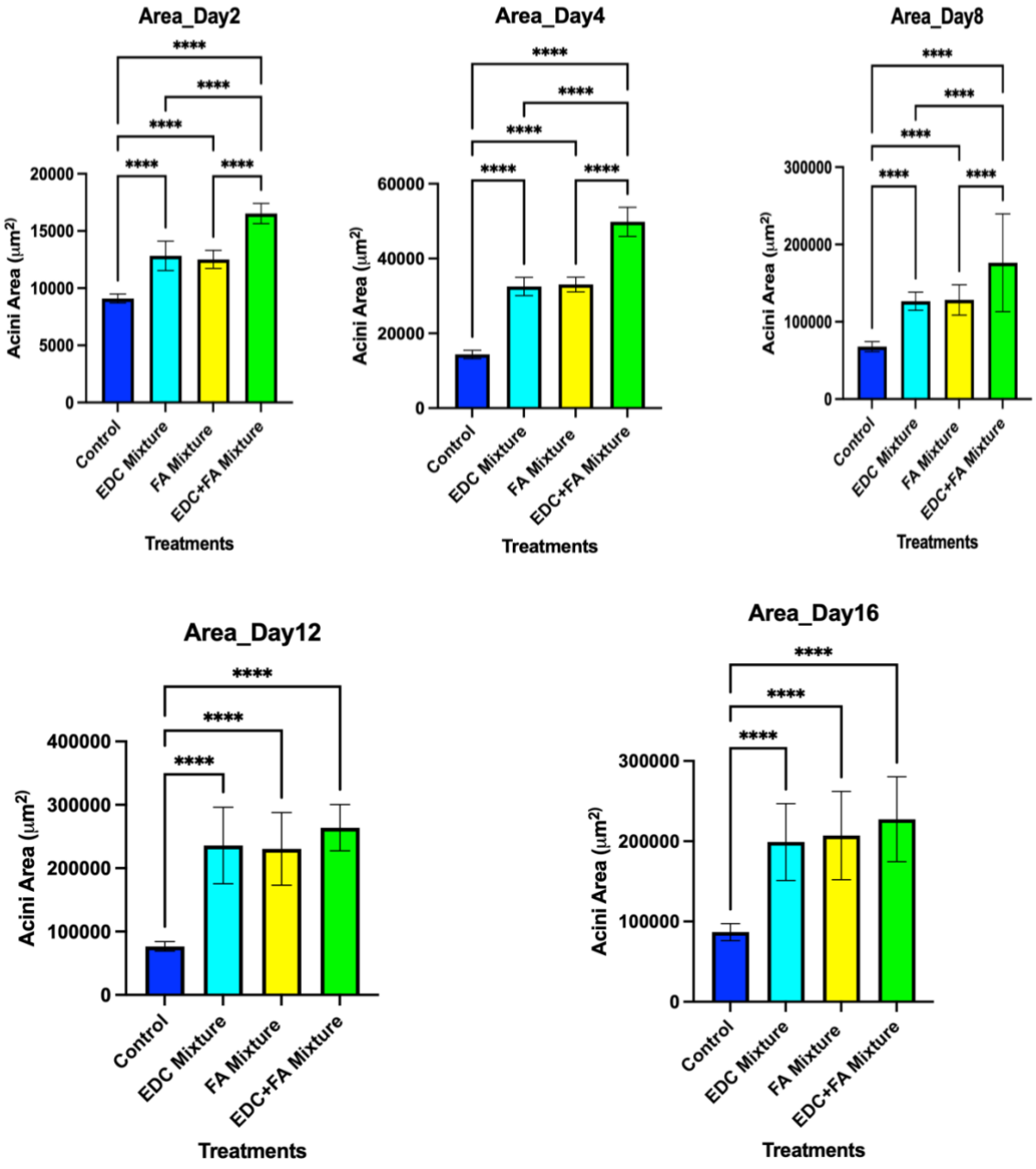
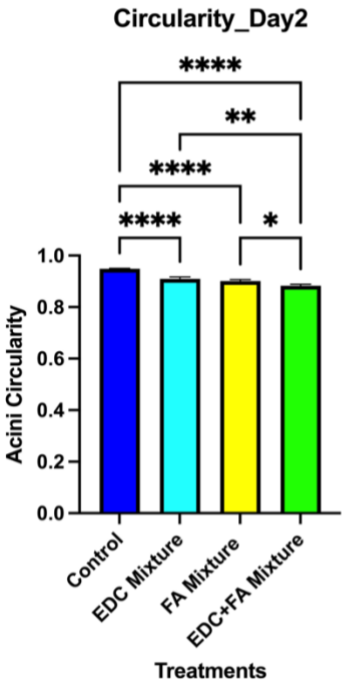
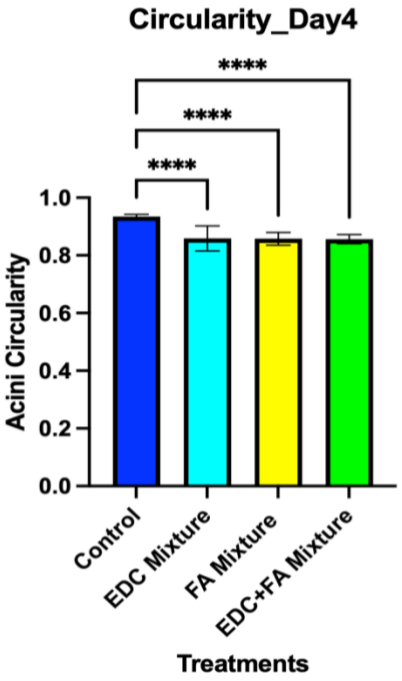
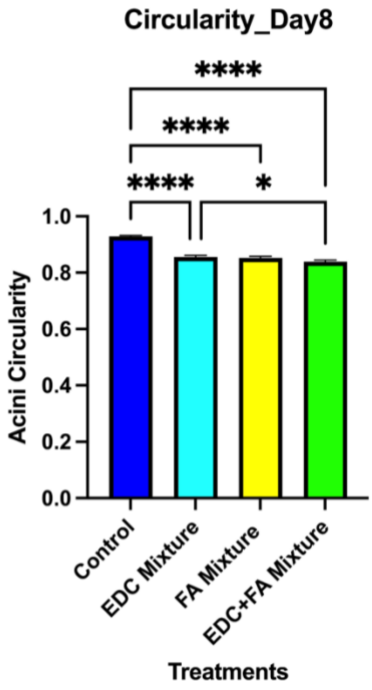


Figure 3.7 Quantification of the acini area in response to a mixture of EDCs, a mixture of Fatty acids and mixtures of EDCs and Fatty combined. *MCF-12A were cultured on Matrigel and treated with a mixture of EDCs, fatty acids and a mixture of EDC and Fatty acids. Light microscopy images of the acini were taken using a Lecia DMi1 microscope fitted with a LEICA MC170 HD screen. Acini areas were measured*

*on Day 2, Day 4, Day 8, Day 12, and Day 16 using ImageJ software. Graphs were plotted using GraphPad Prism. Data represent 10 random acini from three independent experiments run in duplicates with values corresponding to the sample mean and SEM. Significance is denoted by ****<0.0001 *** 0.0002 ** 0.0021 * 0.0323 as determined by an ordinary one-way ANOVA.*







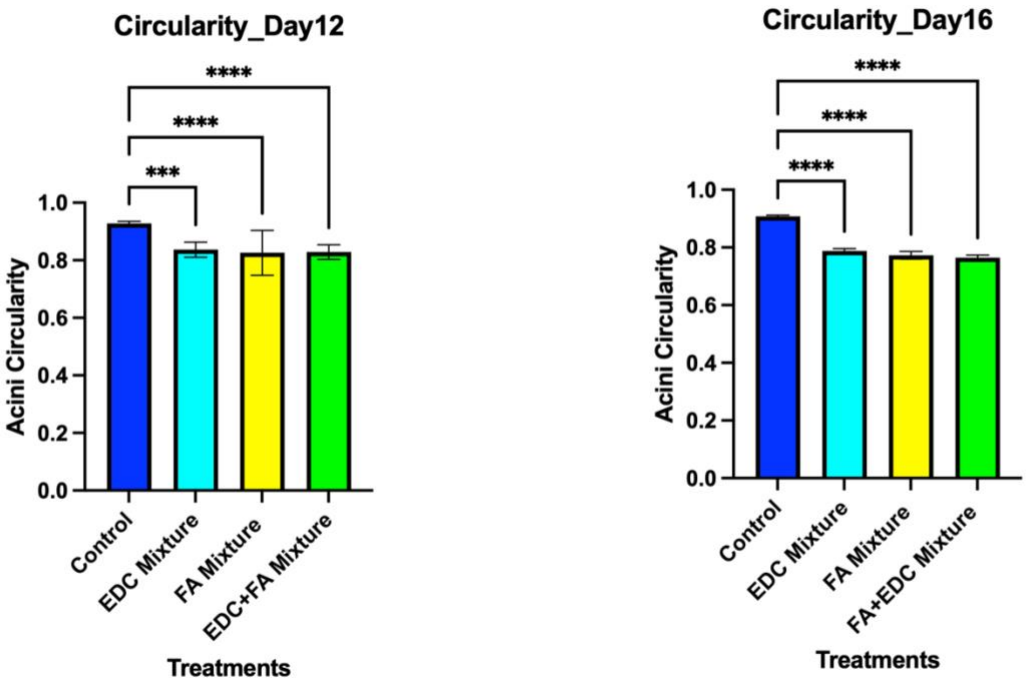


Figure 3.8 Quantification of the acini circularity in response to a mixture of EDCs, a mixture of Fatty acids and mixtures of EDCs and Fatty combined. *MCF-12A were cultured on Matrigel and treated with a mixture of EDCs, fatty acids and a mixture of EDC and Fatty acids. Light microscopy images of the acini were taken using a Lecia DMi1 microscope fitted with a LEICA MC170 HD screen. Acini circularity was measured on Day 2, Day 4, Day 8, Day 12, and Day 16 using ImageJ software. Graphs were plotted using GraphPad Prisms. Data represent 10 random acini from three independent experiments run in duplicates with values corresponding to the sample mean and SEM. Significance is denoted by ****<0.0001 *** 0.0002 ** 0.0021 * 0.0323 as determined by an ordinary one-way ANOVA.*

3.3.2 Uniform Manifold Approximation and Projection of Geneset Signatures Shows Contrast Between Samples

In order to investigate the impact of our test chemicals on the gene expression profile of MCF12A cells cultured in 3D, we analysed RNAseq data. Firstly, we constructed a Uniform Manifold Approximation and Projection (UMAP) to elucidate the differences

in geneset among the samples using the control (0.5%) as a reference (Figure 3.9). The display shows up-regulation (red) and down-regulation (blue) of genesets. The results show slight differences in phenotypic clustering patterns between the samples treated with EDCs mixture and FA mixture, but this was not very marked. However, the samples treated with the FA+EDC mixture showed an inverted geneset expression when compared to the other mixture (EDC mixture and FA mixtures. Overall, while the EDC and FA mixtures up-regulated (Red) similar genesets, the FA+EDC mixture down-regulated those genes, suggesting a combined effect of the two exposures that was not seen with them individually.

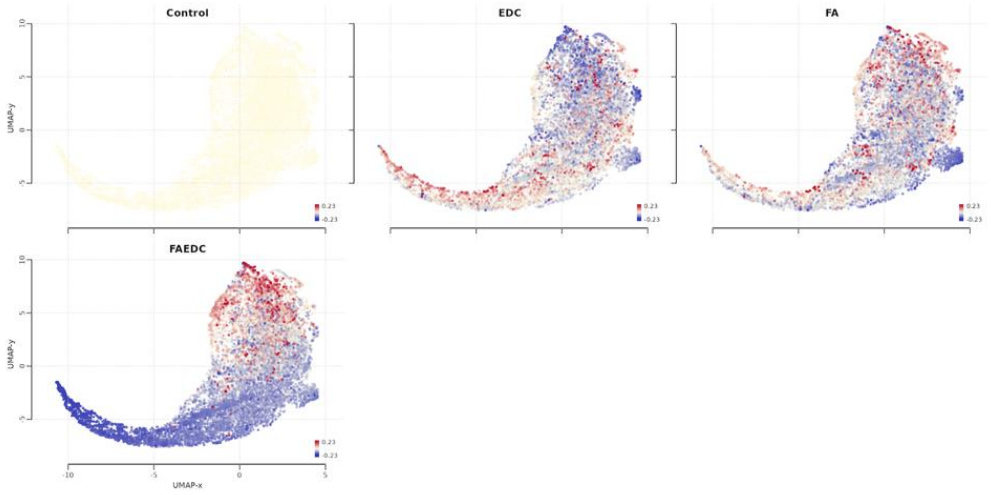


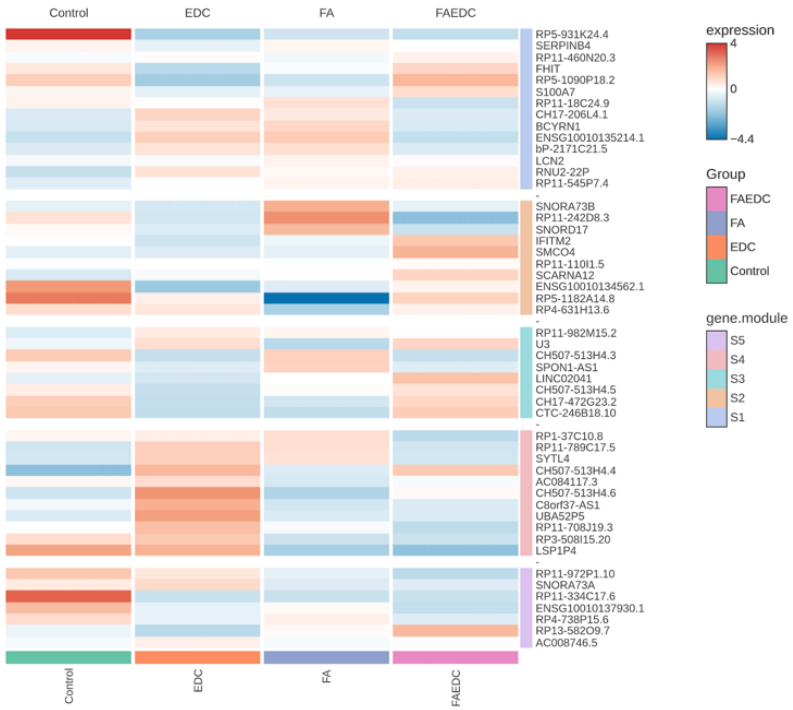
Figure 3.9 Geneset Signature Uniform Manifold Approximation and Projection Showing Up and Down-Regulated Genes in Different Samples.

UMAPs show genes clustered by relative log expression, up (red) or down (blue), regulated in samples treated with an EDC mixture, an EDC mixture, or FA+EDC mixture. The plots show differences in gene expression between the samples. Visualisations were made with Omics Playground (Akhmedov et al., 2020).

3.3.3 Heat Map of Top 50 Differentially Expressed Genes In Different Treatments Identified Five Clusters

We then generated a heat map (Figure 3.10A) of the 50 top differentially expressed genes across the various treatments (Control, EDC, FA, EDC+FA). The relative expression scale of the genes was used for the hierarchical clustering. Five clusters were identified, S1-S5. The functional annotation (Figure 3.10B) of the genes was generated by correlating the genes to 42 reference databases such as KEGG and Gene Ontology (Akhmedov *et al.*, 2020). Some of the processes identified in the functional annotation include PI3K/Akt/MTORC1 signalling, MYC target V1 in S4, fatty acid metabolism, adipogenesis, KRAS signalling, IL6/JAK/STAT3 signalling in S1, G2M checkpoint, E2F targets, DNA repair in S2 and Estrogen response, apoptosis, epithelial-mesenchymal in S3. The heatmap and functional annotations were made with Omics Playground.

A



B

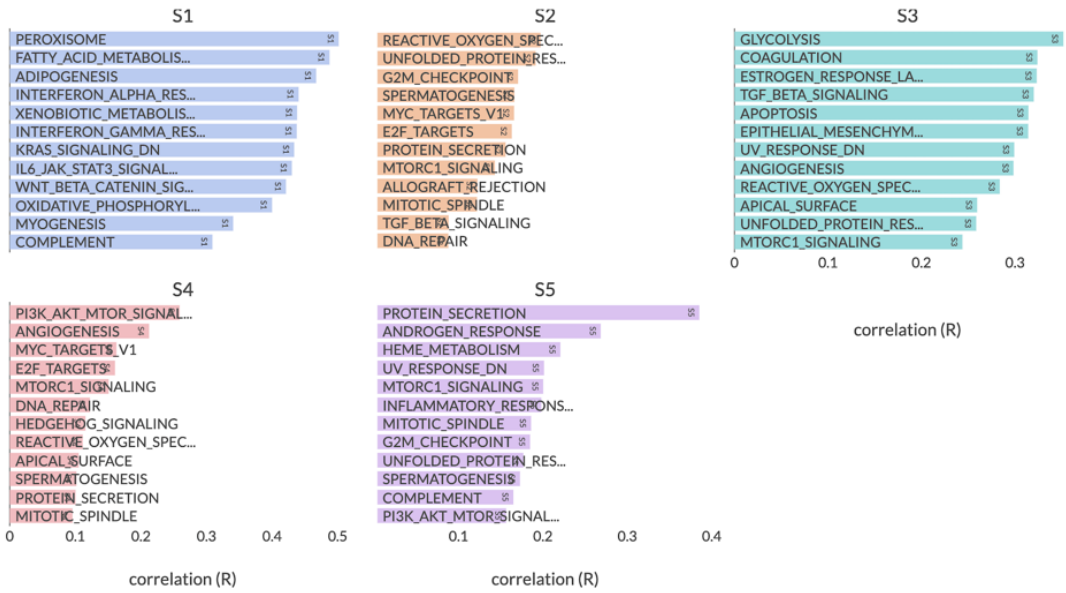


Figure 3.10 Clustered Heatmap and Functional Annotation

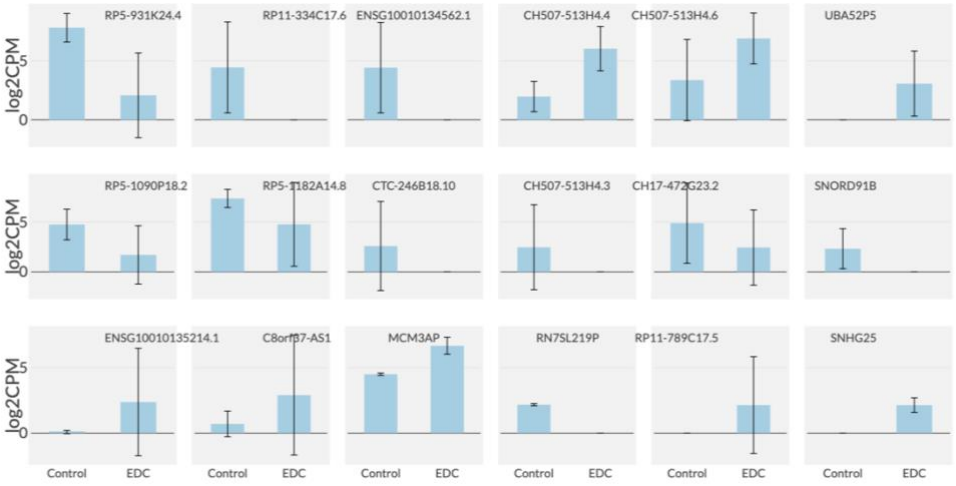
A) *Functional heatmap of 50 genes identified through standard deviation across the treatments (Control, EDC, FA, and FAEDC). Gene-level hierarchical clustering is based on the relative expression scale. In the heatmap, red represents over-expression, while blue denotes under-expression. Five annotated clusters were identified: S1 (blue), S2 (orange), S3 (green), S4 (red), and S5 (pink).* ***B)*** *The functional annotation terms for the five clusters identified in* ***A****. The graphs were generated using the Omics Playground Software, which employs machine learning algorithms to correlate each gene set with over 42 reference databases, including KEGG and Gene Ontology*

3.3.4 Expression of Top Differentially Expressed Genes across the Treatment.

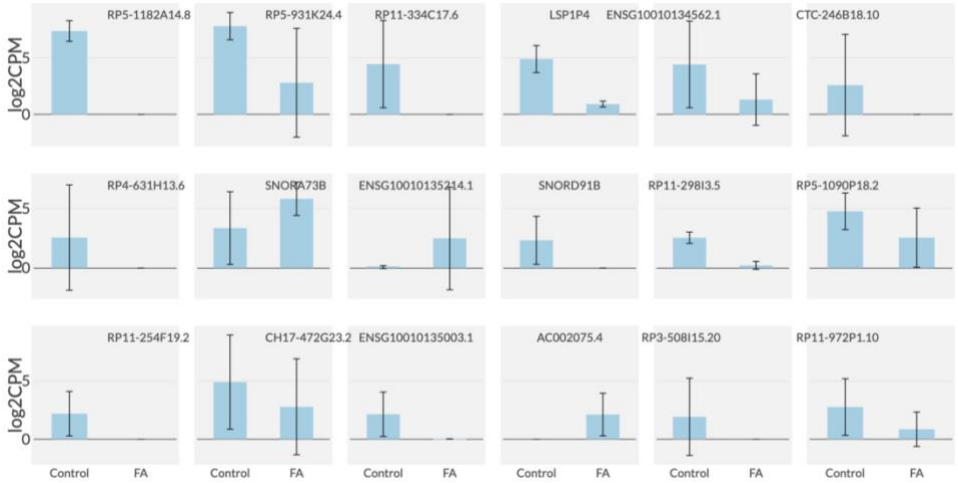
Next, we separated the top differentially expressed genes by comparing each treatment with the controls (Figure 3.8A-C). Using Omics Playground, we selected the top 16 genes regulated differently in the samples compared to the controls based on their False Discovery Ratio (FDR). Due to the use of a few experimental replicates, a

False Discovery Rate (FDR) of 1 was used to compute the top 16 differentially expressed genes. Some of the genes up-regulated by the EDC mixture include *UBAS2P5*, *SNHG25*, and *MCM3AP* (Figure 3.11A). Fatty Acid mixture up-regulated *SNORA73B* (Figure 3.11B). FA+EDC mixture up-regulated *RN75SKP175, SMCO4,* and *RN7SL75P* (Figure 3.11C).

A



B



C

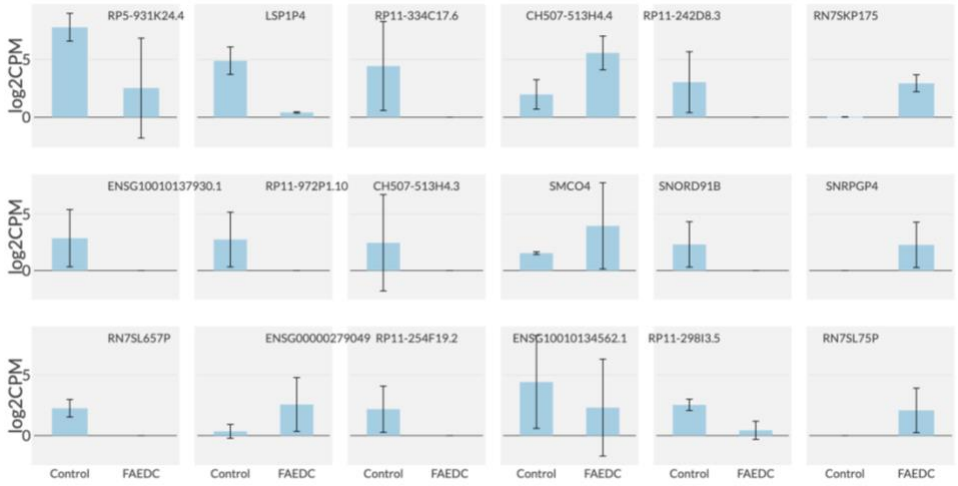
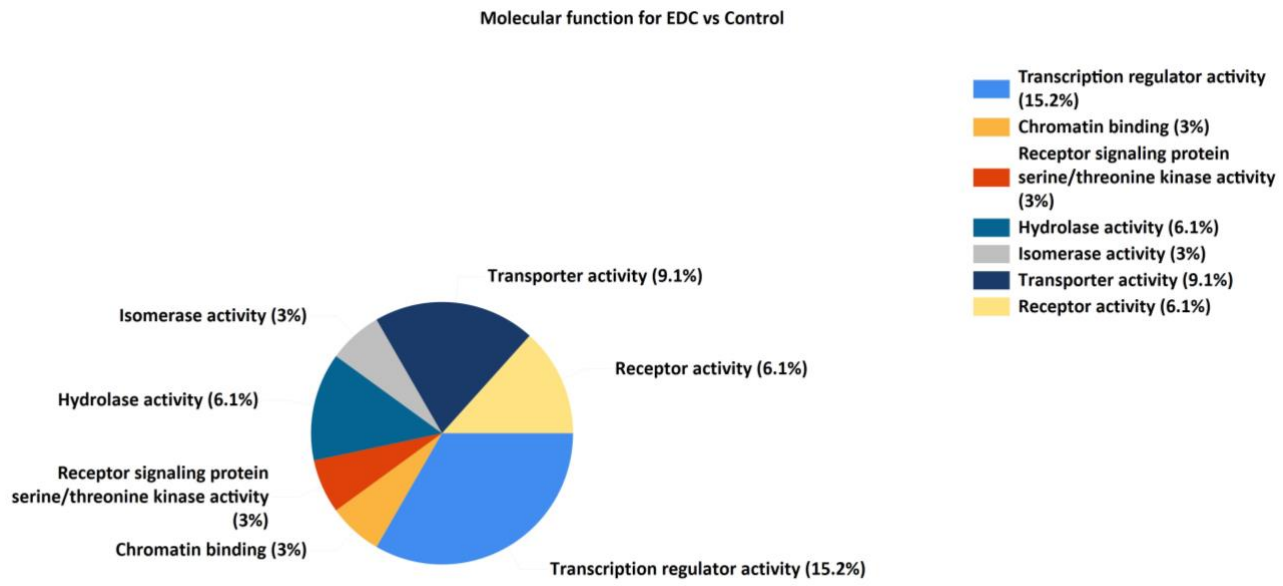


Figure 3.11 Differentiallt Expressed Genes. *Top differentially expressed genes in samples treated with a mixture of EDCs* ***(A),*** *mixtures of Fas* ***(B),*** *and a mixture of FA+EDC* ***(C).***

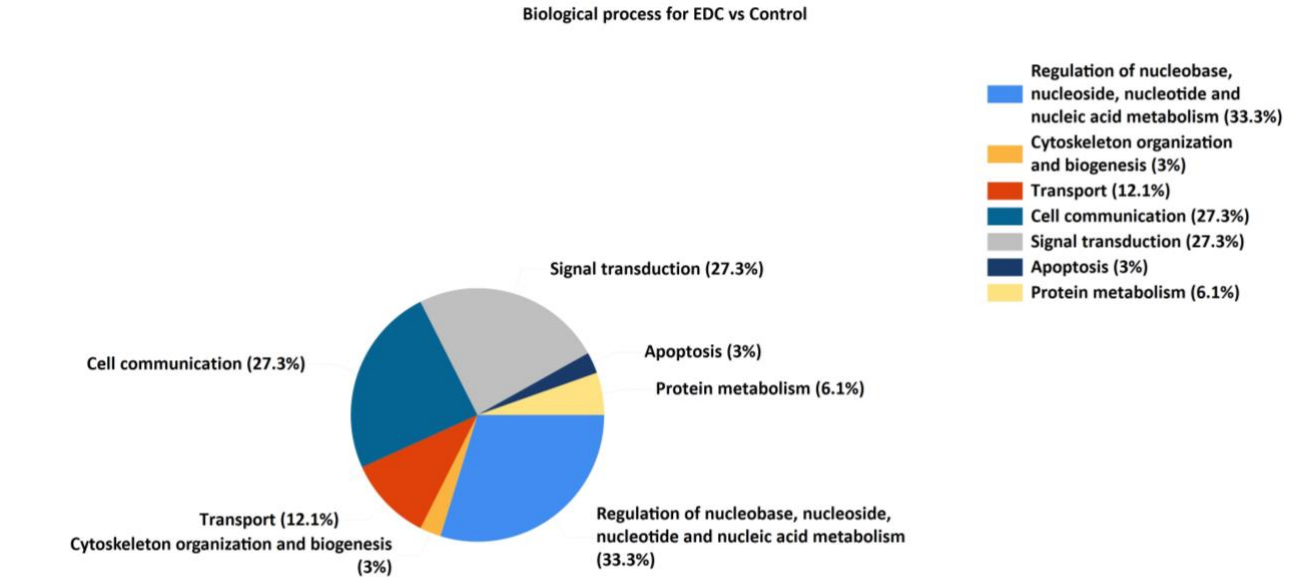
3.3.5 Functional Enrichment of the Top 100 Differentially Expressed Genes (DEGS)

Using Funrich (Pathan *et al.*, 2017), we analysed the top 100 up-regulated and 100 down-regulated genes for their enrichment in biological pathways, biological processes and molecular function for all the treatments. The molecular function refers to the molecular activities initiated by the genes in the biological pathways, while biological processes result from the various molecular activities (Peerapen and Thongboonkerd, 2023). The top 7 processes for each sample are shown in the (Figures 3.12A-I). Molecular activity enriched in the samples includes transcription regulator activity, transcription factor activity and RNA binding. Biological processes enriched in the samples were signal transduction, regulation of nucleic acid metabolism and cell communication. Various biological pathways were enriched in the samples, including biological oxidation, regulation of CDC42 signalling events, estrogen biosynthesis and cytochrome p450 activity.

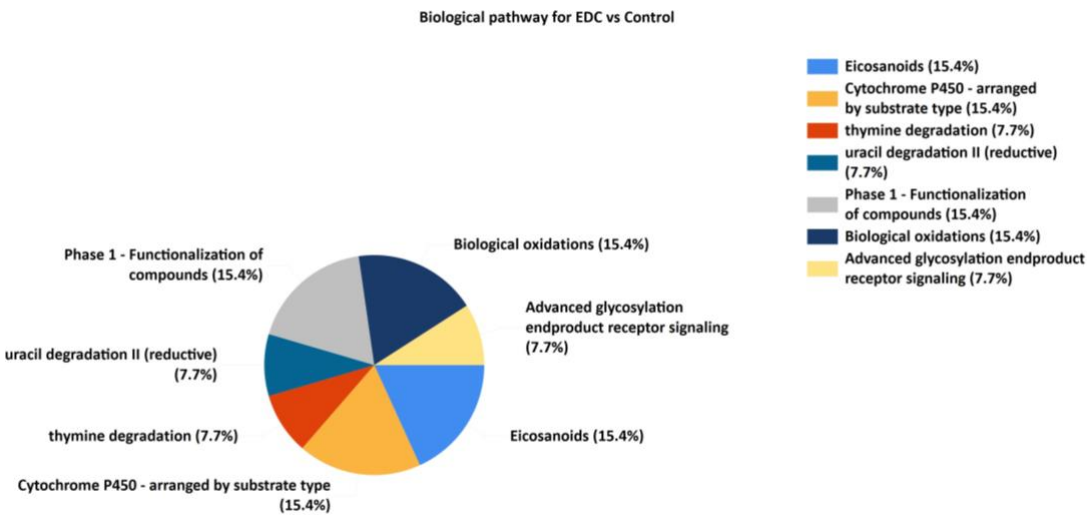
A


Molecular function for EDC vs Control
Transcription regulator activity (15.2%)
Chromatin binding (3%)
Receptor signaling protein serine/threonine kinase activity (3%)
Hydrolase activity (6.1%)
Isomerase activity (3%)
Transporter activity (9.1%)
Receptor activity (6.1%)
Transporter activity (9.1%)
Isomerase activity (3%)
Receptor activity (6.1%)
Hydrolase activity (6.1%)
Receptor signaling protein serine/threonine kinase activity (3%)
Chromatin binding (3%)
Transcription regulator activity (15.2%)

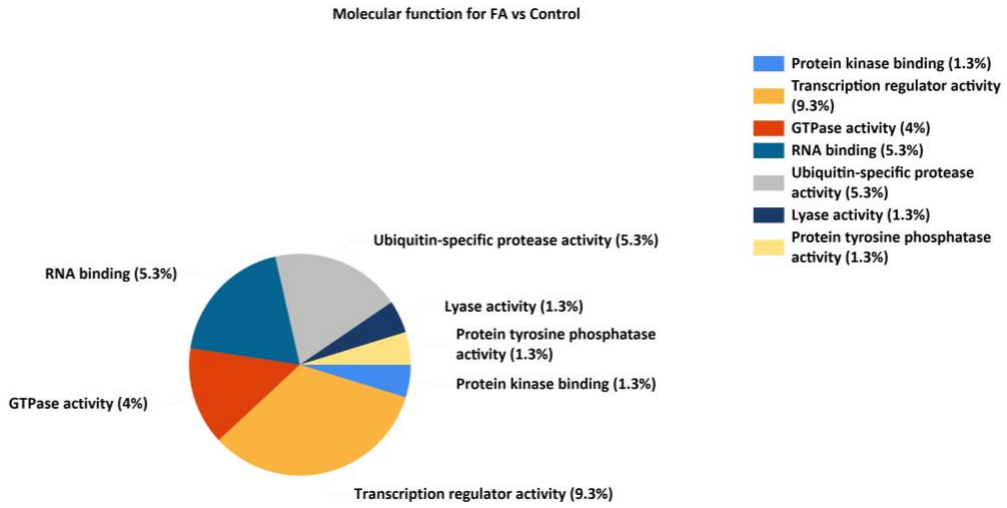
B


Biological process for EDC vs Control
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism (33.3%)
Cytoskeleton organization and biogenesis (3%)
Transport (12.1%)
Cell communication (27.3%)
Signal transduction (27.3%)
Apoptosis (3%)
Protein metabolism (6.1%)
Signal transduction (27.3%)
Cell communication (27.3%)
Apoptosis (3%)
Protein metabolism (6.1%)
Transport (12.1%)
Cytoskeleton organization and biogenesis (3%)
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism (33.3%)

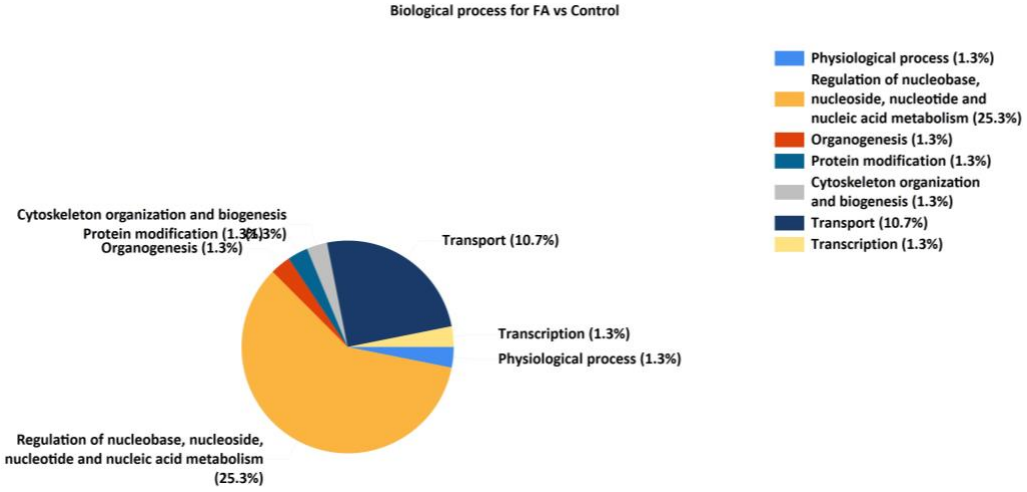
C


Biological pathway for EDC vs Control
Eicosanoids (15.4%)
Cytochrome P450 - arranged by substrate type (15.4%)
thymine degradation (7.7%)
uracil degradation II (reductive) (7.7%)
Phase 1 - Functionalization of compounds (15.4%)
Biological oxidations (15.4%)
Advanced glycosylation endproduct receptor signaling (7.7%)
Biological oxidations (15.4%)
Advanced glycosylation endproduct receptor signaling (7.7%)
Eicosanoids (15.4%)
thymine degradation (7.7%)
uracil degradation II (reductive) (7.7%)
Phase 1 - Functionalization of compounds (15.4%)
Cytochrome P450 - arranged by substrate type (15.4%)

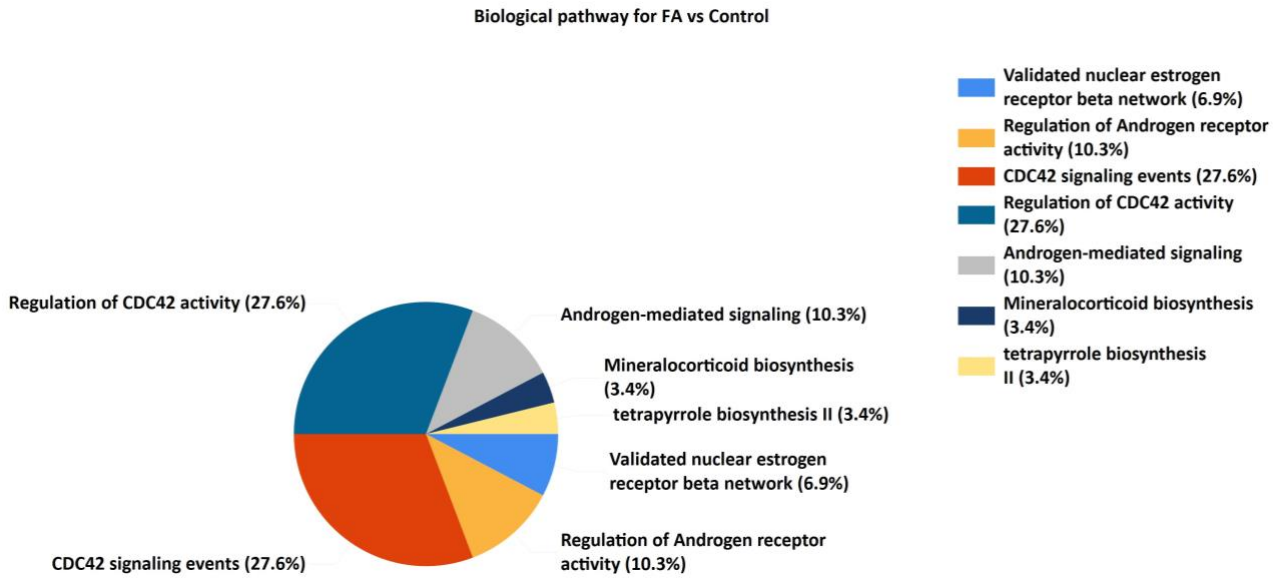
D


Molecular function for FA vs Control
Protein kinase binding (1.3%)
Transcription regulator activity (9.3%)
GTPase activity (4%)
RNA binding (5.3%)
Ubiquitin-specific protease activity (5.3%)
Lyase activity (1.3%)
Protein tyrosine phosphatase activity (1.3%)
Ubiquitin-specific protease activity (5.3%)
Lyase activity (1.3%)
Protein tyrosine phosphatase activity (1.3%)
Protein kinase binding (1.3%)
RNA binding (5.3%)
GTPase activity (4%)
Transcription regulator activity (9.3%)

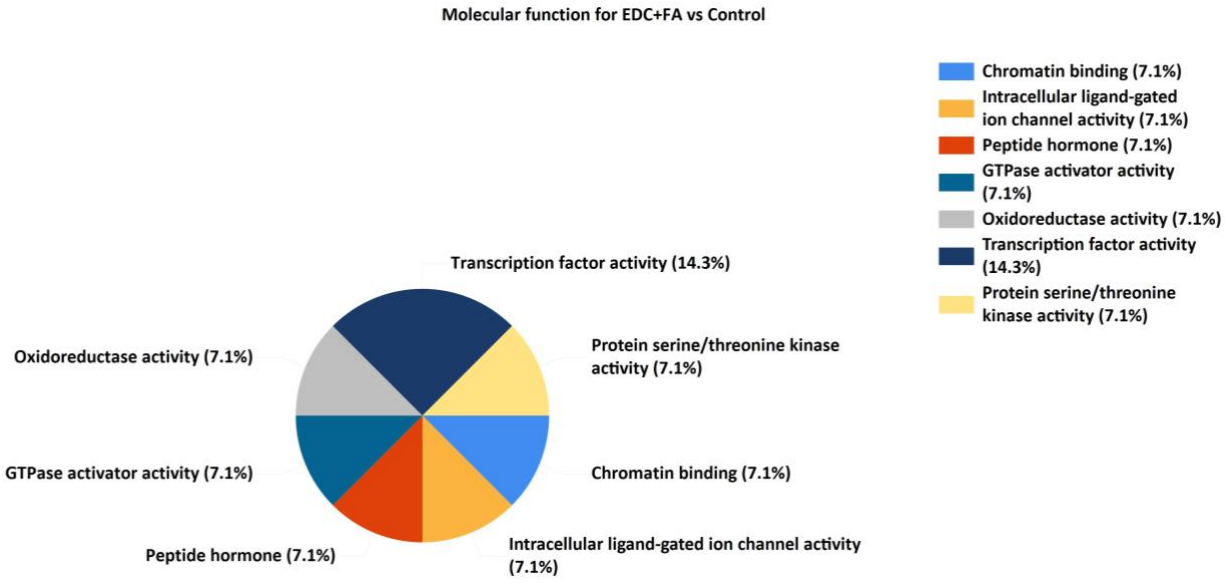
E


Biological process for FA vs Control
Physiological process (1.3%)
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism (25.3%)
Organogenesis (1.3%)
Protein modification (1.3%)
Cytoskeleton organization and biogenesis (1.3%)
Transport (10.7%)
Transcription (1.3%)
Transport (10.7%)
Transcription (1.3%)
Physiological process (1.3%)
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism (25.3%)
Cytoskeleton organization and biogenesis
Protein modification (1.3%)
Organogenesis (1.3%)

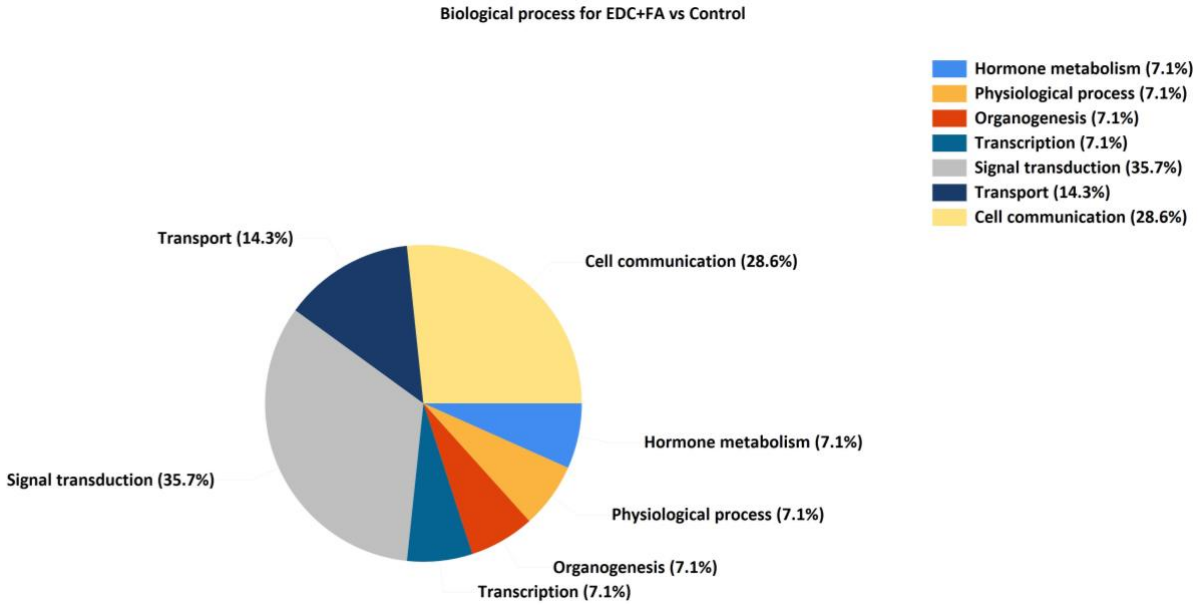
F


Biological pathway for FA vs Control
Validated nuclear estrogen receptor beta network (6.9%)
Regulation of Androgen receptor activity (10.3%)
CDC42 signaling events (27.6%)
Regulation of CDC42 activity (27.6%)
Androgen-mediated signaling (10.3%)
Mineralocorticoid biosynthesis (3.4%)
tetrapyrrole biosynthesis II (3.4%)
Validated nuclear estrogen receptor beta network (6.9%)
Regulation of Androgen receptor activity (10.3%)
CDC42 signaling events (27.6%)
Regulation of CDC42 activity (27.6%)
Androgen-mediated signaling (10.3%)
Mineralocorticoid biosynthesis (3.4%)
tetrapyrrole biosynthesis II (3.4%)

G


Molecular function for EDC+FA vs Control
Chromatin binding (7.1%)
Intracellular ligand-gated ion channel activity (7.1%)
Peptide hormone (7.1%)
GTPase activator activity (7.1%)
Oxidoreductase activity (7.1%)
Transcription factor activity (14.3%)
Protein serine/threonine kinase activity (7.1%)
Transcription factor activity (14.3%)
Protein serine/threonine kinase activity (7.1%)
Oxidoreductase activity (7.1%)
GTPase activator activity (7.1%)
Peptide hormone (7.1%)
Intracellular ligand-gated ion channel activity (7.1%)
Chromatin binding (7.1%)

H



I

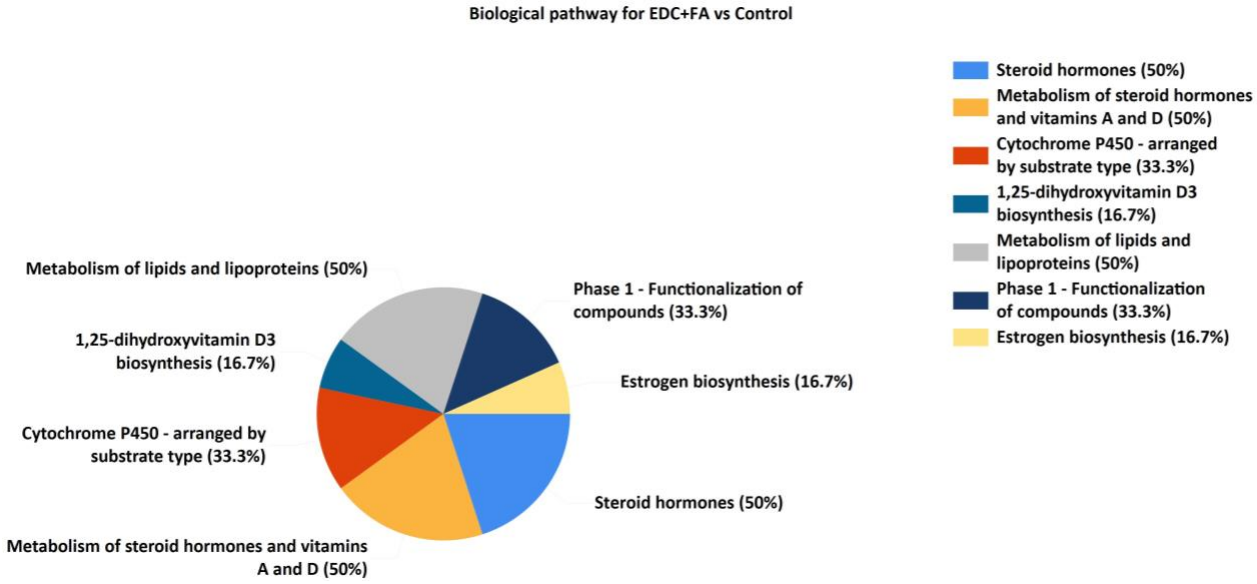
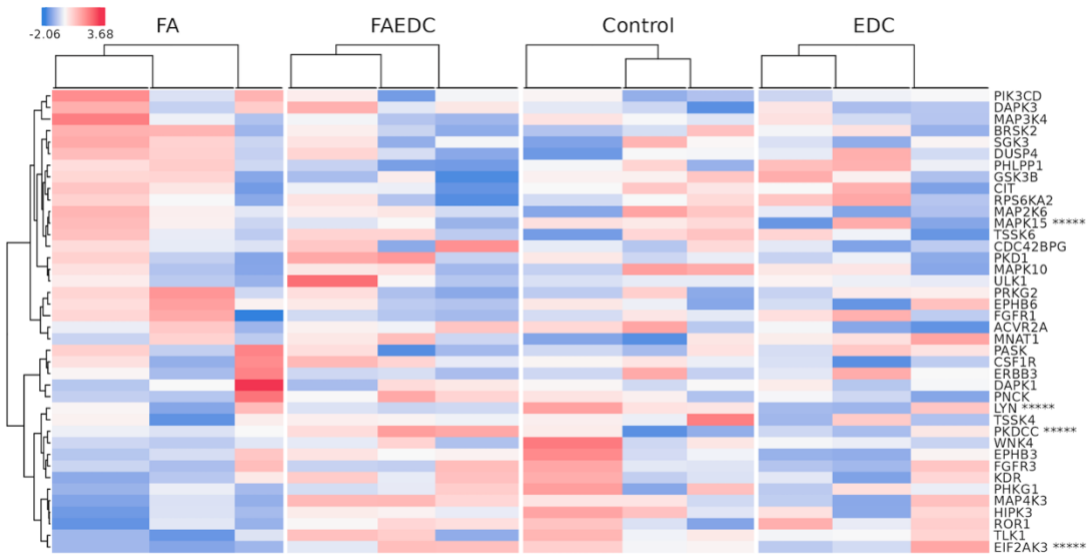


Figure 3.12 Enriched Biological Pathways, Processes and Molecular Functions associated with treatment with the mixtures. *Using the top 100 up and down-regulated genes, Funrich was used to analyse the top 7 enriched pathways.* ***A-C)*** *Molecular pathway, biological process and biological pathway for EDC mixture* ***D-F)*** *Molecular pathway, biological process and biological pathway for FA mixture* ***G-I)*** *Molecular pathway, biological process and biological pathway for FA+EDC mixture*

3.3.6 Exposures to Treatments Causes the Of Expression Different Biomarkers

Across the sample groups, different biomarkers are expressed in response to the treatments. Omics Playground employs machine learning algorithms Sparse Spatial Least Squares (sPLS), Glmnet, and Random Forest (RF) to compute the biomarkers expressed in MCF-12A cells due to the treatment. The expression of these biomarkers can be used to predict and classify treatments. We focused on the expression of kinases because they are essential in cell proliferation and processes leading to breast cancer (Milletti, Colicchia and Cecconi, 2023; Miricescu *et al.*, 2020). A heatmap (Figure 3.13**A**) shows the expression of the biomarkers across the treatment. A box plot (Figure 3.13**B**) of the top 8 biomarkers across the treatment. Some of the important biomarkers expressed include MAPK15, PKDCC, and EIF2AK3. Some of these biomarkers have been implicated in carcinogenesis.

A



B

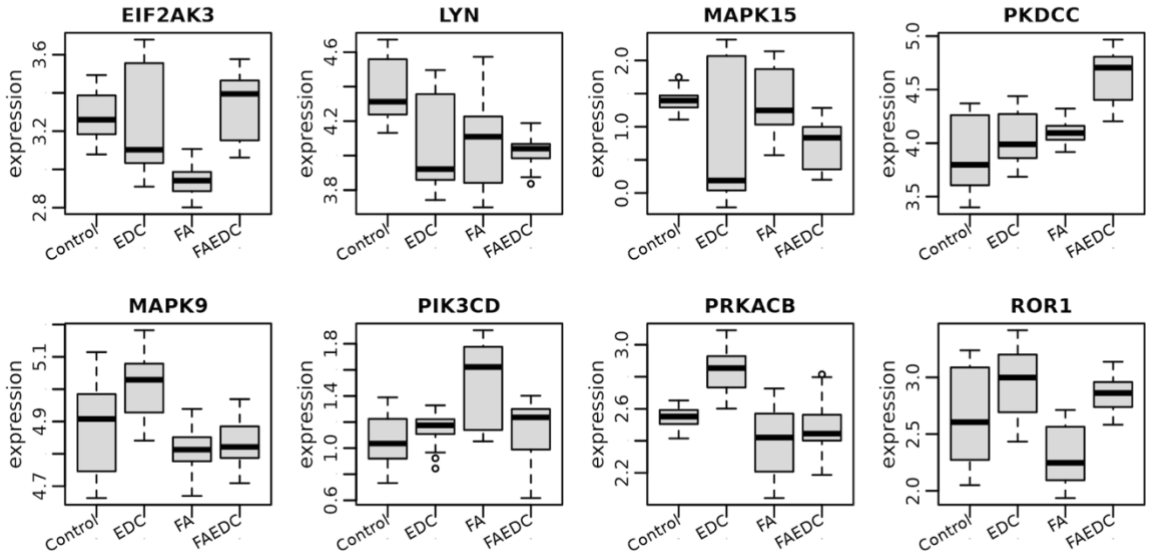


Figure 3.13 Biomarkers Expression across samples A) *A heatmap of the biomarkers expressed across the treatments.* ***B)*** *Box plots of the top eight biomarkers expressed by the treatments. Analysis and images were done with Omics Playground.*

3.4 DISCUSSION

The ability of EDCs to impact overall breast cancer risk has been inconclusive, with harmful effects seen predominantly at concentrations higher than those found in human tissues. These assumptions have been made without taking into consideration the presence of human exposure to mixtures of environmental chemicals that can potentially impact breast cancer risk. Epidemiological studies have been inconclusive on the impact of diet, especially a high-fat diet, on the risk of breast cancer initiation and progression. Here, we present data on the effect of mixtures of endocrine disruptors, mixtures of fatty acids, and then the two mixtures combined on breast epithelial cells, MCF-12A on a 3D cell culture model in order to try to elucidate the potential impact of these combined exposures on breast cancer risk.

Firstly, we showed that MCF-12A cells were organised to form acini when cultured on Matrigel, recapitulating *in vivo* mammary architecture (Marchese, 2013; Marchese and Silva, 2012; Debnath and Brugge, 2005; Atlas and Dimitrova, 2019). This has been a useful model for studying carcinogenesis *in vitro* (Lo *et al.*, 2012). We demonstrated that throughout the 16 days of the culture, MCF-12A acini showed alterations in area and circularity when exposed to the mixtures, suggestive of the initial stages of neoplastic transformation (Debnath *et al.*, 2002; Russo *et al.*, 2010). Another study also demonstrated the disruption of acini morphology of MCF-12A cells cultured on Matrigel (Atlas and Dimitrova, 2019). Confocal images of the acini showed well-formed structures surrounded by a basement membrane under control conditions. These structures are similar to those produced by (Marchese and Silva, 2012). The acini treated with the combined mixtures of EDC and Fatty acids showed a partial hollow after 16 days of incubation, suggesting apoptosis (Marchese, 2013).

The circularity of the control samples was closer to 1 throughout the incubation period. All the treatments significantly decreased circularity. An increase in proliferation and decrease in circularity, as shown by MCF-10A cells cultured on collagen, have been suggested as the causes of the observed acini disorganisation (Carey, Martin and Reinhart-King, 2017; Shi *et al.*, 2014). Mechanistically, the decrease in circularity of acini has been associated with an alteration in the PI3K/Akt, as demonstrated in MCF-10A cultured in a 3D model (Liu *et al.*, 2004). Guo and colleagues demonstrated that E2 activated the PI3K/Akt pathway in endometrial cell lines (Guo *et al.*, 2006) , MCF10 cells (Chen, Chien and Lee, 2023), and MCF-7 cells (Pesiri *et al.*, 2014). EDCs such as phthalates (benzyl butyl phthalates (BPP), di-n-butyl phthalate, (DBP), di-2-ethylhexyl phthalate(DHEP)), BPA, Bisphenol AF (BPAF), tetrachlorobisphenol A, parabens (methyl and propylparaben) have been shown to activate the PI3K/Akt cascade in MCF-7, SK-BR-3, and MCF-10A (Chen and Chien, 2014; Wróbel and Gregoraszczuk, 2014; Lei *et al.*, 2019; Yu *et al.*, 2023; Goodson III *et al.*, 2011). Jackson and colleagues reported that fatty acids (palmitic acid, linoleic acid, oleic acid and stearic acid), both individually and in a mixture, had a significant effect on the PI3K/Akt pathway (Jackson *et al.*, 2022). We suggest that the results from our studies may have been caused by the activation of this cascade by the mixtures of EDCs, FAs and the two mixtures combined. For the first time, we present data that looks specifically at the changes to acini circularity due to exposure of MCF12A cells to mixtures of fatty acid and EDCs combined.

There were significant increases in the area of the acini during the incubation period after exposure to the mixtures. The EDC+FA mixture significantly affected the area of the acini compared to the mixtures of EDCs and FA alone. Marchese and Silva showed that exposure of MCF-12A cells to 10μM of BPA and propylparaben on 3D resulted in

large, deformed acini at the end of 16 days of incubation (Marchese and Silva, 2012). Phoebe Maund's work showed that EDCs such as BPA, *o,p'*-DDT, propylparaben, and BP-3 increased the area of MCF-12A cells grown on Matrigel (Maund, 2018). It has also been demonstrated that BPA, BBP, and E2 induced neoplastic transformation in MCF-10F cells cultured on collagen. The authors reported that treated cells resulted in the formation of a high percentage of ductless solid structures when compared to the controls (Fernandez and Russo, 2010). As discussed earlier in the circularity acini, increased proliferation of cells plays a role in increasing the acini size as the incubation period increases. Mechanistically, increased activation of receptor kinases such as type I insulin-like growth factor receptors (IGFIR) (Jones *et al.*, 2007) and epidermal growth factors (EGFR) (Dimri *et al.*, 2007), downstream signalling pathways such as PI3K/Akt, and Ras/Raf/MAPK (Yanochko and Eckhart, 2006) and oncoprotein such as cyclin D1 (Debnath *et al.*, 2002) promote cell proliferation and increase acini size. It has been demonstrated in MCF10A spheroids that these mechanisms are responsible for uncontrolled proliferation (Debnath *et al.*, 2002). For the first time, we present evidence that a mixture of thirteen EDCs and four fatty acids present in their tissue concentrations can increase the size of the acini. The effect of the combination of both factors (EDC+FA) is more significant than that of EDC and FAs. The structures observed in our studies recapitulate the events of early mammary carcinogenesis (Hebner, Weaver and Debnath, 2008). We, therefore, suggest that a high-fat diet and exposure to EDCs could increase the risk of breast cancer.

The UMAP of the sequencing data showed a difference in the samples. The geneset analysis showed large portions of the UMAP that showed up-regulation in the samples treated with mixtures of EDC and FA, which were down-regulated in the samples treated with the FA+EDC mixture. This observation suggests that the combination of

the two mixtures (FA mixture + EDC mixture) had a greater effect in down-regulating the geneset than the individual mixtures.

Functional analysis of the top 150 differentially expressed genes showed variation in gene expression across treatments. Some differentially expressed genes included among the different treatments include *UBA52P5, SNORD918, SNORA73B, LIP1P4, MCM3AP, SNH925, SMCO4*, and *SNRPGP4*. The gene *SNORD73B* was up-regulated in the sample treated with FA compared to controls. This gene is a variant of *SNORD73,* which has been shown to be involved in fatty acid metabolism and lipotoxicity (Sletten *et al.*, 2021). Chen and colleagues demonstrated that SNORD73B induced cell proliferation, migration, and invasion and inhibited apoptosis in endometrial cancer cells (Chen *et al.*, 2023). It has also been shown that *SNORA73B* is up-regulated in breast cancer cells and is a potential prognostic marker for early breast cancer (Li *et al.*, 2024). *LSP1P4* is downloaded in both FA and FA+EDC treatments. It has been demonstrated that this gene is a marker of DNA damage and DNA repair. Therefore, a down-regulation of the gene may increase the accumulation of double-strand breaks that can lead to the initiation of carcinogenesis (Wang *et al.*, 2021b). *MCM3AP* is a long non-coding RNA (LncRNAs) that is up-regulated in the samples treated with mixtures of EDCs. An up-regulation of MCM3AP has been reported to promote proliferation, invasion, migration, and invasion in breast cancer cell lines such as ZR-75-30 and MDA-MB-231 (Tang, Qin and Yu, 2021). Functional annotation of the top 50 genes showed that they are involved in processes such as PI3K_AKT_mTOR signalling, fatty acid metabolism, production of reactive oxygen species, KRAS signalling, IL6_JAK_STAT3 signalling, and up-regulation of peroxisome activity. These processes have been commonly altered in breast cancer (Xu *et al.*, 2021; Oshi *et al.*, 2022; Liang *et al.*, 2021; Siersbæk *et al.*, 2020; Shen *et*

al., 2020). As discussed earlier, PI3K_Akt_mTOR signalling is involved in the proliferation of breast cancer cells and is activated by both EDCs and Fatty Acids (Liu *et al.*, 2004; Jackson *et al.*, 2022).

To further understand the effect of the mixtures on MCF-12A cells, FunRich analysis showed enrichment of molecular processes such as transcription regulation activity. It has been demonstrated that EDCs such as BPA regulate the transcription factor activity of estrogen receptors, which results in increased proliferation, migration, and invasion of cancer cells (Ma *et al.*, 2016). Biological pathway analysis showed that the mixture of fatty acids enriched the regulation of *CDC42* activity and signalling. It has been demonstrated in yeast cells that inhibiting *CDC42* by anticancer agent reduced migration and polarity of cells, suggesting the roles of *CDC42* activity in migration, invasion, and spread in tumour cells (Rivera-Robles *et al.*, 2020). *CDC42* signalling has also been implicated in breast cancer metastasis in the MDA-MB-231 and MCF-7 cell lines (Zhang *et al.*, 2023). The combined mixture (EDC mixture + FA mixture) and the EDC mixture enriched the cytochrome P450 (CYP) biological pathway; CYP19A1 and CYP27B1 were the two CYP polymorphisms involved. CYP19A1 is elevated in postmenopausal breast cancer patients with an estrogen receptor-positive status (Friesenhengst *et al.*, 2018). Studies have shown that CYP27B1 is expressed in breast cancer lines and plays a role in the synthesis of Vitamin D in breast cancer cells (Dennis *et al.*, 2023).

Examining kinases as biomarkers of exposure showed significant expression of *MAPK15, MAPK9, PKDCC, EIF2AK3*, and *LYN*. *MAPK15* is implicated in breast cancer and has been found to regulate the activity of ERα (Zhong, Lau and Xu, 2024). Similarly, *MAPK9*, which encodes JNK2, is involved in breast cancer and modulates chemotherapeutic agents (Itah *et al.*, 2023; Ashenden *et al.*, 2017). LYN is a kinase

that is significantly expressed in triple-negative and basal-like cancers and aggressive and invasive forms of cancer (Choi *et al.*, 2010; Tornillo *et al.*, 2018). EIF2AK3, also known as PERK, is involved in cell survival in chemotherapeutic treatments for breast cancer (Chen *et al.*, 2019; Alasiri *et al.*, 2020). Wang and colleagues showed that inhibition of EIF2AK3 stops breast cancer and invasion and migration in BT549 and MDA-MB-231 breast cancer cell lines (Wang *et al.*, 2023). These kinases are expressed in our treatments at different levels, and further work has to be done to understand their role in breast cancer.

In conclusion, we showed that under control conditions, MCF12A cells cultured on Matrigel organised to form acini, which remained organised, with visible lumen and controlled proliferation. For the first time, we have shown that both mixtures of EDCs and FA had an impact on the growth of the acini that is different from controls. In both cases, the acini are larger and disorganised (as seen by the area and circularity). Interestingly, the increase in acini size remains similar between EDCs and FA throughout the incubation period. The effect of EDC+FA is always higher than the other two treatments from the start of the incubation period until day 16. This demonstrates that there is a combination effect between the two treatments, indicating a potential increase in cancer risk when these conditions are present together. RNA sequencing data showed that the mixtures up-regulated various genes and enriched molecular and biological pathways involved in breast cancer. Further work can be done to elucidate further how those genes affect breast cancer.

Chapter Four: The Effect of Mixtures of Endocrine Disruptors and Fatty Acids on BRCA1-Silenced MCF-12A Cells.

4 Multifactorial Impacts: The Effect of Mixtures of Endocrine Disruptors and Fatty Acids on BRCA1-Silenced MCF-12A Cells.

4.1 Introduction

As discussed in Chapter One and Chapter Two, germline mutations of the BRCA1/BRCA2 gene significantly increase the risk of developing breast cancer in the lifetime of a woman (Bernstein-Molho *et al.*, 2020). BRCA1 gene mutations confer a slightly higher risk than those in the BRCA2 gene (Collins and Isaacs, 2020). Structurally, BRCA1 is a 1,863 amino acid protein containing two main domains: a zinc finger zinc RING(Really Interesting New Gene) domain at the amino terminus (N-terminus) made up of the first 100 amino acids and two BRCT (BRCA1 C-Terminus) domains at the carboxyl-terminus (C-terminus) (Figure 5.1) (Wang, 2012; Masso *et al.*, 2020). BRCA1 and BARDI form a heterodimer at their N-terminal RING domains that possess an E3 ubiquitin ligase activity (Witus *et al.*, 2021). The role of the E3 ubiquitin ligase activity in the DNA damage repair and tumour suppression function of BRCA1 is still not fully understood (Wang, 2012). The middle section of the protein, which comprises 60% of the total amino acids of BRCA1, houses two nuclear localisation signals (NLS). This middle section is followed by the coiled-coiled (CC) domain, which directly interacts with the Partner and Localizer of BRCA2 (PALB2) and enables the transfer of BRCA2 and RAD51 to DNA double-strand break (DSB) sites (Liu and Lu, 2020). BRCT occurs as tandem repeats and consists of many clusters of conserved hydrophobic amino acid residues (73-128 amino acids) (Bork *et al.*, 1997; Mota *et al.*, 2023). BRCT domains interact with phosphopeptide motifs in partner proteins to produce functional macromolecular complexes involved in DNA damage responses (Venkitaraman, 2019). These phosphopeptides include Abraxas, CtBP

interacting protein (CtIP) and BRCA1-interacting protein C-terminal helicase 1 (BRIP1) (Tarsounas and Sung, 2020).

There are more than 1800 BRCA1 mutations, ranging from frameshift deletions to insertions and nonsense mutations. These mutations lead to premature protein truncation and loss of tumour suppressor function (Godet and Gilkes, 2017). A study by the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA) showed that the specific location of a mutation significantly impacts the risk of developing breast cancer (Rebbeck *et al.*, 2015).

Clinically significant mutations of BRCA1 occur mainly in the RING and BRCT domains. These mutations stop its E3 ubiquitin ligase activity, thereby predisposing mutation carriers to various cancers (Drost *et al.*, 2011).

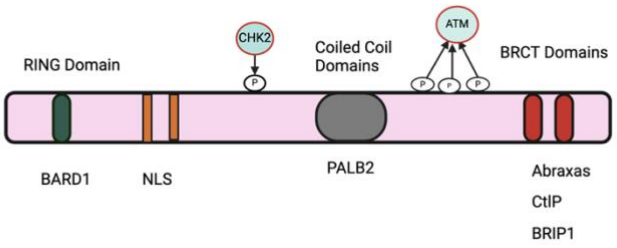


Figure 4.1 Structure of BRCA1 showing its functional domains and interacting proteins. *The BRCA1 amino terminus houses the RING domain, which interacts with the BRCA1-associated RING domain protein 1 (BARD1) and a nuclear localisation sequence (NIL). The carboxyl terminus contains the coiled-coil domain that interacts with PALB2. The BRCT domains bind with ATM-phosphorylated abraxas, CtBP-interacting protein (CtIP) and BRCA1 interacting protein C-terminal helicase-1 (BRIP1) (Roy, Chun and Powell, 2012). Image created with Biorender* (https://biorender.com/).

4.1.1 Mechanisms of BRCA1-Associated Tumourigenesis

As already stated, the loss of the tumour suppression function of BRCA1 is strongly correlated with the initiation of breast cancer. The mechanism underpinning the tumourigenesis in BRCA1 mutation and tissue specificity has not been completely elucidated (Yoshino *et al.*, 2021).

One of the earliest hypotheses put forward to explain carcinogenesis due to mutations in tumour suppressor genes is the Knudson “two-hit” hypothesis. In his seminal work on retinoblastoma gene Rb1, Alfred Knudson concluded that two mutational events are needed for tumorigenesis. He explained that the inheritance of a germline mutation of the heterozygote tumour suppressor gene allele will only lead to cancer when the second allele is also inactivated through somatic or epigenetic mutation (Knudson Jr, 1971). This loss of heterozygosity (LOH) has been seen in BRCA1-associated cancers, where the loss of wild-type alleles was lost in the early onset of breast cancer (Merajver *et al.*, 1995). However, a growing body of work has shown that heterozygosity in BRCA1 confers haploinsufficiency, where a single copy of the gene is insufficient to suppress tumour formation completely. Tumour formation can, therefore, occur despite the presence of a single functional gene, and the loss of the second wild-type is unnecessary (Minello and Carreira, 2023). A study by Hiroyuki and colleagues showed that a mutation of a single allele in BRCA1 resulted in impaired homology-mediated DNA repair and genomic instability in MCF10A cell lines (Konishi *et al.*, 2011). Quantitative allelotyping of tissue specimens from women with germline deleterious mutation in BRCA1 showed that the wild-type BRCA1 allele is not required for BRCA1-associated breast tumourigenesis (King *et al.*, 2007).

4.1.2 RNAi and siRNA

RNA interference (RNAi) is a regulatory process in plants and animals where double-strand RNA (dsRNA) silences post-transcriptional homologous target genes (Ozcan *et al.*, 2015). RNAi does not confer germline mutations, but because of its high specificity and selectivity, it has become a valuable tool for individualised disease therapies (Rao *et al.*, 2009b; Mysore *et al.*, 2019). RNAi effect can be achieved through synthetic small interfering RNAs (siRNA) and vector-based short hairpin RNAs (Mysore *et al.*, 2019; Rao *et al.*, 2009a). The degree and effectiveness of the gene silencing depend on the target gene, tissue type and population differences (Mysore *et al.*, 2019). However, their use is hampered by their polyanionic nature, high molecular weight, short half-life, quick enzymatic degradation, and possible induction of off-target effects (Hu *et al.*, 2020a; Chernikov, Vlassov and Chernolovskaya, 2019). These disadvantages have been overcome by chemical modification and the development of new delivery methods for the RNAi mechanisms (Traber and Yu, 2023; Salim, Goss and Desaulniers, 2021).

siRNAs (small interfering RNAs) are a potent and versatile tool in biological research because of their ability to suppress any gene by a base sequence alone (Setten, Rossi and Han, 2019). They can “silence” the expression of specific genes with complementary sequences (Dong, Siegwart and Anderson, 2019). Mechanistically, (Figure 4.2) siRNAs are produced in the cytoplasm when longer double-stranded RNA (dsRNA) is cleaved by endoribonuclease dicer. The siRNA produced has 21-23 bases long with 2 overhanging phosphorylated bases at the 3’ end of each strand (Zhang *et al.*, 2021a). siRNA then complexes with RNA-induced silencing complex (RISC), which consists of a Dicer and Argonaute 2 (Argo-2). The siRNA is then divided into two strands: the sense and anti-sense (guide), with the anti-sense acting as a guide to

align the siRNA-RISC complex to the target mRNA sequence. The sense strand (also called passenger strand) is discarded and degraded under the mediation of Ago-2 endonuclease, complementary binding of the anti-sense guide to the target causes a disintegration of the sequence and a loss of activity, that is, “silencing” of the gene (Salguero-Aranda *et al.*, 2019).

Synthetic siRNAs mimic the products of the Dicer and are integrated into the downstream of the RNAi mechanism (Kanasty *et al.*, 2013). They have been used in vivo to target genes in diseases such as hypercholesterolemia, liver cirrhosis, hepatitis B, and human papillomavirus. However, the full use of siRNA has been limited predominantly to *in vitro* systems (Raval *et al.*, 2019).

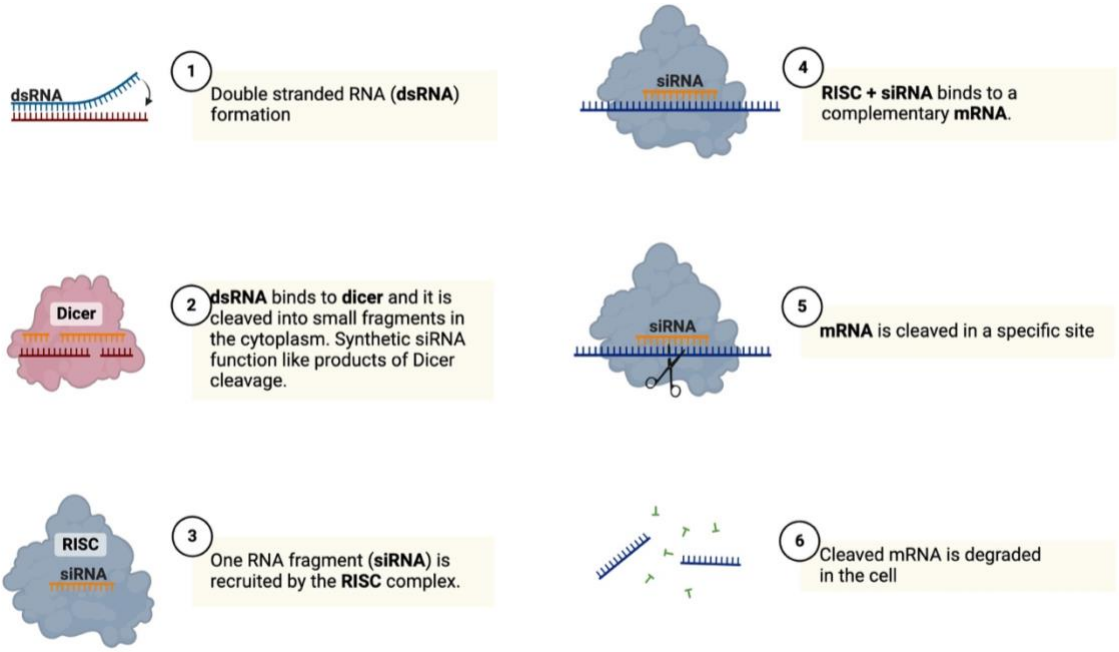


Figure 4.2 *Schematic representation of the mechanism of siRNA-mediated RNA interference*. Image created with (https://biorender.com/).

4.1.3 Effects of multifactorial risk factors

As discussed in Chapter One, interactions between different risk factors are possibly a reason for increased breast carcinogenesis events. Research has revealed significant discrepancies in cancer risk conferred to women who have inherited germline mutation of BRCA1/2 (Friebel, Domchek and Rebbeck, 2014). A prospective cohort study of 6036 BRCA1 and 3820 BRCA2 mutation carriers showed that the risk of developing breast cancer by the age of 80 years is 72% for BRCA1 and 69% for BRCA1. The study also showed a variation in breast cancer risk by family history of breast cancer, suggesting possible genetic modification in mutation carriers (Kuchenbaecker *et al.*, 2017).

Environmental and lifestyle factors such as oral contraceptives, alcohol consumption, reproductive history, physical inactivity, and obesity have been shown to modulate the penetrance of BRCA1/2 genes (Milne and Antoniou, 2016; Daniele *et al.*, 2021), but there is still some conflicting evidence. For example, the effects of the use of oral contraceptives on breast cancer risk for BRCA mutation carriers have been mixed. Iodice and colleagues did not find any evidence that the use of oral contraceptives increased breast cancer risk in BRCA1/2 carriers (Iodice *et al.*, 2010). However, another study reported that the use of oral contraceptives increases (Relative Risk 1.17-1.27) breast cancer risk BRCA1/2 mutation carriers, especially in young people (Schrijver *et al.*, 2022). Rieder and colleagues also confirmed that the use of oral contraceptives caused an earlier induction of breast cancer as compared to non-users among BRCA1/2 mutated gene carriers (Rieder *et al.*, 2016).

Reproductive factors such as higher parity were found to modify breast cancer risk BRCA1/2 mutation carriers. Higher parity decreased (HR=0.27, 95% CI 0.09-0.83, p=0.045) breast cancer risk in BRCA1 mutation carriers (Park *et al.*, 2017). This is

further buttressed by an earlier study, which showed that full-term births decreased breast cancer risk in BRCA1 mutation carriers (HR =0.81 CI 0.7-0.94, p=0.005) (Rieder *et al.*, 2016).

A study of height and body mass index (BMI) as modifiers of breast cancer risk in BRCA1/2 mutation carriers reported that each $5kg^2$ increase in BMI resulted in a 6% (0.92, 95 CI 0.90-0.98, p=0.007) decrease in risk of premenopausal breast cancer in BRCA1/2 carriers (Qian *et al.*, 2019). A prospective multicentre randomised control trial of 502 women with deleterious BRCA1/2 mutation to determine the modulatory effect of the Mediterranean diet revealed that women with high-fat mass (HR 1.87, 95% ci 1.21-2.88) and metabolic risk factors (HR 1.87, 95% CI 1.11-3.19) had a significant increase in the risk of BRCA-associated cancer (Bruno *et al.*, 2021). Mechanistically, obesity increases the penetrance of mutated BRCA genes by regulating insulin and insulin-like growth factor I (IGF-I) (Dumais *et al.*, 2017; Bordeleau *et al.*, 2011).

A randomised prospective trial of 68 participants showed smoking and high physical inactivity during adolescence increased the risk of developing breast cancer for women with deleterious mutation of BRCA1/2 (Grill *et al.*, 2017). An international cohort study of BRC1/2 mutation carriers showed that smoking in pre-productive years increases the risk of breast cancer (HR 1.3 95% CI 0.83-2.01) (Li *et al.*, 2020a). In the LIBRE-1 study, the effect of a 3-month intensive intervention of physical activity and a Mediterranean diet on the progesterone-mediated receptor activator of nuclear factor κB (RANK)/soluble RANK ligand (sRANKL)/osteoprotegerin (OPG) pathway was assessed. This pathway is hyperactivated in germline BRCA1/2 mutation carriers and plays a vital role in mammary carcinogenesis. Activation of the pathway leads to breast tissue proliferation and induction of breast cancer. The study revealed that physical

activity and a Mediterranean diet significantly modulated this pathway (Neirich *et al.*, 2021). Intake of high isoflavone reduced the risk of developing triple-negative breast cancer in BRCA1 mutated gene carriers (Sim *et al.*, 2020).

To date, research has examined the effect of endocrine disruptors and a high-fat diet on BRCA1-silenced breast cells. However, understanding the ability of lifestyle and environmental factors to modulate the risk of Brca1 mutation carriers is essential in risk prediction and effective management of these predisposed people. As already discussed, because of the high lifetime risk of BRCA1 mutation carriers, any additional factors that compound this risk can significantly impact the absolute risk of developing breast cancer. Within this thesis, we have shown that Bisphenol A can cause changes to BRCA1 expression in MCF-12A cells. It is established that BRCA-associated cancers are predominantly found in estrogen-responsive tissues such as breasts and ovaries (Kim *et al.*, 2015). A retrospective cohort study of BRCA1/2 mutation carriers showed that BRCA1/2 carriers with estrogen-positive tumours showed poor prognosis compared to non-carriers. They are 2.3 times more likely to have a recurrence and 3.4 times higher mortality risk (Vocka *et al.*, 2019).

Based on the evidence presented above, it is conceivable that these independent risk factors could act in combination to affect the overall cancer risk. This chapter aims to investigate this specific hypothesis.

4.1.4 Chapter Scope

In this chapter, we aim to understand the effect of mixtures of endocrine disruptors and fatty acids acting alone and in combination on MCF-12A with silenced BRCA1. Our specific research questions are:

1. Does the BRCA1 siRNA (siBRCA1) silence the expression of the BRCA1 gene in the MCF-12A cell line?
2. Does exposure to a mixture of EDC, a mixture of fatty acids and a combination of EDCs and fatty acids induce differential gene expression in MCF-12A cells with and without BRCA1 mutation?
3. Could the gene expression profile in the different exposure scenarios indicate a multifactorial breast cancer risk?

4.2 Methodology

4.2.1 siRNA Transfection

100,000 MCF12A cells were seeded in a 6-well plate. Culture was maintained, as explained in Chapter Two. When the cells were 50% confluent, transfection was done using siRBCA1 and scrambled siRNA control (Sigma Aldrich). The sequence for the siBRCA1 is shown in Table 3.2. Cell transfection was done with the jetPRIME®transfection reagent. siRNA BRCA1 (siBRCA1) and scrambled control were dissolved in nuclease-free water into 100 μM per manufacturer instruction.20μM dilutions were prepared and stored at -20°C for future experiments. The cells were initially transfected with two concentrations of siRNA: 10 nM and 50 nM. Details of the reagents and volumes of reaction are shown in Table (Table 4.1). jetPRIME®transfection reagent was used according to the manufacturer's instructions. Briefly, to prepare the transfection mix, the required volume of siRNA was diluted in jetPRIME®buffer, vortexed for 10 seconds and spun down. The required volume of jetPRIME®reagent was added to the mix and vortexed for 1 second. The mix was then incubated for 10 minutes at room temperature.

Table 4.1 Reagents and volumes for siRNA transfection mix per well (in a 6-well plate).

Volume of siRNA added (10nM)	**Volume of siRNA added (50nM)**	**Volume of jetPRIME buffer added**	**Volume of jetPRIME reagent added**	**Volume of Growth Medium**
0.5 μl	5 μl	200 μl	4 μl	2000 μl

The transfection mix was added to the cells in a serum-containing growth medium (DMEM-F12). Four hours after transfection, the growth medium was replaced with fresh medium. Cells were transfected for 24 hours and 48 hours.

Table 4.2 Sequence of siBRCA1 used for transfection

Oligo Name	**Sequence (5'-3')**
SASI_HS01_00179500	CUACUGUCCUGGCUACUAA
SASI_HS01_00179500_AS	UUAGUAGCCAGGACAGUAG

4.2.2 Real-Time PCR

100, 000 MCF-12A cells were transfected with 10nM and 50nM siRNA, respectively. Scramble siRNA was used as the negative control. After 24 and 48 hours, samples were prepared for RT-PCR, as explained in Chapter Two.

4.2.3 Chemical Handling for RNA Sequencing

100 000 MCF-12A cells were seeded in 6 well plates and allowed to attach for 24 hours. As explained above, the cells were transfected with 50nM of siBRCA1 and 50nM scrambled siRNA. After 24 hours of transfection, cells were treated with an EDC mixture (1.89×10^{-8}M), a fatty acids mixture (5.4×10^{-5} M), and a combination of EDC mixture and Fatty Acid mixtures. For each treatment, the solvent control was not above 0.05%. The negative control was Ethanol (0.5%). In addition to the treatments, one well of 100,000 cells was left untreated. Three independent experiments were done in triplicates. The experiment was stopped 24 hours after chemical treatments were added, and RNA was extracted. The samples were pooled per experiment, and RNA was extracted using the Qiagen Minieasy® Kit according to the manufacturer's instructions.

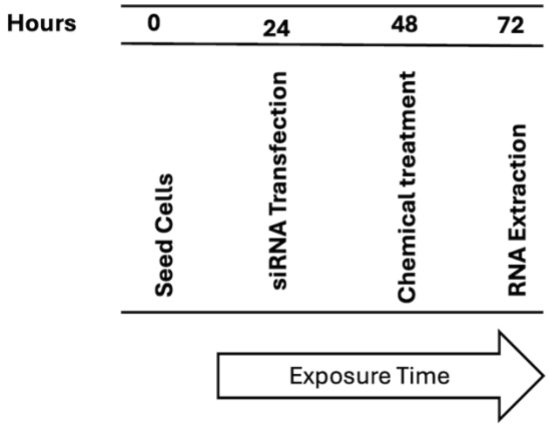


Figure 4.3 Chemical handling for RNA Sequencing. *MCF-12A cells were seeded in 6 well plates at 0 hours and allowed to attach. After 24 hours, cells are transfected with siBRCA1 for 24 hours. At 48 hours, silenced cells were treated with test mixtures. RNA is extracted at 72 hours.*

4.2.4 Western Blot

100 000 cells MCF12A were seeded in a 6-well plate. After leaving the cells to attach for 24 hours, the cells were transfected with 50nM for 24 hours as described above. After 24 hours of transfection, the experiment is stopped. The cells were washed three times with PBS and kept on ice. In a fume hood, 250μl of Laemmli buffer (MerckMillipore) containing 10% v/v sodium dodecyl (SDS), 250mM Tris pH 8.0, 5% v/v glycerol and 0.01% w/v bromophenol blue with 10 μL of protease inhibitor (x7) and 10μL of beta-mercaptoethanol (BME) was pipetted into each well. The cells were dislodged using a scrapper and transferred into an Eppendorf tube. The samples were boiled on a heating block at 90°C for 10 minutes to denature the proteins. To avoid repeated thawing and freezing, samples were aliquoted into smaller tubes on ice and stored at -80°C.

4.2.5 Protein Gel Electrophoresis

In this step, 10% SDS-PAGE resolving gel and 5% stacking gels were prepared using the reagents and volumes below (see Table 3.3). The resolving gel was first pipetted into an already-made cassette (Bio-Rad), and cold 100% methanol was poured on top and allowed to set. When the resolving gel is set, the methanol is poured out, the stacking gel (components and quantities shown in Table 3.4) is pipetted on top of the resolving gel, and a comb is inserted into the gel to create a well to hold samples. The stacking gel was allowed to set for about 15 minutes.

Table 4.3 Components and quantities for preparing 10% SDS-PAGE resolving gel (2 gels)

Component	Amount for 2 Gels (10ml)
Distilled Water	4.0
30% Acrylamide Mix	3.3
1.5M tris(hydroxymethyl)aminomethane (pH 8.8)	2.5
10% Sodium dodecyl sulfate (SDS)	0.1
10% Ammonium Persulfate (APS)	0.1
Tetramethylethylenediamine (TEMED)	0.004

Table 4.4 Components and quantities for preparing 5% stacking gel (2 gels)

Component	Amount for 2 Gels (4ml)
Distilled Water	2.7
30% Acrylamide Mix	0.67
1 M tris(hydroxymethyl)aminomethane (pH 6.8)	0.5
10% Sodium dodecyl sulfate (SDS)	0.1
10% Ammonium Persulfate (APS)	0.04
Tetramethylethylenediamine (TEMED)	

A tank filled with a 10% SDS-PAGE running buffer (see Table 3.4) was set up. The gels were loaded into the tank. The samples were removed from the -80°C refrigerator, warmed for 5 minutes at 90°C and centrifuged. 5μl of the ladder (PageRuler™ – Prestained Protein Ladder (Life Technologies) were loaded into the first well. The samples (7μl) were then loaded into the wells. Gel electrophoresis was done at 40mA per gel and at 300V in a 10% SDS-PAGE running buffer for approximately 45 or more until all the proteins were clearly separated and bands could be seen.

Table 4.5 Components and quantities to prepare 1X SDS-PAGE running buffer.

Component	Amount
Tris base	3.02g
Glycine	14.4g
SDS	1g
Distilled water	1000ml

4.2.6 Blotting and Transfer

Once the proteins had been separated, the next step was to transfer them onto a Nitrocellulose membrane. The Nitrocellulose membrane, which serves as a blotting paper, has a high protein-binding affinity. A cassette "sandwich" was then put together, as shown in Figure 5.3

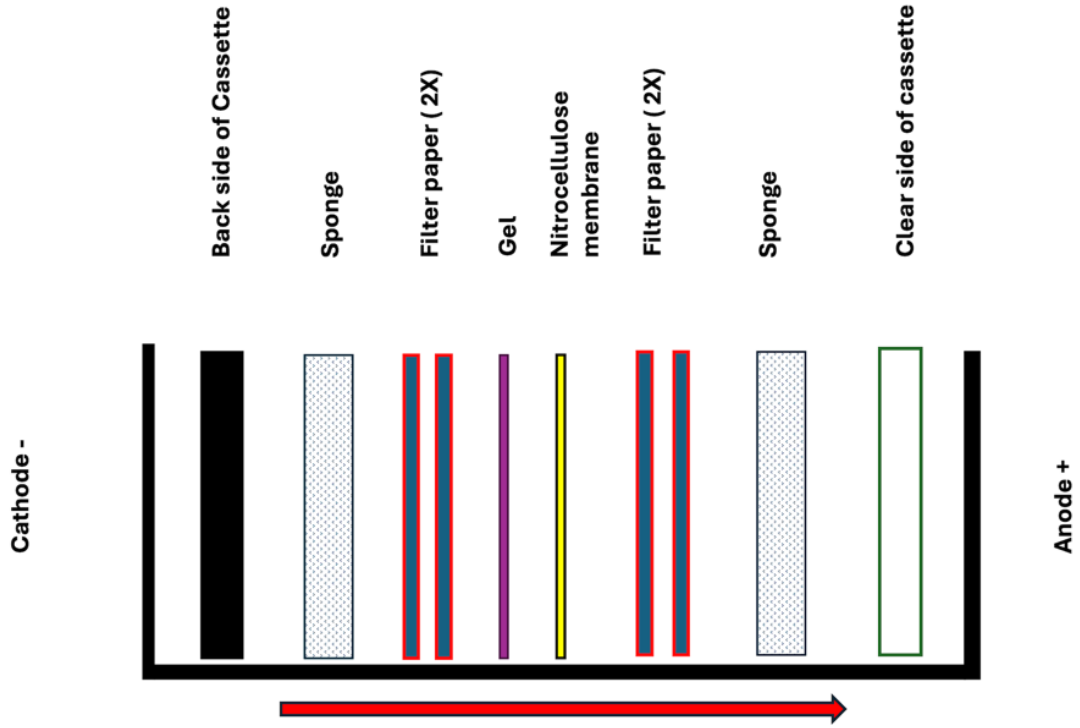


Figure 4.4 Schematic diagram of the electro-blot transfer procedure using an immersion process in a transfer buffer. The red arrow at the bottom of the tank shows the transfer direction.

The “sandwich cassette” is placed in the tank containing 1X transfer buffer (components and quantities seen in Table 3.5). The tank was run at 400mA and 300V for 1 hour and 30 minutes. A block of ice is placed in the tank to raise the level of the transfer buffer solution and avoid overheating the buffer solution. The entire set-up is then placed in a tray surrounded by ice.

Table 4.6 Components and quantities to prepare 1X transfer buffer

Component	Amount
Tris base	2.41g
Glycine	11.25g
Distilled Water	800ml

4.2.7 Membrane blocking and antibody incubation

After the protein transfer, the Nitrocellulose membrane was blocked for an hour in a blocking buffer consisting of 1g milk powder in 20 mL 1X Tris buffer saline-Tween (TBS-Tween). This was done to prevent unspecific binding of the antibody to the membrane. After an hour of blocking, the membranes were rinsed in TBS-Tween and then incubated with the primary antibody diluted at 1:1000 in 5% BSA and left overnight on an orbital rocker at 4C. The membranes were washed 3X times (15 mins per wash) with 1X TBS-Tween and then incubated with the secondary antibody (diluted at 1:2000), prepared the same way as the primary antibody for an hour on a shaker. The membranes were washed three times, 15 minutes per wash in TBS-Tween. For protein detection, the Clarity Western ECL Substrate developer solution (Bio-Rad) was used. 5 ml of each solution was mixed and poured on the membrane

and developed in the G:Box Chemi Multiple Chemiluminescence Image Capture (Syngene).

4.2.8 RNA Sequencing

Illumina PE150 sequencing was used to sequence the samples, and the total resultant reads of each replicate of the three experiments were taken Table (Table 4.3)

Table 4.7 Samples and reads obtained after RNA sequencing. RNA sequencing was carried out using Illumina Sequencing PE150.

Samples	Reads
siRNA Control	206633858
siRNA+ Ethanol	215490654
siRNA Alone	233382618
No Treatments	226937466
siRNA + EDC Mixture	216463502
siRNA + Fatty Acid Mixture	223571590
siRNA+ EDC Mixture + Fatty Acid Mixture	226159274

4.2.9 RNA-seq Data Processing

The pipeline for processing the RNA-seq data was designed as previously described (Zahra *et al.*, 2022). Briefly, the data was aligned to the reference human genome GRCH38 using TopHat2 (v2.1.1) via the ultra-high throughput short-read aligner Bowtie (v2.2.6). Subsequently, the experimental replicates of the three experiments were merged using Samtools (v.0.1.19), where a minimum of 30 high-quality reads were selected. Cufflinks (v.2.2.1) was used to assemble each sample's transcripts and

expression quantification. Finally, Cuffdiff (v.2.2.1) was used to obtain the differential expression between the treated and control samples. Omics Playground (v3.4.4), an online analytics and visualisation platform, was used to analyse and produce graphs for the RNAseq results. The platform was also used to identify DEGs, gene signatures, functional annotation, and biomarkers. FunRich (Pathan *et al.*, 2017) was utilised for gene enrichment to analyse parameters such as biological processes, molecular function, and biological pathways.

4.3 RESULTS

4.3.1 qPCR Analysis showed Down-Regulation of BRCA1 After Silencing BRCA1 In MCF12A Cells

As clearly demonstrated in Figure 4.5, BRCA1 was successfully silenced in MCF12A cells after treatment with 10nM and 50nM for 24 hours. The level of BRCA1 silencing efficiency was determined by qPCR analysis, which showed a down-regulation of BRCA1 after the transfection with the siRNA in both concentrations (10nM, p=0.0032, 50nM, p<0.0001). Seeing that the most significant down-regulation of the BRCA1 gene was observed with 50 nM siBRCA1, this concentration was selected for subsequent experiments with the different treatments.

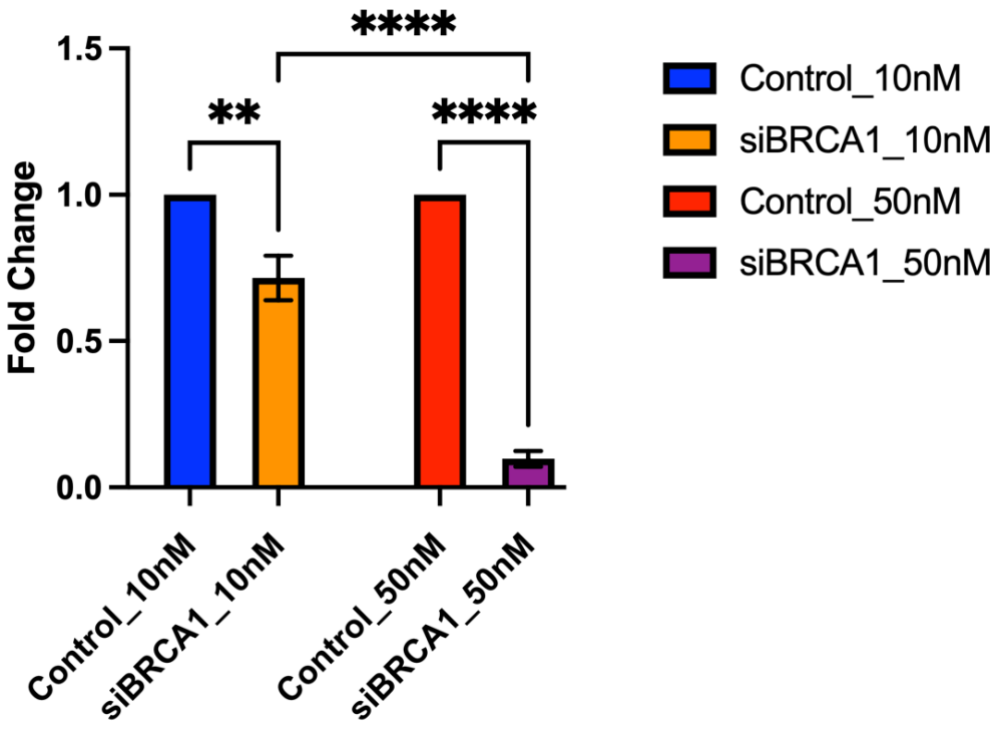
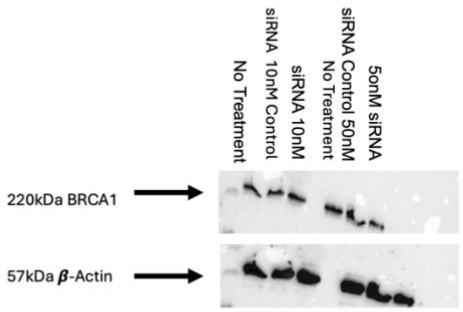


Figure 4.5 Bar plots showing the expression fold changes of the *BRCA1* gene in MCF-12A treated with 10nM and 50nM siBRCA1 with scrambled siRNA as control for 24 hours. Error bars represent SEM. Significance denoted by ****<0.0001, ***<0.0002, **<0.002, *<0.03 as determined by ordinary one-way ANOVA followed by Bonferroni correction. N=3.

4.3.2 Western Blots Showed Down-Regulation BRCA1 After Silencing of BRCA1 in MCF12A Cells

Western blot and densitometry analyses were performed to evaluate the effectiveness of BRCA1 silencing in MCF12A cell lines. The results indicated a degree of down-regulation of the BRCA1 protein. The absence of more significant down-regulation compared to the qPCR results may be attributed to the brief duration of silencing. The samples treated with 10nM siBRCA1 were not statistically significant compared to the no treatments. The samples treated with 50nM showed significant down-regulation compared to no treatments.

A



B

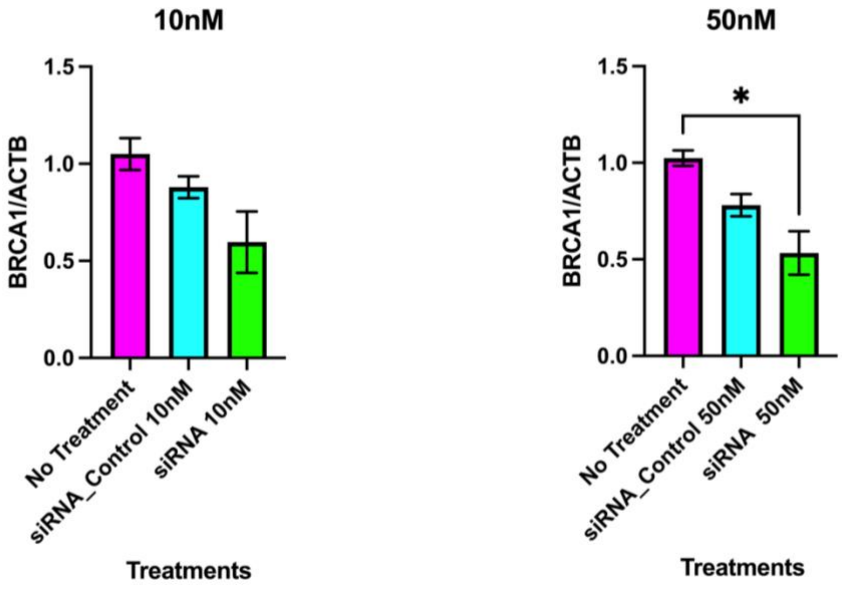
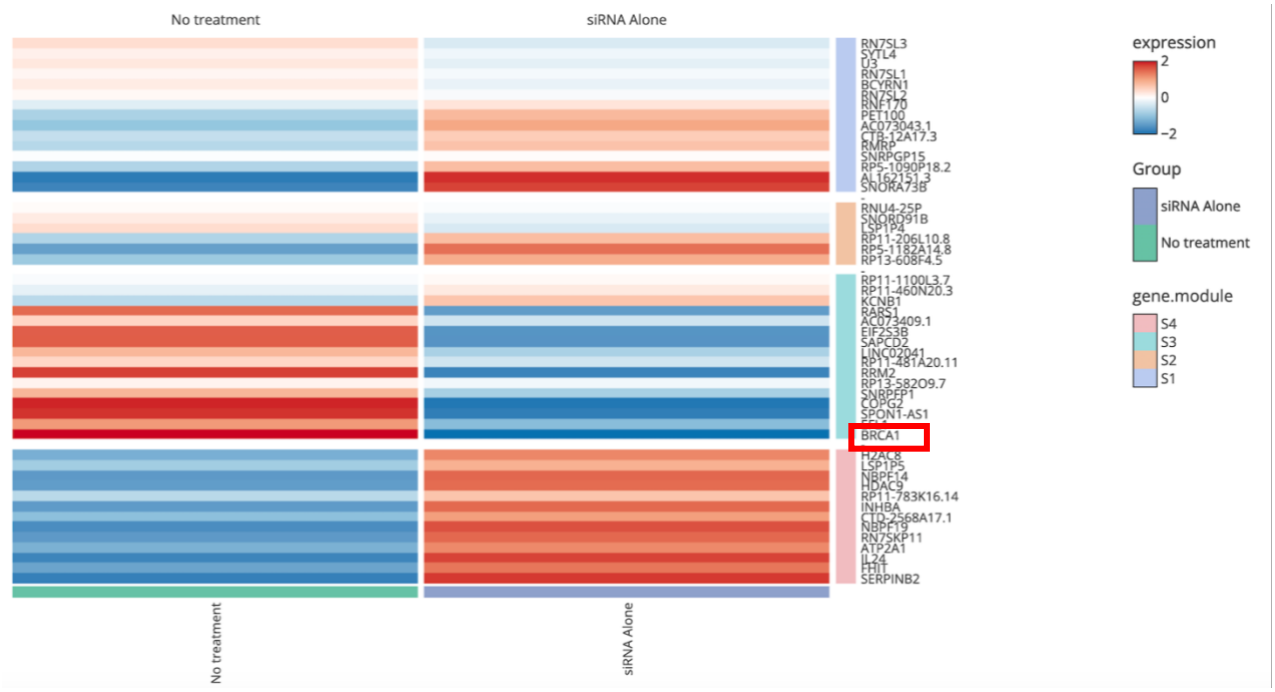


Figure 4.6 Silencing of BRCA1 using 10nM and 50nM siRNA. *Western blots were performed to determine the effectiveness of (**A**) 10nM and 50nM siBRCA1. Densitometry analysis was performed for (**B**) BRCA1/ACTB. Error bars represent SEM, * p< 0.05*

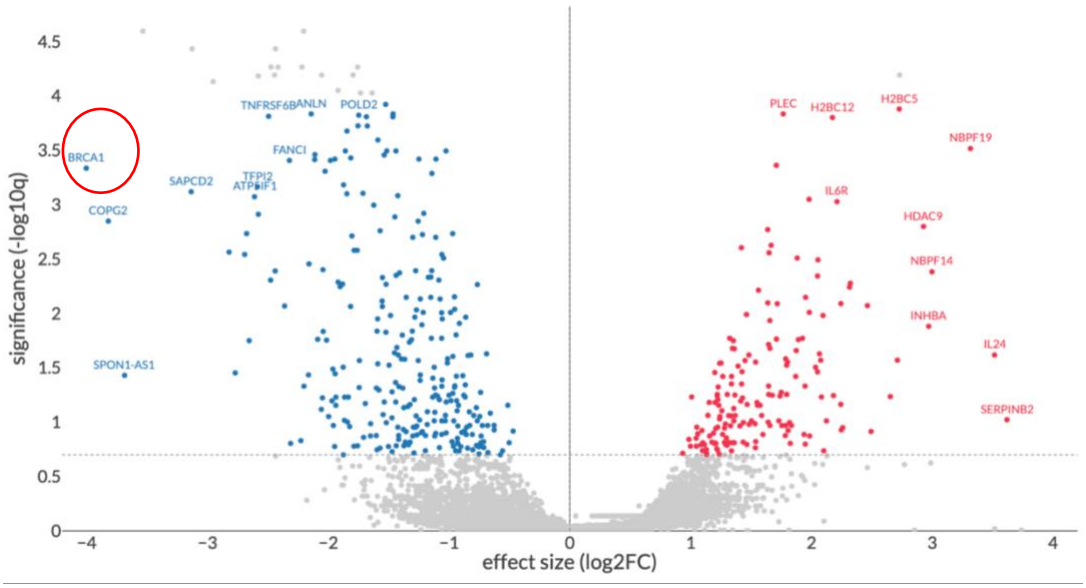
4.3.3 Analysis of RNA-Sequencing Data Showed A Down-regulation of BRCA1 in siRNA treated samples

In order to further confirm that the siRNA treatment had an impact on BRCA1 expression, we compared the data of the samples treated with BRCA1 siRNA (50nM) and those without any treatment (only MCF-12A cells). Analysis of the RNAseq data showed that the sample treated with siBRCA1 exhibited clear down-regulated of the BRCA1 gene, further confirming our findings in the qPCR experiments. Figure 4.6A shows the clustering of the top 50 genes, the up-regulated genes (red), and the down-regulated genes (blue). Figure 4.6B shows the differentially expressed genes in samples treated with siRNA.

A


No treatment
siRNA Alone
RN7SL3
SYTL4
U3
RN7SL1
BCYRN1
RN7SL2
RNF170
PET100
AC073043.1
CTB-12A17.3
RMRP
SNRPGP15
RP5-1090P18.2
AL162151.3
SNORA73B
RNU4-25P
SNORD91B
TSPYL2
RP11-206L10.8
RP5-1182A14.8
RP13-608F4.5
RP11-1100L3.7
RP11-460N20.3
KCNB1
RARS1
AC073409.1
EIF2S3B
SAPCD2
LINC02041
RP11-481A20.11
RRM2
RP13-582O9.7
SNRPFP1
COPG2
SPON1-AS1
BRCA1
H2AC8
LSP1P5
NBPF14
HDAC9
RP11-783K16.14
INHBA
CTD-2568A17.1
NBPF19
RN7SKP11
ATP2A1
IL24
FHIT
SERPINB2
No treatment
siRNA Alone
expression
2
0
−2
Group
siRNA Alone
No treatment
gene.module
S4
S3
S2
S1

B


significance (-log10q)
effect size (log2FC)
BRCA1
COPG2
SPON1-AS1
SAPCD2
TFPI2
ATP8IF1
FANCI
ANLN
TNFRSF6B
POLD2
PLEC
H2BC12
H2BC5
NBPF19
IL6R
HDAC9
NBPF14
INHBA
IL24
SERPINB2

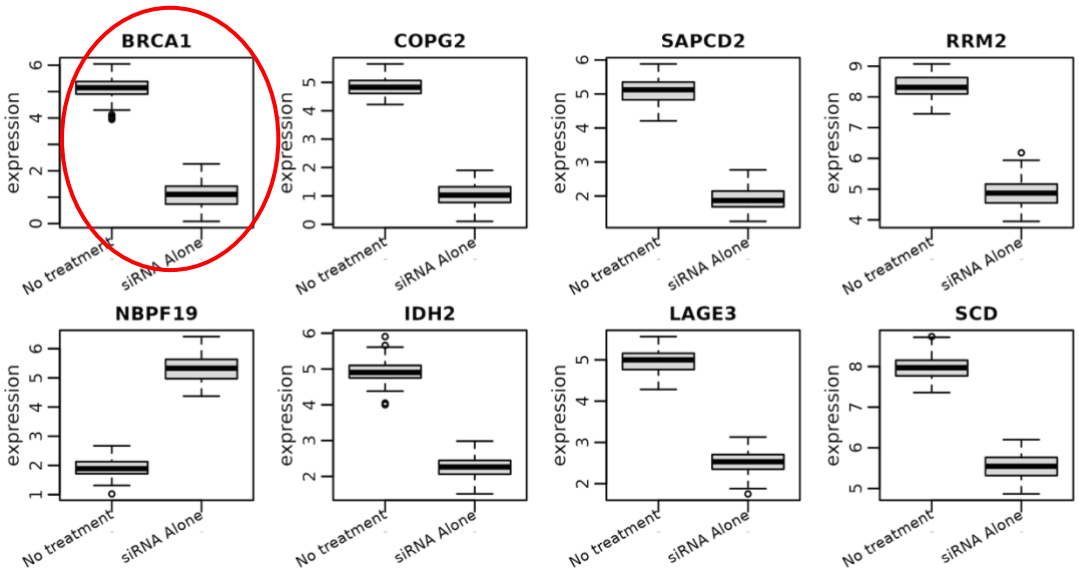


Figure 4.7 The BRCA1 gene was down-regulated by the siBRCA1 in MCF12A cells. **A**) *Heatmap showing the top 50 differentially expressed genes. Data shows a down-regulation of BRCA1. BRCA1 is indicated by the red rectangle* ***B****) Volcano plot showing differentially expressed genes, showing a down-regulation of BRCA1. BRCA1 is circled in red on the volcano plot. The graphs were obtained by using OmicsPlayground* (Akhmedov *et al.*, 2020).

4.3.4 Uniform Manifold Approximation and Projection (UMAP) of Geneset Signature Showed a Clear Contrast between untreated sample and BRCA1 silenced samples

We examined the distribution of geneset between MCF-12A cells treated with 50 nM of siRNABRCA1 and those not treated. The Uniform Manifold Approximation and Projection (UMAP) (Figure 4.6) showed that there was a difference in gene expression between the samples. The untreated samples differed more between the up-regulated (red) and down-regulated genes (blue). The samples treated with siRNA had a more

diffused gene expression, with the up and down-regulated genes having less intensity than the untreated samples.

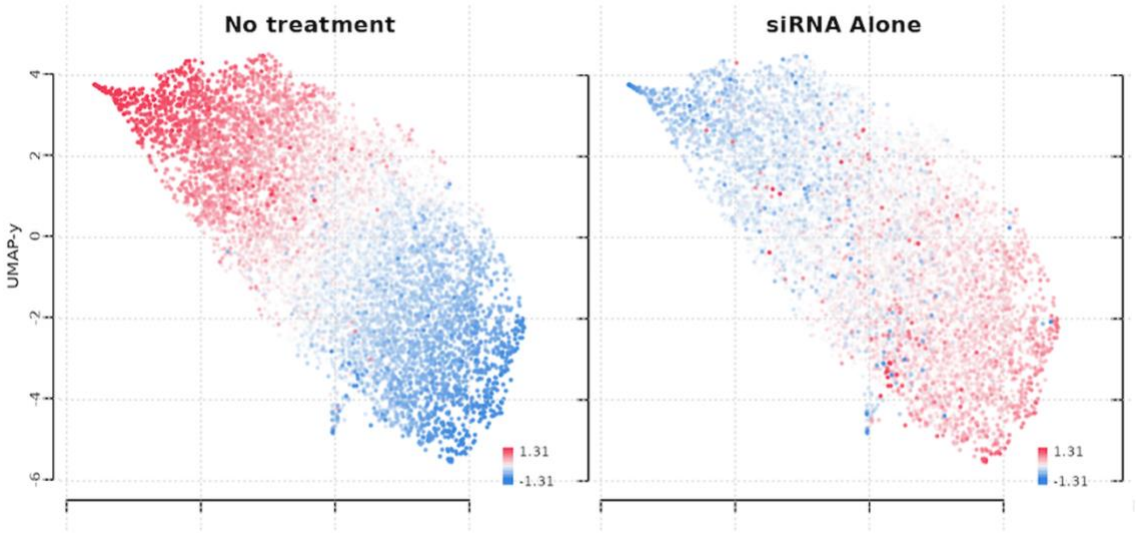


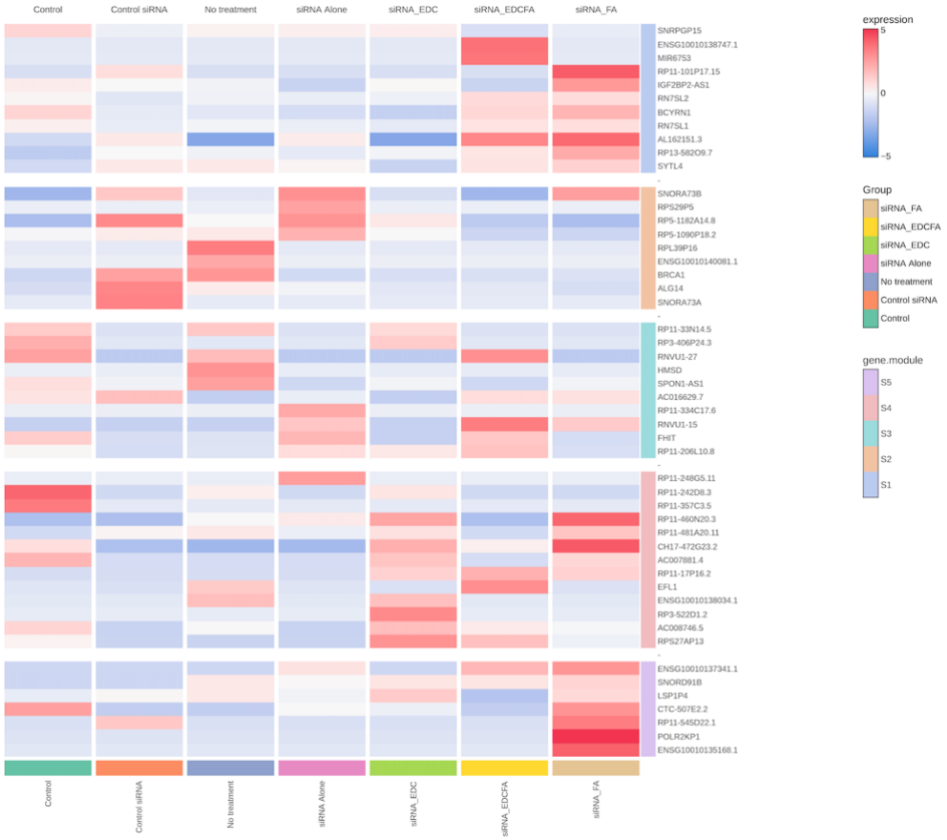
Figure 4.8 Uniform Manifold Approximation and Projection (UMAP) of Geneset Signatures showing Up and Down-Regulated Genes in Different Samples
The UMAPs show genes clustered by log expression, which were up-regulated (red) and down-regulated (blue) in untreated cells and siRNABRCA1-treated cells. The UMAPs were generated with OmicsPlayground (Akhmedov *et al.*, 2020).

4.3.5 Heat Map of Top 50 Differentially Expressed Genes (DEGs) in Different Treatments Identifies Five Clusters

Using the Omics playground platform, expression of the top 50 genes was identified using the highest standard deviation across all samples (siRNA_FA, siRNA_EDC, siRNA_EDCFA) against the control (siRNA_Ethanol). Five hierarchical clusters (S1-S5) were identified at the gene level using a relative expression scale. Functional annotation of the five clusters was analysed by correlating the top 50 genes to reference databases such as KEGG and Gene Ontology. Some functional annotations

are related to fatty acid metabolism, cholesterol homeostasis, glycolysis and adipogenesis. The functional annotation also identified cancer pathways, including G2M, MYC targets, PI3K_Akt_MTOR signalling in the S2 cluster, DNA repair, p53 pathway and apoptosis in the S5 cluster.

A



B

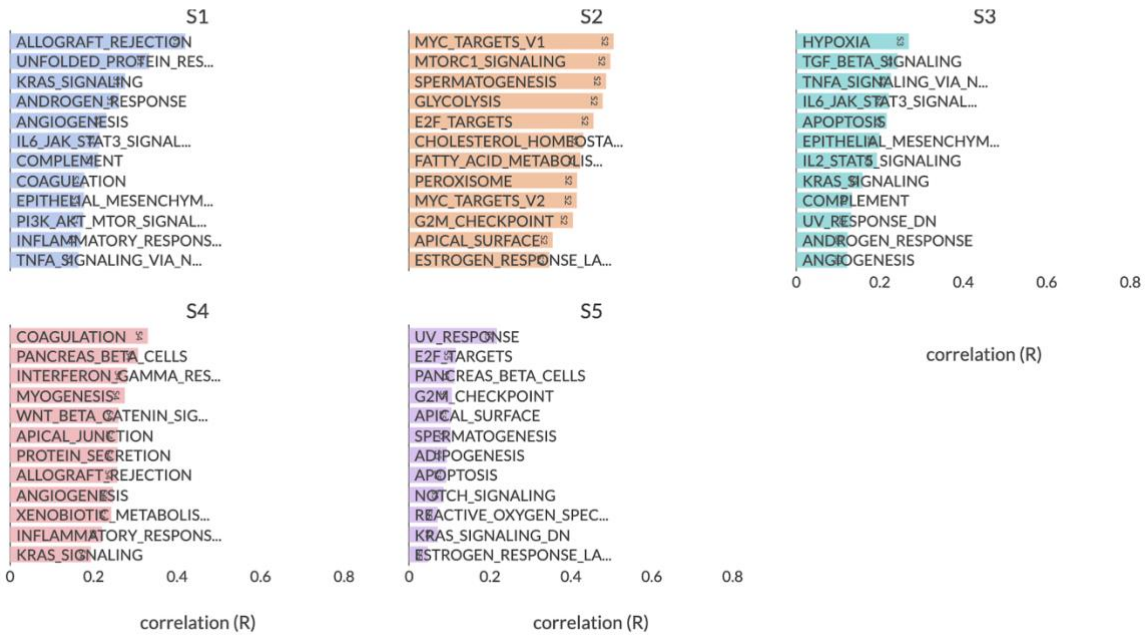


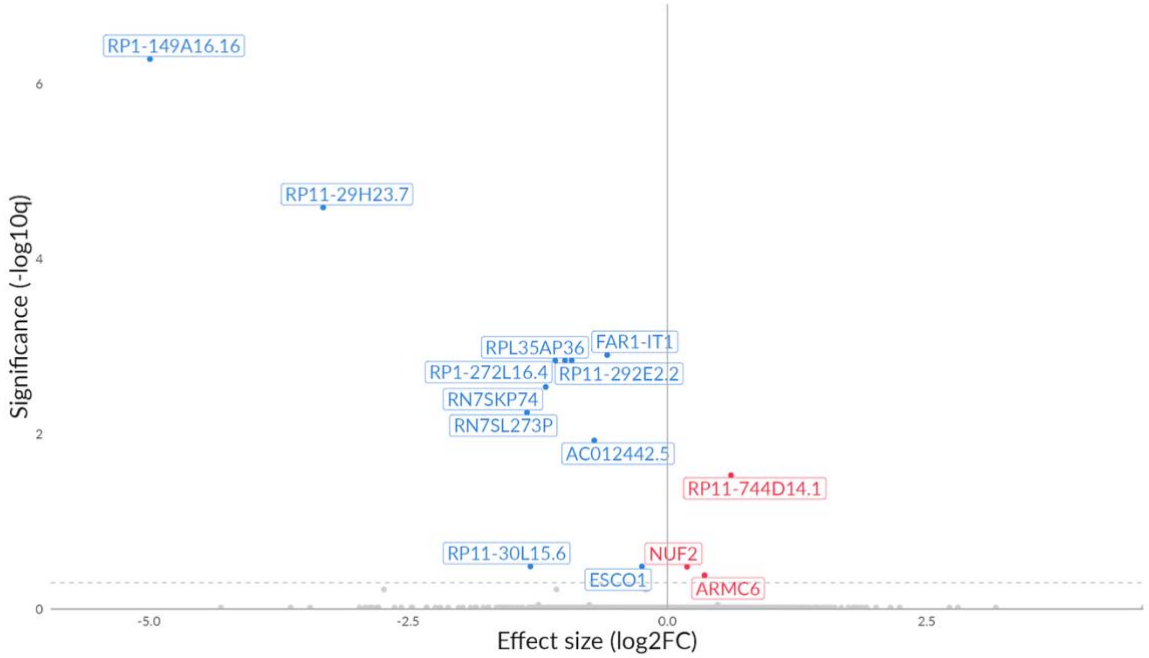
Figure 4.9 Clustered Heatmap and Functional Annotation

A) *Functional heat map of 50 genes identified using the highest standard deviation across all the samples (siRNA_EDC, siRNA_FA and siRNA_FA+EDC). The relative expression scale was utilised for the hierarchical clustering. Red denotes over-expression, and blue denotes under-expression. Five clusters S1(blue), S2 (orange), S3 (green), S4 (red) S5 (Pink).* ***B****) The functional annotation terms for each of the five clusters analysed in A. The functional annotations were generated through Omics Playground, which uses machine learning algorithms to correlate each gene to more than 42 reference databases, such as KEGG and Gene Ontology.*

4.3.6 Significantly Expressed Genes in the treated samples.

We further wanted to analyse which differentially expressed genes were statistically significantly expressed between each treatment and controls. Volcano plots were created to show the most significantly up (red) and down (blue) regulated genes. A False Discovery Rate (FDR) of 0.2 was used in the analysis. Figure 5.8 shows the differential expressions of (A) siRNA+EDC, (B) siRNA+FA, and (C) siRNA+EDCFA in comparison to solvent controls. Genes up-regulated by EDC mixture include *NUF2* and *ARMC6* (Figure 5.8A); Fatty Mixture up-regulated *KLHL22*, *SCML1* and *CRIM1-DT* genes (Figure 5.8b) and EDC+FA up-regulated *ARMC6, NUF2, PTPRN2* and *RTN3P1* genes (Figure 5.8C).

A



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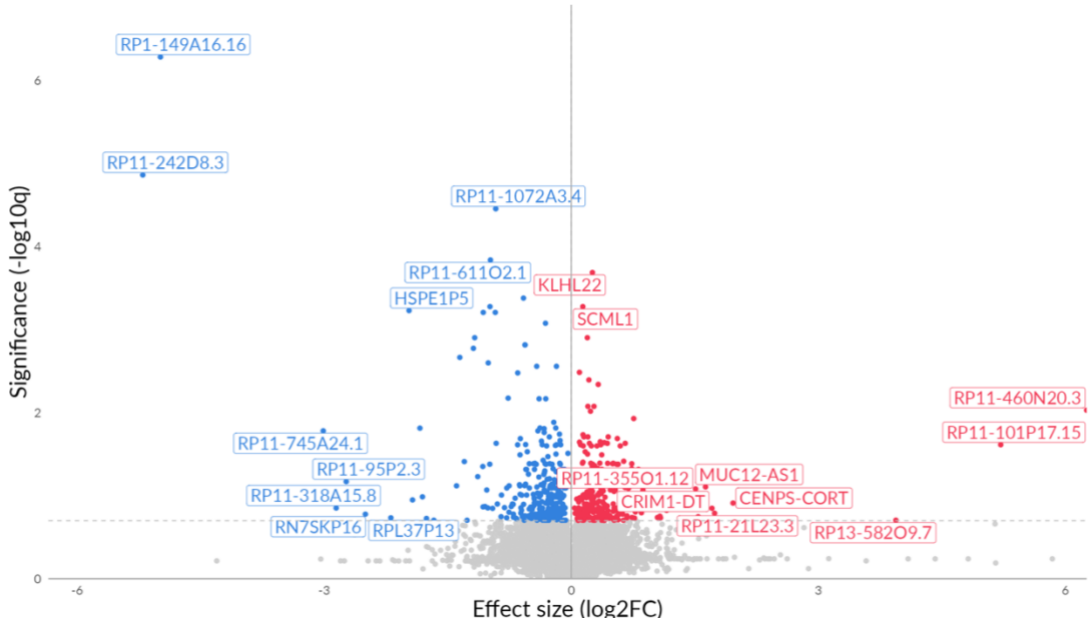


C

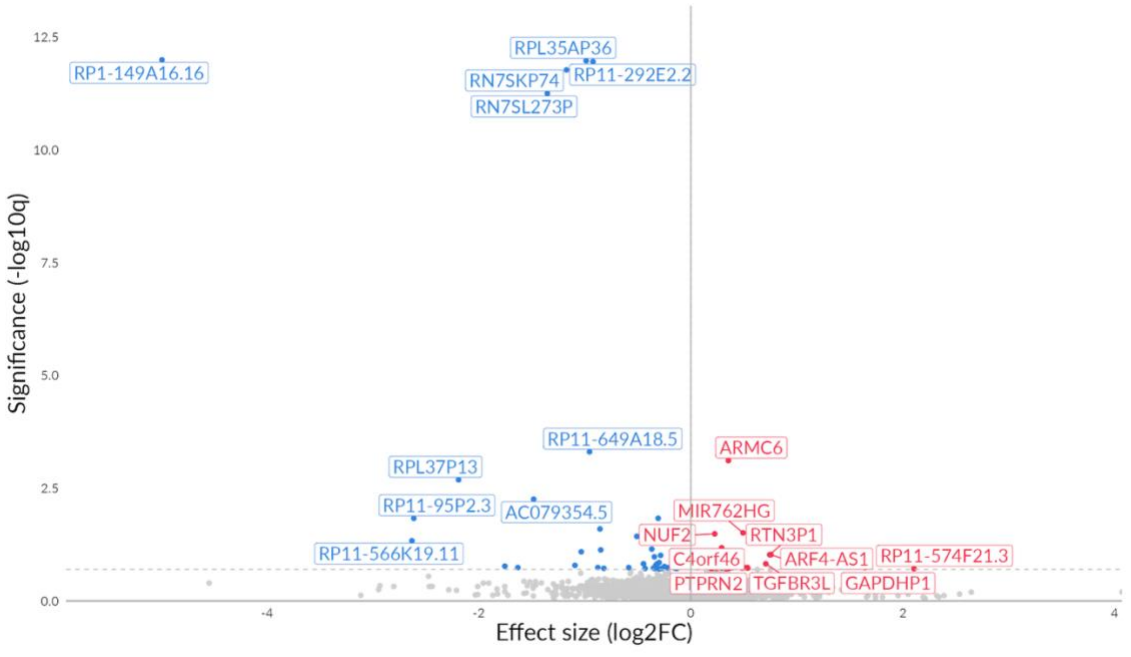


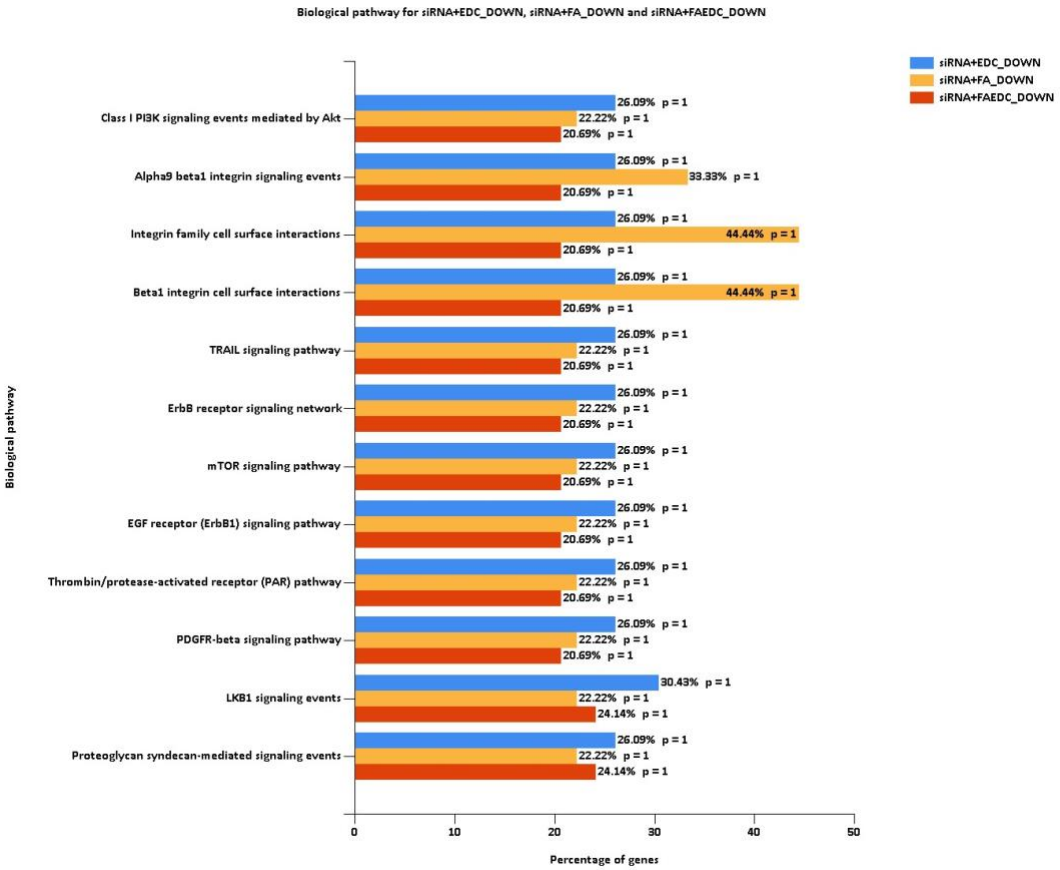
Figure 4.10 Differentially Expressed Genes and Functional Analysis

*Volcano plots showing differentially expressed genes, up-regulated (red) and down-regulated (blue) genes in A) siRNA+EDC, **B**) siRNA+FA, and D) siRNA+EDCFA. Volcano plots were made with OmicsPlayground* (Akhmedov *et al.*, 2020)

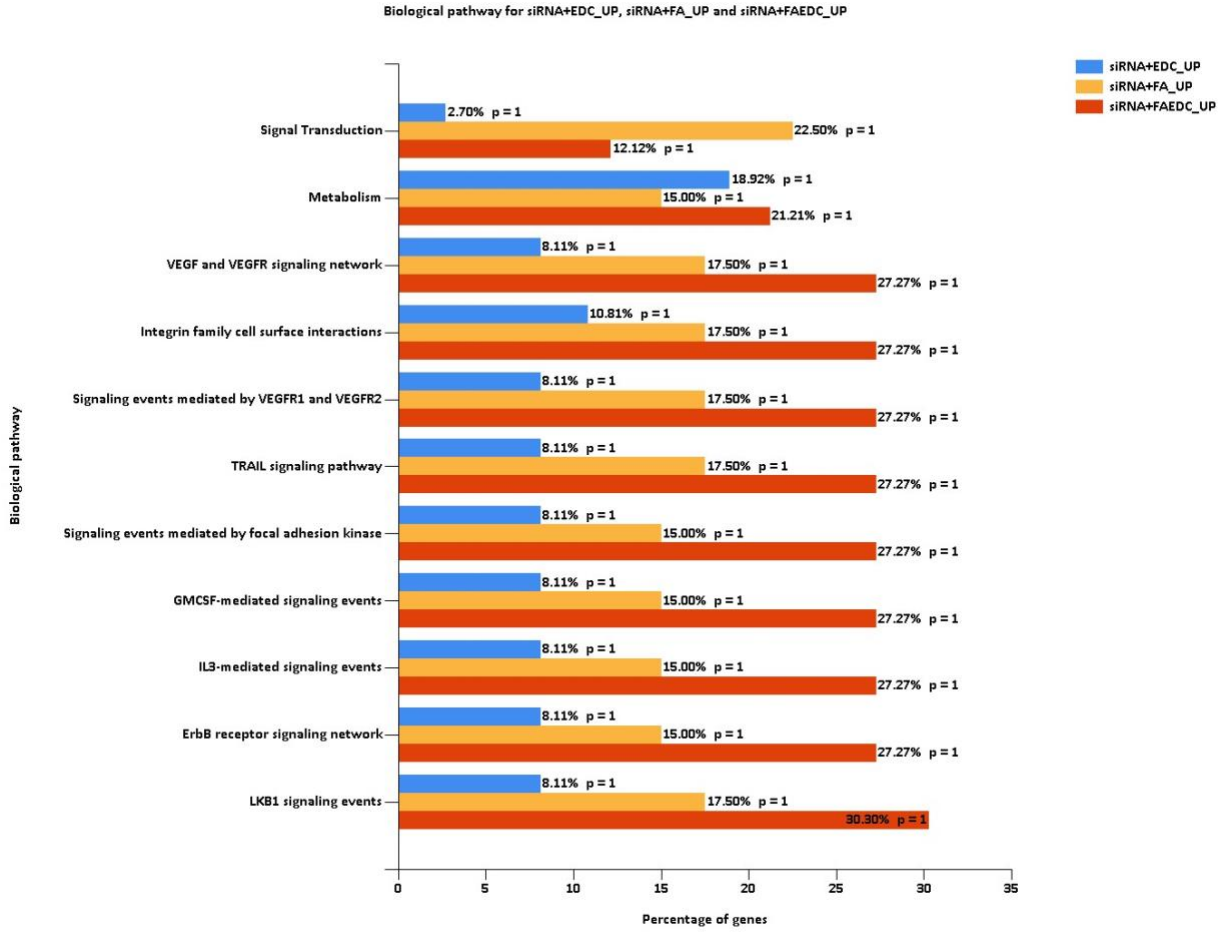
4.3.7 Functional Enrichment of Differentially Expressed Genes (DEGs)

Following the expression analysis, the top 100 up-regulated and down-regulated genes were used for further enrichment analysis using FunRich (Pathan *et al.*, 2017). We analysed the biological pathways such as LKB1 signalling, the S1P1 pathway, Arf6 signalling, and mTOR signalling enriched by down-regulated (**A**), up-regulated genes (**B**), and the total genes affected (**C**). We compared the percentage of genes enriched in the pathways between the three treatments: siRNA+EDC, siRNA+FA, and siRNA+FAEDC. The graphs show the biological pathways and the percentage of genes in each pathway.

A



B


Biological pathway for siRNA+EDC_UP, siRNA+FA_UP and siRNA+FAEDC_UP
siRNA+EDC_UP
siRNA+FA_UP
siRNA+FAEDC_UP
Biological pathway
Signal Transduction
2.70% p = 1
22.50% p = 1
12.12% p = 1
Metabolism
18.92% p = 1
15.00% p = 1
21.21% p = 1
VEGF and VEGFR signaling network
8.11% p = 1
17.50% p = 1
27.27% p = 1
Integrin family cell surface interactions
10.81% p = 1
17.50% p = 1
27.27% p = 1
Signaling events mediated by VEGFR1 and VEGFR2
8.11% p = 1
17.50% p = 1
27.27% p = 1
TRAIL signaling pathway
8.11% p = 1
17.50% p = 1
27.27% p = 1
Signaling events mediated by focal adhesion kinase
8.11% p = 1
15.00% p = 1
27.27% p = 1
GMCSF-mediated signaling events
8.11% p = 1
15.00% p = 1
27.27% p = 1
IL3-mediated signaling events
8.11% p = 1
15.00% p = 1
27.27% p = 1
ErbB receptor signaling network
8.11% p = 1
15.00% p = 1
27.27% p = 1
LKB1 signaling events
8.11% p = 1
17.50% p = 1
30.30% p = 1
0
5
10
15
20
25
30
35
Percentage of genes

C

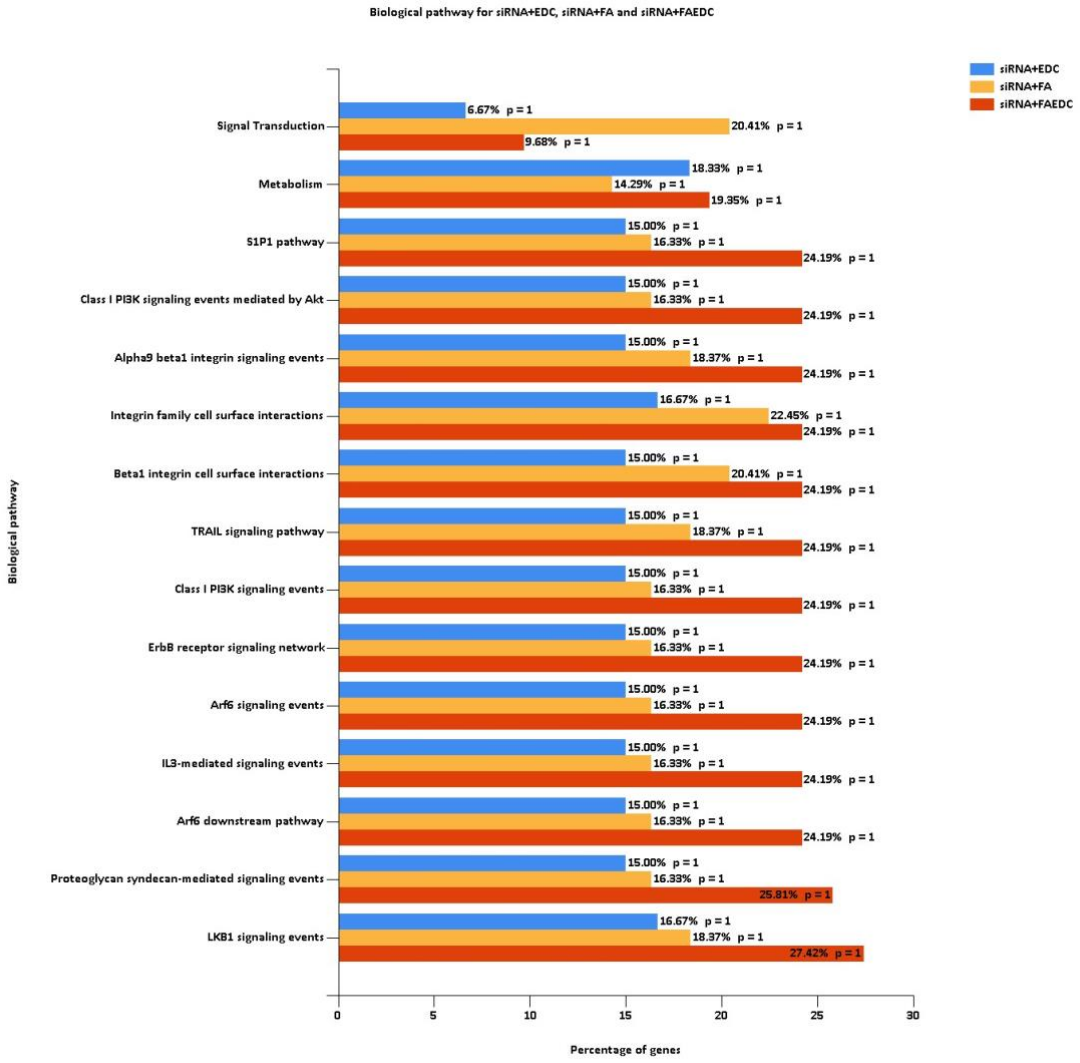
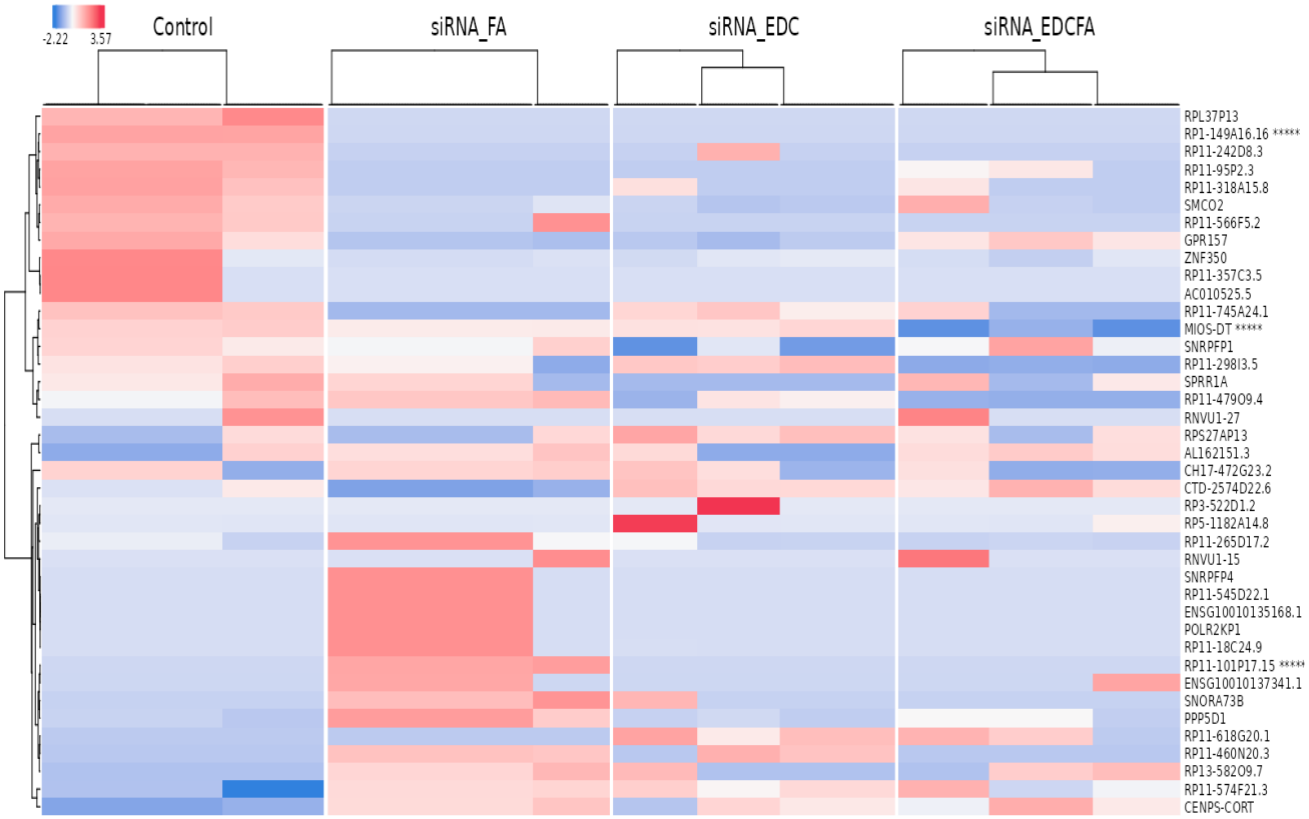


Figure 4.11 Comparison of biological pathway enrichment. *Biological pathways enriched by (A) down-regulated, (B) up-regulated genes, and (C) top 100 genes differentially expressed genes. Comparisons were made between the three treatments, siRNA+EDC, siRNA+FA and siRNA+FAEDC. Bar charts created with FunRich* (Pathan *et al.*, 2017).

4.3.8 Predicted Biomarkers for Exposure to Mixtures of EDCs and Fatty Acid After BRCA1 Silencing

The predicted biomarkers of exposure were generated using Omics Playground. A heat map (**A**) showing the expression of the biomarkers was generated for siRNA+EDC, siRNA+FA, and siRNA+EDC+FA. Eight biomarkers were selected across the phenotypic groups. From the boxplots (Figure 5.11B) of the biomarkers, RP11-101P17.15, RN7SL113P, and POLR2KP1 were up-regulated in siRNA_FA, suggesting that those biomarkers are specific to that treatment. Also, RP1-149A16.16, RPL37P13, and RN7SL23P were all up-regulated in the control samples and down-regulated in the treated sample.

A



B

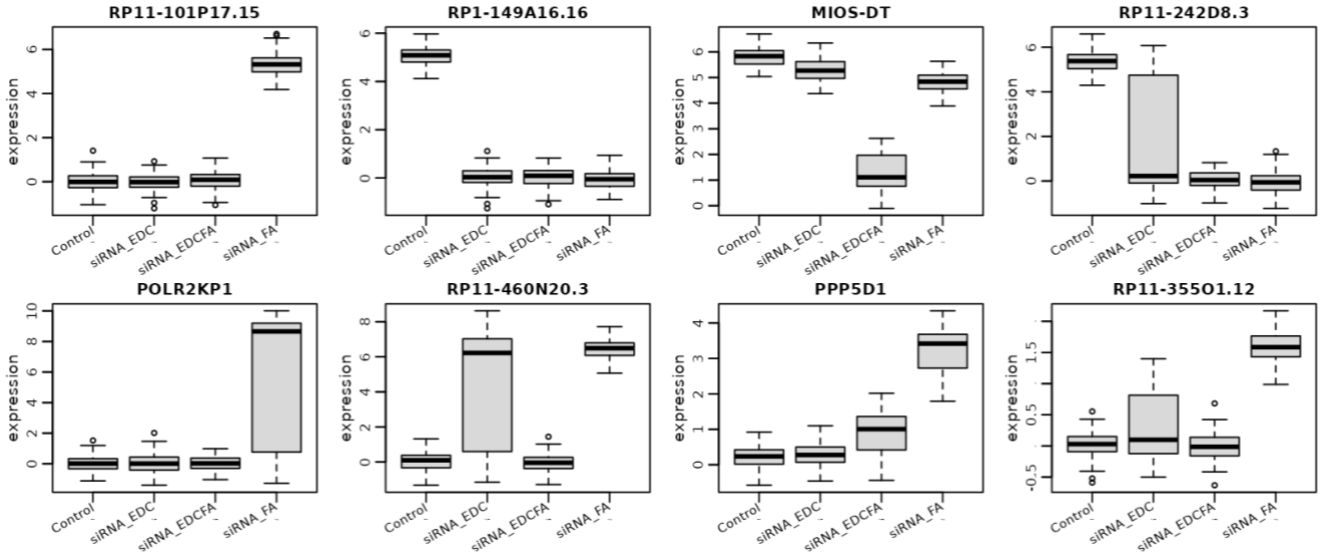


Figure 4.12 Biomarkers expressed in the different treatments.

***A**) Biomarker heat map between the various samples **B**) Top 8 biomarkers associated with the different treatments. These were generated using Omics Playground, where importance scores were calculated using multiple machine learning algorithms.*

4.3.9 Expression of Breast Cancer Genes Before and After Exposure of MCF-12A Cells to EDC+FA Mixture.

Finally, we examined the expression of genes that are well documented as biomarkers for breast cancer risk (Consortium, 2021; Hu *et al.*, 2021) in BRCA1-silenced MCF-12A cells before and after they had been treated with the EDC+FA mixture. We chose the combined mixture for this analysis because it represents a situation where patients are exposed to multiple risk factors. EDCs are ubiquitous in human tissues and fatty acids are a major diet component, so these combined conditions can be considered a representation of real-life exposures. This is also to replicate the potential multifactorial scenario where a person with a mutation in their BRCA1 gene is exposed to EDCs and consumes high amounts of fatty acids. We analysed the LogFC (Log Fold Change) in the expression of these genes to determine if the addition of the EDC+FA to BRCA1 silencing significantly impacted gene expression when compared to BRCA1 silencing alone.

The results showed the down-regulation of genes in the silenced cells, which were then further down-regulated after exposure to the EDC+FA mixture (Table 4.9). Some genes were also slightly up-regulated in the silenced genes; however, when exposed to the EDC+FA mixture, those genes were down-regulated (Table 4.10). However, only some of the genes followed this pattern.

Table 4.8 Down-regulated Breast Cancer genes, which were further down-regulated after exposure to EDC+FA mixture compared with treatment with siRNA Alone.

Genes	siRNA ALONE (LogFC)	siRNA+EDC+FA (LogFC)	Function
CDH1	-0.2425	-1.113	Tumour suppressor gene
CEBPA	-0.2785	-06689	Tumour suppressor gene
CHEK2	-0.1916	-0.6145	Tumour suppressor gene
ERCC2	-0.005642	-0.2101	DNA Repair
ERCC4	-0.01904	-0.5099	DNA Repair
RB1	-0.04841	-1.032	Tumour suppressor gene
EPCAM	-0.05947	-2.587	Oncogene
FANCI	-0.2133	-2.33	DNA Repair

Table 4.9 Up-regulated Breast Cancer genes, which were down-regulated after exposure to EDC+FA mixture compared to treatment siRNA Alone.

Genes	siRNA ALONE (LogFC)	siRNA+EDC+FA Log (FC)	Function
CYLD	1.306	-0.2588	Tumour suppressor gene
PTENP1	0.3908	-0.0424	Tumour suppressor gene
STK11	0.2124	-0.009445	Tumour suppressor gene
WRN	0.01942	-0.121	Tumour suppressor gene
NF1	0.2319	-0.2188	Tumour suppressor gene
NSD1	0.5241	-0.0004259	Oncogene/Tumour suppressor
TSC1	0.4762	-0.5924	Tumour suppressor gene
SMAD4	0.07043	-0.04332	Tumour suppressor gene
VHL	0.6039	-0.07931	Tumour suppressor gene
ATM	1.093	-0.1827	Tumour suppressor gene

4.4 DISCUSSION

Firstly, qPCR (Figure 4.5), western blot (Figure 4.6), and RNAseq (Figure 4.7A and 4.7B) data showed a down-regulation of BRCA1. This showed that the siRNA silencing of BRCA1 in MCF-12A cells was successful. The use of sRNA silencing as a proxy for the silencing of BRCA1 when mutations occur has been used in various studies (Sberna *et al.*, 2023; Gu *et al.*, 2014; Morrone *et al.*, 2019). The western blot did not show a greater level of knockdown. The disparity between qPCR and western blot in determining the efficiency of siRNA knockdown has been reported. While western blot is the best way to confirm siRNA knock, qPCR is alternative approach (Holmes *et al.*, 2010). The successful silencing of BRCA1 in the MCF-12A cells made it possible to carry out the second part of the study.

This chapter focus the differential expression of genes (DEGs) when BRCA1-silenced MCF-12A cells were exposed to EDCs, fatty acids, and the two risk factors combined. The overall aim was to understand how the different treatments could further impact the effect of BRCA1 mutation on potential breast cancer risk. One gene that is down-regulated in all the treatments is *RP-149A16.16*. No work has been done on this gene, which is likely to be a non-coding gene, and further work will have to be done to elucidate its function. When analysing the individual treatments, the mixture of EDCs resulted in the significant up-regulation of *ARMC6* and *NUF2*, which are both expressed in breast cancer (Xu *et al.*, 2019; Adámik *et al.*, 2024). However, no other genes were identified as being significantly regulated by this treatment.

Fatty acid mixture resulted in the up-regulation of *KLHL22, SCML1, MUC12-AS1* and *CRM1-DT* and the down-regulation of *HSPE1P5*. KLHL22 overexpression is a biomarker for poor prognosis for triple-negative breast cancer (Zhang *et al.*, 2024a; Chen *et al.*, 2018). It is also implicated in other cancers, such as colorectal cancer and

melanoma (Song *et al.*, 2020; Liu, Wang and Li, 2020). SCML1 is implicated in cancers such as lung cancer development (Nan, Chen et al. 2019). However, not much work has been done on its role in breast cancer. CRIM1-DT is a long noncoding RNA (LncRNA) that is a prognostic marker for lung adenocarcinoma and has been shown to increase breast cancer risk (Zhao *et al.*, 2024; Di *et al.*, 2022).

The combined mixture (EDC+FA) up-regulated *ARMC6* and *NUF2*, which were also up-regulated by the mixture of EDCs. The two genes were more up-regulated by the EDC+FA mixture than their expressions by the EDC mixture alone. The combined mixture up-regulated *PTPRN2*, a gene which studies have found to promote metastatic breast cancer cell migration (Sengelaub *et al.*, 2016; Sorokin *et al.*, 2015). Overall, the data showed that BRCA1 deficient MCF-12A cells, when exposed to mixtures of EDCs and Fatty Acids, up-regulated genes that play crucial roles in breast cancer initiation and progression. These genes are also implicated in other cancers, such as colorectal, melanoma and pancreatic cancers.

The top biological processes associated with the top 50 DEGs among all treatments include myc targets V1 and V2, mTORC1 signalling, KRAS signalling, fatty acid metabolism, PI3K and apoptosis. Studies have shown that these signalling pathways are activated in breast cancer. Xiang and colleagues showed that the Akt/mTOR pathway is involved in BRCA1-deficient breast cancers (Xiang *et al.*, 2011). Joyce and colleagues also showed that mTORC1 inhibitors significantly decreased tumorigenesis in BRCA1-deficient breast cancer (Joyce *et al.*, 2024). Increased glycolysis has been reported in BRCA1-deficient breast cancer cells, leading to an increase in oxidative stress and glycolytic metabolism in the tumour microenvironment (Martinez-Outschoorn *et al.*, 2012). Chiyoda and colleagues have also reported this phenomenon in ovarian cancer cells (Chiyoda *et al.*, 2017).

We focused on the up-regulated genes and the total genes affected in the functional enrichment of biological pathways. Some of the pathways enriched by the up-regulated genes include signal transduction, TRAIL signalling, LKB1 signalling cascade, IL3-mediated cascade and ErbB receptor signalling networks. These pathways have been shown to be involved in breast cancer development (Sirek *et al.*, 2024; Naimo *et al.*, 2023; Sieber *et al.*, 2022). For example, LKB1 has been shown to promote breast cancer growth and progression in MCF-7 cells by complexing with ERα (Naimo *et al.*, 2023). This pathway is also targeted in chemoprevention in breast cancer (Maharjan *et al.*, 2024; Cao *et al.*, 2023). This pathway was highly enriched in the samples treated with mixtures of a combined mixture of EDC and FA. The genes affected in this pathway include *PRKCD*, *EXOC3,* and *MARK2.* These genes are involved in cancer imitation and progression and chemoresistance (Ponnusamy, Natarajan and Manoharan, 2022; Ming *et al.*, 2024; Zhang *et al.*, 2019).

The treatments enriched SIP1 among the combined total of genes differentially expressed. The combined mixture of EDC and fatty acids highly enriched genes in this pathway, which is highly up-regulated in aggressive triple-negative and basal-like breast cancer (Karihtala *et al.*, 2013). This pathway also promotes breast cancer by forming a positive feedback loop with ACSL4 to regulate lipid metabolism and increase breast cancer metastasis (Lin *et al.*, 2023). Juanjuan He and colleagues reported that targeting this pathway suppresses the proliferation of breast cancer (He *et al.*, 2020). Some genes mapped to this biological pathway are *RAPGEF1*, *MSH2,* and *NCOA1*. Further work is needed to elucidate the role of these genes in breast cancer after BRCA1 mutation.

We also examined the biomarkers of exposure expressed across the samples. Specifically, we saw that *PPP5D1, RP11-101P17-15, RP11-35501.12*, *SNRPFP4*,

and *POLR2KP1* were up-regulated only in samples treated with fatty acids. PPP5D1 is a related gene that is up-regulated in pancreatic cancer (Gu *et al.*, 2019). Further work is needed to elucidate how fatty acids up-regulate these biomarkers and their role in breast cancer

Finally, the analysis of genes, which are biomarkers of breast cancer risk, showed a slight up-regulation of genes such as *CYLD, VHL, ATM* and *NF1* in the BRCA1 silenced cells. However, exposure of these BRCA1 silenced cells to the EDC+FA mixture down-regulated these genes. Some genes down-regulated in BRCA1-silenced cells were further down-regulated after exposure to the EDC+FA mixture. Some of these genes are *CDH1*, *CEBPA*, *CHEK2* and *RB1*. The cylindromatosis (CYLD) gene is a tumour suppressor gene implicated in the development and progression of breast carcinogenesis (Pseftogas *et al.*, 2020). Down-regulation of *CYLD* is involved in mammary cancer development in mice (Pseftogas *et al.*, 2024). It has also been shown in breast cancer cells and human tissues that down-regulation of CYLD is associated with breast cancer (Hayashi *et al.*, 2014). Von Hippel Lindau (VHL) is a tumour suppressor gene that is dysregulated in breast cancer development (Wang *et al.*, 2024; Gervin *et al.*, 2020). Studies have shown that microRNA-155 (miR-155) plays a crucial role in tumour angiogenesis and poor breast cancer by down-regulating *VHL* (Kong *et al.*, 2014). *ATM* is a tumour suppressor gene that plays a role in DNA repair and DNA damage response (Stucci *et al.*, 2021). Down-regulation of *ATM* is implicated in breast cancer development (Raposo-Ferreira *et al.*, 2016). Neurofibromin 1 (NF1) is a tumour suppressor gene associated with elevated cancer development. NF1 depletion is associated with elevated breast cancer risk (Pacot *et al.*, 2024; Tao *et al.*, 2020a). NF1 down-regulation leads to the incidence of contralateral breast cancers with poor prognosis (Evans *et al.*, 2020). *CHEK2* is a

tumour suppressor gene and a moderate penetrance breast cancer risk gene involved in DNA repair, cell cycle arrest and apoptotic processes in response to DNA damage (Graffeo *et al.*, 2022). Dysregulation of *CHEK2* is a breast cancer risk and is implicated in the development of increased tumour sizes with poor prognosis (Bergstrom *et al.*, 2021; Schwartz *et al.*, 2024). *CEBPA* down-regulation has been implicated in cancers such as ovarian cancer and breast cancer (Tan *et al.*, 2024; Cheng *et al.*, 2023). It has been shown that tri-o-cresyl phosphate interacted with ERα to promote cell growth by down-regulating genes such as *CEBPA* (Böckers, Paul and Efferth, 2020). The retinoblastoma susceptibility gene (*RB1*) is a tumour suppressor gene that regulates cellular functions such as apoptosis, cell cycle progression, DNA damage response, and metabolic programming (Yao *et al.*, 2022; Xie *et al.*, 2022). Down-regulation of RB1 has also been implicated in the development of breast cancer (Tao *et al.*, 2020b; Palomar-Siles *et al.*, 2023). Overall, we show that even though these genes are slightly up-regulated in the BRCA1 silenced cells, they were significantly down-regulated when the silenced cells were exposed to EDC+FA. In the real world, these results show that a patient with BRCA1 mutation who is exposed to a mixture of EDC and consumes a high-fat diet could increase their breast cancer risk as a result of the dysregulation of important tumour-suppressor genes.

In conclusion, our data showed that exposing BRCA1 deficient MCF-12A cells to mixtures of EDCs and fatty acids led to the up-regulation of genes and pathways involved in carcinogenesis. The data suggests a possible increase in absolute risk in people with germline mutation of BRCA1 and exposed to mixtures of endocrine-disrupting chemicals and have a high intake of fatty acids.

Chapter Five: A meta-analysis of the transcriptional landscape of 2D vs 3D MCF10 Breast Models

5 A meta-analysis of the transcriptional landscape of 2D vs 3D MCF10 Breast Models

5.1 Introduction

The Extracellular matrix (ECM) is the acellular component of the tumour microenvironment (TME) and comprises different networks of structural and instructional molecules that interact with tumour cells (Schaefer and Reinhardt, 2016). The composition of the breast stromal ECM includes collagens, fibronectin, Laminins, proteoglycans, osteopontin, and hyaluronic acid, which play roles in the process of carcinogenesis (Tamayo-Angorrilla *et al.*, 2022). The components of the ECM provide cells with biochemical and biomechanical impulses that play essential biological roles such as cell adhesion, cell proliferation, migration, autophagy, angiogenesis, immunity, and matrix remodelling (Karamanos *et al.*, 2021). They also play essential roles in breast cancer progression and metastasis (Insua-Rodríguez and Oskarsson, 2016).

As discussed in Chapter One, one of the aims of 3D models is to recapitulate the *in vivo* ECM. Different scaffolds, both natural and synthetic hydrogels, have been developed and used over the years to achieve this. Hydrogels are 3D hydrophilic polymer networks forming 3D matrices with high water contents, which maintain mechanical stability. They have been extensively used in the biomedical industry as drug transporters, dissolvable sutures, and injectables (Rajabi *et al.*, 2021). They are used due to their biocompatibility, biodegradability, cytocompatibility, and tunability (Monfared *et al.*, 2021; Catoira *et al.*, 2019).

While significant work has been carried out developing suitable synthetic hydrogels, given that this project was carried out using natural scaffolds, we focused our attention on comparing only natural scaffolds, such as alginate, collagen, and Matrigel. We

compared the effect of these scaffolds on the genomic and transcriptomic landscape of breast cells.

5.1.1 Alginate

Alginates are biodegradable, non-toxic, non-immunogenic, anionic polymers produced from brown seaweed, forming gels with good biocompatibility and rheological properties (Le *et al.*, 2021; Remaggi *et al.*, 2022). Alginates are used in tissue engineering, drug delivery, as a biocide and as implant materials (Varaprasad *et al.*, 2022). Globally, 38,000 tons of alginates are produced yearly and used in the pharmaceutical and food industries. Structurally, alginates are hydrophilic, linear polysaccharides made of D-mannuronic acid residues and a C5-epimer α-L-glucuronic acid. The hydrogels are formed by the interaction of the polysaccharide units with divalent cations such as Ca^{2+}. Alginate hydrogels are pH-sensitive and have chemical flexibility and crosslinking capabilities. The amount of gel and ions used to form the gel impacts its characteristics, such as elasticity, swelling, porosity, and stability. Alginates can be used as scaffolds in 3D cultures, as their stiffness and elasticity can be tuned through crosslinking to match those of human tissues (Andersen, Auk-Emblem and Dornish, 2015; Abasalizadeh *et al.*, 2020; Shaikh *et al.*, 2022). MCF-7 cell lines cultured on alginate hydrogels have been shown to form circular and organised spheroids similar to in vivo structures (Cavo *et al.*, 2016). In another study, MDA-MB-231 cell lines were cultured on an alginate matrix to study the expression levels of CD44, C24 and ALDHI mRNA markers. The results showed an increased expression of the markers in the 3D alginate cultures compared to 2D cultures (e Souza *et al.*, 2017).

5.1.2 Collagen

Collagen hydrogels have gained prominence in biomedical research as a scaffold because they are the most abundant protein in mammals and are a significant component of the extracellular matrix (Antoine, Vlachos and Rylander, 2014; Walimbe and Panitch, 2020) . It is a triple-helix protein formed from repeated tripeptide proline domains. It is degraded by collagenase-1 in the ECM, an essential process in morphogenesis, development, tissue remodelling and repair (Jabłońska-Trypuć, Matejczyk and Rosochacki, 2016). The collagen family consists of at least 28 members in mammals, divided into fibrillar and non-fibrillar. Fibrillar collagen I, II and III are the most abundant collagen in ECM, with collagen I being the most used type in biomedical industries (Antoine, Vlachos and Rylander, 2014; Copes *et al.*, 2019). Integrins and immunoglobins are incorporated into collagen hydrogels to increase cell adhesion (Luparello, 2013). Collagen hydrogels are widely used because they are cheap, biocompatible, low toxicity, versatile, and highly mimicking the native ECM. To improve the mechanical strength of collagen hydrogels, they are crosslinked using formaldehyde, glutaraldehyde, tyrosinase, transglutaminase, and UV radiation (Sarrigiannidis *et al.*, 2021; Thiele *et al.*, 2014).

5.1.3 Matrigel

Matrigel was developed in the 1970s by Hynda Kleinman and named by John Hassel. The basement membrane was extracted and purified from Engelbreth-Holm-Swarm mouse tumours. Matrigel contains the major essential protein of the ECM basement, including laminin (~60%), collagen IV (~30%), entactin (~8%) and heparin sulfate proteoglycan perlecan (~2-3%). Additionally, Matrigel contains growth factors such as transforming growth factors (TGG-β), fibroblast growth factors (FGFs), epidermal

growth factors (EGF), basic fibroblast growth factors (bFGF), platelets derived growth factors (PDGF) and matrix metalloproteinases (MMPs). The basement proteins and growth factors in their various isoforms provide integrins and binding sites for cell adhesion and differentiation, angiogenesis, but also involved in cancer initiation and progression (Ferreira, Gaspar and Mano, 2018; Aisenbrey and Murphy, 2020).

Matrigel has been widely used in the 3D culture of cultures of various cell types and cell lines. Specifically, a large amount of work has been published where the scaffold was successfully used to culture breast tissues and breast cancer cell lines such as MCF-10A MCF-12, both in mono and co-cultures (Debnath, Muthuswamy and Brugge, 2003; Marchese and Silva, 2012; Wessels *et al.*, 2019).

However, Matrigel exhibits inter- and intra-batch variability in physical, biochemical, and mechanical properties and the presence of endotoxins such as lactate dehydrogenase-elevating virus (LDV), which causes immunotoxicity. The presence of growth factors may also confound biological effects. These factors lead to reduced data reproducibility and suitability for pharmacological studies (Langhans, 2018; Aisenbrey and Murphy, 2020; Kim *et al.*, 2022). The variability in the concentration of the over 2000 proteins in Matrigel affects the quality of 3D structures (Aisenbrey and Murphy, 2020). The protein concentration and number of growth factors can be adjusted for in vitro cell culture, such as in growth factor reduced (GFR) Matrigel (Hughes, Postovit and Lajoie, 2010).

5.1.4 MCF-10A cell line

The initial aim of the chapter was to examine the genetic profile of MCF-12A cell lines on different scaffolds. However, due to the paucity of published data on the MCF-12A cell line, we settled on one of the most used non-tumorigenic cell lines, MCF10A. MCF-12A has been reviewed in Chapter Two. MCF-12A and MCF10A cell lines are both

human breast epithelial cell lines. While MCF-10A cells are estrogen receptor-negative, MCF-12A has been shown to be both estrogen receptor-positive and negative (Engel *et al.*, 2019; Marchese and Silva, 2012; Sweeney, Sonnenschein and Soto, 2018). In 3D, both cell lines form structures resembling mammary acini (Marchese and Silva, 2012; Marchese, 2013). Therefore, MCF10A can be a suitable surrogate for MCF-12A, and the genetic profile seen in MCF10A could potentially be seen in MCF-12A cells.

MCF10A is an immortalised human mammary epithelial cell line regularly used in *in vitro* models to elucidate normal breast function and transformation. These cells have been used to elucidate the transformational activities of oncogenes and tumour suppressor genes (Kadota *et al.*, 2010; Liu *et al.*, 2019a; Qu *et al.*, 2015; Maguire *et al.*, 2016; Gross *et al.*, 2022). They were established by Soule and colleagues in 1990 from human fibrocystic tissue and classified as normal breast cell lines due to their lack of tumourigenesis in nude mice, three-dimensional (3D) growth in collagen, growth in culture medium regulated by hormones and growth factors, and lack of anchorage-independent growth and dome formation in confluent cultures (Soule *et al.*, 1990). When single-cell suspensions of MCF10A cell lines are cultured on hydrogels such as Matrigel, they proliferate and organise into 3D acini structures with a hollow lumen that mimic normal apicobasal polarity and secrete endogenous basement membrane (laminin V and type IV collagen) (Puleo and Polyak, 2021).

5.1.5 Chapter Scope

As discussed in Chapter One, 2D models have been seen as the traditional approach for cell culture; however, they have limitations as they do not recapitulate 3D architecture. Different hydrogels used to replicate the ECM in cancer biology affect the genetic profile expressed. In this study, we used available novel transcriptomics

and clinical data to assess the impact of different scaffolds and growth conditions on MCF10A cells and to evaluate whether they better recapitulate the behaviour of MCF10A cells compared to the traditional 2D models. To achieve this, we addressed the following question:

1. What are the differences between the gene expression profile between 2D and 3D models?
2. What are the effects of the various scaffolds on the gene expression profile of the MCF10A cell line?

5.2 Methodology

5.2.1 Study Design

The study aimed to search extant literature for research modelling breast cancer using the 3D culture technique and examine the differences in gene expression profiles between 2D and 3D cultures. Bioprojects were searched in the National Centre for Biotechnology Information (NCBI) database (Figure 5.1). No limitations were applied to the original publication language as long as English translations were accessible. Search terms used to assess the Bioprojects and the number of Bioprojects accessed are shown in Table 5.1.

Inclusion criteria: Studies were included if they used a 3D and a 2D breast model using MCF10A cells.

Exclusion criteria: Studies that did not satisfy the original search criteria were removed. These included studies that lacked 2D cultures, were not open access, were not human cell lines (MCF10A), and did not have associated data.

Table 5.1 Search Term, Number of Bioprojects and Number of Human Studies

SEARCH TERMS	NUMBER OF BIOPROJECTS	Number of Human studies
“Carcinoma” AND “Breast” AND “3D”	15	10
“Mammary” AND “3D”	110	47
”Mammary “AND “Cancer” AND “3D”	63	29
“Breast” AND “Cancer” AND “3D”	247	201
“Breast” AND “Cancer” AND “3D” NOT “Ultrasound” NOT “SOUND” NOT “ultra” NOT “Imagining”	240	194

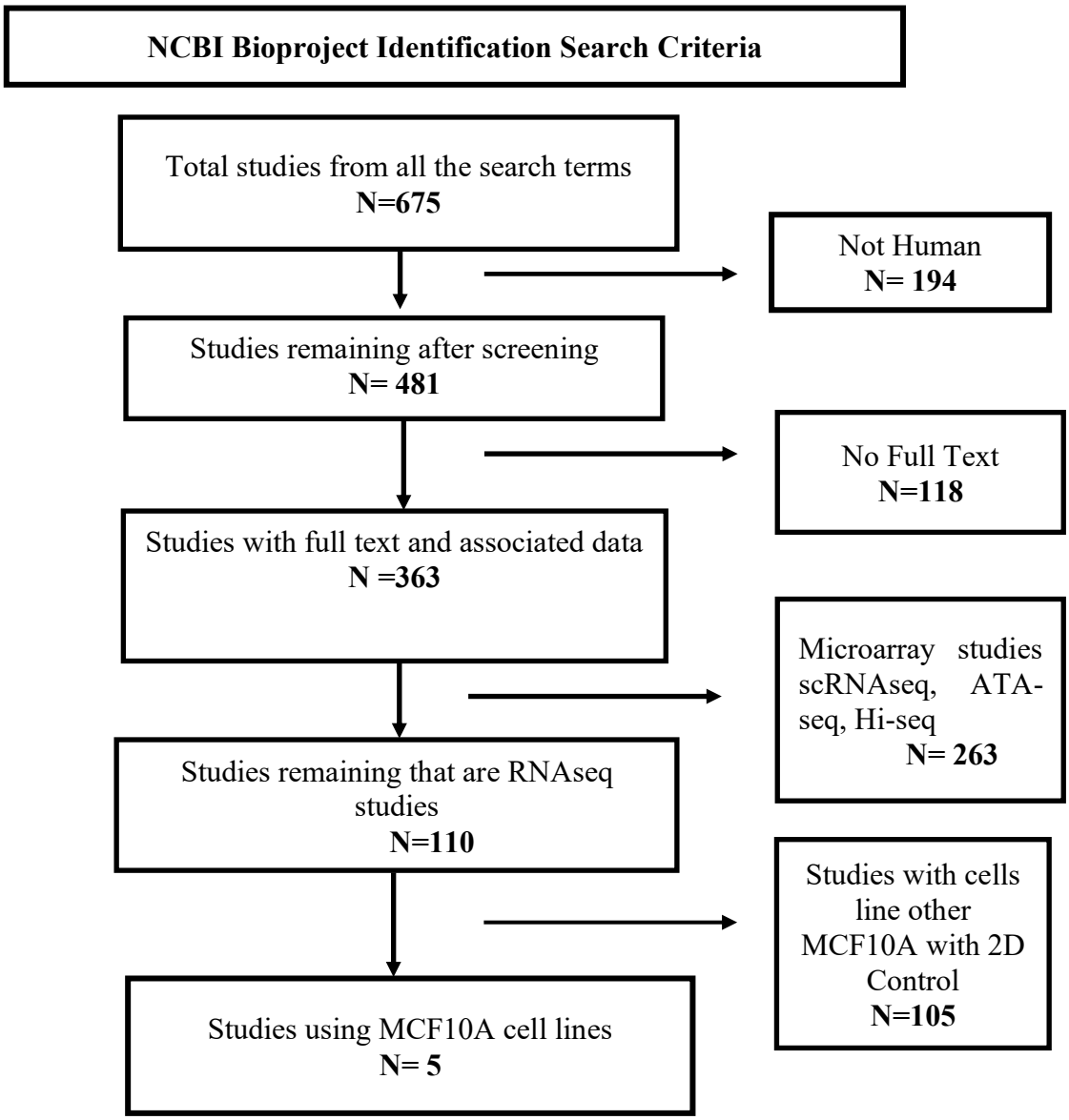


Figure 5.1. Literature search workflow. *Studies were accessed through Pubmed.gov on 3 April 2023 using pre-defined search terms. Studies were subjected to two rounds of screening by two PhD students. Studies that fulfilled the criteria were used for the analysis. The "*" utilised in the search criteria is a wild card enabling all words matching the associated string to be retrieved*

Table 5.2 Information on the Bioprojects chosen for the analysis showing their accession numbers, SRA code, conditions and scaffolds used in the experiments.

Accession Number	SRA code	Cell line	Condition	Scaffold
PRJNA397902	SRR5925772	MCF10A	3D	Soft Alginate and rBM
	SRR5925773	MCF10A	3D	Soft Alginate and rBM
	SRR5925774	MCF10A	3D	Stiff Alginate and rBM
	SRR5925775	MCF10A	3D	Stiff Alginate and rBM
	SRR5925776	MCF10A	3D	Soft col-1 and rBM
	SRR5925777	MCF10A	3D	Soft col-1 and rBM
	SRR5925778	MCF10A	3D	stiff col-1
	SRR5925779	MCF10A	3D	stiff col-1
PRJNA565439	SRR10119384	MCF10A	3D	Matrigel
	SRR10119385	MCF10A	3D	Matrigel
	SRR10119387	MCF10A	3D	Matrigel
	SRR10119388	MCF10A	3D	Matrigel
PRJNA633168	SRR11799648	MCF10A	2D	/
	SRR11799649	MCF10A	2D	/
	SRR11799650	MCF10A	2D	/
	SRR11799651	MCF10A	2D	/
	SRR11799652	MCF10A	2D	/
	SRR11799653	MCF10A	2D	/
	SRR11799654	MCF10A	2D	/
	SRR11799655	MCF10A	2D	/
	SRR11799656	MCF10A	2D	/
	SRR11799657	MCF10A	3D	rBM
	SRR11799658	MCF10A	3D	rBM
	SRR11799659	MCF10A	3D	rBM
	SRR11799660	MCF10A	3D	rBM
	SRR11799661	MCF10A	3D	rBM
	SRR11799662	MCF10A	3D	rBM
	SRR11799663	MCF10A	3D	rBM
	SRR11799664	MCF10A	3D	rBM
	SRR11799665	MCF10A	3D	rBM
PRJNA694979	SRR13537440	MCF10A	3D	Matrigel
	SRR13537441	MCF10A	3D	Matrigel
	SRR13537442	MCF10A	3D	Matrigel
	SRR13537443	MCF10A	2D	/
	SRR13537444	MCF10A	2D	/
	SRR13537445	MCF10A	2D	/

Accession Number	SRA Code	Cell line	Condition	Scaffold
PRJNA860838	SRR20340080	MCF10A	3D	Matrigel
	SRR20340081	MCF10A	3D	Matrigel
	SRR20340082	MCF10A	3D	Matrigel
	SRR20340083	MCF10A	3D	Matrigel
	SRR20340084	MCF10A	3D	Matrigel
	SRR20340088	MCF10A	2D	/
	SRR20340089	MCF10A	2D	/
	SRR20340090	MCF10A	2D	/
	SRR20340091	MCF10A	2D	/
	SRR20340092	MCF10A	2D	/
	SRR20340093	MCF10A	2D	/
	SRR20340097	MCF10A	3D	Matrigel
	SRR20340098	MCF10A	3D	Matrigel
	SRR20340099	MCF10A	3D	Matrigel
	SRR20340103	MCF10A	2D	/
	SRR20340104	MCF10A	2D	/
	SRR20340105	MCF10A	2D	/

In the analysis, Alginate was used to denote both soft and stiff alginate, and collagen was used for both soft and stiff collagen. In PRJNA633168, different scaffolds were used collectively, denoted as reconstituted basement membrane (rBM). rBM used were laminin/collagen-rich gel and polyacrylamide gel (PA), which was prepared by combining polyacrylamide gel and rBM and laminin.

The RNAseq data used in this chapter were processed and analysed using the previously described method to standardise results for comparison (Zahra *et al.*, 2022). Briefly, TopHat2 (v.2.1.1) was used to align the reads to the reference human genome GRCH38 using Bowtie 2 (v.2.2.6), an ultra-high-throughput short-read aligner. Samtools (v.0.1.19) was used to merge the replicates according to a set criterion, selecting only high-quality mapped reads (<30). Cufflinks (v2.2.1) was used to assemble and quantify the transcripts. Cuffdiffs (v2.2.1) was applied to obtain the differential expression profiles. The false discovery rate (FDR) used for the analysis was set at 0.2. We used the online application OmicsPlayground (version 3.4.4,

BigOmics Analytics, Bellinzona, Switzerland) (Akhmedov *et al.*, 2020) to analyse the transcriptional landscape of breast cells grown in 2D and different 3D systems using scaffolds such as collagen, Matrigel and alginate. The analysis includes differentially expressed genes (DEGs), biomarkers and hallmarks of enrichment.

5.3 RESULTS

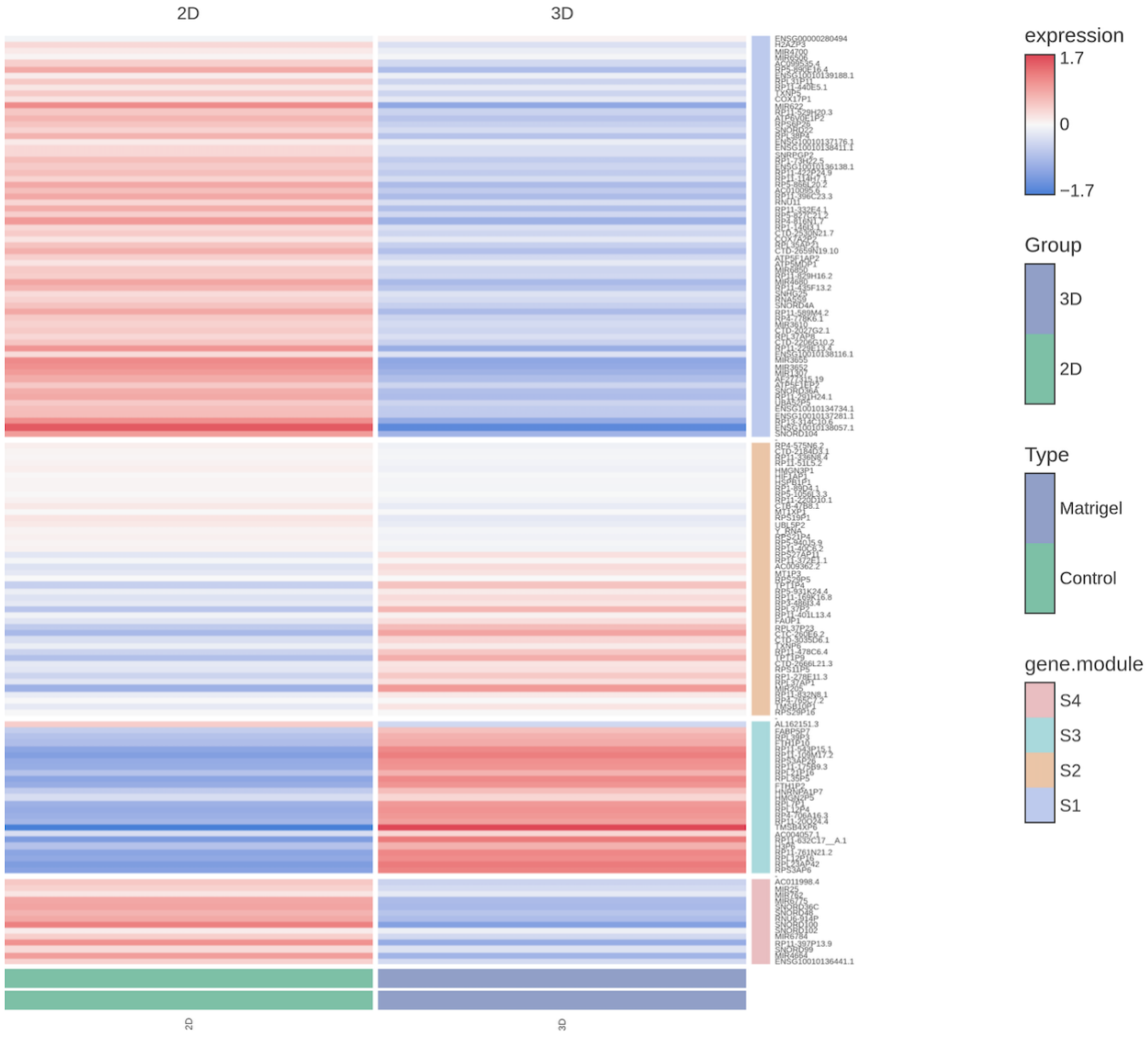
Previous studies have elucidated the differences in the genetic landscape between 2D and 3D cultures (Kerslake *et al.*, 2023; Liu *et al.*, 2022; Arutyunyan *et al.*, 2023). Here, we present our findings regarding these differences and the potential impact of various scaffolds on the genomic and transcriptomic profiles. We also examine pathways affected by differentially expressed genes.

5.3.1 Heat Map of Top 150 Differentially Expressed Genes (DEGs) Identifies Two Different Clusters

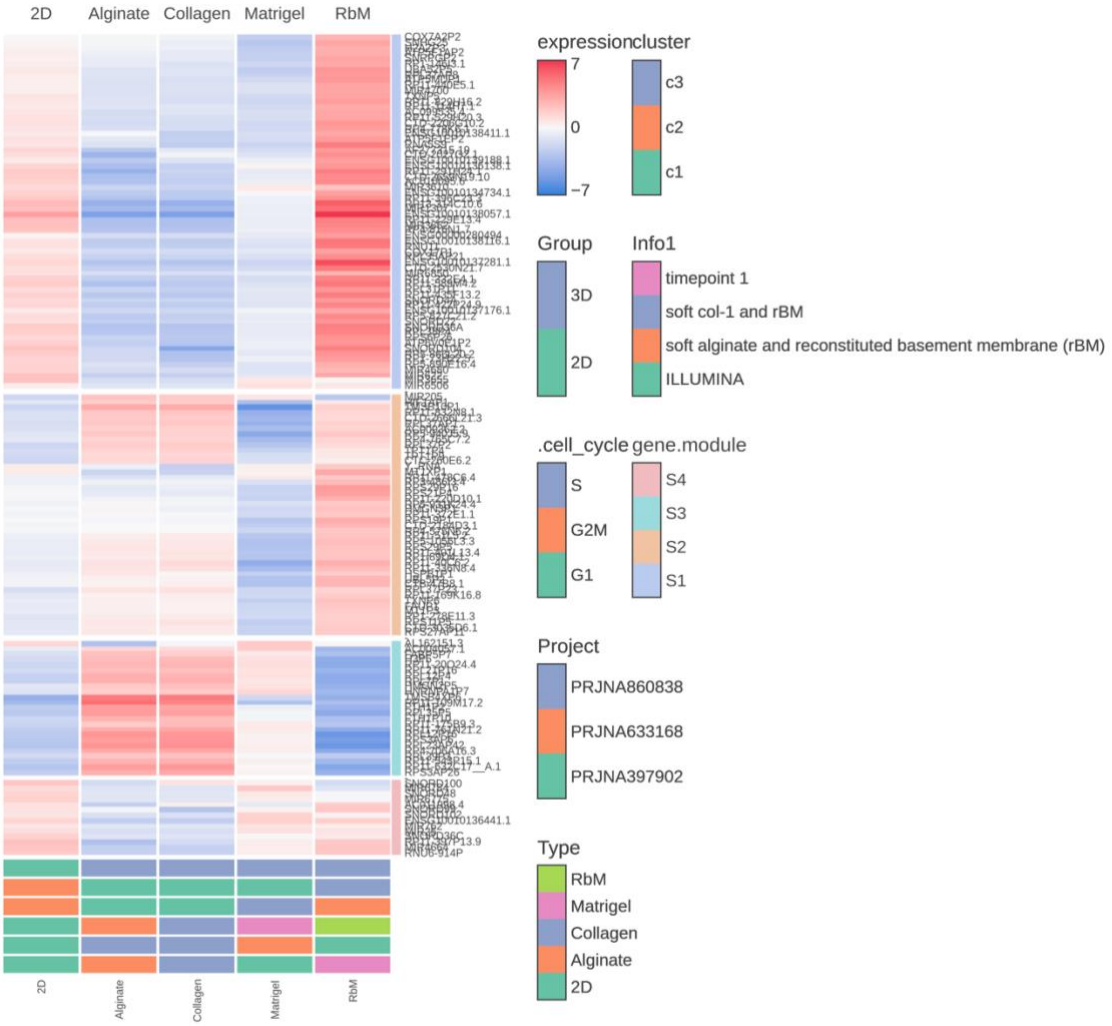
Figure 5.1A is a functional heatmap of the top 150 differentially expressed genes according to their highest standard deviation across 2D and overall 3D cultures. A functional heatmap of the top 150 differentially expressed genes across the scaffold and 2D culture was also performed to understand the expression of genes across various scaffolds (Figure 5.1B). We then used a relative expression scale to perform hierarchical clustering at the gene level. Two clusters were identified, S1-S2 for the two functional heat maps (Figure 5.1C). The pathways were selected based on a number of genes enriched in the particular pathways. The top 150 differentially expressed genes were used as the threshold in this analysis because they account for the top 0.25% of the human genes, ensuring a selective analysis approach and research sensitivity. This was done to find the differences in gene expression between 2D and the overall 3D models, and the impact of the specific scaffolds on gene expression. Functional annotation for each cluster was determined using 42 reference databases, including MsigDB, Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Gene Ontology (GO) (Akhmedov *et al.*, 2020). Some of the key pathways the

genes are involved in include bile metabolism, p53 pathway, IL2_STAT5 signalling, E2F targets and oxidative phosphorylation.

A



B


2D
Alginate
Collagen
Matrigel
RbM
expressioncluster
7
0
−7
c3
c2
c1
Group
3D
2D
Info1
timepoint 1
soft col-1 and rBM
soft alginate and reconstituted basement membrane (rBM)
ILLUMINA
.cell_cycle
S
G2M
G1
gene.module
S4
S3
S2
S1
Project
PRJNA860838
PRJNA633168
PRJNA397902
Type
RbM
Matrigel
Collagen
Alginate
2D

C

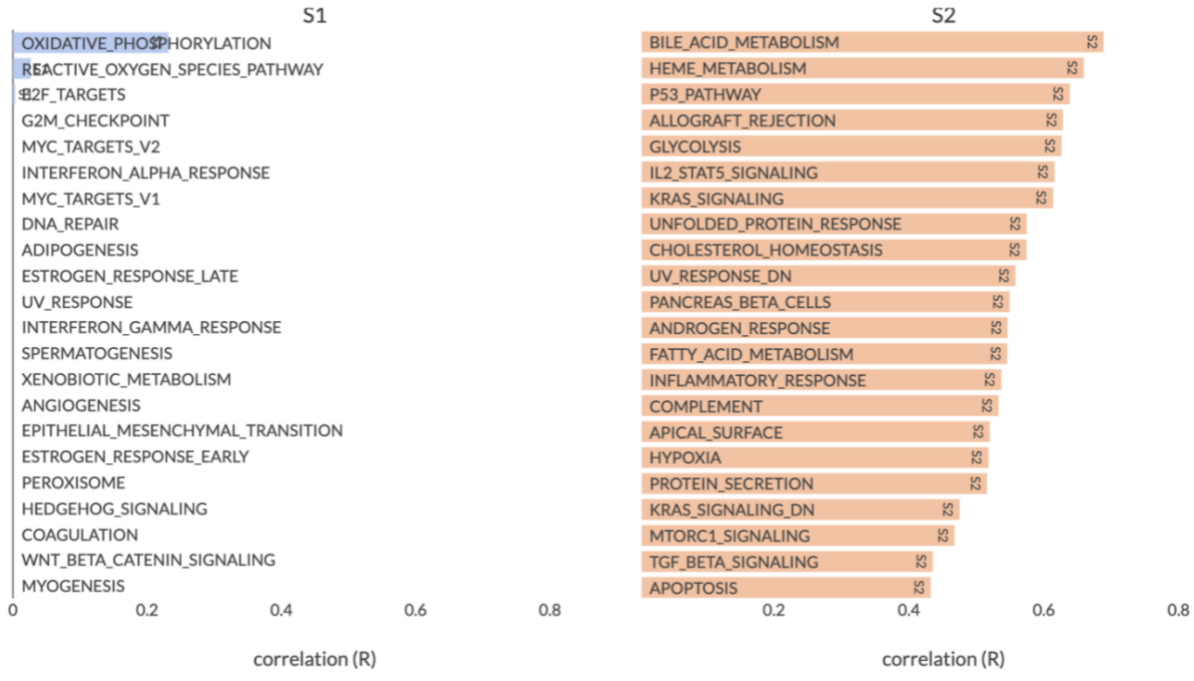

S1
OXIDATIVE_PHOSPHORYLATION
REACTIVE_OXYGEN_SPECIES_PATHWAY
E2F_TARGETS
G2M_CHECKPOINT
MYC_TARGETS_V2
INTERFERON_ALPHA_RESPONSE
MYC_TARGETS_V1
DNA_REPAIR
ADIPOGENESIS
ESTROGEN_RESPONSE_LATE
UV_RESPONSE
INTERFERON_GAMMA_RESPONSE
SPERMATOGENESIS
XENOBIOTIC_METABOLISM
ANGIOGENESIS
EPITHELIAL_MESENCHYMAL_TRANSITION
ESTROGEN_RESPONSE_EARLY
PEROXISOME
HEDGEHOG_SIGNALING
COAGULATION
WNT_BETA_CATENIN_SIGNALING
MYOGENESIS
0
0.2
0.4
0.6
0.8
correlation (R)
S2
BILE_ACID_METABOLISM
HEME_METABOLISM
P53_PATHWAY
ALLOGRAFT_REJECTION
GLYCOLYSIS
IL2_STAT5_SIGNALING
KRAS_SIGNALING
UNFOLDED_PROTEIN_RESPONSE
CHOLESTEROL_HOMEOSTASIS
UV_RESPONSE_DN
PANCREAS_BETA_CELLS
ANDROGEN_RESPONSE
FATTY_ACID_METABOLISM
INFLAMMATORY_RESPONSE
COMPLEMENT
APICAL_SURFACE
HYPOXIA
PROTEIN_SECRETION
KRAS_SIGNALING_DN
MTORC1_SIGNALING
TGF_BETA_SIGNALING
APOPTOSIS
0.2
0.4
0.6
0.8
correlation (R)

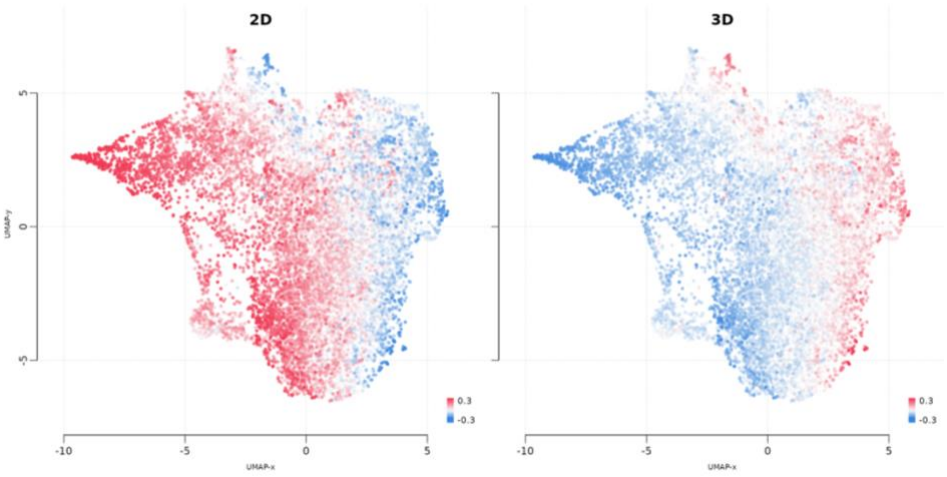
Figure 5.2 Clustered Heatmap and Functional Annotation

*Functional heat map of 150 genes identified using the highest standard deviation across (**A**)2D and 3D models and across the scaffold (**B**). The relative expression scale was utilised for the hierarchical clustering. Red denotes over-expression, and blue denotes under-expression. **C**) Hierarchical clustering identified 2 clusters (S1-S2). The functional annotations were generated through Omics Playground, which uses machine learning algorithms to correlate each gene to more than 42 reference databases, such as KEGG and Gene Ontology.*

5.3.2 Uniform Manifold Approximation and Projection of Geneset Signature of MCF10A cells in 2D and 3D and the different scaffold.

Next, we used Uniform Manifold Approximation and Projection (UMAP), a machine learning tool, to visualise the clustered expression of geneset signature. UMAP of geneset signature of MCF10A cultures in 2D and 3D models (A) showed a complete contrast between the models. This contrast is important as it highlights the differences in genes that are down-regulated (blue) and up-regulated (red) in 2D in relation to 3D model and vice versa. Secondly, the UMAP of the geneset signature of MCF10A on the various scaffolds (**B**) also showed differences in the expression. There were also differences in the expression when the expression on the scaffold was compared to the 2D.

A



B

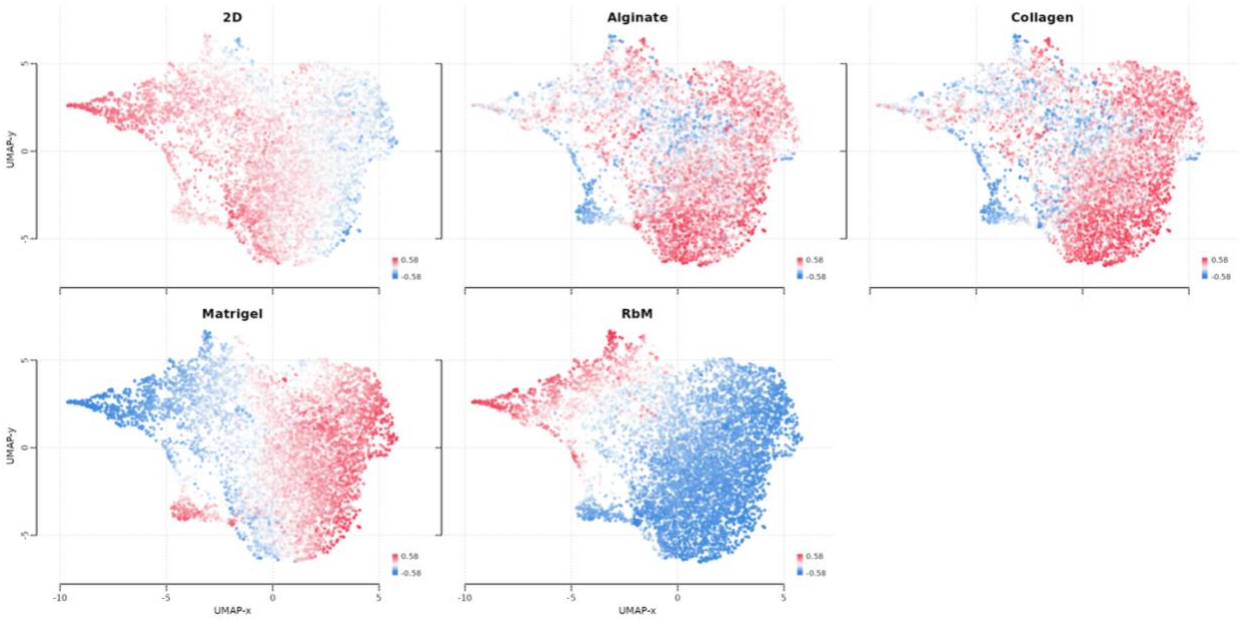


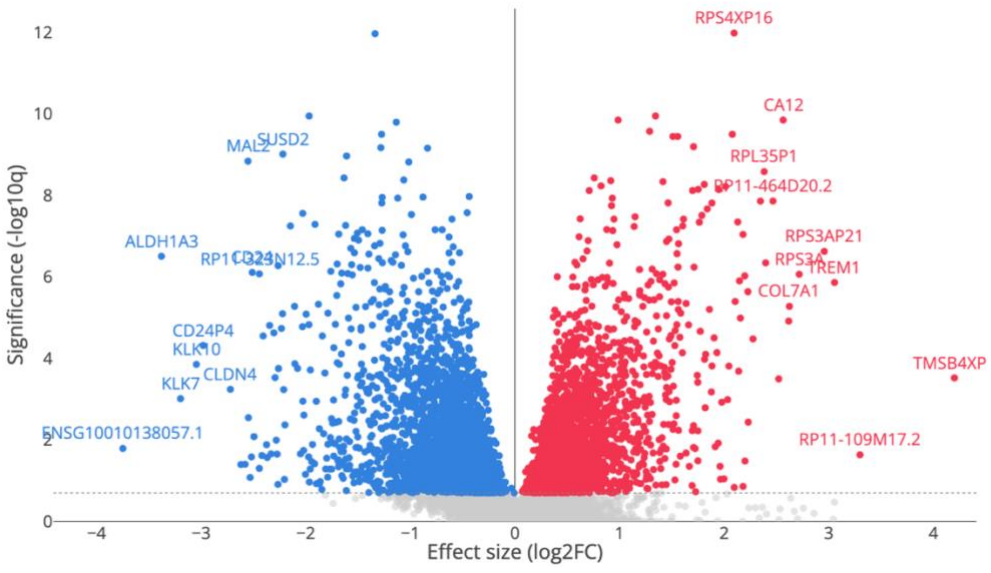
Figure 5.3 Geneset Signatures Uniform Manifold Approximation and Projection Displaying Up and Down-Regulated Genes in 2D and 3D Models and the various scaffolds.

The UMAPs above show genes clustered by relative log-expression, which is up (red) or down (blue) regulated in (A) 2D and 3D models (B) Alginate, Collagen, Matrigel and Reconstituted Basement membrane (RbM). These graphs were made using Omics Playground.

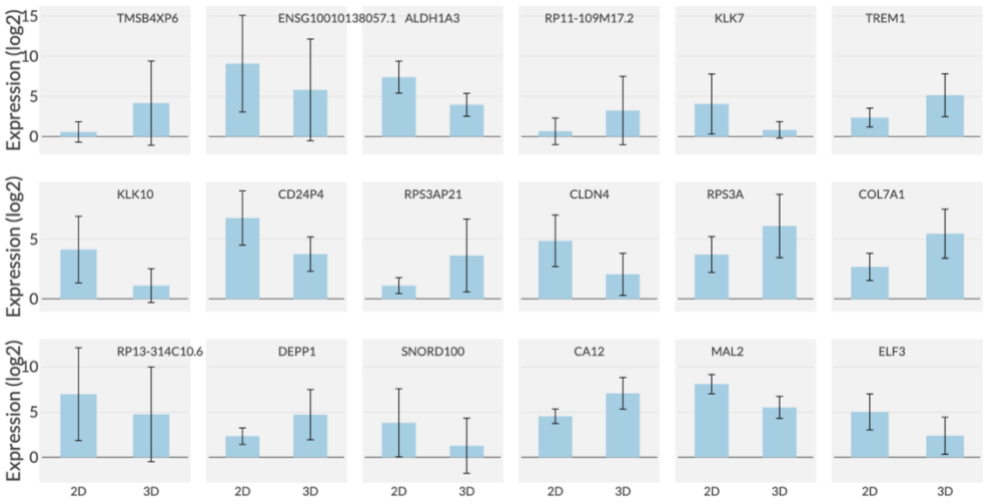
5.4.3 Differential Expression

Volcano plot shows all the differentially expressed genes between 2D and 3D data. Up-regulated genes are shown in red, and down-regulated genes are in blue. The most up-regulated gene is *TMSB4XP6,* and the most down-regulated gene is *KLK7,* shown in Figure 5.3A. Figure 5.3B presents a panel showing the top 16 differentially expressed genes (up and down-regulated). We also looked at differential gene expression between 2D and Matrigel. We chose Matrigel in this comparison because that is the scaffold we used in our experiment in Chapter Three. A volcano plot showing up and down-regulated genes is presented in Figure 5.3C. A panel of 16 of the top differentially expressed genes is also shown in Figure 5.3 D. Some genes significantly up-regulated in Matrigel include *COL7A1, GPNM, CA12* and *CD36*. When comparing the DEGs of all the scaffolds were compared to 2D cultures, 2339 genes were found to be common among them, as shown in Figure 5.3E. Some of the common up-regulated genes in all the scaffolds include *TMSB4XP6, TREM1, FTH1P20, DEPP1* and *CA12*. The common down-regulated genes include *ALDH1A3, KLK10, CD24P4, MAL2, CD24* and *KLF1.*

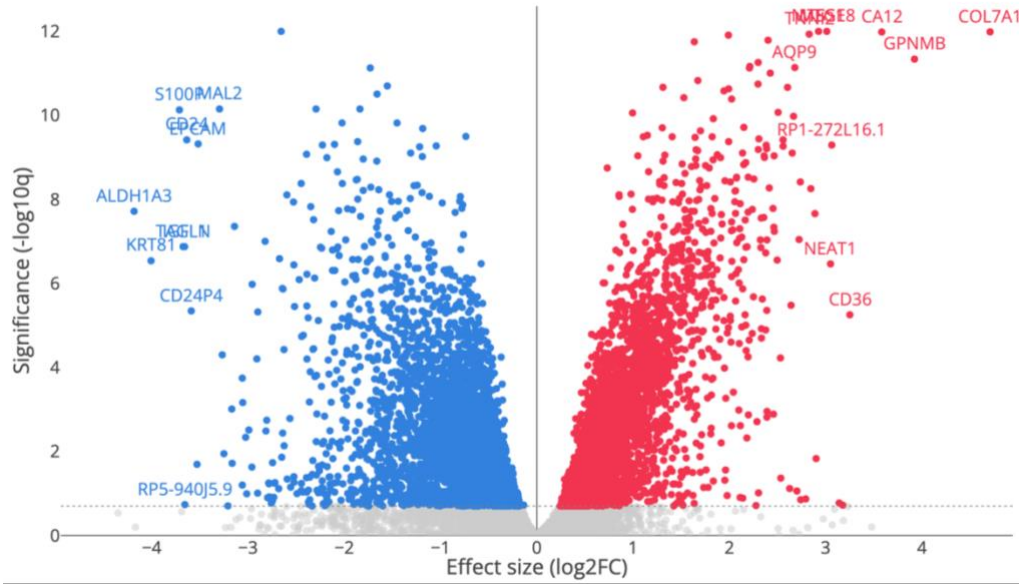
A



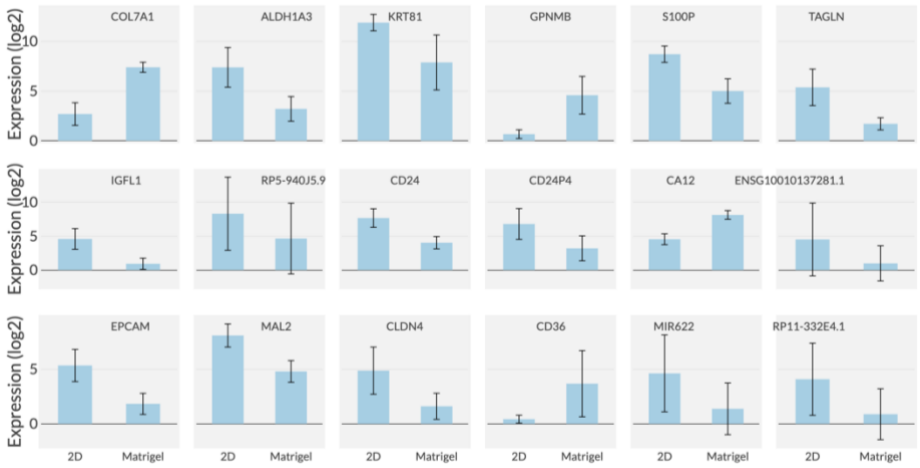
B


TMSB4XP6
ENSG10010138057.1
ALDH1A3
RP11-109M17.2
KLK7
TREM1
KLK10
CD24P4
RPS3AP21
CLDN4
RPS3A
COL7A1
RP13-314C10.6
DEPP1
SNORD100
CA12
MAL2
ELF3
Expression (log2)
2D
3D

C


COL7A1
CA12
GPNMB
AQP9
RP1-272L16.1
NEAT1
CD36
MAL2
CD24
EPCAM
ALDH1A3
KRT81
CD24P4
RPS-940J5.9
Significance (-log10q)
Effect size (log2FC)

D



E

A = Alginate_vs_2D
B = Matrigel_vs_2D
C = Collagen_vs_2D

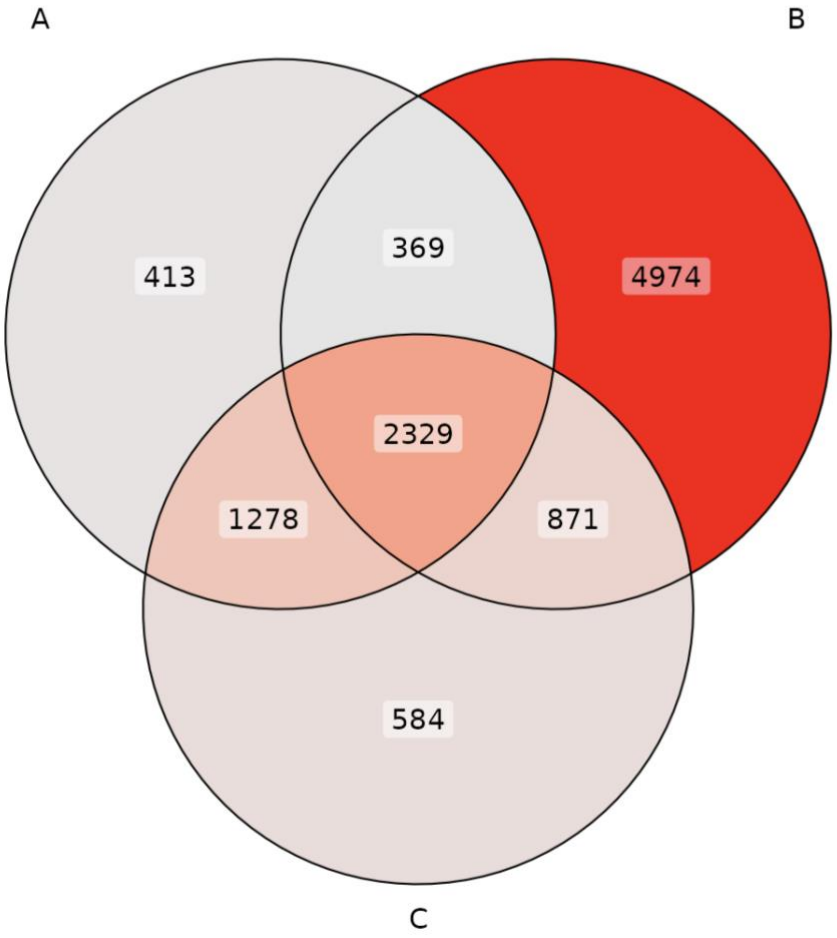


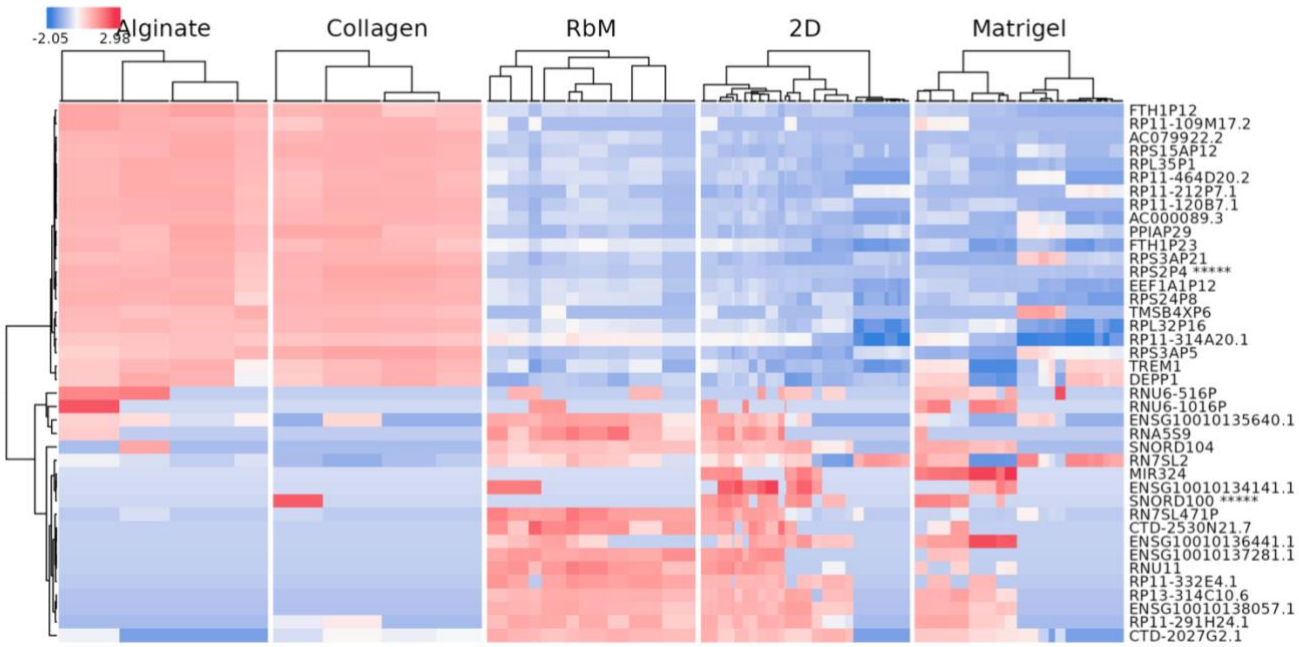
Figure 5.4 Differentially Expressed Genes

Differentially expressed genes (DEGs) in MCF10A cells grown in 3D in comparison to 2D. ***A****) Volcano plot showing DEGs between 2D and 3D* ***B****) Top 18 DEGs expressed between 2D and 3D* ***C)*** *Volcano plot showing DEGs in Matrigel vs 2D* ***D****) Top 18 genes differentially expressed in Matrigel compared to 2D* ***E)*** *Venn diagram showing common genes expressed among when compared to 2D cultures, Alginate (A), Matrigel (B), and Collagen (C). These graphs were produced using Omics Playground.*

5.3.4 Scaffold-Specific Biomarkers – 3D Scaffolds vs 2D

We also explored the transcriptional landscape to determine potential biomarkers of the different scaffolds and 2D cultures (Figure 5). We used machine learning algorithms such as Sparse Partial Least Square (sPLS), Glmnet, and Random Forest (RF) implemented in OmicsPlayground to compute a cumulative importance score for all differentially expressed genes (DEGs). Figure 5.4A is a heat map showing the differences in the expression of biomarkers between scaffolds and 2D culture. Figure 5.4B is a box plot showing eight key genes that can be used as biomarkers in scaffolds. We found four biomarkers that were specific to Alginate and Collagen, namely *FTH1P12, FTH1P3*, and *RP11.109M17.2*. Interestingly, *SNORD100, MIR147B* and *KLK7* were down-regulated in all the scaffolds compared to 2D and, therefore, can be used as specific biomarkers.

A



B

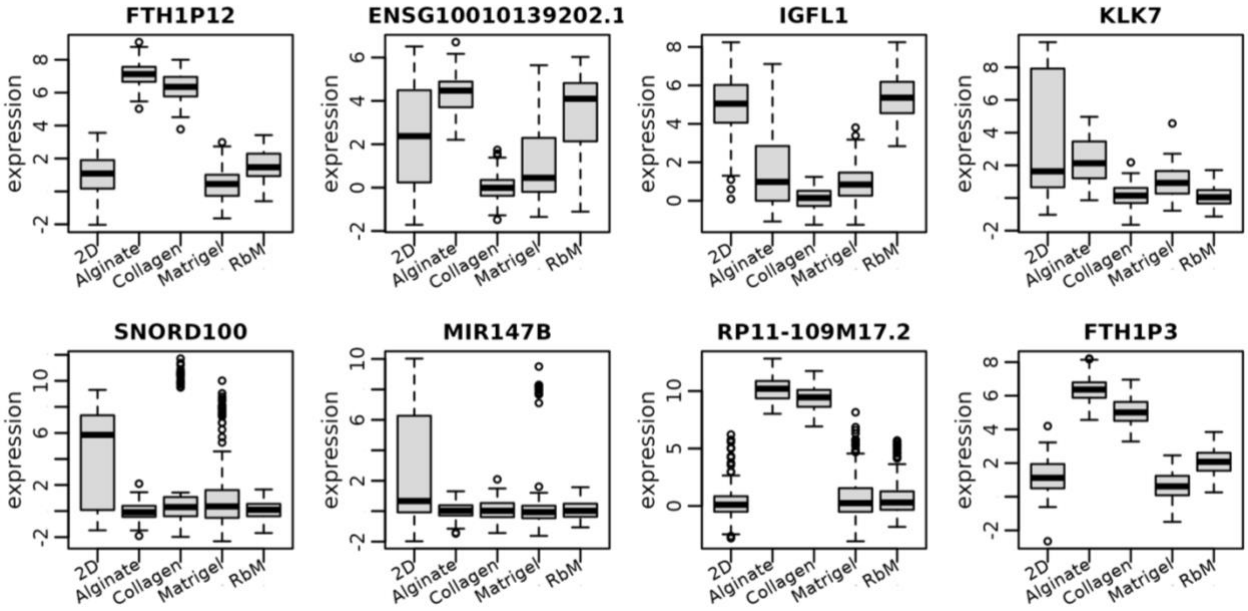


Figure 5.5 Biomarkers expressed in 2D and 3D models and scaffold-specific biomarkers

A) *Biomarker heat map between the different scaffolds and 2D* ***B****) Top 8 key genes associated with the different scaffolds and 2D that can be used as biomarkers. These were generated using Omics Playground, where cumulative importance scores were calculated using multiple machine learning algorithms such as Glmnet, Random Forest and sPLS.*

5.4 Discussion

Breast cancer is the most prevalent and deadly female cancer. Therefore, it is important to develop appropriate models to study the biological mechanisms that drive disease development, proliferation, invasion, and responses to therapeutic agents. Over the years, the study of cancer biology has transitioned from simple monolayer and monoculture to 3D *in vitro* methods that can recapitulate in vivo conditions and the surrounding ECM. In this study, we examined the effect of different scaffolds on the transcriptional landscape of the breast epithelial cell line, MCF10A, including the difference between 3D cultures and the traditional 2D models. Using the Sequence Read Achieves (SRAs) data from the six selected studies, we computed the expression profiles of the genes in 2D and 3D systems. Visual representation of the change in expression using UMAP showed significant differences in the transcriptomic profile potentially related to the differences in growth environment and variation in scaffold.

Functional enrichment of the top 150 differentially expressed genes (DEGs) between 2D and 3D and the specific scaffolds revealed biological processes, including the P53 pathway, glycolysis, KRAS signalling, oxidative phosphorylation and heme metabolism. These biological pathways are involved in normal cellular functions such as cell proliferation, apoptosis, differentiation, glucose transport, energy production, and the production of biomolecules (Simon-Molas *et al.*, 2016; Zhang *et al.*, 2013; Feng *et al.*, 2017; Castruccio Castracani *et al.*, 2020). Specifically, Wnt/β-catenin-mediated inhibition of p53 in hepatocyte cells, which is essential for proliferation, has been suitably modelled in a 3D system (Oliva-Vilarnau *et al.*, 2020). These pathways have also been reported to be involved in the initiation and progression of cancer (Patankar *et al.*, 2019; Ikari *et al.*, 2021; Tidwell *et al.*, 2022). Specifically, glycolytic

proteins are up-regulated in colorectal cancer cell line SW480, which recapitulates more suitably what happens in vivo than in 2D cultures (Kim *et al.*, 2018). Tidwell and colleagues showed that glycolysis and oxidative phosphorylation during glucose metabolism in 3D spheroids of colorectal and pancreatic cancer cells differ significantly from 2D cultures, suggesting the suitability of 3D models in studying tumour metabolism (Tidwell *et al.*, 2022).

We examined the differentially expressed genes between the overall 2D and 3D data. We also chose to compare the DEGs expressed in Matrigel with the 2D results. Matrigel was chosen for more in-depth analysis because it was the scaffold we used in this thesis (see Chapter Three), and we would like to have a better understanding of how the scaffold impacts cellular function and behaviour in comparison with traditional monolayer cultures. We highlight some important genes differentially up-regulated in 3D compared to 2D, including *COL7A1, CA12, TREM1* and *TMSB4XP6*. *COL7A1* is expressed in the ECM and essential for anchoring and attachment of cells (Bornert *et al.*, 2016). *CA12* is expressed in the testis, prostate, kidney, colon, lungs, pancreas and lungs (Kallio *et al.*, 2010). It plays an essential role in the regulation of pH and the homeostasis of bicarbonate and carbon dioxides in tissues such as epithelial cells and cardiac muscles (Lee *et al.*, 2016). In breast cancer, CA12 is regulated by ERα and interacts with TFF3 to decrease the efficacy of neoadjuvant chemotherapy (Shen *et al.*, 2022). When Matrigel data was compared to 2D data, we highlighted the up-regulation of *GPNMB* and *CD36*. GPNMB is expressed in tissues such as the skin, brain, breasts, muscle, and bone. It plays critical roles in cellular functions such as cell adhesion, stress response, and stem cell maintenance (Biswas *et al.*, 2020). Suda and colleagues reported that *GPNMB* prolonged the lifespan of senescent endothelial cells in both in vivo and in vitro conditions (Suda *et al.*, 2022).

CD36 is a receptor for multiple ligands such as lipids, phospholipids and low-density lipoproteins (Liang *et al.*, 2018). GPNMB is expressed in breast cancer, promotes tumour growth and has been identified as a biomarker for poor prognosis (Rose *et al.*, 2010; Liguori *et al.*, 2021). CD36 has been extensively studied for its role in proliferation, migration and mediating resistance to chemotherapeutic agents in breast cancer cell lines (Ligorio *et al.*, 2022; Liang *et al.*, 2018).

We examined the biomarkers expressed in the various scaffolds used to culture the MCF10A cell line. In the scaffold-specific biomarkers, alginate and collagen had similar genetic biomarkers. These include *FTH1P12, FTH1P3*, and *RP11-109M17.2*. *MIR147B, KLK7*, and *SNORD100* were down-regulated in all the scaffolds when compared to 2D culture. The genes were selected using the importance score ranking by OmicsPlayground (Akhmedov *et al.*, 2020). *FTH1P12* and *FTH1P3* are noncoding RNAs (lncRNAs) belonging to the ferritin heavy chain (FHC) family (Darvish, 2024). Their roles in normal cellular function are yet to be fully elucidated. In cancer cells, *FTH1P3* has been shown to increase proliferation in cervical cancer cells via the activation of microRNA-145 (Lv and Zhang, 2020). *KLK7* is mostly expressed in squamous epithelial cells and is crucial In maintaining skin homeostasis (Kind *et al.*, 2024). KLK is expressed in breast cancer tissues and has been found to be a prognostic marker for TNBC (Geng *et al.*, 2020). Further work will be needed to define the functions of these biomarkers in normal cell function and breast cancer.

In conclusion, this meta-analysis examined the transcriptional landscape of MCF10A cells in 3D and 2D models in the literature. We showed the variability of gene biomarkers in specific scaffolds, suggesting that the scaffold of choice in an experiment affect the genetic profile the cells. Our study also showed the up-regulation of genes, biomarkers, and pathways involved in normal cell functions and cancer

development, suggesting the suitability of 3D models to study these phenomena. While the role of some genes has been elucidated, further study is needed on some of them.

CHAPTER SIX: GENERAL DISCUSSION

6 GENERAL DISCUSSION

6.1 Introduction

Breast cancer incidence and mortality are set to increase to an estimated 3 million and 1 million by 2040, respectively (Arnold *et al.*, 2022). The percentage of breast cancer incidence attributed to genetic factors is estimated to be between 5%-10% (Nolan, Lindeman and Visvader, 2023). Lifestyle factors such as smoking (Jones *et al.*, 2017; Del Riccio *et al.*, 2023), BMI (Von Holle *et al.*, 2024; Noureen *et al.*, 2023), physical activities (Dixon-Suen *et al.*, 2022; Hermelink *et al.*, 2022) and alcohol (Jordahl *et al.*, 2022) have been studied for the impact on breast cancer risk. However, little is known about exposure to exogenous chemicals, such as mixtures of endocrine-disrupting chemicals which are ubiquitously present in human tissues and how they interact with other risk factors, such as food and genetic mutations.

It is widely accepted that humans are exposed to a myriad of endocrine-disrupting chemicals (Varticovski *et al.*, 2022). It has been shown that these estrogen-that mimic compounds that can act together to produce an effect (Silva, Rajapakse and Kortenkamp, 2002). EDCs have been implicated in the development of many diseases, including breast cancer (Wan, Co and El-Nezami, 2022; Bimonte *et al.*, 2024). However, most of the studies on the effect of EDCs on breast cancer initiation have been done with individual chemicals. We present data on the effect of a mixture of 12 EDCs on markers of breast cancer initiation and progression. The effect of diet on breast cancer depends on the type of food consumed. While consumption of red and processed meat, sugary food, and eggs positively correlates to increased breast cancer risk, consumption of vegetables, citrus fruits and nutrients such as calcium, vitamin D, and folate decreases breast cancer risk (Buja *et al.*, 2020). The effect of fatty acids on breast cancer has, however, been inconclusive (Yang *et al.*, 2014; Zhou

et al., 2016; Mourouti *et al.*, 2015). The effect of mixtures of fatty acids and how they interact with other risk factors, such as endocrine-disrupting chemicals, has not been studied. Loss of BRCA1 makes breast cancer cells sensitive to endocrine disruption activity (Jones *et al.*, 2010). The interaction between mixtures of EDCs and fatty acids on BRCA1 mutated cells will give us an insight into the multifactorial impact of breast cancer. The aim of this thesis was to address the crucial gaps in our knowledge of our lifestyle factors, such as exposure to EDCs and consumption of a high-fat diet, which interact with factors such as BRCA1 mutation and the overall effect on breast cancer risk. We also determine the differences in genomic and transcriptomic landscape between 2D and 3D cultures of MCF10A cells. In this final chapter, we highlight the main findings of the set of experiments already discussed in this thesis and make recommendations for future works to further expand on these results.

6.1.1 Multifactorial Impacts On Breast Cancer Initiation

As stated earlier, it is established that genetic mutations increase the risk of breast cancer. However, our knowledge of how this risk is modulated by other factors that have been hypothesised to increase breast cancer risk is limited. We aimed to study a scenario where risk factors are increasingly more complex. Our multifactorial approach involved silencing BRCA1 in MCF12A cells (as a proxy for BRCA1 mutation) and exposing those cells to EDC mixture, FA mixture and all three factors combined. We also studied the impact of these realistic mixtures on acinar morphology and gene expression. This work will add to the body of knowledge regarding the interplay of different breast cancer risk and how their interaction increases the overall breast cancer risk.

6.3 Main Findings

The work in this thesis has significantly contributed to our understanding of how a mixture of EDC and Fatty Acids contributes to breast cancer risk in cell culture models that recapitulate the human breast architecture. We also present data on multi-factorial effects on breast cancer.

6.3.1 Mixtures of EDCs and Fatty Acids induce changes in acini morphology

In Chapter Two, we showed that the batch of MCF-12A cell lines used in this thesis were both ER and GPER competent and could represent a non-tumourigenic and hormonally responsive human mammary epithelial cell line suitable for the aims and objectives of this study. We also established that our mixture of fatty acids could cross the cell membrane of the MCF-12A cells and induce effects. Chapter Three used the MCF-12A cells in a 3D culture, which enabled the development of acini-like structures that recapitulated the structures in the human breast. We were able to show that a mixture of EDCs and fatty acids disrupted acini development. Marchese and Silva demonstrated that E2, BPA and propylparaben disrupt the acini development of MCF-12A cells in 3D (Marchese, 2013). We argue that our mixture of EDCs disrupted acini using the same mechanism. We presented data for the first time that a mixture of fatty acids disrupted the formation of acini in MCF-12A cells.

We tested 12 ubiquitous EDCs (BDE 100, propylparaben, methylparaben, ethylparaben, butylparaben, Tonalide, Bisphenol A, 3-benzylidene camphor, Galaxolide, Benzophenone-2, Benzophenone-3, and *p,p'*-DDT) and four fatty acids (Elaidic acid, linoleic acid, palmitic acid and stearic acid) at tissue concentrations and observed how the affected acini development. Changes in parameters, such as

increases in area and decreases in circularity of the acini, are markers of early breast carcinogenesis. Mechanistically, we suggest that the changes we observed in acini development were caused by the activation of the PI3K/Akt cascade (Guo *et al.*, 2006; Chen, Chien and Lee, 2023; Pesiri *et al.*, 2014; Jackson *et al.*, 2022). Analysis of RNA sequencing data showed that the mixtures of EDCs and fatty acids up-regulated genes and pathways implicated in breast cancer. FunRich analysis revealed the enrichment of biological and molecular pathways implicated in breast cancer.

Overall, we report findings that mixtures of endocrine disruptors can induce morphological and genetic alteration in acini formation. These morphological changes are suggestive of neoplastic transformation, supporting the claims these environmental chemicals possess carcinogenic properties and exposure could increase breast cancer risk. The significant increase in acini size and decreased circularity of the combined mixture of EDCs and fatty acids, when compared to the individual mixtures, showed a possible combined effect of the two mixtures. We further suggest that a high bio-burden of EDC and consumption of a high-fat diet could potentially increase a person's breast cancer risk.

6.3.2 Mixtures of EDCs and Fatty Acids up-regulate cancer genes and pathways in BRCA1-deficient MCF-12A cells.

In our quest to elucidate the multifactorial effect of breast cancer, we examined the effect of mixtures of EDCs and fatty acids on BRCA1-deficient MCF-12A cells. Mutation in the BRCA1 gene is one of the most studied genetic risk factors for breast cancer. By the age of 70 years, the BRCA1 mutation confers a 47-66% chance of developing breast cancer in a woman (Krishnan, Patel and Hakem, 2021). Any external factors that can impact this high-risk population can result in a significant

increase in absolute breast cancer risk for individuals (Milne and Antoniou, 2016). For example, Jones and colleagues reported that loss of BRCA1 activity made the breast cancer cell line (MCF-7) susceptible to the bisphenol A effect. This effect was also seen in mice (Jones *et al.*, 2010). However, no work has been done on the impact of a mixture of endocrine disruptors and fatty acids will have a non-tumorigenic cell line like MCF-12A.

Our results showed that the siRNA used in our study could down-regulate the BRCA1 gene. This was an important step, as the chapter aimed to examine the effect of mixtures of EDCs and fatty acids on BRCA1-deficient MCF12A cells. Our results showed that the mixtures of up-regulated genes such as *ARMC6, NUF2, KLHL22, SCML1, MUC12-AS1* and PTPRN2 are implicated in the initiation and progression of cancers such as breast cancer (Xu *et al.*, 2019; Adámik *et al.*, 2024; Sorokin *et al.*, 2015; Sengelaub *et al.*, 2016). Pathways such as mTORC1 and glycolysis, which are involved in carcinogenesis after BRCA1 silencing, were up-regulated (Joyce *et al.*, 2024; Martinez-Outschoorn *et al.*, 2012; Xiang *et al.*, 2011). Overall, we reported that there is a possible interaction between BRCA1 mutation, exposure to mixtures of EDCs and consumption of a fatty acid diet on breast cancer risk.

6.3.3 A comparison of MCF10A cells grown on 2D and 3D cell culture models shows the up-regulation of genes and pathways involved in normal cell functions

Due to the limitations of the 2D cell culture model, 3D was developed to better imitate in vivo conditions. Consequently, there are differences in morphology and gene expression when cells are grown in 2D compared to 3D. For example, when MCF10A cells are grown in 2D cultures, they spread to form a cuboidal epithelial morphology

(Qu *et al.*, 2015) ; however, in 3D cultures, they organise into acini, which mimics in vivo mammary acini (Debnath, Muthuswamy and Brugge, 2003; Marchese and Silva, 2012).

In Chapter Five, the initial objective was to conduct a meta-analysis of published data on MCF-12A in 2D and 3D. However, due to the paucity of published data, we used MCF10A, another human epithelial non-tumorigenic breast cell line, as a surrogate. We wanted to examine the genetic profile of MCF10A when cultured on 2D and 3D models.

The analysis showed up-regulation of genes such as COL7A1 and CA12, which are involved in normal cellular functions (Kallio *et al.*, 2010; Lee *et al.*, 2016; Bornert *et al.*, 2016). We compared the gene profile of MCF10A cultured in 2D to that of MCF10A cultured on Matrigel, the scaffold used in this thesis. Regulatory genes such as CD36 and GPNMB were up-regulated in Matrigel compared to 2D (Biswas *et al.*, 2020; Suda *et al.*, 2022; Liang *et al.*, 2018). We also saw the up-regulation of pathways involved in cellular functions such as cell proliferation, apoptosis, differentiation, glucose transport, energy production, and the production of biomolecules (Zhang *et al.*, 2013; Simon-Molas *et al.*, 2016; Feng *et al.*, 2017) . This also shows the appropriateness of 3D cell culture in modelling these biological processes and producing results that are more likely to recapitulate in vivo conditions than 2D.

6.4 Future Works

This current work has increased our knowledge and understanding of breast risk due to exposure to mixtures of EDC fatty acids. These experiments have also highlighted areas for possible future research that would help us understand how these risk factors interact to increase breast cancer risk.

6.4.1 Effect of EDC and fatty acid mixtures on MCF-12A using a microfluidic device.

Static 3D models have been invaluable in understanding various biological processes. However, the lack of dynamic flow of cell culture media means static 3D cell cultures cannot fully recapitulate the *in vivo* environment. An organ-on-a-chip device facilitates a constant flow of cell culture medium and nutrients around the 3D cultures, better representing what happens *in vivo*. The organ-on-chip system will allow different cells to be cultured, thereby facilitating cell interactions as they occur in the *in vivo* environment. To further examine the effect of a mixture of EDCs and fatty acids on breast cancer initiation and progression, a breast-on-a-chip model will present a more realistic view of breast tissues and their behaviour when exposed to the test chemicals.

6.4.2 Primary BRCA1-deficient exposure to mixtures

In Chapter Four, we used silenced BRCA1 in MCF-12A cells and exposed them to a mixture of EDCs and fatty acids. This work can be extended to BRCA1-silenced primary cells, which will provide further insight into our understanding of the increase in risk when cells are exposed to the mixtures. While immortalised cells have been extensively used in the study of cancer biology, primary cells are more representative of human tissue (Neimark, 2015). Therefore, expanding this work by using primary cells will produce results which are relevant to humans.

6.5 Conclusion

In conclusion, this thesis answered the objectives set out in Chapter One and made critical contributions to the body of current knowledge on how mixtures of EDCs and

fatty acids may cause changes that indicate early breast carcinogenesis. We showed through different endpoints that mixtures of EDCs and fatty acids could disrupt the normal cellular, biological and molecular functions that are implicated in breast cancer initiation and progression. This alteration occurred both individually and in combination, further giving support to the knowledge that different factors can interact to potentially increase breast cancer risk. We also showed using MCF-12A cells that the lack of the *BRCA1* gene causes the up-regulation of Various genes and pathways implicated in breast cancer, showing the risks involved when BRCA1 mutation careers are exposed to mixtures of EDC and consume a high-fat diet. Lastly, we found the suitability of the 3D model to appropriately model various processes and pathways involved in normal breast cell functions. Further research is, however, needed to understand the multifactorial effects of breast cancer fully.

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