Chemoresistance and Fatty Acids: Intricate Interplay Between Breast Tumour Cells and Adipocytes in the Tumour Microenvironment

By

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DECLARATION

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ABSTRACT

Background: Globally an increase in obesity incidence represent a major health concern amongst a rising incidence of impaired treatment outcomes of breast cancer patients. Adipose tissue and/or adipocytes, in the tumour microenvironment serve as an exogenous energy source for the survival of breast cancer cells, especially since adipose tissue is abundant in breast tissue. Breast cancer cells modulate lipid metabolism *(de novo* fatty acid synthesis and lipolysis), by altering the secretion of adipokines through adipocytes, resulting in the release of free fatty acids to provide energy substrates for breast cancer cells to sustain its high proliferation demand for survival. Evidence on the role of obesity and lipid metabolism especially fatty acids in breast cancer treatment resistance is lacking. This motivates investigation to identify and understand the underlying physiological and molecular mechanisms by which chemotherapeutic treatment resistance is achieved. We therefore hypothesise that obesity-induced inflammation alters lipid metabolism in adipose tissue/adipocytes and contribute to the development of doxorubicin chemotherapeutic treatment resistance in breast cancer cells.

Methods: A diet induced obesity animal model was established by feeding female C57BL6 mice a high fat diet for 12 weeks. After developing the diet induced obesity phenotype, breast tumour xenographs were induced by subcutaneous inoculation in the fourth mammary gland with E0771 triple negative breast cancer cells.Once tumours became palpable, mice received either vehicle treatment (Hanks balance salt solution) or doxorubicin treatment (cumulative dose of 12 mg/kg). Plasma inflammatory markers, fatty acid profiles and protein expression of lipid metabolism markers (*de novo* fatty acid synthesis and lipolysis) was determined in mammary adipose and tumour tissue. To validate the *in vivo* model findings, we developed an *in vitro* model using a conditioned media approach. A human adipose tissue derived stem cell line was used for the differentiation of mature adipocytes after which conditioned media was collected to assess the paracrine effect between adipocytes and doxorubicin treated MDA-MB-231 triple negative breast cancer cells. Cell viability was assessed with WST-1 assays. Western blots were used to determined alterations in protein expression of apoptotic and lipid metabolism markers (*de novo* fatty acid synthesis and lipolysis). An Inflammatory marker as well as free fatty acid profile was also analysed in treatment conditioned media.

Results: Diet induced obesity significantly increased tumour growth and decreased doxorubicin treatment efficacy in E0771 triple negative breast tumours ($p<0.0001$), resulting in treatment resistance. Our findings also showed that diet induced obesity supressed *de novo* fatty acid synthesis (decreased SCD-1) and lipolysis (decreased HSL) in mammary adipose tissue of doxorubicin treated mice. Conversely an increase in *de novo* fatty acid synthesis (increased SCD-1) and lipolysis (increased ATGL) was found in tumour tissue, leading to significant changes in FAs composition of both tissues. Diet induced obesity also significantly increased plasma leptin ($p=0.025$) and resistin levels ($p=0.046$) and increased NFκB protein expression in mammary fat of doxorubicin treated mice, thereby inducing systemic and local inflammation. Furthermore, we also report that adipocytes promoted acquired breast cancer treatment resistance by significantly increasing the cell viability of doxorubicin treated MDA-MB-231 triple negative breast cancer cells (Dox+CM *vs* Dox, $p=<0.0001$). This was achieved by attenuating doxorubicin's efficacy to induce apoptosis (decreased cleaved-caspase-3, $p<0.05$), in a paracrine manner. Adipocytes also induced inflammation (increased leptin and MCP-1) as well as lipolysis (increased HSL) in doxorubicin treated breast cancer cells (Dox *vs* Dox+CM $p=0.03$), thereby altering the free fatty acid profile of breast cancer cells.

Conclusion: Our data suggest that adipose tissue/adipocytes significantly contribute to treatment resistance in triple negative breast cancer cells. We have demonstrated in both *in vivo* and *in vitro* models that adipose tissue/adipocytes secretory factors induce inflammation in the breast tumour microenvironment, which leads to the induction of lipolysis in triple negative breast cancer cells. This resulted in altered metabolic behaviour i.e. increased free fatty acid utilization, which can be utilized as energy substrates or induce lipid saturation in order confer to acquired treatment resistance by evading apoptosis We propose that this could be a novel mechanism by which adipose tissue/adipocytes within the tumour microenvironment can contribute to the development of breast cancer treatment resistance under obesogenic conditions. This study also significantly contributed to the identification and understanding of molecular mechanisms underlying breast cancer treatment resistance in obese patients.

OPSOMMING

Agtergrond: Wêreldwyd verteenwoordig die toenemende insidensie van vetsugtigheid ‘n groot gesondheids risikoen beperkook behandelings uitkomste van borskankerpasiënte. Vetweefsel, en/of adiposiete in die tumormikro-omgewing dien as eksogene energiebron vir die oorlewing van borskankerselle, veral omrede daar ‘n oorvloed van vetweefsel in borsweefsel voorkom. Borskankerselle moduleer lipiedmetabolisme *(de novo* vetsuur sintese en lipolise), deur die wysiging van adipokiensekresie *via* adiposiete wat vryvetsure lewer om as energiesubstraat vir borskankerselle dien om sodoende die hoë proliferasie tempo van hierdie selle vir oorlewing te handhaaf. Daar is min inligting oor die rol van lipiedmetabolisme, spesifieke verwysing na vryvetsure in borskanker-behandelingsweerstandigheid. Hierdie feit dien as motivering om die onderliggende fisiologiese en molekulêre meganismes te identifiseer en om die rol van vetsure in chemoterapeutiese behandelingsweerstandigheid te ondersoek. Ons hipotese is dus dat vetsug-geïnduseerde inflammasie, lipiedmetabolisme in vetweefsel/adiposiete wysig en ‘n bydrae lewer tot die ontwikkeling van doksorubisien chemoterapeutieseweerstand in borskankerselle.

Metodes: ‘n Dieetgeïnduseerde vetsug-diermodel is gevestig deur vroulike C57BL6 muise met ‘n hoë vet dieet vir 12 weke te voer. Nadat ‘n dieetgeïnduseerde vetsug fenotipe ontwikkel is, is hererologe borskanker gewasse geïnduseer deur middel van ‘n subkutane inokulering in die vierde borsklier met E0771 trippel negatiewe borskankerselle.Nadat die gewassesigbaar begin word het, het die muise òf ‘n draerbehandeling (Hanks gebalanseerde soutoplossing), òf doksorubisienbehandeling (kumulatiewe dosis van 12 mg/kg) ontvang. Plasma inflammatoriese merkers, vetsuurprofiele en proteïenuitdrukking van lipiedmetabolisme merkers (*de novo* vetsuur sintese en lipoliese) is in borsvetweefsel en tumorweefsel ondersoek. Om die *in vivo* model se bevindinge te bevestig, is ‘n *in vitro* model ontwikkel waarby kondisionerings media ingesluit is. ‘n Stamsellyn vanuit menslike vetweefsel is gebruik om volwasse adiposiete te differensieer waarna kondisionerings media versamel is om die parakrien effek tussen adiposiete en doksorubisien behandelde MDA-MB-231 trippel negatiewe kankerselle te ondersoek. Die aantal lewende selle is met behulp van die WST-1 toets bepaal. Westerse kladtegniek is gebruik om wysigings in proteïenuitdrukking van apoptotiese en lipiedmetabolisme merkers te bepaal (*de novo* vetsuur sintese en lipolise). ‘n Inflammatoriese merker en vryvetsuurprofiel is vir die behandelings-kondisionerings media ondersoek.

Resultate: Die vetsuggeïnduseerde dieet het gewas groei betekenisvol versnel en die doksorubisien behandelings effektiwiteit in E0771 trippel negatiewe borskanker gewasse verlaag ($p<0.0001$), wat tot behandelingsweerstand gelei het. Ons resultate toon verder dat dieetgeïnduseerde vetsug beide *de novo* vetsuur sintese (verlaagde SCD-1) asook lipolise (verlaagde HSL) in borsweefsel van doksorubisien behandelde muise onderdruk. Die teenoorgestelde is by tumorweefsel waargeneem waar *de novo* vetsuur sintese (verhoogde SCD-1) en lipolise (verhoogde ATGL) tot betekenisvolle veranderinge in vetsuursamestelling van beide weefsels aanleiding gegee het. Dieetgeïnduseerde vetsug het plasma leptien ($p=0.025$) en resistienvlakke ($p=0.046$) betekenisvol verhoog, en NFκB proteïenuitdrukking in borsvetweefsel van die doksorubisien behandelde muise verhoogwat tot sistemiese en locale inflammasie gelei het. Adiposiete bevorder ook borskanker behandelingsweerstand deur die aantal lewende selle van doksorubisien behandelde MDA-MB-231 trippel negatiewe borskankerselle (Dox+CM *vs* Dox, $p=<0.0001$) te verhoog. Hierdie effek is verkry deur die effektiwiteit van doksorubisien te verlaag om sodoende sel dood (apoptose) te induseer (verlaagde gesplyte-kaspase-3, $p<0.05$), in ‘n parakriene wyse. Adiposiete het inflammasie induseer (verhoogde leptien en MCP-1) asook lipolise (verhoogde HSL) in die doksorubisien behandelde borskankerselle (Dox *vs* Dox+CM $p=0.03$), en hierdeur die vryvetsuurprofiel van borskankerselle gewysig.

Gevolgtrekking: Ons data wys dat vetweefsel/adiposiete ‘n betekenisvolle bydrae lewer tot behandelingsweerstandigheid in trippel negatiewe borskanker selle. Ons het beide *in vivo* en *in vitro* aangetoon dat vetweefsel/adiposiete sekretoriese faktore induseer om inflammasie in die borskanker mikro-omgewing te veroorsaak, en sodoende lipolise in trippel negatiewe borskankerselle induseer. Dit het die metaboliese gedrag gewysig, i.e. verhoogde vetsuur verbruik wat of kan dien as energiesubstraat of lipiedversadiging induseer om apoptose te ontwyk. Ons is van mening dat hierdie ‘n nuwe meganisme is waarby vetweefsel/adiposiete binne die tumormikro-omgewing ‘n bydrae lewer tot die ontwikkeling van borskankerbehandelingsweerstand. Hierdie studie het verder ‘n betekenisvolle bydrae gelewer om die molekulêre meganismes te identifiseer en te verklaar wat onderliggend tot borskankerbehandelingsweerstand in vetsugtige pasiënte is.

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LIST OF PEER REVIEWED PUBLICATIONS

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LIST OF ABBREVIATIONS

∑ MUFAs	Total Monounsaturated fatty acids
∑ n-3 PUFA	Total omega-3 polyunsaturated fatty acids
∑ n-6 PUFAs	Total omega-6 polyunsaturated fatty acids
∑ n-7 MUFAs	Total omega-7 monounsaturated fatty acids
∑ n-9 MUFAs	Total omega-9 monounsaturated fatty acids
∑ PUFAs	Total polyunsaturated fatty acids
∑ SFAs	Total saturated fatty acids
AA (20:4n-6)	Arachidonic Acid
ABC	ATP-binding cassette
ACC	Acetyl-CoA carboxylase
ADA (22:4n-6)	Adrenic Acid
AKR	Aldo-keto reductase
ALA (18:3n-3)	α-Linolenic Acid
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ARA (20:0)	Arachidic Acid
ATGL	Adipose triglyceride lipase
C	Carbon
CD36	Fatty acid translocase
CEBPα	CCAAT enhancer binding protein-α
Cleaved-PARP	Cleaved-poly (ADP-ribose) polymerase
CM	Conditioned Media

CMS	Chloroform: Methanol: Saline
COX	Cyclooxygenase
CXCL5	C-C Motif ligand 5
DGLA (20:3n-6)	Dihomo-γ-Linolenic Acid
DHA (22:6n-3)	Docosahexaenoic Acid
DIO	Diet-induced obesity
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
Dox+CM	Conditioned media and doxorubicin
Dox-H	Tumour-bearing mice on high fat diet treated with doxorubicin
Dox-L	Tumour-bearing mice on low fat diet treated with doxorubicin
DPA (22:5n-6)	Docosapentaenoic Acid
EA (22:1 n-9)	Erucic Acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDA (20:2n-6)	Eicosadienoic Acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
EPA (20:5n-3)	Eicosapentaenoic Acid
ER^-	Oestrogen receptor negative
ER^+	Oestrogen receptor positive
FA	Fatty acid
FABP-4	Fatty acid binding protein-4

FAMEs	Fatty acid methyl esters
FAs	Fatty Acids
FAS	Fatty acid synthase
FBS	Foetal Bovine Serum
FFA	Free fatty acids
GA (20:1n-9)	Gondoic Acid
GLC	Gas-liquid chromatography
GLUT-4	Glucose transporter-4
H	Hydrogen
hADSC	Human adipose tissue derived stem cells
HBSS	Hanks Balanced Salt Solution
HER-2	Human epidermal growth factor receptor-2
HFD	High fat diet
HIF-1α	Hypoxia inducible factor-1α
HOMA-IR	Homeostatic model assessment of insulin resistance
HSL	Hormone sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like-growth factor-receptor-1
IL-10	Interleukin-10
IL-15	Interleukin-15
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8

JNK	c-Jun N-terminal kinases
LA (18:2n-6)	Linoleic Acid
LDL-c	Low density lipoprotein-cholesterol
LFD	Low fat diet
LOX	Lipoxygenase
LPA	Lysophosphatidic acid
MA (14:0)	Myristic Acid
MAGL	Monoacylglycerol lipase
MAPK	Mitogen activated protein kinase
MCP-1	Macrophage chemoattractant protein-1
MDR-1	Multi-drug resistance protein-1
MDRP-1	Multi-drug resistance associated protein-1
MET	Mesenchymal-Epithelial-Transition
MGA (17:0)	Margaric Acid
mTOR	Mammalian target of rapamycin
MUFAs	Monounsaturated fatty acids
MβCD	Methyl-β-cyclodextrin
n-3	Omega-3
n-3: n-6	Omega-3: Omega-6 ratio
n-6	Omega-6
n-7	Omega-7
n-9	Omega-9
NA (24:1n-9)	Nervonic Acid
NCDRU	Non-Communicable Disease Research Unit

NFκB	Nuclear factor kappa B
OA (18:1n-9)	Oleic Acid
PA (16:0)	Palmitic Acid
PAI-1	Plasminogen activator inhibitor-1
pAkt	Phosphorylated Akt
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCA	Principle component analysis
PE	Percent energy
PenStrep	Penicillin Streptomycin
pERK1	Phosphorylated ERK1
pERK2	Phosphorylated ERK2
P-gp	P-glycoprotein
PI3K	Phosphoinositide-3-kinase
PIP_2	Phosphatidylinositol bisphosphate
PIP_3	Phosphatidylinositol triphosphate
PPAR-α	Peroxisome proliferator-activated receptor-α
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PTA (16:1n-7)	Palmitoleic Acid
PUFAs	Polyunsaturated fatty acids
PVDF	Polyvinylidine fluoride
RIPA	Radio-immunoprecipitation assay buffer
ROS	Reactive oxygen species

SA (18:0)	Stearic Acid
SAMRC	South African Medical Research Council
SCD-1	Stearoyl-CoA desaturase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFAs	Saturated fatty acids
STAT-3	Signal transducer and activator of transcription-3
TAGs	Triglycerides
TBS-T	Tris Buffered Saline-Tween 20
TCA	Tricarboxylic acid cycle
TGF-β	Transforming growth factor-beta
TLC	Thin-layer chromatography
TNBC	Triple negative breast cancer
TNF-α	Tumour necrosis factor-alpha
TPL	Total phospholipid
VA (18:1n-7)	Vaccenic Acid
VEGF	Vascular endothelial growth factor
VEGF-R	Vascular endothelial growth factor-receptor
Vehicle-H	Tumour vehicle high fat diet
Vehicle-L	Tumour vehicle low fat diet
γ-LA (18:3n-6)	γ-Linolenic Acid

CHAPTER 1: LITERATURE REVIEW

1. INTRODUCTION

Breast cancer continues to be a major health risk for women globally (Bray *et al*., 2018). Lifestyle-related factors such as overweight and obesity (adiposity) have reached epidemic proportions (Nagrani *et al*., 2016; Ng *et al*., 2014) and are considered major risk factors for the development and progression of breast cancer (Sparano *et al*., 2012). Obesity results in adipose tissue dysfunction, which is mainly characterized by rapid adipose tissue remodelling (hypertrophy and hyperplasia) (Choe *et al*., 2016). This results in dysfunctional synthesis of several adipokines in coordination with immune cell infiltration leads to a state of sustained low-grade inflammation that activates downstream signalling pathways favouring cancer cell survival (increased proliferation and decreased apoptosis) and hence contributes to breast cancer progression and metastasis (Stone *et al*., 2018; Renehan *et al*., 2015; Toren *et al*., 2013). Furthermore, adipose tissue and/or adipocytes, in the tumour microenvironment serve as an exogenous energy source for the survival of breast cancer cells (Balaban *et al*., 2018; Dirat *et al*., 2011), especially since adipose tissue is abundant in breast tissue (Lin *et al*., 2013). It is further proposed that breast cancer cells modulate lipid metabolism (*de novo* fatty acid synthesis and lipolysis) (Balaban *et al*., 2018), by altering the secretion of adipokines through adipocytes, resulting in the release of free fatty acid (FFA) to provide energy substrates for cancer cells and sustain its high proliferation demand.

Recently, pre-clinical evidence also highlighted obesity as a key role player in breast cancer chemotherapeutic drug resistance (Bousquenaud *et al*., 2018; Incio *et al*., 2018; Iwase *et al*., 2016). This discovery is of clinical significance for overweight/obese breast cancer patients receiving chemotherapeutic agents such as doxorubicin (Sirin *et al*., 2013). This was also confirmed in studies where obesity was associated with poor clinical outcomesin breast cancer patients treated with doxorubicin (Gevorgyan *et al*., 2016; Karpińska *et al*., 2015).

Doxorubicin is a highly sensitive alkylating antineoplastic agent used as a first line adjuvant regimen for breast cancer patients (Guenancia *et al*., 2017). Despite its high sensitivity as a chemotherapeutic agent, it is also associated with a diverse range of cellular toxicities (i.e. disrupting adipose tissue's storage function) and the development of treatment resistance(Arunachalam *et al*., 2012; Biondo *et al*., 2016; Nagendran *et al*., 2013; Xiang *et al*., 2009). Identifying molecular mechanisms in which doxorubicin affect adipose

tissue/adipocytes could contribute towards molecular mechanisms and identifying potential novel pharmacologic targets.This could lead to the development of the appropriate treatment management protocols of doxorubicin related toxicities in order to improve over-all survival of breast cancer patients.

It is also argued that since adipose tissue is a storage site for fatty acids (FAs), tumour cells may utilizethese FAs to promote tumour growth.Fatty acids exhibit a diverse range of physiological functions therefor its' dysfunction and/or dysregulation, might therefore be implicated in breast cancer progression and treatment resistance.The role of FAs in cancer progression is proposed to be achieved by *(i) alterations in cell membrane composition, (ii) biosynthesis of lipid-signalling molecules, (iii) its role in metabolic reprogramming, and (iii) as an energy source*(Mentoor *et al*., 2019).

However, few studies exist in which the effects of doxorubicin on adipose tissue/adipocytes in the context of obesity are investigated. We propose that doxorubicin treatment may exacerbate the negative effects of obesity *per se* and may also thus promote breast cancer treatment resistance (Mentoor *et al*., 2018; Mentoor *et al*., 2019).This literature review will mainly focus on (i) the pathological links between obesity and breast cancer in the context of inflammation as an underlying mechanism, and (ii) the role of obesity in breast cancer treatment resistance (doxorubicin) and the possible mechanisms of how lipid metabolism including FAs can contribute to breast treatment resistance.

1.1OBESITY AND BREAST CANCER

Globally, populations are adoptingto a more westernised lifestyle that is characterised by an increased dietary saturated and *trans* fat intake, refined (added) sugar, and a decrease in high quality protein and fibre intake (Bhurosy *et al*., 2015; van Zyl *et al*., 2012).Over time imbalances in energy homeostasis eventually result in adiposity,that is defined as the abnormal and excessive accumulation of adipose tissue resulting in an increased body weight and a state of overweight or obesity (Zhao *et al*., 2013).

Despite many efforts to address and reduce cancer mortality by implementing lifestyle-related modifications, limited progress has been made due to the very complicated interplay between dietary behaviours and other lifestyle modifications (Ghonchehe*t al*., 2016). This is especially problematic since epidemiological studies strongly suggested that obesity is a significant risk

factor in many lifestyle-associated cancers including breast cancer (Donohoe *et al*., 2011; White *et al*., 2015). Globally, the increasing burden of breast cancer is considered the second most prevalent cancer diagnosed amongst women, in both developed and developing countries (Bray *et al*., 2018). Estimations rank breast cancer as the fifth leading cause of death globallyat 626,679 deaths per annum in females (Bray *et al*., 2018).

1.1.1 Evidence Linking Obesity and Breast Cancer

Strong correlations are reported between obesity and poor clinical outcomes in breast cancer patients (Ewertz *et al*., 2011; White *et al*., 2015). Some of these outcomes include, (i) increased risk in the incidence and mortality of breast cancer (Azrad *et al*., 2013; Pierobon & Frankenfeld, 2013), (ii) inferior disease free survival, (iii) increased risk of cancer recurrence (Sparano *et al*., 2012; Pierobon & Frankenfeld, 2013; Sun *et al*., 2015), and (iv) poor prognosis i.e. larger tumour size, progressive disease stages and more positive lymph nodes (Chen *et al*., 2016; Ewertz *et al*., 2011;White *et al*., 2015). Additionally, weight loss interventions provide indirect evidence that a reduction in excess body weight through bariatric surgery, caloric restriction, and/or exercise significantly decreases overall cancer risk (Wolin *et al*., 2008). Several animal models are used to simulate human breast cancer and diet-induced obesity (DIO), demonstrating an increase in tumour growth that support epidemiological evidence (Cowen *et al*., 2015; Khalid *et al*., 2010; Stemmer *et al*., 2012).Obesity is associated with an increased risk for aggressive basal breast carcinomas including triple negative breast cancer (TNBC), which lacks the expression of molecular markers (ER^-, PR^- and $HER\text{-}2^-$), often presented by high-grade tumours, poor clinical outcomes and treatment insensitive (Chen *et al*., 2016). Triple negative breast cancerdevelops independent from hormonal related pathways (Pierobon & Frankenfeld, 2013), and is associated with the overexpression of growth factors and adipokines favouring breast cancer cell survival (Dietze*et al*., 2018; Sun *et al*., 2017).

1.1.2. Adipose Tissue is a Complex Functional Tissue

Adipose tissue is a complex and vital endocrine organ impacting various physiological systems (Divella *et al*., 2016). It functions as both an energy storage compartment and a metabolic active endocrine organ (Rezaee & Dashty, 2013), secreting various pro- and anti-inflammatory bioactive substances known as adipokines. These include but not limited to leptin, adiponectin, resistin, tumour necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and

macrophage chemoattractant protein-1 (MCP-1) (Gade *et al*., 2010; Mendonça *et al*., 2015). Adipose tissue also functions to regulate the release of FFA to other tissues to provide energy, regulating metabolism and storage of lipids and glucose to maintain energy homeostasis (Stephens, 2012). Apart from these functions, adipose tissue contributes towards steroid sex hormone and growth factor production, as well as the development of insulin resistance, hyperglycaemia and breast cancer (Cefalu, 2009). Epidemiological and experimental models support the role of a dysregulation in adipokine synthesis and their actions in relation to obesity and adipose tissue dysfunction, to the development of various diseases, including breast cancer (Allott&Hursting, 2015; Azrad & Demark-Wahnefried, 2014; Ewertz*et al*., 2011; Kyrgiou *et al*., 2017; Sun *et al*., 2015; Witt *et al*., 2009).

1.1.2.1 Dysfunctional Adipose Tissue-induced Inflammation and Breast Carcinogenesis

A complex pathophysiology underlying breast cancer and obesity-associated adipose tissue dysfunction is proposed to include multiple cellular pathways. This ranges from the bioavailability of sex hormones (increased oestrogen and testosterone), growth factors (insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF), inflammatory markers, as well as FAs and its lipid derived metabolites as evident in diet-induced-obesity animal models (Cowen *et al*., 2015; Khalid *et al*., 2010; Lautenbach *et al*., 2009; Orbetzova *et al*., 2012; Stemmer *et al*., 2012; Weinberg *et al*., 2006). Collectively, sex hormones, growth factors and inflammatory markers regulate major signalling pathways (i.e. nuclear factor kappa B (NFκB), mitogen activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K) and epithelial-to-mesenchymal transition (EMT)) that are involved in cell-proliferation, apoptosis, angiogenesis, invasion and metastasis to influence carcinogenesis (Gordon *et al*., 2017; Iyengar *et al*., 2015). Hyperinsulinemia and upregulated IGF-1 synthesis are characteristic in excess adipose tissue states (Brahmkhatri *et al*., 2015; Lewitt *et al*., 2014). Insulin and IGF-1 binds to the insulin/IGF-1 receptors that activate MAPK and the PI3K/Akt/mammalian target of rapamycin (mTOR) signalling pathways that are linked to pro-carcinogenic events including cell proliferation, growth and survival (Lamming, 2014).

A positive energy state leads to excessive storage of triglycerides (TAGs) in the lipid droplets of adipocytes (lipogenesis) (Jung & Choi, 2014). Adipose tissue undergoes remodelling including adipocyte hyperplasia and hypertrophy (Bjørndal *et al*., 2011) (Figure 1.1), which deregulatesthe secretion of various adipokines including MCP-1 and colony stimulating factor-1.

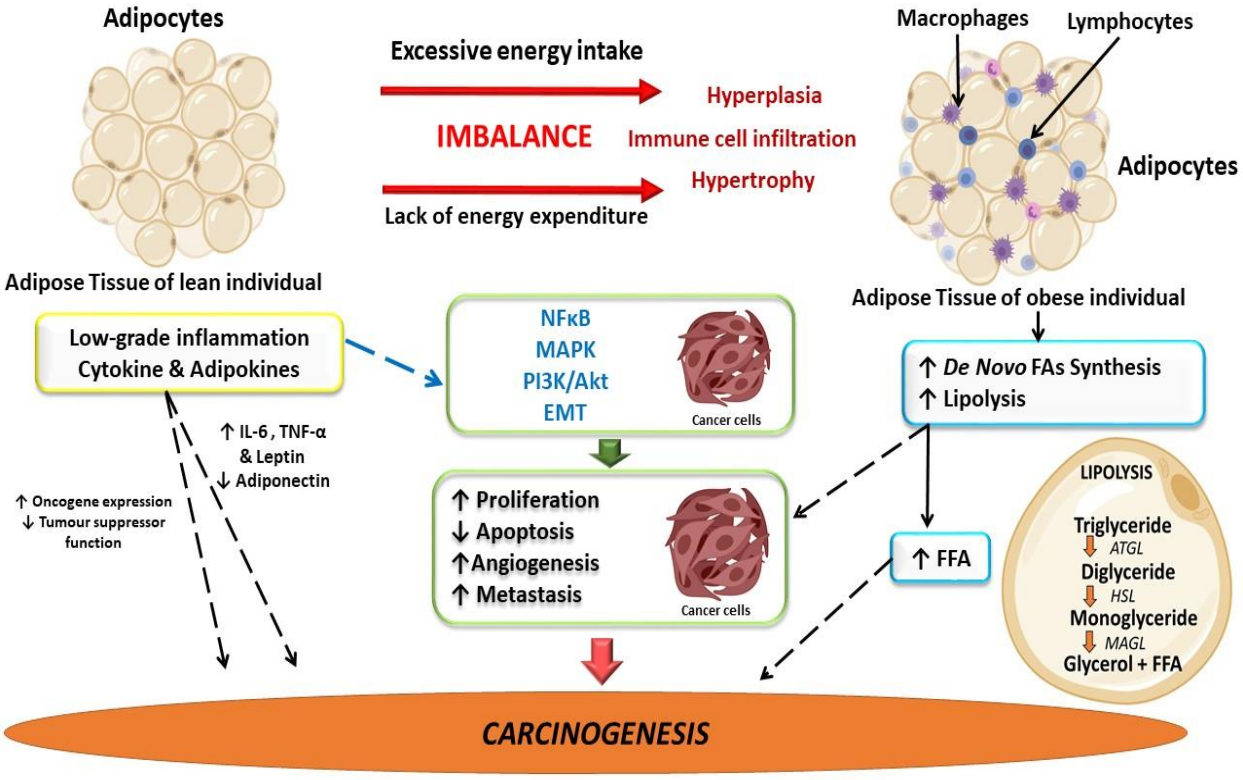

Figure 1.1: The link between adipose-induced inflammation and cancer.

Adipose tissue dysfunction is associated with sustained low-grade inflammation which promotes breast cancer progression. Several inflammatory mediators are implicated in tumour development and progression, possibly as a result of the sustained inflammatory signalling having downstream effects on major pathways involved in angiogenesis, cell-proliferation, apoptosis and metastasis. Hypoxia in adipose tissue also induces the release of inflammatory mediators, thus further exacerbating inflammation. [ATGL, adipose triglyceride lipase; EMT,epithelial-to-mesenchymal transition; FAs, Fatty acids; FFA, Free fatty acids; HSL, hormone sensitive lipase; IL-6, interleukin-6; MAGL, monoacylglycerol lipase; MAPK, mitogen activated protein kinase; NFκB, nuclear factor kappa B; PI3K/Akt, phosphoinositide-3-kinase; STAT-3, Signal transducer and activator of transcription-3; TNF-α, tumour necrosis factor-α] (Mentoor *et al*., 2018; Mentoor *et al*., 2019).

This leads to immune cell infiltration (i.e. monocytes and macrophages) (Gilbert & Slingerland, 2013) which is responsible for the secretion of several pro-inflammatory adipokines, including TNF-α, IL-1β, IL-6, transforming growth factor-beta (TGF-β), leptin, resistin, and downregulate adiponectin and interleukin-10 (IL-10). These actions contribute to a chronic cycle that sustains an inflammatory *milieu* and the development of sustained low-grade inflammation (Figure 1.1) (Divella*et al*., 2016).

Various alterations in signalling pathways are linked to obesity-induced low-grade inflammation, including adenosine monophosphate-activated protein kinase (AMPK), NFκB and c-Jun N-terminal kinases (JNK) (Harvey *et al*., 2011; Yao *et al*., 2017). For example, increased IL-6 and leptin levels has been shown to suppress AMPK, well known for its' anti-inflammatory effects in adipose tissue (Lindholm *et al*., 2013). Additionally, adipose tissue-induced inflammation attenuates the suppression of NFκB phosphorylation and induces M1 to M2 macrophage phenotype switching. The latter is due to the pro-inflammatory state in which saturated fatty acids (SFAs) bind to the toll-like receptors on macrophages (Suganami &Ogawa, 2010) and upregulate the secretion of various pro-inflammatory mediators (IL-1β and TNF-α) in adipose tissue (Galic *et al*., 2011; Roberts *et al*., 2010). This creates a state of chronic systemic low-grade inflammation, which is a well-known hallmark of cancer and predisposing risk factor for tumorigenesis (Grivennikov *et al.*, 2010, Hanahan & Weinberg, 2011). Pre-existing pro-inflammatory microenvironments are associated with an increased risk for cancer, including inflammatory breast cancer (Yao *et al*., 2017). Remarkable similarities exist between dysfunctional adipose tissue and the mammary adipose tissue tumour microenvironment where infiltration of immune cells initiate the secretion of pro-inflammatory molecules, thereby sustaining and promoting the progression of both obesity and breast cancer (Nieman *et al*., 2013; Tan *et al*., 2011) (Figure 1.1).

The complicated pathophysiology existing between adipose tissue, inflammation and breast cancer involves inflammatory mediators such as IL-6 and TNF-α, which enhances tumour progression and survival (Pérez-Hernández *et al*., 2014). Constitutive activation of NFκB target genes such as IL-6 and IL-8 has been found in oestrogen receptor negative (ER^-) basal like breast cancer cells (Freund *et al*., 2003; Sansone *et al*., 2007). Moreover, IL-6 secreted by adipose tissue binds to IL-6 receptor on breast cancer cells and activates the Janus family of kinases that phosphorylates signal transducer and activator of transcription-3 (STAT-3) (Khan *et al*., 2013). These events induce the expression of pro-survival genes (i.e. bcl-x) (Murray,

2007), characteristic of a pro-carcinogenic state promoting breast cancer cell survival and proliferation (Figure 1.1).

Obesity-induced adipokine secretions (IL-6, IL-8, TNF-α, and VEGF) are detected in local adipose tissue and serum (Balistreri *et al*., 2010) and exert physiological effects at distant regions (Landskron *et al*., 2014) promoting breast cancer development through upregulation of inflammatory mediator synthesis and increased immune cell infiltration as well as angiogenesis (Gupta *et al*., 2017). Additionally, overweight/obese patients display numerous crown-like-structures (necrotic adipocytes surrounded by immune cells) in mammary adipose tissue compared to normal weight breast cancer patients. These crown-like structures are characteristic of local inflammation (Khan *et al*., 2013; Vaysse *et al*., 2017) and are associated with an upregulation of pro-inflammatory cytokines and aromatase expression (Choi *et al*., 2018). Although the role of cytokines in the context of obesity and breast cancer development have been reported, the effects of other adipokines should also be considered as possible role players.

Leptin and adiponectin have been antagonistically implicated for their roles in inflammation and tumorigenesis (Figure 1.1) (Surmacz *et al*., 2013). Leptin upregulates pro-inflammatory cytokine secretions and increases cellular proliferation and angiogenesis (Rodríguez *et al*., 2013). Elevated serum leptin levels and increased expression of leptin receptors are also observed in breast cancer patients, and is often associated with higher pathological grade tumours and cancer treatment resistance (Aleksandrova *et al*., 2012; Sultana *et al*., 2017). Adiponectin is decreased in obese (metabolic syndrome) patients and in breast cancer patients, which has been linkedto an increased risk of cancer development. This may be due adiponectin's pro-apoptotic functions as well as anti-inflammatory properties being attenuated at low concentration levels (Otake *et al*., 2010; Zhao & Liu, 2013; Nalabolu *et al*., 2014). Evidence suggests that higher levels of resistin in obese and breast cancer patients respectively (Zeidan *et al*., 2018),promote cell growth and invasiveness through increased STAT-3 expression and phosphorylation (Wang *et al*., 2018; Lee *et al*., 2016). Resistin stimulates the secretion of other pro-inflammatory mediators through upregulation of NFκB expression to promote carcinogenesis (Wang *et al*., 2018).

Additionally, loss of tumour suppressor function, increased cell cycling and stimulation of oncogenes increase inflammation and exacerbates inflammatory related signalling pathways (Dibra *et al*., 2014; Sethi *et al*., 2012; Yang *et al*., 2014) (Figure 1.1). For example, the p53

gene mutation promotes inflammation in the tumour microenvironment by inducing the synthesis of IL-1α, IL-6, TNF-α, and activates NFκB (Cooks *et al*., 2013; Komarova *et al*., 2005) in the tumour microenvironment to enhance genomic instability (Bristow & Hill, 2008; Yang & Karin, 2014). Additionally, mutated p53 induces the PI3k/Akt/mTOR pathway and the synthesis of pro-inflammatory mediators (Wang *et al*., 2015). As a result of rapid hypertrophy and hyperplasia (Trayhurn, 2013) (Figure 1.1), hypoxia inducible factor-1α (HIF-1α) is upregulated (Rausch *et al*., 2017), that binds to transcription factors on VEGF and angiopoietin-2 target genes, stimulating angiogenesis in the microenvironment to exacerbate local inflammation (Rausch *et al*., 2017).

Obesity-induced inflammation may also play a role in breast cancer tumour invasion and metastasis. A proposed underlying mechanism of action for metastasis includes cells migrating through the extracellular matrix (ECM) as a result of epithelial-to-mesenchymal transition (EMT) (Bidard *et al*., 2008), to facilitate the migration of cancer cells away from the primary tumour, as well as mesenchymal-to-epithelial transition (MET) to form tumours at distant sites (Jiang *et al*., 2015) (Figure 1.2).

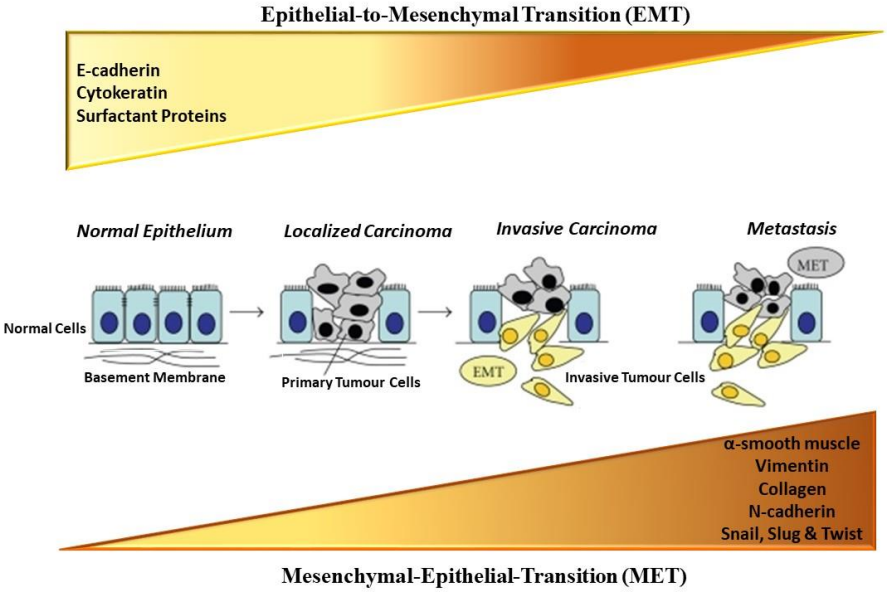

Figure 1.2: Epithelial-to-mesenchymal transition (EMT) and its reversibleprocess mesenchymal-to-epithelial transition (MET). EMT and METis implicated as an underlying mechanism in the invasion and metastasis of breast tumours towards secondary sites. Induction of EMT results in the expression of mesenchymal markers which promotes a mesenchymal phenotype in epithelial cell. During EMT, cells become motile and develop invasive capacities. An increase in the expression of epithelial markers induces the development of an epithelial phenotype via induction MET. (Adapted from Bartis *et al*., 2014; Dongre & Weinberg, 2019; Palena & Schlom, 2010)

Epithelial-to-mesenchymal transition is part of normal developmental and remodelling processes within tissue including the mammary gland. However, when dysfunctional as in the case of breast cancer, it can favour the invasion of breast cancer cells to adjacent tissue (Wang *et al*., 2018). The EMT process includes the degradation of the epithelial basement membrane and ultimately the development of a mesenchymal cell phenotype (Lee *et al*., 2015) (Figure 1.2). During EMT, tumour epithelial cells (which are attached to the basement membrane) undergo both molecular and genetic alterations which results in the loss of cellular junctions, acquisition of a mesenchymal phenotype with enhanced migration and invasion to distant sites (Singh & Settleman, 2010). Regulation of EMT is accomplished by altered expression of proteins present in the cytoskeleton (i.e. α-smooth muscle actin and vimentin), as well as various transcription factors (snail, slug and twist), cell junction proteins (E-cadherin), and ECM-degrading enzymes (Gyamfi *et al*., 2018) (Figure 1.2). During EMT epithelial cells downregulate the expression of E-cadherin and upregulate expression of mesenchymal proteins like vimentin (Gyamfi *et al*., 2018) (Figure 1.2). Cells displaying a mesenchymal phenotype are known to evade cell-death, induce cell migration and induce ECM component alterations that favour breast cancer cell survival (Singh & Settleman, 2010). EMT can be stimulated by various pro-inflammatory markers such as IL-6, IL-8, and TNF-α and MCP-1 derived from cancer-associated adipocytes (Hwang *et al*., 2011; Qian *et al*., 2011; Xie *et al*., 2012).

Furthermore, adipose tissue remodelling *via* inflammation, also leads to increased adipose tissue lipolysis, which is the catabolic breakdown of TAGs [catalysed by adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MAGL)], resulting in the upregulation of FFA release (Bolsoni-Lopes & Alonso, 2015) (Figure 1.1).Increased palmitic acid (PA) (an abundant saturated FA esterified to TAGs in adipose tissue), either as a result of increased dietary intake or adipose tissue lipolysis (Figure 1.1), is subsequently transported to the liver *via* the hepatic portal vein (bound in circulation to albumin) and oxidised to yield energy substrates (β-oxidation), or used as a precursor for *de novo* fatty acid synthesis (lipogenesis) (Cha & Lee, 2016; Mashima *et al*., 2009) (Figure 1.1).Furthermore, TNF-α induce adipocyte lipolysis, which further exacerbates the release of SFAs (Laurencikiene *et al*., 2007), including palmitic-, lauric- and stearic acid (SA) (Lee *et al*., 2001; Lee *et al*., 2004). These saturated FAs bind to toll-like receptor-4 on macrophages, and upregulate synthesis of pro-inflammatory cytokines (i.e. TNF-α), which contributes to adipose tissue inflammation (Suganami & Ogawa, 2010). A phenotypic switch from anti-inflammatory

(M2) to pro-inflammatory macrophages (M1) also occurs due to this pro-inflammatory state (Suganami & Ogawa, 2010).

Under homeostatic conditions, *de novo* FAs synthesis is only responsible for five percent of all TAGs stored in the liver, but can increase during obesogenic states to 30% (Ni *et al*., 2015). Physiologically, this is significant not only because FAs form part of the architecture of cell membrane structure (phosphoglycerides) and play a role in membrane fluidity (phospholipids), but it also serves as cell-membrane signalling molecules within and between cells (Hussein, 2013). The long chain FAs can also act as a source, and/or precursor, of endogenous anti- and-pro-inflammatory lipid mediators. Therefore, any dysfunctional and/or deregulation of FAs metabolism, in relation to both dietary intake and *de novo* synthesis, could be implicated in the development of breast cancer (discussed in section 1.3).

It is evident from these findings that obesity is a casual factor in the development of breast cancer, involving molecular mechanisms in relation to inflammation (including FAs), immune cell infiltration and adipokine dysfunction. Supporting evidence includes obesity as a negative prognostic factor for breast cancer independent of menopausal status, tumour stage, and tumour hormone–binding characteristics (Chen *et al*., 2016; Pierobon & Frankenfeld, 2013). However, experimental breast cancer animal models reveal that DIO not only increases tumour development, progression and metastasis, but subsequently leads to a decrease in the efficacy of chemotherapeutic agents (Cowen *et al*., 2015; Khalid *et al*., 2010; Stemmer *et al*., 2012; Thomas *et al*., 2016).

1.2BREAST CANCER TREATMENT: DOXORUBICIN

Chemotherapy is still considered one of the conventional treatment options in addition to radiotherapy and surgery, which significantly improves cancer patients' overall-survival (Kabel & Baali, 2015; Matsen & Neumayer, 2013). Several chemotherapeutic drug classes exist which are associated with beneficial clinical outcomes for cancer patients (Hassan *et al*., 2010).

Doxorubicin, also known as Adriamycin, or hydroxyl daunorubicin (Meredith & Dass, 2016), is classified as an anthracycline antibiotic, exhibiting broad-spectrum anti-neoplastic activity (Guenancia *et al*., 2017; Meredith & Dass, 2016). It is used to treat a range of malignancies such as that of the breast (used as first line adjuvant chemotherapeutic agent), bladder, stomach,

lung, ovaries, thyroid as well as multiple myeloma, Hodgkin- and non-Hodgkin's lymphoma, due to its poor tumour selectivity (Rivankar, 2014).

Doxorubicin interacts with deoxyribonucleic acid (DNA) by intercalation, thereby inhibiting macromolecule biosynthesis (Thorn *et al*., 2011), which inhibits topoisomerase II (DNA repair function) and relaxes DNA transcription supercoils (Anampa *et al*., 2015; Arunachalam *et al*., 2013). Secondly, doxorubicin generates reactive oxygen species (ROS) that damages cell membranes, DNA and proteins (Anampa *et al*., 2015; Thorn *et al*., 2011) through stimulation of p53-DNA binding to initiates caspase signalling and DNA cross-linking (de Lima Junior *et al*., 2016). Lastly, doxorubicin also induces cell death by increasing mitochondrial membrane permeability to release cytochrome c from mitochondria into the cytosol where it activates the caspase family of proteases, thereby inducing the intrinsic apoptotic pathway (Gorini *et al*., 2018) (Figure 1.3).

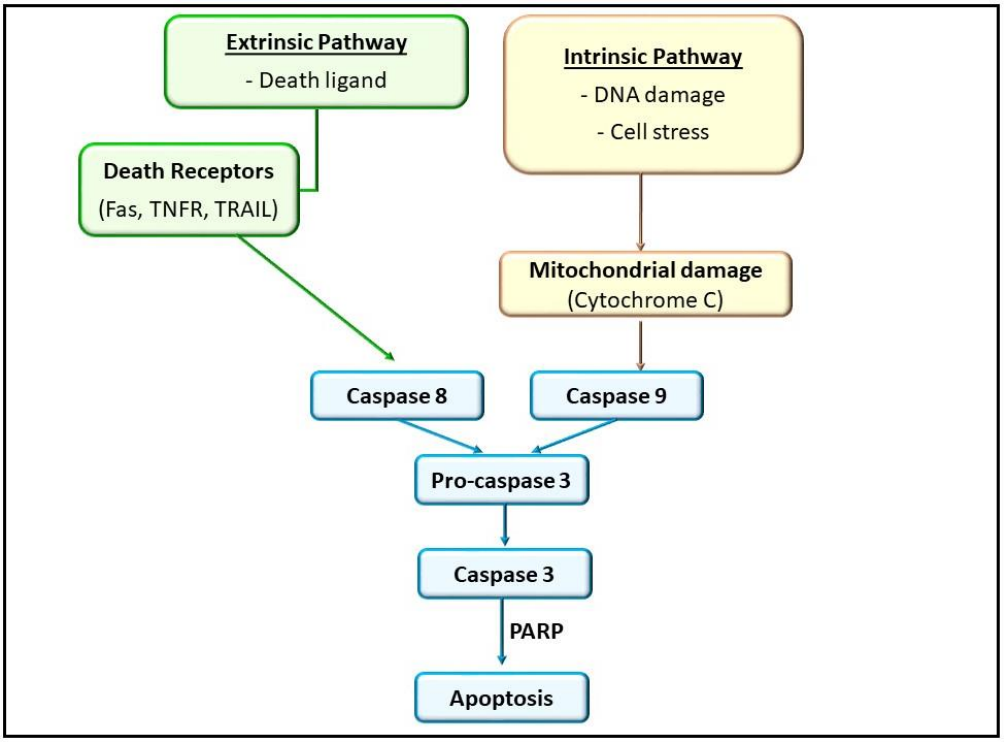

Figure 1.3: Overview of the extrinsic and intrinsic apoptosis pathways. PARP, poly (ADP-ribose) polymerase. (Adapted from Cheng *et al*., 2016; Elmore, 2007; Fulda & Debatin, 2006).

Apoptosis is a cell death mechanism initiated to discard old, and/or damaged cells. It is a highly regulated physiological process which includes several proteases, known as caspases undergoing proteolysis for cleavage and subsequent activation (Matsuura *et al*., 2016).

Apoptosis is executed by two distinct pathways, namely the extrinsic death receptor and intrinsic mitochondrial pathway, which induces the execution pathway (Figure 1.3, extensively reviewed in Elmore, 2007 and Fulda & Debatin, 2006).

Both pathways are induced by proteolysis *via* the cleavage of caspase-8 and caspase-9 respectively, to activate pro-caspase-3 *via* cleavage forming cleaved-caspase-3 (Elmore, 2007, Nagata, 2018) (Figure 1.3). Once cleaved, caspase-3 becomes active and leads to the execution of apoptosis (Bai, 2015). Cellular caspase-3 acts as executioner caspase by obstructing the activation of poly (ADP-ribose) polymerase (PARP) through cleavage of PARP resulting in the inhibition of DNA repair mechanisms (Pistritto *et al*., 2013). PARP is a nucleoprotein that detects alterations and/or damages in single-strand DNA to promote apoptosis, genomic stability, and/or DNA repair. Cleavage of PARP by caspase-3 causes inactivation of PARP activity which increases DNA damage in cancer cells to sustain cancer cell growth (Morales *et al*., 2014) (Figure 1.3).

Furthermore, doxorubicin treatment is often associated with adverse side effects such as nephrotoxicity, hepatotoxicity, sarcopenia, cardiotoxicity (Rivankar, 2014), as well changes in body composition (decreased body weight and lipoatrophy, discussed in section 1.4) (Thivat *et al*., 2010; Vagenas *et al*., 2015). These effects contribute toward cancer recurrence as well as metastasis in obese breast cancer patients, making doxorubicin treatment protocols ineffective and prone to develop treatment resistance for this group of patients (de Visser & Jonkers, 2009; Xu *et al*., 2014).

1.2.1 Evidence Linking Obesity and Cancer Treatment Resistance

Experimental animal models indicated that DIO increases tumour development, progression and metastasis with decreased chemotherapeutic effectiveness in breast cancer (Cowen *et al*., 2015; Guiu *et al*., 2010; Lashinger *et al*., 2014; Khalid *et al*., 2010; Stemmer *et al*., 2012; Thomas *et al*., 2016; Bousquenaud *et al*., 2018; Incio *et al*., 2018). Obesity is also associated with larger tumour sizes and positive lymph node involvement compared to non-obese breast cancer patients (De Azambuja *et al*., 2010; Haakinson *et al*., 2012). These studies have also shown that obesity is linked to poor clinical outcomes in breast cancer patients treated with chemotherapeutic, hormonal-based chemotherapy agents and radiotherapy (Bochet *et al*., 2011; Gevorgyan *et al*., 2016). Obesity was associated with lower pathological complete response, disease free survival, clinical benefit rate and worse overall-survival (Gevorgyan *et*

al., 2016). This was also evident in patients receiving neo-adjuvant chemotherapy (anthracycline followed by taxane) treatment regimens (Iwase *et al*., 2016). In fact, treatment protocols for overweight and obese cancer patients includes prescribed lower doses of chemotherapeutic agents to avoid co-morbidities, side effects and adverse toxicities (Lyman & Sparreboom, 2013). This could also compromise treatment efficacy and contribute to the development of treatment resistance (Ritzmo *et al*., 2007). However, alterations in dosages cannot clarify all occurrences of treatment resistance in relation to obesity (Chen *et al*., 2016). This is especially significant since, mesenchymal like-TNBC is associated with poorer prognosis since only approximately 20% of cases respond to therapy and there is no molecular based regimen to treat this breast cancer subtype currently (Park *et al*., 2018).

1.2.2 Cancer Treatment Resistance Mechanisms

Cancer cells have developed both intrinsic (pre-treatment) and acquired (post-treatment) mechanisms to achieve drug resistance. Currently, known cancer drug resistance mechanisms in obese states include alterations in drug uptake (decreased) and efflux (increased), increased drug sequestering (endo-lysosome and exocytosis) as well as metabolic inactivation, enhanced DNA repair mechanisms, evading therapy-induced apoptosis and altering receptor expression, highlighting the complexity of drug resistance (Wind & Holen, 2011; Zahreddine & Borden, 2013) (Figure 1.4).

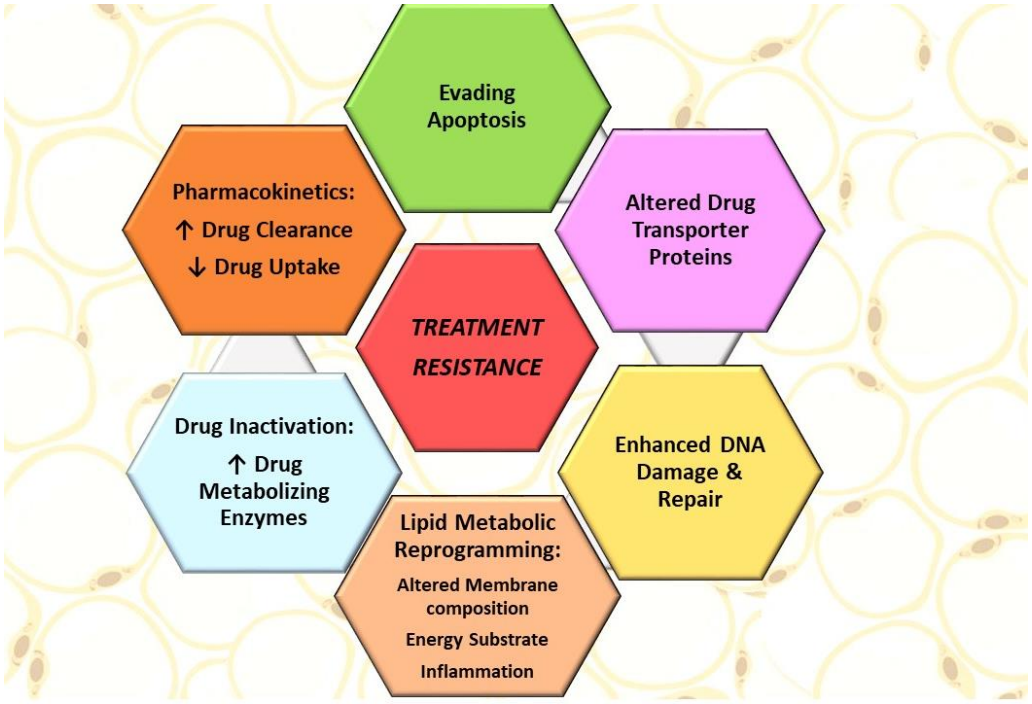

Figure1.4: Overview of proposed cancer drug resistance mechanisms in relation to obesity.

1.2.2.1 Cellular Drug Resistance Mechanisms Linked to Obesity

Treatment resistance can develop due to the evasion of apoptotic pathways (cell-death) by increased anti-apoptotic protein (blc-2) and decreased pro-apoptotic protein (bax) expression (Behan *et al*., 2009) (Figure 1.4). Adipocytes shield cancer cells from chemotherapeutic agents (i.e. vincristine and daunorubicin) by upregulating anti-apoptotic bcl-2, and downregulate the pro-apoptotic bad and pim-2 family members (an oncogene which phosphorylates bad) (Behan *et al*., 2010). The mechanisms by which adipocytes achieved this 'protection of cancer cells' was however not assessed. Nevertheless, a recent *in vitro* study identified resistin (an adipokine mainly secreted from adipose tissue) as a causal factor for acquiring resistance to doxorubicin treatment in both the MCF-7 human oestrogen receptor positive (ER^+) breast cancer cell line as well as in the MDA-MB-231 human TNBC cell line. Here, doxorubicin induced apoptosis (increased cytochrome-c, cleaved caspase-9, cleaved-poly (ADP-ribose) polymerase (PARP) expression) in a time and dose dependant manner. Addition of recombinant resistin to the treatment protocol, downregulated apoptosis through induction of autophagy to evade cell death (Liu *et al*., 2017). Although resistin receptor expression was not assessed with no supporting evidence for animal or human models provided, it would be plausible to motivate for more experimental research to investigate potential mechanisms and causal factors involved

in acquiring doxorubicin treatment resistance in relation to obesity/adipose tissue related secretory factors.

It is probable that treatment resistance can be the result of gene mutations coding for apoptotic proteins. For example, the mutation in p53 that has been associated with acquired resistance to doxorubicin in breast cancer patients, possibly due to inhibition of apoptosis by activating Bax/Bak (pro-apoptotic factors) (Ochs-Balcom *et al*., 2015). General and central obesity showed a positive association with mutations in p53 of tumour tissue, which was further associated with less favourable tumour characteristics including poorly differentiated and higher nuclear grade tumours in breast cancer patients (Park *et al*., 2011).

Modifications in the activation and expression of drug transporter proteins alters drug responses by reducing intracellular drug concentration and thereby promotes treatment resistance (Jones *et al*., 2016) (Figure 1.4). A few examples include (i) P-glycoprotein (P-gp), (ii) multi-drug resistance protein-1 (MDR-1), (iii) multi-drug resistance associated protein-1 (MDRP-1), and (iv) breast cancer resistance protein, which are ATP-binding cassette (ABC) transmembrane pumps responsible for the elimination of toxic compounds from cells (Jones *et al*., 2016).Otherwise normally expressed in healthy tissue, overexpression of P-gp, MDR-1, MDRP-1 and breast cancer resistance protein are present in breast cancer cells in relation to doxorubicin resistance (Hembruff *et al*., 2008; Pajic *et al*., 2009; Rottenberg *et al*., 2007). P-glycoprotein expression can be upregulated by inflammation (NFκB), resulting in an altered expression of MDRP-1 to increase the expression of P-gp and consequently modify drug responses (Xu *et al*., 2014; Malvi *et al*., 2016). Evidence on drug transporter proteins under obesogenic conditions in breast cancer are lacking and further research is needed to provide some understanding on this involvement.

Adipose tissue provides a source of mesenchymal stem cells which has similar characteristics to tumour-initiating stem cells (Onstad *et al*., 2016) and can be recruited to the tumour microenvironment to support breast tumour growth and proliferation (Zhao *et al*., 2018). Tumour-initiating stem cells have the ability to self-renew and/or differentiate, tolerate high levels of DNA damage and induce repair, increase ABC transmembrane transporter protein expression and induce the synthesis of various cytokines and growth factors (increased IL-6 and C-C motif ligand 5 (CXCL5) levels) (Candelaria *et al*., 2017; Schweizer *et al*., 2015; Yao *et al*., 2017), and therefore may be an alternative treatment resistance mechanism (Figure 1.4). Elevated leptin concentrations and leptin receptor expression (increased in adiposity) is

associated with the promotion of cancer stem cells survival and self-renewal, by inducing JAK2/STAT-3 signalling pathways that increase stem cell renewal transcription factors (NANOG, OCT-4 and SOX-2) expression in breast cancer cells (Zheng *et al*., 2013; Sultana *et al*., 2017).

Obesity is also characterised by increased synthesis of pro-inflammatory cytokines (IL-6 and TNF-α) and growth factors (IGF-1), leading to the induction of mitogenic related signalling pathways to promote and sustain cancer cell survival (Iyengar *et al*., 2013). Additional growth factors secreted by adipocytes, and implicated in treatment resistance, include IGF-1 and IGF-1R (insulin-like-growth factor-receptor-1) (increased systemic bioavailability in obesity and adipocytes also secrete IGF-1). These growth factors are linked to decreased apoptosis, increased cancer cell proliferation and pro-inflammatory mediator secretion, which are directly associated with breast cancer risk and progression (Brahmkhatri *et al*., 2015; Lewitt *et al*., 2014; van Bunderen *et al*., 2013). Upregulation of IGF-1R was associated with poor disease prognosis and chemotherapy resistance through increased expression of MDR-1 and MDRP-1 affecting drug transportation and delivery to cancer cells (Vigneri *et al*., 2015).

Acquired resistance to doxorubicin and docetaxel in breast cancer cells was also attributed to the transfer of microRNA present in exosomes (nano-vesicles which mediates cell-cell transfer of DNA, mRNA, microRNA, proteins and lipids) (Chen *et al*., 2014). Adipocyte derived exosomes has been associated with increased migration in breast cancer cells (Lin *et al*., 2013), immune cell recruitment of macrophages and chronic inflammation (Eguchi *et al*., 2015; Kranendonk *et al*., 2014). Resistance to paclitaxel in ovarian cancer cells was attributed to the transfer of microRNA (miR21) present in adipocyte derived exosomes (Au *et al*., 2016; Jia *et al*., 2017), which downregulated the expression of apoptotic protease activating factor-1, a key protein involved in apoptosome formation, thereby inhibiting apoptosis (Au *et al*., 2016). Additionally, adipocyte derived exosomes increased the invasion of melanoma cancer cells and induced metabolic reprogramming by transferring proteins (ECHA (subunit of *mitochondrial trifunctional protein) and* hydroxyacyl-coenzyme A dehydrogenase), involved in FA oxidation to these cancer cells. Interestingly, these effects were found exacerbated in obese adipocytes (Lazar *et al*., 2016). However, evidence on the exact role of adipocyte, and/or obese adipocytes derived factors in breast cancer on treatment resistance are lacking and therefore motivates experimental models to investigate potential mechanisms and causal factors involved in acquiring doxorubicin treatment resistance.

1.2.2.2 Drug Metabolism Treatment Resistance Mechanisms Linked to Obesity

It is reported that obesity alters chemotherapeutic pharmacokinetics by; (i) *increasing drug distribution*, (ii) *altering drug clearance*, and (iii) *modifying the drug-protein binding process* (Thompson *et al*., 2009) (Figure 1.4). Obesity increases the distribution volume of lipolytic drugs by increasing its accumulation in excess adipose tissue (Thompson *et al*., 2009), thereby decreasing exposure of cancer cells to treatment agents. Behan *et al*. (2009) showed that excess adipose tissue could act as a "*shelter*" for protection against treatment toxicity, as cancer cells migrate into adipose tissue.

In obese patient'sdrug clearance is negatively affected *via* the liver, which primarily metabolises, detoxifies and clears drugs from circulation (Griggs *et al*., 2012). Hepatosteatosis impairs hepatic microcirculation, whereas the glomerular filtration, tubular secretion, and reabsorption in the kidneys leads to increased drug clearance (Lyman & Sparreboom, 2013). Using a high fat mice model, Ghose *et al*. (2010) showed a decreased expression of key hepatic drug metabolizing enzymes (i.e. CYP3A11, CYP2B10 and CYP2A4), resulting in high levels of IL-1β, IL-6 and TNF-α (pro-inflammatory markers), as well as increased phosphorylation of JNK and NFκB. CYP34 activity has also been shown to be increased in leptin knockout induced obesity (Behan *et al*., 2010). In addition, the elimination, or the half-life of a drug may also be altered in obese individuals (Hanley *et al*., 2010). Lastly, obesity is also associated with an increase in alpha-1 acid glycoprotein concentration that increase the binding of drugs in the plasma and lowering its bioavailability (Sheng & Mittelman, 2014).

Furthermore, cytarabine (treatment agent in acute myeloid leukaemia), is only toxic to cancer cells in its phosphorylated form (cytarabine triphosphate) (Mansoori *et al*., 2017). Cancer cells disrupt the phosphorylation reactions by altering the expression of enzymes involved in the metabolic activation of cytarabine i.e. aldo-keto reductase (AKR) and carbonyl reductase (CBR) (Wind & Holen, 2011). Sheng *et al*. (2017), showed that adipocytes metabolised daunorubicin by increasing expression of daunorubicin-metabolizing enzymes (such as AKR-1C1, AKR-1C2, AKR-1C3 and CBR-1), which lead to the inactivation of daunorubicin and acquired treatment resistance (Figure 1.4). This directly implicates adipocytes/adipose tissue as a co-factor to decrease certain drug concentration in lipid-enriched tumour microenvironments (Sheng *et al*., 2017). Recent evidence suggests that cancer cells 'manipulate' adipocytes in the tumour microenvironment to survive, but also alter drug

pharmacokinetics and induce drug resistance through the disruption of lipid storage and metabolism (Balaban *et al*., 2017; Duong *et al*., 2015) (Figure 1.4).

Taken together, several mechanisms exist which can result in the modification of drug metabolism, drug transport and the failure of tumour cells to respond to chemotherapeutic drugs, due to overexpression of drug export proteins in cancer cells (Thompson *et al*., 2009). It should be emphasized that limited evidence for the role of overweight/obesity on pharmacokinetics of the majority of anti-cancer drugs in clinical trials exists (Griggs *et al*., 2012; Horowitz & Wright, 2015). This is mainly attributed to participant inclusion criteria into phase I clinical trials and pharmacokinetic analyses, that exclude patients with co-morbidities, which is highly prevalent in overweight and obese cases (Horowitz & Wright, 2015).

1.3 BREAST CANCER TREATMENT RESISTANCE: Role of Lipids and Fatty Acid Metabolism

1.3.1 Basic Physiology of Fatty Acids

Mammals acquire a wide range of dietary fats and oils, including FAs, known as long-chain hydrocarbons molecules (Kihara *et al*., 2012). Post prandially; FAs are subjected to various biochemical processes ranging from β-oxidation for energy, storage within lipid droplets of adipose depots in the form of TAGs as well as incorporation into phospholipids as major structural components of all cellular membranes (Hussein, 2013). Additionally, FAs are involved in autoimmune responses and regulation of transcription and cellular signalling due to active lipid derivative molecules (Fritsche *et al*., 2015), thus implicating any dysregulation or dysfunction of FAs in various diseases including obesity and breast cancer (Fabian *et al*., 2015).

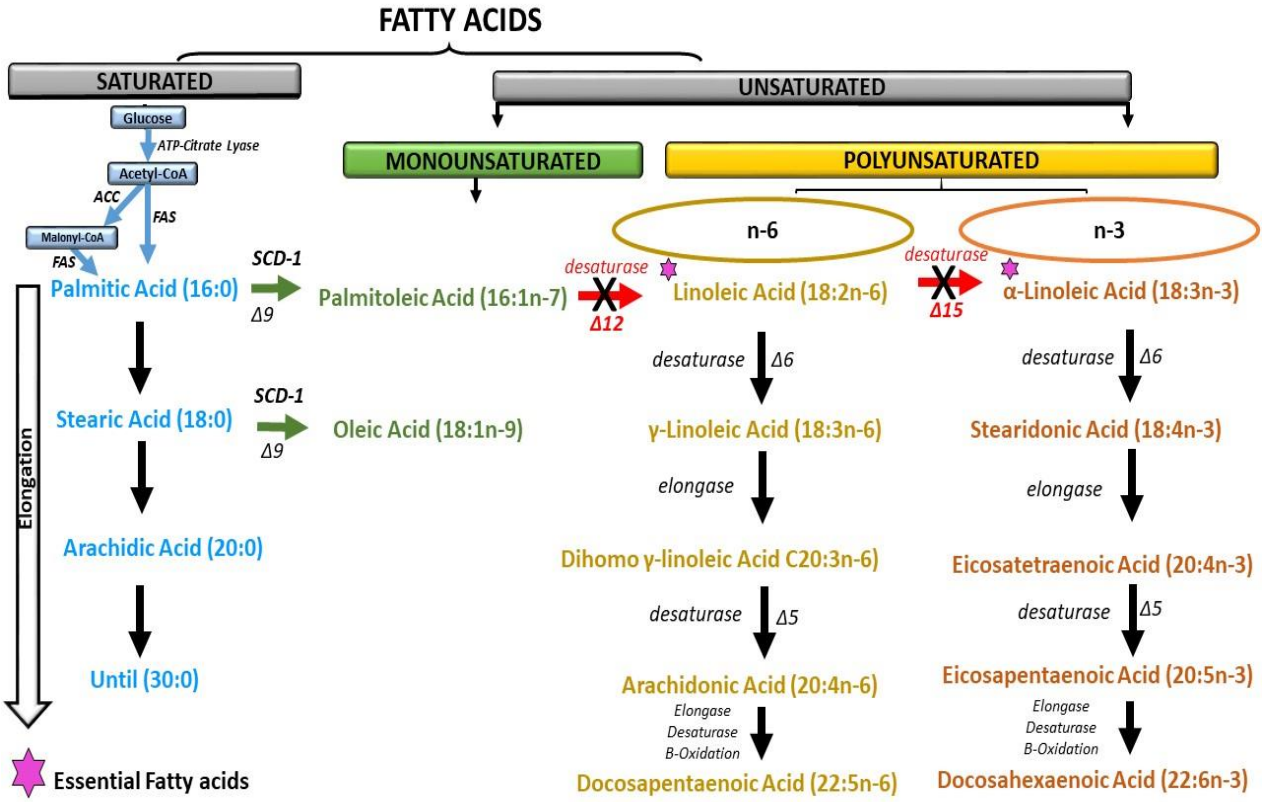

Figure 1.5: Fatty acid classification and the repertoire of fatty acids with different saturation levels. n-3, omega-3 PUFA; n-6, omega-6 PUFA; ACC, Acetyl-CoA carboxylase enzyme; FAS, fatty acid synthase enzyme; SCD-1, stearoyl-CoA desaturase 1 (Adapted from Chajès *et al*., 2011; Jump *et al*., 2012).

Fatty acids can be classified either as *saturated* or *unsaturated*, and further sub-classified as monounsaturated (MUFAs) or polyunsaturated FA (PUFAs) (Hussein, 2013) (Figure 1.5). Saturated fatty acids (SFAs), are characterised by a carbon (C) skeleton, where every single C-molecule has the maximum number of hydrogen (H) atoms allowed present, whereas in unsaturated FAs, a H-pair is missing due to the presence of a double bond (Kihara, 2012). Monounsaturated FAs only has one double bond, whereas PUFAs has multiple double bonds present in the C-back bone. This double bond can also be in a specific configuration either *cis* or *trans*. When the two H-atoms are on opposite sides of the double bond, the configuration is described as *trans*, and when the H-atoms are present on the same side of the double bond the configuration is known as *cis* (Kihara, 2012).

Fatty acids can either be newly synthesised (*de novo*), or it can be absorbed through the diet (Mashima *et al*., 2009). The biochemical process by which FAs are produced from diet-derived carbons is known as *de novo* FA synthesis (Figure 1.6) (Cha & Lee, 2016). Fatty acid

biosynthesis occurs in a limited number of tissues including the liver, adipose and lactating breast tissues (Baenke *et al*., 2013).

The first step in this complex biosynthesis involves the conversion of pyruvate to citrate *via* the tricarboxylic acid (TCA) cycle from diet-derived carbons (Cha & Lee, 2016) (Figure 1.6). Next, glucose-or glutamine-derived citrate being converted to Acetyl-CoA, catalysed by the ATP-citrate lyase enzyme (Mashima *et al*., 2009) (Figure 1.6). Acetyl-CoA is then converted to malonyl-CoA, a process catalysed by the Acetyl-CoA carboxylase (ACC) enzyme (Figure 1.6). Next, both Acetyl-CoA and malonyl-CoA are coupled to the acyl-carrier protein domain of the fatty acid synthase (FAS) enzyme, where these acyl groups undergo condensation reactions, which results in a 16-C SFA known as palmitic acid (PA) (Figure 1.6) (Currie *et al*., 2013).

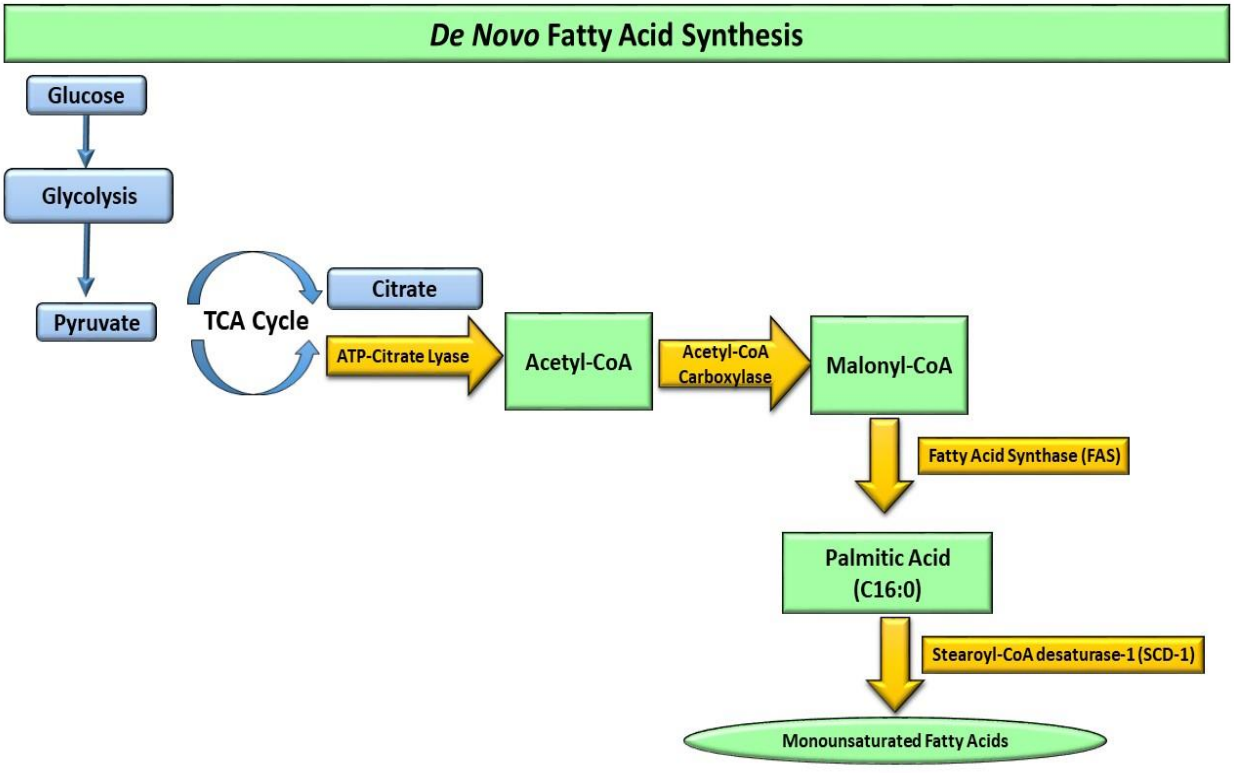

Figure 1.6: Figure illustrating *de novo* fatty acid synthesis. FAS, fatty acid synthase enzyme; SCD-1, stearoyl-CoA desaturase 1; TCA, tricarboxylic acid cycle. (Adapted from Song *et al*., 2018).

Palmitic acid (PA) (16:0) (can also be acquired from the diet), is subjected to subsequent elongation as well as desaturation reactions, which produces a repertoire of saturated and unsaturated FAs with different saturation levels (Kihara, 2012) (Figure 1.5 and Figure 1.6). One of the most prominent desaturases enzymes known as stearoyl-CoA desaturase 1 (SCD-1) (Figure 1.5), functions to incorporate a double bond at the Δ9 position of both PA and SA to generate MUFAs namely palmitoleic acid (16:1n-7, PTA) and oleic acid (18:1n-9, OA) respectively (Huang *et al*., 2016) (Figure 1.5). Mammalian cells have a limited ability to synthesize PUFAs *de novo*, due to a lack in Δ 12 desaturase enzyme (Zaidi *et al*., 2013) (Figure 1.5), thereforelinoleic acid (LA, n-6) and α-linolenic acid (ALA, n-3) are regarded as essential FAs have to be acquired through the diet (Kalupahana *et al*., 2011).

1.3.2 Evidence of Fatty Acid Profiling in Breast Cancer

Little is known on the significance of changes occurring in FA composition in breast tumours as well as its adjacent adipose tissue. Studies investigating adipose tissue FA profiles in breast cancer have yielded some noteworthy results and are summarised in Table 1.1.

Evidence associates increased SFA intake with increased breast cancer risk (Hirko*et al*., 2018; Xia *et al*., 2015). Both SFAs and MUFAs (omega-7 (n-7), and omega-9 (n-9)), have been implicated for their roles in cell membrane composition, as they are more resistant to lipid peroxidation, thereby directly protecting cancer cells against oxidative stress (either immunological and/or therapy induced) (Rysman *et al*., 2010).Both animal and cell cultured based models have also provided sufficient evidence implicating two PUFA families, namely omega-6 (n-6) and omega-3 (n-3), derived from essential dietary FAs (i.e. LA (n-6) and ALA (n-3), which humans cannot synthesize endogenously) (Das, 2006), in breast cancer development, progression as well as treatment resistance (Zalba & Ten Hagen,2017). One primary mechanism involves inflammation, where n-6 PUFAs exhibit pro-inflammatory, and the n-3 PUFA anti-inflammatory effects (Fabian *et al*., 2015). The pro-inflammatory effects of n-6 PUFA are due to the diversity of functions associated with eicosanoids, prostaglandins and leukotrienes (Kremmyda *et al*., 2011). These lipid-derived bioactive mediators upregulates signalling pathways that are involved in angiogenesis, cell-proliferation and inflammation (Wang *et al*., 2006), to contribute to an ideal microenvironment favouring mammary carcinogenesis.

Table 1.1: Fatty acid and lipodomic profiling in cancer patients.

Reference	Cancer Type	Sample Type	Findings
Maillard *et al*., 2002	Breast cancer cases (n=241) *vs* controls (n=88)	Breast adipose tissue collected during lumpectomy or mastectomy	▪ Majority of FAs were MUFAs n-9 (OA), SFAs (PA & SA) & n-6 PUFAs (LA).
Witt *et al*., 2009	Breast cancer cases (n=463) *vs* controls (n=685)	Gluteal adipose tissue	▪ Majority of FAs were MUFAs n-9, SFAs (PA & SA) & n-6 PUFAs (LA).
Bree *et al*., 2013	Breast cancer (n=94) *vs* benign breast (n=57) tumours	Breast and subcutaneous gluteal adipose tissue	▪ Breast cancer cases displayed significantly higher total MUFAs levels *vs* benign cases. ▪ Total PUFAs, n-3, n-6 & n-6: n-3 ratio lower in buttock adipose tissue of benign *vs* breast cancer cases. ▪ Total PUFAs, n-6 & n-6: n-3 ratio in breast adipose tissue, were significantly higher in benign cases compared to breast cancer cases.
Mohammadzadeh *et al*., 2014	Breast cancer cases (n=12)	Tumour samples *vs* adjacent normal appearing tissue	▪ Majority of FAs were SFAs, MUFAs and PUFAs n-6. ▪ Increased MUFAs (OA), n-6 PUFAs (AA) and MUFAs: SFA ratio found in tumour *vs* adjacent tissue.
Cottet *et al*., 2015	Colorectal cancer cases (n=203) *vs* controls (n=233)	Gluteal adipose tissue	▪ Majority of FAs were MUFAs in cancer cases.
Shaikh *et al*., 2017	Breast cancer cases (n=135) *vs* controls (n=50)	Serum lipid fractions	▪ Breast cancer cases displayed significantly higher SFAs (PA & SA) and MUFAs (OA) levels in breast cancer cases *vs* controls. ▪ Decreased n-3: n-6 ratio in breast cancer cases *vs* controls. ▪ Serum total cholesterol and LDL-c was increased in breast cancer patients *vs* controls.
Kang *et al*., 2011	Breast cancer cases (n= 34)	Tumour samples *vs* adjacent normal epithelial tissue	▪ Phosphatidylcholines levels were higher in breast tumour tissue *vs* to adjacent tissue. ▪ Found distinct lipid profiles for breast cancer subtypes.
He *et al*., 2015	Breast cancer cells	Breast cancer cell lines (MCF-7, MDA-MB-231, and MDA-MB-361) vs normal breast epithelial cells (MCF-10A)	▪ Membrane lipid composition differs between breast cancer and normal cell line.
More *et al*., 2017	Breast cancer cases (n=25) *vs* benign (n=28) and age-matched controls (n=28)	Serum lipid fractions	▪ Serum phospholipid composition differed between breast cancer patients, compared to benign and healthy counterparts.

Abbreviations: AA, arachidonic acid; FAs, fatty acids; LDL-c, low density lipoprotein-cholesterol; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; n-3, omega-3; n-6, omega-6; n-9, omega-9; n-3: n-6, omega-3: omega-6 ratio; PA, palmitic acid; OA, oleic acid; LA, linoleic acid; SFAs, saturated fatty acids; SA, stearic acid.

Increased FFA (palmitic-, lauric-, stearic- and behenic acid) (Lee *et al*., 2004; Milanski *et al*., 2009), stimulate the secretion of pro-inflammatory mediators *via* NFκB signalling pathway to further enhance the inflammatory state (Choe *et al*., 2016). In contrast, n-3 PUFAs, display inhibitory effects towards mammary tumorigenesis (Vandersluis *et al*., 2017), however human investigations to date provide contradictory results to breast cancer on these n-3 PUFAs (Azrad *et al*., 2013). For example, epidemiological studies report on an inverse association between n-3 PUFAs intake and breast cancer risk (Murff *et al*., 2011; Sczaniecka *et al*., 2012), while others reported no association (Kiyabu *et al*., 2015; Zheng *et al*., 2018).These differences can be ascribed and related to various factors such as the heterogeneous nature of diseases like obesity and breast cancer and all its associated subtypes, methodological limitations, study designs, statistical analysis, inadequate sample sizes, heterogeneity in fat intake, and dietary assessment methodology. However, credible clinical trials are limited (extensively reviewed in Serini *et al*., 2016).

1.3.3 Role of Fatty Acids in Carcinogenesis: Underlying Mechanisms

The role of FAs in breast cancer risk, progression and anti-cancer treatment resistance is proposed to be achieved through multiple mechanisms (Figure 1.7). Some of these mechanisms include, but are not limited to, the role of FAs in cell membrane composition, source of bioactive lipids (inflammation), metabolic reprogramming, and utilization as a potential energy substrate (Zaidi *et al*., 2013; Wang *et al*., 2016) (Figure 1.7), which will be discussed broadly in the following sections.

1.3.3.1 Role of Lipids in Metabolic Reprogramming

Sufficient evidence demonstrates that cancer drug resistance may be due to cellular metabolic reprogramming (Reviewed in Gandhi & Das, 2019), which is now considered a hallmark of cancer. This is not questionable since cancer cells are characterised by unique metabolic properties including enhanced aerobic glycolysis, glutaminolysis, *de novo* fatty acid synthesis and exogenous lipid uptake, resulting in increased synthesis of proteins, nucleic acids and lipids, which cancer cells can utilize to sustain survival (Bansal e*t al*., 2013; Bhattacharya *et al*., 2016)(Figure 1.7).

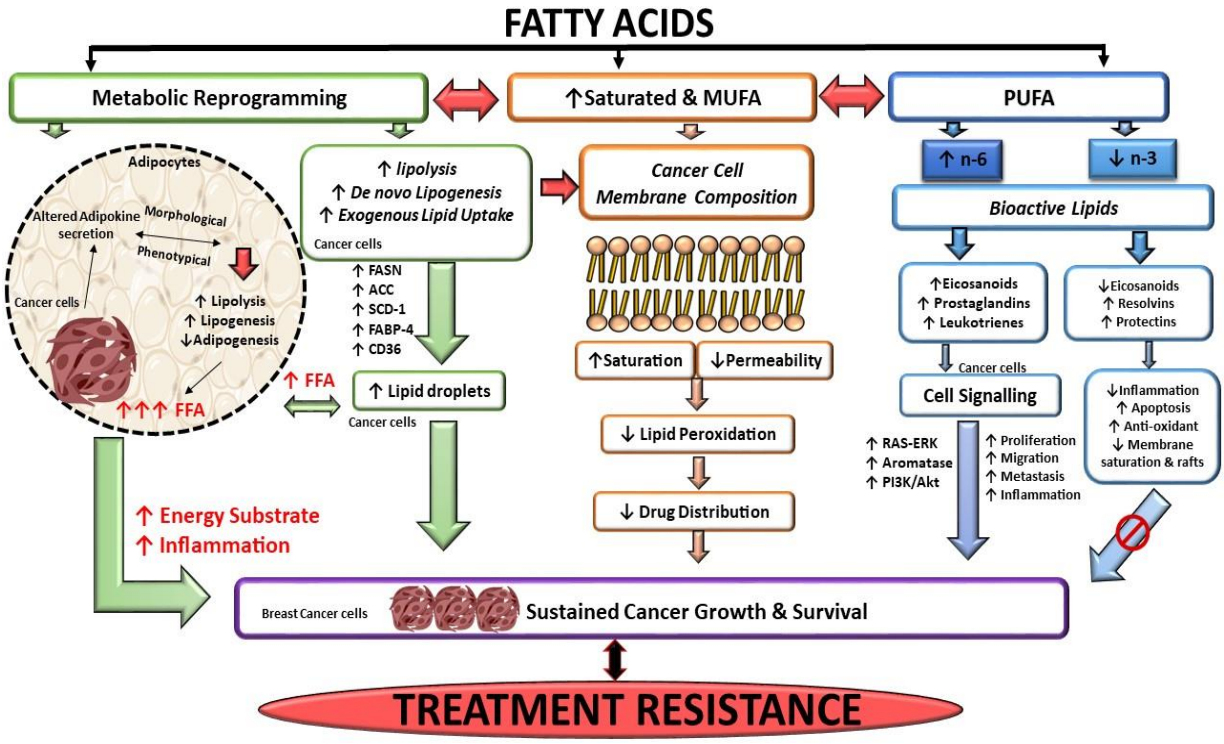

Figure 1.7: Overview of the role of FAs in cancer progression and anti-cancer drug resistance. The multifaceted role of FAs in cancer progression and anti-cancer treatment resistance is proposed to be achieved through multiple mechanisms. This includes the role of fatty acids in cell membrane composition (SFAs and MUFA), source of bioactive lipids [PUFAs (n-3 and n-6)], and role in metabolic reprogramming and can serve as an additional energy source that cancer cells can utilize to sustain survival and treatment resistance.n-3, omega-3 PUFA; n-6, omega-6 PUFA; ACC, Acetyl-CoA carboxylase enzyme; FAs, fatty acids; FABP-4, fatty acid binding protein-4; FAS, fatty acid synthase enzyme; FFA, free fatty acids; MUFAs, monounsaturated fatty acids; PI3K, phosphoinositide-3-kinase; PUFAs, polyunsaturated fatty acids; SCD-1, stearoyl-CoA desaturase 1 (Mentoor *et al*., 2019).

Metabolic reprogramming is pivotal to cancer cells to sustain the high-energy demand required for cellular proliferation (Long *et al*., 2016). The most well-known reprogramming mechanism is known as the Warburg effect where cancer cells increase aerobic glycolysis even in the presence of oxygen, which is characterised by an altered expression of various glucose transporters and glycolysis enzymes i.e. hexokinase and lactate dehydrogenase-A (Bansal e*t al*., 2013). Focusing on metabolic reprogramming in the context of carcinogenesis has shifted from glycolytic metabolic pathway alterations to alterations in other metabolic pathways i.e. protein, nucleic acid and lately an increased emphasis on lipid biosynthesis and uptake. Lipid metabolism is now recognized as a fundamental part of metabolic reprogramming, where cancer cells disrupt normal lipid metabolism by increasing exogenous lipid uptake and *de novo*

synthesis of lipids and cholesterol respectively (Guo *et al*., 2014; Veigel *et al*., 2015) (Figure 1.7).

While the majority of tumour types are dependent on high glucose uptake and consumption, some tumour types i.e. prostate tumours and leukaemia cells have been shown to utilize FAs as the main energy source *via* β-oxidation (Liu, 2006; Tabe *et al*., 2017). Breast cancer cells utilize lipids as an energy source for survival. Evidence to support these findings include studies reporting on adipocytes providing FAs to breast cancer cells by inducing morphological and phenotypical changes in adipocytes by inducing dedifferentiation, or activation of lipogenesis, lipolysis or inhibition of adipogenesis (Balaban *et al*., 2017; Park *et al*., 2011) (Figure 1.7). These FAs become available metabolic substrates for the benefit of cancer cell survival, either by storage in the form of lipid droplets, membrane lipids or energy production *via* β-oxidation (Lengyel *et al*., 2018).

1.3.3.1.1 Adipocytes in the Tumour Microenvironment: Lipid-Related Mechanisms

Breast cancer cells co-exist in a sophisticated microenvironment with various adjacent cell types including adipocytes, macrophages, fibroblast and endothelial cells (Hefetz-Sela & Scherer, 2013). Although existing evidence show beneficial roles of fibroblasts, endothelial cells and macrophages in the tumour microenvironment (Hoy *et al*., 2017; Liao *et al*., 2018; Tao *et al*., 2017), the exact role of adipocytes in the breast tumour microenvironment in breast cancer treatment resistance remains unclear in the context of obesity.

The presence of adipocytes in the tumour microenvironment revealed that breast tumour cells utilize adipocytes to their advantage to promote its survival, growth as well as proliferation and metastasis (Balaban *et al*., 2017; Duong *et al*., 2015). In addition, the presence of adipocytes in the tumour microenvironment also reduces the toxic effects of breast cancer treatment agents (Incio *et al*., 2018). For example, Trastuzumab® treatment (a monoclonal antibody targeting human epidermal growth factor receptor-2 (HER-2)) inhibited breast cancer cell growth in the absence of a lipoma. However, this inhibition was hindered in the presence of a lipoma suggesting that adipose tissue/adipocytes may have an impact on resistance to breast cancer therapy (Duong *et al*., 2015).

Adipocytes in the breast tumour microenvironment is also characterised by both morphological and phenotypical changes. This finding is supported by histological analysis of human mammary tumour biopsies showing no, or very few adipocytes present (Sheng & Mittelman, 2014), with characteristic smaller cell size (Dirat *et al*., 2011). Adipocytes in the breast tumour microenvironment also display a more fibroblast like morphology known as cancer-associated adipocytes (Bochet *et al*., 2013; Park *et al*., 2011). These phenotypical and morphological alterations induce functional changes in adipocytes to yield FFA from TAGs stored in lipid droplets (Figure 1.8) (Choi *et al*., 2018). This is proposed to be as a result of tumour growth inducing lipolysis in adipocytes, which can result in adipose tissue mass reduction (Arner & Langin, 2014).

It was shown that breast cancer cells induce adipocytes to release stored FAs by inducinglipolysis (Balaban *et al*., 2017), a reaction catalysed by adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL), which hydrolyses TAGs from adipocytes to render FFA (Choi *et al*., 2018; Nomura*et al*., 2010).

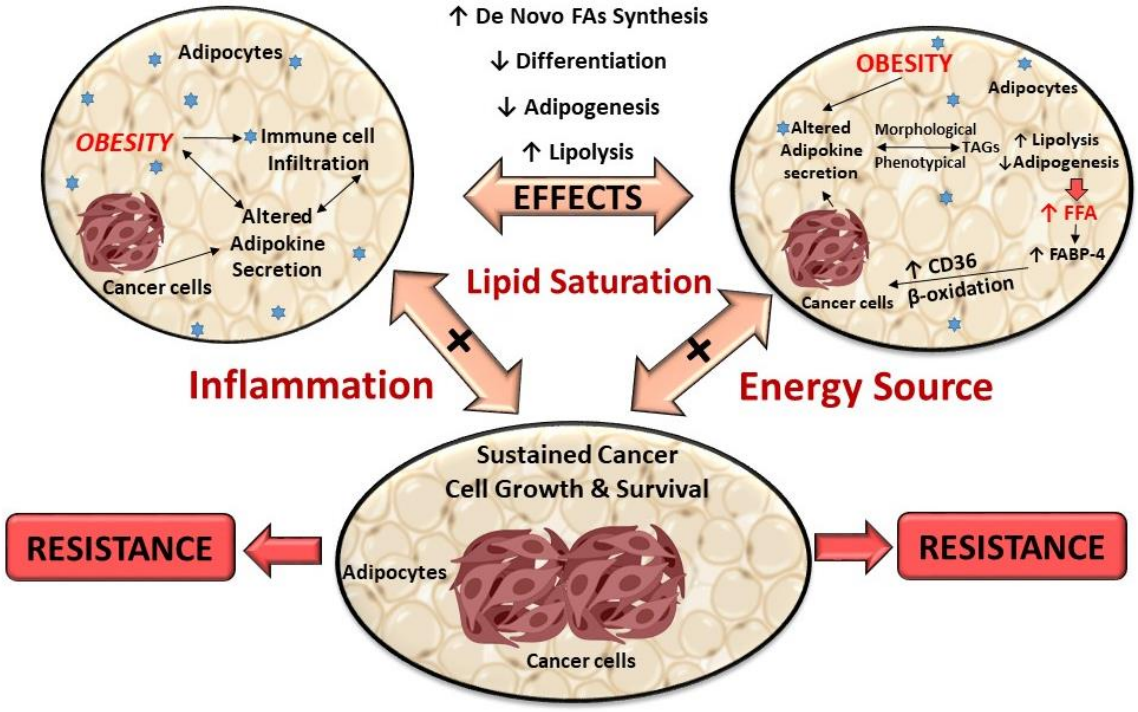

Figure 1.8: Proposed effects of breast cancer cells on adipocytes and its role in treatment resistance. Breast cancer cells dysregulate metabolic pathways by altering the secretion of adipokines from adipocytes which results in inflammation. FABP-4, fatty acid binding protein-4; FAs, fatty acids; FFA, free fatty acids; TAGs, triglycerides; CD36, fatty acid translocase.

Balaban *et al*. (2017) showed that FFA release was exacerbated in 'obese' adipocytes, which breast cancer cells used for proliferation and migration, however treatment resistance was not assessed in this obese breast cancer model. It is proposed that adipocyte-derived FFA are either used as metabolic substrates for energy (β-oxidation) (Lengyel *et al*., 2018), or stored in lipid droplets and/or membranes within tumours (Steuwe *et al*., 2014; You *et al*., 2016) to sustain survival. Fatty acids and its derivatives serve as building blocks for various membrane lipids (i.e. phospholipids and sterol esters) and signalling molecules that are involved in carcinogenesis and the development of breast cancer treatment resistance (Greene *et al*., 2011; Nakajima *et al*., 2017; Rysman *et al*., 2010).

An increase in exogenous FA uptake and consumption *via* FA β-oxidation in breast cancer cells is evident, a finding reported by Kuemmerle *et al.* [2011] in triple-negative breast cancer cell lines, which expressed lipoprotein lipase, which plays a role in the breakdown of TAGs into FFA in lipoproteins (Figure 1.8) (Choi *et al*., 2018).Additional supporting evidence include breast cancer cells increasing exogenous FA uptake and utilization (FFA derived from mammary adipocytes), by altering the expression of various proteins in FA uptake (i.e. increased fatty acid binding protein-4 (FABP-4, which acts as an intracellular lipid chaperone), CD36 (fatty acid translocase, transmembrane channel responsible for exogenous FA uptake) (Guaita-Esteruelas *et al*., 2017; Nath *et al*., 2015; Nieman *et al*., 2011) and β-oxidation (i.e. increased carnitine palmitoyltransferase I expression) (Balaban *et al*., 2017; Luo *et al*., 2017; Wang *et al*., 2018).

Adipocytes supply FFA to breast cancer cells by dedifferentiation and/or inhibition of adipogenesis (Figure 1.8). This is evident in adipocytes showing decreased expression of adipogenic markers such as (CCAAT) enhancer binding protein-α (CEBPα), peroxisome proliferator-activated receptor-γ (PPAR-γ) and FABP-4 in adipocytes co-cultured with cancer cells (Dirat *et al*., 2011). This is corroborated in studies where cancer-associated adipocytes displayedmorphological changes characterised by a more fibroblast like morphology as well as the presence of a higher percentage of fibroblast to adipocytes in the tumour microenvironment (Park *et al*., 2011).

Increased levels of FFA are also stored in tumours in the form of lipid droplets in order to avert lipotoxicity, and/or to serve as an energy reserve (Scalfi-Happ*et al*., 2011). This is also supported by lipid depositions found in tumours (Steuwe *et al*., 2014), including breast tumours where it is associated with cancer aggressiveness (de Gonzalo-Calvo *et al*., 2015). Innovative

imaging technology (Raman-based imaging) is used to define tumour lipid droplet content, which can be utilized both as a monitoring or predicting tool for drug treatment response in cancer patients (Steuwe *et al*., 2014; You *et al*., 2016).

Under normal physiological conditions, *de novo* FA synthesis is suppressed, and only occurs in a small percentage of specific cell types including adipocytes (Weylandt *et al*., 2015). However, cancer cells show a strong dependence on endogenous FA synthesis due to their highly proliferative nature. Under these conditions, cancer cells increase the activation of *de novo* FA synthesis to benefit cancer cell survival. This is supported by increased expression of various enzymes involved in *de novo* FA synthesis such asAcetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in breast cancer cells (Long *et al*., 2016; Menendez & Lupu, 2017; Veigel *et al*., 2015; Yoon *et al*., 2007; Zhao*et al*., 2017).

Fatty acid synthase (FAS) expression was found to be significantly associated with increased expression of both epidermal growth factor and HER-2, which could further implicate FA synthesis in breast cancer treatment resistance (Omabe *et al*., 2015; Vazquez-Martin *et al*., 2007). Molecular analysis of tumour tissuefrom mice on a high fat diet revealed that increased FAS expression was associated with drug resistance (Malvi *et al*., 2016). Supporting evidence alsoshow that transcription factors like sterol regulatory element-binding protein 1 and adipokines, regulate FAS (Guo *et al*., 2014). Additionally, inhibition of FAS inhibits cancer cell growth and induces apoptosis (Alwarawrah *et al*., 2016; Fan *et al*., 2016; Lee *et al*., 2009). Co-treatment of breast cancer cells with Orlistat® (an inhibitor of FAS) and trastuzumab (monoclonal antibody against HER-2) also inhibited cell growth and induced apoptosis (Menendez *et al*., 2005).

Although the mechanisms remain unclear, it is proposed that cancer cells alteradipocyte-signalling pathways and gene expression in the tumour microenvironmentto induce stromal cells to produce adipokines (Nieman *et al*., 2013; Pramanik *et al*., 2013). Adipocytes secrete numerous adipokines (i.e. IL-6, leptin, IGF-1), which acts in a paracrine manner and binds to its respective receptors present on cancer cells (Divella *et al*., 2016), activating various signalling pathways (i.e. PI3K/Akt and MAPK), regulating cancer cell proliferation, migration and apoptosis (Roberts *et al*., 2010). Additionally, the dysregulation of adipokines (i.e. increased IL-6 and decreased adiponectin), has also been shown to regulate the expression of proteinsand transcription factors (HSL, FABP-4 and CEBPα) that are involved in lipid metabolism (Vaysse *et al*., 2017).

Cytokines also play a major role in drug resistance. Although ER^+ sensitive breast cancer cell lines including MCF-7 do not normally express IL-6, high levels of IL-6 were observed in a drug resistant breast cancer cell line (MCF-7/R) (Conze *et al*., 2001; Shi *et al*., 2012). Elevated IL-6 is linked to doxorubicin resistance in breast cancer cells, by increasing cytosine-cytosine-adenosine-adenosine-thymidine (CCAAT) enhancer binding protein (CEBP) activity, which leads to an increased expression of MDRP-1 (Conze *et al*., 2001; Tan *et al*., 2018). Additionally, *ex vivo* mature adipocytes significantly increased the proliferation of both mammary cancer cells (MCF-7) and normal mammary cells (184B5) (Bougaret *et al*., 2018). Adipocytes derived from obese patient's diminished Tamoxifen® treatment efficacy compared to adipocytes derived from normal weight patients. The authors identified IL-6, TNF-α and leptin as potential mediators (Bougaret *et al*., 2018). In agreement, Incio *et al*. (2018) demonstrated that the efficacy of anti-VEGF treatment was decreased in both obese breast cancer patients and in diet-induced obese mice. The authors proposed that inflammation (increased IL-6) and angiogenesis (increased fibroblast-growth factor-2) in adipocyte dense hypoxic microenvironments within tumours, which sustained tumour survival (Incio *et al*., 2018).

It is also proposed that the recruitment of immune cells to the tumour microenvironment, promotes inflammation, stimulating the secretion of matrix metalloproteinase-9 (role in matrix degradation) and evading the host's immune responses (Koti *et al*., 2015). Additionally, MCP-1 benefits vascular endothelial cell survival and activate the JAK2/STAT5 and p38 MAPK pathways, inducing angiogenesis (Nagarsheth *et al.*, 2017). Macrophage chemoattractant protein-1 (MCP-1) and CCLC-5 may also be active in the host microenvironment promoting survival, metastasis, and unfavourable drug responses. Lastly, adipocytes are also stimulated by breast tumour cells to increase expression of matrix metalloproteinase-11, a negative regulator of adipogenesis, by decreasing pre-adipocyte differentiation and reversing mature adipocyte differentiation (Toren *et al*., 2013).

To summarise, delipidation (increased FFA) as a result of increased pro-inflammatory cytokines and a deregulated adipokine profile in adipose tissue/adipocytes, may be responsible for breast tumour enhancing effects of adipocytes, providing a potential mechanism for cancer treatment resistance. However, the role and exact contribution of adipose tissue/adipocytes derived FA in the tumour microenvironment and pathogenesis of breast cancer remains unclear. The exact molecular mechanisms in which breast cancer cells in an obesogenic environment

use adipocyte to their physiological advantage to induce treatment resistance, needs to be explored extensively.

Breast cancer cells use endogenous and exogenous (adipocyte derived) FAs to induce treatment resistance by either using FFA as metabolic substrates for energy (β-oxidation), or stored in lipid droplets within tumours (Steuwe *et al*., 2014), to sustain survival. Additionally, FAs and its derivatives serve as building blocks for various membrane lipids (i.e. phospholipids and sterol esters) and signalling molecules, both implicated to favour carcinogenesis and promote the development of treatment resistance (Greene *et al*., 2011; Nakajima *et al*., 2017; Rysman *et al*., 2010).

1.3.4 Fatty Acids: Membrane Saturation and Drug Resistance

Fatty acids are essential components of cell membranesand play a major role in membrane fluidity (phospholipids), as well as in cell signalling pathways within and between cells (Hussein, 2013). The type of FA consumed in the diet therefore affects phospholipid FA composition and physical-chemical properties of plasma membrane fluidity in breast cancer cells (del Genio *et al*., 2015).

Cancer cells manipulate lipid metabolism in distinct ways in order to survive. For example, an increase in *de novo* FA synthesis leads to the enrichment of cancer cell membranes with either saturated, and/or MUFA (Zalba & Ten Hagen, 2017) (Figure 1.7). This concept is known as *membrane lipid saturation* which protect cancer cells from oxidative damage by reducing lipid peroxidation (Beloribi-Djefaflia *et al*., 2016; Rysman *et al*., 2010) (Figure 1.7). This phenomenon is due to the FAs (saturated and/or MUFA) being more resistant to lipid peroxidation when compared to polyunsaturated acyl chains, thus partly contributing to carcinogenesis by protecting breast cancer cells and increase survival of these cells (Rysman *et al*., 2010). Evidence indicates that an increase in membrane saturated FAs are present in the more aggressive breast cancer subtypes (Luo *et al*., 2017).

Fatty acid synthase (FAS) is one of the primary enzymes responsible for the biosynthesis of saturated FAs (Alwarawrah *et al*., 2016). Yan *et al*. (2014), showed that FAS was overexpressed in both MCF-7 and HER-2 breast cancer cells (SKBR3) and was induced by the PI3K/Akt/mTOR signalling cascade. Inhibition of both FAS (Cerulenin) and mTOR

(rapamycin) resulted in reduced tumour growth in MCF-7/HER-2 xenografts (Yan *et al*., 2014). Liu *et al.* (2008), showed that increased FAS protein expression was associated with drug resistance in the MCF-7 (ER^+) breast cancer cell line. Importantly, the study showed that FAS overexpression-mediated drug resistance in both MCF-7 (ER^+) and MDA-MB-468 (ER^-) breast cancer cells.Orlistat®(an inhibitor of FAS) reversed the drug resistance in MCF-7 cells (Liu *et al*., 2008). Therefore, it may be possible that altered FAS expression could have an effect on the FA composition and lipid profiles in breast cancer cells. This leads to changes in cell membrane lipid composition (densely packed membranes), and a decrease in transmembrane permeability to chemotherapeutic drugs inducing drug tolerance and resistance (Figure 1.7),protecting cancer cells from drug-induced apoptosis (Zaidi *et al*., 2013). In fact, tumours enriched with lipid droplets (TAGs and sterol esters) were found to be more resistant towards chemotherapeutic agents (Steuwe *et al*., 2014).

Stearoyl-CoA desaturase 1 (SCD-1), the enzyme that catalyses the conversion of SFAs to MUFAs (Manni *et al*., 2017), has been identified as a potential biomarker of breast cancer risk specifically in obese women. It has been shown that eicosapentaenoic Acid (EPA) and docosahexaenoic Acid (DHA) n-3 supplementation inhibits lipogenesis by downregulation of SCD-1, thereby attenuating the formation of MUFAs from precursor SFAs (Manni *et al*., 2017). In addition, SCD-1 expression was also found to be associated with advanced disease stage, grade as well as metastasis (Huang *et al*., 2016). It is possible that cancer cells induce changes in plasma membrane lipid composition by increasing *de novo* FA synthesis, which induces changes in signalling molecules i.e. membrane receptors (Hilvo & Orešič, 2012), that may affect several cellular processes such as signal transduction, gene expression and inducing treatment resistance.

Furthermore, PUFA also exert important effects on the structure and physical properties of localized membrane domains (lipid rafts) by modulating enzyme activities and membrane receptors (Turk *et al*., 2012). Here, lipid rafts which are characterised by a distinct lipid composition (high cholesterol and sphingolipidcontent) (Simons & Sampaio, 2011), have been implicated in membrane structure, cholesterol metabolism, protein and drug transmembrane pump localization, as well as signal transduction and T-lymphocyte activation (inflammation) that are related to carcinogenesis (Gelsomino *et al*., 2013; Murai, 2012). These lipid rafts also modulate protein activity, including receptors (Corsetto *et al*., 2012), such as IGF-1R and vascular endothelial growth factor-receptor (VEGF-R), which are linked to cellular

proliferation and apoptosis as well as angiogenesis (Li *et al*., 2006;Mollinedo & Gajate, 2015). Lipid rafts are higher in quantity in breast cancer cell membranes compared to normal breast epithelial cells, and are predominantly enriched withSFA-containing sphingolipid and cholesterol.The incorporation of PUFAs, especially docosahexaenoic acid (DHA), in breast cancer cell membranes disrupt lipid rafts and result in PUFA-rich/cholesterol-poor domains. This implies that alterations in cholesterol metabolism may also be linked to the regulation of lipid rafts within membranes of cancer cells. This is plausible, since a dysfunctional lipid fractions have been observed in breast cancer patients (Shaikh *et al*., 2017), where 27-hydroxycholesterol (metabolite of cholesterol) promotes tumour growth and metastasis (Nelson *et al*., 2013) and mediates aromatase treatment resistance in ER^+ breast cancer cells (Simigdala *et al*., 2016).

Alterations in cholesterol metabolism includes changes in physical-chemical properties, decreasing cholesterol content as well as altering the activity of transmembrane proteins such as G-protein coupled membrane receptors (Turk & Chapkin,2013). Corsetto *et al*. (2012), showed that PUFA (DHA and EPA), reduced cholesterol content in lipid rafts of MDA-MB-231 breast cancer cells (Corsetto *et al*., 2012) and induced changes in membrane composition, which functionally altered cell signalling (Turk *et al*., 2012; Ma *et al*., 2004). DHA and EPA administration also decreased the activation of epidermal growth factor receptor (EGFR) in lipid rafts. This lead to downregulation of downstream signalling pathways i.e. PI3K/Akt and MAPK that are involved in cellular survival and proliferation (Corsetto *et al*., 2011; Schley *et al*., 2007). Badana *et al*. (2016), showed that the disruption of lipid raft integrity by methyl-β-cyclodextrin (MβCD) treatment (a cholesterol sequestering agent), leads to the inhibition of cellular proliferation and induced apoptosis in MDA-MB-231 breast cancer cells. Co-treatment of MβCD with tamoxifen® and cisplatin, increased cellular toxicity of these treatments in both MDA-MB 231 and MDA-MB 468 breast cancer cells (Badana *et al*., 2016). Importantly, since adipocytes can also store large quantities of cholesterol, Lu *et al*. (2017) showed that cholesterol depletion (MβCD treatment) in adipocytes resulted in the disruption of lipid rafts which induced increased synthesis of MCP-1 by these adipocytes, providing evidenceof a link between lipid rafts, adipocytes and chronic inflammation (immune infiltration).

More recently, a high fat diet (HFD) significantly altered both the TAGs and phospholipid profile in the primary tumour of a Lewis Lung Carcinoma in vivo model. The HFD significantly increased PUFA-containing phosphatidylethanolamines and phosphatidylcholine

lipids and decreased both MUFA and SFA containing phosphatidylcholines and phosphatidylethanolamines present in tumours compared to mice on a low fat diet (LFD). This study provided evidence that a HFD (DIO), can lead to the incorporation of dietary FAs into TAGs in tumours. This in turn suggests that cancer cells can acquire lipids via exogenous routes (Sundaram *et al*., 2018), however the role of these lipids in treatment resistance was not assessed and is therefore still unclear.

1.3.5 Fatty Acids and Their Metabolites in Signalling Pathways

Fatty acids, apart from their cellular structural role, are utilised for the biosynthesis of lipid-signalling molecules that can benefit breast tumorigenesis (Fritsche *et al*., 2015). Lipids can also be hydrolysed into other bioactive lipid mediators such as eicosanoids and prostaglandins, that can regulate a variety of carcinogenic processes, including inflammation, cell growth, cell migration and metastasis (Wang *et al*., 2010) (Figure 1.7). Eicosanoids, generated from FAs with a 20-carbon skeleton, plays an important role in cellular signalling pathways (Hilvo *et al*., 2012). They are predominately synthesised from the n-6 PUFA arachidonic acid (AA) which are subjected to two major enzymatic pathways; (i) cyclooxygenase (COX), and (ii) lipoxygenase (LOX) pathways. The COX pathway metabolizes AA to produce prostaglandins, whereas the LOX pathway is involved in generating leukotrienes. Prostaglandins can be further converted into prostaglandin E_2, D_2, $F_{2\alpha}$, $I_2$and thromboxane that mainly act as important second-messenger molecules for cellular signalling (Wang *et al*., 2010).

The exact role of these bioactive lipids in relation to breast cancer is still debated, however, prostaglandin E2 has been implicated in breast cancer supporting migration and tumour-host interactions (Baenke *et al*., 2013). Prostaglandin E2 activate downstream intracellular signalling pathways, including the RAS-ERK pathway that is often mutated in cancer cells inducing sustained cell proliferation (Iyengar *et al*., 2015). Additionally, Prostaglandin E2 is a known inducer of cellular proliferation in breast cancer cells by increasing aromatase expression in stromal adipocytes as well as promoting the invasion of breast cancer cells to lymph nodes *via* an upregulated chemokine expression (Clària *et al*., 2010), and inhibiting apoptosis by upregulating pro-apoptotic marker blc-2 (Wang & DuBois, 2006; Wang & DuBois, 2010).

The role of eicosanoids (i.e. COX derived prostaglandins D2, E2, F2α, I2 and LOX derived *leukotriene* B4) in the regulation of inflammation is well recognized in promoting tumour

initiation and progression (Greene *et al*., 2011; Johnson *et al*., 2015; Sharma & Mohammed, 2006). Evidence on eicosanoids in relation to inflammation and breast cancer has predominantly focused on COX and LOX derived lipid mediators, however eicosanoids can also be derived from cytochrome P450 dependent metabolism subject to the ω-hydroxylase, and epoxygenase pathways (Johnson *et al*., 2015).

Cancer cells evolved to evade immune responses by either inhibiting the cytotoxic effects of T-lymphocytes as well as induce phenotypic alterations of resident immune cells in the tumour-microenvironment (Cohen & Blasberg, 2017). Evidence shows that mammary adipose tissue derived FFA inhibits cytotoxic T-cell anti-tumour functions (Kleinfeld *et al*., 2005), implicating FFA in immunosuppressive functions. High concentrations of prostaglandin E2, assists in facilitating these immunosuppressive functions by inducing phenotype switching from resident T-helper-1 cells in the tumour microenvironment to T-helper-2 cells, known for its immunosuppressive responses (Wang & DuBois, 2016). These responses are characterised by high levels of anti-inflammatory cytokines (IL-4 and IL-10), that suppresses T-helper-1 cell immune responses (i.e. antigen presentation to effector immune cells like B-cells and cytotoxic-T-cells), by favouring T-helper-2 cell responses (persistent tissue repair, angiogenesis and decreased antigen presentation to effector cells) (Ho*et al*., 2016). Prostaglandin E2 that is derived from tumour cells can hinder anti-tumour cytotoxic effects of effector T-lymphocytes by downregulating antigen presentation of tumour cells to dendritic cells (Ahmadi *et al*., 2008). Increased FFA metabolism (FA uptake and β-oxidation) by immune cells affects their functional state. For example, FA β-oxidation is characteristic of anti-inflammatory M2 macrophages, regulatory T cells and is required for the transition from effector to memory T cells (Ho*et al*., 2016).

Other signalling molecules such as lysophospholipids (i.e. lysophosphatidic acid (LPA)) derived from membrane phospholipids in response to extracellular stimuli, have been linked to breast cancer (Röhrig & Schulze, 2016). Adipose tissue derived LPA and autotaxin exacerbate inflammation and induce tumour development in mammary glands, by increasing immune cell infiltration and increasing production of TNF-α and IL-6, whereas the inhibition of autotaxin results in a significant reduction in these inflammatory markers as well as tumour growth (Benesch *et al*., 2015).

1.4. DOXORUBICIN TOXICITY: Lipid Metabolism and Adipose Tissue/Adipocytes

An Anthracycline-based regime (doxorubicin) is associated with adverse side effects and toxicities in multiple tissues and systems, cardiotoxicity being most clinically relevant (Meredith & Dass, 2016; Rivankar, 2014; Tacar *et al*., 2013). Recently, it was reported that doxorubicin has negative effects on adipose tissue/adipocytes (Arunachalam *et al*., 2012; Biondo *et al*., 2016; de Lima Junior *et al*., 2016) (Table 1.2). These effects range from inflammatory, metabolic dysfunction, morphological and phenotypical changes, all contributing to the disruption of adipose tissue/adipocyte homeostasis and lipid storage (Arunachalam *et al*., 2012; Biondo *et al*., 2016).

Since adipose tissue is one of the main storage sites of FAs (Omabe *et al*., 2015), the composition of these FAs may be disrupted and/or altered due to chemotherapeutic agents which therefore might implicate FAs in cancer treatment resistance. It should be noted that the majority of these findings (in Table 1.2) were not based on a cancer model but rather on normal functioning adipose tissue, and not in an obesogenic state were adipose tissue /adipocytes are dysfunctional with chronic low-grade inflammation. This finding is of clinical significance since obesity has also been linked to treatment resistance in cancer patients (De Azambuja *et al*., 2010; Gevorgyan *et al*., 2016; Karpińska *et al*., 2015).

The molecular mechanisms underlying doxorubicin's adverse negative effect on adipose tissue/adipocytes is proposed to involve adipokine dysregulation. For example, decreasing, and/or inhibition of adipogenesis (decreased PPAR-γ and FABP expression) and lipogenesis (decreased FAS expression) as well as the induction of lipolysis (increased HSL expression) (Biondo *et al*., 2016; Xiang *et al*., 2009), induces an increase in FFA release as the result of the phenotypical changes (Biondo *et al*., 2016), thereby disrupting lipid storage. Doxorubicin induced metabolic dysfunction (increased FFA levels) (Arunachalam *et al*., 2012; Biondo *et al*., 2016; de Lima Junior *et al*., 2016; Xiang *et al*., 2009), could potentially increase the availability of FFA energy substrates for cancer cells to utilize and sustain both its' survival and proliferation demands, and indirectly contribute to breast cancer treatment resistance (Figure 1.9).

Table 1.2: Effect of doxorubicin on adipose tissue and/or adipocytes.

Model	Findings	Proposed Mechanism	Reference
In vivo Male dawley Sprague rats epididymal fat Doxorubicin: 2.5 mg/kg/body weight, once a week for 11 weeks.	Doxorubicin was found to be a negative regulator of body weight as it resulted in a significant decrease in the body weight of animals on doxorubicin *vs* untreated controls. The decrease in body weight was specifically due to a loss in adipose tissue.	**Necrosis:** Adipose tissue undergoes necrosis as a result of chemotherapy. However, there is very limited proposed molecular mechanisms by which doxorubicin exerts its effects on a molecular level and to what extent the damage is and is unclear if it is only due to necrosis or not.	Xiang *et al*., 2009
In vivo Male wistar albino rats Doxorubicin: 2 mg/kg/body weight for 7 weeks.	A significant increase in FABP concentration was observed in rats treated with doxorubicin compared to control animals,	**Disrupt lipid-related pathways:** Doxorubicin treatment affects markers regulating adipogenesis.	Dudka *et al*., 2012
In vivo 3T3-L1 cells (differentiated into mature adipocytes)	Doxorubicin treatment resulted in the inhibition of adipogenesis i.e. ↑ expression of PPAR-α, and ↓ PPAR-γ and FABP-4 expression in a dose-dependent manner. Adipocytes treated with doxorubicin which overexpressed PPAR-γ counter acted all the above effects of doxorubicin.	**Disrupt lipid-related pathways:** Doxorubicin acts as an inhibitor of adipogenesis, by being an antagonist to PPAR-γ expression, which may ultimately lead to a lack of fat accumulation.	Arunachalam *et al*., 2012 Arunachalam *et al*., 2013
In vivo Mice Doxorubicin: 8 mg/kg body weight, for four weeks.	Doxorubicin treatment resulted in a significant ↓ in bodyweight and serum TAG concentration compared to saline treated mice.	**Changes in body composition:** Proposed by authors to be the underlying reason for cardio-dysfunction in this animal model.	Nagendran *et al*., 2013
In vivo Rat retroperitoneal adipose tissue Doxorubicin: 15 mg/kg/body weight, 72 hours before sacrifice. ***In vitro*** 3T3-L1 cells (differentiated into mature adipocytes)	*In vitro*: Doxorubicin (10 nM & 100 nM) was toxic to adipocytes, thereby inducing over 90% cellular apoptosis. *In vivo*: Doxorubicin disrupted adipocyte homeostasis: ↓ lipogenesis, ↑ glucose uptake and ↑ lipolysis thereby increasing FFA availability.	**Disrupt lipid-related pathways:** The molecular mechanism by which doxorubicin exerts its toxic effects on adipose tissue was still unknown at this point and warranted further investigation	Batatinha *et al*., 2014
In vivo Male wistar rats retroperitoneal adipose tissue Doxorubicin: 15 mg/kg/body weight, 72 hours prior to sacrifice. ***In vitro*** Primary adipocytes isolated from retroperitoneal fat and 3T3-L1 cells (differentiated into mature adipocytes)	Both *in vivo* and *invitro* models: doxorubicin treatment ↓ adipocyte size compared to controls. *In vivo*: doxorubicin treatment disrupted lipogenesis, i.e. ↓ FAS and ACC expression. In addition, primary adipocytes treated with doxorubicin showed a decrease in insulin-stimulated glucose uptake.	**Phenotypical and metabolic dysfunction**: This may have been the result of decreased expression of proteins regulating lipogenesis and therefore decreased lipid storage.	Biondo *et al*., 2016
In vitro Male wistar rats treated with doxorubicin (15 mg/kg/body weight, 72 hours prior to sacrifice).	Doxorubicin treatment caused a significant ↓ epididymal adipose tissue weight and adiponectin an increase in serum insulin, glucose, FFA concentration levels compared to saline controls. Doxorubicin treatment caused a decreased HOMA-IR (measurement of insulin resistance) and glucose uptake *vs* control animals, which is indicative of impaired insulin sensitivity, and these animals displayed insulin resistance, hyperglycaemia, and hyperinsulinemia.	**Metabolic Dysfunction:** These findings were the result of decreased expression of both AMKP and GLUT-4 in skeletal muscle, which was confirmed by the *in vitro* experiments. The authors concluded that doxorubicin treatment caused hyperglycaemia and insulin resistance mediated by inhibition of AMPK.	de Lima Junior *et al*., 2016
In vivo Type 2 diabetes mice model (db/db, leptin knockout) treated with doxorubicin (15 mg/kg/body weight, 5 days before sacrifice)	Doxorubicin treatment induced an inflammatory milieu in diabetic muscle by exacerbating a pro-inflammatory microenvironment (upregulating transcription factor HIF-1α, NFκB, and TNF-α) as well as decreasing anti-inflammatory actions (downregulating regulatory molecule AMPK and IL-15). Doxorubicin treatment induced a dysregulation in glycolytic metabolism in diabetic skeletal muscle by upregulating *pyruvate dehydrogenase kinase-4* and lactate dehydrogenase and downregulating phosphorylation of ACC.	**Metabolic Dysfunction:** Results suggest that doxorubicin treatment in the context of diabetes may cause an environment which can worsen diabetes related effects.	Supriya *et al*., 2016
In vitro Human HepG2 and colorectal cancer cells were treated with 5-fluorouracil and doxorubicin (2 μg/mL) for 72 hours.	Doxorubicin and 5-fluorouracil treatment significantly increased lipid droplets within HepG2 cancer cells. An increase in SFAs and PUFAs and decrease in MUFAs was found following chemotherapy treatments in the phospholipid membranes of cancer cells.	**Disrupt lipid-related pathways:** Doxorubicin and 5-fluorouracil induced cancer cell invasion and metastasis by increasing lipid accumulation and membrane fluidity in cancer cells by altering fatty acid composition.	Mehdizadeh *et al*., 2017
In vivo Colorectal cancer rat model treated with 5-fluorouracil and Irinotecan for two cycles (50 mg/kg/body weight).	Chemotherapy treatment (5-fluorouracil and Irinotecan), diminished peri-uterine adipose tissues' function to store lipids by downregulating the expression of ACC, FAS and HSL and markers of β-oxidation, compared to treatment naïve rats. Additionally, they also showed that SFAs and MUFAs significantly decreased in chemotherapy threated mice.	**Disrupt lipid-related pathways:** Chemotherapy treatment affects markers regulating lipid and fatty acid metabolism, which could be responsible for altered fatty acid composition in peri-uterine adipose tissue.	Ebadi *et al*., 2017

Abbreviations: ACC, Acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; FABP-4, fatty acid binding protein-4; FAS, fatty acid synthase; FFA, free fatty acids; GLUT-4, G; HOMA-IR, homeostatic model assessment of insulin resistance; HSL, hormone sensitive lipase; HIF-1α, hypoxia inducible factor-1α; IL-15, interleukin-15; MUFA, monounsaturated fatty acids; NFκB, nuclear factor kappa B; PPAR-α, peroxisome proliferator-activated receptor-α; PPAR-peroxisome proliferator-activated receptor-γ; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TAGs, triglycerides; TNF-α, tumour necrosis factor-α(Mentoor *et al*., 2018).

Hong and colleagues, (2002) conducted one of very few studies investigating the effect of doxorubicin treatment on serum lipids and FA composition in an animal model. Here, they revealed that doxorubicin treatment caused an increase in serum total cholesterol as well as serum low density lipoprotein-cholesterol (LDL-c) levels in a concentration-dependent manner. Serum total FFAs were significantly increased in the doxorubicin treatment group compared to the control group (Hong *et al*., 2002). Others reported a dysfunctional lipid fraction has also been documented in breast cancer cases i.e. increased total cholesterol, total lipids and LDL-c (Shaikh *et a*l., 2017). Serum FFA profiles also revealed that doxorubicin treatment caused significant increases in SFAs (PA and SA), n-6 PUFA (LA) and the MUFA (OA) (Hong *et al*., 2002). One hypothesis is that the mechanism here may be due to the disruption and/or inhibition of long-chain FA oxidation, as evident in a doxorubicin-induced cardiotoxicity model (Hong *et al*., 2002). A limitation for this study was that findings were based on serum samples, which reflects a short-term FA profile, whereas adipose tissue is considered the gold standard.

In agreement, with the above mentioned results a recent report showed that chemotherapeutic treatment (5-fluorouracil and Irinotecan) in a colorectal cancer model diminished peri-uterine adipose tissues' function to store lipids by significantly downregulating the expression of lipogenesis (ACC and FAS) and lipolysis (HSL) as well as β-oxidation (CPT-2). It was noted that SFAs (PA) and MUFAs (PTA) significantly decreased in chemotherapy treated mice but it is still unknown whether the suppression of adipose tissue lipid storage capacity induced by the chemotherapy is a result of decreased HSL expression, or due to mitochondrial dysfunction induced by the chemotherapy itself (Ebadi *et al*., 2017). Mehdizadeh *et al*. (2017) showed that doxorubicin and 5-fluorouracil altered FA metabolism and thereby induced cancer cell invasion and metastasis by increasing lipid accumulation. Firstly, they showed that doxorubicin and 5-fluorouracil treatment significantly increased the number of lipid droplets within HepG2 cancer cellsas well as increasing the SFAs (PA) and PUFAs, and significantlydecreeingthe MUFAs (OA and PTA) in the phospholipid membranes of cancer cells. It is suggested that the lipid enriched tumour cells may be the result of lipophagy, and/or the inhibition of mitochondrial FAs oxidation, that is induced *via* increased production of ROS (Schulze *et al*., 2017; Singh & Cuervo, 2012). This shifts the direction of FAs away from oxidation and indirectly promotes TAG synthesis (You *et al*., 2016). Doxorubicin is known to induce both oxidative stress (increased ROS) and inhibit FA oxidation. It may be possible that doxorubicin treatment contributes towards TAG synthesis and subsequent lipid storage or even membrane saturation

in tumour cells (Figure 1.9). However, evidence on the effects of doxorubicin on breast cancer in the context of obesity, where adipose tissue is dysfunctional is lacking.

1.5. SUMMARY AND RATIONALE

Adipose tissue performs an important physiological role as a metabolically active storage compartment and endocrine organ. Any disruption in adipose tissue homeostasis can result in potentially serious health and clinical-related outcomes. Obesity is a risk factor in the development and progression of breast cancer, which involve mechanisms in relation to adipose tissue dysfunction, inflammation and dysfunctional FA metabolism. During obesogenic states, lipid metabolism becomes dysregulated that may further exacerbate breast cancer progression.

Low grade inflammation, another hallmark of an obese state, result in the release of adipokineswhich induced the infiltration of immune cells leading to a sustained inflammatory *milieu*. These inflammatory mediators activate downstream signalling pathways (PI3K, MAPK, NFκB and EMT) in breast cancer cells that favours cancer cell survival, to promote breast cancer development and progression. Recent evidence implicates obesity as a causal factor for reduced chemotherapy efficacy, resulting in treatment resistance. Obesity-driven changes may contribute to chemotherapy resistance by altering drug pharmacokinetics, impairing drug metabolism/delivery; induce chronic inflammation, as well as altering tumour-associated adipocyte adipokine secretion and FA composition. It still remains unclear what the exact mechanism(s) are in which obesity achieves this.

The role of FAs in breast cancer progression is proposed to be achieved by alterations in cell membrane composition, being a source of lipid-signalling molecules, its role in metabolic reprogramming and its role as an additional energy source. Cancer cells manipulate FA metabolism by increasing *de novo* FA synthesis (increased ACC, FAS and SCD-1 enzymes expression), increasing lipolysis (HSL) as well as exogenous FA uptake (increased FABP-4 and CD36 expression). This can confer to membrane lipid saturation which in turn protects cancer cells against the cytotoxic effects of chemotherapeutic drugs. Fatty acids serve as a source of eicosanoids and prostaglandins to activate downstream signalling pathways that regulate carcinogenic events such as cell growth, migration, angiogenesis, metastasis and inflammation. These adipose tissue/adipocytes provide breast cancer cells (abundant adipose

tissue) energy to sustain their survival. Fatty acids(FAs) provide energy for breast cancer cells to sustain its high proliferation rates, or can be stored in tumours as lipid droplets and/or in membrane lipids to evade lipotoxicity and protects against the cytotoxic effects of chemotherapeutic drugs.

The latter have also been shown to impact the endocrine and storage functions of adipose tissue, highlighting this as problematic as adipose tissue is a major storage site for FAs and it is likely that these negative effects may disrupt adipose tissue homeostasis. The toxic effects of doxorubicin, both *in vitro* and *in vivo* with regard to adipose tissue/adipocytes, and/or lipid metabolism, is achieved by inhibition of adipogenesis, downregulation of lipogenesis, inducing lipolysis, and subsequently disrupting lipid storage. The end result is a phenotypical change of adipocytes to produces more "bioavailable" energy substrates (increased FFA) for breast cancer cells to utilize to sustain survival and proliferation demands.Limited evidence exists where the effects of doxorubicin on breast cancer in the context of obesity is explored.

Since there is convincing evidence that both obesity and FAs play a role in both breast cancer progression and drug resistance, it is of significance to elucidate whether FAs play a role in breast cancer treatment resistance in an obesogenic context. This may also identify specific FA classes which might be implicated. Further investigation is therefore needed in order to contribute to the understanding of underlying molecular mechanism by which breast cancer resistance is achieved, as well as to identifying novel targets for intervention to counteract and overcome breast cancer resistance.The molecular mechanism elucidating the role of FA composition in obesity linked to drug resistance still remains unexplored, thus we propose a hypothesis driven investigation. A schematic presentation of the proposed mechanism is illustrated in Figure 1.9.

We propose that doxorubicin treatment in an obesogenic environment characterised by dysfunctional adipose tissue will be less effective possibly due to the fact that doxorubicin can further exacerbate adipose tissue dysfunction (inflammation) (Figure 1.9). This in turn will produce more bioavailable FAs to the survival advantage of breast cancer cells and thereby lead to acquired treatment resistance. Lastly, the role of lipid and FA metabolism in breast cancer also remains understudied as well as the cytotoxic effects of chemotherapeutic drugs on adipose tissue/adipocytes, both of which may contribute to the promotion of treatment resistance.

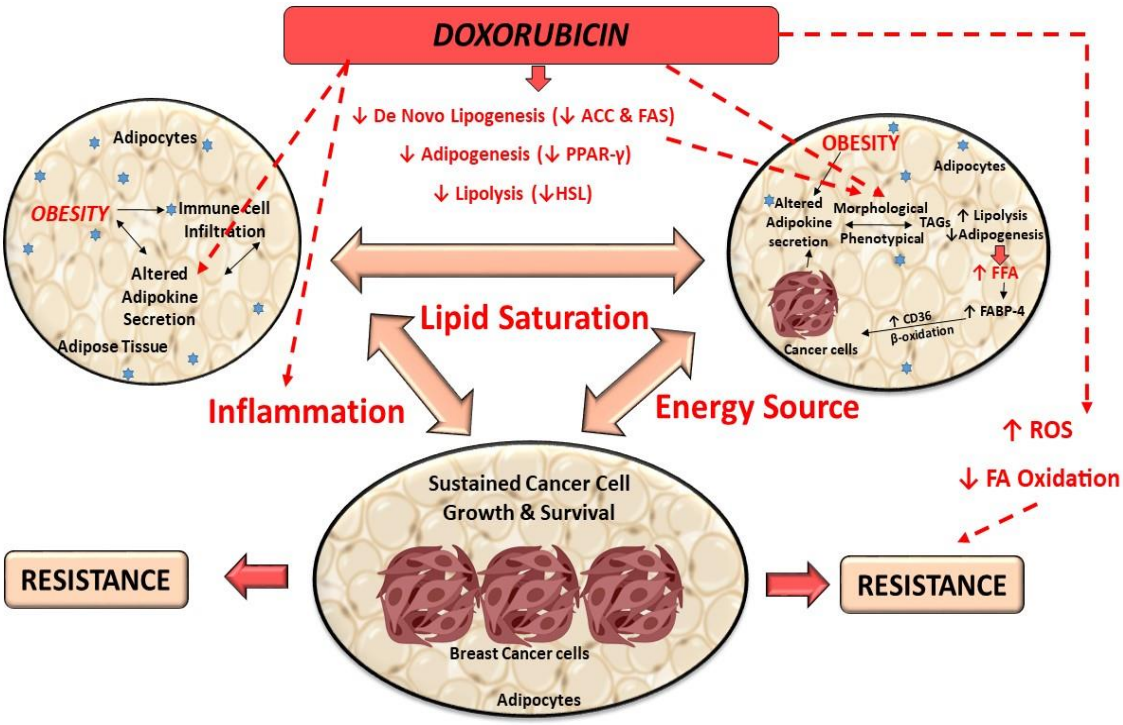

Figure 1.9: Proposed role of doxorubicin in an obesogenic breast cancer model. ACC, Acetyl-CoA carboxylase; CD36, fatty acid translocase; FFA, free fatty acids; FABP, fatty acid binding protein; FABP-4, fatty acid binding protein-4; FAS, fatty acid synthase; HSL, hormone sensitive lipase; PPAR-γ, peroxisome proliferator-activated receptor-γ; TAGs, triglycerides (Mentoor *et al*., 2018).

Since there is an increase in the prevalence of overweight/obese breast cancer patients who receive doxorubicin, it is essential to explore the effect of doxorubicin treatment in this context.Additionally, how obesity may aggravate factors playing a role in the development of doxorubicin treatment resistance. Specifically, since, obese and normal weight patients receive the same treatment regimen. Therefore, extensive investigation is needed to elucidate the underlying mechanism by which obesity contributes to treatment resistance. The identification of molecular mechanisms underlying both the effects of a neoplastic state and doxorubicin treatment on mammary adipose tissue within the tumour microenvironment, will promote the identification of novel pharmacologic targets and the development of appropriate management protocols for obesity driven chemotherapeutic drug resistance as well as doxorubicin related toxicities in order to improve over-all survival of breast cancer patients.

CHAPTER 2: *IN VIVO* MODEL

2. *In Vivo* Model

Problem Statement

Diet-induced obesity not only increases breast tumour development, progression and metastasis, but it also leads to a decrease in chemotherapeutic treatment efficacy. The specific role of obesity in breast cancer resistance remains understudied. Additionally, there appears to be a general lack of evidence that support the role of adipose tissue/adipocytes and FA profiles in breast cancer treatment resistance under obesogenic conditions. The outcomes from this investigation may reveal novel targets for potential interventions to counteract cancer treatment resistance and could be essential towards improving the prognosis of obese breast cancer patients.

Hypotheses

Two hypotheses are tested in this model:

1) Diet-induced obesityinduces inflammation and altersFA profiles in the breast tumour microenvironment in order to sustain cancer cell survival and promotes acquired chemotherapeutic treatment resistance.
2) Doxorubicin treatment may exacerbate the obesogenic effects on breast cancer cells and thus contributing to the development of obesity induced chemotherapeutic treatment resistance.

Aims and Objectives

Since limited evidence exists regarding the effect of obesity on doxorubicin treatment resistance in a xenograft mouse model, where breast cancer is induced, we formulated the following aims:

1. To establish a physiologically obese breast tumor-bearing mouse model.

- *Here, the objective is to employ a diet-induce obesity model through a high fat diet (HFD) and compare differences in body weight and food consumption against a low fat diet (LFD).*

2. To assess the effect of doxorubicin treatment on neoplastic growth in an obesogenic environment (DIO).

- *The objective here was to induce breast xenographstumors and compare differences in tumor volume/weight between LFD and HFD vehicle treatment experimental groups.*

3. To assess DIO, as a contributing factor for doxorubicin chemotherapy treatment resistance.

- *Breast tumors were treated with a chemotherapeutic agent and differences in tumor volume/weight between LFD compared to HFD doxorubicin experimental treatment groups were compared.*

4. To characterize the role of potential causal factors including inflammatory markers, FA profiles as well as markers involved in lipid metabolism.

- *Inflammatory markers, lipid metabolism markers and FAtissue profiles were analyzed between groups for LFD and HFD fed mice treated with vehicle and doxorubicin.*

2.1 MATERIALS AND METHODS*: IN VIVO* MODEL

To determine whether DIO could affect the efficacy of a chemotherapeutic agent (doxorubicin) in breast cancer, we developed a diet-induced obesogenic animal model by nourishing female C57BL6 mice a HFD. After the development of the DIO phenotype, breast tumour xenographs were induced, followed by respective treatments.

2.1.1Animals and Handling

All animal interventions and handling was carried out under the supervision of the animal unit manager at Stellenbosch University. Ethical approval was obtained for this investigation from the animal research committee of Stellenbosch University (Clearance Nr: SU-ACUM13-00015). All protocols were carried out according to the standard guidelines for care and use of laboratory animals implemented at Stellenbosch University as well as accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008.

A total of n=40 three weeks old female C57BL6 mice were housed in the animal research facility at the University of Stellenbosch in static micro-isolation sterilized cages (n=5 per cage) with filtered air. The mice were provided with chow and tap water ad libitum in a regular 12h: 12h light-dark cycle. Previous evidence illustrated when mice are housed in groups, weight gain occurs faster, possibly due to the fact that the mice eats/absorbs/metabolizes the food better and display improved social cohesion compared to when mice are caged in isolation (Dogan *et al*., 2007). All animals were acclimated for a week, after which thespecific diet regimens was assigned and followed. The general welfare of all animals was monitored daily.

2.1.2 Diet Regimens

A HFD was used to induce obesity since reported evidence showed that genetic models of obesity (i.e. ob/ob, db/db and leptin/ leptin receptor deficient mice) demonstrated resistance in developing mammary cancer (Cleary *et al*., 2004).C57BL6 mice is particularly sensitive to DIO and known to develop obesity when fed a HFD (Wang & Liao, 2012).Mice (n=40) were randomly assigned into two equal groups (n=20) and placed on one of two respective diets for

12 weeks (Figure 2.1).The HFD's (HFD, D12492, Research diet Inc., New Jersey, USA) caloric content consisted of 60 percent energy (PE)from fat, 20 PEfrom protein and 20 PE from carbohydrates, compared to the LFD (LFD, D12450J, Research diet Inc., New Jersey, USA), receiving 10 PE from fat, 20 PE from protein and 70 PE fromcarbohydrates (Table 2.1). In order to minimize the possibility of developing various phenotypes, a HFD (60 PE from fat) was used to induce obesity in an E0771 breast cancer tumour model. The fat percentage used in the HFD (60 PE from fat) was justified as a report suggested the proportion of specific FA classes i.e. SFAs, MUFAs, and PUFAs consumed in humans are similar to the HFD we used in our *in vivo* model (Ervin *et al*., 2004). Additionally, a 20-40 PE from fat HFD can take approximately 16-40 weeks for the DIO phenotype to develop. In light of this we also needed to take time of tumour induction as well as treatments into account to establish humane endpoints.

A 12-week study duration was chosen to ensure tumour development needed to be taken into consideration, since obesity has been shown to stimulate tumour growth, therefore justifying a humane end point at week 12. The dietary FA composition according to the manufacturer of both respective diets is summarized in the supplementary section (Supplementary Table 1). Body weight was monitored weekly over twelve weeks and the DIO phenotype was confirmed after eight weeks, after which tumour induction commenced.

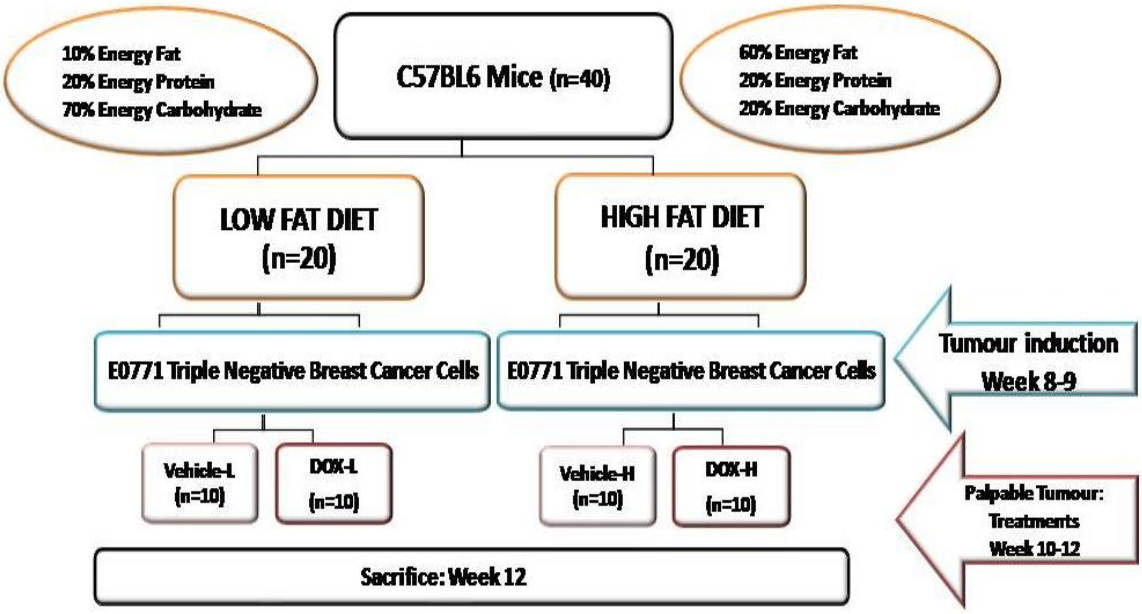

Figure 2.1: Flow diagram illustrating the *in vivo* model and respective experimental groups.

Table 2.1: Dietary composition of the low fat diet (D12450J) and the high fat diet (D12492).

	Low Fat Diet		High Fat Diet	
	Research Diet D12450J		Research Diet D12492	
	gram (%)	**kcal (%)**	**gram (%)**	**kcal (%)**
Protein	19.2	20	26.2	20
Carbohydrates	67.3	70	26.3	20
Fat	4.3	10	34.9	60
Total		100		100
kcal/gm	3.9		5.2	
INGREDIENTS	**gram**	**kcal**	**gram**	**kcal**
Casein, 30 Mesh 200	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	506.2	2024.8	0	0
Maltodextrin 10	125	500	125	500
Sucrose	68.8	275.2	68.8	275.2
Cellulose BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	245	2205
Mineral Mix S10026	10	0	10	0
Dicalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate,1 H_2O	6.5	0	6.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Yellow Dye #5	0.04	0		
FD&C Blue Dye #1	0.01	0	0.05	0
TOTAL	1055.1	4057	773.9	4057.0
Cholesterol(mg)/4057 kcal	-	54.4	-	216.4
Cholesterol (mg)/kg	-	51.6	-	279.6

As per manufacturer product data sheet (Research diet Inc., New Jersey, USA)

2.1.3 Tumours

2.1.3.1 Cell Culture

A very aggressive triple negative breast cancer cell line with metastatic capabilities, E0771 breast cancer cellline, thatoriginatedfromatumour as a result of a spontaneous mutation in aC57BL6 mouse, was used in this *in vivo* model. The E0771 breast cancer cells were cultured under standard conditions in Dulbecco's Modified Eagle's medium (DMEM) in T75 flasks (75 cm^2, SPL Life Sciences), supplemented with 10% Foetal Bovine Serum (FBS) and 1% Penicillin Streptomycin (Penstrep) (growth medium) in an incubator (temperature, 37°C and 5% CO_2 humidity). Growth media was replaced every second day. Regular sub-culturing was performed once cultures reached confluency between 70-80%.Sub-culturing of cells was

achieved by discarding all growth medium, followed by subsequent incubation with 0.25% Trypsin at 37°C until the cells were detached from the flask. Cells were checked daily for any morphological and growth changes.

2.1.3.2 Induction of Tumours

E0771 TNBC cells were prepared for each animal. Mice were restrained and anaesthetized under 3% (v/v) isoflurane (Isofor, Safeline Pharmaceuticals, Florida, South Africa) in an anaesthetic chamber. Mice were inoculated subcutaneously using a 23-gauge needle syringe on the left fat pad of the fourth mammary gland with 1.2 $x10^5$ E0771 TNBC cells, suspended in Hanks Balanced Salt Solution (HBSS) (Sigma Chemical Co., St Louis, MO, USA) (Figure 2.1). Inoculation of tumours commenced after 8 weeks when the DIO phenotype was established in the HFD mice. The inoculation day was designated as day zero.

2.1.4 Drug Administration

Once tumours became palpable (200-300 mm^2, week 10-11), mice were randomly assigned into the respective treatment groups for both LFD and HFD (Figure 2.1). The treatment groups included: (1) *vehicle control (isovolumetric intra-peritoneal injection of HBSS), and (2) doxorubicin treatment* (D5794, LKT® laboratories, Minnesota, USA).

Mice were restrained and doxorubicin treatment was administered in three successive dosages of 4 mg/kg every three days (cumulative dosage of 12 mg/kg) *via* intraperitoneal injection. The dosage of 12 mg/kg doxorubicin is equivalent to 36 mg/m^2 in humans which is within the clinically relevant dosage range of doxorubicin treatment (15 and 90 mg/m^2) (Reagan-Shaw *et al*., 2007). Finally, four respective experimental groups were rendered which included; (i) tumour vehicle-LFD (vehicle-L), (ii) tumour vehicle-HFD (vehicle-H), (iii) tumour doxorubicin-LFD (Dox-L), and (iv) tumour doxorubicin-HFD (Dox-H).

Humane endpoints were implemented when tumour growth influenced general welfare or restricted mobility of the mice, mice began to bite their tumours and mice exhibited changes in posture and facial expression, as determined using the grimace scale. At the end the final total

per experimental group were as follow, vehicle-L (n=8), vehicle-H (n=9), Dox-L (n=10) and Dox-H (n=9).

2.1.5 Measurements, Blood Collection and Tumour-and Fat Tissue Excision

Mice were weighed and the tumour location and time of detection were recorded weekly. The final body weight was calculated after subtracting tumour weight. Tumour growth was measured using a Harpenden calliper (in mm) to determine tumour volume using the following equation:

$$Tumour\ Volume\ (mm^3) = \frac{1}{2}\ (length\ x\ width^2)$$(Tomayko & Reynolds, 1989)

The mice were euthanized, 72 hours after the last scheduled doxorubicin administration. Mice were anesthetized under 3% isoflurane and a deep sleep was confirmed by the absence of the pedal reflex. Mice were sacrificed by cervical dislocation. Whole blood was collected in EDTA tubes (Lasec) from the thoracic cavity using a syringe and immediately placed on ice followed by centrifugation (1000 x g for 10 minutes), plasma was collected, aliquoted and stored at -80˚C for analyses. Mammary adipose tissue (located in the third and fourth quadrant of the mice) and tumour tissue were harvested, weighed, and snap frozen using liquid nitrogen, and stored at -80°C until further examination and analyses.

2.1.6 Blood Analysis

Plasma samples were analysed to quantify the concentration of TNF-α, IL-6, IL-10, leptin (PPX-04-MXCE327), IL-1β and VEGF-A (PPX-02-MXFVKXT) using Custom panel ProcartaPlex mix and match mouse luminex kits. Milliplex mouse adipokine magnetic bead panel MAP kit was used to quantify MCP-1, insulin, plasminogen activator inhibitor-1 (PAI-1) and resistin (MADMAG-71K). All analyses were performed according to the manufacturer's protocols and specifications. Analytes were measured simultaneously using a MAGPIX system plate reader (Bio-Rad, APX1042) and results (expressed in pg/ml) were reported on Bioplex Software 6.1 (Bio-Rad, 2016).

2.1.7 Determination of Tissue Fatty Acid Profiles

For all four treatment groups, determination of the FA composition of the tumour tissue included the total phospholipid (TPL) and FFA fractions, whereas for the mammary adipose tissue, the total lipid FA composition was determined. All FA analyses were conducted by gas-liquid chromatography (GLC) at the Non-Communicable Diseases Research Unit (NCDRU) of the South African Medical Research Council (SAMRC).

Frozen tumour and mammary adipose tissuewereallowed to thaw at room temperature, where approximately 100 mg of tumour tissue and 30 mg of adipose tissue were weighed out for lipid extraction using chloroform: methanol (C:M; 2:1; v:v; Sigma-Aldrich) according to an adapted method by Folch *et al*. (1957) as previously described by Hon *et al*., (2009). The extraction solvent contained 0.01% butylated hydroxytoluene, acting as an antioxidant.

Briefly, lipids of tumour tissue was extracted with 9 mL of C:M (2:1; v:v) by homogenisation for one minute using a Polytron® PT-MR 3100D (Switzerland) homogeniser. The homogenate was filtered through a sintered glass funnel (lined with filter pads) with a glass microfiber filter disk (GF/A, Whatman, England) into a round bottom flask. The Polytron® shaft was rinsed with another 7 mL of the extracting solvent and filtered, collecting the rinse into a round bottom flask. The microfiber filter disk containing the homogenised tissue was removed and placed into an extraction tube and extracted again with 10 mL C:M (2:1; v:v), followed by a 20-minute shaking and filter step as previously mentioned, this was repeated twice. The combined extraction phases containing the lipids were concentrated to dryness using a water bath (37°C, Buchi Rotavapour, Switzerland). Lipids were transferred from the round bottom flask to a 12 mL glass tube with screw cap using 5 × 2 mL chloroform: methanol: saline (CMS; 86:14:1; v:v:v; Sigma-Aldrich) transfer volumes. Saline saturated with CMS (1 mL) was added, mixed and centrifuged, after which the top saline layer was completely removed in order to concentrate the bottom phase to dryness under nitrogen gas-flow using a water bath at 37°C.

Neutral lipids were separated from the TPL fraction using thin-layer chromatography (TLC) silica gel 60 plates (10 × 10 cm; No. 1.05626.0001; Merck, Darmstadt, Germany), and eluted with the solvent system petroleum ether (B&M Scientific): diethyl ether (Merck): acetic acid (Merck) (90:30:1; v:v:v). The lipid bands containing the TPL fraction and FFA fraction was demarcated by visualization under long-wave UV light after plates were sprayed with C:M (1:1; v:v) containing 2,5-bis-(5´-tert-butylbenzoxazolyl-[2´])thiophene (10 mg/100 mL;

Sigma-Aldrich). These lipid bands were scraped off the plates into glass tubes with screw caps. The lipids were trans-esterified by trans-methylation with 2 mL methanol: sulphuric acid (H2SO4; BDH Chemicals, Poole, England) (95:5; v:v) at 70°C for 2 hours to produce FA methyl esters (FAMEs). After cooling, the resulting FAMEs were extracted with 1 mL water and 3 mL n-hexane (Sigma-Aldrich). The upper hexane layer containing the sample FAMEs was then collected and evaporated to dryness for subsequent GLC analysis.

Mammary adipose tissue total lipids were extracted with 9 mL of C:M (2:1; v:v) by shaking for 20 min (mechanical shaker). Subsequently, 1.8 mL saline saturated with CMS was added, mixed and centrifuged at 500 rpm for 10 minutes at 4 °C. The bottom phase was collected and transferred to a 12 mL glass tube with screw cap and the lipid extract was evaporated to dryness under nitrogen gas-flow using a water bath at 37°C. The dried lipids were redissolved in 3 mL C:M (2:1; v:v) of which a 50 µL aliquot was transferred to a clean 12 mL glass tube. The lipid aliquot was evaporated to dryness as described before. These lipids were trans-methylated with 2 mL methanol: sulphuric acid (70°C for 2 hours) with subsequent sample FAMEs isolation for GLC analysis as previously described.

The FAMEs of all samples were re-dissolved in a small volume of n-hexane and analysed (sample injection volume 1 µl) by GLC (Finnigan Focus Gas Chromatograph, Thermo Electron Corporation, Austin, TX, USA) equipped with a flame-ionization detector and a 30 m capillary column of 0.32 mm internal diameter (BPX70 0.25 µm; SGE International Pty Ltd, Ringwood, Victoria, Australia). Gas flow rates were: N_2 (make up gas), 25 mL/min; synthetic air, 250 mL/min; H_2 (carrier gas), 25 mL/min, with a split ratio of 20:1. Oven temperature programming was linear at 4.5°C/min, initial temperature 140°C (hold-time 1min), final temperature 220°C (hold-time 5min), injector temperature 220°C, and detector temperature 250°C. The FAMEs in all samples were identified by comparing the retention times with those of the standard FAME mixture (27 FAMEs, NuChek Prep Inc., Elysian, MN, USA). Relative percentages of individual FAMEs were calculated by taking the area count of a given FAME as a percentage of the total area count of all FAMEs identified in the sample. Desaturase indexes were estimated by product to precursor fatty acid ratios. Desaturase activity of SCD-16 was calculated as the function of palmitoleic acid (C16:1n-7) to palmitic acid (C16:0) and SCD-18 the function of oleic acid (C18:1n-9) to stearic acid (C18:0) (Manni *et al*., 2017; Petrus *et al*., 2017).

2.1.8 Protein Analysis and Western Blotting

Frozen mammary adipose tissue and tumour tissue samples were placed on ice and allowed to thaw at 4°C. For total protein extraction, samples were suspended in 300 µl of cold modified radioprecipitation (RIPA) buffer containing protease and phosphatase inhibitors (2.5 mM Tris-HCL, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 50 mM NaF, 4 mg/ml SBTI, 10 mg/ml leupeptin, 0.1% SDS, 0.5% Na deoxycholate and 1% NP40, adjusted to pH 7.4).

All samples were homogenised on ice under sterile conditions in order to prevent protein cross contamination between samples. Next, all samples were centrifuged at 14 000 rpm for 1 hour at 4°C, which resulted in the formation of distinct layers. The supernatant layer was removed using a sterile 23-guage needle and syringe and placed into a new sterile Eppendorf tube followed by another centrifugation step (14 000 rpm for 30 minutes at 4°C). The process of removing the supernatant was repeated and samples were stored at -80ºC for protein quantification. Protein quantification of the supernatants was performed using a Direct Detect® infrared spectrometer (DDHW00010-WW, Merck). Preparation of protein aliquots containing 20-50 µg protein diluted with Laemmli sample buffer was prepared and boiled for 5 minutes (to denature proteins) before being loaded onto 4-15% polyacrylamide fast cast gels (mini-PROTEAN® TGX™ Gels, Bio-Rad) for separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run at 100 V (constant) and 400 mA for approximately 120 minutes (Power Pac 300, BioRad).

The electro-transfer of proteins from the gel to polyvinylidine fluoride (PVDF) membranes was achieved using a semi-dry electro-transfer system (TransBlot® Turbo™ v1.02, BioRad) for 30 minutes at 25 V and 1.0 A. Transfer efficiency was evaluated using the stain free blot protocol provided on a Chemi-Doc™ MP (BioRad) system. Subsequently, all membranes were washed with 0.1% Tris Buffered Saline-Tween20 (TBS-T) and blocked for one hour in 5% (w/v) non-fat milk and TBS-T at room temperature to prevent non-specific binding of proteins.

The PVDF membranes were then incubated in primary antibody solution (1:1000, diluted in 5% w/v BSA, 1X TBS-T, see Table 2.2) overnight at 4°C. Next, membranes were washed three times each for 5 minutes with TBS-T, prior to incubation with anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (1:10000) (Cell Signalling Technologies) for one hour at room temperature (22°C). A wash step followed using TBS-T (5 times for 5 minutes each) before specific bands were visualized and detected using the (enhanced chemiluminescence (ECL)Western blotting substrate detection kit (BioRad) with the use of

ImageLab 4.0 software on a Chemi-Doc™ MP (BioRad) imaging system. Samples were normalized to total protein present on the same membrane. Bands for each specific protein were quantified as normalized readings comparative to the control sample present on the same blot and are expressed as a percentage of the control.

Table 2.2: Antibodies used for the *in vivo* model Western blotting.

Primary Antibody	Size	Concentration	Supplier
Fatty acid synthase (FAS)	273 kDa	1:1000	Cell Signalling (#3180)
Stearoyl-CoA desaturase-1 (SCD-1)	37 kDa	1:1000	Cell Signalling (#2794)
Adipose tissue triglyceride lipase (ATGL)	54 kDa	1:1000	Cell Signalling (#2439)
Hormone sensitive lipase (HSL)	83 kDa	1:1000	Cell Signalling (#4107)
Fatty acid binding protein-4 (FABP-4)	15 kDa	1:1000	Cell Signalling (#3544)
Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB-p65)	65 kDa	1:1000	Cell Signalling (#8242)
Secondary Antibody			
Anti-rabbit		1:10 000	Cell Signalling (#7074)

2.1.9 Statistical Analysis

Statistical analysis was performed using Statistica version 13.3 (TIBCO Software, Inc, USA). Normality was assessed using the Shapiro-Wilks test and results were reported as mean ± standard error of the mean (SEM). T-tests were used to describe differences between two groups. A mixed model two or three-way ANOVA (analysis of variance) was used to describe differences between three/more groups followed by the Fishers LSD *post hoc* test. Pearson correlations were used on selected parameters in each group and 2D scatter plots were constructedusing GraphPad Prism version 7. Principle component analysis (PCA) was done to generate bi-plot graphs displacing tissue FA profile results of all four respective treatment groups. All graphs were generated in GraphPad Prism version 7. Statistical significance was accepted at $p<0.05$.

2.2 RESULTS: *IN VIVO* MODEL

To determine whether DIO could affect the efficacy of doxorubicin treatment in breast cancer, we developed a DIO model, where female C57BL6 mice were fed a HFD for twelve weeks. After the DIO phenotype was established, tumours were induced by subcutaneous inoculation in the fourth mammary gland with E0771 triple negative breast cancer cells. Control animals received HBSS inoculations. Once tumours became palpable mice were randomly assigned to either receive doxorubicin treatment (cumulative dose of 12 mg/kg) or vehicle treatment (HBSS) (see methods section 2.1). Four respective experimental groups were rendered, which includes tumour vehicle-LFD (vehicle-L), tumour vehicle-HFD (vehicle-H), tumour doxorubicin-LFD (Dox-L) and tumour doxorubicin-HFD (Dox-H).

2.2.1 HFD Increases Body Weight and Mammary Adipose Tissue Weight

2.2.1.1 Body Weight and Food Consumption

To determine whether the HFD induced the obesity phenotype, differences in body weight and food consumption were assessed between LFD (n=20) and HFD (n=20) mice for the first eight weeks.Mice fed a HFD showed significantly higher body weight at week 6 ($p=0.0013$), week 7 ($p=0.00013$) and week 8 ($p=0.0000020$), compared to LFD mice (Figure 2.2A). Asignificantly lower food consumption per cage was observed for mice fed a HFD at week 2 ($p=0.00053$), week 3 ($p=0.0050$) and week 4 ($p=0.014$) compared to LFD mice (Figure 2.2B).

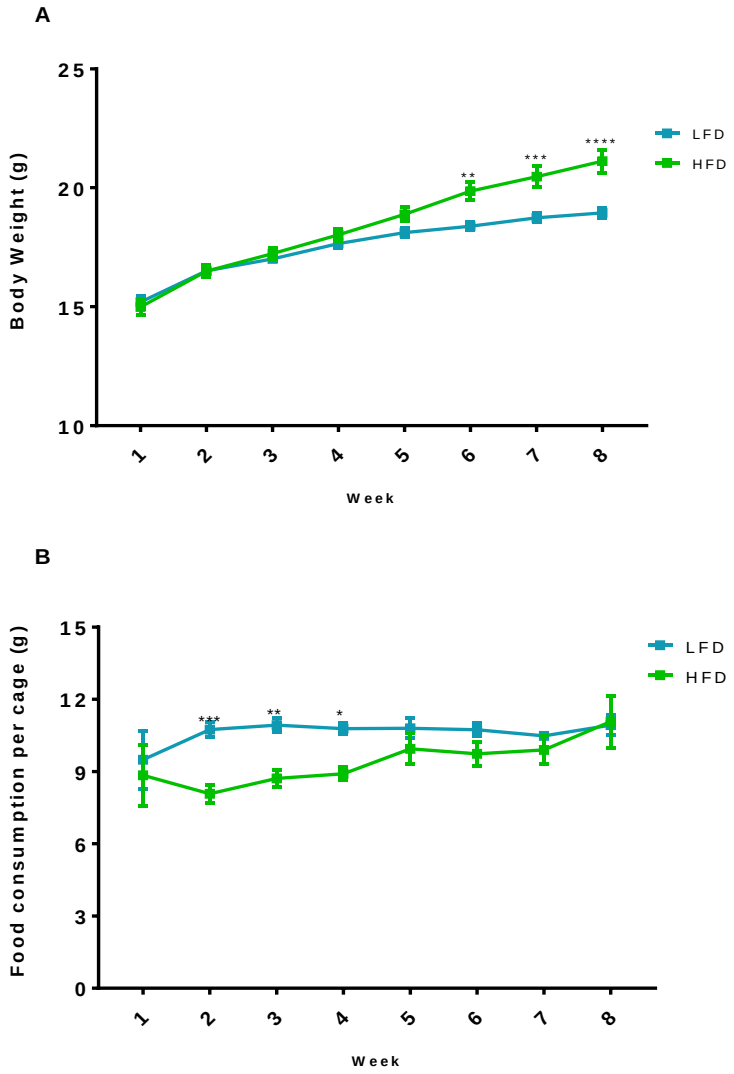

Figure 2.2: Difference in (A) body weight, and (B) food consumption in mice (n=5 per cage) on LFD and HFD for 8 weeks. Results are presented as mean ± SEM (n=20 per group). T-tests were employed for comparison between groups for all weeks. $p<0.05$ was considered as statistically significant. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ and **** $p<0.0001$. HFD, high fat diet; LFD, low fat diet.

To establish whether tumour induction, and/or doxorubicin treatment has an effect on body weight, differences in body weight were assessed from week 8 to week 12 (tumour induction week 8-9, and treatment week 10-12), for all four experimental treatment groups. Here the vehicle-H showed significantly higher body weights compared to Dox-H mice at week 8-12 ($p<0.0001$) (Figure 2.3A). Dox-H mice showed similar significantly higher body weights compared toDox-L mice at week 8-12 ($p<0.0001$) (Figure 2.3A).

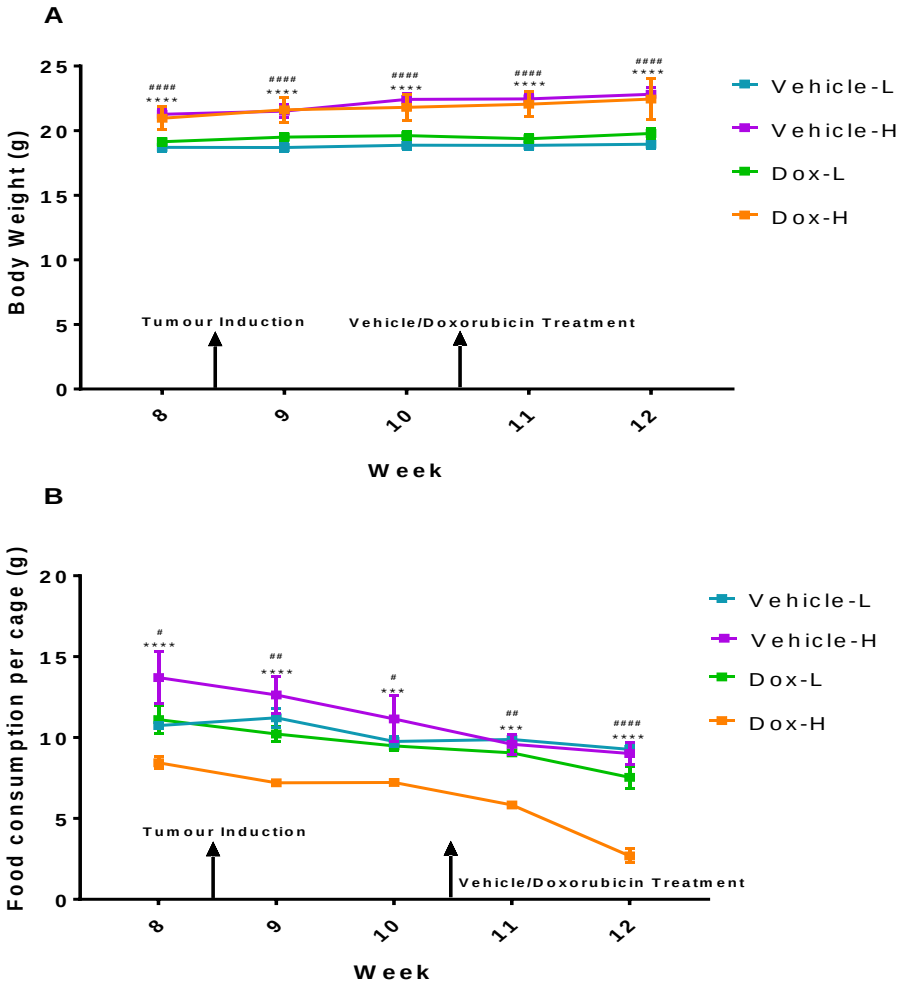

Figure 2.3: (A) Mean body weight, and (B) food consumption of mice (n=5 per cage) receiving vehicle or doxorubicin treatment on LFD or HFD. Results are presented as mean ± SEM (n=10 per group). Three-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. *** = $p<0.001$ and *** = $p<0.0001$. $^{\#}$ = $p<0.05$, $^{\#\#}$ = $p<0.01$, $^{\#\#\#\#}$ = $p<0.0001$. * Vehicle-H *vs* Dox-H, $^{\#}$ = Dox-L *vs* Dox-H.

It was noted that the Dox-H mice showed significantly lower food consumption compared to vehicle-H mice at week 8 ($p<0.0001$), week 9 ($p<0.0001$), week 10 ($p=0.00031$), week 11 ($p=0.00056$) and week 12 ($p<0.0001$) (Figure 2.3B). The Dox-H mice showed significantly lower food consumption compared to Dox-L mice at week 8 ($p=0.014$), week 9 ($p=0.0053$), week 10 ($p=0.037$), week 11 ($p=0.0029$) and week 12 ($p=0.000039$) (Figure 2.3B).

2.2.1.2 HFD Increases Mammary Adipose-and Tumour Tissue Weight

The vehicle-H mice showed significantly higher mammary adipose tissue weight (p=0.0072) and tumour weight (p=0.023) compared to vehicle-L mice (Figure 2.4). Mice from the Dox-H group also showed significantly higher mammary adipose tissue (p=0.011) and tumour weight (p=0.0089) compared toDox-L group (Figure 2.4).

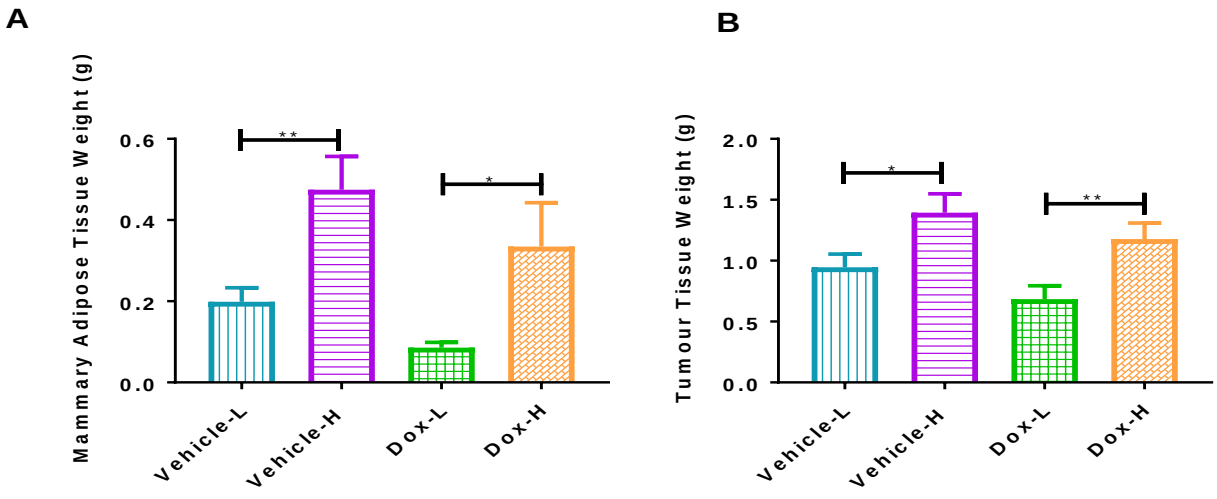

Figure 2.4: Differences in (A) mammary adipose tissue weight, and (B) tumour weight of vehicle and doxorubicin treatment groups on LFD *vs* HFD. Results are presented as mean ± SEM (n=9-10 per group). Two-way ANOVA with Fishers LSD *post hoc* correction.p<0.05 was considered as statistically significant. * = p<0.05; ** = p<0.01.

2.2.2 Diet-Induced Obesity Promotes Tumour Progression by Decreasing Doxorubicin Treatment Efficacy in Breast Cancer Tumours

To determine whether DIO is a causal factor for treatment resistance differences in tumour volume were assessed between all four experimental groups. Vehicle treated mice on the HFD (vehicle-H) showed a significantly higher tumour volume compared to corresponding mice on the LFD (vehicle-L), at day 18 (p=0.04), day 19 (p=0.03), day 20 (p<0.0001), day 21 (p<0.0001), day 22 (p=0.01), day 23 (p<0.0001), day 24 (p=0.02), day 25 (p=0.01), day 26 (p<0.0001), and day 27 (p<0.0001) (Figure 2.5). Therefore, obesity is associated with enhanced tumour growth in our model.

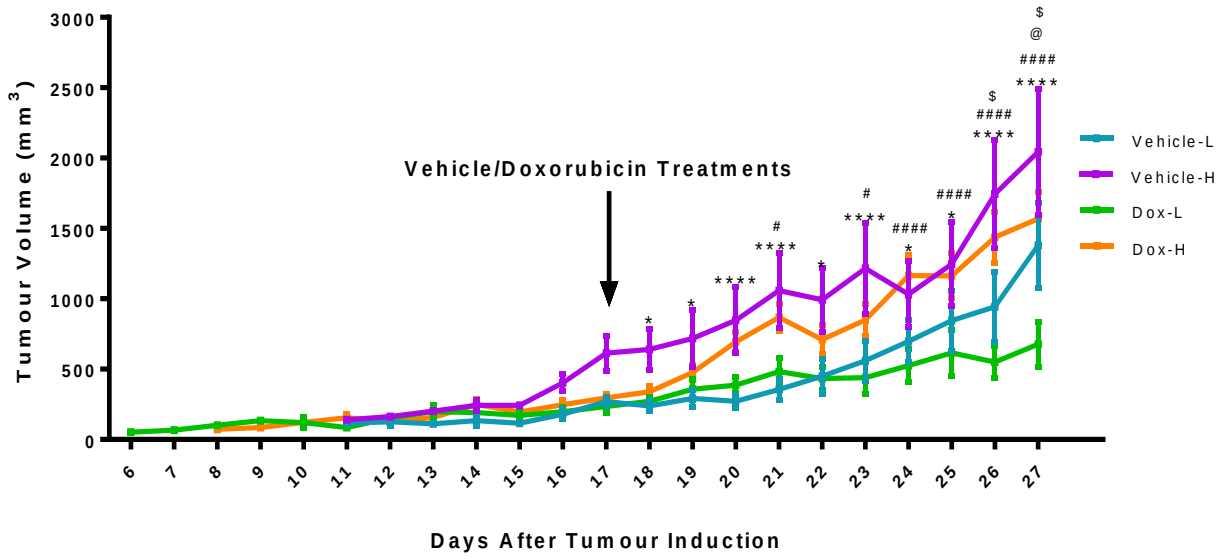

Figure 2.5: Differences in tumour volume for the vehicle and doxorubicin treatment groups on LFD *vs* HFD. Results are presented as mean ± SEM (n=10 per group). Three-way ANOVA with Fishers LSD *post hoc* correction. $p<0.05$ was considered as statistically significant. * = $p<0.05$ and **** = $p<0.0001$. $^{\#}$ = $p<0.05$ and $^{\#\#\#\#}$ = $p<0.0001$, $^{@ \text{ and } \$}$ = $p<0.05$. * Vehicle-L *vs* Vehicle-H, $^{\#}$ = Dox-L *vs*Dox-H, $^{@}$ Vehicle-L *vs* Dox-L, $^{\$}$ Vehicle-H *vs* Dox-H.

Interestingly, mice fed a HFD treated with doxorubicin (Dox-H), showed a significantly higher tumour volume compared to corresponding mice on the LFD (Dox-L), at day 21 ($p=0.04$), day 23 ($p=0.03$), day 24 ($p<0.0001$), day 25 ($p<0.0001$), day 26 ($p<0.0001$), and day 27 ($p<0.0001$) (Figure 2.5). It was also noted that the Dox-L mice showed a significantly lower tumour volume at day 27 compared to Vehicle-L mice ($p<0.0001$), and Dox-H mice yielded a significantly lower tumour volume at day 26 ($p=0.02$) and day 27 ($p=0.01$) compared to the vehicle-H mice (Figure 2.5). Therefore, DIO is associated withbreast cancer treatment resistance in our model. In light of these results, we further aimed to identify potential causal factors that could play a role in DIO-treatment resistance within our model.

2.2.3 Diet-Induced Obesity Induces Systemic Inflammation and Local Inflammation in Mammary Adipose Tissue of Obese Mice treated with Doxorubicin

Evidence support the role of obesity induced inflammation in breast cancer progression and acquired breast cancer drug resistance (Bougaret *et al*., 2018; Incio *et al*., 2018). We therefore determined if obesity induced inflammation (systemic and local) is associated with treatment resistance by determining differences in inflammatory markerconcentrationsbetween all four experimental groups. Interleukin-6 (IL-6) was significantly higher in Dox-H mice compared to vehicle-H mice (p=0.067, Figure 2.6A). A trend towards significance (p=0.07, Figure 2.6A) was observed for IL-6 in the Dox-H compared to Dox-L mice.

The doxorubicin treated HFD mice (Dox-H) showed significantly higher resistin (p=0.046, Figure 2.6D) and lower VEGF-A concentration (p=0.013, Figure 2.6F) compared to Dox-L mice. No significant differences were reported for TNF-α (Figure 2.6B), MCP-1 (Figure 2.6E), PAI-1 (Figure 2.6G) and insulin (Figure 2.6H), between any of the respective experimental groups.Leptin concentration were significantly higher in vehicle-H compared to vehicle-L mice (p=0.024, Figure 2.6C) and Dox-H compared to Dox-L mice (p=0.025, Figure 2.6C). In addition, a positive correlationbetween leptin and mammary adipose tissue weight was found (Figure 2.7). Significant strong positive correlations were only observed for the doxorubicin treatment groups (Dox-L, r=0.78, p=0.008, and Dox-H, r=0.92, p=0.001) (Figure 2.7C&D). IL-10 and IL-1β was undetectable within all samples of all the experimental groups.

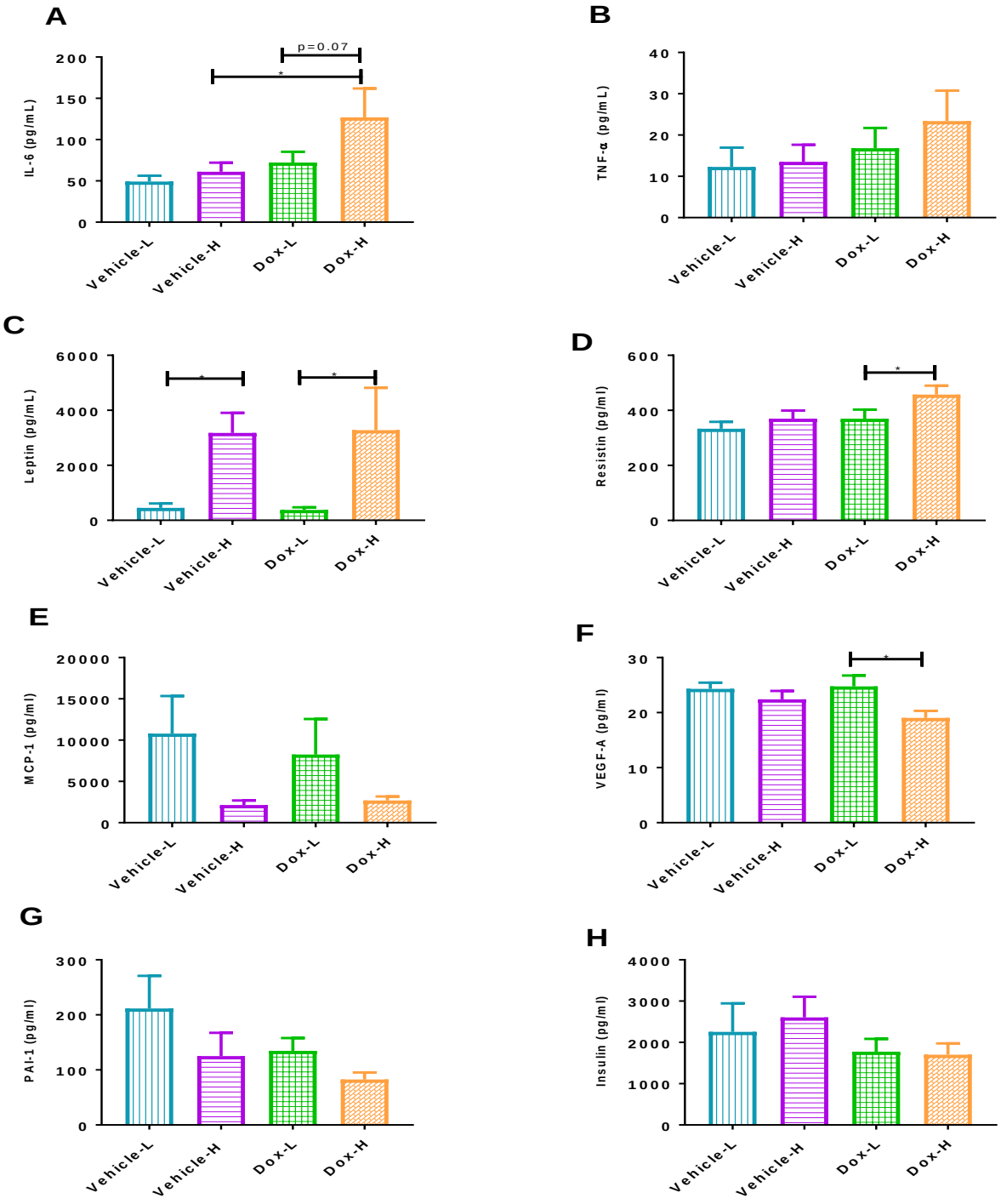

Figure 2.6: Mean inflammatory marker concentrations for all treatment groups. (A) IL-6 (B) TNF-α, (C) Leptin and (D) Resistin, (E) MCP-1, (F) VEGF-A, (G) PAI-1 and (H) Insulin.Results are presented as mean ± SEM (n=6-9) where two-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. *= $p<0.05$.

Since mammary adipose tissue is in close proximity to breast tumours, it is hypothesized that mammary adipose tissue, could display inflammation similar to what is observed in visceral adipose tissue of obese individuals and may therefore play a significant role in obesity linked treatment resistance. NFκB-p65 protein expression in mammary adipose tissue of all experimental treatment groups, revealed that Dox-H mice showed significantly higher protein expression of NFκB-p65 compared to both VC-H (p=0.0073) and Dox-L (p=0.025) mice, respectively (Figure 2.8F).

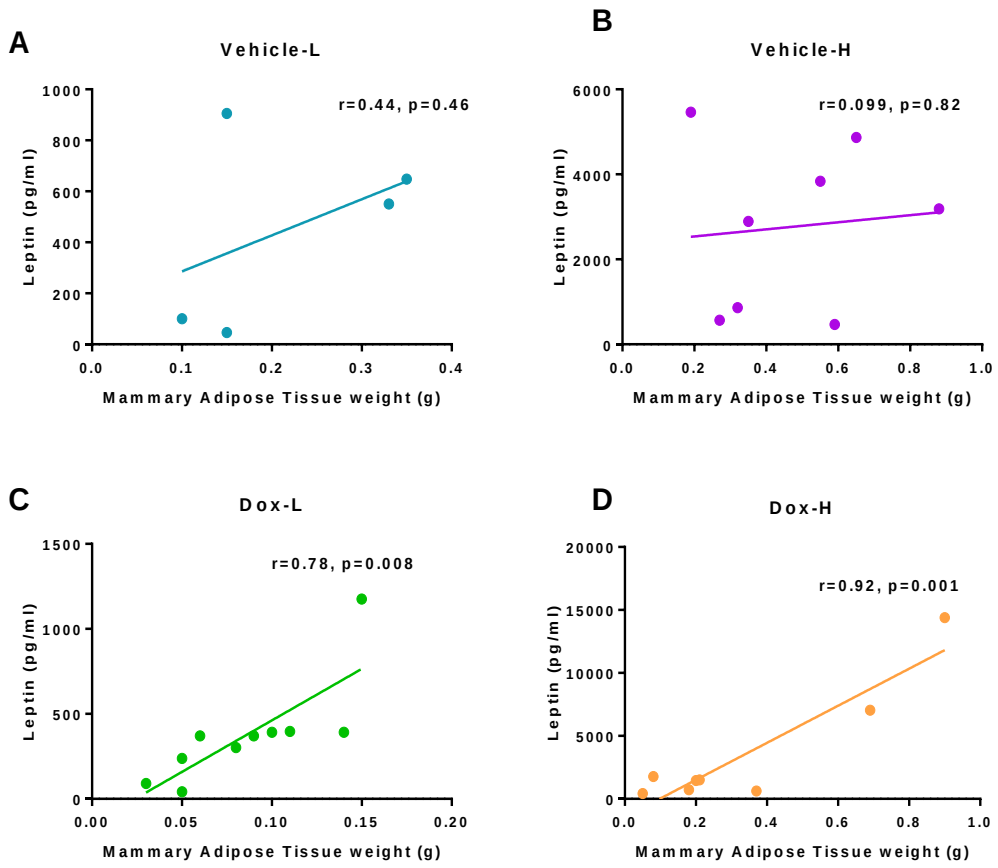

Figure 2.7: Pearson correlations between the leptin concentration and mammary adipose tissue weight for (A&B) vehicle and doxorubicin treated groups (C&D) on LFD and HFD**.**

2.2.4 Diet-Induced Obesity and Doxorubicin Supress *De Novo* Lipogenesis and Lipolysis in Mammary Adipose Tissue

To determine whether DIO alters lipid metabolism protein expression in adjacent mammary adipose tissue and in turn exhibit pro-tumour effects, we measured protein expression levels of markers of *de novo* FA synthesis (FAS and SCD-1), lipolysis (ATGL and HSL) as well as FA transport (FABP-4) within mammary adipose tissue.

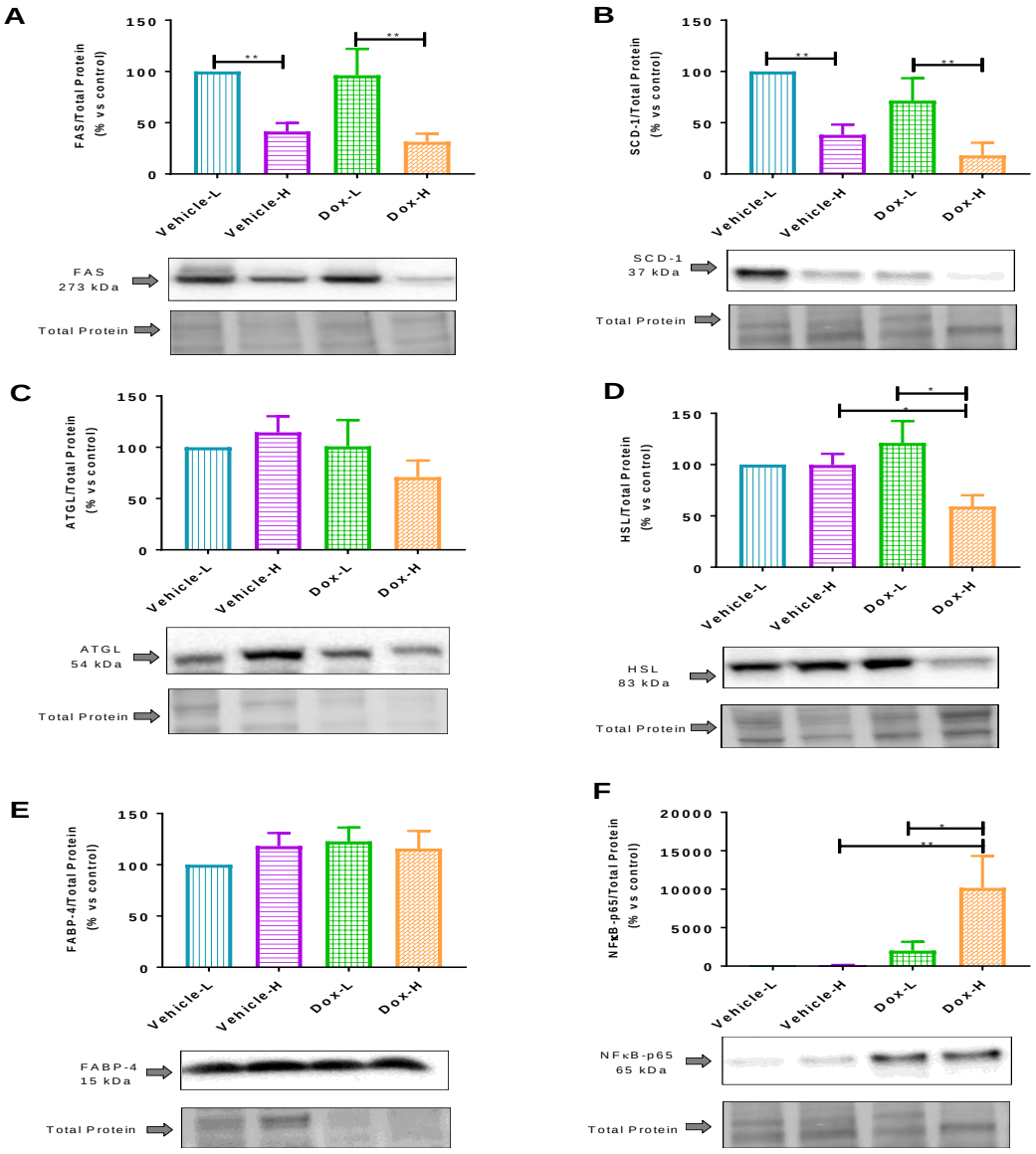

Figure 2.8: Western blot analyses of lipid metabolism marker protein expression in mammary adipose tissue, (A) FAS, (B) SCD-1, (C) ATGL, (D) HSL, (E) FABP-4 and (F) NFκB-p65. Results are presented as mean ± SEM (n=6-8) where two-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. *= $p<0.05$, **= $p<0.01$.

Fatty acid synthase (FAS) and SCD-1 was found to be significantly decreased in the vehicle-H compared to vehicle-L mice (FAS, p=0.0081 and SCD-1, p=0.0035 Figure 2.8 A&B) and Dox-H compared to Dox-L mice (FAS, p=0.0039 and SCD-1, p=0.0099 Figure 2.8 A&B).Moreover, HSL was significantly lower in Dox-H compared to both vehicle-H (p=0.038, Figure 2.8D) and Dox-L mice (p=0.025, Figure 2.8D). No significant differences were observed for ATGL (Figure 2.8C) and FABP-4 (Figure 2.8E), between any of the respective experimental groups.

2.2.5 Diet-Induced Obesity Increases *De Novo* Lipogenesis and Lipolysis in Breast Cancer Tumours Treated with Doxorubicin

In order to establishwhether DIO altered lipid metabolism within the tumour microenvironment, we determined protein expression of markers regulating *de novo* FA synthesis (SCD-1) and lipolysis (ATGL) within tumour tissue. Interestingly, we found that Dox-H mice showed a significant increase in the protein expression of SCD-1 and ATGL compared to vehicle-H (SCD-1, p=0.011 and ATGL, p=0.0030) and Dox-L mice (SCD-1, p=0.031 and ATGL, p=0.0014) in tumour tissue (Figure 2.9).

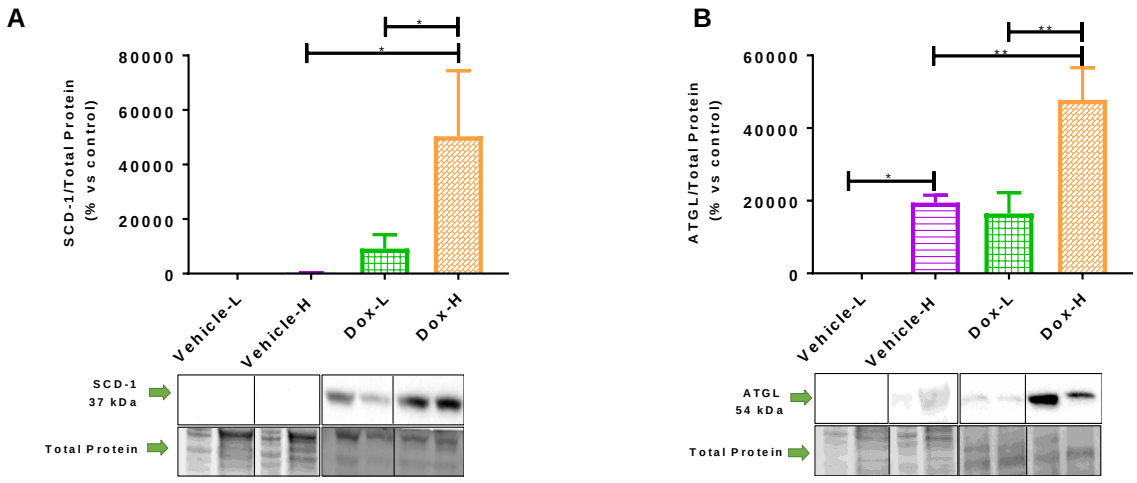

Figure 2.9: Western blot analysis of lipid metabolism protein expression in tumour tissue. (A) SCD-1 (n=5) and (B) ATGL (n=4). Results are presented as mean ± SEM, where two-way ANOVA with Fishers LSD *post hoc* correction was employed. p<0.05 was considered as statistically significant. *= p<0.05, **= p<0.01.

A significant strong negative correlation was also found between mammary adipose tissue HSL protein expression and plasma resistin concentration in the HFD doxorubicin treatment group (Dox-H, r= -0.73, p=0.04) (Figure 2.10D).

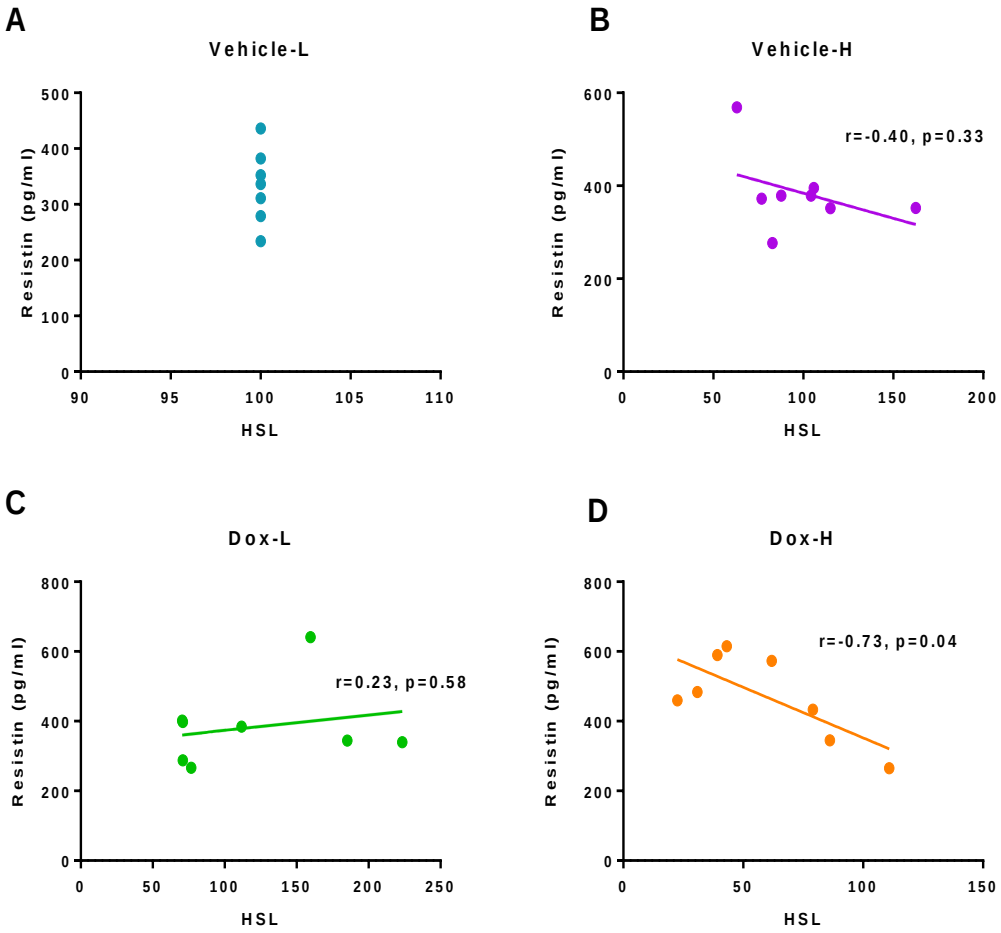

Figure 2.10: Pearson correlations between the resistin concentration and HSL mammary adipose protein expression for (A&B) vehicle and (C&D) doxorubicin treated groups.

2.2.6 Mammary Adipose-and Tumour Tissue Fatty Acid Profiles

Dysfunctional FAs and/or dysregulation in relation to dietary intake as well as newly synthesised FAs might be implicated in breast cancer progression and/or chemotherapeutic treatment resistance. The FA composition of both mammary adipose tissue and tumour tissue, in a mice model on a LFD compared to a HFD was determined. This was done to explore any alterations in mammary adipose and tumour tissue FA profiles in a neoplastic environment as well as to understand if/how changes in tumour and adipose FA profiles could impact chemotherapy treatment outcomes.

A total lipid fatty acid profile of mammary adipose tissue was determined as well as both the TPLand FFA profile of tumour tissue. All FAs for both mammary adipose tissue (total lipid) and tumour tissue (TPL) are summarised in Tables 2.3 and 2.4, where only some FAs are presented in graphs. To determine whether the LFD and/or HFD could have an effect on mammary adipose-and-tumour tissue FA profiles in mice receiving vehicle or doxorubicin treatment.

The predominant FA classes in mammary fat were MUFAs, followed by SFAs and PUFAs in all treatment groups (Supplementary Figure 1). In the tumour TPL fraction, the predominant FA classes were SFAs, followed by PUFAs and MUFAs in all experimental groups (Supplementary Figure 2).

Table 2.3: Mammary adipose tissue total fatty acid percentage composition of mice from the different experimental groups.

Adipose Tissue total fatty acid percentage					Significance			
Fatty Acid	**Vehicle-L (%)**	**Vehicle-H (%)**	**Dox-L (%)**	**Dox-H (%)**	**Vehicle-L *vs* Vehicle-H**	**Vehicle-L *vs* Dox-L**	**Vehicle-H *vs* Dox-H**	**Dox-H *vs* Dox-L**
Saturated Fatty Acids								
∑ SFAs	29,29 ± 0,43	30,03 ± 0,68	27,18 ± 0,40	28,26 ± 0,62	NS	*	*	NS
14:0 (Myristic Acid, MA)	1,93 ± 0,095	1,25± 0,041	1,48 ± 0,055	1,11 ± 0,062	****	***	NS	**
16:0 (Palmitic Acid, PA)	23,27 ± 0,30	21,13 ± 0,45	20,6 ± 0,14	19,73 ± 0,86	**	**	*	NS
17:0 (Margaric Acid, MGA)	0,24 ± 0,057	0,49 ± 0,032	0,32± 0,042	0,48 ± 0,036	NS	NS	NS	NS
18:0 (Stearic Acid, SA)	3,62 ± 0,25	6,88 ± 0,22	4,48 ± 0,33	6,69 ± 0,46	****	*	NS	****
20:0 (Arachidic Acid, ARA)	0,13 ± 0,015	0,17 ± 0,025	0,20 ± 0,022	0,14 ± 0,027	NS	*	NS	NS
Monounsaturated Fatty Acids								
∑ MUFAs	49,65 ± 0,46	43,36 ± 0,18	50,64 ± 0,61	44,95 ± 0.80	****	NS	NS	****
∑ n-7 MUFAs	10,41 ± 0.22	4,94 ± 0,23	9,102 ± 0,34	4,574 ± 0,29	****	**	NS	****
16:1n-7 (Palmitoleic Acid, PTA)	6,86 ± 0,15	2,65 ± 0,15	4,98 ± 0,27	2,32 ± 0,26	****	****	NS	****
18:1n-7 (Vaccenic Acid, VA)	3,54 ± 0,10	2,29 ± 0,16	4,13 ± 0,11	2,26 ± 0,13	**	****	NS	****
∑ n-9 MUFAs	39,24 ± 0,66	38,42 ± 0,37	41,53 ± 0,70	40,37 ± 1,06	NS	*	NS	NS
18:1n-9 (Oleic Acid, OA)	38,29 ± 0,71	37,57 ± 0,37	40,2 ± 0,72	39,55 ± 1,00	NS	NS	NS	NS
20:1n-9 (Gondoic Acid, GA)	0,94 ± 0,04	0,80 ± 0,04	1,21 ± 0,09	0,79 ± 0,08	NS	**	NS	***
Polyunsaturated Fatty Acids								
∑ PUFAs	21,09 ± 0,69	26,61 ± 0,68	22,18 ± 0,35	26,79 ± 0,24	****	NS	NS	****
∑ n-3 PUFA	1,07 ± 0.054	1,36 ± 0,039	0,89 ± 0,13	1,25 ± 0,096	*	NS	NS	**
18:3n-3 (α-Linolenic Acid, ALA)	0,85 ± 0,041	0,76 ± 0,032	0,64 ± 0,029	0,72 ± 0,065	NS	**	NS	NS
22:6n-3 (Docosahexaenoic Acid, DHA)	0,19 ± 0,016	0,34 ± 0,018	0,18 ± 0,042	0,28 ± 0,038	**	NS	NS	*
∑ n-6 PUFAs	20,00 ± 0,66	25,25 ± 0,64	21,29 ± 0,23	25,54 ± 0,19	****	NS	NS	****

18:2n-6 (Linoleic Acid, LA)	18,82 ± 0,70	23,27 ± 0,61	19,99 ± 0,11	23,71 ± 0,20	****	NS	NS	****
18:3n-6 (γ-Linolenic Acid, γ-LA)	0,17 ± 0,018	0,16 ± 0,0055	0,17 ± 0,011	0,16 ± 0,014	NS	NS	NS	NS
20:2n-6 (Eicosadienoic Acid, EDA)	0,25 ± 0,013	0,65 ± 0,019	0,31 ± 0,022	0,66 ± 0,038	****	NS	NS	****
20:3n-6 (Dihomo-γ-Linolenic Acid, DGLA)	0,16 ± 0,010	0,24 ± 0,010	0,17 ± 0,015	0,23 ± 0,013	***	NS	NS	**
20:4n-6 (Arachidonic Acid, AA)	0,044 ± 0,25	0,58 ± 0,0098	0,46 ± 0,043	0,56 ± 0,069	*	NS	NS	*
22:4n-6 (Adrenic Acid, ADA)	0,11 ± 0,017	0,20 ± 0,0073	0,12 ± 0,038	0,18 ± 0,045	*	NS	NS	NS
22:5n-6 (Docosapentaenoic Acid, DPA)	0,080 ± 0,0058	0,12 ± 0,0037	0,090 ± 0,040	0,042 ± 0,014	*	NS	NS	*
Ratios								
SCD-16 =16:1n-7/16:0	0,30 ± 0,010	0,13 ± 0,007	0,24 ± 0,012	0,12 ± 0,009	****	**	NS	****
SCD-18=18:1n-9/18:0	10,83 ± 0.90	5,49 ± 0,19	9,18 ± 0.72	6, 00 ± 0.36	****	NS	NS	**

Results are presented as mean ± SEM (n=5 per group) where two-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. *= $p<0.05$, **= $p<0.01$, *** = $p<0.001$ and **** $p<0.0001$. Abbreviations: Dox-L, tumour doxorubicin-LFD; Dox-H, tumour doxorubicin-HFD; NS, Non-Significant; Vehicle-L, tumour vehicle-LFD; Vehicle-H, tumour vehicle-HFD; SCD, stearoyl-CoA desaturase (estimated desaturase activity using product-to-precursor FA ratios: SCD-16 = 16:1n-7:16:0 ratio; SCD-18 = 18:1n-9:18:0 ratio).

Table 2.4: Tumour tissue total phospholipid fatty acid percentage composition of all experimental groups.

Tumour TPL					Significance			
Fatty Acid	**Vehicle-L (%)**	**Vehicle-H (%)**	**Dox-L (%)**	**Dox-H (%)**	**Vehicle-L *vs* Vehicle-H**	**Vehicle-L *vs* Dox-L**	**Vehicle-H *vs* Dox-H**	**Dox-H *vs* Dox-L**
Saturated Fatty Acids								
∑ SFAs	39,67 ± 0,17	41,57 ± 0,46	40,52 ± 0,27	42,38 ± 0,17	***	NS	NS	***
14:0 (Myristic Acid, MA)	1,53 ± 0,050	1,59 ± 0,079	1,49 ± 0,053	1,30 ± 0,061	NS	NS	**	*
16:0 (Palmitic Acid, PA)	19,76 ± 0,22	19,47 ± 0,30	20,65 ± 0,20	20,53 ± 0,24	NS	*	**	NS
18:0 (Stearic Acid, SA)	17,3 ± 0,067	19,52 ± 0,19	17,28 ± 0,10	19,46 ± 0,10	****	NS	NS	****
20:0 (Arachidic Acid, ARA)	0,033 ± 0,033	0,11 ± 0,077	0,097 ± 0,097	0,11 ± 0,069	NS	NS	NS	NS
Monounsaturated Fatty Acids								
∑ MUFAs	27,76 ± 0,31	21,88 ± 0,28	24,24 ± 0,17	19,28 ± 0,20	****	****	****	****
∑ n-7 MUFAs	6,49 ± 0,047	4,13 ± 0,035	5,53 ± 0,084	3,45 ± 0,064	****	****	****	****
16:1n-7 (Palmitoleic Acid, PTA)	1,84 ± 0,027	0,98 ± 0,016	1,54 ± 0,010	0,72 ± 0,015	****	****	****	****
18:1n-7 (Vaccenic Acid, VA)	4,65 ± 0,026	3,15 ± 0,025	4,30 ± 0,079	2,72 ± 0,050	****	***	****	****
∑ n-9 MUFAs	21,27 ± 0,28	17,75 ± 0,27	18,41 ± 0,14	15,84 ± 0,16	****	****	****	****
18:1n-9 (Oleic Acid, OA)	17,47 ± 0,25	14,69 ± 0,21	15,04 ± 0,10	12,85 ± 0,13	****	****	****	****
20:1n-9 (Gondoic Acid, GA)	1,11 ± 0,025	1,07 ± 0,021	0,93 ± 0,023	0,80 ± 0,027	NS	****	****	**
22:1 n-9 (Erucic Acid, EA)	0,30 ± 0,0066	0,27 ± 0,018	0,25 ± 0,0072	0,24 ± 0,016	NS	*	NS	NS
24:1n-9 (Nervonic Acid, NA)	2,39 ± 0,062	1,73 ± 0,093	2,18 ± 0,072	2,00 ± 0,083	****	NS	*	NS
Polyunsaturated Fatty Acids								
∑ PUFAs	32,57 ± 0,25	36,55 ± 0,24	35,24 ± 0,31	38,34 ± 0,12	****	****	***	****
∑ n-3 PUFA	6,00 ± 0,14	6,64 ± 0,15	7,06 ± 0,27	6,86 ± 0,10	*	***	NS	NS

22:5n-3 (Docosapentaenoic Acid, DPA)	1,04 ± 0,0079	1,20 ± 0,027	1,08 ± 0,019	1,32 ± 0,12	NS	NS	NS	*
22:6n-3 (Docosahexaenoic Acid, DHA)	4,95 ± 0,14	5,31 ± 0,10	5,89 ± 0,33	5,5 ± 0,09	NS	**	NS	NS
∑ n-6 PUFAs	26,57 ± 0,14	29,92 ± 0,23	28,18 ± 0,10	31,48 ± 0,14	****	****	****	****
18:2n-6 (Linoleic Acid, LA)	8,51 ± 0,05	10,5 ± 0,12	9,02 ± 0,01	10,63 ± 0,09	****	***	NS	****
20:2n-6 (Eicosadienoic Acid, EDA)	0,65 ± 0,011	1,30 ± 0,027	0,71 ± 0,059	1,31 ± 0,034	****	NS	NS	****
20:3n-6 (Dihomo-γ-Linolenic Acid, DGLA)	1,73 ± 0,030	1,64 ± 0,026	1,59 ± 0,017	1,47 ± 0,025	*	**	***	**
20:4n-6 (Arachidonic Acid, AA)	12,27 ± 0,12	12,31 ± 0,087	13,11 ± 0,18	13,36 ± 0,092	NS	****	****	NS
22:4n-6 (Adrenic Acid, ADA)	2,98 ± 0,025	3,76 ± 0,053	3,19 ± 0,060	4,33 ± 0,057	****	**	****	****
22:5n-6 (Docosapentaenoic Acid, DPA)	0,43 ± 0,02	0,40 ± 0,02	0,49 ± 0,01	0,38 ± 0,01	*	NS	NS	***
Ratios								
SCD-16 =16:1n-7/16:0	0,093 ± 0,0021	0,050 ± 0,0010	0,074 ± 0,00061	0,035 ± 0,0011	****	****	****	****
SCD-18=18:1n-9/18:0	1,01 ± 0,012	0,75 ± 0,014	0,87 ± 0,0048	0,66 ± 0,081	****	****	****	****

Results are presented as mean ± SEM (n=5) where two-way ANOVA with *Fishers* LSD *post hoc* correction was employed, $p<0.05$ was considered as statistically significant. *= $p<0.05$, **= $p<0.01$, *** = $p<0.001$ and **** $p<0.0001$. Abbreviations: Dox-L, tumour doxorubicin-LFD; Dox-H, tumour doxorubicin-HFD; NS, Non-Significant; Vehicle-L, tumour vehicle-LFD; Vehicle-H, tumour vehicle-HFD; SCD, stearoyl-CoA desaturase (estimated desaturase activity using product-to-precursor FA ratios: SCD-16 = 16:1n-7:16:0 ratio; SCD-18 = 18:1n-9:18:0 ratio).

2.2.6.1 Diet-Induced Obesity and Doxorubicin Differentially Alters Saturated Fatty Acids in the Tumour Microenvironment

The total saturated fatty acid (∑ SFAs) present in the TPL of tumour tissue, was significantly higher in vehicle-H compared to vehicle-L mice (p=0.00027) and higher in Dox-H compared to Dox-L mice (0.00034) (Figure 2.11A), however this was not true for mammary adipose tissue (Figure 2.11A).

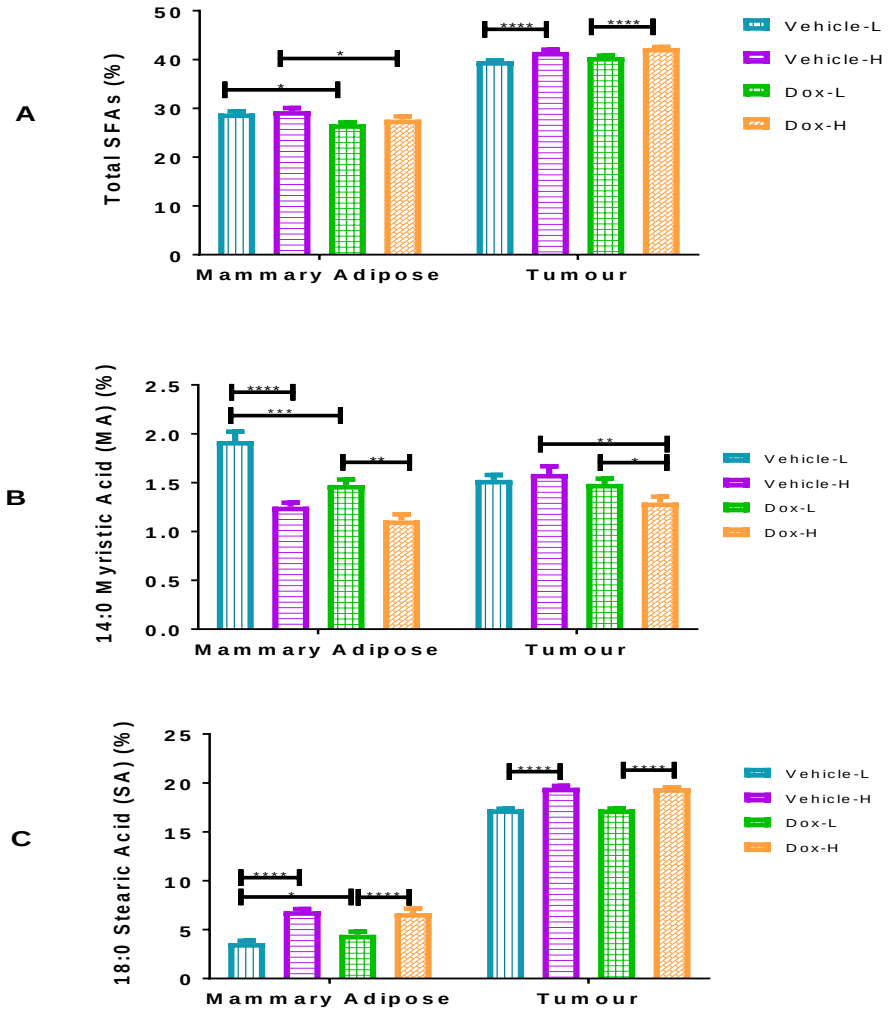

Figure 2.11: Saturatedfatty acid composition (A) Total SFAs, (B) Myristic Acid (MA) and (C) Stearic Acid (SA) of mammary adipose-and-tumour tissue of mice fed a LFD or HFD with either vehicle or doxorubicin treatment. Results are presented as mean ± SEM (n=5) where two-way ANOVA with Fishers LSD *post hoc* correction was employed. p<0.05 was considered as statistically significant. *= p<0.05, **= p<0.01, *** = p<0.001 and **** p<0.0001.

In mammary adipose tissue, myristic acid (MA) was significantly lower in the vehicle-H mice compared to vehicle-L ($p<0.0001$) and lower in vehicle-L compared to Dox-L mice ($p=0.0014$) (Figure 2.11B). Myristic acid was also significantly lower in the Dox-H mice compared to Dox-L mice in tumour tissue ($p=0.041$) (Figure 2.11B).

Stearic Acid (SA) was significantly higher in vehicle-H mice compared to vehicle-L ($p<0.0001$) in mammary adipose tissue. In addition, SA was also found to be significantly higher in the Dox-H mice compared to Dox-L mice ($p<0.0001$) (Figure 2.11C). Similar, in tumour tissue, SA was also higher in vehicle-H compared to vehicle-L mice ($p<0.0001$) and higher in Dox-H compared to Dox-L mice ($p<0.0001$), respectively (Figure 2.11C).

2.2.6.2 Diet-Induced Obesity and Doxorubicin SupressMonounsaturated Fatty Acids in the Tumour and Surrounding Mammary Fat

A similar and significant trend was observed for various MUFAs in both mammary adipose tissue and tumour phospholipid FAs where the total MUFAs (∑ MUFAs), palmitoleic acid (PTA) and oleic acid (OA), were found to be significantly lower in vehicle-H compared to vehicle-L mice, and significantly lower in Dox-H compared to Dox-L mice, respectively (Figure 2.12).However, no significant differences were observed for OA in mammary adipose tissue (Figure 12C).

2.2.6.3 Diet-induced Obesity and Doxorubicin Selectively Increases Polyunsaturated Fatty Acids in the Tumour Microenvironment

The total n-6 PUFAs (∑ n-6 PUFAs), linoleic acid (LA) and eicosadienoic acid (EDA), were significantly higher in the mammary adipose tissue vehicle-H compared to vehicle-L mice (∑ n-6 PUFAs, $p<0.0001$, LA, $p<0.0001$, EDA, $p<0.0001$), and higher in Dox-H compared to Dox-L mice (∑ n-6 PUFAs, $p<0.0001$, LA, $p<0.0001$, EDA, $p<0.0001$) (Figure 2.13).

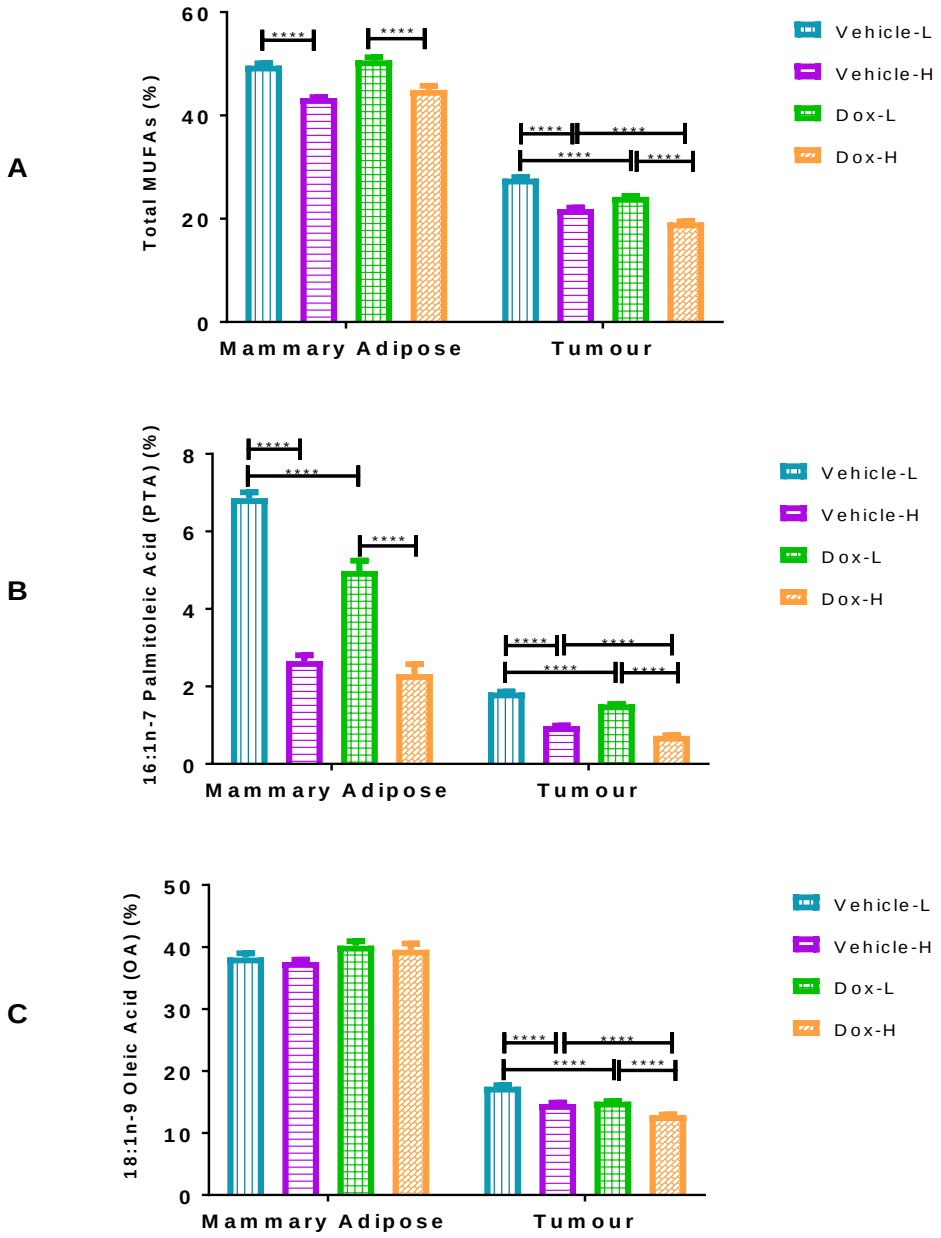

Figure 2.12: Monounsaturated fatty acid composition (A) Total MUFAs (∑ MUFAs), (B) Palmitoleic Acid (PTA) and (C) Oleic acid (OA)of mammary adipose-and-tumour tissue of mice fed a LFD or HFD diet with either vehicle or doxorubicin treatment. Results are presented as mean ± SEM (n=5) where two-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. *= $p<0.05$, **= $p<0.01$, *** = $p<0.001$ and **** $p<0.0001$.

Similar results were observed in the tumour phospholipid FA fraction i.e. higher ∑ n-6 PUFAs, LA and EDA levels in vehicle-H compared to vehicle-L (∑ n-6 PUFAs, $p<0.0001$, LA, $p<0.0001$ and EDA, $p<0.0001$) as well as higher levels of these FAs in Dox-H compared to Dox-L mice (Figure 2.13).

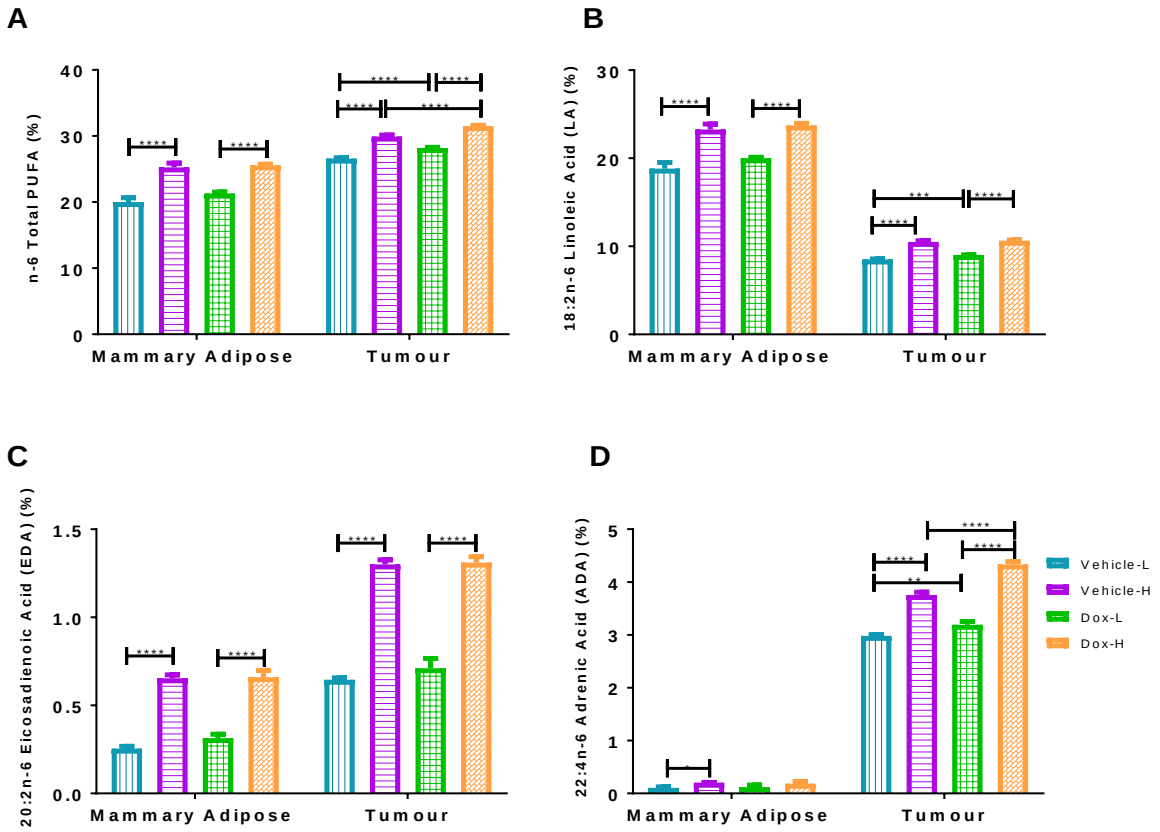

Figure 2.13: Polyunsaturated fatty acid composition (A) ∑ n-6 PUFAs, (B) Linoleic Acid (LA), (C) Eicosadienoic Acid (EDA), and (D) Adrenic Acid (ADA)of mammary adipose-and-tumour tissue of mice fed a LFD or HFD with either vehicle or doxorubicin treatment. Results are presented as mean ± SEM (n=5) where two-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. *= $p<0.05$, **= $p<0.01$, *** = $p<0.001$ and **** $p<0.0001$.

Moreover, in mammary adipose tissue total n-3 PUFAs (∑ n-3 PUFAs) and DHAwere significantly higher in the vehicle-H compared to vehicle-L mice (∑ n-3 PUFAs, $p=0.029$ and DHA, $p=0.0034$) and higher in Dox-H compared to Dox-L mice (∑ n-3 PUFAs, $p=0.0095$ and DHA, $p=0.037$), respectively (Table 2.3). The only significant difference observed for ALA was decreased levels were present in Dox-L compared to vehicle-L ($p=0.0040$,

Table2.3) in mammary adipose tissue, whereas ALA was not detected in any of the treatment groups in the tumour tissue. In the TPL of tumour tissue, ∑ n-3 PUFAs was only found to be significantly higher for vehicle-H compared to vehicle-L mice (p=0.021) (Table 2.4).

It was also noted that in the tumour tissue, adrenic acid (ADA) was significantly higher in Dox-H compared to Dox-L mice (p<0.0001, Figure 2.13D). Higher levels of ADAwerealso observed for vehicle-H treated mice compared to vehicle-L mice in mammary adipose tissue (p=0.043, Figure 2.13D) and tumour tissue (p<0.0001, Figure 2.13D). The opposite was found to be true for docosapentaenoic acid (DPA, 22:5n-6) in mammary adipose tissue (p=0.049, Table 2.3) and tumour tissue (p=0.014, Table 2.4).

2.2.7 Desaturation Indexes

In mammary adipose tissue stearoyl-CoA desaturase-16 (SCD-16) and stearoyl-CoA desaturase-18 (SCD-18) ratios were significantly lower in vehicle-H compared to vehicle-L mice (SCD-16, p<0.0001 and SCD-18, p<0.0001) and lower in Dox-H compared to Dox-L mice (SCD-16, p<0.0001 and SCD-18, p=0.0020) (Table 2.3). Similar results were observed in the tumour tissue i.e. SCD-16 and SCD-18 were significantly lower in vehicle-H compared to vehicle-L (SCD-16, p<0.0001and SCD-18, p<0.0001) and lower in Dox-H compared to Dox-L (SCD-16, p<0.0001 and SCD-18, p<0.0001) (Table 2.4).

2.2.8 Fatty Acid Principal Component Profiles

Principle component analysis (PCA) was performed on both total lipid mammary and tumour total phospholipid FA profile data. Based on PCA, two principle components are compared to generate bi-plot graphs displacing FA profile data of all four respective treatment groupsfor both mammary adipose tissue and tumour tissue.

For mammary adipose tissue, the first principle component (PC 1) is responsible for 51% of the total variation and the second principle component (PC2) accounts for 20% of the total variation. The mammary adipose tissue bi-plot shows distinct differences in the FA profile between the four treatment groups. For example, vehicle-L treated mice correlated positively

with MA and total n-7 MUFAs (∑ n-7 MUFAs) and PTA, whereas vehicle-H correlated positively with PUFAs i.e. n-3 (DHA) and n-6 (DGLA, AA and ADA) (Figure 2.14). Moreover, Dox-L treated mice correlated positively with SFAs (MA and PA) and MUFAs (VA and GA), whereas Dox-H mice correlated positively with n-3 (DHA) and n-6 (LA, EDA, DGLA, AA and ADA) (Figure 2.14).

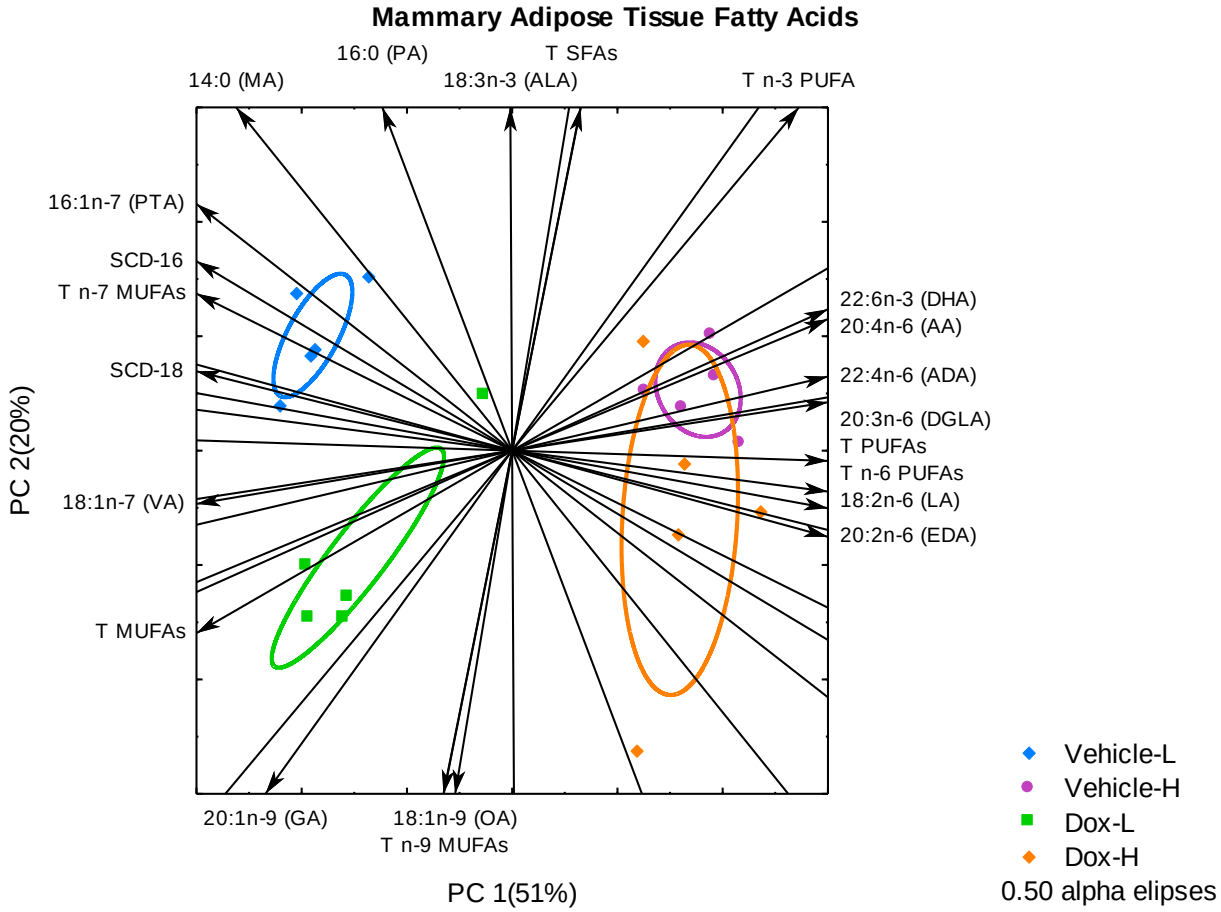

Figure 2.14: Bi-plot of mammary adipose tissue determined by PCA of FA profile results.

For tumour tissue total phospholipids, the PC1 is responsible for 64% of the total variation, and the PC2 accounts for 18% of the total variation, respectively. The tumour tissue bi-plot shows distinct differences in the FA profile between the four treatment groups. Moreover, vehicle-L treated mice showed a positive correlation for MUFAs (PA, VA and OA), whereas vehicle-H showed a positive correlation with SFAs (SA) and n-6 PUFA (LA and EDA) (Figure 2.15). Doxorubicin treated mice on the LFD (Dox-L) showed a positive correlation for MUFAs (PTA, VA and NA) and n-6 PUFAs (DPA), whereas Dox-H mice correlated

positively with total SFA, SA and n-6 (ADA). Our results provide a unique perspective to the distinct FA profiles in mice fed a LFD or HFD receiving vehicle or doxorubicin treatment in a tumour bearing model (Figure 2.15).

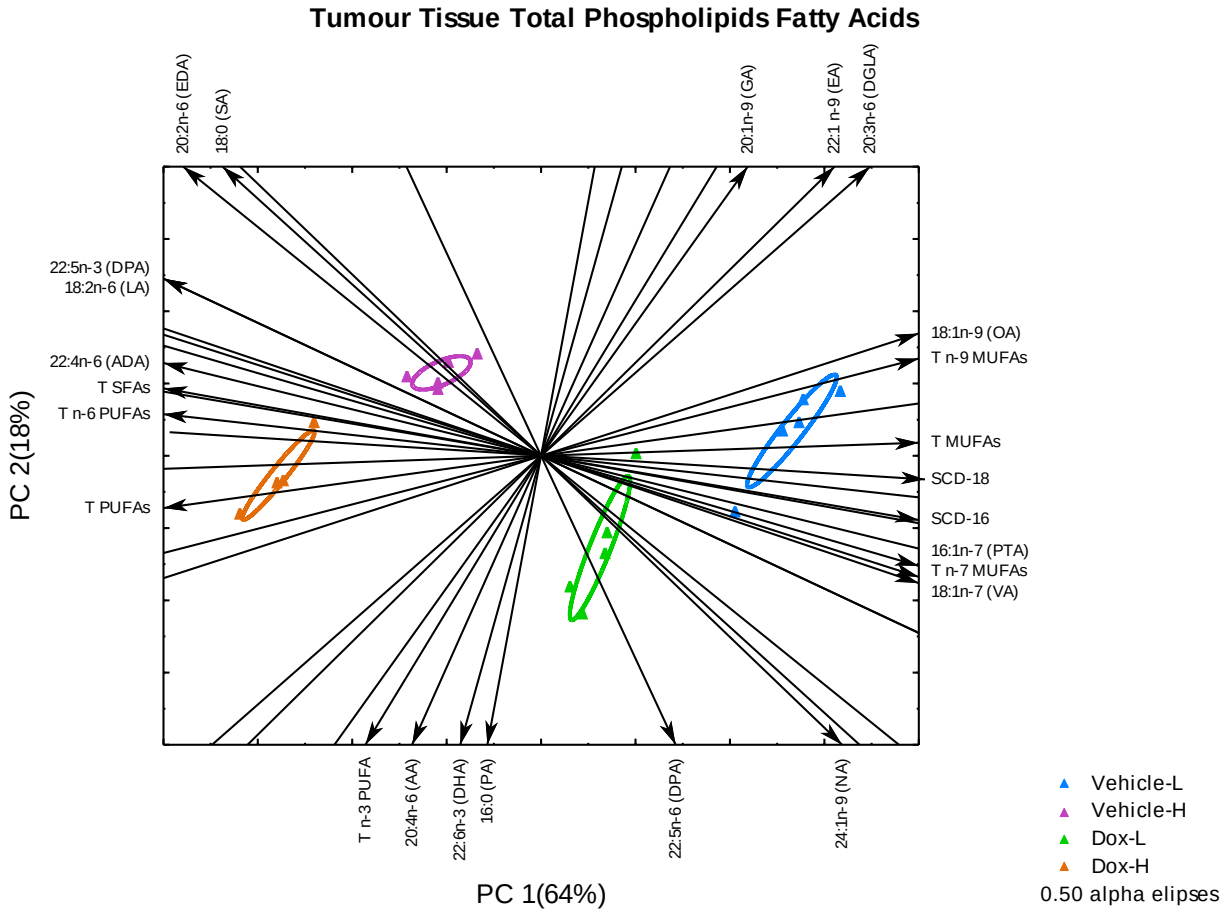

Figure 2.15: Bi-plot of tumour tissue phospholipids determined by PCA of FA profile results.

2.3 DISCUSSION: *IN VIVO* MODEL

To determine whether DIO could affect the efficacy of a chemotherapeutic agent (doxorubicin), we developed an obesogenic breast cancer model, where female C57BL6 mice were fed a HFD. After developing the DIO phenotype, tumour xenographs were induced, followed by either vehicle or doxorubicin treatment.

2.3.1 Body Weight and Tumour Volume: Diet-Induced Obesity Significantly Decreases Doxorubicin Treatment Efficacy in Breast Tumours

As with human studies establishing strong correlations between obesity, larger tumour sizes and positive lymph node involvement (De Azambuja *et al*., 2010, Gevorgyan *et al*., 2016; Haakinson *et al*., 2012), animal models also report on DIO promoting tumour growth, progression and metastasis (Guiu *et al*., 2010, Lashinger *et al*., 2014; Stemmer *et al*., 2012,), specifically in breast cancer (Bousquenaud *et al*., 2018, Dong *et al*., 2017, Incio *et al*., 2018).

In our study we established that mice fed a HFD showed significantly increased body weight after six to eight weeks (effect of diet alone) compared to LFD controls (Figure 2.2A).Additionally, a continued increase in body weight was found in Dox-H mice compared to LFD control mice, confirming an obese phenotype (Figure 2.3A). The obesity phenotype observed in HFD fed mice was corroborated by a significantly higher mammary adipose tissue weight observed in the HFD groups of both vehicle and doxorubicin treated mice compared to the LFD mice respectively (Figure 2.4A). Similar to findings of Hu *et al*. (2004) and Santander *et al* (2015), who showed that a HFD resulted in weight gain in C57BL/6 mice (as a result of increased fat mass), by assessing body composition using nuclear magnetic resonance. As expected, the Dox-H mice showed a significant decrease in body weigh compared to vehicle-H mice (Figure 2.3A), which may be as a result of a well-known side-effect of doxorubicin treatment i.e. weight loss due to loss of appetite (Thivat *et al*., 2010).

Furthermore, we report on higher food consumption in both vehicle-H and Dox-L mice compared to Dox-H mice (Figure 2.3B). This further supports the obesity phenotype observed in HFD mice that was not as a result of increased food consumption. This is specifically true for the Dox-H micewhich displayed hypophagia, but still showed continued

increase in body weight possibly, as a result of insulin insensitivity induced by high leptin levels in Dox-H mice (Figure2.6C).Hypophagia can be explained by high leptin concentrations which has been shown to inhibit food intake by a negative feedback mechanism of action in order to regulate body weight and energy intake (Lin *et al.*, 2000; Klok *et al*., 2006). Furthermore,a side effect of doxorubicin treatment is loss of appetite (Thivat *et al*., 2010).

We found that vehicle-H mice presented with significantly increased tumour weight and tumour volume compared to vehicle-L mice (Figure 2.4&Figure 2.5) and also observed that doxorubicin treated mice on the HFD showed significantly higher tumour weight and tumour volume compared to doxorubicin treated LFD mice (Dox-H *vs* Dox-L, Figure 2.4 & Figure 2.5). Previous studies support these findings that DIO promotestumour growth and decreases treatment efficacy. For example, Lautenbach *et al*. (2009) showed that female obese Sprague Dawley rats (HFD, 60 PE from fat for 8 weeks) were more susceptible to tumour induction by dimethylbenzathraceneand also displayed increased tumour growth compared to controls. This was corroborated by Khalid *et al*. (2010) who found that a HFD (45 PE from fat) significantly increased body weight and fat mass compared to mice on a LFD (10 PE from fat) in a MMTV-HER2/Neu transgenic breast cancer model, and that obesity promoted tumour growth, reflected by an increase in tumour size. A breast cancer model by Cowen *et al*. (2015) reported that female MMTV-PyMT mice on a HFD (35.7 PE fromfat), showed a higher body weight compared to mice on the low fat diet (10 PE from fat) even after adjusting for tumour weight, and tumour volume was also found to be significantly increased in HFD mice compared to LFD mice.

Obesity correlates with poor clinical outcomes in breast cancer patients treated with chemotherapy (doxorubicin), hormonal-based chemotherapy agents and radiotherapy (Bochet *et al*., 2011, Karpińska *et al*., 2015; Gevorgyan *et al*., 2016). Iwase *et al*. (2016) reported that a high visceral fat area is associated with negative clinical outcomes for patients receiving neo-adjuvant chemotherapy treatment regimens. It is also reported in pre-clinical animal models that DIO decreased the efficacy of breast cancer treatment protocols (Incio *et al*., 2018; Lehuédé *et al*., 2019)

Adipose tissue undergoes remodelling which includes adipocyte hyperplasia and hypertrophy (Jung & Choi, 2014; Rezaee & Dashty, 2013), which leads to an increase in fat storage in the form of TAGs in the lipid droplets of adipocytes which then ultimately induces weight gain.

Additionally, diets high in fat has an effect on the secretion of leptin which regulates food intake and satiety functions in the hypothalamus, which contributes to weight gain (Assiri *et al*., 2015). Dysfunctional adipose tissue is characterised by sustained low grade inflammation as a result of abnormal synthesis of various adipokines (increased pro-inflammatory and decreased anti-inflammatory) and immune cell infiltration (Cowen *et al*., 2015), and an increased release of FFA (Bjørndal *et al*., 2011). These FAs can be stored in lipid droplets to be oxidised for energy substrates or be used as precursors for newly synthesised FAs (Zaidi *et al*., 2013), to further promote and accelerate breast cancer tumour cell growth under obesogenic conditions (Currie *et al*., 2013). We have also established that obesity is associatedwith breast cancer treatment resistance in this current DIO mouse model.

2.3.2 Inflammatory Markers: Diet-induced Obesity Induces Systemic and Mammary Fat Inflammation

We have demonstrated that leptin was significantly increased in both vehicle and doxorubicin treated HFD mice compared to LFD control groups (Figure 2.6C). Leptin concentrations also correlated positively with mammary adipose tissue weight in the doxorubicin treated groups. A strong positive correlation was evident in Dox-L and a very strong positive correlation in the Dox-H group (Figure 2.7). These findings indirectly implicate mammary adipose tissue, specifically adipocytes in the tumour microenvironment as a source of leptin secretion in obese mice which showed greater mammary adipose weight.Especially since E0771 breast cancer cells does not produce leptin, even when co-cultured with adipocytes (Santander *et al*., 2015). The increased mammary adipose tissue weight as the result of the HFD could possibly be one of the primary sources of leptin in our study.

Obesity-induced adipokine secretions are detected in local adipose tissue and serum (Popko *et al*., 2010; Santander*et al*., 2015), whereas mRNA expression levels, showed that adipocytes co-cultivated with breast cancer cells showed significantly higher IL-6, IL-1β and TNF-α concentrations (Dirat *et al*., 2011). These elevated circulating cytokines (i.e. IL-6 and IL-8), exert effects at distant sites (Deng *et al*., 2016; Kolb *et al*., 2016), promoting breast cancer progression. This is achieved through upregulation of inflammatory mediator synthesis and increased immune cell infiltration and thereby induce cellular proliferation, angiogenesis as well as inhibition of apoptosis (Cowen *et al*., 2015; Guzik *et al*., 2017; Liu *et al*., 2013;

Lautenbach *et al*., 2009; Khalid *et al*., 2010; Pérez-Hernández *et al*., 2014; Vucenik & Stains, 2012). Evidence also support the role of obesity-induced inflammation in acquired breast cancer drug resistance. For example, adipocytes derived from obese patients diminished Tamoxifen^{R} treatment efficacy compared to adipocytes derived from normal weight patients. Interleukin-6, TNF-α and leptin were identified as potential mediators (Bougaret *et al*., 2018). In agreement, Incio *et al*. (2018) demonstrated that obesity decreased the efficacy of anti-VEGF treatment in both breast cancer patients and in diet-induced obese mice. It was argued that increased inflammatory markers in adipocyte dense microenvironments within tumours can sustain tumour survival (Incio *et al*., 2018).

Leptin and resistin are well-known adipokines that are linked to breast cancer (Surmacz *et al*., 2013). High concentrations of leptin and resistin favours cancer cell proliferation (Liu *et al*., 2013; Liu*et al*., 2017). Leptin is secreted primarily by adipose tissue and increases with higher degrees of adiposity and has been implicated for its role in obesity, inflammation and breast tumorigenesis (Surmacz *et al*., 2013). Leptin increases the synthesis of other pro-inflammatory cytokines and also plays a role in breast cancer progression by increasing cellular proliferation through binding to its receptor and downstream signalling through Jak2/STAT-3, ERK1/2 and PI3K pathways (Rodríguez *et al*., 2013). Elevated serum leptin concentrations and increased expression of leptin receptors are also reported in breast cancer patients, often associated with higher pathological grade tumours and treatment resistance (Assiri *et al*., 2015; Sultana *et al*., 2017). It has been established thatelevated leptin concentrations and leptin receptor expression are associated with the promotion of cancer stem cells survival and self-renewal in breast cancer cells (Sultana *et al*., 2017; Zheng *et al*., 2013), thus contributing to the development of treatment resistance.

Interestingly, resistin was also found to be significantly higher in Dox-H compared Dox-L mice (Figure 2.6D). Resistin is primarily synthesised by adipose tissue in rodents, in contrast to humans where reports indicate that immune cells may also be a potential source (Rosendahl*et al*., 2018). Studies report on higher concentrations of resistin in obese and breast cancer patients (Gharibeh *et al*., 2010; Zeidan *et al*., 2018), which stimulates the secretion of other pro-inflammatory mediators by increasing NFκB expression (Wang *et al*., 2018), thereby favouring breast carcinogenesis. Resistin was also shown to promote cell growth and invasiveness through increased STAT-3 expression and phosphorylation (Wang *et al*., 2018), which was associated with increased invasion and migration *via* epithelial to mesenchymal transition (Lee *et al*., 2016; Wang *et al*., 2018).

A well-known mechanism of developing breast cancer treatment resistance includes the evasion of apoptotic pathways (de Visser & Jonkers, 2009; Wind and Holen *et al*., 2011). Adipocytes protect cancer cells from doxorubicin-induced apoptosis by upregulating the expression of pro-survival markers in the apoptotic pathway (blc-2) as well as by increasing the synthesis of pro-inflammatory markers specifically resistin (Behan *et al*., 2009; Liu *et al*., 2017). Resistin has been identified as a causal factor for acquiring resistance to doxorubicin treatment in both MCF-7 and MDA-MB-231 breast cancer cells by inducing autophagy (Liu *et al*., 2017).

NFκB is an important transcription factor regulating local inflammation in mammary epithelium and adipose tissue (Barham *et al*., 2015). Our results confirmed local inflammation by means of significantly higher levels of NFκB-p65protein expression in mammary adipose tissue of the Dox-H compared to both VC-H and Dox-L mice (Figure 2.8F) in this model. White adipose tissue exists in different anatomical compartments such as subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) (Rezaee & Dashty, 2013). These different adipose tissue depots have its own unique metabolic and adipokine profile (Nieman *et al*., 2013), where VAT is mostly associated with higher relative expression and concentrations of various adipokines i.e. IL-6, TNF-α and immune cell infiltration in an obese state (Saely *et al*., 2012). Theoretically, mammary adipose tissue is classified as SAT, however when considering the volume of fat and its' close proximity to breast tumours, it shares striking similarities to VAT (Rosendahl*et al*., 2018). Additionally, mammary adipose tissue in the tumour microenvironment displays persistent inflammation and also harbour crown like structures which are well known inflammatory *foci* (Cha *et al*., 2018; Vaysse *et al*., 2017). It is therefore our understandingthat mammary adipose tissue displays local inflammation as a result of the DIO similar to that in visceral adipose tissue of obese individuals and as a result may play a significant role in obesity induced breast cancer treatment resistance. It may wellbe that treatment resistance results from obesity induced systemic inflammation. This is further corroborated by inflammatory signalling found in the mammary adipose tissue, as a result of the HFD and subsequent exacerbation by the doxorubicin treatment itself since, where it has been shown to induce inflammation in metabolic tissues (Supriya *et al*., 2016). Therefore, we propose that treatment resistance observed in the obese doxorubicin treated mice may in part be as a result of inflammation. IL-6 was significantly increased in Dox-H compared to vehicle-H mice, whereas no significant differences were observed for TNF-α, MCP-1, PAI and insulin in our model.

We have provided evidence that obesity induces inflammation as a result of adipokine dysfunction in obese vehicle treated mice and to a greater extent in obese doxorubicin treated mice. We propose that DIO drives both systemic and local inflammation in mammary adipose tissue thereby promoting breast cancer growth. This may be achieved by inducing signalling pathways regulating cell growth, inhibition of apoptosis and invasion all of which ultimately contributes to the development of breast cancer treatment resistance. It is evident from these findings that DIO is associated with the development of breast cancer treatment resistance which is mediated bylocal and systemic and inflammation.

2.3.3 Diet-Induced Obesity Distinctly Alters Lipid Metabolism in the Tumour Microenvironment Leading to Changes in Fatty Acid Composition in Mammary Adipose and Tumour Tissue

Under normal physiological conditions, *de novo* FA synthesis is suppressed and only occurs in a small percentage of specific cell types including adipocytes (Zaidi *et al*., 2013). Given the abundance and close proximity of breast cancer tumours to mammary adipose tissue (a source of FAs), breast cancer cells show a strong dependence on exogenous and endogenous lipids to maintain survival. This is accomplished by an increased expression of various enzymes involved in *de novo* FA synthesis including ACC, FAS and SCD-1 enzymes in breast cancer cells (Huang *et al*., 2016; Veigel *et al*., 2015; Yoon *et al*., 2007). Supporting evidence reports on exogenous lipid utilization, where breast cancer cells induce adipocytes to release FFA, *via* activation of lipolysis (increased expression of ATGL and HSL), or inhibition of adipogenesis (PPAR-γ) (Balaban *et al*., 2017; Balaban *et al*., 2018). Adipocyte derived FFA can be taken up by breast cancer cells (Balaban *et al*., 2017) which become available metabolic substrates for the benefit of breast cancer cell survival either by storage in the form of lipid droplets, or energy production *via* β-oxidation and importantly could also be incorporated into phospholipids and cholesterol esters in cell membranes (Shyu *et al*., 2018), and thereby confer to a more lipid saturated membrane. This metabolic behaviour protects breast cancer cells from the cytotoxic effects of chemotherapeutic agents. We propose that the excess lipids storage/utilization in mammary adipose tissue of obese patients, could explain the resistance to treatment protocols found in breast cancer patients. Especially since "obese" adipocytes compared to normal adipocytes provide higher concentrations of FFA to breast cancer cells in order to sustain survival and migration (Balaban *et al*., 2017).

In our model we found that tumour Σ SFA was increased in both HFD vehicle and doxorubicin treated mice compared to LFD mice (Figure 2.11A). Stearic acid (SA) was increased in vehicle-H and Dox-H mice of mammary adipose tissue and tumour tissue compared to LFD control mice (Figure 2.11C).Furthermore, we also report that the HFD compared to LFD, decreased the percentage of various MUFAs (PTA, OA and VA) in both mammary adipose and tumour tissue, and that the decrease is more profound in the doxorubicin treated mice (Figure 2.12). It was also illustrated with PCA that irrespective of treatments,that the LFD compared to the HFD has distinct associations with certain FA classes independent of treatment i.e. in mammary adipose tissue and tumour tissue the LFD is positively correlated with MUFAs (i.e. PTA, OA and VA) (Figure 2.14& 2.15).

Reports on alterations occurring in FA composition of breast tumours as well as its adjacent adipose tissue, within the tumour microenvironment in both human and animal models are limited. Our findings are in agreement withMaillard *et al*. (2002), who showed that the most abundant FAs present in breast cancer adipose tissue of breast cancer patients were SA, PA, OAandLA compared to controls. Breast cancer cases presented with significantly higher Σ MUFAs levels in tumour tissue as well as lower Σ PUFA and n-6 PUFAs in breast adipose tissue, when compared to benign cases (Bree *et al*., 2013).Mohammadzadeh *et al*. (2014), confirmed an increased OA, arachidonic acid (AA) and MUFAs: SFAs ratio found in breast tumour compared to adjacent tissue.

Strong correlations exist between increased SFA dietary intake and increased breast cancer risk (Hirko *et al*., 2018; Xia *et al*., 2015). Reports also strongly suggests that breast cancer tumours rely on lipid metabolism in order to favour survival and to diminish the cytotoxic effects of administered treatments by increasing the expression of various proteins regulating lipid metabolism (Du *et al*., 2018; Iwamoto *et al*., 2018). Cancer cells are metabolically very active to provide energy for its rapid development and growth, and since FAs are stored in lipid droplets within tumours, it serves as a reservoir of FAs which can be utilized for energy generation (Antalis *et al*., 2011). This is supported by the increased amount and size of lipid droplets found in breast cancer tumours, specifically more aggressive phenotypes (Antalis *et al*., 2011; Balaban *et al*., 2018). Additionally, a shift from *de novo* synthesis to exogenous lipid uptake leads to membrane lipid saturation (Hussein, 2013). Fatty acids are essential components of cell membrane structure and fluidity and it is known that the type of FA (i.e. increased SFAs characteristic of obesity) derived from the diet, modify phospholipid FA composition (densely packed membranes) and physical-chemical properties (decrease in

transmembrane permeability) of plasma membranes in cancer cells, which explains the increased SFA profile found in both the vehicle and doxorubicin treated HFD mice. Thismight be to protect cancer cells from oxidative damage induced by chemotherapy by decreasing lipid peroxidation, ultimately leading to acquired treatment resistance (Rysman *et al*., 2010; Zhao *et al*., 2017).

The decreased MUFA profile observed in mammary adipose tissue could possibly be as a result of alterations in the expression of enzymes regulating lipogenesis, since PA (16:0)can be desaturated (catalysed by SCD-1) to produce palmitoleic acid (PTA, 16:1n-7) (Kihara, 2012). We found a decrease in FAS and SCD-1 protein expression in the HFD fed mice (both vehicle and doxorubicin treated) within mammary adipose tissue (Figure 2.8A&B), which translate to a decrease in lipogenic activity in mammary adipose tissue of the HFD (obese) animals. This was further supported by SCD-16 and SCD-18 (desaturation indexes) reported in the HFD compared to LFD animals in mammary adipose tissue, specifically in the doxorubicin treated mice (Table 2.3). Our findings can be explained by the high dietary carbohydrate content of the LFD i.e. 70 PEfrom carbohydrates, which might partially explain why SCD-1 and FAS expression was higher in the LFD mice, irrespective of treatments, as dietary carbohydrates are substrates for *de novo* FA synthesis. Our results are in agreement with Liu *et al*. (2015), who showed that rats fed a HFD (60 PE from fat) compared to control diet (10 PE from fat), showed decreased SCD-1 expression in adipose tissue total lipid and serum FFA fractions. Additionally, it may also be that a HFD supresses SCD-1 expression to prevent exogenous accumulation of FAs in adipose tissue depots in order to promote β-oxidation. This could have implications for tumour survival since an increase in β-oxidation is linked to increased energy production which breast cancer cells utilize for survival, and/or to evade the toxic effects of cancer treatments. This provides a plausible explanationfor the decreased lipogenic/lipolytic activity in mammary adipose tissue (HFD) to increase the FFA "pool" by preventing fat storage, which may also be exacerbated by doxorubicin treatment itself and all of which may contribute to the development of breast cancer treatment resistance.

However, the increased SCD-1 expression illustrated in Dox-H *vs* Dox-L mice in tumour tissue (Figure 2.9A) does not explain the decreased MUFAs observed in Dox-H mice (Figure 2.12). Firstly, the decreased MUFA profile may be the result of increased lipolysis of lipid droplets within the tumour itself, as evident by the increased expression of ATGL in the tumour tissue of the HFD mice i.e. vehicle-H *vs* vehicle-L and Dox-H *vs* Dox-L mice, respectively (Figure 2.9B).It could also be the result of breast tumour cells utilizing these

MUFAs to achieve treatment resistance, by increasing the release of MUFAs from the cell membrane. This is supported by the decreased MUFAs within the FFA tumour tissue fraction i.e. PA, VA, gondoic acid (GA) and nervonic acid (NA) found in the Dox-H compared to Dox-L mice, (supplementary Table 2). Lastly, the decreased MUFAs found in tumour tissue can also be explained by the preferential release and low re-uptake of MUFA in specific tissues as well as the selective preference of SFA above that of MUFA or the selective rerouting of MUFA to other lipid pools within the tumour tissue under obesogenic conditions. Taken together, the HFD (DIO) induces both *de novo* FA synthesis and lipolysis in the tumour, which is exacerbated by doxorubicin treatment and might therefor confer to acquire treatment resistance under obesogenic conditions.

Furthermore, a dysregulation of cytokines (i.e. increased IL-6, TNF-α and IL-1β) and adipokine secretion (increased leptin and decreased adiponectin) (Koti *et al*., 2015; Mahon *et al*., 2015; Nieman *et al*., 2013; Vyas *et al*., 2014), has also been shown to regulate the expression of transcription factors involved in lipid metabolism i.e. leptin inhibiting lipogenesis (Coelho *et al*., 2013; Dirat *et al*., 2011). The outcome is altered adipocyte endocrine functionality that favours tumour cells to produce more adipokines (Vyas *et al*., 2015). This could result in breast cancer having the ability to further exacerbate dysfunction in the lipid storage function of adipose tissue. In turn these breast cancer cells can utilize these FFA to favour tumour growth (Balaban *et al*., 2017) and potentially lead to acquired treatment resistance. Elevated TNF-α concentration has been shown to inhibit adipocyte lipolysis (Laurencikiene *et al*., 2007) and high leptin concentration have been shown to decrease adipose tissue SCD-1 expression (Miyazaki *et al*., 2009). We argue that obesity induced inflammation (increased resistin levels), may lead to lipolysis inhibition (decreased HSL in Dox-H *vs* Dox-L mice) in mammary adipose tissue (Figure 2.8D). A significant negative correlation was observed between resistin concentration and HSL protein expression in mammary adipose tissue in the Dox-H mice (Figure 2.10). Thiscan be explained by high SFA levels inducing the secretion of pro-inflammatory mediators (*via* NFκB signalling pathway) (Lee *et al*., 2004, Milanski *et al*., 2009), which in turn may inhibit lipolysis (decreased HSL).Additionally, we confirmed a higher NFκB-p65 protein expression in mammary adipose tissue of Dox-H compared Dox-L mice (Figure 2.8F), which indicates a state of inflammation in mammary adipose tissue. All of which could be as a result of DIO induced inflammation inhibiting lipolysis in mammary adipose tissue and thereby increasing the FFA

"pool" which can favourbreast tumour growth and induce treatment resistance in a paracrine manner.

Mammary adipose tissue and tumour tissue showed a significant increase in various n-6 PUFAs (LA, EDA and adrenic acid (ADA)) in vehicle-H and Dox-H mice compared to LFD mice (Figure 2.13).This is supported by PCA of tumour tissue TPL FAs,illustrating that the HFD irrespective of treatments, is positively associated with n-6 PUFAs (Figure 2.15). Linoleic acid and ALA are essential FAs derived from the diet (Di Pasquale, 2009). These FAs are desaturated (FA desaturases) and elongated (Elovl2 and Elovl5) to form other major PUFAs i.e. AA and DHA (Di Pasquale, 2009). Both experimental diets in our study used soybean oil, which is rich in both LA and ALA. Therefore, the increase in PUFAs found in both the mammary adipose tissue as well as the tumour tissue of the HFD mice, may be reflective of the higher total PUFA intake of the HFD.

The proportions of FAs within the two respective diets differed significantly. Linoleic acid (LA) and AA accounted for the elevation of the n-6 PUFA in both mammary and tumour tissue of HFD fed mice, suggesting an increased pro-inflammatory profile, specifically in the obese doxorubicin treated mice. This is further supported by a significant decrease found in AA in the tumour FFA fraction of the Dox-H compared to Dox-L mice (Supplementary Table 2). The pro-inflammatory effects of n-6-PUFA is due to the diversity of functions associatedwith lipid derived bioactive mediators i.e. eicosanoids, prostaglandins and leukotrienes (Currie*et al*., 2013; Kremmyda *et al*., 2011).

Eicosanoids are predominately synthesised from the n-6 PUFA AA, where the cyclooxygenase pathway generates prostaglandins and the lipoxygenase generates leukotrienes (Baenke *et al*., 2013).

Prostaglandin can be further converted into prostaglandin E2, prostaglandin I2 and thromboxanes, functioning as important second messenger molecules for cell signalling (Green *et al*., 2011). These bioactive lipids have been implicated in breast cancer supporting angiogenesis, cell-proliferation, cell migration, metastasis and inflammation (Clària *et al*., 2010; Fritsche *et al*., 2015; Wang *et al*., 2006). For example, prostaglandin E2 has been implicated in breast cancer supporting migration and cellular proliferation (Baenke *et al*., 2013), by inducing the RAS-ERK pathway (Iyengar *et al*., 2015) and increasing aromatase expression in stromal adipocytes as well as promoting the invasion of breast cancer cells to

lymph nodes *via* an upregulated chemokine expression (Clària *et al*., 2010), and inhibiting apoptosis by upregulating pro-apoptotic marker blc-2 (Wang & DuBois, 2006; Wang & DuBois, 2010). COX derived prostaglandins D2, E2, F2α, I2 and LOX derived *leukotriene* B4 also excerbates inflammation well known to promoting tumour initiation and progression (Greene *et al*., 2011; Johnson *et al*., 2015; Sharma & Mohammed, 2006).

More importantly, doxorubicin treatment itself has been shown to negatively impact adipose tissue and/or adipocytes functionality, by altering lipogenesis (decreased FAS) and lipolysis (increased HSL) (Arunachalam *et al*., 2012; Batatinha *et al*., 2014; Biondo *et al*., 2016; de Lima Junior *et al*., 2016; Supriya *et al*., 2016), which participate towards the disruption of adipose tissue homeostasis. The consequence is an increase in FFA release that disrupts lipid storage (Biondo *et al*., 2016). Doxorubicin-induced FFA release could potentially further increase the availability of energy substrates (FFA) for cancer cells to utilize to sustain both its' survival and proliferation demands, and thereby indirectly promote breast cancer treatment resistance. Recently, Ebadi *et al*. (2017) indicated that chemotherapy treatment (5-fluorouracil and Irinotecan) in a colorectal cancer model can diminish peri-uterine adipose tissues' function to store lipids by significantly downregulating the expression of ACC, FAS and HSL as well as markers of β-oxidation (i.e. CPT-2). It was further noted that SFAs (PA) and MUFAs (PTA) was significantly decreased in chemotherapy treated mice. However, it remains unclear whether the suppression of adipose tissue lipid storage capacity induced by chemotherapy is a result of decreased HSL expression, or due to mitochondrial dysfunction induced by the chemotherapy. Doxorubicin and 5-fluorouracil has the ability to induce cancer cell invasion and metastasis by increasing lipid accumulation and membrane fluidity, by altering lipid metabolism (Mehdizadeh *et al*., 2017).Treatment with doxorubicin and 5-fluorouracil significantly increased the number of lipid droplets within HepG2 cancer cells. Additionally, a significant increase in SFAs (PA) and PUFAs and significant decrease in MUFAs (OA and PTA), was evident in the phospholipid profile of cancer cells following chemotherapy treatment. However, these studies were not under obesogenic conditions.

In summary, FA profiles within the tumour microenvironment have not yet been explored in an obese breast cancer animal model that illustrates its role in breast cancer treatment resistance. We provide evidence that obesity altered the FAs profile of both tumour tissue and its adjacent surrounding mammary adipose tissue. The expression of lipid metabolism enzymes in this study was also differentially altered by obesity, and it is very likely that the altered FA composition observed in both mammary adipose tissue as well as tumours is the

result of alterations in lipogenesis and/or lipolysis, which is associated with the development of breast cancer resistance to doxorubicin treatment.

2.4 SUMMARY OF *IN VIVO* MODEL FINDINGS

We successfully showed that DIO significantly decreased the treatment efficacy of doxorubicin in TNBC tumours. Our findings have showed that DIO selectively supresses *de novo* FA synthesis and lipolysis in mammary adipose tissue, but increased lipogenesis and lipolysis in tumour tissue. This precedes changes in the incorporation of dietary FAs into total FA lipid profile within mammary adipose tissue as well as the phospholipid membranes of breast tumour cells. This might suggest that exogenous dietary lipids can alter the energy metabolism ofTNBCtumours in this current in *vivo* model. Alterations in FAs composition in both mammary adipose and tumour tissue could be a mechanism by which FAs composition can be altered in response to DIO within the tumour microenvironment and thereby contributing to the development of breast cancer treatment resistance within our current model (Figure 2.16).

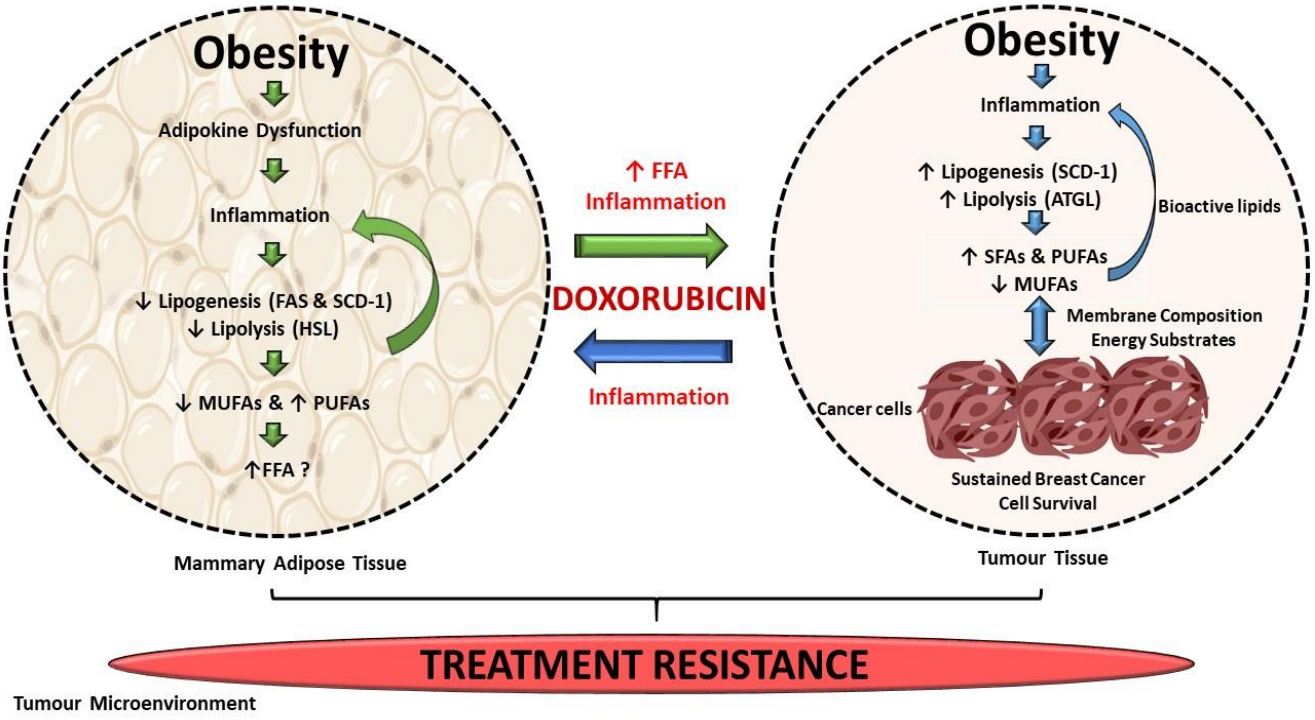

Figure 2.16: Summary of *in vivo* model findings.

We also propose that DIO induces inflammation (adipocyte dysfunction), leads to the suppression of lipogenesis and lipolysis in mammary adipose tissue (increased FFA "pool") thereby preventing FA storage in mammary adipose tissue. The consequence is an altered mammary adipose tissue total lipid FA composition (decreased MUFAs and increased PUFAs) that exacerbates local inflammation in mammary adipose tissue and favours breast cancer cell survival in a paracrine manner. These adipose tissue derived-FFA could become available metabolic substrates for the benefit of breast cancer cell survival either by storage in the form of lipid droplets, or energy production *via* β-oxidation and could also be incorporated into phospholipids and cholesterol esters in cell membranes. Additionally, we propose that the above mentioned events are exacerbated by doxorubicin treatment itself (Mentoor *et al*., 2018; Mentoor *et al*., 2019) resulting in breast cancer treatment resistance (Figure 2.16).

Furthermore, we also showed that DIO as a result of consuming a HFD induces *de novo* FA synthesis and lipolysis in breast tumour tissue that increases the abundance of SFAs in the breast cancer cell membranes. This can induce acquired resistance to doxorubicin by conferring to a more lipid-saturated cell membrane, known to protect cancer cells from the cytotoxic effects of chemotherapeutic agents. We speculate that increased breast cancer tumour growth is also the result of increased pro-inflammatory mediators i.e. n-6 PUFAs (precursors of bioactive lipids) in the tumour cell membrane phospholipids. These pro-inflammatory can also exacerbate inflammation (paracrine manner) in the tumour microenvironment, all of which promotes the survival of breast cancer cells by inducing downstream changes in carcinogenic signalling pathways i.e. cell growth, migration and metastasis (Figure 2.16).

Our results provide a unique perspective to the distinct FA profiles within the tumour microenvironment and prove that consuming a HFD (DIO), can distinctly alter the FA composition of tissues within the tumour microenvironmentwhich may contribute towards treatment resistance. Here, we report for the first time that *de novo* FA synthesis and lipolysis protein expression is suppressed in mammary adipose tissue and increased in tumour tissue in an obese xenograft breast cancer model treated with doxorubicin. We propose that this might be considered a novel mechanism in which breast cancer cells use to suppress the storage of FAs as TAG in mammary adipose tissue, and thereby increasing the availability of FFA as well as exacerbating inflammation under obesogenic conditions (Figure 2.16).

CHAPTER 3: *IN VITRO* MODEL

3. *In Vitro* Model

Obesity is associated with an increased risk for TNBC that develop independently of hormonal related pathways (Pierobon & Frankenfeld, 2013), involvingdysfunctional synthesis of growth factors and adipokines (Dietze*et al*., 2018; Sun *et al*., 2017). Triple negative breast cancer often present with metastasis and treatment resistance compared to other breast cancer subtypes, thus highlighting the need to identify the underlying molecular markers that are influencing and contributing to treatment resistance (Al-Mahmood *et al*., 2018). This is especially significant since mesenchymal like-TNBC is associated with poorer prognosis which may be due to enhanced epithelial-to-mesenchymal transition (EMT) as a result of dysfunctional, and/or altered FA metabolism (Wang *et al*., 2018).

To validate the *in vivo* model findings, we have developed an *in vitro* model to investigate the effect of adipocytes on breast cancer cells treated with, or without doxorubicin treatment in a paracrine manner, using a conditioned media approach to simulate the mammary tumour microenvironment.

Hypothesis

Adipocytes contribute to the development of doxorubicin resistance in breast cancer cells *via* a paracrine mechanism.

Aims and Objectives

- The first objective of the *in vitro* model was to successfully differentiate a human adipose tissue derived mesenchymal stem cell line into mature adipocytes.
- The second objective was to characterize the effect of adipocyte conditioned media on MDA-MB-231 TNBC cells.
- Subsequently, the effect of adipocyte conditioned media on MDA-MB-231 TNBC cells was evaluated in the presence or absence of doxorubicin treatment.
- We also characterized the role of potential causal factors in breast cancer treatment resistance including, (i) apoptosis, (ii) proliferation, (iii) inflammatory, and (iv) lipid metabolism markers, (v) FA profiles and, (vi) EMT markers.

3.1 MATERIAL AND METHODS: *IN VITRO* MODEL

3.1.1 Adipocyte Culturing

A human adipose tissue derived stem cells (hADSC) line (hADSCs; Donor 26508, #0000364977, Poietics, Lonza, Basel, Switzerland, Supplementary Table 3) was used for the differentiation of mature adipocytes (Figure 3.1). Human adipose tissue derived stem cells were cultured in 100 mm tissue cultureplates (75 cm^2, SPL Life Sciences) under standard conditions in an incubator (37°C and 5% CO_2 humidity).

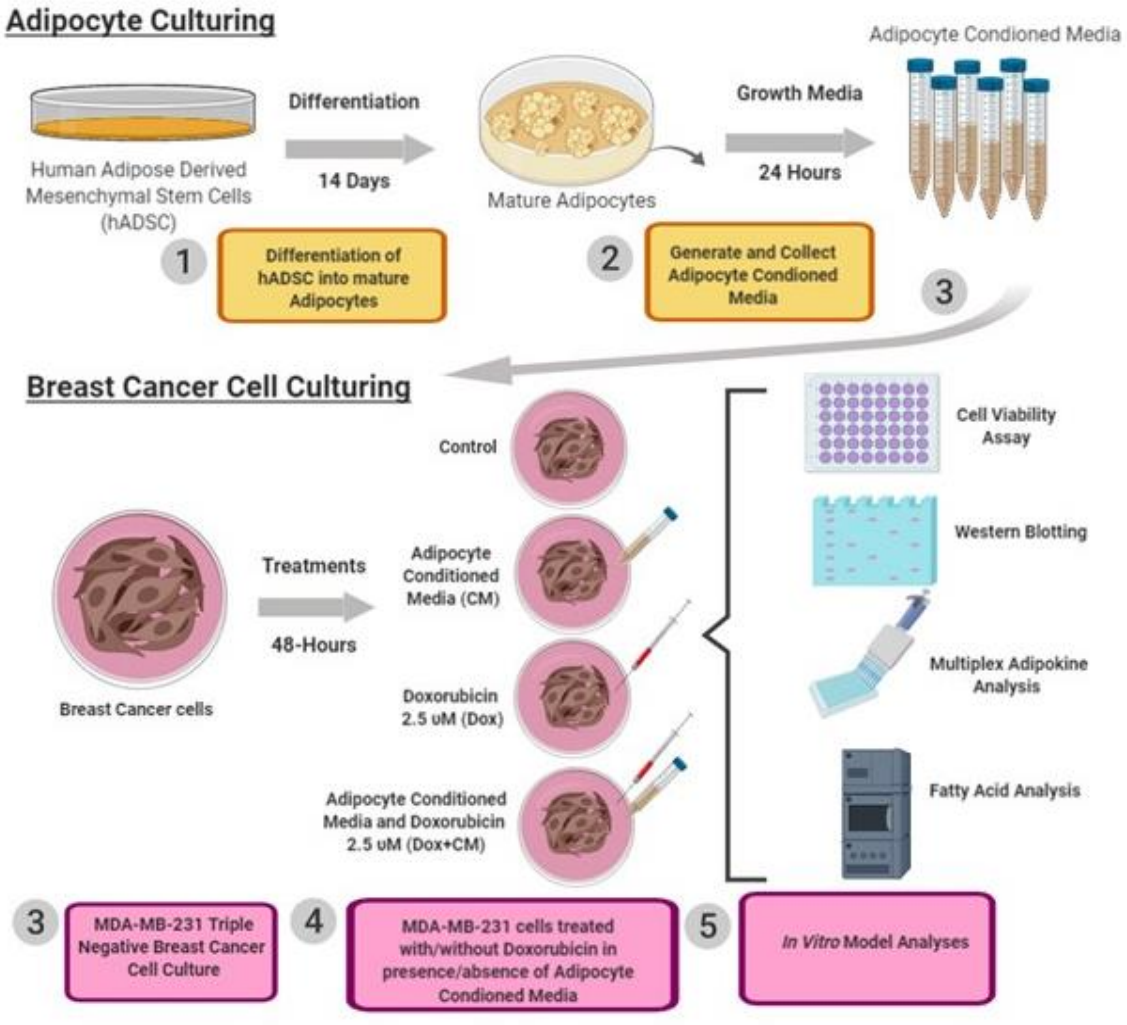

Figure 3.1: Illustration of the *in vitro* model experimental treatment protocol (created with BioRender®).CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media.

Growth media used for adipocyte culturing consisted of DMEM with high-glucose (4.5 g/L) and ultra-glutamine (Life Technologies), supplemented with 10% FBS (Scientific Group) and 1% PenStrep (Life Technologies). Growth media was replaced on a regular basis until cells reached a confluency of 70-80%. Cells were spilt and seeded into various plates, with fresh growth medium subjected to various experiments and treatment protocols. Seeding of cells commenced once cells reached a confluency of 70-80%. Seeding was accomplished by washing the cell monolayer with pre-heated phosphate buffered saline (1X Phosphate buffered saline (PBS)). This was followed by an incubation step with 0.5% Trypsin EDTA (1X) (Life Technologies) until all cells detached from the flasks surface. A centrifugation step (5 minutes at 1500 rpm), allowed all the cells to pellet, where after it was resuspended in fresh growth media for seeding purposes. Passage number for hADSC differentiation ranged between four to six.

3.1.1.1 Differentiation of Adipocytes

Once the hADSC reached two days' post confluency, and was in the second passage, differentiation experiments were initiated (Figure 3.1). In short, adipogenic differentiation medium consisted of DMEM (Life Technologies), supplemented with 10µM insulin (Sigma-Aldrich®), 0.5mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich®), 1µM dexamethasone (Sigma-Aldrich®) and 56 µM indomethacin (Sigma-Aldrich®) and 1 ml FBS (Scientific Group) (van de Vyver *et al.*, 2014). Adipogenic differentiation medium was replaced every second day. The differentiation process from hADSC to mature adipocytes took approximately 14 days, based on visual inspection of cultures daily and was confirmed with Oil Red O staining (described in section 3.1.1.2).

3.1.1.2 Generation of Adipocyte Condition Medium

To produce adipocyte conditioned media, all adipogenic differentiation medium was collected and discarded, followed by the replacement with normal growth media (DMEM with high-glucose (4.5 g/L), 10% FBS and 1% PenStrep) for 24 hours (Figure 3.1). After 24-hours, all media was collected (which will be referred to as adipocyte conditioned media), pooled and filtered, using a 0.2 µm filter. The adipocyte conditioned media was snap frozen in liquid nitrogen and stored at -80°C until experimental procedures and analyses. Evaluation of

successful differentiation for mature adipocytes was done by quantitation of triglycerides by the Oil Red O staining method (described in van de Vyver *et al*., 2014). In short, all medium was discarded and mature adipocytes was stained with Oil Red O stain (0.7% red oil diluted in 70% (v/v) isopropanol), followed by a 30-minute incubation step at room temperature (22°C). Cells were washed three times with distilled water and photographed for image analysis. All experiments were carried out in triplicate and at least four random images were taken per well using a light microscope (Olympus CKX41, CachN 10/0.25 PhP objective) and EOS600D Canon digital camera.

3.1.2 Breast Cancer Cell Culturing

A human adenocarcinoma triple negative breast cancer cell line (MDA-MB-231) was used for all breast cancer cell culture experiments. The MDA-MB-231 breast cancer cells were cultured in growth medium consisting of DMEM (Life Technologies) supplemented with 10% FBS (Scientific Group) and 1% PenStrep (Life Technologies) (Figure 3.1). All breast cancer cells were cultured in cell culture flasks (25 cm^2 and 75 cm^2 flasks, SPL, Life Sciences), under standard conditions in an incubator (37°C and 5% CO_2 humidity). Growth media was replaced on a regular basis and sub-culturing was done once cultures reached a confluency of 70-80%, after which cells were sub-cultured and/or seeded as previously described (see section 3.1.1)

3.1.3 Experimental Protocol

The effect of adipocyte condition medium, on MDA-MB-231 breast cancer cells was evaluated with or without doxorubicin treatment (Figure 3.1). In short, MDA-MB-231 breast cancer cells were seeded in 6-well tissue culture plates (9.5 cm^2, SPL Life Sciences) at a cell density of 250 000 cells per well, and allowed to grow and attach. Prior to any treatment, growth medium was aspirated and cells were washed with pre-heated PBS, to ensure that all cell debris were removed. A summary of the experimental treatment protocol is provided in Table 3.1, which included four experimental treatment groups; (i) control, (ii) conditioned media (CM), (iii) doxorubicin (Dox) and, (iv) the combination of conditioned media and doxorubicin (Dox+CM). The conditioned media treatment consisted of a 30% adipocyte conditioned media and 70 % growth media ratio. All experimental protocols were repeated in triplicate.

Table 3.1: *In vitro* model experimental treatment protocol.

EXPERIMENTAL PROTOCOL		
Breast Cancer Cells	**Treatment**	**Groups**
MDA-MB-231 cells	-	Control
MDA-MB-231 cells	Adipocyte Conditioned Media	Conditioned Media (CM)
MDA-MB-231 cells	Doxorubicin (2.5 µM)	Doxorubicin (Dox)
MDA-MB-231 cells	Adipocyte Conditioned Media + Doxorubicin (2.5 µM)	Conditioned Media + Doxorubicin (Dox+CM)

Abbreviations: CM, conditioned media; Dox, doxorubicin.

3.1.3.1 Doxorubicin Treatment

A stock solution of doxorubicin hydrochloride (D5794, LKT® laboratories, Minnesota, USA) was dissolved in DMEM and aliquoted for later use to avoid freeze thaw cycles (stored at -20°C). The desired concentration of doxorubicin was diluted into complete growth/treatment media before each experiment. The optimal concentration of 2.5 µM doxorubicin treatments was selected based on assessment by dose-response experiments and cell-viability assays previously established (Davis, 2016).

3.1.4 *In Vitro* Model Analyses

Figure 3.2 illustrates all molecular analyses of the *in vitro* model which included cell viability assays and western blotting of MDA-MB-231 breast cancer cells as well as multiplex inflammatory marker immunoassay and FFA profile determination of treatment conditioned media of all four experimental treatment groups.

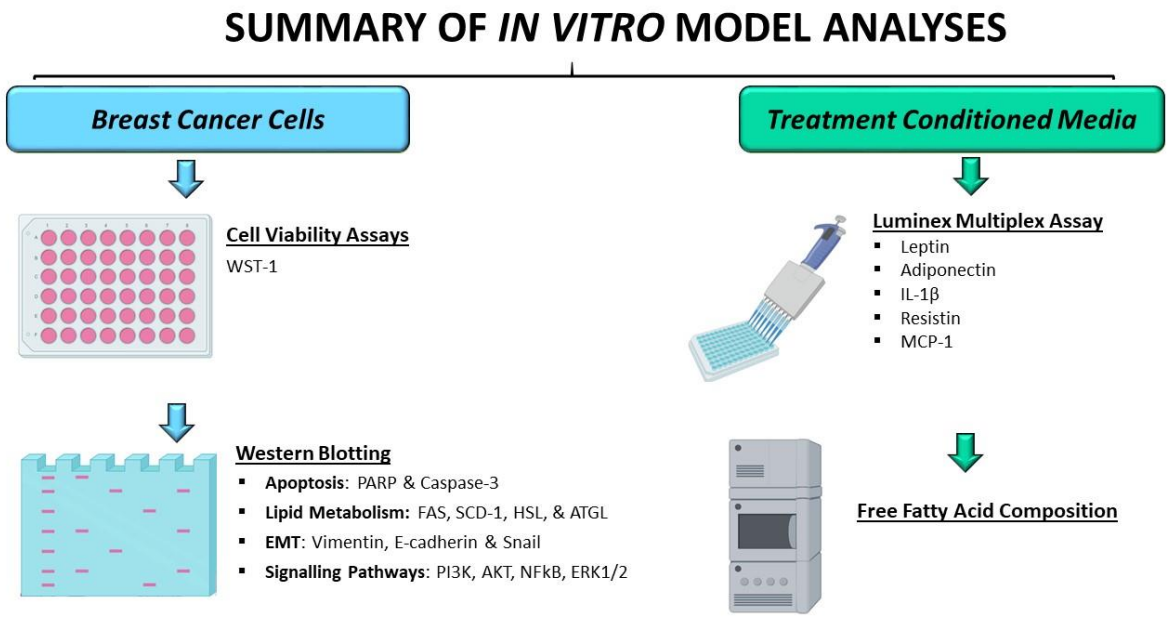

Figure 3.2: A summary highlighting the *in vitro* model analyses.

3.1.4.1 Cell Viability Assay: WST-1

Cell viability was assessed using the tetrazoliumsalt, WST-1 (Roche®, Merck®, #5015944001), as a percentage of metabolically healthy cells (Figure 3.2). The assay is based on the principle that cleavage of the tetrazolium salt, WST-1 to formazan by mitochondrial dehydrogenases activity increase the quantity of formazan dye produced.This translates to an increase in metabolically viable cells, which leads to an increase of dehydrogenase enzymes present in mitochondria. This ultimately results in the formation of purple formazan crystals from WST-1, which is solubilized and can be quantified spectrophotometrically at anoptical density of 440nm.

Briefly, MDA-MB-231 breast cancer cells were seeded at a cell density of 25 000 cells per well into 48 well plates (0.95 cm^2, SPL Life Sciences), and incubated with 250 µl growth medium to allow to grow and attach, followed by subsequent treatment administration (see Table 3.1). Next, all media was collected and a 10% WST-1 solution was added to the growth media, which was added to each well. Cells were incubated for 85 minutes at a 37ºC, 5% CO_2 humidified environment (C01901R, Snijders Scientific), followed by a 1-minute shaking step (200rpm) at room temperature, until all formazan crystals dissolved. All experiments were

conducted under dark conditions and plates were covered with foil, due to the light sensitivity of WST-1. Absorbance values were determined at 440 nm using a universal micro plate reader (EL800, Bio-Tek Instruments Inc.). All experimental groups were analyzed in triplicate in three independent experiments. Absorbance values were expressed as a percentage of WST-1 *versus* the treated control group. The optimal treatment duration for all experimental groups following treatment, was based on assessment by cell-viability assays (see results section 3.2.2).

3.1.4.2 Protein Analysis and Western blots

In order to extract total protein, all cell flasks/plates from the experimental treatment groups were immediately place on ice, and media was collected and filtered using a 0.2 µm filter, which will now be referred to as treatment conditioned media. The treatment conditioned media was snap frozen in liquid nitrogen and stored at -80°C until subsequent analysis.

After collection of treatment conditioned media, all flasks/plates were washed with 1x PBS three times, in order to remove cellular debris. 80 µl of cold modified RIPA buffer, containing protease and phosphatase inhibitors (2.5 mM Tris-HCL, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 50 mM NaF, 4 mg/ml SBTI, 10 mg/ml leupeptin, 0.1% SDS 0.5%, Na deoxycholate and 1% NP40, calibrated to pH 7.4), was added to each flask, followed by a 5-minute incubation on ice. Cells were then detached from the flask/plate using a sterile cell scraper (100 % ethanol) between all samples. Whole cell lysates were collected from the flask/plate, sonificated three times at 3Hz for three seconds, and centrifuged at 14000 rpm for five minutes at 4°C. The supernatant was collected into Eppendorf tubes and stored at -80 ºC for protein quantification. A Direct Detect® infrared spectrometer (DDHW00010-WW, Merck) was used to quantify the protein content of samples.

Sample preparation of protein aliquots containing 30 µg protein were diluted with Laemmli sample buffer and boiled for five minutes to denature proteins before being loaded into 4-15 % polyacrylamide fast cast gels (mini-PROTEAN® TGX™ Gels, Bio-Rad) for separation by SDS-PAGE. Gels were run at 110 V (constant) and 400 mA for approximately 120 minutes (Power Pac 300, BioRad). The electro-transfer of proteins from the gel to preparedPVDF membranes was achieved using a semi-dry electro-transfer system (TransBlot® Turbo™ v1.02,

BioRad) for 30 minutes at 25 V and 1.0 A. Transfer efficiency was evaluated using the stain free blot protocol provided on a Chemi-Doc™ MP (BioRad) system. All membranes were washed with 0.1% Tris Buffered Saline-Tween20 (TBS-T) and blocked for one hour in 5% (w/v) non-fat milk and TBS-T at room temperature, to prevent non-specific binding. The PVDF membranes were then incubated at 4°C overnight with a primary antibody (see Table 3.2).

Table 3.2: Antibodies used in *in vitro* model for western blotting.

Primary Antibody	**Size**	**Concentration**	**Company**
Apoptosis			
PARP and Cleaved-PARP	89, 116 kDa	1:1000	Cell Signalling (#9532)
Caspase-3 and Cleaved-caspase 3	35, 17 kDa	1:1000	Cell Signalling (# 9662)
Proliferation			
Total PI3K	85 kDa	1:1000	Abcam (#ab86714)
Phosphorylated PI3K p85/p55 (Tyr467/Tyr 199)	85,55 kDa	1:1000	Elabscience (#ENP0224)
Total AKT	60 kDa	1:1000	Cell Signalling (#9272)
Phosphorylated AKT (Ser473)	60 kDa	1:1000	Cell Signalling (#4060)
Total ERK 1/2	44,42 kDa	1:1000	Abcam (#ab184699)
Phosphorylated ERK1/2 (Thr202/Tyr214, Thr185/Tyr187)	44,42 kDa	1:2000	Cell Signalling (#4370)
Epithelial -to-Mesenchymal Transition (EMT)			
Snail	29 kDa	1:1000	Cell Signalling (#3879)
Vimentin	57 kDa	1:1000	Cell Signalling (#5741)
E-cadherin	135 kDa	1:1000	Cell Signalling (#3195)
Lipid Metabolism			
Fatty acid synthase (FAS)	273 kDa	1:1000	Cell Signalling (#3180)
Stearoyl-CoA desaturase-1 (SCD-1)	37 kDa	1:1000	Cell Signalling (#2794)
Adipose tissue triglyceride lipase (ATGL)	54 kDa	1:1000	Cell Signalling (# 2439)
Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB-p65)	65 kDa	1:1000	Cell Signalling (#8242)
Hormone sensitive lipase (HSL)	83 kDa	1:1000	Cell Signalling (# 4107)
Secondary Antibodies			
Anti-mouse		1:10 000	Cell Signalling (#7076S)
Anti-rabbit		1:10 000	Cell Signalling (#7074)

Next, the membranes were washed three times for five minutes each with TBS-T, prior to incubation with anti-rabbit/mouse IgG horseradish peroxidase conjugated secondary antibody (1:10000) (Cell Signalling Technologies), for one hour at room temperature. This was followed by a wash step in TBS-T (five times for five minutes each), before specific bands were visualized and detected using the ECL western blotting substrate detection kit (BioRad)

with the use of ImageLab 4.0 software on a Chemi-Doc™ MP (BioRad) imaging system. Samples were normalized to total protein present on the same membrane. Bands for each specific protein were quantified as a percentage comparative to the control sample present on the same blot.

3.1.4.3 Treatment Conditioned Media Inflammatory Markers Quantification

Treatment conditioned media collected from all experimental treatment groups were used to quantify the concentration levels of adiponectin, leptin, IL-1β, resistin and MCP-1 using a custom panel milliplex luminex kit (HADCYMAG-61K, Merck). Analyses were performed according to the manufacturer's protocols and specifications. Analytes were measured simultaneously using MAGPIX system plate reader (Bio-Rad, APX1042) and the values (expressed in pg/ml) were reported on Bioplex Software 6.1 (Bio-Rad, 2016).

3.1.4.4 Analysis of Treatment Conditioned Media to Determine Free Fatty Acid Profiles

Lipids of 1 mL aliquots of treatment conditioned media were extracted with chloroform: methanol (21 mL; C:M; 2:1; v:v; Sigma-Aldrich) according to an adapted method by Folch *et al.* (1957). The extraction solvent contained 0.01% butylated hydroxytoluene as an antioxidant. Briefly, the extraction solvent was added to each sample followed by a 20-minute shaking step. Saline saturated with chloroform: methanol: saline (4.2 mL; CMS; 86:14:1; v:v:v; Sigma-Aldrich) was added, mixed and centrifuged at 500 rpm for 10 minutes at 4 °C. The bottom phase was collected and transferred to a 12 mL glass tube with screw cap. To the remaining upper layer, 10 mL CMS was added and was vigorously shaken for 1 minute using a vortex mixer followed by a centrifugation step. The bottom layer was collected as previously mentioned before and combined with the first partially evaporated collection, which was evaporated to dryness under nitrogen gas-flow and water bath set at 37 °C. The FFA fraction was separated from other lipid fractions using thin-layer chromatography (TLC) silica gel 60 plates (10 × 10 cm; No. 1.05626.0001; Merck, Darmstadt, Germany), and eluted with the solvent system petroleum ether (B&M Scientific): diethyl ether (Merck): acetic acid (Merck)

(90:30:1; v:v:v). The lipid band containing the separated FFA fraction was demarcated by visualization under long-wave UV light after plates were sprayed with chloroform: methanol (1:1; v:v) containing 2,5-bis-(5´-tert-butylbenzoxazolyl-[2´]) thiophene (10 mg/100 mL; Sigma-Aldrich). These regions were scraped off the plates into glass tubes with screw caps followed by FA methyl esters (FAMEs) production by adding 2 mL methanol: sulphuric acid (H_2SO_4; BDH Chemicals, Poole, England) (95:5; v:v) and applying heat (70 °C) for 2 hours. After cooling, the resulting FAMEs were extracted with 1 mL water and 3 mL *n*-hexane (Sigma-Aldrich). The upper hexane layer was collected and evaporated to dryness. The FAMEs were re-dissolved in a small volume of *n*-hexane and analysed (sample injection volume 1 µl) by GLC (Trace 1300 Gas Chromatograph; Thermo Fisher Scientific, MA, USA) equipped with a flame-ionization detector and a 30 m capillary column of 0.32 mm internal diameter (BPX70 0.25 µm; SGE International Pty Ltd, Victoria, Australia). Gas flow rates were: N_2 (make up gas), 25 mL/min; synthetic air, 250 mL/min; H_2, 25 mL/min, with a split ratio of 15:1 and column flow (H_2, carrier gas) was set at 1.0 mL/min. Oven temperature programming was linear at 3.5°C/min, initial temperature 140°C (hold-time 1min), final temperature 220°C (hold-time 5 min), injector temperature 220°C, and detector temperature 250°C.

The FAMEs in all samples were identified by comparing the retention times with those of the standard FAME mixture (27 FAMEs, NuChek Prep Inc., Elysian, MN, USA). The individual FAMEs were quantified using heptadecanoic acid (C17:0; Sigma-Aldrich) as an internal standard and are expressed as µg FAME/mL spent medium. The total µg FAMEs/mL treatment conditioned medium was expressed as percentage of the total mass of FAMEs. Relative percentages of individual FAMEs were calculated by taking the area count of a given FAME as a percentage of the total area count of all FAMEs identified in the sample.

3.1.5 Statistical Analysis

Statistical analysis was performed using Statistica version 13.3 (TIBCO Software, Inc, USA). Normality was assessed using the Shapiro-Wilks test, and results were reported as means ± SEM. A one-way ANOVA was used to describe differences between three or more groups, followed by the Fishers LSD *post hoc* test. Pearson correlations were also used on selected parameters in each group. All graphs were generatedusing GraphPad Prism version 7. All analyses statistical significance was accepted at $p<0.05$.

3.2 RESULTS: *IN VITRO* MODEL

3.2.1 Adipocyte Differentiation

Human adipose tissue derived stem cells (hADSC) was successfully differentiated into mature adipocytes (Figure 3.3A&B).

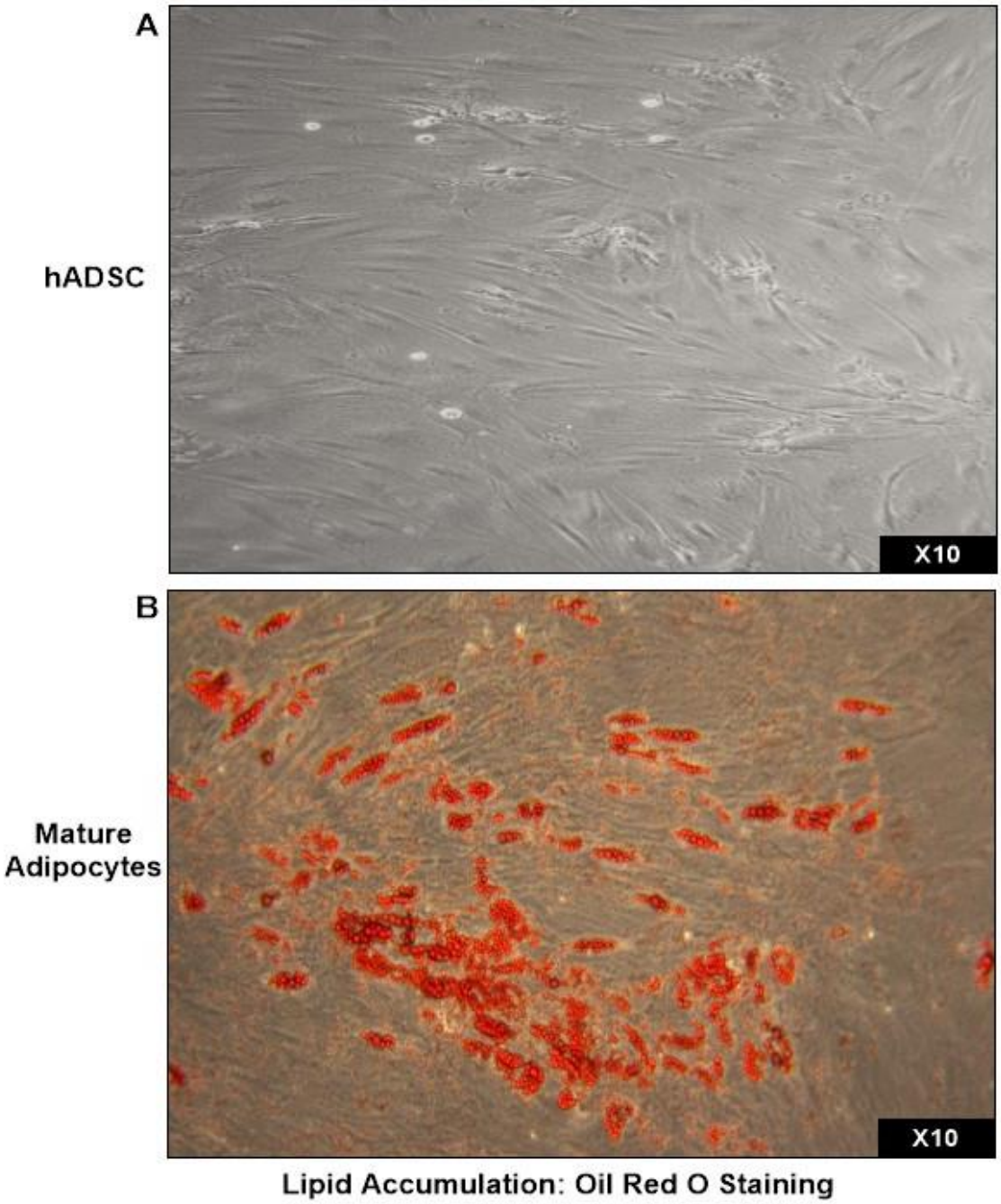

Figure 3.3: (A) Human adipose tissue derived stem cells differentiation into mature adipocytes. (B) Lipid accumulation (n=6) assessed with Oil Red O staining (day 14). hADSC, human adipose tissue derived stem cells.

3.2.2 Time Dependent Increase in Cell Viability After Adipocyte Conditioned Media Treatment On Doxorubicin Treated Breast Cancer Cells

To determine the effect of adipocyte conditioned media on MDA-MB-231 breast cancer cells treated with/without doxorubicin, WST-1 cell viability assays were performed following 24 hours and 48-hour treatment, respectively. Following a 24-hour doxorubicin treatment, cell viability significantly decreased compared to the control treated cells (control *vs* Dox, $p<0.0001$), whereas Dox+CM compared toCM treated cells showed a significant decrease in cell viability (CM *vs* Dox+CM, $p<0.0001$) (Figure 3.4A). After 48-hours of treatment we observed a significant increase in cell viability of Dox+CM compared to Dox treated cells (Dox+CM *vs* Dox, $p<0.0001$, Figure 3.4B). Therefore, the treatment duration for all experiments were assessed at 48-hours following treatment.

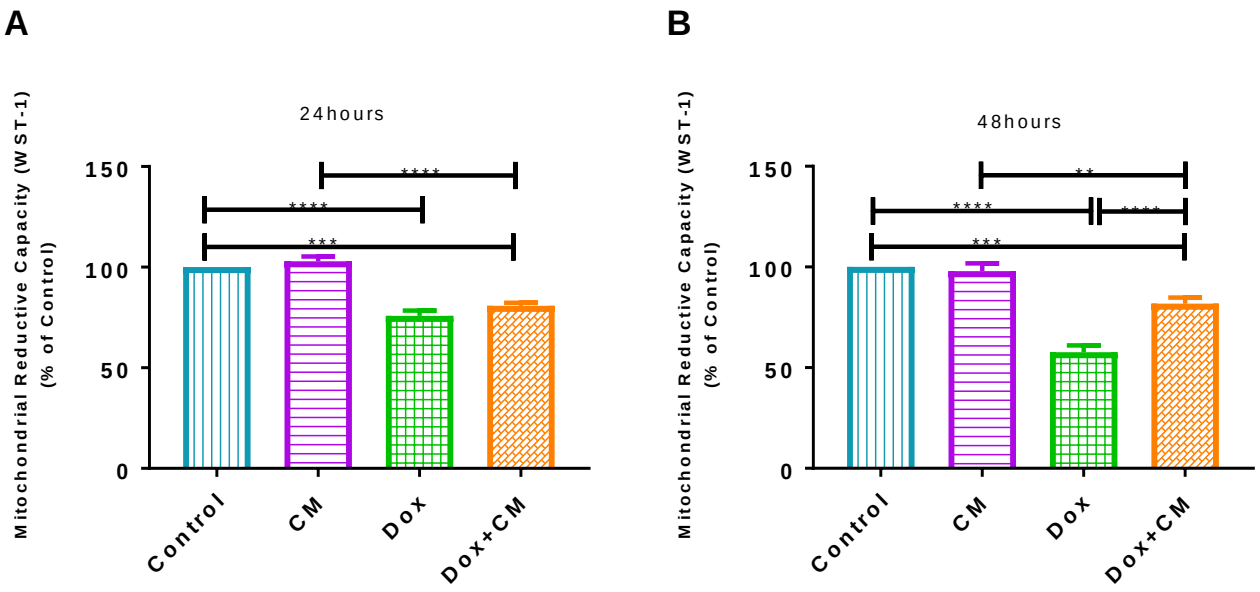

Figure 3.4: The effect of adipocyte conditioned media on cell viability in MDA-MB-231 breast cancer cells treated with/without doxorubicin (2.5 µM) for (A) 24 hours and (B) 48 hours. Results are presented as mean ± SEM (n=3). One-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. ** = $p<0.01$, *** = $p<0.001$ and **** = $p<0.0001$. CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media.

3.2.3 Doxorubicin-Induced Apoptosis is Attenuated by Adipocyte Conditioned Media Treatment in Breast Cancer Cells

Doxorubicin is a well-known anti-neoplastic agent used for the treatment of breast cancer patients. It induces cell death by inducing DNA damage and mitochondrial membrane permeabilisation. This in turn leads to an increased release of cytochrome C (mitochondria) in the cytosol which initiates the caspase-family signalling leading to apoptosis (Rivankar, 2014; Thorn *et al*., 2011). To evaluate if adipocyte conditioned media could alter doxorubicin induced apoptosis, we assessed the protein expression of poly (ADP-ribose) polymerase (PARP) activation (116 kDa) and cleavage (89 kDa), which regulates DNA repair. Additionally, we also assessed capase-3 activation (35 kDa) and cleavage (15kDa). Caspase-3 is an executioner caspase induced by both the intrinsic pathway and extrinsic death receptor pathway, once cleaved; caspase-3 becomes active and leads to the execution of apoptosis (Bai, 2015). In our model, we found that Dox treatment significantly increased cleaved-PARP protein expression compared to control cells (p=0.044. Figure 3.5), whereas cleaved-PARP expression was significantly increased in Dox+CM compared to CM treated cells alone (p=0.027, Figure 3.5).

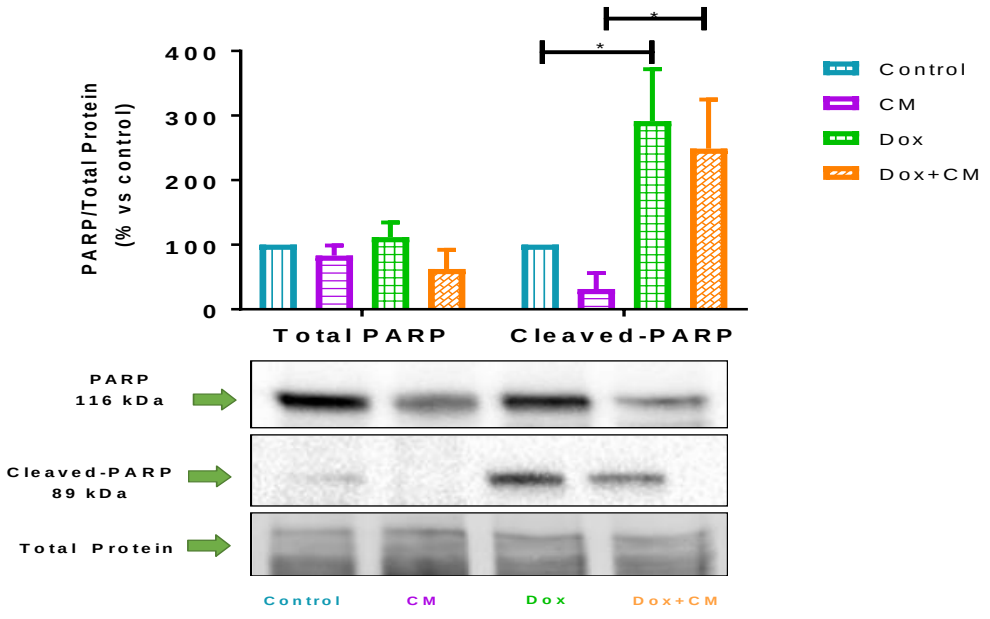

Figure 3.5: The effect of adipocyte conditioned media on PARP and cleaved-PARP protein expression in MDA-MB-231 breast cancer cells treated with/without doxorubicin for 48-hours. Results are presented as mean ± SEM (n=3). One-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. * = $p<0.05$. CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media.

Additionally, post 48-hours doxorubicin treatment, cleaved-caspase-3 expression was significantly increased in the Dox treatment group (control *vs* Dox p=0.0054) (Figure 3.6), confirming that doxorubicin treatment induced apoptosis in MDA-MB-231 breast cancer cells. Cleaved-caspase-3 expression was significantly decreased in the Dox+CM treated group compared to Dox treatment (Dox *vs* Dox+CM, p=0.043), suggesting that adipocytes significantly decreased doxorubicin's efficacy to induce apoptosis in a paracrine manner (Figure 3.6).

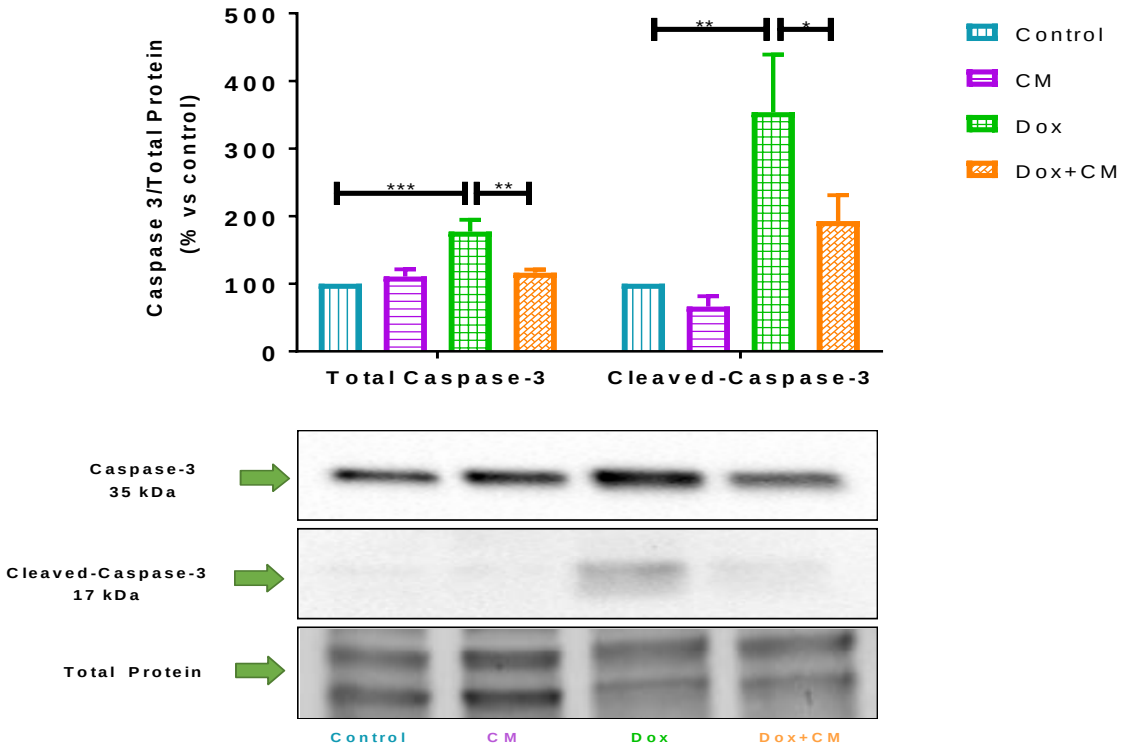

Figure 3.6: The effect of adipocyte conditioned media on caspase-3 and cleaved-caspase-3 in MDA-MB-231 breast cancer cells treated with/without doxorubicin for 48-hours. Results are presented as mean ± SEM (n=3). One-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media.

3.2.4 Signalling Pathways

Cellular proliferation is a complex process regulated by several signalling pathways including the PI3K/Akt and ERK/MAPK pathways, both of which have been shown to be overexpressed/hyperactive in breast cancer (Chua *et al*., 2009; McCubrey *et al*., 2007). Western blot analysis was done to analyse the protein expression of both PI3K/Akt and ERK pathways to corroborate cell viability results observed in our model.

3.2.4.1 PI3K Pathway: Adipocyte Conditioned Media Induces PI3K-Phosphorylation in Breast Cancer Cells

Phosphoinositide 3-kinases are well-known lipid kinases which phosphorylate the 3-hydroxyl group of phosphoinositides. These kinases have a heterodimer structure consisting of a catalytic (p110) and regulatory subunit (p85), which is frequently mutated and/or overexpressed in breast cancer (Hemmings & Restuccia, 2015). The PI3K pathway is induced upon binding of growth factors and/or ligands to the receptor tyrosine kinase. Once activated, phosphatidylinositol bisphosphate (PIP_2) is phosphorylated to phosphatidylinositol triphosphate (PIP_3), during which PIP_3 acts as a binding site for Akt (Yu & Cui, 2016). This binding leads to the translocation of inactive Akt from the cytosol to the plasma membrane, where Akt is phosphorylated at Ser 473 by mTOR complex 2 and at Thr 308 by pyruvate dehydrogenase kinase isoform 2, resulting in the induction of downstream signalling pathways that regulate cellular growth and proliferation signals, cell migration, angiogenesis and evasion of apoptosis (Hemmings & Restuccia, 2015). Akt (activated by growth factors i.e. EGF, IGF and VEGF) is a significant protein in the PI3K-Akt pathway which has numerous downstream effects that promote cell growth and proliferation (Yu & Cui, 2016).

We established that phosphorylated PI3K (Tyr 467, 85 kDa) protein expression was significantly increased in CM treated cells compared to both control ($p= 0.020$) and Dox+CM ($p=0.017$), (Figure 3.7). Similar results were observed for phosphorylated PI3K (Tyr 199, 55 kDa) i.e. control *vs* CM ($p= 0.038$) and CM *vs* Dox+CM ($p=0.018$) (Figure 3.7).

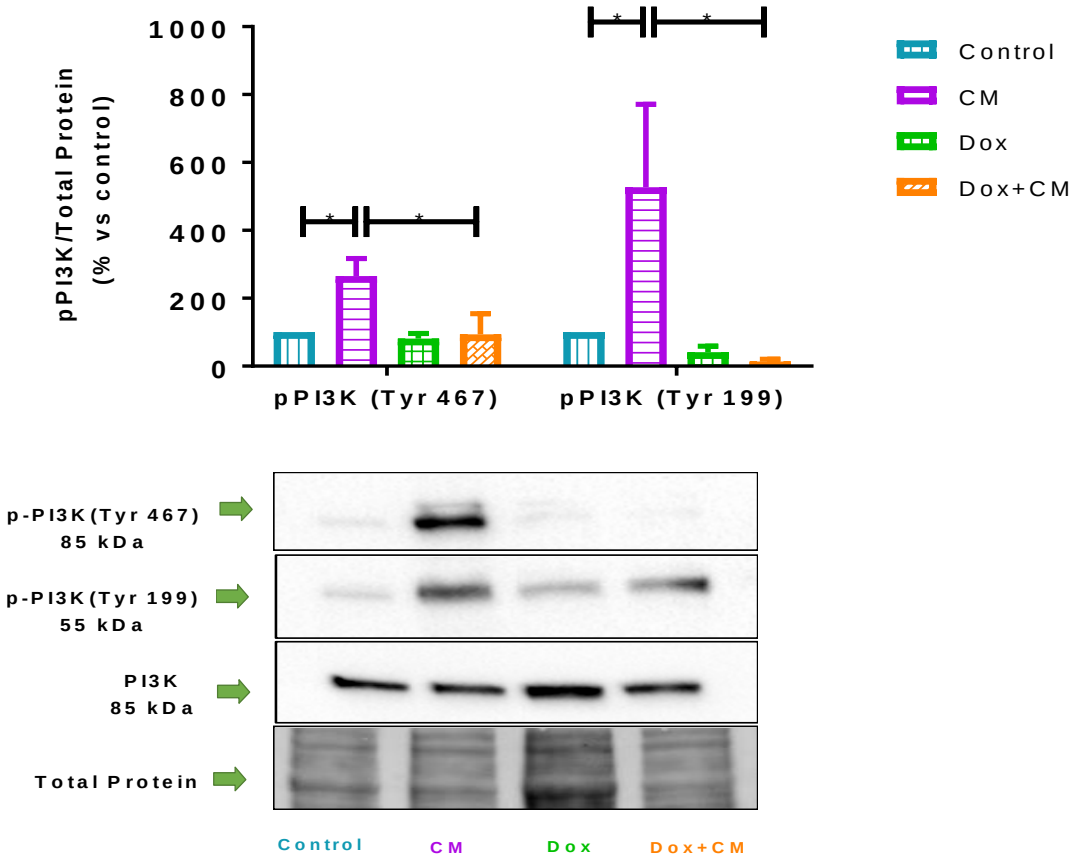

Figure 3.7: The effect of adipocyte conditioned media on PI3K and phosphorylated-PI3K (Tyr 467, 85 kDa and Tyr 199, 55 kDa) cellular proliferation pathway in MDA-MB-231 breast cancer cells treated with/without doxorubicin for 48-hours. Results are presented as mean± SEM (n=3). One-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. * = $p<0.05$. CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media.

3.2.4.2 Akt Pathway: Adipocyte Conditioned Media Induces Akt-Phosphorylation in Breast Cancer Cells

Akt is a serine/threonine specific protein kinase that regulates apoptosis and cellular proliferation. In its inactive conformation, Akt is present within the cytosol and upon activation translocates to the cell membrane (Hemmings & Restuccia, 2015), followed by an interaction between PIP_3 and Akt which induces a conformational change in Akt exposing the Thr308 and Ser473 phosphorylation sites. Full phosphorylation of Ser473 is required for Akt activation (Yu & Cui, 2016). We found that phosphorylated Akt (pAkt, Ser473) expression was significantly increased in CM ($p=0.0003$) and significantly decreased in Dox treated cells

(p=0.014) compared to control cells (Figure 3.8). A significant increase was also observed in CM treated cells when compared to Dox+CM treated cells for both total Akt (p=0.033, Figure 3.8) and pAkt (p<0.001, Figure 3.8), respectively.

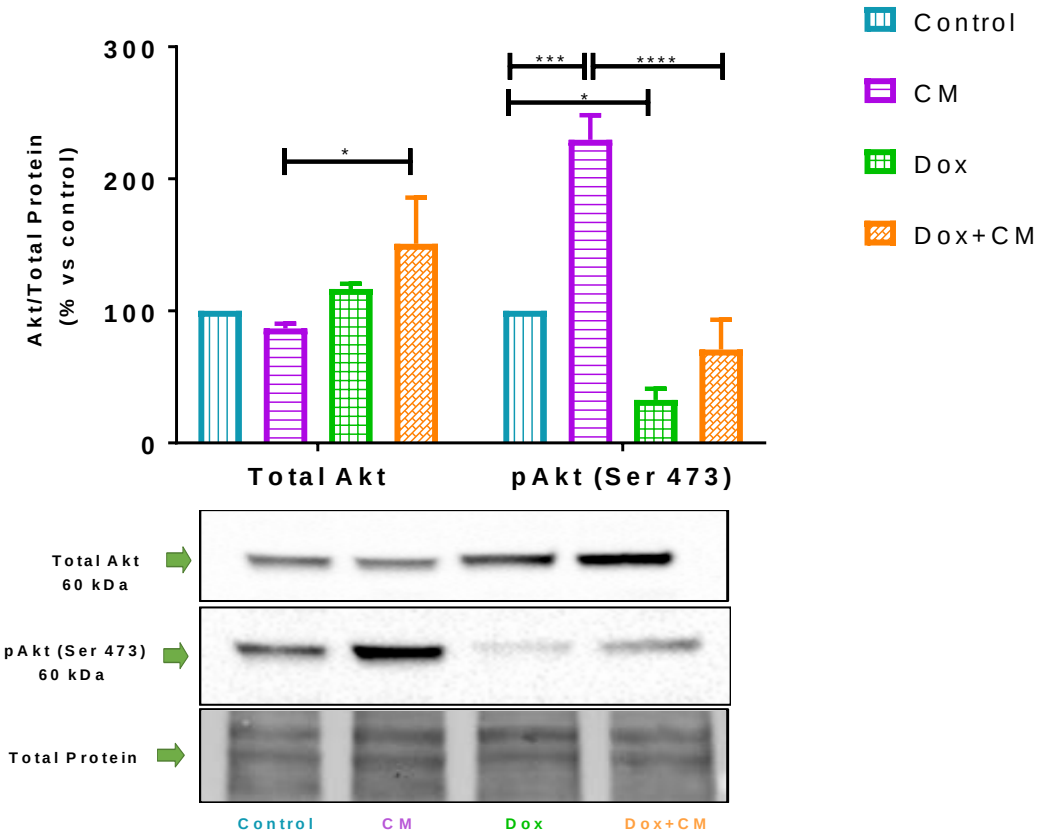

Figure 3.8: The effect of adipocyte conditioned media on Akt and phosphorylated-Akt (Ser 473) in MDA-MB-231 breast cancer cells treated with/without doxorubicin for 48-hours. Results are presented as mean ± SEM (n=3). One-way ANOVA with Fishers LSD *post hoc* correction was employed. p<0.05 was considered as statistically significant. * = p<0.05, *** = p<0.001 and **** = p<0.0001. CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media.

3.2.4.3 ERK Pathway: Adipocyte Conditioned Media decreases ERK-Phosphorylation in Breast Cancer Cells treated with Doxorubicin

The ERK/MAPK pathway is well known to induce several cellular processes including cell division, proliferation, differentiation and survival. Once ERK1/2 is phosphorylated by MEK1/2, it becomes activated and translocate to the nucleus to activate these processes (McCubrey *et al*., 2007).

In our model phosphorylated ERK1 (pERK1, Thr202/Tyr204) protein expression was significantly decreased in the CM treated group ($p<0.0001$), and significantly increased in Dox ($p<0.0001$) and Dox+CM ($p=0.030$) treated cells compared to control cells, respectively (Figure 3.9). Additionally, we observed that pERK1 expression was increased in Dox+CM compared to CM only cells ($p<0.0001$), and lower when compared to Dox treated cells ($p=0.012$) (Figure 3.9). Phosphorylated ERK2 (pERK2, Thr185/Tyr185) was significantly decreased in the CM treated compared to both control ($p=0.0028$) and Dox+CM treated cells ($p=0.0063$), respectively (Figure 3.9).

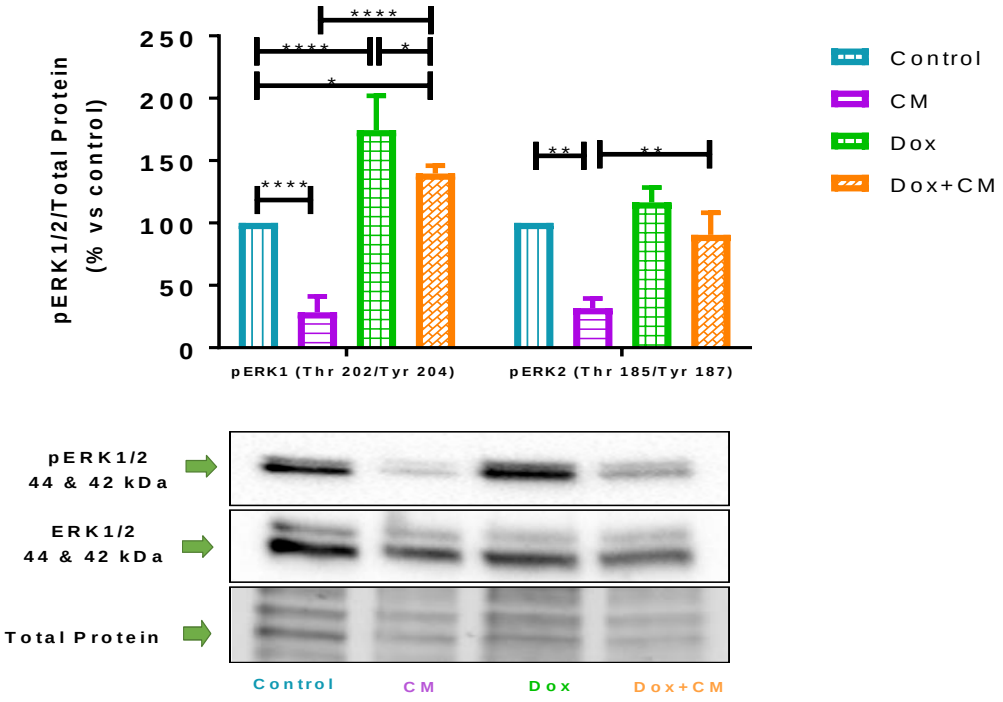

Figure 3.9: The effect of adipocyte conditioned media on ERK and phosphorylated-ERK1/2 in MDA-MB-231 breast cancer cells treated with/without doxorubicin for 48-hours. Results are presented as mean ± SEM (n=3). One-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. * = $p<0.05$, ** = $p<0.01$ and **** = $p<0.0001$. CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media.

3.2.5 Mesenchymal -to-Epithelial Transition (MET): Hybrid Phenotype

Adipocytes in the tumour microenvironment have been shown to induce mesenchymal -to-epithelial transition (MET) in breast cancer cells which promotes invasion and metastasis (Pallegar *et al*., 2018). In light of this, we determined the protein expression of both epithelial (E-cadherin) and mesenchymal markers (snail and vimentin) using Western blotting, in order to determine if adipocytes can induce any alterations in the expression of these markers in breast cancer cells in a paracrine manner.

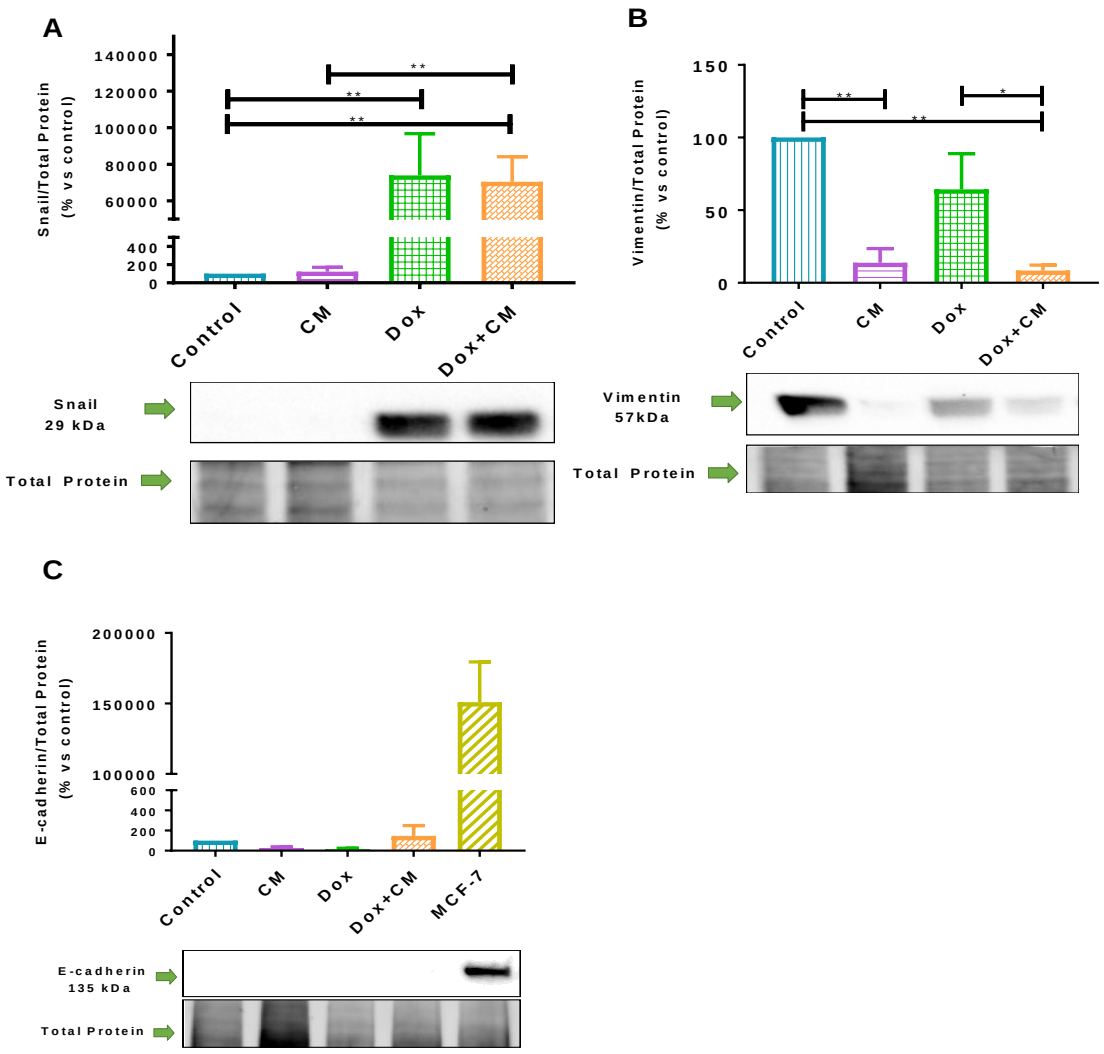

Figure 3.10: The effect of adipocyte conditioned media on EMT markers in MDA-MB-231 breast cancer cells treated with/without doxorubicin for 48-hours. (A) Snail, (B) Vimentin, and (C) E-cadherin. Results are presented as mean ± SEM (n=3). One-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. * = $p<0.05$ and ** = $p<0.01$. CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media.

Snail expression was significantly increased in both Dox (p=0.044) and Dox+CM (p=0.0058) treated cells compared to the control group, respectively (Figure 3.10A). Additionally, snail was significantly increased in Dox+CM cells compared to CM treated cells (p=0.0058) (Figure 3.10A). Vimentin was found to be significantly higher in the control compared to CM treated cells (p=0.0018) and Dox compared to Dox+CM treated cells (p=0.018) (Figure 3.10B). No significant differences were observed for E-cadherin protein expression between any of the respective treatment groups (Figure 3.10C).

3.2.6 Lipolysis is Induced by Adipocyte Conditioned Media in Doxorubicin Treated Breast Cancer Cells

To ascertain whether adipocytes induce breast cancer cells to dysregulate lipid metabolism, we assessed protein expression levels of lipid metabolism markers which regulates *de novo* FA synthesis (FAS and SCD-1) and lipolysis (ATGL and HSL) with/without doxorubicin treatment in the presence, and/or absence of adipocyte conditioned media treatments.

Compared to the control group, HSL protein expression was significantly upregulated after Dox treatment (Control *vs* Dox, p<0.011, Figure 3.11D), whereas both HSL and ATGL protein expression were significantly higher in the Dox+CM treatment group compared to the control group (ATGL, p=0.021 and HSL, p=0.0002), respectively (Figure 3.11C and 3.11D). Additionally, HSL expression was also significantly higher Dox+CM compared to Dox treatment (Dox *vs* Dox+CM, p=0.03) and CM treatment (p=0.0018), respectively (Figure 3.11D). No significant differences were observed for FAS and SCD-1 between any of the experimental treatment groups (Figure 3.11A&B).

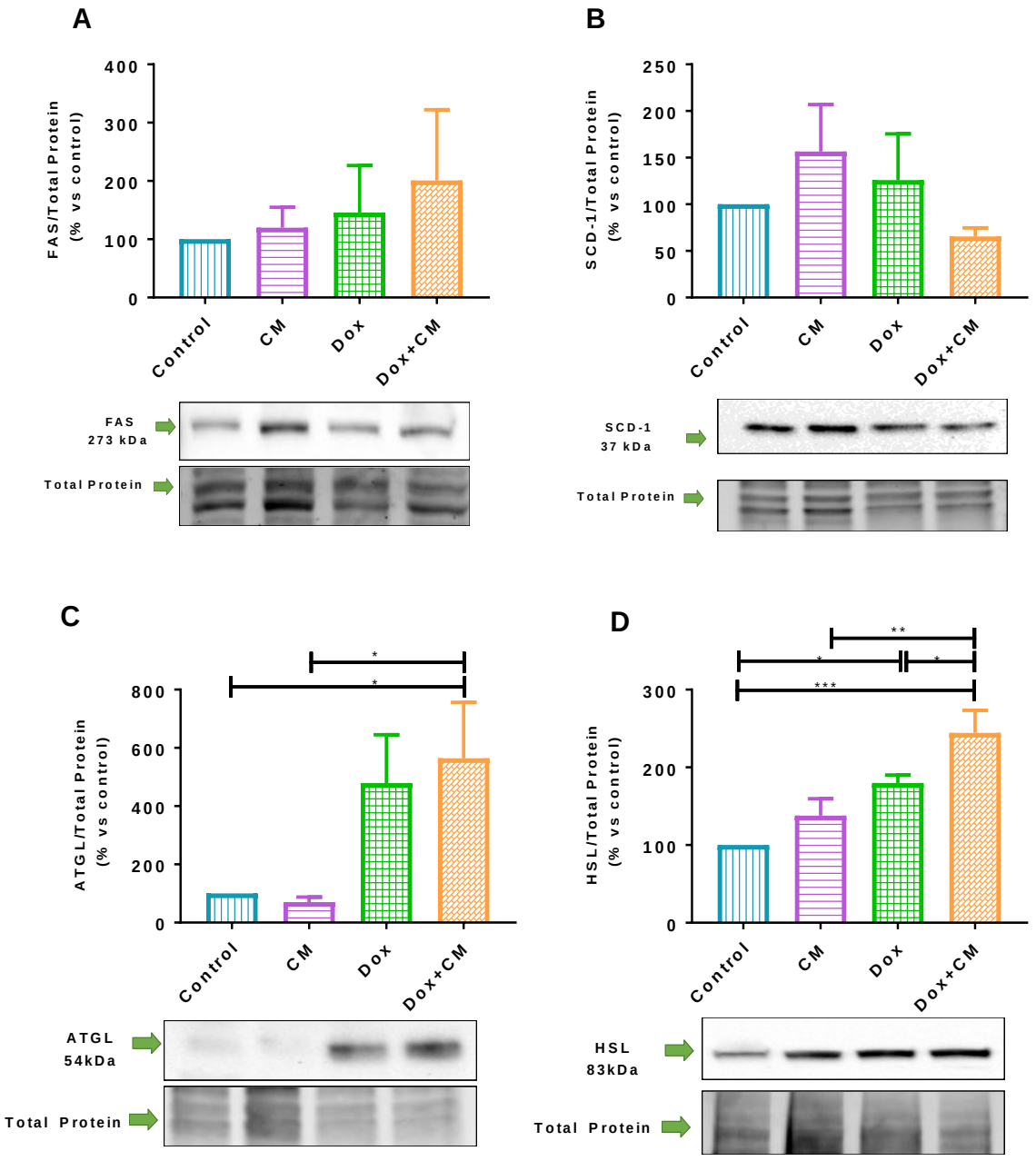

Figure 3.11: The effect of adipocyte conditioned media on markers of lipid metabolism in MDA-MB-231 breast cancer cells treated with/without doxorubicin for 48-hours (A) FAS, (B) SCD-1, (C) ATGL and (D) HSL. Results are presented as mean ± SEM (n=3, except HSL n=4). One-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. ATGL, Adipose triglyceride lipase; CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media; FAS, fatty acid synthase; HSL, hormone sensitive lipase; SCD-1, Stearoyl-CoA desaturase-1.

3.2.7 Adipocyte Conditioned Media and Doxorubicin Treatment Alters FFA Profile Evident in Treatment Conditioned Media

D*e novo* FAs synthesis and lipolysis is dysregulated within breast cancer cells, additionally breast cancer cells also manipulate adipocytes in the tumour microenvironment to release FAs (Balaban *et al*., 2017). These FFA are utilized by breast cancer cells to avoid the cytotoxic effects of anti-cancer drugs (Du *et al*., 2018; Iwamoto *et al*., 2018). We therefore determined the FFA composition of treatment conditioned media of all experimental treatment groups to identify whether changes in the FFA composition in conditioned media could account for chemotherapy treatment outcomes in breast cancer cells.

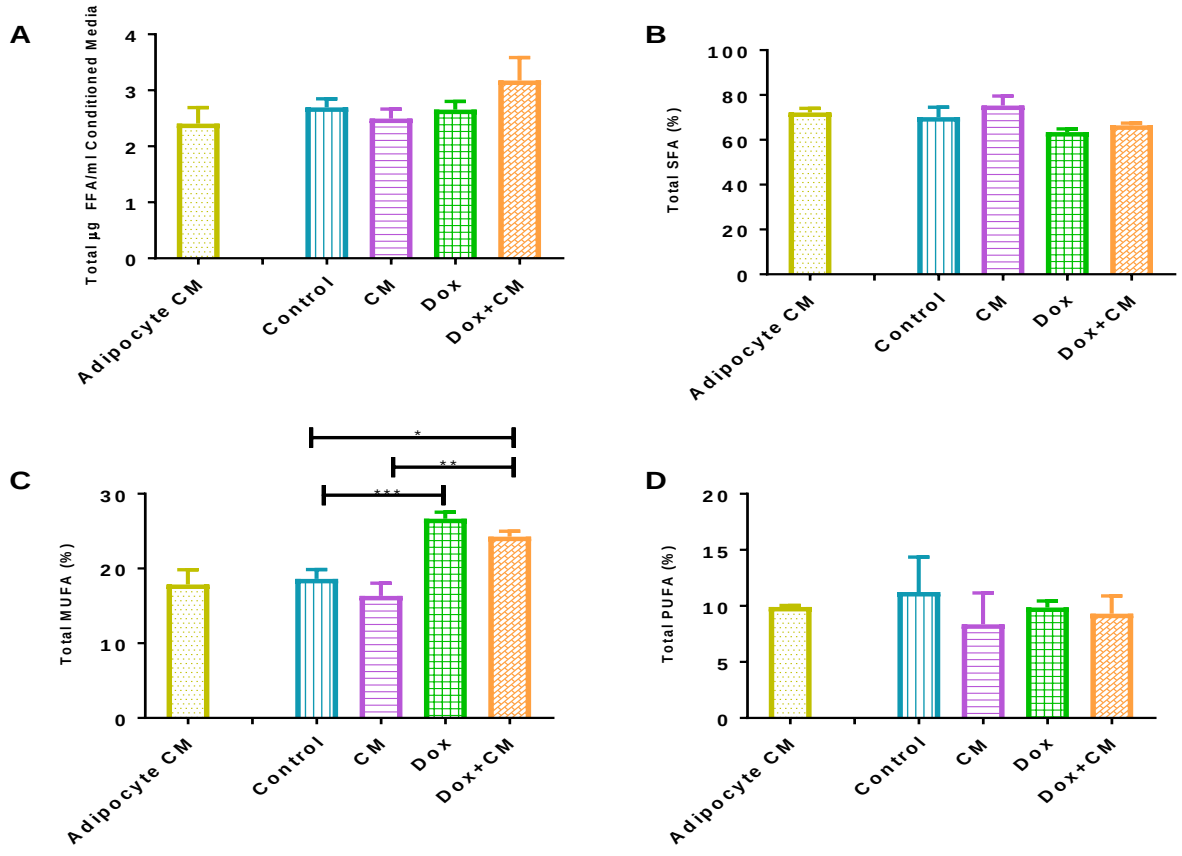

Figure 3.12: The Total FFA classes in treatment conditioned media of all experimental treatment groups. (A) Total FFA, (B) Total SFAs, (C) Total MUFAs and (D) Total PUFAs. Results are presented as mean ± SEM (n=3). One-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media; FFA, free fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

The total µg FFA per 1ml adipocyte conditioned media as well as treatment conditioned media is illustrated in Figure 3.12A, each FA is expressed as a percentage of the total FAs. In all experimental groups, the most abundant FAs type was SFAs, followed by MUFAs and lastly PUFAs (Supplementary Figure 6). No significant difference was observed for Σ SFAs (Figure 3.12B) and Σ PUFAs (Figure 3.12D), between any of the treatment groups. Moreover, both Dox ($p=0.015$) and Dox+CM ($p=0.010$) treatment conditioned media showed statistically higher Σ MUFAs compared to treatment conditioned media from the control group (Figure 3.12C), whereas Dox+CM showed significantly higher Σ MUFAs compared to the CM groups treatment conditioned media ($p=0.0015$, Figure 3.12C).

Similar results were observed for both VA and OA, in both Dox (VA, $p=0.0068$ and OA, $p=0.0007$) and Dox+CM (VA, $p=0.024$ and OA, $p=0.013$) treatment groups, showing statistically higher VA and OA levels compared to controls (Figure 3.13C&D). In addition, Dox+CM showed significantly higher VA and OA compared to CM group (VA, $p=0.014$ and OA, $p=0.0022$, Figure 3.13C&D). Additionally, OA was found to be lower in Dox+CM compared to Dox treatment conditioned media, however only a trend towards statistical significance was observed (Figure 3.13D), whereas LA was significantly lower in Dox+CM compared to Dox treatment group ($p=0.03$, Figure 3.13E).

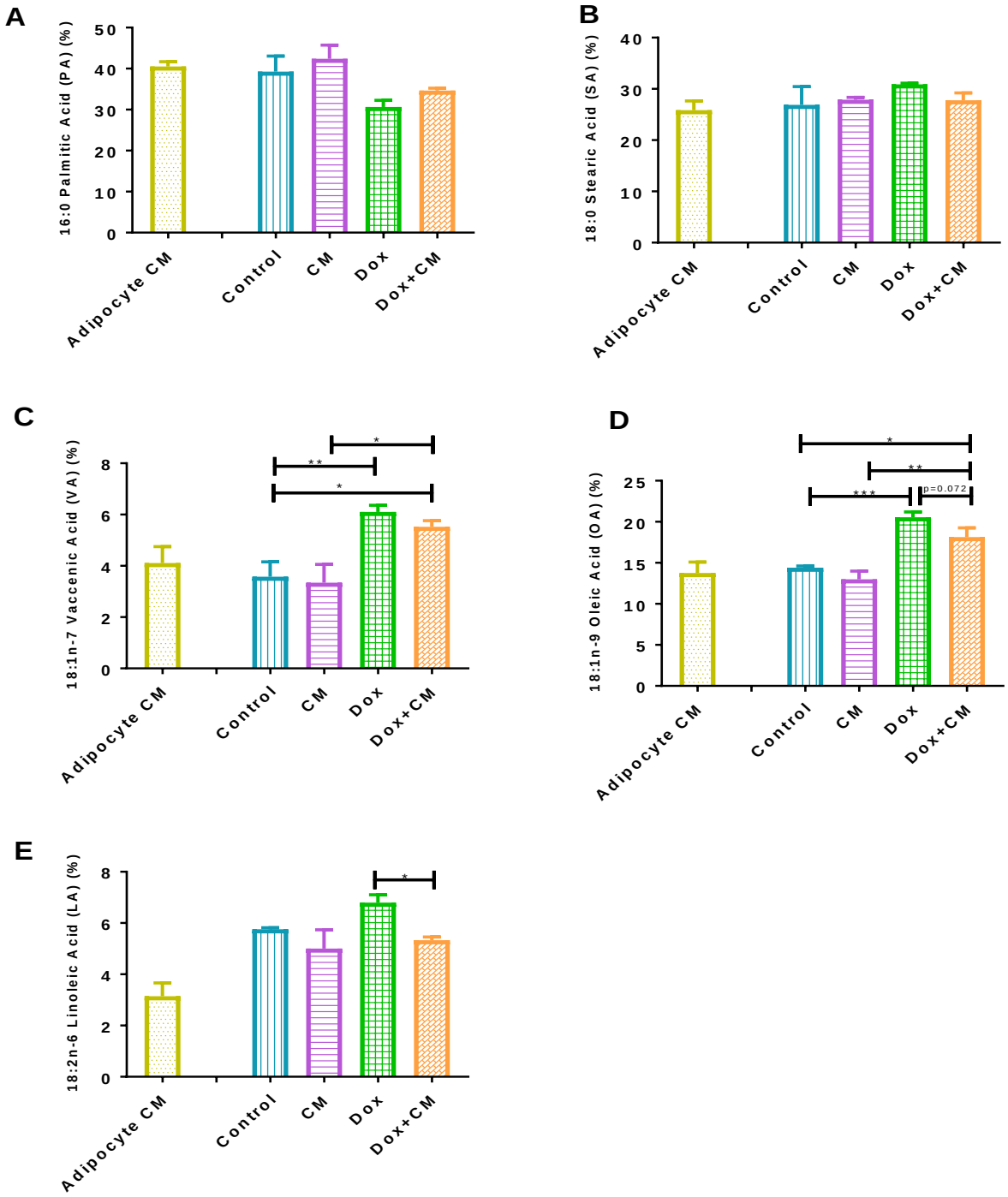

Figure 3.13: Individual FFAs of treatment conditioned media of breast cancer cells treated with/without adipocyte conditioned media and/or doxorubicin for 48-hours. (A) Palmitic Acid (PA), (B) Stearic Acid (SA), (C) Vaccenic Acid (VA) and (D) Oleic Acid (OA) and (E) Linoleic Acid (LA). Results are presented as mean ± SEM (n=3). One-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media.

3.2.8Adipocyte Conditioned Media Induces an Increasein Inflammatory MarkersConcentration in Doxorubicin Treated Breast Cancer Cells

Adipocytes have been shown to favour breast cancer cell proliferation, migration and invasion in the TME through the paracrine secretion of various adipokines. Therefore, to determine if adipocytes can induce doxorubicin treatment resistance in MDA-MB-231 TNBC cells, we determined the concentrations of various adipokines in the treatment conditioned medium of all experimental groups. Various adipokines were identified in the adipocyte conditioned media ranging from highest to lowest concentration: these included adiponectin, leptin, MCP-1, resistin, and IL-1β (SupplementaryFigure 8).

Adiponectin, leptin, MCP-1, and IL-1β concentrations were found to be significantly higher in the CM, Dox and Dox+CM groups compared to the control group (all $p<0.0001$, Figure 3.14). Adiponectin, leptin and MCP-1 concentrations were significant higher in CM compared to Dox+CM group ($p<0.0001$) (Figure 3.14), whereas the opposite was true for IL-1β ($p<0.0001$, Figure 3.14D). Interestingly, Leptin ($p=0.025$) and MCP-1 ($p=0.0022$) concentrations were also significantly higher in Dox+CM compared to the Dox treated group (Figure 3.14B&C). Lastly, resistin concentration was found to be significantly higher in the CM group compared to both control and Dox+CM groups ($p<0.0001$, Figure 3.14E).

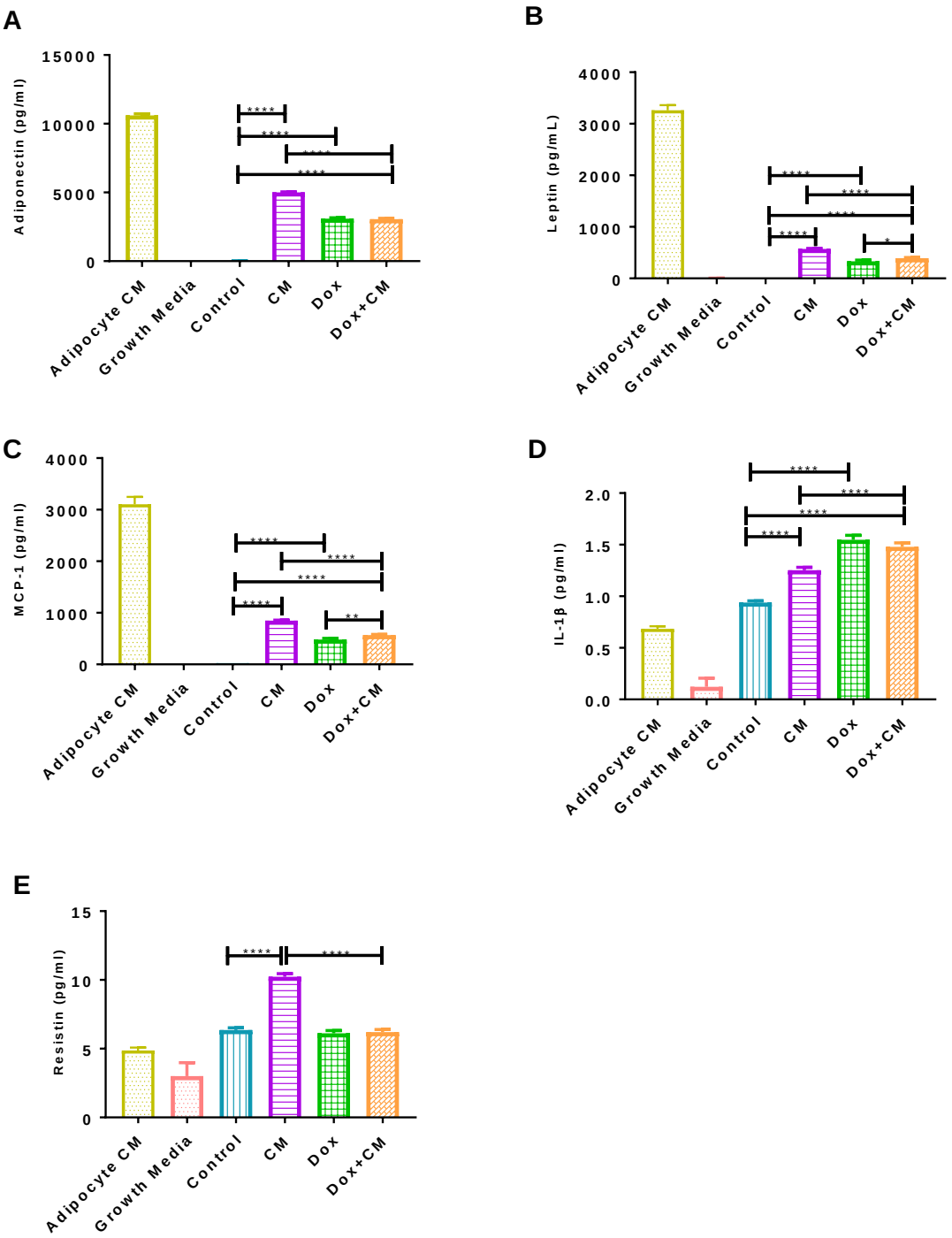

Figure 3.14: Inflammatory marker concentrationsin treatment conditioned media of MDA-MB-231 breast cancer cells treated with/without adipocyte CM and/or Dox for 48-hours. (A) Adiponectin, (B) Leptin, (C) MCP-1 and (D) IL-1β and (E) Resistin. Results are presented as mean ± SEM (n=4). One-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. * = $p<0.05$, ** = $p<0.01$ and **** = $p<0.0001$. Adipocyte CM, Adipocyte Conditioned Media, CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media.

3.3 DISCUSSION: *IN VITRO* MODEL

3.3.1 Doxorubicin induced Apoptosis Attenuated in Breast Cancer Cells Treated with Adipocyte Conditioned Media

We measured protein expression levels of PARP and cleaved-PARP as well as caspase-3 and cleaved-caspase-3 to evaluate whether adipocyte conditioned media could decrease the efficacy of doxorubicin induced apoptosis in a paracrine manner. Here, we found that following 48-hours of Dox treatment, cleaved-caspase-3 protein expression was significantly increased by doxorubicin treatment (control *vs* Dox, Figure 3.6), confirming that doxorubicin treatment induced apoptosis in MDA-MB-231 breast cancer cells. Cleaved-caspase-3 expression was significantly decreased in Dox+CM *vs* Doxtreated cells, suggesting that adipocytes significantly decreased doxorubicin's efficacy to induce apoptosis in MDA-MB-231 TNBC cells in a paracrine manner (Figure 3.6), which is an indication of acquired treatment resistance.

Treatment with doxorubicin induces DNA damage, generatesROS, initiates mitochondrial membrane dysfunction, as well as induces apoptosis in response to topoisomerase II inhibition to promote apoptotic cell death in cancer cells through activation of caspases (Anampa *et al*., 2015; Thorn *et al*., 2011; Gorini *et al*., 2018). Supporting evidence shows doxorubicin treatment decreased MDA-MB-231 breast cancer cell viability, and significantly increasedcaspase-3 expression and induced DNA fragmentation in a time-dependent manner (48-72-hour treatment) (Nestal *et al*., 2013; Pilco-Ferreto & Calaf, 2016). Importantly, breast cancer treatment resistance can develop due to the evasion of apoptotic pathways (cell-death) by increasing the anti-apoptotic protein blc-2 and decreasing the pro-apoptotic protein, bax (Behan *et al*., 2009). It has also been shown that adipocytes protect cancer cells from daunorubicin by upregulating anti-apoptotic bcl-2, and downregulation of pro-apoptotic bad and pim-2 family members (Behan *et al*., 2009; Behan *et al*., 2010). Liu *et al*. (2017) showed that the adipokine, resistin, that is mainly secreted from adipose tissue acts as a causal factor in doxorubicin treatment resistance in both MCF-7 and MDA-MB-231 breast cancer cells. They proposed that doxorubicin induced apoptosis (increased cytochrome-c, cleaved caspase-9, cleaved PARP expression) was suppressed by resistin treatment which lead to autophagy (Liu *et al*., 2017).

We also report that doxorubicin treatment significantly increased cleaved-PARP protein expression compared to the control treatment group, whereas cleaved-PARP was also

significantly higher in Dox+CM compared to CM treatment group (Figure 3.5). PARP functions by binding to damaged single stranded DNA in order to repair DNA damage (Morales *et al*., 2014). This is in agreement with previous work on breast cancer cells showing that doxorubicin induced PARP activation (Wang *et al*., 2014).However, the significant decrease in caspase-3 and cleaved-caspase-3 in the Dox+CM compared to Dox treated cells (Figure 3.6) was not supported by a significant increase in PARP activation and cleavage (Figure 3.5) in our model. This was a surprising result since caspase-3 cleavage induces PARP inactivation and cleavage. Our results can be explained by our treatment duration of 48-hours since it has been previously reported that PARP is activated and cleaved in MDA-MB-231 cells 72-hours post doxorubicin treatment (Muñoz-Gámez *et al*., 2005). This might suggest that our treatment duration of 48-hours might be insufficient to induce PARP cleavage. Regardless, since doxorubicin induced apoptosis (decreased caspase-3 and cleaved-caspase-3) was decreased by adipocyte conditioned media treatment in MDA-MB-231 breast cancer cells, this might explain the time dependant increase in cell viability of the Dox+CM treated cell in our model (Figure 3.4), which is characteristic of acquired breast treatment resistance.

Evidence support the role of obesity induced inflammation (IL-6, TNF-α and leptin) in acquired breast cancer drug resistance (Bougaret *et al*., 2018; Incio *et al*., 2018). Therefore, we propose that the increased cell viability observed in the Dox+CM group may in part be as a result of adipocyte derived adipokines inducing inflammation. We report that leptin and MCP-1 concentrations were significantly higher in Dox+CM compared to the Dox treated group (Figure 3.14B&C). In agreement to our results, leptin derived from obese adipocytes has been shown to attenuate Tamoxifen^{R} treatment efficacy compared to adipocytes derived from normal weight patients (Bougaret *et al*., 2018). Additionally, adipocytes supress doxorubicin-induced apoptosis by upregulating the expression of pro-survival apoptotic proteins (i.e. blc-2) as well as increasing the synthesis of various pro-inflammatory markers (Behan *et al*., 2009; Liu *et al*., 2017). Moreover, MCP-1 has been shown to protect breast cancer cells from chemotherapeutic (5-fluorouracil) induced apoptosis, as a result of treatment-induced inflammation (Vyas *et al*., 2014). It is proposed that adipocyte dysfunction (obesity) leads to dysfunctional adipokine synthesis, which inturn results in increased immune cell infiltration and inflammatory mediator synthesis. All of these factorsfavour sustained cellular proliferation, angiogenesis as well as the inhibition of apoptosis, ultimately favouring the development of breast cancer treatment resistance (Cowen *et al*., 2015; Pérez-Hernández *et al*., 2014).

3.3.2 Adipocyte Conditioned Media May Induce a Partial Mesenchymal-to-Epithelial Transition (MET)

Adipocytes have been shown to induce signalling pathways in cancer cells which favourinvasion and metastasis which is associated with acquired treatment resistance in breast cancer patients (Gyamfi *et al*., 2018; Wang *et al*., 2018; Wei *et al*., 2016; Bousquenaud *et al*., 2018; Hanahan & Weinberg, 2011). A complex process involving various cellular processes i.e. proliferation, cell-cell adhesion, cell matrix adhesion, proteolysis, migration, and evasion of apoptosis (cell-death) contributes towards invasion and metastasis (Jiang *et al*., 2015). A proposed underlying mechanism of action for metastasis includes cells migrating through the extracellular matrix (ECM) as a result of EMT (Bidard *et al*., 2008) to facilitate the migration of cancer cells away from the primary tumour, as well as mesenchymal-to-epithelial transition (MET) to form tumours at distant sites (Jiang *et al*., 2015).

Metastatic cancer cell lines which have undergone EMT has been shown not to maintain their mesenchymal phenotype (Olea-Flores *et al*., 2018). Reports also show that cancer cells can display a hybrid phenotype characterised by the expression of both epithelial and mesenchymal markers (Jolly *et al*., 2017; Mittal, 2018), or it could be the result of breast cancer cells undergoing partial transitions of MET and EMT (Pallegar *et al*., 2018). This hybrid phenotype has been found to be present in TNBC patients (Yu *et al.,* 2019), and it is proposed that cancer cells with this hybrid phenotype are able to rapidly enter back into the cell cycle and proliferate after metastasis to secondary sites and may also have different responses to signalling pathways for cell growth, cell death, metastasis and drug resistance (D'Esposito *et al*., 2016).

Interestingly, adipocytes induce this hybrid/partial phenotype in BC cells (Wang *et al*., 2017), and inducing morphological changes (loss of stellate and gain of round/mass colony phenotype) in MDA-MB-231 breast cancer cells, as well as inducing a partial MET protein marker expression profile (increased E-cadherin & Claudin-7 expression) (Pallegar *et al*., 2018). In addition, adipocyte conditioned media affected cell morphology immediately, but did not affect EMT biomarkers, suggesting that mature adipocytes promote a partial MET in mesenchymal MDA-MB-231, which was not the case for conditioned media. We speculate that partial MET may be plausible for the results obtained in our model since decreased

vimentin expression was induced by adipocyte condition media in breast cancer cells treated with and without doxorubicin (Figure 3.10B). Vimentin is a major cytoskeletal structural protein responsible for maintaining cellular shape as well as the stabilization of cytoskeletal interactions (Huber *et al.*, 2004; Wu *et al.*, 2007). High protein expression of vimentin is found in mesenchymal type cells including that of aggressive breast cancer cell lines i.e. MDA-MB-231 (Pallegar *et al*., 2018), that supports findings from our results (Figure 3.10B).

E-cadherin is a cell surface protein responsible for the formation and maintenance of adherent junctions within cell-cell adhesion (Lee *et al*., 2015). We did not observe any significant differences for E-cadherin protein expression between any of the respective treatment groups (Figure 3.10C), which is in agreement with literature where mesenchymal-like cells shows low protein expression of E-cadherin (Lee *et al*., 2015). Snail is a transcription factor regulating EMT, where increased snail expression is linked to advanced tumour grade and metastasis in breast cancer (Olea-Flores *et al*., 2018). In our model snail protein expression was significantly increased in both Dox and Dox+CM treated cells compared to the control group, respectively (Figure 3.10A). Additionally, snail protein expression was also significantly increased in Dox+CM cells compared to CM treated cells alone (Figure 3.10A), suggesting that doxorubicin treatment itself induced snail expression, which has been reported in MDA-MB-231 TNBC cells (Chen *et al*., 2013). Lee *et al*. (2015), showed that MDA-MB-231 co-cultured with adipocytes presented with morphological changes (dispersed features, elongated and spindle-like structures) as well as increased vimentin protein expression, whereas E-cadherin was repressed and snail expression was not affected. It was proposed that other regulators of EMT might be responsible for the EMT induction by adipocytes, for example twist, which is a key transition factor regulating EMT. It is well known that the PI3K-Akt pathway and the phosphorylation of NFκBinduce downstream effects on growth factors leading to upregulation of snail activity (Wang *et al.*, 2013; Julien *et al.*, 2007). Therefore, this may be plausible that the increased snail protein expression observed in the Dox treatment group, could be due to the significant increase in pERK1 protein expression, observed in the Dox and Dox+CM treatment groups compared to the control group (Figure 3.9).

However, conflicting results are reported on the role of adipocytes in EMT and MET. For example, Wang *et al*. (2015) reports that leptin and IL-8 induced EMT in MCF-7 and SK-BR-3 breast cancer cells through PI3K/Akt pathway induction, however this was not the case in MDA-MB-231 cells. Wei *et al*. (2016) found that high leptin concentrations increased

vimentin and decreased E-cadherin protein expression in MCF-7 and MDA-MB-468 breast cancer cells.Wang *et al*. (2018), showed that synthetic resistin increased the expression of EMT transcription factors i.e. zeb, snail and twist as well as vimentin and supressed the expression of E-cadherin in breast cancer cells, thereby inducing EMT.Dutta *et al*. (2018) showed that higher MCP-1 levels are associated with cell invasion and metastasis in TNBC cells (BT549 and HCC1395) by inducing p44/42 MAP kinase (MAPK) pathway. Fujisaki *et al*. (2015), showed that conditioned medium from human mammary cancer associated adipocytes, showed increased MCP-1 levels, which decreased vimentin expression in MDA-MB-231 TNBC cells, which is in agreement with the increased MCP-1 concentration (Figure 3.14C) and decreased vimentin protein expression observed in the Dox+CM compared to the Dox group alone (Figure 3.10B) in our current model. Gyamfi *et al*. (2018) reported a decreased E-cadherin and increased vimentin protein expression in MDA-MB-468 breast cancer cells after co-culture with adipocytes. Here it is suggested that adipocytes induce and/or favour breast cancer migration and invasion through EMT as a result of adipocyte derived adipokine sectored factors (resistin, TGF-β, IL-6, leptin and IL-8), that phosphorylate and activate STAT-3 signalling in a paracrine manner (D'Esposito *et al*. 2016; Wang *et al*., 2018; Wei *et al*., 2016). This is not the case in our model as we found no significant differences in proliferation signalling pathways (i.e. PI3K, Akt, ERK2, MAPK and its phosphorylation as well as NFκB (Supplementary Figure 5)) between the Dox and Dox+CM treated MDA-MB-231 breast cancer cells, where we observed acquired doxorubicin treatment resistance. Therefore, it might be plausible that MET could be induced*via* another signalling pathway i.e. STAT-3. Additionally, the contradictory results between studies can be ascribed to the following, different breast cancer cell lines used, co-culture *vs* conditioned medium models and differences in treatment and treatment duration.

3.3.3 Adipocytes Do Not Promote Treatment Resistance *via* Induction of Cellular Proliferation Signalling Pathways

Cellular proliferation includes complex processes regulated by several signalling pathways such as the PI3K/Akt and ERK/MAPK pathways, both of which is overexpressed/hyperactive in breast cancer (Chang *et al*., 2002; Chua *et al*., 2009; McCubrey *et al*., 2007). From our model we found that phosphorylated PI3K (both Tyrosine 467 and 199) was significantly

increased in CM treated cells compared to both control and Dox+CM groups, respectively (Figure 3.7). Additionally, Akt phosphorylation (Ser473) was significantly increased in both CM and doxorubicin treated cells compared to the control group (Figure 3.8).

The increased cell viability in Dox+CM *vs* Dox treatment groupswe observed did not correlate with increased activation of the PI3K/Akt pathway or the MAPK pathway (Figure 3.7 and Figure 3.9). The ERK1/2/MAPK signalling pathway plays an important role in the regulation of cellular survival and proliferation. The MAPKsare serine/threonine kinases, where dual phosphorylation of both Thr202/Tyr204 residue are needed to activate ERK1 and phosphorylation of Thr185/Tyr187 is needed to activate ERK2 in humans (McCubrey *et al*., 2012). We showed that ERK1 phosphorylation was significantly decreased in CM treated cells and increased significantly in Dox and Dox+CM treated cells compared to control group (Figure 3.9). Additionally, we also observed that pERK1 expression was increased in Dox+CM compared to CM only cells, and decreased when compared to Dox only treated cells (Figure 3.9). Phosphorylated ERK2 was significantly decreased in the CM treated compared to both control and Dox combined with conditioned media treated cells, respectively (Figure 3.9). This suggests that adipocyte conditioned media, even in the presence of doxorubicin treatment decreased pERK1, showing a significant downregulation of ERK signalling occurred. In agreement, Taherian & Mazoochi. (2012) observed that the protein expression of total ERK1/2 and pERK1/2 was decreased in MDA-MB-231 cells after doxorubicin treatment, suggesting that the decreased expression of ERK in MDA-MB-231 cells could therefore induce the apoptosis pathway. However, in our model the Dox+CM compared to the Dox treated group was able to evade apoptotic cell death as evident by decreased caspase-3 and cleaved-caspase-3 activation in the Dox+CM group (Figure 3.6). The increased expression of Akt and decreased ERK in the CM group might suggest that Akt alone is sufficient to induce cell proliferation and increased cell viability in the CM treated group.

Our results are in accordance with previous findings where adipocyte conditioned media increased cellular proliferation in MDA-MB-231 cells after 24-72 hours of treatment (Balaban *et al*., 2017). Additionally, Bougaret *et al*. (2018) showed that adipocyte conditioned media derived from obese adipocytes increased cell viability, leading to decreased Tamoxifen® treatment efficacy in MCF-7 cells. However, it should be highlighted that these studies made use of different breast cancer and pre-adipocyte cell lines, differed in treatment and treatment duration and also used different methods to quantify cellular proliferation (MTT and cell

count proliferation assays (incuCyte)), and also did not assess cellular proliferation signalling pathways. This could explain in part our contradictory results.

We do report similar findings with Lapeire *et al*. (2017), who showed that pERK1/2, ERK, Akt and pAkt was not activated in MDA-MB-231 breast cancer cells treated with conditioned media derived from cancer associated adipose tissue in breast cancer patients. Supporting evidence includes Pallegar *et al*. (2018), showing that adipocytes and adipocyte conditioned media, did not increase cellular proliferation marker ki67 expression in MDA-MB-231, but induced a partial/hybrid of MET in these TNBC cells. This in turn may explain the lack of significance observed for these proliferation signalling pathways (i.e. PI3K, AKT and ERK2 and its phosphorylation as well as NFκB (Supplementary Figure 5)) between the Dox and Dox+CM treated MDA-MB-231 breast cancer cells. It might be plausible that cellular proliferation could be induce *via* another signalling pathway i.e. STAT-3. It may also be possible that adipocyte conditioned media can induce cellular proliferation/survival *via* other mechanisms of action. For example, increased FFA can induced proliferation *via* Akt activation in MDA-MB-231 cells (Hardy *et al*., 2000). A recent report showed that lipid accumulation (adipocyte derived FFA) leads to uncoupled FA oxidation, which favoured invasion due to EMT, but not proliferation (Wang *et al*., 2017). This was the rationale to assess the role of lipid metabolism proteins as well as FFA profilesin treatment conditioned media obtained from this *in vitro* model.

3.3.4 Adipocyte Derived Factors May Induce Lipolysis in Breast Cancer Cells Treated with Doxorubicin in a Paracrine Manner

Sufficient evidence demonstrates breast cancer cells have a strong dependence on both endogenous synthesis of lipids as well as exogenous lipid uptake to sustain survival and development (Balaban *et al*., 2017; Balaban *et al*., 2018; Wang *et al*., 2017). This is not questionable since breast tumours are in close proximity to mammary adipose tissue, which is a major source of FAs (Park *et al*., 2011). To determine if adipocytes can induce breast cancer cells to dysregulate lipid metabolism in a paracrine manner, and whether this could be a potential mechanism through which breast cancer cells develop acquired treatment resistance, we determined the protein expression levels of lipid metabolism markers which

regulate of *de novo* FA synthesis (FAS and SCD-1), and lipolysis (ATGL and HSL) following doxorubicin and/or adipocyte conditioned media treatments.

In our *in vitro* model we found that compared to the control group both ATGL and HSL protein expression was significantly increased following Dox and Dox+CM treatment (Figure 3.11C&D). Interestingly, HSL protein expression was also significantly higher in Dox+CM compared toDox treated cells (Figure 3.11D), thereby increasing lipolysis. Additionally, we also found a higher adiponectin, leptin, MCP-1, IL-1β and resistin concentrations in the CM treated cells compared to the control group, suggesting that adipocytes induce MDA-MB-231 TNBC cells to increase the production of these inflammatory mediators in a paracrine manner. Additionally, adiponectin, leptin, MCP-1 and resistin concentrations were also significant higher in CM compared to Dox+CM group (Figure 14). In agreement, doxorubicin treatment has been shown to induce inflammation in metabolic tissues (Supriya *et al*., 2016). Lastly, we propose that adipocyte derived factors (increased leptin and MCP-1 levels, Dox+CM *vs* Dox group Figure 3.14), leads to lipolysis (increased HSL, Dox+CM *vs* Dox group Figure 3.11D) in MDA-MB-231 TNBC cells.

In light of this we propose that adipocyte secretory factors induce MDA-MB-231 TNBC cells to induce lipolysis and inflammation to counteract the cytotoxic effects of doxorubicin treatment. A dysregulation of cytokines (i.e. increased IL-6, TNF-α and IL-1β) and adipokines (increased leptin and decreased adiponectin) (Koti *et al*., 2015; Mahon *et al*., 2015; Nieman *et al*., 2013; Vyas *et al*., 2014) have also been shown to regulate the expression of transcription factors involved in lipid metabolism. For example, elevated leptin and TNF-α concentration has been shown to inhibit adipocyte lipogenesis and lipolysis, respectively (Coelho *et al*., 2013; Laurencikiene *et al*., 2007), however this was not under obesogenic conditions.

Evidence strongly supports the role of adipocytes in the tumour microenvironment to exacerbate local inflammation, which favours tumour cell survival, progression and treatment resistance. Obesity-induced adipokine secretions are detected in local adipose tissue and serum (Popko *et al*., 2010; Santander *et al*., 2015), whereas mRNA expression levelsshowed that adipocytes co-cultivated with breast cancer cells also displayed significantly higher IL-6, IL-1β and TNF-α concentrations (Dirat *et al*., 2011), promoting breast cancer progression. Increased leptin, IL-8, IL-6 and IL-1β concentrations were found in MDA-MB-231 and MDA-MB-468 TNBC cells after co-cultivation with adipocytes or adipocyte conditioned

medium (Nickel *et al*., 2017; He *et al*., 2018).Adipokines from obese adipocytes also favoured drug resistance in breast cancer cells (Bougaret *et al*., 2018; Incio *et al*., 2018).

Both adipocytes and breast cancer cells in the tumour microenvironment are known to produce increased MCP-1 levels, which act as a chemoattractant for macrophages to the tumour microenvironment. These resident macrophages, in turn induce the synthesis of various pro-inflammatory cytokines (i.e. leptin) and chemokines thereby exacerbating local inflammation as well as anti-inflammatory cytokines which induces tissue repair, all of which favour tumour progression (Dutta *et al*., 2018; Fujisaki *et al*., 2015). Santander *et al*. (2015) showed that co-cultivation of macrophages with E0771 TNBC cells and adipocytes resulted in increased leptin and MCP-1 concentrationswhereintegrated actions promoted macrophage recruitment and breast tumour promotion. High levels of MCP-1 and leptin is associated with obesity, advanced breast cancer disease stage and macrophage tumour infiltration (Dutta *et al*., 2018; Sultana *et al*., 2017). Macrophage chemoattractant protein-1 (MCP-1) may also be active in the host microenvironment promoting survival, metastasis, and unfavourable drug responses. Here, it is proposed that the recruitment of immune cells to the tumour microenvironment, promotes inflammation, stimulating the secretion of matrix metalloproteinase-9 (role in matrix degradation), evading the host's immune responses and induces angiogenesis (Koti *et al*., 2015), all of which favours treatment resistance(Nagarsheth *et al*., 2017).

Moreover, both Dox and Dox+CM treatment groups also showed statistically higher Σ MUFAs, VA and OA compared to control groups' treatment conditioned media (Figure 3.12&Figure 3.13). TheDox+CM group showed significantly higher Σ MUFAs, VA and OA compared to CM groups' treatment conditioned media (Figure 3.12& 3.13), suggesting that doxorubicin treatment itself increases MUFAs levels. In agreement, evidence on exogenous lipid utilization, where breast cancer cells induce adipocytes to release FFA, *via* activation of lipolysis (increased expression of ATGL and HSL), or inhibition of adipogenesis (PPAR-γ) (Balaban *et al*., 2017; Balaban *et al*., 2018) has been reported. Adipocyte derived FFA can be assimilated by breast cancer cells (Wang *et al*., 2017), which become available metabolic substrates for the benefit of breast cancer cell survival either by storage in the form of lipid droplets, or energy production *via* β-oxidation. Importantly it could also be incorporated into phospholipids and cholesterol esters in cell membranes (Shyu *et al*., 2018) to confer to a more

lipid saturated membrane. This metabolic behaviour protects breast cancer cells from the cytotoxic effects of chemotherapeutic agents, and thereby favours treatment resistance.

Furthermore, the decreased MUFAs profile observed in our *in vitro* model is similar to what was observed in our*in vivo* model. i.e. various MUFAs in the *in vivo* model were also decreased in the tumour FFA fraction, in Dox-H compared to Dox-L mice i.e. ∑ MUFA ($p=0.0080$), PA ($p<0.0001$), VA ($p<0.0001$), GA ($p=0.0034$) and NA ($p=0.022$) (Supplementary Table 2).Firstly, the decreased MUFAs profile may be the result of increased lipolysis (as a result of inflammation) of lipid droplets within breast cancer cells as evident by the increased expression of HSL in the breast cancer cells treated with doxorubicin i.e. control *vs* Dox and Dox *vs* Dox+CM, respectively (Figure 3.11D). Additionally, it could also be the result of breast cancer cells utilizing these MUFAs to achieve treatment resistance by increasing the release of MUFAs from the cell membrane. This is all plausible since doxorubicin treatment itself increases lipolytic activity in cancer cells.Thereby increasing the FFA "pool" in order to prevent fat storage (Ebadi *et al*., 2017; Mehdizadeh *et al*., 2017), which may be exacerbated by adipocyte conditioned medium, all of which may ultimately contribute to the development of breast cancer treatment resistance in our model.

We propose that the doxorubicin treatment resistance found in our *in vitro* model may be as a result of adipocyte derived inflammation (increased leptin and MCP-1) inducing lipolysis which in turn leads to increased FFA availability for breast cancer cells to utilize to sustain survival by evading apoptosis. This is supported by a very strong negative correlation found between HSL and cleaved-caspase-3 protein expression in the Dox+CM treatment group ($r=-1.00$, $p=0.0017$, Supplementary Figure 7).

Moreover, breast cancer cells also increase *de novo* FA synthesis, confirmed by an increased expression of ACC, FAS and SCD-1 enzymes in breast cancer cells (Huang *et al*., 2016, Veigel *et al*., 2015, Yoon *et al*., 2007). However, no significant difference wasobserved in both *de novo* fatty acid synthesis markers FAS and SCD-1, between any of the experimental treatment groups (Figure 3.11A &3.11B). This could explain the lack of significant differences observed in Σ SFAs, PA and SA between any of the experimental treatment groups (Figure 3.12& 3.13). We report contrasting findings to Wang *et al*. (2017), who showed significantly higher SFAs (PA and SA) in breast cancer cells co-cultured with adipocytes and no significant differences for MUFAs, and PUFAs. However, these authors used a co-cultivation model whereas we used a conditioned media approach. Additionally, our models

also different in breast cancer cell lines used.Our model also included the use of a chemotherapeutic agent, all of which could account for the contradictory results.

The only significant difference observed for the PUFAs class, was decreased LA levels in the Dox+CM compared to Dox treated cells alone (Figure 3.13E). Linoleic acid (LA) is an essential FA derived from the diet (Di Pasquale, 2009), and is subjected to desaturation (FA desaturases) as well as elongation (Elovl2 and Elovl5) to form other major PUFAs such as arachidonic acid, which is well-known for its pro-inflammatory and pro-carcinogenic effects in breast cancer (Di Pasquale, 2009). We speculate that the decrease in PUFAs is as a result of LA being used as a precursor to pro-inflammatory PUFAs including AA. The pro-inflammatory effects of n-6-PUFA is due to the diversity of functions associated lipid derived bioactive mediators i.e. eicosanoids, prostaglandins and leukotrienes (Baenke *et al*., 2013).Lipid derived bioactive mediators acts as second messengers in cellular signalling (Green *et al*., 2011), regulating angiogenesis, cell-proliferation, cell migration, metastasis and inflammation (Clària *et al*., 2010; Wang &DuBois, 2006), all of which may contribute to an ideal microenvironment favouring carcinogenesis and thereby promoting acquired cancer resistance to therapies. Taken together we propose that adipocyte secretory factors induce MDA-MB-231 TNBC cells to induce lipolysis as well as inflammation in the presence of doxorubicin treatment. This in turn can lead to increase available FFA substrates which can be utilized as an energy substrate to sustain survival by evading apoptosis, induced a hybrid MET phenotype and/or induce lipid saturation in order confer to acquired breast cancer treatment resistance.

3.4 SUMMARY OF *IN VITRO* MODEL FINDINGS

To summarize, we have successfully shown that adipocytes decreased doxorubicin's treatment efficacy on MDA-MB-231 TNBC cells in an *in vitro* paracrine model. Our findings indicate that doxorubicin induced apoptosis is attenuated by adipocyte conditioned media, which was associated with increased cell viability of MDA-MB-231 TNBC cells. We propose that adipocyte secretory factors induce TNBC cells to induce lipolysis and inflammation to counteract the cytotoxic effects of doxorubicin treatment (Figure 3.15).

We propose that adipocyte induce inflammation in the breast tumour microenvironment leads to the induction of lipolysis (increased HSL) in MDA-MB-231 TNBC cells *via* paracrine factors (increased leptin and MCP-1) (Figure 3.15). This results to altered metabolic behaviour i.e. increased FFA utilization, which further exacerbates inflammation (Figure

3.15). These FFA substrates can be utilized for energy purposes in sustain survival and additionally, may induce a hybrid MET phenotype in order confer to acquire treatment resistance by evading apoptosis. We further propose that the above mentioned events are exacerbated by doxorubicin treatment itself (Mentoor *et al*., 2018; Mentoor *et al*., 2019) resulting in breast cancer treatment resistance (Figure 3.15).

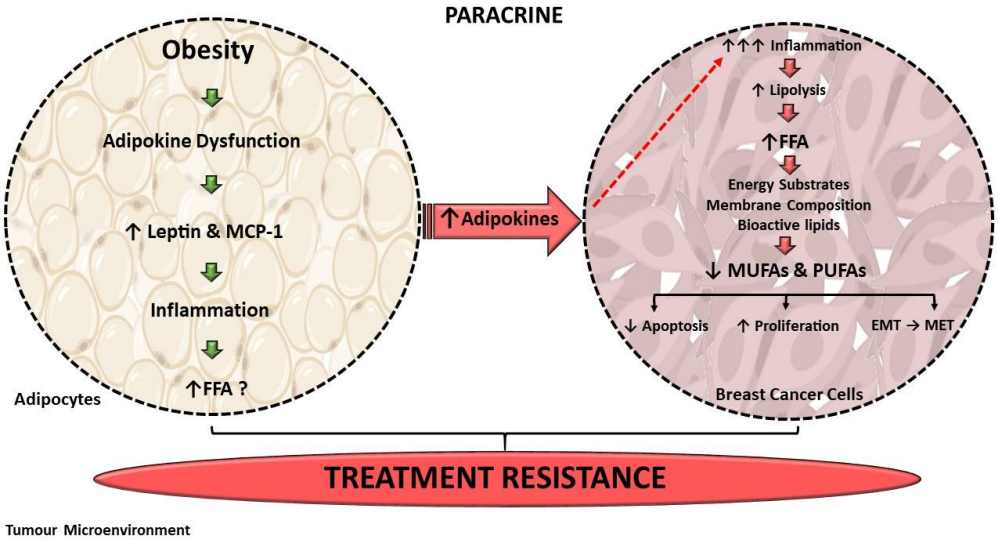

Figure 3.15: Summary of *in vitro* model findings. EMT, epithelial-to-mesenchymal transition (EMT); FFA, free fatty acids; MCP-1, macrophage chemoattractant protein-1; MET, mesenchymal-to-epithelial transition; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.

Lastly, we speculate that breast cancer treatment resistance is also the result of increased n-6 PUFAs utilization (precursors of bioactive lipids) in the tumour microenvironment. These bioactive lipids, exacerbates the inflammatory tumour microenvironment, which promotes the survival of breast cancer cells by inducing downstream changes in carcinogenic signalling pathways i.e. cell growth, migration and metastasis.Here, we report for the first time that lipolysis is increased in MDA-MB-231 TNBC cells treated with doxorubicin in a paracrine manner. It is suggested that this might be considered a novel mechanism in which breast cancer cells dysregulate lipid metabolism and thereby increasing the utilization of available FFA as well as exacerbating inflammation to evade apoptosis and achieve acquired treatment resistance (Figure 3.15).

CHAPTER 4: CONCLUSION

The complex pathophysiology underlying obesity and breast cancer includes obesity favouring breast tumour development, progression and metastasis, but importantly it decreases the efficacy of chemotherapeutic agents. Adipose tissue in the tumour microenvironment serves as an exogenous energy source for the survival of breast cancer cells. Breast cancer cells modulate lipid metabolism (*de novo* FA synthesis and lipolysis) by altering the secretion of adipokines through adipocytes, resulting in the release of FFA.

The specific role of obesity in breast cancer treatment resistance remains understudied. Additionally, there appears to be a general lack of evidence that support the role of adipose tissue/adipocytes,especially the role of FA composition in breast cancer treatment resistance.This therefore warrants investigations to elucidate, understand and identify the underlying physiological, and molecular mechanisms involved by which chemotherapeutic treatment resistance is achieved. This in turn may reveal novel targets for potential interventions to counteract breast cancer treatment resistance. We proposed that doxorubicin treatment on dysfunctional adipose tissue and/or adipocytes (in the tumour microenvironment), may exacerbate the negative effects of DIO *per se*, and further promote breast cancer treatment resistance.

In light of this, we employed aphysiologically obese breast tumor-bearing mouse model. We utilized a DIO approach in order to assess the role of obesity in treatment resistanceto determine the molecular mechanisms underlying the role of adipose tissue and fatty acids in treatment resistance within the tumour microenvironment.To validate the *in vivo* model findings, we developed an *in vitro* model to investigate the effect of adipocytes on breast cancer cells and its role in treatment resistance using a conditioned media approach to simulate the mammary tumour microenvironment.

We successfully showed that DIO significantly increased tumour growth and decreased the treatment efficacy of doxorubicin on triple negative E0771 breast cancer tumours, resulting in treatment resistance. Furthermore, our findings also showed that DIO selectively supressed lipogenesis and lipolysis in mammary adipose tissue, but increased *de novo* FA synthesis and lipolysis in breast tumour tissue. This in turn led to changes in the incorporation of dietary FAs into TAGs within mammary adipose tissue andbreast tumour phospholipids, whichsuggests that exogenous dietary lipids can alter the energy metabolism of E0771 TNBC

cells. These alterations in FAs composition in both mammary adipose and tumour tissue could result to more lipid saturated cell membranes (increased SFA), known to protect breast cancer cells from the cytotoxic effects of chemotherapeutic agents. Therefore, we propose that this could be a novel mechanism by which FAs composition can be altered in response to DIO within the tumour microenvironment and thereby contribute to the development of breast cancer treatment resistance within our current model (Figure 4.1).

We also speculate that increased breast cancer tumour growth is also the result of increased pro-inflammatory mediators i.e. n-6 PUFAs (precursors of bioactive lipids) and mammary adipose tissue derived inflammatory adipokines (paracrine manner) which exacerbates inflammation in the tumour microenvironment.All of these factors promote the survival of breast cancer cells, by preventing FAs storage in mammary adipose tissue (decreased SFAs and MUFAs), which in turn increases the FFA "pool". This in turn favours breast cancer cell survival in a paracrine manner. We propose that the above mentioned events are exacerbated by doxorubicin treatment and thereby ultimately results in breast cancer treatment resistance (Figure 4.1).

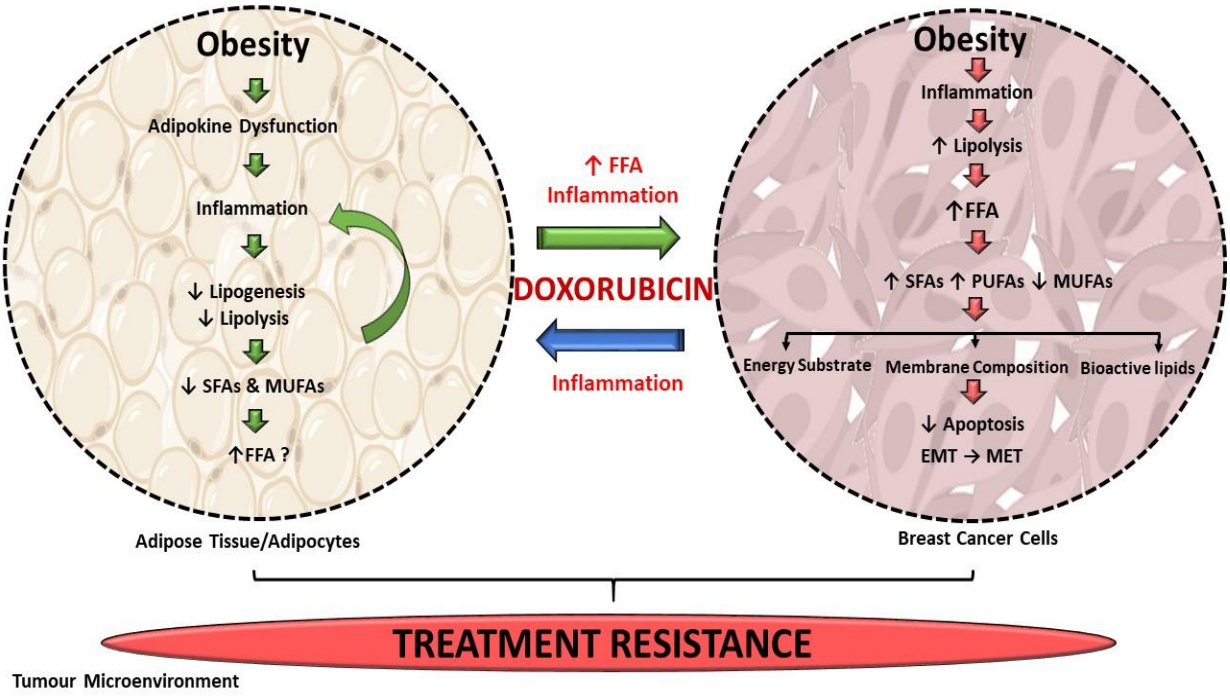

Figure 4.1: Summary of main findings. EMT, epithelial-to-mesenchymal transition (EMT); FFA, free fatty acids; MET, mesenchymal-to-epithelial transition; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids.

Our results provide a unique perspective at the distinct FA profiles within the tumour microenvironment and suggest that DIO can distinctly alter the FA composition of tissues within the TME and thereby achieve treatment resistance. Here, we report for the first time that lipid metabolism protein expression is suppressed in mammary adipose tissue and increased in tumour tissue in an obese xenograft breast cancer model treated with doxorubicin. We propose that this might be considered a mechanism through which breast cancer cells suppress the storage of FAs in mammary adipose tissue, to increase the availability of FFA as well as exacerbating inflammation under obesogenic conditions.

Furthermore, we have also successfully shown that adipocytes decreased doxorubicin's treatment efficacy on MDA-MB-231 TNBC cells in an *in vitro* paracrine model. Our findings indicate that doxorubicin induced apoptosis is attenuated by adipocyte conditioned media, which increased cell viability of doxorubicin treated TNBC cells, thereby promoting acquired breast cancer treatment resistance. We have demonstratedin both models that adipose tissue/adipocytes induce inflammation in the breast tumour microenvironment, which leads to the induction of lipolysis in TNBC cells. This resulted in altered metabolic behaviour i.e. increased FFA utilization, which further exacerbates inflammation. These FFA can be utilized as an energy substrate to sustain survival and additionally, may induce a hybrid MET phenotype and/or lipid saturation in order confer to acquired treatment resistance through the evasion of apoptosis.Here, we report for the first time that lipolysis is increased in TNBC cells treated with doxorubicin in both models. This might be considered a novel mechanism in which breast cancer cells dysregulate lipid metabolism and thereby increasing the utilization of available FFA as well as exacerbate inflammation to evade apoptosis to achieve acquired treatment resistance (Figure 4.1).

This study however had some limitations. Firstly, the fat percentage used in the HFD of the *in vivo model* (60 PE from fat) is higher compared to human consumption. However, a report suggests that although the total fat percentage is high, the proportion of specific FA classes i.e. SFAs, MUFAs, and PUFAs consumed in humans is similar to the HFD we used in our *in vivo* model (Ervin *et al*., 2004). However, metabolic differences between rodents and humans should be noted, therefore results and conclusion obtained in the *in vivo* model is specific to this model. Additionally, we did not quantify adipokine markers in both mammary and tumour tissue and did not assess any markers of apoptosis, cellular signalling and EMT in tumour tissue of the *in vivo* model. For future investigation and analysis, we propose to assess the above mentioned limitations as well as to include the assessment of additional FA transport

markers i.e. FABP-5 and CD36, including the fatty acid oxidation marker i.e. carnitine palmitoyltransferase I (CPT-1). We also propose to include adipocytes derived from normal weight donors and compare it to obese adipocytes for an *in vitro* model. We also propose to investigate modifications in the activation and expression of various drug transporter proteins i.e. P-glycoprotein, multi-drug resistance protein-1 as well as multi-drug resistance associated protein-1. Additionally, investigating alterations in key hepatic drug metabolizing enzymes i.e. CYP3A11, CYP2B10, CYP2A4 as well as aldo-keto reductase. Determining if adipocytes could also hinder drug induced apoptosis *via* other mechanisms of cell death for example autophagy by including various markers of autophagy i.e. LC3 and p62.

Regardless, our models had several advantages. Firstly, in both models we assessed full fatty acid profiles in both tumour and mammary adipose tissue, therefore a comprehensive FA assessment of the breast TME. Additionally, we also assessed several markers regulating lipid metabolism as well multiple inflammatory markers in both models. We also included a comprehensive assessment of apoptosis, proliferation, EMT and inflammatory markers in the *in vitro* model. All of which were assessed under obesogenic conditions in the presence of doxorubicin treatment in both models.

In conclusion, we demonstrated that adipose tissue/adipocytes significantly contribute to treatment resistance in triple negative E0771 and MDA-MB-231 breast cancer cells. This study also significantly contributed to the identification and understanding of molecular mechanisms underlying breast cancer treatment resistance in obese patients. Despite this, future investigations and research are still needed to elucidate and enable the development of novel therapeutic strategies to implement appropriate management protocols of doxorubicin related toxicities as well as to the improve prognosis and over-all survival of obese breast cancer patients. A potential intervention may be n-3 fatty acid supplementation, as n-3 PUFA have shown anti-cancer and anti-obesogenic effects in both humans and animal models (Kalupahana *et al*., 2011; Pirillo *et al*., 2013; Sulciner *et al*., 2018). Omega-3 FAs has also been shown to down-regulate *HER-2/neu* expression, induce apoptosis as well as inhibit FAS (Huang *et al*., 2016). Furthermore, DHA has also been shown to induce apoptosis by down regulating the expression of anti-apoptotic proteins (Blc) in MCF-7 human breast cancer cells (Chiu *et al*., 2004). Eicosapentaenoic acid (EPA), DHA, and their derivatives are also characterized by minimal inflammatory eicosanoid production and increased resolvins and protectins production, which act as mediators to resolve inflammation cascades (Moro *et al*.,

2016). Both EPA and DHA bind to G protein-coupled receptor-120 thereby inhibiting NFκB and c-Jun N-terminal kinase (JNK) pathways, which inhibits inflammation indirectly (Kalupahana *et al*., 2011). Omega-3 derived resolvins also suppresses tumor growth by suppressing inflammation caused by tumor cell debris and by suppressing AA derived eicosanoid synthesis (Moro *et al*., 2016; Sulciner *et al*., 2018).

To conclude, evidence on lipid metabolism especially FA profiles within the tumour microenvironment has not yet been explored in an obese breast cancer animal model, specifically illustrating its role in breast cancer treatment resistance. Here, we provide for the first time to our knowledge, evidence that DIO altered the FAprofiles of both tumour tissue and its adjacent surrounding mammary adipose tissue.

SUPPLEMENTARY

Supplementary Tables

Supplementary Table 1: Typical fatty acid composition of LFD (Research diets, D12450J) and HFD (Research Diets, D12492).

Fatty acid composition	**LFD** (D12450J)	**HFD** (D12492)
Saturated (g)	10,1	81,5
Monounsaturated (g)	12,8	91,5
Polyunsaturated (g)	20,2	81,5
Saturated (%)	23,5	32
Monounsaturated (%)	29,7	35,9
Polyunsaturated (%)	46,8	32
Fatty Acid (g)		
Acetic Acid	0	0
Butyric acid	0	0
Caproic acid	0	0
Caprylic acid	0	0
Capric acid	0	0,1
Lauric acid	0	0,2
Myristic acid	0,3	2,8
Pentadecylic acid	0	0,2
Palmitic acid	6,4	49,9
Palmitoleic acid	0,3	3,4
Margaric acid	0,1	0,9
Stearic acid	3,1	26,9
Oleic acid	12,3	86,6
Linoleic acid	17,8	73,1
Linolenic acid	2,1	5,2
Arachidic acid	0,1	0,4
	0,2	1,5
	0,2	2
Dihomo-γ-Linolenic acid	0	0,3
Arachidonic acid	0,1	0,7
Eicosapentaenoic acid	0	0
Behenic acid	0,1	0
Docosapentaenoic acid	0	0,2
Total	43,1	254,5

As per manufacturer product data sheet (Research diet Inc., New Jersey, USA)

Supplementary Table 2:FFA percentage composition of tumour tissue of mice from the different experimental groups.

Tumour FFA					Significance			
Fatty Acid	**Vehicle-L (%) (n=5)**	**Vehicle-H (%) (n=5)**	**Dox-L (%) (n=5)**	**Dox-H (%) (n=5)**	**Vehicle-L *vs* Vehicle-H**	**Vehicle-L *vs* Dox-L**	**Vehicle-H *vs* Dox-H**	**Dox-H *vs* Dox-L**
SFAs								
∑ Total SFA	38,88 ± 1,01	44,38 ± 1,36	42,02 ± 0,52	44,38 ± 1,09	**	*	NS	NS
14:0 (Myristic Acid, MA)	1,56 ± 0,083	1,13 ± 0,21	1,23 ± 0,078	1,31 ± 0,077	*	NS	NS	NS
16:0 (Palmitic Acid, PA)	21,77 ± 0,50	21,33 ± 0,70	21,7 ± 0,43	22,91 ± 0,49	NS	NS	*	NS
18:0 (Stearic Acid, SA)	15,17 ± 0,36	21,81 ± 1,01	19,09 ± 0,78	20,16 ± 0,91	****	**	NS	NS
MUFAs								
∑ MUFAs	39,09 ± 0,41	30,28 ± 0,80	34,07 ± 0,65	31,22 ± 0,74	****	****	NS	**
∑ n-7 MUFAs	8,18 ± 0,06	3,96 ± 0,23	6,59 ± 0,13	3,68 ± 0,07	****	****	NS	****
16:1n-7 (Palmitoleic Acid, PTA)	37,9 ± 0,012	1,43 ± 0,11	2,54 ± 0,14	1,45 ± 0,054	****	****	NS	****
18:1n-7 (Vaccenic Acid, VA)	4,39 ± 0,061	2,53 ± 0,14	4,05 ± 0,045	2,23 ± 0,044	****	**	*	****
∑ n-9 MUFAs	30,91 ± 0,36	26,32 ± 0,69	27,48 ± 0,56	27,54 ± 0,69	****	***	NS	NS
18:1n-9 (Oleic Acid, OA)	28,97 ± 0,34	24,54 ± 0,79	25,24 ± 0,58	26,07 ± 0,66	***	***	NS	NS
20:1n-9 (Gondoic Acid, GA)	1,07 ± 0,03	1,07 ± 0,04	1,11 ± 0,04	0,94 ± 0,03	NS	NS	*	**
24:1n-9 (Nervonic Acid, NA)	0,88 ± 0,025	0,89 ± 0,078	1,12 ± 0,076	0,88 ± 0,020	NS	**	NS	*
PUFAs								
∑ PUFAs	22,03 ± 0,64	25,34 ± 0,86	23,91 ± 0,45	24,41 ± 0,45	**	*	NS	NS
∑ n-3 PUFA	2,04 ± 0,18	2,38 ± 0,33	2,61 ± 0,21	1,82 ± 0,035	NS	NS	NS	*
22:6n-3 (Docosahexaenoic Acid, DHA)	1,92 ± 0,06	2,22 ± 0,18	2,35 ± 0,08	1,82 ± 0,04	NS	*	*	**
∑ n-6 PUFAs	19,99 ± 0,48	22,96 ± 0,56	21,3 ± 0,31	22,59 ± 0,43	***	NS	NS	NS
18:2n-6 (Linoleic Acid, LA)	10,59 ± 0,24	12,07 ± 0,48	9,69 ± 0,30	12,90 ± 0,24	**	*	NS	****
20:2n-6 (Eicosadienoic Acid, EDA)	0,71 ± 0,018	1,38 ± 0,055	0,85 ± 0,042	1,41 ± 0,041	****	*	NS	****
20:3n-6 (Dihomo-γ-Linolenic Acid, DGLA)	0,92 ± 0,04	0,92 ± 0,06	1,04 ± 0,04	0,88 ± 0,05	NS	NS	NS	0.062
20:4n-6 (Arachidonic Acid, AA)	6,30 ± 0,11	6,62 ± 0,42	7,62 ± 0,33	6,59 ± 0,02	NS	**	NS	*
22:4n-6 (Adrenic Acid, ADA)	1,61 ± 0,039	1,97 ± 0,13	2,11 ± 0,083	1,98 ± 0,073	***	***	NS	NS
RATIOS								
SCD-16 =16:1n-7/16:0	0,17 ± 0,0042	0,067 ± 0,0056	0,12 ± 0,0047	0,063 ± 0,0020	****	****	NS	****
SCD-18=18:1n-9/18:0	1,92 ± 0,067	1,14 ± 0,077	1,34 ± 0,082	1,31 ± 0,089	*****	****	NS	NS

Results are presented as mean ± SEM (n=5) where two-way ANOVA with Fishers LSD post hoc correction was employed, $p<0.05$ was considered as statistically significant. *= $p<0.05$, **= $p<0.01$, *** = $p<0.001$ and **** $p<0.0001$. Abbreviations: Dox-L, tumour doxorubicin-LFD; Dox-H, tumour doxorubicin-HFD; NS, Non-Significant; Vehicle-L, tumour vehicle-LFD; Vehicle-H, tumour vehicle-HFD; SCD, stearoyl-CoA desaturase (estimated desaturase activity using product-to-precursor FA ratios: SCD-16 = 16:1n-7:16:0 ratio; SCD-18 = 18:1n-9:18:0 ratio).

Supplementary Table 3: Human adipose tissue derived stem cells (hADSC) Donor information of *in vitro* model.

Donor 26508, #0000364977 Poietics, Lonza, Basel, Switzerland	
Age	40 years
Sex	Female
Race	Black
Height	1.52 m
Weight	63.50 kg
BMI	27 kg/m^2 (Overweight)
Diabetes	No
Smoking	No

Lonza

Certificate of Analysis

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Print Date: 13-Jun-2019

Product Name: Adipose-Derived Stem Cells (Amp)
Material Number: PT-5006
Batch No: 0000364977
Manufacturing Date: 25-Jun-2013

Test	*RESULT*	*SPECIFICATION MIN*	*MAX*	*UNIT*
Tissue Acquisition Number	26508			
DONOR CHARACTERISTICS				
Age	40 Y			
Sex	FEMALE			
Race	B			
BMI	27			
VIRUS TESTING				
HIV Test	Not Detected			
HBV Test	Not Detected			
HCV Test	Not Detected			
MICROBIAL TESTING				
Sterility Test		***	***	
Mycoplasma		***	***	
CELL PERFORMANCE TESTING				
Cell Passage Frozen	1			
Viability	85	>=70%	*****	%
Cell Count (Viable Cells/ml)	1750000	>=1,000,000	*****	
Seeding Efficiency	89	>=20%	*****	%
Doubling Time (hours)	29	15	70	hrs

Lonza Walkersville Inc.
8830 Biggs Ford Road
Walkersville, MD 21792 8415
Tel (301) 898 7025
Fax (301) 845 4024

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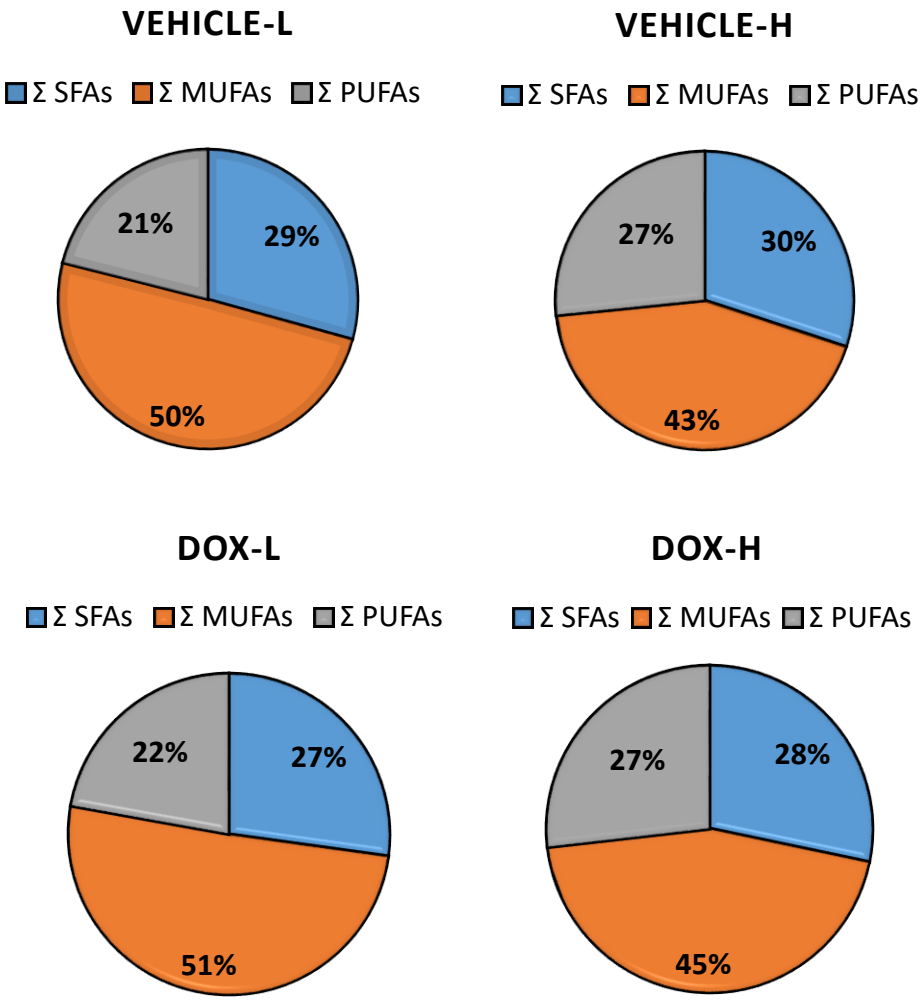
Print Date: 13-Jun-2019

Product Name: Adipose-Derived Stem Cells (Amp)
Material Number: PT-5006
Batch No: 0000364977
Manufacturing Date: 25-Jun-2013

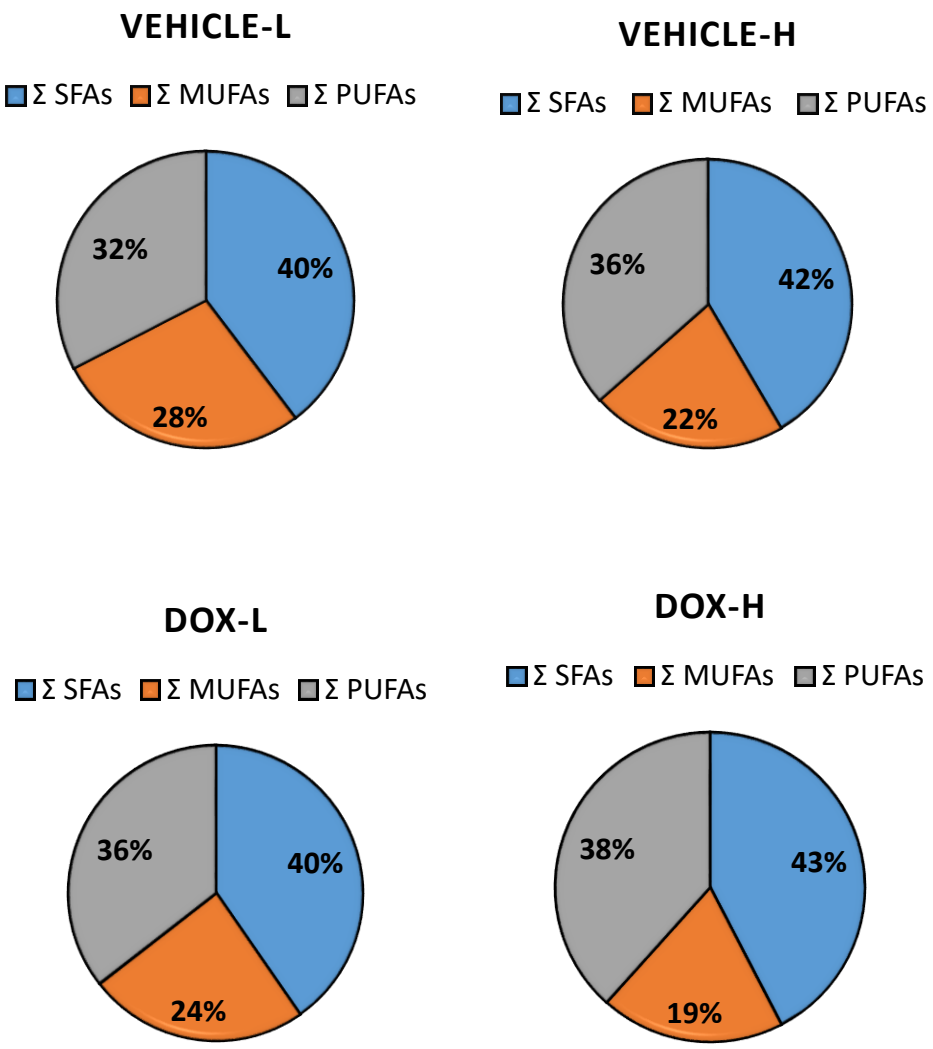
Test	*RESULT*	*SPECIFICATION MIN*	*MAX*	*UNIT*
CD13,CD29,CD44,CD73,CD90,CD105,CD166	Pass	>=90% Pos.	*****	
CD14, CD31, CD45		<=5% Pos.	*****	
CD34		% Pos. FIO	*****	

Additional Information:
These cells were isolated from donated human tissue after obtaining permission for their use in research applications by informed consent or legal authorization. This product is for research use only. Details concerning the use of our cell and media products can be downloaded from our website at www.lonza.com/cell-protocols.

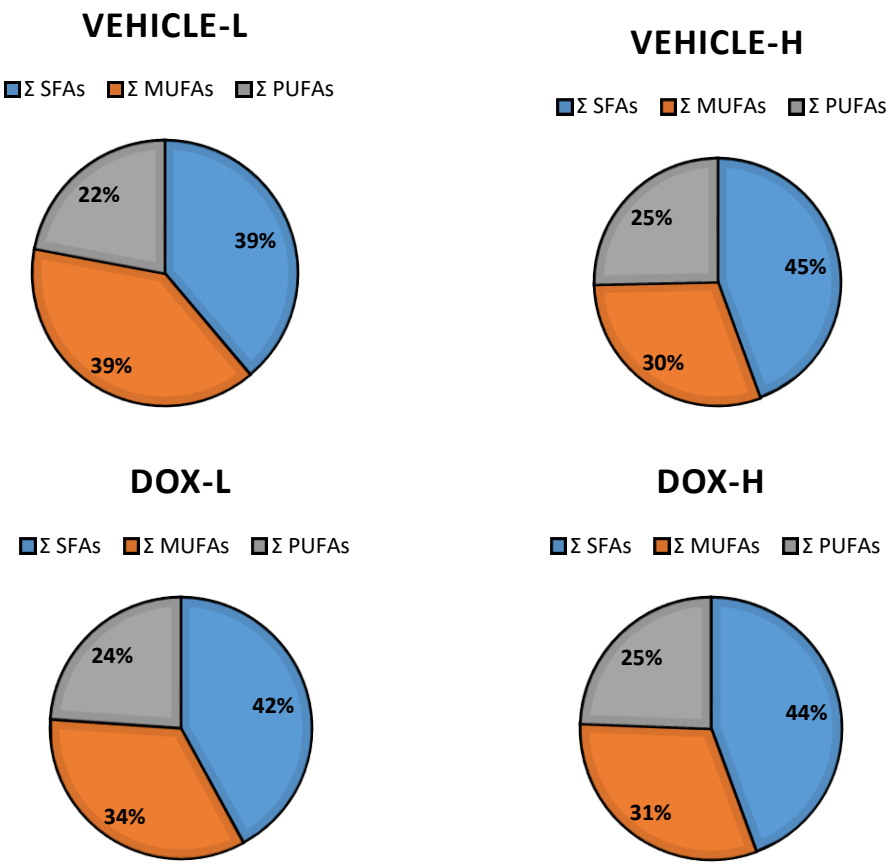
Supplementary Figures



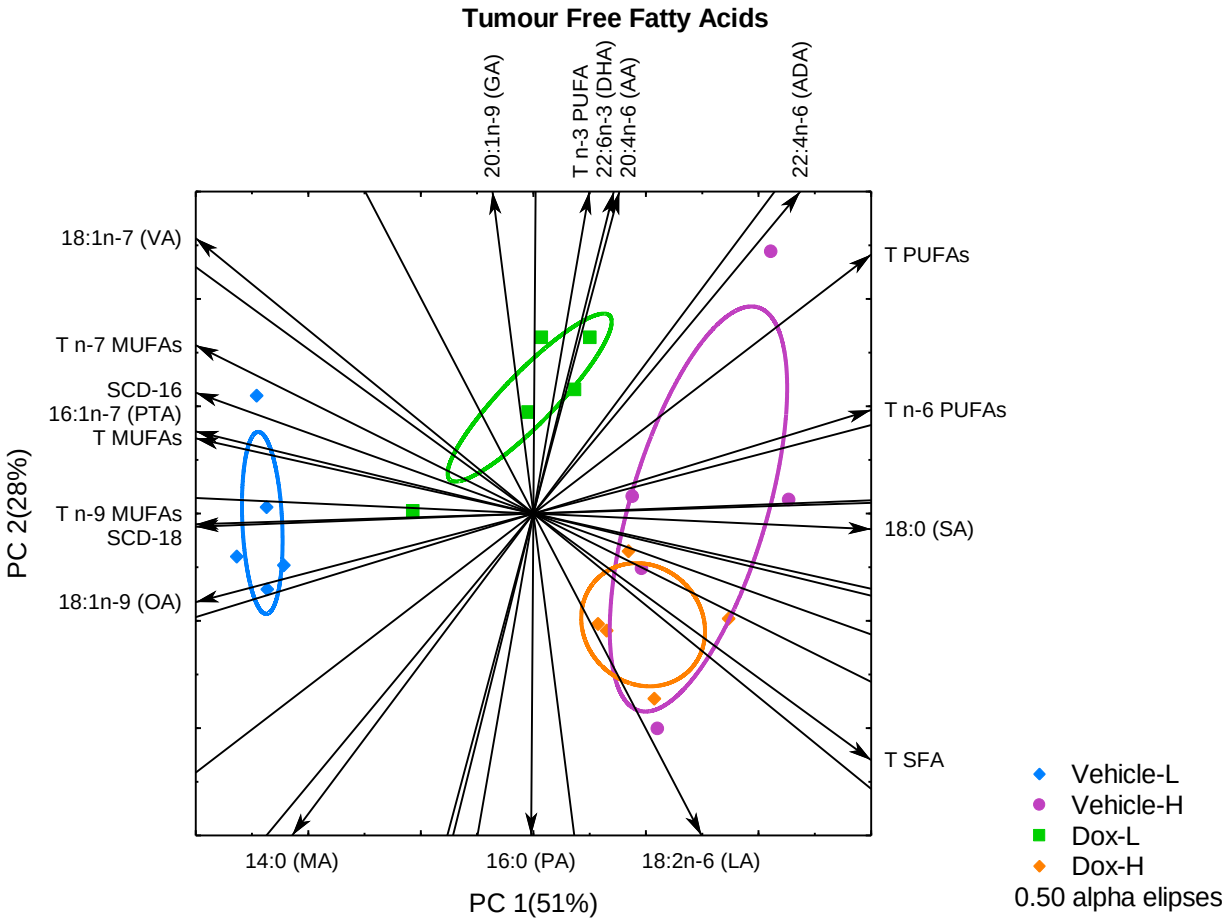
Supplementary Figure 1: Summary of total fatty acid class percentages in mammary adipose tissue total lipid profileper experimental group of *in vivo* model.



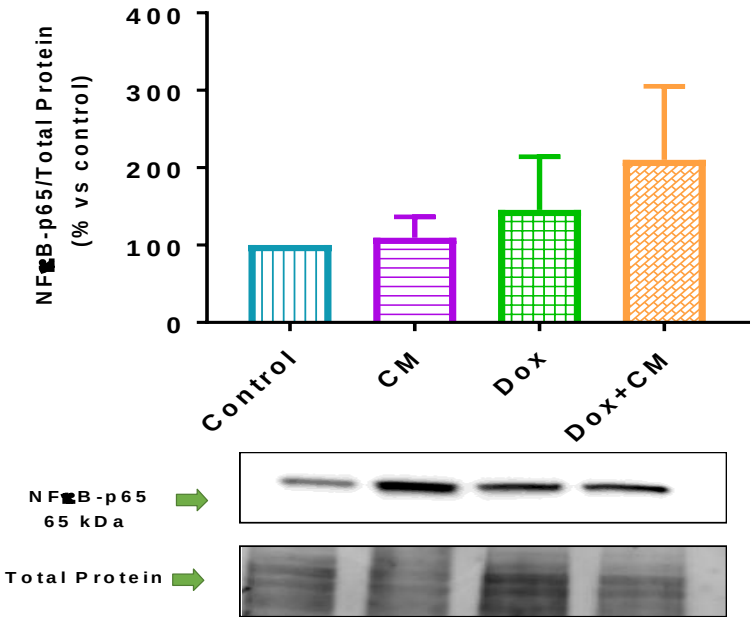
Supplementary Figure 2: Summary of total fatty acid class percentages in tumour tissue total phospholipids per experimental group of *in vivo* model.



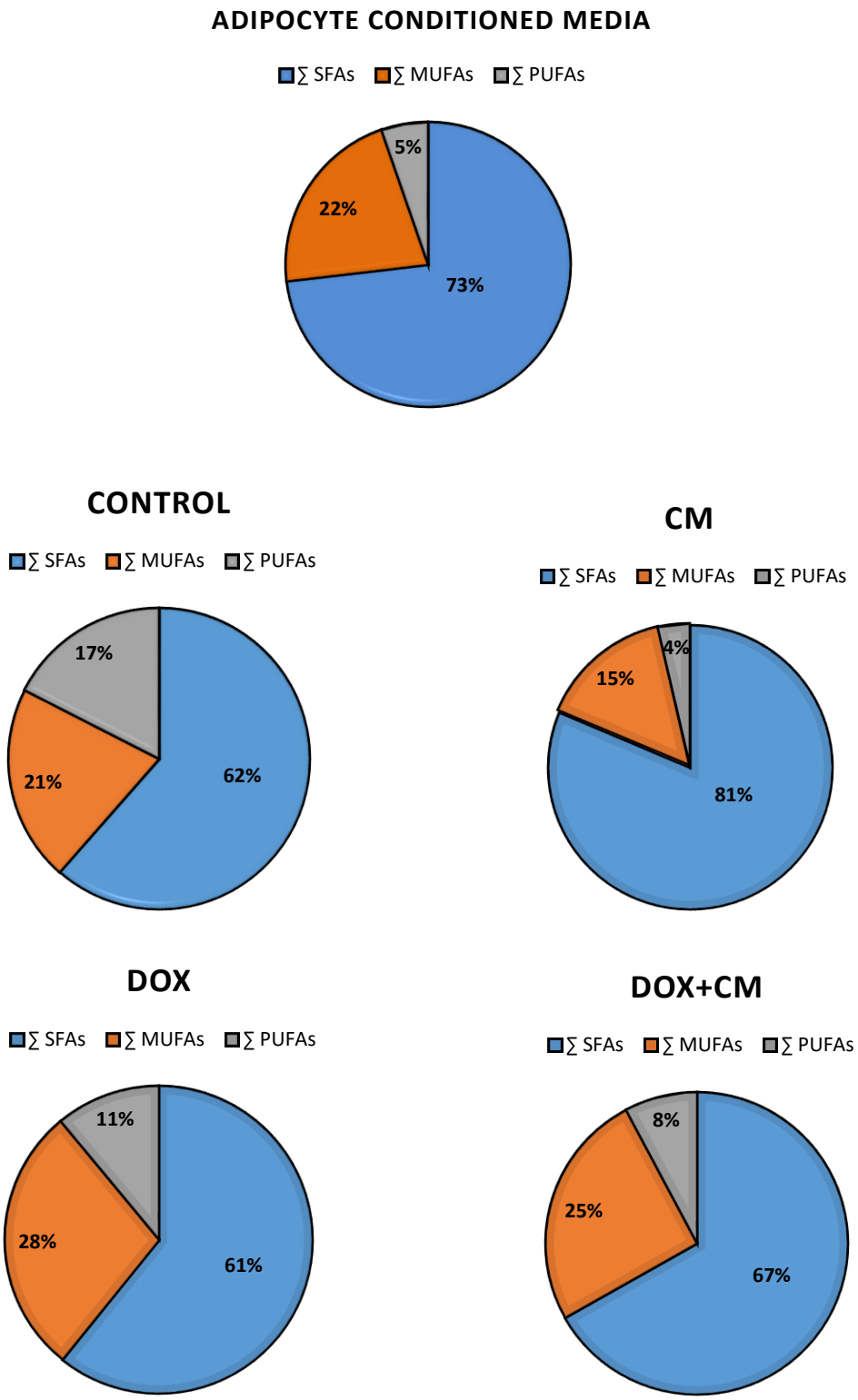
Supplementary Figure 3: Summary of tumour tissue FFA class percentage per experimental group of *in vivo* model.



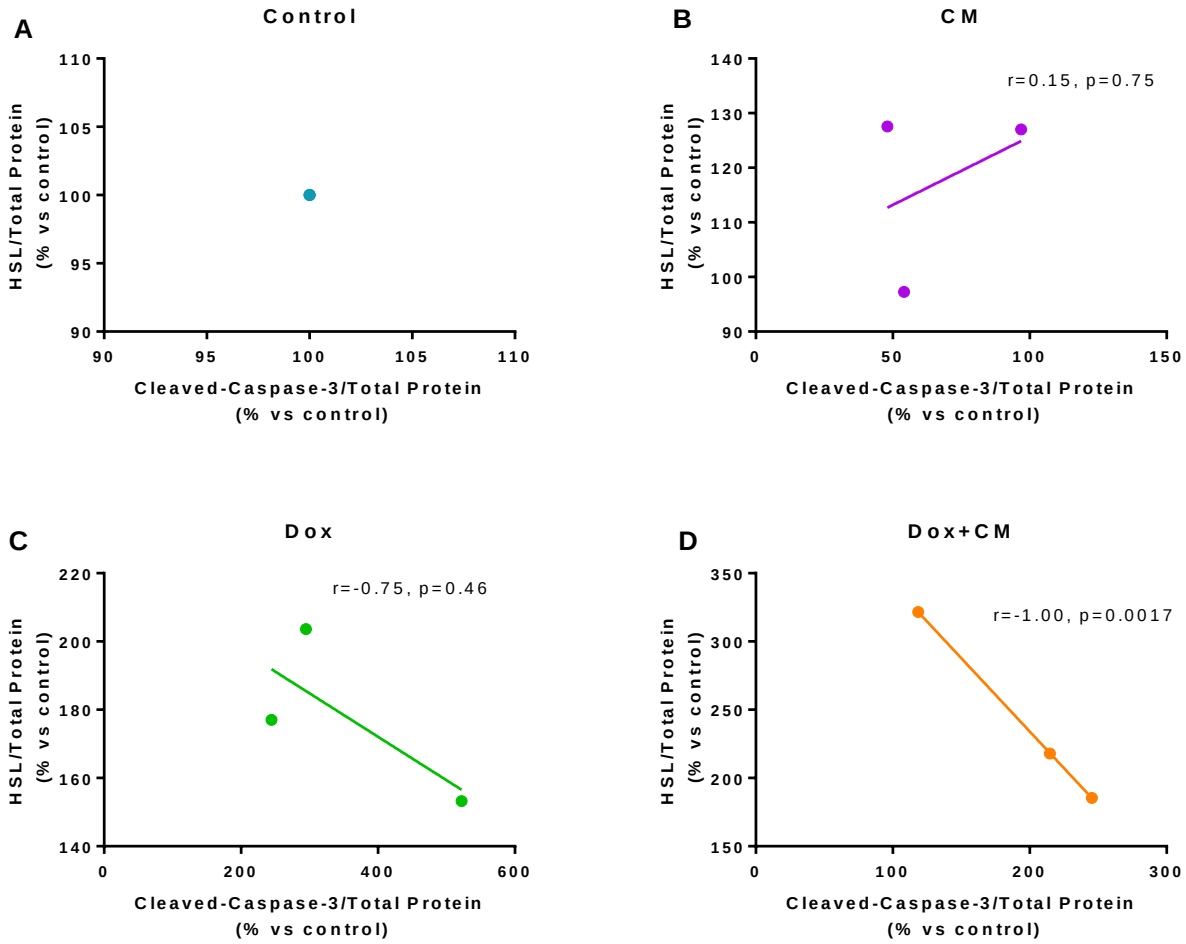
Supplementary Figure 4: Tumour FFA composition principle component analysis (PCA) bi-plot of *in vivo* model.



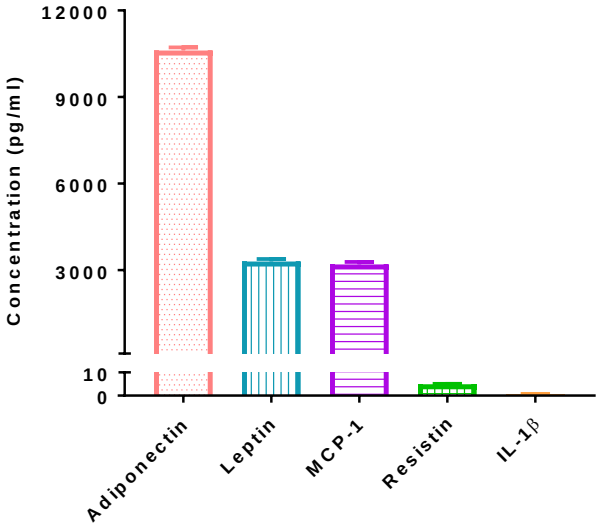
Supplementary Figure 5: The effect of adipocyte conditioned media on NFκB-p65 protein expression in MDA-MB-231 breast cancer cells treated with/without doxorubicin treatment for 48-hours. Results are presented as mean ± SEM (n=3). One-way ANOVA with Fishers LSD *post hoc* correction was employed. $p<0.05$ was considered as statistically significant. CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media



Supplementary Figure 6: Summary of total FA percentages in treatment conditioned media FFA fraction for all experimental groups. CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids.



Supplementary Figure 7: Correlations between HSLand cleaved-caspase-3 MDA-MB-231 TNBC cells protein expression. (A) control, (B) CM, (C) Dox and (D) Dox+CM treated breast cancer cells. CM, Conditioned media; Dox, Doxorubicin; Dox+CM, Doxorubicin + conditioned media.



Supplementary Figure 8: Adipokine concentrations of adipocyte conditioned media. Results are presented as mean ± SEM (n=4). Adipocyte CM, Adipocyte Conditioned Media.

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