Inhibition of Hypoxia and EGFR Sensitizes TNBC to Cisplatin and Suppresses Bulk and Cancer Stem Cells

Sarah McGarry

Thesis submitted to the University of Ottawa in partial Fulfillment of the requirement for the Master of Science in Biochemistry Degree

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine

University of Ottawa

ABSTRACT

Despite progress being made in our understanding of triple negative breast cancer (TNBC), the overall survival and disease-free survival for TNBC patients continues to be considerably poorer than their ER/PR/HER2+ counterparts. Metastasis and chemoresistance are the pivotal issues holding back the long-term success of TNBC treatments. In addition to the bulk tumor cells, cancer stem cells (CSCs) have emerged as important targets for alleviating TNBC progression and relapse.

Cisplatin, a platinum based chemotherapeutic agent, has shown promising potential for the treatment of TNBC in clinical trials; however, cisplatin treatment is associated with tumor hypoxia that in turn promotes CSC enrichment and drug resistance. My work is to develop a combinational treatment to improve the long-term therapeutic potential of cisplatin that not only targeted the bulk TNBC population but also $ALDH^{high}$ and $CD44^{+}/CD24^{-}$ CSC populations.

Through clinical dataset analysis, I found that patient TNBC tumors expressed high levels of epidermal growth factor receptor (EGFR) and hypoxia genes. A similar expression pattern was demonstrated in cisplatin-resistant ovarian cancer. I therefore developed a combinational therapeutic to co-inhibit EGFR and hypoxia using metformin (an AMPK activator) and gefitinib (an EGFR inhibitor), which sensitized bulk TNBC cells to cisplatin and also led to the effective inhibition of both $CD44^{+}/CD24^{-}$ and $ALDH^{high}$ CSCs. I obtained similar results by using clinically relevant TNBC patient samples *ex vivo*. Since these drugs are already frequently used in the clinic, this study illustrates a novel, clinically translatable therapeutic approach to improve the long-term therapeutic outcome of cisplatin for TNBC treatment.

ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. Lisheng Wang, for being such a fantastic source of guidance and knowledge. As a non-traditional student, Dr. Wangs remarkable trust and belief in my ability to successfully pursue my Masters in Biochemistry within his lab is something I will always be grateful for.

I would also like to thank my Thesis Advisory Committee members Dr. Marceline Côté and Dr. Tommy Alain, who provided priceless feedback and thoughtful suggestions for the design of my project.

Thank you to Dr. Sulaiman for his assistance in problem solving and advice on how to design a project. Thank you to the undergraduate thesis students from our lab, especially Jason, Emil, and Alex who were vital members of our lab and assisted me with experimental repeats. To all of my fellow lab members, thank you for your patience and guidance through these last two years of grad school and my three years prior as a volunteer.

Most importantly, thank you to my family and friends. Thank you to my parents for their unconditional love and support through this demanding time. Shoutout to my cat Ari for constantly working to distract me. To my fiancé Andrew, there are no words that can adequately express my gratitude for your incredible patience, love and support always.

DEDICATION

I am proud to dedicate this work in memory of my loving Grandma and best friend, Roberta Evelyn Mattocks, who bravely fought ovarian cancer for three years and passed away during my graduate studies. She was so incredibly proud and encouraging of my pursuit of cancer research, and I know she is looking down on me today with great pride.

I would also like to dedicate this work to my mom, Patricia McGarry, who was diagnosed with TNBC in 2011, underwent surgery followed by months of chemotherapy and radiation and has now been cancer free for 8 years. She is a true breast cancer warrior. Mom, I love you to the moon and back.

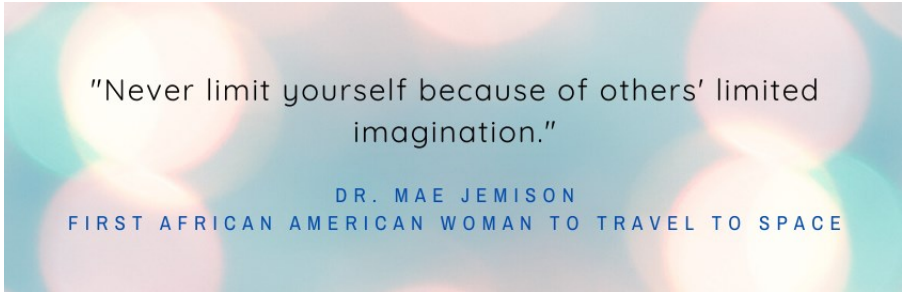


TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SUPPLEMENTARY FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

7-AAD	7-aminoactinomycin D
ALDH	aldehyde dehydrogenase
AMPK	5' adenosine monophosphate-activated protein kinase
ATP	adenosine triphosphate
CD24	cluster of differentiation 24
CD44	cluster of differentiation 44 (hyaluronic acid receptor)
CI	confidence interval
CMG	cisplatin, metformin and gefitinib combination
CM	cisplatin and metformin combination
CG	cisplatin and gefitinib combination
CSC	cancer stem cell
DEAB	N,N‐diethylaminobenzaldehyde
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
EGFR	epidermal growth factor receptor
EMT	epithelial‐to‐mesenchymal transition
ER	estrogen receptor
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FDA	Food and Drug Administration
FIH	factor inhibiting HIF
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

HDAC	histone deacetylase
HER2	human epidermal growth factor receptor type 2
HIF	hypoxia-inducible factor
HR	hazard ratio
HRE	hypoxia-response element
IL6	interleukin 6
LKB1	liver kinase B1
LDH1	L-lactate dehydrogenase
MG	metformin and gefitinib combination
MET	mesenchymal to epithelial transition
mTORC1	mammalian target of rapamycin complex 1
NCS	newborn calf serum
MCL1	induced myeloid leukemia cell differentiation protein
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
OD	optical density
ODD	oxygen-dependent degradation
OS	overall survival
PBS	phosphate-buffered saline
PDX	patient‐derived xenograft
PE	phycoerythrin
PDK1	pyruvate dehydrogenase kinase 1
PHD	prolyl hydroxylase
PR	progesterone receptor

RT-qPCR	reverse transcription polymerase chain reaction
SD	standard deviation
STAT 3	signal transducer and activator of transcription 3
TCGA	The Cancer Genome Atlas
TNBC	triple negative breast cancer

1. INTRODUCTION

1.1 Breast Cancer

Cancer has now surpassed heart disease as the number one cause of mortality in developed countries [1]. Despite continual research being performed to understand the mechanisms and develop therapeutics to treat cancer patients, this trend has not been halted. The most frequently diagnosed form of cancer amongst women is breast cancer, making up 1 in 4 cancer diagnosis worldwide [2]. In 2018, there were over 2,000,000 new cases diagnosed worldwide, with 22,261 of those cases in Canada [2]. Sadly over 600,000 women die from breast cancer every year making it the leading cause of cancer-related deaths in women throughout the world [2].

1.2 Breast Cancer Subtypes

Breast cancer has been divided into four main subtypes based on the presence or absence of specific tumor markers, with patient prognosis varying based on the subtype, and stage of the disease. Over 65% of breast cancer cases are estrogen receptor (ER+) positive enabling hormonal signaling. Luminal A/B breast cancer is identified by the presence of the estrogen receptor with +/- the progesterone receptor (PR). Over 65% of ER+ breast cancers are positive for the progesterone receptor (PR+) [3]. ER+ breast cancer are often treated with specific selective estrogen receptor modulators (SERMs) which block estrogen signalling, inhibit cell proliferation and promote senescence [3].

Human epidermal growth factor receptor type 2 (HER2+) breast cancer makes up 15-20% of breast cancer incidences and through the presence of HER2 demonstrates elevated EGFR signalling [4]. HER2 is a type 1 transmembrane growth factor receptor which can activate a variety of signal pathways including MAPK, STAT and P13K/AKT that are associated with disease

progression, proliferation, aggressiveness, and metastasis. HER2+ breast cancer is an aggressive subtype; however specific monoclonal antibodies such as herceptin and tratuzumab which target and inhibit HER2 protein and subsequent signalling has been proven to be an effective strategy [5-7].

Triple-negative breast cancer (TNBC) is characterized by negative for ER, PR and HER2 receptors [8]. As TNBC does not possess these commonly targeted receptors, treatment is limited to non-specific chemotherapy, surgery, and radiotherapy. TNBC is extremely aggressive. Despite only making up 15-20% of breast cancer diagnosed, TNBC disproportionately accounts for most breast cancer-related deaths due to lack of specific targets for effective treatment [8]. As such, the development of targeted therapies for TNBC remains an unmet medical need.

1.3 Prognosis

Prognosis for patients diagnosed with breast cancer vary widely depending on factors such as tumor grade, stage, and subtype. A retroactive clinicopathological by Dent *et al* studied a cohort of over 1,600 women with breast cancer at Women's College Hospital in Toronto, where 11% had TNBC. They found that compared to other women with breast cancer, those with TNBC had an increased likelihood of death within 5 years of diagnosis (hazard ration (HR) 3.2; $P<0.001$) [9, 10]. Relative to HR+/HER2− breast cancers, women with TNBC also suffered an increase in disease recurrence (HR 2.6; $P<0.0001$) [9].

Similar results were presented in a retroactive clinicopathological study by Lin *et al* where they reviewed over 15,000 breast cancer patients' medical records using the (American) National Comprehensive Cancer Network database [11]. They revealed that relative to HR+/HER2− tumors, TNBC was associated with a higher risk of brain or lung metastases [11]. Lin *et al*

determined that even after adjusting for age, stage, race, grade, and delivery of chemotherapy, ultimately women with TNBC and had worse overall survival, (adjusted HR 2.72, 95% CI 2.39–3.10, p<0.0001) [11]. Lin *et al* highlighted that the difference in risk of death between TNBC and HR+/HER2− women was most dramatic within the first two years after diagnosis (HR for OS for 0 to 2 years 6.10 [95% CI 4.81, 7.74]) [11].

1.4 Cancer Stem Cells

While chemotherapy and surgery are the current mainstays for the medical treatment of TNBC, chemotherapy is associated with drug resistance and enrichment of cancer stem cells (CSCs) over time which facilitate tumorigenesis and disease relapse [12]. CSCs make up the minority of cells in a tumor and are at the top of the cellular hierarchy, maintaining stem-cell like characteristics (self renewal and differentiation) to produce bulk tumor cells, expand tumor mass, and influence tumor progression [13-15]. CSCs play a critical role in drug resistance, cancer metastasis and disease relapse.

In the literature, there have been two main populations of breast cancer CSCs characterized based on the expression of $CD44^{+}/CD24^{-}$ and $ALDH^{high}$ markers [14, 16, 17]. A major barrier preventing long-term effective treatment of TNBC is the enrichment of these CSCs populations following chemotherapeutic exposure. As such new strategies to circumvent chemotherapy induced CSC enrichment is necessary for effective patient treatment.

1.4.1 $CD44^{+}/CD24^{-}$ CSCs

In a pioneering report in 2003, Al Hajj *at al* demonstrated that the fractionated $CD44^{+}/CD24^{-}$ subpopulation based on surface markers from breast cancer patients exhibited a

greater than 100 fold increase in tumorigenicity the ability to form new tumors compared to unsorted cells [13]. Using a serial dilution assay with various subpopulations on Al Hajj *et al* determined that $CD44^{+}/CD24^{-}$ subpopulation was able to reconstitute it tumor with as few as 100 fractionated cells compared to the 10,000 cells from the tumor control group [13]. Further studies have demonstrated that $CD44^{+}/CD24^{-}$ CSCs possess a mesenchymal phenotype, reside at the edge of the tumor and are highly metastatic and aggressive [14].

CD44 (cluster of differentiation 44) is a cell-adhesion receptor that is found ubiquitously throughout the body and has many processes in normal life (such as haematopoiesis) and in pathological situations (such as cancer and metastasis) [18, 19]. CD44 has been found to be highly expressed in many cancers including TNBC [19, 20]. It is a class 1 transmembrane glycoprotein which act as a hyaluronic acid receptor associated with controlling mesenchymal-like processes including cell motility, proliferation, invasion, and migration [19, 21]. In contrast, CD24+ (cluster of differentiation 24) is associated with a more epithelial-like phenotype involved in carbohydrate metabolism and RNA splicing [21, 22]. CD24 expression is inversely associated with CSC enrichment and is used in CSC analysis as a marker for differentiation.

1.4.1 $ALDH^{high}$ CSCs

Aldehyde dehydrogenase (ALDH) is an important enzyme in chemotherapeutic detoxification and has been used in the characterization of breast CSCs [23]. ALDH was originally used as an indicator of hematopoietic stem cells, but high expression of ALDH has been associated with CSCs in various cancers including breast cancer [24, 25]. Increased expression of ALDH in tumor tissue is strongly associated with significantly reduced patient prognosis, with the 5-year overall survival rate of $ALDH^{high}$ patients at just 19.8% compared to $ALDH^{low}$ patients at 58.7%

[23]. In breast cancer, $ALDH^{high}$ CSCs reside within the core of the tumor and are demonstrated to possess an epithelial-like phenotype [14].

In a 2008 landmark study, Ginester *et al* studied the tumorgenicity of ALDH-positive CSCs. They fractionated ALDH-positive and ALDH-negative breast cancer cells and performed serial dilution assay *in vivo* (injecting 50,000 cells; 25,000 cells; 5,000 cells; 500 cells into immunocompromised mice after which recording the frequency of novel tumor formation) [23]. Ginester *et al* observed that the unsorted cells (control) were not tumorigenic even at 50,000 cells [23]. The ALDH-positive cells saw a dramatic increased tumorgenicity, capable of reforming tumors when implanted with more than 1,500 cells [23].

Other breast CSCs have been identified (such as CD133, EpCAM, and CD90) however, $CD44^{+}/CD24^{-}$ and $ALDH^{high}$ are the most characterized and established breast CSC markers and as such will be the focus in this study [26-31].

1.5 Hypoxia and Cancer

Hypoxia (low concentrations of oxygen in cells or tissues) is one the most pervasive microenvironment stresses in solid tumors and has been fundamentally linked with the development and aggressiveness of a large variety of solid cancers, including TNBC [32]. In brief, the cells within the tumor centre undergo necrosis due to the poor blood supply and low oxygen, while the cells in-between oxygenation and necrosis areas are alive under hypoxic conditions. These hypoxic cells have been found to be more resistant to chemotherapy and radiotherapy. Hypoxia has been found to promote angiogenesis, glycolysis, influence CSC plasticity, epithelial CSC enrichment and tumorgenicity [33-37].

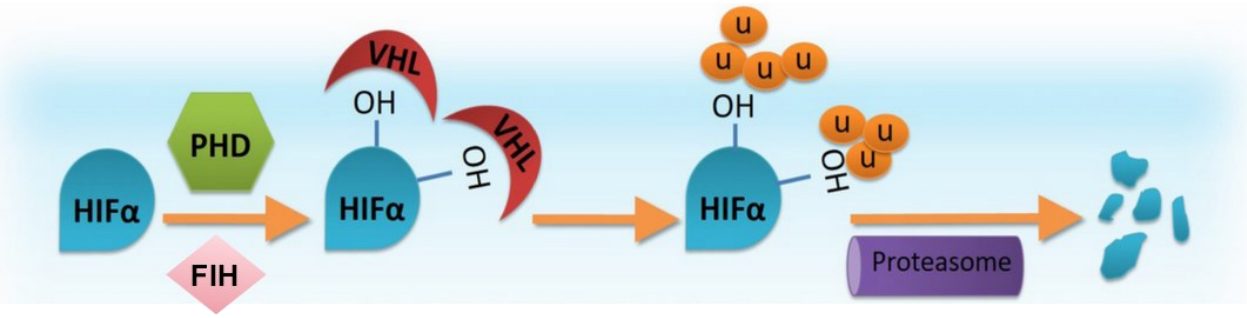
1.5.1 Hypoxia-Inducible Factor (HIF) Pathway

The hypoxia-inducible factor (HIF) pathway is an ubiquitous cellular mechanism which promotes transcriptional adaptation to hypoxic conditions [38]. In the presence of oxygen (normoxia), HIF-prolyl hydroxylases (PHDs) and the factor inhibiting HIF (FIH) bind to HIF-α and hydroxylate the proline residues leading to their destabilization by promoting ubiquitination by the von-Hippel Lindau (VHL) ubiquitin ligase (Figure 1A) [39]. This targets HIF-α for proteasomal degradation [38, 40]. Ultimately showing that in normoxic conditions, HIFs are continuously degraded and thereby unable to induce transcription of hypoxia related genes.

The HIF transcriptional complex is made up of highly conserved transcription factors which respond to hypoxic conditions. In hypoxia, the oxygen-dependent hydroxylation of HIF-α subunits by PHDs and FIH is reduced. This results in an accumulation of HIF-α that dimerizes with HIF-β and trans-locates to the nucleus to activate the transcription of genes containing hypoxia related elements (HREs) including angiogenesis, altered metabolism (decreased reliance on oxidative phosphorylation for energy production and increased reliance on glycolysis), and inflammation (such as *PDK1*, *TNF* etc.) (Figure 1B) [41].

A

Normoxia



B

Hypoxia

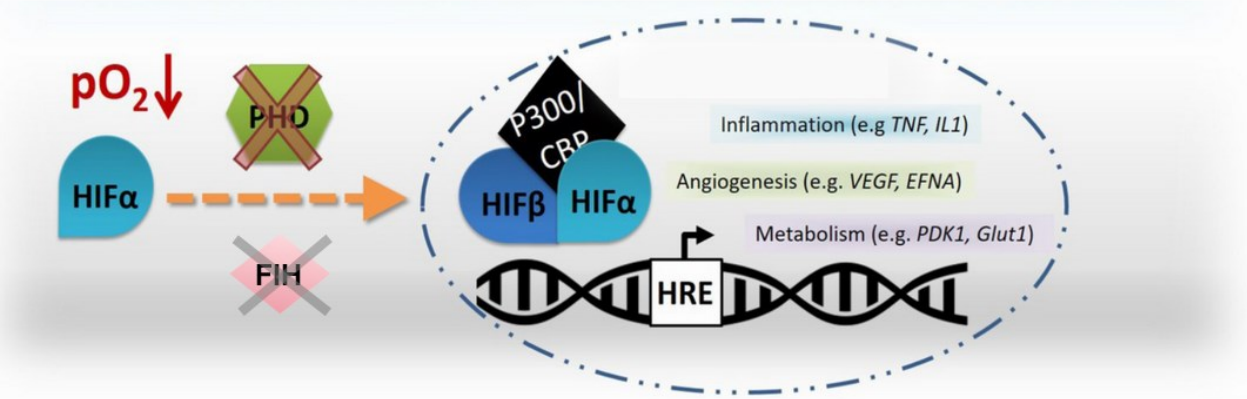


Figure 1: Hypoxia-Inducible Factor (HIF) Pathway. The HIF-pathway is oxygen dependent. **(A)** In the presence of oxygen (normoxia), HIF-prolyl hydroxylases (PHDs) and the factor inhibiting HIF (FIH) bind to HIF-α and hydroxylate the proline residues leading to their destabilization by promoting ubiquitination by the von-Hippel Lindau (VHL) ubiquitin ligase. This targets HIF-α for proteasomal degradation and thereby unable to induce transcription of hypoxia related genes. **(B)** In hypoxia, the oxygen-dependent hydroxylation of HIF-α subunits by PHDs and FIH is reduced, resulting in an accumulation of HIF-α which dimerizes with HIF-β and translocate to the nucleus where it activates the transcription of genes containing hypoxia related elements (HREs) including angiogenesis, altered metabolism, and inflammation. [Modified from Sormendi and Wielockx. 2016 Transl Cancer Res *5:*S1503-S1508 (Suppl 7) [41]]

1.5.2 Hypoxia and CSCs

HIF-1α is a transcriptional factor which is predominantly detected in hypoxic tissues and significantly elevated HIF-1α activity has been found in the $ALDH^{high}$ CSC population in breast cancer [42, 43]. HIF-1α may partially account for TNBCs metastatic, invasive and chemoresistant properties as well as the Warburg effect; a metabolic change found in solid tumors where metabolism switches to a pro-glycolytic state for energy production making conventional inhibitors which target oxidative phosphorylation or the mitochondria, less effective [44]. Unfortunately, chemotherapeutic agents used to treat TNBC have been demonstrated to stimulate HIF-1α, making targeting the hypoxia pathway of particular interest in the hopes to use a combinational therapy to supress the epithelial $ALDH^{high}$ CSCs to improve treatment efficacy of TNBC [33, 45-47].

1.6 Pharmaceutical Inhibitors

1.6.1 Cisplatin

Many frontline chemotherapy treatments are effective on the bulk tumor population in TNBC but are often ineffective on the CSC subpopulations. Cisplatin is a platinum 2 complex capable of creating adducts, causing DNA damage, and subsequently inducing apoptosis in a multitude of cancers (Figure 2) [48-50]. Recent clinical trials have demonstrated increased efficacy of cisplatin combinational chemotherapy in comparison with conventional chemotherapeutic approaches for the treatment of TNBC [51-53]. Ferreira *et al* demonstrated in their Phase II clinical trial that cisplatin in association with doxorubicin and cyclophosphamide was associated with a pathological complete response rate of 19.5% in patients with principally stage III tumors; however they found the tolerability of their treatment profile was a limitation [53]. While cisplatin

is effective at targeting the bulk tumor population, it has been demonstrated to augment hypoxia which in turn promotes chemoresistance and the enrichment of cancer stem cells (CSCs) through the mechanism that remains convoluted [54, 55].

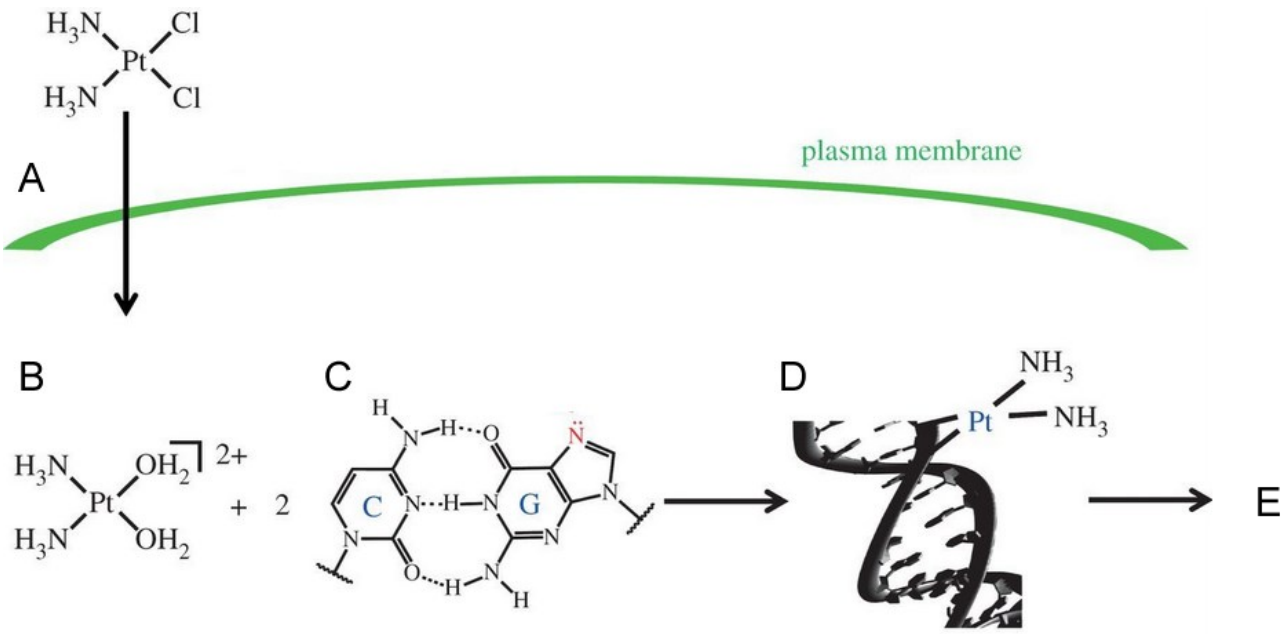

A
plasma membrane
B
C
D
E
2+
+ 2
C
G
Pt
NH3
NH3

Figure 2: Mechanism of Action of Cisplatin. (A) Cisplatin passive uptake across the cell membrane. **(B)** Cisplatin activation via hydrolysis. **(C)** DNA interaction/binding at C-G base pairs. **(D)** Intrastrand DNA adduct formation. **(E)** Transcription inhibition resulting in apoptosis/cell death. [Modified from Johnstone et al. 2015 Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences 373:2037 [49, 50]].

1.6.2 AMPK Signalling and Metformin

The AMP-activated protein kinase (AMPK) signalling pathway is a central regulator of cellular metabolism and plays a critical role in regulating growth, metabolism, autophagy and cell polarity. Thus, the AMPK pathway is currently an area of active research in the field of oncology [56, 57]. AMPK is activated when AMP and ADP levels within the cell rise in response to physiological stresses (Figure 3). Activated AMPK negatively regulates ATP consuming processes and stimulates ATP producing processes. Metformin, a front-line medication for the treatment of type 2 diabetes, has been shown to directly activate AMPK through phosphorylation at Thr-172 [58, 59]. Upstream of AMPK, liver kinase B1 (LKB1) phosphorylates and activates AMPK. AMPK carries out key tumor suppressing functions of LKB1 including inhibiting mTORC1 [56, 60]. Metformin has been shown to lower blood glucose levels in a LKB1 dependent fashion. Moreover, it has been shown that metformin even at low concentrations, phosphorylates LKB1 further stimulating its activity and subsequent AMPK activation [58, 59, 61]. As such, though the precise mechanisms in which metformin activates AMPK remains convoluted there has been substantial evidence indicating multiple pathways that may be involved.

Metformin was recently reported to enhance the anticancer effects of cisplatin by targeting RAD51, demonstrating decreased cell viability and metastatic effect than cisplatin alone [62]. Cisplatin and metformin were further found to exhibit a synergistic anticancer effect on an orthotopic murine model of 4T1 breast cancer *in vivo* [62].

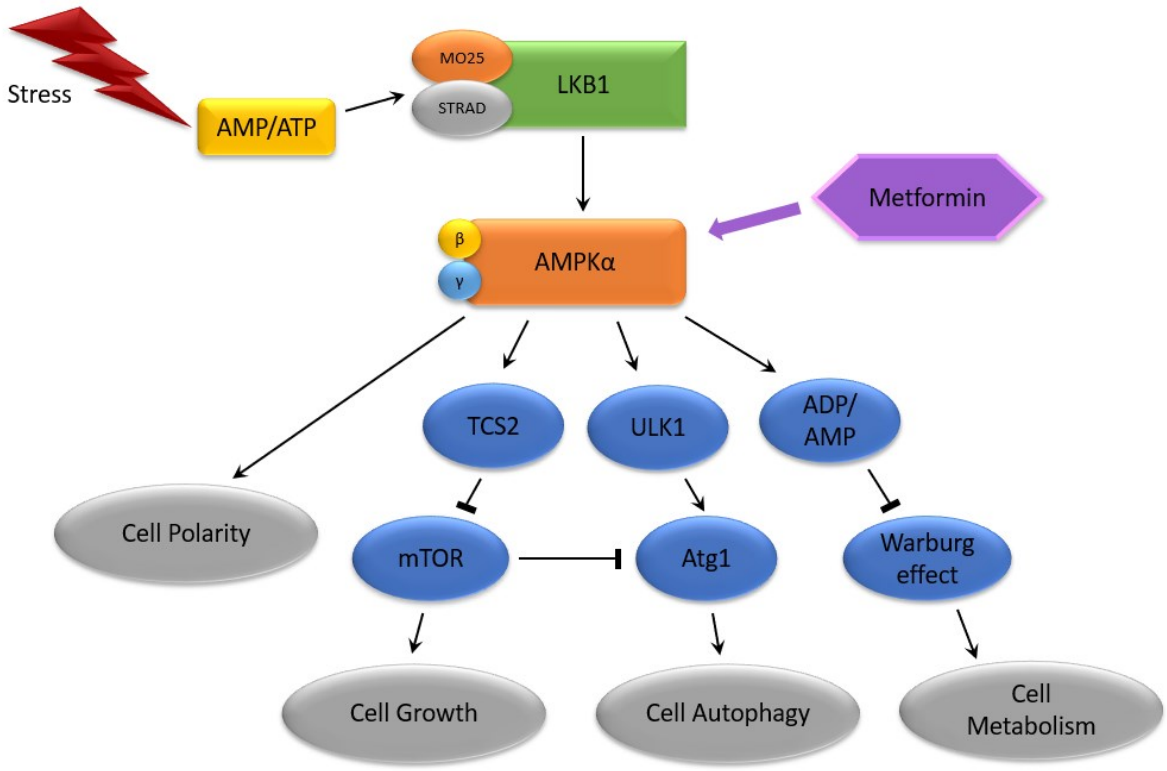

Stress
AMP/ATP
MO25
STRAD
LKB1
Metformin
β
γ
AMPKα
TCS2
ULK1
ADP/
AMP
Cell Polarity
mTOR
Atg1
Warburg
effect
Cell Growth
Cell Autophagy
Cell
Metabolism

Figure 3: AMPK Signalling Pathway. The AMP-activated protein kinase (AMPK) signalling pathway is a central regulator of cellular metabolism and plays a critical role in regulating growth, metabolism, autophagy and cell polarity. AMPK is activated when AMP and ADP levels in the cells rise in response to physiological stresses or the presence of pharmacological inducers. Liver kinase B1 (LKB1) is the upstream kinase that directly mediates AMPK in response to AMP or ADP increase. Activated AMPK directly phosphorylates several substrates to intensely affect metabolism and growth. [Modified from Li et al. 2015 Oncology Reports 34:6 [63]].

1.6.3 EGFR Signalling and Gefitinib

The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor which has been strongly implicated in many epithelial tumors and is frequently overexpressed in TNBC compared to other breast cancer subtypes [64, 65]. Activation of the epidermal growth factor receptor (EGFR) pathway (Figure 4) lead to the dimerization of the EGFR-receptor which causes the phosphorylation of specific tyrosine residues. This allows the EGFR receptor to act as a docking site for proteins with Src homology 2 and phosphotyrosine-binding residues (STAT3, Ras/Raf/MAPK, PI3K, etc.) [65-67]. These proteins get phosphorylated, activated and can mediate their secondary messengers. These pathways either act as direct transcriptional regulators or indirectly to influence cell proliferation, apoptosis, migration, survival and complex processes, including angiogenesis and tumorigenesis [65, 67].

Accordingly, targeting EGFR is now an active area of research. Gefitinib is a tyrosine kinase inhibitor (TKI) approved by FDA in 2003. Gefitinib inhibits EGFR by binding to the ATP-binding site of the EGFR enzyme, inhibiting ATP from being able to phosphorylate tyrosine thereby preventing EGFR activation (Figure 4) [66]. Gefitinib has also been found to elicit anti-hypoxic properties and has been demonstrated to overcome cisplatin-induced hypoxia resistance through the modulation of HIF1α in ovarian cancer cell lines and non-small cell lung cancer [68, 69]. This led to gefitinib entering clinical trials for TNBC; however, gefitinib alone is prone to resistance via stimulation of bypass signalling which in turn promotes Akt/mTORC1 and subsequent hypoxia, diminishing the efficacy of gefitinib for long-term treatment [64, 67, 70-72]. Gefitinib has shown significant synergism with metformin in lung and bladder cancers [73, 74]; however, the mechanisms behind is still under investigation,

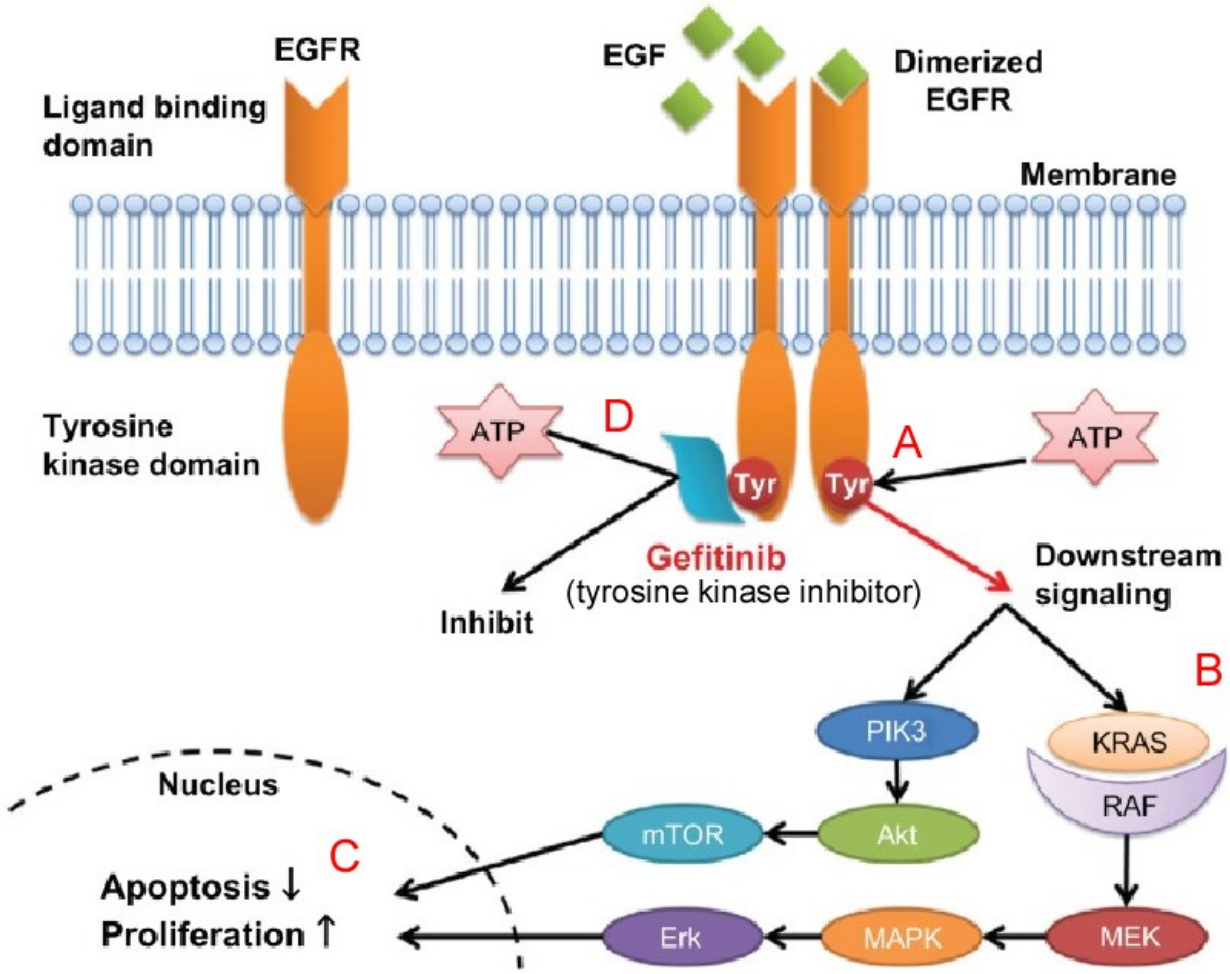

EGFR
EGF
Dimerized EGFR
Ligand binding domain
Membrane
Tyrosine kinase domain
ATP
D
A
ATP
Tyr
Tyr
Gefitinib
(tyrosine kinase inhibitor)
Inhibit
Downstream signaling
B
PIK3
KRAS
RAF
Nucleus
mTOR
Akt
C
Apoptosis ↓
Proliferation ↑
Erk
MAPK
MEK

Figure 4: EGFR Signaling Pathway and Gefitinib. (A) Activation of the epidermal growth factor receptor (EGFR) pathway leads to the dimerization of the EGFR-receptor which causes the phosphorylation of specific tyrosine residues. **(B)** The EGFR receptor acts as a docking site for proteins with Src homology 2 and phosphotyrosine-binding residues (Ras/Raf/MAPK, PI3K, etc.). These proteins get phosphorylated, activated and can mediate their secondary messengers **(C)** who either act as direct transcriptional regulators or indirectly to influence cell proliferation, apoptosis, migration, survival and complex processes, including angiogenesis and tumorigenesis. **(D)** Gefitinib (a tyrosine kinase inhibitor) binds to the ATP-binding site of the EGFR receptor, inhibiting ATP from binding and phosphorylating tyrosine, thereby preventing EGFR activation. [Modified from Araki et al. 2013 Clin Med Insights Oncol 7:85 407 - 421 [66]].

1.7 Research Rationale, Hypothesis and Objectives

1.7.1 Research Rationale

Ultimately the goal of my work was to develop a combinational treatment for TNBC that not only targeted the bulk tumor population but also both $ALDH^{high}$ and $CD44^{+}/CD24^{-}$ CSC populations. This would hopefully demonstrate an approach to improve the long-term therapeutic potential of cisplatin for TNBC treatment, lower the chance of relapse, chemoresistance and metastasis in TNBC patients.

1.7.2 Hypothesis

I hypothesized that the combination of EGFR inhibition and AMPK activation through gefitinib and metformin would sensitize TNBC cells to cisplatin treatment and reverse/reduce cisplatin-induced hypoxia and CSC enrichment.

1.7.3 Objectives

Aim 1: Identify potential therapeutic approach for the development of a combinational therapy by using database analysis of breast cancer patients.

Aim 2: Investigate the identified combinational therapy in conjunction with cisplatin and assess the effect of combinational (CMG) treatment on hypoxia-related gene expression.

Aim 3: Assess the combinational efficacy on cell viability/apoptosis of bulk and $CD44^{+}/CD24^{-}$ and $ALDH^{high}$ CSCs in TNBC MDA-MB 231 and SUM 149-PT cell lines.

Aim 4: Utilize a clinically translatable model (*ex vivo* organotypic cultures of TNBC patient tumor samples and patient-derived xenografts) to further evaluate the treatment effects of CMG on cell viability (Alamar Blue assays) and $CD44^{+}/CD24^{-}$ and $ALDH^{high}$ CSCs (flow cytometry).

2. MATERIALS AND METHODS

2.1 Cell culture and reagents

MDA-MB-231 breast cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM-F12 media supplemented with 10% Fetal Bovine Serum (FBS, HyClone, Logan, UT, USA), and 1% penicillin/streptomycin. SUM 149-PT breast cancer cells were purchased from Asterand (Detroit, MI, USA) and maintained in Hams F-12 medium supplemented with 5 $\mu g/mL^{-1}$ insulin, 10 mM HEPES, 1 $\mu G/mL^{-1}$ hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin/streptomycin, and 5% of Fetal Bovine Serum (FBS, HyClone, Logan, UT, USA). MCF-10A, immortalized human mammary cell line, was purchased from ATCC and maintained in DMEM-F12 media supplemented with 10% horse serum, 20 ng/mL^{-1} epithelial growth factor (RD Systems, Minneapolis, MN, USA), 0.5 $\mu g/mL^{-1}$ hydrocortisone, 10 $\mu g/mL^{-1}$ insulin, 100 ng/mL^{-1} Cholera Toxin (Sigma-Aldrich, St. Louis, MO, USA), and 1% penicillin/streptomycin. Cells were cultured at 37 °C in a 5% CO_2 incubator. Cisplatin was purchased from Caymen Chemical Company (Ann Arbor, Michigan, USA), gefitinib from LC labs- G-4408 (Woburn, Massachusetts, USA) and metformin from Caymen Chemical Company (Ann Arbor, Michigan, USA).

2.2 Ex vivo organotypic cultures of TNBC breast cancer tissue and patient-derived xenograft fragments

Tumor tissues from 2 TNBC patients undergoing routine surgical procedures were obtained. The protocol was approved by The Ottawa Hospital Research Ethics Board (Protocol# 20120559-01H). Tumor cores were sliced with a scalpel to obtain approximately 2 × 1 mm tumor slices. The slices were randomized, placed into a well of 24-well plate and cultured in DMEM-

F12 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1 μg/ml insulin, 0.5 ng/ml hydrocortisol and 3 ng/ml epidermal growth factor. These primary tissue fragments were treated with the same concentrations of inhibitors as described in the figures, followed by a viability assay and flow cytometric analysis as previously described [75, 76]. The TNBC patient-derived xenograft samples HCI-001, HCI-002, HCI-016 were obtained from University of Utah, cut similarly to the patient tumor samples, and cultured in the same conditions as the clinical samples. For flow cytometry analysis, patient tumor samples and PDX tumor cells were enzymatically digested into single-cell suspension using 1 x collagenase/hyaluronidase in DMEM (Stem Cell Technology).

Table 1: Brief Characterization of PDX Samples

Sample	Primary Diagnosis	Pre-collection treatment	Clinical Metastasis	Reference
HCI-001	IDC; Stage 4	Paclitaxel, zometa	Lung	DeRose 2011
HCI-002	IDC; Stage 3A	None	Lung	DeRose 2011
HCI-016	IDC; BRCA+	None	n/a	

2.3 Flow cytometry analysis

Cancer cells, patient tumor samples or PDX tumor cells were dissociated and then filtered through a 40-μm strainer and suspended in PBS supplemented with 2% FBS and 2 mM/L EDTA (FACS buffer) as previously described by our lab [76]. 1 μL of mouse IgG (1 mg/mL) was added and incubated at 4 °C for 10 minutes. The cells were then re-suspended in 1× binding buffer (eBioscience) and cell apoptosis was determined using Annexin-V-V450 Apoptosis Detection Kit (BD Bioscience).

Cells were then incubated with the ALDEFLUOR Assay Buffer at 4 °C for 30 minutes. Anti-CD44 (APC) and anti-CD24 (PE; BD Pharmingen) antibodies were added according to the manufacturer's instructions. ALDH activity was determined using ALDEFLUOR (Stem Cell

Technology) with a DEAB control according to the manufacturer's instructions. Cells were washed twice with 7-aminoactinomycin D (7-AAD), eBioscience) was added to exclude dead cells.

Flow cytometry was performed on a MD LSRFortessa. Gating for controls can be seen in Supplementary Figure 4 and Supplementary Figure 6. Data was analyzed with FlowJo Software (Ashland).

2.4 Quantitative real-time PCR

Total RNAs were extracted using RNeasy kit (QIAGEN) and real-time qPCR (RT-qPCR) analysis was performed using Bio-Rad MyiQ (Bio-Rad, Hercules, CA, USA) as previously describe by our lab [75]. The conditions for RT-qPCR reactions were: one cycle at 95°C for 20 seconds followed by 45 cycles at 95°C for three seconds and annealing at 60°C for 30 seconds. Results were normalized to the housekeeping gene 18S ribosomal RNA (18S) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative expression level of genes from different groups were calculated with the 2ΔΔCT method and compared with the expression level of appropriate control cells. Specific primer sequences for individual genes are listed in Table 1. All primers were obtained from Eurofin genetics.

Table 2: List of primer sequences.

Genes	Forward	Reverse
18S	AACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
GAPDH	ACAGTCAGCCGCATCTTCTT	GACAAGCTTCCCGTTCTCAG
PDK1	CAACAGAGGTGTTTACCCCC	ATTTTCCTCAAAGGAACGCC
LDH1	GGCCTGTGCCATCAGTATCT	GGAGATCCATCATCTCTCCC

2.5 Luciferase Reporter

MDA-MB-231 TNBC cells were seeded into 24-well plates and transfected with 500 ng of a ODD-Luciferase-pcDNA3 (Addgene Plasmid # 18965, a gift from William Kaelin) [77], or HRE-luciferase (Addgene Plasmid # 26731, a gift from Navdeep Chandel) [78] constructs in conjunction with 500 ng *Renilla* pRL-SV40P (Addgene Plasmid #27163, a gift from Dr. Ron Prywes) [79] construct using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 18 hours, cells were treated with either DMSO (vehicle) cisplatin (5uM), metformin (25uM), gefitinib (5uM) and their combinations for 24 hours, after which cells were lysed and both Firefly and *Renilla* luciferase activity was quantified using a Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions.

2.6 Cell viability Assays

MDA MB-231 or SUM 149-PT cells were seeded into 24 well plates (5×10^4 cells/well). After 120 hours of treatment, viability analysis was performed by incubating 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 1 mg/ml) for 4 hours. Absorbance was read at 570 nm.

For patient tumor samples and PDX tumors, tumor fragments were incubated in 24-well plates (i.e., organotypic slice culture). After 144 hours of treatment, Alamar blue viability analysis was performed by incubation with 10% Alamar blue reagent (Thermo Fisher Scientific) for 4 hours. Florescence was measured at 560 nm excitation and 590 nm emission.

2.7 Clinical database analysis

Gene Expression Omnibus2R database [80, 81] was used to analyze various datasets. Dataset: GSE38959 was used to compare 30 TNBC patient samples to 13 normal mammary tissue samples [82]: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38959. Dataset: GSE15709 was used to compare cisplatin resistant and sensitive ovarian cancer cell lines [83]: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15709.

2.8 Western blot analysis

Cells were harvested, washed with PBS, and lysed with lysis buffer supplemented with protease inhibitors (Roche, Sainte-Agathe-Nord, QC, Canada). After the protein concentrations were determined using a Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA), samples were then normalized and denatured. The samples were then loaded into an 8% polyacrylamide gel and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by transference to a poly difluoride membrane. Proteins were identified by incubation with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence solution (Thermo Scientific, Waltham, MA, USA). Antibodies used in this study include the following: AMPKα (1 : 1,000, Cat: 2532; Cell Signalling Technologies), Phospho-AMPKα (Thr172) (1 : 1,000, Cat: 2535; Cell Signalling Technologies) and anti-a-tubulin monoclonal antibody (1 : 500, Cat: T9026; Sigma-Aldrich).

2.9. Statistical analysis

For all clinical database data, the log rank test was performed to determine whether observed differences between groups were statistically significant. Data are expressed as means +/-Standard Deviation (SD) or Standard Error (SE). Statistical significance was determined using ANOVA or Student's t test wherever appropriate. Results were considered significant when * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

3. RESULTS

3.1 TNBC patient tumor samples vs normal breast tissue gene expression

Through a database of 30 TNBC and 13 normal mammary tissue samples, I assessed gene overexpression in TNBC, attempting to identify some potential targets related to my hypothesis.

3.1.1 TNBC patient tumor samples overexpress hypoxia related genes in comparison to normal breast tissue

Using the NCBI Gene Expression Omnibus (GEO2R), I found that EGFR and hypoxia related genes were overexpressed in the majority of TNBC samples compared to normal mammary tissue samples (Figure 5). *AKT1* (protein kinase B) encodes the serine/threonine protein kinase which accelerates cell proliferation while also inhibiting cell motility (thereby supressing tumor metastasis) [84-87]. As expected, higher levels of EGFR and phosphorylated Akt were found in TNBC cells than in non-TNBC cells (Figure 5A) [88].

Downstream of EGFR, the STAT3 transcription factor (signal transducer and activator of transcription 3) drives cell proliferation and survival, and plays an important role as a transcription modulator for mitochondrial respiration and oxidative metabolism [89-91]. Using the GEO2R dataset, I found that the majority of TNBC samples have enriched *STAT3* signalling compared to normal mammary tissue (Figure 5A).

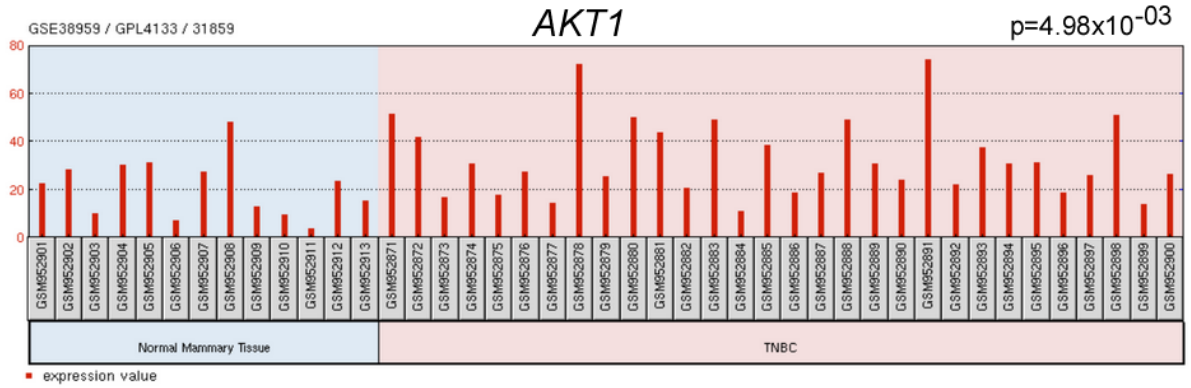
Hypoxic conditions activate the hypoxia signalling cascade which is primarily governed by hypoxia-inducible factor 1 alpha (*HIF1A*) [92]. HIF1A promotes primary tumor growth, maintains CSCs and regulates CD44, which is associated with cancer aggressiveness and metastasis [93-95]. I found that *HIF1A* expression was elevated in TNBC patient samples

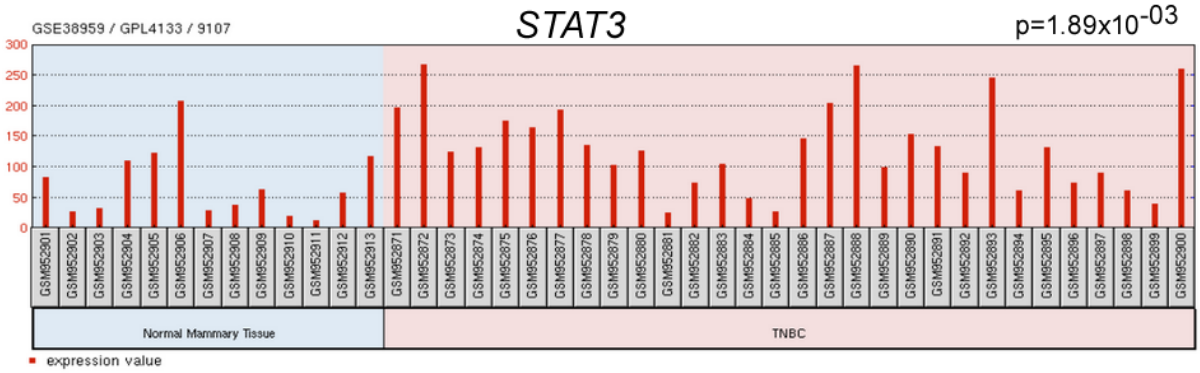
compared to normal mammary tissue (Figure 5B). This result was expected as HIF1A has been historically linked to local tumor hypoxia [96].

3.1.2 TNBC is associated with apoptosis resistance compared to normal breast tissue

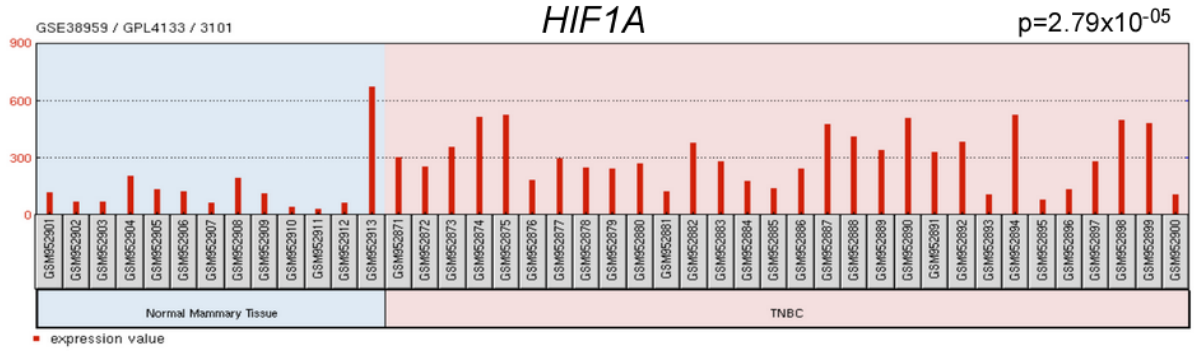
Apoptosis is an orchestrated cellular process which allows the body to balance cell division and cell death [35]. When defects occur along the pathways involved with apoptosis, cancer and other pathological conditions can form [35]. Abnormal increases in the levels of anti-apoptotic BCL-2 proteins (BCL-2, MCL-1 or BCL-XL) prevent apoptosis, thus promoting cancer and allows resistance to cancer therapy-induced cell killing [97, 98]. I found *MCL-1* expression to be elevated in TNBC patient samples compared to normal mammary tissue demonstrating that TNBC tumors are not undergoing apoptosis (Figure 5C).

A EGFR





B Hypoxia



C Apoptosis

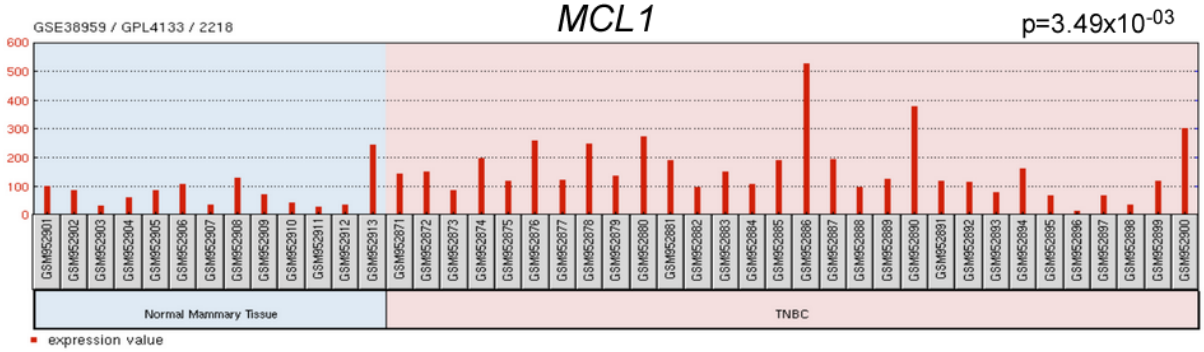


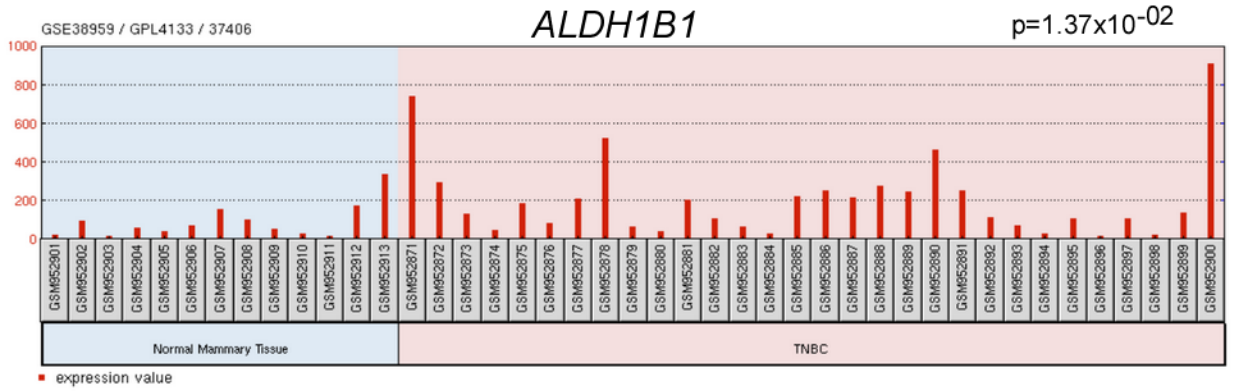
Figure 5: Upregulated EGFR and hypoxia signalling and apoptosis-resistance related gene expression in TNBC tumors in comparison to normal breast tissues. The relative expression levels (A.U arbitrary unit) of genes in 30 TNBC and 13 normal mammary tissue samples using the NCBI Gene Expression Omnibus (GEO2R). GSE38959 samples were assessed using the GPL4133 Agilent-014850 Whole Human Genome Microarray 4x44K and genes relating to **(A)** EGFR, **(B)** Hypoxia and **(C)** Apoptosis were assessed.

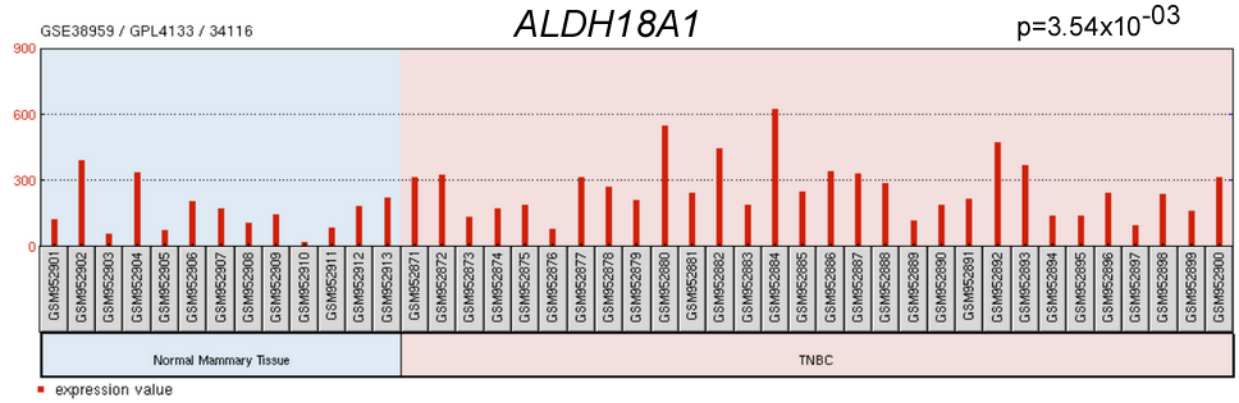
3.1.3 TNBC is associated with increased stemness and CSC related genes compared to normal breast tissue

To look at CSC gene expression in TNBC and normal mammary tissues, I analyzed ALDH and CD44. Aldehyde dehydrogenase (ALDH) is an important enzyme in chemotherapeutic detoxification and has been used in the characterization of breast CSCs [23-25]. *ALDH18A1* and *ALDH1B1* are two isomers of ALDH1 and both were upregulated in TNBC in the GEO2R dataset (Figure 6A-B). CD44 is a CSC marker and associated with mesenchymal-like processes including cellular adhesion, invasion, and migration [19]. As expected, I found that CD44 was highly expressed in TNBC (Figure 6B) [19].

I also observed that high expressions of EGFR and hypoxia genes were correlated with increased CSC-related genes (Figure 5 and Figure 6). As cisplatin is a first line inhibitor for ovarian cancer, results from a dataset of cisplatin-sensitive and cisplatin-resistant A2780 ovarian cancer cells were compared. Similar to the TNBC samples the cisplatin-resistant ovarian cancer samples were found to overexpress EGFR and hypoxia related genes, suggesting an association between EGFR/hypoxia and cisplatin resistance (Supplementary Figure 1). As such, I reasoned that the inhibition of EGFR and hypoxia may sensitize to cisplatin treatment.

A CSC Related Genes





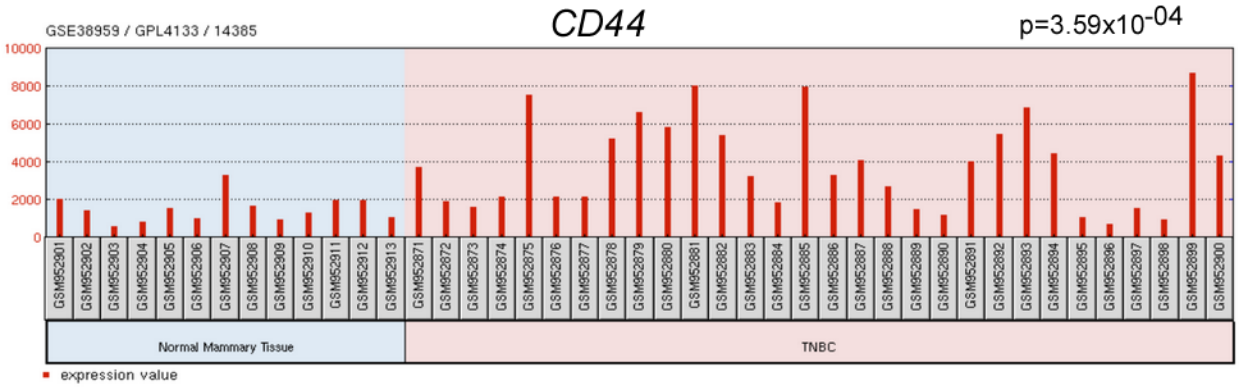


Figure 6: **Upregulated CSC gene signalling in TNBC.** The relative expression levels (A.U: arbitrary unit) of genes in 30 TNBC and 13 normal mammary tissue samples using the NCBI Gene Expression Omnibus (GEO2R). GSE38959 samples were analyzed using the GPL4133 Agilent-014850 Whole Human Genome Microarray 4×44K.

3.2 Combination of metformin and gefitinib effectively represses cisplatin-induced hypoxia in TNBC

Results from the analyses of clinical datasets (TNBC and ovarian cancer) suggest that gene expressions of hypoxia and stemness are upregulated, which is associated with cisplatin-resistance. The goal of this study was to develop a new therapy to be used in conjunction with cisplatin for effective treatment of TNBC bulk and CSC populations. Cisplatin has been used to treat a variety of cancers due to its ability to cause DNA damage and cell apoptosis. However, while cisplatin is effective on inhibiting the bulk tumor population, it has been shown to promote hypoxia, which may in turn promotes chemoresistance and spares growth of CSC [99].

Based on literature and the aforementioned results, I speculated that a combinational therapy of gefitinib (an EGFR inhibitor) and metformin (an AMPK activator) at clinically relevant concentrations [100-102]. I assessed how hypoxia-related gene expression was altered by cisplatin and the combinational therapy in MDA MB-231 cells using luciferase assays and qPCR analysis. MDA MB-231 cells were transfected with either ODD-luciferase or HRE-luciferase plasmids in concurrence with a Renilla SV40 construct using Lipofectamine 2000. After 18 hours, cells were treated with either DMSO (vehicle), cisplatin (5μM), metformin (25μM), gefitinib (5μM) or different combinations for 24 hours. After that, cells were lysed and both Firefly and Renilla luciferase activity was quantified using a Dual-Luciferase® Reporter Assay System.

As expected, I found that cisplatin upregulated hypoxia-related gene expression (Figure 7A-B). Hypoxia response element (*HRE*) expression increased with the luciferase assay by 1.5-fold after MDA MB-231 TNBC cells were treated with cisplatin (Figure 7A). Oxygen dependent deprivation (*ODD*) luciferase expression was also increased over 20% (Figure 7B).

For RT-qPCR analysis, I treated MDA MB-231 cells for 48 hours and then extracted the mRNA, converted mRNA to cDNA using RT transcriptase. After normalization and quantification of cDNA samples, I performed RT-qPCR analysis using primers for *PDK1* and *LDH1* to assess hypoxia in the treated and control groups and normalized the samples to the housekeeping gene 18S ribosomal RNA (18S) or GAPDH. *PDK1* expression increased nearly 2-fold (Figure 7C) and *LDH1* increased 1.5-fold (Figure 7D).

To inhibit cisplatin-upregulated hypoxia and CSCs, gefitinib was incorporated [102]. Gefitinib is an EGFR inhibitor which elicits anti-hypoxic properties and has been demonstrated to overcome cisplatin-induced hypoxia resistance through the modulation of HIF1α in ovarian cancer cell lines [68, 69], although little are known in TNBC. I found that gefitinib reduced cisplatin-upregulated hypoxia on TNBC MDA MB-231 cells (Figure 7B). Metformin was also incorporated into the combinational therapy as it has been shown to enhance the anticancer effects of cisplatin [62] and exhibit synergy with gefitinib in lung and bladder cancers [73, 74]. Our luciferase result showed that metformin also exhibited the anti-hypoxic effects in MDA MB-231 cells (Figure 7A-B).

While cisplatin increased the transcriptional activity of HIF-1 through HRE, the combination of metformin and gefitinib (MG) inhibition reduced HIF-1 activity. Moreover, the three drug combination (CMG) effectively inhibited HIF-1 activity (Figure 7A). Similarly, ODD expression was reduced 50% by the combination treatment CMG (Figure 7B). Similar findings were observed for the inhibition of *PDK1* and *LDH1* gene expression following CMG treatment (Figure 7C-D). Overall, I found that the combination of metformin and gefitinib was able to prevent cisplatin-upregulated hypoxia in TNBC cells, which has not yet been reported (Figure 7A-D).

Hypoxia Gene Expression

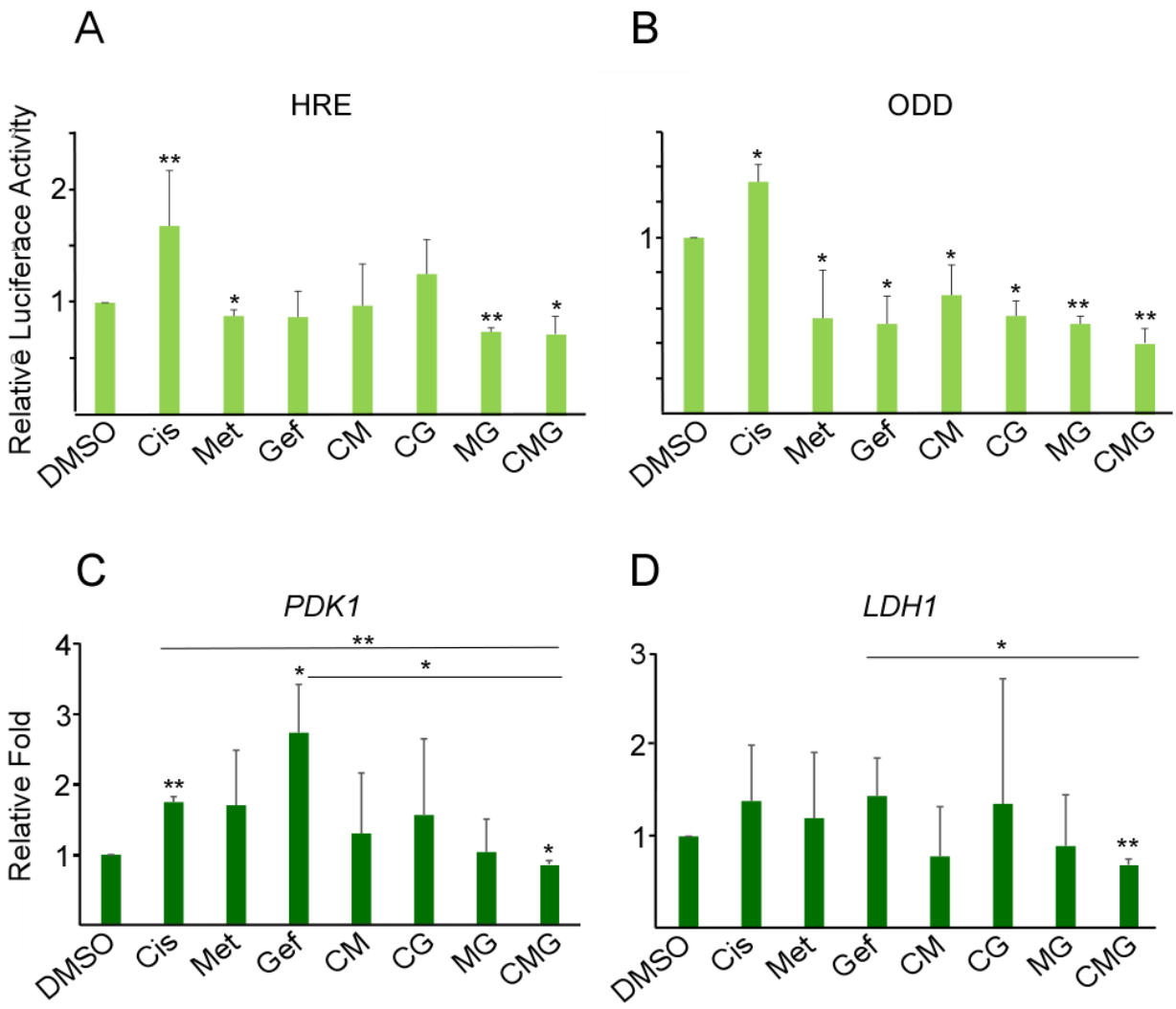
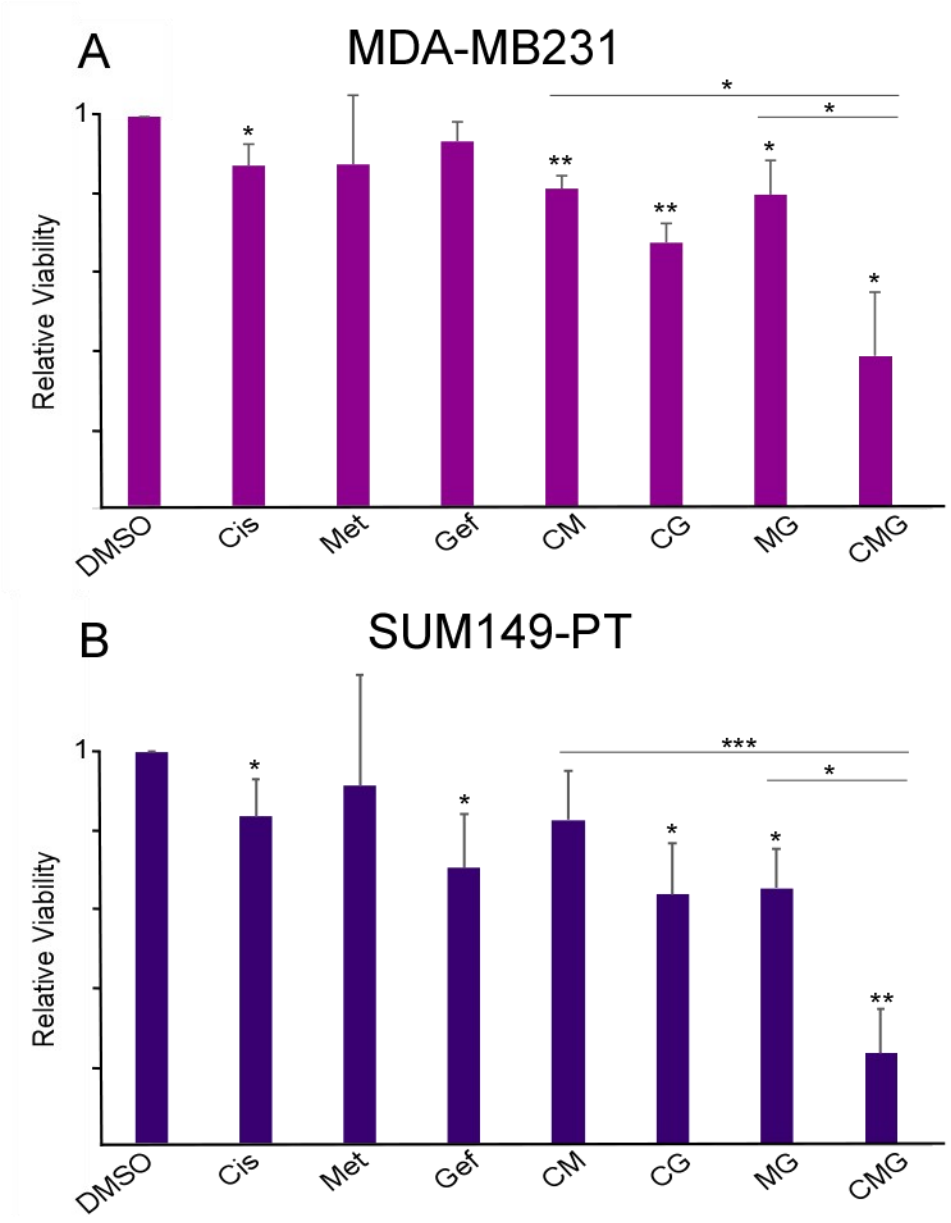


Figure 7. Combination of metformin and gefitinib effectively represses cisplatin-upregulated hypoxia in TNBC. (A) Relative Luciferase Reporter Expression of HRE in MDA MB-231 TNBC cells after 24 hours of treatment. Cells were transfected with HRE-Luciferase and normalized with the pRL-SV40P Renilla construct. Cells were exposed to the following concentrations: 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib, the control was treated with DMSO vehicle (n=3). **(B)** Relative Luciferase Reporter Expression of ODD in MDA MB-231 TNBC cells after 24 hours of treatment. Cells were transfected with ODD-Luciferase and normalized with the pRL-SV40P Renilla construct. Cells were exposed to the following concentrations: 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib, the control was treated with DMSO vehicle. **(C)** RT-qPCR analysis and comparison of relative mRNA levels of hypoxia gene PDK1 48 hours post treatment with DMSO vehicle control or the following drugs in different combinations: 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib (n=3). **(D)** RT-qPCR analysis and comparison of relative mRNA levels of hypoxia gene LDH1 48 hours post treatment with 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib, the control was treated with DMSO (n=3).

3.3 CMG Combination reduces viability of TNBC cell lines

Cell viability is frequently assessed using the MTT assay. In a MTT assay, the yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is metabolized by live cells, resulting in formation of intracellular purple formazan, which is then dissolved and quantified using a spectrometer [103]. To evaluate CMGs effects on cell viability, I conducted a MTT analysis and performed cell counting using a hemocytometer and trypsin blue exclusion dye on the two different TNBC cell lines, MDA MB-231 and SUM149-PT (inflammatory TNBC cell line) are both highly aggressive and invasive TNBC cell lines, frequently used in TNBC research.

MDA MB-231 and SUM 149-PT were seeded into 48-well plates (500 cells/well) and treated with individual drug, double drugs, or triple drug CMG (CMG = cisplatin 5μM, metformin 25μM, gefitinib 5μM) at 0 hours and 72 hours. After 120 hours of treatment, MTT was added and absorbance was read at 570 nm. The individual treatments (cisplatin, metformin or gefitinib alone) as well as the double-dug treatments (CM, CG and MG) only moderately decreased cell viability (Figure 8). The combination of CMG, however, showed a significant decrease in cell viability (Figure 8C). MDA MB-231 cells treated with CMG showed a decrease by over 60% in cell viability compared to the control (Figure 8A) and SUM 149-PT treated cells showed a decrease of over 75% (Figure 8B).


A
MDA-MB231
Relative Viability
1
DMSO
Cis
Met
Gef
CM
CG
MG
CMG
B
SUM149-PT
Relative Viability
1
DMSO
Cis
Met
Gef
CM
CG
MG
CMG

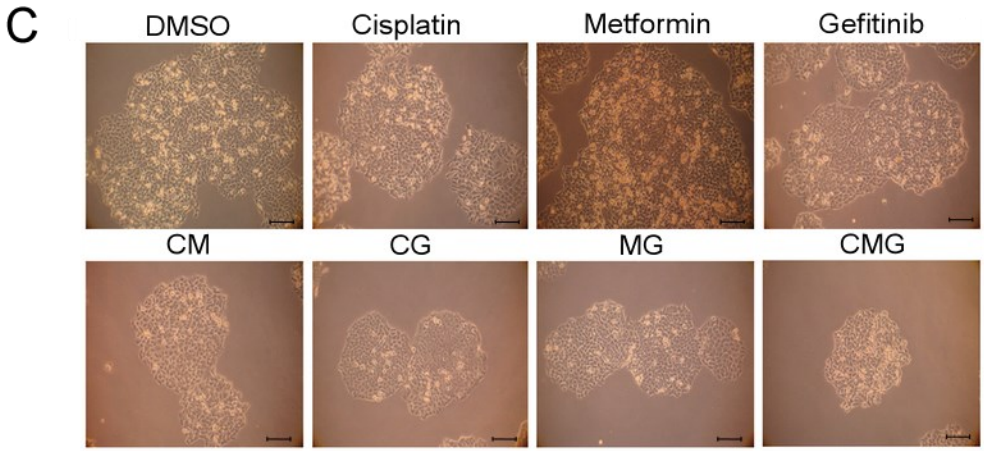

C
DMSO
Cisplatin
Metformin
Gefitinib
CM
CG
MG
CMG

Figure 8: CMG combination reduces viability on TNBC cell lines. (A) Cell viability of MDA MB-231 cells after 120 hours of exposure to DMSO vehicle control or the following drugs in different concentrations: 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib. Drugs were added at 0 hours and 72 hours (n=3). **(B)** Relative cell viability (measured by cell counting using a hemocytometer and trypsin blue exclusion dye) of the CMG combinational treatment on SUM 149-PT cells after 144 hours of exposure with the same concentrations as above, drugs were added at 0 hours, 72 hours and 120 hours. (n=3). **(C)** Representative photos 120 hours after CMG treatment on MDA MB-231 cells. Scale bar representing 100μm. Photos taken at 10x magnification.

3.4 CMG combination promotes apoptosis of TNBC cell lines

From the literature and my own findings (Figure 5C), altered apoptosis in TNBC increases drug resistance [35]. I asked whether the CMG combination was able to enhance apoptosis in MDA MB-231 and SUM149-PT cells. This was assessed using flow cytometry. Within the early stages of apoptosis there are changes that occur at the surface of the cell, one of the key changes is the translocation of phosphatidylserine from the inner side of the plasma membrane to the external surface of the cell [104]. Staining cells with Annexin V allows for the detection of phosphatidylserine on the surface of the cell and is used as a marker of apoptosis [104, 105]. 7-AAD is a DNA intercellular dye which penetrates and stains cells when there has been a loss of the integrity of the plasma membrane (i.e. cell death) thereby indicating cell death [106, 107]. CMG-treated MDA MB-231 cells were stained with Annexin V and 7-AAD and assessed via flow cytometry, and then divided into four categories (A) dead (7-AAD+/Annexin V-), (B) late apoptosis (7-AAD+/Annexin V+), (C) alive (7-AAD-/Annexin V-), and (D) early apoptosis (7-AAD-/Annexin V+) (Figure 9E).

Using flow cytometric analysis, I found the combination of CMG had the most significant increase in early apoptosis (7-AAD-/Annexin V+) with a 24-fold increase (Figure 9D), and late apoptosis (7-AAD+/Annexin V+) with an almost 6-fold increase (Figure 9B). None of the individual treatments showed significant effect on apoptosis. The double treatments with CG, CM, and MG induced early apoptosis and apoptosis respectively (Figure 9B and 9D).

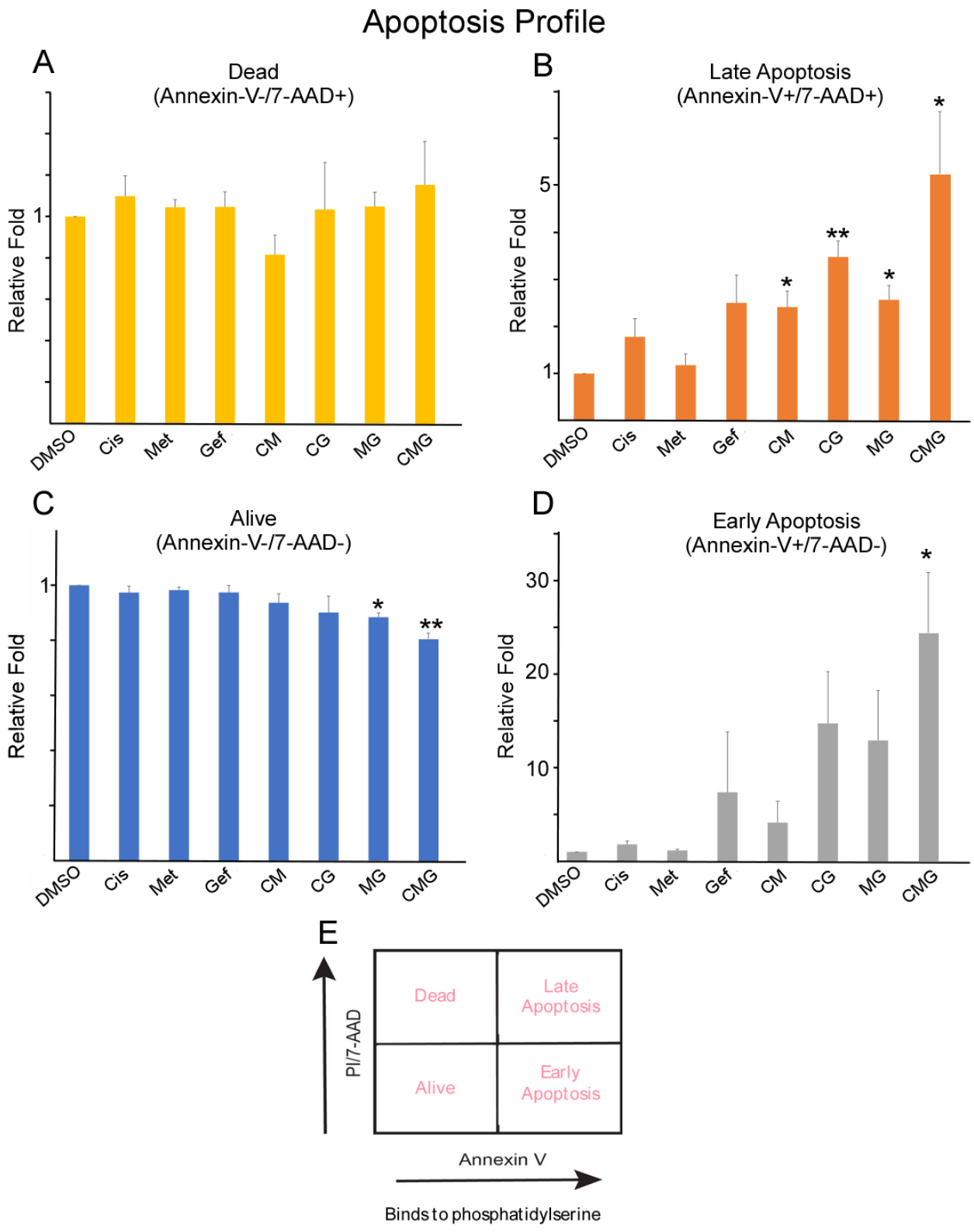

Apoptosis Profile
A
Dead
(Annexin-V-/7-AAD+)
Relative Fold
1
DMSO
Cis
Met
Gef
CM
CG
MG
CMG
B
Late Apoptosis
(Annexin-V+/7-AAD+)
Relative Fold
5
1
*
**
*
*
C
Alive
(Annexin-V-/7-AAD-)
Relative Fold
1
*
**
D
Early Apoptosis
(Annexin-V+/7-AAD-)
Relative Fold
30
20
10
*
E
PI/7-AAD
Dead
Late Apoptosis
Alive
Early Apoptosis
Annexin V
Binds to phosphatidylserine

Figure 9: CMG combination induces apoptosis in TNBC cell lines. Relative fold profile of cell death and apoptosis analyzed by flow cytometry. Based on Annexin-V/7-AAD staining, **(A)** dead (7-AAD+/Annexin V-), **(B)** late apoptosis (7-AAD+/Annexin V+), **(C)** alive (7-AAD-/Annexin V-), and **(D)** early apoptosis (7-AAD-/Annexin V+). MDA MB-231 cells were treated with DMSO vehicle control or the following drugs in different combinations for 144 hours prior to flow cytometric analyses: 5µM of cisplatin, 25µM of metformin and/or 5µM of gefitinib. Drugs were added at 0-hour, 72 hour, and 120 hr (n=3). Annexin V conjugated with V450 fluorophore and 7-AAD were added to the cells, followed by analysis using the BD LSRFortessa. **(E)** Legend of the gating strategy for apoptosis. Diagram adapted from https://bitesizebio.com/28356/locating-your-cellular-apoptosis-squad-annexin-v-staining-assays/

3.4 Combination of hypoxia and EGFR inhibition with cisplatin (CMG) suppresses the $CD44^+/CD24^-$ CSC population in TNBC cell lines

$CD44^+/CD24^-$ CSCs play a critical role in drug resistance, cancer metastasis and disease relapse in TNBC [14, 16, 108]. A major challenge preventing long-term effective treatment of TNBC is the enrichment of CSC populations following chemotherapy. An important focus of this study was to investigate whether the CMG combination was able to significantly reduce the $CD44^+/CD24^-$ CSC subpopulation in TNBC. I analyzed the CMG effects on $CD44^+/CD24^-$ subpopulation in MDA MB-231 and SUM149-PT cells using flow cytometry. The cells were treated with single, double and CMG combination for 120 hours, then stained with PE and APC conjugated anti-CD24 and anti-CD44 antibodies, respectively, followed by flow cytometric analysis.

3.4.1 Combination of hypoxia and EGFR Inhibition with cisplatin (CMG) supresses the $CD44^+/CD24^-$ CSC population in MDA MB-231 cells

The absolute living $CD44^+/CD24^-$ CSCs in MDA MB-231 cells decreased from $36.14x10^4$ to $2.8x10^4$ cells following 120 hours of CMG treatment (Figure 10A). While the double treatments were moderately effective ($23.5\ x10^4$ cells, $29.5\ x10^4$ cells, and $14.8x10^4$ cells for CM, CG and MG respectively), the triple combination of CMG was the most effective for suppressing $CD44^+/CD24^-$ subpopulation in MDA MB-231 cells (Figure 10A). Metformin alone was more effective than other single drug treatments, although cisplatin also showed moderate inhibitory effect on $CD44^+/CD24^-$ subpopulation in MDA MB-231 (Figure 10A).

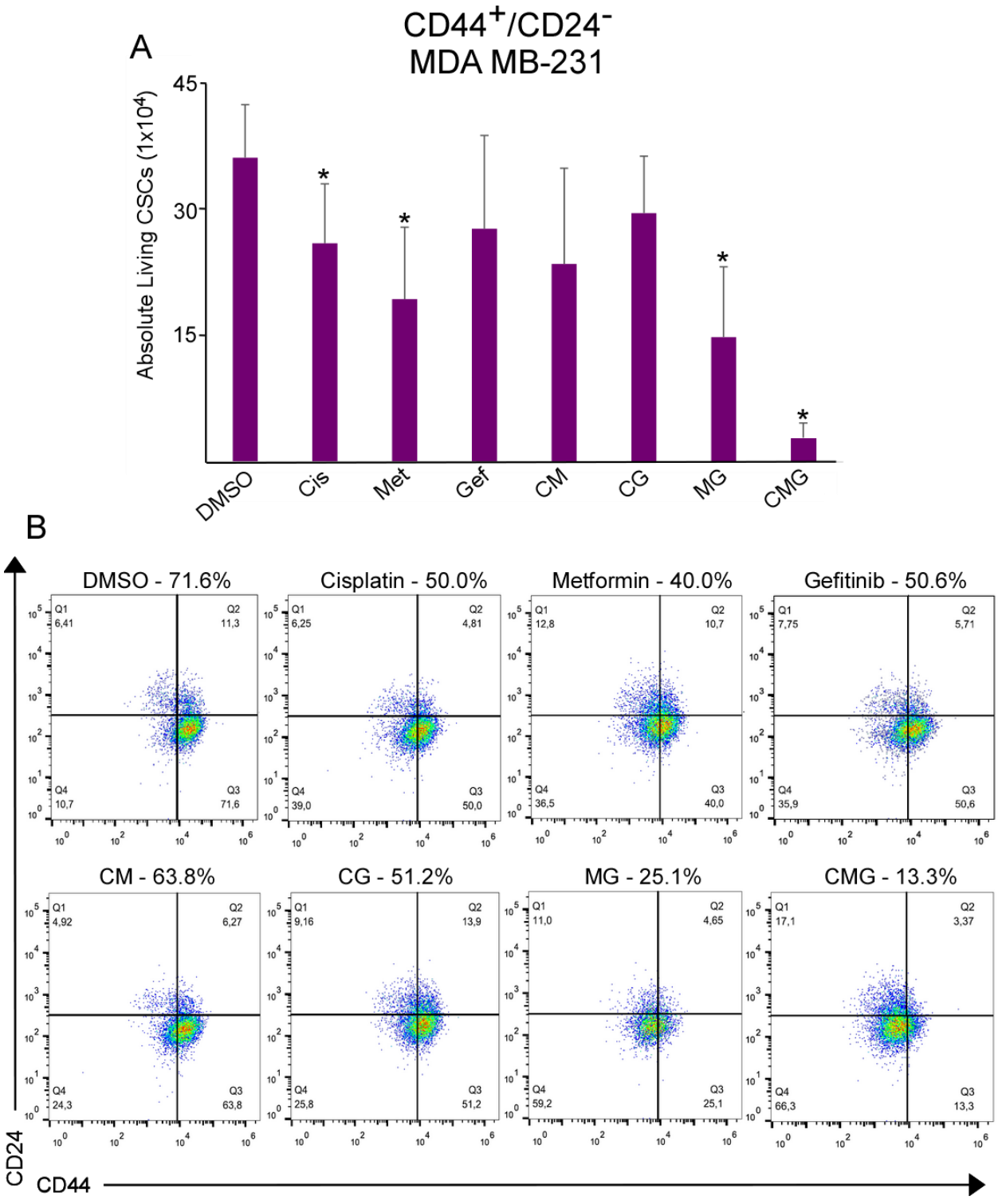

A
CD44+/CD24-
MDA MB-231
Absolute Living CSCs (1x10⁴)
45
30
15
*
DMSO
Cis
Met
Gef
CM
CG
MG
CMG
B
DMSO - 71.6%
Cisplatin - 50.0%
Metformin - 40.0%
Gefitinib - 50.6%
CM - 63.8%
CG - 51.2%
MG - 25.1%
CMG - 13.3%
CD24
CD44

Figure 10: CMG combination reduces $CD44^+/CD24^-$ CSCs in MDA MB-231 cells. (A) Absolute living $CD44^+/CD24^-$ CSC subpopulation in MDA MB-231 cells after 120 hours of exposure to DMSO vehicle control or the following drugs in different combinations: 5μM of cisplatin, 25μM of metformin, and/or 5μM of gefitinib. Drugs were added at 0- hour, and 72-hour. At 120-hour, the cells were stained with PE- or APC-conjugated anti-CD24 and/or anti-CD44 antibodies, and analyzed using the BD LSRFortessa (n=3). **(B)** Representative flow plots of MDA MB-231 cells after 120 hours of treatment with the drug combinations listed above. Controls and gating for MDA flow plots were showed in Supplementary Figure 4.

3.4.2 Combination of hypoxia and EGFR Inhibition with cisplatin (CMG) suppresses the $CD44^+/CD24^-$ CSC population in SUM 149-PT cells

To exclude cell line specificity for CMG treatment, I treated a different TNBC cell line SUM 149-PT cells and found a similar trend. The absolute living $CD44^+/CD24^-$ CSCs in SUM 149-PT cells decreased from $5.87x10^4$ cells to $0.20x10^4$ cells following 120 hours of CMG treatment (Figure 11A). The double treatments (CG and MG) on SUM 149-PT cells were more effective than the MDA MB-231 counterparts for the inhibition of $CD44^+/CD24^-$ CSCs ($14.8x10^4$ cells, and $6.8x10^4$ cells for CG and MG respectively). In contrast to what we saw with MDA MB-231 cells, gefitinib as a single treatment was the most potent single-therapy for the inhibition of $CD44^+/CD24^-$ CSCs in comparison to cisplatin and metformin ($9.5x10^4$, $40.2x10^4$, and $38.0x10^4$ cells respectively) (Figure 11A). However, although the double treatment of MG and single treatment of gefitinib reduced the absolute numbers of living $CD44^+/CD24^-$ CSC subpopulation in SUM 149-PT cells, the triple combination of CMG was the most significant treatment (Figure 11A).

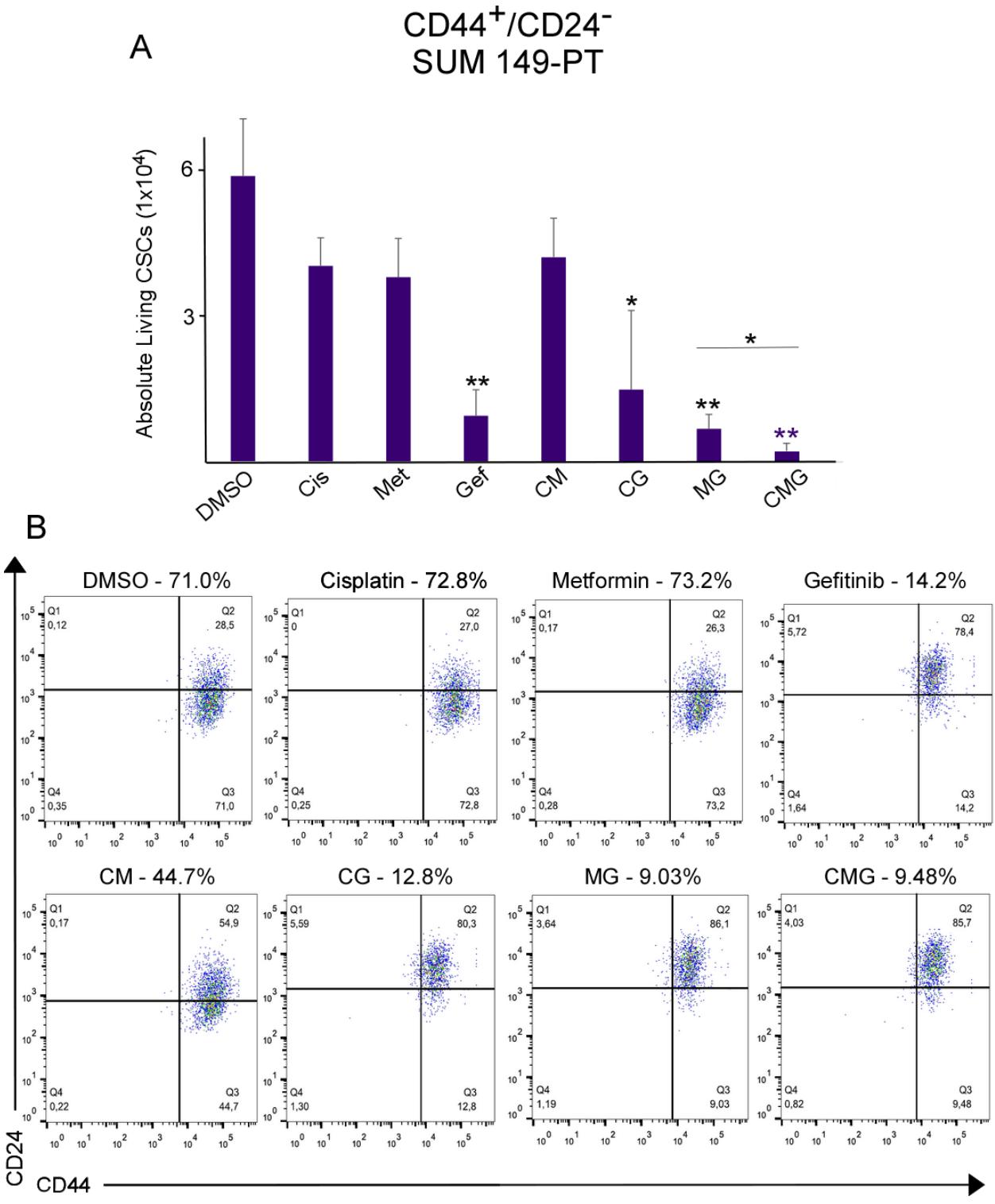

A
CD44+/CD24-
SUM 149-PT
Absolute Living CSCs (1x10⁴)
6
3
DMSO
Cis
Met
Gef
CM
CG
MG
CMG
B
DMSO - 71.0%
Cisplatin - 72.8%
Metformin - 73.2%
Gefitinib - 14.2%
CM - 44.7%
CG - 12.8%
MG - 9.03%
CMG - 9.48%
CD24
CD44

Figure 11: CMG combination significantly reduces CD44+/CD24- CSCs in SUM 149-PT cells. (A) Absolute numbers of living $CD44^{+}/CD24^{-}$ CSC subpopulation in SUM 149-PT cells after 120 hours of exposure to DMSO vehicle control or the following drugs in different combinations: 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib. Drugs were added at 0 hour and 72 hours, and then stained with PE- and APC-conjugated anti-CD24 and anti-CD44 antibodies, followed by flow cytometric analysis using the BD LSRFortessa (n=3). **(B)** Representative flow plots of percentages of $CD44^{+}/CD24^{-}$ CSCs in SUM 149-PT cells after 120 hours of exposure to the drug combinations listed above.

3.5 Combination of hypoxia and EGFR Inhibition with cisplatin (CMG) suppresses the ALDH^{high} CSC population in TNBC cell lines

ALDH^{high} CSCs play a critical role in drug resistance, cancer metastasis and dramatic increased tumorgenicity in TNBC [23-25]. To investigate whether the CMG combination was able to significantly affect the ALDH^{high} subpopulation in MDA MB-231 and SUM149-PT cells flow cytometric analysis was utilized. The cells were treated with single, double and CMG combination for 120 hours, then then incubated with ALDEFLUOR assay buffer +/- a DEAB control followed by flow cytometric analysis.

3.5.1 Combination of hypoxia and EGFR Inhibition with cisplatin (CMG) suppresses the ALDH^{high} CSC population in MDA MB-231 cells

Following MDA MB-231 cells treatment with cisplatin, the ALDH^{high} CSC subpopulation increased 50% (Figure 12A). This increase was opposite to the results found of the CD44^{+}/CD24^{-} CSCs population where cisplatin inhibited them (Figure 10A). As single therapies metformin and gefitinib reduced the ALDH^{high} population to 38% and 45% respectively (Figure 12A). The double therapies were more effective than the single therapies with a reduction to 36.4%, 38.7% and 9.2% for CM, CG and MG respectively. Significantly, the CMG treatment demonstrated over a 90% reduction in ALDH^{high} CSC MDA MB-231 cells (Figure 12).

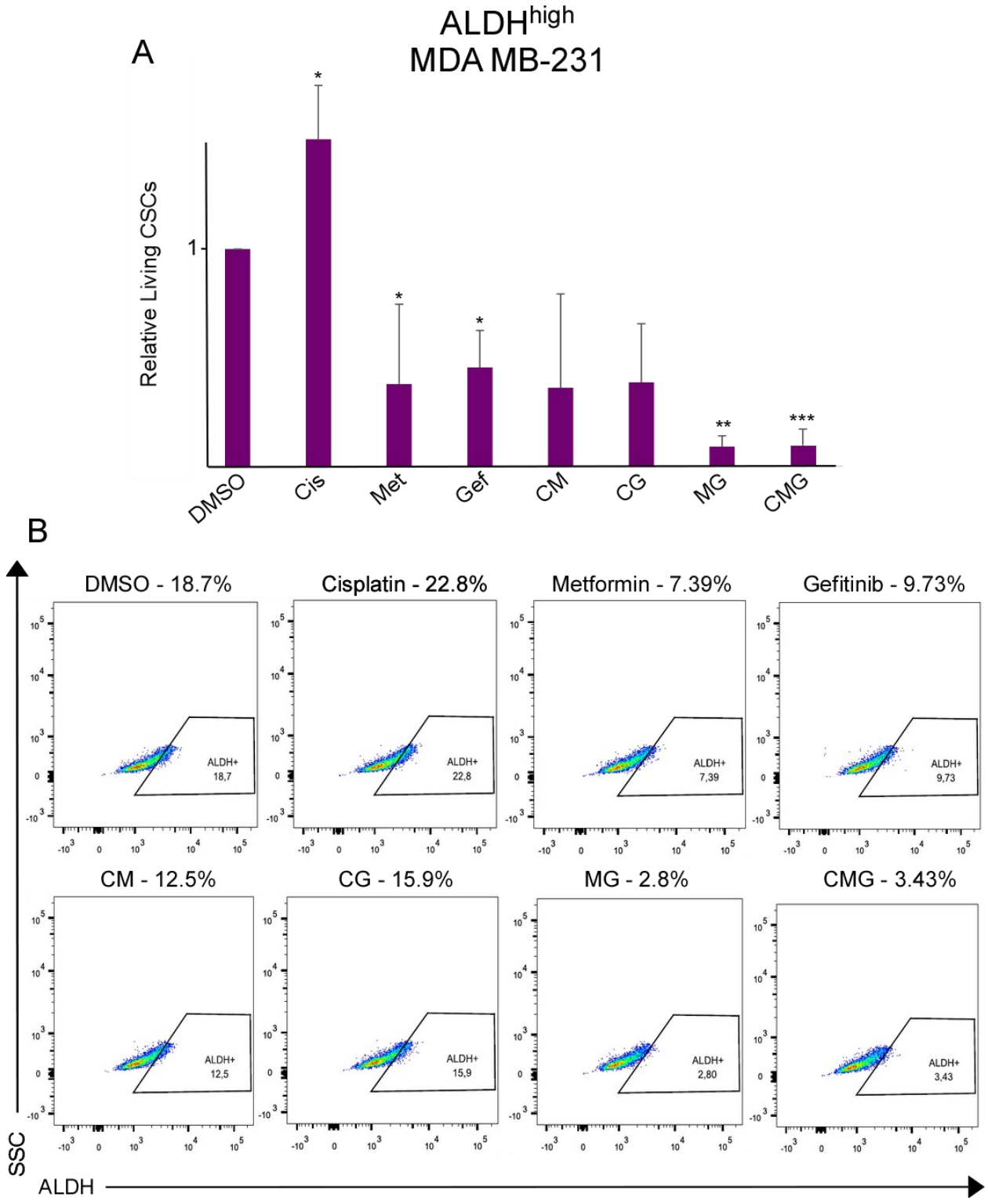

A
ALDH^high
MDA MB-231
Relative Living CSCs
1
*
*
*
**
***
DMSO
Cis
Met
Gef
CM
CG
MG
CMG
B
DMSO - 18.7%
Cisplatin - 22.8%
Metformin - 7.39%
Gefitinib - 9.73%
CM - 12.5%
CG - 15.9%
MG - 2.8%
CMG - 3.43%
ALDH+ 18,7
ALDH+ 22,8
ALDH+ 7,39
ALDH+ 9,73
ALDH+ 12,5
ALDH+ 15,9
ALDH+ 2,80
ALDH+ 3,43
SSC
ALDH

Figure 12: CMG combination significantly reduces ALDH^{high} CSCs whereas cisplatin increases ALDH^{high} CSCs in MDA MB-231 cells. (A) Relative living ALDH^{high} CSC subpopulation in MDA MB-231 cells after 120 hours of exposure to DMSO vehicle control or the following drugs in different combinations: 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib. Drugs were added at 0 hour and 72 hours, and then incubated with ALDEFLUOR assay buffer +/- DEAB, followed by flow cytometric analysis using the BD LSRFortessa (n=3). DEAB Control is depicted in Supplementary Figure 4 **(B)** Representative flow cytometry plots of ALDH^{high} CSC subpopulation (percentages) in MDA MB-231 cells after 120 hours of exposure to the drug combinations listed above.

3.5.2 Combination of hypoxia and EGFR Inhibition with cisplatin (CMG) suppresses the ALDH^{high} CSC population in SUM 149-PT cells

Again, to exclude cell line specificity, I treated SUM 149-PT cells with the CMG combinational treatment. On SUM 149-PT cells, cisplatin did not increase the ALDH^{high} CSC subpopulation but also did not show any inhibitory effect, equal to the vehicle control with $28x10^4$ absolute living ALDH^{high} CSCs (Figure 13A). Following the same trend as seen on the MDA MB-232 cells, metformin and gefitinib were fairly potent as single therapies against the ALDH^{high} CSC subpopulation with $13.1x10^4$ and $9.2x10^4$ cells respectively. The double therapies of CM and MG were more effective than CG ($3.1x10^4$ cells, $4.8x10^4$ cells and $9.5x10^4$ cells respectively). Significantly, the CMG treatment was the most effective, reducing ALDH^{high} CSC in SUM 149-PT cells from $28.1x10^4$ to $1.2x10^4$ cells (Figure 13).

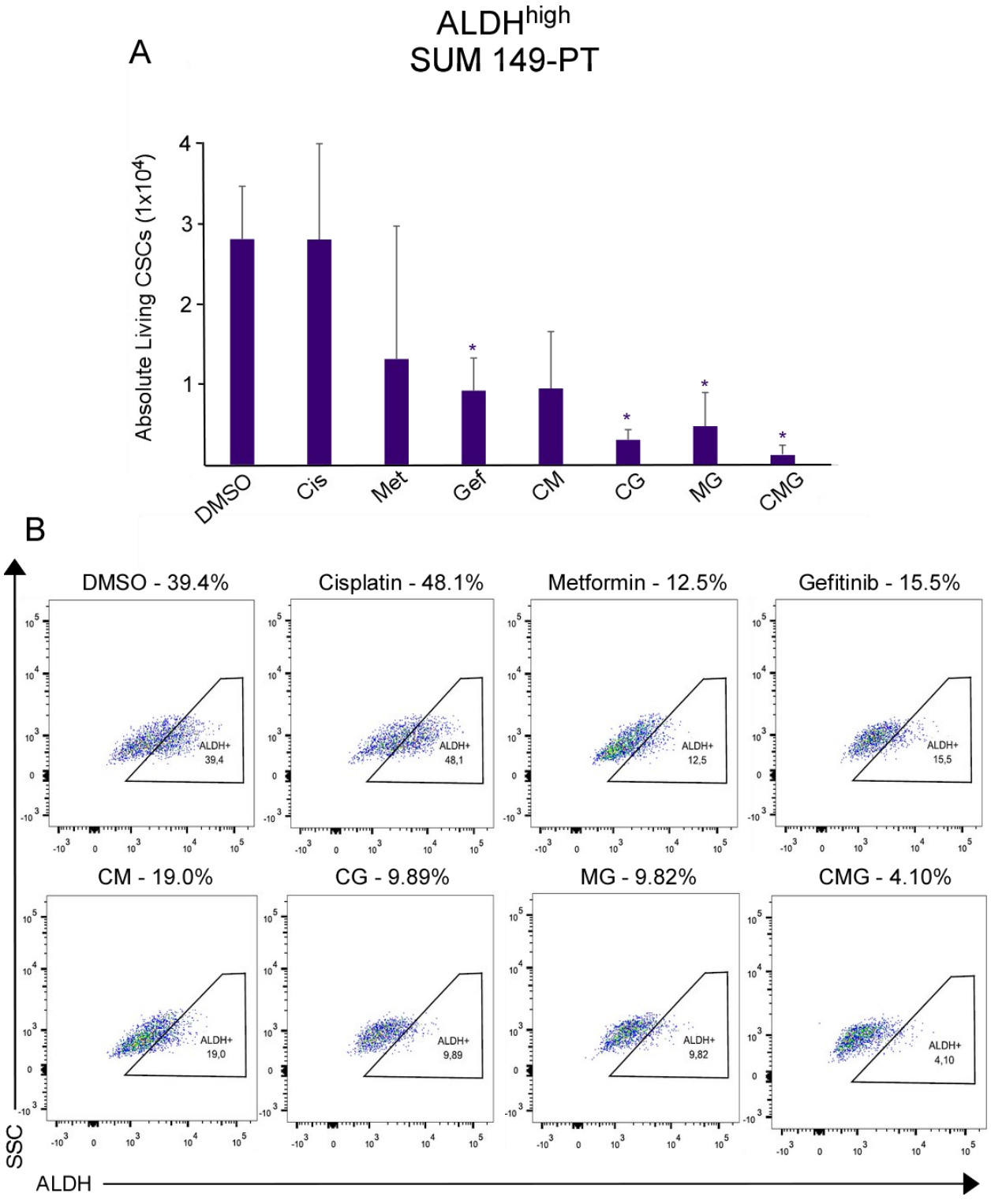

A
ALDH^high
SUM 149-PT
Absolute Living CSCs (1x10^4)
4
3
2
1
DMSO
Cis
Met
Gef
CM
CG
MG
CMG
B
DMSO - 39.4%
Cisplatin - 48.1%
Metformin - 12.5%
Gefitinib - 15.5%
CM - 19.0%
CG - 9.89%
MG - 9.82%
CMG - 4.10%
ALDH+ 39,4
ALDH+ 48,1
ALDH+ 12,5
ALDH+ 15,5
ALDH+ 19,0
ALDH+ 9,89
ALDH+ 9,82
ALDH+ 4,10
SSC
ALDH

Figure 13: CMG combination reduces ALDH^{high} CSCs in SUM 149-PT cells. (A) Absolute living ALDH^{high} CSC subpopulation in SUM 149-PT cells after 120 hours of exposure to the DMSO vehicle control or following drugs in different combinations: 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib. Drugs were added at 0 hour and 72 hours, and then incubated with ALDEFLUOR assay buffer containing DEAB control and reagent, followed by flow cytometric analysis using the BD LSRFortessa (n=3). **(B)** Representative flow cytometry plots of ALDH^{high} CSC subpopulation (percentages) in SUM 149-PT cells after 120 hours of exposure to the drug combinations listed above.

3.6 CMG Combination reduces viability of organotypic cultures prepared from TNBC patient-derived xenografts and patient samples

To verify the aforementioned *in vitro* findings using conventional TNBC cell lines for potential clinical translation, I moved to an *ex-vivo* patient-derived xenograft (PDX) model. PDXs are tumor fragments removed from patient tumors, followed by directly implantation into immunodeficient mice. This model allows the tumors to retain their structures, micro-environments and heterogeneous cell populations [109]. PDX tumors can be cut into thin slices and cultured *ex-vivo* (i.e. organotypic culture) for short term experiments.

I utilized the Alamar Blue to analyze cell viability after drug treatment of organotypic cultures of PDX and patient tumor samples. Alamar blue (resazurin), a blue and nonfluorescent dye, was added to three TNBC PDX samples and two patient TNBC tumor samples freshly obtained from The Ottawa Hospital. The resazurin was metabolised by live cells in the medium and turned to pink and highly fluorescent resorufin [110, 111]. I then used a spectrometer to measure the fluorescence and determine the baseline viability of the PDX and tumor samples prior to drug treatment. The samples were treated with drugs in different combinations at 0 hour, 72 hours and 120 hours. The Alamar Blue analysis was then preformed at 144 hours to determine drug effect on the viability of PDX and tumor samples and normalized with the initial baseline prior to drug treatments (Figure 14, Supplementary Figure 6).

Cisplatin and metformin alone had little to no effect on all five PDX and tumor viability (Figure 14, Supplementary Figure 6). Depending on the sample gefitinib reduced viability of PDX/tumor organotypic cultures between 10% to 40% but was statistically insignificant in comparison to vehicle control. In concordance with our *in vitro* cell line viability results, the

combination of CMG was the only treatment to significantly decrease viability of in the PDX/tumor organotypic cultures up to 60% (Figure 14, Supplementary Figure 6).

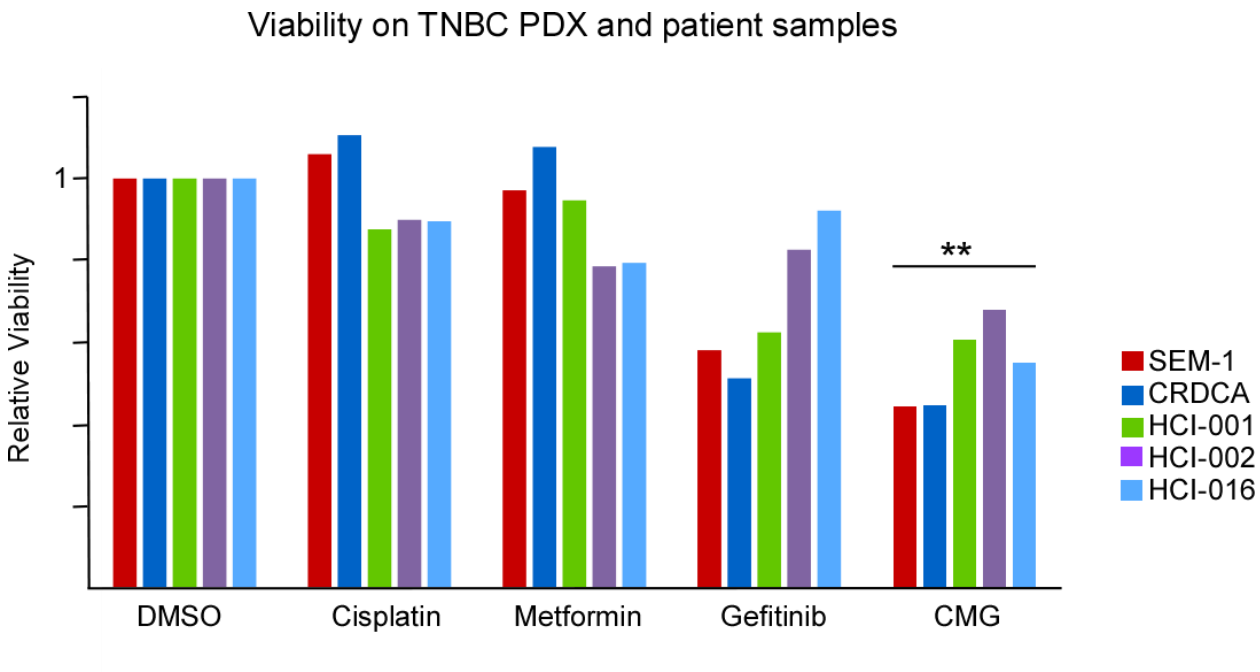
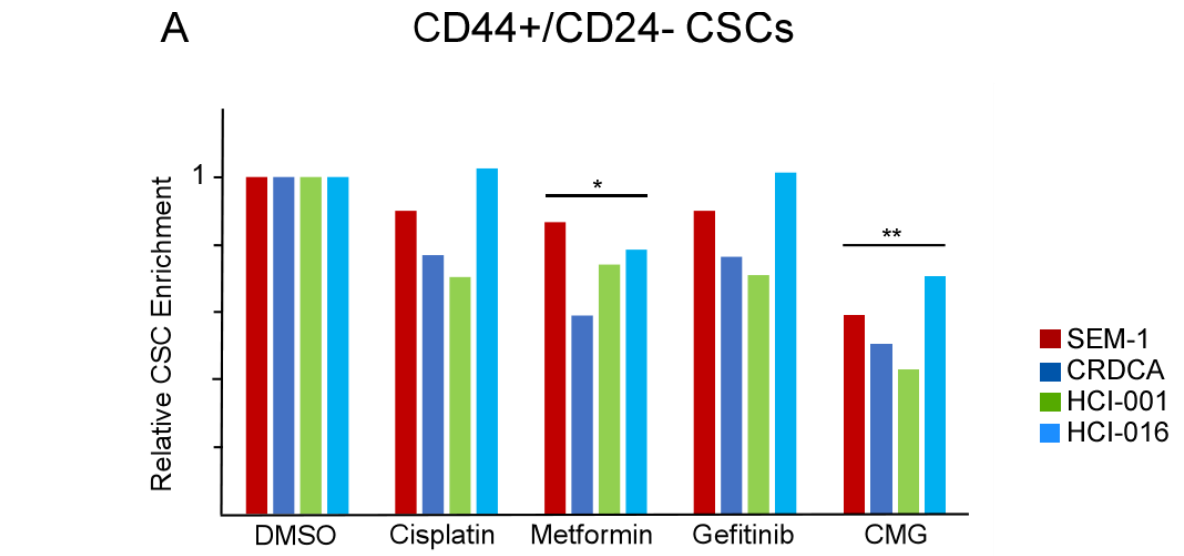

Viability on TNBC PDX and patient samples
Relative Viability
1
**
DMSO
Cisplatin
Metformin
Gefitinib
CMG
SEM-1
CRDCA
HCI-001
HCI-002
HCI-016

Figure 14: CMG combination significantly reduces viability of organotypic cultures of TNBC PDX and patient samples. Relative Alamar blue viability analysis of two primary patient TNBC tumors (CRDCA and SEM-1 samples) and three patient-derived xenograft samples (HCI-001, HCI-002, HCI-016) after 144 hours of exposure to the DMSO vehicle control and drugs in different combinations: 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib. Drugs were added at 0 hour and 72 hours. Tumor samples were cut into approximately 2 × 1 mm tumor slices and cultured *ex vivo*. Alamar Blue (resazurin) was added and the resazurin is metabolized by live cells in the medium and turned to pink and highly fluorescent resorufin that was measured using a spectrometer at 560 excitation and 590 emission. Viability was calculated based on OD values at 144 hours – OD values at 0 hour.

3.7 CMG Combination inhibits the $CD44^+/CD24^-$ CSC population in TNBC PDX and patient samples

I further assessed the $CD44^+/CD24^-$ CSCs population after drug treatment using flow cytometry. PDX and patient tumor samples were cut into thin slices and cultured *ex-vivo* (i.e. organotypic culture) and treated for 144 hours with DMSO vehicle control or the drug in different combinations (adding drugs at 0 hour, 72 hours and 120 hours). After 144 hours, the tumor samples were disassociated into a single cell suspension and stained with PE- and APC-conjugated anti-CD24 and anti-CD44 antibodies.

The flow cytometric data showed that the CMG combination significantly reduced the $CD44^+/CD24^-$ CSC population (Figure 15A). In the representative flow plots of PDX/clinical samples, we reveal that CMG significantly reduced the $CD44^+/CD24^-$ CSC subpopulation from 15.8% to 8.17% in the SEM-1 tumor after treatment for 144 hours (Figure 15B). Metformin moderately decreased CD44+/CD24- CSCs without inhibiting the viability of PDX and patient samples (Figure 15).


A
CD44+/CD24- CSCs
Relative CSC Enrichment
1
*
**
DMSO
Cisplatin
Metformin
Gefitinib
CMG
SEM-1
CRDCA
HCI-001
HCI-016

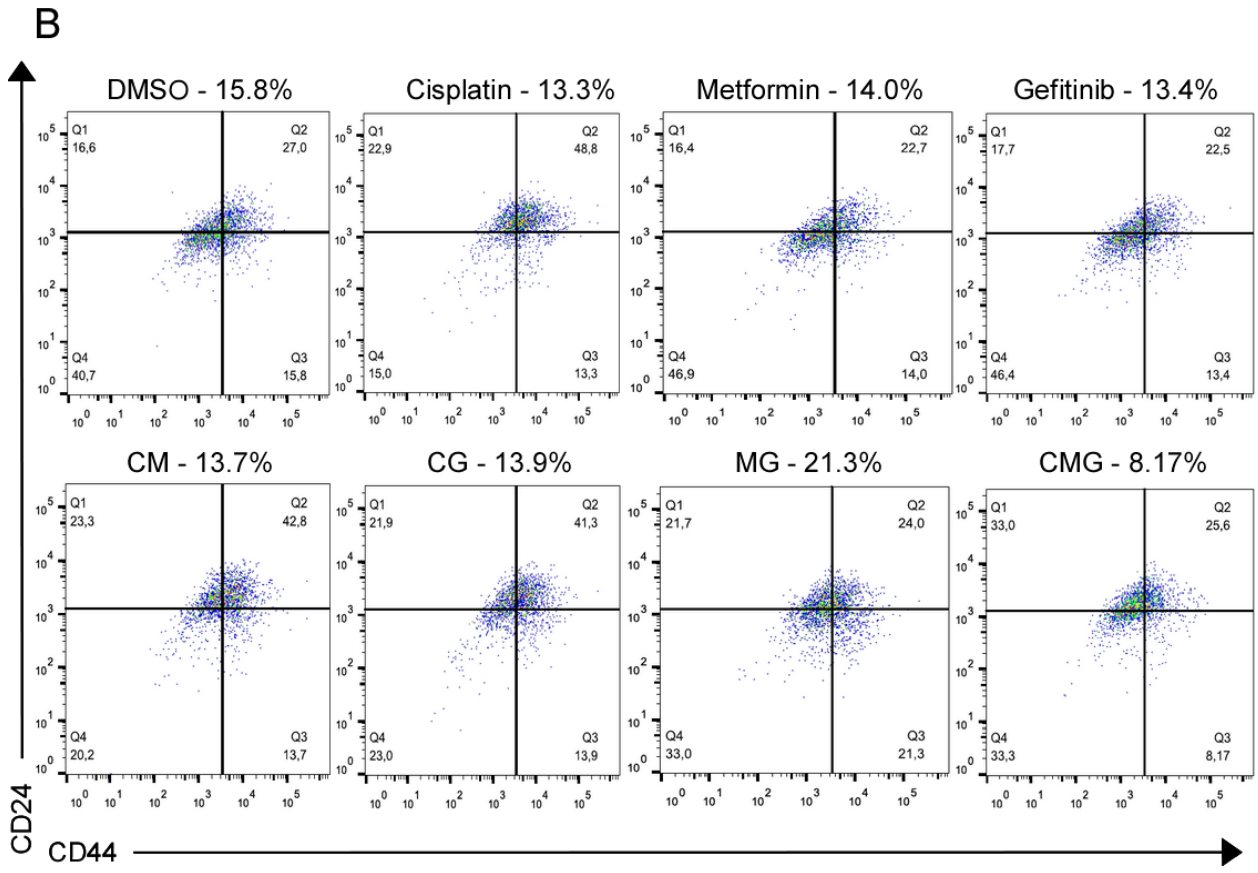

B
DMSO - 15.8%
Cisplatin - 13.3%
Metformin - 14.0%
Gefitinib - 13.4%
CM - 13.7%
CG - 13.9%
MG - 21.3%
CMG - 8.17%
CD24
CD44

Figure 15: CMG combination effectively reduces $CD44^+/CD24^-$ CSCs in TNBC PDX and patient samples. (A) the $CD44^+/CD24^-$ CSC populations in SEM-1, CRDCA, HCI-016 and HCI-001 TNBC tumor samples after *ex vivo* organotypic culture and exposure to DMSO vehicle control or the following drugs in different combination for 144 hours: 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib. Drugs were added at 0 hour, 72 hours and 120 hours. After that, the cells were mechanically and enzymatically disassociated into single cells and then stained with PE- and APC-conjugated anti-CD24 and anti-CD44 antibodies, followed by flow cytometric analysis using the BD LSRFortessa (n=3). **(B)** Representative flow plots of the $CD44^+/CD24^-$ CSC subpopulations (percentages) in SEM-1 cells after treatment with drug in different combinations. Controls and gating for PDX flow plots were showed in Supplementary Figure 7.

3.8 CMG combination more effectively inhibits the ALDH^{high} CSC subpopulation in TNBC PDX and patient samples

Consistent with the results obtained from the MDA MB-231 cell line (Figure 12), cisplatin alone significantly increased the ALDH^{high} population in all four PDX and patient samples (Figure 16A). In the representative plots of the HCI-001 PDX sample, cisplatin increased the ALDH^{high} population from 17% to 37% (Figure 16B). This is in contrast to the *in vitro* data from SUM 149-PT cells where cisplatin did not significantly impact the ALDH^{high} population (Figure 13).

Both metformin and gefitinib were able to reduce the ALDH^{high} CSC population in contrast to the cisplatin-induced increase of ALDH^{high} CSCs (data not shown). This also supports *in vitro* data from both MDA MB-231 and SUM 149-PT lines where we saw metformin and gefitinib reduce the ALDH^{high} population (Figure 12, Figure 13).

Importantly, the CMG combination significantly reduced the ALDH^{high} population in all four PDX and patient tumor samples up to 93% (from 47% to 93%) (Figure 16A). In the representative flow plots of PDX/clinical samples, we reveal that CMG reduced the ALDH^{high} CSC subpopulation in the HCI-001 PDX tumor from 25.1 % to 16.4% in 144 hours (Figure 16B). This data reinforces the *in vitro* results from both MDA MB-231 cell and SUM 149-PT lines where the combination of CMG was the most significant treatment group at reducing the ALDH^{high} population (Figure 12 and Figure 13).

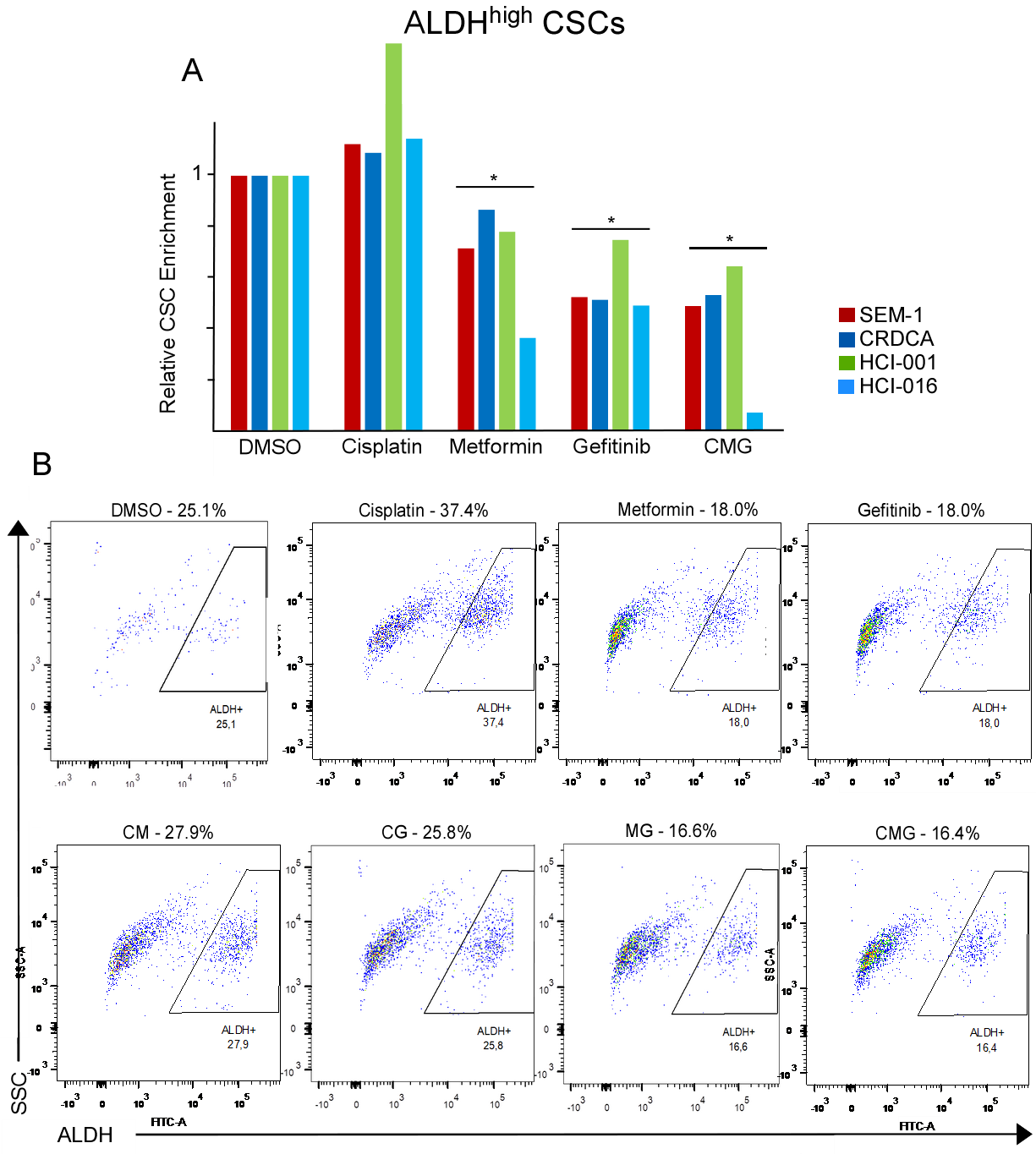

ALDH^high CSCs
A
Relative CSC Enrichment
1
*
*
*
DMSO
Cisplatin
Metformin
Gefitinib
CMG
SEM-1
CRDCA
HCI-001
HCI-016
B
DMSO - 25.1%
ALDH+
25,1
Cisplatin - 37.4%
ALDH+
37,4
Metformin - 18.0%
ALDH+
18,0
Gefitinib - 18.0%
ALDH+
18,0
CM - 27.9%
ALDH+
27,9
CG - 25.8%
ALDH+
25,8
MG - 16.6%
ALDH+
16,6
CMG - 16.4%
ALDH+
16,4
SSC-A
SSC
FITC-A
ALDH
FITC-A

Figure 16: CMG combination reduces ALDH^{high} CSCs in TNBC PDX and patient samples. Flow cytometric data of the ALDH^{high} CSC populations in SEM-1, CRDCA, HCI-016 and HCI-001 TNBC tumors (cultured *ex vivo*) 144 hours after exposure to the following concentrations: 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib, the control was treated with DMSO. Drugs were added at 0 hr, 72 hr and 120 hr, tumor slices were then disassociated and were then incubated with ALDEFLUOR assay buffer from which an aliquot was incubated with DEAB (an ALDH inhibitor) and analyzed using the BD LSRFortessa (n=3). DEAB controls depicted in Supplemental Figure 7. **(B)** Representative flow plots of the ALDH^{high} CSC subpopulations in HCI-001 cells after 144 hours of treatment with the combinational therapy.

4. DISCUSSION

In 2020, Canada is projected to have 27,000 women diagnosed with breast cancer and sadly over 5,000 women will pass away from the disease [112]. Triple negative breast cancer (TNBC) is an extremely aggressive subtype of breast cancer which does not possess the target receptors used to treat other subtypes of breast cancer (estrogen, progesterone and HER2 receptors) and therefore treatment for patients with TNBC is limited to non-specific chemotherapy, surgery and radiotherapy. Despite making up only 15-20% of breast cancer diagnosis', TNBC disproportionately for the majority of breast cancer deaths. Even after adjusting for age, stage, race, grade, and delivery of chemotherapy, Lin *et al* found that ultimately women with TNBC were 2.72 times more likely to die of the disease than their ER+ counterparts [11]. As such, the development of targeted therapies for TNBC remains an unmet medical need.

4.1 CMG combination reduces viability on TNBC cell lines and induces apoptosis

One of the most fundamental hallmarks of cancer cells involves its ability to sustain chronic proliferation [113]. Chemotherapy treatments generally work by killing these highly proliferative and regenerative cells [114]. Proliferation and apoptotic assays are some of the primary methods/experiments utilized to test if a treatment will be able to successfully kill the type of cancer being tested.

To evaluate how the CMG combinations would affect the viability of TNBC cells *in vitro* MTT assays were performed MDA MB-231 cells (mesenchymal-phenotype) and SUM 149-PT (epithelial-phenotype) TNBC cell lines. Following 120 hours of treatment (cisplatin 5µM, metformin 25µM and/or gefitinib 5µM) viability was moderately affected by the individual and double treatments (Figure 8 and Figure 9). In contrast, the CMG combination significantly reduced

cell viability in both TNBC cell lines, in MDA MB-231 cells viability decreased 50% and in SUM 149-PT cells viability decreased 70% (Figure 8). Notably, concentrations of cisplatin, metformin and gefitinib used in these experiments were clinically relevant, suggesting a tangible therapeutic approach to kill TNBC cells [13, 100-102].

MCF-10A cells has been often used in breast cancer research as a model for normal mammary epithelial cells function and transformation; however recent literature has reported that MCF-10A cells exhibit different profiles compared to normal mammary tissue bringing into question the suitability of this model [115, 116]. MTT analysis on MCF-10A cells treated with CMG found that compared to the MDA MB-231 and SUM 149-PT TNBC cells, gefitinib (5μM) as an individual treatment significantly reduced viability (Supplementary Figure 2). Subik *et al* analyzed breast cancer cell lines with immunohistochemistry and found that MCF-10A cells that EGFR expression is strongly present compared to MDA MB-231 cells [117]. It is reasonable to conclude that because gefitinib is an EGFR inhibitor and MCF-10A cells are so high in EGFR, gefitinib would affect MCF-10A cells viability more dramatically. Ultimately, from the literature and our own data, we judged that MCF-10A cells are not a viable model of general normal cell toxicity when being treated with gefitinib or other EGFR inhibitors [116].

Apoptosis is an orchestrated cellular process that balances cellular turnover, cell division and cell death [118]. In cancer, the mechanism of apoptosis has been found to be altered allowing for a pathological imbalance of cell death and division and increase in drug resistance [35]. Campbell *et al* used cBioportal analysis on 816 breast cancer patient samples generated by The Cancer Genome Atlas (TCGA) and observed that gene amplification of anti-apoptotic gene (*MCL-1*) was found in 20% of breast cancer cases [17]. Campbell *et al* also found that high MCL-1 protein expression was correlated with poorer patient survival in 420 patients [97]. My database

results found that TNBC has an abnormal increase in anti-apoptotic gene expression (*MCL-1*) compared to normal mammary tissue (Figure 5C), associated with apoptotic-resistance [97, 98].

Annexin V allows for the detection of phosphatidylserine which is found on the surface of the cell as a marker of apoptosis [104, 105] and 7-AAD is a DNA intercellular dye which penetrates and stains cells when there has been cell death [106, 107]. CMG had the most significant increase in early apoptosis (7-AAD-/Annexin V+) with a 25-fold increase (Figure 9D) and dead by apoptosis (7-AAD+/Annexin V+) population, with an almost 6-fold increase (Figure 9B). None of the individual treatments were shown to meaningfully effect apoptosis, and of the double treatments CG was the most substantial at inducing early apoptosis and apoptosis with a 14.7- and 3.5-fold increase respectively (Figure 9B, Figure 9D). Again, it is noteworthy that CMG at clinically relevant dosages was able to significantly induce apoptosis of MDA-MB-231 cells.

4.2 Cisplatin increases hypoxia in TNBC cell lines but metformin and gefitinib reduce it

Cisplatin is a chemotherapeutic agent which has been found to be effective at targeting the bulk tumor population of multiple types of cancer including TNBC; however, cisplatin has been demonstrated to enrich for hypoxia which promotes chemoresistance and the enrichment of CSCs [33, 34, 36, 37]. Though the mechanism through which this effect is mediated through remains is not fully understood [54, 55]. In my study, I looked to reduce/reverse cisplatin's hypoxia enrichment so as to better capitalize on its ability to reduce the bulk tumor population.

Gefitinib (an EGFR inhibitor) has been demonstrated to overcome cisplatin-induced hypoxia; however resistance has been seen through the stimulation of bypass signalling promoting Akt/mTORC1 and subsequent hypoxia [64, 67-72]. Metformin (an AMPK activator)

has been found to overcome cisplatin resistance in TNBC through the downregulation of RAD51 [62] and has shown synergism with gefitinib in lung and bladder cancer [73, 74].

As was supported from the literature and in conjunction with my database work (Figure 5B), cisplatin was found to increase the transcriptional activity of HIF-1 related gene expression (Figure 7A-B). This is important because cisplatin's substantial increase in hypoxia signalling is detrimental as it hinders clinician's ability to use it as a single-treatment therapy for TNBC as resistance is rapidly generated.

While Rho *et al* reported that gefitinib reduced cisplatin-induced hypoxia, our results for the combination of CG show a less robust anti-hypoxia response (Figure 7); however, Rho *et al* found results at 30μM which is 6 times higher than the clinically relevant concentration of 5μM that we utilized [68]. Increased hypoxia has been reported to be promote gefitinib resistance [71]. As anti-hypoxic effects were not seen at our clinically relevant dosage of CG and our goal for clinical translation, supports the addition of metformin to the combinational treatment. The combination of metformin and gefitinib, but neither individually, significantly reduced the cisplatin increased HIF-1 transcriptional activity (Figure 7A).

Similar findings were demonstrated in *PDK1* and *LDH1* gene expression following CMG treatment (Figure 7C-D). Where cisplatin alone increased HIF-1 related gene expression, CMG ablated this resulting in a significant reduction in HIF-1 activity compared to the control (Figure 7B). Overall, I observed that the combination of metformin and gefitinib was able to prevent cisplatin-induced hypoxia in TNBC, which has not yet been reported (Figure 7A-D).

4.3 Cisplatin increases ALDH^{high} CSCs in TNBC cell lines and PDX tumors but metformin and gefitinib reduce/reverse it

Aldehyde dehydrogenase (ALDH) has been used in the characterization of breast CSCs in TNBC with increased ALDH expression being strongly associated with significantly reduced patient prognosis (5-year OS rate: 19.8% ALDH^{high} vs 58.7% ALDH^{low}) [23-25]. ALDH^{high} CSCs are found within the core of breast cancer tumors and have an epithelial-like phenotype [14]. Silva *et al* found when treating SKOV3 ovarian cancer cells with cisplatin, that as the bulk tumor population (absolute number) was reduced as a result of increasing concentrations of cisplatin (1µg/mL - 3µg/mL), the percentage of ALDH^{high} SKOV3 cells increased dramatically from 5% to 30% [31]. This correlates with our flow cytometry data which revealed that MDA MB-231 cells, SUM 149-PT cells and our clinical/PDX samples treated with cisplatin (5µM) alone demonstrated significant increases in the ALDH^{high} population relative to the DMSO treated control (Figure 12A, Figure 13A, Figure 16A).

The gold standard of studying the tumorgenicity of a population is through *in vivo* serial dilution assay in immunocompromised mice. Through this method, Ginester *et al* detected ALDH-positive cells capable of reforming tumors with more than 1,500 cells compared to the unsorted cells (control) which were not tumorigenic even at 50,000 cells [23], gives us further evidence that the ALDH^{high} CSC population is directly associated with a dramatic increased in tumorgenicity [23].

Importantly, the ALDH^{high} CSC population in breast cancer has been found to have elevated HIF-1 activity [42, 43], and as we have seen with our previously discussed results, chemotherapeutic agents used to treat TNBC including cisplatin stimulate HIF-1 [33, 45-47]. Zhang *et al* shed light on the mechanisms through which cisplatin mediates CSC enrichment via

enrichment of IL-6. HEk293 cells upon exposure to IL-6 using a luciferase assay (HRE-luc) demonstrated potent upregulation [119]. They put forward a potential mechanism where IL-6 mediates cisplatin induced hypoxia and associated ALDH^{high} CSC enrichment. They proved their mechanism through siRNA-IL6 knockdowns [119]. Our results support their hypothesis where cisplatin induces hypoxia and associated ALDH^{high} CSC enrichment (Figure 7, Figure 12A, Figure 13A, Figure 16A), which is why the addition of specific inhibitors in, conjunction with cisplatin, are essential to prevent this response which is responsible for increased hypoxia.

Metformin (an AMPK activator) has been found to significantly reduce the growth of ovarian cancer cell lines [120]. Shank *et al* studied its effect when in conjunction with cisplatin treatment *in vitro* with SKOV3 and A2780 ovarian cancer cell lines, and *in vivo* using both tumor cell lines and ALDH(+) CSC tumor xenografts. Their *in vitro* FACS analysis revealed that metformin (1mM) alone reduced the ALDH(+) CSCs from 0.39% to 0.17%, cisplatin (1.5µg/mL) increased to 2.13%, and cisplatin with metformin reduced the ALDH(+) CSCs to 0.33% [120]. My results show very similar trends among TNBC cell lines when looking at the ALDH^{high} population in MDA MB-231 and SUM 149-PT cells treated with cisplatin with/without metformin (Figure 12 and Figure 13). Shank *et al* then injected 1,000 fractionated ALDH(+) or ALDH(-) SKOV3 cells into nude mice and treated them. They found the combination of metformin and cisplatin on the ALDH(+) tumors led to a significant reduction in tumor growth and importantly the FACS analysis of these tumors demonstrated that the combination of metformin and cisplatin demonstrated a 4.4 fold reduction in ALDH(+) cells [120]. While our project did not include an *in vivo* study, our *ex vivo* model shows a reduction in ALDH^{high} populations within the broad-spectrum patient samples/PDX tumors (Figure 16).

Gefitinib has been demonstrated to enhance tumor inhibition when combined with cisplatin short-term through concurrent EGFR inhibition [121]. However, it has also been reported that gefitinib and cisplatin antagonize each other in regards to drug treatment in NSCLC tumor models [122]. To explain such apparent contradiction; a study by An *et al* demonstrated that gefitinib similarly to cisplatin mediates hypoxia and HIF1α enrichment through the secretion of IL6 [123]. This is similar to the mechanism of cisplatin induced hypoxia and ALDH enrichment. Thus this may explain why short-term gefitinib treatment with cisplatin is effective but prone to resistance. Our results show elevated *PDK1* gene expression upon gefitinib exposure, supporting this mechanism of hypoxia resistance over time (Figure 7C). Our results with short-term cisplatin + gefitinib (CG) are effective; however, from the above literature; long-term treatment via IL6 secretion may mediate resistance. Thus metformin is required to combat the generation of resistance and increase anti-tumor efficacy through AMPK activation [120]. Additionally, research by Mishra *et al* further highlighted the ability of metformin to suppress IL6 mediated hypoxia and ALDH enrichment [124]. Such findings suggest that through the inhibition of metformin, the anti-tumorigenic properties of gefitinib may be maintained for long-term treatment and the cisplatin mediated hypoxia and ALDH enrichment can be ablated.

Our results demonstrate that the combination of CMG was successful at inhibiting the $ALDH^{high}$ population at clinically relevant dosages (Figure 12, Figure 13, Figure 16). Overall, the results of CMG on TNBC cell lines were optimistic enough to move forward to a more clinically translatable model.

4.4 Combinational treatment of cisplatin, metformin and gefitinib significantly reduce the CD44+/CD24- subpopulation of TNBC cell lines and PDX tumors

Wang *et al* studied tumor samples from a cohort of 145 TNBC patients who had not been treated with radiotherapy, chemotherapy, targeted therapy or adjuvant endocrine treatment prior to surgery, and tumor samples underwent double-staining immunohistochemistry with antibodies for CD44 and CD24 [125]. Notably, for patients with a $CD44^{+}/CD24^{-}$ phenotype, the risk of death was increased over 4-fold compared with patients with the $CD44^{-}/CD24^{-}$ counterparts (est HR: $CD44^{+}/CD24^{-}$ in CD44/CD24 status 4.38 (95% CI, 1.57–12.18))[125]. Based on our knowledge of the highly tumorigenic properties of $CD44^{+}/CD24^{-}$ subpopulation in TNBC from the pioneering work by Al Hajj *et al*, the significant increase in mortality found in Wang *et al*'s work can be reasoned, highlighting the intense need for TNBC treatments which are capable of targeting the $CD44^{+}/CD24^{-}$ population [13].

Our flow cytometry data revealed that the combination of CMG led to a robust decrease in the $CD44^{+}/CD24^{-}$ CSC subpopulation of both MDA MB-231 and SUM 149-PT TNBC cells (Figure 10, Figure 11). While the individual treatments of cisplatin, metformin and gefitinib were able to slightly reduce the $CD44^{+}/CD24^{-}$ as single treatments, and the double treatment combinations saw further moderately improved results (CM, CG, MG), it was only when all three drugs were used in combination that the effects were so magnified.

Variations in results between TNBC cell lines were found in the double treatments. For example in the combination of metformin and gefitinib (MG) reduced the $CD44^{+}/CD24^{-}$ CSCs in MDA MB-231 to 40%, but in SUM 149-PT cells the MG combination reduced it to 12% (Figure 10A, Figure 11A). These variations are not surprising among these two cell lines, SUM 149-PT cells are often used to characterize epithelial-like TNBC cells and MDA MB-231 cells

mesenchymal-like [20]. These difference in the single and double treatments between the two cell lines emphasizes the important of looking at multiple cell lines when researching a more consistent treatment.

4.5 Patient derived xenograft (PDX) and ex vivo slice culture is a more clinically translatable model to assess drug efficacy and CSC targeting

In vitro and *in vivo* preclinical research has for decades commonly used dozens of different TNBC cancer cell lines to study cancer pathogenesis, progression, and drug effectiveness [109]. Recent literature has shone a light on the growing disconnection between results generated using TNBC cell lines and what has been observed in clinical trials [109, 126-128]. This is not to say that there are not benefits to cell line work during preclinical research, in fact it is a productive first step, however *in vitro* cell lines have limitations as preclinical models because culturing cells selects for cell types that can grow in these foreign and artificial environments long term [109, 128].

Patient derived xenograft (PDX) models are an excellent resource available to researchers. PDXs are generated by transplanting patients' tumor tissue into an immunocompromised mouse, and from there they are expanded and serially passaged in mice [109, 129]. Unlike cell lines which lead to a loss of tumor architecture and heterogeneity, the PDX model can preserve tumor heterogeneity, architecture, stromal and extracellular components of the original patient tumor [109]. Zhang *et al* found that TNBC in comparison to hormone-receptor positive breast cancer worked particularly well at engrafting to the PDX model (53.8% vs. 15.6%, $p = 0.02$) [130, 131]. PDX tumors can be passaged and expanded 4-5 times, after that point the tumor stroma has been found to be replaced by the host mouse stroma which can influence results [127, 132].

Short-term *ex vivo* cultured PDXs have had success when used for pre-clinical drug screening, Bruna *et al* demonstrated that of 40 *ex vivo* cultures PDX tissues, over 80% were verified by *in vivo* PDX models [133]. These short-term *ex vivo* cultured PDXs retained the tissue architecture, as well as molecular and genetic features of the *in vivo* PDX models [133]. Our lab has previously found substantial result using both *in vivo* and *ex vivo* PDX models [108].

To verify my *in vitro* results from CMG treated cells in MDA MB-231 and Sum 149-PT cells I performed *ex vivo* organotypic slice cultures with different combinations on three TNBC patient-derived xenograft tumor fragments (HCI-001, HCI-002 and HCI-016) as well as two TNBC patient tumors (CRDCA and SEM-1) obtained from The Ottawa Hospital (Protocol# 20120559-01H) (Table 1). These specific PDX samples were selected to represent a range of patient drug sensitivity including: paclitaxel-resistant HCI-001 (obtained from a paclitaxel-treated patient with disease progression and paclitaxel-resistance) and paclitaxel-sensitive HCI-002 and HCI-016 (obtained from patient without prior exposure to chemotherapy). Tumor histology/immunohistology, clinical markers, drug treatment and response, whole exome sequencing, RNA sequencing, RPPA analysis, and growth curve for HCI-001, HCI-002 and HCI-016 have been described in detail in the spreadsheet on PDXNet [134] and in the supplemental results published elsewhere [135].

Cell viability results showed that neither cisplatin or metformin had much effect on any of the PDX and tumor samples viability (Figure 14, Supplementary Figure 6), while gefitinib as a single treatment demonstrated a decrease in viability depending on the sample but was not significant. Corresponding with our *in vitro* results, CMG effectively inhibited viability in all five of the slice cultures from TNBC patients (Figure 14).

To continue assessing CMG's effects on TNBC the more a clinically translatable model, I measured CSC enrichment following treatment on PDX and patient tumor *ex vivo* samples via

flow cytometry. CMG was able to inhibit the $CD44^{+}/CD24^{-}$ and $ALDH^{high}$ CSC population consistently across all patient tumor samples and PDX tumor fragments while single inhibitors demonstrated varied results. Conversely to the cell line results which demonstrated moderate efficacy of single inhibitors (Figures 10-13); the results generated between the models highlights key differences between 2D homologous cell line culture and 3D *ex vivo* tumor fragment response to CMG, highlighting the heterogeneity of clinical cancer tumors and the importance of using patient tumor samples for *in vitro* analyses to improve applicability. It is reasonable to expect that the future of preclinical work will include massive PDX libraries as described by Gao *et al* in 2015 with the Novartis Institutes for Biomedical Research patient-derived tumor xenograft encyclopedia (NIBR PDXE) of over 1,000 PDX samples [136]. Researchers have also begun using the predictive power of PDX models in co-clinical trials and personalized medicine [127].

5. CONCLUSION AND PERSPECTIVES

Despite progress being made in our understanding of triple negative breast cancer (TNBC), the overall survival and disease-free survival continues to be poorer than ER/PR/HER2+ breast cancer patients. Metastasis and chemoresistance are the pivotal issues holding back the long-term success of TNBC treatments. Over the past two decades, our understanding on the mechanisms behind chemoresistance and metastasis have expanded greatly. Cancer stem cells (CSCs) have emerged as major players in these mechanisms, making them important targets to destroy in addition to the bulk tumor cells during patients' first-line treatment. My work focused on demonstrating a combinational treatment approach to improve the long-term therapeutic potential of cisplatin for TNBC patients that not only targeted the bulk tumor population but also $ALDH^{high}$ and $CD44^{+}/CD24^{-}$ CSC populations.

Using database analysis, I identified hypoxia as a potential target as it was upregulated in TNBC compared to normal mammary tissue as well as in cisplatin-resistant ovarian cancer (Figure 5B and Supplementary Figure 1). I confirmed that hypoxia was upregulated following cisplatin treatment through gene analysis on treated MDA MB-231 cells (Figure 7). Consulting the literature, metformin (an AMPK activator) and gefitinib (an EGFR inhibitor) were added together and the combination was able to significantly reduce the cisplatin-induced hypoxia (Figure 7).

Viability analysis on TNBC cell lines (MDA MB 231 and SUM 149-PT) demonstrated that the triple-combination treatment of CMG (cisplatin 5μM, metformin 25μM, gefitinib 5μM) significantly reduced viability (Figure 4). Based on the literature and my database analysis TNBC has upregulated anti-apoptotic gene expression compared to normal mammary tissue (Figure 5C); however through flow cytometry analysis for Annexin V and 7-AAD I confirmed that CMG significantly induced apoptosis *in vitro* (Figure 9).

These viability and apoptosis findings were important in showing CMG's ability to kill bulk tumor cells, however to determine if CMG was able to target both epithelial and mesenchymal CSC populations. Using flow cytometry on SUM 149-PT and MDA MB-231 cells following treatment with CMG demonstrated potent inhibition of CD44+/CD24- CSCs populations. As previous reports have linked hypoxia to $ALDH^{high}$ CSC enrichment, it was not surprising that this finding was demonstrated in TNBC cells following cisplatin treatment leading to the enrichment of $ALDH^{high}$ cells (Figure 12 and Figure 13). The addition of metformin and gefitinib to the cisplatin reduced the $ALDH^{high}$ population demonstrating that CMG was not only killing the bulk tumor cells but it was also targeting the two main CSC populations identified in TNBC.

Upon our optimistic *in vitro* results using TNBC cell lines (MDA MB-231 and SUM 149-PT), I employed a patient derived xenograft (PDX) model and conducted viability and CSC analysis *ex vivo* on three PDX tumor slices and two patient tumor samples. In an *ex vivo* short-term experiments, I demonstrated that CMG significantly reduced the viability of all 5 PDX/clinical samples (Figure 14, Supplementary Figure 6). Flow cytometry showed that the $CD44^{+}/CD24^{-}$ and $ALDH^{high}$ populations were significantly reduced. These positive results are important as these samples represent the heterogeneity and variations between patients' response to treatment.

Future experiments could include an *in vivo* PDX mouse experiment where a serial dilution assay is performed following treatment as this is the gold standard to prove a reduction in tumorgenicity. An *in vivo* experiment would also be an improved model to assess CMGs effects on normal mammary cells as MCF-10A cells possessed substantially elevated EGFR protein expression making them unsuitable to be an appropriate control. Conducting further protein expression analysis, siRNA to knockdown EGFR and alternative AMPK activation agents (ex.

AICAR) would potentially facilitate our understanding of the mechanism behind how EGFR inhibition and AMPK activation together are able to reduce cisplatin induced hypoxia.

Overall, my project demonstrated both *in vitro* and *ex vivo* that cisplatin with the addition of metformin and gefitinib (CMG) was able to successfully kill the bulk tumor cells and target the $CD44^{+}/CD24^{-}$ and $ALDH^{high}$ CSC populations, demonstrating a potential approach to improve the long term-therapeutic potential of cisplatin, lowering the chance of relapse, chemoresistance and metastasis which could lead to increased patient prognosis for TNBC patients.

6. REFERENCES

1. Dagenais GR, Leong DP, Rangarajan S, Lanas F, Lopez-Jaramillo P, Gupta R, Diaz R, Avezum A, Oliveira GB, Wielgosz A: **Variations in common diseases, hospital admissions, and deaths in middle-aged adults in 21 countries from five continents (PURE): a prospective cohort study**. *The Lancet* 2020, **395**(10226):785-794.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A: **Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries**. *CA: a cancer journal for clinicians* 2018, **68**(6):394-424.
3. Burstein HJ, Temin S, Anderson H, Buchholz TA, Davidson NE, Gelmon KE, Giordano SH, Hudis CA, Rowden D, Solky AJ: **Adjuvant endocrine therapy for women with hormone receptor–positive breast cancer: American Society of Clinical Oncology clinical practice guideline focused update**. *Journal of Clinical Oncology* 2014, **32**(21):2255.
4. Loibl S, Gianni L: **HER2-positive breast cancer**. *The Lancet* 2017, **389**(10087):2415-2429.
5. Von Minckwitz G, Procter M, De Azambuja E, Zardavas D, Benyunes M, Viale G, Suter T, Arahmani A, Rouchet N, Clark E: **Adjuvant pertuzumab and trastuzumab in early HER2-positive breast cancer**. *New England Journal of Medicine* 2017, **377**(2):122-131.
6. Cameron D, Piccart-Gebhart MJ, Gelber RD, Procter M, Goldhirsch A, de Azambuja E, Castro Jr G, Untch M, Smith I, Gianni L: **11 years' follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive early breast cancer: final analysis of the HERceptin Adjuvant (HERA) trial**. *The Lancet* 2017, **389**(10075):1195-1205.
7. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, Gianni L, Baselga J, Bell R, Jackisch C: **Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer**. *New England Journal of Medicine* 2005, **353**(16):1659-1672.
8. Liedtke C, Mazouni C, Hess KR, André F, Tordai A, Mejia JA, Symmans WF, Gonzalez-Angulo AM, Hennessy B, Green M: **Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer**. *Journal of clinical oncology* 2008, **26**(8):1275-1281.
9. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Narod SA: **Triple-negative breast cancer: clinical features and patterns of recurrence**. *Clinical cancer research* 2007, **13**(15):4429-4434.
10. Haffty BG, Yang Q, Reiss M, Kearney T, Higgins SA, Weidhaas J, Harris L, Hait W, Toppmeyer D: **Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer**. *Journal of clinical oncology* 2006, **24**(36):5652-5657.
11. Lin NU, Vanderplas A, Hughes ME, Theriault RL, Edge SB, Wong YN, Blayney DW, Niland JC, Winer EP, Weeks JC: **Clinicopathologic features, patterns of recurrence, and survival among women with triple‐negative breast cancer in the National Comprehensive Cancer Network**. *Cancer* 2012, **118**(22):5463-5472.
12. Wahba HA, El-Hadaad HA: **Current approaches in treatment of triple-negative breast cancer**. *Cancer biology medicine* 2015, **12**(2):106.
13. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF: **Prospective identification of tumorigenic breast cancer cells**. *Proceedings of the National Academy of Sciences* 2003, **100**(7):3983-3988.
14. Liu S, Cong Y, Wang D, Sun Y, Deng L, Liu Y, Martin-Trevino R, Shang L, McDermott SP, Landis MD: **Breast cancer stem cells transition between epithelial and mesenchymal states reflective of their normal counterparts**. *Stem cell reports* 2014, **2**(1):78-91.
15. Turashvili G, Brogi E: **Tumor heterogeneity in breast cancer**. *Frontiers in medicine* 2017, **4**:227.

16. Li W, Ma H, Zhang J, Zhu L, Wang C, Yang Y: **Unraveling the roles of CD44/CD24 and ALDH1 as cancer stem cell markers in tumorigenesis and metastasis**. *Scientific reports* 2017, **7**(1):1-15.
17. Sulaiman A, McGarry S, Lam KM, El-Sahli S, Chambers J, Kaczmarek S, Li L, Addison C, Dimitroulakos J, Arnaout A: **Co-inhibition of mTORC1, HDAC and ESR1α retards the growth of triple-negative breast cancer and suppresses cancer stem cells**. *Cell death disease* 2018, **9**(8):1-14.
18. Ponta H, Sherman L, Herrlich PA: **CD44: from adhesion molecules to signalling regulators**. *Nature reviews Molecular cell biology* 2003, **4**(1):33-45.
19. Senbanjo LT, Chellaiah MA: **CD44: a multifunctional cell surface adhesion receptor is a regulator of progression and metastasis of cancer cells**. *Frontiers in cell developmental biology* 2017, **5**:18.
20. Sulaiman A, McGarry S, Han X, Liu S, Wang L: **CSCs in Breast Cancer—One Size Does Not Fit All: Therapeutic Advances in Targeting Heterogeneous Epithelial and Mesenchymal CSCs**. *Cancers* 2019, **11**(8):1128.
21. Park SY, Lee HE, Li H, Shipitsin M, Gelman R, Polyak K: **Heterogeneity for stem cell–related markers according to tumor subtype and histologic stage in breast cancer**. *Clinical Cancer Research* 2010, **16**(3):876-887.
22. Jaggupilli A, Elkord E: **Significance of CD44 and CD24 as cancer stem cell markers: an enduring ambiguity**. *Clinical and Developmental Immunology* 2012, **2012**.
23. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S: **ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome**. *Cell stem cell* 2007, **1**(5):555-567.
24. Marcato P, Dean CA, Giacomantonio CA, Lee PW: **Aldehyde dehydrogenase: its role as a cancer stem cell marker comes down to the specific isoform**. *Cell cycle* 2011, **10**(9):1378-1384.
25. Kastan MB, Schlaffer E, Russo J-E, Colvin OM, Civin CI, Hilton J: **Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells**. ***Blood*** 1990.
26. Wright MH, Calcagno AM, Salcido CD, Carlson MD, Ambudkar SV, Varticovski L: **Brca1 breast tumors contain distinct CD44+/CD24-and CD133+ cells with cancer stem cell characteristics**. *Breast Cancer Research* 2008, **10**(1):R10.
27. Liu T, Sun B, Zhao X, Zhao X, Sun T, Gu Q, Yao Z, Dong X, Zhao N, Liu N: **CD133+ cells with cancer stem cell characteristics associates with vasculogenic mimicry in triple-negative breast cancer**. *Oncogene* 2013, **32**(5):544-553.
28. Amoury M, Kolberg K, Pham A-T, Hristodorov D, Mladenov R, Di Fiore S, Helfrich W, Kiessling F, Fischer R, Pardo A: **Granzyme B-based cytolytic fusion protein targeting EpCAM specifically kills triple negative breast cancer cells in vitro and inhibits tumor growth in a subcutaneous mouse tumor model**. *Cancer letters* 2016, **372**(2):201-209.
29. Brugnoli F, Grassilli S, Lanuti P, Marchisio M, Al-Qassab Y, Vezzali F, Capitani S, Bertagnolo V: **Up-modulation of PLC-β2 reduces the number and malignancy of triple-negative breast tumor cells with a CD133+/EpCAM+ phenotype: a promising target for preventing progression of TNBC**. *BMC Cancer* 2017, **17**(1):617.
30. Lobba ARM, Carreira ACO, Cerqueira OLD, Fujita A, DeOcesano-Pereira C, Osorio CAB, Soares FA, Rameshwar P, Sogayar MC: **High CD90 (THY-1) expression positively correlates with cell transformation and worse prognosis in basal-like breast cancer tumors**. *PloS one* 2018, **13**(6).
31. Silva IA, Bai S, McLean K, Yang K, Griffith K, Thomas D, Ginestier C, Johnston C, Kueck A, Reynolds RK: **Aldehyde dehydrogenase in combination with CD133 defines angiogenic ovarian cancer stem cells that portend poor patient survival**. *Cancer research* 2011, **71**(11):3991-4001.
32. Bao B, Azmi AS, Ali S, Ahmad A, Li Y, Banerjee S, Kong D, Sarkar FH: **The biological kinship of hypoxia with CSC and EMT and their relationship with deregulated expression of miRNAs and**

tumor aggressiveness. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* 2012, **1826**(2):272-296.
33. Lu H, Samanta D, Xiang L, Zhang H, Hu H, Chen I, Bullen JW, Semenza GL: **Chemotherapy triggers HIF-1–dependent glutathione synthesis and copper chelation that induces the breast cancer stem cell phenotype**. *Proceedings of the National Academy of Sciences* 2015, **112**(33):E4600-E4609.
34. Semenza GL: **HIF-1: upstream and downstream of cancer metabolism**. *Current opinion in genetics development* 2010, **20**(1):51-56.
35. Wong RS: **Apoptosis in cancer: from pathogenesis to treatment**. *Journal of Experimental Clinical Cancer Research*

2011, **30**(1):87.
36. Kuo C-Y, Cheng C-T, Hou P, Lin Y-P, Ma H, Chung Y, Chi K, Chen Y, Li W, Kung H-J: **HIF-1-alpha links mitochondrial perturbation to the dynamic acquisition of breast cancer tumorigenicity**. *Oncotarget* 2016, **7**(23):34052.
37. Kim H, Lin Q, Yun Z: **BRCA1 regulates the cancer stem cell fate of breast cancer cells in the context of hypoxia and histone deacetylase inhibitors**. *Scientific reports* 2019, **9**(1):1-11.
38. Strowitzki MJ, Cummins EP, Taylor CT: **Protein hydroxylation by hypoxia-inducible factor (HIF) hydroxylases: unique or ubiquitous?** *Cells* 2019, **8**(5):384.
39. Sim J, Cowburn AS, Palazon A, Madhu B, Tyrakis PA, Macías D, Bargiela DM, Pietsch S, Gralla M, Evans CE: **The factor inhibiting HIF asparaginyl hydroxylase regulates oxidative metabolism and accelerates metabolic adaptation to hypoxia**. *Cell metabolism* 2018, **27**(4):898-913. e897.
40. Biddlestone J, Bandarra D, Rocha S: **The role of hypoxia in inflammatory disease**. *International journal of molecular medicine* 2015, **35**(4):859-869.
41. Sormendi S, Wielockx B: **HIF-pathway proteins: central regulators of tumor immunology**. *Transl Cancer Res* 2016, **5**(suppl 7):S1503-S1508.
42. Ziello JE, Jovin IS, Huang YJTYjob, medicine: **Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia**. 2007, **80**(2):51.
43. Shiraishi A, Tachi K, Essid N, Tsuboi I, Nagano M, Kato T, Yamashita T, Bando H, Hara H, Ohneda O: **Hypoxia promotes the phenotypic change of aldehyde dehydrogenase activity of breast cancer stem cells**. *Cancer science* 2017, **108**(3):362-372.
44. El Hout M, Cosialls E, Mehrpour M, Hamaï A: **Crosstalk between autophagy and metabolic regulation of cancer stem cells**. *Molecular Cancer* 2020, **19**(1):27.
45. Badowska-Kozakiewicz AM, Budzik MP: **Triple-Negative Breast Cancer: Expression of Hypoxia-Inducible Factor 1α in Triple-Negative Breast Cancer with Metastasis to Lymph Nodes**. In: *Breast Cancer and Surgery.* edn.: IntechOpen; 2018.
46. Badowska-Kozakiewicz A, Sobol M, Patera J: **Expression of hypoxia-inducible factor 1α in invasive breast Cancer with metastasis to lymph nodes: correlation with steroid receptors, HER2 and EPO-R**. *Advances in Clinical and Experimental Medicine* 2016, **25**(4):741-750.
47. Chen Y, Zhang B, Bao L, Jin L, Yang M, Peng Y, Kumar A, Wang JE, Wang C, Zou X: **ZMYND8 acetylation mediates HIF-dependent breast cancer progression and metastasis**. *The Journal of clinical investigation* 2018, **128**(5):1937-1955.
48. Go RS, Adjei AA: **Review of the comparative pharmacology and clinical activity of cisplatin and carboplatin**. *Journal of Clinical Oncology* 1999, **17**(1):409-409.
49. Johnstone TC, Suntharalingam K, Lippard SJ: **Third row transition metals for the treatment of cancer**. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences* 2015, **373**(2037):20140185.

50. Browning RJ, Reardon PJT, Parhizkar M, Pedley RB, Edirisinghe M, Knowles JC, Stride EJAn: **Drug delivery strategies for platinum-based chemotherapy**. 2017, **11**(9):8560-8578.
51. Hu X-C, Zhang J, Xu B-H, Cai L, Ragaz J, Wang Z-H, Wang B-Y, Teng Y-E, Tong Z-S, Pan Y-Y: **Cisplatin plus gemcitabine versus paclitaxel plus gemcitabine as first-line therapy for metastatic triple-negative breast cancer (CBCSG006): a randomised, open-label, multicentre, phase 3 trial**. *The lancet oncology* 2015, **16**(4):436-446.
52. Rodler ET, Kurland BF, Griffin M, Gralow JR, Porter P, Yeh RF, Gadi VK, Guenthoer J, Beumer JH, Korde L: **Phase I study of veliparib (ABT-888) combined with cisplatin and vinorelbine in advanced triple-negative breast cancer and/or BRCA mutation–associated breast cancer**. *Clinical Cancer Research* 2016, **22**(12):2855-2864.
53. Ferreira AR, Metzger-Filho O, Sarmento R, Bines J: **Neoadjuvant Treatment of Stage IIB/III Triple Negative Breast Cancer with Cyclophosphamide, Doxorubicin, and Cisplatin (CAP Regimen): A Single Arm, Single Center Phase II Study (GBECAM 2008/02)**. *Frontiers in oncology* 2018, **7**:329.
54. Deben C, Deschoolmeester V, De Waele J, Jacobs J, Van den Bossche J, Wouters A, Peeters M, Rolfo C, Smits E, Lardon F: **Hypoxia-induced cisplatin resistance in non-small cell lung cancer cells is mediated by HIF-1α and mutant p53 and can be overcome by induction of oxidative stress**. *Cancers* 2018, **10**(4):126.
55. Clark DW, Palle K: **Aldehyde dehydrogenases in cancer stem cells: potential as therapeutic targets**. *Annals of translational medicine* 2016, **4**(24).
56. Faubert B, Vincent EE, Poffenberger MC, Jones RG: **The AMP-activated protein kinase (AMPK) and cancer: many faces of a metabolic regulator**. *Cancer letters* 2015, **356**(2):165-170.
57. Mihaylova MM, Shaw RJ: **The AMPK signalling pathway coordinates cell growth, autophagy and metabolism**. *Nature cell biology* 2011, **13**(9):1016-1023.
58. Li M, Li X, Zhang H, Lu Y: **Molecular mechanisms of metformin for diabetes and cancer treatment**. *Frontiers in physiology* 2018, **9**:1039.
59. Meng S, Cao J, He Q, Xiong L, Chang E, Radovick S, Wondisford FE, He L: **Metformin activates AMP-activated protein kinase by promoting formation of the αβγ heterotrimeric complex**. *Journal of Biological Chemistry* 2015, **290**(6):3793-3802.
60. Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Mäkelä TP, Alessi DR, Hardie DG: **Complexes between the LKB1 tumor suppressor, STRADα/β and MO25α/β are upstream kinases in the AMP-activated protein kinase cascade**. *Journal of biology* 2003, **2**(4):28.
61. Inoki K, Zhu T, Guan K-L: **TSC2 mediates cellular energy response to control cell growth and survival**. *Cell* 2003, **115**(5):577-590.
62. Lee JO, Kang MJ, Byun WS, Kim SA, Seo IH, Han JA, Moon JW, Kim JH, Kim SJ, Lee EJ: **Metformin overcomes resistance to cisplatin in triple-negative breast cancer (TNBC) cells by targeting RAD51**. *Breast Cancer Research* 2019, **21**(1):1-18.
63. Li N, Huang D, Lu N, Luo LJOr: **Role of the LKB1/AMPK pathway in tumor invasion and metastasis of cancer cells**. 2015, **34**(6):2821-2826.
64. Nakai K, Hung M-C, Yamaguchi H: **A perspective on anti-EGFR therapies targeting triple-negative breast cancer**. *American journal of cancer research* 2016, **6**(8):1609.
65. Scaltriti M, Baselga J: **The epidermal growth factor receptor pathway: a model for targeted therapy**. *Clinical Cancer Research* 2006, **12**(18):5268-5272.
66. Araki T, Yashima H, Shimizu K, Aomori T, Hashita T, Kaira K, Nakamura T, Yamamoto K: **Review of the treatment of non-small cell lung cancer with gefitinib**. *Clinical Medicine Insights: Oncology* 2012, **6**:CMO. S7340.
67. Wheeler DL, Dunn EF, Harari PMJNrCo: **Understanding resistance to EGFR inhibitors—impact on future treatment strategies**. *Nature reviews Clinical oncology* 2010, **7**(9):493.

68. Rho JK, Choi YJ, Lee JK, Ryoo B-Y, Na II, Yang SH, Kim CH, Yoo YD, Lee JC: **Gefitinib circumvents hypoxia-induced drug resistance by the modulation of HIF-1α**. *Oncology reports* 2009, **21**(3):801-807.
69. Ohta T, Ohmichi M, Shibuya T, Takahashi T, Tsutsumi S, Takahashi K, Kurachi H: **Gefitinib (ZD1839) increases the efficacy of cisplatin in ovarian cancer cells**. *Cancer biology therapy* 2012, **13**(6):408-416.
70. Liu S, Yang H, Ge X, Su L, Zhang A, Liang L: **Drug resistance analysis of gefitinib-targeted therapy in non-small cell lung cancer**. *Oncology letters* 2016, **12**(5):3941-3943.
71. Minakata K, Takahashi F, Nara T, Hashimoto M, Tajima K, Murakami A, Nurwidya F, Yae S, Koizumi F, Moriyama H: **Hypoxia induces gefitinib resistance in non‐small‐cell lung cancer with both mutant and wild‐type epidermal growth factor receptors**. *Cancer science* 2012, **103**(11):1946-1954.
72. Huang W-C, Chen Y-J, Li L-Y, Wei Y-L, Hsu S-C, Tsai S-L, Chiu P-C, Huang W-P, Wang Y-N, Chen C-HJJoBC: **Nuclear translocation of epidermal growth factor receptor by Akt-dependent phosphorylation enhances breast cancer-resistant protein expression in gefitinib-resistant cells**. *Journal of Biological Chemistry* 2011, **286**(23):20558-20568.
73. Morgillo F, Sasso FC, Della Corte CM, Vitagliano D, D'aiuto E, Troiani T, Martinelli E, De Vita F, Orditura M, De Palma R: **Synergistic effects of metformin treatment in combination with gefitinib, a selective EGFR tyrosine kinase inhibitor, in LKB1 wild-type NSCLC cell lines**. *Clinical Cancer Research* 2013, **19**(13):3508-3519.
74. Peng M, Huang Y, Tao T, Peng C-Y, Su Q, Xu W, Darko KO, Tao X, Yang XJSr: **Metformin and gefitinib cooperate to inhibit bladder cancer growth via both AMPK and EGFR pathways joining at Akt and Erk**. *Scientific reports* 2016, **6**(1):1-10.
75. Sulaiman A, Sulaiman B, Khouri L, McGarry S, Nessim C, Arnaout A, Li X, Addison C, Dimitroulakos J, Wang L: **Both bulk and cancer stem cell subpopulations in triple‐negative breast cancer are susceptible to Wnt, HDAC, and ERα coinhibition**. *FEBS letters* 2016, **590**(24):4606-4616.
76. Sulaiman A, McGarry S, Lam KM, El-Sahli S, Chambers J, Kaczmarek S, Li L, Addison C, Dimitroulakos J, Arnaout A: **Co-inhibition of mTORC1, HDAC and ESR1α retards the growth of triple-negative breast cancer and suppresses cancer stem cells**. *Cell death & disease* 2018, **9**(8):815.
77. Safran M, Kim WY, O'Connell F, Flippin L, Günzler V, Horner JW, DePinho RA, Kaelin WG: **Mouse model for noninvasive imaging of HIF prolyl hydroxylase activity: assessment of an oral agent that stimulates erythropoietin production**. *Proceedings of the National Academy of Sciences* 2006, **103**(1):105-110.
78. Emerling BM, Weinberg F, Liu J-L, Mak TW, Chandel NS: **PTEN regulates p300-dependent hypoxia-inducible factor 1 transcriptional activity through Forkhead transcription factor 3a (FOXO3a)**. *Proceedings of the National Academy of Sciences* 2008, **105**(7):2622-2627.
79. Chen X, Prywes R: **Serum-induced expression of the cdc25AGene by relief of E2F-mediated repression**. *Molecular and Cellular Biology* 1999, **19**(7):4695-4702.
80. Edgar R, Domrachev M, Lash AE: **Gene Expression Omnibus: NCBI gene expression and hybridization array data repository**. *Nucleic acids research* 2002, **30**(1):207-210.
81. Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Holko M: **NCBI GEO: archive for functional genomics data sets—update**. *Nucleic acids research* 2012, **41**(D1):D991-D995.
82. Komatsu M, Yoshimaru T, Matsuo T, Kiyotani K, Miyoshi Y, Tanahashi T, Rokutan K, Yamaguchi R, Saito A, Imoto S: **Molecular features of triple negative breast cancer cells by genome-wide gene expression profiling analysis**. *International journal of oncology* 2013, **42**(2):478-506.

83. Li M, Balch C, Montgomery JS, Jeong M, Chung JH, Yan P, Huang TH, Kim S, Nephew KP: **Integrated analysis of DNA methylation and gene expression reveals specific signaling pathways associated with platinum resistance in ovarian cancer**. *BMC medical genomics* 2009, **2**(1):34.
84. Li W, Hou J-Z, Niu J, Xi Z-Q, Ma C, Sun H, Wang C-J, Fang D, Li Q, Xie S-Q: **Akt1 inhibition promotes breast cancer metastasis through EGFR-mediated β-catenin nuclear accumulation**. *Cell Communication and Signaling* 2018, **16**(1):1-13.
85. Saji M, Narahara K, McCarty SK, Vasko VV, La Perle KM, Porter K, Jarjoura D, Lu C, Cheng S-Y, Ringel MD: **Akt1 deficiency delays tumor progression, vascular invasion, and distant metastasis in a murine model of thyroid cancer**. *Oncogene* 2011, **30**(42):4307-4315.
86. Li C-W, Xia W, Lim S-O, Hsu JL, Huo L, Wu Y, Li L-Y, Lai C-C, Chang S-S, Hsu Y-H: **AKT1 inhibits epithelial-to-mesenchymal transition in breast cancer through phosphorylation-dependent Twist1 degradation**. *Cancer research* 2016, **76**(6):1451-1462.
87. Ooms LM, Binge LC, Davies EM, Rahman P, Conway JR, Gurung R, Ferguson DT, Papa A, Fedele CG, Vieusseux JL: **The inositol polyphosphate 5-phosphatase PIPP regulates AKT1-dependent breast cancer growth and metastasis**. *Cancer cell* 2015, **28**(2):155-169.
88. Ignacio RMC, Gibbs CR, Lee E-S, Son D-S: **The TGFα-EGFR-Akt signaling axis plays a role in enhancing proinflammatory chemokines in triple-negative breast cancer cells**. *Oncotarget* 2018, **9**(50):29286.
89. K Lau Y-T, Ramaiyer M, E Johnson D, R Grandis J: **Targeting STAT3 in Cancer with Nucleotide Therapeutics**. *Cancers* 2019, **11**(11):1681.
90. Zhong Z, Wen Z, Darnell JE: **Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6**. *Science* 1994, **264**(5155):95-98.
91. Carbognin E, Betto RM, Soriano ME, Smith AG, Martello G: **Stat3 promotes mitochondrial transcription and oxidative respiration during maintenance and induction of naive pluripotency**. *The EMBO journal* 2016, **35**(6):618-634.
92. Lee JW, Ko J, Ju C, Eltzschig HK: **Hypoxia signaling in human diseases and therapeutic targets**. *Experimental & molecular medicine* 2019, **51**(6):68.
93. Krishnamachary B, Penet M-F, Nimmagadda S, Mironchik Y, Raman V, Solaiyappan M, Semenza GL, Pomper MG, Bhujwalla ZM: **Hypoxia regulates CD44 and its variant isoforms through HIF-1α in triple negative breast cancer**. *PLoS One* 2012, **7**(8).
94. Peng S-T, Su C-H, Kuo C-C, Shaw C-F, Wang H-S: **CD44 crosslinking-mediated matrix metalloproteinase-9 relocation in breast tumor cells leads to enhanced metastasis**. *International journal of oncology* 2007, **31**(5):1119-1126.
95. Schwab LP, Peacock DL, Majumdar D, Ingels JF, Jensen LC, Smith KD, Cushing RC, Seagroves TN: **Hypoxia-inducible factor 1α promotes primary tumor growth and tumor-initiating cell activity in breast cancer**. *Breast Cancer Research* 2012, **14**(1):R6.
96. Bernardi R, Gianni L: **Hallmarks of triple negative breast cancer emerging at last?** *Cell research* 2014, **24**(8):904-905.
97. Campbell KJ, Dhayade S, Ferrari N, Sims AH, Johnson E, Mason SM, Dickson A, Ryan KM, Kalna G, Edwards J: **MCL-1 is a prognostic indicator and drug target in breast cancer**. *Cell Death & Disease* 2018, **9**(2):1-14.
98. Kelly GL, Strasser A: **The essential role of evasion from cell death in cancer**. In: *Advances in cancer research. Volume 111*, edn.: Elsevier; 2011: 39-96.
99. Wang L, Liu X, Ren Y, Zhang J, Chen J, Zhou W, Guo W, Wang X, Chen H, Li M: **Cisplatin-enriching cancer stem cells confer multidrug resistance in non-small cell lung cancer via enhancing TRIB1/HDAC activity**. *Cell Death & Disease* 2017, **8**(4):e2746-e2746.

100. McKillop D, Partridge EA, Kemp JV, Spence MP, Kendrew J, Barnett S, Wood PG, Giles PB, Patterson AB, Bichat FJMct: **Tumor penetration of gefitinib (Iressa), an epidermal growth factor receptor tyrosine kinase inhibitor**. *Molecular cancer therapeutics* 2005, **4**(4):641-649.
101. McCreight LJ, Stage TB, Connelly P, Lonergan M, Nielsen F, Prehn C, Adamski J, Brøsen K, Pearson ER: **Pharmacokinetics of metformin in patients with gastrointestinal intolerance**. *Diabetes, Obesity Metabolism*

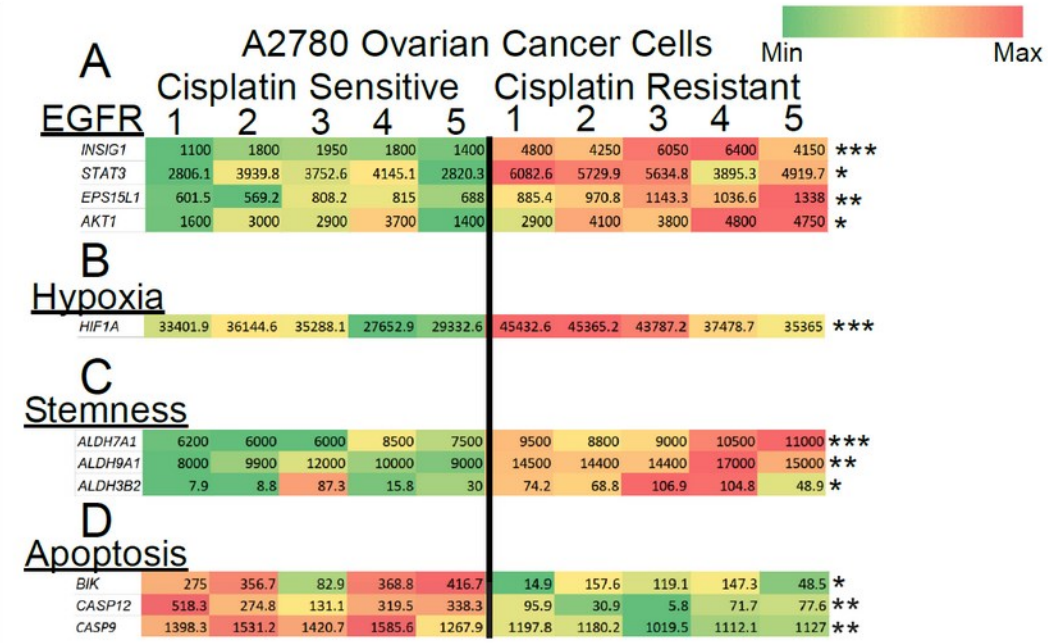
2018, **20**(7):1593-1601.
102. Cohen MH, Williams GA, Sridhara R, Chen G, Pazdur R: **FDA drug approval summary: gefitinib (ZD1839)(Iressa) tablets**. *The oncologist* 2003, **8**(4):303-306.
103. Van Meerloo J, Kaspers GJ, Cloos J: **Cell sensitivity assays: the MTT assay**. In: *Cancer cell culture.* edn.: Springer; 2011: 237-245.
104. Vermes I, Haanen C, Steffens-Nakken H, Reutellingsperger C: **A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V**. *Journal of immunological methods* 1995, **184**(1):39-51.
105. Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RAB, Henson PM: **A receptor for phosphatidylserine-specific clearance of apoptotic cells**. *Nature* 2000, **405**(6782):85-90.
106. Lecoeur H, Ledru E, Prévost M-C, Gougeon M-L: **Strategies for phenotyping apoptotic peripheral human lymphocytes comparing ISNT, annexin-V and 7-AAD cytofluorometric staining methods**. *Journal of immunological methods* 1997, **209**(2):111-123.
107. Dong HP, Holth A, Kleinberg L, Ruud MG, Elstrand MB, Tropé CG, Davidson B, Risberg B: **Evaluation of cell surface expression of phosphatidylserine in ovarian carcinoma effusions using the annexin-V/7-AAD assay: clinical relevance and comparison with other apoptosis parameters**. *American journal of clinical pathology* 2009, **132**(5):756-762.
108. Sulaiman A, McGarry S, Li L, Jia D, Ooi S, Addison C, Dimitroulakos J, Arnaout A, Nessim C, Yao Z: **Dual inhibition of Wnt and Yes‐associated protein signaling retards the growth of triple‐negative breast cancer in both mesenchymal and epithelial states**. *Molecular oncology* 2018, **12**(4):423-440.
109. Sulaiman A, Wang L: **Bridging the divide: preclinical research discrepancies between triple-negative breast cancer cell lines and patient tumors**. *Oncotarget* 2017, **8**(68):113269.
110. O'brien J, Wilson I, Orton T, Pognan F: **Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity**. *European journal of biochemistry* 2000, **267**(17):5421-5426.
111. Rampersad SN: **Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays**. *Sensors* 2012, **12**(9):12347-12360.
112. Brenner DR, Weir HK, Demers AA, Ellison LF, Louzado C, Shaw A, Turner D, Woods RR, Smith LM: **Projected estimates of cancer in Canada in 2020**. *Cmaj* 2020, **192**(9):E199-E205.
113. Hanahan D, Weinberg RA: **Hallmarks of cancer: the next generation**. *Cell* 2011, **144**(5):646-674.
114. Feitelson MA, Arzumanyan A, Kulathinal RJ, Blain SW, Holcombe RF, Mahajna J, Marino M, Martinez-Chantar ML, Nawroth R, Sanchez-Garcia I: **Sustained proliferation in cancer: Mechanisms and novel therapeutic targets**. In: *Seminars in cancer biology: 2015*: Elsevier; 2015: S25-S54.
115. Qu Y, Han B, Yu Y, Yao W, Bose S, Karlan BY, Giuliano AE, Cui X: **Evaluation of MCF10A as a reliable model for normal human mammary epithelial cells**. *PloS one* 2015, **10**(7):e0131285.
116. Rosenbluth JM, Schackmann RC, Gray GK, Selfors LM, Li CM-C, Boedicker M, Kuiken HJ, Richardson A, Brock J, Garber J: **Organoid cultures from normal and cancer-prone human breast tissues preserve complex epithelial lineages**. *Nature communications* 2020, **11**(1):1-14.

117. Subik K, Lee J-F, Baxter L, Strzepek T, Costello D, Crowley P, Xing L, Hung M-C, Bonfiglio T, Hicks DG: **The expression patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by immunohistochemical analysis in breast cancer cell lines**. *Breast cancer: basic clinical research* 2010, **4**:117822341000400004.
118. Kerr JF, Wyllie AH, Currie AR: **Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics**. *British journal of cancer* 1972, **26**(4):239-257.
119. Zhang F, Duan S, Tsai Y, Keng PC, Chen Y, Lee SO, Chen Y: **Cisplatin treatment increases stemness through upregulation of hypoxia‐inducible factors by interleukin‐6 in non‐small cell lung cancer**. *Cancer Science* 2016, **107**(6):746-754.
120. Shank JJ, Yang K, Ghannam J, Cabrera L, Johnston CJ, Reynolds RK, Buckanovich RJ: **Metformin targets ovarian cancer stem cells in vitro and in vivo**. *Gynecologic oncology* 2012, **127**(2):390-397.
121. Khalil A, Jameson MJ: **The EGFR inhibitor gefitinib enhanced the response of human oral squamous cell carcinoma to cisplatin in vitro**. *British journal of cancer* 2017, **17**(4):545-555.
122. Tsai C-M, Chen J-T, Stewart DJ, Chiu C-H, Lai C-L, Hsiao S-Y, Chen Y-M, Chang K-T: **Antagonism between gefitinib and cisplatin in non-small cell lung cancer cells: why randomized trials failed?** *Journal of Thoracic Oncology* 2011, **6**(3):559-568.
123. An SM, Lei HM, Ding XP, Sun F, Zhang C, Tang YB, Chen HZ, Shen Y, Zhu L: **Interleukin-6 identified as an important factor in hypoxia-and aldehyde dehydrogenase-based gefitinib adaptive resistance in non-small cell lung cancer cells**. *Oncology Letters* 2017, **14**(3):3445-3454.
124. Mishra AK, Dingli D: **Metformin inhibits IL-6 signaling by decreasing IL-6R expression on multiple myeloma cells**. *Leukemia* 2019, **33**(11):2695-2709.
125. Wang H, Wang L, Song Y, Wang S, Huang X, Xuan Q, Kang X, Zhang Q: **CD44+/CD24-phenotype predicts a poor prognosis in triple-negative breast cancer**. *Oncology letters* 2017, **14**(5):5890-5898.
126. Savage P, Pacis A, Kuasne H, Liu L, Lai D, Wan A, Dankner M, Martinez C, Muñoz-Ramos V, Pilon V: **Chemogenomic profiling of breast cancer patient-derived xenografts reveals targetable vulnerabilities for difficult-to-treat tumors**. *Communications Biology* 2020, **3**(1):1-15.
127. Hidalgo M, Amant F, Biankin AV, Budinská E, Byrne AT, Caldas C, Clarke RB, de Jong S, Jonkers J, Mælandsmo GM: **Patient-derived xenograft models: an emerging platform for translational cancer research**. *Cancer discovery* 2014, **4**(9):998-1013.
128. Kerbel RS: **Human tumor xenografts as predictive preclinical models for anticancer drug activity in humans: better than commonly perceived—but they can be improved**

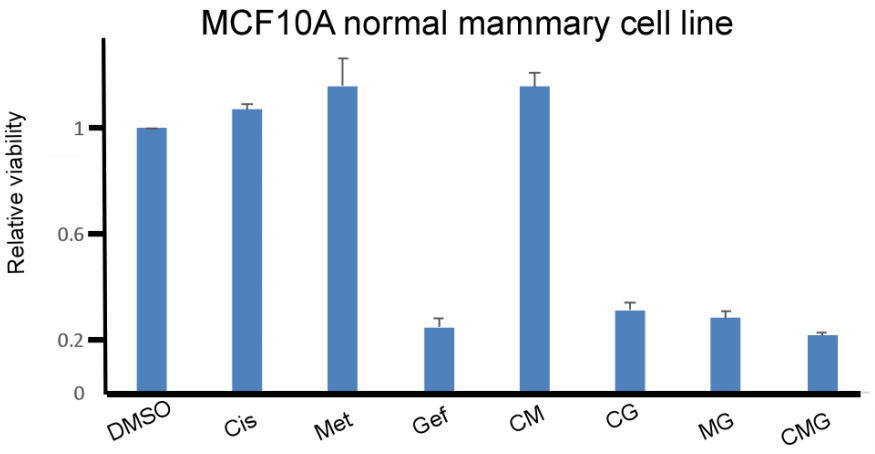
Cancer biology & therapy 2003, **2**(sup1):133-138.
129. Kim MP, Evans DB, Wang H, Abbrusseze JL, Fleming JB, Gallick GE: **Orthotopic and heterotopic generation of murine pancreatic cancer xenografts**. *Nature protocols* 2009, **4**(11):1670.
130. Zhang X, Claerhout S, Prat A, Dobrolecki LE, Petrovic I, Lai Q, Landis MD, Wiechmann L, Schiff R, Giuliano M: **A renewable tissue resource of phenotypically stable, biologically and ethnically diverse, patient-derived human breast cancer xenograft models**. *Cancer research* 2013, **73**(15):4885-4897.
131. McAuliffe PF, Evans KW, Akcakanat A, Chen K, Zheng X, Zhao H, Eterovic AK, Sangai T, Holder AM, Sharma CJPo: **Ability to generate patient-derived breast cancer xenografts is enhanced in chemoresistant disease and predicts poor patient outcomes**. *PloS one* 2015, **10**(9):e0136851.
132. McAuliffe PF, Evans KW, Akcakanat A, Chen K, Zheng X, Zhao H, Eterovic AK, Sangai T, Holder AM, Sharma C: **Ability to generate patient-derived breast cancer xenografts is enhanced in chemoresistant disease and predicts poor patient outcomes**. *PloS one* 2015, **10**(9):e0136851.

133. Bruna A, Rueda OM, Greenwood W, Batra AS, Callari M, Batra RN, Pogrebniak K, Sandoval J, Cassidy JW, Tufegdzic-Vidakovic A: **A biobank of breast cancer explants with preserved intra-tumor heterogeneity to screen anticancer compounds**. *Cell* 2016, **167**(1):260-274. e222.
134. PDXNet: **PDX DEVELOPMENT AND TRIAL CENTERS RESEARCH NETWORK**. *https://brandi-davis-7wsfsquarespacecom/hcibcm*.
135. DeRose YS, Wang G, Lin YC, Bernard PS, Buys SS, Ebbert MT, Factor R, Matsen C, Milash BA, Nelson E *et al*: **Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes**. *Nat Med* 2011, **17**(11):1514-1520.
136. Gao H, Korn JM, Ferretti S, Monahan JE, Wang Y, Singh M, Zhang C, Schnell C, Yang G, Zhang Y: **High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response**. *Nature medicine* 2015, **21**(11):1318-1325.

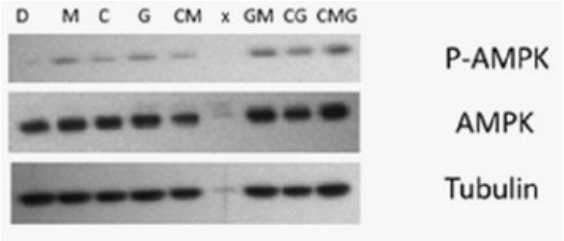
7. SUPPLEMENTARY FIGURES



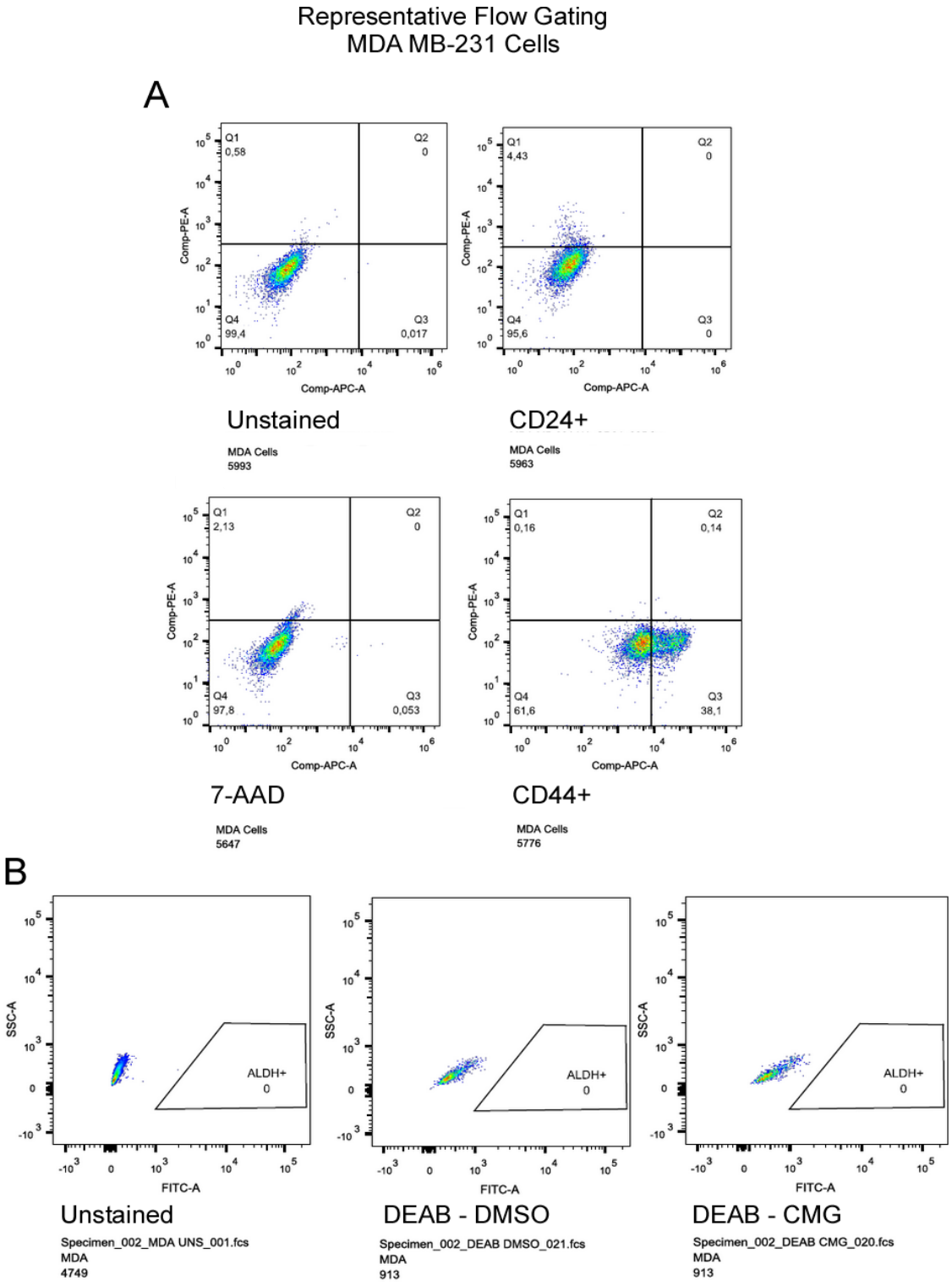
Supplementary Figure 1: Cisplatin resistance is associated with an enrichment of hypoxia, EGFR, stemness and anti-apoptosis related gene expression in ovarian cancer. (A-D) The relative expression levels (A.U arbitrary unit) of genes in 5 cisplatin sensitive and 5 cisplatin resistant A2780 Ovarian cancer samples were compared using the NCBI Gene Expression Omnibus (GEO2R). The GSE15709 samples were analyzed using the GPL570 Affymetrix Human Genome U133 Plus 2.0 Array and the values were profiled in a heat map with green representing the row minimum and red representing the row maximum per gene analysis.



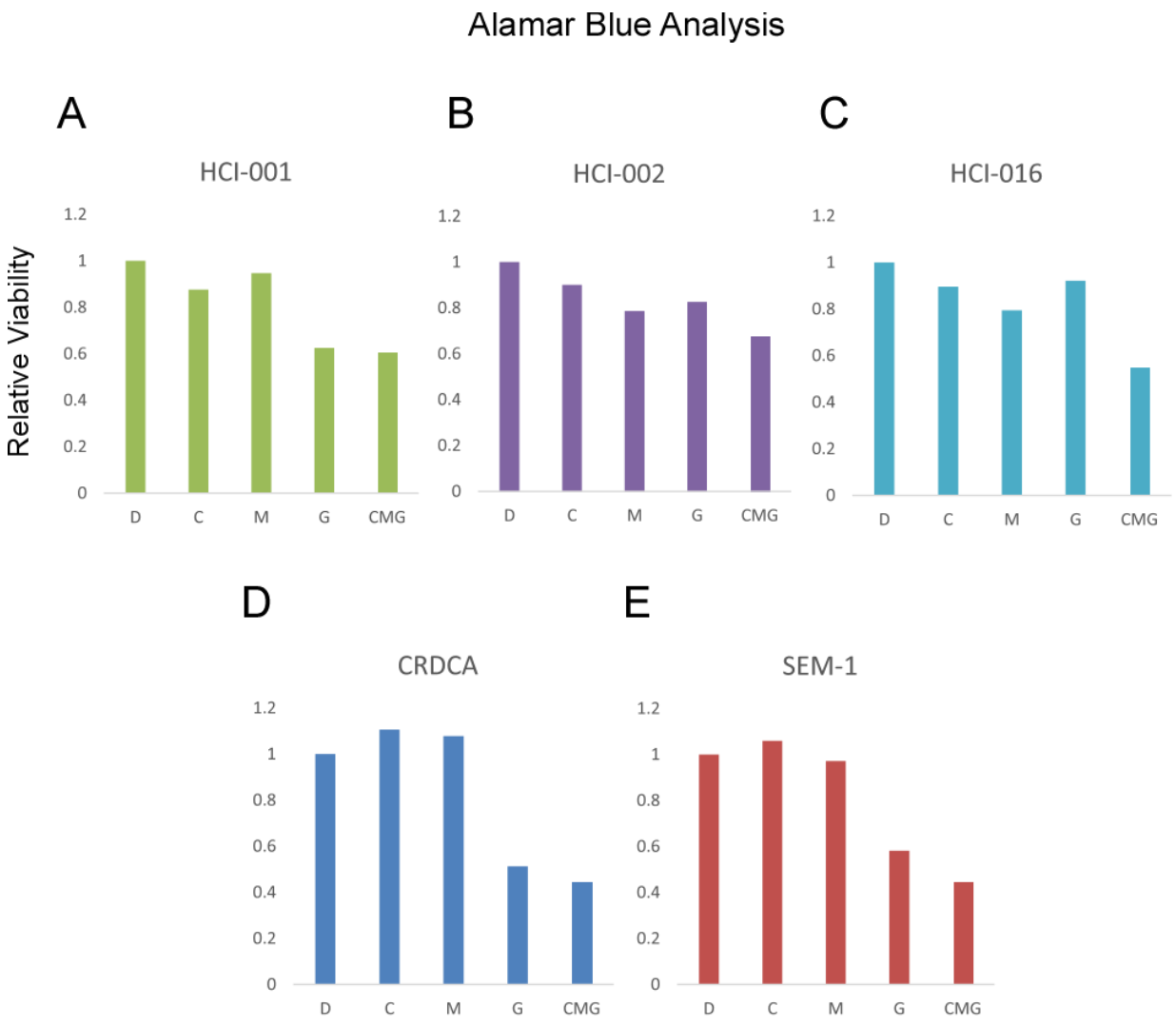
Supplementary Figure 2: CMG combination effect on viability of MCF-10A normal mammary cell line. Cell viability of MCF-10A normal mammary cell line after 120 hours of exposure to DMSO vehicle control or the following drugs in different concentrations: 5µM of cisplatin, 25µM of metformin and/or 5µM of gefitinib. Drugs were added at 0 hours and 72 hours (n=3).



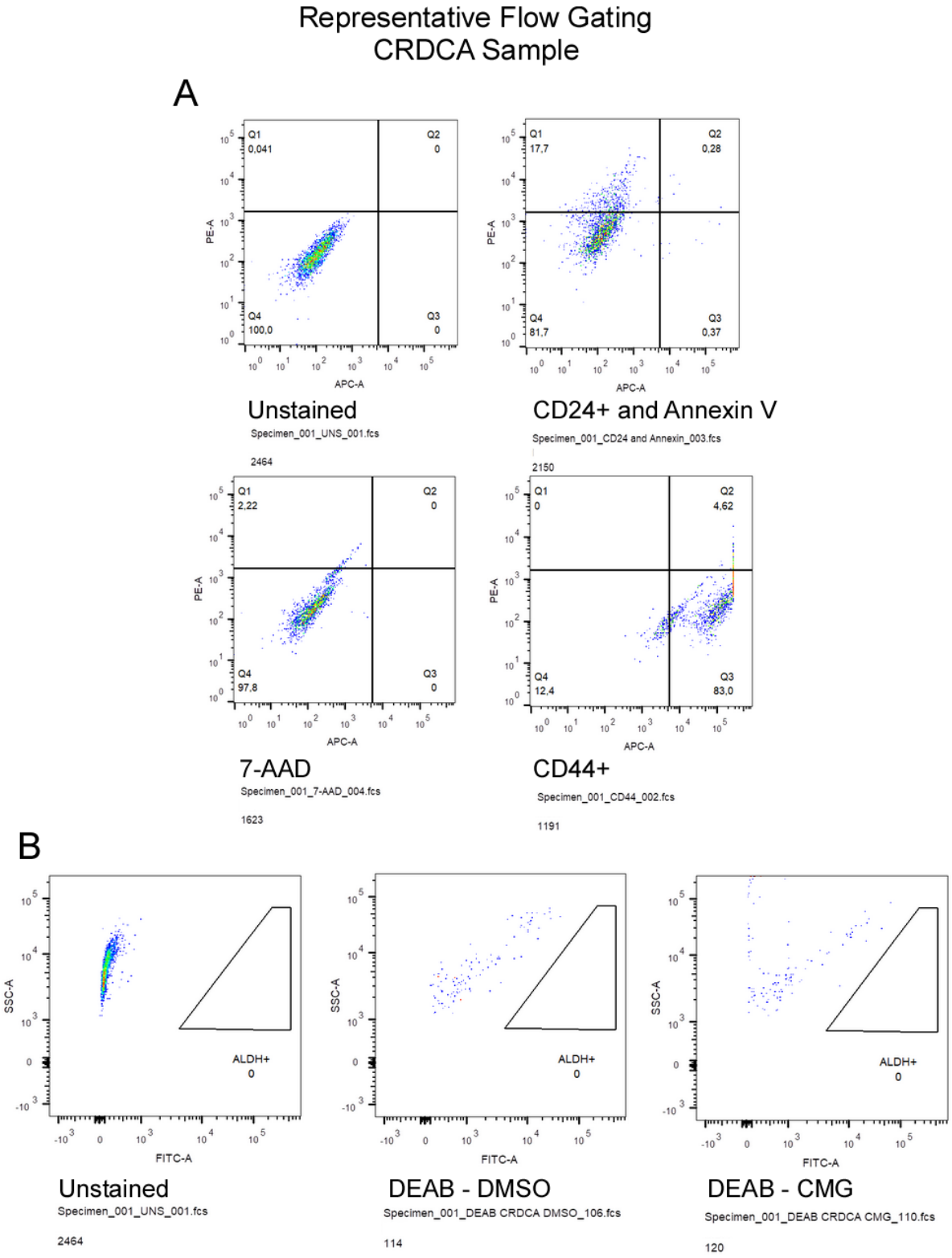
Supplementary Figure 3: Metformin induces AMPK and this is magnified in the combination treatment of CMG. Representative western blots of AMPK and p-AMPK expression in MDA MB-231 cells after 48 hours of exposure to the DMSO vehicle control and drugs in different combinations: 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib. Drugs were added at 0 hour and 72 hours.0



Supplementary Figure 4: Flow gating of MDA MB-231 cell line. Representative flow gating plots of MDA MB-231 cells after 120 hours of treatment with single, double or CMG combinations. Cells were single stained with PE- or APC-conjugated anti-CD24 and/or anti-CD44 antibodies and analyzed using the BD LSRFortessa.



Supplementary Figure 5: CMG combination significantly reduces viability of organotypic cultures of TNBC PDX and patient samples. Relative viability of three patient-derived xenograft samples **(A)** HCI-001, **(B)** HCI-002, **(C)** HCI-026, and two primary patient TNBC tumors **(D)** CRDCA, **(E)** SEM-1. Relative Alamar blue viability analysis after 144 hours of exposure to the DMSO vehicle control and drugs in different combinations: 5μM of cisplatin, 25μM of metformin and/or 5μM of gefitinib. Drugs were added at 0 hour and 72 hours. Tumor samples were cut into approximately 2 × 1 mm tumor slices and cultured *ex vivo*. Alamar Blue (resazurin) was added and the resazurin is metabolized by live cells in the medium and turned to pink and highly fluorescent resorufin that was measured using a spectrometer at 560 excitation and 590 emission. Viability was calculated based on OD values at 144 hours – OD values at 0 hour.



***Supplementary Figure* 6: Flow gating of PDX/clinical samples.** Representative flow gating plots of CRDCA clinical sample after 120 hours of treatment with single, double or CMG combinations. Cells were single stained with PE- or APC-conjugated anti-CD24 and/or anti-CD44 antibodies and analyzed using the BD LSRFortessa.

8. APPENDIX

8.1 Curriculum Vitae

EDUCATION

Master of Science, Biochemistry, University of Ottawa, Ottawa, 2018-present
- Thesis: Inhibition of Hypoxia Sensitizes TNBC to Cisplatin and Promotes Bulk and CSC Inhibition
- Supervisor: Dr. Lisheng Wang

Master of Arts, History, Wilfrid Laurier University, Waterloo, 2015
- Major Research Project: Everyday 'Martyrs' of the Great War: The Moral Regulation of War Widows in Ontario, 1914 - 1938
- Supervisor: Dr. Cynthia Comacchio

Bachelor of Arts with Honours, History, Carleton University, Ottawa, 2014
- Undergraduate Project: A Tightrope Walk Towards the Easter Rising Centenary in 2016: Northern Ireland and the Republic of Ireland Debate the Issues Through Newspapers
- Supervisor: Dr. Aleksandra Bennett

RESEARCH EXPERIENCE

Graduate Researcher
University of Ottawa, Faculty of Medicine, Ottawa
Primary Investigator: Dr. Lisheng Wang
2018 to present
- Adept at cell culture, patient derived xenograft culture, primary patient tumor cultivation, western blotting, and qPCR experiments to generate data in triple negative breast cancer models.
- Experienced with transfection for gene enrichment/knockdown as well as reporter analysis.
- Trained and proficient at intraperitoneal, subcutaneous and intravenous tail injections in mice (both immunocompetent and immunocompromised)
- Capable of cell line and patient derived xenograft implantation mice surgery. Additionally, preformed autopsies, took biopsies and performed *in vivo* perfusion.
- Experienced in Flow Cytometry preparation, FACs sorting, and *in vivo* imaging system (IVIS) for fluorophore detection *in vivo*.

Visiting Graduate Researcher
Dr. Guang Ji, Longhua Hospital, Shanghai
May- June 2019
- Collaborated with Dr. Ji, Dr. Han and Dr. Song in establishing a clinically relevant liver and breast cancer models *in vivo*.

- Worked to engraft current patient tumors into immunocompromised mice for the development of patient derived xenograft models.
- Mentored graduate students in various laboratory procedures (eg. Mouse dissections, tumor implantations, plasmid extractions, transfection, FACs, etc)
- Delivered two seminar presentations to discuss current research and the field of oncology and graduate life in Canada

Volunteer Lab Member
University of Ottawa, Faculty of Medicine, Ottawa
Supervisor: Andrew Sulaiman
Primary Investigator: Dr. Lisheng Wang
2015 to 2018

- Assisted in primary breast cancer stem cells research *in vitro* through qPCR, western blot, FACS preparation, viability analysis and transfection.
- Aided in literature search, project design and data interpretation.
- Provided editing and feedback for manuscripts in preparation of submission.
- Assisted in *in vivo* experimentation through organ excision, dissection, FACS preparation of organs and tumors, weighing tumors. Aid in preparation for IVIS analysis of flourophores within mouse organs/tumors.
- Trained and supervise the work of undergraduate honors students and volunteers
- Conducted screening and interviews for potential undergraduate thesis and volunteer candidates
- Presented research at conferences.

Master of Arts (History) Candidate
Wilfrid Laurier University, Waterloo
Supervisor: Dr. Cynthia Comacchio
2014 to 2015

- Conducted graduate level research on various topics including, but not limited to: class and gender as it pertained to the moral regulation of First World War widow pensions, race and psychiatry in nineteenth-century American and Canadian asylums, gender and the Temperance movement, the civilian evacuation of Britain during the Second World War, and Waterloo regions experiences during the First World War

Co-Leader, Researcher and Social Media Coordinator
Waterloo Region During the First World War Website, Waterloo
Supervisor: Dr. Heather MacDougall
2015

- Responsible for co-leading a group of graduate students, setting objectives, priorities, managing resources, preparing briefing material and summaries
- Conducted individual research on Waterloo Region war dead from 1915 and wrote short summaries on each for the new “Commemoration” section of the website
- Consulted with the media on a news article containing my original research
- The group was the recipient of the Waterloo Regional Heritage Research Award, worth $3000

Research Assistant
Laurier Centre for Military Strategic and Disarmament Studies, Waterloo
Supervisor: Dr. Mark Humphries
2014 to 2015

- Transcribed handwritten diaries of a First World War Medical Officer for the SSHRC-funded "Through Veterans' Eyes" project
- Attended presentations given by guest lecturers on a variety of military history research topics

LEADERSHIP EXPERIENCE

Research Supervisor
Dr. Lisheng Wang Lab, University of Ottawa, Faculty of Medicine, Ottawa
2018 to 2020

- Train and supervise an undergraduate thesis students' honors project

Vice President of Finance
BMIGSA, University of Ottawa, Faculty of Medicine, Ottawa
2018 to 2020

- Ensure that BMIGSA funds are spent responsibly and in the best interests of the students
- Organize and plan both academic and social events for BMI students
- Represented graduate students at the Faculty of Medicine meeting in September 2018

Graduate Research Assistant
University of Ottawa, Faculty of Medicine, Ottawa
2019

- Conducted research for the Faculty of Medicine Alumni Trajectory Project under the supervision of the Biochemistry Program Director

Undergraduate Thesis Student Recruitment
University of Ottawa, Faculty of Science, Ottawa
2016

- Presented at Student Recruitment Seminar
- Conducted screening and interviews for potential candidates

PUBLICATIONS

Peer Reviewed Publications

1. **Sarah McGarry***, Andrew Sulaiman*, Jason Chambers, Emil Al-Kadi, Alex Phan, Li Li, Karan Mediratta, Jim Dimitroulakos, Christina Addison, Xuguang Li and Lisheng Wang. Inhibition of Hypoxia Sensitizes TNBC to Cisplatin and Promotes Inhibition of Bulk Cancer Cells and Cancer Stem Cells. *International Journal of Molecular Sciences* 2020; doi: 10.3390/ijms21165788. **Impact Factor 4.1**
2. Andrew Sulaiman, **Sarah McGarry**, Sara El-Sahli, Li Li, Jason Chambers, Alexandra Phan, Emil Al-Kadi, Zaina Kahiel, Eliya Farah, Guang Ji, Seung-Hwan Lee, Krishna K

Inampudi, Tommy Alain, Suresh Gadde, Lisheng Wang. Retardation of TNBC PDX Tumors growth and diminution of Tumorigenesis via Nanotherapy. *Advanced Therapeutics* 2020; doi: 10.1002/adtp.202000123.

3. Andrew Sulaiman, **Sarah McGarry**, Xianghui Han, Sheng Liu, Lisheng Wang. CSCs in TNBC - One Size Does Not Fit All: Therapeutic Advances in Targeting Heterogeneous Epithelial and Mesenchymal CSCs. *Cancers* 2019; *11*(8), 1128, doi: 10.3390/cancers11081128. **Impact Factor 6.162**
4. Andrew Sulaiman, **Sarah McGarry***, Sara El-Sahli*, Li Li, Greg Cron, Christina Addison, Jim Dimitroulakos, Zemin Yao, Xuguang Li, Suresh Gadde° and Lisheng Wang°. Co-Targeting Bulk Tumor and CSCs in Clinically Translatable TNBC Patient-Derived Xenografts via Combination Nanotherapy. *Molecular Cancer Therapeutics* 2019; 18:1755–64 doi: 10.1158/1535-7163.MCT-18-0873. **Impact Factor 4.9**
5. Andrew Sulaiman, **Sarah McGarry**, Sara El-Sahli, Ka Mien Lam, Jason Chambers, Shelby Kaczmarek, Li Li, Christina Addison, Jim Dimitroulakos, Angel Arnaout, Carolyn Nessim, Zemin Yao, Guang Ji, Haiyan Song, Suresh Gadde, Xuguang Li, Lisheng Wang. Co-inhibition of mTORC1, HDAC and ESR1α Regards the Growth of Triple Negative Breast Cancer and Suppresses Cancer Stem Cells. *Cell Death and Disease* 2018; 9(8): 815 doi: 10.1038/s41419-018-0811-7. **Impact Factor 5.9**
6. Andrew Sulaiman, **Sarah McGarry**, Li Li, Deyong Jia, Sarah Ooi, Christina Addison, Jim Dimitroulakos, Angel Arnaout, Carolyn Nessim, Zemin Yao, Guang Ji, Haiyan Song, Suresh Gadde, Xuguang Li, Lisheng Wang. Dual inhibition of Wnt and YAP signaling retards the growth of both mesenchymal and epithelial TNBC. *Molecular Oncology,* 2018; 12(4):423-440. **Impact Factor 5.3**
7. Andrew Sulaiman, Brandon Sulaiman, Lara Khouri, **Sarah McGarry**, Carolyn Nessim, Angel Arnaout, Sean Xuguang Li, Christina Addison, Jim Dimitroulakos, and Lisheng Wang. Both bulk and CSC subpopulations in TNBC are susceptible to Wnt, HDAC and ERα co-inhibition. *FEBS Letters*, 2017;590(24):4606-4616. **Impact Factor 3, 20 Citations**
8. **Sarah E. McGarry**, A Tightrope Walk Towards the Easter Rising Centenary in 2016. *The Mirror*, 2015.

PRESENTATIONS

Oral Presentations

- Sarah McGarry. (May 2020) "Inhibition of Hypoxia Sensitizes TNBC to Cisplatin and Promotes Bulk and CSC Inhibition." *University of Ottawa Faculty of Medicine, Seminar Day (Virtual)*. Ottawa, ON. Canada.
- Sarah McGarry. (June 2019) "Graduate School: Perspectives from a New MSc Candidate from the University of Ottawa, Canada." *Longhua Hospital Research Seminar Series*, Shanghai, China.
- Sarah McGarry. (May 2019) "Triple Negative Breast Cancer: Cancer Stem Cells and Development of Novel Approaches to Target Both Bulk and CSC Populations." *Longhua Hospital Research Seminar Series*, Shanghai, China.
- Sarah McGarry. (February 2019) "Sensitizing TNBC to Cisplatin Treatment via EGFR and Hypoxia Inhibition." *University of Ottawa Faculty of Medicine, Seminar Day*. Ottawa, ON. Canada.

- Sarah E. McGarry. (March 2015) "Caging the Leprechaun: Criminally Insane and 'Lunatic' Irish Patients Admitted to Rockwood Asylum from 1857-1878." *Tri-U Colloquium: From Shattered Nerves to Shell Shock*. Waterloo, Canada.
- Sarah E. McGarry. (March 2015) "Deconstructing Ideal Widowhood: A Sample Analysis of Canadian Veterans' Widows Pension Files." *New Trajectories in War and Society: A Graduate Student Colloquium*. Waterloo, Canada.
- Sarah E. McGarry. (April 2014) "A Tightrope Walk Towards the Easter Rising Centenary in 2016: Northern Ireland and the Republic of Ireland Debate the Issues Through Newspapers." *Underhill Undergraduate Colloquium*. Ottawa, Canada.

Poster Presentations (* indicates presenting author)

- A. Sulaiman, J. Chambers*, S. McGarry, S. Gadde, L. Wang. (May 2019) "Targeting both Mesenchymal and Epithelial TNBC via Dual Inhibition of Wnt and YAP Signaling in Clinically Translatable PDX Models" *OISB Scientific Meeting, 2019*. Ottawa, Canada
- Sarah McGarry*, Andrew Sulaiman, Dr. Wang. (November 2018) "Co-Inhibition of Metabolic Pathways to Re-Sensitize Triple Negative Breast Cancer Bulk and CSC Populations to Chemotherapeutic Agents." *Journee Pharee.* Bromont, Canada.
- Andrew Sulaiman, Sarah McGarry*, Dr. Wang. (May 2018) "Dual Inhibition of Wnt and YAP pathways retards the growth of both mesenchymal and epithelial TNBC." *BMI Scientific Symposium,* Montebello, Canada.
- Sarah McGarry*, Dr. Wang. (November 2017) "Targeting HDACs, mTORc1, and ESR1 to Target Triple Negative Breast Cancer Cancer Stem Cells." *Journee Pharee*. Bromont, Canada.
- Sarah McGarry*. (May 2017) "An autocrine inflammatory loop after chemotherapy withdrawal facilitates the repopulation of drug-resistant breast cancer cells." *60th annual Canadian Society for Molecular Biosciences conference*. Ottawa, Canada
- Andrew Sulaiman, Sarah McGarry*. (November 2016) "Modeling epithelial and mesenchymal conversion in TNBC to identify sensitivities within breast cancer stem cell populations." *University of Ottawa, Student Recruitment Seminar*. Ottawa, Canada.

CONFERENCES ATTENDED

- Journee Pharee. Bromont, Canada. 2018
- BMI Scientific Symposium, Montebello, Canada. 2018
- Journee Pharee. Bromont, Canada. 2017
- 60th annual Canadian Society for Molecular Biosciences Conference. Ottawa, Canada. 2017.
- University of Ottawa Student Recruitment Seminar. Ottawa, Canada. 2016.
- World Life Science Conference. Beijing, China. 2016.
- Tri-U Colloquium: From Shattered Nerves to Shell Shock. Waterloo, Canada. 2015.
- New Trajectories in War and Society: A Graduate Student Colloquium. Waterloo, Canada. 2015.
- Graduate & Professional Studies Symposium. Waterloo, Canada. 2014.
- Underhill Undergraduate Colloquium. Ottawa, Canada. 2014.

HONOURS AND AWARDS

- Nominated for Thesis Award, 2020
- Charles Sean Alger Memorial Scholarship Fund, 2019 $1,500
- Recognized as publishing one of the Top 20 Most Read Manuscript in Molecular Oncology, 2019
- Admission Scholarship, University of Ottawa, 2018-2020 $14,000
- Admission Scholarship, University of Ottawa, 2018 $7,500
- Heritage Research Award, Waterloo Regional Heritage Foundation, 2015 $3,000
- North American Conference on British Studies Undergraduate Essay Contest, North American Conference on British Studies, 2014 $100
- Admission Scholarship, Wilfrid Laurier University, 2014 $3,000
- J. Carlisle Hanson Award, Carleton University, Department of History, 2014 $1,500
- Underhill Undergraduate Colloquium Award, Carleton University, 2014 $150
- Admission Scholarship, Carleton University, 2009 $4,000
- Ontario Scholar, St. Joseph's Catholic High School, 2009
- Canada Cord Award, Girl Guides of Canada, 2007

VOLUNTEER EXPERIENCE

- Vice President of Finance, BMIGSA, University of Ottawa, Faculty of Medicine. 2018 to 2020
- Graduate Student Mentee, BMIGSA Graduate Student Mentorship Program, University of Ottawa Faculty of Medicine. 2018 to 2019.
- Volunteer Lab Member, University of Ottawa, Faculty of Medicine. 2015 to 2018.
- Volunteer Notetaker, Paul Menton Centre Notetaking Services for Students with Disabilities. 2013.
- Varsity Athlete, Carleton University Rowing Team. 2010.
- Assistant Coach, St. Joseph's High School Rowing Team. 2010.

WORK EXPERIENCE

- Graduate Research Assistant, University of Ottawa, Faculty of Medicine, Ottawa, 2019.
- Ward Clerk (casual), The Ottawa Hospital. 2015 to 2018.
- Administrative Assistant (casual), The Robin Easey Centre, The Ottawa Hospital. 2016 to 2018.

TEACHING EXPERIENCE

Teaching Assistant
Wilfrid Laurier University, History Department, Waterloo
Course Title: Science in the Modern World, 1800 to the Present (HP202)
Supervisor: Dr. Suzanne Zeller
2015

- Organized and guided weekly tutorial discussions on topics in the history and philosophy of science
- Graded term papers, tutorial participation, and final examinations

Teaching Assistant
Wilfrid Laurier University, History Department, Waterloo
Course title: History of Vikings (HI299J)
Supervisor: Dr. Alicia Mackenzie
2014

- Attended weekly lectures and assisted the Professor and fellow teaching assistants in facilitating active learning exercises within the lecture hall
  Graded midterms, reflection papers, and final exams