The Influence of Administrative Timing in Triple-Negative Breast Cancer Treatments

Emily Mailloux
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The Influence of Administrative Timing in Triple-Negative Breast Cancer Treatments

By

Emily Mailloux

A Thesis
Submitted to the Faculty of Graduate Studies through the Department of Biomedical Sciences and the Department of Integrative Biology in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

2023

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The Influence of Administrative Timing in Triple-Negative Breast Cancer Treatments

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DECLARATION OF CO-AUTHORSHIP/PREVIOUS PUBLICATIONS

I. Co-Authorship

I hereby declare that this thesis incorporates material that is the result of joint research, as follows:

Appendix A incorporates the outcome of a joint research undertaken in collaboration with Bre-Anne, Fifield, Amin Kay, Swati Kulkarni, Rasna Gupta, John Mathews, Rosa-Maria Ferraiuolo, Huda Al-Wahsh, and Abdulkadir Hussein, and the lead researches Dr. Lisa Porter at the University of Windsor and Dr. Caroline Hamm at the Windsor Regional Hospital Cancer Centre. In all cases the key ideas, primary contributions, experimental designs, data analysis, interpretation, and writing were performed by Lisa Porter, Caroline Hamm, and Bre-Anne Fifield, with help from all other co-authors. The collaboration is covered in appendix A of the thesis.

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ABSTRACT

Breast cancer accounts for 25% of all cancers in Canadian women, and 15-20% of these are triple-negative breast cancers (TNBC), which have a poorer prognosis than other breast cancer subtypes. TNBC lacks expression of the estrogen receptor, progesterone receptor, and the human epidermal growth factor receptor 2 (HER2), which are common therapeutic targets in breast cancer. Due to the lack of target therapy, generalized chemotherapy treatments are used instead. The standard of care for treatment of TNBC instead consists of doxorubicin (A), cyclophosphamide (C), paclitaxel (T), and carboplatin (Carbo), that target various aspects of the cell cycle to induce cell cycle arrest. Pre-clinical models may be tested to determine how the administrative timing of ACT+Carbo may affect efficacy of treatments. The purpose of this study was to determine how the addition and timing of TNBC treatments influence cell cycle progression and how pre-clinical models can be used to optimize current ACT+Carbo treatments. MDA-MB-231 and MDA-MB-468 TNBC cells were treated with AC, T, TCarbo, or Carbo at various time points in vitro. Flow cytometry, trypan blue exclusion assay, and MTT were used to determine cell cycle progression, proliferation rate, and synergy. Casper zebrafish were used as an in vivo model. It was found that the combination pattern of T/TCarbo resulted in increased efficacy comparable to all other combinations via pre-clinical models. This information may be appliable to current TNBC treatments to improve efficacy, lower toxicity, and increase the 5-year survival rate of TNBC patients.
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TABLE OF CONTENTS

DECLARATION OF CO-AUTHORSHIP/PREVIOUS PUBLICATIONS .................. iii

ABSTRACT ........................................................................................................ v

ACKNOWLEDGEMENTS ............................................................................... vi

LIST OF FIGURES ......................................................................................... ix

LIST OF APPENDICES .................................................................................. x

LIST OF ABBREVIATIONS/SYMBOLS ......................................................... xi

INTRODUCTION .............................................................................................. 1

1. Triple-Negative Breast Cancer ................................................................. 1
2. Mitotic Cell Cycle Progression ................................................................. 2
3. TNBC Treatment and Obstacles .............................................................. 7
   3.1 Chemotherapy Drug Mechanisms and Treatments ............................. 7
   3.2 Cell Resistance to Chemotherapy Drugs .......................................... 11
   3.3 Synergism/Antagonism .................................................................... 12
4. Pre-Clinical Models Advantages ............................................................. 14
5. Hypothesis and Objectives ................................................................... 16

MATERIALS AND METHODS .................................................................... 17

1. Cell Culture ............................................................................................. 17
2. Drug Treatment ....................................................................................... 17
3. Flow Cytometry ..................................................................................... 18
4. Animal Care and Handling ..................................................................... 18
5. Egg Collection and Injections ............................................................... 18
6. Embryo Image Analysis ........................................................................ 19
7. MTT Assay ............................................................................................. 19
8. Bliss Independence Model .................................................................... 20
9. Statistical Analysis ................................................................................ 20

RESULTS ..................................................................................................... 22

1. Therapeutic Drugs Interfere With Cellular Replication at Varying Stages of The Cell Cycle ........................................................................................................... 22
2. Alteration in Administrative Timing Leads to Increased Efficacy of T and TCarbo Treatments ............................................ 26

3. TNBC Cells Cycle Back into the Cell Cycle After Drug Treatments ............................................. 30

4. Synergistic Relationship Between TCarbo Varies Among Concentrations While AC Exhibits An Overall Synergistic Relationship ................................................................................ 32

5. Synergy Influences Efficacy of TCarbo combination ................................................................. 36

6. Fold Change Variation Among T and Carbo Treatments ....................................................... 39

DISCUSSION ........................................................................................................................................ 43

REFERENCES/BIBLIOGRAPHY .................................................................................................. 52

APPENDICES ............................................................................................................................... 61

VITA AUCTORIS ........................................................................................................................... 75
LIST OF FIGURES

Figure 1: Mitotic cell cycle progression, CDKs, and cyclins. ................................. 5
Figure 2: Hallmarks of cancer. .................................................................................. 7
Figure 3: ACT chemotherapy treatment layout of TNBC patients. ....................... 11
Figure 4: IC_{50} values and dose curves of MDA-MB-231 and MDA-MB-468 cell lines. .. 24
Figure 5: Cell cycle profile after a single treatment................................................ 25
Figure 6: 4-day proliferation trial results of independent or combined therapeutic agent
after a single dose. ...................................................................................................... 28
Figure 7: Double treatment of T and TCarbo proliferation rate trials. ...................... 29
Figure 8: Quantification of live cells when treated with AC precursor to T and TCarbo
results. ...................................................................................................................... 31
Figure 9: T vs Carbo synergy depicted in surface plots with corresponding matrix plots. 34
Figure 10: A vs C synergy depicted in surface plots with corresponding matrix plots..... 35
Figure 11: Difference in proliferation rates between treatments of IC_{50} versus synergy
concentrations of T and Carbo patterns..................................................................... 38
Figure 12: Casper zebrafish injection schematic....................................................... 41
Figure 13: Casper zebrafish tumour fold change among treatments. ......................... 42
Figure 14: Clinical trial layout of ACT+Carbo treatments in TNBC. ......................... 47
LIST OF APPENDICES

Appendix A: A prospective phase II clinical trial identifying the optimal regimen for carboplatin plus standard backbone of anthracycline and taxane-based chemotherapy in triple negative breast cancer ................................................................. 61

Appendix B: Permissions .................................................................................. 74
LIST OF ABBREVIATIONS/SYMBOLS

A……………………………………………………………………………………………………...Doxorubicin

ACT……………………………………………………………………………………………………….Doxorubicin+Cyclophosphamide & Paclitaxel Treatment

BC……………………………………………………………………………………………………….Breast Cancer

BL1……………………………………………………………………………………………………….Basal-like 1

BL2……………………………………………………………………………………………………….Basal-like 2

BLBC…………………………………………………………………………………………………… Basal-like Breast Cancer

C…………………………………………………………………………………………………………Cyclophosphamide

Carbo………………………………………………………………………………………………………Carboplatin

CDK……………………………………………………………………………………………………….Cyclin-Dependent Kinase

CKI……………………………………………………………………………………………………….Cyclin-Dependent Kinase Inhibitor

DMEM……………………………………………………………………………………………………….Dulbecco’s Modified Eagle’s Medium

DMSO……………………………………………………………………………………………………….Dimethyl Sulfoxide

dpf…………………………………………………………………………………………………………Days Post-Fertilization

dpi…………………………………………………………………………………………………………Days Post-Injection

ECM……………………………………………………………………………………………………….Extracellular Matrix

EDTA……………………………………………………………………………………………………….Ethylenediaminetetraacetic Acid

ER……………………………………………………………………………………………………….Estrogen Receptor

FBS……………………………………………………………………………………………………….Fetal Bovine Serum

G0……………………………………………………………………………………………………….Non-Growth Phase

G1……………………………………………………………………………………………………….Gap I

G2……………………………………………………………………………………………………….Gap II

HER2……………………………………………………………………………………………………….Human Epidermal Growth Factor Receptor Type 2

IC_{50}……………………………………………………………………………………………………….Half Maximal Inhibitory Concentration

IHC……………………………………………………………………………………………………….Immunohistochemistry

LD_{10}……………………………………………………………………………………………………….Lethal Dose For 10%
Mitotic
Thiazolyl Blue Tetrazolium Bromide
Penicillin-Streptomycin
Phosphate Buffered Solution
Pathological Complete Response
Propidium Iodide
Progesterone Receptor
Reactive Oxygen Species
Revolutions Per Minute
Synthesis
Paclitaxel
Tumour Microenvironment
Triple-Negative Breast Cancer
INTRODUCTION

1. Triple-Negative Breast Cancer

Breast cancer (BC) is the leading cancer in Canadian women, accounting for 25% of all cancers and accounts for 13% of cancer deaths in Canadian women annually (Hsu et al., 2022). 15-20% of all BC cases in women are triple-negative breast cancer (TNBC) (Seung et al., 2020). There are higher incidences of TNBC among young African-American and Hispanic women who contain a mutation in the BRAC1 gene (Aysola et al., 2013). While the 5-year survival rate of breast cancer patients in Canada is 89%, TNBC is lower at only 77% (Hsu et al., 2022). TNBC is a poorly differentiated cancer that is associated with high recurrence rates, metastases, and overall poor survival rates in patients, as is demonstrated in the 5-year survival rate previously stated (Nedeljković & Damjanović, 2019). TNBC metastases commonly occurs in the brain and lungs (Nedeljković & Damjanović, 2019). The diagnoses of TNBC is often accomplished using immunohistochemistry (IHC), a staining process that utilizes targeted antibodies to test for specific markers or antigens that make up a tumour. The identifying characteristics that mark a subtype as TNBC is the lack of expression of three receptors: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Nedeljković & Damjanović, 2019). These three receptors are commonly used in targeted therapy for breast cancers, but due to the lack of expression of these receptors in TNBC, traditional targeted therapy treatment cannot be utilized, and generalized chemotherapy treatments must be implemented instead.

There are six subtypes of TNBC as classified by Lehmann et al. in 2011. These subtypes are basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal, mesenchymal stem-like, immunomodulatory, and luminal androgen receptor (Lehmann et al., 2011). However, those that are classified as ‘basal-like’ contain gene expression that is similarly found in classic basal/myoepithelial breast cancer (Lehmann & Pietenpol, 2014). 70-80% of TNBC are basal-like,
while only 70% of basal-like tumours are triple-negative (Nedeljković & Damjanović, 2019); TNBC and basal-like breast cancers (BLBC) overlap in gene expression profiles by 56%, while profiles between BLBC and non-TNBC tumours only share 11.5% overlap (L. Yin et al., 2020). The BL1 subtype contains abnormal levels of DNA repair and cell cycle-regulating genes, such as high amplification of MYC, CDK6, and PIK3Ca, and heter- or homozygous deletion in DNA repair-related genes such as PTEN, RB1, and TP53 (L. Yin et al., 2020). TNBC patients that contained the BL1 subtype were found to be more sensitive to cisplatin treatments than other subtypes (Lehmann & Pietenpol, 2014). The BL2 subtype contains abnormal activation of many signalling pathways, which include EGFR, MET, Wnt/β-catenin, and IGF-1R pathways; the therapeutic drugs potentially used for targeting include mTOR inhibitors and growth factor inhibitors, such as the drugs gefitinib, lapatinib, and cetuximab (L. Yin et al., 2020).

2. Mitotic Cell Cycle Progression

Proliferating cells go through stages of growth and division, known as the cell cycle. The purpose of the mitotic cell cycle is to allow turnover in a tissue while maintaining homeostasis by creating a copy of the original cell, known as a daughter cell. This entire process takes 24 hours in a typical human cell and, as seen in Figure 1, is divided into four different phases: Gap I (G1), Synthesis phase (S), Gap II (G2), and Mitotic phase (M) (Alberts et al., 2002). G1, S, and G2 are collectively called interphase (I-phase), which most proliferating cells spend 95% of their life in, accounting for 23 hours out of a 24 hour cell cycle, leaving 1 hour for M phase (Alberts et al., 2002). Each phase plays an important role in the duplication of the cell. The two Gap phases (G1 and G2) are responsible for the duplication of organelles, increase in cell size, protein creation, and self-organization for chromosomal segregation (Barnum & O’Connell, 2014). S phase is located between both Gap phases, and it is in this phase when DNA duplication occurs within the cell. The last and final phase of the cell cycle is the M phase, where chromosomal segregation occurs, and cytokinesis divides the cell to form two daughter cells. If cells divide to form two
genetically identical daughter cells it is called symmetric cell division, but if the daughter cells are not genetically identical, such as a stem cell and differentiating cells, is it known as asymmetric cell division (Majumdar & Liu, 2020). Another phase related to the cell cycle is called G zero (G0) (Harper & Brooks, 2005). G0 occurs when a cell goes dormant, or quiescent, for long periods of time; they do not progress further in the cell cycle, nor do they undergo cell division. However, quiescent cells can be stimulated by external factors, such as mitogens, which prompt the quiescent cells in G0 to re-enter the cell cycle, progressing until division. Cells that are stuck in the G0 phase permanently are known as being in senescence (Ding et al., 2020).

There are three checkpoints in the cell cycle: G1/S, G2/M, and mitotic spindle checkpoint (Barnum & O’Connell, 2014). Both G1/S and G2/M checkpoints check for DNA damage, while the mitotic spindle checkpoint validates chromosome alignment. The purpose of each checkpoint is to confirm the cell is in the proper condition before passing onto the next phase. If there is any form of error within the cell, such as DNA damage, the checkpoints will prohibit the cell from proceeding onto the next phase and either implement error repair or promote cell apoptosis.

Engrossed in the cell cycle are regulators and checkpoints that mimic border patrols to affirm the cell is properly equipped before the cell is allowed to proceed to the next phase(s) of the cell cycle (Barnum & O’Connell, 2014). The two major classes of proteins that highly regulate the cell cycle are cyclins and cyclin-dependent kinases (CDK). CDKs are the central drivers of cell cycle progression and are serine/threonine protein kinases that phosphorylate key substrates which in sequence promote DNA synthesis and mitotic progression (Figure 1). Active CDKs drive cell cycle transition points between phases, such as G1, S phase, and G2/M. CDKs are activated via cyclin subunits that bind to the inactive CDKs and promote an active configuration. Active CDKs phosphorylate target proteins that help regulate key events in the cell cycle. Cyclins are tightly regulated at the level of synthesis and ubiquitin-dependent proteolysis, and concentrations of cyclins vary throughout the cell cycle, allowing activation of particular
CDKs at varying times (Ding et al., 2020). Cyclins contain a cyclin box region of homology used as a domain for binding and activating CDKs. In contrast to cyclins, CDK inhibitors (CKI) negatively regulate cyclin/CDK complex activity (Barnum & O’Connell, 2014). Two major groups of CKIs include the INK4 family, which binds to CDK4/6 proteins, and the Cip/Kip family, which binds to cyclin/CDK complexes (Schirripa et al., 2022). Within human cells, there are 29 cyclins and 20 CDKs; CDK1, CDK2, CDK3, CDK4, and CDK6 are the main CDKs that regulate transition between cell cycle phases, while CDK7-11 mediate gene transcription (Lim & Kaldis, 2013). The CDK that regulates the transition of G1 to S phase and S phase itself in the cell cycle is CDK2 with cyclin E and A, respectively. CDK4 and CDK6 are found within the G1 phase of the cell cycle with cyclin D; lastly, CDK1 is responsible for the G2/M phase with cyclin A and B (Figure 1) (Lim & Kaldis, 2013). Each CDK and cyclin work in tandem to promote proper cell formation within the cell cycle and are essential for the formation of genetically identical daughter cells without defective DNA or organelles. The cell cycle works to maintain cell homeostasis, but disruption within this process may lead to apoptosis of the cell, which is the goal when treating cancer cells by targeting specific aspect of the cell cycle.
Majority of cancer cells contain overlapping properties that are used to define common characteristics of cancer such as: maintaining proliferative signals, resistance to growth suppressors, invasion and metastasis, inducing angiogenesis, resistance to apoptosis, and replicative immortality (Gutschner & Diederichs, 2012). These 6 hallmarks of cancer were proposed by Hanahan and Weinberg in 2000, and since then the number of hallmarks has expanded (Gutschner & Diederichs, 2012; Hanahan & Weinberg, 2000). More recent additions of the hallmarks of cancer include: genome instability and mutation, deregulating cellular energetics, tumour-promoting inflammation, and avoiding immune damage (Figure 2) (Garnham et al., 2019). While these hallmarks portray characteristics shared by nearly all cancer cells, it fails to depict the two precursor molecular and cellular mechanisms that give rise to the remaining eight traits; these two enabling process are tumour-promoting inflammation and genomic instability (Hanahan, 2022). Genomic instability shapes the genetic composition of cancer cells, shepherding their morphology, behaviour, and response to treatment and tumour
microenvironment (TME); this may result in defects in DNA repair, oncogene-induced replication stress, and error in spindle assemble checkpoints (Chen et al., 2022). Although genetically unstable cells may exhibit various cancer characteristics, they will not automatically thrive in a tissue due to targeting by immune cells, often provoking an immune response that will eradicate abnormal cells. There are numerous ways cancer cells may bypass ‘immune checkpoints,’ allowing tumours to proliferate in a tissue. Immune checkpoints are essential to maintain immune homeostasis and prevent the body’s immune system from attacking healthy cells and tissues; this known as autoimmunity (Chen et al., 2022). Tumour-promoting inflammation occurs when tumour cells cause necrosis of healthy cells, provoking inflammation (Khusnurrokhman & Wati, 2022). As the healthy cells die, the contents of the cells are released into interstitial tissue. This release triggers the cultivation of proinflammatory mediators. As the tissue environment remains inflamed, prolonged exposure will affect DNA damage, mutation, or formation of reactive oxygen species (ROS), increasing the prospect of tumour formation and growth (Khusnurrokhman & Wati, 2022). The gathered hallmarks generalize the characteristics in a clear and concise summary of how cells can exhibit cancerous traits.
3. TNBC Treatment and Obstacles

3.1 Chemotherapy Drug Mechanisms and Treatments

Treatment for TNBC is not a simple solution. Due to the unique characteristics of TNBC, there is no lone treatment that works for all tumour subtypes. Adjuvant and neoadjuvant chemotherapy are two strategies for treating TNBC. Adjuvant chemotherapy involves the removal of the tumour, via lumpectomy or mastectomy, followed by chemotherapy to kill undetected, residual cancer cells to prevent future recurrences (Information et al., 2019; Saifi et al., 2022). Neoadjuvant chemotherapy is the use of chemotherapy to downgrade the primary tumour prior to
removal. The agents used in chemotherapy treatments target various aspects of the cell cycle to promote apoptosis of the cell. The first and most commonly used agent is doxorubicin (A). A is an anthracycline antibiotic derived from Streptomyces peucetius var. caesius, and is used to treat a diverse range of cancers including breast, lung, ovarian, and thyroid (Thorn et al., 2011a). The primary target of A is the inhibition of topoisomerase II, an enzyme responsible for the tension release on DNA strands during DNA replication in S-phase. Topoisomerase II is a critical enzyme in DNA synthesis. With inhibition of topoisomerase II, DNA replication is severely stalled, hindering the cell from further proceeding in the cell cycle, invoking cell apoptosis in the G2/M phase of the cell cycle. Cyclophosphamide (C) is another common chemotherapy agent used to treat various types of cancers, including breast cancer, retinoblastomas, and ovarian adenocarcinomas (Ogino & Tadi, 2022; Thorn et al., 2011b). C is a form of nitrogen mustard drug that acts as a DNA alkylating agent, meaning it adds alkylating groups to biological molecules containing negative charges, thus interfering with DNA. While C is not cell-cycle phase specific, it interferes with the division of cells by creating cross-links in DNA and RNA stands, eliciting major influence in the S-phase of the cell cycle (Jones et al., 2009; Ogino & Tadi, 2022). A and C are frequently used in combination to optimize their influence on the cell cycle and promote apoptosis in actively dividing cancer cells. As both drugs target DNA replication processes, they target primarily within the S-phase of the cell cycle.

Paclitaxel (T), a taxane derived from the pacific yew tree (Taxus brevifolia), is an effective chemotherapy agent used to treat various types of cancer, including breast, ovarian, and lung cancers (Skubitz, 2011). T prevents depolymerization of microtubules by attaching to the beta-subunits of tubulin, inhibiting the cell during G2/M phase. The accumulation of microtubule bundles and asters force the cell to arrest and promotes apoptosis (Skubitz, 2011); (Schwab, 2011a). Lastly, carboplatin (Carbo) is a second-generation platinum compound often used in a variety of cancers including testis, ovary, breast, and lung cancer. Platinum agents have
promising potential in TNBC treatments. As TNBC itself is heterogeneous with a variety of subtypes; those with a BRCA1 mutation display a particular sensitivity to platinum drugs, posing as a promising addition to TNBC treatments (Isakoff, 2010). Studies have found that the addition of platinum-based drugs in TNBC increased pathological complete response (pCR) from 27.7% to 40.1%, demonstrating how the addition of platinum agents will increase response in TNBC patients (Pandy et al., 2019). Previous studies on cisplatin, a platinum drug, have shown that BC patients who received cisplatin alone resulted in partial or complete response (objective response) rates to treatment ranging from 42-54% (Isakoff, 2010). However, when cisplatin was given following previous chemotherapy, the objective response rate was lowered by 0-9% (Isakoff, 2010). This is mainly due to the high cytotoxicity of cisplatin. To lower toxicity of treatments associated with cisplatin, the derivative Carbo was created (Sousa et al., 2014a). Carbo is lower in toxicity, and is able to be more easily tolerated by TNBC patients, primarily replacing cisplatin in many treatments (Schwab, 2011b). Carbo binds to GC-rich sites in DNA, creating intra- and interstrand DNA cross-links, and DNA-protein cross-links, thus inhibiting DNA replication and inducing apoptosis of the cell while being cell cycle phase non-specific (Sousa et al., 2014b). When used in chemotherapy treatments, Carbo is used in combination with T during the second half of treatments (Figure 3). Chemotherapy drugs, when used appropriately, work synergistically to prevent rapid proliferation of cancer, helping to irradiate cancer cells within TNBC patients.

While most breast cancer treatments include AC, not all include T or Carbo, but studies have shown that the use of TCarbo increased TNBC patients 5-year disease-free survival (DSF) by 6.2% (Yu et al., 2020). While the set timing of AC is well established within TNBC treatments, the optimal pattern and timing of T and Carbo has not been well established. A study done on ovarian cancer compared the efficacy of weekly Carbo vs every-3-week TCarbo combination, determining that patients receiving only the weekly Carbo contained significantly worse prognosis and overall survival outcomes, forcing a premature termination of treatments.
(Falandry et al., 2021). This suggests that the TCarbo treatment combination contains a higher efficacy comparable to only Carbo. These results hint towards a pattern-based optimization of T and Carbo treatments that may directly influence efficacy of TNBC therapy.

A recent clinical trial conducted through a collaboration between Windsor Regional Hospital Cancer Centre and the University of Windsor researched the varying patterns of T vs TCarbo administrative timing in TNBC patients and how this influenced results (Hamm et al., 2022). Patients were administered AC biweekly for four rounds followed by either T or TCarbo biweekly for four rounds (ACT+Carbo). Patients who received T followed by TCarbo were able to tolerate the treatment better and contained the most promising outcome (Figure 3) (Hamm et al., 2022). This suggests that a particular pattern of T and Carbo may contain the highest efficacy within TNBC therapy. The novel results from this clinical trial provide insight into the relationship of T and Carbo and the optimal timing of administration in TNBC patients that will result in the highest efficacy of treatments. Further research into confirmation and mechanistic aspect of if and why T followed by TCarbo provide the highest efficacy and how we can utilize this knowledge for future TNBC treatments will provide novel ways of viewing how drug administration within cancer patients should be taken into consideration. Optimizing chemotherapy drug administrative timing of TNBC treatments may increase the 5-year survival rate of TNBC patients in the future.

Side-effects of chemotherapy are that healthy dividing cells are also targeted in addition to the cancer cells, such as myelopoietic bone marrow precursor cells, mucous membrane cells in throat and mouth, intestine epithetical cells, and hair follicle resulting in hair loss, anemia, vomiting, infection in the mouth, nausea, and diarrhea whilst on treatments (Information et al., 2019). Although severity of side-effects may vary among patients, drug types and doses also impact the degree of side-effects. The optimization of drugs and regimen for TNBC patients is an extremely important aspect of treatment, not only to increase the 5-year survival rate of TNBC
patients, but to also lower side-effects of chemotherapy treatments while optimizing drug therapies.

![Clinical TNBC Timeline of ACT+Carbo Treatment](image)

**Figure 3: ACT chemotherapy treatment layout of TNBC patients.**

Image created using BioRender.

3.2 Cell Resistance to Chemotherapy Drugs

Chemotherapy is currently the most successful way to treat cancer, 90% of failures in this therapy occur due to metastasis of cancer in relation to drug resistance (Mansoori et al., 2017); (Nedeljković & Damjanović, 2019). Many cancer patients that receive chemotherapy often contain drug resistant cancer cells, posing a serious problem. Cancer cells can acquire drug resistance via multiple mechanisms: drug inactivation, apoptosis inhibition, multi-drug resistance, alteration in drug metabolism, epigenetic and drug targets, increased DNA repair and amplification of genes (Mansoori et al., 2017). There are other intrinsic and extrinsic factors that relate to cell resistance: tumour heterogeneity, tumour microenvironment, and cancer stem cells. TNBC is a prime example of tumour heterogeneity, as the cancer is made up of a variety of different subtypes in a single tumour, resulting in a variety of genetic, epigenetic, transcriptomic,
and proteomic properties. This variety within a tumour may lead to drug resistance in some subtypes while other subtypes remain responsive. The microenvironment and its role in chemoresistance is one of the main causes for relapse in cancer patients, and involves normal stromal cells, extracellular matrix (ECM), and other soluble factors including cytokines and growth factors (Mansoori et al., 2017). Due to the large influence the TME has on chemotherapy treatments and drug resistance, mimicking the environment is a key factor in studying and developing chemotherapies that can efficiently treat TNBC while preventing relapse in patients. The mimicry of the TME can be accomplished through in vivo treatments, such as casper zebrafish, and 3D models, such as organoids. Another aspect of the TME is the cancer stem cells present that have been shown to contain special mechanisms, including overexpression of the ATP-binding cassette (ABC) that are able to avoid chemotherapeutic agents. Cancer stem cells can become dormant, among other aspects, and can remain after chemotherapy treatments in patients only to metastasize to distant organs and form tumours there. Possible ways to bypass resistance in cancer cells is to use alternative patterns of chemotherapy agents to avoid continuous exposure to particular types of drug(s). This will force the cancer cell to use alternative mechanisms to fight the anti-cancer drugs, avoiding the development of resistance (Shenfield, 1982). This can easily be done by frequently switching between drugs in TNBC patients. Another strategy is utilizing combination drug therapy, allowing a lower amount of chemotherapy agents to be used in combination, not only lowering cytotoxicity side-effects in patients, but lowering the chance of the chemoresistance in cells (Shenfield, 1982). Chemoresistance is a prevalent issue when treating TNBC that is difficult to overcome, but utilizing strategies to work around resistance is a key aspect to increasing efficacy of TNBC treatments.

3.3 Synergism/Antagonism

Mono-therapy and combination therapy are two main ways cancer is treated via therapeutic agents; the difference between these approaches is that mono-therapy is the use of a single agent,
while combination therapy is the use of two or more agents (Mokhtari et al., 2017). Combination therapy is generally deemed more effective than mono-therapy due to the targeted effect the combination of drugs has on rapidly dividing cells, lowering the chance of harming healthy cells. In contrast, mono-therapy agents commonly contain non-selective targeting of actively proliferating cells, causing death of both cancerous and healthy cells (Mokhtari et al., 2017). Furthermore, cancer cells treated with two or more drugs are less likely to become resistant to drugs used, while in mono-therapy drug resistance is a major issue. Combination therapy is an effective tool for treating cancer patients, but there are dangers in creating a cocktail of drugs, including antagonism among the agents used. Using drugs that are antagonistic to each other may produce cytotoxic effects on cancer cells (Mokhtari et al., 2017).

When using a cocktail of drugs in treating cancer, their synergistic or antagonist relationship must be taken into account. A synergistic relationship between drugs is when the combined effect is larger than the additive effects of each drug, while an antagonistic relationship between drugs is when the combined effect of drugs is lower than the total additive effects of each drug (N. Yin et al., 2014). A synergistic relationship within a drug cocktail of treatments is sought after due to the high efficacy of treatments, delayed drug resistance, and lower toxicity and treatment side-effects in cancer patients. Antagonistic drugs are often undesirable in treatments; however, they may provide use in selecting against specific drug-resistant mutations within cancer patients. While various chemotherapy agents target diverse aspects of the cell or cell cycle, randomly or strategically selecting agents to use in cancer treatments does not automatically convey that said agents are synergistic in nature. Calculating drug interactions and determining the synergistic/antagonistic relationship can be done in several ways, including using the Bliss independence model and the Loewe Additivity model (Lederer et al., 2019). Both of these models are important in determining the synergistic relationship between drugs used in chemotherapy, differing in the viewpoint of analysis but answering the same question. The Bliss
independence model focuses on the enhancement of drug treatments due to drug interaction, while the Loewe Additivity model focuses on dose reduction. The Bliss independence model is a better model of analysis in association with chemotherapy drug combinations (Zhao et al., 2014). The Bliss independence model analyzes data under the assumption of no interaction between drugs or combined drugs containing differing mechanisms of target sites from one another (Pemovska et al., 2018). Using this model, levels of synergy between therapeutic agents can be measured to understand how doses of each drug influences efficacy of treatments.

4. Pre-Clinical Models Advantages

Pre-clinical models are commonly used to test and develop cancer treatments by utilizing both in vitro and in vivo models (Sajjad et al., 2021). In vitro models are a method to test and optimize chemotherapy treatments outside of clinical trials by using cell lines derived from tumours of previous cancer patients to study various responses within cancer cells, such as drug interactions within tumours, including inhibition/promotion of cellular processes. While using cell lines is a quick, reliable, and effective in vitro model to test tumour response to chemotherapy agents, it is limited in its mimicry as it does not take into account the TME normally found within a tumour. There are multiple ways to overcome this obstacle, including utilizing 3D models or in vivo models. The use of zebrafish (Danio rerio) as an in vivo model in cancer is not a new concept. It was introduced in the 1990s and is still commonly used today. Zebrafish provide a quick, inexpensive, and easy way to use in vivo models for research and contains 70% genetic similarity to humans (Teame et al., 2019). Zebrafish also metabolize drugs similarly to humans, allowing further avenues of research in relation to toxicity and drug discovery (Park et al., 2020). Zebrafish are commonly used as cancer models, where human tumour cells are transplanted into zebrafish embryos (xenografts) to research tumour formation, progression, treatment, and metastasis (Tavares & Santos Lopes, 2013). However, the downsides of using zebrafish models is the black melanophores and reflective iridophores (White et al., 2008). Black melanophores make
up the black stripe seen down the sides of the fish, and the reflective iridophores are the reflective scales of the fish; both of these may inhibit research within the zebrafish, such as the study of cancer migration or tumour formation. To overcome these hindrances, other models such as casper zebrafish were developed (White et al., 2008). Casper zebrafish is a unique and effective model to use. As a double mutant, casper zebrafish lack black melanophores and reflective iridophores, making them transparent. This allows for direct observation and study of internals organs and injected cells when studying cell migrations within the fish, providing a unique and effective in vivo model to utilize within research. The combined use of in vitro and in vivo models provides a solid base in replicating tumours normally found in cancer patients to test current and novel drug treatments.
5. Hypothesis and Objectives

Hypothesis: The efficacy of ACT+Carbo treatments of triple-negative breast cancer is influenced by the administrative timing of T and Carbo treatments by altering cellular response to chemotherapy drugs, including drug-related cell resistance and cell cycle progression and arrest.

Objectives: The first objective is to determine how the administrative timing of each drug relates to cell cycle progression, cell cycle arrest, and how this relates to the optimal timing of T and TCarbo administrations. The second objective is to assess the synergistic/antagonistic relation among ACT+Carbo agents.
MATERIALS AND METHODS

1. Cell Culture

MDA-MB-231 (HTB26; ATCC) and MDA-MB-468 (HTB132; ATCC) TNBC cells were purchased from ATCC and cultured in growth media composed of Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS: Thermo-Fisher), and 1% Penicillin-Streptomycin (P/S: 15140-1322; Thermo-Fisher). Once cells reached 70-80% confluency, an appropriate amount of 0.25% trypsin (25200-072; Life Technologies) was added to the plate and incubated at 37°C for 3-5 minutes to detach cells from the plate before being collected. All cells were maintained at 37°C with 5% CO₂. Cell passage numbers were kept between 18-39 to avoid replication errors of experiments (Passage Number Effects in Cell Lines, n.d.). Cells were counted using Trypan Blue Exclusion Assay; 50 μl of Trypan Blue was combined with 50 μl of cell suspension, and 10 μl was placed onto hemocytometer and manually counted.

2. Drug Treatment

Drug treatments used included: Paclitaxel (T) (P3456; ThermoFisher), carboplatin (Carbo) (C2538; Sigma-Aldrich), doxorubicin (A) (J64000; ThermoFisher), and cyclophosphamide (C) (C0768; Sigma). Stock concentrations of each drug used were as follows: A; 17.24 mM, C; 197 mM, T; 5.8 mM, Carbo; 26.9 mM. T, A, and C were reconstituted in DMSO and Carbo was reconstituted in water. Seeding densities of 5,000 cells per well in a 96-well plate, 10,000 cells per well in a 24-well plate, 25,000 cells per well in a 12-well plate, 380,000 cells on a 6 cm plate, and 1,000,000 cells on a 10 cm plate were used. In single treatments, IC₅₀ concentrations of drugs were used to treat MDA-MB-231 and MDA-MB-468 cells for 24 hours, and 48 hours after cells were collected and analyzed. In extended time courses where two or more treatments of drugs were
were used, cells were treated with appropriate drugs at the IC₅₀ concentration for 24 hours for each treatment, with 48 hours between treatments.

3. Flow Cytometry

Cells were collected and centrifuged at 1000RPM for 5 minutes at 4°C before the supernatant was removed and the pellet fixed in 1 ml of ice cold 70% ethanol and stored at -20°C. The fixed samples were centrifuged at 1000RPM for 5 minutes at 4°C, and the supernatant was removed. The cells were then washed with 500 μl of 1xPBS before being centrifugation at 1000RPM for 5 minutes at 4°C and the supernatant removed. The pellets were then stained using 2 mM EDTA PBS with 50 μg/ml propidium iodide (PI) (P4170; Sigma) for a minimum of 20 minutes. Cell cycle profiles were analyzed based on the DNA content. Data was collected by BD LSR Fortessa™ X-20 (Flow Cytometry Facility; U of Windsor).

4. Animal Care and Handling

Casper zebrafish (Danio rerio) were handled in compliance with local animal care guidelines and standard protocols of Canada and following the University of Windsor Animal Utilization Project Proposal #19-03. Adult zebrafish were kept at 28.5°C. Breeding of casper zebrafish were done in accordance with “The Zebrafish Book” (Westerfield, 2000).

5. Egg Collection and Injections

Eggs were collected from casper zebrafish and kept in E3 embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 10-5% Methylene Blue) at 28°C. At 2 days post fertilization (2dpf), embryos were moved to 12-well plates with 3 embryos per well and maintained at 33°C. Prior to injection, 1,000,000 MDA-MB-231 cells were resuspended in 500 μl of serum-free media with 10μl of Vybrant DiI Cell-Labeling Solution (V22885; Invitrogen) for cell labeling and incubated for 20 minutes at 37°C. Cells were washed with serum-free media twice and resuspended in 100 μl of serum-free media and placed on ice until injection. A 2%
agarose gel was prepared on a 10 cm plate and 0.168 mg/ml of Tricaine (Sigma, MS222) solution was formulated. The Nanoject III microinjector (Drummond Scientific) was set up with a glass needle, and oil was taken up into the needle, followed by the uptake of labelled cells. Casper zebrafish embryos at 2dpf were anesthetized with tricaine prior to injections. Embryos were placed on agarose gel and 30 nl of cell suspension was injected directly into the embryo yolk sac. Images of successfully injected embryos were taken at 35x magnification with a Leica inverted microscope (Leica fluorescence stereomicroscope M205). Embryos were maintained in E3 and placed back in 12-well plates at 3 embryos per well and the water was changed daily starting 5dpf. Embryos were treated with T, TCarbo, or DMSO in E3 at 3 days post-injection (3dpi) and 6dpi for 24 hours. The LD$_{10}$ concentrations of T and Carbo used were 1 μM and 250 μM, respectively. Embryos were imaged on the Leica every day (3dpi-9dpi) at the same exposure and intensity until final date of experiment.

6. Embryo Image Analysis

All image analysis was accomplished using ImageJ software. Images of embryos were imported into ImageJ and converted to 8-bit greyscale, and the image threshold adjusted to eliminate background noise. Integrated density of florescence was measured to represent tumour burden. Measurements were transferred to Excel files and tumour burden fold change calculated.

7. MTT Assay

Cell viability was determined using the MTT (Thiazolyl Blue Tetrazolium Bromide) assay. Cells were seeded in 96-well plates at 5,000 cells per well in 100 μl of growth media. 24 hours after seeding, media was replaced with fresh growth media containing increasing concentrations of paclitaxel, carboplatin, doxorubicin, cyclophosphamide, or DMSO. Cell were treated for 24 hours, growth media was then removed, and cells washed gently with 100 μl of pre-warmed 1xPBS. Once 1xPBS was removed, 100 μl of MTT working solution (1 ml of MTT solution (5
mg/ml Thiazolyl Blue Tetrazolium Bromide in PBS and sterilized through 0.22 \( \mu \)m filter) (M5655; Sigma) and 9 ml of serum-free DMEM) were added to each well. The plate was wrapped in foil to minimize light exposure and incubated at 37°C for 2 hours and 15 minutes. MTT and media were carefully removed and 100 \( \mu \)l of DMSO added to each well and gently pipetted up and down 3 times to thoroughly mix while avoiding bubbles. Plates were re-wrapped in foil and left at room temperature for 15 minutes. Plates were then read on the Cytation 5 Cell Imaging Multimode Reader (BioTek Instruments) for absorbance at OD=570nm. IC\(_{50}\) values were calculated using the free dose-response fitting software Dr-Fit (Di Veroli et al., 2015).

8. Bliss Independence Model

The Bliss independence model was utilized to analyze synergism/antagonism of combination drug treatments. Combenefit software (Di Veroli et al., 2016) was used to calculate drug interactions via the Bliss independence model. The Bliss equation is:

\[
Y_{ab,P} = Y_a + Y_b - Y_a Y_b
\]

Where \( Y_a \) is percent inhibition from drug A at dose \( a \), \( Y_b \) is percent inhibition from drug B at dose \( b \), and \( Y_{ab,P} \) is predicted percent inhibition. \( Y_{ab,O} \) is the observed percentage inhibition, which is compared to the \( Y_{ab,P} \) value. An additive effect is when \( Y_{ab,O} = Y_{ab,P} \). A synergistic relationship between the two drugs is when \( Y_{ab,O} > Y_{ab,P} \). An antagonistic relationship between the two drugs is when \( Y_{ab,O} < Y_{ab,P} \) (Zhao et al., 2014).

9. Statistical Analysis

Excel was used for all statistical analysis (Student’s t-test and one-way ANOVA with Tukey post-hoc test), with exception of dose-curve graphs, IC\(_{50}\) value, and synergy between drug combinations. The ImageJ software was used for analysis of integrated density from images of casper zebrafish. Dr-Fit software was used for all dose-curve graphs and IC\(_{50}\) calculations. Drug
combination and synergy assessment was accomplished using the Combenefit software (Di Veroli et al., 2016).
RESULTS

1. Therapeutic Drugs Interfere With Cellular Replication at Varying Stages of The Cell Cycle

Chemotherapeutic drugs work by targeting specific aspects of the cell cycle, leading to cellular arrest at different phases of the cell cycle. Understanding the effects of combining therapeutic agents on cell cycle arrest is critical to determine the most effective treatment timing. To assess this, the IC\(_{50}\) concentration for TNBC standard of care chemotherapeutic agents, paclitaxel (T), carboplatin (Carbo), doxorubicin (A), and cyclophosphamide (C), were assessed in TNBC cell lines MDA-MB-231 and MDA-MB-468. IC\(_{50}\) values were determined by cell viability via the MTT assay (Figure 4A & B). A drastic IC\(_{50}\) concentration difference between MDA-MB-231 and MDA-MB-468 were seen in Carbo at 962 \(\mu\)M and 64.3 \(\mu\)M, respectively (Figure 4C). A more moderate difference was seen in T and A between the two cell lines: 63.3 \(\mu\)M in MDA-MB-231 and 29.7 \(\mu\)M in MDA-MB-468 for T and 2.76 \(\mu\)M in MDA-MB-231 and 1.06 \(\mu\)M in MDA-MB-468 for A (Figure 4C). IC\(_{50}\) concentrations for C between cell lines only differed slightly at 4.05 mM and 4.36 mM for MDA-MB-231 and MDA-MB-468, respectively. The IC\(_{50}\) concentrations displayed in Figure 4C were used throughout in vitro experiments. TNBC subtypes are known to vary in their sensitivity to chemotherapy agents, with certain chemotherapy agents not being as effective in more resistant cell lines than subtypes with higher sensitivity to the drug.

Once the IC\(_{50}\) values were determined, flow cytometry was used to acquire cell cycle profiles after a single treatment of each drug. Following the schema in Figure 5A, the MDA-MB-231 cell line was treated with a single treatment of T, TCarbo, Carbo, or AC for 24 hours and, 48 hours post-treatment, the cell cycle profile was analyzed via flow cytometry. Bar charts depict the breakdown of each phase of the cell cycle, while pie charts depict the percentage of dead and live cells within each treatment group (Figure 5B-C). In all treatments, the percentage of cells in the S phase were the lowest among all cell phases (Figure 5B), but the percentage of cells in G1 and
G2/M varied by drug treatment. Carbo arrested the majority of cells in the G1 phase of the cell cycle at 48% (Figure 5B). While all treatments, aside from Carbo and DMSO, lead to higher percentages of cells arrested in G2/M, when Carbo was used in combination with T more cells were arrested in the G2/M phase than when T was used alone (Figure 5B). The distribution of cells among the phases of the mitotic cell cycle after therapeutic treatments provides key insight to understanding how ACT+Carbo agents work in relation to each other and where they arrest cells within the cell cycle.
Figure 4: IC₅₀ values and dose curves of MDA-MB-231 and MDA-MB-468 cell lines.

TNBC cell lines, A) MDA-MB-231 and B) MDA-MB-468, were treated with paclitaxel, carboplatin, doxorubicin, or cyclophosphamide for 24 hours. Cell viability was determined via MTT assay (n=4-14). All IC₅₀ values and graphs were calculated with Dr-Fit software. C) IC₅₀ values of ACT+Carbo drugs in both cell lines.
Figure 5: Cell cycle profile after a single treatment

A) Treatment schematic of 4-day single treatment trial. MDA-MB-231 cells were seeded at the appropriate density and treated with DMSO, T, Carbo, TCarbo, or AC for 24 hours; 48 hours post-treatment, cells were collected, fixed, and analyzed via flow cytometry. Analysis is represented as cell percentages found in G1, S, or G2/M phase of the cell cycle. B) MDA-MB-231 cell cycle profile analysis (n=3). C) Pie charts represent percentage of dead and live cells of individual drugs (DMSO, T, TCarbo, Carbo, or AC). Error bars reflect SE; Student’s t-test, **p<0.01, ***p<0.001.
2. Alteration in Administrative Timing Leads to Increased Efficacy of T and TCarbo Treatments

Treatment for TNBC administers either a single chemotherapy agent or a combination of two or more within the standard of care. Pre-clinical models can be used to determine how to optimize treatments, but the reliability and mimicry of these models within TNBC has not been fully studied in relation to ACT+Carbo treatments. To determine if in vitro models can predict clinical response, single and combined agents of A, C, T and Carbo were first tested. MDA-MB-231 and MDA-MB-468 cells were treated with DMSO, T, TCarbo, Carbo, AC, A, or C for 24 hours, and 48 hours post-treatment cells were collected and proliferation rates assessed using the trypan blue exclusion assay (Figure 6A). Results show that both cell lines exhibit differences in proliferation rates between single and combined agents. In MDA-MB-231 cells, the quantification of live cells between T and TCarbo differed only by 13% (Figure 6B), while they differed in MDA-MB-468 cells by 2.3% (Figure 6D). There was no significance between T and TCarbo treatments. MDA-MB-231 and MDA-MB-468 cells treated with only Carbo contained relatively high proliferation rates of 29% and 16.5% respectively, as opposed to TCarbo that exhibited proliferation rates of 33.5% and 13% respectively (Figure 6B & D). TNBC cells were treated with AC, A, or C to confirm the additive effect of these two drugs in combination, as per previous methods (Figure 6A). Cells treated with A or C contained elevated proliferation rates of live cells comparable to AC. While there is no significant difference between A and AC in MDA-MB-231 cells, the percentage of live cells in those treated with only C is significantly higher at 40.5% than both A and AC at 26% and 21%, respectively (Figure 6C). In MDA-MB-468 cells, A and C treatments contained higher percentages of live cells at 35.5% and 30% respectively, while the AC treatment only resulted in 11.5% of live cells (Figure 6E).

Within the standard of care, TNBC patients receive ordered treatments of T and TCarbo over a period of time, however, the timing pattern of these agents are often not taken into consideration. To determine if the order of T and TCarbo influence efficacy of treatments while
using predictive models, ordered patterns of T and TCarbo were tested using *in vitro* models. MDA-MB-231 and MDA-MB-468 cell lines were treated with T or TCarbo for 24 hours, and 48 hours later the treatment was repeated (Figure 7B); 7 days after the start of the first treatment, quantification of live cells were determined via trypan blue exclusion assay. Among all combinations used (Figure 7A), a distinction in those treated with T/TCarbo between cell lines was evident. Proliferation rates of cells administered with T/TCarbo were the lowest at 11% and 6% in MDA-MB-231 and MDA-MB-468 cell lines, respectively (Figure 7C & D). No significance was found among treatments, with the exception between T/T and T/TCarbo in MDA-MB-231 cells and between T/TCarbo and TCarbo/T in MDA-MB-468 cells (Figure 7C & D). Proliferation rates of T/T, T/TCarbo, and TCarbo/TCarbo were relatively even in MDA-MB-231 cells; the order with the highest quantification of live cells at 19% was T/T, 8% higher than T/TCarbo (Figure 7C). Variation in proliferation rates among treatments were apparent in MDA-MB-468 cells; TCarbo/T resulted in the highest proliferation rate at 19.5%, a 13.5% difference compared to T/TCarbo (Figure 7D).
Figure 6: 4-day proliferation trial results of independent or combined therapeutic agent after a single dose.

A) Treatment schematic of the 4-day proliferation trial. Cells were seeded at the appropriate density and treated with DMSO, T, Carbo, TCarbo, AC, A, or C for 24 hours. 48-hours post-treatment cell were collected and counted via trypan blue exclusion. Graphs denote MDA-MB-231 cells treated with B) DMSO, T, Carbo, or TCarbo (n=4, DMSO n=3) and C) DMSO, AC, A, and C (n=3). Graphs also denote MDA-MB-468 cells treated with D) DMSO, T, Carbo, or TCarbo (n=3) and E) DMSO, AC, A, and C (n=3). Error bars reflect SE; Student’s t-test, *p<0.05, **p<0.01, ***p<0.001.
Figure 7: Double treatment of T and TCarbo proliferation rate trials.

A) Four combination patterns of T and TCarbo. B) Treatment schematic of the 7-day proliferation trial. Cells were seeded at the appropriate density and treated with DMSO, T, or TCarbo for 24 hours on day 1 and day 4 with 48 hours between treatments. 48 hours after the last treatment, cells were collected and counted via trypan blue exclusion. Graphs denote cell viability of **C** MDA-MB-231 \((n=12, \text{ DMSO } n=10)\) and **D** MDA-MB-468 \((n=4)\). Error bars reflect SE; Student’s t-test, \(*p<0.05, **p<0.01, ***p<0.001.\)
Combination therapy of AC is a common precursor to other drugs within TNBC treatments; ACT chemotherapy is no different (Taherkhani et al., 2017; Yardley et al., 2017). The administration of AC prior to T or Carbo treatments is accepted in TNBC therapy, but how the prior addition of AC influences T and Carbo efficacy has not been thoroughly investigated. To determine the effects of AC on the efficacy of T and Carbo treatments, a 12-day proliferation assay was conducted; following the standard timeline, AC was administered twice to MDA-MB-231 cells, followed by T or TCarbo twice (Figure 8A). Cells were collected 12 days after the start of the initial treatment and proliferation quantified via trypan blue exclusion assay. The lowest amount of live cells was found when using T/TCarbo and TCarbo/TCarbo. These two drug patterns contained almost equal percentages of live cells at 11.6% and 11%, respectively (Figure 8B). Cells treated with T/T contained the highest quantification of live cells at 14.7% and TCarbo/T the second highest at 16.4% (Figure 8B). Significance was found between TCarbo/T and TCarbo/TCarbo (Figure 8B). These results demonstrate how AC prior to T and Carbo treatments influence efficacy of optimized treatment.
Table 1: Experiments performed in the 12-day proliferation trial.

<table>
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<tr>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
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<th>Day 5</th>
<th>Day 6</th>
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<td>Cells treated with AC</td>
<td>Removed drug(s)</td>
<td>---</td>
<td>Cells treated with AC</td>
<td>Removed drug(s)</td>
<td>---</td>
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<table>
<thead>
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<th>Day 8</th>
<th>Day 9</th>
<th>Day 10</th>
<th>Day 11</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells treated with T or TCarbo</td>
<td>Removed drug(s)</td>
<td>---</td>
<td>Cells treated with T or TCarbo</td>
<td>Removed drug(s)</td>
<td>Collected/Counted via Trypan Blue Exclusion Assay</td>
</tr>
</tbody>
</table>

Figure 8: Quantification of live cells when treated with AC precursor to T and TCarbo results.

A) Treatment schematic of the 12-day proliferation trial. MDA-MB-231 cells were seeded at the appropriate density and treated twice with DMSO or AC for 24 hours followed twice by DMSO, T, or TCarbo for 24 hours with 48 hours between treatments. B) Graph denotes cell viability of MDA-MB-231, n=4. Error bars reflect SE; Student’s t-test, *p<0.05.
4. Synergistic Relationship Between TCarbo Varies Among Concentrations While AC Exhibits An Overall Synergistic Relationship

Utilizing a cocktail of drugs for chemotherapy is a common practice in TNBC. When merging therapeutic agents together, all agents should improve and compliment each other to result in the highest efficacy. However, not all drugs work synergistically, but often may be antagonistic, which is detrimental for treatments. To determine the synergy level between the two main combined drugs, TCarbo and AC, the Bliss independence model was utilized. Using the Combenefit software and the Bliss independence model, matrix and surface plots of synergy distribution were acquired for MDA-MB-231 (Figure 9A & 10A) and MDA-MB-468 (Figure 9B & 10B) cell lines. The colour spectrum of all plots represent levels of synergy between the two agents: blue indicating high synergism, green indicating slight synergism, yellow indicating slight antagonism, and red indicating high levels of antagonism. The shift in tones represents the level of synergy within that hue, where dark blue indicates strong synergism while light blue indicates medium synergism. Matrix plot values represent the percentage difference comparable to the control, where the control is the expected additive value of both drugs without direct drug interaction. The matrix plots compare the interaction of T and Carbo at varying concentrations, displaying areas of synergy within each cell line. Both MDA-MB-231 and MDA-MB-468 cells show increased levels of antagonism at higher doses of either T or Carbo, but higher levels of synergism at medium doses of T and lower doses of Carbo (Figure 9A & B). Some of the highest levels of synergy in Carbo are at concentrations of 30 µM and 35 µM in MDA-MB-231 and MDA-MB-468, respectively (Figure 9A & B). The highest synergy level of T is at the concentration of 40 µM in MDA-MB-231 cells (Figure 9A), and high synergy of T in MDA-MB-468 cells is at 15 µM (Figure 9B). Results of AC synergy exhibits high levels of synergistic activity between A and C in both cell lines (Figure 10A & B). Synergism is seen between A and C in MDA-MB-231 cells at increasing doses, the highest synergy peaking at 1 µM of A and 6
mM of C. The highest levels of synergy between A and C in the MDA-MB-468 cells is at a concentration of 0.8 μM of A regardless of C concentration (Figure 10B). Medium to low synergy is seen at lower doses of C in both cell lines (Figure 10A). All surface and matrix plots display variation in beneficial interaction between two drugs, where T and Carbo display high synergy at specific concentrations, while A and C exhibit blanket synergism between drugs.
Figure 9: T vs Carbo synergy depicted in surface plots with corresponding matrix plots.

Surface plots (left) with the corresponding matrix plots (right) of the synergy distribution of drug combination T and Carbo. A) MDA-MB-231 and B) MDA-MB-468 cells lines were exposed to increasing concentrations of either T or Carbo for 24 hours; cell viability was determined via MTT. n=4-8. Values in matrix plot boxes represent predicted percentage difference from the additive effect of TCarbo; values range from 100 (synergy) to -100 (antagonism). Statistical significance was evaluated by Combenefit Software using a one-sample t-test, *p<0.05, **p<0.01, ***p<0.001.
Figure 10: A vs C synergy depicted in surface plots with corresponding matrix plots.

Surface plots (left) with the corresponding matrix plots (right) of the synergy distribution of drug combination A and C. A) MDA-MB-231 (n=4-8) and B) MDA-MB-468 (n=4) cells lines were exposed to increasing concentrations of either A or C for 24 hours; cell viability was determined via MTT. Values in matrix plot boxes represent predicted percentage difference from the additive effect of AC; values range from 100 (synergy) to -100 (antagonism). Statistical significance was evaluated by Combenefit Software using a one-sample t-test, *p<0.05, **p<0.01, ***p<0.001.
5. Synergy Influences Efficacy of TCarbo combination

Patients with TNBC are administered T and Carbo at individually determined concentrations. These concentrations do not take into account the synergistic relationship between the two drugs and how it may impact dosage. Increased synergism between drugs allows for lower concentrations of those drugs to be used while achieving optimized results; how this relates to T and Carbo in TNBC patients is not known. In vitro models were utilized to determine how the normal concentration of T and Carbo compares to the synergistically-derived concentration relative to proliferation rates and T and Carbo patterns. This provides a beneficial way to theorize how synergistic concentrations correlate to the efficacy of treatments in TNBC patients. While previous experiments have looked into the administrative timing and synergistic relation of T and Carbo, doses relative to each individual drug (IC₅₀) were used. However, the synergism of TCarbo may be lower at those concentrations. To determine if the synergism between T and Carbo plays a major part in treatment efficacy, IC₅₀ concentrations (IC₅₀: 63 μM T and 962 μM Carbo) or synergic-derived concentrations (Synergy: 40 μM T and 30 μM Carbo) were administered twice to MDA-MB-231 cells in varying patterns of T and TCarbo. Figure 7A denotes the regimens used. Cells were treated twice with either T or TCarbo for 24 hours with 48 hours between treatments; 7-days after the start of the initial treatment, the quantification of live cells was determined via trypan blue exclusion assay (Figure 11A). The graph in Figure 11B denotes a trend between cells treated with the IC₅₀ concentration versus synergistic-derived concentration: both result in similar live cell percentages within each treatment pattern. The overall difference between the two concentration groups (IC₅₀ and synergy) ranges from the smallest difference in TCarbo/T treatments at 2.5%, to, and the largest difference in TCarbo/TCarbo treatments at 12% (Figure 11B). Half of the regimen patterns display a greater decrease in live cells when treated with synergistic-derived concentrations versus the IC₅₀ concentrations. No significance is shown among treatments, but all treatments are significant.
relative to the control. Results demonstrate the minute difference between using the higher IC$_{50}$ concentrations of drugs versus utilizing the synergistic concentration between drugs to elicit effective treatment outcomes.
Figure 11: Difference in proliferation rates between treatments of IC$_{50}$ versus synergy concentrations of T and Carbo patterns.

A) Treatment schematic of the 7-day proliferation trial between IC$_{50}$ and synergy concentrations. MDA-MB-231 cells were seeded at the appropriate density and treated twice with DMSO, T, or TCarbo for 24 hours with 48 hours between treatments. IC$_{50}$ samples were treated at concentrations of 63 $\mu$M (T) and 962 $\mu$M (Carbo), and synergy samples at concentrations of 40 $\mu$M (T) and 30 $\mu$M (Carbo). B) Graph denotes cell viability of MDA-MB-231, $n=3$. Error bars reflect SE; Student’s t-test, ***p<0.001.
6. Fold Change Variation Among T and Carbo Treatments

*Casper* zebrafish are a beneficial pre-clinical model that can be used to observe tumour formation, migration, and treatment response to simulate tumours in cancer patients. This is accomplished by engrafting cancer cells into the zebrafish and observing the growth of the tumour after treatments (Figure 12). To further understand how the administrative timing of T and Carbo relate to efficacy in TNBC treatments, fluorescently labelled MDA-MB-231 cells were injected into the yolk sac of *casper* zebrafish 2dpf and treated with T or TCarbo for 24 hours at 3dpi and 6dpi (Figure 13A). Zebrafish were imaged everyday for 7 days (3dpi-9dpi). Integrated density of tumours were measured using ImageJ and fold change of each group determined comparable to day 1. Fold change is the increase/decrease in the tumour burden comparable to the initial burden; it is a ratio of tumour size relative to day 1 tumour burden. Results in Figure 13B display variability among all combination patterns of T and TCarbo listed on Figure 7A. Figure 13B displays tumour growth in TCarbo/T and TCarbo/TCarbo in the first day before steadily declining until day 6, from which tumour burden levels out in TCarbo/T until day 7 and TCarbo/TCarbo continues to decline. Within the 7 days, T/T and T/TCarbo treatments display an overall decline in tumour burden (Figure 13B). Zebrafish initially treated with T display the lowest tumour growth, while groups initially treated with TCarbo show an increase in tumour burden before declining. All treatments are significant on day 2 with TCarbo/T treatments exhibiting the largest increase in tumour size on day 2 (Figure 13B). Tumour size in T/TCarbo treatments contain the least amount of change in all groups on days 2-5 until the tumour size in majority of treatments decline to near zero and level out on day 6-7 (Figure 13B). While tumour growth may differ among treatments within the first few days, from day 4 onward, all groups show a steady decline in tumour burden. 18% of zebrafish were dead by day 6, and 61% by day 7. The control group, DMSO, exhibits odd engraftment with staggering growth between days 1-3, leveling out on day 4, and steadily declining for the remaining time. The increase or decrease in tumour burden can be used to monitor the growth rate of engrafted cells and, as a pre-clinical
model, provides insight into how the TME and the immune system may influence efficacy of TNBC treatments.
Figure 12: *Casper zebrafish injection schematic.*

Fluorescently labelled MDA-MB-231 cells were injected into anesthetized *casper* zebrafish embryos (2dpf). 3dpi fish were placed in 12-well plates at 3 fish per well and treated for 24 hours with T or TCarbo with DMSO as the control. 48 hours after initial treatment (6dpi), zebrafish were treated with drugs again for 24 hours. Zebrafish were imaged using inverted microscope (Lecia) every day (3dpi – 9dpi). Image created using BioRender.
Figure 13: *Casper* zebrafish tumour fold change among treatments.

A) Treatment schematic of *casper* zebrafish engrafted with MDA-MB-231 cells, where day 1-7 represents 3dpi-9dpi. LD_{10} concentrations of T and Carbo were added to E3 water for 24 hours on 3dpi and 6dpi. Zebrafish were imaged daily. n=18-24. B) Tumour integrated density was measured each day, normalized to first day of treatment, and fold change calculated. Error bars reflect SE. Statistical significance calculated using one-way ANOVA and Tukey post-hoc test, *p<0.05.
DISCUSSION

The term ‘triple-negative breast cancer’ was coined in 2005 referring to a subset of an aggressive and invasive BC that lacked the three main receptors: ER, PR, and HER2 (Pareja et al., 2016). Due to the loss of these receptors, targeted therapies cannot be used to treat TNBC, and general chemotherapy treatments must be used instead, making TNBC difficult to treat. The 5-year survival rate of TNBC is 77% and the relapse rate of stage 1, 2 and 3 TNBC is 40% (Stewart et al., 2019). Improvements in current TNBC treatments are required to increase patient survival and efficiently prevent future relapse of TNBC; one way to accomplish this is to optimize the efficacy of the current standard of care in TNBC through pre-clinical models. These in vitro and in vivo models may be utilized to optimize treatments of TNBC, which can be directly implemented in TNBC regimens to increase pCR rates of patients, lower toxicity, and elevate overall efficacy of administered treatments. This can be accomplished by using pre-clinical models to test chemotherapy treatments, followed by confirmation via clinical trials. This is an effective and ethical way to come up with novel treatments and optimize current regimens while decreasing TNBC patient risk. This research aims to optimize ACT+Carbo treatments of TNBC using predictive pre-clinical models for clinical application.

This study used TNBC cell lines from 2 different subtypes of TNBC to compare the effects of chemotherapy across a spectrum of subtypes, as many subtypes of cancer vary in sensitivity to chemotherapy agents. The resistance of MDA-MB-231 cells is highly correlated to the expression of mesenchymal markers and cancer stem cell profiles of the cell line found in the mesenchymal subtype of TNBC (Garcia et al., 2021). MDA-MB-468 is a basal-like subtype of TNBC that displays high responsiveness to chemotherapy, particularly to platinum-based drugs and targeted DNA-repair deficiency therapy (Garcia et al., 2021). These characteristics of both cell lines are clearly displayed through the IC_{50} concentrations; higher IC_{50} values in majority of the drugs were correlated with MDA-MB-231 cells, while lower IC_{50} concentrations were found
in MDA-MB-468 cells (Figure 4). The largest difference was observed in Carbo, where the IC\textsubscript{50} value of MDA-MB-231 cells was 962 µM, significantly higher than the IC\textsubscript{50} value of MDA-MB-468 at 64.3 µM (Figure 4). Carbo is a platinum-based chemotherapy agent that targets GC-rich areas in the DNA, and is expected that the MDA-MB-468 cell line would be more sensitive to Carbo treatments compared to other cell lines. Understanding how subtypes differ in chemosensitivity is an important factor in developing future TNBC regimens to optimize anti-cancer treatments.

Majority of therapeutic agents target the cell cycle at specific phases, however, some target general aspects of the cell and are cell cycle non-specific; this includes C and Carbo (Ogino & Tadi, 2022; Sousa et al., 2014b). Results from the 4-day cell cycle profile of MDA-MB-231 cells display exactly where and at what percentage the cells are arrested within the cell cycle after a single treatment. The cell cycle profiles confirmed T arrests cells in G2/M, and Carbo was non-specific in cell cycle targeting (Figure 5B). When these two agents were used in combination, cells arrested in the G2/M phase of the cell cycle are higher than either T or Carbo alone (Figure 5B). This highlights the difference between individual versus combination treatment of T and Carbo. Results demonstrate how T may be a useful therapeutic agent for TNBC, but when partnered with Carbo enhances the influence of the platinum drug and promotes G2/M arrest within the cell cycle at a higher rate than when individually used. Repetitive treatments of TCarbo may appear be a productive anti-cancer strategy, however a study conducted on neoadjuvant TNBC compared the pCR rates of patients that received weekly doses of TCarbo versus weekly T and every 3-week Carbo in treatments found pCR rates of TNBC patients given weekly Carbo was 85%, and every 3-week Carbo was 92% (Landry et al., 2022). While statistical significance was not reached for this study, these initial results suggest the delayed addition of Carbo may possess an advantage when treating TNBC. It is notable that the patients that received weekly Carbo were more likely to miss chemotherapy doses or require a reduction in doses of Carbo due
to high hematologic toxicity (Landry et al., 2022). High toxicity is a consequence of constant use and high doses of Carbo and may lead to increased cellular resistance to treatments and harsh physical side-effects in patients, such as bone marrow suppression, nephrotoxicity, and neurotoxicity (CARBOplatin, 2017).

Chemoresistance is a serious and difficult problem within TNBC. Resistance to chemotherapy agents, especially platinum-based drugs, may be achieved by more efficient DNA repair, increased drug efflux via membrane transport system, initiation of pro-survival signals, and antioxidant upregulation (Moens et al., 2021). A way to combat and prevent resistance to platinum-based agents in TNBC is to reduce constant exposure to associated chemotherapy agents or to lower doses of platinum-based drugs. One way to achieve this is to stagger treatments of platinum-based agents, as previously discussed. Another way is to lower doses of these agents during treatment; this relates to the synergy of chemotherapy drugs. Understanding the additive effect two or more drugs have on each other within TNBC and optimizing treatments using those results is an important strategy to prevent chemoresistance. Figure 9 displays the synergistic relationship between T and Carbo at varying concentrations using the Bliss independence model. The TNBC cell lines used in this work displays high synergy of combined drugs at low concentrations relative to the IC50, particularly in the MDA-MB-231 mesenchymal subtype. Mesenchymal-like cells within TNBC are more resistant to platinum-based treatments, as clearly evident with the higher IC50 concentration of Carbo (962 μM); however, Figure 9A demonstrates that the Carbo sensitivity of MDA-MB-231 cells, when used in combination with T, is significantly increased at synergistic concentrations of Carbo (30 μM). Taking these results a step further, two doses of T+/Carbo at either IC50 or synergistic values previously determined were used on MDA-MB-231 cells and proliferation rates compared (Figure 11). The proliferation rate among all combinations of T and Carbo between IC50 and synergistic concentrations displays a relatively even outcome (Figure 11B). No significant difference was found between IC50 and
synergistic treatments. However, taking into consideration the concentration of Carbo differed by 18.8% between concentrations, the suppressed proliferation within the synergistic combination of TCarbo illustrates the importance of synergy within TNBC treatments. Figure 11B results demonstrates how synergistic concentrations of TCarbo in TNBC will result in high efficacy of treatments while requiring lower doses of TCarbo; consequently, lower doses will lessen the chance of chemoresistance, lower toxic side-effects, and increase pCR rates of TNBC patients.

Pre-clinical models are a powerful and efficient way to develop and improve cancer chemotherapy treatments by understanding how drug response differs among subtypes of TNBC. This can be achieved via proliferation, gene expression, resistance, and cell cycle analysis within \textit{in vitro} models. Creating a pre-clinical model that not only mimics \textit{in vitro} and \textit{in vivo} results, but also clinical trial results is required to demonstrate the reliability of lab based pre-clinical models that could be used to test cancer treatments that accurately reflect patient outcomes. Further testing of these pre-clinical models is required to determine if they can accurately be used as the first step in developing cancer treatments prior to clinical trials. To accomplish this, proliferation rates of live cells treated with T and TCarbo were tested. Figure 6B & D displays cell viability 72-hours post-treatment, where there is no significant difference between T and TCarbo treatments, but slight significance between monotherapies of Carbo and T in MDA-MB-231 cells. It has been previously discussed that platinum-based agents are effective in treating TNBC (Garcia et al., 2021), but while initially these results in Figure 6B & D appear contradictory, it is not indicative of the additive effect that TCarbo has on the cells, as after a single treatment cells may be arrested in the G2/M phase but are able to cycle back into a proliferative state. To confirm this was the case, a 7-day proliferation trial consisting of two doses of drug treatments with 48 hours between treatments was conducted. In Figure 7, an optimized sequence of therapeutic agents was observed where cells treated with T followed by TCarbo proved the most effective combination of these drugs. Similar results were observed in a clinical trial on TNBC patients,
where those that received T followed by TCarbo in a repetitive regimen had the most promising outcomes and tolerated the chemotherapy better than any other combination of T and TCarbo (Figure 14) (Hamm et al., 2022). These results suggest that the efficacy of ACT+Carbo treatments can be increased by optimizing the administrative timing of T and TCarbo within treatments.

**Optimal ACT+Carbo Treatment**

![Optimal ACT+Carbo Treatment](image)

**Figure 14: Clinical trial layout of ACT+Carbo treatments in TNBC.**
Image created using BioRender.

The use of pre-clinical models is beneficial to test and predict clinical outcomes to novel or optimized treatments. One example is the optimization of ACT+Carbo treatments, where *in vitro* models accurately mimicked the high efficacy within the T/TCarbo pattern, as was found in a previous clinical study (Hamm et al., 2022). However, TNBC patients on ACT+Carbo treatments commonly receive 4 treatments of AC prior to T or TCarbo agents, and whether this has an impact on T and Carbo efficacy is not well understood. The use of topoisomerase inhibitors (A) and alkylating agents (C) as an anti-cancer therapy are known to work well, as the topoisomerase inhibitors prevents DNA duplication and many alkylating agents create DNA cross-links (Sun et al., 2021). Together, these provide an effective strategy to target and promote apoptosis in cancer cells. In combination, AC arrests cells in the G2/M phase of the cell cycle (Figure 5B), even though C is cell cycle phase non-specific and A arrests cells in the S phase.
When proliferation rates were quantified 72-hours post-treatment of A, C, or AC, it was determined that the combination of AC resulted in the lowest amount of live cells (Figure 6C & E) and high synergism at many concentrations was detected using the Bliss independence model (Figure 10). This is indicative of the additive effect that occurs when A and C are amalgamated in cancer treatments (Sun et al., 2021). Taking these results into consideration, the next step was to determine whether the use of AC influences the optimized pattern of T and TCarbo treatments. Figure 8 denotes results from the 12-day proliferation assay, where two treatments of AC were followed by two treatments of either T or TCarbo. Results clearly mirror the same pattern of T/TCarbo found in the 7-day proliferation trial (Figure 7C & D). This suggests that AC agents, while an effective anti-cancer treatment, do not exhibit noticeable influence on T and TCarbo treatment efficacy in this experimental design. These findings insinuate that AC does not significantly influence T and TCarbo interactions in the cell, and the administrative patterns of T and Carbo are solely due to the two drugs themselves.

Pre-clinical in vivo models, including mice and zebrafish, can be engrafted with patient tumours and cell lines to be studied in an environment that simulates the TME and other factors naturally occurring in the cancer patients. Zebrafish contain 70% genomic similarity to human in addition to high similarity in drug metabolism (Park et al., 2020). These features prove extremely beneficial to using zebrafish in various research fields, including drug discovery, toxicology, and disease models (Park et al., 2020). Engraftment of human cells in the zebrafish can occur in a variety of different organs. One well established engraftment site for cancer cells is the yolk sac at early stages of zebrafish development (Póvoa et al., 2021). Zebrafish have a highly conserved vertebrate innate immune system that begins to develop at 1 day post-fertilization (dpf) (Liao et al., 2021). However, the adaptive immune system in zebrafish is not functional until 2-3 weeks post-fertilization; this provides a window of opportunity to study the response of the innate
immune system to treatments *in vivo* without the interference of the adaptive immune system (Póvoa et al., 2021).

In this work, MDA-MB-231 cells were engrafted into *casper* zebrafish to determine how T and TCarbo combinations relate to tumour burden, and if the optimal treatment mimics *in vitro* results. Figure 13B displays the tumour fold change among all T and TCarbo patterns displayed in Figure 7A, where fold change is the increase/decrease in the tumour burden comparable to the initial burden. After a single treatment of either T or TCarbo, a large discrepancy was observed between the two treatments. The large increase in tumour size when initially treated with TCarbo vs the decline in tumour burden seen in initial treatments of T gives insight into the differences noted between these two treatments. After the second dose of T or TCarbo was administered on day 4, a steady decline and overlap in tumour burden among all samples was seen on day 6. A pattern of T and TCarbo is suggested by the overall lowest tumour burden associated with T/TCarbo treatments (Figure 13B). This pattern correlates to previous *in vitro* MDA-MB-231 results in Figure 7C and Figure 8B. In addition, *in vivo* results mimic the optimized order of T/TCarbo found in previous clinical trials, further establishing the benefit of using zebrafish as a pre-clinical model to simulate the TME of cancer patients to test and understand drug interaction and increase treatment efficacy (Hamm et al., 2022). Due to the similarity of drug metabolism in human and zebrafish, the utilization of *casper* zebrafish to test drug combinations to increase treatment efficacy is a strong bases to creating a reliable pre-clinical model (Park et al., 2020). Figure 13B further solidifies this by exhibiting the same optimized pattern of T/TCarbo found in previous *in vitro* models and further manifesting the correlation between *in vitro* and *in vivo* experiments as pre-clinical models.

The steady decline in tumour burden from day 4 onward in all treatments is a consequence of starvation. Due to fluorescently labeled MDA-MB-231 cells that were engrafted in the embryos, zebrafish were not able to be fed due to the bioluminescence found in zebrafish
food. Embryos are normally fed daily starting 5dpf, but this may be delayed until 8dpf without adversely impacting larval growth and development (Hernandez et al., 2018). However, if embryos are not fed they usually die between 10-12dpf. Within the in vivo experiment, 61% of zebrafish were dead by day 7 (11dpf) and 96% dead by 12dpf (not shown); this is due to starvation, which may cause an increase in the production of reactive oxygen species (ROS) and cause autophagy within embryos (Liao et al., 2021). Regardless of the decline in live zebrafish larva within the experiment, the control group (DMSO) maintained a relatively even tumour burden from days 1-4, but declines from days 5-7 (Figure 13B). The engraftment success rate of MDA-MB-231 cells within the casper zebrafish may have influenced tumour burden throughout the experiment. The engraftment efficiency in zebrafish varies among cell lines. A study done in 2021 compared the engraftment efficiency in zebrafish among several cell lines; those with engraftment rates between 20-30% were labelled ‘regressors’ while cell lines with engraftment rates above 80% were labelled ‘progressor’ (Póvoa et al., 2021). MDA-MB-231 cells contained low engraftment rates and therefore were labelled as a ‘regressor’, while MDA-MB-468 cells were found to be a ‘progressor’ cell line.

Taking this into account, the next step is to use the MDA-MB-468 cell line within zebrafish xenografts to solidify the association between T and Carbo treatment patterns, the correlation to the TME and innate immunity, and how these results may differ from MDA-MB-231 cells due to engraftment efficiency. Assuming MDA-MB-468 cells will result in higher engraftment rates and sustainable food sources lacking in fluorescence will be developed for the zebrafish, it is speculated that clear and defined trends in T and TCarbo combination patterns will occur, mimicking the optimal pattern of T/TCarbo found in MDA-MB-231 cells, further establishing the creditability of pre-clinical models are a predictive tool in TNBC treatments. Zebrafish are a beneficial model that can be used to study various aspects of TNBC treatments, such as efficacy, immune influence, and toxicity. Casper zebrafish xenograft models can be
utilized to determine how various subtypes of TNBC differ in response to chemotherapy treatments, providing a fast, effective, and ethical pre-clinical model to develop and enhance current and novel TNBC treatments.

In conclusion, this study was used to determine how pre-clinical models can be utilized to improve current treatments, predict patient outcomes, and understand the plausible mechanisms. ACT+Carbo treatments are commonly used on TNBC patients, and this research suggests the order of T/TCarbo within patient regimen will result in the highest response rate, lowest toxicity, and lower chemoresistance comparable to all other T and Carbo orders. Applying this knowledge to the current TNBC standard of care may increase the 5-year survival rate of TNBC patients.
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APPENDICES

Appendix A: A prospective phase II clinical trial identifying the optimal regimen for carboplatin plus standard backbone of anthracycline and taxane-based chemotherapy in triple negative breast cancer

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A prospective phase II clinical trial identifying the optimal regimen for carboplatin plus standard backbone of anthracycline and taxane-based chemotherapy in triple negative breast cancer

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Abstract
Addition of platinum to combination chemotherapy for triple negative breast cancer (TNBC) has shown efficacy and is increasingly accepted in the clinic, yet optimal delivery is unknown. A prospective clinical trial with TNBC patients was conducted to determine the optimal chemotherapy regimen to deliver carboplatin with standard dose dense ACT. Tissue microarray was conducted to isolate markers indicative of response to treatment. 90 TNBC patients were enrolled onto our trial. The most successful version placed the carboplatin on the second and final paclitaxel treatment with liberal hematological parameters. Our final regimen had the lowest grade 3 or 4 toxicities, no delays, no dose reductions of carboplatin, and 32% reduction in paclitaxel doses. Stage I (AJCC7) patients did well with carboplatin-based chemotherapy with zero relapse rate. Reduction in protein levels of androgen receptor and PD-L1 were found to be potential indicators of patient relapse. We have optimized a protocol for the addition of carboplatin to standard of care chemotherapy in TNBC patients. Early data indicates reduced protein levels of androgen receptor and PD-L1 as indicators of response to treatment.

Trial registration This trial was registered at Canadian Cancer Trials. http://www.canadiancancertrials.ca/

Keywords Triple negative breast cancer · Carboplatin · Chemotherapy · Adjuvant · Androgen receptor · PD-L1 · Regimen

Abbreviations

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<td>TNBC</td>
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<tr>
<td>HR</td>
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<td>pCR</td>
<td>Pathological complete remission</td>
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<td>BRCA-1</td>
<td>Breast cancer gene 1</td>
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<td>CTCAE</td>
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Background
Triple negative breast cancer (TNBC) comprises approximately 12–17% of breast cancers, with incidence being higher in the African American population [1]. Median presenting age for TNBC is 56 years, representing an overall younger cohort of patients [2–4]. TNBC is estrogen and progesterone receptor negative by immunohistochemistry staining and has low levels of the human epidermal growth factor (HER2) receptor, eliminating the option of current hormone and targeted therapies. Chemotherapy remains

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the only standard systemic treatment for early stage TNBC. A TNBC diagnosis is associated with shorter overall and disease-free survival compared to non-TNBC, stressing the priority to improve options for these patients [1, 5].

Two landmark neoadjuvant trials demonstrated that addition of platinum increased pathological complete response (pCR) [6, 7]. A meta-analysis of thirteen randomized controlled studies confirmed that platinum addition increases pCR in TNBC and suggested it should be considered as standard first line therapy for curative intent treatment in TNBC [8]. In the neoadjuvant setting, platinum-based chemotherapy showed a pCR benefit of 40% versus 27% [8]. For non-TNBC, pCR has not reliably translated into improved relapse-free survival (RFS) and overall survival (OS), but has been suggested to improve survival benefit in the TNBC population [9]. Despite accumulating evidence to support the benefit of platinums in TNBC, consensus guidelines to date have not universally recommended using platinum agents in the curative setting outside of a clinical trial [10]. Concerns regarding the use of platinums in the adjuvant setting include increased toxicities and low regimen completion rates [7].

This study was designed to identify an optimal chemotherapy regimen for TNBC, that would allow for minimal toxicity and maximum tolerance and protocol completion. Additionally, tissue microarray (TMA) analysis was conducted to standardize the discovery of protein alterations that could be used in predicting response to treatment and to better stratify TNBC patient populations.

Methods

Study design

This was a single arm prospective clinical trial investigating the use of carboplatin in TNBC. The primary endpoint was to identify a carboplatin containing regimen that allowed minimal dose reductions, delays and maximal completion of all chemotherapy. The chemotherapy regimen was modified twice with REB approval. The first modification allowed for weekly paclitaxel. The second protocol change was designed to minimize dose reductions and delays in chemotherapy. It was designed primarily as an adjuvant clinical trial study, but neoadjuvant chemotherapy was allowed in locally advanced

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<td></td>
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tumors. The American Joint Commission on Cancer Seventh Edition was used for staging (Table 1). Our results were compared to our previously published historical controls [5].

Study population

A total of 90 TNBC patients were enrolled from a single site in Windsor, Ontario. BRCA status of patients was collected when available. Patients were Her-2 negative, ER <10% and PR <10% as the low ER and PR positivity was more commonly accepted at the beginning of this study as TNBC.

Treatment program

The regimen was modified twice to improve tolerance and completion rates. Following consent, patients were treated with dose dense adriamycin, cyclophosphamide and paclitaxel (dAdACT) every two weeks for a total of 8 treatments [11]. They received Adriamycin 60 mg/m² intravenous (IV) every 2 weeks for four cycles with cyclophosphamide 600 mg/m² IV for four cycles. Following this, they continued with four cycles of paclitaxel at 175 mg/m² every two weeks combined with carboplatin.

In version 1, patients received carboplatin with an AUC of 6 on the first and third paclitaxel. Patients required a platelet count of 100 x 10⁹ to proceed to the next chemotherapy. Either the carboplatin or the paclitaxel could be modified for neuropathy. In version 2 of the protocol, the study was modified to allow weekly paclitaxel. Paclitaxel was given at 80 mg/m² weekly x 12 weeks [12]. During weekly paclitaxel, carboplatin was administered with an AUC of 6 every 4 weeks for three treatments. In version 3, we used the first dose dense ACT regimen, but moved the carboplatin to the second and the final paclitaxel. In addition, we changed the parameters of continuing chemotherapy to allowing platelet counts ≥ 70,000 x 10⁹. Only the paclitaxel could be modified for neuropathy (Fig. 1).

RFS and OS along with toxicities were collected. Both neoadjuvant and adjuvant patients were included in the PFS and OS. Literature supports that no difference in outcomes is expected based on the timing of the chemotherapy [13]. Toxicity scores were obtained using the common terminology criteria for adverse events (CTCAE) scoring system in the following: fever, anorexia, vomiting, taste alteration, stomatitis, dermatitis, constipation, diarrhea, urinary frequency, sleep alterations, motor neuropathy, dyspea, cough, fatigue, headache, hearing changes, sensory neuropathy, pain, alopecia, irregular menses, hot flashes, other. This study was approved by The Windsor Regional Hospital Research Ethics Board (CC-11-114) and the University of Windsor Research Ethics Board under protocol number 11-115.

TMA construction

Cores used for TMA analysis were taken from each patient’s original diagnostic biopsy. Control “normal” tissue used in the TMAs was taken from the surrounding tissue shown not to have cancer. Paraffin embedded tissue blocks were received from Windsor Regional Cancer Centre and constructed into TMAs using an Arraymold Inc. TMA mold with 72 1.5 mm cores (20015D). Briefly, paraffin X-tra paraffin (Sigma) was added to the mold, an embedding ring was placed on top and filled with paraffin. After an hour incubation on ice the mold was separated from the embedding ring. 3-4 cores were taken from each embedded sample and placed into the TMA mold. Following 10 min of incubation in 65 °C oven, the mold was placed on ice. The

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Fig. 1 Schematic of treatment regimens. Overview of the three different treatment regimens used in the clinical trial. Version 1: dAdACT with AUC6 Carb on 1st and 3rd pacl (dose dense adriamycin, cyclophosphamide, taxol with area under the curve carboplatin 6; with carboplatin administered on the first and third of four Taxol (paclitaxel)). Version 2: dAdACT with AUC6 Carb on 1st, 5th, 9th pacl (dose dense adriamycin, cyclophosphamide, taxol with area under the curve carboplatin 6; with carboplatin administered on the first and third and ninth of twelve weekly taxol (paclitaxel)). Version 3: dAdACT with AUC6 Carb on 2nd and 4th pacl (dose dense adriamycin, cyclophosphamide, taxol with area under the curve carboplatin 6; with carboplatin administered on the second and fourth of four taxol (paclitaxel), with the additional parameter of proceeding with chemotherapy if the platelet count was greater than 70,000 x 10⁹). A Adriamycin, C Cyclophosphamide, T paclitaxel, Ca Carboplatin
cores were left overnight prior to sectioning. TMAs were sectioned using a Leica microtome at 10 μm and placed on Fisherbrand Superfrost Plus microscope slides (12-550-15) and heated for 10 min. Cores used for TMA analysis were taken from each patient’s original diagnostic biopsy. Control ‘normal’ tissue used in the TMAs was taken from the surrounding tissue shown not to have cancer.

**Immunofluorescence (IF) staining**

TMA sectioned slides were deparaffinized and rehydrated using xylene and decreasing concentrations of EtOH. Slides were washed in 1×PBS, followed by sodium citrate (pH 6.0) antigen retrieval. Slides were washed in distilled water and blocked using blocking buffer (3% BSA/PBS, 0.1% Tween) at room temperature, followed by incubation with primary antibody (1:200) for 1 h at room temperature (Supplemental Table 1). Slides were washed in 1×PBS, followed by incubation with a fluorescence-conjugated secondary antibody (ThermoFisher Scientific, T3601; 1:1300) for 20 min at room temperature. Following washing in 1×PBS, slides were incubated with Toto-3 nuclear stain (1:1300) for 20 min. After incubation, slides were washed in 1×PBS, then dehydrated in increasing EtOH concentrations and immersed in xylene prior to cover-slipping.

**Statistical analysis**

Descriptive univariate analysis was used to compare differences among the historical (with and without chemotherapy) groups and the groups in the three regimens with respect to their demographic variables. Survival times (overall and relapse-free times) were analyzed by using Kaplan-Meier survival curves along with log-rank tests. Differences among the survival times, restricted to the first 4.5 years were also examined by using restricted mean survival estimates approach. This part of the analysis was carried out by using the SAS software’s PROC LIFETEST and UNIVAR macro.

TMA analysis was performed using R software. We modeled the outcome “y” using multiple linear mixed models, we used mixed models to accommodate for the non-independence of observations since we have multiple observations from the same patient and multiple observations from the same slide. Interaction terms were also added to the models to detect if the protein effect is uniform across age, relapse and stage categories. We computed the 95% confidence interval for the estimated marginal mean of “y” and used heat map charts to display the unadjusted and adjusted mean of “y”. For cell line data analysis, a Student’s T-Test was performed with assumed equal variance. Significance was scored as *p < 0.05, **p < 0.01, ***p < 0.001.

**Results**

**Patient demographics**

Between 2011 and 2017, 90 patients with TNBC were enrolled on our trial. Two patients had more than zero positivity of ER and three patients had PR positivity of greater than zero. All patients had <10% ER and PR positivity and all were HER-2 negative. Eight patients were excluded from analysis as four were lost to follow-up, one was retrospectively found to be stage IV at presentation, and three did not take at least one study drug. Ages ranged from 26 to 72; 46% of patients were less than 50 years of age, while the median age was 51 years. BRCA testing was a recent protocol change, however, twenty-six tested negative for BRCA-1 and BRCA-2 and four patients tested positive for BRCA-1 or BRCA-2. Fifty-two have not been tested or the results are unknown. Table 1 Demographics carries the detailed information. [See Additional file 1].

**Protocol delay and completion rates**

Percent of patients experiencing delays in their chemotherapy regimens is shown in Table 2. In version 1 28% of patients had delays in their chemotherapy. The weekly paclitaxel regimen had highest percentage of dose delays at 67% and was abandoned because of this. In the first two versions, isolated thrombocytopenia was reason for 45% of the dose delays. Version 3 had no-chemotherapy delays when the carboplatin was moved to the second and the last

<table>
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<th>Table 2</th>
<th>Protocol completion rates</th>
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<td></td>
<td>Version 1 d(d)ACT with AUC6 carbo on 1st and 3rd paclitaxel</td>
</tr>
<tr>
<td>n =</td>
<td>58</td>
</tr>
<tr>
<td>Delays in chemotherapy n (%)</td>
<td>16 (28%)</td>
</tr>
<tr>
<td>Paclitaxel dose reductions n (%)</td>
<td>14 (26%)</td>
</tr>
<tr>
<td>Carboplatin dose reductions n (%)</td>
<td>28 (48%)</td>
</tr>
</tbody>
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paclitaxel, and the patients proceeded with chemotherapy if the platelet count was greater than $70,000 \times 10^3/L$.

The initial version of the protocol had the highest percent of carboplatin dose reductions. Reasons for dose reductions of paclitaxel and/or carboplatin were recorded. Peripheral neuropathy was the most common reason for dose reduction for both chemotherapies: 50% of reductions with carboplatin and 42% of dose reductions with paclitaxel. There were no carboplatin dose decreases in version 3, but slightly higher paclitaxel dose reduction than the first version of the protocol. Version 3 of the protocol had the lowest number of delays in chemotherapy and fewest carboplatin dose reductions. The weekly taxol regimen had the highest percent delays, and version 1 had the highest carboplatin dose reduction. Overall, patients on version 3 displayed the greatest protocol compliance.

Toxicities

Toxicities with an incidence greater than 10% are listed in Table 3. Overall, all three versions reported high overall toxicity scores in sensory neuropathy, fatigue, pain, constipation, taste alteration, nausea and anemia; however, very little grade 3 or 4 toxicity was reported in any regimen. Version 1 and 3 had the fewest serious adverse events (ASE). There were only 10% serious adverse events in version 1 and 16% in version 3, with pain being the most common ASE in 11% of patients in version 3. Version 3 had the lowest incidence of toxicity.

Progression-free survival (PFS) and overall survival (OS)

Patients on all 3 versions of the treatment protocol were followed for OS and PFS. Table 4 summarizes rates of relapse and survival over the entire follow-up period for each group. Although it has a smaller sample size and shorter follow-up time window, patients on version 3 of the treatment protocol have the best OS and PFS, as compared to those on version 1 or 2 of the protocol, with no relapses to date [1].

Analysis of PFS and OS was conducted between the three regimens and compared with historical chemotherapy and ‘no-chemotherapy’ control groups. Kaplan-Meier estimates of OS (Fig. 2A) indicate a statistically significant difference among the five groups (log-rank statistic $= 12.94$, $p$ value $= 0.014$). From the survival curves it is clear that regimen 2 and 3 have the best OS with no deaths among the 18 patients in regimen 3 or the 6 in regimen 2. However, due to the small sample size and the shorter follow-up time window (median 2-year follow-up), it was not feasible to use techniques such as pairwise comparisons with adjustment of the significance level to look at statistical difference between treatment regimens. There was no statistically significant difference in PFS among the three groups (log-rank $= 5.9$, $p$ value $= 0.23$). Nevertheless, the Kaplan-Meier estimated curves in Fig. 2B indicate that regimen 1 and 3

---

**Table 3** Toxicities reported at an incidence of greater than 10%

<table>
<thead>
<tr>
<th></th>
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<tr>
<td></td>
<td>All (%)</td>
<td>Grade 3/4 (%)</td>
<td>All (%)</td>
<td>Grade 3/4 (%)</td>
<td>All (%)</td>
<td>Grade 3/4 (%)</td>
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<tr>
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<td>5</td>
<td>80</td>
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<tr>
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<td>3</td>
<td>40</td>
<td>40</td>
<td>11</td>
<td>3</td>
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<tr>
<td>Nausea</td>
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<td>40</td>
<td>0</td>
<td>26</td>
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<tr>
<td>Vomiting</td>
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<td>0</td>
<td>0</td>
<td>21</td>
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<tr>
<td>Change in taste</td>
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<td>0</td>
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<tr>
<td>Sensory neuropathy</td>
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<td>0</td>
<td>60</td>
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**Table 4** Overall survival and progression-free survival

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<td>$N$</td>
<td>Number Died</td>
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<tr>
<td>Version 1</td>
<td>58</td>
<td>9</td>
</tr>
<tr>
<td>Version 2</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Version 3</td>
<td>18</td>
<td>0</td>
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Cell cycle mediators are differentially expressed across TNBC stages

To determine whether correlations existed between patient response and the protein levels of select cell cycle regulators, we constructed TMAs from paraffin-embedded samples...
collected at diagnosis for each patient. While patient numbers were low for individual versions of the protocol, we examined changes in protein levels between stages, between patients who relapse within the study and those who did not, and between different age groups of patients. When comparing protein levels between stages, a number of individual proteins were found to be altered (Fig. 3). Patients presenting with higher stage of disease were found to have decreased levels of cyclin dependent kinase inhibitors (CKIs) p16, p21 and p27 as well as cyclin E (Fig. 3A–D). Similarly, higher stage of disease correlated with lower levels of the receptor for the steroid hormone, androgen (androgen receptor: AR) (Fig. 3F). Levels of c-Myc phosphorylation at residues Thr59Ser62 were significantly lower in stage 2 patients versus normal breast tissue (Fig. 3E).

**Stratification of TNBC patients based on age**

Since our study group was comprised of a wide age range, we sought to determine whether protein levels in the TMA analysis were different based on age of the patient. Samples were analyzed for the following cohorts: ≤ 50, 50–64, > 64 years. Only 2 proteins showed significant differences in levels between age groups: p21 was significantly higher in patients ≤ 50 years compared to older cohorts (Fig. 4A). Phosphorylated ERK (Thr202/Tyr204) was also significantly higher in patients ≤ 50 years (Fig. 4B). In patients ≤ 50 years, AR levels were significantly lower in those patients that had relapsed (Fig. 4D). Interestingly, while < 50 years group had significantly higher levels of p21 over all, the levels were even higher in the subgroup that relapsed (Fig. 4E).

**PD-L1 is significantly decreased in patients who relapse**

The ability to predict patients at higher risk of relapse is of utmost importance. Differences in levels of proteins at diagnosis was assessed for patients that relapsed, regardless of age or stage. Only one protein tested was significantly different at diagnosis for patients that would relapse after treatment. PD-L1, a membrane bound ligand upregulated during inflammation and/or oncogenic signals [14] was significantly lower for patients that relapsed (Fig. 5).

**Discussion**

**Identification of a novel treatment protocol with improved outcomes, tolerability and completion rates**

We have identified a carboplatin containing regimen for TNBC with improved completion rates and tolerability. The meta-analysis by Pandy et al. [8] provides evidence to support the routine use of carboplatin in TNBC. Different regimens are being used based on either the GeparSixto [7] trial or the CALGB 40,603 trial [15]. In the GeparSixto study, patients with TNBC demonstrated statistically significant higher pCR rate if carboplatin was added (p = 0.005). However, 48% of the patients treated on the carboplatin arm did not complete their regimen. Even after dose adjustments, 41% in the AUC 1.5 group discontinued therapy, mainly because of adverse events with > 10% grade 3 or 4 hematological, diarrhea, mucositis, fatigue, infection and other toxicities, the majority being grade 3 toxicities. Surprisingly, the follow-up study, Geparuroto [16], which used 18 weeks of weekly carboplatin with paclitaxel and liposomal doxorubicin did not demonstrate improvement in the pCR over the non-carboplatin containing regimen. 88% of patients on the carboplatin arm experienced delays in their chemotherapy, and 57% had dose reductions in their chemotherapy doses. The lack of benefit in the carboplatin arm in this trial was felt to be secondary to dose delays, dose reductions and treatment discontinuations. The CALGB 40,603 (Alliance) trial added Carboplatin AUC 6 to weekly paclitaxel [8]. In this trial the adriamycin/cyclophosphamide treatment followed the carboplatin/paclitaxel. Unfortunately, dose delays and cancellations plagued this arm of the trial with only 80% of those on the carboplatin arm receiving all four doses of ddAC. Despite this, pCR was higher in the carboplatin containing arm of the trial.

Our trial demonstrates the difficulty in identifying a carboplatin containing regimen that can be delivered to patients without delays and cancellations of treatments. Our trial starts with ddAC of which all patients completed this part of the regimen. We also experienced significant delays with weekly paclitaxel and AUC 6 carboplatin, with 1 of 6 patients relapsing. Our third version of the protocol, which used ddPac with Carbo AUC 6 on the second and last paclitaxel demonstrated excellent tolerance and completion. In this final version, we had no delays in chemotherapy with everyone completing the entire regimen. There were no dose reductions in the carboplatin and dose reductions only in paclitaxel for neurotoxicity. Thrombocytopenia and pain were the only grade 3 or 4 toxicities in this regimen. We demonstrated that with this change in timing of the
Fig. 3 Stage specific differences in biomarker expression. Average expression of A p16, B p21, C p27, D Cyclin E1, E phosphorlyated c-Myc, and F AR corrected for nuclear control Toto3 across different stages of TNBC. G Heat map showing expression of all biomarkers across all stages of TNBC as compared to normal breast tissue. Error bars reflect SD. Normal n = 23, Stage 1 n = 19, Stage 2 n = 46, Stage 3 n = 10. *p < 0.05, **p < 0.01, ***p < 0.001.

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Fig. 4 Younger TNBC patients have unique expression profile. Average expression of A p21 and B phosphorylated ERK corrected for nuclear control Toto3 across age groups of TNBC patients (<50 n=32, 50-65 n=37, >65 n=7). C Heat map showing all biomarkers assessed in 3 different age groups of TNBC patients (<50 n=32, 50-65 n=37, >65 n=7). D Heat map showing expression of AR and E p21 corrected for nuclear control Toto3 in patients younger than 50 years of age with and without relapse. **p<0.01, ***p<0.001

Heat map showing all biomarkers in patients under 50 years of age with and without relapse (upper heat map) and patients between 50 and 65 years of age with and without relapse (lower heat map). (<50 no relapse n=29, <50 relapse n=29, 50-65 no relapse n=22, 50-65 relapse n=5) Error bars reflect SE. **p<0.01, ***p<0.001
carboplatin, patients were able to complete their treatment regimen with manageable toxicity and good outcomes, with no relapses in the 18 patients treated on this protocol with a median of two years follow-up.

In our study, we found that chemotherapy delays, most often for thrombocytopenia appeared to impact patient outcome. In the second regimen, significant and prolonged delays occurred and 1 of the 6 patients relapsed. In the first two versions, 45% of the delays were secondary to isolated thrombocytopenia, using strict criteria requiring the platelet count to be $\geq 100,000 \times 10^9/L$. By moving the carboplatin to the second and last paclitaxel and allowing patients to continue with chemotherapy as long as the platelet count was $\geq 70,000 \times 10^9$ allowed patients to remain on regimen with no delays and expected 30% dose reductions in the paclitaxel for neuropathy.

It is promising that none of the 18 patients treated on our third version relapsed, however follow-up is short and numbers are small. We have identified a final regimen that offers manageable toxicity and excellent completion rates.

**Molecular profiles that predict response**

Stratifying the TNBC population by molecular targets has promise in both guiding treatment and prognosis. Low sample size and the changes in protocol introduced considerable variability for this study. Despite this, determining whether any proteins emerged as strong predictive indicators of response to treatment was of interest. Since TNBC patients present at a younger age, many studies have focused on patient prognosis at various ages with specific subtypes of these TNBC [17, 18]. In our study, the median age of relapsed patients was 53 years, and 4 of the 12 that relapsed (33%) were less than age 50 (36-62), hence within this study there was no statistical correlation between population age and relapse. AR has gained increasing attention as a potential target [19, 20]. We found AR protein levels to be lower in breast cancer tissue of all stages, as compared to normal breast tissue, especially in those <50 years who relapsed. This data supports previous evidence which suggest AR expression is associated with better prognosis [19].

Cell cycle proteins mediate a wide variety of cellular functions including proliferation and drug resistance and are known to be altered across cancers. In this study, three CKIs, p21, p27 and p16, were found at lower levels in breast cancer tissues as compared to normal breast tissue. Decreased levels of p27 have predicted higher risk of relapse and lower survival [21–23]. The importance of p16 has been controversial, with some studies demonstrating higher levels found, but no correlation to outcome to date [24–26], while elevated levels of p21 have been correlated with an increase in relapse and drug resistance [27–29]. Cyclin E1 was significantly reduced in stage two patients. Recent evidence suggests Cyclin E1 amplification is associated with poor clinical outcomes [30] consistent with the improved outcome seen in our study for stage II patients. Significant research in the ERK pathway has found a low frequency of ERK pathway mutations in TNBC but evidence of its importance in TNBC [31–33]. Elevated levels of phosphorylated ERK were found in the youngest group, suggesting increased activation of the...
ERK pathways. This group may have had a better overall outcome, though not statistically significant. Immunotherapy targeting PD-L1 in TNBC has been identified as beneficial in the metastatic setting [14]. Atezolizumab with nab-paclitaxel has demonstrated improved progression-free survival in patients with PD-L1 expression on their tumors [34]. Higher expression of PD-L1 has been associated with longer overall and disease-free survival [35–37]. In our study, we demonstrated that patients who relapsed had an initially lower PD-L1 level. Thus, expression of the immune checkpoint regulator PD-L1 may prove to be a useful biomarker in identifying patients at higher risk of relapse.

Conclusion

We recommend our final protocol to be tested in a larger population: carboplatin AUC 6 given on the second and last paclitaxel in dose dense ACT, while using liberal parameters of allowing platelets to drop to 70,000 x 10⁹/L. This protocol allowed all patients to complete the protocol with no dose reductions of carboplatin and reasonable dose reductions of paclitaxel because of neuropathy. In this final group of patients, there were no relapses to date. Molecular analysis of the cancer specimens revealed potentially predictive and prognostic markers that warrant further investigation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12302-021-01637-0.

Acknowledgements We thank the Windsor Regional Cancer Program for in-kind support in completing this study. Special thanks to Pa Dupuis, previous director of the Windsor Regional Cancer Clinical Trials Department. We appreciate the time and efforts of Krista Naccarato, Karen Metcalfe and members of the Porter Lab for ongoing support. heartfelt appreciation to all of the patients who enrolled in this trial. Thanks to the Great Lakes Environmental Institute for use of their TMA scanner.

Author contributions CH, RF, HA, EM, and BF contributed to data acquisition. CH, RF, HA, AH, BF, EM and LAP contributed to data analysis. CH, SK, RG, AK, JM all accrued and supported patients during this clinical trial. CH, RF, HA, AH, BF and LAP prepared the manuscript. CH and LAP secured the funding for this study.

Funding This work was supported by Canadian Institutes Health Research to L.A.P. (Grant#146189) and the Windsor Cancer Centre Foundation’s Seeds4Hope Program (CH).

Data availability All data generated from this study are included in the manuscript and additional files.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflict to disclose.

Ethical approval This study was completed with approval from the Windsor Regional Hospital Research Ethics Board Number CC-11-114.

Consent for publication All authors have agreed to publish this manuscript.

References

Appendix B: Permissions

To Whom It May Concern

Dear Sir/Mam

Please accept this letter as my authorization for Ms. Emily Mailloux to use our co-authored publication entitled “A prospective phase II clinical trial identifying the optimal regimen for carboplatin plus standard backbone of anthracycline and taxane-based chemotherapy in triple negative breast cancer” in her theses as an appendix. Please do not hesitate to contact me if you require further clarification.

Sincerely,

Lisa Porter, PhD

Executive Director of WE-SPARK Health Institute and a Professor in the Department of Biomedical Sciences

University of Windsor
VITA AUCTORIS

NAME: Emily Mailloux

PLACE OF BIRTH: Windsor, ON

YEAR OF BIRTH: 1997

EDUCATION: University of Windsor, B.Sc., Windsor, ON, 2020

University of Windsor, M.Sc., Windsor, ON, 2023