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Quantitative proteomics reveals mechanisms of methotrexate resistance (MTX-R) in Gestational Trophoblastic Neoplasia (GTN) and other cancer types

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A thesis submitted for the degree of Doctor of Philosophy

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Statement of Originality

I certify that, unless otherwise stated in the text, this thesis represents original research undertaken during my doctoral studies in the Department of Surgery and Cancer at Imperial College London. Acknowledgment is given to collaborators in Gemini Biosciences Ltd (Liverpool, UK) who conducted the Mass Spectrometry experiments. Some illustrations within this thesis were created by me using the BioRender App under a purchased license, while others have been licensed from journals where applicable.

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Abstract

Patients with low-risk gestational trophoblastic neoplasia (GTN) after a molar/non-molar pregnancy are mostly treated with methotrexate (MTX), but many develop resistance (MTX-R), requiring alternative or more toxic combination therapies to achieve remission. Understanding the molecular mechanisms of MTX-R is crucial for improving treatment. This study employed proteomics profiling of patient tissue samples to explore alterations linked to MTX-R in patients and human choriocarcinoma cell lines.

Bioinformatics analysis identified the Serine Synthesis Pathway (SSP) as significantly downregulated in MTX-R patients and the resistant choriocarcinoma cell line JEG3-R. Phosphoserine aminotransferase 1 (PSAT1), a key SSP enzyme, emerged as a potential prognostic marker for MTX sensitivity in a small patient cohort following Immunohistochemistry (IHC) analysis. Functional studies showed that simultaneous serine deprivation and inhibition of *de novo* serine synthesis increased MTX resistance in choriocarcinoma cells. Conversely, overexpression of PHGDH and PSAT1, combined with serine and glycine depletion, reversed resistance in JEG3-R cells, restoring MTX sensitivity.

Further experimentation revealed that MTX-R cells exhibit enhanced detoxification capacity, redirecting serine towards glutathione synthesis while downregulating energy-demanding processes like proliferation. This adaptation helps resistant cells mitigate MTX-induced oxidative stress contributing to MTX-R. Investigation into the underlying mechanisms suggested that activation of the Integrated Stress Response (ISR) pathway in JEG3-R cells facilitates translational blockage of non-essential pathways while promoting survival mechanisms such as autophagy. Moreover, dose-specific inhibition of the ISR managed to sensitize JEG3-R cells to MTX indicating a potential therapeutic strategy in the resistant choriocarcinoma.

Finally, SSP downregulation was examined in MTX-sensitive and MTX-resistant breast, colon, and osteosarcoma cell lines. Notably, four of seven MTX-R lines displayed similar SSP downregulation as choriocarcinoma MTX-R cells, reinforcing the role of serine metabolism in MTX

response. These findings highlight the potential of SSP as a target for overcoming MTX-R and advancing clinical research.

Acknowledgments

First and foremost, I would like to express my deepest gratitude to my supervisors, Dr. Olivier Pardo and Professor Michael Seckl, for their insightful guidance and support throughout my PhD journey. Their expertise, encouragement, and patience were instrumental in shaping this thesis. I would also like to extend my sincere thanks to our collaborators from Gemini Biosciences company for our smooth collaboration and for performing the Mass Spectrometry experiments on our behalf. Also, many thanks to Dr. Florian Rothweiler for providing us with the cell lines which served as a valuable tool for the purposes of this project. I should also thank Dr David J. Pinato and his lab members who performed the analysis of the immunohistochemical staining.

To my lab partners, Dr. Geoffrey Maher, Emily Hayes, Chai Peng, and Dr. Nirosha Suraweera, I am thankful not only for their scientific insights but also for the enjoyable atmosphere they helped create in the lab. Special thanks to Dr Yakinthi Chrisochidou, whose warm welcome in the lab grew into a genuine friendship and made my time in the lab even more pleasant. I also would like to thank former lab members, Dr. Lucksamon Thamlikitkul and Dr. Rajat Roy, with whom I shared many memorable moments.

To the new friends I made during my time in London, and the mutual support and fun moments we shared. A big thank you to all my friends in Greece who patiently stood by me, despite my many absences and late replies. Special thanks to Stelios, for his endless patience, support, and understanding during this intense period. I am grateful to him for ensuring I never gave up.

And lastly, I am deeply grateful to my parents, as I wouldn't be where I am today without them. Their unconditional love, faith in me, and encouragement throughout my life have meant everything to me. Also, I am thankful for my beloved older siblings who have been a source of inspiration to me. And of course, a special thank you to Thanos for helping me begin this journey and get settled in London.

Dedicated to my Parents

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List of Abbreviations

1C	One-carbon
3' UTR	Three prime untranslated region
3-PG	3-phosphoglycerate
3-PS	3-phosphoserine
5,10-Mthf	5,10-methylenetetrahydrofolate
5-FU	5-fluorouracil
5-Mthf	5-methyltetrahydrofolate
AARE	Amino Acid Response Element
ACT-D	Actinomycin-D
AHCY	Adenosylhomocysteinase
AMPK	AMP-activated protein kinase
AMPKα1	5'-AMP-Activated Protein Kinase Catalytic Subunit Alpha-1
ASCT1 (SLC1A4)	Alanine/Serine/Cysteine/Threonine Transporter 1
ASCT2 (SLC1A5)	Alanine/Serine/Cysteine/Threonine Transporter 2
ATF3	Activating Transcription Factor 3
ATF4	Activating Transcription Factor 4
ATIC	5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase/IMP Cyclohydrolase
ATP	Adenosine triphosphate
biCHMs	biparental Complete Hydatidiform moles
CARE	C/EBP-ATF response element
CBS	Cystathione Synthase
CDA	Cytidine Deaminase
ChIP	Chromatin Immunoprecipitation
CHM	Complete hydatidiform moles
CHMs	Complete Hydatidiform moles
CHOP	C/EBP homologous protein
CRC	Colorectal cancer
CREB	cyclic AMP response element (CRE)-binding protein
DHF	Dihydrofolic acid
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DR5	Death receptor 5
DTNB	(5,5'-dithiobis-(2-nitrobenzoic acid)
ECAR	Extracellular acidification rate
EHMT2	Euchromatic Histone Lysine Methyltransferase 2
eIF2a	Eukaryotic Translation Initiation Factor 2A
EMA/CO	Etoposide, methotrexate, actinomycin-D / cyclophosphamide, vincristine

EMT	Epithelial to Mesenchymal Transition
ER	Endoplasmic Reticulum
ETT	Epithelioid trophoblastic tumours
EZH2 (KMT6A)	Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit
FDA	Food and drug administration
FDR	False Discovery Rate
FFPE	Formalin-Fixed Paraffin-Embedded
FIGO	International federation of gynaecology and obstetrics
G6PD	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MA	Gas chromatography-mass spectrometry
GCN2	General Control Nonderepressible 2
GFP	Green fluorescent protein
GLS-2	Glutaminase-2
Gly	Glycine
GO	Gene ontology
GSH	Glutathione
GSTT1	Glutathione S-Transferase Theta 1
GTD	Gestational trophoblastic disease
GTN	Gestational trophoblastic neoplasia
GTT	Gestational trophoblastic tumour
H&E	Haematoxylin and Eosin
hCG	Human chorionic gonadotropin
Hcy	Homocysteine
HER2-	Human epidermal growth factor receptor 2 negative
HIPK1	Homeodomain-interacting protein kinase 1
HMs	Hydatidiform moles
HRI	Heme-regulated eIF2a kinase
ISR	Integrated Stress Response
JMJD2C (KDM4C)	Jumonji Domain-Containing Protein 2C
KDM4C (JMJD2C)	Lysine (K)-Specific Demethylase 4C
KEGG	Kyoto encyclopaedia of gene and genomes
KHDC3L	KH Domain Containing 3 Like, Subcortical Maternal Complex Member
KMT6A (EZH2)	Lysine N-Methyltransferase 6
LKB1	Liver kinase B1
lncRNA	long noncoding RNA
MAT1A	Methionine Adenosyltransferase 1A
MAT2A	Methionine Adenosyltransferase 2A
MDM2	Monocarboxylate transporter
MEK	Mouse double minute 2
me-THF	5,10-methylene-THF
MID	Mitogen-activated protein kinase

MRPL9	Mitochondrial Ribosomal Protein L9
MS	Mass spectrometry
MS	Methionine Synthase
MTHFD1	Methylenetetrahydrofolate Dehydrogenase, Cyclohydrolase And Formyltetrahydrofolate Synthetase 1
MTHFD1L	Methylenetetrahydrofolate Dehydrogenase (NADP+ Dependent) 1 Like
MTHFD2	Methylenetetrahydrofolate Dehydrogenase (NADP+ Dependent) 2, Methenyltetrahydrofolate Cyclohydrolase
MTHFR	Methylenetetrahydrofolate Reductase
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MTX	Methotrexate
MTX-PGs	Polyglutaminated MTX
MTX-R	MTX-Resistant
MTX-S	MTX-Sensitive
MYC / c-MYC	Myc proto-oncogene protein
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NLRP7	Nucleotide-Binding Oligomerization Domain, Leucine Rich Repeat And Pyrin Domain Containing 7
NRF2	Nuclear Factor Erythroid 2-Related Factor 2
NSCLC	Non-small cell lung cancer
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
PARP	Poly (ADP-ribose) polymerase PCNA Proliferating cell nuclear antigen
PD-L1	Programmed Cell Death 1 Ligand 1
PERK	PKR-like ER kinase
PHGDH	Phosphoglycerate dehydrogenase
PHMs	Partial Hydatidiform moles
PI	Propidium iodine
PKR	Double-stranded RNA-dependent protein kinase
PP1	Protein phosphatase 1
PPP	Pentose Phosphate Pathway
PSAT1	Phosphoserine Aminotransferase 1
PSPH	Phosphoserine Phosphatase
PSTT	Placental-site trophoblastic tumours
RFC1	Reduced folate carrier 1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
Ser	Serine

SFXN1	Sideroflexin 1
SG-	Serine and Glycine absent
SG+	Serine and Glycine present
SHMT1	Serine Hydroxymethyltransferase 1
SHMT2	Serine Hydroxymethyltransferase 2
SILAC	Stable isotope labelling by amino acids in cell culture
siRNA	Small interfering RNA
SLC1A4 (ASCT1)	Solute Carrier Family 1 Member 4
SLC1A5 (ASCT2)	Solute Carrier Family 1 Member 5
SLC38A1 (SNAT1)	Solute Carrier Family 38 Member 1
SLC38A2 (SNAT2)	Solute Carrier Family 38 Member 2
SLC7A10	Solute Carrier Family 7 Member 10
SMC2	Structural Maintenance Of Chromosomes 2
SNAT1 (SLC38A1)	Sodium-Coupled Neutral Amino Acid Symporter 1
SNAT2 (SLC38A2)	Sodium-Coupled Neutral Amino Acid Symporter 2
SSP	Serine Synthesis Pathway
TCA	Tricarboxylic acid
TCEP	(Tris(2-carboxyethyl)phosphine)
TF	Transcription factor
THF	Tetrahydrofolate
TIGAR	TP53 Induced Glycolysis Regulatory Phosphatase
TK1	Thymidine Kinase 1 (TK1)
TMP	Thymidine monophosphate
TNM	Tumour, Node, Metastasis
TTP	Thymidine triphosphate
TYMS	Thymidylate Synthase (TYMS)
TYMS (TS)	Thymidylate synthase
UAPI	Uterine Artery Pulsatility Index
Uorf	upstream open reading frame
WHO	World Health Organisation
ZIP	Zero interaction potency

1. Introduction

1.1 Cancer

Cancer is a multifaceted and complex disease that is defined by the body's aberrant cells having escaped from central endogenous regulatory systems, growing and spreading out of control. Fundamentally, genetic mutations that interfere with the regular processes that control cell division, growth, and death are the root causes of cancer. These mutations can be acquired over time due to a range of factors including exposure to chemical carcinogens, genetic predisposition, or errors in DNA replication (Cooper, 2000). As a result, these mutated cells proliferate uncontrollably and form tumours that can be characterized either as benign or malignant. Malignant tumours are the only cancerous types of tumours and have the ability to invade nearby normal tissue and to metastasize, or spread throughout the body by the lymphatic or blood circulatory systems (Martin T. et al., 2013).

Cancers may be classified based on the organ or tissue of origin and also based on the molecular traits of the respective cancer cells, enabling the development of numerous treatment strategies (Carbone, 2020). Despite the accumulating knowledge regarding the onset and spread of cancer, it continues to rank among the world's leading causes of death, accounting for over 20 million new cases and 9.7 million deaths in 2022. This number is predicted to climb to an estimated 35 million new cases annually by 2050 (Morgan *et al.*, 2023).

The complexity of cancer has been efficiently addressed by D. Hanahan and R. Weinberg in 2000 by outlining the six hallmarks of cancer cells: the ability to induce angiogenesis, promote tissue invasion and metastasis, resist cell death by evading apoptosis, maintain self-sufficiency in growth signals, show insensitivity to anti-growth signals and possess limitless replicative potential (Hanahan and Weinberg, 2000). These distinguishing features offered back then a foundation for comprehending the basic biological skills that cancer cells have gained. Over the years, more cancer cell traits have been identified and in 2011, Hanahan and Weinberg updated the hallmarks and included two emerging principles: reprogramming of energy metabolism and avoidance of immune system destruction. Additionally, they defined the underlying mechanisms of these hallmarks and these are genome instability/mutations that contribute to tumour heterogeneity

and tumour-promoting inflammation that supports tumour progression (Hanahan and Weinberg, 2011). Eleven years later, Douglas Hanahan disclosed several newly proposed emerging hallmarks and enabling characteristics, such as unlocking phenotypic plasticity, non-mutational epigenetic reprogramming, polymorphic microbiomes, and the role of senescent cells (**Figure 1.1**) (Hanahan, 2022). Lastly, apart from malignant cells, tumors have an additional layer of intricacy as they comprise a range of seemingly healthy cells that aid in the acquisition of the aforementioned hallmarks by forming the “tumour microenvironment” (Hanahan and Weinberg, 2011).

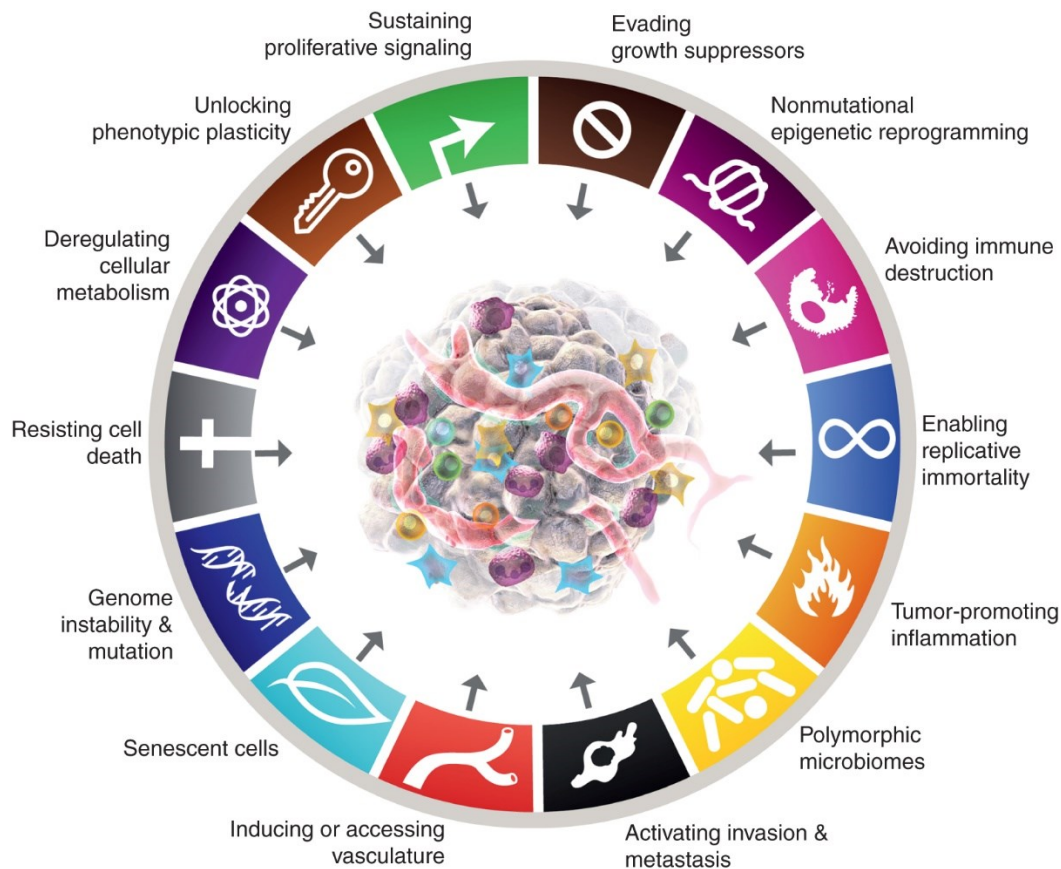


Figure 1.1: The Hallmarks of Cancer-new additions and dimensions

The established hallmarks of cancer as defined in 2000 and 2011 and the new additions as suggested in 2022 by Hanahan and Weinberg. (Image taken from (Hanahan, 2022), Copyright © 2022, American Association for Cancer Research.

1.2 Gestational Trophoblastic Disease

Gestational Trophoblastic Disease (GTD) is a group of disorders associated with pregnancy that arise from either genetically abnormal or normal conceptions (Seckl, Sebire and Berkowitz, 2010). During the initial stages of embryonic development, cells from the outer layer of the blastocyst, known as trophoblasts, form small cell projections called villi, which later develop into the placenta (Knöfler *et al.*, 2019). Nevertheless, irregular developmental processes that cause dysregulated cell proliferation can result in an anomalous pregnancy known as hydatidiform mole ((Seckl, Sebire and Berkowitz, 2010), (Froeling and Seckl, 2014)) (**Figure 1.2**). Hydatidiform moles (HMs), the most prevalent form of GTD, are classified into two types: complete (CHMs) and partial (PHMs) (Mangili *et al.*, 2014). Malignant types of GTD consist of invasive moles, choriocarcinoma, and the less common placental site trophoblastic tumors (PSTT) and epithelioid trophoblastic tumors (ETT) (Seckl, Sebire and Berkowitz, 2010).

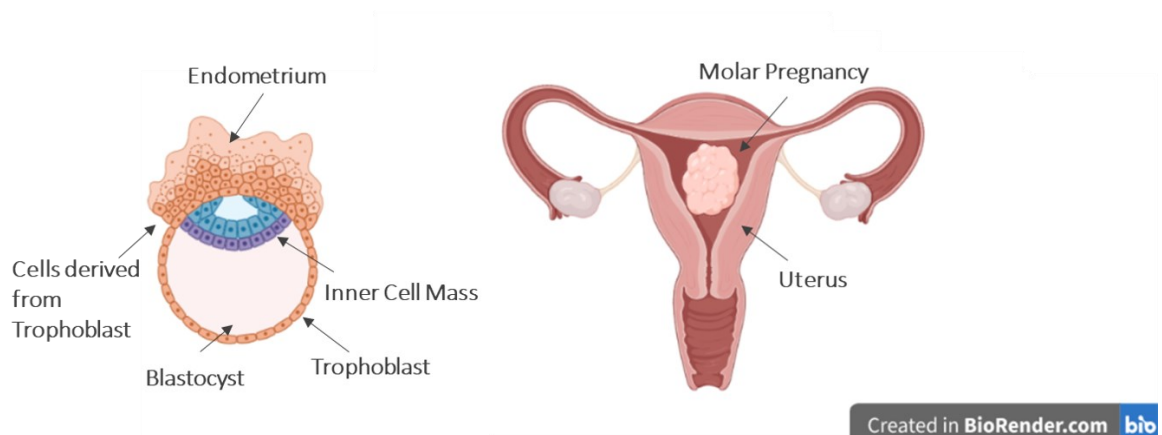


Figure 1.2: Molar Pregnancy

Left: Illustration depicting early embryonic development, where trophoblast cells form the placenta, while the inner cell mass develops into fetal structures. Right: Depiction of a molar pregnancy. (created in BioRender.com)

1.2.1 Incidence rate

Overall, more cases of GTD are reported in Asian countries compared to North America or Europe. This could be attributed to genetic factors, differences in hospital-based as opposed to national registration systems, misdiagnosis, socioeconomic status, or possibly dietary habits (Palmer, 1994). In the United Kingdom, there is a centralized national registry system to track and manage GTD patients (Sebire and Seckl, 2008). Statistics indicate that 1-3 in 1,000 pregnancies is a complete hydatidiform mole, while 1 in 1,000 pregnancies are partial hydatidiform moles ((Candelier, 2016),(Florea *et al.*, 2023)). Nearly 10% of these hydatidiform moles progress to malignant trophoblastic tumors. (Sebire and Seckl, 2008). Remarkably, the occurrence of molar pregnancies is higher in both upper and lower ends of maternal age spectrum, specifically in women over 45 years and under 16 years (Sebire *et al.*, 2002). Furthermore, following one molar pregnancy, the likelihood of a second one increases to 1-2%, while the risk of a third one climbs to 15-20%. This is likely associated with a rare form of biparental origin which is either familial or sporadic and not the classical androgenetic complete mole (Seckl, Sebire and Berkowitz, 2010).

The exact incidence of gestational trophoblastic tumors is less clear, as they can arise from any type of pregnancy (Seckl, Sebire and Berkowitz, 2010). Choriocarcinoma may occur in 1 out of 50,000 pregnancies, while placental site trophoblastic tumors (PSTT) account for 0.2% of all recorded GTD cases in the UK (Fisher *et al.*, 1988), (Schmid *et al.*, 2009).

1.2.2 Hydatidiform moles (HMs)

A hydatidiform mole is considered non-malignant, as it represents the non-invasive form of GTD. However, it can progress to a malignant state, making it pre-malignant as well. Molar pregnancies are characterized by an abnormal overgrowth of placental tissue with little to no fetal tissue present (Sebire and Seckl, 2008). Complete and partial hydatidiform moles, the two forms of hydatidiform moles are genetically distinct. Complete moles have a diploid chromosome number, while partial moles have a triploid chromosome number (LINDOR *et al.*, 1992).

Complete moles (CHM) occur when an oocyte lacking maternal chromosomes is fertilized by either one or two sperm cells. In the monospermic case, the single spermatocyte duplicates its DNA resulting in an androgenetic diploid 46XX or 46YY karyotype ((Kajii and Ohama, 1977), (Fisher RA, 1998)). In contrast, a dispermic fertilization may result in an androgenetic 46 XY as well as YY or XX diploid karyotype and represents 1 out of 10 complete moles (Berkowitz *et al.*, 1986). In both cases, the nuclear DNA derives from the father, while the mitochondrial DNA originates from the mother (Azuma *et al.*, 1991). A rare form, the biparental complete hydatidiform mole (biCHM) is linked with recurrent molar pregnancies (**Figure 1.3**). This mole is not the typical androgenetic type, even though it is often characterized as a CHM in appearance. Genetic studies on these moles have identified mutations primarily in the *NLRP7* gene located at 19q13.3–13.4 region and the *KHDC3L* gene at the 6q13 region. These mutations can be either sporadic or inherited in an autosomal recessive manner. ((Slim and Wallace, 2013), (Murdoch *et al.*, 2006), (Kalogiannidis *et al.*, 2018)). Moreover, familial recurrent biCHMs have been linked to imprinting errors in which paternally imprinted genes are overexpressed and maternally imprinted genes are silenced (El-Maarri *et al.*, 2003). Partial moles (PHMs) originate from the fertilization of a normal haploid egg by two sperm cells, resulting in a biparental triploid karyotype (69XXY or 69XXX) (Lawler, Fisher and Dent, 1991) . These moles are usually misdiagnosed as non-molar miscarriages (Froeling and Seckl, 2014) (**Figure 1.3**).

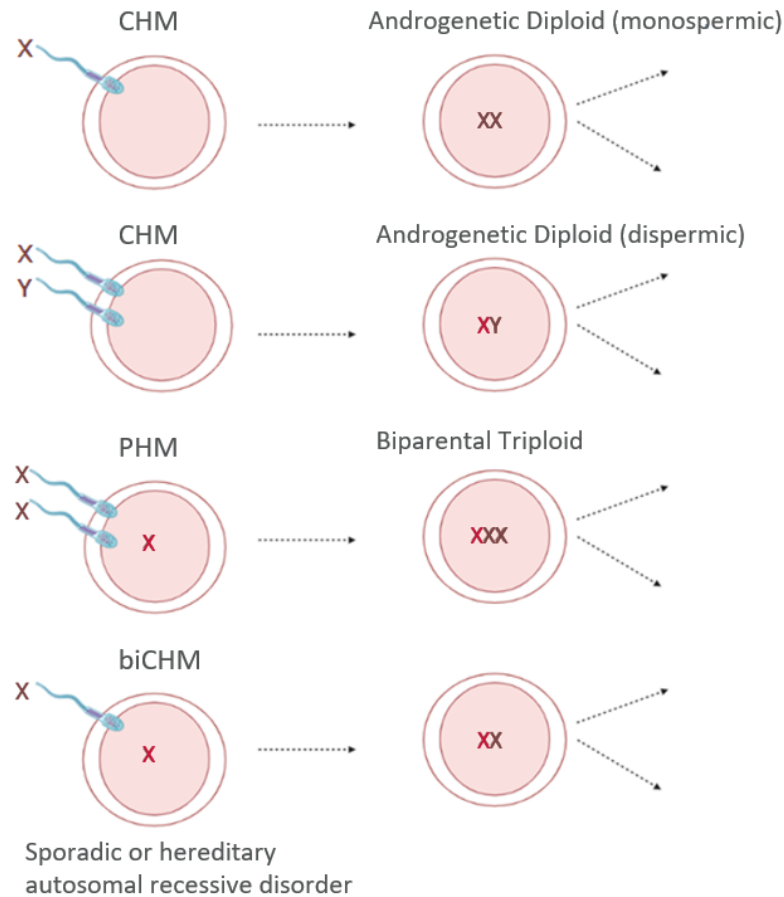


Figure 1.3: Karyotype origin of types of Hydatidiform Moles

Illustration of the karyotype origin of CHMs, PHMs and rare biCHMs, with paternal (brown) and maternal (red) chromosomes highlighted. CHMs are primarily androgenetic and result from the fertilization of an egg lacking maternal chromosomes by one (monospermic) or two (dispermic) sperms. Biparental CHMs are rare and arise from autosomal recessive mutations in *NLRP7* gene. PHMs occur when a healthy oocyte is fertilized by two sperm cells, resulting in triploid biparental moles. (Figure adapted from: (Seckl, Sebire and Berkowitz, 2010), created in BioRender.com).

The hydatidiform moles are defined by trophoblast hyperplasia, which includes the proliferation of both cytotrophoblast and syncytiotrophoblast cells, along with the swelling of placental villi (Szulman and Surti, 1978). Complete moles exhibit a more diffused villous structure, whereas partial moles have focal and irregularly shaped villi (Ronnett, 2019). In partial hydatidiform moles, fetal parts may be present, while in complete hydatidiform moles, they are

absent. (Sebire, Fisher and Rees, 2003) (**Figure 1.4**). Methods such as immunostaining and DNA genotyping are useful for distinguishing between complete moles, partial moles, as well as non-molar specimens. The lack of p57^{kip2} immunostaining, an imprinted gene of paternal origin that preferentially expresses the maternal allele, suggests the presence of complete moles, as they lack maternal genes (Castrillon *et al.*, 2001). The distinguishment between androgenetic (complete) diploids from biparental (partial) triploids, as well as from non-molar cases is conducted by DNA genotyping which compares the ratios of polymorphic alleles (Ronnett, 2019). Furthermore, the overexpression of genes derived from the father in both complete and partial moles is a key factor in genetic analysis, helping differentiate these cases from non-gestational ones (Fisher *et al.*, 2007).

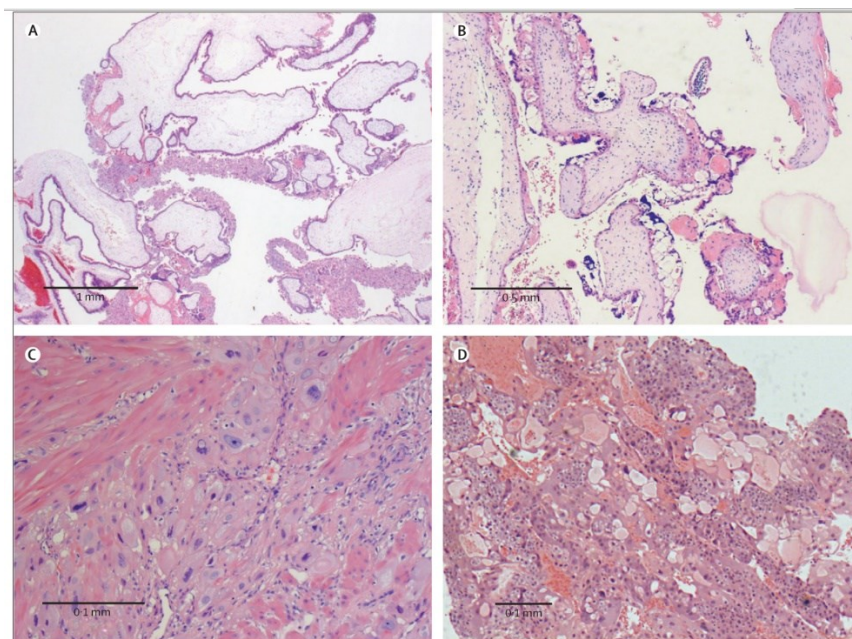


Figure 1.4: Cellular morphology of GTD forms

Haematoxylin and eosin (H&E) -stained tissue sections showing: A) Complete hydatidiform mole (CHM) (x20 magnification), B) Partial hydatidiform mole (PHM) (x40 magnification), C) Placental-site trophoblastic tumour (PSTT) (x200 magnification) and D) Choriocarcinoma (x100 magnification). All GTD forms exhibit abnormal trophoblast proliferation, with dysmorphic chorionic villi seen in CHMs and PHMs. However, villi and anomalous trophoblast invasion do not appear neither in PSTT nor in choriocarcinoma (Image adapted from: (Seckl, Sebire and Berkowitz, 2010).

1.2.3 Gestational Trophoblastic Neoplasia (GTN)

Gestational Trophoblastic Neoplasia (GTN) encompasses all malignant forms of GTD including invasive moles and metastatic conditions such as choriocarcinoma, and the rarest types, epithelioid trophoblastic tumours (ETT) and placental-site trophoblastic tumours (PSTT). Each of these types exhibits distinct immunohistochemical and pathological features (Seckl, Sebire and Berkowitz, 2010). Following a molar pregnancy, an invasive mole is the most probable malignant form and generally presents a lower risk of becoming resistant to single-agent chemotherapy. The diagnosis is usually not pathological but made clinically as a consequence of plateaued or rising serial hCG (serum) levels, a biomarker used to assess disease progress after a molar pregnancy (Mangili *et al.*, 2014).

Gestational choriocarcinoma is a highly aggressive GTN type that originates from the villous trophoblasts of the placenta (Seckl, Sebire and Berkowitz, 2010) (**Figure 1.4**). This type of gestational trophoblastic tumor can develop after various types of gestation, including molar pregnancies, normal non-molar pregnancies resulting in live births (whether pre-term or term), ectopic pregnancies, or even following a miscarriage ((Noal, Joly and Leblanc, 2010), (Lurain, 2010)). Choriocarcinoma is characterized by trophoblast hyperplasia, tissue necrosis, the absence of chorionic villi, and a tendency to metastasize, primarily to the vagina, lungs, and brain, depending on the disease stage (Seckl, Sebire and Berkowitz, 2010).

Placental-site trophoblastic tumor (PSTT) is a rare GTN form that originates from the placental implantation site. It is characterized by the absence of chorionic villi, predominantly containing mononuclear intermediate. In comparison to choriocarcinoma, PSTT has less tissue necrosis, vascular invasion, and hemorrhage ((Lurain, 2010), (Seckl, Sebire and Berkowitz, 2010)) (**Figure 1.4**). Epithelioid trophoblastic tumor (ETT) is pathologically distinct from PSTT although it seems to behave in a similar clinical fashion. It resembles carcinoma and appears to originate from chorionic-type intermediate trophoblasts (Shih IM, 1998). For both PSTT and ETT, having biopsy material to enable histopathology is necessary for a definitive diagnosis.

1.3 Clinical Investigation and Diagnosis of molar pregnancies and post-molar GTN

An initial sign of a molar gestation may be vaginal bleeding and a uterus larger than expected for the gestational age. This is typically followed by an ultrasound conducted in the first trimester. (Seckl, Sebire and Berkowitz, 2010). An ultrasound examination of a complete hydatidiform mole reveals a heterogeneous mass in the uterine cavity absent of any fetal parts, with a placenta displaying cystic formations and a distorted gestational sac resembling a spontaneous abortion (Santos-Ramos R, Forney JP, 1980). Partial moles progress more slowly than complete moles and are typically detected in the second trimester, often accompanied by vaginal bleeding and/or spontaneous abortion (Berkowitz RS, Goldstein DP, 1985). Whilst the ultrasound changes may be highly suggestive of a molar pregnancy, they are not diagnostic and instead a histopathological examination of the material evacuated from the uterus is required (Seckl *et al.*, 2000). Finally, since conducting a histological analysis after every termination is impractical, the most effective approach to ensure a molar pregnancy is not overlooked is to monitor hCG serum levels for at least 4 weeks post-treatment, continuing until they return to normal (Seckl *et al.*, 2004).

Once a pathological diagnosis of a molar pregnancy has been made, all patients in the UK are registered for hCG monitoring. In approximately 16% of cases of CHM and 1% of PHM, the disease continues to persist and is said to have undergone a malignant change that will typically require chemotherapy treatment to induce remission. The onset of malignant change is detected by a plateaued serum hCG over at least 3 consecutive values or a rising hCG over at least 2 serial values (Ngan and Seckl, 2007). It follows that the measurement of hCG is essential so the next section will briefly explore this.

1.3.1 hCG

The monitoring of human chorionic gonadotropin (hCG) serum levels is employed to diagnose Gestational Trophoblastic Disease (GTD). hCG consists of two subunits, α and β , with the β subunit being unique and specific. An assay that measures hCG levels (whether intact or hyperglycosylated) during a normal pregnancy relies on targeting the β subunit. (Mitchell and

Seckl, 2007). In malignant cases, these assays can also identify various forms of hCG, including free β -hCG, β -core hCG, nicked free β -hCG, and nicked hCG lacking the β subunit's c-terminal peptide, as well as nicked hyperglycosylated hCG. ((Cole, 2010), (Cole LA, Shahabi S, Butler SA, Mitchell H, Newlands ES, Behrman HR, 2001)). There is a hyperglycosylated hCG assay that suggests the presence of gestational trophoblastic neoplasia when a high ratio of this variant to total hCG levels is observed ((Cole *et al.*, 2006), (Palmieri *et al.*, 2007)). In addition, a study (Cole *et al.*, 2006) found that the invasive phenotype in trophoblasts and choriocarcinoma cells is promoted by hyperglycosylated hCG. Furthermore, free β -hCG has been proposed as a potential biomarker for PSTT (Nola *et al.*, 2018).

However, these and other commercial assays are not yet fully optimised and can produce false-negative results, potentially resulting in delayed diagnosis or early cessation of treatment (Mitchell and Seckl, 2007). False-positive results are typically ruled out through further urine testing (Cole *et al.*, 2001). This is because the large interfering molecules responsible for false positives such as human anti-mouse antibodies do not cross the renal glomerulus into the urine. In contrast, true hCG, being much smaller, is detectable in urine (Racek *et al.*, 2023). Thus, it is crucial to improve existing hCG assays or develop assays for novel or additional markers.

1.3.2 FIGO/WHO Staging and Prognostic Scoring System

Once a diagnosis of post-molar GTN is made patients undergo evaluation to determine the disease's severity and initiate treatment. For GTN, a patient stratification system is available to predict the risk of a patient becoming resistant to single-agent chemotherapy with either methotrexate or actinomycin D. In particular, the risk scoring and anatomical system created by the World Health Organization (WHO) combined with the staging system from the International Federation of Obstetrics and Gynecology (FIGO), have led to the development of a prognostic scoring and anatomical tool ((Figo Oncology Committee, 2002), (Kohorn, 2001)) (**Table 1.1**). This tool incorporates eight distinct risk factors to assist in assigning each patient to the most appropriate treatment strategy (Taylor *et al.*, 2015).

Gestational trophoblastic neoplasia is classified into four clinical stages based on the FIGO anatomical staging. Stage 1 refers to tumors that are limited to the uterus. In Stage 2, the tumor extends beyond the uterus to nearby genital regions. Stage 3 indicates that the tumors have spread to the lungs, with or without involvement of the genital area. Finally, in Stage 4, the most advanced stage, the tumor involves metastasis to distant organs such as the liver or brain. (Figo Oncology Committee, 2002) (**Table 1.1**).

The scoring system is only applicable to patients with a post-molar GTN or a histological diagnosis of choriocarcinoma. It is not used for assessing PSTT/ETT due to fundamental differences in cellular origin compared to HMs, which influence their clinical behaviour and resistance to conventional chemotherapy (Liu *et al.*, 2022). Each risk factor is assigned a specific score, and the total of all these scores yields the WHO or FIGO prognostic score. Based on this score, patients are categorized as either low risk (score < 6) or high risk (score ≥ 7) for developing resistance to single-agent chemotherapy. ((Figo Oncology Committee, 2002), (Kohorn, 2001)). Most patients (90-95%) fall into the low-risk category and their prognosis is generally favorable. Single-agent chemotherapy, primarily methotrexate but also actinomycin-D, is regarded as effective for curing the disease (Khan *et al.*, 2003). High-risk patients need a more aggressive treatment approach, which involves combination chemotherapy using the EMA/CO regimen. This includes etoposide, methotrexate, and actinomycin-D, switched weekly with cyclophosphamide and vincristine (Ngan and Seckl, 2007).

Table 1.1: FIGO/WHO Staging and Prognostic Scoring System

At the top, the FIGO anatomical staging outlines the four distinct clinical stages of gestational trophoblastic neoplasia. At the bottom, the eight risk factors for GTN are listed along with their associated scores. (Table sourced from: (Braga *et al.*, 2019).

GTN: FIGO staging and classification (Washington, 2000)				
FIGO anatomic staging				
Stage I: Disease confined to the uterus				
Stage II: GTN extends outside of the uterus, but is limited to the genital structures (adnexa, vagina, broad ligament)				
Stage III: GTN extends to the lungs, with or without known genital tract involvement				
Stage IV: All other metastatic sites				
Modified WHO prognostic scoring system as adapted by FIGO				
Prognostic factors	Score			
	0	1	2	4
Age	< 40	≥ 40	-	-
Antecedent gestation	Mole	Abortion	Term	-
Interval (mo)	< 4	4-6	7-12	> 12
Pretreatment serum hCG (IU/L)	< 10 ³	10 ³ to < 10 ⁴	10 ⁴ to < 10 ⁵	> 10 ⁵
Largest tumor size (including uterus)	< 3	3 to 4	≥ 5	-
Site of metastases	Lung	spleen, kidney	gastro intestinal tract	brain, liver
Number of metastases	-	1-4	5-8	> 8
Previous failed chemotherapy	-	-	single drug	2 or more drugs

1.3.3 Need for better biomarkers

The majority of GTN patients respond well to one of the chemotherapy treatments available. Nevertheless, a few of these women ultimately succumb to their condition, often due to delayed diagnosis, early misdiagnosis, or late presentation. Early deaths can also occur as a result of severe bleeding, metabolic acidosis, myelosuppression or sepsis, while late deaths are typically associated with drug resistance (Ngan *et al.*, 2021). Additionally, while MTX monotherapy is considered an effective treatment, about one in three women develop resistance to this drug. At present, the FIGO/WHO Staging and Prognostic Scoring System inaccurately categorizes approximately 33% of low-risk cases, with most having a FIGO score of 5 or 6 ((Mangili *et al.*, 2014),(Seckl, Sebire and Berkowitz, 2010), (Braga *et al.*, 2021)). This misclassification results in a postponement of combination therapy in favor of monotherapy. Some progress has recently been made for women with a FIGO score of 5 or 6 where 3 subgroups have been identified who could start combination agent chemotherapy immediately (Braga *et al.*, 2021). Nevertheless, for the vast majority of low-risk cases starting on single agent MTX, predicting who will fail therapy is still

based on detecting a serially plateaued or rising hCG whilst on therapy. Several studies have tried to identify ways to predict MTX resistance early and these are discussed in the section below. However, none are universally adopted and none can predict resistance in advance of starting therapy. As a result, there is an immediate requirement for improved stratification biomarkers at the time of initial diagnosis of a post-molar GTN.

1.3.4 Current approaches for diagnosis of resistance to single agent chemotherapy

The recent PREDICT-GTN 1 and PREDICT-GTN 2 studies aim to enhance the predictive accuracy and clinical utility of the FIGO scoring system in diagnosing resistance to single-agent chemotherapy in GTN. PREDICT-GTN 1 study explored six models using logistic regression (LR) and multilayer perceptron (MLP) methods, incorporating combinations of scored and raw FIGO data, nonimaging variables, imaging variables and pretreatment hCG levels. While some models marginally improved true positive rates and decision-curve analysis, significant patient misclassifications occurred (Parker *et al.*, 2023). PREDICT-GTN 2 reduced the computational complexity of the first study by developing three streamlined, two-factor models integrating pretreatment hCG with other key variables such as history of failed chemotherapy, site of metastases and number of metastases. These models matched FIGO's predictive performance proving that simplified models can maintain diagnostic accuracy while improving accessibility and clinical efficiency (Parker *et al.*, 2024).

You *et. al* attempted to model the decreasing levels of hCG during the MTX treatment, using a population-based kinetic approach to estimate the parameters of hCG kinetics, aiming to evaluate their predictive value and relationship with MTX resistance. (You *et al.*, 2010). The predictive value of this approach was confirmed by the same research group that utilized the Charing Cross Hospital database. The modeled residual concentration (hCG_{res}) emerged as the most strongly correlated factor with the highest predictive capability for MTX resistance, following three cycles of MTX doses (You *et al.*, 2013). An online tool is also available that

incorporates the clinical use of this kinetic parameter to predict MTX resistance (<https://www.biomarker-kinetics.org/hCG>) (Dekeister *et al.*, 2020).

A recent study highlighted HLA-G as an important predictive biomarker for resistance to single-agent therapy in choriocarcinoma (Bolze *et al.*, 2020). The researchers compared gene expression patterns between complete hydatidiform moles and gestational choriocarcinoma samples, discovering that HLA-G, along with COL27A1, IL1R2, and GLI3, showed significantly lower expression at both mRNA and protein levels in mono-chemoresistant choriocarcinoma compared to mono-chemosensitive cases (Bolze *et al.*, 2020).

The uterine artery pulsatility index (UAPI) measures tumor vascularity in patients undergoing Doppler sonography. Agarwal *et al.* found that this index is capable of predicting resistance to methotrexate, revealing that patients resistant to MTX had reduced uterine blood flow (indicating higher vascularity) compared to those sensitive to the drug. (Agarwal *et al.*, 2012).

Building on the pulsatility index, there is an ongoing prospective clinical trial called “TITANIUM” (Gestational Trophoblastic Neoplasia Ultrasound Assessment) designed to evaluate various ultrasound morphologic features of GTN and their correlation with resistance to single-agent chemotherapy in low-risk patients. This method could enhance the sensitivity of the FIGO scoring system and lead to a more precise treatment approach for patients with GTD (Verri *et al.*, 2019). Moreover, patients who show resistance to combination therapy might need alternative combinations, including high-dose chemotherapy or even adjuvant treatments like immune checkpoint therapy. Nevertheless, effective and specific biomarkers for accurately stratifying these patients into the best treatment option are still lacking.

1.3.5 Current and upcoming Treatment Strategies

Most patients with gestational trophoblastic disease receive treatment with single-agent Methotrexate, an antifolate drug that impedes nucleotide synthesis either directly or by

disrupting the folate cycle. This mechanism halts cell progression in the synthesis phase (S phase) of the cell cycle, eventually leading to cell death (Bertino *et al.*, 1996). Patients who develop resistance to monotherapy may achieve remission with a further single agent, but they frequently need more intensive and potentially toxic combination therapies, such as the EMA/CO regimen. Besides chemotherapy options, various immunotherapy strategies are currently being evaluated in clinical trials. A phase II clinical trial (TROPHIMMUN trial) tried to assess the effectiveness of Avelumab (anti-PD-L1) in patients who are resistant to single-agent chemotherapy (cohort A), or to polychemotherapy (cohort B) ([NCT03135769](#)), and the results were promising for cohort A but not for cohort B ((You *et al.*, 2020), (You *et al.*, 2023)). Finally, Ghorani E. *et al.* propose that Pembrolizumab (anti-PD-1) represents a promising new strategy for managing drug-resistant gestational trophoblastic neoplasia, potentially serving as an alternative to high-dose chemotherapy (Ghorani *et al.*, 2017). Several other studies have now highlighted the importance of immunotherapy (Baas *et al.*, 2023), but it's exact role in the management of post-molar GTN is still to be established.

1.4 Cancer Metabolism

Metabolic reprogramming is one of the hallmarks of cancer cells (Hanahan, 2022) and one of the drug resistance strategies enabling them to evade the effect of anticancer agents (Zaal and Berkers, 2018). Cancer cells remodel their metabolism during tumourigenesis and tumour progression, to facilitate uncontrolled proliferation and survival, especially under nutrient-deprived conditions (Cairns, R., Harris, I. & Mak, 2011). To achieve this, cancer cells favor aerobic glycolysis over mitochondrial oxidative phosphorylation (OXPHOS), a well-established phenomenon called Warburg effect (Vander Heiden MG, Cantley LC, 2009). Glycolysis is not as efficient as oxidative phosphorylation for energy production. Nevertheless, glycolysis provides cancer cells with more than enough glycolytic intermediates, to supply multiple biosynthetic pathways essential for fulfilling their high anabolic needs (DeBerardinis, R.J., Chandel, 2020).

Besides the rewiring of the balance between OXPHOS and glycolysis, other components of the metabolic network such as lipids (Bacci, 2020) and amino acids (Pranzini *et al.*, 2021) homeostasis are subject to change to facilitate cancer progression and drug resistance. More specifically, amino acid metabolism has a direct impact not only in supplying necessary building blocks for the production of proteins, but also on the cellular epigenetic status, redox balance and maintenance of cancer stem cells. Indirect impact of altered amino acid metabolism includes anticancer therapy resistance and cancer immune escape (Pranzini *et al.*, 2021).

1.4.1 Serine synthesis pathway (SSP)

Within amino acid-related anabolic processes, the serine synthesis pathway holds a pivotal role in redirecting glucose metabolism (Kalhan and Hanson, 2012). Serine is considered a non-essential amino acid as it can either be imported from the extracellular environment or synthesized *de novo* via the Serine Synthesis Pathway (SSP) (Metcalf JS, Dunlop RA, Powell JT, Banack SA, 2018). SSP is linked to glycolysis as it is one of the glycolysis-diverting pathways along with the Pentose Phosphate Pathway (PPP), TriCarboxylic Acid (TCA) and Lactate cycles (Amelio *et al.*, 2014).

1.4.2 De novo SSP

De novo serine biosynthesis is the major source of serine (Ayman W. El-Hattab, 2016). Cells utilize intracellular glucose to make serine through the three-step *de novo* serine synthesis pathway (Locasale, 2013). Firstly, the glycolytic intermediate 3- phosphoglycerate (3-PG), is oxidized to 3-phosphohydroxypyruvate (3-PH) in a NAD⁺-dependent way, via the enzymatic activity of Phosphoglycerate dehydrogenase (PHGDH). Then, Phosphoserine aminotransferase 1 (PSAT1) catalyzes the conversion of 3-PH into 3-phosphoserine (3-PS) with a parallel transamination reaction of glutamate to α -ketoglutarate. Finally, phosphoserine phosphatase (PSPH) catalyzes the hydrolysis of 3PS into serine (Eagle, 1959) (**Figure 1.5**).

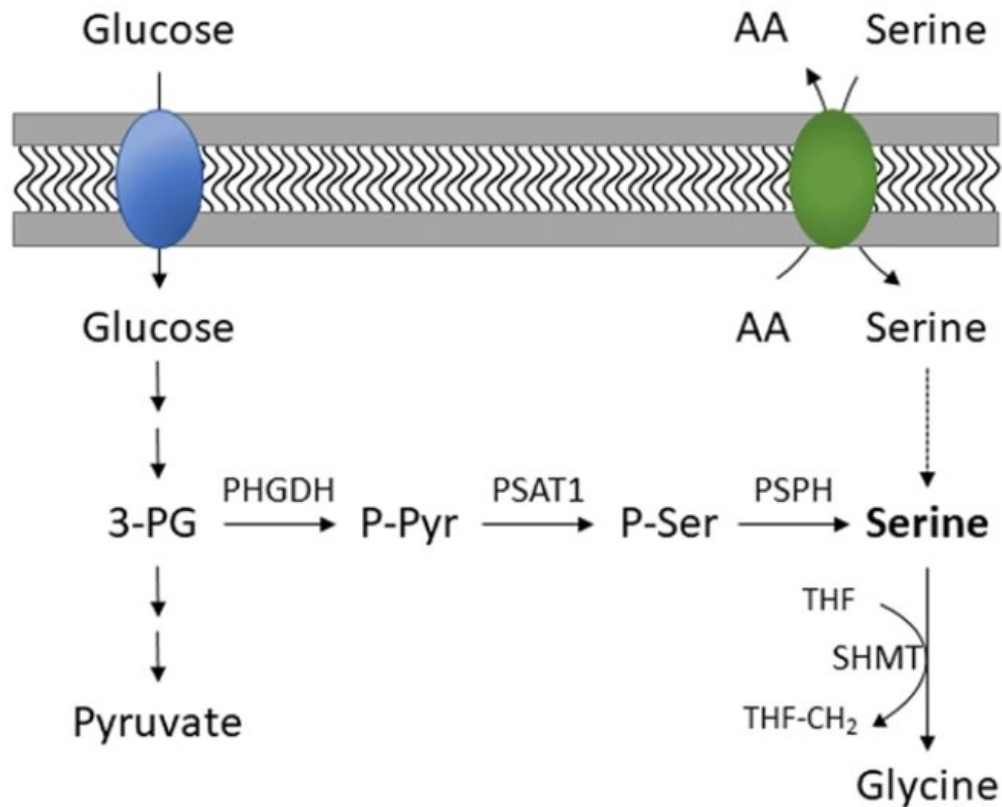


Figure 1.5: Serine synthesis pathway

Serine can be acquired from the extracellular environment through amino acid exchangers or synthesized *de novo* from glucose. The first step of its synthesis is catalyzed by the rate-limiting enzyme Phosphoglycerate dehydrogenase (PHGDH), which oxidizes 3-phosphoglycerate (3-PG) using NAD⁺ as a cofactor to generate phosphohydroxy pyruvate (P-Pyr). Next, phosphoserine aminotransferase (PSAT1) transfers an amino group from glutamate (Glu) to P-Pyr, forming phosphoserine (P-Ser). Finally, phosphoserine phosphatase (PSPH) hydrolyzes P-Ser to produce serine. (Image taken from: (McNamee, Michod and Niklison-Chirou, 2021).

1.4.3 Serine importers and exporters

Besides glucose-derived synthesis of Serine, this small amino acid can also be transported into cells via sodium-dependent transporters. These transporters include members of ASCT family such as ASCT1 (SLC1A4) and ASCT2 (SLC1A5), system A transporters such as SNAT1 (SLC38A1) and SNAT2 (SLC38A2) and members of ASC family such as Asc-1 (SLC7A10) and (SLC38A2) ((Conger *et al.*, 2023), (Wei *et al.*, 2021), (Ayman W. El-Hattab, 2016)). ASCT2 is considered the main serine transporter in cancer cells (Conger *et al.*, 2023) while ASCT1 is the main serine transporter in

neuronal cells. SNAT1 and SNAT2 have higher affinity for alanine, (El-Hattab, 2016). Last but not least, SFXN1 transports serine into mitochondria where serine is converted into glycine and formate (Kory N et. al, 2018).

1.4.4 Serine metabolism and cancer

Serine, as the main one-carbon donor, is necessary for the synthesis of other amino acids, proteins, nucleotides, and glutathione, among other metabolic functions that are essential for the development and survival of proliferating cells. Serine metabolism is crucial in cancer development and progression as cancer cells exhibit increased demand for serine to drive their growth (Amelio *et al.*, 2014). Abnormalities in serine metabolism, including elevated serine synthesis and uptake, are prevalent in a variety of cancer types and are linked to aggressive tumor characteristics. Preclinical studies targeting serine metabolism have been proven promising for developing novel antineoplastic therapies (Albert M. Li, 2021).

1.4.5 Contribution of Serine in 1-Carbon metabolism

De novo glucose-derived or/and imported serine along with glycine are considered the main carbon units supplying 1-Carbon metabolism and the methionine and folate cycles (Locasale, 2013). Serine, which is mostly catabolized in mitochondria (Tibbetts and Appling, 2010), is utilized via the folate cycle for *de novo* production of adenosine, guanosine and thymidylate with a parallel regeneration of mitochondrial cofactors NADPH, NADH and ATP (Tedeschi *et al.*, 2013). Mitochondrial serine hydroxymethyltransferase 2 (SHMT2) is an enzyme that facilitates the movement of the α -carbon of serine to tetrahydrofolate (THF), giving rise to 5,10-methylene-THF. Then, mitochondrial methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) acts on 5,10-methylene-THF converting it to 10-formyl-THF (Xiu and Field, 2020). MTHFD1-like (MTHFD1L) cleaves 10-formyl-THF in an intermediate molecule which will be able to pass through the mitochondrial membrane and be exported in the cytosol and is called formate. The trifunctional cytosolic Methylenetetrahydrofolate Dehydrogenase (MTHFD1) is then converting the exported formate back to 10-formyl-THF for use in purine synthesis and 5,10-methylene-THF for use in thymidylate synthesis (Tibbetts and Appling, 2010). Finally, Serine

Hydroxymethyltransferase 1 (SHMT1) transforms glycine into serine and Methylenetetrahydrofolate reductase (MTHFR) converts 5,10-methylene-THF to 5-methyltetrahydrofolate, completing this way the folate cycle ((Girgis *et al.*, 1998), (Froese, Fowler and Baumgartner, 2019)).

The coupling of folate and methionine cycles is happening with the demethylation of 5-methyltetrahydrofolate by Methionine Synthase (MS) and the carbon donation into the methionine cycle. The donated carbon is used in the methylation of homocysteine (Hcy) by MS and its cofactor Vitamin B12. Serine contributes to the initiation of the methionine cycle as the carbon unit that homocysteine (Hcy) accepts during its re-methylation and conversion to methionine (Fowler, B., Whitehouse, C., Wenzel, 1997). Methionine is converted to S-adenosylmethionine (SAM) by the methionine adenytransferase (MAT). SAM serves as a methyl donor, generating S-adenosylhomocysteine (SAH) as a byproduct. SAH is hydrolyzed by adenosylhomocysteinase (AHCY) to homocysteine which will be converted back to methionine, completing a full methionine cycle. Alternatively, homocysteine can be channeled to the transsulfuration pathway for glutathione (GSH) synthesis (Sanderson *et al.*, 2019) (**Figure 1.6**).

Through the transsulfuration pathway, serine serves as a precursor for cysteine. More specifically, homocysteine along with serine are processed enzymatically by cystathionine synthase (CBS) leading to the production of cystathionine. Following its cleavage by cystathionine lyase (CGL), cystathionine yields α -ketobutyrate and cysteine. Cysteine is then diverted towards synthesis of glutathione (GSH) (Davis *et al.*, 2004) (**Figure 1.6**).

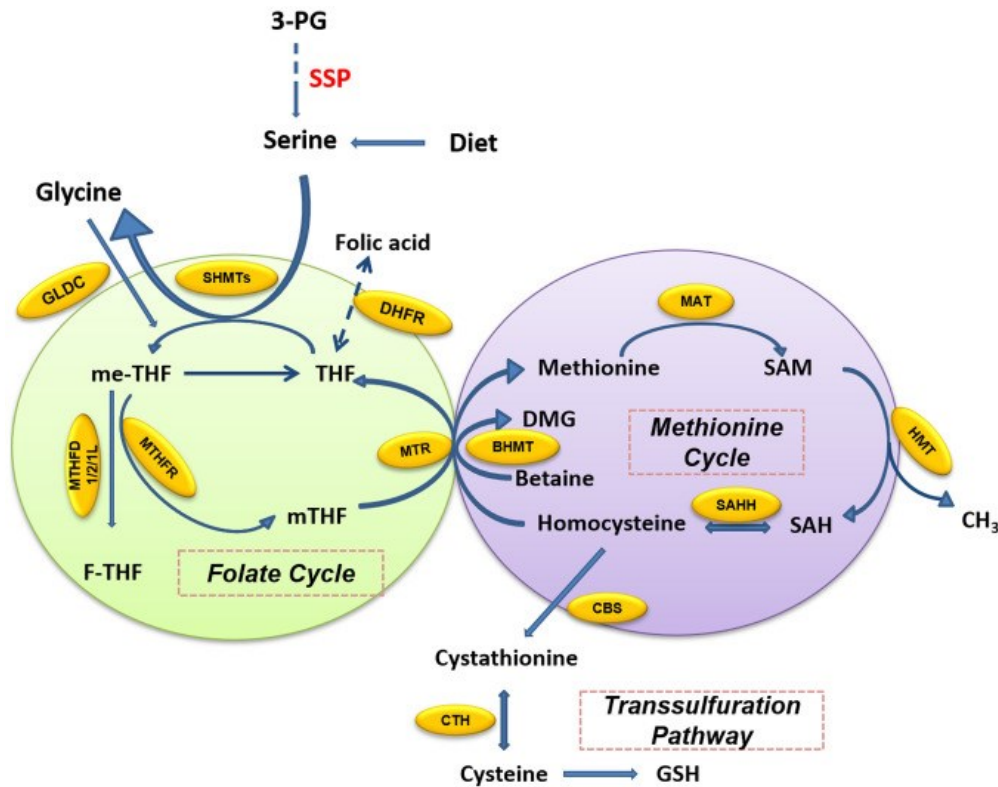


Figure 1.6: 1-Carbon metabolism

One-carbon metabolism encompasses the interconnected folate cycle, methionine cycle, and transsulfuration pathway. The folate cycle supplies one-carbon units to the methionine cycle, which produces homocysteine as an intermediate. Homocysteine can be further processed into glutathione (GSH) via the transsulfuration pathway. Notable components include 3-PG (3-phosphoglycerate), BHMT (betaine homocysteine methyltransferase), CBS (cystathionine β-synthase), CTH (cystathionase), DHFR (dihydrofolate reductase), DMG (dimethylglycine), F-THF (10-formyltetrahydrofolate), GLDC (glycine dehydrogenase), GSH (glutathione), HMT (histone methyltransferase), MAT (methionine adenosyltransferase), me-THF (5,10-methylenetetrahydrofolate), mTHF (5-methyl-tetrahydrofolate), MTHFD (methylenetetrahydrofolate dehydrogenase), MTHFR (methylenetetrahydrofolate reductase), MTR (methionine synthase), SAH (S-adenosyl homocysteine), SAHH (SAH hydrolase), SAM (S-adenosylmethionine), SHMT (serine hydroxymethyltransferase), SSP (serine synthesis pathway), and THF (tetrahydrofolate). (Image taken from: (Pan et al., 2021).

1.4.6 Outputs of 1-Carbon metabolism

Overall, one-carbon metabolism has three important outcomes encompassing cellular processes essential for growth and maintenance. Firstly, it facilitates the production of macromolecules such as nucleic acids for DNA and RNA synthesis, amino acids for protein synthesis, and lipids which serve as energy repositories and structural components of cell

membranes (Ducker and Rabinowitz, 2017). Secondly, 1-Carbon metabolism helps preserve cellular redox homeostasis and shield cells against oxidative stress via reduction of NADPH and oxidation of NADP⁺ and GSH production. GSH reduces reactive oxygen species (ROS) and preserves the proper NADPH/NADP⁺ ratio for anabolic metabolic processes (Petrova *et al.*, 2023). Lastly, one-carbon metabolism plays a pivotal role in methylation reactions, since methyl groups responsible for DNA and histone methylation originate from SAM which is synthesized during the methionine cycle (Mentch SJ, 2016).

1.4.7 One carbon metabolism and cancer treatment

Several chemotherapeutic agents serve as folate antagonists such as methotrexate and pemetrexed and are used as first line treatment in various and diverse types of cancer such as breast cancer, acute lymphoblastic leukemia, bladder cancer and lymphomas ((Cheson BD, 1992), (Bertino, 1975)). The direct target of these agents is DHFR/THF which results in hindering of folate cycle and subsequently of one-carbon metabolism ((Baram *et al.*, 1987),(Allegra *et al.*, 1986)). Moreover, pathways derived from 1-C metabolism have been targeted by chemotherapeutic agents, such as nucleotide synthesis. For instance, 5-fluorouracil (5-FU) which is an analog of pyrimidine, blocks thymidine synthase activity preventing the methylation of dUMP to dTMP thereby blocking nucleotide synthesis (Peters, 1988). Other examples of pathways targeted downstream of 1-C metabolism include epigenetic regulation of histones and DNA and more specifically inhibitors targeting the methyltransferase activity of particular enzymes (Wagner and Jung, 2012). These inhibitors disrupt SAM-mediated methylation of histones and DNA and subsequently the activation/repression pattern of the genes, radically changing the epigenetic signature of the cell (Goldberg, Allis and Bernstein, 2007).

1.5 Methotrexate

1.5.1 Methotrexate-Mechanism of action

The folate analog Methotrexate (MTX) is an antimetabolite used as both a chemotherapeutic and immunosuppressant agent ((Sidney Farber, 1949),(R. Gubner, S. August, 1951)). It was initially designed to inhibit DHFR blocking this way the folate cycle and, by extension, the *de novo* purine and thymidine nucleotide synthesis (Jacques Jolivet et. al, 1983). Dihydrofolate reductase (DHFR) is an essential enzyme implicated in the folate pathway and in the regeneration of tetrahydrofolic acid (THF) from dihydrofolic acid (DHF) in a NADPH-dependent way. The reduced folate or else THF acts as a carbon donor/co-factor in many methyl transfer reactions in 1-carbon metabolism (Chen *et al.*, 1984). Methotrexate is a molecule with a chemical structure similar to folic acid, with a 1000-fold greater binding affinity to DHFR (Bertino, 2009).

The incorporation of Methotrexate into the cell is facilitated either mainly via the reduced folate carrier 1 (RFC1) or via the folate receptor alpha (FRa) ((Wright *et al.*, 2022), (Sánchez-Del-Campo *et al.*, 2009)). The export is facilitated by ATP-binding cassette (ABC) efflux transporters (Murakami and Mori, 2012). Once inside the cell, MTX undergoes polyglutamination catalyzed by folylpolyglutamate synthase (FPGS) which enhances its stability and intracellular retention. Conversely, folylpolyglutamate hydrolase (FPGH) catalyzes the reverse reaction, potentially reducing MTX levels within the cell. The MTX polyglutamination also inhibits other folate-dependent enzymes such as thymidylate synthase (TYMS) and 5-aminoimidazole-4-carboxamide ribotide transformylase (AICAR transformylase/ATIC), an enzyme responsible for catalyzing the last stages of *de novo* purine synthesis, affecting DNA replication (Visentin M, Zhao R, 2012). Thus, upon MTX treatment, DNA synthesis is blocked since cells undergo cell cycle arrest in the synthesis phase (S phase), resulting in cellular death (Tsurusawa M et al., 1990).

1.5.2 Methotrexate-Mechanisms of Resistance

Resistance to methotrexate (MTX) can arise through several mechanisms. There are four commonly encountered mechanisms. Firstly, the membrane transport of MTX. Reduced drug uptake into cells, possibly due to low expression or loss-of-function mutations of transporters RFC1 and FRa, hinders the import of MTX (Gorlick *et al.*, 1997). Additionally, overexpression of ABC-transporters such as MRP1 (ABCC1), MRP2 (ABCC2) and MRP3 (ABCC3) facilitate more efficient export of MTX, decreasing the intracellular MTX levels ((Hooijberg JH *et al.*, 1999), (Kool *et al.*, 1999)). Secondly, impaired retention within cells can result from defective polyglutamylation or enhanced breakdown of polyglutamate, diminishing the prolonged presence of MTX necessary for its therapeutic effects ((Rhee MS *et al.*, 1993), (Weigand *et al.*, 1999)). Thirdly, the inhibitory effect of MTX may be offset by increased folate metabolism caused by increased activity of DHFR, the enzyme that MTX targets. The increased activity of this enzyme results from the amplification of the *DHFR* gene ((Srimatkandada S *et al.*, 1983)). Fourthly, the diminished binding affinity of MTX to DHFR, due to mutations, can mitigate MTX's ability to disrupt folate-dependent pathways crucial for cell survival (Chunduru *et al.*, 1994).

1.6 Transcriptional and epigenetic regulation of Serine synthesis pathway

There are several transcription factors regulating the serine synthesis pathway. One key factor is ATF4 (Activating Transcription Factor 4) which is a downstream target of the Integrated Stress Response (ISR) pathway (Lu, Harding and Ron, 2004). ATF4 is selectively activated by ISR signaling, upon stressors such as nutrient depletion and deregulation of ATF4, allowing cancer cells to overcome such stress triggers (Wortel *et al.*, 2017). ATF4 is a member of the ATF/cyclic AMP response element-binding (CREB) family of transcription factors, that binds to specific DNA sequences, including the C/EBP-ATF response elements (CAREs), present in the promoters of its

target genes *PHGDH* and *PSAT1*, thereby promoting serine biosynthesis (Adams, 2007). (The interconnection of ATF4 and ISR signaling is extensively reported in paragraphs §1.7.2-1.7.3).

Apart from ATF4, another member of the ATF/ CREB family, ATF3 plays a role in regulating the serine synthesis pathway and tumour growth upon serine-limiting conditions. ATF3 is induced in an ATF4-dependent way by binding to ATF4 and regulating its stability. Moreover, ATF3 directly binds to the promoters/enhancers of the SSP genes *PHGDH*, *PSAT1*, and *PSPH* and subsequently recruits the transcriptional co-activator p300 to enhance the transcription of these genes (Li *et al.*, 2021).

c-Myc directly regulates the transcription not only of the SSP genes *PHGDH*, *PSAT1*, *PSPH* but also that of the folate cycle *SHMT1* and *SHMT2*, the responsible enzymes for the glycine-serine interconversion (Bauwe and Kolukisaoglu, 2003). More specifically, there is a reported overlap of MYC and ATF4 in Burkitt lymphoma cells in which ATF4 is under the transcriptional control of MYC, promoting the overproduction of PHGDH and PSAT1 (Białopiotrowicz *et al.*, 2020).

The serine synthesis pathway is also under the transcriptional control of NRF2 (Nuclear factor erythroid-2–related factor 2) in an ATF4-dependent manner. It has been observed that Non-small cell lung cancer (NSCLC) cells upregulate PHGDH, PSAT1, and SHMT2 via ATF4 in order to promote one-carbon metabolic flow towards glutathione and nucleotide synthesis (Denicola *et al.*, 2016). It has been established that NRF2 is a direct target of ATF4 in addition to forming heterodimers and interacting with NRF2 ((Ye *et al.*, 2014), (He *et al.*, 2001)). In the case of the NSCLC cells, NRF2 doesn't bind to the ATF4 binding sites of its target genes but its silencing can affect the binding of ATF4 to these sites, supporting the transcriptional regulatory role of NRF2 in SSP (Denicola *et al.*, 2016).

AMPK (AMP-activated protein kinase) interacts with mTORC1 (mammalian target of rapamycin complex 1) and HIF-1a (hypoxia-inducible factor-1 alpha) to regulate the serine synthesis pathway. AMPK is a cellular energy sensor that gets activated upon energy loss and in order to restore energy balance, it inhibits anabolic pathways and activates catabolic pathways (Hardie, 2011). Serine depletion can trigger AMP-activated protein kinase (AMPK) activation via

reactive oxygen species (ROS) accumulation and subsequent AMPK-dependent HIF-1a stability and transactivation. HIF-1a induces then the expression of not only the SSP enzymes but also of the glycolytic genes (*GLUT1*, *GLUT3*, *HK2*, and *PFKFB2*) leading to enhanced glucose uptake, glycolysis and serine and glycine biosynthesis (Yun *et al.*, 2023). On the other hand, mTORC1 is not reportedly triggered upon serine depletion and transcriptional activation of the SSP is an event of the broader anabolic processes driven by mTORC1. The AMP-activated protein kinase-mTORC1 axis can promote the upregulation of *PSAT1*, *PSPH*, *SHMT1* and *SHMT2*, as a result of LKB1 (Liver kinase B1) downregulation, the main activating kinase of AMPK. This cascade leads to elevated SAM levels, and subsequent increased DNA methyltransferase activity supporting tumourigenesis (Kottakis F *et al.*, 2019).

Additionally, ATF4 collaborates with a member of the Jumonji domain 2 (JMJD2) family, KDM4C (Lysine demethylase 4C) for the transcriptional activation of *PHGDH* and *PSAT1*. Under normal/serine deficient conditions, KDM4C epigenetically activates the SSP genes by removing the repressive histone modification H3 lysine 9 (H3K9) trimethylation. KDM4C is also activating ATF4, further promoting the positive transcription of these SSP genes (Zhao *et al.*, 2016).

The Euchromatic Histone Lysine Methyltransferase 2 (EHMT2) or else G9A epigenetically regulates the SSP. G9A mono-/di-methylates histone H3 at lysine 9 (H3K9). Upon serine depletion, G9A mono-methylates H3 at lysine 9 of *PHGDH*, *PSAT1*, *PSPH*, *SHMT1* and *SHMT2*, maintaining these enzymes in an active state. According to the same article, G9A inactivation led to serine deprivation, inducing cell death while G9A overexpression has been associated with enhanced serine synthesis in cancer cells, an increase in their proliferation rate and tumourigenicity as well as higher mortality rates in cancer patients (Ding, 2013).

Last but not least, p53 is the major negative regulator of the transcription of *PHGDH* and *PSAT1* (Ou *et al.*, 2015). p53 holds a central role in normal and cancer cell homeostasis as a regulator of the cellular stress response which gets triggered by various stressors including oxidative stress and serine deficiency ((Maddocks *et al.*, 2013), (Vousden and Prives, 2009)). In particular, p53 facilitates the antioxidant capability of cancer cells by helping them overcome serine shortage. Cells lacking p53 are unable to counteract serine deficiency-induced oxidative

stress, resulting in decreased viability and proliferation rate. Initiation of the p53–p21 pathway induces cell cycle arrest under serine depletion, leading to redirection of serine supplies towards glutathione synthesis, achieving cell survival ((Maddocks *et al.*, 2013), (Amelio *et al.*, 2014)). Moreover, p53 family members directly affect various pathways linked to glycolysis, which gives cancer cells the ability to react to metabolic stress. Specifically, the pentose phosphate pathway (PPP) and nucleotide biosynthesis are supported by p53, TIGAR (TP53-inducible glycolysis and apoptosis regulator), p73, and G6PD (glucose-6-phosphate dehydrogenase) ((Bensaad *et al.*, 2006),(Du W. *et al.*, 2013)). Furthermore, the expression of GLS-2 (glutaminase-2) is activated by p53 and p73, leading to glutaminolysis, which interferes with glucose-derived serine synthesis ((Hu *et al.*, 2010),(Velletri *et al.*, 2013)).

1.7 ISR Signaling - Overview

The integrated stress response (ISR) pathway is an evolutionarily conserved defense mechanism that cells deploy upon various extrinsic or intrinsic stressors, ensuring cellular survival and adaptation in adverse conditions. These stressors include hypoxia, nutrient deprivation, viral infection, or endoplasmic reticulum (ER) stress and can initiate a cascade of signaling events regulating global protein synthesis ((Wek RC, Jiang HY, 2006), (Ron, 2002), (Harding *et al.*, 2003), (Brostrom CO, Prostko CR, Kaufman RJ, 1996)). The phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 alpha (eIF2 α) by one of the four eIF2 α kinase family members serves as the activation and commencement step of the ISR pathway (Ron, 2002).

The four members of the eIF2 α kinase family are PKR (Double-stranded RNA-dependent protein kinase), PERK (PKR-like ER kinase), HRI (Heme-regulated eIF2 α kinase) and GCN2 (General control non-depressible protein 2) (Pakos-Zebrucka K *et al.*, 2016) (**Figure 1.7**). Each eIF2 α kinase is activated by a distinct stress trigger. PKR is activated by dsRNA during viral infection, blocking viral and host protein synthesis through eIF2 α phosphorylation (Balachandran *et al.*, 2000). PERK is triggered by endoplasmic reticulum (ER) stress resulting from the accumulation of unfolded proteins in the ER ((Gardner and Walter, 2011), (Wang and Kaufman, 2016)). HRI has been

reported to be triggered not only by heme deprivation but also by oxidative and osmotic stress, heat shock and proteasome inhibition ((McEwen *et al.*, 2005), (Yerlikaya A, Kimball SR, 2008)). GCN2 is activated upon amino acid deprivation (Vazquez De Aldana *et al.*, 1994). Eventually, all four eIF2 α kinases converge on the phosphorylation of eIF2 α , which is central to the ISR and halts global protein translation, while selectively activating specific stress-responsive genes, like activating transcription factor 4 (ATF4). ATF4 then initiates a transcriptional program that promotes antioxidant responses, amino acid metabolism, autophagy, and other adaptive mechanisms with the ultimate goal of restoring cellular homeostasis (Lu, Harding and Ron, 2004).

1.7.1 The role of eIF2 α in ISR

The phosphorylation and RNA binding sites of eIF2 α make it the primary regulatory component of the eIF2 complex, which is composed of eIF2 α , eIF2b, and eIF2c. Under normal circumstances, eIF2 is essential for the commencement of mRNA translation and the identification of the AUG start codon (Pain, 1996),(Jackson RJ et al., 2010). eIF2 along the initiation factors, eIF1 and eIF1A, methionyl-tRNA (Met-tRNAⁱ) and GTP build the 43S pre-initiation complex (PIC) which is recruited to the 5'methylguanine Cap of mRNA (Aitken and Lorsch, 2012). Upon phosphorylation of eIF2 α at Serine 51, and subsequent activation of ISR signaling, eIF2 blocks the formation of the PIC complex and the translation of mRNAs with 5'methylguanine Cap. The only mRNAs that manage to get translated are those with a short upstream open reading frame (uORF) in their 5' untranslated region (5'UTR). ATF4, ATF5 and CHOP mRNAs are among these, with ATF4 being the most well-characterized effector of the ISR pathway (Hinnebusch and Lorsch, 2012).

1.7.2 ATF4 is the central transcriptional regulator of ISR Signaling

ATF4 is an essential transcription factor in various cellular stress response pathways, and especially in ISR signaling, that helps cells adapt to a restricted nutrients supply (Ryoo, 2024). Elevated ATF4 protein levels facilitate the transcription of its stress-responsive target genes via binding of ATF4 to C/EBP-ATF response element (CARE) sequences (Kilberg *et al.*, 2012). These

CARE sequences serve as amino acid response element (AARE) sequences during amino acid starvation and ATF4 is the sole transcription factor that is known to bind all known AARE sequences ((Siu *et al.*, 2002), (Averous *et al.*, 2004), (B'Chir *et al.*, 2013)). ATF4 interacts with other key transcription factors orchestrating cellular adaptation to stress, which are also its target genes, such as C/EBP homologous protein (CHOP) (Siu *et al.*, 2002). Both ATF4 and CHOP can bind under stress conditions to the AARE sequences of autophagy-related genes and genes related to amino acid metabolism (B'Chir *et al.*, 2013). Moreover, PERK mediated activation of ISR and subsequent activation and cooperation of both ATF4 and CHOP has been reported as a way to induce apoptosis as a response to ER stress (Cao *et al.*, 2012).

1.7.3 ICR regulates amino acid metabolism via ATF4 activation

Among the metabolic pathways stimulated by ISR are glycolysis, *de novo* serine synthesis, 1-carbon metabolism (folate and methionine cycles) and the transsulfuration pathway (Ryoo, 2024). ISR-mediated activation of ATF4 and ATF3 induces the transcription of PHGDH, PSAT1 and PSPH, the three enzymes of the Serine Synthesis pathway ((Han *et al.*, 2013), (Ye *et al.*, 2012), (Li *et al.*, 2021)). Folate enzymes such as SHMT and MTHFD2 are activated by ATF4, ATF3 and CHOP ((Khan *et al.*, 2017), (Li *et al.*, 2021)). Moreover, ATF4 facilitates cysteine production, which is essential for the production of glutathione, the role of which is to shield cells from oxidative stress. Besides serine being a precursor for cysteine, ATF4 positively regulates cystathionine gamma-lyase, an enzyme necessary for the conversion of the methionine cycle's metabolite homocysteine to cysteine (Sbodio, Snyder and Paul, 2019).

1.7.4 GCN2 activation during amino acid deprivation

Phosphorylation of eIF2 α by GCN2 not only inhibits global protein synthesis but also specifically enhances the translation of certain mRNAs, such as that of ATF4. Following ATF4 transcription and translation, ATF4 moves into the nucleus and stimulates the transcription of genes related to amino acid metabolism, including those that code for enzymes implicated in amino acid transport, biosynthesis, and antioxidant defense (Lange *et al.*, 2008). In parallel, ATF4 promotes the expression of genes associated with autophagy, a process through which cells break

down and recycle biological components to produce amino acids and other vital nutrients (B'Chir *et al.*, 2013). Therefore, the adaptive metabolic reprogramming required for cell survival under conditions of amino acid stress is facilitated by the selective translation of ATF4 in response to GCN2-mediated eIF2 α phosphorylation.

1.7.5 Induction of autophagy via ISR signaling

Autophagy is a highly conserved cellular process essential for maintaining cellular homeostasis and promoting cell survival during times of stress. It involves the lysosomal degradation and recycling of damaged or unnecessary cellular components, including organelles, proteins, and macromolecules. The creation of an autophagosome, a double-membraned structure that engulfs cytoplasmic cargo intended for breakdown, is the first step in the process of autophagy. The next step is the fusion of the autophagosome with lysosomes, forming the autolysosome, where lysosomal enzymes degrade the cargo (Mizushima, 2007). Amino acids, nucleotides, and fatty acids are among the breakdown products that are produced as a result of the autophagy process; these recycled breakdown products are re-used in cellular metabolism and energy production (Feng *et al.*, 2014). Numerous cellular stressors, including deficiency in nutrients, hypoxia, DNA damage and reactive oxygen species (ROS) can trigger autophagy ((Clementi *et al.*, 2020), (Pakos-Zebrucka K *et al.*, 2016),(Akman *et al.*, 2021)). As a consequence, ISR signaling is induced and can orchestrate cell death or cell survival pathways to cope with the stress conditions (Pakos-Zebrucka K *et al.*, 2016).

GCN2 is the eIF2 α kinase activated by sensing uncharged (deacetylated) tRNAs, which accumulate under conditions of amino acid deficiency. As a response to this trigger, GCN2 can induce the upregulation of many autophagy-related genes via eIF2 α phosphorylation and selective activation of its downstream target, ATF4 (B'Chir *et al.*, 2013). As a transcription factor, ATF4 in turn stimulates the expression of genes involved in different stress responses, such as those controlling autophagy (Wortel *et al.*, 2017). Intriguingly, according to Anne-Catherine Maurin *et al.*, phosphorylation of eIF2 α is necessary for the GCN2-dependent initiation of

autophagy during short-term depletion of any essential amino acid, while expression of ATF4 is not, indicating the potential involvement of translational regulatory mechanisms (Maurin *et al.*, 2022). According to other studies, upon amino acid starvation, ISR can be activated by mTORC1 (mammalian target of rapamycin complex 1) independently of eIF2 α kinases and with subsequent mTORC1 mediated activation of ATF4 and autophagy ((Suraweera *et al.*, 2012), (Lehman *et al.*, 2015), (Denoyelle S *et al.*, 2011)).

1.7.6 Activation of Apoptosis Through Integrated Stress Response

The Integrated Stress Response (ISR) pathway is essential not only for promoting cell survival under stress conditions but also for orchestrating programmed cell death in situations where cellular damage is irreversible (Pakos-Zebrucka K *et al.*, 2016). ATF4 greatly contributes via multiple mechanisms to ISR-induced cell death, if homeostasis is not restored. The most studied is through ATF4-mediated activation of CHOP. CHOP is a pro-apoptotic factor upregulating BH3-only BCL-2 family and death receptor 5 (DR5), leading to ER stress-induced apoptosis ((Puthalakath *et al.*, 2007), (Galehdar *et al.*, 2010)). Moreover, CHOP forms protein complexes with ATF4 or ATF3, facilitating the transcription of pro-apoptotic genes like *PMAIP1* (Phorbol-12-Myristate-13-Acetate-Induced Protein 1) and the induction of the intrinsic apoptotic pathway (Teske *et al.*, 2013). It is worth mentioning that CHOP alone is not able to induce cell death, in contrast to ATF4 which can activate it. It has also been reported that ATF4-mediated oxidative stress and cellular redox homeostasis disturbances contribute to the apoptotic signaling cascade in neurons both *in vitro* and *in vivo* (Lange *et al.*, 2008). Finally, the ATF4 response triggers p53-mediated apoptosis in the event of persistent mitochondrial insufficiency. Upon transient inhibition of the electron transport chain, pro-survival mechanisms are orchestrated by ATF4, helping the cells recover from deficiency stress. However, sustained inhibition of the respiratory chain, which is crucial for the biosynthesis of pyrimidines, makes cells prone to genetic instability. A feedback mechanism enables p53 to suppress ATF4 and its pro-survival activities and promotes cell death. As a result, the interplay between ATF4 and p53 is crucial in determining how cells will react to mitochondrial dysfunction (Evstafieva *et al.*, 2014).

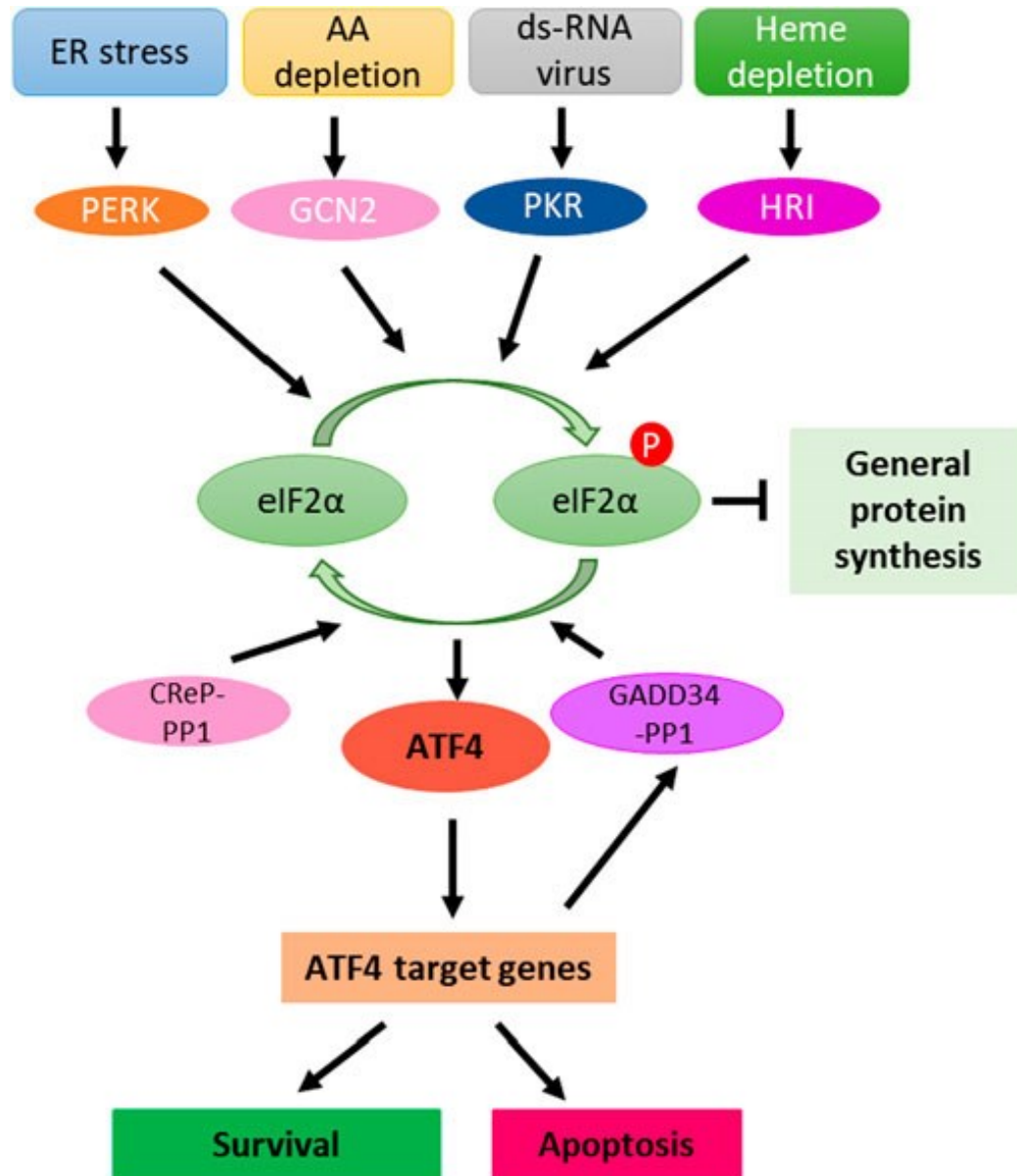


Figure 1.7: Integrated stress responses signaling pathway

The integrated stress response (ISR) signaling pathway is activated upon various cellular stress conditions, including ER stress, amino acid deficiency, double-stranded RNA virus infection and heme depletion. These stresses activate sensor kinases—PERK, GCN2, PKR and HRI—which phosphorylate the eukaryotic translation initiation factor eIF2 α . Phosphorylation of eIF2 α suppresses global protein synthesis while enabling the selective translation of ATF4 mRNA. ATF4 functions as a transcription factor, binding to DNA to regulate genes involved in cellular adaptation, survival, and apoptosis. The ISR is modulated by feedback mechanisms through constitutively expressed phosphatase complex CReP-PP1 and inducible phosphatase GADD34-PP1, which dephosphorylate eIF2 α to attenuate or terminate the stress response. (Image taken from: (Tian et al., 2021))

1.7.7 ISR-induced chemoresistance

While the ISR pathway serves as a mechanism for normal cells to quickly adjust to stimuli that are non-oncogenic, it can also facilitate the neoplastic transformation of cells. ISR can be induced as a result of chemotherapy-induced cytotoxicity and may function as an oncogenic-driven program that promotes tumor growth, metastasis, and therapy resistance (Lines *et al.*, 2023). The most characteristic oncogenic-related reaction upon ISR activation is the selective upregulation of p21, resulting in cell cycle arrest and cancer cell survival (Lehman *et al.*, 2015). Moreover, it has been reported that GCN2-mediated p21 induction upon nutrient depletion contributes to drug resistance in treated liver cancer cells (Skuli *et al.*, 2023). Additionally, epithelial–mesenchymal transition transcription factors were selectively translated in breast cancer cells upon ISR activation, facilitating cancer cell plasticity (Jewer *et al.*, 2020). Finally, chronic ER stress in BRAF-mutated melanoma increases phosphorylated levels of eIF2 α which activates ISR and promotes chemoresistance by dysregulating autophagy (Corazzari *et al.*, 2015). These examples of aberrant activation of ISR indicate it as an obstacle in cancer treatment but concurrently, the fact that a tumour might be over-reliant on ISR activation, makes it a targetable approach.

1.7.8 Targeting GCN2/ISR Signaling Pathway - Dual Roles of the Integrated Stress

ISR signaling has a dual role in balancing cell status, promoting either its survival or its death and its role has been studied in tumorigenesis and tumour progression (Tian *et al.*, 2021). Although the ISR aims to restore homeostasis and cell survival throughout cancer growth, persistent ISR activation can lead to cell death (Lines *et al.*, 2023). Targeting components of the ISR pathway, leading to its activation or inhibition, has been proposed as a promising anti-cancer strategy, particularly when combined with existing therapies, such as immunotherapy. Enhanced ISR signaling can be achieved by increasing the activity of the eIF2 α kinases, GCN2, PERK, and HR. The majority of eIF2 α activators are small molecules such as Arginine deiminase and Halofuginone which target GCN2 ((Castilho *et al.*, 2014), (Long *et al.*, 2013)). Alternatively, eIF2 α

activation can be achieved by preventing its dephosphorylation by phosphatases, as with Salubrinal which targets the recruiters of Protein phosphatase 1 (PP1) (Boyce *et al.*, 2005).

It has been suggested that drug resistance in cancer may be overcome by inhibiting ISR signaling. A way to manage this is by inhibiting the eIF2 α kinases mostly with small-molecule ATP-competitors. Representative examples are the GCN2 inhibitors, GCN2iB, and GZD824 (Olverembatinib). GZD824 is a multikinase inhibitor that is actively being tested in several clinical trials for hematological cancers while it has shown efficient antitumour activity in *in vitro* models of non-small cell lung cancer (Ren *et al.*, 2013). As for GCN2iB, it was reported to suppress the growth of prostate cancer xenografts (Cordova *et al.*, 2022). Finally, another more refined strategy is to terminate the eIF2 α signaling cascade further downstream of eIF2 α to circumvent redundancy between kinases and the wider effects of upstream kinase inhibition. This can, for instance, be achieved by impeding ATF4 synthesis (Sidrauski *et al.*, 2015).

1.8 Methotrexate Resistance beyond GTN

1.8.1 Osteosarcoma

Osteosarcoma is a type of bone cancer deriving from primitive bone-forming mesenchymal cells. It affects primarily children, adolescents, and young adults representing about 20% of all primary bone tumours. (Zhao *et al.*, 2021). Osteosarcoma is usually treated with a multimodal strategy that includes surgery, chemotherapy, and radiotherapy when it is considered necessary. Combining chemotherapy with surgical resection has tackled the occurrence of micrometastases, and survival rates have been significantly improved (Wadhwa, 2014). Methotrexate is one of the agents included in the adjuvant/neoadjuvant chemotherapy regimen strategies for the initial occurrence of osteosarcoma (Yu *et al.*, 2019). The occurrence of resistance to methotrexate in osteosarcoma patients has been reported as the result of impaired intercellular transport of MTX via the reduced folate carrier (RFC) and elevated dihydrofolate reductase (DHFR) expression, in correlation to retinoblastoma (RB1) gene status ((Guo W. *et al.*,

1999), (Serra *et al.*, 2004)). More specifically, MTX resistance in RB1-positive osteosarcoma cell lines (such as U2OS-MTX-Resistant cells) and RB1-positive osteosarcoma patients was linked to DHFR overexpression while in the case of RB1-negative osteosarcoma MTX-resistant cell lines (such as SAOS-2-MTX-Resistant cells), the reduced expression of RFC was the determinant (Serra *et al.*, 2004).

1.8.2 Breast Cancer

Breast cancer remains one of the most widespread and challenging malignancies impacting women on a global scale with more than 2 million new cases registered per year (Łukasiewicz S. *et al.*, 2021). Treatment for breast cancer is determined by the cancer's subtype and the extent to which it has metastasized to lymph nodes or other regions of the body. Breast cancer subtypes that express neither the HER-2, PR, or ER receptors are known as triple-negative breast cancers (TNBC) and are considered more aggressive than other subtypes. Especially in such cases, chemotherapy remains an essential component of the treatment approach and is often administrated alongside targeted therapies, before or after radiotherapy or surgery (Akram *et al.*, 2017). More specifically, the FDA has authorized the use of immunotherapeutic atezolizumab (Tecentriq) in combination with chemotherapy for treating patients with TNBC (Schmid *et al.*, 2018) and the immunotherapeutic Keytruda (pembrolizumab) recently received fast-track approval for the treatment of metastatic TNBC (Cortes *et al.*, 2022). Methotrexate (MTX) is not a first-line treatment for breast cancer but can be used in patients whose cancer has metastasised and/or in patients whose cancer was previously treated with radiotherapy and surgery (Yang *et al.*, 2020). Development of resistance to MTX in breast cancer has been reported to occur as a result of decreased expression of the reduced folate carrier (RFC) (Lindgren *et al.*, 2006).

1.8.3 Colon Cancer

Colorectal cancer (CRC) ranks as the third most frequently diagnosed cancer globally with almost 2 million new cases per year, and the second deadliest type of cancer with close to 1 million deaths annually (Morgan *et al.*, 2023). CRC is often identified at an advanced stage when therapeutic choices are limited as existing treatments have a greater chance of curing patient at

early stages of the disease. Surgical excision is the main treatment for CRC at early stages and is often combined with adjuvant or neoadjuvant chemotherapy. The first line of treatment for advanced metastatic CRC is usually chemotherapy either alone or in combination with targeted therapy (Cunningham *et al.*, 2010). MTX is an important component of the chemotherapy regimen (Marsh *et al.*, 1991). The long noncoding RNA H19 (lncRNA H19) has been implicated in MTX resistance in CRC. lncRNA H19 overpresentation and subsequent activation of the Wnt/ β -catenin pathway were linked to enhanced MTX resistance in the MTX-resistant CRC cell line HT-29-R and its silencing sensitized these cells to MTX (Wu *et al.*, 2017). Moreover, the upregulation of miR-770-5p has been reported to affect MTX resistance in human CRC cells by downregulating HIPK1 (homeodomain-interacting protein kinase 1) which regulates the activity of several transcription factors (Zhang, Li and Sun, 2019).

1.9 Serine synthesis metabolism beyond GTN

As mentioned above, serine metabolism plays a crucial role during tumorigenesis and tumour progression. Since serine is a precursor for the protein, nucleotides, and glutathione synthesis, cancer cells benefit from high rates of serine uptake and synthesis to support these processes. (Locasale, 2013). Therefore, targeting serine metabolism enzymes has emerged as a promising anticancer therapeutic approach.

In the case of osteosarcoma, the mTORC1 pathway seems to promote amino acid metabolism by upregulating glycolysis and glycolysis-derived *de novo* serine and glycine synthesis. The mTORC1-mediated serine/glycine pathway functions as a defense mechanism in osteosarcoma cells as it ensures antioxidant capability in osteosarcoma cells and maintenance of their proliferation (Wang *et al.*, 2017). As mTORC1 activity was found increased in clinical osteosarcoma samples, mTORC1 inhibition may be a potential therapeutic strategy through impairment of serine metabolism in osteosarcoma patients.

PHGDH overexpression is identified in certain cancer subtypes, particularly in breast cancer. Triple-negative breast cancers are more likely than other breast cancer subtypes to have

PHGDH copy number gain (Locasale *et al.*, 2011). Moreover, *PHGDH* expression is also more elevated in ER-negative than in ER-positive tumors (Possemato *et al.*, 2011) and both *PHGDH* and *PSAT1* protein levels were found to be increased in metastatic versus non-metastatic ER-negative breast cancer cells (Pollari *et al.*, 2011).

In CRC, *PHGDH* mRNA and protein levels were found significantly overexpressed in tumours in comparison with paired healthy tissue, and *PHGDH* protein expression was linked to advanced TNM (Tumour, Node, Metastasis; TNM staging system) status and larger tumour size. Furthermore, *PHGDH* expression could help define a high-risk subset of CRC patients as *PHGDH* high levels were correlated with poor prognosis (Jia *et al.*, 2016). The overrepresentation of *PHGDH* in CRC cells under serine depletion has been explained by an increase in ATF4-mediated transcription via GCN2-eIF2 α signaling (Dai *et al.*, 2023). Finally, the ability of CRC cells to withstand extracellular serine depletion is significantly influenced by the presence or lack of the tumor suppressor protein p53. Both p53^{+/+} and p53^{-/-} human CRC cells exhibit a drop in ATP levels and an increase in serine synthesis from glycolytic intermediates when grown in a serine- and glycine-free medium. In the case of p53^{+/+} cells, their adaptation to serine depletion is achieved by undergoing p53/p21 cell cycle arrest and by channeling serine towards GSH synthesis to reduce oxidative damage rather than towards nucleotide synthesis as in p53^{-/-} cells (Maddocks *et al.*, 2019).

1.10 Aims of this research project

The purpose of this project is to explore the mechanisms behind MTX resistance in post-molar GTN, with the potential of identifying novel proteins that can be used as biomarkers of MTX response and assess the prospect of targeting the MTX-resistance-driven pathways. The steps are the following:

1. Identification of pathways involved in MTX resistance, via proteomic analysis in samples derived from GTD patients sensitive or resistant to MTX utilizing Nano-LC Mass Spectrometry (Nano LC-MS/MS).
2. Bioinformatics analysis for the identification of differentially expressed proteins and their corresponding pathways implicated in resistance.
3. Validation of MS findings utilizing a pair of human choriocarcinoma cells mimicking the patients' treatment response (sensitivity/resistance to MTX).
4. Identification of key proteins with common expression patterns at both patient and cell line levels regarding MTX resistance/sensitivity and their assessment as biomarkers of treatment response via Immunohistochemical staining.
5. Further exploration of the pathways that drive MTX resistance and potential therapeutic strategies to target them.
6. Investigation of common MTX-Resistance mechanisms across different cancer types using other pairs of MTX-R/MTX-S cell lines.

2. Methods

2.1 Cell lines

The human choriocarcinoma cell lines, JEG3 (MTX- Sensitive) and JEG3-R (MTX- Resistant) were gifted from Dr. Kevin Elias (Harvard Medical School, Boston, MA, USA). The JEG3 cell line is an epithelial, hypertriploid, clonally-derived, adherent one with high-producing levels of hCG. It was isolated from the Woods strain of the Erwin-Turner tumour (Kohler and Bridson, 1971). The JEG3-R cell line was generated after prolonged (over a 14 month-timeframe) and repeated exposure of JEG3 cells to increasing concentrations of MTX (Elias *et al.*, 2017).

The human osteosarcoma cell line U2OS-R was generated by me after extended (over an 18-month time frame) and repeated exposure of U2OS cells to increasing concentrations of MTX. U2OS cells are derived from a moderately differentiated sarcoma of the tibia and are epithelial, hypertriploid, and adherent (Ponten and Saksela, 1967). U2OS cells were exposed to progressively increasing concentrations of MTX starting from 0.5nM up to 40µM. The continuous selection process lasted 18 months and involved multiple cycles of MTX treatment, removal of the dead cells and a short “resting” period of the surviving cells after transferring them to a smaller culture flask. Soon after the cells looked healthy again and after reaching 60% confluency, the cells were treated with an increased concentration of MTX compared to last time (0.5uM -1µM -2µM - 3µM - 5µM - 7µM - 10µM - 12µM - 15µM - 17.5µM - 20µM - 25µM - 30µM – 40µM). The response of these cells to MTX was tested regularly and always compared to the sensitive U2OS cells.

Other human MTX-Sensitive and MTX-Resistant cell lines used for the research purposes of this thesis were obtained from Dr. Florian Rothweiler (Goethe-Universität Frankfurt, Germany). These cell lines were established in the framework of The Resistant Cancer Cell Line (RCCL) collection and are listed in the table below:

Table 2.1: Cell lines

Name	Type of Cancer	Parental	Resistance to MTX	Histopathological classification
SW480	Colorectal Cancer	Yes	No	Epithelial-Adherent
SW480-R	Colorectal Cancer	No	Yes	Epithelial-Adherent
LoVo	Colorectal Cancer	Yes	No	Epithelial-Adherent
LoVo-R	Colorectal Cancer	No	Yes	Epithelial-Adherent
HCC1806	Breast Cancer	Yes	No	Epithelial-Adherent
HCC1806-R	Breast Cancer	No	Yes	Epithelial-Adherent
BT-20	Breast Cancer	Yes	No	Epithelial-Adherent
BT-20-R	Breast Cancer	No	Yes	Epithelial-Adherent
MDA-MB-436	Breast Cancer	Yes	No	Epithelial-Adherent
MDA-MB-436-R	Breast Cancer	No	Yes	Epithelial-Adherent
CAL-51	Breast Cancer	Yes	No	Epithelial-Adherent
CAL-51-R	Breast Cancer	No	Yes	Epithelial-Adherent
Saos-2	Osteosarcoma	Yes	No	Epithelial-Adherent
Saos-2-R	Osteosarcoma	No	Yes	Epithelial-Adherent

2.2 Cell culture

JEG3, JEG3-R, U2OS and U2OS-R cell lines were maintained at 37 °C in a humidified environment at 10% CO₂. The above cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin (Full-DMEM). JEG3-R and U2OS-R cells were treated with 20 µM MTX (Sigma Aldrich, USA) every two weeks for 72 hours to maintain resistance properties and allowed to grow in MTX-free media for at least one-week prior to experimental use.

MDA-MB-436 and MDA-MB-436-R cell lines were cultivated in DMEM/Ham's F12 (Thermo Scientific, 11330057), supplemented with 10% (v/v) foetal calf serum (FCS), 20 mM HEPES, 4 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin (Full DMEM-F12). All the other MTX-Resistant cell lines and their sensitive counterparts listed in **Table 2.1** were cultured in

Iscove's Modified Dulbecco's Medium (IMDM, Thermo Scientific, 12440061) supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin (Full-IMDM). MTX was added to the MTX-Resistant cell lines for 72 hours every fortnight to a concentration which the cells are adapted to. All the cell lines grown in Full DMEM-F12 or Full-IMDM were maintained at 37 °C in a humidified environment at 5% CO₂.

All cell culture work was performed using sterile material under a laminar flow hood. A 37 °C water bath was used to pre-warm media and buffers prior cell culture. Cells were sub-cultured to approximately 80% confluency. As for passaging, media and cellular debris were aspirated and cells were rinsed with PBS once. Next, cells were incubated with a Trypsin solution [2.5 mg/ml trypsin in 0.02% (w/v) EDTA, 0.09% (w/v) NaCl] at 37 °C for 5 minutes to detach cells from the culture plates. Cells were collected using Full-DMEM/Full-DMEM-F12/Full-IMDM prior transfer to new culture flasks. All cells were tested monthly for mycoplasma contamination.

For long term storage in liquid nitrogen (LN₂), cells were re-suspended after trypsinization into freezing medium; FCS (foetal calf serum) supplemented with 10% (v/v) DMSO, transferred into freezing vials (>1x10⁶ cells/vial) and frozen at -80 °C using a Mr. Frosty Nalgene® freezing container. Three days later, cells were transferred into vapour-phase liquid nitrogen storage tanks. For cell revival the frozen vials were quickly thawed in a water bath at 37 °C and cells were re-suspended in Full-DMEM/Full-DMEM-F12/Full-IMDM (37 °C). Cells were then spun down at 1000 g for 2 minutes to remove DMSO from the freezing media, re-suspended in Full-DMEM/Full-DMEM-F12/Full-IMDM and allowed to grow in a humidified incubator.

2.3 Serine and glycine depletion

For the Serine and Glycine (SG) depletion experiments, SG+ and SG- reconstituted media were prepared. The SG+ medium contains equivalent levels of Serine and Glycine as the off-the-shelf DMEM in which the choriocarcinoma cells are normally grown, whereas the SG- lacks serine and glycine amino acids. The recipe of the SG+/SG- media is the following: MEM (Gibco, 21090) supplemented with 10% dialysed FBS (Hyclone, Thermo Scientific), 1% penicillin–streptomycin,

D-glucose (5 mM), sodium pyruvate (65 μ M), 1X MEM vitamin solution (Gibco, 11120), L-Glutamine (2 mM), L-Proline (0.15 mM), L-Alanine (0.15 mM), L-Aspartic acid (0.15 mM), L-Glutamic acid (0.15 mM) and L-Asparagine (0.34 mM). The SG+ medium only contained 0.4 mM L-Serine and 0.4 mM L-Glycine (Tajan *et al.*, 2021).

2.4 Cell Survival Assays

2.4.1 Crystal Violet

Cells were seeded in quadruplicates in 96-well plates, subjected to the desired treatment and prior to being fixed at specific timepoints, the culture medium was carefully removed using an aspirator with an 8-channel tip adapter. The cells were fixed with Crystal Violet aqueous solution (Crystal violet powder 0.5% w/v, Methanol 25% v/v) for 30 minutes at room temperature. The plates were then thoroughly washed with running tap water and air-dried overnight. The day after, the addition of Acetic Acid aqueous solution (Acetic Acid 10% v/v) in each well for 30 minutes at room temperature on an orbital shaker led to the solubilization of the formed crystals. After that, the absorbance was measured at 595 nm using the Sunrise™ microplate reader (Tecan, Männedorf, Switzerland). The data were analysed using Microsoft 365 Excel version 2304 (Microsoft, WA, USA).

2.5 Drug Treatments

Table 2.2: List of drugs

Drug Name	Drug Type	Function/Target	Provider	Product ID
Methotrexate	Chemotherapy agent and immune-system suppressant	Folate Antagonist/DHFR	Sigma-Aldrich	454126
NCT-503	Inhibitor	Phosphoglycerate dehydrogenase (PHGDH)	Stratech	S8619
SGI-1027	DNA methyltransferase (DNMT) inhibitor	DNMT3B, DNMT3A, and DNMT1	Bio-Techne Ltd	5155
Zebularine	Substrate analog inhibitor	Cytidine deaminase (CDA) and DNA Methylation	Bio-Techne Ltd	2293
Hygromycin B (hydrate)	Antibiotic	Selection of transformed cells expressing <i>E. coli hyg</i> resistance gene	Cayman Chemical	14291
GCN-IN-1	Inhibitor	General control nonderepressible 2 kinase (GCN2)	Cambridge Bioscience Ltd	HY-100877
GZD824 (Olverembatinib dimesylate)	Inhibitor	Bcr-Abl, GCN2	Stratech Scientific Ltd	S7194-SEL
Perhexiline maleate	Metabolic modulator	Carnitine palmitoyltransferase 1 and 2 (CPT-1, CPT-2), Mammalian target of rapamycin complex 1 (mTORC1)	Merck Life Science UK Limited	SML0120
GSK2656157	Inhibitor	PERK	Stratech Scientific Ltd	B2175-APE

2.5.1 Calculation of IC₅₀ values

All the MTX-Sensitive and MRX-Resistant cell lines underwent drug treatment with serial dilutions to determine a relative IC₅₀ value. Cells (1.5-6 x 10³/well) were seeded with four technical replicates (wells) per condition in 96-well plates (Corning, NY, USA) and were drug-treated 24h after seeding. Calculation of IC₅₀ values was performed by linear regression of the survival percentage/drug concentration with serial dilutions, using the crystal violet method to measure cell viability (methodology described in Section §2.4.1). IC₅₀ values were calculated in GraphPad based on plotted cell viability measurements by the equation:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log \text{IC}_{50} - X) \cdot \text{HillSlope}}}$$

Y: The measured cell viability (%) at a given drug concentration.

X: The log-transformed concentration of the drug.

Bottom: The minimum response observed at the highest drug concentrations, where the drug fully inhibits cell viability.

Top: The maximum response observed at the lowest drug concentrations, typically the untreated or control condition.

\logIC₅₀: The logarithmic concentration of the drug that produces 50% of the maximal response.

HillSlope: A parameter that controls the steepness of the curve.

All relevant graphs were generated using the Prism software (GraphPad Software, INC, CA, USA).

2.5.2 Synergy analysis of pairwise drug combination treatments

Results from two-drug combination treatments were analysed using the SynergyFinder 3.0 web-application (<https://synergyfinder.fimm.fi>) (Ianevski, Giri and Aittokallio, 2022). Based on the Zero Interaction Potency (ZIP) model, pairwise drug combination efficacy was systematically evaluated. In order to determine if there is a synergistic, antagonistic, or additive effect between the two compounds, the model assumes no interaction between agents, unless demonstrated otherwise. The model defines the drug interaction relationship between single drugs and their combinations by contrasting the distinct dose-response potency curves (Yadav *et al.*, 2015). Ultimately, the software reveals the ideal concentration for each individual agent that enhances pairwise drug efficacy.

2.6 RNA Extraction

Total RNA was extracted using the PureLink RNA Mini Kit (Invitrogen, Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. Briefly, collected cell pellets were PBS washed followed by cell lysis with Lysis Buffer containing 1% (v/v) of β -mercaptoethanol to neutralize any enzymatic activity of intracellular RNases. Following thorough vortexing, to induce pellet lysis, the lysates were then loaded into a homogenisation tube and centrifuged at 13,000 x g on a bench-top Eppendorf 5810 centrifuge for 2 minutes. The flow-through cellular extract liquid was mixed with an equal volume of 70% ethanol (EtOH) and vortexed until there were no visible precipitates, prior to loading onto a spin cartridge. The spin cartridges were then centrifuged at 13,000 x g on a bench-top centrifuge for 30 s to allow RNA binding onto the matrix of the cartridge, with any flow-through discarded. Subsequently, matrix-bound RNA underwent three washing steps, (once with wash buffer I and twice with wash buffer II), each one followed by a 30 s centrifugation to remove remaining cellular molecules, salts, and ethanol. Matrix-bound RNA was eluted in RNase-free water in a recovery tube and RNA concentration was determined

using the NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA) and ND-1000 software, prior to storage of samples at -80 °C or conversion to cDNA.

2.7 cDNA Reverse Transcription

Total purified RNA was converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. Each reverse transcription reaction (20 µL) contained 0.5 µg of RNA, dNTP mix, reverse transcriptase random primers, and MultiScribe™ reverse transcriptase. The reverse transcription reaction was performed under the following PCR thermal-cycling conditions: 25 °C for 10 minutes, 37 °C for 120 minutes, and 85 °C for 5 minutes. Then, the converted cDNA was stored at -20 °C or processed for RT-qPCR. The generated cDNA was diluted 12.5-fold to a final concentration 2ng/µl and stored at -20 °C.

2.8 Real Time-quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was performed using the Fast SYBR Green Master Mix, 2 ng/µl cDNA and 0.4 µM of each specific primer pair (**Table 2.3**). It was carried out on a StepOnePlus™ Real-Time PCR System with the following conditions: initial activation at 95 °C for 20 sec, followed by 40 cycles of: denaturation step at 95 °C for 1 minute and then annealing/extension step at 60 °C for 20 sec. The final melting curve was carried out at 95 °C for 15 sec and then for 15 sec at 60 °C.

The mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method in Microsoft 365 Excel version 2304 (Microsoft, WA, USA). Briefly, CT values were first normalised to the housekeeping reference gene, HPRT1 or GAPDH, and then normalised to the CT values of a control group. The obtained value was converted into a fold change using the equation: 2^{-x} , where x is the $\Delta\Delta CT$. All primer pairs were previously tested for target specificity and appropriate amplification efficiency. Primers

were purchased from Life Technologies Limited. All primers were diluted in RNase-free water to a final concentration of 10 μ M and stored at -20 °C according to the manufacturer's instructions.

Table 2.3: Oligonucleotide primers for RT-qPCR

Target gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
ATF3	GTGCCGAAACAAGAAGAAGG	TCTGAGCCTTCAGTTCAGCA
ATF4 (1)	TTCTCCAGCGACAAGGCTAAGG	CTCCAACATCCAATCTGTCCCG
ATF4 (2)	TCGATGCTCTGTTTCGAATG	AGAATGTAAAGGGGGCAACC
ACTB	TCCTCCTGAGCGCAAGTACTC	CTGCTTGCTGATCCACATCTG
CBS	CGGTGGTGGATAAGTGGTTC	AGTCCTTCCTGTGCGATGAG
CHOP	CCTAGCTTGGCTGACAGAGG	CTGCTCCTTCTCCTTCATGC
CREBBP	CACAGCTAATGGCAGCTTTCAT	CTGGCACTATTTGTTGTTGCT
DHFR	CATGGTCTGGATAGTTGGTGGC	GTGTCACTTTCAAAGTCTTGCATG
EIF2A	AAGCATGCAGTCTCAGACCC	GTGGGGTCAAGCGCCTATTA
EP300	GCGGCCTAAACTCTCATCTC	TCTGGTAAGTCGTGCTCCAA
EZH2	CCCTGACCTCTGTCTTACTTGTGGA	ACGTCAGATGGTGCCAGCAATA
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA
GCN2	GGAGCAACAGTGGTCCCAT	GGCAAGGGAGGTCTGAAGTC
HPRT1	CATTATGCTGAGGATTTGAAAGG	CTTGAGCACACAGAGGGCTACA
KDM4C	CCGATGACTCTTGTAAGCAGC	GACTTCGTCTGCCAAAGGTGGA
MAT1A	TCATGTTACATCGGAGTCTGT	CATGCCGGTCTTGCACT
MAT2A	TAGCCTTGGAGCAACAGTCA	CCATTACGGCGTAGTTCTGC
MTHFD1	TTTGGCTTGAAGAGGGACATGAGG	AGGACCTTAGAGGACTAGCAGGGT
MTHFD1L	CAACATCAAGTGCCGAGCTT	AAGAGGAACACCAGCCGTTA
MTHFD2	GATCCTGGTTGGCGAGAATCC	TCTGGAAGAGGCAACTGAACA
MTHFR	AGTGGGGGCTATGTCTTCCA	TCAGGGGCGTTGGTGATATTT
MYC	TCTCTCCGTCCTCGGATTCT	TTGTTCTCCTCAGAGTCGCT
NRF2	GGTTGCCCACATTCCAAAT	AGCAATGAAGACTGGGCTCT
P53	TAACAGTTCCTGCATGGGCGGC	AGGACAGGCACAAACACGCACC
PERK	TGGGACCAAGACCGTGAAAG	TCGTCACT ATCCCATTGGCG
PHGDH (1)	CACGACAGGCTTGCTGAATGA	CTTCCGTAAACACGTCCAGTG
PHGDH (2)	GCCCTTACCAAGTGCCTTCTC	GACAATGACTGCGGGGCTTA
PKR	GTGGACCTCTACGCTTTGGG	TGGGCTTTTCTT CCACACAGT
PSAT1 (1)	CGGTCCTGGAATACAAGGTG	AACCAAGCC CATGACGTAGA
PSAT1 (2)	ACTTCCTGTCCAAGCCAGTGGA	CTGCACCTTGATTCCAGGACC
PSPH	TGGAGATGGTGCCACAGATA	CCTCCAAATCCAATGAAAGC
RFC1	CCTCGTGTGCTACCTTTGCTT	TGATCTCGTTCGTGACCTGCT
SAHH	ATTCTGGATGATGGTGGTGAC	GTGGGTATTTGGTGTGGATGA
SHMT1	TGAACACTGCCATGTGGTGACC	CTCTTTGCCAGTCTTGGGATCC

SHMT2	GCCTCATTGACTACAACCAGCTG	ATGTCTGCCAGCAGGTGTGCTT
SLC1A4	TGTGGTTGCAGCTTTCCGTACG	CCAGAGCAAACAGGACCAATCC
SLC1A5	TCCTCTTCACCCGCAAAAACCC	CCACGCCATTATTCTCCTCCAC
SLC38A1	GGCATCTGTATTTGCTGCTG	CGTTGCTGACGTTGTCATCT
SLC38A2	TAATCTGAGCAATGCGATTGTGG	AGATGGACGGAGTATAGCGAAAA
TK1	GGGGCAGATCCAGGTGATTC	CCATGGTGTTCCGGTCATGT
TYMS	CCTCTGCTGACAACCAAACG	GAAGACAGCTCTTTAGCATTTG

2.9 Small interfering RNA (siRNA) transfection

For the highest transfection efficiency on the JEG3 and JEG3-R cells, Lipofectamine™RNAiMax transfection reagent was used according to the manufacturer's instructions (Thermo Fisher Scientific, MA, USA) and the transfection solution was mixed in the Opti-MEM™ Reduced Serum medium (Thermo Fisher Scientific, MA, USA). Cells were seeded 24 hours before transfection at a density of 2×10^5 /well for JEG3 and 2.3×10^5 /well for JEG3R in 6-well plates to achieve 60% confluency at 24 hours after seeding. For single knockdowns the siRNA concentration was 20 nM and for double knockdowns (silencing of two different genes) the total concentration was also 20 nM (10 nM of each target siRNA). siGENOME pooled siRNAs (4 individual siRNAs targeting the same gene) were used to target the genes of interest (Horizon Discovery, Cambridge, UK) (**Table 2.4**). The non-targeting (siGENOME™ RISC-free control) was used at a final concentration of 20 nM (**Table 2.4**). Transfected cells were harvested for assessment of the transfection efficiency and for downstream analysis via western Blotting at 72 hours post siRNA transfection.

In the case of survival assays, cells were seeded at a density of 1×10^6 for JEG3 and 1.5×10^6 for JEG3R in 10 cm plates followed by transfection 24 hours later. Then, the day after transfection, cells were trypsinized, measured and seeded in 96-wells (3×10^3 /well for JEG3 and 6×10^3 /well for JEG3R) and were treated with Methotrexate (with concentrations ranging from

~2nM to 50 μ M) for 72 hours the day after. The transfection efficiency here was evaluated in duplicate wells from the experimental ones, in 6-well plates 72 hours post transfection.

Table 2.4: siRNA oligonucleotides

Target gene	Contents	Catalog ID
ATF4	SMARTpool, 5nmol	M-005125-02-0005
MYC	SMARTpool, 5nmol	M-003282-07-0005
PHGDH	Set of 4, 2 nmol	MQ-009518-01-0002
PSAT1	Set of 4, 2 nmol	MQ-010398-02-0002
TP53	SMARTpool, 5nmol	M-003329-03-0005
siGENOME RISC-Free Control	SMARTpool, 5nmol	D-001220-01-05

2.10 Bacterial transformation and Plasmid preparation

Plasmids were amplified in chemically competent bacteria through heat shock-mediated transformation. Shortly, 1-5 μ l (~50ng) plasmid DNA was incubated with 25 μ l of bacterial suspension (DH5 α competent (*E. coli*) cells) for 30 minutes on ice. Then, the bacteria and plasmid mix were heated for 45 seconds at 42 $^{\circ}$ C to introduce the plasmid into the bacteria by heat shock and placed back on ice for 2 minutes. The transformed bacteria were then mixed with 1000 μ l of LB Broth media and incubated for one hour at 37 $^{\circ}$ C on a thermal-controlled tube shaker. Then, this mixture was added to a big (1000ml) shake flask containing 100 ml of LB Broth media and 100 μ g/ml of Kanamycin, the antibiotic for which the plasmids used in this project contain a resistance gene for. The shake flask was left overnight at 37 $^{\circ}$ C on a thermal-controlled tube shaker. The day after, plasmids were extracted and purified using the PureLink™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen, MA, USA). Plasmid concentration was determined using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, MA, USA). The plasmids were then stored at -20 $^{\circ}$ C.

2.11 Plasmid DNA Transfections

To achieve 60-70% confluency the day after seeding, 2×10^5 JEG3 cells or 2.5×10^5 JEG3-R cells were seeded into each well of a 6-well cell culture plate 24 hours prior transfection with the expression vector of interest (Table 2.5). Lipofectamine® 3000 transfection Reagent was used as per the manufacturer's protocol (Invitrogen, Thermo Fisher Scientific). Cells medium was replaced with Opti-MEM Reduced Serum medium (Phenol red-, HEPES+, L-Glutamine+). Expression vectors were transfected at 0.5 µg per well of a 6-well plate. Transfection mixes were prepared in Opti-MEM Medium and incubated for 5 min prior their addition to the cells. After 5 hours of transfection, the culture medium was replaced with fresh Full-DMEM to reduce the toxicity to the cells. Transfected cells were treated with 100 µM of Hygromycin for selection cells efficiently transfected with the vector containing the hygromycin resistance gene. 48 hours later the medium was replaced with Hygromycin-free fresh medium and cells grown in this medium for 2 days prior to experimental use. The cells were harvested for downstream analyses and successful transgene expression evaluated via western Blotting.

Table 2.5: Plasmids used in the study. All plasmids are based on the pCMV3-C-FLAG backbone, a versatile expression vector designed for high-level expression in mammalian cells under the control of the CMV promoter. The backbone also includes a FLAG tag for convenient detection and purification of expressed proteins.

Plasmid name	Backbone	Insert	Source
pCMV3-C-FLAG Negative Control Vector	pCMV3-C-FLAG	FLAG	Sino Biological, China
pCMV3-C-FLAG-PHGDH	pCMV3-C-FLAG	PHGDH	Sino Biological, China
pCMV3-C-FLAG-PSAT1	pCMV3-C-FLAG	PSAT1	Sino Biological, China

2.12 Cell Lysis and Protein Extraction

Cells were collected in the form of cell pellet by trypsinization and centrifugation. Cell scraping in lysis buffer was used instead of trypsinization only when membrane associated proteins had to be subsequently detected via western Blotting, to avoid damage of proteins caused by trypsin's protease activity. In a nutshell, the culture medium was removed from the cell culture plate/flask and then the cells were washed with PBS. Addition of trypsin solution (10% trypsin (BioWhittaker™, Lonza, MD, USA) in EDTA (Sigma-Aldrich, MO, USA)) on the cells for 5 minutes at 37 °C made them detach from the surface of the plate/flask which was confirmed by observation under a light microscope. Addition of fresh culture medium into the cell culture plate/flask inactivated trypsin and cells were collected after centrifugation at 1200 rpm for 3 minutes and removal of the supernatant. The cell pellet was collected and ice cold RIPA buffer (Radioimmunoprecipitation assay buffer; 50 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 2% Triton-X100, 1% Sodium Deoxycholate, 0.1% Sodium dodecyl sulfate (SDS), 2 mM EDTA) supplemented with 10mM β -Glycerophosphate, 10 mM fluoride (NaF), 2 mM Dithiothreitol (DTT), 1 mM pre-activated Sodium orthovanadate (Na₃VO₄) and 1X Complete EDTA-free Protease Inhibitor Cocktail, (Sigma Life Sciences, MO, USA) was added for the lysis of the cells. Cell lysates were incubated on ice for 20 minutes (vortexing every 5 minutes) and insoluble fraction removed following centrifugation (13,000 g for 25 minutes at 4 °C, Eppendorf 5810 centrifuge). Protein samples were prepared by mixing the cell lysates with Bolt™ lithium dodecyl sulfate (LDS) sample buffer (Thermo Fisher Scientific, MA, USA) and 200 mM DTT. Protein samples were subjected to western blotting or stored at -20 °C.

2.13 Protein Quantification

Protein concentration was measured using the Bradford protein assay (Bio-Rad Laboratories California, USA), according to the manufacturer's protocol. The protein lysate (2 μ l) was mixed with 498 μ l of a diluted assay reagent. Bovine serum albumin (BSA) and 0.4% v/v lysis

buffer were added into the diluted assay reagent to make standard protein solutions ranging from 0 to 10 µg/µl. Each sample or standard protein solution was then loaded in triplicates (100 µl/well) in wells of a 96-well microtitre plate for a colorimetric assay. The absorbance at 595 nm was recorded using the Sunrise™ microplate reader (Tecan, Männedorf, Switzerland). The protein concentration of the experimental samples was calculated manually using Microsoft 365 Excel version 2304 (Microsoft, WA, USA) after a standard curve was generated using measurements from the BSA standards.

2.14 Western blotting

Protein samples were analysed by a polyacrylamide gel SDS/PAGE electrophoresis using Mini-PROTEAN® 3 Cell gel tanks. The samples were firstly heat-denatured at 95 °C for 5 minutes and briefly spun down. 25-40 µg of protein for each sample were loaded and resolved by 6 %-15 % SDS-PAGE at 80 V for 20 minutes and 60-80 minutes at 120 V in Tris-glycine running buffer (250 mM Tris base, 1.92 M glycine, 1 % (v/v) 10 % SDS). Proteins were transferred from the gels onto 0.2 µm nitrocellulose membranes using either the Trans-Blot® Turbo™ Transfer System, or wet-transfer (Tris-glycine running buffer with 20 % (v/v) methanol), using the Mini Trans-Blot module (90 V for 1-2 hours). The membranes were blocked in 5% (w/v) non-fat dried milk powder in 0.05 % Tween-20 PBS for 1 hour at room temperature and immunoblotted with the appropriate primary antibodies at 4 °C overnight (**Table 2.6**) On the following day, membranes were washed 3X with PBST (0.05 % Tween-20), for 5 minute each per wash, before applying HRP-conjugated secondary antibodies (**Table 2.7**) Blotting membranes were washed three times with PBST as before and were developed with Pierce™ ECL (enhanced chemiluminescence) western blotting substrate or SuperSignal™ extended duration substrate for 1 minute. Proteins were visualised using the quantitative iBright™ FL1500 Imaging System and image analysis was performed using FIJI ImageJ version 1.52q (National Institute of Health, MD, USA)) software.

All primary antibodies were diluted in 5% bovine serum albumin (BSA) supplemented with 0.02% sodium azide (NaN₃) in PBS (or TBS-see exceptions), at 1:1.000 ratio, except for Phospho-

AMPK α (Thr172), Phospho-eIF2 α (Ser51), Tri-Methyl-Histone H3 (Lys27), and Tri-Methyl-Histone H3 (Lys9) that were diluted at 1:500 ratio and TBS was used instead of PBS. Both secondary antibodies were diluted at 1:5.000 ratio in 5% dried skim milk powder in 0.05% Tween-20 PBS.

Table 2.6: List of primary Antibodies for western blotting

Primary Antibodies					
Target Protein	Species Reactivity	Company	Clone	Catalogue Number	Dilution
AMPK α 1	Rabbit	Cell Signaling	-	2795S	1:1000
ASCT1 (SLC1A4)	Rabbit	Cell Signaling	-	8442S	1:1000
ASCT2 (SLC1A5)	Rabbit	Cell Signaling	D7C12	8057S	1:1000
ATF4	Rabbit	Cell Signaling	D4B8	11815S	1:1000
CDA	Rabbit	Invitrogen	-	PA589551	1:1000
DHFR	Mouse	antibodies-online.com	6G7	ABIN1497822	1:1000
eIF2 α	Rabbit	Cell Signaling	-	9722S	1:1000
FLAG	Mouse	Sigma	M2	F1804	1:1000
GCN2	Rabbit	Cell Signaling	-	3302S	1:1000
GSTT1	Rabbit	Invitrogen	-	PA5115333	1:1000
KDM4C	Rabbit	Bio-Techne Ltd	-	NB110-38884	1:1000
Lamin B1	Rabbit	Cell Signaling	D4Q4Z	12586S	1:1000
MAT2A + MAT1A	Rabbit	Abcam	EPR10496	ab177484	1:1000
Methionine Synthase	Rabbit	Cell Signaling	D3D6H	68796S	1:1000
MRPL9	Rabbit	Sigma-Aldrich	-	HPA008579	1:1000
MTHFD1	Rabbit	Invitrogen	-	PA5-81889	1:1000
MTHFD1L	Rabbit	Cell Signaling	D7O8E	14998S	1:1000
MTHFD2	Rabbit	Cell Signaling	D8W9U	41377S	1:1000
MTHFR	Rabbit	Cell Signaling	D1E4V	25164S	1:1000
p53	Mouse	Santa Cruz	DO-1	sc-126	1:1000
PHGDH	Rabbit	Sigma-Aldrich	-	HPA024031	1:1000
Phospho-AMPK α (Thr172)	Rabbit	Cell Signaling	D4D6D	50081S	1:500
Phospho-eIF2 α (Ser51)	Rabbit	Cell Signaling	-	9721S	1:500
PSAT1	Rabbit	Invitrogen	-	PA5-100027	1:1000
RFC1	Rabbit	Invitrogen	-	PA5-35965	1:1000

SFXN1	Rabbit	Sigma-Aldrich	-	HPA019543	1:1000
SHMT1	Rabbit	Cell Signaling	D3B3J	80715S	1:1000
SHMT2	Rabbit	Cell Signaling	E7F4Q	33443S	1:1000
SLC1A4	Rabbit	Cell Signaling	-	8442S	1:1000
SNAT1 (SLC38A1)	Rabbit	Cell Signaling	D9L2P	36057S	1:1000
SNAT2 (SLC38A2)	Rabbit	Invitrogen	-	PA5-106786	1:1000
Thymidine Kinase 1 (TK1)	Rabbit	Cell Signaling	-	8960S	1:1000
Thymidylate Synthase (TYMS)	Rabbit	Cell Signaling	D5B3	9045S	1:1000
Tri-Methyl- Histone H3 (Lys27)	Rabbit	Cell Signaling	C36B11	9733S	1:500
Tri-Methyl- Histone H3 (Lys9)	Rabbit	Cell Signaling	D4W1U	13969S	1:500
Vinculin	Mouse	Sigma-Aldrich	-	V9131	1: 10000

Table 2.7: List of secondary Antibodies for western blotting

Secondary Antibodies					
Target Protein	Species Reactivity	Company	Clone	Catalogue Number	Dilution
Anti-mouse secondary	Mouse	Agilent DAKO	-	P0447	1:5000
Anti-rabbit secondary	Rabbit	Agilent DAKO	-	P0448	1:5000
Anti-goat secondary	Goat	Sigma-Aldrich	-	AP106P	1:5000

2.15 Cell Cycle Analysis: Propidium Iodide Staining

JEG3 and JEG3-R cells were harvested 48 hours post incubation on either SG+/SG- medium via trypsinization. Prior to fixation with ice-cold 70% ethanol, cells were washed with PBS once. 200 µl of ethanol was added onto the cell pellet dropwise while vortexing to mitigate clumping of cells and the fixation process allowed to take place for 30 minutes at 4 °C. Cells were then washed with PBS twice before they were incubated with 50 µl of ribonuclease A (100 µg/ml) (Sigma Aldrich, Dorset, UK) for 15 minutes at room temperature to degrade RNA so that only the DNA could be stained. Then, the DNA was stained by the addition of 200 µl of 50 µg/mL propidium iodide (Sigma-Aldrich, MO, USA) into the sample. As stated below, flow cytometry was used to analyze DNA profiling.

2.16 Flow cytometry acquisition

The flow cytometry analysis was conducted at the Flow Cytometry Facility of the MRC London Institute of Medical Sciences (London, UK) using the BD™ LSR II Flow cytometer and BD™ FACS Diva software (BD Biosciences, NJ, USA). Based on the forward scatter area (FSC-A) and the side scatter area (SSC-A), the samples were initially gated to exclude cellular debris. The single cells were then chosen using the second gating method based on the forward scatter area (FSC-A) and the forward scatter height (FSC-H). The unstained and single stained samples were used to optimise the fluorescent signal detection. At least 10,000 events were recorded for each sample. The optical filter used to detect the fluorescent signal from propidium iodide (PI) was 610/20 nm. Data were processed and analysed using FlowJo version 10.7.1 (BD Life Sciences, NJ, USA).

2.17 Immunofluorescence staining and Confocal microscopy

Cells were seeded the day before staining at optimal densities (2×10^5 for JEG3 and 2.3×10^5 for JEG3-R) on Poly-L-Lysine coated glass coverslips in wells of a 6-well culture plate. Fixation of the cells happened by adding a 4% paraformaldehyde solution (final concentration 2%) in the medium of the cells for 10 minutes at 37 °C. After removal of the fixative, the cells were washed with PBS three times and were then permeabilised with 0.25% Triton-X100 (Sigma-Aldrich, MO, USA) in PBS for 10 minutes at room temperature. Prior blocking of non-specific staining, cells were washed three times with PBS and the coverslips were transferred on a flat surface. The blocking buffer was freshly prepared (10% Serum from the host [here goat] of the secondary antibody in PBS) and the blocking step lasted 1 hour at room temperature. Overnight incubation with primary antibodies at 4 °C followed. The primary antibodies were diluted 1:1,000-1:200, as per the manufacturer's instructions, in blocking buffer and 200 µl of primary antibody working solution was added on the coverslips. The following day, coverslips were washed in PBS three times prior to incubation with 200 µl of the appropriate secondary antibodies diluted in blocking buffer, for 1 hour at room temperature in the dark. Then the coverslips were washed again in PBS and rinsed with water prior to counterstaining with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride, ThermoFisher Scientific) diluted 1:10,000 in PBS for 10 minutes at room temperature in the dark to stain nucleic DNA. Finally, the coverslips were mounted on microscope slides (Superfrost microscope slides; VWR, PA USA) using 1 drop of ProLong™ Glass Antifade Mountant (Invitrogen™). Cell imaging took place 24 hours post immunostaining. For the capture of high-resolution images of cells grown on coverslips, the Zeiss LSM-780 inverted laser scanning confocal microscope (FILM facility, Imperial College London, UK) was used. The acquired images were then processed using Fiji version 1.0 (Schindelin *et al.*, 2012). For the quantification of nuclei foci on ImageJ, a script which automated the process was used and was written by colleague Dr. Sotirios Zervopoulos (Imperial College London, London, UK).

2.18 Metabolic Assays

2.18.1 Glutathione Assay

Reduced and total glutathione levels were measured with a Glutathione Colorimetric Assay Kit (BioVision, K261-100). The assay contains DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) that reacts with reduced glutathione (GSH), to generate yellow coloured 2-nitro-5-thiobenzoic acid which can be measured. 750,000 cells from each condition were harvested for the assay. The cells were pelleted at 700 x g for 5 minutes at 4 °C and washed using ice-cold PBS. Following removal of supernatant, cell pellets were lysed in 80 µl ice-cold glutathione buffer and incubated on ice for 10 minutes. Then, 20 µl of 5% Sulfosalicylic acid (SSA), which protect the glutathione from oxidation and γ -glutamyl transpeptidase reaction, was added and mixed with the cell pellet. The mixture was centrifuged at 8000 x g for 10 minutes. Supernatant containing glutathione was transferred to a fresh tube for glutathione assay.

The standard curves for detecting reduced glutathione (GSH) and total glutathione were prepared by diluting the 1 µg/µl GSH standard, to generate the standard for GSH and total glutathione, respectively. The dilution was carried out in 1% SSA. For the detection of reduced form of glutathione, 20 µl of NADPH Generating Mix and 120 µl of Glutathione Reaction Buffer was added to each well of a 96-well. For the detection of total glutathione level, in addition to 20 µl of NADPH Generating Mix and 120 µl of Glutathione Reaction Buffer, an extra 20 µl of Glutathione reductase was added to each well. Glutathione reductase reduces all the oxidised glutathione (GSSG) back to GSH, enabling us to detect the total glutathione level when DTNB is converted into 2-nitro-5-thiobenzoic acid during the GSH oxidation process. The reaction mix was incubated in the wells for 10 minutes to generate NADPH. After 10 minutes, 20 µl of either GSH standard solution or the sample was added to the respective well and incubated for 5 to 10 minutes. Following that, 20 µl of substrate solution was added, and the sample was read for absorbance immediately after 10 minutes at 412 nm using the Sunrise™ microplate reader (Tecan, Mannedorf, Switzerland).

2.18.2 ATP quantification

The ATP levels of JEG3 and JEG3R cell lines were determined using a Luminescent ATP Detection Assay Kit (Abcam, ab113849), according to the manufacturer's instructions. Briefly, the cells were collected and resuspended in full medium at a concentration of 2.0×10^3 /100 μ l and seeded into a white flat bottomed 96-well plate. 50 μ l of detergent were added into each well to lyse the cells. Then, 50 μ l of Substrate Solution were added which contains the luciferase enzyme and luciferin. The produced luminescent product was measured using a PHERAstar Plus microplate reader.

2.19 Dissection and Deparaffinization of Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections

The paraffin-embedded tissue samples were acquired from the Trophoblastic Disease Centre at Imperial College. Patient material used in the present study was approved by the Imperial College Healthcare Tissue Bank (ICHTB) under number 12275 and the Research Ethics Committee (REC) 17/WA/0161. The tissue samples were complete hydatidiform moles with clinical data from patients who were treated later for malignant forms of GTD. The samples were grouped into two distinct groups based on patients' response status to Methotrexate (MTX) and to combination therapy: 1) MTX-sensitive patients and 2) MTX-resistant/combo-sensitive patients. 10 μ m thick sections were cut from each FFPE block and mounted on glass microscope slides. Before the dissection process, training with Dr Geoffrey Maher (Department of Surgery and Cancer, Imperial College London, London, United Kingdom) was invaluable in accurately identifying the molar tissue for dissection from the tissue slides, as well as distinguishing and avoiding the maternal regions of the tissue. Hematoxylin and eosin (H&E)-stained sections (3 μ m) for each block were also obtained, which served as a reference to the dissection process. Sectioning and H&E staining procedures were obtained from the ECMC/ICBRC Laboratory of

Imperial College London at Hammersmith Hospital. The areas of interest (moles) within the tissue (occasionally intermixed with maternal tissue) were isolated by microdissection using a surgical scalpel and placed in microcentrifuge tubes. The combined size of the dissected area should be at least 1,5cm² in total. The tubes contained Histo-Clear (replacement for xylene) which assisted in removing the paraffin. Deparaffinization occurred by three changes of incubation of dissected tissue in Histo-Clear, for 10 minutes each. Then, rehydration was achieved through a series of graded ethanol double changes for 5 minutes each (100%, 96%, 70%). The tubes were subsequently centrifuged at full speed and the supernatant was carefully removed without disturbing the pellets.

2.20 Protein Extraction from FFPE tissue sections

2.20.1 Extraction of Total Protein from FFPE Tissue Sections

The tissue samples were resuspended in 94 µl of Extraction Buffer (EXB Plus, Qiagen) supplied with 6 µl of β-mercaptoethanol and mixed by vortexing. The homogenates were firstly incubated on ice for 10 minutes, followed by 20 minutes in Ultrasonic Water Bath Sonicator (at 4 °C, 30 seconds sonication followed by 30 seconds without sonication x 20 cycles). Then the samples were heated at 100 °C for 20 minutes and then at 80 °C for 2 hours, using a thermomixer. After heating incubation, the samples were incubated on ice for 5 minutes. Following centrifugation of the tubes, the supernatant containing the extracted protein was transferred to a new collection tube. For quantification of protein yield, the Bradford assay was used.

2.20.2 Purification of protein from FFPE tissue samples

Protein samples were purified by sequential addition of methanol (4 volumes), chloroform (1 volume) and water (3 volumes) followed by vortexing and centrifugation at 9000g for 1 min

after each step. After the final centrifugation, three distinctive phases are obtained: a lower, colorless, organic (chloroform) phase; a white interphase containing the extracted proteins; and an upper, colorless, aqueous (methanol) phase. The upper aqueous phase was carefully discarded and 3 volumes of methanol was added to the remaining sample to denature the proteins, followed by vortexing and centrifugation, to achieve precipitation of the protein material in a visible pellet. Then, the supernatant with the rest cellular components was carefully discarded and the protein pellet was washed in 100% ethanol and stored at -80°C. The purified samples were then sent to our SME partner, Gemini Biosciences.

2.21 Proteomic analysis

2.21.1 TMT Labelling and High pH reversed-phase chromatography (Gemini Biosciences Ltd)

Samples were reduced (10mM TCEP (Tris(2-carboxyethyl)phosphine), 55 °C for 1 h), alkylated (18.75mM iodoacetamide, room temperature for 30minutes) and digested with trypsin (2.5 µg trypsin per 100 µg protein; 37 °C, overnight). Peptide levels were then measured using a quantitative colorimetric peptide assay kit (Pierce) and 50 µg of each sample labeled with Tandem Mass Tag (TMT) ten plex reagents according to the manufacturer's protocol (Thermo Fisher Scientific, Loughborough, LE11 5RG, UK) and the labeled samples pooled.

An aliquot of 50 µg of the pooled sample was desalted using a SepPak cartridge according to the manufacturer's instructions (Waters, Milford, Massachusetts, USA). Eluate from the SepPak cartridge was evaporated to dryness and resuspended in buffer A (20 mM ammonium hydroxide, pH 10) prior to fractionation by high pH reversed-phase chromatography using an Ultimate 3000 liquid chromatography system (Thermo Fisher Scientific). In brief, the sample was loaded onto an XBridge BEH C18 Column (130 Å, 3.5 µm, 2.1 mm X 150 mm, Waters, UK) in buffer A and peptides eluted with an increasing gradient of buffer B (20 mM Ammonium Hydroxide in

acetonitrile, pH 10) from 0-95% over 60 minutes. The resulting fractions (15 in total) were evaporated to dryness and resuspended in 1% formic acid prior to analysis by nano-LC MSMS using an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific).

2.21.2 Nano-LC Mass Spectrometry (Gemini Biosciences Ltd)

High pH RP fractions were further fractionated using an Ultimate 3000 nano-LC system in line with an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). In brief, peptides in 1% (vol/vol) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (vol/vol) acetonitrile 0.1% (vol/vol) formic acid peptides were resolved on a 250 mm × 75 µm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using 7 gradient segments (1-6% solvent B over 1 minute, 6-15% B over 58 minutes, 15-32%B over 58 minutes, 32-40%B over 5 minutes, 40-90%B over 1 minute, held at 90%B for 6 minutes and then reduced to 1%B over 1 minute) with a flow rate of 300 nl min⁻¹. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization at 2.0kV using a stainless-steel emitter with an internal diameter of 30 µm (Thermo Scientific) and a capillary temperature of 300 °C.

All spectra were acquired using an Orbitrap Fusion Lumos mass spectrometer controlled by Xcalibur 3.0 software (Thermo Scientific) and operated in data-dependent acquisition mode using an SPS-MS3 workflow. FTMS1 spectra were collected at a resolution of 120 000, with an automatic gain control (AGC) target of 200 000 and a max injection time of 50 ms. Precursors were filtered with an intensity threshold of 5000, according to charge state (to include charge states 2-7) and with monoisotopic peak determination set to Peptide. Previously interrogated precursors were excluded using a dynamic window (60s +/-10ppm). The MS2 precursors were isolated with a quadrupole isolation window of 0.7m/z. ITMS2 spectra were collected with an AGC target of 10 000, max injection time of 70ms and CID collision energy of 35%.

For FTMS3 analysis, the Orbitrap was operated at 50 000 resolution with an AGC target of 50 000 and a max injection time of 105 ms. Precursors were fragmented by high energy collision dissociation (HCD) at a normalised collision energy of 60% to ensure maximal TMT reporter ion yield. Synchronous Precursor Selection (SPS) was enabled to include up to 10 MS2 fragment ions in the FTMS3 scan.

2.21.3 Data Analysis (Gemini Biosciences Ltd)

The raw data files were processed and quantified using Proteome Discoverer software v2.1 (Thermo Scientific) and searched against the UniProt Human database (downloaded August 2020: 167789 entries) using the SEQUEST HT algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.6 Da. Search criteria included oxidation of methionine (+15.995 Da), acetylation of the protein N-terminus (+42.011 Da) and Methionine loss plus acetylation of the protein N-terminus (-89.03Da) as variable modifications and carbamidomethylation of cysteine (+57.021Da) and the addition of the TMT mass tag (+229.163 Da) to peptide N-termini and lysine as fixed modifications. Searches were performed with full tryptic digestion and a maximum of 2 missed cleavages were allowed. The reverse database search option was enabled and all data was filtered to satisfy a false discovery rate (FDR) of 5%.

2.22 Bioinformatics analysis – Cytoscape

Prior bioinformatic analysis, sensitive (MTX-S) and resistant (MTX-R) tissue samples were subjected to statistical analysis (unpaired Student t-test) with proteins filtered by p-value < 0.05 (-Log₁₀pvalue=1.3). Bioinformatic analysis was conducted via Cytoscape and highlighted enriched pathways and biological processes within the compiled lists of proteins found to be differentially expressed between our patient groups (Log₂ ratio MTX-R/MTX-S $\pm$ 1.5) by the Mass Spectrometry analysis. Utilizing the Reactome FI plugin, the entire proteome results were processed, and linkers

were added to make network semantic. Modularisation of the network was then performed based on module community centrality (Newman, 2006). Nodes were coloured using continuous red-green mapping of the Log2 ratio in expression changes. ReactomeFI plugin was then used to cluster the obtained functional interaction network and uncover groups of functionally related proteins with distinct biological processes (Wu, Feng and Stein, 2010). These processes were then subjected to gene ontology (GO) analysis under Reactome FI and this analysis was independently validated using the BinGO plugin.

2.23 Statistical analysis

Unless indicated otherwise, the statistical analysis was performed using Prism 9 version 9.2.0 (GraphPad Software, CA, USA). Normally distributed continuous data were analysed using the Unpaired Student's t-test for single comparisons or one-way ANOVA for multiple comparisons. For qPCR experiments, western blots and cell survival assays, each independent experiment consists of at least three technical replicates. The data were considered statistically significant if the p-value was less than 0.05. The asterisks were used to indicate the level of significance; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. The results were presented as mean $\pm$ standard error of mean (SEM).

3. Mass Spectrometry based identification of proteins and pathways associated with MTX-Resistance in GTD patients

3.1 Introduction

Although significant advancements have been made in the clinical management of Gestational Trophoblastic Disease (GTD), challenges persist, including the absence of reliable biomarkers for predicting chemoresistance. In parallel, identification of the underlying mechanisms promoting chemoresistance is a necessary approach which will give holistic insights into the treatment landscape of GTD patients. The existing FIGO/WHO serves as a predictive tool for assessing patients' responses to single-agent chemotherapy (Figo Oncology Committee, 2002). Nevertheless, misclassification of approximately one-third of the patients causes delays in delivering optimal treatment strategies, and the emergence of resistance to first-line therapy—typically Methotrexate (MTX)—further postpones successful treatment (Ngan *et al.*, 2021). Hence, there is considerable scope for improving the accurate prediction of MTX resistance in patients. Introduction of novel biomarkers could efficiently stratify GTD patients at an earlier stage of diagnosis into existing/new treatment options. Furthermore, discovery of new targetable pathways responsible for MTX resistance could lead to more effective and less toxic replacement or combination therapies to optimize patient care.

For the above reasons, proteomics profiling of clinically annotated formalin-fixed paraffin-embedded (FFPE) patient tissue samples was carried out using quantitative Mass Spectrometry (MS) analysis. The tissue samples were complete hydatidiform moles evacuated from women diagnosed with GTD, whose subsequent post-molar GTN was treated with MTX. Two groups of 5 patients chosen based on their response status to MTX, MTX-Sensitive (MTX-S) and MTX-Resistant (MTX-R), were analysed following tissue dissection and subsequent protein extraction. Data generated using these tissue samples were complemented by the proteomics profiling (previously performed in our lab) of the MTX sensitive JEG3, and resistant JEG3-R human choriocarcinoma cell lines. The overlay in molecular pathways/proteins associated with therapy resistance in the two datasets helped us select molecular changes for further phenotypic validation.

Pathway-based analysis revealed that most downregulated pathways in the MTX-Resistance group are metabolism-related while pathways associated with mRNA splicing and processing are upregulated.

Then cross-comparison of the patient and cell line proteomics datasets, enabled us to focus on pathways and molecules that were common alterations in both settings. We then validated the impact of modulating some of these changes on MTX response using choriocarcinoma cell lines as experimental models. In particular, we show that decrease in Serine Synthesis Pathway (SSP) activity is associated with MTX resistance and will be thoroughly analysed in Chapter 4 along with other connected metabolic and signaling pathways. Finally, five hits common to the two proteomics datasets were validated by Immunohistochemistry (IHC) analysis leading us to propose PSAT1 expression as a valuable prognostic marker of MTX sensitivity and MRPL9 as a valuable prognostic marker of MTX resistance in GTD.

3.2 Results

3.2.1 Identification of Differentially Expressed Proteins (DEPs)

Following the Mass Spectrometry (MS) analysis of the patient tissue samples by our collaborators in Gemini Biosciences Ltd, analysis of the extracted data was carried out using the Proteome Discoverer v2.1 software. In total, almost 6500 proteins were detected and false discovery rate (FDR) calculation used to ascertain the statistical significance of their detection using a 5% threshold. The protein abundances were normalized to the total peptide count in each sample. Log2 fold changes in protein abundance (Log2FC) between the two patient groups (MTX-R/MTX-S) and associated statistical significance (unpaired Student t-test) were calculated. Hits for further analysis were chosen based on a p-value < 0.05 and a Log2FC ≥ 0.378 or ≤ -0.51 (FC ≥ 1.3 OR ≤ 0.7 , respectively). Changes in protein expression between sensitive and resistant tissue samples were depicted as a volcano plot (**Figure 3.1**). In total, 135 proteins were found to be significantly upregulated and 96 downregulated in MTX-resistant as compared to sensitive patients.

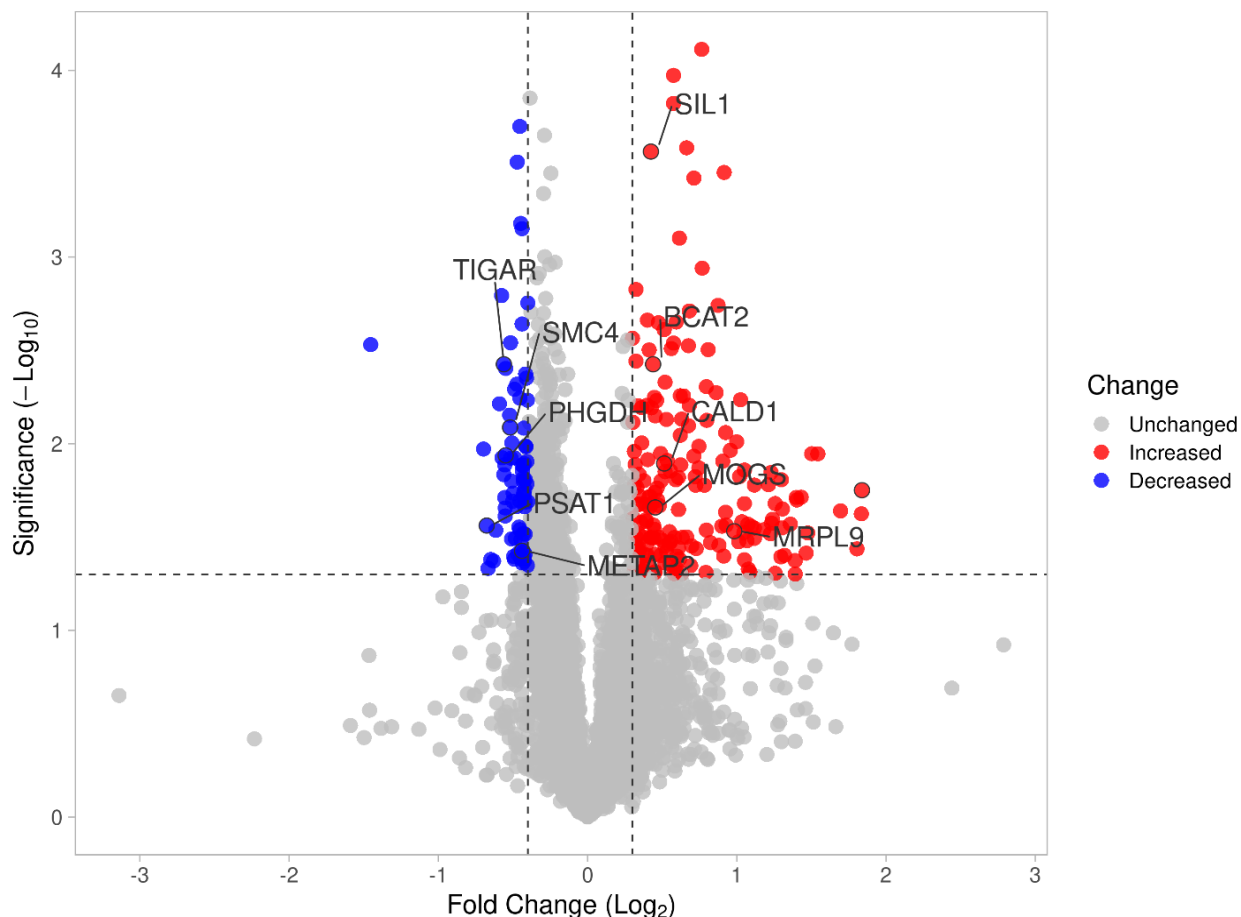


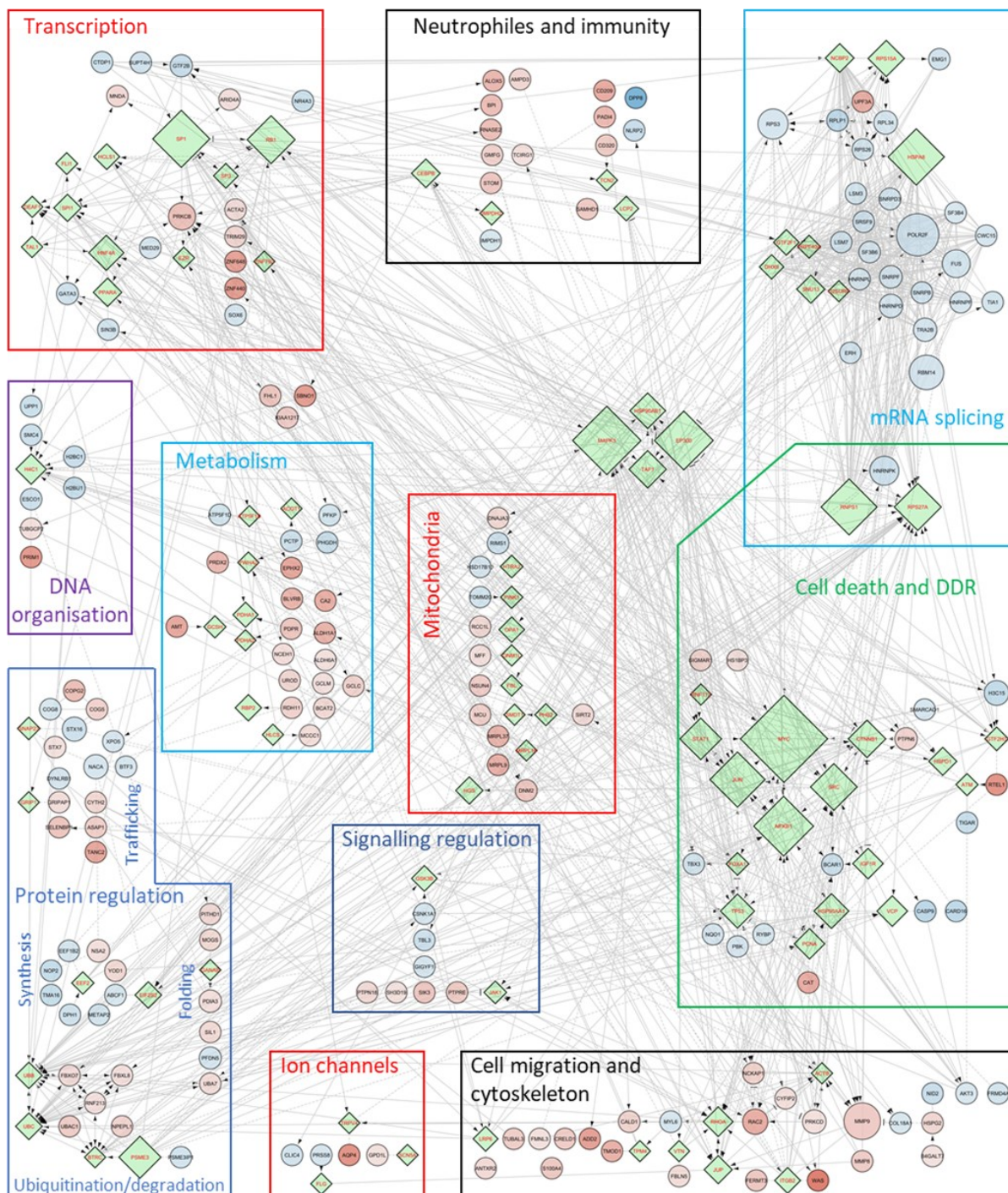
Figure 3.1: Visualization of significantly up/down-regulated hits in the MTX-Resistant group of patients compared to the MTX-Sensitive group in the patient proteomics data set.

The volcano plot displays the significantly up/down-regulated hits in the MTX-R group of patients in comparison to MTX-Sensitive patients. The annotated hits (TIGAR, SMC4, PHGDH, PSAT1, METAP2, SIL1, BCAT2, CALD1, MOGS, MRPL9) are the commonly differentiated hits in both two data sets (patient- and cell lines- proteomics data sets). Hits were filtered by $p\text{-value} < 0.05$ ($-\text{Log}_{10}p\text{value}=1.3$) and by $\text{Log}_2\text{Fold_Change_Ratio} \geq 0.378$ OR ≤ -0.51 (Fold Change ratio MTX-R/MTX-S ≥ 1.3 OR ≤ 0.7 respectively). Volcano plots were created on VolcanoR web tool.

3.2.2 Functional and pathway enrichment analysis

To visualize complex networks and highlight enriched pathways and biological processes implicated in MTX resistance, the DEPs (differentially expressed proteins) identified above were subjected to bioinformatics analysis in the open-source software platform Cytoscape (Paul Shannon *et al.*, 1971) utilizing the Reactome FI plugin (**Figure 3.2.A**). The total number of hits recognized by ReactomeFI and subjected to this analysis was 191 (Over-represented or Top hits: 104, Under-represented or Bottom hits: 87). Log2 Fold Change Ratios in differential expression were mapped onto the network, indicating over-represented proteins in MTX-R as red, under-represented ones as blue, and linkers (additional nodes added by Reactome FI to enhance network connectivity) as green-coloured. The size of each node is representative of its Degree Centrality. The analysis separated 11 interconnected modules based on distinct associated biological processes as revealed by gene ontology (GO) analysis using Reactome FI (**Figure 3.2.B**) which was further validated utilizing the BinGO (Biological Networks Gene Ontology) plugin (**Supplementary Table 3.1**). Interestingly, most of the revealed pathways shown in **Figure 3.2.B** are related to Metabolism (red font) or to RNA processing (green font). More specifically, proteins that appear to be downregulated in the MTX-R group in comparison with the S group are involved in mRNA splicing and processing whereas upregulated proteins are implicated in amino acid and nucleotides metabolism. Intriguingly, only one metabolism-related pathway seems to be underrepresented in the MTX-Resistant group of patients, that of “Serine and glycine biosynthesis”.

A)



Mass Spectrometry based identification of proteins and pathways associated with MTX-Resistance in GTD patients

B)

Upregulated pathways in MTX-R

Term	FDR	DB
Heme Metabolism	3.07E-08	MSDB
Branched-chain amino acid catabolism	2.59E-05	BP
Metabolism	3.17E-05	BP
Valine, leucine and isoleucine degradation	1.30E-04	K
mRNA Splicing - Major Pathway Homo sapiens R-HSA-72163	3.39E-04	R
mRNA Splicing Homo sapiens R-HSA-72172	4.35E-04	R
Nucleotide metabolism	8.75E-04	BP
Xenobiotic Metabolism	1.11E-03	MSDB
Processing of Capped Intron-Containing Pre-mRNA Homo sapiens R-HSA-72203	2.42E-03	R
Purine metabolism Homo sapiens R-HSA-73847	3.47E-03	R
Amino acid metabolism	3.57E-03	BP
Reactive Oxygen Species Pathway	4.07E-03	MSDB
Keap1-Nrf2 pathway	4.66E-03	BP
Porphyrin metabolism	4.66E-03	BP
Fatty Acid Metabolism	6.38E-03	MSDB
Propanoate metabolism	6.49E-03	K
leukotriene biosynthesis Homo sapiens PWY66-375	9.83E-03	HC
γ-glutamyl cycle Homo sapiens PWY-4041	9.83E-03	HC

Downregulated pathways in MTX-R

Term	FDR	DB
mRNA Splicing Homo sapiens R-HSA-72172	5.54E-05	R
Processing of Capped Intron-Containing Pre-mRNA Homo sapiens R-HSA-72203	3.76E-04	R
Spliceosome	4.90E-04	BC
Messenger RNA processing	5.65E-04	BC
RNA polymerase II transcription	6.84E-04	BC
Gene expression	8.63E-04	BC
SLBP independent Processing of Histone Pre-mRNAs Homo sapiens R-HSA-111367	9.04E-04	R
SLBP-dependent processing of replication-dependent histone pre-mRNAs	9.16E-04	BC
Spliceosomal assembly	1.94E-03	BC
Cleavage of growing transcript in the termination region	1.99E-03	BC
Serine and glycine biosynthesis Homo sapiens SER-GLYSYN-PWY	4.02E-03	HC
heme biosynthesis Homo sapiens PWY-5920	4.02E-03	HC
Nonsense-Mediated Decay (NMD) Homo sapiens R-HSA-927802	4.84E-03	R
RNA Polymerase II Transcription Termination Homo sapiens R-HSA-73856	4.84E-03	R
Post-elongation processing of intronless pre-mRNA	6.00E-03	BC
B cell activation Homo sapiens P00010	6.48E-03	P

Figure 3.2: Cytoscape analysis for patient-proteomics data.

A) In total 191 proteins with differential expression were subjected to bioinformatics analysis in Cytoscape. Gene ontology (GO) analysis using Reactome FI identified 11 interconnected modules, each associated with distinct biological processes. Hits were filtered by p -value < 0.05 ($-\text{Log}_{10}p\text{value}=1.3$) and by $\text{Log}_2\text{Fold_Change_Ratio} \geq 0.378$ OR ≤ -0.51 . Proteins over-represented in MTX-R are indicated as red nodes, under-represented as blue nodes, and linkers as green nodes. The size of each node is representative of its statistical significance. **B)** Tables showing enriched (left table) and downregulated (right table) pathways and in the MTX-Resistant group of patients compared to the MTX-sensitive one, as revealed by gene ontology (GO) analysis using Reactome FI in Cytoscape. Most of the pathways were related to Metabolism (red labeled) or to RNA processing (green-labeled) and were grouped by the expression pattern of their proteins in the MTX-Resistant group of patients (Upregulated or Downregulated).

3.2.2.1 Comparison between the patients' and the cell lines' proteomic datasets

The MS analysis previously performed in the choriocarcinoma cell lines JEG3 and JEG3-R provided us with a valuable proteomic dataset (hereinafter referred to as “cell lines’ dataset”). The MTX-Resistant cell line, JEG3-R, has been generated after extended exposure of JEG3 cells to progressively higher concentrations of MTX. Here, we used the cell lines’ dataset to filter results from the patients’ dataset to prioritise hits for validation.

Firstly, comparison of pathway analyses performed separately for each proteomic dataset gave us the opportunity to observe overlapping identified pathways associated with MTX-resistance. For the cell lines’ dataset, the majority of the revealed pathways are also metabolism-related, and most of them are downregulated in the MTX-resistant cells, such as Amino Acid Metabolism, glycolysis and Glycine, serine and threonine metabolism (**Supplementary Figure 3.1**).

Comparison of over- and under-expressed proteins in both the MTX-R group of patient samples and in JEG3-R cells disclosed ten common hits, five in each category (**Figure 3.3.A-B**). The 5 common over-expressed (top) hits are MRPL9, CALD1, MOGS, BCAT2, and SIL1. The 5 common under-expressed (bottom) hits are METAP2, PHGDH, PSAT1, TIGAR, and SMC4.

Interestingly, 3 out of 5 downregulated hits, Phosphoglycerate dehydrogenase (PHGDH), Phosphoserine aminotransferase (PSAT1), and TP53 Induced Glycolysis Regulatory Phosphatase (TIGAR) are implicated in the glucose-derived *de novo* serine synthesis. Serine is a non-essential amino acid that can either be produced *de novo* within the cell or taken up from the extracellular environment (Reitzer, 2014). *De novo* serine synthesis pathway derives from glycolysis in which the glycolytic intermediate molecule, 3-phosphoglycerate (3PG), is converted into serine through a 3-step enzymatic process. The three enzymes participating in this process are PHGDH (phosphoglycerate dehydrogenase), PSAT1 (phosphoserine aminotransferase 1), and PSPH (phosphoserine phosphatase) (Locasale, 2013) **Figure 3.3.C**. TIGAR has been indirectly implicated in the Serine synthesis pathway (SSP) through its role in modulating glycolysis, as it inhibits glycolysis and promotes the pentose phosphate pathway (PPP). SSP intersects with PPP as both

pathways utilize glucose-derived precursors. TIGAR can influence the availability of these precursors, thereby impacting the SSP (Amelio *et al.*, 2014).

3.2.2.2 *Serine synthesis pathway downregulation in MTX-Resistant patients and in choriocarcinoma MTX-Resistant cells*

The expression of the SSP enzymes (PHGDH and PSAT1) and TIGAR was tested in the choriocarcinoma cells JEG3 and JEG3-R. The downregulation of PHGDH and PSAT1 was validated at protein **Figure 3.3.D** and mRNA **Figure 3.3.F** level in the JEG3-R choriocarcinoma cell line as compared to its sensitive counterpart. In contrast, TIGAR levels were unchanged between the two cell lines (**Supplementary Figure 3.2.A**). This may be explained by the fact that MTX resistance in JEG3R cells is not a stable phenotype as these cells need to be cyclically treated (every two weeks for 72h) with high-dose MTX to maintain resistance. TIGAR, the TP53-inducible glycolysis and apoptosis regulator, is a target of p53, and while both p53 and TIGAR levels are increased following MTX treatment, these return to baseline levels 6 days later (**Supplementary Figure 3.2.B**). The expression of p21, a transcriptional target of p53 participating in cell cycle arrest, also increased upon MTX treatment, consistent with the delayed cell cycle progression previously observed in JEG3R (Georgiou *et al.*, 2022). Hence, our failure to validate increased TIGAR expression in JEG3R compared to JEG3 cells could be influenced by the instability of the resistance phenotype in JEG3-R cells and the time point at which these were analysed.

Mass Spectrometry based identification of proteins and pathways associated with MTX-Resistance in GTD patients

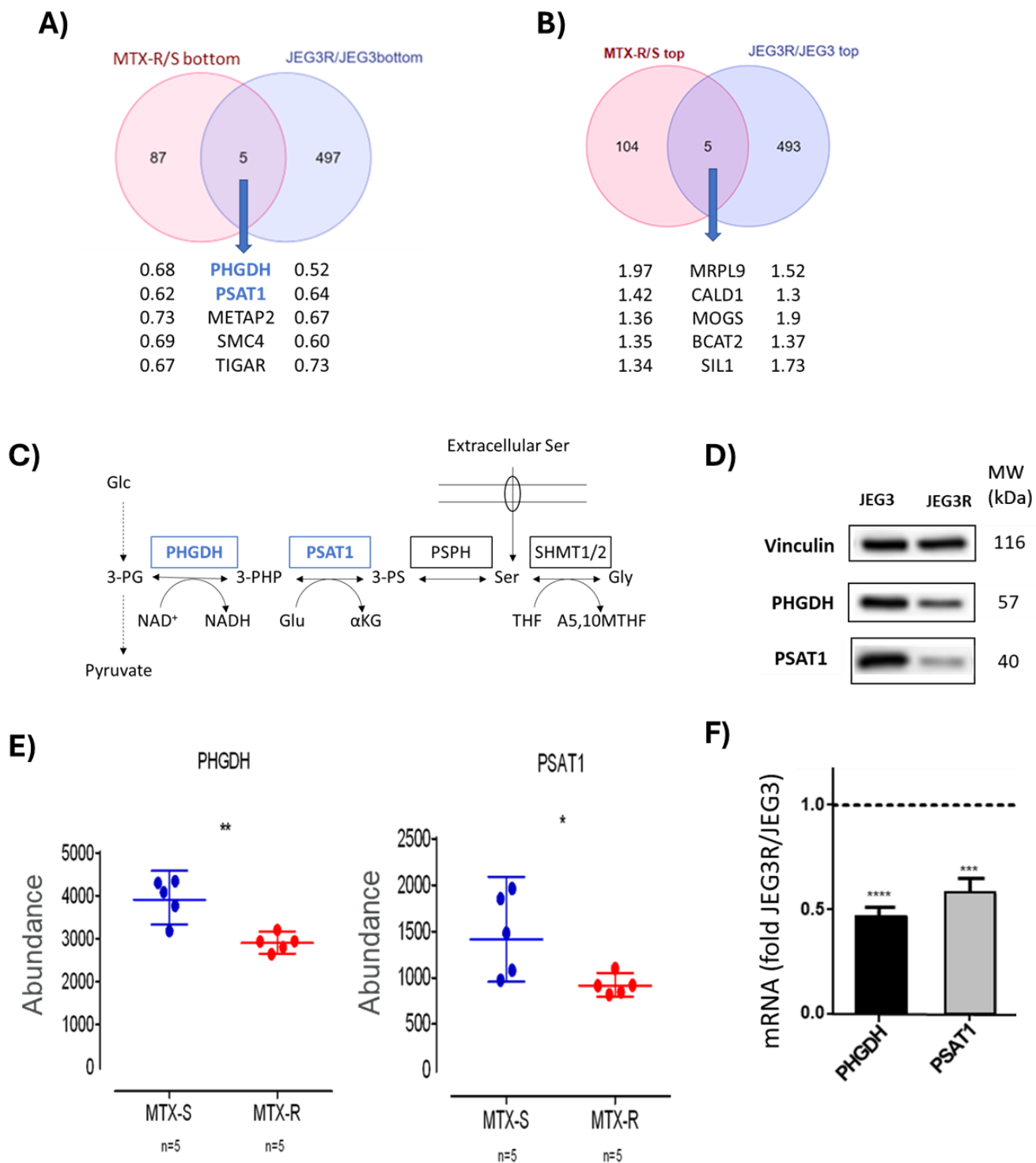


Figure 3.3: PHGDH and PSAT1 are downregulated in both MTX-Resistant patients and in choriocarcinoma MTX-Resistant cells

A) Top (Over-represented, Fold Change ≥ 1.3) and **B)** bottom (Under-represented, Fold Change ≤ 0.7) hits that derived after the comparison along with the fold change ratios indicated in each data set. Out of 104 MTX-R/S top and 493 JEG3R/JEG3 top hits, 5 hits were mapped. Out of 87 MTX-R/S bottom and 497 JEG3R/JEG3 bottom hits, 5 hits were mapped. **C)** PHGDH and PSAT1 are the first two enzymes of serine synthesis pathway. **D)** Immunoblots for bottom hits PHGDH and PSAT1 involved in SSP in the choriocarcinoma cell lines JEG3 and JEG3R. Vinculin was utilized as a loading control. The data represent 3 independent biological replicates. **E)** Scatter plots indicating the abundance values for PHGDH and PSAT1 in the tested patient samples. **F)** Bar graphs showing the relative mRNA expression of PHGDH and PSAT1 in JEG3 and JEG3R cells. The data show the mean fold changes $\pm$ SEM from 3 independent experiments. Statistical significance was determined using the Student's t-test in scatter plots and bar graph (* $p < 0.005$, ** $p < 0.001$, *** $p < 0.005$, **** $p < 0.001$).

3.2.3 Immunohistochemistry (IHC) analysis

Finally, Immunohistochemistry (IHC) analysis was conducted for five proteins differentially expressed amongst our resistant and sensitive patient samples. Three were from under-expressed (PHGDH, PSAT1, and TIGAR) while two were from upregulated (MRPL9 and CDA) proteins in resistant versus sensitive samples. All proteins, except for CDA, were common differentially expressed hits between MTX-R patient samples and JEG3-R cells.

Cytidine Deaminase (CDA) was chosen in view of data that will be described in the next chapter. CDA is involved in the pyrimidine salvage nucleotide pathway, which MTX-resistant cells seem to favor instead of the more energy-demanding *de novo* pyrimidine synthetic pathway (Frances and Cordelier, 2020). Consistent with this, CDA has previously been linked to Methotrexate resistance (Ye *et al.*, 2015), (Rajabpour *et al.*, 2017)]. Peptides for CDA were detected in the patients' proteomic dataset [fold change ratio MTX-R/MTX-S=1.43, p-value=0.035 (*)] but not in the cell lines' analysis (**Supplementary Table 4.1**). Nevertheless, CDA protein levels were found to be increased in JEG3-R cells compared to JEG3 cells using western blotting (**Figure 4.1.A**).

As for MRPL9, there is no published evidence associating this protein with Methotrexate response. High MRPL9 expression has however been connected to poor survival in patients with lung cancer (Xin-Yuan Li, Xin-Yu He, Hong Zhao, Lu Qi, 2023). High serum levels for MRPL9 were

also correlated with the progression of hepatocellular carcinoma and revealed the potential of this protein as a prognostic biomarker in this disease (Xie *et al.*, 2023). Lastly, overexpression of MRPL9 in papillary thyroid cancer cells and interplay with Gamma-glutamylcyclotransferase (GGCT) was shown to be involved in malignant progression of the disease via activation of MAPK/ERK pathway and to have prognostic power (Zhang *et al.*, 2022).

Following immunohistochemistry, the histopathologist Dr David J. Pinato (Department of Surgery & Cancer, Imperial College London, Hammersmith Hospital, London, United Kingdom), performed semiquantitative analysis of the staining. The average intensity of the signal was rated from 0 (negative) to 3 (strong) and multiplied by the percentage of cells showing expression (0 to 100), resulting in a score ranging from 0 to 300, as formerly described (Dhillon *et al.*, 2010).

In total, 10 tissues samples were assessed, including 5 from MTX-Sensitive and 5 from MTX-Resistant tumours. The immunohistochemical scores (IHSs) are listed in **Table 3.1** with representative images in **Figure 3.4.** and **Supplementary Figure 3.3.** The trends observed for PSAT1 and MRPL9, although not statistically significant, are consistent with our previous results and suggest that these proteins may validate better as prognosis biomarkers for MTX sensitivity and resistance, respectively, in a larger population of patient samples. However, analysis of a larger cohort of patients is now required to confirm this.

Table 3.1: Immunohistochemical Analysis

	MTX-S				MTX-R				
Candidate Biomarkers	Intensity (+)	% Cells+	Hscore	SEM ±	Intensity (+)	% Cells+	Hscore	SEM ±	p-value
PHGDH	2.8	90	258	35.0	2.8	86	242	23.3	0.72
PSAT1	1.8	46	84	22.0	1.2	45	56	25.0	0.31
MRPL9	1.2	54	72	28.0	1.8	70	132	34.4	0.35
CDA	1.6	31.6	43	25.8	1	56.6	52	23.3	0.83
TIGAR	1.8	88	162	40.8	1.8	86	158	39.8	0.94

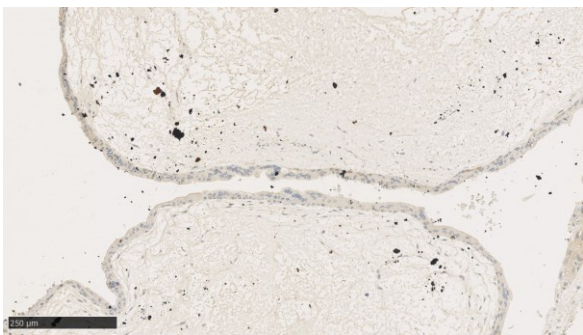
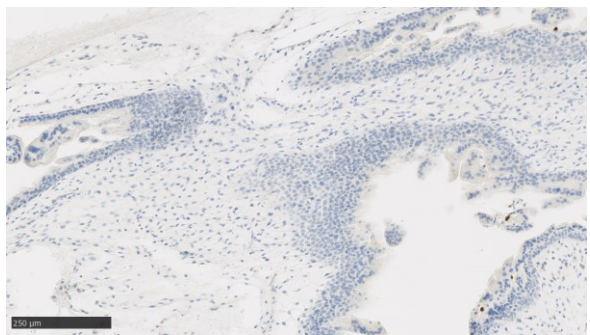
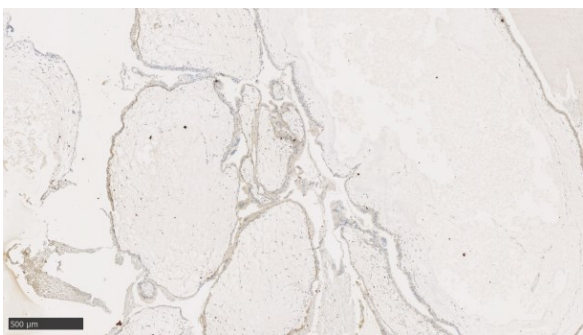
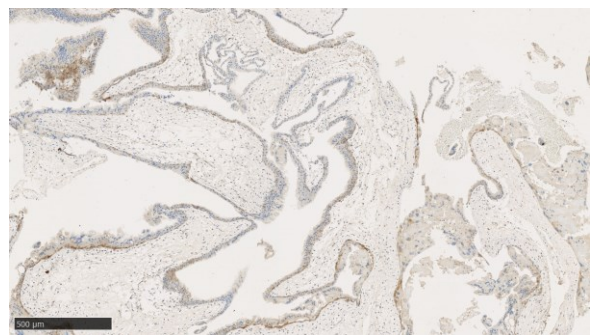
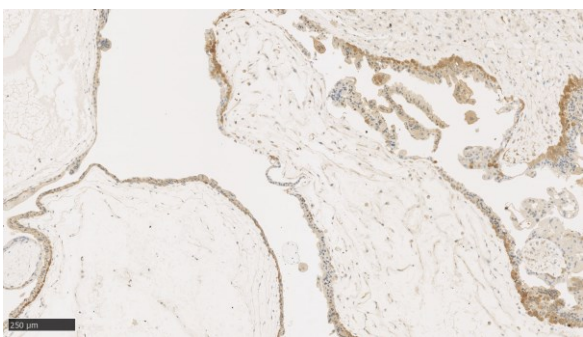
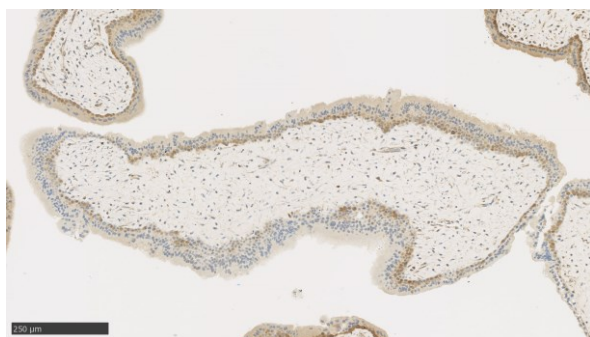
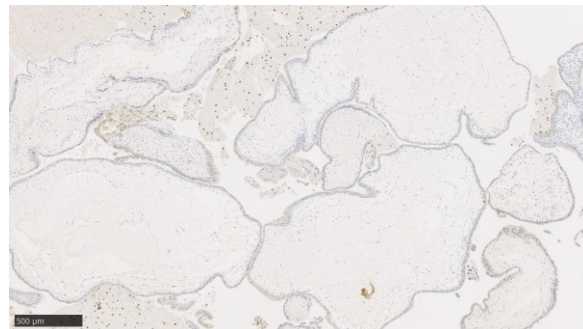
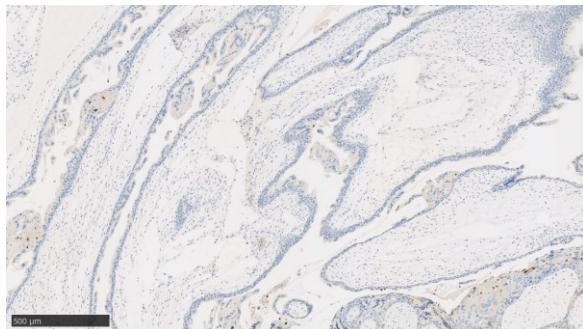
Immunohistochemical scores (IHSs) of 5 candidate biomarkers, PHGDH, PSAT1, MRPL9, CDA and TIGAR, as provided by the histopathologist. These scores represent the average values from 5 MTX-Sensitive (MTX-S) and 5 MTX-Resistant (MTX-R) samples. Hscore is the product of Intensity (1-3) multiplied by the percentage (%) of stained cells. ± SEM for each set of 5 samples was calculated. Statistical significance was determined using the Student's t-test.

A)

PSAT1

MTX-S

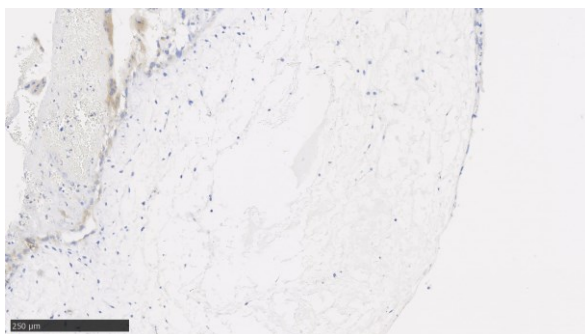
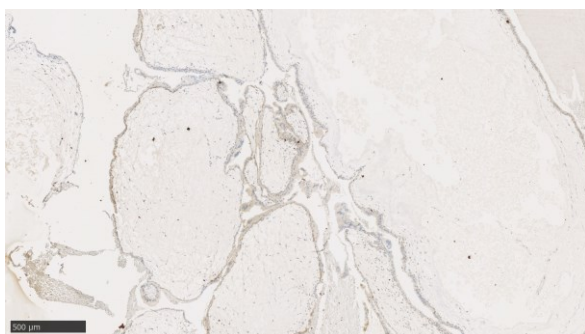
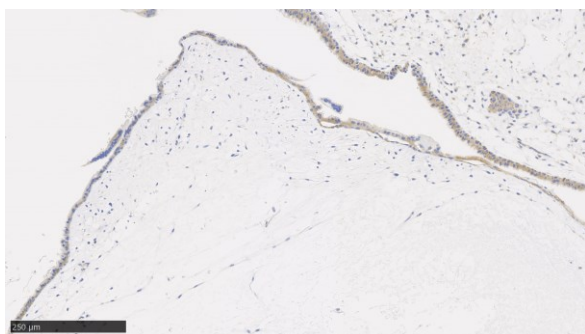
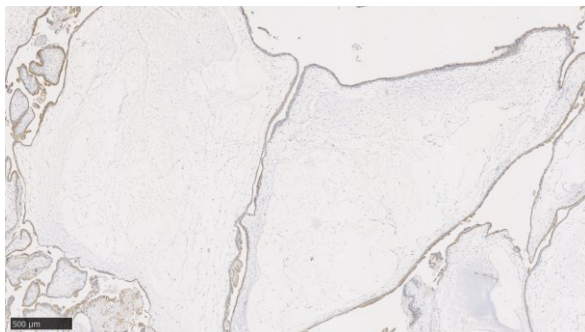
MTX-R



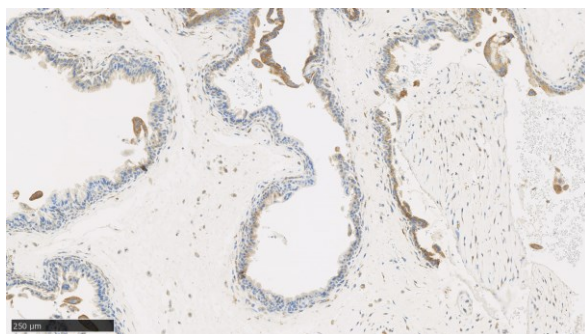
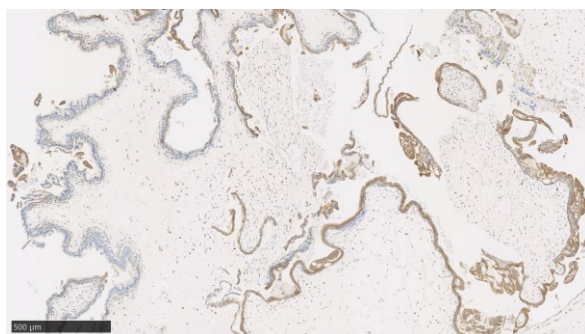
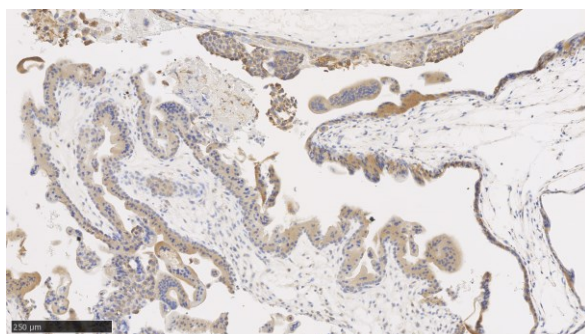
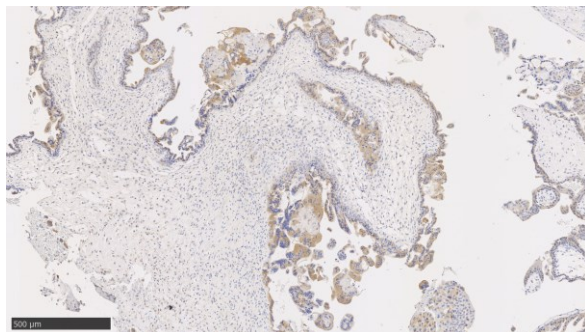
B)

MRPL9

MTX-S



MTX-R



Mass Spectrometry based identification of proteins and pathways associated with MTX-Resistance in GTD patients

Figure 3.4: The potential of PSAT1 and MRPL9 as prognosis biomarkers for MTX sensitivity and resistance, respectively.

Representative IHC images of PSAT1 **(A)** and MRPL9 **(B)** expression in GTD patient sections from the MTX-Sensitive (MTX-S) and the MTX-Resistant (MTX-R) groups. Original magnification: $\times 4.18$. Scale bar: 250 μ m (1st and 3rd pairs of images in (A) and (B)) or 500 μ m (2nd and 4th pairs of images in (A) and (B)).

3.3 Discussion

Resistance to a chemotherapeutic agent is often associated with global alterations in the proteomic profile of cancer cells and their identifications could uncover biomarkers of response. Here we applied Mass Spectrometry (MS) to identify proteomic changes associated with MTX resistance in Gestational Trophoblastic Disease through comparative analysis of five MTX-Sensitive and five MTX-Resistant patient samples. 135 proteins significantly overrepresented, and 95 proteins significantly underrepresented in the MTX-Resistant in comparison with the MTX-Sensitive group were highlighted. Pathway analysis revealed that the upregulated proteins in the MTX-Resistant group are associated with amino acid and nucleotide metabolism. This was anticipated since changes in amino acid metabolism are associated with antineoplastic drug resistance through a variety of mechanisms such as energy-efficient persistence of biosynthetic processes, maintenance of redox balance and epigenetic alterations (Bhingarkar *et al.*, 2021). Pathway analysis of proteins downregulated in the MTX-Resistant group revealed a single metabolism-related pathway: Serine and glycine biosynthesis.

Comparison of the patients and choriocarcinoma cell line proteomics datasets highlighted overlapping hits over or under-represented in the resistant setting. Three out of five overlapping underrepresented hits, PHGDH, PSAT1 and TIGAR, were members of the glucose-derived *de novo* serine synthesis pathway. The downregulation of PHGDH and PSAT1 associated with MTX resistance was validated by western Blotting in JEG3-R as compared to JEG3 cells. In contrast, TIGAR levels were unchanged between the two cell lines. This may be explained by the instability of the resistance phenotype in JEG3-R cells and the time point at which the cells were analysed as explained above.

Following *de novo* serine synthesis or its import from the extracellular environment, serine can be converted reversibly into glycine and both amino acids are considered major contributors of 1-C units for 1-C metabolism, or else the folate and methionine cycles (Amelio *et al.*, 2014). DHFR, the main target of Methotrexate, converts folic acid into the biologically active

THF (Newman and Maddocks, 2017). At the same time, the reversible transformation of serine and THF to glycine and 5,10-methylene THF is catalyzed by SHMT (MacFarlane *et al.*, 2008). This interconnection between serine metabolism and folate metabolism is vital for providing the necessary building blocks for DNA replication and repair (Geeraerts *et al.*, 2021). Moreover, the serine biosynthesis pathway and 1-C metabolism are cooperating in the context of cellular metabolic reprogramming under nutrient-deprived or stressful conditions such as chemotherapy treatment altering the redox balance and the epigenetic signature of the cell ((Campbell and Wellen, 2018), (Ducker and Rabinowitz, 2017)).

Serine metabolism has been previously connected to response to chemotherapy either by promoting or inhibiting it. More specifically, decrease of PHGDH expression was observed in a group of patients with resistant ovarian cancer following platinum-based chemotherapy (Van Nyen *et al.*, 2022). This observation was described as a result of adjustment of the resistant cells to preserve intracellular nicotinamide adenine dinucleotide (NAD⁺) levels. PHGDH downregulation is affecting carbon metabolism and subsequently NAD⁺ availability. This aids tumor cells maintain Poly (ADP-ribose) polymerase (PARP) activity when subjected to platinum treatment. PARP activity is involved in DNA repair mechanisms and its maintenance contributes to resistance against certain chemotherapeutic drugs ((Skidmore CJ, Davies MI, Goodwin PM, Halldorsson H, Lewis PJ, Shall S, 1979), (Morales *et al.*, 2014)).

Finally, we performed immunohistochemistry on additional patient tissue samples to validate the potential for our common differentially expressed proteins to serve as biomarkers of resistance to MTX. The proteins were chosen from the Venn diagrams in **Figure 3.3.A-B**. In addition to the downregulated serine synthesis pathway proteins (PHGDH, PSAT1 and TIGAR), the upregulated MRPL9 and CDA were tested because of their previously reported involvement as biomarkers of chemotherapy resistance. This analysis suggests that PSAT1 and MRPL9 may be prognostic markers for MTX sensitivity and MTX resistance respectively, but a larger panel of tissue samples needs to be investigated to provide statistical power to this conclusion.

4. Unveiling the interplay between MTX-resistance, Serine Synthesis, 1-Carbon Metabolism, and Integrated Stress Response (ISR) signaling pathway in Choriocarcinoma cells

4.1 Introduction

The Serine Synthesis Pathway (SSP) provides cells with serine and glycine, the fuels of 1-carbon Metabolism. These fundamental building blocks give rise to cellular macromolecules contributing to nucleotide biosynthesis, methylation reactions, and the maintenance of cellular redox equilibrium (Geeraerts *et al.*, 2021). Methotrexate is an antimetabolite that disrupts the folate cycle and subsequently impacts 1-carbon metabolism, a network intimately connected with the SSP (Pranzini *et al.*, 2021).

According to the Cytoscape analysis of Chapter 3, the Serine Synthesis Pathway is one of the most downregulated pathways in the group of the MTX-Resistant patients compared to the sensitive ones. Resistant choriocarcinoma cells JEG3-R show similar changes as compared to the sensitive counterparts. This similarity led us to explore this specific pathway and its interconnection with downstream and upstream networks in the choriocarcinoma cell lines JEG3 and JEG3-R.

In the present chapter, we examined whether *de novo* serine synthesis suppression in JEG3-R cells is related to its MTX resistance status, focusing on pathways downstream to SSP. Folate and methionine cycles were among the first pathways tested, leading us to important observations. Channeling of serine towards glutathione synthesis and skipping of nucleotide synthesis matches with previous findings from our lab, suggesting a focus on more efficient detoxifying capacity of the resistant cells and cell survival rather than on energy-demanding processes, such as proliferation. Also, the observed increased levels of methionine cycle enzymes led us to the finding that the PSAT1 gene is regulated epigenetically in the JEG3-R cells. In addition, a series of experiments using serine and glycine deprivation confirmed the involvement of SSP in MTX-resistance.

Then, to answer why and how JEG3-R cells downregulate SSP, we examined the expression of transcription factors affecting the transcription of the SSP enzymes, PHGDH and PSAT1. The most crucial transcription factor involved appears to be p53. However, the overexpression of ATF4 in resistant cells along with the fact that its downregulation made the JEG3-R cells even more

resistant, led us to investigate even further upstream. The GCN2-mediated phosphorylation of eIF2 α and activation of the Integrated Stress Response (ISR) signaling pathway in JEG3-R could explain the selective overexpression of ATF4 and activation of some of its targets as well as the translational block of 5'Cap-dependent protein synthesis of non-essential for the survival pathways such as proliferation or even apoptosis. In particular, under stress conditions, prioritization of biological processes that favor cell survival, like autophagy, may contribute to MTX resistance. The combination of MTX with GCN-2 inhibitors in JEG3-R cells demonstrated a synergistic interaction at specific concentrations, supporting our hypothesis.

4.2 Results

4.2.1 Variations between JEG3 and JEG3-R cells in the Serine Synthesis Pathway and 1-Carbon Metabolism

Following the results of Chapter 3 regarding the downregulated *de novo* Serine Synthesis Pathway (SSP) in the MTX-Resistant group of patients and the choriocarcinoma JEG3-R cell line compared to the MTX-Sensitive group of patients and JEG3 cells, respectively, we explored this pathway and its association to methotrexate resistance. Firstly, the mRNA and protein expression of other members of the SSP pathway, apart from PHGDH and PSAT1, was measured. Because of the connection between SSP and 1-Carbon (1-C) metabolism, the expression of enzymes of the folate and methionine cycles was also assessed (**Figure 4.1.A-B**).

Phosphoserine phosphatase (PSPH) is the third major enzyme of the SSP pathway which catalyzes the conversion of phosphoserine to serine (Amelio *et al.*, 2014). Unlike the two first enzymes of the pathway, the mRNA and protein levels for PSPH were unchanged between the JEG3 and JEG3-R cell lines (**Figure 4.1.A-B**). In contrast, JEG3 cells show higher expression of the cytosolic enzymes TYMS (Thymidylate synthase) and SHMT1 (serine hydroxymethyltransferase) at the protein level than their resistant counterparts (**Figure 4.1.A-B**). SHMT1 initiates serine catabolism by transferring the γ -carbon amino acid side chain to THF, generating 5,10-methylene-THF (me-THF) and glycine (Albert M. Li, 2021). TYMS then facilitates the conversion of methylene-THF into dTMP (by adding a single carbon atom onto dUMP) (Chen *et al.*, 2017). This pattern observed in JEG3 cells suggests a utilization of serine metabolism towards thymidine synthesis and subsequently DNA synthesis and cell proliferation. This finding aligns with the slower proliferation rate observed for JEG3-R compared to JEG3 cells (**Supplementary Figure 4.1**).

The preceding results may also suggest that JEG3-R cells rely more heavily on the salvage pathway of nucleotide biosynthesis, saving energy and cellular resources. Two enzymes involved in this salvage pathway were found overexpressed in JEG3-R as compared to JEG3 cells; TK1 (Thymidine kinase 1) and CDA (Cytidine Deaminase) (**Figure 4.1.A-B**). CDA was also found to be upregulated in the MTX-R group of patients (**Figure 4.1.C**), (**Supplementary table 4.1**)). TK1 is

responsible for the conversion of thymidine into thymidine monophosphate (TMP), a precursor of thymidine triphosphate (TTP), one of the four nucleotide triphosphates that are required for DNA synthesis (Walter and Herr, 2022). CDA is essential for the catabolism of pyrimidine nucleotides. It catalyzes the deamination of cytidine into uridine which can then be converted to uridine monophosphate (UMP) and incorporated into the pyrimidine salvage pathway for the synthesis of other pyrimidine nucleotides (Frances and Cordelier, 2020). CDA has been connected to resistance to chemotherapy ((Ye *et al.*, 2015),(Rajabpour *et al.*, 2017)). An upregulation of the pyrimidine salvage pathway could be an additional obstacle to the activity of MTX which indirectly targets the *de novo* biosynthesis of the purines and pyrimidines. Therefore, we initially decided to test the combination of MTX and CDA inhibitors (Zebularine and Tetrahydrouridine) to see if this approach may re-sensitise resistant cells to MTX. However, CDA inhibition did not deliver a significant increase in MTX response (**Supplementary Figure 4.2**).

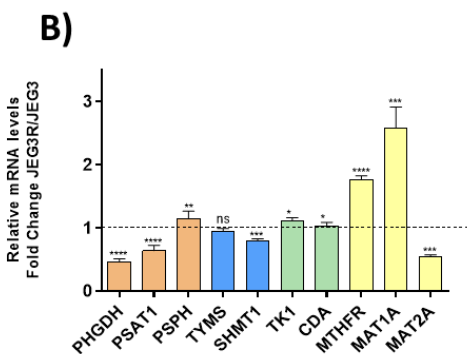
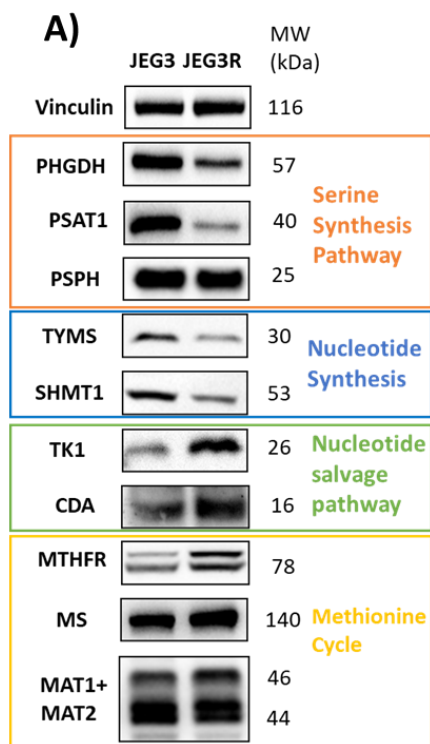
To test another therapeutic route exploiting this shift towards the salvage pathway in resistant cells, we next tested the combination of MTX with the uridine import inhibitor, Dipyridamole. To estimate the effectiveness of this combination, we used SynergyFinder 2.0, a web-tool for interactive analysis and visualization of synergy in multi-drug treatments. Here, to determine synergy or antagonism, the ZIP reference model was used which assumes no interaction between drugs (Yadav *et al.*, 2015). The summary synergy score represents the average additional response resulting from drug interactions, with a score > 10 indicating synergistic, and < -10 antagonistic, interactions. A score between -10 to +10 reflects additive interaction (Ianevski, Giri and Aittokallio, 2021). As shown in **Figure 4.1.E**, Dipyridamole promoted the response to MTX in both choriocarcinoma cell lines, especially in JEG3-R where synergy was clearly observed (average ZIP score of +24.16%).

Besides the overexpression of DHFR (**Supplementary Figure 4.3**) (Georgiou *et al.*, 2022), three other important enzymes of the folate/methionine cycles were found significantly upregulated in JEG3-R cells. The first enzyme is MTHFR (Methylenetetrahydrofolate reductase) (**Figure 4.1.A-B**) and is an essential enzyme of 1-C metabolism as it converts 5,10-methylenetetrahydrofolate (5,10-Mthf) to 5-methyltetrahydrofolate (5-Mthf) (Locasale, 2013). 5-

Mthf then donates its CH₃-group for re-methylation of homocysteine to methionine via MS (Methionine Synthase), the second 1-C cycle enzyme which was also found overexpressed in JEG3-R cells (**Figure 4.1.A-B**), linking thereby the folate and methionine cycles. Methionine is then converted to S-adenosylmethionine (SAM), a major reactive CH₃- carrier, by MAT (Methionine Adenosyltransferase), the third 1-C enzyme found overexpressed JEG3-R cells. More specifically, two isoforms of MAT were tested, MAT1A which primarily functions in the liver and MAT2A which is broadly distributed, encoding for two catalytic subunits, $\alpha 1$ (MAT1A) and $\alpha 2$ (MAT2A) (Kotb *et al.*, 1997). MAT1A was found upregulated in the JEG3-R cells at both mRNA and protein level whereas MAT2A was found slightly upregulated in JEG3 cells (**Figure 4.1. A-B**). DNA and histone methyltransferases use SAM for the methylation of DNA and histones. Alternatively, demethylation of SAM produces S-adenosylhomocysteine (SAH) which is then converted to homocysteine. Meanwhile, serine along with Cystathione Synthase (CBS) will give rise to cysteine which will be further used for Glutathione (GSH) production (Geeraerts *et al.*, 2021). Hence, GSH production rather than nucleotide synthesis could be favored through the switch of the SSP in JEG3R cells (**Figure 4.1.D**).

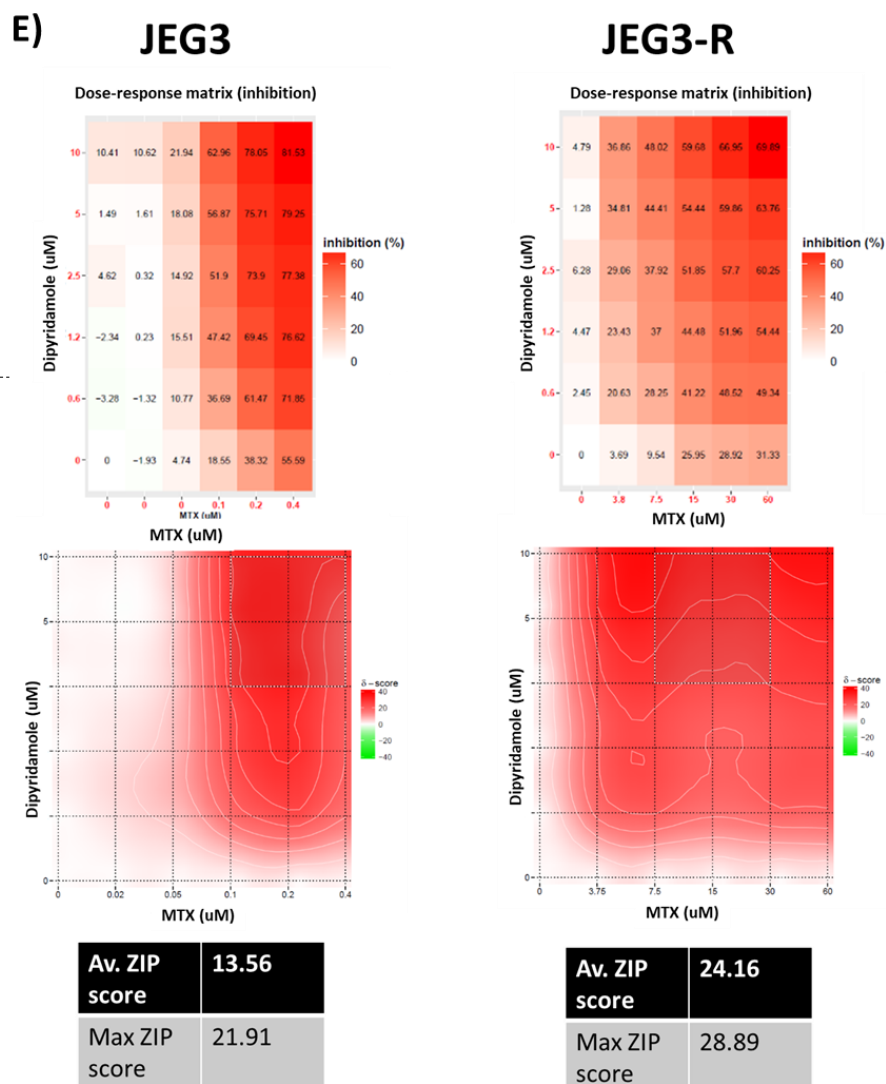
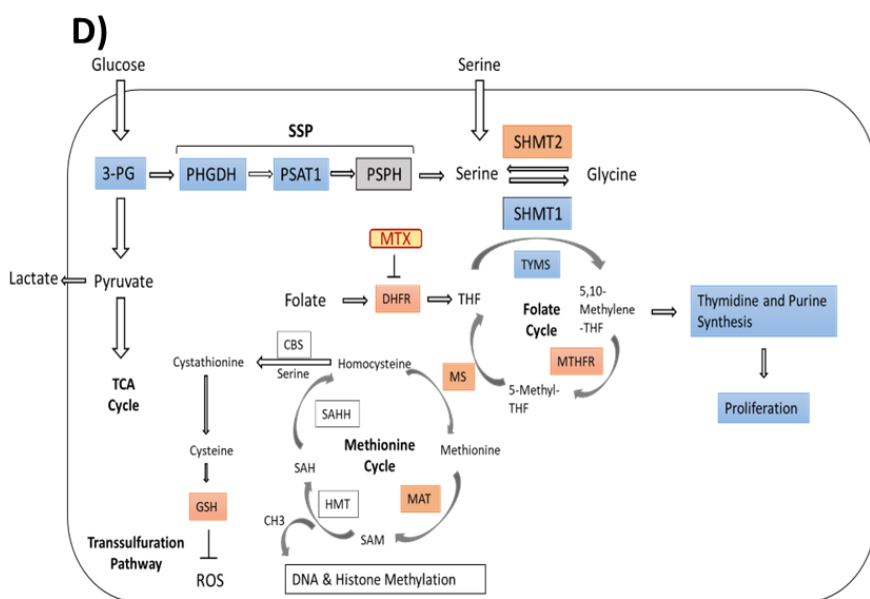
In addition, to further validate our results, we cross-checked the normalized fold change ratios of the protein abundances in two separate proteomic datasets; the patient proteomic dataset (Chapter 3) provided us the fold change MTX-R/MTX-S ratio and that of the choriocarcinoma cell lines JEG3 and JEG3-R (analysis previously performed in our lab) gave us the fold change JEG3-R/JEG3 ratio (**Figure 4.1.C**). Among the proteins tested here, only the SSP enzymes PHGDH and PSAT1 seem to follow the same downregulation trend in the resistant parts of any dataset of any origin (either proteomic or cellular or patient-derived). Interestingly, both MTHFR (not statistically significant) and CDA (p-value=0.035 (*), unpaired Student t-test). In the MTX-R group of the patient proteomic dataset seems to resemble the overexpression pattern observed in the resistant cells (**Figure 4.1.C**).

Unveiling the interplay between MTX-resistance, Serine Synthesis, 1-Carbon Metabolism, and Integrated Stress Response (ISR) signaling pathway in Choriocarcinoma cells



C)

Fold Change Ratio	MTX-R/MTX-S	JEG3R/JEG3
Protein		
PHGDH	0.68	0.52
PSAT1	0.62	0.64
PSPH	0.86	-
TYMS	1.15 ns	0.85
SHMT1	0.99 ns	0.33
TK1	0.94 ns	-
CDA	1.43	-
MTHFR	1.27 ns	-
MS	-	-
MAT1A	-	-
MAT2A	0.94	0.82



Unveiling the interplay between MTX-resistance, Serine Synthesis, 1-Carbon Metabolism, and Integrated Stress Response (ISR) signaling pathway in Choriocarcinoma cells

Figure 4.1: Serine and Glycine are utilized differently by JEG3 and JEG3-R cells.

A) Immunoblots indicating the expression of proteins involved in the Serine Synthesis pathway, Nucleotide Synthesis, Nucleotide salvage pathway, and Methionine Cycle in JEG3 and JEG3-R cells. Vinculin was utilized as a loading control. The data represent 3 independent biological replicates. The data show the mean fold changes $\pm$ SEM from 3 independent experiments **B)** Relative mRNA expression of genes involved in the Serine Synthesis pathway, Nucleotide Synthesis, Nucleotide salvage pathway, and Methionine Cycle in JEG3 and JEG3-R cells. The data represent the mean fold changes $\pm$ SEM of at least 3 independent experiments. Statistical significance was determined using an unpaired Student's t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, ns=not significant). **C)** The table contains the normalized fold change ratios of the protein abundances in the two separate proteomic datasets; the patient proteomic dataset (fold change MTX-R/MTX-S ratio) and that of the choriocarcinoma cell lines JEG3 and JEG3-R (fold change JEG3-R/JEG3 ratio). All the values derived from the patient proteomic analysis are statistically significant unless it is stated otherwise. In the case of the choriocarcinoma cell lines proteomic analysis, there was only 1 technical replicate for each cell line. **D)** Schematic representation of how serine is utilized in the 1-C metabolism. Orange-coloured nodes represent upregulation, whereas blue-coloured ones represent downregulation of proteins/molecules in JEG3-R as compared to JEG3 cells. Grey-coloured nodes were tested and showed no difference between the two cell lines. White coloured nodes were not tested. **E)** The effects of combinations of Dipyridamole and MTX on the viability of JEG3 and JEG3-R cells were analysed using SynergyFinder 2.0 online web portal to highlight synergistic interactions between the two compounds. The Zero interaction potency (ZIP) model was utilized and ZIP scores < -10 highlight antagonism, while ZIP scores > 10 demonstrate synergism. The upper panels depict cell viability results as a function of the concentration of both compounds. The lower panels depict the results of synergy calculations. The average ZIP score was calculated by collecting data from three independent experiments.

4.2.2 GSH and ROS levels contribution to the MTX resistance of JEG3-R cells

The above statement regarding JEG3-R cells' preference in GSH synthesis rather than that of nucleotides is consistent with our findings that GSH levels are higher in JEG3-R than in JEG3 cells (**Figure 4.2.A**). This may participate in MTX resistance either through increased detoxification of the drug itself through conjugation with GSH or through neutralizing chemotherapy-induced Reactive Oxygen Species (ROS) synthesis and shielding cells from oxidative stress ((Kwon *et al.*, 2019), (Hess and Khasawneh, 2015)). In support of the first possibility, the protein levels of Glutathione S-Transferase Theta 1 (GSTT1), a member of the glutathione S-transferase family, were found elevated in JEG3-R cells in comparison with JEG3 cells (**Figure 4.2.B-C**). Additionally, this enzyme was among the most highly upregulated protein hits in the MTX-R group in the proteomics analysis (**Supplementary Table 4.2**). GSTT1 participates in the detoxification process by coupling different chemicals, such as methotrexate, with glutathione and this conjugation makes the drugs highly-soluble and facilitates their export from the cell [(Townsend, Tew and Chase, 2019) (Dasari *et al.*, 2018)].

Interestingly, previous studies from our lab have shown higher ROS levels in JEG3-R than in JEG3 cells. To further examine these observations, a time-course experiment was carried out. ROS levels were tested upon Methotrexate treatment in both cell lines at 3 different time points; 24h, 48h, and 72h and in comparison with those found in the untreated cells (**Figure 4.2.D**). The ROS levels in JEG3-R were comparable to the untreated condition at all timepoints. On the other hand, the ROS levels of JEG3 started decreasing gradually at 24h and 48h but then dropped dramatically at 72h compared to the untreated condition. One explanation for this result would be that the constantly increased ROS levels in JEG3-R compared to JEG3 may be a consequence of JEG3-R cells' adaptation to methotrexate exposure and could contribute to resistance mechanisms. Increased ROS levels have the ability to trigger a number of survival pathways that support resistance against methotrexate-induced cell death (Frieze C, Yang J, 2019).

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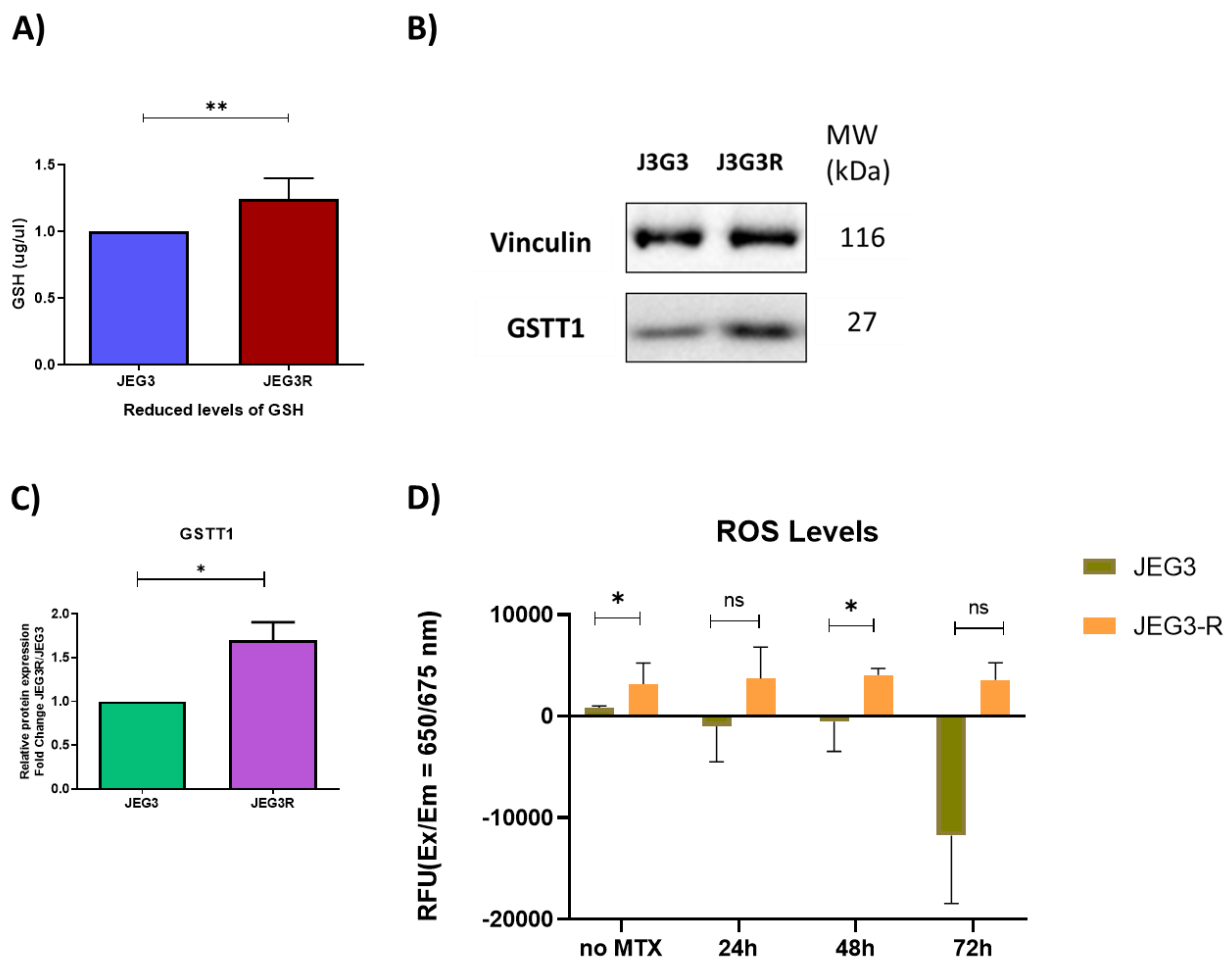


Figure 4.2: JEG3-R cells seem to have better detoxification capacity against Methotrexate

A) Assessment of glutathione levels was carried out by a colorimetric assay. Bars show the mean $\pm$ SEM from 3 independent experiments. Statistical significance was determined using the Unpaired Student's t-test ($p \leq 0.01$). **B)** Immunoblots showing the expression of GSTT1 in JEG3 and JEG3-R cells. Vinculin was utilized as a loading control. **C)** Quantification of western blotting data from 3 independent biological replicates (B). Statistical significance was determined using an unpaired Student's t-test ($*p \leq 0.05$). **D)** Time-course experiment quantifying the levels of Reactive Oxygen species (ROS) upon MTX treatment, measured at 3 time points 24h, 48h, and 72h. Fluorescence of the fluorescent product, 2',7'-dichlorofluorescein (DCF) post oxidation by ROS induction, was measured and is displayed as relative fluorescence units (RFU). The mean $\pm$ SEM of the DCF fluorescence dye at every timepoint was normalized relative to the 0-hour time point. The experiment was performed twice (2 biological replicates). Each condition in every biological replicate had at least 3 technical replicates. Unpaired student t-test was employed to determine the significance of differences between JEG3 and JEG3-R in each concentration separately ($*p \leq 0.05$, ns=not significant).

4.2.3 Epigenetic regulation of the SSP genes

As mentioned above, DNA and histone methyltransferases utilize SAM produced during the methionine cycle for the methylation of DNA and histones (Geeraerts *et al.*, 2021). The increased MS and MAT levels in JEG3-R cells may indicate a strategy to maintain methionine levels, which is crucial for the regeneration of SAM. We therefore investigated possible epigenetic modifications as a determinant of the differential transcription of the two main SSP enzymes, PHGDH and PSAT1. Firstly, the levels of two epigenetic marks, methylation of lysine 9 (H3K9me3) and lysine 27 (H3K27me3) of histone H3 were tested in both cell lines. H3K9me3 and H3K27me3 are known to promote chromatin compaction in promoter regions, leading to the repression of transcription of the corresponding genes (Zhang, Cooper and Brockdorff, 2015). Changes in overall H3K9me3 and H3K27me3 levels were assessed by quantitative western blotting in JEG3 and JEG3-R cells (**Figure 4.3.A-B**). Regarding H3K9me3 mark, no significant change was detected between the two cell lines. The levels of H3K27me3 mark were found to be elevated in the case of JEG3-R cells compared to JEG3, indicating that these resistant cells have more genomic regions protected from DNA methylation compared to the sensitive ones. Additionally, the mRNA levels of the histone methyltransferase Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2, aka KMT6A), were found to be elevated in JEG3-R cells compared to JEG3 (**Figure 4.3.C**). EZH2 has a repressive role in gene expression by participating to the H3K27 methylation (Tan *et al.*, 2014). To further test how and if these histone modifications affect PHGDH and PSAT1 transcription, the cells were treated with the DNA methyltransferase inhibitor, Zebularine. Upon Zebularine treatment (100uM) the levels of both H3K27me3 and H3K9me3 were surprisingly elevated, but protein expression of PSAT1 was upregulated in JEG3-R cells in comparison with the untreated control (**Figure 4.3.D**). This may mean that other epigenetic marks, not tested here, are responsible for the repression of PSAT1 expression and these could be inhibited by Zebularine. It is also possible that other chromatin-modifying enzymes are recruited to counteract the loss of DNA methylation and maintain transcription of this gene. If one of these hypotheses is correct, the

transcription of PSAT1 may be regulated epigenetically in JEG3R cells. This should be further tested in the future.

The increased levels of H3K9me3 could be explained by the decrease in the levels of the histone lysine demethylase KDM4C upon Zebularine treatment. KDM4C acts on lysine residues of histone proteins and more specifically is known to demethylate H3K9me3 (Tan *et al.*, 2014) (**Figure 4.3.E**). Interestingly, this demethylase has been linked to the transcription factor Activating transcription factor 4 (ATF4) and their cooperation is required for the transcription of the SSP enzymes. (Zhao *et al.*, 2016). Indeed, KDM4C acts on the promoter regions of both PHGDH and PSAT1 in a human esophageal squamous cell carcinoma cell line (Zhao *et al.*, 2016). ATF4 is a known activating transcription factor of these enzymes and KDM4C depends on ATF4 binding on the promoter region of these genes so that maximum transcription efficiency of SSP genes can be achieved through KDM4C/ATF4 interaction. While the levels of ATF4 in JEG3-R upon Zebularine treatment increased slightly, the accompanying downregulation of KDM4C could explain why PHGDH levels remain unchanged following drug treatment (**Figure 4.3.D**). Obviously, activation/repression of genes is the result of multiple combinatorial events and is cell-context dependent. Hence, DNA methylation and activity of transcription factors can be impacted through this complex interplay to recruit other chromatin-modifying enzymes and modulate the overall state of the chromatin (Marques and Outeiro, 2013). We therefore decided to examine further the interplay between the transcription factors impacting the SSP pathway.

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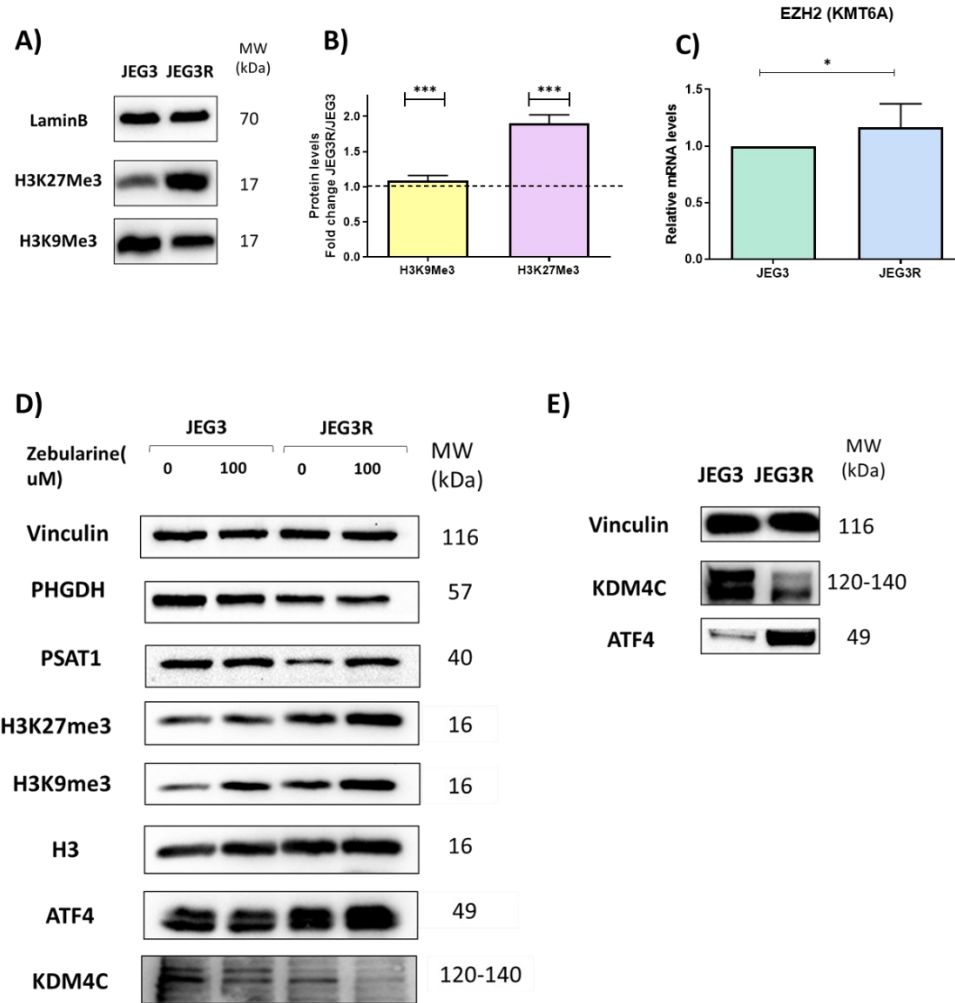


Figure 4.3: The expression of the PSAT1 gene is regulated epigenetically in the JEG3-R cells.

Immunoblots indicating the expression of epigenetic marks H3K9me3 and H3K27me3 in JEG3 and JEG3-R cells. LaminB served as a loading control in the western blot analysis. The data represent 3 independent biological replicates. **B)** Quantification of western blotting data from 3 independent biological replicates. Statistical significance was determined using the Student's t-test (**p < 0.01, ***p < 0.001). **C)** Relative mRNA expression of EZH2 (KMT6A) in JEG3 and JEG3-R cells. The data show the mean fold changes $\pm$ SEM from 3 independent experiments. Unpaired Statistical significance was determined using the Student's t-test (*p < 0.05). **D)** Immunoblots showing the effect of Zebularine treatment in JEG3 and JEG3-R cells in SSP enzymes PHGDH and PSAT1, in epigenetic marks H3K9me3 and H3K27me3 and in ATF4 and KDM4C. **E)** Immunoblots indicating the expression of ATF4 and KDM4C in JEG3 and JEG3-R cell lines. Detection of Vinculin or LaminB served as a loading control in the western blot analysis. Data are representative of 3 independent biological replicates. The data show the mean fold changes $\pm$ SEM from 3 independent experiments. Statistical significance was determined using the unpaired Student's t-test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns=not significant).

4.2.4 p53 surpasses other transcription factors in regulating the SSP genes in our cell lines

Multiple transcription factors have been reported to affect the transcription of the SSP enzymes (Li and Ye, 2020). Those that upregulate the transcription of SSP genes are ATF4 (Adams, 2007), ATF3 (Li *et al.*, 2021), c-MYC (Sun *et al.*, 2015) and NRF2 (Denicola *et al.*, 2016). The transcriptional factor responsible for the downregulation of the transcription of SSP genes is p53 (Maddocks *et al.*, 2013). The mRNA and protein levels of these transcription factors are depicted in the figure **Figure 4.4.A-B**. p53 mRNA and protein levels are elevated in JEG3-R cells compared to JEG3 (as previously observed in our lab (Georgiou *et al.*, 2022) and upon siRNA-mediated downregulation of p53, the levels of both PHGDH and PSAT1 are increased in both cell lines (**Figure 4.4.C**), further reinforcing the role of p53 as a negative modulator of SSP enzymes in our cells. In parallel, c-MYC was found downregulated in resistant cells compared to their sensitive counterpart, in agreement with our published results (Georgiou *et al.*, 2022), and this may in turn decrease the ability of this transcription factor to promote PHGDH and PSAT1 transcription. In contrast, no difference in expression between the two cell lines was detected for the protein levels of NRF2. While ATF3 mRNA levels were found elevated in JEG3-R compared to JEG3 cells the corresponding protein levels could not be investigated due to poor antibody reagents. However, it has been reported that ATF3 is activated upon serine deprivation, leading to the stabilization of ATF4 and recruitment of the transcriptional co-activator p300. Notably, p300 levels are decreased in JEG3- cells compared to JEG3 cells (**Figure 4.4.B**). These events collectively contribute to the induction of the SSP pathway (Li *et al.*, 2021). As for ATF4, one of the major transcription factors of the SSP pathway, it was found to be overexpressed in JEG3-R in comparison to JEG3 cells. However, as discussed in the preceding section, the ability of this increase to be translated to downstream upregulation of PHGDH and PSAT1 may be thwarted by the decrease in KDM4C protein levels in resistant cells.

To establish the relevance of ATF4 and p53 to the Serine metabolism phenotype in our cells, we investigated how targeting these transcription factors could modulate the expression of SSP enzymes in our cells. Upon siRNA-mediated downregulation of ATF4, the protein levels of

both PHGDH and PSAT1 were decreased in both cell lines compared to the non-targeting condition (**Figure 4.4.C**). The parallel increase of the protein levels of p53 could explain the downregulation of the SSP enzymes and also indicate the particular importance of p53 as compared to other transcription factors in the regulation of PHGDH and PSAT1 expression in our culture conditions. This scenario is further supported by the fact that siRNA-mediated downregulation of p53 led to the inhibition of the expression of SSP enzymes, without affecting the expression levels of ATF4 (**Figure 4.4.D**).

Experiments were additionally carried out to evaluate the impact of siRNA-mediated downregulation of ATF4 on the response of JEG3 and JEG3-R cells to MTX. In the case of JEG3-R cells, ablation of ATF4 made the cells even more resistant as indicated by the doubling of the IC₅₀ value for MTX in ATF4-silenced as compared to the non-targeted cells (**Figure 4.4.F**). On the contrary, the downregulation of ATF4 in JEG3 cells did not alter their sensitivity to MTX (**Figure 4.4.E**). Therefore, it seems that ATF4 plays a more crucial role in JEG3-R than in JEG3 cells and the further decrease in PHGDH and PSAT1 expression downstream of ATF4 downregulation in JEG3-R cells exacerbates resistant phenotype.

An experiment to test the subcellular localization of ATF4 was carried out, as nuclear localization is critical to its activity as a transcription factor and mis-localization could explain the inability of ATF4 to positively regulate the transcription of the SSP enzymes. We performed this using immunofluorescence staining followed by confocal microscopy. The nuclear levels of ATF4 were found to be increased in JEG3-R as compared to JEG3 cells (**Supplementary Figure 4.4.A**). In addition, the proportion of ATF4 within the nucleus versus the cytoplasm was higher in JEG3-R than in JEG3 cells (**Supplementary Figure 4.4.B**). This finding excludes the potential that the lack of nuclear localization of ATF4 might explain the decrease in the expression of SSP enzymes. Post-translational modifications of ATF4, such as phosphorylation, acetylation, or methylation, can affect the functional state of ATF4 and may be responsible instead. Additionally, interplay with other transcription factors and partners could limit or even cancel the ability of ATF4 to activate the transcription of SSP enzymes. Indeed, our results may suggest that increased p53 and/or decreased KDM4C levels could be counteracting ATF4's activity (**Figure 4.3.C**).

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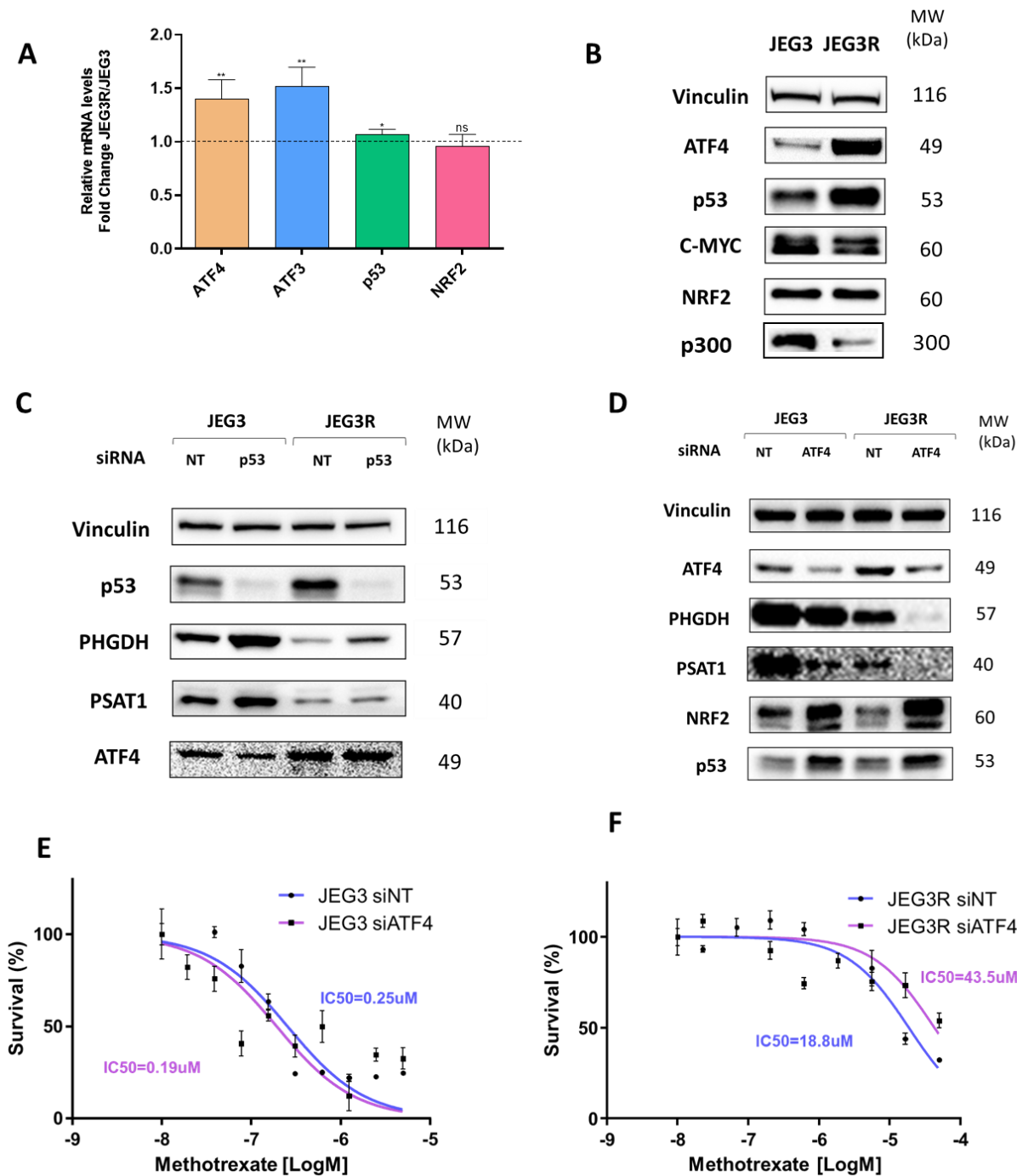


Figure 4.4: ATF4 affects MTX resistance of JEG3-R but not directly through downregulation of PHGDH and PSAT1 which are mostly under negative control of p53.

A) Relative mRNA expression of transcription factors ATF4, p53, c-MYC and NRF2 in JEG3 and JEG3R cells. The data show the mean fold changes $\pm$ SEM from 3 independent experiments. Statistical significance was determined using the Unpaired Student's t-test (* $p < 0.005$, ** $p < 0.001$, *** $p < 0.005$, **** $p < 0.001$, ns=not significant). **B)** Immunoblots showing the effect of siRNA-mediated p53 downregulation in JEG3 and JEG3-R in SSP enzymes, PHGDH and PSAT1, and in the transcription factor ATF4. **C)** Immunoblots showing the expression of transcription factors ATF4, p53, c-MYC and NRF2. **D)** Immunoblots showing the effect of siRNA-mediated ATF4 downregulation in JEG3 and JEG3-R in SSP enzymes, PHGDH and PSAT1, and in the transcription factors ATF4 and NRF2. For the above immunoblots, Vinculin was utilized as a loading control. The data represent 3 independent biological replicates. The data represent the mean fold changes $\pm$ SEM from at least 3 independent experiments. Statistical significance was determined using the Student's t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, ns=not significant). **E)+F)** IC_{50} Graph of MTX at siATF4 treated versus siNT treated JEG3 (**E**) and JEG3-R (**F**) cells. IC_{50} values for MTX were evaluated from the curve utilizing GraphPad Prism. Data represent the average from 3 biological replicates.

4.2.5 ISR activation potentially leads JEG3-R cells toward autophagy contributing to the resistance phenotype

Another possibility was also examined to explain the reduced levels of PHGDH and PSAT1 in JEG3-R compared to JEG3 cells, despite the overexpression of ATF4. The Integrated Stress Response (ISR), which is triggered through a range of stressors such as nutrient deprivation, ER stress, oxidative stress, and exposure to chemotherapy drugs, depends heavily on eukaryotic initiation factor 2 alpha (eIF2 α) (Pakos-Zebrucka K et al., 2016). Under normal conditions, eIF2 α is involved in the commencement of protein biosynthesis by delivering initiator tRNA onto the ribosome (Vanselow *et al.*, 2022). Upon stress, phosphorylation of eIF2 α at Serine 51 occurs, resulting in decreased global protein synthesis and selective translation of particular mRNAs. ATF4 is one of these specific mRNAs which modulates the expression of genes implicated in multiple cellular processes, such as amino acid metabolism (Tian *et al.*, 2021). The ratio phosphorylated(Ser51)-eIF2 α to total eIF2 α under normal conditions was tested and was found to be higher in JEG3-R than in JEG3 cells (**Figure 4.5.A-B**), indicating activation of ISR in the

resistant cells. General control nonderepressible 2 (GCN2) is one of the kinases which phosphorylates eIF2 α and gets activated upon amino acid deprivation (Deval *et al.*, 2009). GCN2 protein levels were found to be overexpressed in JEG3-R cells suggesting that amino acid limitation could be activating ISR (**Figure 4.5.C**). GCN2 mRNA levels were though decreased in JEG3-R compared to JEG3 suggesting a post-translational modification affecting protein stability (**Figure 4.5.D**).

As mentioned earlier, ATF4 is one of the mRNAs selectively transcribed upon eIF2 α phosphorylation. Target genes regulated by ATF4 control cell survival and autophagy (Ye *et al.*, 2010). While transcription of serine synthesis enzymes is not exclusively regulated by ATF4, this transcription factor modulates the expression of some of the amino acid transporters which facilitate the cellular import of serine and other amino acids ((Wang *et al.*, 2013),(Wei *et al.*, 2021)). The protein levels of ASCT2 (SLC1A5) and ASCT1 (SLC1A4) were found increased in JEG3-R cells compared to JEG3, but this was found not to be due to increased steady state mRNA levels (**Figure 4.5.D-E-H**). This could help resistant cells mitigate their decreased ability to produce serine *de novo*. Moreover, Autophagy related 5 (ATG5), a downstream transcriptional target of ATF4 (B'Chir *et al.*, 2013), which induces autophagy by promoting autophagosome biogenesis, (Zheng *et al.*, 2019), was found upregulated in the resistant cells (**Figure 4.5.C-H**). In addition, C/EBP homologous protein (CHOP) interacts and cooperates with ATF4 to induce the expression of autophagy genes including ATG5 (B'Chir *et al.*, 2013). CHOP protein levels were found elevated in the JEG3-R cells (**Figure 4.5.C-H**) suggesting that JEG3-R cells may further overcome decreased serine synthesis through the recycling of existing protein content.

AMPK (AMP-activated protein kinase) is another metabolic stress-sensor that gets activated upon the increase of the ratio of AMP to ATP. The protein levels of the catalytic subunit AMPK α and its phosphorylation at Threonine-172 (indicator of AMPK activation) were tested. The increased ratio of phospho(T172)-AMPK α to total AMPK in JEG3-R cells versus JEG3 suggests the activation of AMPK in the resistant cells (**Figure 4.5.F-G**). AMPK not only halts processes that consume ATP (anabolic pathways) like protein and nucleotide synthesis but also promotes

autophagy ((Ke *et al.*, 2018),(Hardie, Ross and Hawley, 2012)). So, activation of AMPK also suggests the possibility of autophagy induction in JEG3-R cells.

The hypothesis of ISR activation by the GCN2-mediated phosphorylation of eIF2 α with the following activation of ATF4 and the regulation of pathways potentially associated to Methotrexate resistance was further tested. The JEG3-R cells were treated with two GCN2 inhibitors, A-92 and GZD824 ((Kato *et al.*, 2020),(Szaruga *et al.*, 2023),(Carlson *et al.*, 2023)). According to the relevant literature, both inhibitors are non-suppressive in lower concentrations and are effective inhibitors of GCN2 at higher concentrations. This was the case for choriocarcinoma-resistant cells as well. Single treatment of JEG3-R cells requires concentrations above 5 μ M and 2 μ M to achieve a 50% inhibition of cell growth in response to A-92 and GZD824, respectively (**Figure 4.5.I-J**). Combination treatment with Methotrexate was only synergistic at high concentrations of the GCN2 inhibitors. On the contrary, combination of lower concentrations of any of the two GCN2 inhibitors with Methotrexate resulted in a non-synergistic effect, as indicated by the % inhibition percentage (**Figure 4.5.I-J**). Thus, GCN2 inhibition manages to sensitize JEG3-R cells to Methotrexate, as long as an appropriate dosing of the GCN-2 inhibitor is chosen, and the Methotrexate dose remains low. However, it is necessary to evaluate the nonspecific toxicity of these inhibitors by performing cell viability assays in normal placenta cells to ensure the *in vitro* safety and efficacy of them.

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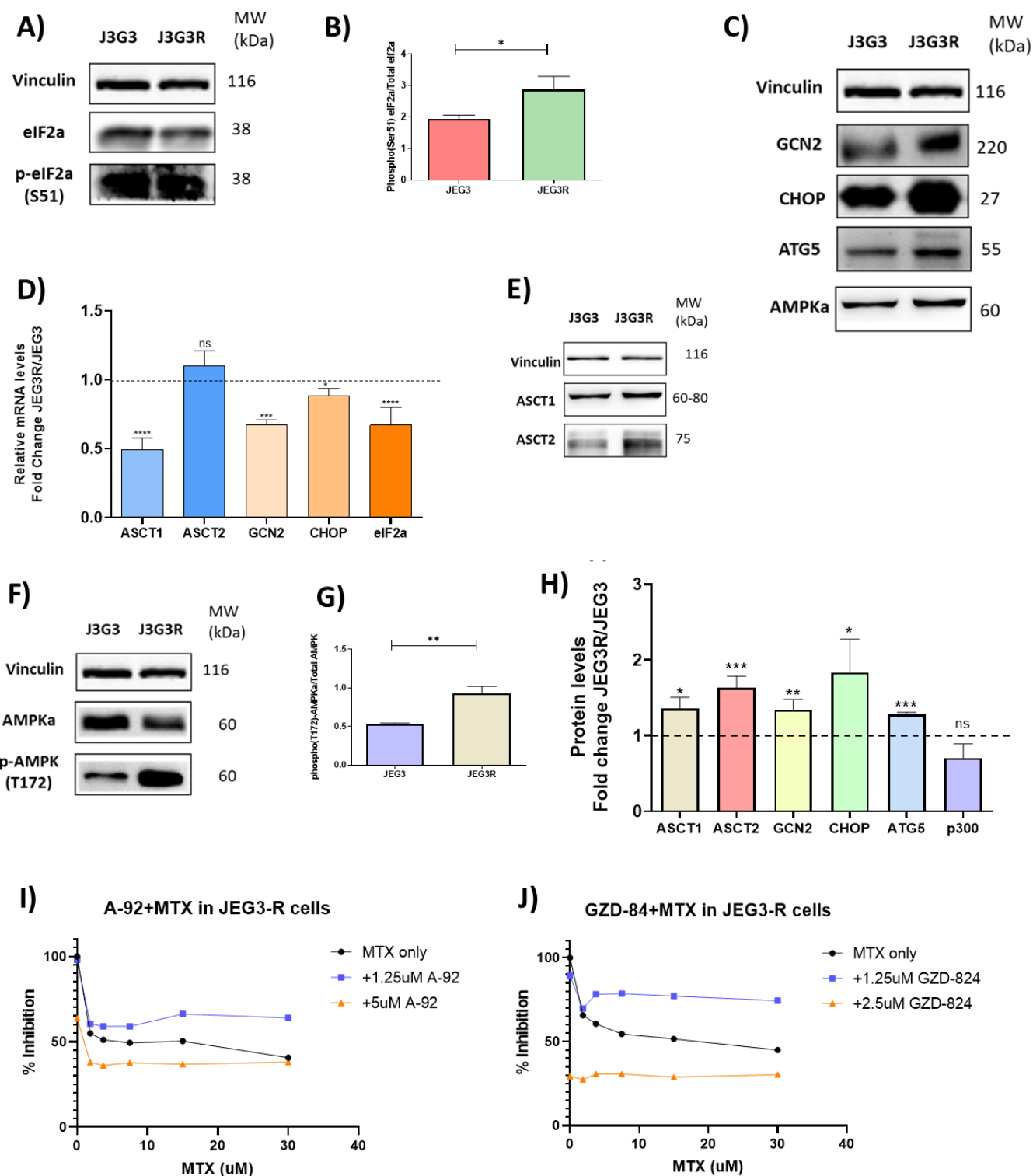


Figure 4.5: GCN 2-mediated ISR activation as an upstream mechanism leads MTX resistance in JEG3-R cells.

A) Immunoblots showing the total and phosphorylated at Serine 51 of eIF2 α in JEG3 and JEG3-R cell lines. **B)** Bar graph showing the ratio phospho(Ser51)-eIF2 α /Total eIF2 α for each cell line. **C)** Immunoblots showing the expression of proteins tested here related to autophagy activation and process such as GCN2, CHOP, AMPK α and phosphorylation of AMPK α . **D)** Immunoblots showing the expression of amino acid transporters, ASCT1 and ASCT2, targets of ATF4. **E)** Relative mRNA expression of amino acid transporters, ASCT1 and ASCT2 and GCN2 and CHOP. **F)** Immunoblots showing the total and phosphorylated at Threonine 172 levels of AMPK in JEG3 and JEG3-R cell lines. **G)** Bar graph showing the ratio phospho(T172)-AMPK α /Total AMPK for each cell line. **I)+J)** Dose-response (%inhibition) for the combination treatment of increasing concentrations of Methotrexate with a low and a high concentration of A-92 (**H**) and with a low and a high concentration of GZD-824 (**I**). The data represent the mean fold changes $\pm$ SEM from two biological replicates (each one with three technical replicates). For the above immunoblots, Vinculin was utilized as a loading control in the western blot analysis. The data show the average fold changes $\pm$ SEM from three independent experiments. The bar graph data show the average fold changes $\pm$ SEM of at least 3 independent experiments. Statistical significance was determined using the Unpaired Student's t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, ns=not significant).

4.2.6 JEG3 cells rely more on extracellular serine despite the more active SSP

Serine and glycine are biosynthetically interconnected as serine can be converted to glycine by SHMT1, and glycine to serine by SHMT2 (Amelio *et al.*, 2014). Changes in the synthesis or cellular import of Serine can in turn impact the intracellular levels of glycine. Hence, we investigated how modulating the levels of both amino acids in the cell culture medium influenced the growth and MTX response of our cell lines.

Two different conditions or else two different growth media were established. The reconstituted SG+ medium contains equivalent levels of serine and glycine as the off-the-shelf DMEM in which the cells are normally grown, whereas the SG- medium lacks both serine and glycine amino acids (**Figure 4.6.A**). Firstly, the levels of intracellular and extracellular L-serine were tested in both cell lines (**Figure 4.6.B-C**). The levels of intracellular serine remained higher in JEG3 compared to JEG3-R cells in both SG+ and SG- conditions, probably due to their more active SSP.

Interestingly, the levels of serine found in both media were quite similar, with a slight increase in the levels of serine in the SG- condition for both cell lines (not statistically significant for JEG3-R cells). A possible explanation for this observation would be the activation of the *de novo* SSP as a counteracting mechanism upon serine and glycine deprivation, as suggested by the increased levels of both PHGDH and PSAT1 in the SG- condition of siNT-treated cells (**Figure 4.7.B**).

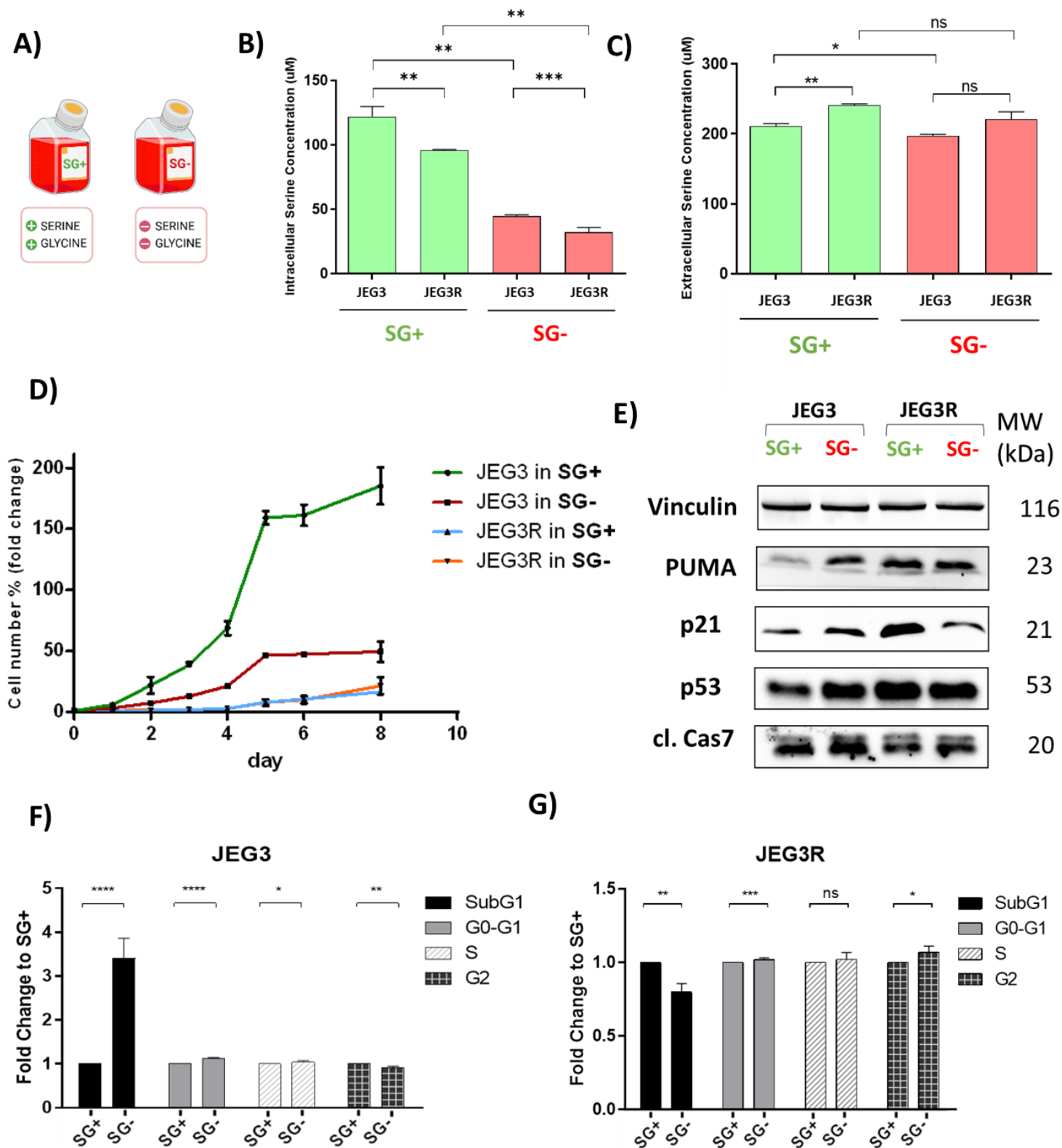
We tested then whether exogenous serine and glycine depletion from the medium would affect the proliferation of these cells. Regarding JEG3-R cells, the absence or presence of these two amino acids had no impact on their proliferation rate (**Figure 4.6.D**). On the contrary, in SG- conditions, JEG3 cells proliferated much slower than in SG+ conditions, despite the fact that JEG3 cells are expected to have a more active SSP (**Figure 4.6.D**). This result suggests that JEG3 cells rely more on the uptake of extracellular serine and glycine than JEG3-R cells, to fulfill their needs.

To check whether this slower growth rate is due to p53-mediated apoptosis or cell cycle arrest, the expression status of critical proteins contributing to these processes was evaluated at the protein level. p53 levels were elevated in both cell lines upon SG deprivation and those for p21, a downstream target of p53 and indicator of cell cycle arrest (El-Deiry *et al.*, 1993), slightly increased in JEG3 cells, but decreased in the case of the resistant cells, when in SG- conditions (**Figure 4.6.E, Supplementary Figure 4.5**). On the other hand, PUMA, the p53-upregulated modulator of apoptosis (Nakano and Vousden, 2001), showed higher protein expression upon SG deprivation in JEG3 cells, but remained unchanged in the JEG3-R cells (**Figure 4.6.E, Supplementary Figure 4.5**). The levels of the cleaved Caspase 7 (executioner Caspase) were also elevated in the SG- condition of JEG3 cells and unchanged in the case of JEG3-R cells (**Figure 4.6.E, Supplementary Figure S4.6**). Collectively these data indicate that the intrinsic apoptotic pathway is activated in JEG3 cells upon SG depletion.

This induction of this apoptotic effect phenotype was confirmed upon the cell cycle profile analysis of JEG3 cells following cellular staining with propidium iodide (PI). This dye binds DNA and the resulting fluorescence intensity measured by flow cytometry is proportional to the cellular DNA content. The apoptotic fragmentation of DNA can be visualized using this stain as the appearance of a fluorescent peak situated below that of the G1 phase of the cell cycle (aka

Sub-G1 peak) (Kastan, 1997). As shown in **Figure 4.6.F**, the percentage of cells in Sub-G1 is elevated in JEG3 cells cultured in SG- medium while that of the cells in the S phase remains unchanged, indicating that JEG3 cells undergo apoptosis in serine and glycine-deprived conditions. On the other hand, Serine/Glycine deprivation doesn't seem to perceptibly disturb any phase of the cell cycle of JEG3-R cells (**Figure 4.6.G**). Overall, JEG3-R cells seem to better adapt to serine/glycine depletion as compared to JEG3 cells and this may be through the channeling of serine into pathways crucial for cell survival.

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Figure 4.6: JEG3 cells undergo apoptosis upon Serine and Glycine starvation while JEG3-R cells adjust more efficiently under such conditions.

A) Two reconstituted growth media, SG+ and SG- representing two different conditions, the availability of Serine and Glycine in the medium and the absence of them respectively. **B)+C)** Intracellular **(B)** and extracellular **(C)** levels of D-Serine in JEG3 and JEG3R in SG+ and SG- conditions. **D)** Proliferation assay for JEG3 and JEG3R in both SG+ and SG- conditions. **E)** Immunoblots of p53, PUMA, p21, and cleaved Caspase-3 and Caspase-7. Vinculin was utilized as a loading control. The data show the mean fold changes $\pm$ SEM from at least 3 independent experiments. **F)** Cell cycle profile of JEG3 and JEG3R with or without SG deprivation. The cells were subjected to propidium iodide (PI) staining prior to flow cytometry analysis. The bars are indicating the percentage population of cells within the SubG1, G0-G1, S and G2 phases of the cell cycle. The data show the average fold changes $\pm$ SEM from at least 3 independent experiments. Statistical significance was determined using the Unpaired Student's t-test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, ns=not significant).

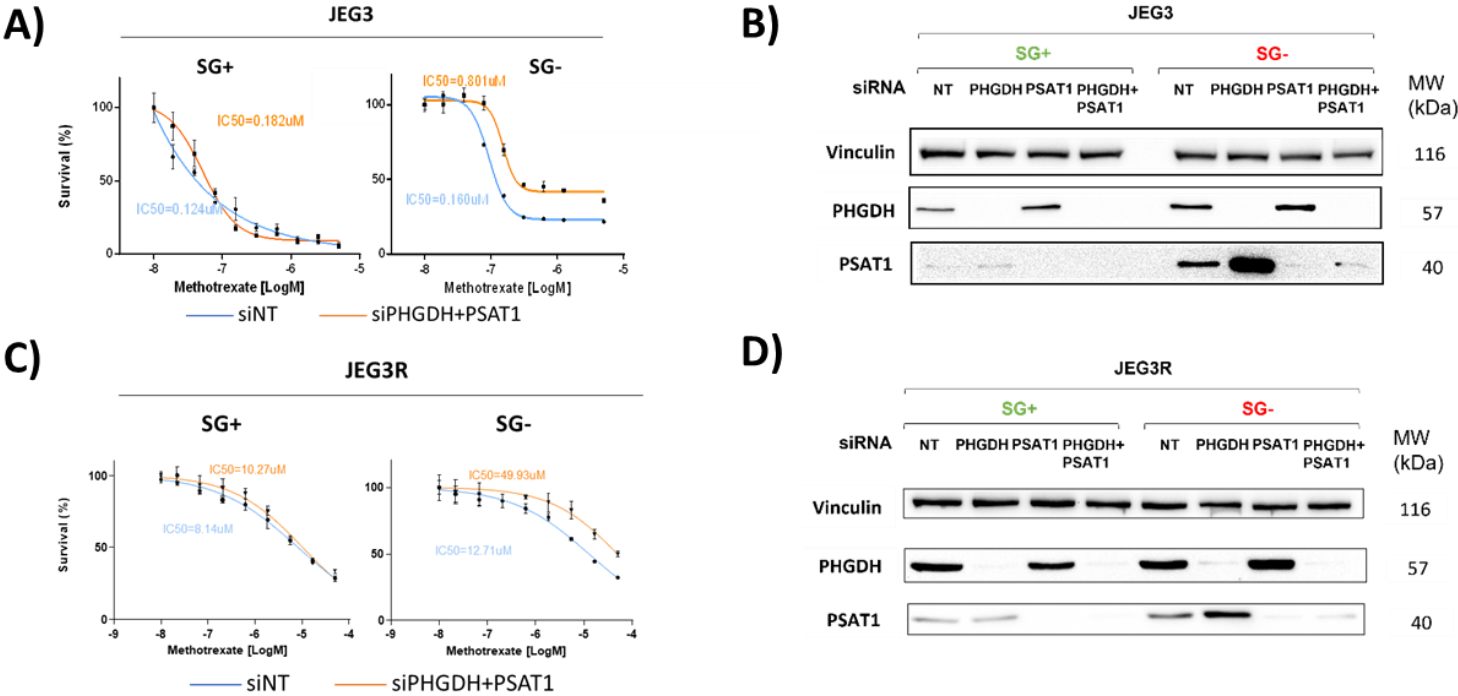
4.2.7 Dual *de novo* serine synthesis inhibition and SG- deprivation from the growth medium promotes MTX resistance in both JEG3 and JEG3-R

We tested next whether SSP inhibition in combination with or without serine starvation affected the MTX sensitivity of our cells. siRNAs were used to downregulate the first two enzymes of the SSP, PHGDH and PSAT1, either as single or together. The siRNA-mediated downregulation of these enzymes had no effect on the MTX sensitivity of either JEG3 or JEG3-R cells in the SG+ condition, indicating that even when *de novo* serine synthesis is inhibited, the cells can rely on extracellular serine and glycine uptake (**Figure 4.7.A**). In contrast, concomitant inhibition of the SSP enzymes and serine and glycine depletion promoted resistance of these cells to MTX. The IC₅₀ of JEG3 cells silenced for both PHGDH and siPSAT1 increased 5 times compared to the non-targeting siRNA-treated cells (JEG3 siNT IC₅₀: 0.16uM, JEG3 siPHGDH+siPSAT1 IC₅₀: 0.801uM) (**Figure 4.7.C**). In the case of JEG3-R cells, the IC₅₀ increased 4 times (JEG3-R siNT IC₅₀: 12.71uM, JEG3-R siPHGDH+siPSAT1 IC₅₀: 49.93uM) (**Figure 4.7.C**). So, intra- and extra-cellular serine levels impact on the activity of MTX with serine/glycine depletion promoting resistance of choriocarcinoma cells to this drug.

To independently confirm results obtained through genetic downregulation, a pharmacological approach was carried out. We combined the PHGDH inhibitor, NCT-503, which

Unveiling the interplay between MTX-resistance, Serine Synthesis, 1-Carbon Metabolism, and Integrated Stress Response (ISR) signaling pathway in Choriocarcinoma cells

has been reported to effectively reduce glucose-derived serine production (Pacold *et al.*, 2016), together with MTX in the presence or absence of extracellular Serine/Glycine. This combination was antagonistic in JEG3 cells, whether in the presence or absence of Serine/Glycine (average ZIP score of -26% for SG+ and of -18.4% for SG- conditions) (**Figure 4.7.E**). In the case of JEG3-R cells, the antagonistic effect was even more pronounced (average ZIP score of -39.71% for SG+ and of -46.15% for SG- conditions) (**Figure 4.7.E**). Hence, pharmacological inhibition of PHGDH reproduces the effects of genetic silencing in SG- conditions and extends the antagonism with MTX to SG+ conditions. This suggests that decreased intracellular availability of Serine/Glycine is associated with resistance to MTX.



Unveiling the interplay between MTX-resistance, Serine Synthesis, 1-Carbon Metabolism, and Integrated Stress Response (ISR) signaling pathway in Choriocarcinoma cells

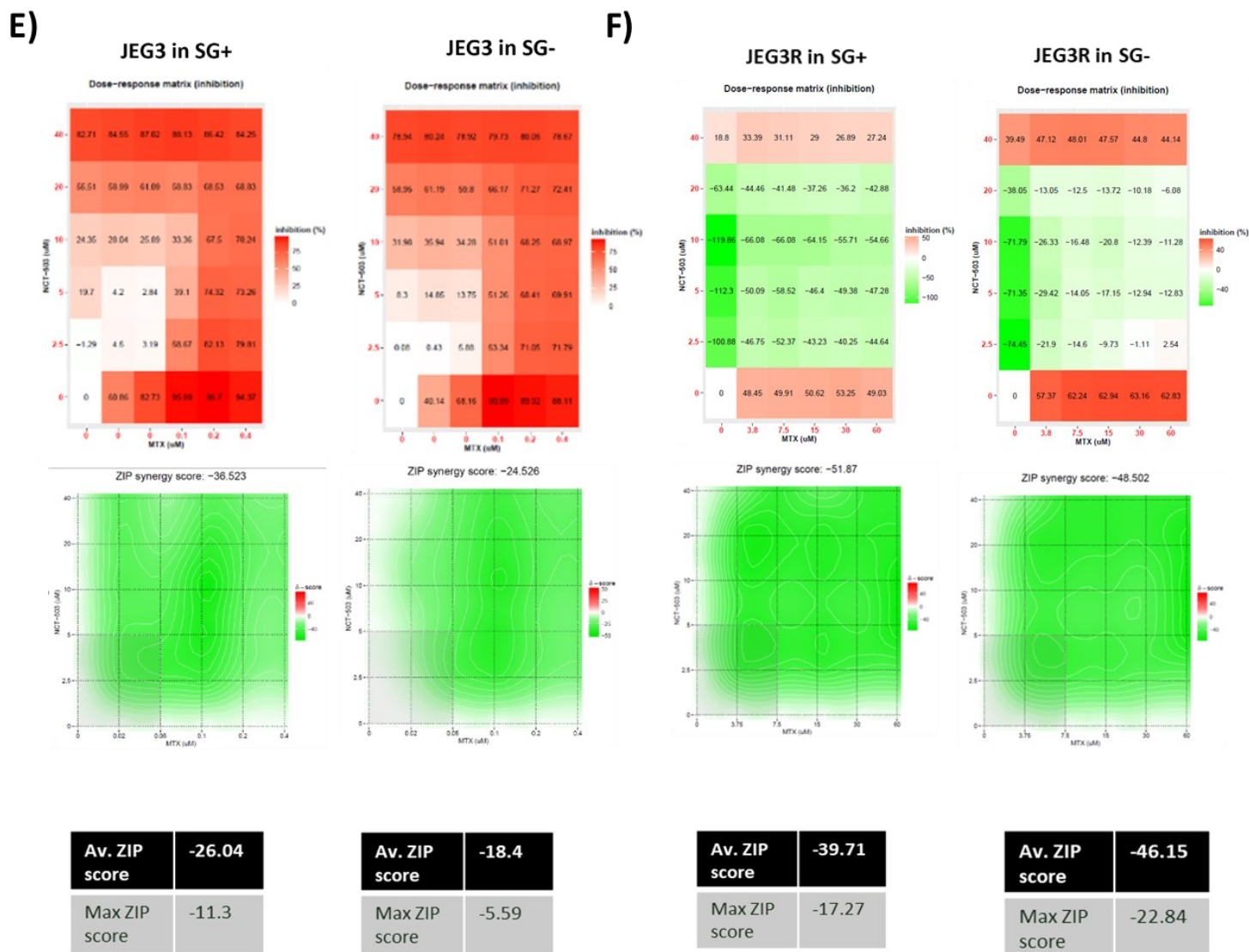


Figure 4.7: Dual genetic or pharmacological de novo serine synthesis inhibition in combination with SG- deprivation from the growth medium increases the MTX resistance of both JEG3 and JEG3-R.

A) and **C)** IC-50 Graph of MTX at siRNA treated JEG3 (**A**) and JEG3-R (**C**) cells. Data show the mean fold changes $\pm$ SEM from at least 3 independent experiments.. **B)** and **D)** Immunoblots depicting the efficacy of siRNA-mediated downregulation of PHGDH+PSAT1 in JEG3 (**B**) and JEG3-R (**D**) cells. Vinculin was utilized as a loading control. The data represent 3 independent biological replicates. **E)** and **F)** Visualization of the combination treatment of NCT-503 and MTX in JEG3 (**E**) and JEG3-R (**F**) in both SG+ and SG- conditions. SunergyFinder 2.0 online web app was used to calculate synergy scores between the two compounds. The Zero interaction potency (ZIP) model was utilized and ZIP scores <-10 highlight antagonism, while ZIP scores >10 demonstrate synergism. The upper panels depict cell viability results as a function of the concentration of both compounds. The lower panels depict the results of synergy calculations. The average ZIP score was calculated by collecting data from three independent experiments.

To further confirm this conclusion, we conducted a rescue experiment through the overexpression of both PHGDH and PSAT1 in JEG3-R cells and parallel deprivation of the medium from serine and glycine. This rescued the sensitivity of these cells to MTX as shown by an ~7-fold decrease in the IC₅₀ values for this compound in cells overexpressing PHGDH and PSAT1 in comparison with cells transfected with the corresponding empty vector (pCMV3) (**Figure 4.8.A**). It was interesting to note that overexpression of PSAT1 was accompanied by a decrease in DHFR levels, which may participate in the observed sensitization to MTX (**Figure 4.8.B**). A similar but lesser effect on MTX sensitivity was observed for cells in the SG+ condition (**Supplementary Figure 4.6**).

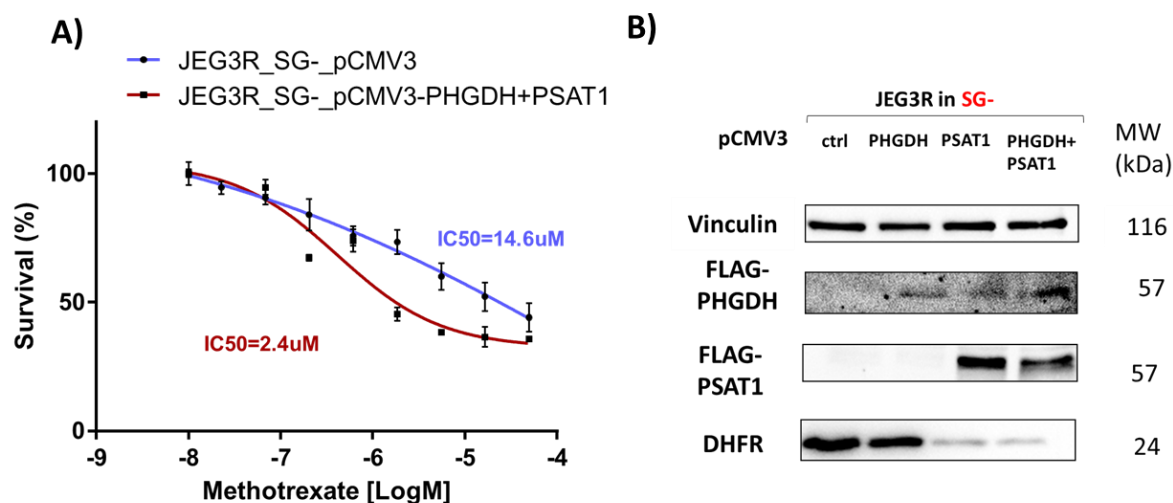


Figure 4.8: pCMV3-mediated overexpression of both PHGDH and PSAT1 in JEG3R cells and concomitant depletion of the medium from serine and glycine rescued the phenotype by reducing the resistance of these cells to MTX.

A) IC₅₀ Graph of MTX at JEG3R_pCMV3_PHGDH+PSAT1 versus JEG3R_pCMV3 cells in SG- condition. Data show the average fold changes $\pm$ SEM from at least 3 independent experiments. **B)** Immunoblots showing the expression of flagged-PHGDH and flagged-PSAT1 and DHFR. The flagged versions of PHGDH and PSAT1 serve as controls here indicating the successful overexpression of each protein. Vinculin was utilized as a loading control. The data represent 3 independent biological replicates.

4.3 Discussion

Our published results so far have shown that, upon MTX resistance, JEG3-R cells undergo cell cycle arrest following p21 upregulation in response to p53 stabilization. This is accompanied by increased DNA repair through non-homologous end-joining pathway (Georgiou *et al.*, 2022). In this chapter, we showed that these cells also rewire their metabolism, with a decrease in the expression of serine *de novo* pathway enzymes and an accompanying decrease in Serine intracellular levels. There is also a possible redirection of extracellular and glucose-derived serine towards GSH production and increased methylation capacity (**Figure 4.1.D**). Concomitantly, the salvage nucleotide pathway is promoted. On the other hand, JEG3 cells appear to spend 1-carbon fuels, serine and glycine for nucleotide synthesis and sustenance of proliferation as indicated by the increased proliferation compared to the resistant cells in normal conditions (**Figure 4.6.D**).

The preference of JEG3-R for the salvage pathway over *de novo* nucleotide synthesis was further validated with a combination treatment of MTX and Dipyridamole, a uridine import inhibitor. This combination was synergistic, sensitising JEG3-R to MTX (**Supplementary Figure 4.2**). Dipyridamole was previously reported to increase the effectiveness of MTX in solid tumours ((Kinsella, Smith and Pickard, 1997),(Higano and Livingston, 1989)). The combination of anti-folates such as MTX with inhibitors of the pyrimidine ribonucleotide salvage pathway now requires further investigation.

Elevated GSH and GSTT1 levels in JEG3-R cells support the hypothesis that these cells use serine to promote GSH synthesis which is then used for detoxification of MTX. Although ROS are often associated with oxidative damage, they can also function as signaling molecules triggering cellular pathways involved in stress response and adaptation ((Raamsdonk, 2015), (Sena and Chandel, 2012)). Maintenance of increased ROS levels in JEG3-R compared to JEG3 cells (**Figure 4.2.D**) suggests a potential mechanism by which these cells can withstand the cytotoxic effects of MTX. The activation of survival pathways in response to increased ROS could contribute to MTX

resistance. Quenching of ROS species in JEG3R should be performed in the future to further test this hypothesis.

We showed that JEG3-R cells have elevated levels of the epigenetic mark H3K27me3 and of the histone methyltransferase which attaches methyl groups to lysine 27 of histone H3 (EZH2). This would suggest that, compared to sensitive cells, JEG3-R cells have more genomic areas shielded by DNA methylation. There is also a reported link between EZH2, MYC and SSP enzymes; in Burkitt lymphoma cells, increased H3K27me3 leads to the blocking of miR-494 and subsequent translational repression of MYC, a transcription factor for PHGDH and PSAT1, through a feedback loop in which MYC maintains high levels of H3K27me3 by upregulating EZH2 methyltransferase (Białopiotrowicz et al., 2020). This is consistent with the decreased levels of c-MYC in JEG3-R compared to JEG3 cells (**Figure 4.4.B**). Treatment with the DNA methyltransferase inhibitor Zebularine increased PSAT1 expression but at the same time increased the levels of both H3K27me3 and H3K9me3 marks in our cells. This may suggest that other epigenetic marks repressing the expression of PSAT1 were demethylated, allowing its transcription. As for PHGDH, other chromatin-modifying enzymes or transcription factors may be involved in its regulation in our system.

Among transcription factors regulating the transcription of the SSP genes, PHGDH and PSAT1, p53 appeared as a master negative regulator. Increased levels and activity of p53 in JEG3-R cells seem responsible for the downregulation of the SSP enzymes, as suggested by our siRNA-mediated downregulation experiments (**Figure 4.4.D**). This occurred independently of any effect on ATF4, a transcription factor repeatedly involved in driving PHGDH and PSAT1 transcription (Wu and Liang, 2024) (**Figure 4.4.C**). However, and in accordance with the literature, silencing of ATF4 made JEG3-R cells even more resistant to MTX (**Figure 4.4.F**). Taken together, our data suggests that, while ATF4 contributes to the MTX resistance of JEG3-R cells, this effect is eclipsed by the role of p53 action in our cell system, and an interplay between these two transcription factors may exist.

To try and clarify this interplay, pathways upstream of SSP and ATF4 were tested. Results of this chapter indicated that ISR signaling is activated in JEG3-R cells as suggested by GCN2-

mediated phosphorylation and activation of eIF2 α . This would suggest that the global translation machinery is downregulated in this cell line, possibly to the benefit of a selective protein translation program (Deval *et al.*, 2009). ATF4 is one of the proteins regulated by such a program and we suggest that autophagy may be induced in JEG3-R cells due to the upregulation of target genes of ATF4 such as ATG5 (**Figure 4.5C,4.5.H**). This hypothesis was supported by the findings that CHOP was upregulated in JEG3-R cells (**Figure 4.5C,4.5.H**), as this protein interacts with ATF4 to promote the expression of ATG5 and the activation of the metabolic stress-sensor AMPK, promoting autophagy (B'Chir *et al.*, 2013). ISR-induced autophagy would be a mechanism through which the resistant choriocarcinoma cells could promote their survival by providing nutrients and energy during chemotherapy-induced stress. Future experiments are necessary to definitively validate this hypothesis.

Finally, the hypothesis that GCN2-mediated activation of ISR signaling is implicated in the MTX resistance of JEG3-R cells was supported by the effects of the combination of MTX with GCN2 inhibitors (**Figure 4.5.I-J**). Both GZD-824 and A-92 sensitized JEG3-R cells to MTX, but only in high concentrations. GCN2 inhibitors as monotherapy or in combination with Methotrexate at an appropriate dosage should be tested further and taken under consideration as a potential therapeutic approach in resistant choriocarcinoma.

Our experiments further demonstrated that JEG3-R cells are more resilient to Serine/Glycine deprivation than JEG3 cells, which triggered the apoptotic pathway in response to this stimulus (**Figure 4.6**). The resilience of JEG3-R cells could result from the channeling of 1-carbon fuels to pathways more crucial to their survival, such as autophagy and GSH synthesis. Upstream signals responsible for this may involve ISR signaling which is activated by GCN2-mediated phosphorylation of eIF2 α upon nutrient deprivation (Pakos-Zebrucka K *et al.*, 2016).

Finally, concomitant Serine/Glycine depletion from the growth medium with either genetic or pharmacological inhibition of the *de novo* SSP triggered MTX-resistance in both JEG3 and JEG3-R cells, with the effect being more pronounced in the former (**Figure 4.7**). Therefore, our data support the notion that intra- and extra-cellular serine levels impact MTX sensitivity. This was further validated by the ability of overexpression of SSP enzymes to sensitise JEG3-R cells to

MTX under Serine/Glycine deprivation (**Figure 4.8**). Hence, one could propose that therapeutically targeting upstream signalling pathways to promote SSP may be a valid strategy to sensitise resistant patients to MTX treatment. More work is now required to understand which upstream pathways could be so targeted.

5. Investigation of common MTX-Resistance mechanisms across different cancer types

5.1 Introduction

Methotrexate (MTX) is a chemotherapy drug commonly used in the treatment of various cancer types, though it is not considered a first-line treatment for most of them. MTX is mainly combined with other drugs in many different neoplasms such as osteosarcoma (Crews *et al.*, 2004), breast cancer (Society, 1995), and colon cancer (Wu *et al.*, 2017). Nevertheless, the emergence of resistance in these cancer cells frequently compromises the efficacy of this chemotherapeutic drug (Schlappack, Zimmermann and Hill, 1991), as we have already seen in choriocarcinoma cells (Sakai *et al.*, 1989).

Following the altered metabolism pattern seen in the MTX-resistant choriocarcinoma cells versus their sensitive counterparts, we wanted to investigate whether this is the typical pattern observed in other MTX-Resistant cells as well. Firstly, we generated a new MTX-Resistant human osteosarcoma cell line, the U2OS-MTX-Resistant. This cell line originated after prolonged exposure of U2OS cells in MTX, in the same way the JEG3-Resistant cells were generated. U2OS cells are epithelial and U2OS-R cells acquired a mesenchymal phenotype suggesting that the cells have undergone Epithelial to Mesenchymal Transition (EMT). Key proteins of the Serine Synthesis Pathway (SSP), folate and methionine cycles were tested in U2OS-R/U2OS and the expression pattern was compared to that of the JEG3-R/JEG3. The similarities observed in the expression of these proteins suggest that choriocarcinoma and osteosarcoma might share common mechanisms underlying MTX Resistance.

Having a pair of sensitive and (lab-made) MTX-resistant osteosarcoma cell lines was convenient for comparisons but not enough. Our collaborators provided us with 7 more pairs of human MTX-Sensitive and MTX-Resistant cell lines. One of them was the osteosarcoma cell line SAOS-2/SAOS-2-MTX-R, four of them were the triple-negative breast cancer cell lines MDA-MB-436/MDA-MB-436-MTX-R, Cal51/Cal51-MTX-R, HCC-1806/HCC-1806-MTX-R and two of them were the colon carcinoma cell lines SW-480/SW-480-MTX-R and LoVo/LoVo-MTX-R. These cell lines were tested to assess whether they displayed similar metabolic changes to those previously seen in the JEG3-R vs JEG-3 cells.

Here we show that one MTX-Resistant breast cancer cell line (*MDA-MB-436-R*) and one MTX-Resistant osteosarcoma cell line (*SAOS-2-R*) had the same expression pattern for the proteins PHGDH, PSAT1, DHFR and MTHFR previously seen in the MTX-Resistant choriocarcinoma cell line JEG3-R. Moreover, four out of seven MTX-Resistant cell lines tested here seem to have downregulated *de novo* Serine synthesis, further supporting our statement that Serine metabolism is affecting response of the cancer cells to Methotrexate.

5.2 Results

5.2.1 Establishment of a new MTX-Resistant osteosarcoma cell line

The MTX-Resistant osteosarcoma cell line, U2OS-MTX-R (or else U2OS-R), derived from its sensitive counterpart, U2OS cell line. U2OS cells were exposed to progressively increasing concentrations of MTX starting from 0.5nM up to 40µM (**Figure 5.1.A**). The selection process spanned 18 months and consisted of repeated cycles of MTX treatment, removal of dead cells, and a brief recovery period for surviving cells in smaller culture flasks. Once the cells regained health and reached 60% confluency, they were exposed to progressively higher MTX concentrations (0.5µM -1µM -2µM - 3µM - 5µM - 7µM - 10µM - 12µM - 15µM - 17.5µM - 20µM - 25µM - 30µM – 40µM). The response of the cells to MTX was periodically tested and compared to the sensitive U2OS cells. According to the MTX IC₅₀ values that were calculated via a cell survival assay, the U2OS-R cells became almost 9 times more resistant to MTX compared to JEG3 (Average values: U2OS MTX-IC₅₀=4.2µM ,U2OS-R MTX- IC₅₀ =37.6uM, p-value=0.06) (**Figure 5.1.B**).

The protein levels of DHFR, the main target of MTX, were tested and did not give the anticipated results given the MTX-resistance level of U2OS-R cells (**Figure 5.1.C-D**). DHFR protein was only slightly elevated in U2OS-R compared to U2OS, not resembling the overexpression observed in JEG3-R cells compared to the sensitive counterparts (**Supplementary Figure 4.3, Figure 5.5.D**). This observation suggests that in the case of these osteosarcoma cells, MTX-resistance and DHFR expression levels do not have a substantial cause-and-effect relationship.

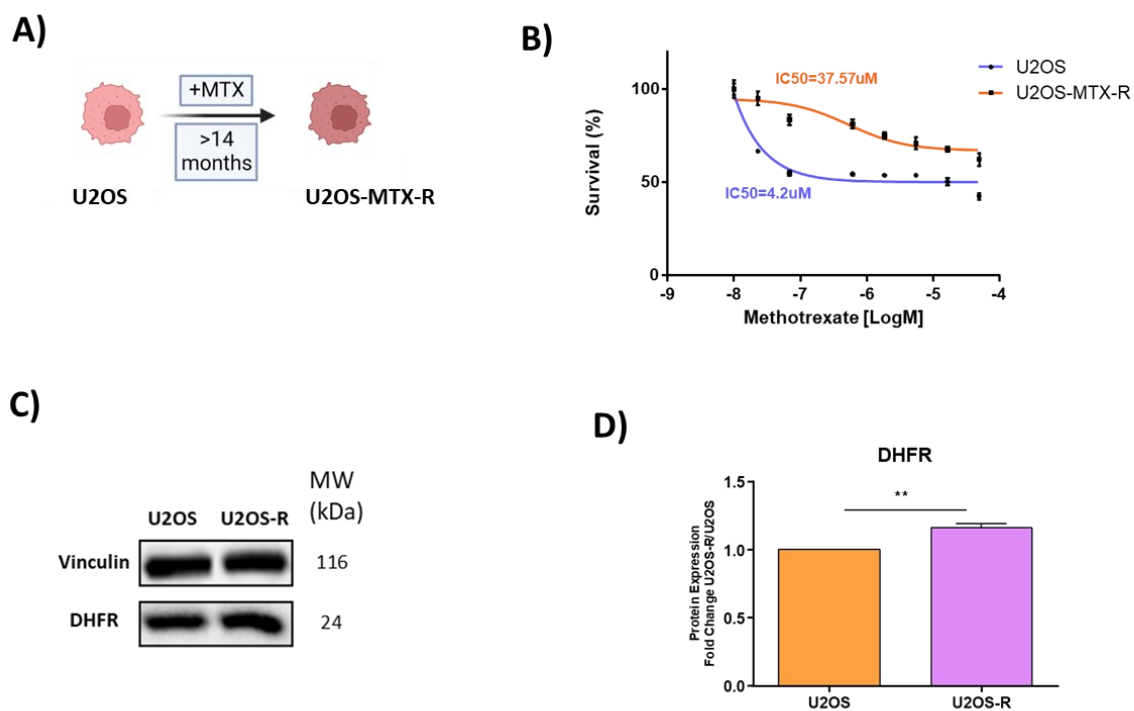


Figure 5.1: Generation of Methotrexate-Resistant Osteosarcoma Cell Line U2-OS .

A. U2OS-MTX-R cells were generated after prolonged exposure of U2OS cells to increasing concentrations of MTX. **B.** IC₅₀ Graph of MTX at U2OS versus U2OS-MTX-R cells. **C.** Immunoblots showing the expression of DHFR in U2OS-R and U2OS cells Vinculin was utilized as a loading control. The data represent 3 independent biological replicates. **D.** Bar plot showing the fold change ratio U2OS-R/U2OS for DHFR protein expression. The data show the mean fold changes $\pm$ SEM from 3 independent experiments. Statistical significance was determined using the Unpaired Student's t-test (** $p \leq 0.01$).

Then, the morphology of the cells under the microscope was tested. U2OS is a cell line with epithelial morphology and organized structure with a flattened, polygonal shape and well-defined edges (**Figure 5.2.A-C**). They are quite adherent and are actively dividing forming a monolayer on the culture surface. The U2OS-R cells exhibited alterations in their morphology compared to their non-resistant counterparts. The resistant cells appear more elongated and spindle-shaped indicating that they have acquired a mesenchymal phenotype (**Figure 5.2.A-C**). This phenomenon is called Epithelial to Mesenchymal Transition (EMT) and is usually observed in cancer cells developing resistance to chemotherapeutic agents ((Fischer *et al.*, 2016),(Zeng *et al.*,

2019)). U2OS-R cells seem also to have acquired the ability of forming colonies (**Figure 5.2.A**) a characteristic which partially explains their survival ability even in high concentrations of MTX (Curt *et al.*, 1985). Additionally, U2OS-R cells lost their cell polarity and cell-cell adhesion that we observe in U2OS cells, suggesting their potential migratory and invasive properties as mesenchymal and not epithelial cells (Thiery *et al.*, 2009). Finally, the resistant osteosarcoma cells proliferated in a slower manner than the sensitive ones based on the visual inspection of both cell lines under the microscope, although this was not quantified experimentally

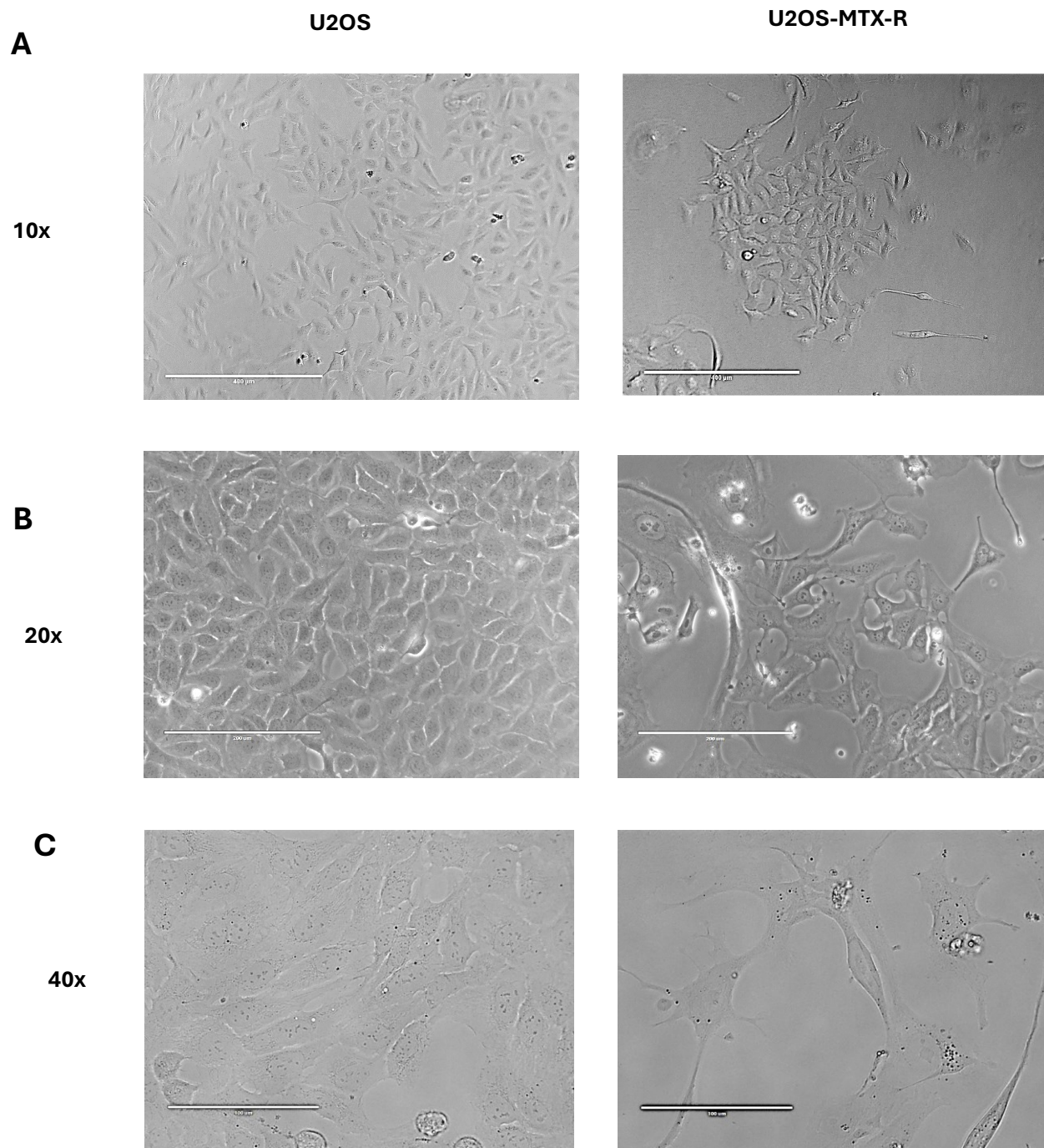


Figure 5.2: U2OS-R cells acquired a mesenchymal phenotype.

Captured images in EVOS M7000 microscope with transmitted light channel (Brightfield) on U2OS and U2OS-MTX-R cells in 10x (A), 20x(B) and 40x (C) magnifications. Scale bar: 400μm (A), 200μm (B), 100μm (C).

In light of our findings in Chapter 4, we tested the protein expression levels of the *de novo* Serine biosynthesis pathway, PHGDH, PSAT1 and PSPH. Interestingly, all three enzymes were found to be statistically downregulated in U2OS-R cells compared to U2OS (**Figure 5.3.A-B**). The fact that downregulation of Serine synthesis pathway is observed in another MTX-Resistant cell line other than JEG3-R cells, further suggests that MTX-Resistance and Serine metabolism may be interconnected.

Apart from these enzymes, important enzymes of 1-Carbon metabolism were also tested to investigate any other similarities to the choriocarcinoma pair of cell lines. Intriguingly TK1 (Thymidine kinase 1) and MTHFR (Methylenetetrahydrofolate reductase) were found to be overexpressed in U2OS-R compared to U2OS while TYMS (Thymidylate synthase) is overregulated in U2OS compared to U2OS-R cells (**Figure 5.3.A-B**). These results are in full alignment with the results in choriocarcinoma cell lines and allow us to hypothesize that both resistant cell lines recruit similar cellular mechanisms to establish their survival and resistance to MTX. Even though further exploration of the metabolism of osteosarcoma cells was not carried out in the context of this thesis, further experimentation in the future could validate the hypothesis or introduce new ones. Based on the results from the protein levels of the SSP and 1-Carbon metabolism enzymes and given the fact that U2OS-R were generated in the same way as the JEG3-R cells, we postulate the following; TK1 overexpression in U2OS-R cells, TYMS overexpression in U2OS cells and the slower proliferation rate of U2OS-R cells could indicate that the sensitive cells prefer to utilize the 1-carbon units in the nucleotide synthesis for the maintenance of their proliferation. On the other hand, U2OS-R cells might rely on less energy-demanding processes for their survival and promote the salvage nucleotide synthesis, like JEG3-R cells do. In addition, MTHFR overexpression is observed in U2OS-R cells (**Figure 5.3.A-B**) as well as in JEG3-R cells (**Figure 4.1.A**). More data is needed to assess the outcome of this enzyme change on the folate and methionine cycles. Nevertheless, there are several reports supporting that the outcome of MTX treatment in patients with osteosarcoma is strongly associated with polymorphisms of MTHFR gene ((Xu *et al.*, 2018), (Zhang *et al.*, 2021), (Xie *et al.*, 2018), (Labarque and Laenen, 2017), (Park and Shin, 2016)).

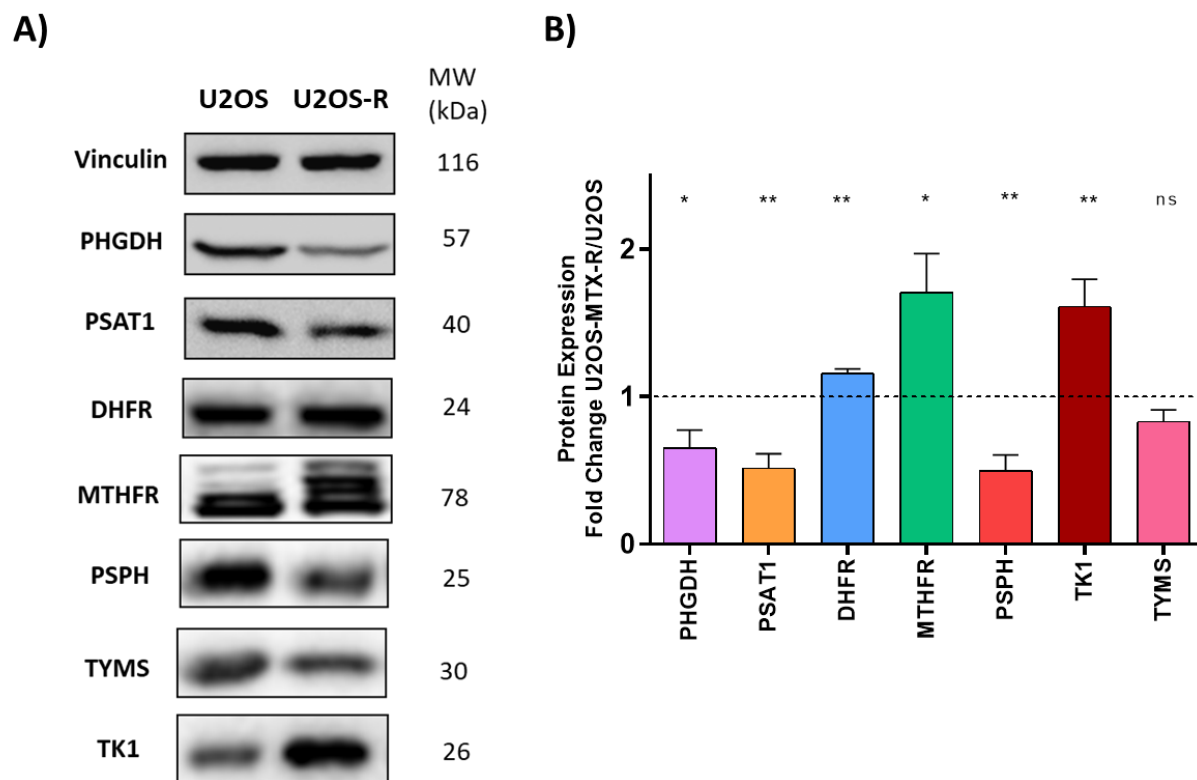


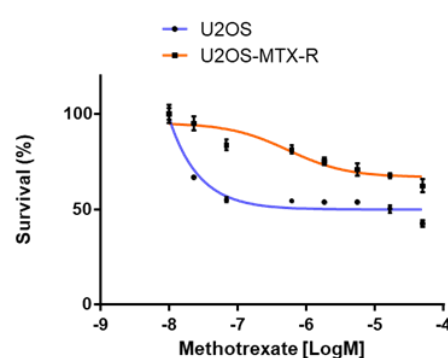
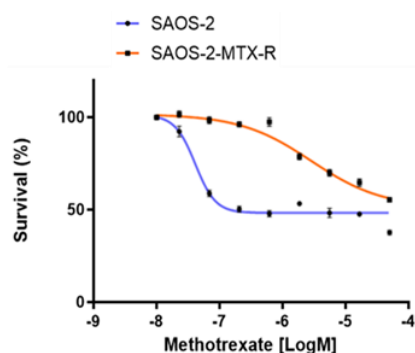
Figure 5.3: U2OS-R cells seem to have a similar metabolic pattern to JEG3-R cells.

A) Immunoblots showing the expression of proteins involved in the Serine Synthesis pathway and 1-Carbon Metabolism. Vinculin was utilized as a loading control. The data represent 3 independent biological replicates. **B)** Bar plots showing the fold change U2OS-R/U2OS ratio in the tested enzymes. The data show the mean fold changes $\pm$ SEM from 3 independent experiments. Statistical significance was determined using the Unpaired Student's t-test (** $p \leq 0.01$).

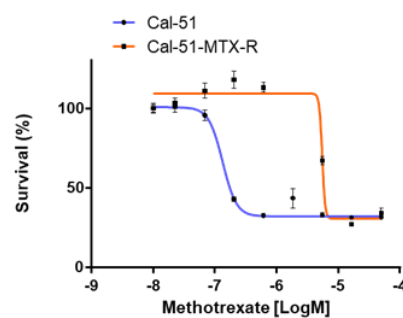
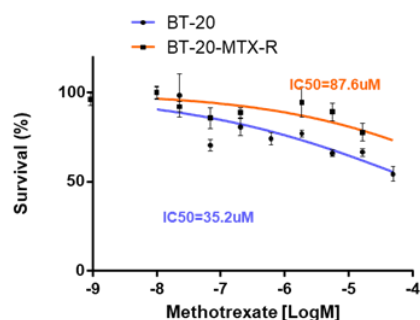
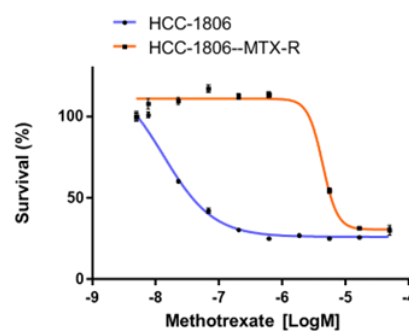
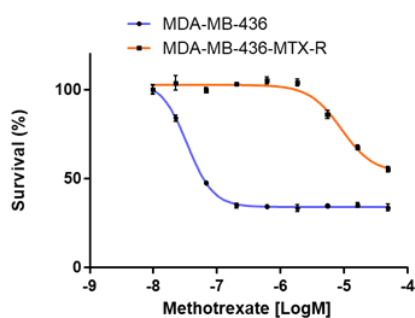
Since we detected the same metabolic pattern in both MTX-resistant choriocarcinoma and osteosarcoma cell lines, we wondered whether we could find any similarities in MTX-resistant cell lines from other cancer types. Our collaborators gifted us with 7 pairs of human sensitive and MTX-Resistant cell lines; one osteosarcoma cell line pair (SAOS-2/SAOS-2-MTX-R), four pairs of breast cancer cell lines (MDA-MB-436/MDA-MB-436-MTX-R, Cal51/Cal51-MTX-R, HCC-1806/HCC-1806-MTX-R) and two colon cancer cell lines (SW-480/SW-480-MTX-R and LoVo/LoVo-MTX-R).

R). The first action carried out after receiving, thawing and growing the cell lines in cell culture, was to determine the MTX response of each cell line (**Figure 5.4 and Table 5.1**). This result provided information on the relative resistance of each cell line for MTX. According to **Table 5.1**, the most resistant cell line is BT-20-MTX-R (87.6uM) and is almost 2.5 times more resistant than BT-20 cells (35.2uM). LoVo-MTX-R cells were the least MTX-resistant (1.88uM) but were 188 times more resistant than their parent non-MTX-R LoVo cells. The biggest shift towards resistance happened in the colon cancer cell line SW-480 since SW-480-MTX-R (16.44uM) increased their resistance to MTX 1644 more times than SW-480 cells (0.01uM). The MTX-resistant osteosarcoma cell line we got from our collaborators, is 72 times more resistant than the sensitive one (SAOS-2-MTX-R: 2.16uM and SAOS-2: 0.03uM). The fold change ratio of the breast cancer cell lines-pair Cal51-MTX-R/Cal-51, equals 47. The other two resistant breast cancer cell lines MDA-MB-436-MTX-R and HCC-1806-MTX-R are 298 and 248 times respectively more resistant than the sensitive cell lines they were derived from (MDA-MB-436-MTX-R: 8.94uM, MDA-MB-436: 0.03uM and HCC-1806-MTX-R: 4.96uM, HCC-1806: 0.02uM).

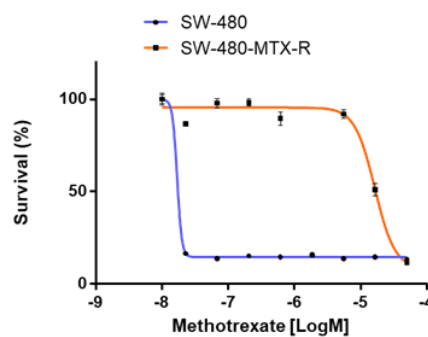
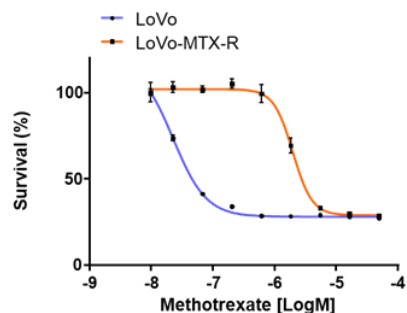
A) Osteosarcoma Cell Lines



B) Breast Cancer Cell Lines



C) Colon Cancer Cell Lines



Investigation of common MTX-Resistance mechanisms across different cancer types

Figure 5.4: Testing of MTX response in MTX-Sensitive and MTX-Resistant cell lines.

IC₅₀ Graphs of MTX at pairs of MTX-Sensitive versus MTX-Resistant osteosarcoma **(A)**, breast cancer **(B)**, and colon cancer **(C)** cell lines. IC₅₀ values for MTX were calculated from the curve utilizing GraphPad Prism. The data represent 3 independent biological replicates.

Table 5.1: MTX IC₅₀ values for MTX-Sensitive and MTX-Resistant cell lines.

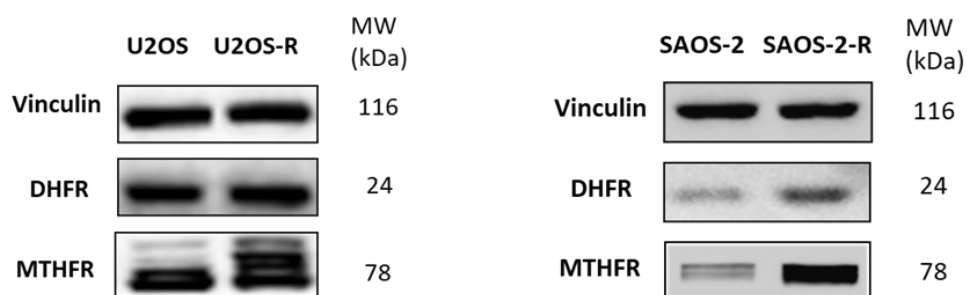
Cancer Type	Cell Line	MTX IC ₅₀ (uM)	Fold Change Ratio MTX IC ₅₀ R/S	P-value
Osteosarcoma	U2OS	4.2	8.92	ns
	U2OS--MTX-R	37.5		
	SAOS-2	0.03	72	*
	SAOS-2-MTX-R	2.16		
Breast Cancer	MDA-MB-436	0.03	298	*
	MDA-MB-436-MTX-R	8.94		
	HCC-1806	0.02	248	**
	HCC-1806-MTX-R	4.96		
	BT-20	35.2	2.48	ns
	BT-20-MTX-R	87.6		
	Cal51	0.09	47.66	*
	Cal51-MTX-R	4.29		
Colon Cancer	LoVo	0.01	188	***
	LoVo--MTX-R	1.88		
	SW480	0.01	1644	****
	SW480-MTX-R	16.44		

The table displays the IC₅₀ values for MTX in pairs of MTX-Sensitive and MTX-Resistant cell lines from osteosarcoma, breast cancer, and colon cancer and the fold change ratio of MTX IC₅₀ Resistant/Sensitive for each pair of tested cell line. It also includes the statistical significance of the differences, (**p*<0.005, ***p*<0.001, ****p*<0.0005, *****p*<0.0001, ns=not significant).

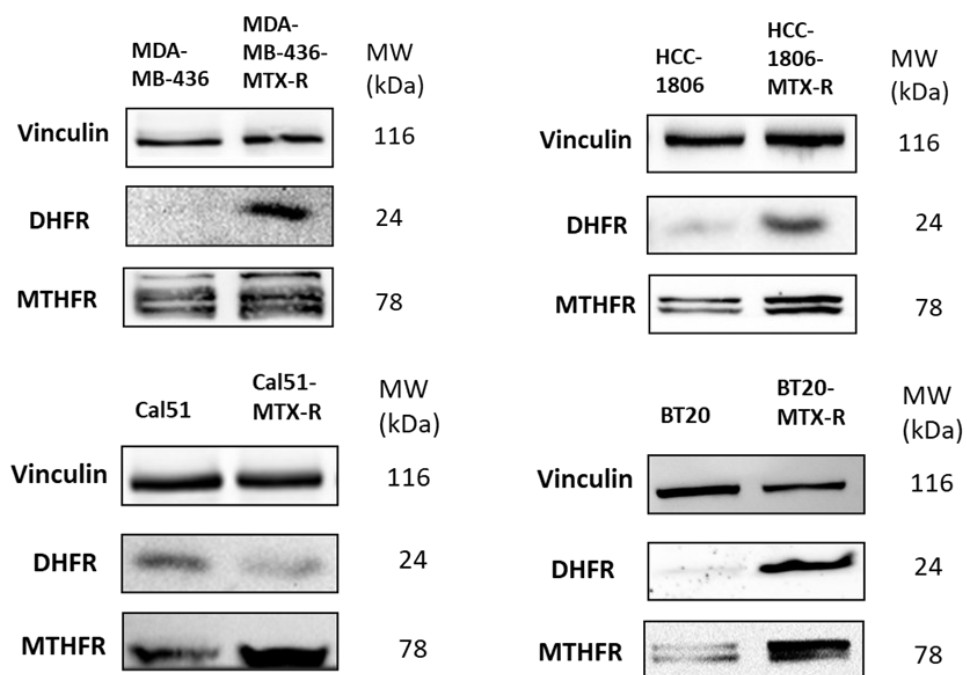
Next, we tested DHFR and MTHFR protein levels in these sensitive and resistant to MTX cell lines (**Figure 5.5.A-E**). Elevated levels of DHFR have been linked to methotrexate resistance in several types of cancer, including osteosarcoma (Eug *et al.*, 2019), breast (Cowan *et al.*, 1982) and colon cancer (Morales *et al.*, 2009). We observed that DHFR levels are elevated in both (tested here) osteosarcoma MTX-resistant cell lines, U2OS-R (1.2) and SAOS-2-R (2.4 fold) compared to their sensitive ones, with that of SAOS-2-R being more prevalent (following image analysis of the bands of the Western blots for the quantification of the relative protein expression levels). In the case of the MTX-resistant breast cancer cell lines (tested here), three out of four (MDA-MB-436-R, HCC-1806-R and BT-20-R) had increased DHFR protein levels compared to their sensitive counterparts. Cal-51-R was the only MTX-resistant breast cancer cell line which had lower DHFR protein levels in comparison to Cal-51. Lastly, both MTX-resistant colon cancer cell lines (tested here), LoVo-R and SW-80-R, had increased DHFR levels compared to LoVo and SW-80, respectively.

MTHFR genetic polymorphisms have been connected to the chemosensitivity of colorectal and breast cancer cells (Sohn *et al.*, 2004). According to our results depicted in **Figure 5.5.D-3**, MTHFR is co-overexpressed with DHFR in the osteosarcoma MTX-resistant U2OS-R and SAOS-2-R cells, and in the MTX-resistant breast cancer MDA-MB-436-R, HCC-1806-R and BT-20-R. On the other hand, the breast cancer Cal51-R cells and the MTX-resistant colon cancer LoVo-R and SW-480-R cells had similar MTHFR protein levels compared to their sensitive counterparts. This observation was necessary to examine whether other MTX-resistant cancer cell lines follow the same pattern with the choriocarcinoma-resistant JEG3-R cells.

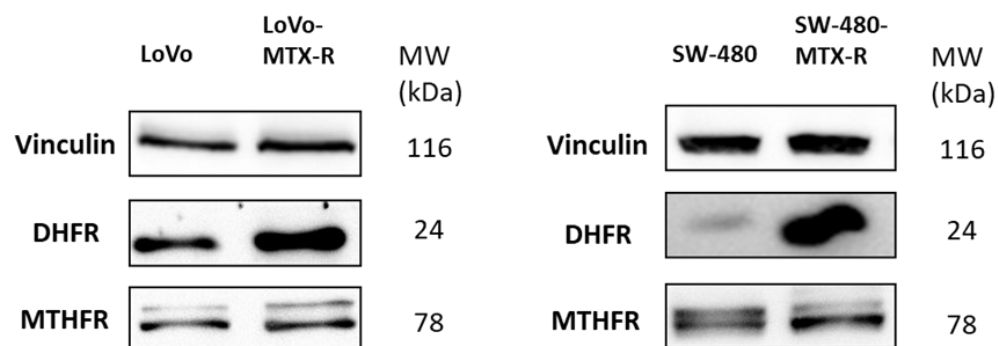
A) Osteosarcoma Cell Lines



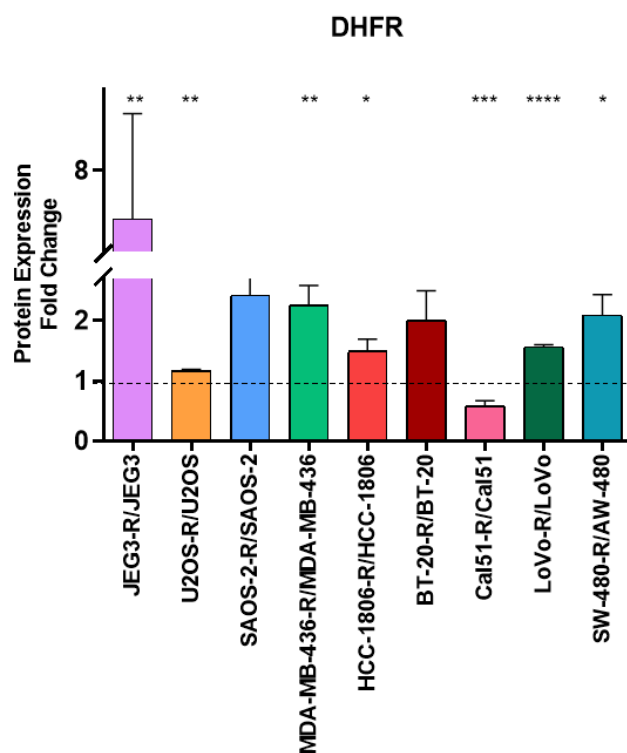
B) Breast Cancer Cell Lines



C) Colon Cancer Cell Lines



D)



E)

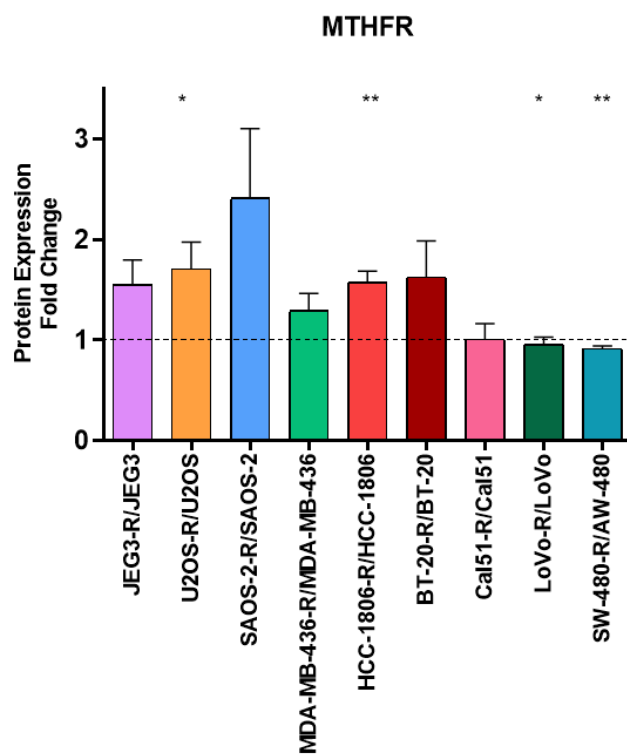
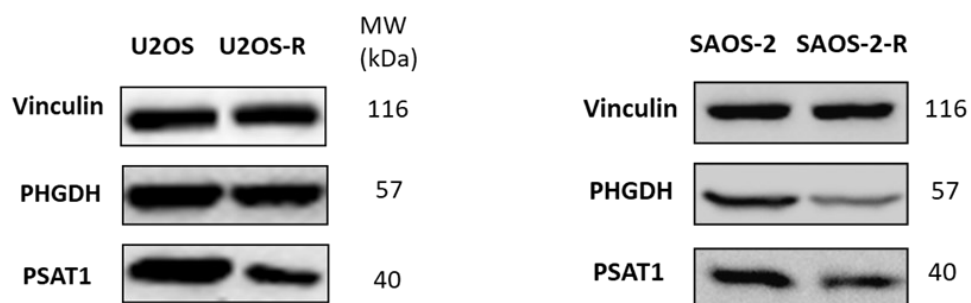


Figure 5.5: Comparison of DHFR and MTHFR protein levels among multiple pairs of MTX-Sensitive and MTX-Resistant cell lines.

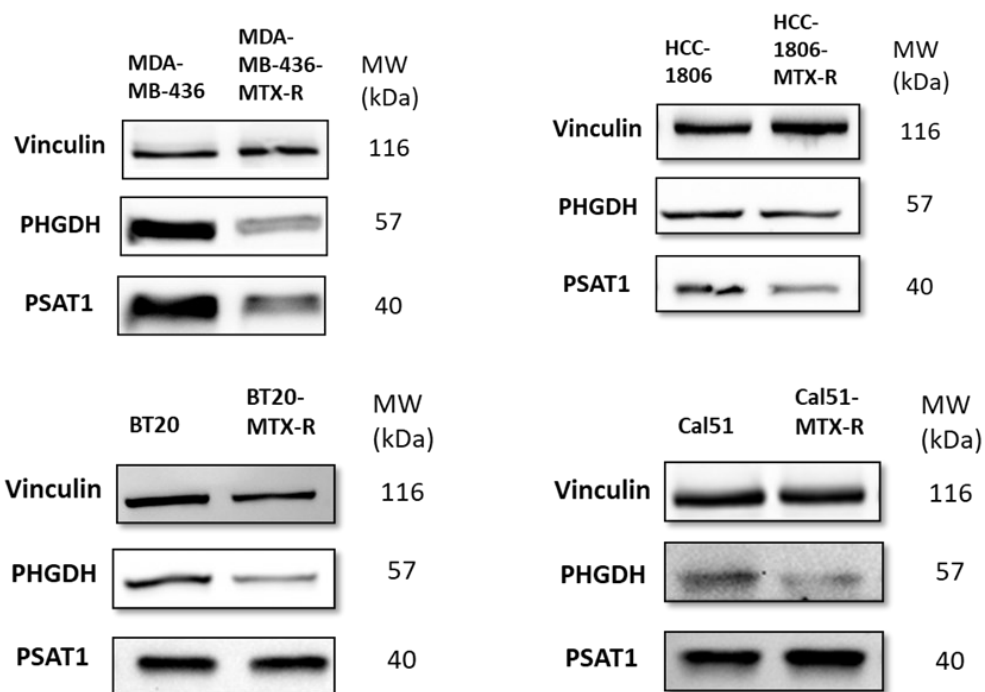
A-C: Immunoblots showing the expression of DHFR and MTHFR proteins in osteosarcoma **(A)**, breast cancer **(B)** and colon cancer cell lines **(C)**. Vinculin was utilized as a loading control. The data represent 3 independent biological replicates. **D-E:** Bar plots showing the fold change (MTX-R/MTX-S) ratios in DHFR **(D)** and MTHFR **(E)**. The data show the mean fold changes $\pm$ SEM from 3 independent experiments. Statistical significance was determined using the Unpaired Student's t-test (* $p < 0.005$, ** $p < 0.001$, *** $p < 0.005$, **** $p < 0.001$).

Following the observed common expression pattern in DHFR and MTHFR of the MTX-resistant cancer cell lines, the protein levels of the Serine Synthesis enzymes were tested as well. PHGDH levels were downregulated in all the MTX-resistant cancer cell lines except for the breast cancer HCC-1806-R cells and the colon cancer SW-480-R cells. SW-480-R cells had intriguingly much higher PHGDH levels than the sensitive SW-480 cells (almost three times more). As for PSAT1 protein, its expression was downregulated in both resistant osteosarcoma cells U2OS-R and SAOS-2-R and in two out of four MTX-resistant breast cancer cells, MDA-MB-436 and HCC-1806. BT-20-R cells had slightly elevated PSAT1 levels compared to BT-20 while Cal-51-R cells had almost double PSAT1 levels compared to sensitive Cal-51. The colon cancer LoVo-R cells had lower PSAT1 expression levels compared to LoVo cells. On the contrary, the other colon cancer MTX-resistant SW-480-R cells had much more elevated levels compared to their sensitive counterpart **(Figure 5.6.A-E)**.

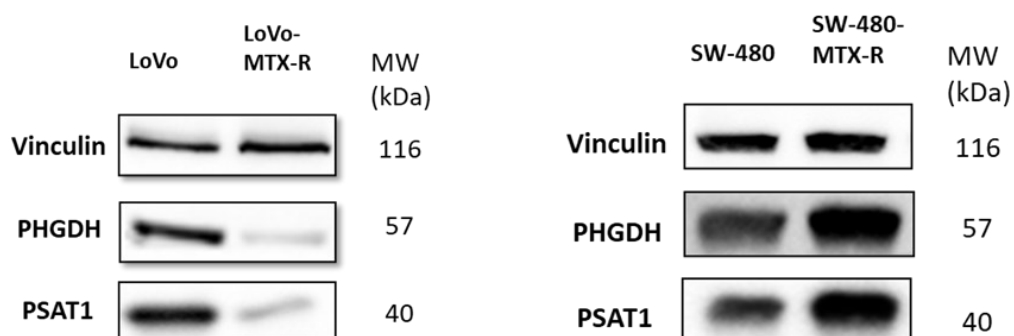
A) Osteosarcoma Cell Lines



B) Breast Cancer Cell Lines



C) Colon Cancer Cell Lines



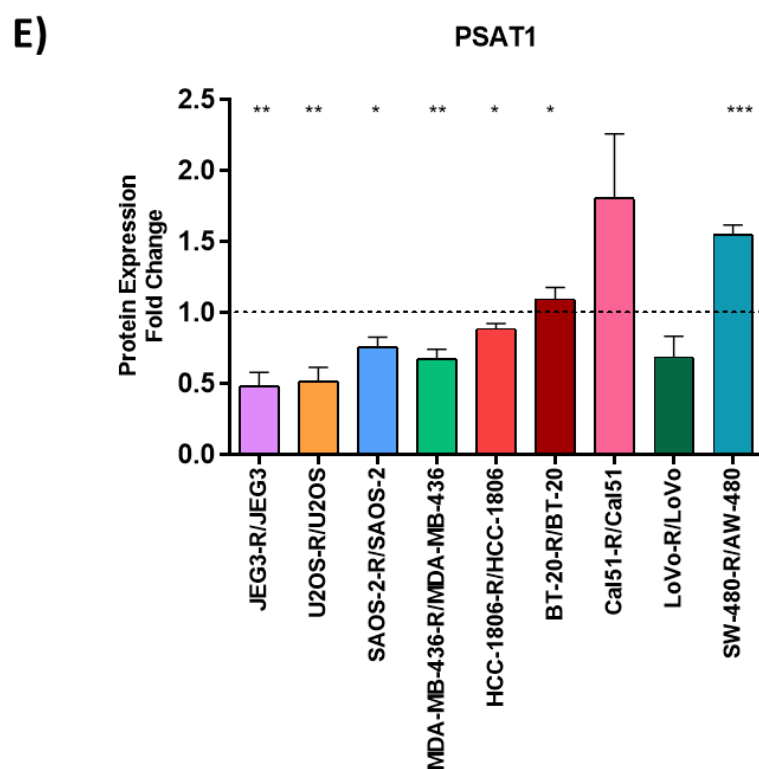
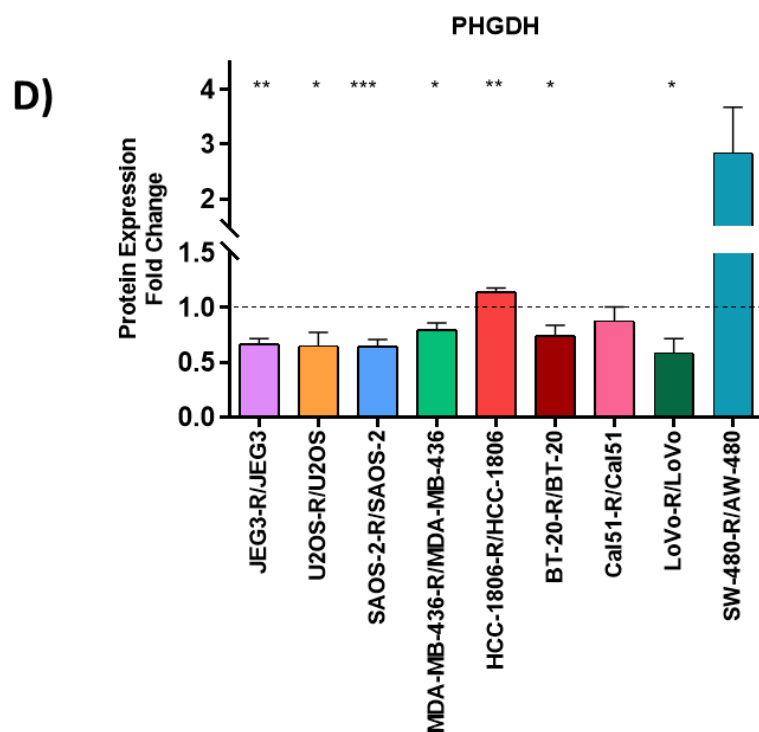


Figure 5.6: Comparison of PHGDH and PSAT1 protein levels among multiple pairs of MTX-Sensitive and MTX-Resistant cell lines.

A-C: Immunoblots showing the expression of PHGDH and PSAT1 proteins in osteosarcoma **(A)**, breast cancer **(B)** and colon cancer cell lines **(C)**. Vinculin was utilized as a loading control. The data represent 3 independent biological replicates. **D-E.** Bar plots showing the fold change (MTX-R/MTX-S) ratios in PHGDH **(D)** and PSAT1 **(E)** The data show the average fold changes $\pm$ SEM from 3 independent experiments. Statistical significance was determined using the Unpaired Student's t-test(* $p < 0.005$, ** $p < 0.001$, *** $p < 0.005$, **** $p < 0.001$).

5.3 Discussion

A major challenge in cancer treatment is the development of either acquired or intrinsic resistance to chemotherapeutic agents. Methotrexate (MTX) is a chemotherapy medication commonly utilized in the treatment of various forms of malignancies, either alone or in combination with other drugs. Unfortunately, a large number of patients relapse as a result of resistance to MTX (Koźmiński *et al.*, 2020). Thus, an investigation of the reasons behind this phenomenon is required.

In this chapter, we tried to understand whether there is a common expression pattern in MTX-resistant cancer cell lines as previously seen for choriocarcinoma JEG3-R cells in order to investigate whether there are common underlying mechanisms of MTX-resistance. Here, we exposed U2OS cells over a prolonged time to increasing concentration of MTX to generate the resistant osteosarcoma cell line, U2OS-R, to serve as a useful tool for the comparison with the sensitive/resistant choriocarcinoma pair, JEG3/JEG3-R. During this long-lasting process the U2OS cells underwent Epithelial to Mesenchymal Transition (EMT) which could be responsible for making U2OS-R cells less prone to undergoing apoptosis, allowing them to survive in the presence of MTX (Shibue and Robert A. Weinberg, 2017).

DHFR levels of U2OS-R were slightly increased compared to U2OS cells, not resembling the significant overexpression observed in the case of JEG3-R cells versus JEG3 cells. This outcome is most likely informing us that MTX-Resistance in U2OS cells is not DHFR-driven. This result though contradicts a prior publication stating that MTX-Resistance in U2OS-Resistant cells is strongly associated with DHFR overexpression (Serra *et al.*, 2004). One explanation to this interesting disagreement could be the fact that our U2OS-MTX-R cells are much more resistant than the U2OS-R cell lines the prior authors were using (the maximum concentration in which they exposed their cells is 2.2 μ M MTX). Our U2OS-R cells have adapted to higher stress triggers and might have employed alternative mechanisms of resistance that do not involve DHFR overexpression and at the same time, allow their survival.

Then, we focused on checking whether U2OS-R cells/U2OS cells have any other similarities with the metabolic fate of JEG3-R cells. Indeed, U2OS-R cells had decreased protein levels of PHGDH, PSAT1 and TYMS and elevated protein levels of MTHFR and TK1 in comparison with U2OS cells **Figure 5.3**. Collectively, we can hypothesize that in the case of U2OS cells, utilization of carbon units is promoted toward nucleotide synthesis and maintenance of the proliferation rate. On the contrary, U2OS-R cells might rely on the salvage nucleotide pathway for the biosynthesis of nucleotides and save carbon units for less energy-consuming processes necessary for their survival. The combination of the above results is not sufficient to fully support the metabolic tendencies of these cells, but our hypothesis relies on the similar observed effects on choriocarcinoma cells (**Chapter 4**).

The observed similarities between the two MTX-resistant cell lines triggered us to explore whether other MTX-resistant cell lines adhere to the same metabolic mechanisms. In total, two osteosarcoma (*U2OS-R/U2OS*, *SAOS-2/SAOS-2-MTX-R*), four breast cancer (*MDA-MB-436/MDA-MB-436-MTX-R*, *Cal51/Cal51-MTX-R*, *HCC-1806/HCC-1806-MTX-R*) and two colon cancer (*SW-480/SW-480-MTX-R* and *LoVo/LoVo-MTX-R*) pairs of MTX-resistant/MTX-sensitive cells were tested. In the 4 enzymes (due to time restriction) examined, PHGDH, PSAT1, DHFR and MTHFR, the MTX-resistant cell lines MDA-MB-436-R and SAOS-2-R cells demonstrated the same expression pattern as previously seen in the JEG3-R cells. It is plausible that these cell lines and these cancer types by extension, possess the same underlying mechanisms driving MTX-resistance but further work is clearly necessary to validate this notion. The MTX-resistant cell lines which had the exact same expression pattern in three out of four enzymes as JEG3-R cells were U2OS-R and LoVo-R. U2OS-R differ from JEG3-R cells in DHFR protein levels. As mentioned above, there is only a slight increase of DHFR in U2OS-R cells compared to U2OS cells while JEG3-R cells have a 30-times fold change ratio compared to JEG3 cells (**Supplementary Figure 4.3**). LoVo-R cells differ from JEG3-R cells in MTHFR protein levels. MTHFR polymorphisms have been associated with MTX sensitivity of colon and breast cancer cells (Sohn *et al.*, 2004). We only tested MTHFR protein levels here but based on our results and those of others ((Eug *et al.*, 2019), (Cowan *et al.*, 1982), (Morales *et al.*, 2009), (Sohn *et al.*, 2004)), it would be interesting to test polymorphisms in this protein to assess their relevance to MTX-R.

Another important outcome from the comparison of multiple MTX-resistant cell lines was the fact that four out of seven tested MTX-resistant cell lines have downregulated serine metabolism as it is indicated by the decreased levels of PHGDH and PSAT1 enzymes compared to their sensitive counterparts. These MTX-Resistant cell lines were both osteosarcoma cell lines U2OS-R and SAOS-2, one MTX-Resistant breast cancer cell line, the MDA-MB-436-R and one colorectal cancer cell line, the LoVo-R one. There aren't so far any published papers connecting low expression of SSP enzymes to development of resistance to Methotrexate in any cancer type. However PHGDH downregulation has been linked to metastatic capacity in primary tumours of patients with breast cancer (Rossi *et al.*, 2022). Additionally, decreased cellular serine availability and PHGDH levels have been linked to platinum-based chemotherapy resistance in ovarian cancer patients (Van Nyen *et al.*, 2022).

In conclusion, this downregulation in the Serine synthesis pathway was a common characteristic in four different resistant types of cancer; choriocarcinoma, breast cancer, colon cancer and osteosarcoma. This result strengthens our statement that Serine metabolism likely has a critical role in MTX-resistance and in the way the MTX-resistant cells rewire their metabolism to overcome MTX-induced stress, avoid apoptosis and induce survival mechanisms.

6. General Discussion and Future Directions

6.1 General Discussion

Gestational Trophoblastic Disease (GTD) includes a variety of pregnancy-related conditions that arise from abnormal fertilization, leading to the deregulated proliferation of trophoblast cells, and giving rise to an anomalous type of pregnancy which is called molar ((Seckl, Sebire and Berkowitz, 2010), (Froeling and Seckl, 2014)). These molar pregnancies can be non-malignant hydatidiform (complete or partial) moles or malignant forms such as invasive moles, choriocarcinoma, and the rarer placental site trophoblastic tumors (PSTT) and epithelioid trophoblastic tumors (ETT) ((Mangili *et al.*, 2014), (Seckl, Sebire and Berkowitz, 2010)).

Despite advances in the clinical management of GTD, significant challenges persist, particularly in predicting chemoresistance. The current FIGO/WHO scoring system predicts patient response to single-agent chemotherapy but often misclassifies patients, delaying optimal treatment and resulting in resistance to first-line therapies like Methotrexate (Ngan *et al.*, 2021). Therefore, there is a pressing need for novel biomarkers to better stratify GTD patients early in diagnosis and identify new pathways responsible for MTX resistance. This could lead to more effective and less toxic treatment options, improving patient outcomes and care.

To address the challenges of chemoresistance in Gestational Trophoblastic Disease (GTD), we conducted proteomics profiling of formalin-fixed paraffin-embedded (FFPE) patient tissue samples using quantitative Mass Spectrometry (MS). The tissue samples were complete hydatidiform moles evacuated from women diagnosed with GTD, whose post-molar GTN was treated with MTX. This study involved two groups of five patients each, categorized based on their response to Methotrexate (MTX) as either MTX-Sensitive (MTX-S) or MTX-Resistant (MTX-R). We identified 135 proteins significantly overrepresented and 96 proteins significantly underrepresented in the MTX-R group compared to the MTX-S group (**Figure 3.1**). Pathway analysis indicated that the upregulated proteins in MTX-R patients are linked to amino acid and nucleotide metabolism, a finding consistent with known mechanisms of antineoplastic drug resistance, including energy-efficient biosynthesis, redox balance maintenance, and epigenetic

alterations (Bhingarkar *et al.*, 2021) (**Figure 3.2**). Intriguingly, the only metabolism-related pathway downregulated in MTX-R patients was the serine and glycine biosynthesis pathway.

Complementary data from our lab's previous proteomics profiling of MTX-sensitive JEG3 and MTX-resistant JEG3-R human choriocarcinoma cell lines were used to identify the overlap in molecular pathways and proteins associated with MTX resistance (**Supplementary Table 3.1, Supplementary Figure 3.2**). Our integrative approach included the comparison of both the patient and choriocarcinoma cell line proteomics datasets revealing several overlapping proteins that were either overrepresented or underrepresented in the MTX-resistant setting. Among the five commonly underrepresented proteins, three—PHGDH, PSAT1, and TIGAR—belong to the glucose-derived *de novo* serine synthesis pathway. The downregulation of PHGDH and PSAT1 in the MTX-resistant group was also confirmed via western Blotting in JEG3-R cells in comparison with JEG3 cells (**Figure 3.2.A-C**). However, TIGAR levels remained unchanged between the two cell lines, possibly due to the instability of the resistance phenotype in JEG3-R cells and the specific timing of the samples' collection (**Supplementary Figure 3.2.A**).

We then conducted an Immunohistochemistry (IHC) analysis on patient tissue samples, focusing on five proteins that were differentially expressed in MTX-R versus MTX-S patient samples. Among these proteins, three—PHGDH, PSAT1, and TIGAR—were underexpressed, while two—MRPL9 and CDA—were overexpressed in resistant samples. Notably, all proteins except CDA were also identified as differentially expressed in both MTX-R patient samples and JEG3-R cells which is why they were chosen. The results suggested that PSAT1 and MRPL9 could serve as prognostic markers for MTX sensitivity and resistance, respectively (**Figure 3.3**). Regarding MRPL9, no published evidence directly links this protein to Methotrexate response. However, high MRPL9 expression has been linked to poor survival in lung cancer patients (Xin-Yuan Li, Xin-Yu He, Hong Zhao, Lu Qi, 2023), malignant progression of hepatocellular carcinoma (Xie *et al.*, 2023), and thyroid cancer (Zhang *et al.*, 2022) indicating its potential as a prognostic biomarker in these diseases. Nevertheless, further investigation with a larger panel of tissue samples is essential to statistically validate these findings and confirm the role of these proteins in predicting MTX resistance in Gestational Trophoblastic Disease. Moreover, testing a broader range of differentially expressed proteins between the sensitive and resistant groups to assess their

prognostic value would have been ideal. However, the limited availability of tissue samples and the need for delicate handling of the tissue slides were the limitations of this study.

The Serine Synthesis Pathway (SSP) supplies cells with serine and glycine, essential for 1-carbon metabolism, which supports nucleotide synthesis, methylation reactions, and cellular redox balance (Geeraerts *et al.*, 2021). Methotrexate interferes with folic acid metabolism, thereby impacting 1-carbon metabolism, which is closely linked to the SSP (Pranzini *et al.*, 2021). In the current thesis, we examined whether reduced serine synthesis in JEG3-R cells is linked to their MTX resistance, focusing on pathways downstream of the SSP. Initial studies on 1-carbon metabolism -or else the folate and methionine cycles- revealed key insights. Along with the overexpression of DHFR (**Supplementary Figure 4.3**), three other key enzymes in the folate/methionine cycles were significantly upregulated in JEG3-R cells: MTHFR (Methylenetetrahydrofolate reductase), MS (Methionine Synthase), and MAT1A (Methionine Adenosyltransferase) (**Figure 4.1.A-B**). The enhanced folate cycle activity counteracts the inhibitory effects of MTX on DHFR, ensuring a steady supply of tetrahydrofolate (THF) derivatives necessary for nucleotide biosynthesis and repair (Lee, Vousden and Hennequart, 2024). The upregulation of MTHFR enhances the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, supporting thymidine and purine synthesis (Locasale, 2013). The upregulation of MS facilitates the conversion of homocysteine to methionine, while MAT1A catalyzes the formation of S-adenosylmethionine (SAM), a universal methyl donor, maintaining this way DNA methylation processes crucial for cell function and survival under stress conditions (Kotb *et al.*, 1997). Alternatively, the demethylation of SAM results in the formation of S-adenosylhomocysteine (SAH), which is subsequently converted to homocysteine. At the same time, serine and Cystathione Synthase (CBS) contribute to the production of cysteine, which is then utilized for Glutathione (GSH) synthesis (Geeraerts *et al.*, 2021). Therefore, in JEG3-R cells, the switch to the SSP might favor GSH production over nucleotide synthesis. Each of the above scenarios was then tested individually.

Previous findings on the slower proliferation rate and maintenance of energy metabolism of JEG3-R cells compared to JEG3 cells (Georgiou *et al.*, 2022) led us to further investigate the nucleotide metabolism preferences of these cells. JEG3 cells exhibited higher expression of the

cytosolic enzymes TYMS and SHMT1 compared to JEG3-R suggesting serine utilization towards thymidine and DNA synthesis and cell proliferation (**Figure 4.1.A-B**). This finding is consistent with the observed faster proliferation rate of JEG3 cells compared to JEG3-R cells (**Supplementary Figure 4.1**). In contrast, JEG3-R cells showed increased expression of TK1 and CDA, enzymes involved in the nucleotide salvage pathway, suggesting a reliance on this pathway to conserve energy and resources. This shift to the salvage pathway highlights a potential mechanism of MTX resistance in JEG3-R cells. Additionally, combining MTX with the uridine import inhibitor Dipyridamole showed synergistic effects, particularly in JEG3-R cells, indicating that targeting the salvage pathway could enhance MTX efficacy and counteract resistance (**Figure 4.1.E**). Here, it would be valuable to quantify the nucleotide pools of each cell line by high-performance liquid chromatography (HPLC) to assess any alterations indicative of a shift towards the salvage pathway in JEG3-R cells before and upon MTX treatment and validate our hypothesis.

Our findings suggest that JEG3-R cells favor Glutathione (GSH) synthesis over nucleotide synthesis, as evidenced by higher GSH levels compared to JEG3 cells (**Figure 4.2.A**). This preference may contribute to methotrexate (MTX) resistance by enhancing the detoxification of MTX through GSH conjugation or by neutralizing chemotherapy-induced Reactive Oxygen Species (ROS) and protecting against oxidative stress (Hess and Khasawneh, 2015). Elevated levels of Glutathione S-Transferase Theta 1 (GSTT1) in JEG3-R cells support the first possibility, as GSTT1 is involved in detoxifying MTX by coupling it with GSH, increasing its solubility and facilitating its export from the cell (**Figure 4.2.B-C**). Additionally, former studies from our lab indicated that JEG3-R cells exhibit higher ROS levels compared to JEG3 cells. A time-course experiment further demonstrated that ROS levels in JEG3-R cells remained stable upon MTX treatment, while in JEG3 cells, ROS levels gradually decreased and then dropped significantly after 72 hours (**Figure 4.2.D**). This suggests that the persistent high ROS levels in JEG3-R cells may be an adaptive response to MTX exposure, activating survival pathways that help resist MTX-induced cell death. Metabolic profiling of these cells by performing a Seahorse assay to measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) upon MTX treatment could show us the metabolic adaptations of JEG3-R cells in response to persistent ROS.

The hypothetical maintenance of SAM levels by upregulated MS and MAT1A in JEG3-R cells led us to test whether epigenetic modifications could influence the transcription of SSP enzymes, PHGDH and PSAT1. We found higher levels of the repressive mark H3K27me3 (Zhang, Cooper and Brockdorff, 2015) in JEG3-R cells, suggesting more regions protected from DNA methylation compared to JEG3 cells, indicating gene repression via chromatin compaction. Also, we found elevated mRNA levels of the histone methyltransferase EZH2 in JEG3-R cells aligning with the increased H3K27me3 levels, pointing to a repressive role in gene expression through histone methylation (**Figure 4.3.A-B-C**). However, treatment with Zebularine, a DNA methyltransferase inhibitor, unexpectedly increased both repressive epigenetic marks H3K27me3 and H3K9me3 levels while still upregulating PSAT1 protein expression in JEG3-R cells, implying that other epigenetic mechanisms in combination with the activity of transcription factors may regulate PSAT1 repression (**Figure 4.3.D**). It would be interesting to deep dive into these epigenetic mechanisms by performing a DNA Methylation Analysis to detect any differences in DNA methylation patterns at the promoter regions of the genes encoding the SSP enzymes. However, in the context of this thesis, we decided to further investigate the interplay between the transcription factors affecting the SSP pathway.

There are multiple transcription factors, including ATF4, ATF3, c-MYC, and NRF2 involved in upregulating the transcription of SSP enzymes, while p53 acts as a negative regulator ((Xu *et al.*, 2020), (Adams, 2007), (Li *et al.*, 2021), (Sun *et al.*, 2015), (Denicola *et al.*, 2016), (Maddocks *et al.*, 2013)). In JEG3-R cells, elevated p53 levels and decreased c-MYC expression were observed, which could lead to decreased PHGDH and PSAT1 transcription (**Figure 4.4.A-B**). ATF4, despite being overexpressed in JEG3-R cells, did not lead to increased SSP enzyme expression, likely due to the presence of counteracting factors like p53 and/or due to decreased KDM4C levels. KDM4C is crucial for ATF4's binding to the promoter regions of serine pathway genes, while ATF4 is also necessary for KDM4C's transcriptional regulation, with their interdependent interaction ensuring optimal transcription of SSP genes (Zhao *et al.*, 2016). The siRNA-mediated downregulation of p53 promoted the expression of SSP enzymes without altering ATF4 expression levels (**Figure 4.4.D**). When ATF4 was downregulated, PHGDH and PSAT1 levels dropped, highlighting the importance of ATF4 in these cells (**Figure 4.4.C**). Furthermore, silencing ATF4 increased

methotrexate resistance in JEG3-R cells, but not in JEG3 cells, indicating ATF4's critical role in the resistant phenotype (**Figure 4.4.E-F**). Nuclear localization studies of ATF4 excluded mislocalization as a cause for the lack of SSP enzyme transcription, suggesting that other factors, such as post-translational modifications or interactions with other proteins, might inhibit ATF4's function (**Supplementary Figure 4.4**). These findings indicate that the regulation of SSP enzymes through transcription factors, especially the interplay between ATF4 and p53, is complex and significantly impacts MTX resistance in JEG3-R cells. This regulatory network potentially facilitates the survival and adaptation of JEG3-R cells to MTX treatment, contributing to their resistant phenotype. Additional experimentation on the interplay between these two transcription factors could provide valuable data. A Chromatin Immunoprecipitation (ChIP) Assay in choriocarcinoma cells JEG3 and JEG3-R could reveal not only whether ATF4 and/or p53 directly bind to the promoters of SSP enzyme genes but also if these factors co-occupy the same or adjacent regions on the DNA suggesting a direct interaction between them and a coordinated regulation of their common target genes.

Then, one more potential explanation was explored for the lower levels of PHGDH and PSAT1 in JEG3-R cells compared to JEG3 cells, despite the overexpression of ATF4. We suggest that the activation of the Integrated Stress Response (ISR) holds a significant role in conferring resistance to Methotrexate in choriocarcinoma cells. This activation is mediated through the phosphorylation of eIF2 α , resulting in the selective translation of specific mRNAs, including ATF4 (Pakos-Zebrucka K et al., 2016). In JEG3-R cells, ATF4's activity leads to the upregulation of amino acid transporters like ASCT2 and ASCT1, which help in the uptake of serine despite its reduced synthesis (**Figure 4.5.D-E-H**). Furthermore, the increased levels of ATG5, an autophagy-related protein regulated by ATF4, along with elevated levels of the autophagy-promoting protein CHOP (B'Chir *et al.*, 2013), suggest that JEG3-R cells are likely utilizing autophagy to compensate for decreased serine production (**Figure 4.5.C-H**). Moreover, AMPK activation, indicated by the increased phosphorylation of its catalytic subunit AMPK α (**Figure 4.5.F-G**), supports the notion of autophagy induction in JEG3-R cells, as AMPK promotes autophagy while inhibiting ATP-consuming processes (Hardie, 2011). Additional experimentation should be conducted to confirm that autophagy has indeed a role in the MTX resistance of JEG3-R cells. An assay (e.g. LC3-II

Turnover Assay) to monitor the accumulation of autophagosomes could determine autophagic flux in JEG3-R cells upon MTX treatment. In addition, a rescue experiment by restoring serine levels in the medium of JEG3-R cells leading to reduction of autophagy and sensitization of these cells to MTX would suggest that autophagy compensates for serine deprivation. Also, targeting central autophagy mediators through genetic silencing in JEG3-R would reveal if autophagy participates in the resistance to MTX and the maintenance of Serine levels.

One of the kinases that phosphorylates eIF2 α is general control nonderepressible 2 (GCN2), which gets activated in response to amino acid deprivation (Deval *et al.*, 2009) and is overexpressed in JEG3-R cells (**Figure 4.5.C**). The impact of ISR activation on Methotrexate resistance was further investigated using GCN2 inhibitors. Although high concentrations of these inhibitors combined with Methotrexate were synergistic in inhibiting cell growth, lower concentrations did not show a significant synergistic effect. This implies that GCN2 inhibition can sensitize JEG3-R cells to Methotrexate, provided that the dosing is carefully selected. However, evaluating the potential nonspecific toxicity of these inhibitors in normal cells in the future is crucial for determining their safety and effectiveness in therapeutic contexts.

We then investigated the effect of serine and glycine level modulation on JEG3 and JEG3-R cells and revealed distinct adaptive mechanisms between the two cell lines. JEG3-R cells exhibit a higher resilience to serine and glycine depletion compared to JEG3 cells. This is evidenced by the JEG3-R cells maintaining their proliferation rate regardless of the presence of these amino acids in the medium, while JEG3 cells show a significant reduction in growth under deprivation conditions (**Figure 4.6.**). This suggests that JEG3 cells are more reliant on extracellular sources of serine and glycine, despite having a more active serine synthesis pathway. Furthermore, JEG3 cells, when deprived of serine and glycine, display markers of p53-mediated apoptosis in contrast with JEG3-R which seem to adapt and survive serine/glycine deprivation (**Figure 4.6**). The metabolic flexibility of JEG3-R cells to withstand serine and glycine depletion likely contributes to their Methotrexate resistance. By efficiently channeling available serine into critical survival pathways (such as autophagy and GSH synthesis) and avoiding apoptosis triggered by nutrient stress, JEG3-R cells maintain their viability and proliferative capacity even in adverse conditions. Conducting a metabolic flux analysis to examine the production and consumption rates of these

metabolites and understand how serine and glycine are utilized would be essential to determine the reason behind this.

The simultaneous depletion of serine and glycine from the growth medium, along with either genetic or pharmacological blockade of *de novo* serine biosynthesis pathway, induced Methotrexate resistance in both JEG3 and JEG3-R cells, with a more pronounced effect in JEG3-R cells (**Figure 4.6**). These results confirmed our hypothesis that reduced intracellular availability of serine and glycine is linked to MTX resistance. Finally, we performed a “rescue” experiment by overexpressing the SSP enzymes in JEG3-R cells through transfection with plasmid vectors. The parallel depletion of serine and glycine from the medium restored their sensitivity to Methotrexate (MTX). This was evidenced by a substantial ~7-fold reduction in the IC₅₀ values for MTX in the cells overexpressing these enzymes compared to those transfected with an empty vector (**Figure 4.8.A**). A similar, albeit lesser, effect was observed in cells grown in serine and glycine-sufficient conditions (**Supplementary Figure 4.6**). These results suggest that targeting upstream signaling pathways to enhance SSP activity could be a viable strategy to sensitize resistant patients to MTX treatment. However, additional research is needed to identify which specific upstream pathways could be targeted for this purpose without disrupting normal cellular functions.

Following our observations in the choriocarcinoma MTX-resistant cell line, we wondered whether the observed patterns are consistent across different forms of cancers such as osteosarcoma and breast and colon cancer. By comparing JEG3 and JEG3-R cell lines pair with other MTX-sensitive and MTX-resistant cell line pairs, we found similar changes in the expression of the serine biosynthesis pathway. Specifically, we observed that four out of seven tested MTX-resistant cell lines showed decreased PHGDH and PSAT1 levels (**Figure 5.6**). These included two osteosarcoma cell lines (U2OS-R and SAOS-2), one breast cancer cell line (MDA-MB-436-R), and one colorectal cancer cell line (LoVo-R). While no published studies directly link low SSP enzyme expression to MTX resistance, PHGDH downregulation is associated with metastatic capacity in breast cancer (Rossi *et al.*, 2022), and decreased cellular serine availability and PHGDH levels have been linked to platinum-based chemotherapy resistance in ovarian cancer (Van Nyen *et al.*, 2022).

We demonstrate that both an MTX-resistant breast cancer cell line (MDA-MB-436-R) and an MTX-resistant osteosarcoma cell line (SAOS-2-R) exhibited identical expression patterns for the proteins PHGDH, PSAT1, DHFR, and MTHFR, as previously observed in the MTX-resistant choriocarcinoma cell line JEG3-R (**Figure 5.5-5.6**). Moreover, elevated DHFR protein levels are commonly associated with methotrexate (MTX) resistance across various cancer cell lines, including osteosarcoma (U2OS-R and SAOS-2-R) and certain breast cancer lines (MDA-MB-436-R, HCC-1806-R, and BT-20-R) (**Figure 5.5**). However, exceptions were observed, such as the Cal-51-R breast cancer line and the SW-480-R colon cancer line, which had lower DHFR levels than their sensitive counterparts. Additionally, MTHFR was co-overexpressed with DHFR in some MTX-resistant lines (like in the case of JEG3-R) but remained unchanged in others (**Figure 5.5**). This suggests that while DHFR overexpression is a common mechanism of MTX resistance, it is not universally applicable, and other mechanisms, such as MTHFR expression and potentially MTHFR polymorphisms, might also play a role. This variability indicates that MTX resistance can be driven by diverse metabolic and genetic factors, necessitating a tailored approach to understanding and overcoming resistance in different cancer types.

6.2 Limitations of the study

The limitations of this study primarily stem from the scarcity and complexity of molar tissue samples available for research. The dissection of molar tissue posed significant challenges due to its frequent intermixing with maternal tissue and the high presence of blood, making it difficult to obtain pure and sufficient samples for analysis. Furthermore, complete hydatidiform moles, are relatively rare. These factors contributed to the limited number of immunohistochemistry (IHC) samples, which in turn reduced the statistical power of the results. Although the IHC findings revealed clear trends, they did not reach statistical significance due to the small sample size. Additionally, JEG3 and JEG3-R cells represented the best and only available cell line model of methotrexate (MTX) sensitivity and resistance for validation experiments following proteomic analysis of tissue samples. Due to the limited tissue availability, the use of this pair of cell lines provided a practical and reliable analog to patient response to MTX treatment. And finally, while these cell lines are derived from partial moles rather than complete moles, they remain the most

relevant tool for modeling MTX response and comparing outcomes between choriocarcinoma and pre-malignant hydatidiform moles.

6.3 Conclusion

In conclusion, our study on Methotrexate resistance in Gestational Trophoblastic Disease and various cancer cell lines has unveiled critical insights into the underlying mechanisms of MTX resistance. Proteomics profiling of both patient tissue samples and choriocarcinoma cell lines revealed the Serine Synthesis Pathway as notably downregulated in MTX-resistant cases, with Phosphoserine Aminotransferase 1 (PSAT1) emerging as a potential prognostic marker for MTX sensitivity. The study uncovered the possibility that MTX-resistant choriocarcinoma JEG3-R cells exhibit enhanced detoxification capabilities through rerouting serine towards glutathione synthesis, which helps them manage MTX-induced reactive oxygen species (ROS) and evade the drug's effects. This cellular adaptation may involve the activation of the Integrated Stress Response (ISR) pathway, which would suppress non-essential processes while promoting survival mechanisms like autophagy. Notably, targeting the ISR pathway showed promise in re-sensitizing resistant cells to MTX, suggesting a potential therapeutic approach. Furthermore, similar patterns of SSP downregulation were observed in several other MTX-resistant cell lines across different cancer types, supporting the notion that alterations in serine metabolism play a crucial role in MTX resistance and highlighting the potential for SSP modulation therapeutic strategies in clinical settings

7. Supplementary

7.1 Supplementary Figures – Chapter 3

Supplementary Table 3.1: GO Biological Process Analysis MTX-R vs MTX-S.

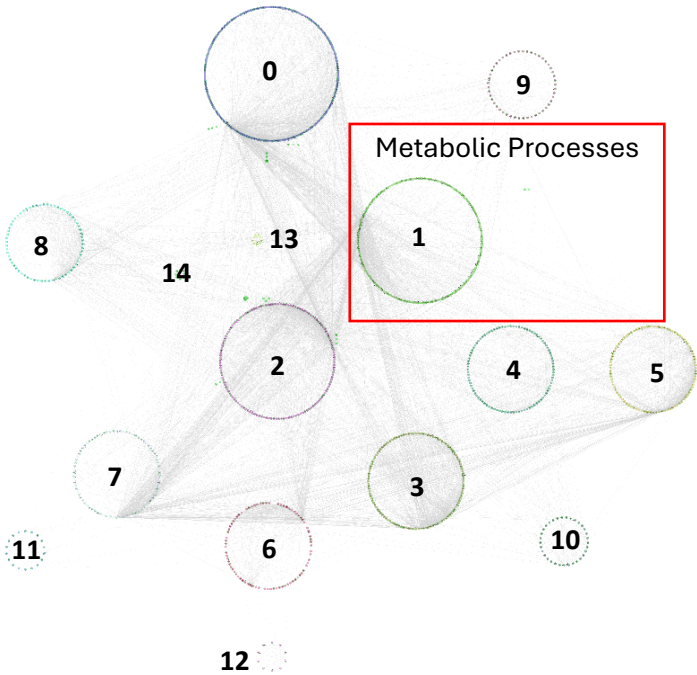
GO Biological Process analysis MTX-R vs S

Upregulated proteinsDownregulated proteins

Term	FDR	Term	FDR
branched-chain amino acid catabolic process (GO:0009083)	8.83E-05	mRNA processing (GO:0006397)	1.50E-03
branched-chain amino acid metabolic process (GO:0009081)	8.83E-05	RNA splicing, via transesterification with bulged adenosine as nucleophile (GO:0000377)	1.50E-03
valine metabolic process (GO:0006573)	1.96E-03	mRNA metabolic process (GO:0016071)	1.53E-03
neutrophil degranulation (GO:0043312)	3.77E-03	mRNA splicing, via spliceosome (GO:0000398)	1.53E-03
neutrophil activation involved in immune response (GO:0002283)	3.77E-03		
neutrophil mediated immunity (GO:0002446)	3.77E-03		
cellular amino acid catabolic process (GO:0009063)	4.43E-03		
glutathione biosynthetic process (GO:0006750)	5.93E-03		
nonribosomal peptide biosynthetic process (GO:0019184)	6.99E-03		

Tables showing enriched (left table) and downregulated (right table) biological processes in the MTX-Resistant group of patients compared to the MTX-sensitive one, as revealed by gene ontology (GO) analysis using the BinGO (Biological Networks Gene Ontology) plugin of Cytoscape.

A



B

Term	FDR	DB
Lipid and lipoprotein metabolism	1.72E-11 BP	
Various types of N-glycan biosynthesis	4.69E-10 K	
Asparagine N-linked glycosylation	1.09E-09 BP	
Carbohydrate metabolism	2.89E-09 BP	
Oxidative Phosphorylation	2.36E-08 MSDB	
Protein processing in endoplasmic reticulum	5.10E-08 K	
Glycolysis	9.24E-08 MSDB	
TCA cycle and respiratory electron transport	2.40E-07 BP	
Fatty Acid Metabolism	8.01E-07 MSDB	
Glycosphingolipid metabolism	6.58E-06 BP	
Mitochondrial fatty acid beta-oxidation	1.16E-05 BP	
Sphingolipid metabolism	1.39E-05 BP	
Glycosaminoglycan metabolism	1.67E-05 BP	
Hyaluronan Uptake And Degradation	2.58E-05 R	
Hypoxia	4.16E-05 MSDB	
Keratan Sulfate/Keratin Metabolism	5.45E-05 R	
Starch and sucrose metabolism	1.19E-04 K	
Vitamin C (Ascorbate) Metabolism	1.73E-04 R	
Amino sugar and nucleotide sugar metabolism	1.89E-04 BP	
Glycogen biosynthesis	2.43E-04 BP	
Branched-chain amino acid catabolism	0.002102 BP	
Valine, leucine and isoleucine degradation	0.002188 BP	
Lysine degradation	0.002188 BP	
Cholesterol metabolism	0.003866 K	
ER quality control compartment (ERQC)	0.005393 BP	
Proton-coupled Monocarboxylate Transport	0.006365 R	
LDL Remodeling	0.006365 R	
Term	FDR	DB
Purine nucleotides de novo biosynthesis	3.77E-16 HC	
Superpathway of purine nucleotide salvage	9.27E-13 HC	
Fatty Acid Metabolism	1.98E-12 MSDB	
Amino Acid metabolism	4.35E-12 WP	
TCA cycle and respiratory electron transport	1.2E-11 BP	
Cholesterol metabolism	1.27E-10 WP	
Glycolysis	4.11E-10 MSDB	
Nucleotide metabolism	5.94E-10 BP	
Adenosine ribonucleotides de novo biosynthesis	8.78E-10 HC	
Cysteine and methionine metabolism	9.93E-10 K	
Trans-sulfuration and one-carbon metabolism	6.75E-09 WP	
Pentose phosphate pathway	6.93E-09 BP	
Pyruvate metabolism	2.64E-07 K	
Cori Cycle	4.92E-07 WP	
Folate metabolism	1.17E-06 BP	
Glycine, serine and threonine metabolism	1.67E-06 K	
Urea cycle and associated pathways	2.49E-06 WP	
Gluconeogenesis	0.00032 BP	
Glyoxylate and dicarboxylate metabolism	0.000327 K	
Alanine, aspartate and glutamate metabolism	0.000354 BP	
Glutathione metabolism	0.002539 K	

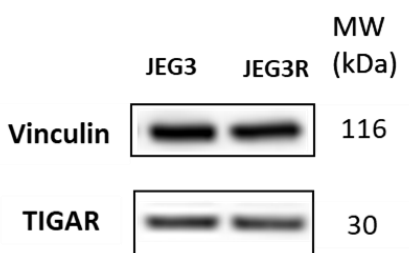
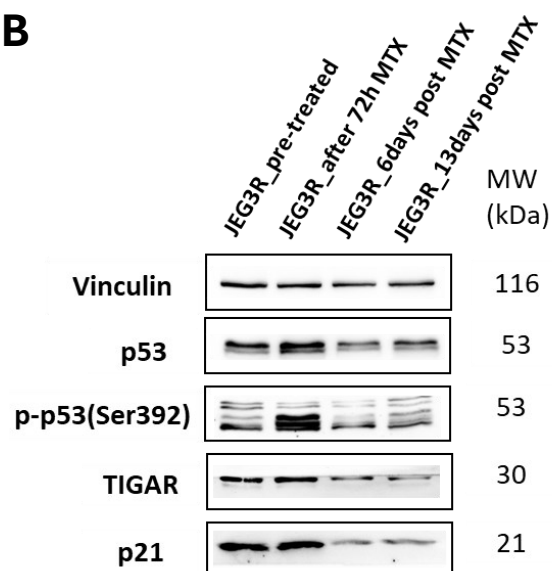
Upregulated proteins

Downregulated proteins

Supplementary Figure 3.1: Cytoscape analysis for cell lines JEG3 and JEG3-R-proteomics data.

These data were generated by Marina Georgiou.

A) Differentially Expressed Proteins (DEPs) were subjected to bioinformatics analysis in Cytoscape and 12 interconnected modules were connected to distinct biological processes as revealed by gene ontology (GO) analysis under Reactome **FI B)** Tables showing enriched pathways (upper table) and biological processes (lower table) in JEG3-R cells compared to JEG3 cells, as revealed by gene ontology (GO) analysis under Reactome **FI** in Cytoscape and independently validated utilizing the BinGO (Biological Networks Gene Ontology) plugin. Most of the pathways were related to Metabolism (red labeled) or to RNA processing (green-labeled) and were grouped by the expression pattern of their proteins in the MTX-Resistant group of patients (Upregulated or Downregulated).

A**B**

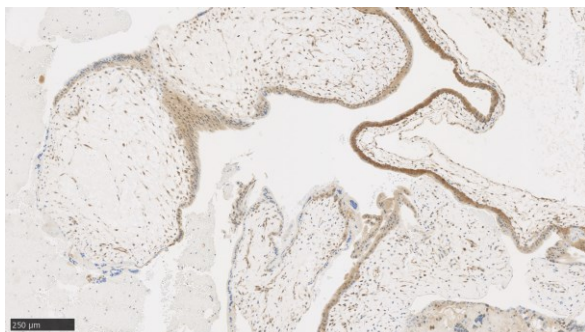
Supplementary Figure 3.2: TIGAR protein levels affected by instability of the resistance phenotype in JEG3-R cells

A): Immunoblot for bottom hit TIGAR involved in the glycolysis. Vinculin was utilized as a loading control. The data represent at least 3 independent biological replicates. **B):** Immunoblots showing the TIGAR, p53, phospho(Ser392)-p53 and p21 levels upon MTX treatment of JEG3R cells. Vinculin was used as a loading control. Data are representative of 2 independent biological replicates.

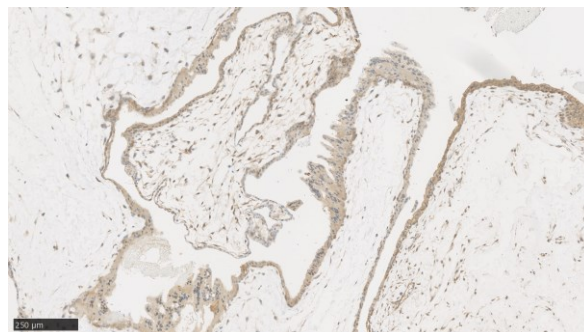
A)

PHGDH

MTX-S



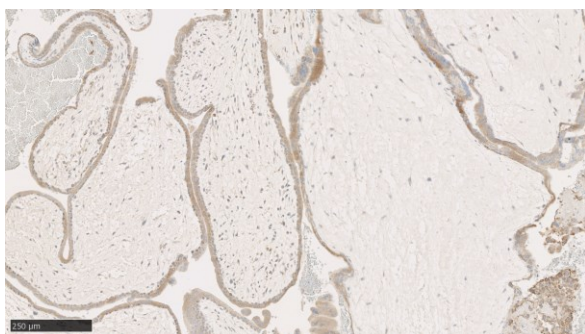
MTX-R



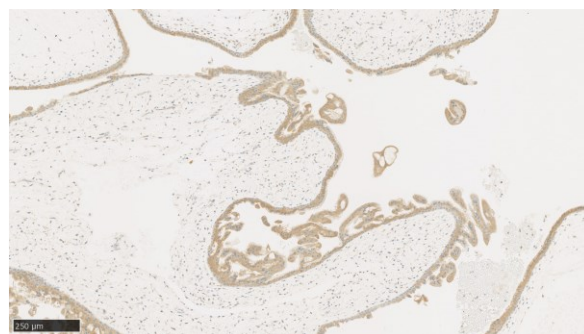
B)

TIGAR

MTX-S



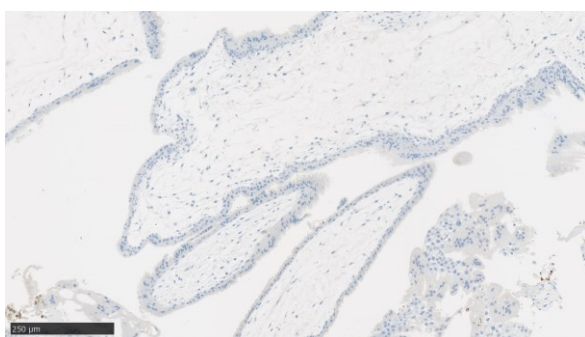
MTX-R



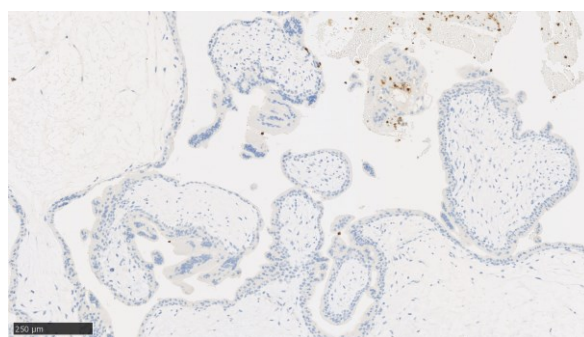
CDA

C)

MTX-S



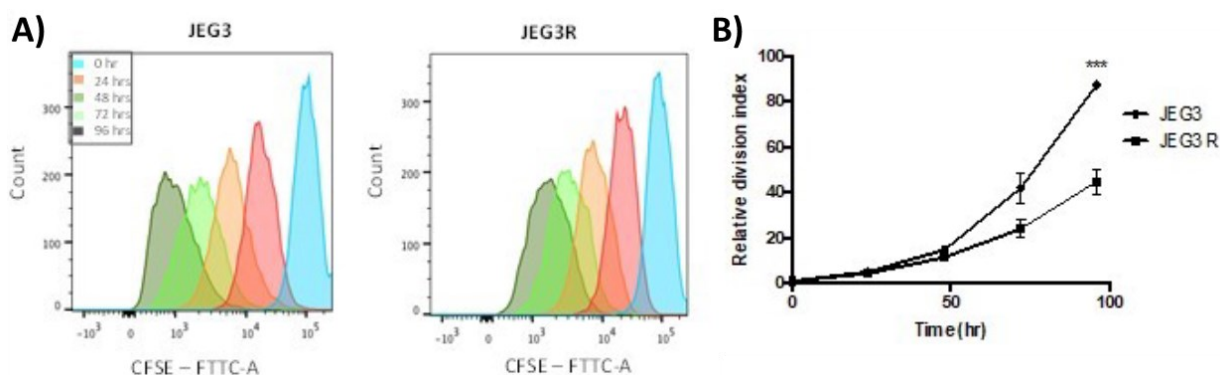
MTX-R



Supplementary Figure 3.3: The assessment of PHGDH, MRPL9 and CDA as prognosis biomarkers for MTX sensitivity and resistance

Representative IHC images of PHGDH (A), TIGAR (B) and CDA (C) expression in GTD patient sections from the MTX-Sensitive (MTX-S) and the MTX-Resistant (MTX-R) groups. Original magnification: $\times 4.18$. Scale bar: $250\mu\text{m}$.

7.2 Supplementary Figures – Chapter 4



Supplementary Figure 4.1: JEG3-R cells have a slower proliferation rate compared to JEG3 cells

For the needs of the proliferation assay, the same number of JEG3 and JEG3-R cells were stained with CFSE (carboxyfluorescein diacetate succinimidyl ester) and analyzed by flow cytometry at 0, 24, 48, 72, and 96 hours post-staining. **A)** Representative distribution of CFSE fluorescence dye across the selected time points for the two choriocarcinoma cell lines tested here. The histograms are representative of three independent experiments. **B)** The geometric means of the CFSE fluorescence dye at each time point was normalized against the 0-hour timepoint and the inversed normalized fluorescence intensity values were utilized to provide the relative division index of both JEG3 and JEG3-R. The graph shows the average $\pm$ SEM of 3 independent experiments. Statistical significance was determined using the two-way ANOVA test ($***p \leq 0.001$).

The above data belong to Dr. Marina Georgiou and are implemented in her PhD Thesis. The experiments were performed by herself with the contribution of her MRes students Ms Josephine Choo and Ms Tsvetana Stoilova (MRes Cancer Biology students).

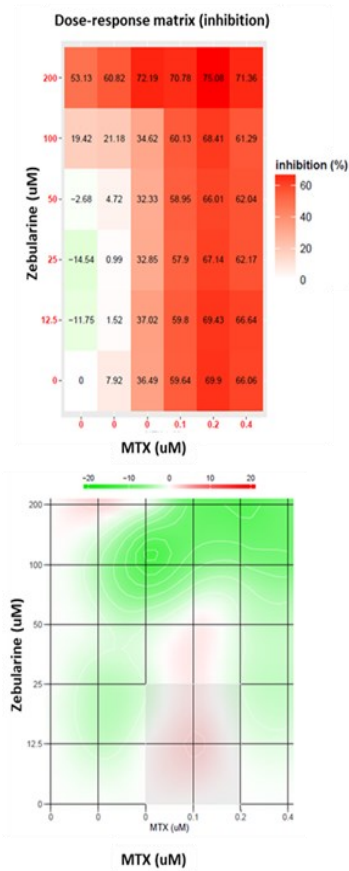
Supplementary Table 4.1: MTX-R/S Fold change ratio of CDA

Fold change ratio of MTX-Resistant / MTX-Sensitive group of patients for CDA. MTX-R group: n=5, MTX-S group: n=5. p-value=0.035 (*), unpaired Student t-test.

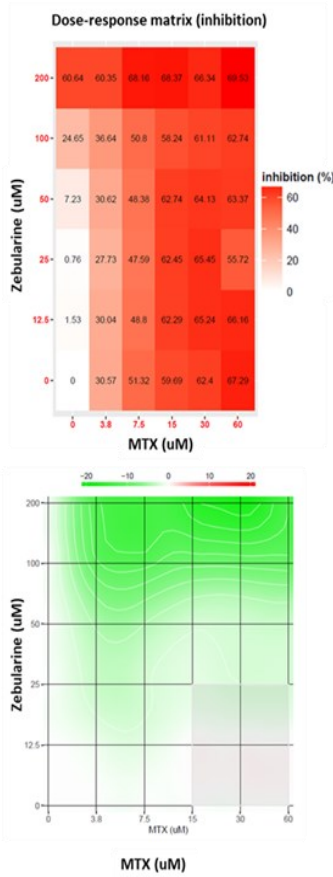
Hit name	Description	MTX-R/S f.c.
CDA	Cytidine deaminase	1.43*

A)

JEG3

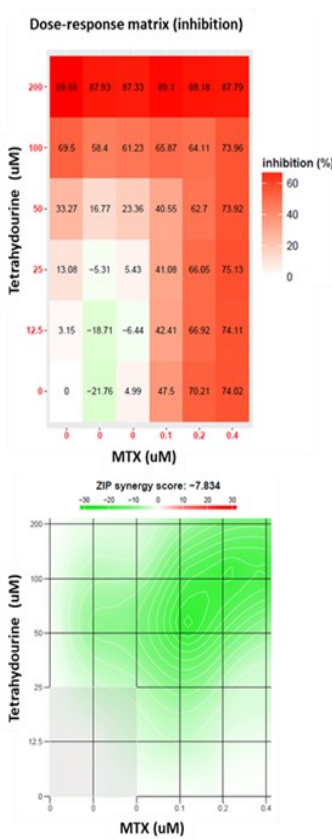


JEG3-R

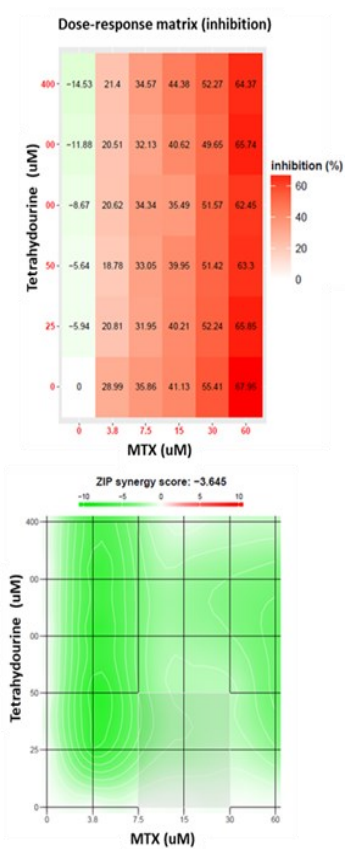


B)

JEG3

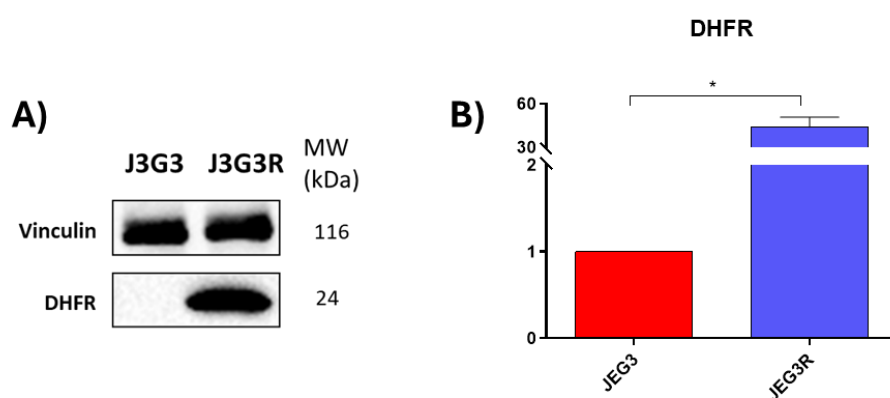


JEG3-R



Supplementary Figure 4.2: Drug synergy analysis for Methotrexate and CDA inhibitors

Visualization of the combination treatment of Methotrexate and Zebularine **(A)** Methotrexate and Tetrahydourine **(B)** in both JEG3 and JEG3-R cells. SunergyFinder 2.0 online web app was used to calculate synergy scores between the two compounds. The Zero interaction potency (ZIP) model was utilized and ZIP scores <-10 highlight antagonism, while ZIP scores >10 demonstrate synergism. The upper panels depict cell viability results as a function of the concentration of both compounds. The lower panels depict the results of synergy calculations. The average ZIP score was calculated by collecting data from three independent experiments.



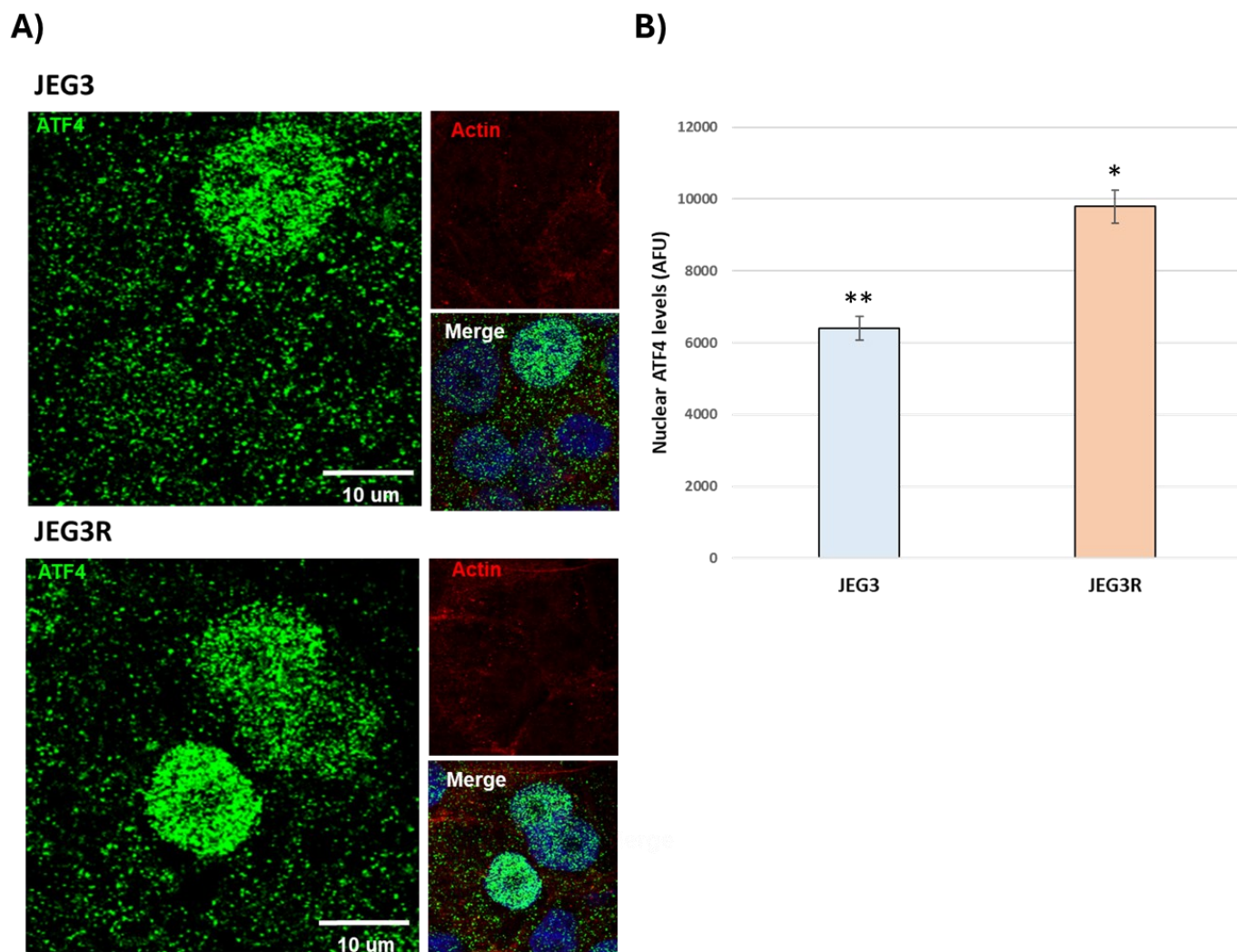
Supplementary Figure 4.3: DHFR overexpression in JEG3 and JEG3-R cells.

A) Immunoblot showing the expression of DHFR. Vinculin was utilized as a loading control. **B)** Relative mRNA expression of DHFR. The data show the mean fold changes $\pm$ SEM from 3 independent experiments. Statistical significance was determined using the Unpaired Student's t-test ($*p \leq 0.05$).

Supplementary Table 4.2: MTX-R/S Fold change ratio of GSTT1.

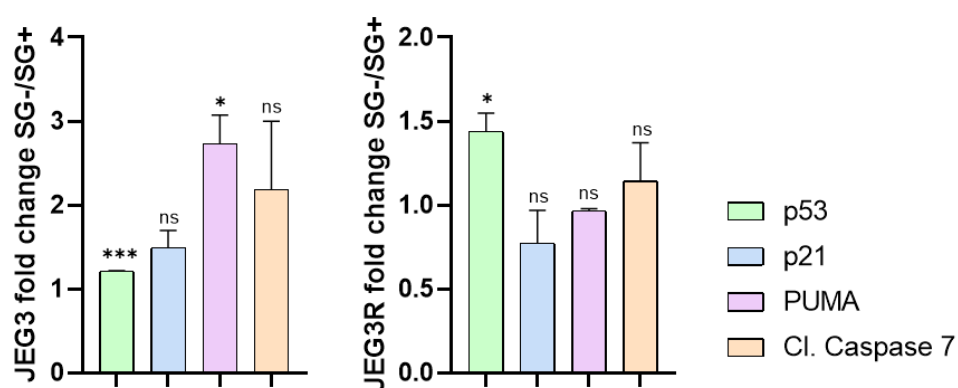
Hit name	Description	MTX-R/S f.c.
GSTT1	Glutathione S-transferase theta-1	1.44 (ns)

Fold change ratio of MTX-Resistant /MTX-Sensitive group of patients for GSTT1. MTX-R group: n=5, MTX-S group: n=5. Statistical significance was determined using the Unpaired Student's test (p -value=0.2, ns=not significant)



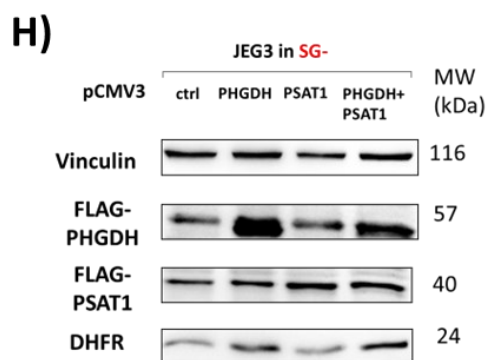
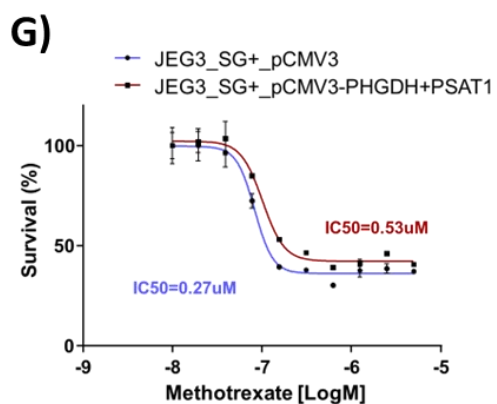
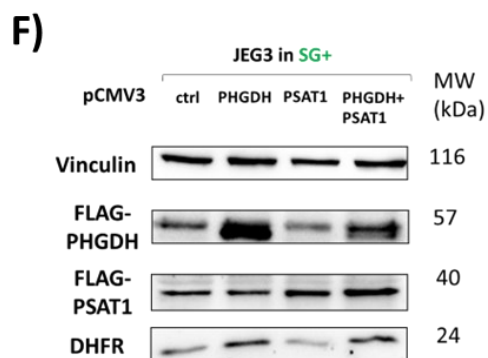
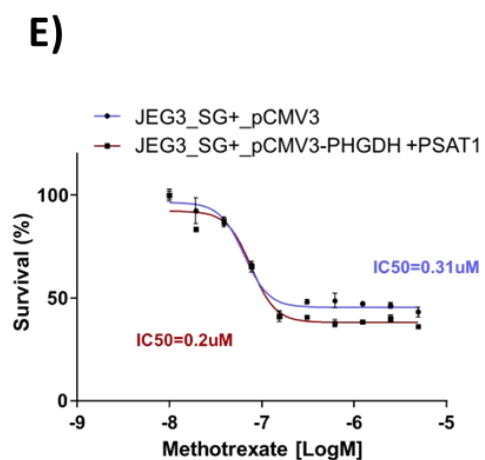
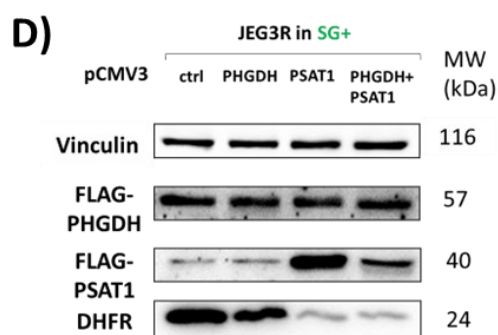
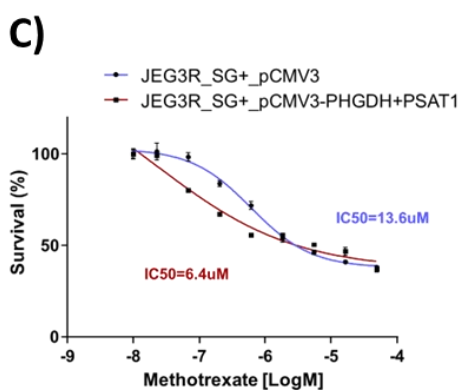
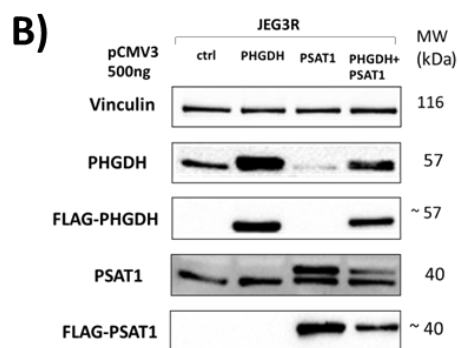
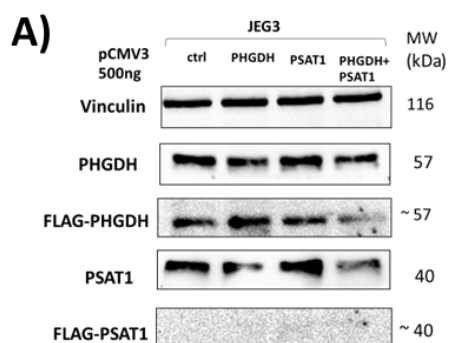
Supplementary Figure 4.4: The functional state of ATF4 in JEG3-R is not affected by mechanisms preventing the translocation into the nucleus.

A) Immunofluorescence staining with ATF4 and Actin in the JEG3 and JEG3-R cells. Images were captured in HCF1 – Zeiss LSM-780 inverted confocal microscope. **B)** Representative graph bars showing the nuclear ATF4 levels in JEG3 and JEG3-R measured in AFU (arbitrary fluorescence units). The data show the mean fold changes $\pm$ SEM from 3 independent experiments.



Supplementary Figure 4.5: JEG3 cells undergo apoptosis upon Serine/Glycine depletion

Bar graphs showing the fold change SG-/SG+ protein expression of p53, p21, PUMA and Cleaved Caspase 7 in JEG3 (left panel) and JEG3-R (right panel). The data show the mean fold changes $\pm$ SEM from at least 3 independent experiments. Statistical significance was determined using the Unpaired Student's t-test in (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, ns=not significant).



Supplementary Figure 4.6: pCMV3-mediated overexpression of both PHGDH and PSAT1 in JEG3R cells in SG+ condition made JEG3R cells more sensitive in a less extent to SG- condition while no condition conferred the same result for JEG3

A)+B) Generation of JEG3 **(A)** and JEG3R **(B)** cell lines overproducing PHGDH and PSAT1 in regular medium. Immunoblots showing the levels of PHGDH and PSAT1 (both flagged and unflagged) as a confirmation of the succession of the experiment. **C)** IC-50 Graph of MTX at **JEG3R_pCMV3_PHGDH+PSAT1** versus **JEG3R-pCMV3** cells in **SG+** condition. **D)** Immunoblots displaying the expression of flagged-PHGDH and flagged-PSAT1 and DHFR in JEG3R in SG+. **E)** IC-50 Graph of MTX at **JEG3_pCMV3_PHGDH+PSAT1** versus **JEG3-pCMV3** cells in **SG+** condition. **F)** Immunoblots displaying the expression of flagged-PHGDH and flagged-PSAT1 and DHFR in JEG3 in SG+. **G)** IC-50 Graph of MTX at **JEG3_pCMV3_PHGDH+PSAT1** versus **JEG3-pCMV3** generated **JEG3** cells in **SG-** condition. **H)** Immunoblots displaying the expression of flagged-PHGDH and flagged-PSAT1 and DHFR in JEG3R in SG+. For the above immunoblots. Vinculin was utilized as a loading control. The above data represent 3 independent biological replicates.

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