Studying genetic factors influencing DNA damage and repair during ageing: drug treatments and models of JAK/STAT pathway-related blood cancers

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Declaration

I affirm that I will comply with the University of Sheffield's policies concerning plagiarism. All submitted work will be original and created by me. Any content derived from external sources will be properly cited or directly quoted within the document.

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Abstract

The ubiquity of DNA damage from both natural and artificial sources poses a constant challenge to all living organisms. This research aims to better understand the processes that modulate DNA damage and how they contribute to disease and ageing, with a particular focus on the JAK/STAT signalling pathway and its inhibition by methotrexate (MTX) in *Drosophila melanogaster* models. The JAK/STAT pathway plays a pivotal role in cellular growth, development, and immune responses, and its dysregulation is implicated in various malignancies and disorders, including myeloproliferative neoplasms (MPNs). MTX, initially developed over seven decades ago as an anti-folate chemotherapeutic agent, is currently a World Health Organization-listed 'essential medicine' and serves as a first-line treatment for autoimmune and inflammatory conditions such as rheumatoid arthritis and psoriasis. Recent research in the Zeidler lab has identified MTX as an inhibitor of the JAK/STAT pathway, which is already targeted by other treatments for rheumatoid arthritis. This study reveals that low-dose MTX does not induce significant DNA damage in *Drosophila melanogaster* and may even extend lifespan and healthspan, suggesting potential applications beyond its traditional use in treating inflammatory diseases.

While high doses of MTX are associated with toxicity and DNA damage, low doses appear to be less harmful and may offer protective effects, opening new avenues for safer and more effective treatments for various diseases. Other findings demonstrates that folinic acid can mitigate the toxic effects of MTX in *Drosophila*. Low doses of MTX were found to inhibit JAK/STAT signalling in the gut of adult *Drosophila*, aligning with previous findings in human cell lines and suggesting a dose-dependent cellular stress response at higher concentrations. The preliminary data from next generation sequencing reveals that low concentrations of MTX (≤100 nM) did not induce inheritable mutations in the X chromosome DNA of *Drosophila*, supporting earlier findings that such concentrations do not cause DNA damage or affect biological processes like eclosion and wing serration. This observation contrasts with the genotoxic effects observed at higher MTX concentrations (>1 μM), which negatively influenced various biological processes and induced DNA damage. The study calls for further research

to explore the transgenerational effects of MTX, and may even help with a new discovery, given the limitations of the current study's sample size and genomic scope.

Publications

1. Alqarni, A.M. and Zeidler, M.P., 2020. How does methotrexate work? *Biochemical Society Transactions*, *48*(2), pp.559-567.
2. Chen, Z., Cordero, J., Alqarni, A.M., Slack, C., Zeidler, M.P. and Bellantuono, I., 2022. Zoledronate extends healthspan and survival via the mevalonate pathway in a FOXO-dependent manner. *The Journals of Gerontology: Series A*, *77*(8), pp.1494-1502.

Poster presentations

1. Drug Repurposing at a Biochemical Society Scientific meeting in Birmingham, UK on 18-19th November 2019.
2. 63rd Annual *Drosophila* Research Conference, San Diego, USA on April 6-10, 2022

Oral presentations

1. Drug Repurposing at a Biochemical Society Scientific meeting in Birmingham, UK on 18-19th November 2019.

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Abbreviations

AICAR-T: aminoimidazole carboxamide ribonucleotide transferase

AICAR-T: aminoimidazole-carboxamide ribonucleotide transformylase

AML: acute myeloid leukaemia

AMPK: activated protein kinase

ASXL1: Additional Sex Combs-Like 1

ATM: ataxia-telangiectasia mutated

BER: base excision repair

BWA: Burrows Wheeler Aligner

CBL: Casitas B-lineage lymphoma proto-oncogene

CML: chronic myeloid leukaemia

CNV: Copy Number Variation

CPDG2: carboxypeptidase G2

DDR: DNA damage response

DHFR: dihydrofolate reductase

DMARD: disease-modifying anti-rheumatic drug

DNA: Deoxyribonucleic acid

DSBs: double strand breaks

DSBR: double-strand break repair

ELISA: Sandwich enzyme-linked immunosorbent assay

Epo: Erythropoietin

ET: essential thrombocythemia

FA: folinic acid

FAIRAR: formamidoimidazole carboxamide ribonucleotide

FPGS: folylpolyglutamate synthase

GFP: Green Fluorescent Protein

GD: GranDad

GGH: Gamma-glutamyl hydrolase

GMR: Glass Multimerised Response element

HDMTX: High-dose methotrexate

hop: hopscotch

HP1: heterochromatin protein 1

HR: homologous recombination

IDH: Isocitrate dehydrogenase

IGF: insulin/insulin-like growth factor

IKZF1: IKAROS family zinc finger 1

IL-6: interleukin-6

in-del: insertion-deletion

InDels: insertions or deletions of small DNA segments

LOF: loss of function

LOH: loss of heterozygosity

MF: myelofibrosis

MPNs: myeloproliferative neoplasms

MTX: Methotrexate

mwh: multiple wing hair

NAD+: Nicotinamide adenine dinucleotide

NGS: next generation sequencing

NHEJ: non-homologous end-joining

NICE: National Institute for Health and Care Excellence

NMN: nicotinamide mononucleotide

NR: nicotinamide riboside

PI3K: phosphatidylinositide 3-kinase

PIKK: phosphatidylinositol 3-kinase-like kinase

PV: polycythemia vera

RING: Rapid iterative negative geotaxis

RNAi: RNA interference

RNR: ribonucleoside diphosphate reductase

RTKs: receptor tyrosine kinases

SASP: senescence-associated secretory phenotype

SLS: Scientific Laboratory Supplies

SNP/InDel: Single Nucleotide Polymorphism/Insertion-Deletion

SOCS: suppressors of cytokine signalling

SSB: single-stranded breaks

SV: Structural Variation

TET2: TET oncogene family member 2

TOR: target of rapamycin

Tpo: thrombopoietin

TYK2: tyrosine kinase 2

UAS: Upstream Activation Sequence

UV: ultraviolet

w: white

Chapter 1: Introduction

1.1 Introduction

[Note: Section 1.5 is based / rewritten from my paper (Alqarni and Zeidler, 2020)]. This review was initially drafted by AA and cowritten in collaboration with MZ. The portions of the review included in this Chapter have been paraphrased. All living organisms are exposed to both naturally occurring and manmade sources of deoxyribonucleic acid (DNA) damage throughout their lives. This includes ionising radiation and chemical mutagens which cause a range of DNA damage including single strand breaks (SSBs), double strand breaks (DSBs), and base modifications) (Iliakis et al., 2003a). In actively dividing cells, the DNA damage response (DDR) (Karantanos and Moliterno, 2018) triggers cell cycle checkpoints and leads to either programmed cell death, senescence or other forms of cell cycle arrest (Iliakis et al., 2003a). However, the ability of non-dividing, post-mitotic cells to respond to DNA damage is less well understood and apoptosis senescence of these post-mitotic cells may compromise the tissues they make up, thereby contributing to the gradual physiological decline associated with ageing. Ultimately, failure to repair DNA damage can lead to the accumulation of potentially oncogenic mutations, that could ultimately lead to cancer. Furthermore, malignancies are themselves often treated with DNA damaging therapies which can lead to the development of therapy-induced secondary cancers (Reuvers et al., 2020).

One class of cancer of particular interest to the Zeidler lab are the myeloproliferative neoplasms (MPNs). The ‘classic’ MPNs are Philadelphia-chromosome negative chronic myeloid leukaemias comprised of the haematological diseases polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis (MF). MPN patients generally have clonally-related populations of haematopoietic stem cells carrying mutations that activate the JAK/STAT signalling pathway. The most common of these is the gain-of-function JAK2 V617F mutation which is present in 95% of PV patients and 40-60% of ET and MF patients (James et al., 2005a; Kralovics et al., 2005a; Levine et al., 2005).

Intriguingly, a number of studies have shown that cells carrying the JAK2 V617F mutation have reduced genomic stability and a higher rate of DNA damage (Karantanos and Moliterno, 2018). However, the mechanism for this effect is not entirely clear. Consistent with this, studies in *Drosophila* have shown that when unphosphorylated, the STAT92E transcription factor can dimerise with heterochromatin protein 1 (HP1) to stabilise heterochromatin and protect DNA from damage (Shi et al., 2008a). This DNA-protective effect is reduced when STAT92E is recruited to the canonical JAK/STAT signalling pathway by JAK-mediated phosphorylation - as would take place constitutively in the presence of a gain-of function JAK mutation such as V617F. Consistent with this, gain-of-function mutations in the *Drosophila* JAK homologue Hopscotch have been shown by the Zeidler lab to reduce genome stability as demonstrated by increased rates of interchromosomal meiotic recombination and non-disjunction in these backgrounds (Zeidler, unpublished data).

In this thesis I will develop tools and techniques to study DNA damage and related organismal aging *in vivo* using *Drosophila* as a model system. I will examine how DNA damage in post-mitotic cells is repaired and accumulates as animals age. I will seek to identify the genetic factors that affect DNA damage rates *in vivo* and whether pharmacological interventions (for example methotrexate that inhibits JAK/STAT activity) is able to modulate DNA damage rates in a way that correlates with changes in lifespan and healthspan.

1.2 Overview

1.2.1 The JAK/STAT signalling pathway

The JAK/STAT signalling pathway is an important and highly conserved signalling cascade which regulates cell proliferation, development, immunity, and inflammation (Brown and Zeidler, 2008). While loss of the pathway is generally lethal, its inappropriate activation is associated with various types of cancers and diseases, including MPNs (Brown and Zeidler, 2008).

The canonical JAK/STAT signalling pathway consists of four main components: extracellular ligands, trans-membrane receptors, JAK kinases and STAT transcription factors. Initially, membrane bound receptors, such as the Erythropoietin (Epo) receptor, bind to the Epo ligand on the extracellular side of the plasma membrane (Tsurumi et al., 2017). In the canonical model of pathway activity, ligand binding to the receptor, causes JAKs associated with the intracellular side of the receptor to be activated, phosphorylating both their own 'regulatory' tyrosine residues and tyrosine residues present in the opposite 'trans-' JAK and in the associated 'trans-'receptor. Cytosolic STATs then bind to the phosphorylated tyrosine residues present within the activated receptors via their own SH2 domains, and are themselves phosphorylated by JAK on an invariant and highly conserved tyrosine residue. Finally, STATs dimerise in a head to tail conformation and translocate to the nucleus, where they bind to a palindromic DNA sequence and activate the transcription of adjacent target genes (Figure1) (Darnell et al., 1994; Levy and Darnell, 2002). This pathway was discovered initially in mammals at the beginning of the 1990s and is widely conserved across evolution including in *Drosophila* (Bromberg and Darnell, 2000; Darnell et al., 1994; Levy and Darnell, 2002).

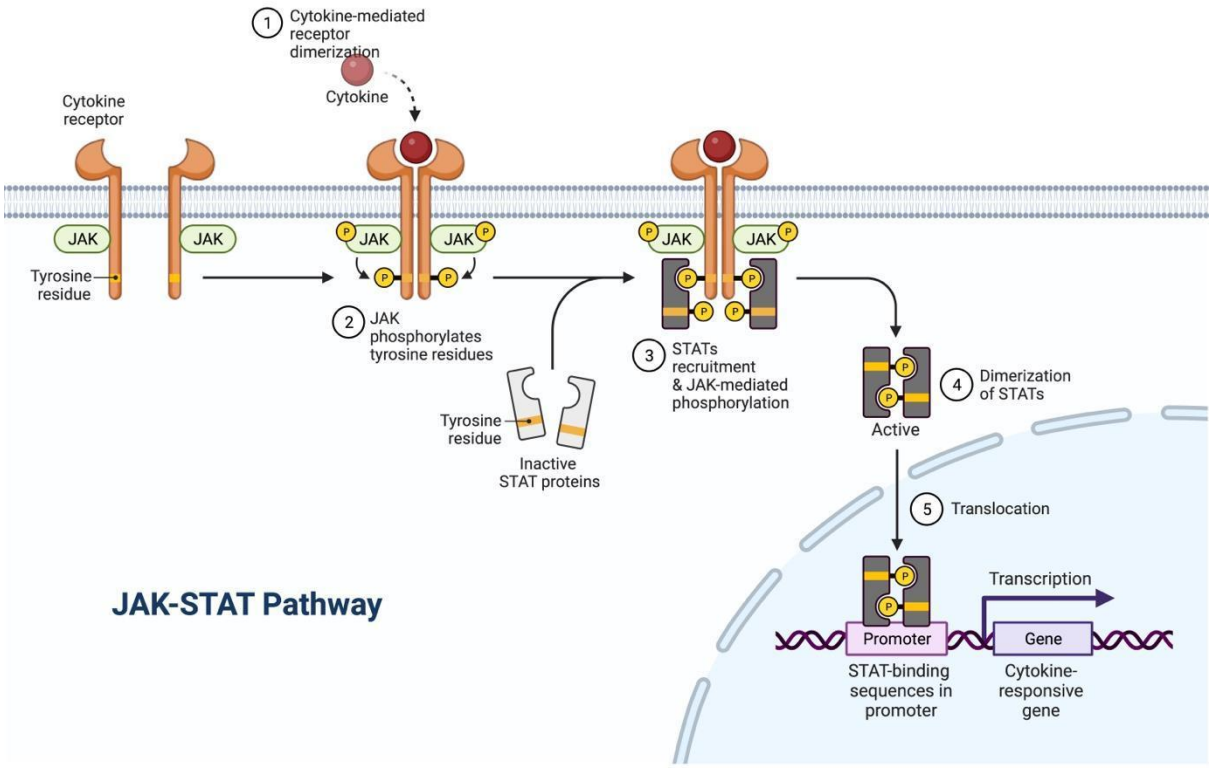


Figure 1: Structure of JAK/STAT signalling pathway: This diagram shows the process of the intracellular signalling pathway. First, inactivation of the JAK complex binds to the receptors. Second, when the JAK is attached to the ligands, JAK becomes phosphorylated leading to activation of the tyrosine residues. Third, STATs are then phosphorylated by binding to the activated tyrosine residues. The STAT complex translocates to the nucleus and drives the transcription of the targeted genes. Created with BioRender.com

The mammalian genome encodes seven STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6), and four JAK kinases (JAK1, JAK2, JAK3 and tyrosine kinase 2 -TYK2) (Tsurumi et al., 2017). However, the *Drosophila melanogaster* genome contains only one JAK and one STAT-like gene. These are *hopscotch (hop)*, which is most similar to JAK2 and STAT92E that is most similar to STAT5. This simplified, yet conserved pathway allows *Drosophila* to provide a potentially similar, but less complicated model to test and understand the general genetic mechanisms underlying JAK/STAT signalling (Grewal and Elgin, 2002; Shi et al., 2006).

One example of insight gained from such research in *Drosophila* was the finding that over-activation of JAK (by the gain-of-function allele $hop^{Tum\text{-}l}$) causes the over-proliferation of blood cells and the formation of haematopoietic tumours (Harrison et al., 1995; Shi et al., 2006). Consistent with this finding, it was subsequently shown that JAK/STAT pathway activating mutations are the primary or 'driver' mutations responsible for causing disease in almost all MPN patients (Harrison et al., 1995; Shi et al., 2006). Furthermore, activating mutations in JAK2 have also associated with DNA damage and repair processes. For example, the JAK2 V617F mutation is associated with increased DNA damage by factors such as reactive oxygen species (ROS) and replication stress disruption leading to an increased mutation rate (Iliakis et al., 2003). Typically, these types of DNA damage result in the activation of cell cycle checkpoints that can trigger programmed cell death or senescence (Iliakis et al., 2003). The cell cycle arrest that results from such as DNA Damage Response (DDR) activation is a major mechanism in preventing cancer.

Given the links between aberrant JAK/STAT pathway activity and diseases such as cancer and autoimmune disorders, the pathway is closely monitored and controlled through numerous mechanisms of negative feedback and signal termination (O'Shea and Plenge, 2012).

1.2.1.1 Negative regulators of JAK/STAT signalling

To ensure cellular homeostasis, the JAK/STAT signalling pathway must be carefully managed. This control is facilitated through various mechanisms of negative feedback:

Suppressors of Cytokine Signalling (SOCS) are transcriptional targets of the JAK/STAT cascade, that have been shown to be negative regulators of pathway signalling. By attaching to JAKs, SOCS proteins inhibit their kinase activity or bind to receptors to block STAT recruitment (Yoshimura et al., 2007). Protein Inhibitors of Activated STATs (PIAS) inhibit STAT function by obstructing their ability to bind DNA or by trapping them in inactive complexes (Shuai and Liu, 2005). Protein Tyrosine Phosphatases (PTPs) are a class of enzymes that remove phosphate groups from phosphorylated tyrosine residues on pathway receptors, JAKs and STATs. PTPs, such as SHP1 (Src homology region 2 domain-containing

phosphatase-1) and SHP2 (Src homology region 2 domain-containing phosphatase-2), counteract their activation and are key in the negative regulation of JAK/STAT signalling (Stark et al., 1998).

Signal termination encompasses not only these negative regulators but also the breakdown of signalling elements. For example, by ubiquitination and proteasomal degradation. The process of ubiquitination marks JAKs and STATs for degradation by the 26S proteasome, which reduces the concentration of these signalling molecules and terminates the signal. SOCS (Suppressor of Cytokine Signalling) proteins play a key role in this process by promoting the ubiquitination of the receptor complex, including JAKs, thus facilitating their degradation and negatively regulating cytokine signalling pathways. (Alexander and Hilton, 2004). Endocytosis and lysosomal degradation of receptors also occurs. Following activation, cytokine receptors may be internalised and either recycled or transported to lysosomes for breakdown, so reducing the cell's sensitivity to subsequent signals (Cendrowski et al., 2016). Transcriptional repression also plays a role. Genes activated by STAT proteins may produce inhibitors of signalling, introducing an additional layer of feedback regulation. The activation of SOCS genes, for instance, can hinder further JAK/STAT activation.

In summary, the JAK/STAT signalling pathway is crucial for transmitting extracellular messages to the nucleus, altering gene expression, and affecting a wide array of biological functions. Its regulation requires a careful equilibrium between activation and suppression, where negative feedback and signal discontinuation are essential in preventing pathological conditions. A deeper understanding of these control mechanisms presents potential avenues for therapeutic interventions in diseases linked to abnormal JAK/STAT signalling.

The identification of the JAK2 V617F mutation in a considerable number of patients with Myeloproliferative Neoplasms (MPNs) was a breakthrough in understanding the molecular mechanisms underlying these conditions. This mutation, alongside other ‘driver’ mutations that activate the JAK/STAT pathway, plays a fundamental role in the development of MPNs by driving the uncontrolled proliferation of hematopoietic progenitor cells (Kralovics et al., 2005).

1.2.2 Mutations leading to MPNs

1.2.2.1 JAK2 V617F mutation

The mutation most frequently associated with MPNs is JAK2 V617F, detected in about 95% of Polycythemia Vera (PV) instances and 50-60% of Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) cases (James et al., 2005; Wan and Coveney, 2012).. This genetic alteration occurs in the pseudokinase (JH2) domain of JAK2, resulting in the substitution of valine for phenylalanine at position 617. Normally, the JH2 domain exerts an autoinhibitory effect on the adjacent kinase (JH1) domain, preventing its activation in the absence of cytokine binding. The V617F mutation disrupts this inhibitory interaction by stabilising the JH2–JH1 interface in a conformation that mimics the active state, allowing for persistent JH1 activation. In addition, the bulky phenylalanine residue at position 617 promotes constitutive JAK2 dimerization via the FERM and SH2-like domains, even without receptor or ligand engagement. This leads to ligand-independent autophosphorylation and activation of downstream signalling pathways. Importantly, the mutant protein remains responsive to ligand stimulation, resulting in exaggerated signalling output when cytokines are present (James et al., 2005; Wan and Coveney, 2012).

1.2.2.2 Mutations in CALR and MPL

Although the JAK2 V617F mutation is predominant, it is not present in all individuals with MPNs. Mutations in CALR (calreticulin) and MPL (thrombopoietin receptor) have been discovered in patients without the JAK2 V617F mutation. +1 frameshift mutations in the golgi chaperone and calcium ion-binding protein CALR result in a modified C-terminus that lacks the proteins original Golgi retention signal and adds a novel peptide. This mutated protein has an increased affinity for interacting with MPL, triggering MPL dimerization and, consequently, JAK2 activation (Shivarov et al., 2014). In a similar manner, mutations in MPL, such as W515L/K, directly stimulate JAK2 activation by promoting receptor dimerisation and autophosphorylation of JAK2 (Nangalia et al., 2013; Pikman et al., 2006).

In summary, mutations that activate the JAK/STAT pathway play a fundamental role in the development of Myeloproliferative Neoplasms (MPNs). Specifically, the JAK2 V617F mutation, as

well as mutations in CALR and MPL, result in the continuous low-level activation of this signalling pathway, which in turn drives the uncontrolled growth of haematopoietic stem cells and blood cell precursors. Gaining an understanding of these molecular dynamics has shed light on the underlying mechanisms of MPNs and opened avenues for the development of targeted treatment options.

Interestingly, MPNs 'driven' by gain-of-function JAK/STAT pathway mutations are also frequently associated with additional mutations in other genes, most notably epigenetic regulators such as TET oncogene family member 2 (*TET2*), Isocitrate dehydrogenase (*IDH*), IKAROS family zinc finger 1 (*IKZF1*), Casitas B-lineage lymphoma proto-oncogene (*CBL*), and Additional Sex Combs-Like 1 (*ASXL1*) (Tefferi, 2010). Many of these secondary mutations are linked to changes in disease severity and prognosis (Tefferi, 2010). It is possible that many of these secondary mutations arise due to increased rates of DNA damage caused by the JAK/STAT-pathway activating driver mutation. Furthermore, this suggests that the normal checkpoint activation and senescence/apoptosis pathways are insufficient to prevent mutated MPN haematopoietic stem cells from multiplying and ultimately taking over the bone marrow niche (Vainchenker and Kralovics, 2017).

Ultimately, if the rate of DNA damage in HSCs carrying gain-of-function JAK/STAT alleles can be reduced, the frequency of developing secondary mutations associated with poorer prognosis may also be decreased. Such a therapy could ultimately provide a therapeutic method, particularly for those patients at risk of progressing to myelofibrosis (MF) or acute myeloid leukaemia (AML).

1.2.3 DNA damage repair

DNA is prone to many alterations and changes caused by both internal factors for instance ROS, and external factors such as carcinogens, pollution, and ionising irradiation (Ciccia and Elledge, 2010). These mutagens commonly lead to single-stranded breaks (SSB). Whilst double-stranded breaks (DSBs) happen less frequently, they can have extremely serious impact and cause chromosomal changes, deletions, and multiple types of cancer. DNA damage response (DDR) is an important system in mammalian cells. It protects the DNA from mutations which could be passed to new offspring, it

prevents transformation of cells that might result in cancer, and stabilizes the genome (Delia and Mizutani, 2017). There are many factors affecting DNA which can cause replication stress.

Endogenous replication stress is one of the major causes of DSBs due to the lack of nutrients inside the cell (Bester et al., 2011). When this results in stalled replication forks, it may cause single strand nicks (Cobb et al., 2005; Lambert et al., 2005), and if these are not repaired, it could result in DSBs (Cortés-Ledesma and Aguilera, 2006). An intra S phase checkpoint is then activated, resulting in either cell cycle arrest to allow for repair of the damage or the initiation of programmed cell death.

There are three essential kinases related to DDR signalling. These are ataxia-telangiectasia mutated (ATM), ATR, and DNA-PK. These proteins make up the PIKK (phosphatidylinositol 3-kinase-like kinase) family (Shiloh and Ziv, 2013). The primary role of these kinases involves the regulation of the DDR through checkpoint activation, chromosomal modifications, cell cycle arrest, or the initiation of programmed cell death in the absence of viable repair options (Cussiol et al., 2020).

These kinases tend to exhibit the same characteristics: ATM is activated by and repairs double strand breaks (DSBs), while ATR repairs single-strand breaks (SSBs). When ATM is activated due to double-strand breaks (DSBs), it facilitates their repair through a process called homologous recombination (HR). On the other hand, DNA-PK primarily addresses DSBs using a different mechanism known as non-homologous end-joining (NHEJ) (Blackford and Jackson, 2017).

The third DNA repair mechanism is termed mismatch repair in which mis-paired bases that remain after DNA replication and proof-reading, as well as small deletions and insertions are recognised by a protein complex (Chang et al., 2002; Modrich and Lahue, 1996).

There are two pathways that are responsible for the repair of DSBs, the NHEJ and HR pathways. DSBs are potentially very dangerous and may cause serious problems if not repaired because large amounts of information within the genome, such as chromosomes and genes, may be deleted or rearranged. In NHEJ, the ends of the two broken chromosomes are joined together, but this may cause loss or increase

of the number of the nucleotides at the break site. As a result, this pathway may cause a mutation in the region of the original break (Rassool, 2003).

In HR, the homologous chromosome that is equivalent to the broken one is used as a template to repair the break. Therefore, the HR pathway is better than the NHEJ because it is more sensitive and generally does not cause mutations (Shrivastav et al., 2008).

1.2.4 JAK2 and DNA damage

A number of studies have shown that activation of JAK/STAT signalling is associated with increased rates of DNA damage (Silver-Morse and Li, 2013). It is important to note that an increase in ROS levels has been implicated in causing greater DNA damage and genomic instability (Nieborowska-Skorska et al., 2012) Additionally, mutations occur either directly following DNA damage or because of a failure in the DNA damage repair. This increase could be the consequence of increased rates of DNA damage, and/or a decrease in the effectiveness of DNA repair mechanisms.

The level of intracellular reactive oxygen species (ROS) are increased in many myeloid malignant diseases including MPNs (Nieborowska-Skorska et al., 2012). Over-activation of JAK2 V617F results in increased ROS through the activation of phosphatidylinositide 3-kinase (PI3K) and the negative regulation of NHE-1, which is crucial for inducing apoptosis following DNA damage (Ahn et al., 2016). A different study illustrated that JAK2 V617F mutation causes non-autonomous DNA damage through the accumulation of ROS in the adjacent normal cell reference. However, ROS induce lipocalin-2, which activates p53, leads to apoptosis specifically in JAK2 V617F mutated cells (Kagoya et al., 2014).

The JAK2 V617F mutation in the Ba/F3 cell line has been shown to increase the frequency of homologous recombination (HR), which is associated with RAD51 activity and the process of sister chromatid exchange (Plo et al., 2008). In addition, the JAK2 V617F mutation has been found to cause replication fork stalling. This can lead to activation of the intra S checkpoint through PI3K signalling. However, in the presence of the JAK2 mutation, the stimulation of the intra S checkpoint by DNA damage was much lower for PV patients (Chen et al., 2014). This is because of a defect of ATR in PV

patients, which results in reducing the phosphorylation of Chk1 to counteract replication errors (Chen et al., 2014a). These data can increase the mutations and disrupt the genome. Eventually, PV cells will experience *P53* suppression - the main cause of PV transforming to AML (Cerquozzi and Tefferi, 2015).

1.2.5 The non-canonical JAK/STAT signalling pathway

Studies in *Drosophila melanogaster* have also described a non-canonical JAK/STAT signalling pathway that regulates and stabilises heterochromatin formation and so indirectly affects genomic stability. In this non-canonical pathway, unphosphorylated STAT binds to Heterochromatin protein 1 (HP1) to generate a STAT:HP1 hetero-dimer which then associates with DNA to promote heterochromatin formation and so leads to genome stabilisation (Shi et al., 2008).

As noted above, in the canonical JAK/STAT pathway over-activation of JAK results in the constitutive phosphorylation of STAT, thereby resulting in STAT homo-dimerization in the nucleus. This reduces the ability of unphosphorylated STATs to form hetero-dimers of STAT:HP1, thus leading to reduced heterochromatinisation and genome instability (Brown and Zeidler, 2008). These findings suggest that the heterochromatin may not be affected by a lower amount of JAK activating STAT, while over activation of JAK may recruit STAT and cause genome instability.

Consistent with the model in which unphosphorylated STAT:HP1 heterodimers promote chromatinisation, JAK gain-of-function mutations, STAT loss-of-function and decreasing levels of heterochromatin all led to increased genome instability compared to controls. Conversely, increasing the level of an unphosphorylatable STAT92E variant (by overexpressing STAT92E with a Y704F mutation) or overexpressing wild type HP1 decreases the DNA damage after irradiation and stabilises the genome (Yan et al., 2011).

In the non-canonical mode of JAK/STAT signalling, either reducing the amount of STAT or over activating JAK decreases the proportion of heterochromatin and inhibits the expression of genes in regions of heterochromatin (Larson et al., 2012). Conversely, in the canonical pathway, JAK

overexpression and reduction of STAT level have opposite effects on STAT target gene expression (Tsurumi et al., 2017).

In another study by Tsurumi, Zhao et al. (2017), the researchers compared the transcriptomic changes induced by canonical and non-canonical JAK/STAT pathways in *Drosophila*. They analysed gene expression during embryogenesis using a microarray, comparing wild type embryos to those with JAK gain-of-function, JAK loss-of-function, and STAT loss-of-function mutations. Their findings revealed that the non-canonical pathway influenced gene expression patterns, leading to a notable increase in the proportion of genes associated with heterochromatin.

Disruption of the genome can lead to serious problems, including tumorigenesis, ageing, or cell death (Jefford and Irminger-Finger, 2006; Lombard et al., 2005). STAT is an important regulator for heterochromatin assembly, and heterochromatin has a significant role in controlling and maintaining the genome (Grewal and Jia, 2007).

Unphosphorylated STAT plays a crucial role in genome stability by managing heterochromatin formation. According to a study by Yan et al. (2011) in the FASEB Journal, maintaining genome stability through heterochromatin formation is essential for normal cellular functions and survival under genotoxic stress (S.-J. Yan et al., 2011). Another study by Shi et al. (2008) in Nature Cell Biology highlighted that the levels of unphosphorylated STAT92E can lead to heterochromatin stability (Shi et al., 2008a). Furthermore, the role of heterochromatin in promoting longevity was emphasised by Larson et al. (2012) in PLoS Genetics, suggesting that proper heterochromatin formation, facilitated by unphosphorylated STAT, not only ensures genome protection but also has implications for extending lifespan (Larson et al., 2012).

1.3 The JAK/STAT pathway is an attractive drug target

The JAK/STAT signalling pathway plays a critical role in multiple cellular processes, including cell proliferation, differentiation, and immune responses. Dysregulation of this pathway has been implicated in a number of pathological conditions, making it an attractive target for therapeutic intervention. Kinase inhibitors, designed to target the ATP pocket of kinases, offer a promising approach to modulate the activity of this pathway. Ruxolitinib, a JAK1/2 inhibitor, exemplifies this category of drugs. Developed after the discovery of the gain-of-function JAK2 V617F mutation in 2005 (Harrison et al., 2012), several clinical trials have showed to its efficacy. The drug has since been licenced to treat patients with primary and secondary MF, showing significant improvement in quality of life scores, reducing spleen volume, and enhancing patient survival (Pardanani et al., 2015). While the EMA has licensed it for treating MF and PV patients resistant to other treatments, its usage in the UK is challenging. The National Institute for Health and Care Excellence (NICE) has cited cost-effectiveness as a concern, given its substantial annual cost of approximately £40,000 per patient (Dakin et al., 2015)

1.4 Introduction to ageing

Ageing is a complex process that affects many living organisms, characterised by a gradual decline in physiological function and an increased vulnerability to death. However, this process is not universal across all species, and examples of species that do not age can be found, such as jellyfish. For instance, *Turritopsis dohrnii* can revert its cells to an earlier life stage, effectively bypassing senescence. This ability has led to its description as a biologically 'immortal' species. It is a complex biological phenomenon that involves a progressive decline in physiological functions and an increased susceptibility to diseases and death. Ageing can be observed at multiple levels, including at the cellular, tissue, and organismal level. At the cellular level, ageing is characterised by a decline in the functionality and regenerative capacity of cells (Mohamad Kamal et al., 2020). This decline is attributed to various factors, including telomere shortening, DNA damage, epigenetic alterations, mitochondrial

dysfunction, and impaired cellular communication. These cellular changes contribute to the overall decline in tissue and organ functions (Rossiello et al., 2022).

Ageing also impacts different tissues and organs. For example, in the cardiovascular system, ageing leads to stiffening of blood vessels, reduced elasticity of the heart, and decreased efficiency of the circulatory system. Similarly, in the nervous system, ageing is associated with a decline in cognitive functions, such as memory and learning abilities (Abdellatif et al., 2023). The study of ageing, known as gerontology, aims to understand the underlying mechanisms and factors that contribute to this complex process. Researchers investigate various aspects of ageing, including genetic factors, environmental influences, lifestyle choices, and the interplay between them. By understanding the underlying mechanisms of ageing, scientists hope to develop strategies to promote healthy ageing and improve the quality of life for older individuals.

A great number of mutations accumulate over time as tissues age. Progeroid syndromes serve as a prime example of how defects in the DNA Damage Response (DDR) system can lead to premature ageing. Essentially, when the DDR system is compromised, as seen in progeroid syndromes, There is an accumulation of DNA damage. This heightened DNA damage accelerates the ageing process, manifesting as rapid age-related physiological decline. Over time, this can predispose individuals to age-associated diseases, including cancer. This implies that the high levels of DNA damage results in accelerated progression of age-related physiological failure and can lead to diseases related to old age such as cancer. Moreover, increased DNA damage can trigger intracellular signalling pathways that result in programmed cell death. This leads to both a direct loss of tissue and also a reduction in the number of stem cells which combine to exhibit the ageing phenotype (Freitas and de Magalhães, 2011).

Consistent with the model in which the accumulation of DNA damage acts as a driver of the ageing phenotype, the three DNA Damage Response (DDR) mechanisms—base excision repair (BER), nucleotide excision repair (NER), and non-homologous end joining (NHEJ)—have been strongly correlated with ageing. A study by Freitas and de Magalhães (2011) demonstrated that alterations in

these three DDR mechanisms can lead to the manifestation of physical characteristics commonly associated with ageing (Freitas and de Magalhães, 2011).

In addition, oxidative stress, resulting from an imbalance between reactive oxygen species production and the body's antioxidant defences, has been shown to play a pivotal role in brain ageing - especially via oxidative damage to DNA (Dröge and Schipper, 2007). A study by Mecocci et al. (1994) in the Annals of Neurology highlighted that increased oxidative damage to mitochondrial DNA is observed in Alzheimer's disease, suggesting its contribution to the neurodegenerative process (Mecocci et al., 1994). Another study emphasized that DNA damage can impact the expression of genes crucial for learning, memory, and neuronal survival, potentially accelerating the brain ageing process. In summary, oxidative DNA damage is intricately linked to cognitive decline and neurodegenerative diseases, underscoring its significance in brain ageing. In addition, the levels of DNA damage can cause less efficient gene expression (Lu et al., 2004).

1.5 How does methotrexate work?

The use of anti-folates, which began over seven decades ago to tackle juvenile acute lymphocytic leukemia, marked the dawn of chemotherapy as a method for battling cancer (Farber and Diamond, 1948). Inadvertently, the team led by Faber conceived a powerful, yet reversible, dihydrofolate reductase (DHFR) inhibitor by substituting the hydroxyl group in the pteridine ring of folic acid with an amino group (Figure 2). The enzyme, DHFR, is integral to the folate pathway and crucial for the production of purines and pyrimidines, which are needed for DNA replication and repair (Figure 3) (Bedoui et al., 2019a). Further efforts to create less toxic anti-folates with reduced side effect profiles that were also easier to synthesise led to the development of methotrexate (MTX) (Figure 2).

Methotrexate (MTX) and its metabolite, 7-hydroxy-MTX, are transported into cells using the Reduced Folate Carrier and Folate Binding Protein. Inside the cell, a significant amount of MTX undergoes polyglutamation, resulting in MTXglu1-7. Both the original MTX and its polyglutamated forms inhibit DHFR; notably, MTXglu5 has an impressive binding affinity with a Ki of 0.004 nM (Shih and

Thornton, 1999). Additionally, MTXglu5 acts on other elements of the folate pathway, including thymidylate synthase, glycinamide adenine ribonucleotide formyl transferase, and aminoimidazole carboxamide ribonucleotide transferase (AICAR-T), but with lesser binding affinities as shown by their higher Ki values. This implies that the primary inhibitory effect on the folate pathway is mainly through DHFR (Shih and Thornton, 1999).

While MTX is no longer widely used as a cancer treatment, it has been instrumental in addressing trophoblastic disease and stomach, bladder, and head and neck cancers. MTX has been a fundamental component of combination therapies for lymphoma and a regular part of adjuvant therapy for breast cancer, as well as a prevalent treatment for metastatic disease. With high-dose treatment reaching up to 12 g/m^2 (Frei et al., 1980), MTX signifies a potent anti-cancer strategy, but it's often associated with severe side effects that usually necessitate comprehensive supportive care.

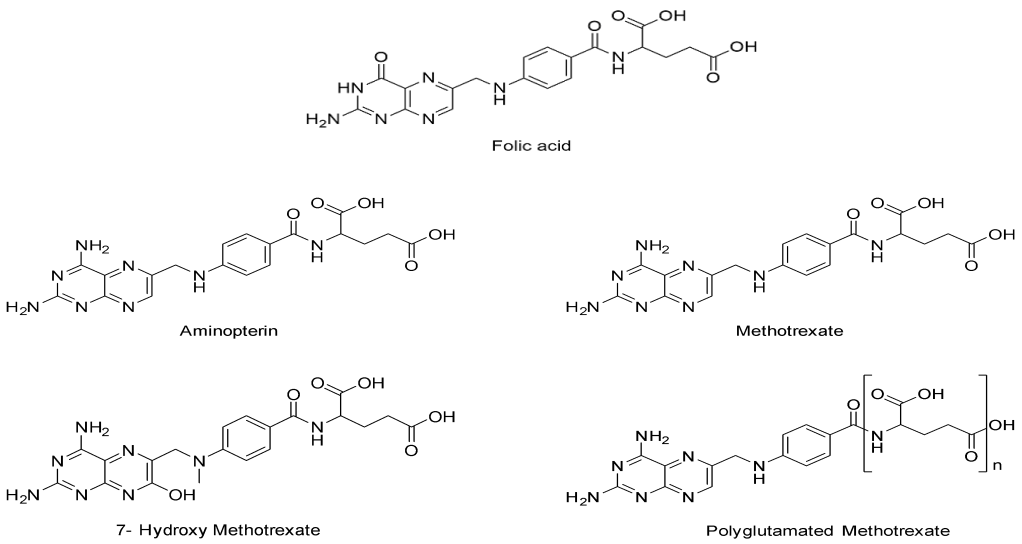


Figure 2: Structure of MTX-related molecules. Molecular structure of folate, aminopterin and MTX as well as 7-hydroxy and polyglutamated forms of MTX (adapted from Alqarni and Zeidler, 2020).

Besides its immediate use in cancer therapy, a study from 1951 noted that aminopterin treatment resulted in a noticeable decrease in inflammatory symptoms in six out of seven patients suffering from rheumatoid arthritis (RA) (Gubner et al., 1951). This knowledge was subsequently applied to psoriasis patients and replicated by the newly developed MTX analogue (Greb et al., 2016). Despite the initial potential of MTX in treating RA, the emergence of anti-inflammatory glucocorticoids temporarily outshone it. Even the success of low-dose MTX (10-15 mg per week) in relieving symptoms in 25 out of 29 patients (Hoffmeister, 1983) did not garner significant attention at the time. However, by the mid-1980s, a number of randomised, placebo-controlled trials began focusing on patients with enduring RA, leading to discoveries of significant and lasting clinical responses, functional improvements, and impressive long-term retention rates (Weinblatt et al., 1985). Further large-scale trials, comparing MTX to other treatments and combination therapies, including biologic agents such as TNFα inhibitors, highlighted its effectiveness in both standalone and combination therapies (Rau, 2010).

MTX has evolved into a standard treatment for RA and is a cornerstone in the field of rheumatology, owing to its proven safety and efficacy and globally accepted treatment protocols (Bedoui et al., 2019a). In this setting, MTX is typically administered at a dose ranging from 7.5 to 25 mg once a week, and two decades of usage have shown that supplementing with 1–5 mg/day of folic or folinic acid can aid in managing side effects. However, supplementation is generally avoided on the day of MTX dosage to prevent overloading cellular folate transporters (Bedoui et al., 2019a).

The anti-cancer action of MTX is attributed to its ability to inhibit DHFR. Given this, and in the absence of other molecular mechanisms, research into its anti-inflammatory and DMARD (disease-modifying anti-rheumatic drug) activity has mainly centred on mechanisms linked to the folate pathway. Suggested mechanisms include inhibiting purine and pyrimidine synthesis, blocking transmethylation reactions, limiting antigen-dependent T-cell proliferation, altering glutathione metabolism - which leads to modified monocyte recruitment, and increasing adenosine release (Cronstein, 2005).

Of these proposed anti-inflammatory mechanisms of action, the most thoroughly researched is increased extracellular adenosine levels. In this hypothesis, the blockage of aminoimidazole-carboxamide by polyglutamated MTX is key.

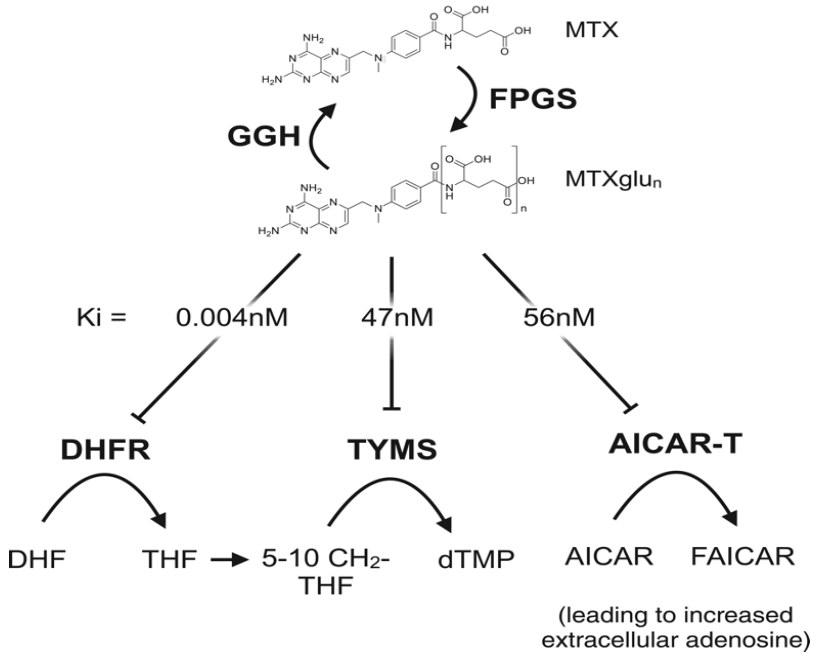


Figure 3: Interactions of MTX with the folate pathway. After entering the cell, MTX is subjected to a process called polyglutamation by the enzyme folylpolyglutamate synthase (FPGS). On the other hand, the enzyme Gamma-glutamyl hydrolase (GGH) performs the opposite function, de-glutamating MTX, ensuring an equilibrium of both MTX forms within the cell. The polyglutamated form of methotrexate ($MTXglu_2$) acts as an inhibitor to DHFR, which is responsible for converting DHF to tetrahydrofolate (THF). Moreover, this polyglutamated version of MTX also blocks thymidylate synthetase (TYMS), a crucial enzyme that facilitates the conversion of 5–10-methyl-tetrahyrofolate into deoxythymidylate monophosphate (dTMP) in the primary pyrimidine production pathway. Additionally, $MTXglu_2$ interferes with AICAR-T, which modifies AICAR into formamidoimidazole carboxamide ribonucleotide (FAICAR) in the primary purine creation route. This inhibition results in a rise in extracellular adenosine concentrations. Ki values are for the $MTXglu_2$ form and taken from (Shih and Thornton, 1999). Enzymes are shown in bold.

The inhibition of aminoimidazole-carboxamide ribonucleotide transformylase (AICAR-T) by polyglutamated MTX leads to a rise in intracellular AICAR levels (as depicted in Figure 3), an occurrence noticed in RA patients undergoing MTX treatment. AICAR then restricts the function of AMP Deaminase, prompting the discharge of intracellular adenine nucleotides (ATP). Once outside the cell, these nucleotides are dephosphorylated into adenosine. This extracellular adenosine attaches to a series of G-protein-coupled adenosine receptors, which subsequently impact neutrophil activity through mechanisms that are not entirely comprehended yet. Even though this complex pathway has been the subject of extensive investigation, there is scant direct evidence supporting the notion that adenosine is the principal mechanism of action of low-dose MTX in biological systems (Shih and Thornton, 1999).

1.5.1 Evidence for MTX as a JAK/STAT inhibitor

Cellular communication is orchestrated through a complex network of signalling pathways, ensuring the proper functioning and regulation of biological processes - including cellular growth, maturation, and immune functions. Anomalies in this pathway are associated with several medical conditions, highlighting its importance for therapeutic research. A pivotal player in this intricate network is the JAK/STAT signalling pathway. The JAK/STAT signalling pathway, conserved across evolution, is fundamental to numerous biological activities, including haematopoiesis, immune reactions, and inflammation (Arbouzova and Zeidler, 2006).

To delve deeper into its mechanisms, a study utilised a *Drosophila* JAK/STAT pathway reporter test, a tool previously validated through extensive genome-wide RNAi screens (Müller et al., 2005). Building on this foundation, another research initiative employed this test to probe a comprehensive library of 2000 substances, encompassing pure natural products, agricultural chemicals, and FDA-approved drugs (S. Thomas et al., 2015b). Notably, this screen identified both aminopterin and MTX as inhibitors of the *Drosophila* JAK/STAT signalling pathway. Further tests confirmed a dose-responsive inhibition by these compounds when the pathway was activated either by its ligand or through overactive JAK gene variants (S. Thomas et al., 2015). This data implies that the inhibitory action likely targets the JAK kinase stage or stages beneath it. Moreover, reducing the expression of folate pathway enzymes like

DHFR, Thymidylate Synthase, and Glycinamide Adenine Ribonucleotide Formyl Transferase using RNAi did not influence JAK/STAT signalling. This observation points towards MTX's inhibitory action being separate from its effects on the folate pathway.

Corroborating these discoveries, other research groups have also independently utilised *Drosophila* to identify MTX as a drug that promotes heterochromatin formation and suppresses JAK/STAT signalling *in vivo* (Loyola et al., 2019).

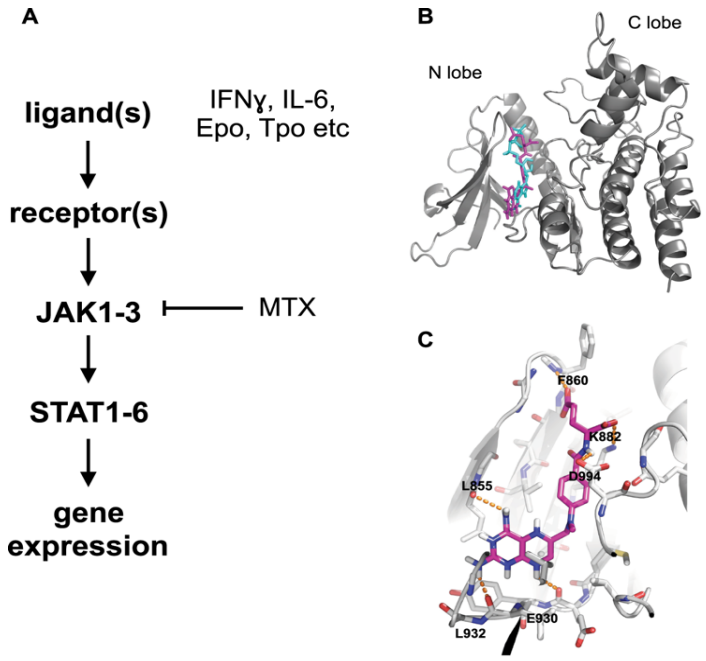


Figure 4: The JAK/STAT signalling pathway and predicted MTX interactions. (A) The JAK/STAT signalling pathway encompasses various ligands such as IFNγ, interleukin-6 (IL-6), erythropoietin (Epo), and thrombopoietin (Tpo). Proposed inhibition of JAKs by MTX is shown. (B and C) There are anticipated interactions between MTX and JAK2. (B) A visual depiction of the JAK2 JH1 (kinase) domain in humans (sourced from 5TQ8.pdb) demonstrates that MTX (depicted in magenta) is anticipated to dock between the N and C lobes of the kinase at the ATP-binding site (represented in cyan). (C) MTX displays expected hydrogen bonding (indicated by orange lines) with specific residues in the binding pocket and an ionic interaction with lysine 882. These residues are situated within a 5 Å radius from the attached ligand. Molecular models were first published as part of Chinnaiya et al. (Chinnaiya et al., 2017) and were obtained from the Haematologica Journal website http://www.haematologica.org.

The initial findings in the *Drosophila* model, were mirrored in human HDLM2 cells, originating from lymphoma, where it reduced the phosphorylation levels of STAT1, STAT5, JAK1, and JAK2. Moreover, the study also observed a reduction in STAT3 and STAT5 phosphorylation in HEL cells, derived from erythroid leukaemia and carrying the gain-of-function JAK2 V617F mutation, post MTX treatment. These effects manifest in a dose-dependent fashion and at concentrations comparable to the 0.4–0.8 μM peak MTX levels detected in the blood of RA patient (Hobl et al., 2012; S. Thomas et al., 2015b). In contrast, it did not observe any alteration in the phosphorylation state of pAKT, pJun, or pERK1/2, suggesting that the decrease in JAK and STAT phosphorylation is pathway-specific and not due to non-specific interactions with kinases in general (S. Thomas et al., 2015b).

Expanding on this initial discovery, an *in vivo* examination of MTX's impact using a mouse model for polycythemia vera (PV) was undertaken. This transgenic model ubiquitously expresses the human JAK2 V617F mutation and so results in continuous JAK/STAT pathway activation, leading to increased haemoglobin and haematocrit concentrations (Zaleskas et al., 2006, Cahu and Constantinescu, 2015; Kleppe et al., 2015). Interestingly, MTX dosages that are effective against induced RA in mice didn't cause a reduction in blood counts of the regular littermates, suggesting MTX doesn't indiscriminately suppress bone marrow activity. However, the mutant mice exhibited notable improvements in both blood metrics and spleen enlargement symptoms after receiving low-dose MTX, showing effects comparable to the JAK1/2 inhibitor, Ruxolitinib, which was used as a positive control. There was also a marked reduction in phosphorylated STAT3 and STAT5 levels and a drop in the mRNA levels of the JAK/STAT target gene PIM1 in spleens from hJAK2 V617F expressing mice previously exposed to MTX (Chinnaiya et al., 2017).

Finally, computational modelling was used to test for the potential of MTX to act as a competitive inhibitor of the JAK tyrosine kinase domain. Consistent with *in vivo* findings, digital docking to a highly detailed model of the JAK2 tyrosine kinase domain suggested that MTX might occupy the ATP-binding pocket, possibly even more preferentially than ATP, hinting at MTX's potential role as a competitive

inhibitor of the kinase (Figure 4B,C) (Chinnaiya et al., 2017). This opens up avenues for further exploration into similar structures and other JAK kinases to understand this interaction better.

In addition to work undertaken in the Zeidler lab on this topic, independent evidence has also been generated by other groups examining patient data. A notable study analysing gene expression in CD4+ cells from RA patients yet to be treated with DMARDs and subsequently assigned different therapies – either tocilizumab with MTX, tocilizumab alone, or just MTX – provided intriguing findings. Analysis of the variant mRNAs in the MTX-treated group against the KEGG database revealed significant changes primarily in the p53 signalling (P = 8.44E–06) and JAK/STAT signalling (P = 2.22E–04) pathways (Teitsma et al., 2017). This highlights the potential of low-dose MTX to dampen the JAK/STAT signalling in individuals with RA.

Lastly, it's crucial to mention that a recent comprehensive study involving 4786 heart patients who received low-dose MTX to diminish inflammation linked to atherosclerotic events revealed a notable rise in non-basal-cell-skin cancer. The rate increased from 0.2 cases per 100 patient-years in the control group to 0.61 cases in the group treated with MTX (Ridker et al., 2019). While the exact reason behind this increase remains unclear, it is speculated that it might be due to a decrease in JAK-driven cancer immune responses. This theory aligns with observations made after using the JAK1/2 inhibitor, ruxolitinib, whose prolonged use is also associated with an increased cancer risk (Elli et al., 2019).

1.5.2 Why might JAK inhibition explain the DMARD activity of MTX?

The JAK/STAT pathway plays a pivotal role in controlling immune responses and signalling, both before and after the secretion of inflammatory cytokines (Elli et al., 2019). For example, Interferon-gamma (IFNγ) and Interleukins (IL)-6 and 10, which are powerful pro-inflammatory cytokines and whose activity and levels are markedly increased in inflammatory diseases, function by directly signalling through receptors linked to JAKs, culminating in STAT activation (Figure 4A) (Owen et al., 2019). Furthermore, the expression of other pro-inflammatory cytokines such as (TNF-α) is also dependent on JAK/STAT signalling (Owen et al., 2019), while the JAK/STAT pathway itself is also

required for the differentiation and control of multiple components and effectors of the immune system (Walker and Smith, 2005).

Given these roles, the link between the JAK/STAT pathway signalling and RA has garnered significant attention, spurring numerous pharmaceutical endeavours focused on this pathway (Walker and Smith, 2005). Such investigations have paved the way for the creation and subsequent approval of the JAK1/3 inhibitor Tofacitinib (Kremer et al., 2009) and Tocilizumab, an antibody designed to inhibit the receptor of the inflammatory-promoting cytokine IL-6 (Mihara et al., 2005; Woo et al., 2005). Comprehensive clinical trials have attested to the efficacy of these treatments, leading to their official endorsement by regulatory bodies. As such, it is clear that inhibition of JAK/STAT pathway signalling can be an effective therapeutic option for the treatment of inflammatory diseases such as RA.

1.5.3 Implications for the repurposing of MTX

MTX is acknowledged as a 'Fundamental Medicine' by the World Health Organization, is globally accessible at an affordable price through healthcare systems and is given weekly to millions of individuals living with RA. MTX is typically effective and well-received, boasting a reliable safety record and high adherence rates among patients. Numerous individuals persist with their MTX monotherapy routine for many years. The mechanism of action for MTX was believed to be primarily through its role as a folate antagonist, inhibiting dihydrofolate reductase and thereby affecting DNA synthesis and cellular replication. However, this simplistic view has been challenged over the years. Given the drug's multifaceted effects on immune responses and its relatively low toxicity profile, especially when compared to other chemotherapeutic agents that target DNA synthesis, the traditional understanding of its mechanism seems somewhat implausible. Recent research suggests that MTX's therapeutic effects in RA might be attributed to more complex immunomodulatory actions, which are still being elucidated.

Considering these factors, the proposition that low-dose MTX may operate by inhibiting JAK/STAT signalling offers a fresh perspective on the previously ambiguous molecular mechanism-of-action, which might potentially pave the way for new uses of this medicine.

Drug reassignment, redirection, and salvage approaches are frequently promoted as quick, affordable, and effective methods for identifying new uses for existing treatments. These strategies typically focus on repurposing recognized medications that already have established safety, pharmacodynamics, and pharmacokinetics profiles for new ailments. These endeavours usually stem from novel insights into a drug's mechanism-of-action, a better understanding of a disease's molecular nature, incidental discoveries, or a targeted exploration of 'big data', encompassing computational modelling and machine learning-based techniques (Koromina et al., 2019).

Given the involvement of the JAK/STAT signalling in MPNs and the identification of MTX as a pathway inhibitor, it has been proposed that MTX could be reassigned to treat this group of conditions (Chinnaiya et al., 2017; S. Thomas et al., 2015b). Mouse models of PV have exhibited a positive response to low-dose MTX (Chinnaiya et al., 2017), and preliminary studies suggest it could be efficacious in PV and ET patients (Francis et al., 2019; Palandri et al., 2016) While clinical trials are necessary to affirm safety and efficacy in the MPN patient population, it will be intriguing to observe, in the long run, if the uncovering of MTX's mechanism-of-action could potentially lead to new therapies for this significant patient group.

1.6 Lifespan and healthspan, and their limitations

Lifespan refers to the maximum number of years an organism can live, while healthspan refers to the period of life during which an individual retains good health and functional abilities (Cutler and Mattson, 2006). Lifespan is determined by a combination of genetic, environmental, and lifestyle factors specific to each species. In the UK, the average life expectancy at birth is approximately 80 years, with slight variations between men and women. This is consistent with the average for many European countries. The limited lifespan and healthspan can be attributed to several factors, including

the involvement of stem cells and DNA damage (Garmany et al., 2021). Stem cells are undifferentiated cells that self-renew and differentiate into specialised cell types. They play a crucial role in tissue regeneration and maintenance throughout an organism's life (Kolios and Moodley, 2013). However, the functionality and regenerative capacity of stem cells decline with age, leading to a reduced ability to repair damaged tissues and organs. This decline in stem cell function contributes to the overall decline in health and the onset of age-related diseases (Sahin and Depinho, 2010).

DNA damage is another significant factor responsible for ageing. Somatic cells accumulate DNA damage due to various endogenous and exogenous factors, including oxidative stress, exposure to harmful chemicals, and radiation (Schumacher et al., 2021). Accumulation of DNA damage impairs cellular functions and leads to genomic instability, cellular senescence and increased susceptibility to diseases. The impairment in the repair of DNA damage, thus, contributes to the ageing process and the overall decline in healthspan.

During the 20th century, there was a remarkable improvement in lifespan across the globe (Giraldo, 2009), transforming public health outcomes and shaping the demographic landscape. Factors that played a crucial role in this trend include:

i. Improved Standards of Living: Advancements in technology, industrialization, and urbanisation led to improved standards of living for many people. Access to clean water, sanitation systems, and better housing conditions reduced the spread of infectious diseases and improved overall health.
ii. Better Nutrition: The understanding of nutrition and the availability of a diverse range of food improved significantly during the 20th century. Adequate nutrition played a vital role in reducing malnutrition, preventing stunted growth, and improving overall health and immune function.
iii. Childhood Disease Prevention: The development and widespread implementation of childhood immunisation programs were instrumental in reducing the impact of infectious diseases on child

mortality rates. Vaccines against diseases such as polio, measles, diphtheria, and pertussis significantly reduced childhood mortality and contributed to increased life expectancy.

iv. Control of Bacterial Infections: The discovery and widespread use of antibiotics revolutionised medicine and played a significant role in combating bacterial infections. Antibiotics, such as penicillin, allowed for effective treatment of previously life-threatening conditions, such as pneumonia, sepsis, and tuberculosis.

v. Improved Healthcare: Advances in medical knowledge, diagnostic techniques, and treatment modalities greatly improved healthcare outcomes. Medical interventions deliver timely and quality care. Waiting times for treatments and delays in accessing essential services for conditions such as heart disease, stroke, and cancer have been significantly reduced, enhancing the effectiveness of medical interventions. This improvement allows individuals to survive and live longer with these diseases.

vi. Social Care and Support: The development of social care systems and support networks for the elderly and individuals with disabilities played a crucial role in extending lifespan. Improved caregiving services, assisted living facilities, and home healthcare services provided essential support for individuals who could no longer care for themselves.

vii. Health Education and Awareness: Increased health education and awareness campaigns focused on promoting healthy lifestyles, preventive measures, and early detection of diseases. This empowered individuals to make informed choices about their health and take proactive steps to prevent illness and maintain well-being.

viii. Advances in Surgical Techniques: The refinement of surgical techniques, anaesthesia, and post-operative care contributed to improved outcomes for surgeries. Procedures such as organ transplants, joint replacements, and cardiac surgeries became more common and successful, prolonging the lives of individuals with severe health conditions.

ix. Social and Economic Progress: Economic growth and social progress led to improvements in living conditions, education, and access to healthcare services. Rising income levels and social welfare programs allowed individuals to afford healthcare and access necessary treatments.

Despite all these advances, over recent years, the UK has observed a concerning shift in the trajectory of life expectancy. Instead of the anticipated growth, there's evidence suggesting a stagnation or even a decline in some instances (Hiam et al., 2020). This unexpected trend has sparked debates and investigations into its potential causes. One notable factor is the austerity measures introduced post the 2008 financial crisis. Aimed at stabilising the economy, these measures inadvertently led to reduced public spending on essential services. The repercussions were felt especially in the social care sector and the NHS, with the elderly and vulnerable populations bearing the brunt of these cutbacks (Seamer et al., 2019). It's crucial to understand the depth of these impacts and their long-term implications on public health. Cuts in funding for social care can limit access to necessary support, resulting in inadequate care and potentially compromising health outcomes. The reduction in funding for social care services has put additional strain on individuals and families who rely on these services for their well-being. Lack of access to appropriate social care can lead to delayed or insufficient support for older adults, especially those with chronic health conditions or disabilities (Abdi et al., 2019). This can contribute to worsening health outcomes and a decline in overall well-being.

Furthermore, the NHS, a publicly funded healthcare system in the UK, has faced significant challenges in recent years. Increased demand, budget constraints, and workforce shortages have put pressure on the system's capacity to deliver timely and quality care. Longer waiting times for treatments and delays in accessing essential healthcare services can have negative consequences on health outcomes, particularly for older individuals with complex medical needs (Prentice and Pizer, 2007). Lastly, the impact of austerity measures and cutbacks in social care has been particularly pronounced in areas with higher levels of deprivation (Stuckler et al., 2017). Health inequalities have widened, leading to poorer health outcomes and shorter lifespans in these regions. Socioeconomic factors, including income inequality and limited access to education, employment, and healthy living conditions, can contribute to these disparities in health and longevity.

Efforts are underway to address these concerns and mitigate the impact on lifespans. Policy discussions are focusing on the need for increased funding for social care, improvements in healthcare delivery, and strategies to tackle health inequalities. Recognising the importance of adequate support for vulnerable

populations, including the elderly, is essential to ensure that lifespans can continue to improve and that all individuals have the opportunity to lead healthy and fulfilling lives.

1.7 Hallmarks of old age

The hallmarks of old age are interconnected pathways and processes that contribute to the progressive decline in physiological functions and increased vulnerability to age-related diseases. These hallmarks represent key biological changes that occur during the ageing process.

i. Genetic instability: Accumulation of genetic mutations and alterations in cells over time contribute to genetic instability. This can result from errors during DNA replication, exposure to environmental mutagens, or a decline in DNA repair mechanisms. Genomic instability leads to impaired cellular function and an increased risk of diseases, particularly cancer associated with old age (Vijg and Suh, 2013).

ii. Telomere attrition: Telomeres are protective caps at the ends of chromosomes that shorten with each cell division. Telomere attrition acts as a mitotic clock, and when telomeres become critically short, cells may undergo senescence or cell death (Koliada et al., 2015). Telomerase, the enzyme which adds telomeric DNA to counteract telomere shortening plays a crucial role in this aspect. However, in most somatic cells, telomerase activity is limited where telomere attrition causes cellular ageing and has implications for tissue regeneration and the development of age-related diseases (Shay and Wright, 2007).

iii. Epigenetics: Epigenetic modifications are heritable changes in gene expression patterns that do not involve alterations to the DNA sequence itself impact ageing. During ageing, there are alterations in DNA methylation patterns, histone modifications, and changes in non-coding RNAs. These epigenetic alterations can affect gene expression and cellular function, contributing to age-related changes in tissues and organs (Wang et al., 2022). Epigenetic regulation is influenced by various pathways, including DNA methyltransferases, histone-modifying enzymes, and chromatin remodelling complexes.

iv. Loss of Proteostasis: Proteostasis refers to the maintenance of protein homeostasis, including protein synthesis, folding, and degradation. During ageing, there is a decline in proteostasis, leading to protein misfolding and aggregation. This impairment is associated with the accumulation of damaged proteins, which can disrupt cellular functions and contribute to age-related diseases, such as Alzheimer's and Parkinson's disease (Hipp et al., 2019). Pathways involved in proteostasis include chaperones, protein degradation systems (such as the ubiquitin-proteasome system and autophagy), and the heat shock response.

v. Deregulated Nutrient Sensing: Nutrient sensing pathways, such as the insulin/insulin-like growth factor (IGF) signalling pathway and the target of rapamycin (TOR) pathway, play a role in regulating cellular metabolism, growth, and ageing. During ageing, there is a dysregulation of these nutrient-sensing pathways, leading to altered nutrient utilisation, reduced energy metabolism, and impaired stress resistance (Johnson, 2018). These changes contribute to the ageing process and age-related diseases.

vi. Mitochondrial Dysfunction: Mitochondria are the powerhouses of cells, responsible for energy production through oxidative phosphorylation. However, during ageing, mitochondrial function declines. This dysfunction can result in increased production of reactive oxygen species (ROS), impaired energy production, and compromised cellular metabolism. Mitochondrial DNA mutations, impaired mitochondrial dynamics, and dysregulation of mitochondrial quality control pathways are implicated in age-related mitochondrial dysfunction (Amorim et al., 2022).

vii. Cellular Senescence: Cellular senescence is a state of irreversible growth arrest in cells. Senescent cells accumulate with age and secrete pro-inflammatory factors, creating a chronic low-grade inflammatory environment known as senescence-associated secretory phenotype (SASP) (Kumari and Jat, 2021). This chronic inflammation contributes to tissue dysfunction and promotes age-related diseases. Senescence is regulated by multiple pathways, including tumour suppressor genes (such as p53 and pRb), DNA damage response pathways (such as

p16INK4a and p21), and the senescence-associated secretory phenotype (SASP) signalling pathway.

viii. Stem Cell Exhaustion: Stem cells are essential for tissue regeneration and maintenance throughout life. However, during ageing, there is a decline in the number and regenerative capacity of stem cells (Sameri et al., 2020). This is attributed to various factors, including the accumulation of DNA damage, alterations in stem cell niche environments, and changes in the signalling pathways that regulate stem cell self-renewal and differentiation. Stem cell exhaustion contributes to the impaired tissue repair and regeneration observed in old age.

ix. Altered Intercellular Communication: Communication between cells and tissues is crucial for maintaining proper physiological functions. However, with age, there are alterations in intercellular communication pathways, including signalling molecules, cell-cell contacts, and extracellular matrix components (Bechtel et al., 2021). These changes can disrupt tissue homeostasis, impair immune responses, and contribute to age-related diseases.

1.8 Cancer and age as the primary risk factor

Age is a primary risk factor for the development of cancer (Niccoli and Partridge, 2012). As individuals age, they become more susceptible to cancer due to several factors. Firstly, there is a higher likelihood of accumulating genetic mutations in cells with increasing age. Over time, exposure to various carcinogens, DNA replication errors, and impaired DNA repair mechanisms can lead to the accumulation of genetic alterations that promote uncontrolled cell growth (Alhmoud et al., 2020). Additionally, the immune system function declines with age, compromising its ability to recognise and eliminate cancer cells. The decline in immune surveillance allows cancer cells to evade detection and proliferate more freely. Age-related changes in the tumour microenvironment also contribute to tumour progression and immune evasion. Moreover, the likelihood of developing cancer increases with age where there is greater chance of exposure to environmental risk factors, such as tobacco smoke, ultraviolet radiation, and certain chemicals (Fane and Weeraratna, 2020). Similarly, lifestyle factors,

including poor diet, sedentary behaviour, and chronic inflammation, have cumulative effects over time and contribute to cancer development.

1.9 Treatment of ageing

Ageing is a complex process influenced by various factors, and currently, there is no specific treatment to halt or reverse the ageing process itself. However, medical advancements and lifestyle interventions can help manage age-related diseases and improve overall health and quality of life in older individuals. Preventive measures, such as regular health screenings, vaccination programs, and lifestyle modifications, can help reduce the risk of age-related diseases (Partridge et al., 2018). Early detection and effective management of conditions such as cardiovascular diseases, diabetes, cancer, and neurodegenerative disorders can improve outcomes and enhance overall healthspan. Similarly, adopting a healthy lifestyle can have a significant impact on ageing. This includes regular physical exercise, maintaining a balanced diet rich in nutrients, avoiding smoking and excessive alcohol consumption, and managing stress (Rippe, 2018). These lifestyle choices promote overall health, reduce the risk of chronic diseases, and contribute to healthy ageing. Geriatric care also has a significant impact on improving ageing as it focuses on addressing the unique healthcare needs of older individuals. This specialised approach considers age-related changes in physiology, cognition, and social factors. Geriatric care teams work collaboratively to manage chronic conditions, optimise medication use, provide rehabilitation services, and offer social support to improve the overall well-being of older adults.

While there are currently no medications approved specifically for anti-ageing purposes, certain drugs have shown potential in targeting specific age-related pathways or diseases associated with ageing. For example, drugs targeting cellular senescence, such as senolytics, aim to selectively eliminate senescent cells and alleviate age-related tissue dysfunction (Chaib et al., 2022; Ellison-Hughes, 2020). Other areas of research include investigating the role of compounds that activate Sirtuins (a class of proteins associated with longevity) and exploring the potential benefits of certain anti-inflammatory and antioxidant agents (Grabowska et al., 2017). Furthermore, geroprotectors which are interventions aimed

at modulating the underlying mechanisms of ageing to promote healthy ageing and extend healthspan are being explored. These interventions may include caloric restriction mimetics, which mimic the effects of reduced calorie intake without actually restricting food intake, or interventions targeting specific signalling pathways involved in ageing, such as the mammalian target of rapamycin (mTOR) pathway (López-Lluch and Navas, 2016). Various compounds and interventions are being studied in preclinical and clinical settings to assess their potential in promoting healthy ageing.

1.10 Drugs to modify lifespan and healthspan

While the concept of drugs that can significantly extend human lifespan is still a topic of ongoing research and debate, there are several compounds that have shown promise in modulating the ageing process and improving healthspan in preclinical studies. Some examples of these compounds include:

i. Rapamycin: Rapamycin is an immunosuppressant drug that inhibits the mTOR pathway, which regulates cellular growth and metabolism. Studies in various model organisms have shown that rapamycin can extend lifespan and improve healthspan. However, more research is needed to determine its safety and efficacy in humans (Harrison et al., 2009).

ii. Metformin: Metformin is a widely used drug for the treatment of type 2 diabetes. It has been found to have potential anti-ageing effects, including the activation of AMP-activated protein kinase (AMPK) and modulation of mitochondrial function. Ongoing clinical trials are investigating its effects on age-related diseases and lifespan extension (Novelle et al., 2016).

iii. NAD+ Precursors: Nicotinamide adenine dinucleotide (NAD+) is a coenzyme involved in energy metabolism and cellular processes. NAD+ levels decline with age, and supplementation with NAD+ precursors, such as nicotinamide riboside (NR) or nicotinamide mononucleotide (NMN), has shown promise in preclinical studies to improve mitochondrial function, delay ageing, and increase healthspan (Covarrubias et al., 2021).

iv. Senolytics: Senolytics are compounds that selectively eliminate senescent cells, which accumulate with age and contribute to tissue dysfunction. These compounds, such as dasatinib and quercetin, have shown positive effects in preclinical models by reducing age-related tissue degeneration and improving healthspan (Ellison-Hughes, 2020).

1.11 Societal benefit from improved healthspan

Improved healthspan, which refers to the period of life spent in good health and free from age-related diseases and disabilities, would have significant societal benefits. Some of these benefits include:

i. Increased Productivity: A population with an extended healthspan would be able to contribute to society for a longer period. Older individuals would remain active and engaged in various sectors, bringing their experience, skills, and knowledge to the workforce. This leads to increased productivity and economic growth (Bloom et al., 2002).

ii. Reduced Healthcare Burden: Age-related diseases and disabilities often require extensive healthcare resources and services. By improving healthspan and delaying the onset of these conditions, the burden on healthcare systems would be reduced. This allows for more efficient allocation of resources and focuses healthcare efforts on preventive measures and managing age-related conditions effectively (Garmany et al., 2021).

iii. Enhanced Quality of Life: Longer periods of healthy and active life mean that individuals can enjoy a higher quality of life. They can participate in social activities, pursue personal interests, maintain independence, and have a positive impact on their communities (Leeuwen et al., 2019). Improved healthspan allows older individuals to continue engaging in meaningful relationships and experiences, leading to overall satisfaction and well-being.

iv. Social Cohesion and Intergenerational Relationships: A society with improved healthspan fosters stronger intergenerational relationships. Older individuals can actively participate in

family life, provide guidance and support to younger generations, and serve as role models (Wong et al., 2018). This promotes social cohesion and strengthens the fabric of communities.

v. Economic Savings: Age-related diseases and disabilities often require significant healthcare expenditures and long-term care services. By delaying the onset of these conditions and promoting healthy ageing, societal costs associated with medical treatments, hospitalizations, and long-term care can be reduced (Okamoto et al., 2023). These savings can be redirected to other areas of societal development.

1.12 Problems with lifespan as a measure of wellbeing

Lifespan is often used as a metric to evaluate population health and wellbeing, but it has limitations in providing a comprehensive understanding of an individual's quality of life. One of the main issues is that lifespan includes the time individuals spend at the end of their lives, which can be marked by significant health challenges and a decline in quality of life (Infurna et al., 2020). Focusing solely on extending lifespan without considering the quality of life during this period may overlook the importance of palliative care, comfort, and maintaining dignity for individuals in their final stages of life. Another challenge arises from the management of chronic conditions that were previously fatal but can now be controlled through medical treatments. While these treatments may increase an individual's overall lifespan, they may also result in a prolonged period of poor quality of life. Individuals may experience pain, disability, or dependence on others for daily activities, significantly impacting their overall wellbeing.

Additionally, individuals may face cumulative frailty caused by diseases associated with old age (Clegg et al., 2013). Conditions such as cardiovascular diseases, respiratory disorders, and neurodegenerative diseases can lead to a decline in physical and cognitive function, affecting independence, mobility, and overall quality of life (Cesari et al., 2014). Lifespan alone may not fully capture the impact of these cumulative health challenges. Furthermore, wellbeing is a subjective experience that encompasses various dimensions, including physical, mental, and social aspects. Lifespan measurements do not

directly reflect an individual's subjective experiences, psychological wellbeing, social connectedness, or fulfilment of personal goals. Factors like happiness, satisfaction, and social relationships play crucial roles in overall wellbeing but are not adequately captured by lifespan measurements (Diener and Suh, 1997). There are also ethical considerations to take into account. Focusing solely on extending lifespan without sufficient attention to the quality of life raises questions about individual preferences, autonomy, and the balance between quantity and quality of life. It is essential to respect a person's right to make choices regarding their healthcare and end-of-life decisions, recognizing that quality of life may hold greater significance for some individuals than simply prolonging their lifespan.

1.13 Healthspan

Healthspan refers to the period of life during which an individual is generally healthy, free from chronic diseases, and able to maintain optimal physical and mental functioning (Garmany et al., 2021; Holmboe et al., 2012). It represents the duration of time in which an individual experiences a high quality of life, without significant impairments or limitations due to health conditions.

1.14 Difficulties in quantifying the quality of life

Quantifying the quality of life is a complex task as it involves subjective aspects that can vary among individuals. Different individuals may prioritise different aspects of their quality of life, such as physical health, mental well-being, social relationships, or personal fulfilment. Objective measurements alone may not capture the multidimensional nature of quality of life, making it challenging to establish a universally applicable quantification method.

1.15 Molecularly 'protecting' DNA and stem cells to increase healthspan

There is growing evidence suggesting that protecting the integrity of DNA and preserving the regenerative capacity of stem cells may contribute to increased healthspan (Mecocci et al., 2018). DNA damage accumulation and decline in stem cell function are associated with ageing and age-related diseases. Strategies aiming at enhancing DNA repair mechanisms, antioxidant interventions, and

regenerative medicine approaches are being explored to enhance the molecular protection of DNA and stem cells, potentially extending healthspan (Garmany et al., 2021).

1.16 Animal models for lifespan and healthspan studies

In order to study lifespan and health-span, researchers employ various biological systems to investigate the ageing process and factors influencing overall health and longevity. Two commonly utilised systems are vertebrate models, particularly mice, and invertebrate models such as yeast, nematodes, and fruit flies.

1.16.1 Vertebrate systems (mice)

Mice are frequently employed in lifespan and health-span studies due to their genetic similarity to humans and the availability of various genetic tools and resources. They share numerous physiological and molecular similarities with humans, making them valuable models for investigating age-related diseases and interventions. However, studying lifespan and health-span in mice can be expensive and time-consuming. Mice have a 2-3 year lifespan, which means that conducting lifespan studies can span several years, making longitudinal studies challenging and extremely costly.

1.16.2 Invertebrate systems (yeast, nematodes, and fruit flies)

Invertebrate models offer several advantages for studying lifespan and health-span (Kumar Chaudhary and Rizvi, 2019). They have shorter lifespans compared to vertebrates, allowing researchers to conduct lifespan studies more rapidly. Additionally, these models are smaller in size, easier to maintain in large numbers, and have well-characterised genomes, making them more amenable to genetic manipulations. While invertebrates may differ in certain aspects from humans, they still share conserved ageing-related mechanisms and provide valuable insights into the basic biology of ageing (Bitto et al., 2016).

1.16.3 Fruit flies (*Drosophila melanogaster*) as a model system

Flies, specifically the species *Drosophila melanogaster* (fruit flies), have become a powerful model system in ageing research due to their unique attributes and genetic tractability. Some of the characteristics of flies as a model system in their applications to study lifespan and health-span include:

i. Short lifespan: Fruit flies have a relatively short lifespan, with an average lifespan of about 60-80 days (Staats et al., 2018). This short lifespan enables researchers to conduct rapid experiments and observe multiple generations within a relatively short time frame.

ii. Genetic tools and resources: Fruit flies have a well-annotated genome and a vast array of genetic tools and resources. The ability to easily manipulate their genetic makeup using techniques such as genetic crosses, transgenic techniques, and RNA interference (RNAi) has greatly contributed to understanding the genetic basis of aging and age-related diseases (Minois et al., 2010). The flies' genome can be selectively modified, or specific genes silenced to investigate their effects on lifespan and health-span.

iii. Conserved ageing mechanisms: Despite the evolutionary distance between fruit flies and humans, many fundamental ageing-related mechanisms are conserved across species (Helfand and Rogina, 2003). Fruit flies share common signalling pathways and biological processes involved in ageing, including insulin/IGF signalling, nutrient sensing, oxidative stress response, and mitochondrial function (Partridge et al., 2011). By studying these conserved pathways in flies, researchers gain valuable insights into the underlying biology of ageing and potential interventions to modulate lifespan and healthspan.

iv. Health-span assessments: Fruit flies exhibit measurable age-related physiological decline and pathologies that resemble certain aspects of human ageing. It is possible to investigate various health parameters in flies, such as locomotor activity, stress resistance, metabolic function, and neurodegeneration (Jones and Grotewiel, 2010). In addition, fruit flies can be used to investigate age-related changes and the evaluation of interventions aimed at improving health-

span. For instance, flies' ability to climb or fly, their sensitivity to stressors, or their resistance to oxidative damage to assess their overall health and functional decline during ageing can be measured (Brandt and Vilcinskas, 2013).

v. High-throughput screening: The short lifespan and ease of maintenance of fruit flies make them amenable to high-throughput screening approaches. Large numbers of flies for potential interventions or genetic manipulations that extend lifespan or improve health-span can be screened. This enables the identification of candidate genes, drugs, or environmental factors that may influence ageing and age-related diseases.

vi. Modelling human diseases: Fruit flies can also be used to model specific human diseases associated with ageing, such as neurodegenerative disorders (Brandt and Vilcinskas, 2013). Human disease-associated genes can be genetically engineered into the flies' genome and study their effects on lifespan, behaviour, and neurodegeneration. This approach provides insights into the underlying mechanisms of age-related diseases and allows for the screening of potential therapeutic interventions.

Fruit flies serve as a valuable model system for studying lifespan and health-span due to their short lifespan, genetic tractability, conserved ageing mechanisms, and the ability to assess various health parameters. Their utilisation has provided significant contributions to our understanding of ageing processes, genetic determinants of lifespan, and potential interventions to promote healthier ageing outcomes.

1.17 Pathways involved in lifespan extension

1.17.1 The insulin signalling pathway

The insulin/insulin-like growth factor (IGF) signalling pathway plays a pivotal role in lifespan regulation across various organisms, from fruit flies to mammals (Partridge et al., 2011). Central to nutrient sensing and energy metabolism, its dysregulation is often tied to age-related diseases and

decreased longevity. Initiated by insulin or insulin-like peptides binding to their receptors, it triggers a cascade of intracellular events activating key components like the insulin/IGF receptors, insulin receptor substrates, and the phosphatidylinositol 3-kinase. The pathway fosters cell growth and nutrient absorption but also impedes stress responses (Siddle, 2011). Persistent IGF signalling in adulthood can, however, undermine health-span and lifespan due to enhanced oxidative stress and inflammation (Bartke, 2008). Conversely, reduced insulin/IGF-1 signalling has been linked to extended lifespan in several organisms, often modulated by the interplay with other pathways like the TOR and AMPK pathways (Partridge et al., 2011).

1.17.2 The TOR signalling pathway

The TOR pathway, conserved across species, regulates cellular growth, metabolism, and aging. Its inhibition has been linked to extended lifespan in multiple organisms, suggesting its potential as a longevity-promoting target (Johnson, 2018). This pathway modulates cellular activities based on nutrient availability, energy, and growth factors. Overactivation, often due to nutrient abundance, leads to increased protein synthesis and cell growth (Gonzalez and Rallis, 2017). Harrison et al. (2009) found that rapamycin, an mTOR pathway inhibitor, extended the lifespan of male and female mice when administered from middle age. This effect was consistent across diverse genetic backgrounds, emphasizing the potential of targeting mTOR in age-related disease interventions.

1.17.3 The Ras/Raf pathway

The Ras/Raf pathway, pivotal in cell growth, differentiation, and survival, is often dysregulated in diseases like cancer (Chappell et al., 2011). Initiated by receptor tyrosine kinases (RTKs) in response to growth factors, this pathway activates a cascade involving Ras, Raf kinases, and downstream proteins like MEK and ERK. While primarily linked to cell proliferation and tumorigenesis, recent studies suggest its role in lifespan regulation (Dorard et al., 2023). It has also recently been shown that inhibition of the Ras/MAPK signalling pathway can promote longevity by preventing cellular senescence and so influence ageing independently of its oncogenic functions (Laskovs et al., 2022). Consistent with this, genetic approaches reducing RAS/MAPK signalling activity extend lifespan and

improve healthspan, delaying age-related issues (Laskovs et al., 2022). Studies in *Caenorhabditis elegans* first demonstrated that reduced activity of *let-60* (the worm Ras homolog) or *mpk-1* (ERK homolog) leads to increased lifespan, suggesting that downregulation of Ras/MAPK signalling promotes longevity (Slack et al., 2015). Taken together, these data strongly support the significant role of Ras signalling in modulating lifespan and ageing processes.

1.17.4 The AMP-activated protein kinase (AMPK) pathway

AMPK is an evolutionary conserved energy-sensing kinase that is activated under conditions of low cellular energy levels (Hardie, 2011). It regulates cellular energy homeostasis by promoting ATP production and inhibiting energy-consuming processes. Activation of the AMPK pathway has been associated with increased lifespan in various organisms, including worms, flies, and mice (Bitto et al., 2015). AMPK regulates cellular metabolism and nutrient sensing, thereby influencing lifespan and health-span.

1.17.5 The sirtuin pathway

Sirtuins are a family of NAD+-dependent enzymes that play a role in cellular metabolism, stress response, and ageing. Sirtuins regulate various processes, including gene expression, DNA repair, and mitochondrial function (Lagunas-Rangel, 2019). In particular, the mammalian sirtuin SIRT1 has been extensively studied for its potential role in lifespan extension. Activation of SIRT1 has been shown to increase lifespan in multiple model organisms, and it is thought to mediate the effects of calorie restriction on lifespan (Park et al., 2013).

1.17.6 DNA damage response pathways

DNA damage is a major contributor to ageing and age-related diseases. Cells have evolved intricate DNA damage response pathways to sense and repair DNA damage. These pathways involve proteins such as ataxia-telangiectasia mutated (ATM), ATR and Rad3-related (ATM), and p53. Activation of DNA damage response pathways leads to the induction of DNA repair mechanisms, cell cycle arrest,

and apoptosis if the damage is too severe (Nowsheen and Yang, 2012). Dysfunction in DNA damage response pathways can lead to accelerated ageing and increased susceptibility to age-related diseases.

1.17.7 The JAK/STAT pathway

While the JAK/STAT pathway isn't traditionally linked to ageing, recent research suggests a potential role in lifespan regulation, notably in the *Drosophila melanogaster* gut (Herrera and Bach, 2019a). In this context, the pathway is vital for gut homeostasis and longevity (Herrera and Bach, 2019; Izumi et al., 2019). Buchon et al. (2014) found that its persistent activation in adult fly guts improved function, infection resistance, and lifespan without hampering bolstered stem cell activity, tissue homeostasis, and antimicrobial peptide expression, ensuring a balanced gut microbiome (Buchon et al., 2014). The authors further demonstrated that these effects were mediated by the Upd cytokines (particularly Upd2 and Upd3), which serve as ligands to activate the JAK/STAT pathway. These cytokines were shown to stimulate ISC proliferation and differentiation, thereby supporting tissue renewal without causing tumorigenic overgrowth (Jiang et al., 2009). Additionally, it optimised nutrient absorption and metabolism, indirectly extending lifespan. Improved gut barrier integrity also contributed to a reduced systemic inflammatory response, which is a known contributor to age-related decline. These combined effects suggest that localised, regulated activation of JAK/STAT signalling in the gut has a systemic impact on ageing by preserving tissue function and enhancing physiological resilience (Rera et al., 2012). The findings highlight the intricate relationship between tissue-specific signalling and overall ageing, warranting further exploration in human contexts. Indeed, given the evolutionary conservation of the JAK/STAT pathway, these *Drosophila*-based findings offer a valuable model for investigating similar mechanisms in mammals, particularly in relation to gastrointestinal health, stem cell maintenance, and inflammatory ageing (Arbouzova & Zeidler, 2006)

1.18 Mechanisms of lifespan/healthspan improvement

The mechanisms underlying the increase in lifespan and healthspan are complex and multifactorial. Various processes and cellular components contribute to the maintenance of these extended health

outcomes. Some of the key mechanisms involved in increasing lifespan and healthspan include DNA damage repair, stem cell maintenance, and metabolic changes.

1.18.1 DNA damage repair

As hinted at earlier, accumulation of DNA damage is a hallmark of ageing and can lead to genomic instability and functional decline. Efficient DNA damage repair mechanisms are crucial for maintaining genomic integrity and preventing age-related diseases. Enhanced DNA repair pathways, such as nucleotide excision repair (NER), base excision repair (BER), and homologous recombination (HR), play a critical role in increasing lifespan and health-span (MacRae et al., 2015). These repair mechanisms help to minimise the accumulation of DNA lesions, prevent mutations, and preserve cellular function.

1.18.2 Stem cell maintenance

Stem cells have the remarkable ability to self-renew and differentiate into various cell types, making them essential for tissue regeneration and homeostasis. With age, stem cell functionality declines, leading to reduced tissue repair and regeneration (Blau et al., 2015). Strategies that promote stem cell maintenance and function can extend lifespan and health-span. Preservation of stem cell populations, activation of quiescent stem cells, and modulation of the stem cell niche microenvironment are some of the mechanisms involved in promoting healthy ageing.

1.18.3 Metabolic changes

Metabolism plays a crucial role in ageing and age-related diseases. Caloric restriction, for example, has been shown to extend lifespan and health-span in various organisms (Vera et al., 2013). It promotes metabolic changes that enhance cellular stress resistance, improve mitochondrial function, and regulate nutrient sensing pathways, such as insulin/IGF-1 signalling and mTOR signalling. These metabolic alterations contribute to increased cellular and organismal resilience and promote healthy ageing.

1.18.4 Hormesis

Hormesis is a phenomenon where exposure to mild stressors can induce beneficial adaptive responses that confer increased resistance to subsequent stress. Hermetic interventions, such as heat shock, oxidative stress, or hermetic compounds, activate stress response pathways, including heat shock response, antioxidant defence pathways, and autophagy (Calabrese et al., 2012). These adaptive responses can improve cellular and organismal health, increase stress resistance, and extend lifespan and health-span.

1.18.5 Epigenetic modifications

Epigenetic modifications, such as DNA methylation, histone modifications, and non-coding RNAs, regulate gene expression patterns without altering the DNA sequence. Age-related changes in the epigenome can impact gene expression and cellular function via epigenetic modifications (Unnikrishnan et al., 2018). As such, there are altered cellular functions and potentially emanation of age-related diseases and conditions. Epigenetic changes in the epigenome can act as a regulatory mechanism to control gene expression and cellular processes, and their dysregulation with age can have profound effects on overall health and well-being (Herceg and Paliwal, 2011). Modulating epigenetic marks through interventions like dietary interventions, exercise, and pharmacological agents can potentially reverse age-associated epigenetic alterations and promote healthier ageing (Szic et al., 2015).

1.19 The aims of this project

The initial impetus for this project stemmed from concerns about the potential harmful effects of low doses of methotrexate (MTX). Given its widespread use in various medical conditions, understanding its impact at a cellular and organismal level was deemed crucial. As the research progressed, intriguing observations suggested that MTX might have implications beyond its immediate therapeutic effects, potentially influencing lifespan and healthspan.

Thus, the specific aims of this project are:

- To delve deeper into the JAK/STAT signalling pathway, DNA damage, and DDR *in vivo*, particularly in the context of MTX exposure.
- To elucidate the interrelationships between these processes and their collective impact on diseases and ageing.
- To specifically assess the effects of methotrexate on the model organism, *Drosophila melanogaster*, providing insights into its broader implications on health and longevity.

Chapter 2: Materials and Methods

2.1 Establishment of *in vivo* assays for DNA damage

At the first stage, fruit flies (*Drosophila melanogaster*) were used to create an *in vivo* assay to detect DNA damage. The eye of the fly is compound and comprised of approximately 800 simple eyes, termed ommatidia. Each ommatidium contains a central group of 8 photoreceptors and is surrounded by 2 primary, 6 secondary, and 3 tertiary pigment cells. This approach uses the fact that each adult *Drosophila* eye contains high levels of red pigment which acts as an optical insulator. Production of red pigment is dependent on the *white (w)* gene which is required for pigment production and when mutated, results in a white-eyed fly. Thus, the frequency of DNA damage can be assessed using an easily scorable, high throughput readout. In this assay, we re-combined existing transgenic strains carrying the *GMR-Gal4* construct (Bloomington Stock 8605, donated by Claude Desplan, New York University) and a *UAS*-white *RNAi* construct.

Even in an otherwise *white+* genetic background, this combination is sufficient to knock down the levels of *white* mRNA, such that very little pigment is made resulting in a light-yellow eye colour (Figure 5). As the reduction in pigment production is dependent on the *w+; GMR-Gal4, UAS-white RNAi*, any DNA damage that affects any of these components will stop the *white* mRNA knockdown. This allows normal levels of white protein to be made and the pigment cells to become red again. By inducing DNA damage during the larval stages when the eye is still growing, small groups of clonally related cells — each of which contains a mutation in the *GMR-Gal4/UAS-white RNAi* system — can produce patches of red tissue in the adult eye that are easily identifiable (Figure 6). Preliminary results also suggest that DNA damage that occurs during adult life in a single pigment cell can also be detected by this assay, suggesting that *white* expression and pigment production are ongoing throughout adult life. However, these single *white+* cells are very small and thin and are therefore difficult to visualize and quantify in adult stages.

Red clones in the eyes of *Drosophila* and serrated wing phenotypes were imaged using a microscope equipped with the NIS-Elements BR imaging software for precise focus and image acquisition. Adult flies were anesthetized by freezing briefly before imaging. For red clones, a neutral white background was used to enhance contrast, and images were captured under optimized lighting conditions. For the serrated wing assay, wings were imaged directly without dissection, to ensure their natural morphology remained intact. Quantification of both phenotypes was scored manually using stereomicroscopy.

2.1.1 Quantification of serrated wing phenotypes

Serrated wing phenotypes were quantified in adult *Drosophila melanogaster* using a stereomicroscope under consistent magnification. Following eclosion, flies from each treatment group were anaesthetised using CO_2 and their wings gently spread for scoring. The number of visible notches or serrations along the distal wing margin was counted for each individual fly. At least 200 flies per MTX concentration and control group were assessed. The mean number of wing notches per fly was calculated for each condition, and comparisons were made between MTX-treated groups and the untreated control to assess the incidence and severity of wing morphology defects.

Figure 5: White eye of *Drosophila melanogaster*. This picture shows a white eye in which very little pigment is made, giving it a light-yellow eye colour. The genotype of this fly is *w+; GMR-Gal4 UAS-white RNAi.*

Figure 6: Mutated eye of *Drosophila melanogaster* with red clones. This picture shows multiple red clones that resulted from induction of DNA damage during larval stages by irradiation. Therefore, the eye contains small groups of clonally related cells, each of which contains a mutation in the *GMR-Gal4/UAS-white RNAi* system, that produce patches of red tissue that are easily identifiable in the adult eye.

2.2 Gal4-UAS system

The Gal4/UAS system in *Drosophila* may be used to overexpress or knock down particular genes in a tissue-specific manner *in vivo* (Brand & Perrimon 1993). In our model, we knocked down a gene to observe a change in phenotype. Gal4 can be used under a tissue-specific promoter, such as GMR which is a promoter that contains a multimerised binding site for the Glass transcription factor which is expressed during eye development (Ellis, Oneill et al., 1993). The Gal4 transcription factor binds to the DNA of the UAS and drives the expression of the target gene, such as RNAi (Li, Li et al., 2012). A summary of the fly lines used in this study is provided in Table 1.

Table 1: Summary of *Drosophila* lines used in this study.

w^+; P{w[+mC]=GAL4-ninaE.GMR}12, P{ w[+mC UAS-white^{RNAi}}GD30033 (*w^+; GMR-Gal4,UAS-white^{RNAi}*)	A recombinant between Bloomington Stock 1104 - w[*]; P{w[+mC]=GAL4-ninaE.GMR}12 and the GD30033 stock from the Vienna *Drosophila* Resource Centre — an *in vivo* UAS-RNAi targeting the mRNA transcribed from the white locus. This recombinant has also been crossed into a w^+ background but is phenotypically very light yellow. Recombinant made by Martin Zeidler (Kalidas and Smith, 2002; Li et al., 2012; Liang et al., 2015).
white^{Dahomey} (*w^{Dah}*)	Laboratory-adapted strain derived from wild-type flies collected in Dahomey (now Benin) in 1970 (Grandison et al., 2009). The *w1118 mutation was backcrossed into the wild-type Dahomey strain.* Gift from Prof Alex Whitworth.
w^{1118}	A widely used laboratory strain of *Drosophila melanogaster* carrying a null mutation in the *white* (w) gene, resulting in a white-eyed phenotype. This strain is often employed as a genetic background for transgene studies and as a control in experiments involving pigmentation or eye development.
w[*]; P{w[+mC]=AyGAL4}25 P{w[+mC]=UAS-GFP.S65T} *(Actin-GFP)*	Bloomington Stock 4411 — a transgenic *Drosophila* line expressing GFP under the indirect control of the Actin5C promoter.
OreR	A standard wild-type laboratory strain of *Drosophila melanogaster*, originally derived from a natural population. Widely used as a control strain in various genetic, developmental, and behavioural studies due to its well-characterized genetic background.

2.3 *Drosophila* media

In this project, a standard food formula for *Drosophila*, as outlined in Table 2, was consistently used unless specified otherwise. This food is typically dried and stored at the bottom of containers at ambient temperature. To administer drugs or small molecules to the *Drosophila*, the substances were usually mixed into the standard food while in a liquid state at 60°C. The food was allowed to solidify at room temperature overnight before being used.

For experiments involving MTX (PQ, Sigma Aldrich), various concentrations of MTX were prepared by diluting the stock solution into the liquid food. The concentrations used ranged from 0 nM to 4000 nM. See Appendix A for the calculations used to generate the appropriate final concentration. The units and pipetting volumes were verified to ensure accuracy. To accurately prepare very low concentrations of MTX (e.g., 0.1 µM, 0.001 µM, and 0.0001 µM), serial 1:100 and 1:1000 dilutions of the stock solution were prepared. This approach allowed me to handle the small volumes more effectively and ensure that the correct low doses of MTX were added. To minimize additional toxicity, the stock solutions of MTX and FA were diluted in PBS rather than DMSO.

Table 2: Standard *Drosophila* food.

Mix the following contents in 1 litre cold H_2O_2
• 80 g Medium Cornmeal (Triple Lion, Beanies)
• 18g Dried Yeast (Kerry Ingredients, BTP Drewitt)
• 10g Soya Flour (Lembas Wholefoods, Beanies)
• 80g Malt Extract (Rayner's Essentials, Beanies)
• 40g Molasses (Rayner's Essentials, Beanies)
• 8g Agar (BTP Drewitt)
Bring to boil and stir constantly until homogeneity reached. Add following contents after cooling to 60°C
• 25ml 10% Nipagen (Fisher Scientific, Loughborough) in 95% Ethanol (Fisher Scientific)
• 4ml Propionic Acid (Fisher Scientific)

2.4 Optimization of the DNA damage assay

The high dose of X-rays used in preliminary experiments may inflict so much damage on the flies that it may induce substantial apoptosis and developmental defects and cause difficulty in quantification. Given this, I repeated the previous experiments and irradiated the *w+; GMR-Gal4, and UAS-white RNAi* flies with different doses of X-rays at the same developmental times to establish optimal conditions.

To undertake these assays, virgin females homozygous for *w+; GMR-Gal4/, UAS-white RNAi* were crossed with males (either wild type or carrying other UAS constructs). Eggs were collected at 24 h

intervals and aged at 25°C until the early third instar larval stage. Larvae were then irradiated; for example, for 18 minutes and 30 seconds at 120 kV and 3 mA using a Torex Cabinet X-ray system (Faxitron) to deliver 200 Gy of ionizing irradiation. Adult flies were allowed to eclose, and the number of red clones were counted approximately 12 days after egg laying (Figure 14).

2.5 Testing the effect of MTX on development and DNA damage

First, the effects of different concentrations of MTX in *Drosophila* food on survival, development, and DNA damage phenotypes were tested. The *w+; GMR-Gal4, UAS-white RNAi* flies were grown on food containing a range of concentrations of MTX (0 nM, 1 nM, 10 nM, 100 nM, 1000 nM, and 4000 nM; Figure 16) and not exposed to any irradiation; 200 flies were used for each concentration. Then, the files were scored for red-eye clones and serrated wings.

After this initial experiment, the same experiments were repeated with the additional step that larvae were also irradiated at the third instar larva stage at 200 Gy. For this experiment, the number of flies was doubled (n=400 for each concentration) and the dose of 10 µM MTX was tested as well (Figure 16).

2.5.1 The *mwh* wing hair assay

The *mwh* wing hair assay was used to evaluate DNA damage in the wings of *Drosophila melanogaster* under different experimental conditions, including exposure to varying doses of MTX and X-ray irradiation. This assay involved outcrossing males from the *mwh[1]* homozygous amorphic allele (kind gift of David Strutt) to *w[1118]* virgin females to generate heterozygous offspring. The flies were subsequently grown on normal or food containing 0.001 µM, 0.01 µM, 0.1 µM, or 1 µM MTX. The control group did not receive MTX or X-ray exposure.

At the early/mid third instar stages, heterozygous larvae were irradiated with X-rays and allowed to continue their development and hatch as adult flies. Flies were exposed to 100 Gy of X-ray radiation using a Faxitron X-ray machine (120 kV, 3 mA for 9 min and 15 sec per thousand rads, shelf 7). The

bottles were randomly located within the cone of X-rays produced by the machine and moved halfway through irradiation in an attempt to provide a uniform exposure to all animals.

Adult wings were prepared and examined under a compound microscope for the presence of patches of *mwh-/-* cells (clones) in the otherwise heterozygous (and phenotypically wild type) background (see Figure 17 for an example of a typical clone). Both surfaces of the wings were systematically screened using 5x and 10x objectives to scan for wing hairs on the slide and 20-40x objectives in a Zeiss Axioskop 2 microscope to identify clones. Representative examples were photographed using a 63x oil objective and Micro-Imager camera system.

The presence of a clone was taken as an indication of either a) loss of heterozygosity in the *mwh* locus caused by X-ray irradiation damage and unsuccessful repair or b) a double-stranded DNA break between the *mwh* locus (at 3-0 on the tip of 3L) and the third chromosome centromere which was not accurately repaired and ultimately resulted in mitotic recombination between the heterozygous chromosomes and the generation of a *mwh* homozygous/wild type clonal pair.

During quantification of the *mwh* clones, it was assumed that the differences in clone size were explained by variations in the developmental stage when irradiated, but that each individual clone was the result of a failed DNA repair event in a single progenitor cell. As such, only the number of clones was counted, and multiple clones in the same wing were only counted when clearly separated from one another. However, it is possible that independent clones that happened to overlap on both the dorsal and the ventral surfaces of the adult wing were undercounted.

2.6 *Drosophila* embryo collection

Two to three hundred flies were placed in collection cages for egg laying. The cage openings were covered with apple juice agar plates (20% *v/v* apple juice, Sainsbury's, London, UK; 3% *w/v* agar, Invitrogen, Warrington, UK; and 0.0015% *w/v* nipagen, Sigma Aldrich) and topped with yeast paste. Flies laid eggs on these plates, which were replaced twice daily early in the morning and the evening around 5 pm. The initial two plates were discarded. Embryos were allowed to accumulate on the plate

for approximately 15-20 hours, primarily during the overnight hours. Subsequently, the eggs were rinsed off the plates using PBS and collected in a conical flask. After several PBS washes, the clear supernatant was decanted. A sample of the eggs (around 30 μL) was introduced into 5-10 food bottles. The bottles were incubated at 25°C under a 12-hour light-dark cycle and 60% humidity, in preparation for the collection of age-synchronized flies.

2.7 Age-matched adult fly collection and sorting

Flies that emerged within the initial 24 hours were discarded, and the bottles were returned to a 25°C incubator to collect the middle batch. Flies that hatched in the subsequent 15-20 hours, which were one-day-old, were gently relocated to fresh food bottles without the use of CO_2. Once relocated, these flies were allowed to mature and mate over a few days inside the incubator.

For the sex separation process, a small batch of flies (less than 50) was transferred onto an anaesthetic pad and quickly sorted by sex using a soft brush. After each sorting session, the separated male and female flies were moved to separate bottles or vials. To avoid potential negative effects on future experiments related to longevity and behaviour, the exposure of flies to CO_2 was kept to a minimum, in accordance with findings by Poon et al. (2010).

2.8 Testing the effect of MTX on *Drosophila* lifespan

In the longevity studies, female $w^{Dahomey}$ flies were examined. The Dahomey strain is a wild type line derived from Dahomey, Africa, that has been widely used in longevity and metabolism studies (Partridge et al., 1997). The w^{1118} mutation was backcrossed into the wild-type Dahomey strain. The lifespan and fecundity of this strain has been extensively characterized under various conditions. These flies were collected without CO_2 and aged together with males until 4-5-days-old, then sorted using minimal CO_2 and kept in groups of 20 in individual vials. These vials contained food mixed with varying concentrations of MTX (0 nM, 1 nM, 10 nM, 100 nM, 1000 nM, and 4000 nM). Unless otherwise noted,

each longevity test involved 200 flies, which required 10 vials of females for each concentration of MTX. Grouping 20 flies per vial facilitated easier tracking during the longevity study.

This experiment was conducted three times, using three separate batches of flies acquired at different times, with each batch consisting of 200 flies. The flies were moved to new vials every Mon, Wed, and Fri, at which point dead flies were counted. The method of transferring was tipping. During each transfer, the number of deceased flies was recorded, along with any censored flies (those that had escaped or died accidentally, such as during handling). The experiments concluded when all flies had died.

Data was processed using the Survival Table in GraphPad Prism Version 9.0. A death was represented as '1' and a censored event as '0'. For instance, if three female flies died and one fly escaped on a given day, the score for that day would be three '1's and one '0'. The generated mortality curve was subsequently analysed using the Gehan-Breslow-Wilcoxon test in GraphPad Prism to enable a comparison of the survival rates across the different treatment groups.

2.9 Rapid iterative negative geotaxis (RING) assay

Eggs and age-matched adult flies were obtained as detailed in sections 2.6 and 2.7. Around 200 $w^{Dahomey}$ flies for each sex were gathered in food bottles which either contained just food or a combination of food and MTX. These bottles were routinely moved to new fly food every two days during the experimental period.

One day prior to the experiment, the flies were sorted by sex and moved from the bottles to food vials using CO_2 anaesthesia. For each concentration of MTX, five sets of vials were created, with each vial housing ten flies. These vials were incubated overnight, to allow the flies to acclimatise to room temperature and humidity and to recover from the CO_2 exposure. On the day of the experiment, after spending 1-2 hours in the vials at room temperature, the flies were transferred to empty polystyrene tubes. As starvation prompts a climbing response in *Drosophila* (Gargano et al., 2005), all flies underwent a starvation period of 50-60 minutes prior to the commencement of the experiment.

To evaluate RING, groups of 10 flies were placed into a 25 mL stripette (Fisher Scientific). Following a 1-minute acclimatization period, the stripette was briskly and firmly tapped against the table surface thrice to trigger the flies' negative geotaxis reactions. The flies were given a 10-second window to climb, after which their respective positions were documented through a photograph. The ascended distance after the 10-second interval for each fly was subsequently determined from the captured image (Refer to Figure 7). Over 7 weeks, ten flies from every condition were evaluated at each age checkpoint, with quintuplicate trials conducted weekly for both male and female flies.

Figure 7: Rapid iterative negative geotaxis (RING) assay: After being knocked to the base of the stripette, flies were allowed to climb for a duration of 10 seconds. A photograph was captured at the conclusion of this 10-second interval to document the climbing achieved by each fly.

2.10 Enzyme-linked immunosorbent assay (ELISA)

The levels of canonical JAK/STAT activity in *Drosophila* were measured using the *10xSTAT-GFP* reporter line, as described by Bach, Ekas et al. (2007). A summary of *10xSTAT-GFP* reporter lines and genotypes has been provided in Appendix D. This reporter line, which is widely used in the lab, allows for the quantification of JAK/STAT signalling activity through GFP expression. A sandwich ELISA assay was employed to quantify GFP levels in adult *Drosophila* carrying the *10xSTAT-GFP* reporter on chromosomes II and III. Flies were fed different doses of MTX to assess the effects of the drug on JAK/STAT pathway activity. This assay was designed to determine whether low doses of MTX suppress JAK/STAT signalling *in vivo*. In addition, a schematic of the *in vivo* STAT activity reporter construct in *Drosophila melanogaster* has been shown in Chapter 4, Figure 29.

On the first day, the capture antibody was prepared by diluting the Abnova goat anti-GFP antibody (Appendix B) to a concentration of 0.0625 µg/mL in 100 units sodium bicarbonate solution. This solution was formulated by dissolving 111 mg of $Na(CO_3)_2$ in 10.5 mL of H_2O. Given that the stock antibody concentration was 1 mg/mL, a total of 66 µL of the stock solution (equivalent to 12 µg of the antibody) was required to achieve the desired final volume of 10.5 mL for a 96-well plate. Subsequently, 100 µL of this diluted antibody was added to each well of a Costar 96-well high-binding EIA plate (Fisher Scientific Ltd, Catalogue No: 10544522, Mfr. Part No: 3590). The plate was incubated overnight at 4˚C. All reagents used in the assays were prepared following the manufacturer's guidelines, as outlined in Appendix C.

On the second day, the plate was washed thrice using the ELISA wash buffer. Excess buffer was removed by flicking the plate over a sink and dabbing the plate on paper. The plate was then blocked using the ELISA wash buffer for 1 hour at room temperature. Concurrently, the protein concentrations of the samples intended for testing were determined and the necessary dilutions were set up. Following the blocking step, 100 µL of each sample was added to the respective well and incubated for either 3

hours at 37˚C or overnight at 4˚C. For assays involving conditioned media concentration calculations, serial dilutions were performed down the plate. Additionally, a serial dilution of recombinant GFP, starting at a concentration of 5 ng/mL, was prepared using the ELISA wash buffer.

At day 3, post-sample incubation, the plate was washed three times with the ELISA wash buffer, then treated with the primary antibody, Rabbit anti-GFP ab290, at a dilution of 1:20,000. This involved adding 0.5 µL of the antibody to 10 mL of the ELISA wash buffer. The plate was incubated with the primary antibody solution overnight at 4˚C.

On the last day, the plate underwent another three washes with the ELISA wash buffer, followed by three washes with PBS. Then, the plate was incubated with the secondary antibody, Anti-Rabbit HRP, at a dilution of 1:5,000 for 1 hour at room temperature. This dilution was prepared by adding 2 µL of the secondary antibody (0.37 mg/mL stock concentration) to 10 mL of the ELISA wash buffer. Following the incubation, the plate was washed three times with the ELISA wash buffer, followed by PBS. Fresh developing solution was prepared by mixing 25 mL of the HRP assay buffer with one 10 mg tablet of o-phenylenediamine and 10 µL of H_2O_2 in a conical flask, then 200 µL of the developing solution was added to each well, and colour development was monitored for approximately 10 minutes. The reaction was halted by adding 50 µL of stop solution to each well, and absorbance was measured at 492 nm.

2.11 Gut dissection

In an attempt to measure JAK/STAT pathway activity *in vivo*, adult female flies of the *10x STAT-GFP* genotype located on chromosomes II and III were grown on MTX-containing food of the indicated concentrations (made as previously described) for 14 days. After this time, adult flies were anesthetised using CO_2, transferred into PBS, and their were guts dissected. Proteins expressed by the gut cells were extracted and the levels of GFP expressed were measured using a quantitative sandwich ELISA assay. Details of this experiment are given below:

2.11.1 Extraction and sample preparation

Protein extraction buffer was prepared (50 mM Tris HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, and 0.003% Triton X-100), with protease inhibitor tablets (cOmplete Mini, EDTA-free, ROCHE, Sussex, UK) added at double the standard concentration (one tablet per 5 mL of buffer) to ensure sample protein stability as high levels of protease activity were anticipated, given the nature of the tissue. Aliquots of 100 µL extraction buffer were pre-prepared in Eppendorf tubes and kept on ice. The guts of three female flies were dissected and immediately transferred into the pre-chilled Eppendorf tubes. The flies were fed food containing different doses of MTX for two weeks; the control group did not receive MTX. The tissues were homogenised using a clean plastic micro-pestle, incubated on ice for 10 minutes to allow lysis to occur, and centrifugated at 4°C for 5 minutes at maximum speed in a cooled benchtop micro-centrifuge. After centrifugation, the supernatant (approximately 60 µL) was carefully removed from above the debris pellet and transferred into fresh Eppendorf tubes. The supernatants were then frozen on dry ice and stored at -80°C. This process was repeated to generate 3–4 experimental replicates for each MTX concentration, with each replicate containing the gut extracts from three flies.

To quantify GFP expression, 50 µL of each prepared gut extract was added to the well of an ELISA plate and serially diluted across the plate, as mentioned before.

2.11.2 Gut dissection for confocal imaging

To directly visualize GFP expression produced by the JAK/STAT pathway reporter, gut dissections were performed on female flies of the *10x STAT-GFP* genotype located on chromosomes II and III. Flies were anesthetized using CO_2 and their midguts were dissected under a dissecting microscope in cold 1x PBS to preserve tissue integrity. The guts were carefully removed and transferred onto glass slides pre-coated with poly-lysine to ensure adherence during imaging. Excess PBS was gently removed, and the samples were stained with 5 µL of phalloidin and mounted on a slide with 30 µL DAPI (Sigma Aldrich). Coverslips were placed over the dissected tissues and sealed with nail polish to prevent drying, and allowed to cure briefly. Samples were imaged using a Nikon A1 confocal

microscope under GFP-specific excitation and emission settings. The imaging parameters, such as laser intensity and exposure time, were standardized across all samples to ensure consistency.

2.12 Animal whole genome sequencing

In this chapter, I present the experiments conducted to evaluate the potential genotoxic effects of exposure to low-dose methotrexate (MTX) on genomic stability in *Drosophila melanogaster*. The study, designed and developed by Dr. Zeidler and Adel Alqarni (AA), involved AA conducting DNA extractions and genetic analysis. Data generation and analysis were performed by Novogen. Bioinformatic analysis of the sequencing data was assisted by Dr. Natalia Bulgakova and Dr. Martin Zeidler. This chapter was written by AA.

Sequencing the complete genetic composition of animals through Illumina platforms has emerged as a highly efficient and rapid technique for discerning genetic variations within individuals of the same species or between closely related species. This approach, referred to as sequencing by synthesis, facilitates the detection of alterations in individual DNA units (SNPs), insertions or deletions of small DNA segments (In-Dels), variations in the number of gene copies (CNVs), and substantial structural modifications in genes (SVs). The insights gleaned from this sequencing process have proven invaluable for investigating the relationships among diverse animal populations and for identifying genetic discrepancies associated with diseases. Additionally, it aids in the selection of optimal plants and animals for agricultural breeding and in identifying shared genetic variations across distinct animal groups. This section will present a comprehensive account of the process of animal whole genome sequencing used in this study, including the sample preparation for *Drosophila melanogaster*, quality assessment, library construction, sequencing procedures, and subsequent bioinformatics analysis (Figure 8).

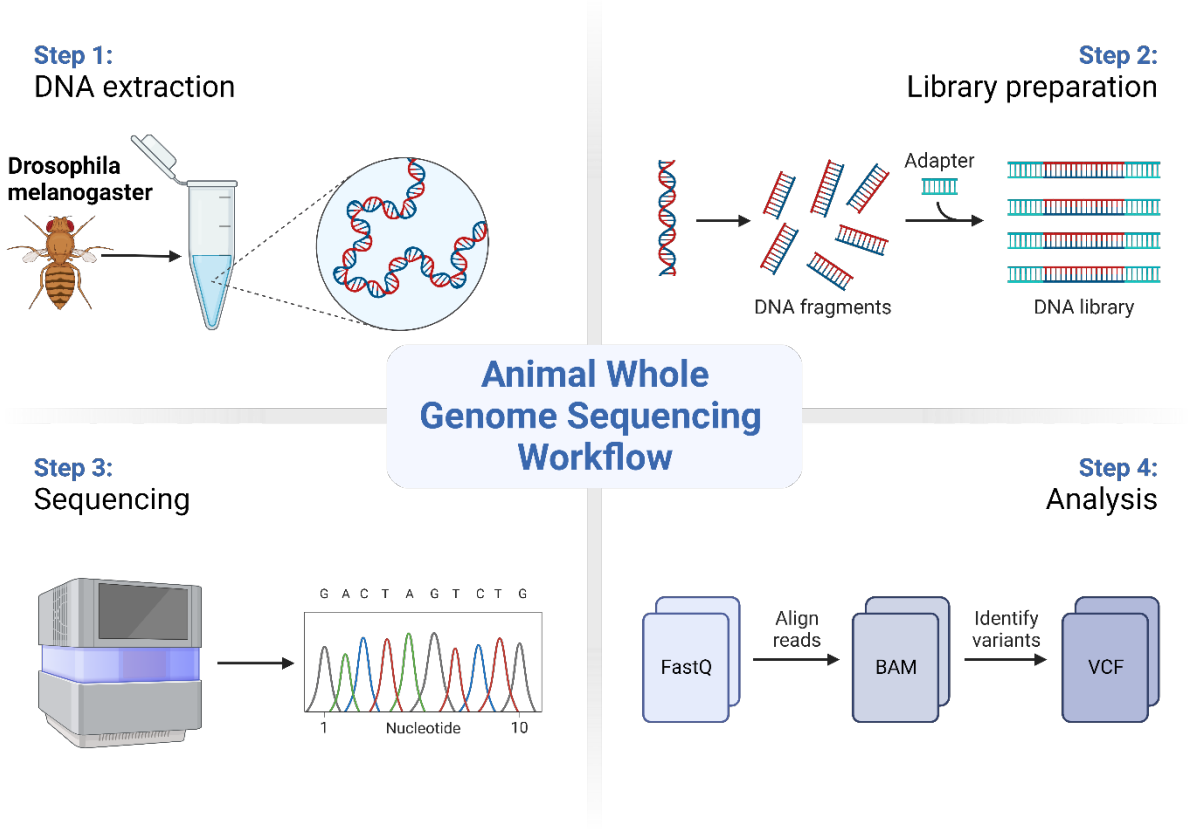


Figure 8: Schematic of the Next-Generation Sequencing (NGS) Experiment.

This schematic outlines the workflow for the next-generation sequencing (NGS) experiment performed on *Drosophila melanogaster*. The process includes DNA extraction, library preparation, sequencing on Illumina platforms, and bioinformatics analysis to identify single nucleotide polymorphisms (SNPs) and structural variants (SVs).

2.12.1 Sample preparation

$w^{Dahomey}$ *Drosophila melanogaster* were sequenced to compare the mutation rate between two generations, i.e., grandfathers and their grandsons.

2.12.2 DNA extraction

The DNA extraction protocol employed the Qiagen DNeasy Blood & Tissue Kit (Catalog Number/ID: 69504) which required the appropriate preparation of ethanol-containing buffers before commencing

the procedure. One fly was homogenized for each sample and the DNA concentration was measured using a NanoDrop ND-1000. Library construction followed the Illumina TruSeq DNA protocol, with 150 bp paired-end reads generated using HiSeq sequencing platforms. The *Drosophila melanogaster* Release 6 (dm6) genome was used as the reference genome for alignment. To safeguard the integrity of the DNA, excessive mixing or vortexing of solutions was carefully avoided to prevent potential DNA shearing. A single *Drosophila* specimen stored at -20°C was gently transferred into a 1.5 mL microcentrifuge tube, followed by the addition of 180 μL of Buffer ATL. Subsequently, the specimen was ground using a pestle to facilitate homogenization. Proteinase K (20 μL) was then introduced into the mixture, which was thoroughly mixed through 2-3 brief vortex pulses. The resultant mixture was incubated at 56°C with gentle agitation (e.g., 300 rpm shaking) for 1-3 hours; one hour was typically deemed adequate. Following this, the sample was centrifuged at 8000 RPM for 1 minute to eliminate wing fragments and cuticle debris. The supernatant was then carefully transferred to a fresh 1.5 mL microcentrifuge tube. RNase A (4 μL of 100 mg/mL, DNAse-free) was carefully added, and the solution was briefly vortexed. After 5 minutes of incubation at room temperature, Buffer AL (200 μL) was introduced into the sample and mixed briefly by vortexing, followed by addition of 200 μL of absolute ethanol, and the mixture was subjected to brief vortexing to ensure proper mixing. The combined mixture, including any precipitate, was accurately pipetted into the DNeasy Mini spin column positioned within a provided 2 mL collection tube. After centrifugation at 8000 RPM for 1 minute, the flow-through and collection tube were discarded. The DNeasy Mini spin column was then transferred to a new 2 mL collection tube, 500 μL of Buffer AW1 was added, and the tube was centrifuged for 1 minute at 8000 RPM to eliminate the flow-through into the collection tube. This process was repeated after adding Buffer AW2 (500 μL) to the spin column, followed by centrifugation at 14,000 RPM for 3 minutes to dry the DNeasy membrane. The previous step was repeated to ensure complete drying of the membrane. Subsequently, the DNeasy Mini spin column was positioned within a clean 1.5 mL microcentrifuge tube and 100 μL of Buffer AE was precisely pipetted directly onto the DNeasy membrane. After incubation for 1 minute at room temperature, the tube was centrifuged for 1 minute at 8000 RPM to facilitate the elution of DNA into the tube.

2.12.3 DNA purity and integrity assessment

The quality of the extracted DNA was assessed using a NanoDrop® ND-1000 spectrophotometer to yielding precise ??? values for each sample. DNA purity and integrity were further assessed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) to ensure the suitability of samples for downstream applications. The DNA was assessed by 1% agarose gel electrophoresis.

The DNA concentration was accurately quantified using a Qubit™ 2.0 fluorometer (Life Technologies, Carlsbad, USA) and further verified using the Bioanalyser 2100 system (Agilent Technologies, USA).

2.12.4 Library construction, quality control, and sequencing

2.12.5 DNA library preparation and quality assessment

DNA sequencing, including library construction, quality control, and sequencing, was performed at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). In short, after passing the QC procedures, a total of 0.2 μg of DNA per sample was used as input material for DNA library preparation. Genomic DNA was fragmented by sonication to an average size of 350 bp. The resulting DNA fragments were then end-polished, A-tailed, and ligated to full-length adapters suitable for Illumina sequencing, followed by size selection and PCR amplification. The amplified PCR products were purified using the AMPure XP system (Company, Beverly, CA USA).

Library quality control was performed in three steps: a Qubit 2.0 (Life Technologies, Carlsbad, USA) was used for preliminary testing of the library concentration, then the Agilent 5400 system (Agilent, USA) was employed to determine the insert size, and qPCR was used for precise quantification of the library's effective concentration.

2.12.6 Sequencing strategy

The libraries that passed quality control were pooled and sequenced on Illumina platforms with the PE150 strategy, according to effective library concentration and data amount required.

2.12.7 Bioinformatics analysis pipeline

2.12.7.1 Data quality control

Data quality control is a critical process in any scientific research or data analysis, including genetic and genomic studies such as SNP/InDel detection and CNV/SV detection in organisms like *Drosophila melanogaster*. It involves a series of steps to ensure that the data is accurate, reliable, and suitable for the intended analyses. The quality of the data directly influences the validity and robustness of the results and conclusions. Quality control is crucial for ensuring the reliability of subsequent bioinformatics analyses, as sequence artifacts can pose challenges. These artifacts may include adapter contamination, low-quality nucleotides, and ambiguous nucleotides (N). To address these issues, the following data processing steps were implemented: paired reads were eliminated if either read showed evidence of adapter contamination, defined as more than 10 nucleotides aligning to the adapter with a mismatch rate of 10% or less; paired reads were removed if either read contained more than 10% ambiguous bases; and paired reads were excluded if the Phred quality score was below 5 for over 50% of bases in either read.

2.12.7.2 Mapping reads to the reference sequence

Reads were mapped to the reference genome using the Burrows-Wheeler Aligner (BWA) to efficiently align short sequencing reads against a large reference sequence, using the default parameters to align paired-end clean reads (Li and Durbin, 2009). The initial mapping results were generated in BAM format, containing all aligned reads, including duplicates and unsorted data. To obtain the final processed BAM file, several steps were undertaken. First, SAMtools was used to sort the BAM file by genomic coordinates, to organise the reads for downstream analysis, and an index file was created to enable quick access to specific genomic regions (Li et al., 2009). For samples with multiple sequencing runs, Picard tools (https://broadinstitute.github.io/picard/) was used to merge the BAM files into a single consolidated file for each sample to ensure consistent data organisation. Finally, SAMtools was employed to identify and remove duplicate reads, likely the results of PCR artefacts, to enhance the accuracy of downstream analyses. These steps yielded high-quality, non-redundant BAM files suitable for further genomic analysis.

2.12.8 Variant detection and annotation

2.12.8.1 SNP/In-Del detection

Variant detection and annotation, particularly focused on SNP/In-Del (Single Nucleotide Polymorphism/Insertion-Deletion) identification in *Drosophila melanogaster*, involves the process of pinpointing and characterizing genetic changes within the genome of this fruit fly species. The process entails comparing an individual fly's DNA sequence to a reference genome to identify potential genetic variations, including SNPs and In-Dels—alterations involving single nucleotides or small insertions/deletions. Annotation follows, with the aim to understand the functional consequences of these variations, such as determining if a variant is positioned within a gene's coding region, assessing its impact on protein-coding sequences, and predicting any structural or functional shifts.

The raw SNP/In-Del sets were called using GATK (DePristo et al., 2011) with the parameters set as '–gcpHMM 10 -stand_emit_conf 10 -stand_call_conf 30'. Then we filtered these sets using the following

criteria: SNP: QD < 2.0, FS > 60.0, MQ < 30.0, HaplotypeScore > 13.0; MappingQualityRankSum < -12.5, ReadPosRankSum < -8.0; and INDEL: QD < 2.0, FS > 200.0, ReadPosRankSum < -20.0/.

Figure 9 shows the quality distribution of SNPs by, from top to bottom, the distribution of reads supporting each SNP. Figure 10 shows the cumulative distribution of SNP quality in the *Drosophila* sequencing data, while figure 11 represents the SNP mutation frequency distribution in the *Drosophila* genome. Therefore, the proportions of different types of mutations detected in the *Drosophila* whole genome sequencing shows in (Figure 12).

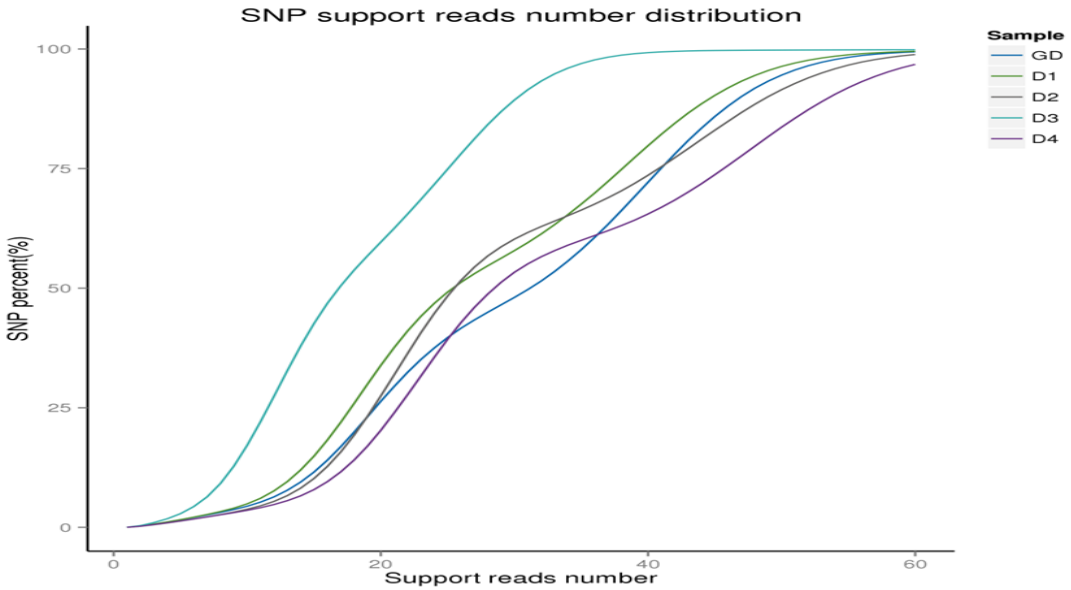


Figure 9: Quality Distribution of SNPs in the *Drosophila* Whole Genome Sequences: This figure shows the quality distribution of single nucleotide polymorphisms (SNPs) detected in the *Drosophila* whole-genome sequencing experiment. It includes the distribution of SNP support reads and adjacent SNP distances.

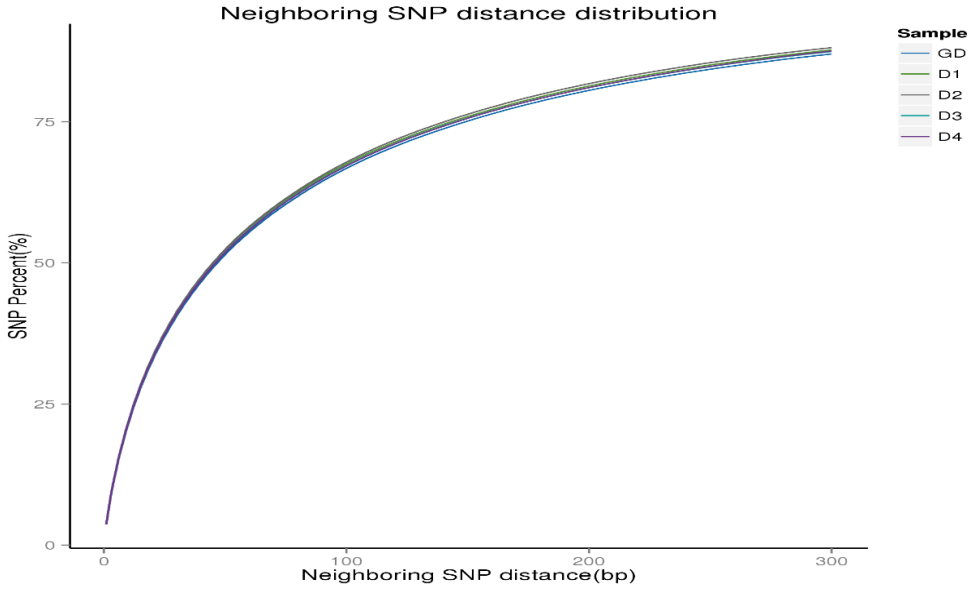


Figure 10: Cumulative Distribution of SNP Quality in the *Drosophila* Sequencing Data: This figure illustrates the cumulative distribution of SNP quality scores for the *Drosophila* sequencing data. The x-axis represents the number of SNPs, and the y-axis indicates the mutation types and associated quality metrics.

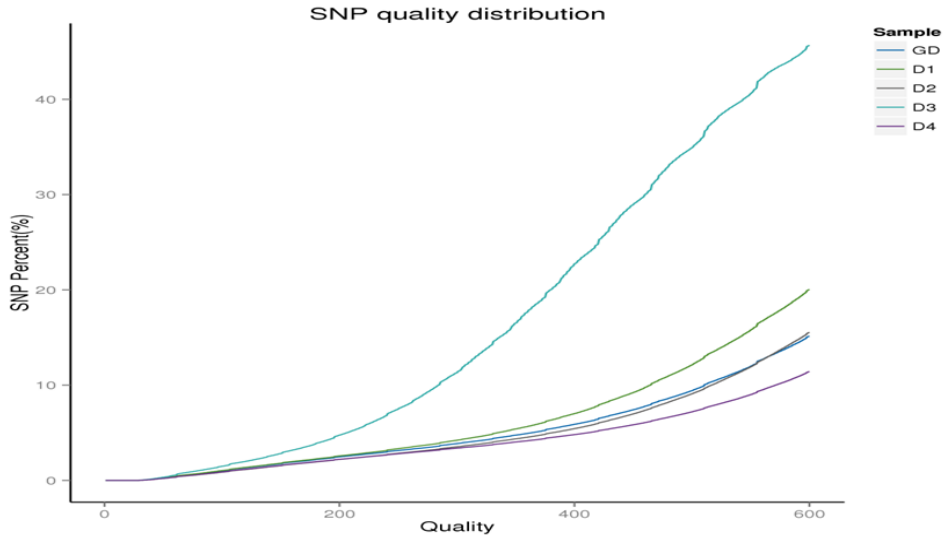


Figure 11: SNP Mutation Frequency Distribution in the *Drosophila* Genome: This figure presents the frequency of SNP mutations across the *Drosophila* genome. The x-axis represents the number of SNPs detected, and the y-axis categorizes the mutation types (e.g., transitions, transversions).

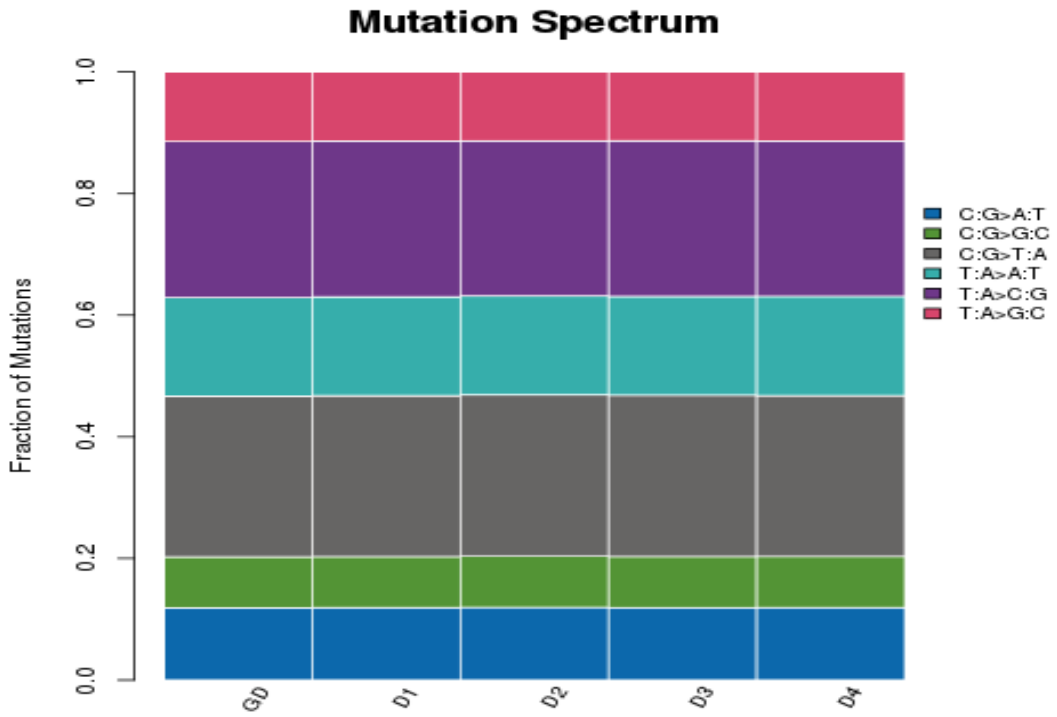


Figure 12: Proportions of Different Types of Mutations Detected in the *Drosophila* Whole Genome Sequencing: This figure shows the relative proportions of different types of mutation (e.g., SNPs, insertions, deletions) detected in the *Drosophila* whole-genome sequencing data. The *x*-axis represents the samples, and the y-axis indicates the proportion of each type of mutation.

2.12.9 CNV and SV detection

CNV (Copy Number Variation) and SV (Structural Variation) detection in *Drosophila melanogaster* entails the identification and characterization of substantial genetic changes that extend beyond single nucleotide alterations. These variations, encompassing CNVs and SVs, offer critical insights into the genetic intricacies of this model organism's genome. CNVs Detection involves discerning differences in the quantity of specific genomic segments among individuals. This encompasses duplications, deletions, and amplifications of DNA. Detecting CNVs requires comparing the read count of specific genome regions in the target genome to a reference, with deviations indicating CNVs. SVs are more extensive genomic modifications like inversions, translocations, and sizable insertions or deletions. These changes can profoundly influence gene expression, genome organization, and observable traits.

Identifying SVs often requires advanced sequencing technologies capable of spanning longer DNA stretches and spotting alterations in genomic arrangement.

CNVs were detected using CNVnator (Abyzov et al., 2011) to identify potential deletions and duplications (parameter: - call 100). SVs were detected using BreakDancer (Chen et al., 2009). Figure 13 shows the distribution of the different types of CNVs detected.

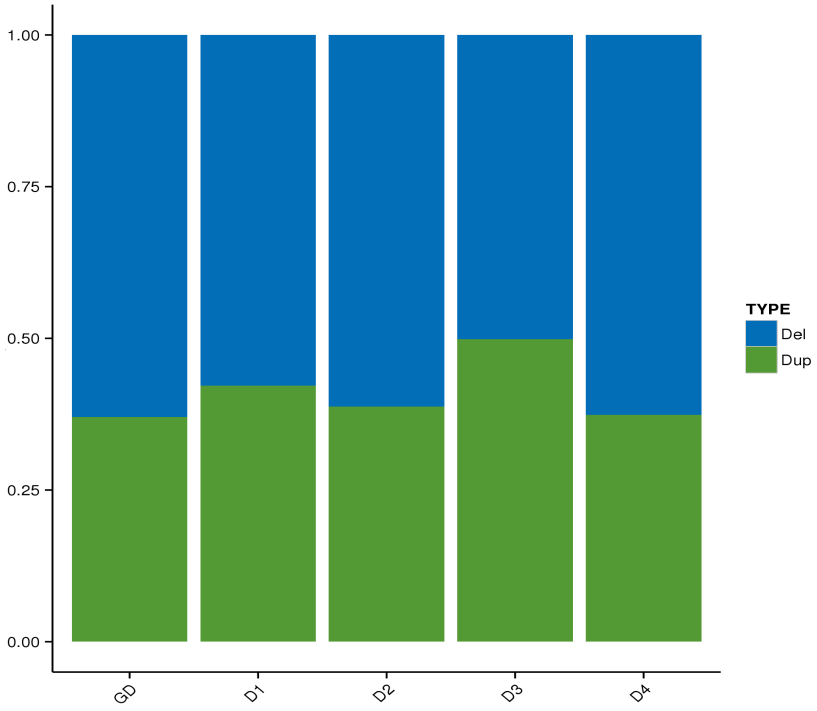


Figure 13: Copy Number Variation (CNV) Type Statistics in *Drosophila* Genome: This figure shows the distribution of different types of copy number variations (CNVs) detected in the *Drosophila* genome. The x-axis shows the proportion of deletions, duplications, and other types of CNVs detected, and the *y*-axis indicates the *Drosophila* samples.

2.12.10 Annotation

In this study, functional annotation of variants was carried out using the ANNOVAR tool introduced by Wang et al. in 2010. ANNOVAR is a pivotal resource in the field of genomics that enables comprehensive and insightful interpretation of genetic variations. By leveraging a diverse array of annotation databases and algorithms, ANNOVAR facilitates the elucidation of the potential functional consequences of genetic alterations. For gene and region annotations, the UCSC known genes database was employed (Wang et al., 2010). This resource, curated by the University of California, Santa Cruz, represents a compendium of well-characterized genes with meticulously annotated features. These features encompass a spectrum of gene-related information, including coding sequences, transcription start and end sites, splice variants, and functional domains. The UCSC known genes database can effectively correlate identified genetic variants with their corresponding gene targets and specific genomic regions. ANNOVAR serves as a bridge between raw variant data and biologically relevant insights, and integrates numerous databases and algorithms to provide multi-faceted view of the consequences of variants. This allows researchers to not only discern the potential impacts of variants on protein-coding regions, but also non-coding elements such as regulatory regions and splice sites.

2.13 Statistical analysis

All numerical results are presented as the mean value plus or minus the standard deviation (SD). Statistical evaluations were conducted using GraphPad Prism 9 software. To compare two groups, the non-parametric unpaired Student's t-test was employed, unless specified otherwise. For analyses involving more than two groups, one-way or two-way ANOVA were utilized, followed by post-hoc assessments using Bonferroni's Sidak's multiple comparison tests. Survival-related data were assessed using the log-rank (Mantel-Cox) test. Statistical significance was declared if the p-value was less than 0.05, with varying levels of significance indicated as follows: *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

Chapter 3: Low-Dose Methotrexate May Prevent DNA Damage and Increase Lifespan and Climbing in *Drosophila melanogaster*

3.1 Introduction

Cancer is a disease characterized by the uncontrolled growth and proliferation of cells. A critical factor driving this uncontrolled growth is the presence of mutations in oncogenes and tumour suppressor genes. These mutations often arise from DNA damage and can lead to aberrant cellular processes that promote cancer progression (Basu, 2018).

DNA damage is any alteration to the normal structure of DNA molecules that can occur as a result of environmental factors, such as exposure to ultraviolet (UV) radiation or chemicals, or from internal metabolic processes (Huang and Zhou, 2021). These alterations can include mutations, which are changes in the DNA sequence, and structural changes, such as breaks in the DNA double helix. Thus, maintaining genome integrity, i.e., the stability and accuracy of the genetic information within cells, is crucial for the proper functioning of cells and the organism as a whole. DNA damage, if left unrepaired, can lead to mutations and chromosomal abnormalities that can result in cancer, developmental disorders, and other diseases (Wiesmüller et al., 2002).

To prevent the negative effects of DNA damage, cells have developed several mechanisms to detect and repair damaged DNA. These mechanisms include base excision repair (BER), which repairs small, isolated damage in the DNA, such as the loss of a single nitrogenous base. Nitrogenous bases are the building blocks of DNA and include adenine (A), thymine (T), cytosine (C), and guanine (G). Nucleotide excision repair (NER) repairs larger, more complex damage in the DNA, such as damage caused by UV radiation; double-strand break repair (DSBR) repairs breaks in both strands of the DNA double helix caused by exposure to ionizing radiation or certain chemicals; and mismatch repair (MMR) detects and correct errors that occur during DNA replication (Chatterjee and Walker, 2017). The failure of these mechanisms to work properly can lead to the accumulation of DNA damage over time, which

can lead to the development of cancer and other diseases (Schumacher et al., 2021). Therefore, the maintenance of genome integrity through the proper functioning of DNA damage detection and repair mechanisms is essential for the health and survival of cells and organisms.

DNA damage and repair also play a significant role in the ageing process. As cells divide and replicate throughout a person's lifetime, DNA molecules are constantly exposed to various types of damage, such as mutations caused by exposure to environmental toxins or errors that occur during DNA replication. DNA damage repair involves a variety of enzymes and proteins that work to fix errors and damage in the DNA. However, with age, the efficiency of DNA repair decreases, leading to an accumulation of unrepaired DNA damage (Petr et al., 2020). This accumulation of DNA damage can also lead to various age-related diseases, such as cancer and neurodegenerative diseases.

Another way that DNA damage and repair impact ageing is through telomeres, the protective end caps of chromosomes. Telomeres shorten as cells divide and replicate, and once they become too short, cells can no longer divide and replicate, and thus become senescent. Overall, this can lead to a decrease in the number of functioning cells, which can contribute to the ageing process (Reichert and Stier, 2017). Additionally, the loss of DNA repair capacity may also contribute to the development of age-related diseases such as cancer. The accumulation of DNA damage can also lead to mutations in genes that regulate cell growth and division such as p53, which plays central role in regulating DNA damage response pathways, or the RAS family of proteins like the KRAS, HRAS and NRAS proto-oncogenes that become activated due to such mutations (Lim and Leprivier, 2019). All of these subsequently result in the development of cancer.

Targeting DNA damage for therapeutic purposes in cancer treatment has gained significant attention in research (Chabanon et al., 2021). The goal is not to decrease DNA damage, but rather to enhance the body's natural DNA repair mechanisms. This can be achieved by using drugs that either promote the existing DNA repair machinery or prevent further DNA damage. An example of this strategy is the use of drugs like PRIMA-1, which activates the p53 tumour suppressor gene. Such drugs have been found to increase DNA repair activity, and could thereby enhance the body's natural repair processes and help

to prevent cancer progression (Lagunas-Rangel and Bermúdez-Cruz, 2020). Another way to target DNA damage is by inhibiting the activity of enzymes that are involved in DNA damage. For example, PARP inhibitors are a class of drugs that target the activity of PARP enzymes, which are involved in repairing DNA damage. These drugs have been shown to be effective in treating cancers such as breast cancer and ovarian cancer (Kim et al., 2021). Radiation therapy is also used to induce DNA damage as a therapeutic strategy in cancer treatment to cause cancer cell death in brain tumours, lung cancer, and prostate cancer (Hosoya and Miyagawa, 2014).

Methotrexate (MTX) was one of the first chemotherapy drugs to be developed and has been extensively used to treat various types of cancer (Alqarni and Zeidler, 2020a). MTX inhibits dihydrofolate reductase (DHFR), a key enzyme in the folate pathway that is ultimately involved in the production of the nucleotides (dNTPs) that make up the nucleic acids (DNA and RNA) in cells. By inhibiting DHFR, MTX diminishes the availability of dNTPs within the cell, and disrupts both the replication and repair of DNA. This leads to the accumulation of DNA damage in rapidly dividing cancer cells. However, this same mechanism also impairs the DNA repair capability in normal, healthy cells. This impairment occurs directly or indirectly because of the drug's action due to a phenomenon known as the 'bystander effect', where cells adjacent to the affected cells also experience damage. It is not clear whether the low doses of MTX, as used by patients with RA, also cause a similar inhibition of DHFR and lead to increased DNA damage. Despite these effects, it is important to note the limitations of MTX. High doses of MTX can lead to resistance in cancer cells, and make the drug less effective. Moreover, these high doses can cause toxicity in normal cells, lead to folate deficiency, and further interfere with DNA damage repair. Such complications can result in systemic toxicity, which poses challenges for the use of MTX in clinical settings (Bertino, 1981).

This chapter aimed to investigate whether low doses of methotrexate (MTX), induce DNA damage or related phenotypes *in vivo* using *Drosophila melanogaster* as a model organism. Given the well-established effects of high-dose MTX on DNA replication and repair, and the uncertainty surrounding the long-term consequences of low-dose exposure, a reliable *in vivo* assay for DNA damage assessment was required. To address this, a DNA damage reporter model was developed and validated in

Drosophila using GFP-based detection systems and phenotypic markers. Various transgenic stocks and genetic systems, including the multiple wing hair (*mwh*) and serrate wing phenotypes, were employed to evaluate the genotoxic potential of low-dose MTX. In addition, key physiological outcomes relevant to ageing and toxicity, such as lifespan, climbing ability, fecundity, and eclosion, were examined. Comparative experiments with hydroxyurea (HU), a known DNA synthesis inhibitor, were also conducted to distinguish MTX-specific effects from general replication stress. These investigations were intended to provide a comprehensive *in vivo* assessment of the potential DNA-damaging and physiological effects of long-term low-dose MTX exposure.

3.2 Results

3.2.1 Development of an *in vivo* DNA damage reporter model

In the first part of this study, a *Drosophila* model to report the levels of *in vivo* DNA damage was developed. This reporter is based on the visibility of red (i.e., *white*+) pigmented regions within a fly eye that is otherwise phenotypically white (*white*-) (Chen et al., 2009). The adult eye consists of around 800 ommatidial subunits, and even small white+ clones (estimated at just 1-2 ommatidia in size) can be readily identified visually under a stereomicroscope, which provides a way to rapidly screen for around 1600 potential mutation events per fly.

In this assay, a promoter based on a Glass Multimerised Response element (GMR) drives the expression of *Gal4*, which is strongly expressed throughout the cells posterior to the morphogenetic furrow in the developing imaginal disc, with expression subsequently maintained in the adult eye (Moses and Rubin, 1991). This drives the yeast *Gal4* transcription factor (Brand and Perrimon, 1993). When expressed in these cells, Gal4 binds to an Upstream Activation Sequence (UAS) located upstream of a gene encoding an mRNA designed to fold into a hairpin structure (Piccin et al., 2001). This hairpin structure is digested into 21 bp RNAi molecules by Dicer, and the RNAi molecules subsequently bind to the RNA Inducing Silencing Complex (RISC) to induce an *in vivo* RNAi response. In this assay, the siRNAs from the hairpin mRNA have been designed to target mRNA from the *white* gene ("Vienna *Drosophila* Resource

Center," n.d.), and thus reduce the levels of an enzyme required for the production of red eye colour pigment. The two transgenes described above have been recombined together on the second chromosome and are present in a *white+* genetic background to generate a stock that is:

w+; p[w+, GMR-Gal4], p[w+,UAS-white RNAi] / CyO.

In this stock, knockdown of the mRNA encoding the *white* gene by the *GMR-Gal4, UAS-white RNAi* cassette reduces the levels of White protein, and hence pigment levels, to a level where only a very light-yellow colouration is visible (Figure 14A).

If the DNA sequences encoding the *GMR, Gal4*, *UAS,* or the *RNAi* are damaged, or possibly when dominant negative mutations in the cellular RNAi machinery occur, then *white* mRNA is no longer degraded, and the resulting cell is able to make red pigment. If the damage occurs during growth of the eye imaginal disc, the cell is likely to divide a number of times to produce a group of clonally related cells, all of which express white protein. These clones form spots containing red pigment that are visible in the adult eye (Figure 14B). As such, the number of spots visible in each eye is presumed to be proportional to the number of incorrectly repaired DNA damage events that occurred within the *GMR, Gal4, UAS,* or *RNAi* genes inserted in the genome during development of the fly.

3.2.2 X-ray radiation induces DNA damage in *Drosophila melanogaster*

In order to test the *GMR-Gal4, UAS-white RNAi* mutation detection assay (hereafter, termed *GMR-white RNAi*), DNA damage was induced by irradiating *w+; GMR-white RNAi /* + larvae [generated by outcrossing *w+; GMR-Gal4, UAS-white RNAi / CyO* virgins to *OreR* wild type males] with different doses of X-rays (0, 25 Gy, 50 Gy, 100 Gy, and 200 Gy). It is worth mentioning that these doses are considerably high when compared to human radiation tolerance. Flies were irradiated with different doses of irradiation to establish the optimal dose of X-rays to induce mutations. After irradiation, larvae were allowed to develop and the number of white+ clones that developed in the eyes were scored for the adult male and female flies that subsequently emerged.

As shown in Figure 14, there was a dose-dependent increase in the number of red clones in both male and female flies. For the males, a significantly higher number of red clones were observed in flies irradiated with 100 Gy and 200 Gy compared with untreated controls ($p < 0.0001$). However, only female flies whose larvae were irradiated with 200 Gy showed a significantly higher number of red clones when compared with the control untreated flies ($p < 0.0001$). This finding suggests that the *GMR-white RNAi* reporter is able to indicate DNA damage *in vivo* and therefore represents a viable tool to investigate the effect of MTX in this system. While the reason for the difference in the results observed between the male and female flies is unknown, it is possible that this may be due to the difference in the physiological and biological make-up of the flies of different sexes.

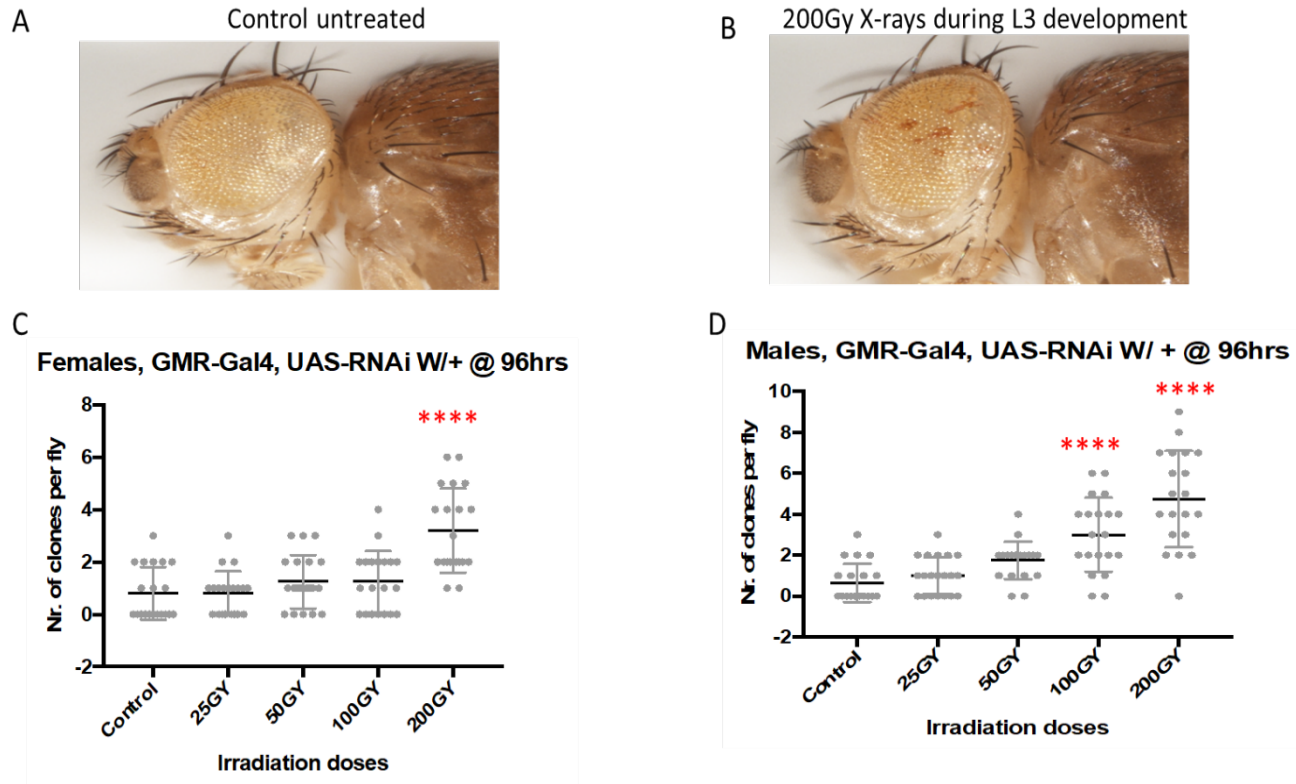


Figure 14. DNA damage assay: (A) Image of a control *GMR-Gal4, UAS-RNAi W/+* unirradiated fly lacking red pigmentation on the eye. (B) Image of *GMR-Gal4, UAS-RNAi W/+* flies irradiated with 200 G at the third instar larvae stage. Red clones are visible in the eye (C & D) Number of red clones following irradiation of *GMR-Gal4, UAS-RNAi W/+* (C) female and (D) male flies with various doses of X-rays. Data is presented as mean ± SD of number of clones per fly (n = 200). Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test; *p <0.05, **p<0.01 and ***p<0.001.

3.2.3 Effects of low-dose MTX on fecundity and larval development/eclosion

To study the toxicity of chemotherapeutic drugs, I tested the effects of MTX on fecundity and development. I therefore made standard fly food supplemented with different concentrations of MTX (1 nM, 10 nM, 100 nM, 1 μM, and 4 μM) and examined the effects on the ability of a 'near wild type' strain of flies commonly used in lifespan experiments - $w^{Dahomey}$ (Gubina et al., 2019) to lay eggs and for the resulting embryos to develop, grow through larval stages, pupate and eclose as adults.

In the first experiment, 100 male and 100 female flies in each bottle were fed with fly food containing different concentrations of MTX (1 nM, 10 nM, 100 nM, 1 μM, and 4 μM) for a total of 7 days to make sure the drug had been absorbed by the flies. The control group had no MTX supplied. The flies were moved to new fresh fly food regularly (Monday, Wednesday, and Friday). After that, the female flies were allowed to lay embryos overnight on a new fresh fly food bottle at 25°C, and then the number of embryos were counted within 20 h under microscope. The finding of this experiment was that a dose-dependent decrease in the number of embryos laid was observed (Figure 15A&C). A significant reduction in the number of embryos was observed for flies exposed to MTX concentrations ≥ 1 μM compared to the control untreated flies ($p < 0.0001$).

Therefore, I conducted a new similar experiment to support and confirm the previous results, with the only difference that the number of adults eclosed was counted for 5 consecutive days (Figure 15B). A large batch of embryos laid overnight by around 200 $w^{Dahomey}$ flies was collected and washed, and a 30 μL bed-volume of embryos was pipetted onto the surface of fly food supplemented with the indicated concentrations of MTX. Bottles were then incubated at 25°C for 11-12 days until the first adults began to eclose, then kept for a further 5 days to allow all viable pupae to hatch. Adult flies were counted and removed from the bottle at regular intervals. In this second experiment, a similar trend was observed as the previous experiments where development from embryo to adult eclosion took place growing on food containing MTX. Consequently, larvae which developed on food containing 1 μM MTX resulted in significantly fewer adults eclosing compared with the control untreated group (Figure 15B). Taken

together, this data suggests that low doses of MTX did not affect the number of adults eclosing or the overall fecundity of the flies in terms of producing embryos or development to adults.

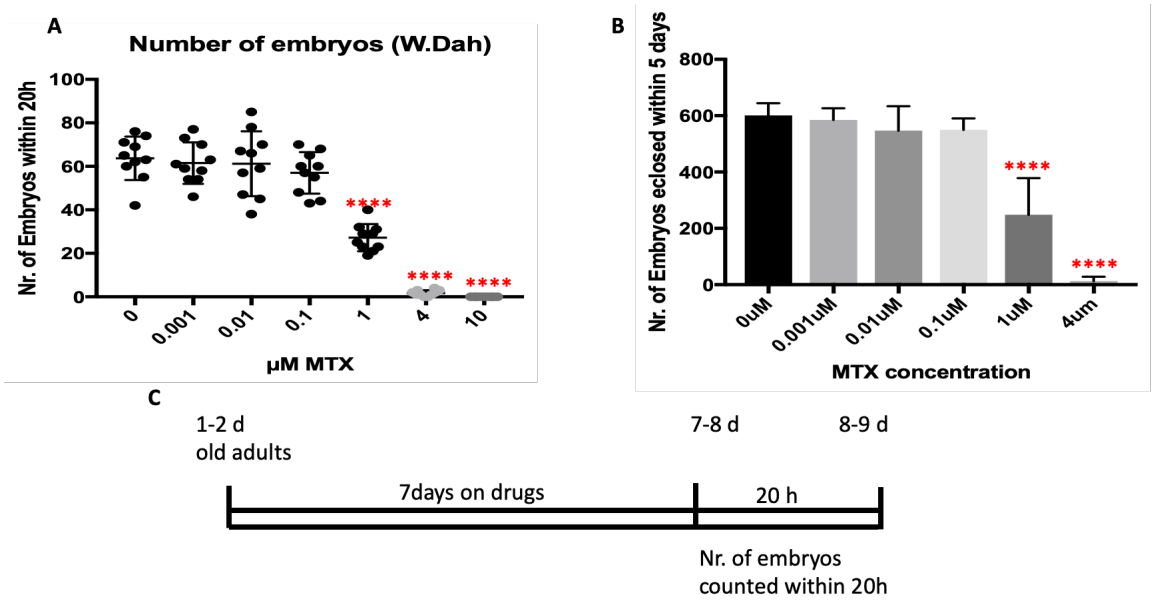


Figure 15. The effect of MTX on egg laying and development to adult stages in $w^{Dahomey}$. These graphs demonstrate the impact of MTX at different concentrations: A) on the number of synchronised eggs collected within 20 h after being on different drug concentrations for 7 days, B) the number of eggs that eclosed to adults within 5 consecutive days. C) Experimental timeline showing how the flies were exposed to MTX for 7 days. Data is presented as mean ± SD of number of flies. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test; *p <0.05, **p<0.01 and ***p<0.001.

3.2.4 MTX, DNA damage and serrated wings

Combination therapy of chemotherapy agents (such as MTX) and radiation is a widely employed treatment approach for cancer (Mokhtari et al., 2017). When used together, these two therapies can complement each other's mechanisms of action to more effectively target cancer cells (Bhatia et al., 2020), although combination therapy may possibly result in an increased risk of recurrence. Simultaneously, the use of low-dose MTX in large populations of patients with long-term chronic

inflammatory disease may also impose a mutational burden on these patients, with potential implications for the development of cancers, ageing etc.

I investigated the effect of different concentrations of MTX in terms of inducing DNA damage or serrated wings in combination with or without X-ray radiation in two separate experiments (Figure 16). The first experiment investigated the effects of various MTX concentrations (Figure 16C&D); 200 *w+; GMR-Gal4, UAS-white RNAi / +* larvae [generated by outcrossing *w+; GMR-Gal4,UAS-white RNAi / CyO* virgins to OreR wild type males] were placed on fly food for two weeks with different concentrations of MTX (1 nM, 10 nM, 100 nM, 1 μM, and 4 μM). In the second experiment, the first experiment was repeated with 400 flies per each concentration, and the third instar larva were irradiated with 200 Gy (Figure 16E&F).

In flies that were only treated with MTX, low concentrations of MTX up to 100 nM did not induce significant levels of DNA damage. However, 1 μM MTX resulted in a significant increase in DNA damage compared to the control untreated group ($p < 0.05$). Unexpectedly, 4 μM MTX alone resulted in a non-significant increase in DNA damage. This observation was probably due to the inability of the flies to withstand the high dose of MTX, as a high level of fly death was found and impacted the number of flies available for DNA damage scoring. In flies exposed to MTX in combination with X-ray radiation, low doses of MTX up to 1 μM did not induce a significant level of DNA damage (Figure 16E&F). However, in combination with 200 Gy, MTX > 4 μM induced significantly higher levels of DNA damage compared with the control group ($p < 0.05$).

The serrated or notched wing phenotype in *Drosophila melanogaster* is a widely recognised indicator of developmental disruption, often arising from perturbations in apoptosis, DNA damage responses, or improper cell proliferation during wing imaginal disc development (Ryoo et al., 2004; Perez-Garijo et al., 2009). Genotoxic stress, such as that induced by radiation or chemotherapeutic agents, can trigger apoptotic signalling cascades in wing disc cells, leading to improper tissue morphogenesis and visible structural abnormalities such as serrated or notched wings. In this study, the serrated wing phenotype was used as a morphological marker of potential DNA damage. Adult wings were scored visually post-

eclosion using stereomicroscopy, and flies were classified as exhibiting serration based on the presence of jagged or uneven edges along the wing margin, in contrast to the smooth appearance seen in untreated controls. The frequency of this phenotype was quantified across treatment groups to evaluate the mutagenic potential of MTX and/or radiation exposure.

Morphologically, flies exposed to MTX concentrations up to 100 nM did not show any visible changes in wing morphology. However, when the MTX concentration was increased to 1 μM or above, significantly higher numbers of flies exhibited serrated wings ($p<0.001$; Figure 16B). When flies were exposed to a combination of X-ray radiation and MTX, low doses of MTX up to 100 nM did not induce significant changes in wing morphology compared with the control fly group, which was consistent with the lack of significant DNA damage observed in this condition. However, increasing the MTX concentration to 1 μM or above resulted in significantly higher number of flies exhibiting serrated wings ($p<0.05$). Taken together, this data suggests that low-dose MTX did not induce DNA damage and did not impact repair of the DNA damage caused by exposure to 200 Gy radiation.

Next, a comprehensive study was conducted to understand the effects of MTX on DNA damage and wing morphology in flies, two distinct experiments were conducted. In the initial experiment, 200 flies of the genotype *w+; GMR-Gal4, UAS-white RNAi / +*, which were generated by outcrossing *w+; GMR-Gal4, UAS-white RNAi/CyO* virgins to OreR wild type males, were subjected to varying MTX concentrations (1 nM, 10 nM, 100 nM, 1 μM, and 4 μM) (Figure 16C&D). The findings revealed that while MTX concentrations up to 100 nM did not induce significant DNA damage, a concentration of 1 μM resulted in a marked increase in DNA damage compared to the untreated group ($p < 0.05$). Interestingly, the 4 μM concentration, despite being higher, showed a non-significant increase in DNA damage, a phenomenon potentially attributed to the elevated mortality rates at this concentration. In terms of wing morphology, only concentrations of 1 μM and above led to a significant number of flies exhibiting serrated wings ($p<0.001$).

To build on these findings, a subsequent experiment was conducted with a larger sample of 400 flies for each MTX concentration. These flies were then exposed to an additional variable: irradiation with

200 Gy (Figure 16E&F). In this combined setting, MTX concentrations up to 1 μM, even in the presence of X-ray radiation, did not show significant DNA damage. However, when combined with 200 Gy radiation, MTX concentrations greater than 4 μM resulted in significantly elevated levels of DNA damage compared to the control group ($p < 0.05$). The wing morphology findings mirrored the DNA damage results, with MTX up to 100 nM having no significant impact, but concentrations of 1 μM and above, when combined with radiation, leading to a significant number of flies with serrated wings ($P<0.05$).

Collectively, these experiments suggest that low doses of MTX, in isolation, do not significantly compromise DNA integrity. Moreover, even when subjected to substantial radiation, these low MTX doses do not appear to hinder the DNA repair mechanisms, emphasizing the potential resilience of these mechanisms in the presence of low MTX concentrations.

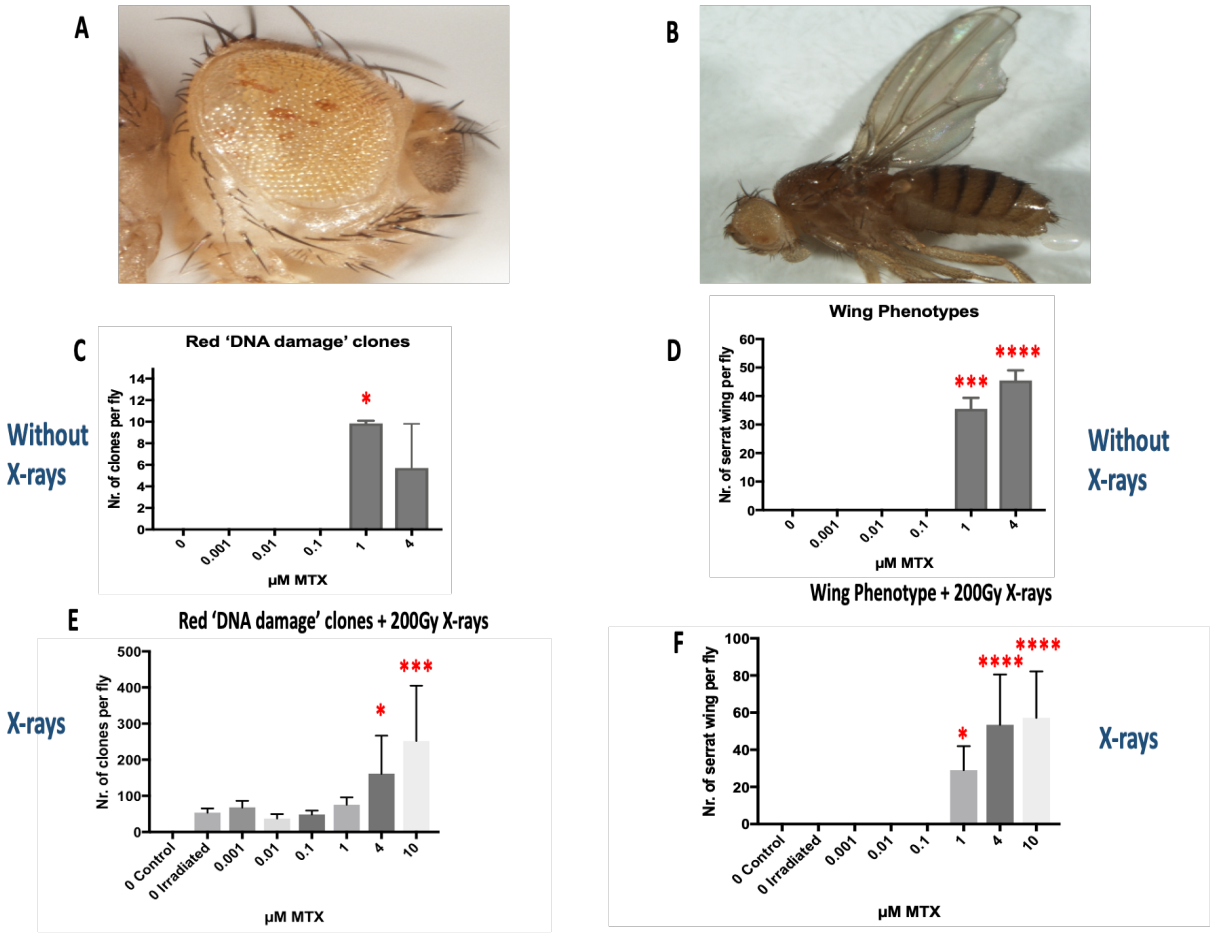


Figure 16. Phenotypic changes induced by MTX: A) Photograph of a mutated eye of *Drosophila melanogaster w+; GMR-Gal4, UAS-white RNAi / +* with multiple red clones. B) Photograph of a mutated notch wing phenotype (i.e., a serrated wing phenotype). (C) Numbers of red clones per group of 200 flies after treatment with various doses of MTX in the absence of X-ray irradiation. D) Number of notched wings per group of 200 flies after treatment with various doses of MTX in the absence of X-ray irradiation. (E) Numbers of red clones per group of 400 flies after treatment with various doses of MTX after X-ray irradiation at 200 Gy. (F) Number of notched wings per group of 400 flies after treatment with various doses of MTX and X-ray irradiation at 200 Gy. The data is depicted as the mean number of flies ± standard deviation (SD). Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test; *p <0.05, **p<0.01 and ***p<0.001.

3.2.5 Low-dose MTX did not cause loss of heterozygosity (LOH) of the *multiple wing hair* (*mwh*)

The *multiple wing hair* (*mwh*) gene in *Drosophila melanogaster* is a known model for studying developmental biology and genetics, especially human hazard development studies (Pitchakarn et al., 2021). To more deeply investigate the genetic effects of MTX on *Drosophila*, a focused analysis was conducted to evaluate the loss of heterozygosity (LOH) in the *mwh* gene, which is associated with the manifestation of the multiple wing hair phenotype.

To understand the rationale behind the experiment investigating the loss of heterozygosity (LOH) at the *mwh* locus in *Drosophila melanogaster*, it is crucial to understand the genetic implications of MTX exposure in this model organism. LOH is the loss of the normal function of one allele of a gene in which the other allele was already inactivated. This phenomenon is particularly significant in cancer biology, as it may lead to the expression of harmful traits that are normally suppressed by the wild type allele. In *Drosophila*, the *mwh* gene serves as an excellent proxy for studying mutagenic events that could be analogous to oncogenic processes in humans due to its visible phenotypic manifestations, namely the formation of multiple wing hairs from a single cell. In this study, LOH at the *mwh* locus specifically refers to the genetic and phenotypic changes induced by MTX that result in the visible manifestation of multiple hairs per cell, which suggests a mutagenic process removed the suppression normally imposed by the wild type allele.

The experiment's design involved treating flies with various concentrations of MTX and subsequently exposing the files to radiation to enhance the mutagenic environment, and thereby increase the probability of observing LOH. Varied concentrations of MTX were tested to determine the threshold at which MTX begins to have a measurable effect on the genetic stability of cells. Observations of increased multiple wing hair occurrences at higher MTX concentrations would provide evidence of induced LOH.

The experimental design involved growing *mwh* flies on varying concentrations of MTX, specifically 0.001 μM, 0.01 μM, 0.1 μM, and 1 μM. After that, they were irradiated with 100 Gy at the third instar larva stage. Additionally, control flies were grown without MTX and not subjected to X-ray exposure, to serve as a negative control. The results in Figure 14 showed that both 100 and 200 Gy produced significant effects. However, for these experiments, the dose was dropped to 100 Gy as Figure 14 indicted 200 Gy caused very high levels of cell death. Post-exposure, the wings of the flies were mounted on cover slides and then analysed using a microscope to assess the presence of multiple wing hairs.

As depicted in Figure 17, flies exposed to MTX concentrations up to 10 nM did not exhibit a significant LOH in the *mwh* gene, as evidenced by the absence of extra hair growth compared with the control unexposed flies. However, flies exposed to MTX concentrations of 100 nM and above displayed a pronounced increase in the rate of LOH in the *mwh* gene, as indicated by a higher incidence of extra hair growth, distinctly visible as red patches in Figure 17A ($p < 0.05$). This data indicated that while low doses of MTX did not induce the *mwh* phenotype in the irradiated flies, higher concentrations of MTX lead to a significant increase in the multiple wing hair phenotype in the flies.

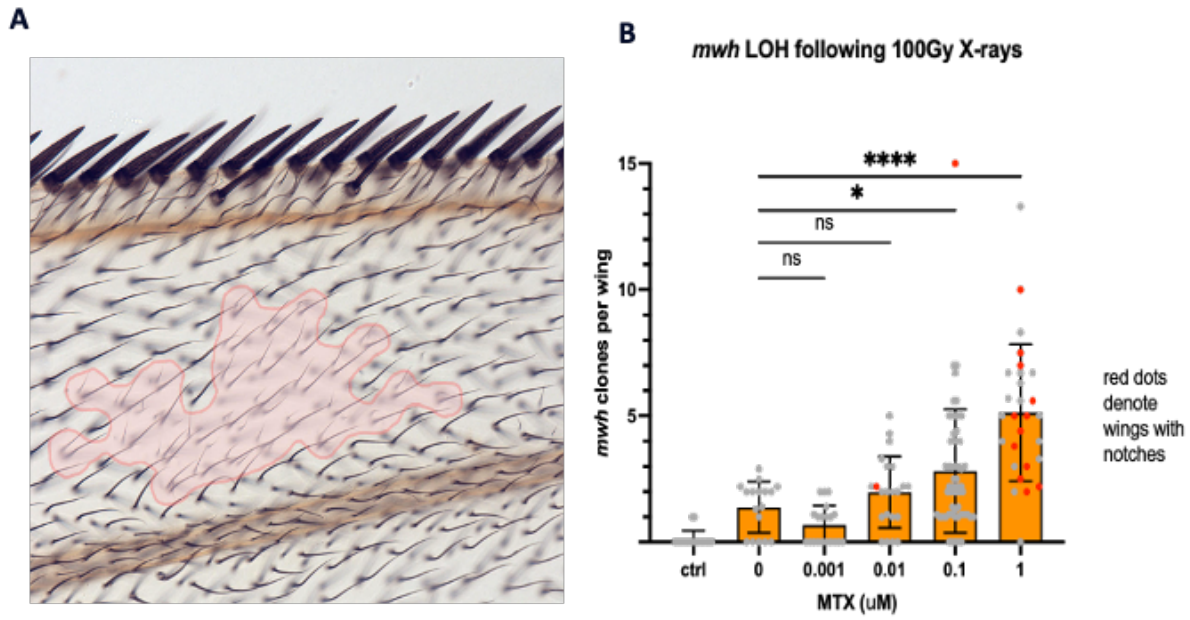


Figure 17. High-dose MTX leads to LOH of the *mwh* gene: Flies grown on food containing the indicated concentrations of MTX and exposed to 100 Gy of X-ray irradiation were evaluated for the presence of clones of cells displaying the *mwh* phenotype. Control flies were grown without MTX and not subjected to X-ray exposure. (A) The region outlined in red indicates a clone in the adult wing containing a *mwh* mutant clone characterised by multiple wing hairs produced by each cell. (B) Number of *mwh* clones per wing in flies grown on food containing the indicated MTX concentrations. Each dot represents a single wing, with grey dots indicating normal morphology and red dots indicating wings with serrated phenotypes, as illustrated in (B). Data is presented as mean ± SD of number of flies. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test; *p <0.05, **p<0.01 and ***p<0.001.

3.2.6 Hydroxyurea (HU) does not induce DNA damage or inhibit eclosion at any doses tested

Hydroxyurea (HU) is a medication that is commonly used for cancer treatment, particularly for myeloproliferative neoplasms (MPNs), chronic myeloid leukaemia (CML), and sickle cell anaemia (Al-Amleh et al., 2022). HU was selected to explore its influence on DNA integrity and developmental processes in *Drosophila melanogaster*, and serve as a comparative agent to MTX. As a parallel to the mechanism of MTX, which inhibits folate pathway metabolism to reduce dNTP levels, HU inhibits the activity of ribonucleoside diphosphate reductase (RNR), an enzyme required for the synthesis of

nucleotides, which are essential for DNA synthesis and repair (Krakoff et al., 1968). HU is well-documented to reduce the available nucleotide pools, and thus provides a model to examine the impacts of nucleotide depletion in biological systems (Koç et al., 2004). Specifically, these experiments aimed to assess whether such inhibition leads to observable phenotypic consequences under controlled experimental conditions.

The effect of HU on the induction of DNA damage was investigated using the *GMR-white RNAi* assay and wing development and eclosion were measured. HU at low concentrations up to 100 μM did not significantly induce DNA damage and did not significantly impact the physiology of the flies with respect to wing phenotype or eclosion from pupa to adult compared with the unexposed controls (Figure 18). However, 1000 μM HU induced DNA damage, as indicated by red clones, but this effect was not significant compared to the control. It was also observed that 10000 μM HU resulted in no flies surviving to count. This is possibly due to the toxicity of the high concentration of HU. Similarly to low dose MTX, low-dose HU did not induce serration of the wings (data not shown).

HU targets nucleotide biosynthesis. In contrast, MTX affects both nucleotide biosynthesis and the JAK/STAT signalling pathway, and potentially other mechanisms. By comparing the effects of MTX to those of HU, we can distinguish whether the observed effects of MTX are primarily attributable to nucleotide depletion or to its impact on the JAK/STAT pathway. This comparison provides valuable insight into the underlying mechanisms of MTX activity.

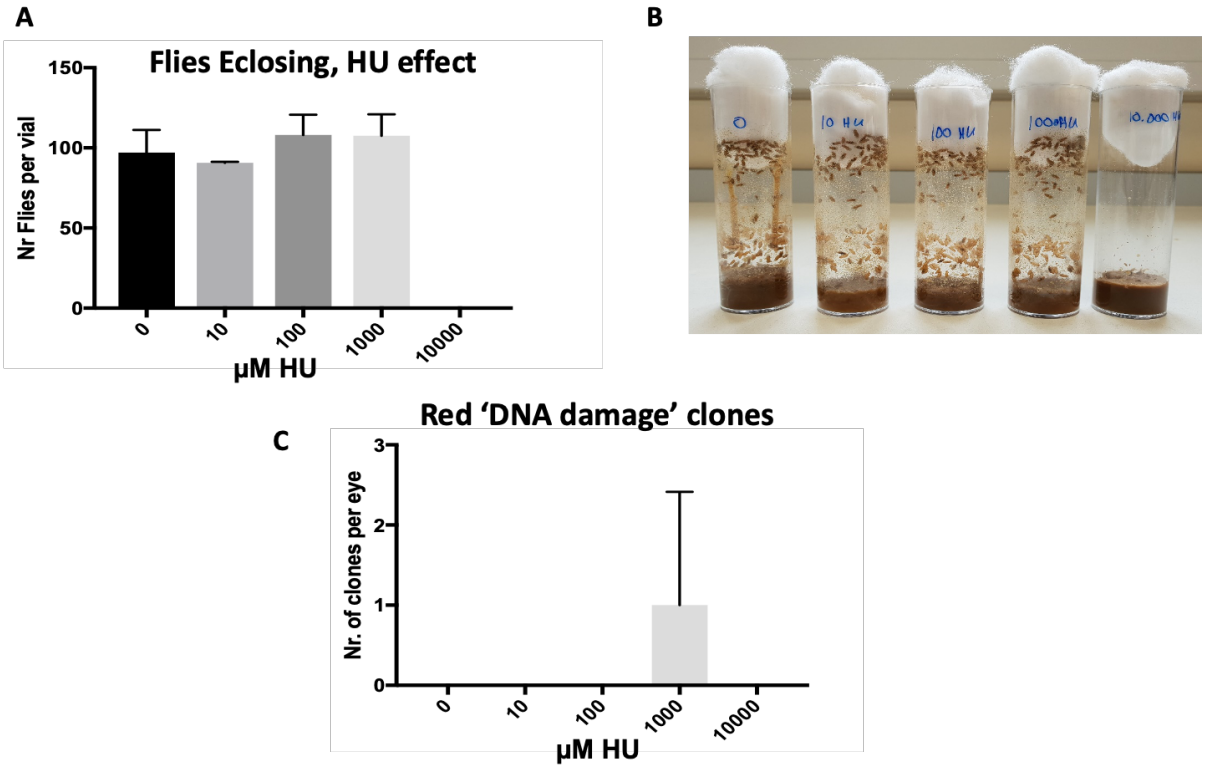


Figure 18. Effects of hydroxyl-urea *w+; GMR-Gal4, UAS-white RNAi / +* in the absence of X-ray irradiation: A) Count of flies that eclosed (n=200). At 10,000 µM HU, none of the flies survived to reach the eclosion stage. B) Photograph of the actual number of surviving flies for each concentration of HU. (C) Number of red clones per eye in flies treated with HU (n=200). Importantly, no alterations were observed in the wing phenotype. The data is depicted as the mean number of flies ± standard deviation (SD). Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test.

3.2.7 Low-dose MTX extends the lifespan of *Drosophila*

The results above indicated low doses of MTX (less than 100 nM) do not induce detectable DNA damage in a discrete number of loci in the *Drosophila melanogaster* genome, as they did not impact the wing phenotype nor inhibit eclosion of embryos. Thus, the possible toxic side effects of MTX were further evaluated by investigating the lifespan of female flies. Flies (n=200/concentration) were exposed to MTX at different concentrations and the average lifespan of the flies was recorded post-exposure (Figure 19A). High concentrations of MTX between 1 and 10 µM significantly reduced the lifespan of

the flies compared with the control flies ($p<0.01$), which is consistent with the previous survival and fecundity experiments (Figure 19A). On the contrary, the low concentrations of 10 and 100 nM MTX significantly increased the lifespan of the flies compared to the control untreated flies ($p < 0.05$).

In order to confirm this important result, this experiment was repeated to provide a completely independent experimental replicate. To increase the statistical power of the experiment, 400 adult flies were tested at only the low doses of MTX. Again, 1 and 100 nM MTX significantly increased the lifespan of the exposed flies compared to unexposed flies ($p < 0.001$; Figure 19B). These findings represent the most pivotal and captivating outcomes throughout the entire project. Their significance not only underscores the core essence of the research but also illuminates potential avenues for further exploration. This suggests that, unlike the high dose, low-dose MTX improved the survival of the flies by increasing their lifespan.

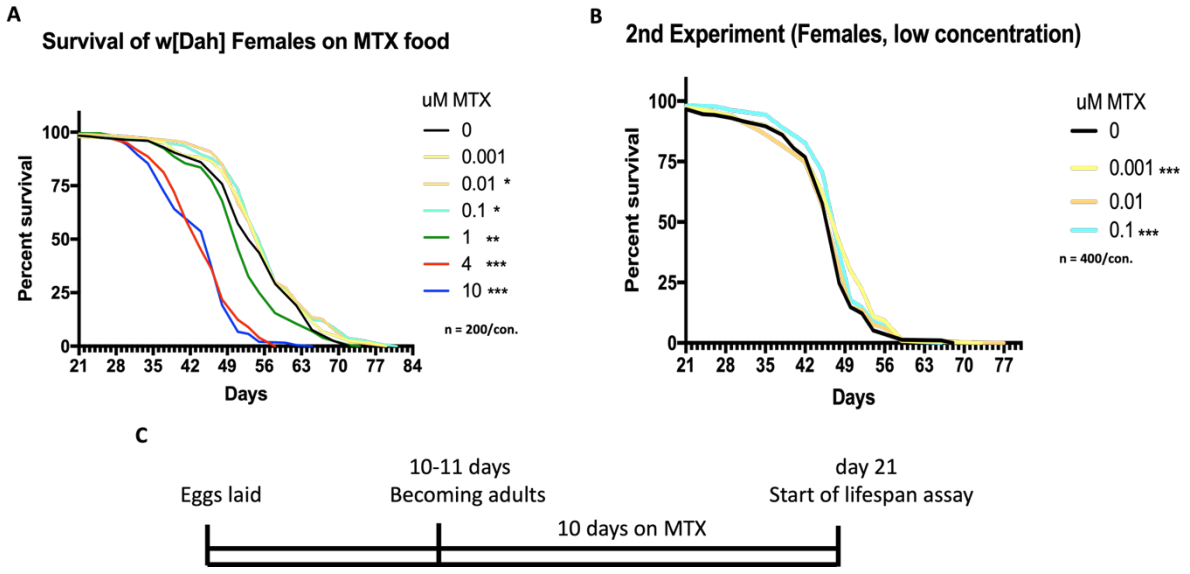


Figure 19. Effects of MTX on the lifespan of female *Drosophila*. The lifespan of females *w[Dah] Drosophila* flies fed different doses of MTX was monitored for ten days post eclosion before lifespan monitoring began. Control flies were not fed MTX. Consequently, the Y-axis begins at day 21, corresponding to the timepoint post eggs being laid. (A) First experiment (n = 200 flies per condition); (B) second experiment (n = 400 flies per condition): (C) Experimental timeline shows how the flies were exposed to MTX. The median lifespan of both experiments has been provided in Appendix E. Data were

analysed using comparison of survival curves using GraphPad Prism 9 and the log-rank (Mantel-Cox) test; *p <0.05, **p<0.01 and ***p<0.001.

3.2.8 Low-dose MTX may increase the climbing ability of *Drosophila*

During the lifespan experiment described above, it was noted that older flies kept on food containing low doses of MTX appeared to be more active than flies on control vials with standard food. Old control flies often stood stationary on the food for long periods, while MTX-treated flies were still actively moving and climbing the sides of the vial. In order to confirm this qualitative observation, experiments were performed to quantitatively measure climbing ability.

The influence of biological differences between male and female *Drosophila* can significantly impact experimental outcomes. Different studies demonstrated these fundamental differences in the biology of male and female *Drosophila* (Magwere et al., 2004). Given this background, an experimental trial was carried out to investigate the difference in motor activities of $w^{Dahomey}$ male and female flies by assessing the climbing of male and female flies without exposure to drugs. As described in the methods chapter, this experimental design used large pools of mixed sex fly populations kept in bottles that were regularly changed and incubated at 25°C. In preparation for a climbing test, groups of 50 randomly selected flies of each sex were removed from the mixed population, placed in vials, and moved into the research lab for at least 2 hours to allow the flies to acclimatise to the lab temperature and recover from CO_2 anaesthesia. The climbing test consisted of a 25 mL stripette held vertically in a clamp and closed with foam at each end. Groups of 10 flies were added to the stripette which was dropped/tapped onto the bench to knock the flies to the bottom. After 10 seconds, a photograph was taken against a white background. This test was repeated three times for each group, for five groups of 10 for each sex. Pictures were then evaluated manually to count the height climbed by individual flies.

The heights climbed over 10 s by each group of flies in weeks 1 through 7 are plotted in Figure 20. Male flies consistently climbed significantly higher than females ($p < 0.05$). The males climbed highest in week 2, to 16.5 cm (which translates to 1.65 cm/s); the females also climbed highest in week 2, to 11 cm (1.1 cm/s). The male flies climbed an average of 33% higher than the females across the weeks

under investigation. This confirms the physiological difference between the sexes which may confound experimental outcomes on lifespan. Interestingly, even at 7 weeks post-eclosion, both male and female flies continued to climb to very similar heights as their peak climbs during week 2. It is unclear why this is the case, as our observation during the lifetime experiments suggested that old flies move less.

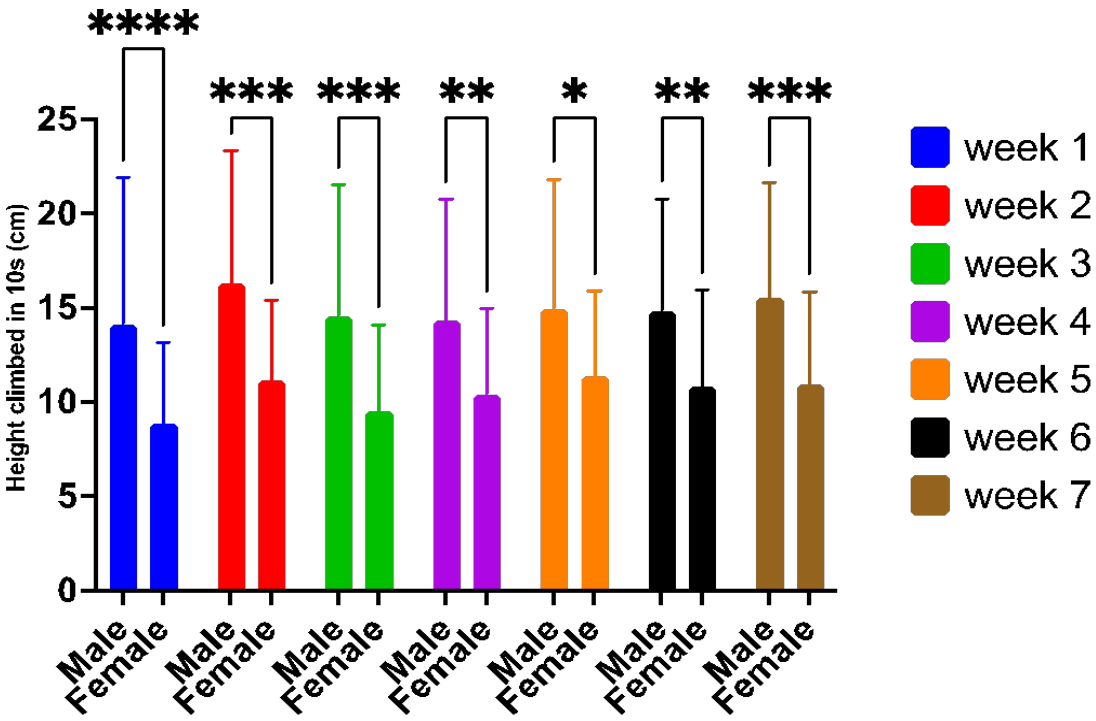


Figure 20. Climbing rate of male and female *Drosophila* flies over a 7-week period. Flies were placed in cylindrical vessels and the height climbed was evaluated each week for a 7-week period. Data is presented as mean height climbed ± SD for n = 50 flies. Data showed male flies climbed higher than females for all weeks under investigation. Statistical significance was tested by Two-way ANOVA, followed by post-hoc assessments using Sidak's multiple comparison tests; *p <0.05, **p<0.01 and ***p<0.001.

To investigate the influence of MTX on climbing behaviour, newly eclosed male flies were grown on food containing 1 nM, 10 nM, or 100 nM MTX for a 7-week period. As before, groups of flies were then tested for their climbing ability at weekly intervals and the results are shown in Figure 21. Although there was no difference in the heights climbed during the first three weeks, the flies exposed to 100 nM

MTX showed a significant increase in height climbed from week 4 through week 7 compared to the control unexposed groups (Figure 21). The flies exposed to 100 nM MTX climbed an average of 17.4 cm (1.74 cm/s) compared to 15 cm (1.5 cm/s) for the control group. However, the flies exposed to 1 nM and 10 nM MTX groups showed no difference in height climbed compared to the control unexposed groups. These findings suggest that MTX at 100 nM seems to enhance the locomotor activity of the male flies.

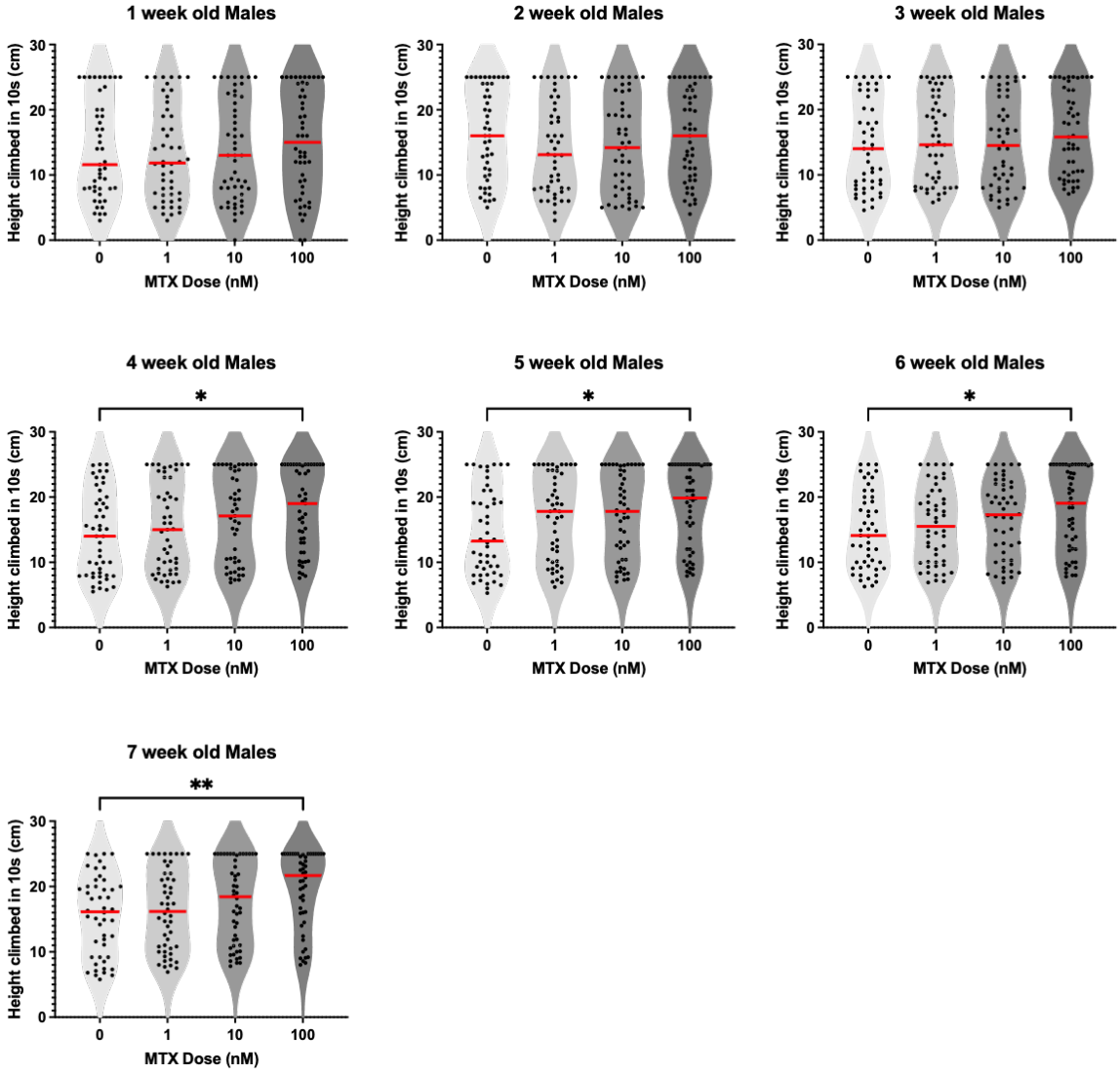


Figure 21. Climbing rate of male *Drosophila* over a 7-week period after exposure to MTX. Flies were fed MTX (1 nM, 10 nM, or 100 nM) containing fly food and were kept in cylindrical vessels. Height climbed after 1 to 7 weeks post exposure to MTX was analysed. Data is presented as mean height climbed ± SD of n

= 50 flies. Data showed the male flies exposed to 100 nM MTX climbed significantly higher than control flies from week 4 through week 7. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test; *p <0.05 and **p<0.01.

Similar experiments were also conducted for female flies exposed to low concentrations of MTX (1 nM, 10 nM, and 100 nM) and the climbing activities of the flies were observed from week 1 to week 7. This experiment showed that there was no significant difference in the climbing height of the flies over the 7-week period, even though a steady time dependent-increase in distance climbed was observed for each concentration range (Figure 22). The reason for the lack of a significant difference in climbing is females is unclear, especially since the increased lifespan observed in females suggests that both sexes can be affected by the drug. However, at week 7, the mean values for females treated with 0 nM and 100 nM MTX tended to be higher, even though the differences were not statistically significant.

Flies were separated based on sex to eliminate the confounding effect of the male's ability to climb farther than females if they are compared together when exposed to MTX. This separation allowed for a more accurate assessment of the impact of low-dose MTX exposure on motor activity. The aim was to determine whether MTX influences locomotion, such as hypokinesia or reduced climbing ability, as a potential contributing factor to changes in lifespan, without sex acting as a confounding variable.

Despite identifying an increase in the climbing ability of flies treated with MTX, one significant weakness of this experimental design was only fully recognised after the experiments had been concluded. This issue is related to the 25 cm height of the stripette which limited the maximum height individual flies could climb. As such, flies photographed at the top of the stripette after 10 s could potentially have climbed further if they had had more space. As such the mean height climbed is likely to be an underestimate of the potential climbing that would have happened in a longer apparatus. Similarly, the standard deviation of the datasets that include many flies at 25 cm is likely to be smaller than would have been the case had the fast climbers been unrestricted, which may also have changed the significance of the differences between the groups.

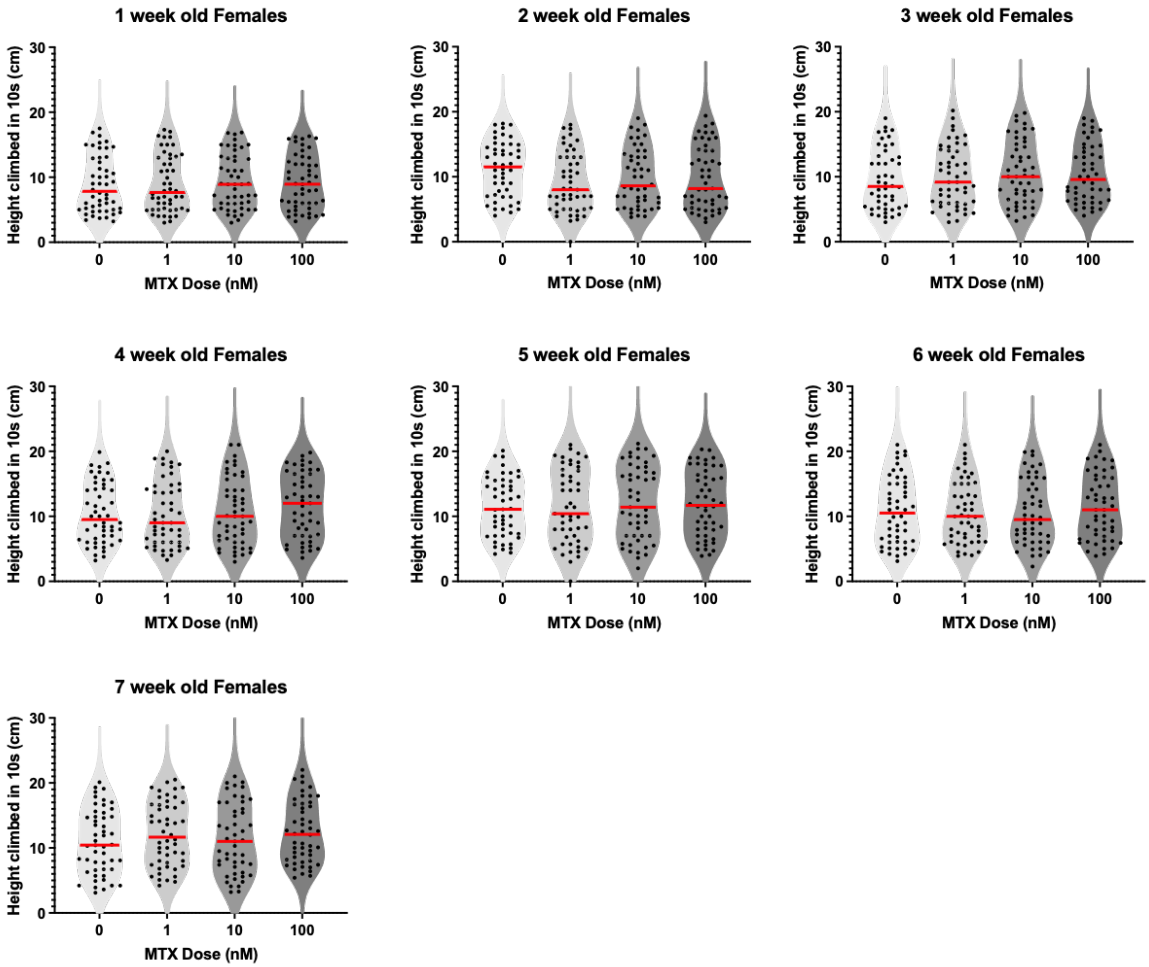


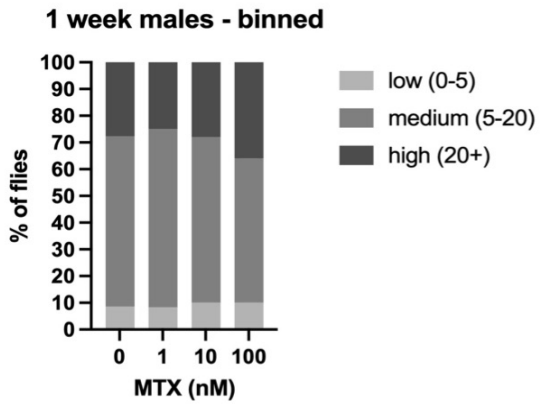
Figure 22. Climbing rate of female *Drosophila* over a 7-week period after exposure to MTX. Flies were fed with MTX (1 nM, 10 nM, and 100 nM) containing fly food and were kept in cylindrical vessels. Height climbed after 1 to 7 weeks post exposure to MTX was analysed Data is presented as mean height climbed ± SD of n = 50 flies. The female flies similar climbed heights during all weeks under investigation. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test.

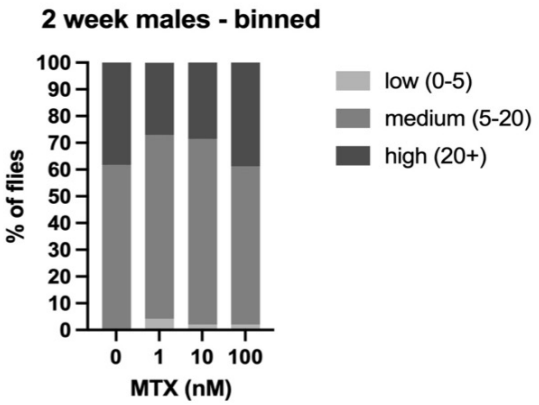
Statistical analysis of the average climbing ability showed that there were no significant changes in the climbing performance of female flies across the different MTX concentrations. As a result, I therefore focused on male flies, where a more pronounced effect was observed. The climbing data generated by male flies is particularly interesting because at higher doses of MTX, the flies tend to climb better than at the beginning of the experiment, indicating that their climbing ability improves as they age; this result is not necessarily what would be expected. In order to better visualise and understand the male-specific data, and in the light of the 25 cm climbing restriction outlined above, I replotted the raw data, binning

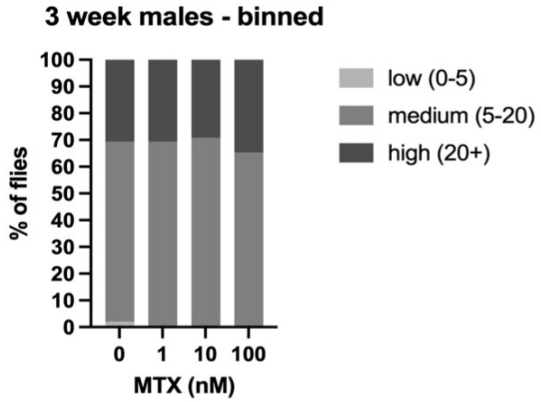
each individual into one of three groups: low climbers which climbed less than 5 cm, medium climbers which climbed between 5 and 20 cm, and high climbers which climbed more than 20 cm in 10 seconds.

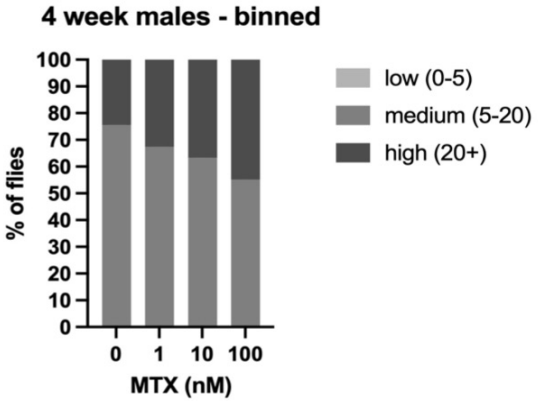
This new analysis provided a clearer visualization of the dose-dependent improvement in male climbing over the 7-week period (Figure 23) as it showed that the control group's climbing ability remained relatively constant with a slight increase over time. In contrast, the MTX-treated groups, particularly at the 100 nM concentration, showed a progressive improvement in climbing performance over time. Starting from week 4, there was a marked increase in the proportion of high climbers in the 100 nM MTX group, with over 50% achieving high climbing status by week 7. Concurrently, after week 4, no flies were classified as low climbers. Moreover, all older flies climbed more than 5 cm across all drug-treated groups, indicating that ageing male flies continue to be able to climb, even in old age. This graphical representation more clearly revealed a dose-response relationship between MTX and climbing performance, especially at the higher doses of MTX.

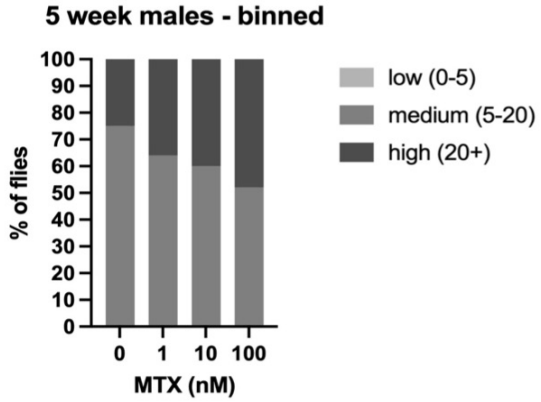
In contrast, the majority of previous studies that assessed climbing did not observe differences in climbing with age (Gargano et al., 2005; Simon et al., 2006; Martinez et al., 2007). Most of these experiments used spontaneous climbing assays, in which climbing ability is assessed under more natural conditions using a laser. In contrast, the experiments in this thesis were conducted by inducing a climbing response by tapping the stripette on the table. This may have induced a shock response that led to different behaviour, even in older flies, compared to the spontaneous assays. Moreover, the flies were exposed to CO_2 for sorting by sex before the experiments, which may have affected the results. Taken together, these methodological differences may explain why the data here are inconsistent with the literature and suggest that the results should be treated with caution.

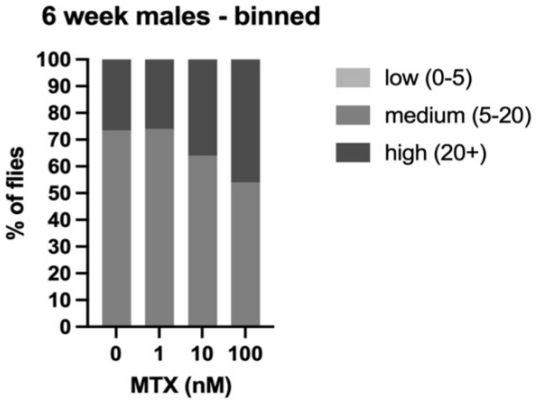

1 week males - binned
% of flies
MTX (nM)
0
1
10
100
low (0-5)
medium (5-20)
high (20+)


2 week males - binned
% of flies
MTX (nM)
low (0-5)
medium (5-20)
high (20+)


3 week males - binned
% of flies
MTX (nM)
low (0-5)
medium (5-20)
high (20+)


4 week males - binned
% of flies
MTX (nM)
low (0-5)
medium (5-20)
high (20+)


5 week males - binned
% of flies
MTX (nM)
low (0-5)
medium (5-20)
high (20+)


6 week males - binned
% of flies
MTX (nM)
low (0-5)
medium (5-20)
high (20+)

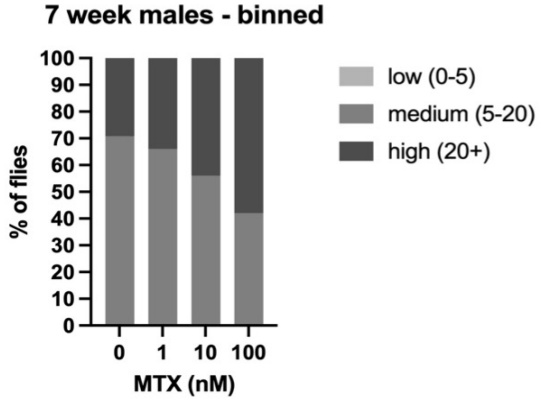

7 week males - binned
% of flies
MTX (nM)
low (0-5)
medium (5-20)
high (20+)

Figure 23: Climbing performance categorization of male *Drosophila* over a 7-week period after exposure to MTX. Flies were fed with MTX-containing food (1 nM, 10 nM, and 100 nM) and kept in a cylindrical vessel. The climbing height of each fly was measured weekly for 7 weeks and categorized into three groups: low climbers (0–5 cm, light grey), medium climbers (6–19 cm, medium grey), and high climbers (≥20 cm, dark grey). The data show a dose-dependent increase in the proportion of high climbers, particularly in the 100 nM MTX group, starting from week 4. The control group maintained a relatively consistent climbing performance with only a slight increase in climbing ability over time, while the MTX-treated groups showed a progressive improvement in climbing ability over time. By week 7, over 50% of flies in the 100 nM group were categorized as high climbers, with a concurrent reduction in low climbers. Data are presented as the percentage of flies in each climbing category for each week.

3.3 Discussion

The major conclusion finding from this chapter is that administering low doses of MTX does not lead to DNA damage in flies. More intriguingly, low doses of MTX seem to have a positive impact and potentially enhance both healthspan and overall lifespan. This finding suggests that MTX, when given in controlled amounts, might offer benefits beyond its traditional applications, and could open avenues for further research into its potential as a longevity-enhancing agent.

In this study, I evaluated the impact of low dose MTX with or without irradiation on the induction of DNA damage, eclosion, and serrated wing formation in *Drosophila melanogaster*. *Drosophila melanogaster*, also known as the fruit fly, is commonly used as a model organism in cancer research, which includes investigations of DNA damage and repair (Mirzoyan et al., 2019). We showed here that low-dose MTX up to 100 nM did not induce significant DNA damage, negatively influence eclosion of embryos, or negatively impact wing formation in *Drosophila*. Although 4 µM MTX without irradiation resulted in insignificant levels of DNA damage, this may be explained by the high number of deaths recorded at this concentration, which considerably reduced in the number of flies that could be assessed for DNA damage. On the contrary, 10 µM MTX in combination with X-ray irradiation induced significant levels of DNA damage. Compared to the previous experiment, double the number of flies were sampled for this experiment, resulting in a considerably higher number of live flies that could be assessed for DNA damage. One striking observation was the development of the notch wing phenotype

in flies exposed to combination of MTX and X-ray radiation, or MTX alone at concentrations above 1 μM. This phenotype was not generated by exposure to HU (Figure 18) which also acts by restricting the production of dNTPs. The appearance of wing serration in response to MTX exposure suggests that MTX may be disrupting developmental pathways involved in wing margin formation. In *Drosophila*, wing serration is often associated with disruptions in the Notch signalling pathway, which plays a critical role in defining the dorsal-ventral boundary and proper margin development (Diaz-Benjumea & Cohen, 1995). One plausible model is that MTX-induced DNA replication stress or folate pathway disruption interferes with Notch pathway signalling during wing disc development. Since folate metabolism is essential for nucleotide synthesis, MTX-induced folate antagonism may impair rapidly dividing cells in the wing margin zone, leading to improper differentiation or apoptosis. This, in turn, may affect cell-cell signalling, resulting in serrated or notched wing edges. Alternatively, MTX may indirectly alter the expression of genes downstream of Notch such as *wingless*, *cut*, or *achaete* which are essential for margin stability (Affleck et al., 2006; Affleck & Walker, 2008). Further experiments, such as immunostaining or genetic interaction tests with Notch mutants, would be necessary to validate this model and pinpoint the molecular targets of MTX in wing serration.

The "mutated notch wing phenotype" is a specific type of phenotypic alteration that affects the development of wings in organisms, and is potentially caused by alterations in the Notch signalling pathway (Penton et al., 2012). Notch is a highly conserved signalling pathway that plays an important role in different developmental processes, including cell fate determination and tissue patterning (Zhou et al., 2022). While there is no explanation for the development of the notch wing phenotype in flies exposed to MTX, there is evidence of crosslink between notch signalling and the JAK/STAT pathway. In a previous study, it was shown that activation of the Notch pathway caused STAT3 to become activated along with the Notch effectors Hes1 and Hes5 (Kamakura et al., 2004). Hes proteins interact with JAK2 and STAT3, to aid in the formation of a complex between JAK2 and STAT3. This complex leads to the phosphorylation and subsequent activation of STAT3 (Hu et al., 2021). Another study demonstrated that, in cells at the posterior boundary, the JAK/STAT signalling cascade works together with Notch signalling to regulate the expression of the *short stop* gene, which encodes a cytoskeletal

protein (Josten et al., 2004). As such activation of JAK/STAT by MTX may have resulted in activation of notch signalling and caused the observed notch wing phenotype.

Studies have shown that mutations in the *mwh* gene lead to the formation of extra wing hairs which can serve as a visible marker for genetic analysis (Pitchakarn et al., 2021). *In vivo* imaging has further elucidated this phenomenon by revealing that, during the early stages of hair growth, cells exhibit multiple actin bundles. These bundles, in conjunction with hairs, eventually coalesce to form a singular, growing hair. The *mwh* gene plays a pivotal role in this context, by functioning as an inhibitor of the actin cytoskeleton (Lu and Adler, 2015). When mutations occur in the *mwh* gene, the initiation of hair growth expands and surpasses the confined region at the distal edge of pupal wing cells. This aberration results in the emergence of multiple hairs, which exhibit disrupted polarity. Thus, the *mwh* gene and its associated mutations were employed as a potential genetic marker in this study. Understanding the intricacies of how the *mwh* gene functions and its impact on wing hair development can provide insights into the broader genetic mechanisms at play. Furthermore, by studying these mutations, we can gain a deeper understanding of the genetic factors that influence wing hair growth, which could be instrumental in our project's objectives related to genetic analysis and mutation impacts.

The findings from this study indicate that low doses of MTX did not lead to the manifestation of the multiple wing hair phenotype, although this characteristic was observed in flies exposed to higher MTX concentrations. The prevailing hypothesis is that the majority of clones associated with the *mwh* wing phenotype in flies exposed to high MTX doses arise from mitotic recombination events. However, this phenotype could also be induced by mutation of the second copy of the *mwh* gene, i.e., by loss of heterozygosity (LOH). Thus, the possibility of LOH cannot be entirely dismissed as the *mwh* gene is relatively small, which reduces the probability of spontaneous mutations and suggests that the observed phenotypic changes are more likely to be associated with specific experimental treatments rather than random genetic variation. Without definitive evidence or targeted experiments to rule out LOH, LOH remains a potential contributor to the observed wing phenotype in the context of MTX exposure (Affleck et al., 2006). By contrast, the GMR>w- appears to be induced by a loss of function (LOF) mutation in the knockdown cassette. Recombination would not result in a clone, which leads to the

inference that high doses of MTX exhibit mutagenic properties. However, the absence of clones in the RNAi-based GMR>w- assay should not be automatically interpreted as an absence of mutations. This assay is limited by its design to detect only mutations within or affecting the specific knockdown cassette, and therefore represents a narrow window of the genome. A zero-clone outcome may simply indicate that no detectable LOF mutations occurred within the limited loci examined (Rong & Golic, 2000), rather than confirming a complete absence of mutagenic events elsewhere in the genome. This highlights the inherent limitations in sensitivity and scope of the RNAi clone assay and underscores the need to interpret negative results with caution.

Conversely, low doses of MTX might protect against formation of aberrant phenotypes. This protective effect is particularly intriguing when considering the impact of the *mwh* mutation on the actin cytoskeleton. The mutation leads to the formation of extra actin bundles or hairs which are not as seamlessly fused as in the wild type. However, low dose MTX seemed to mitigate this effect and prevent the formation of additional hairs. As development progresses, the propensity for the formation of these extra hairs due to the additional actin bundles persists, leading to an increase in hair count. It's essential to note that while our observations suggest a potential relationship between MTX and actin bundling, direct evidence linking MTX to actin bundling alterations is required to substantiate this claim further. As development proceeded, the additional hairs consequent of the extra actin bundles continued to form, increasing the hair number.

Our assessment showed that low-dose MTX significantly increased the lifespan of *Drosophila,* while higher doses resulted in significant reduction in lifespan (Burhans and Weinberger, 2009). These findings from our study suggest that low-dose MTX may have lifespan-extending effects in *Drosophila melanogaster*, possibly due to the MTX reducing the rate of DNA synthesis and slowing the rate of DNA damage due to ageing. While it is challenging to make direct quantitative comparisons between MTX doses in flies and humans due to differences in metabolism, body size, and drug pharmacokinetics, the nanomolar concentrations used in this study (e.g., 100 nM) are substantially lower than the micromolar to millimolar plasma concentrations typically achieved in high-dose MTX therapy in patients (Howard et al., 2016). In clinical settings, therapeutic plasma levels often reach up

to 1000 μM in high-dose regimens (Howard et al., 2016). Therefore, the low doses administered in *Drosophila* are more analogous to sub-therapeutic or maintenance doses in humans, such as those used for anti-inflammatory treatments (e.g., in rheumatoid arthritis), rather than cytotoxic cancer doses. This comparison supports the translational potential of low-dose MTX as a modulator of longevity-related processes, though further pharmacodynamic modelling would be needed to draw more precise interspecies parallels. It is also important to consider the differences in drug bioavailability between humans and *Drosophila*. In humans, MTX is typically administered orally or intravenously, with absorption influenced by factors such as dosage form, renal function, and plasma protein binding. In contrast, flies are exposed to MTX through feeding, and the actual internal concentration achieved depends on variables such as gut absorption, cuticular permeability, enzymatic breakdown, and excretion (Rand et al., 2010). This route likely results in lower and more variable systemic bioavailability compared to controlled dosing in humans. As a result, the internal exposure in flies may not directly reflect human pharmacokinetics, and interpretation of MTX's effects in *Drosophila* must account for these physiological and metabolic differences. Nevertheless, *Drosophila* remains a useful in vivo model to study general mechanisms of drug action and toxicity.

Studies in budding yeast have shown that a reduction in growth signalling molecules promotes increased lifespan, mainly due to reduced replication stress (Burhans and Weinberger, 2009). Furthermore, the JAK/STAT pathway regulates a variety of cellular processes which include proliferation and survival of immune cells. The overactivation of JAK/STAT proteins, in addition to the reduced expression of various suppressors of cytokine signalling (SOCS) proteins, have been linked to increased cancer cell proliferation, cancer progression, metastasis, and tumour cells survival in different cancers (Gutiérrez-Hoya and Soto-Cruz, 2020). MTX has been documented to inhibit the JAK/STAT pathway in several previous studies, which is the basis of administration of MTX in the treatment of inflammatory diseases, like rheumatoid arthritis, and myeloproliferative neoplasms (Thomas et al., 2015; Wang et al., 2022). Thus, coupled with previous reports that inhibition of the JAK/STAT pathway activity in the gut stem cell niche of the adult fly is able to extend lifespan, these results indicate that MTX increases lifespan

through deactivation or inhibition of JAKT/STAT signalling. Taken together, this provide insights into the potential lifespan-extending effects of low dose MTX in other organisms, including humans.

In the climbing experiment, female flies consistently climbed to a lower height compared to male flies. It was also found that MTX did not affect the climbing height of female flies. However, interestingly, 100 nM MTX enhanced the locomotion of the male flies from week 4 through week 7 compared to the untreated control groups. A previous study showed that calorific restriction differentially affects both locomotion and lifespan in male and female *Drosophila* (Magwere et al., 2004). Magwere et al. (2004) demonstrated that dietary restriction (40% required diet) resulted in a peak lifespan in males, while females showed a peak lifespan at 60% of the dietary requirement. Female *Drosophila* have larger body size with higher fat storage compared to males (Wat et al., 2021) and thus require a higher dietary intake, which may explain the reason for the peak lifespan at higher dietary intake. Taken together, these factors may explain the reason why male flies are more active than females. However, low-dose MTX seemed to enhance locomotion in male flies from 4th week post-exposure to MTX, and the reason for this finding is not currently understood. This may likely be due to activation of pathways by low dose MTX, such as the JAK/STAT pathway (Thomas et al., 2015); this may have promoted survival pathways in the male flies but not in the females, possibly maybe due to hormonal influences.

The restructured graphical representation of the climbing ability of male *Drosophila* exposed to MTX provided an alternative understanding of the dose-dependent effects of MTX on locomotor performance. By categorising the flies into low, medium, and high climbers, this visualisation clearly showed that MTX treatment, especially 100 nM, enhanced motor activity over time, with a significant proportion of flies transitioning to the high climbing category from week 4 onward (Figure 23). The disappearance of low climbers from the drug-treated groups further supports the idea that MTX might confer resilience against age-related declines in motor function. The gradual improvement in climbing ability with higher doses suggests that MTX may not only prevent age-related decline, but may actually enhance responsiveness.

Mechanistically, the observed effects of MTX on locomotion in male *Drosophila melanogaster* may involve the JAK/STAT signalling pathway. Previous studies suggest that MTX inhibits the JAK/STAT pathway (Thomas et al., 2015), a critical regulator of cellular proliferation, differentiation, and tissue maintenance. The JAK/STAT pathway plays a well-established role in maintaining stem cell populations in various tissues, including those critical for muscle integrity and regeneration (e.g., somatic stem cells in flies) (Hombría and Brown, 2002). If MTX influences JAK/STAT signalling in a dose-dependent manner, it may enhance the preservation or functionality of muscle-associated stem cells. While specific studies on muscle stem cells in *Drosophila* are sparse, it is worth noting that vertebrate studies have demonstrated the importance of JAK/STAT in maintaining muscle satellite cells, which are key for muscle repair and regeneration (Herrera and Bach, 2019). The potential involvement of muscle stem cells in *Drosophila* locomotion may provide a plausible explanation for the sex-specific effects observed in this study. Male flies might potentially experience greater MTX-induced preservation or activation of muscle-associated stem cells compared to females due to hormonal differences or differential baseline JAK/STAT activity between the sexes (Doles and Olwin, 2014). This preservation of stem cell pools could prevent or mitigate age-related muscle decline, and thereby maintain locomotion and spontaneous activity in MTX-treated males. To test this hypothesis, future experiments could focus on examining the effect of MTX on muscle-associated stem cells in flies. Specifically, genetic reporter lines such as *Zfh1-Gal4* or *Mef2-Gal4* could be used to label myogenic progenitors, while proliferation can be quantified using EdU incorporation or phospho-H3 staining. Functional assays—such as flight muscle histology or RNAi-mediated knockdown of JAK/STAT components in muscle stem cells—would help determine whether MTX alters stem cell maintenance or differentiation. Combining MTX treatment with muscle degeneration mutants (e.g., *Mhc* or *Act88F*) may clarify if MTX preserves locomotor ability under muscle stress. These approaches would clarify whether the improved climbing performance in MTX-treated males is mediated by stem cell–dependent preservation of muscle function, which is consistent with previous evidence showing that JAK/STAT signalling is required for muscle homeostasis (Kierdorf et al., 2020) and for the maintenance of adult stem cells in *Drosophila* (Herrera & Bach, 2019).

One limitation of the experimental design, recognized only after the experiments were concluded, was the 25 cm height of the stripette used to measure climbing ability. This restricted the maximum height to which individual flies could climb. Flies that reached the top of the stripette within the 10-second timeframe could have potentially climbed higher if the apparatus had provided for more space. Consequently, the mean climbing height is likely an underestimate of the flies' true climbing potential. Furthermore, this limitation likely reduced the standard deviation of the datasets, as many flies clustered at the 25 cm maximum height. This restriction may have influenced the significance and p-values of the statistical analysis, and potentially altered the interpretation of the results. Future experiments should consider using a taller apparatus to better capture the full climbing capacity of the flies.

Furthermore, females did not show a significant difference in climbing, and the reason for this is unclear, especially since the increased lifespan observed in females suggests that both sexes are affected by MTX. However, at week 7, the mean values for females treated with 100 nM MTX tended to be higher than those treated with 0 nM, though the differences were not statistically significant. Observing these means is still valuable, as it looks as if the drug treated flies are trending higher.

The effects of low doses of MTX alone or in combination with X-ray radiation on DNA damage and the serrated wing phenotype were also studied in this model. MTX and radiation are often investigated as combinational therapeutic strategy for cancer. MTX can sensitize cancer cells to radiation, making them more susceptible to the DNA damage caused by radiation therapy (Costantini et al., 2010). This can enhance the effectiveness of the radiation therapy and reduce the required radiation dose. This combinatorial therapy has been studied in various types of cancer such as head and neck cancer, breast cancer, and lung cancer, with promising results (Bhatia et al., 2020). However, despite this potential positive effect of combinational treatment, concerns related to the negative toxic effect of MTX have long hindered its wide application in different cancers.

High-dose methotrexate (HDMTX) therapy, which is commonly used in the treatment of various types of cancer, can lead to significant toxicity in patients. The most well-known toxicities associated with HDMTX include renal dysfunction, mucositis, and myelosuppression (Howard et al., 2016).

Additionally, HDMTX can cause neurotoxicity, nephrotoxicity, and dermatologic toxicity, and hepatotoxicity (Howard et al., 2016). These toxicities can lead to a number of complications, including treatment delays and decreased quality of life for patients. There are several strategies that can be used to mitigate the toxicities associated with HDMTX. One approach is to use lower doses of the drug, which can reduce the incidence and severity of toxicities.

3.4 Conclusion

In this chapter, I have shown that high doses of MTX induce DNA damage, inhibit fecundity and larval development, and reduce adult lifespan. High doses of HU had similar effects on larval development. These findings are consistent with the known roles of both drugs in the cellular production of dNTPs, and this conclusion is supported by rescue of the MTX-induced larval development phenotype by the addition of folinic acid, which will be presented in the next chapter (Figure 25).

In contrast to the results observed at high doses of MTX above 1 μM, lower doses of MTX did not induce DNA damage or impact the repair of damaged DNA (Figure 16). Furthermore, low-dose MTX did not inhibit fecundity or eclosion. It was also observed that low-dose MTX conferred an increase in the average lifespan of the flies, which coupled with the findings on the effect of the dose on *mwh* phenotype, may indicate that low dose MTX confers a protective effect. This study adds to the growing body of evidence that suggests low-dose MTX may have a different impact on cellular processes and biology compared to high-dose MTX. Moreover, the lack of DNA damage and the absence of alterations in DNA repair pathways observed after low-dose MTX treatment suggest that low-dose MTX therapy, as given for long term inflammatory diseases such as rheumatoid arthritis, may be less toxic compared to high-dose treatments. This is a significant finding, as it may open new avenues for the development of safer and more effective treatments for various diseases.

Chapter 4: Folinic Acid Bypasses the Toxic Effect of MTX on Fly Eclosion, and Low-Dose MTX May Inhibit JAK/STAT Activity in the *Drosophila* Gut

4.1 Introduction

Methotrexate (MTX) is a chemotherapeutic agent widely used in the treatment of various types of cancer, including leukaemia, lymphoma, breast cancer, and lung cancer (Chen et al., 2014; Khodashenas et al., 2021). In this role, MTX works by inhibiting the enzyme dihydrofolate reductase (DHFR), a central component of the folate pathway that is ultimately essential for the synthesis of nucleotides required for DNA repair and synthesis. As a result, MTX can slow or stop the growth of cancer cells, and thus increase patient survival rates. However, MTX is also known to cause a variety of toxic side effects, including damage to healthy cells and tissues (Gunyeli et al., 2021). The severity of these side effects can vary depending on the dose of MTX, the duration of treatment, and the type of cancer being treated. The most common side effects of MTX in humans include nausea, vomiting, and hair loss. In some cases, MTX toxicity can lead to more serious side effects or life-threatening complications such as neurotoxicity, liver damage, renal failure, and myelosuppression (Hamed et al., 2022).

In animal models, MTX has also been shown to be effective at inhibiting the growth and proliferation of cancer cells (Wei et al., 2019). In one study, MTX was found to significantly reduce tumour growth in a mouse model of colon cancer (Nakase et al., 2004), while another study showed that MTX was effective at inhibiting the growth of lung cancer cells in rats (Yan et al., 2013). However, the use of MTX in animal models is also associated with several toxic effects. For example, MTX can cause myelosuppression and decrease production of white blood cells, red blood cells, and platelets in mice (Góra-Tybor and Robak, 1993). MTX can also lead to intestinal damage and inflammation, as well as liver and kidney toxicity in rats (El-Sheikh et al., 2016).

One important factor to consider in cancer therapy is to mitigate MTX toxicity to reduce the side effects of treatment. These side effects can be extremely debilitating for patients and may interfere with their ability to complete their course of treatment, and thus lead to suboptimal outcomes. In addition to its toxic effects, the effectiveness of MTX can be limited by the development of resistance in cancer cells, which can lead to treatment failure (Nikolaou et al., 2018). To address this issue, studies have explored the use of combination therapy, where MTX is used in combination with other chemotherapeutic agents to enhance its efficacy and reduce toxicity. By minimising the toxicity of MTX, clinicians can help to reduce the side effects of treatment and improve patient quality of life. In addition to reducing side effects, mitigating MTX toxicity can also help to improve treatment efficacy. High levels of toxicity may require clinicians to reduce the dose or frequency of MTX, which can lead to suboptimal treatment outcomes. By minimising toxicity, clinicians may be able to administer higher doses of MTX or more frequent treatment cycles, and thus potentially improve response rates and overall survival.

One treatment that has been used to suppress MTX toxicity is glucarpidase, a recombinant form of the carboxypeptidase G2 (CPDG2) enzyme that breaks down MTX in the body (Zobeck et al., 2021). Glucarpidase can be given to patients who experience severe MTX toxicity or who have high levels of the drug in their system through catabolism of MTX, with the aim to reduce the risk of side effects and minimise the associated toxicity.

Corticosteroids such as dexamethasone or prednisone are other forms of drugs used to reduce the toxicity of MTX. Corticosteroids have anti-inflammatory properties, which can help to reduce the inflammation and mucositis caused by MTX (Mahendran et al., 2018). In addition, corticosteroids may also help to reduce bone marrow suppression, a common side effect of MTX treatment (Sosin and Handa, 2003).

However, the most widely used drug to suppress MTX toxicity and side effects is folinic acid, also known as leucovorin (Shea et al., 2013). Folinic acid is the metabolic product of DHFR reduction of folic acid, and its supplementation bypasses the inhibition of DHFR by MTX, and thus counteracts the

effect of MTX through the provision of an alternative source of folinic acid which is necessary for DNA synthesis.

While many of the toxicities listed above are associated with high doses of MTX used in chemotherapy, low doses of MTX can exert an immunomodulatory function by suppressing the activity of the immune system during cancer treatment (Vial and Descotes, 2003). Low-dose MTX can reduce inflammation and control the symptoms associated with autoimmune and inflammatory diseases, which may be linked to the finding that MTX inhibits JAK/STAT signalling, a signalling pathway involved in the regulation of IL-6-mediated inflammation (S. Thomas et al., 2015a). However, despite the fact that low doses of MTX are used to treat autoimmune and inflammatory diseases in the clinical setting, concerns have been raised regarding the potential for low-dose MTX to cause DNA damage and increase the risk of developing cancer. This concern stems from the fact that MTX disrupts folate metabolism and leads to the inhibition of purine and pyrimidine synthesis, which are required for DNA replication and repair (Bökkerink et al., 1988).

Thus, this chapter aimed to examine the ability of low-dose MTX to induce DNA damage and its role in the regulation of JAK/STAT signalling. This chapter further investigates the potential molecular mechanisms that might be responsible for the reproductive, survival, lifespan and DNA damaging effects of MTX described in Chapter 3. I show that the toxicity caused by high doses of MTX can be rescued by folinic acid to demonstrate that MTX is likely to act via DHFR in *Drosophila*. I also attempt to demonstrate that inhibition of the endogenous JAK/STAT pathway by MTX previously described in cultured cells also occurs *in vivo*, in both whole adult flies and within the adult gut.

4.2 Results

4.2.1 Folinic acid mitigates MTX-induced inhibition of fly eclosion

The aim of Chapter 3 was to demonstrate that low doses of MTX could extend lifespan, while higher doses are fatal. In the previous chapter, I demonstrated the effects of MTX on the DNA damage and overall survival and development of *Drosophila*. I showed that high-dose MTX induced DNA damage

and suppressed eclosion of the flies, whereas low MTX doses did not induce significant DNA damage or influence the fly eclosion rates.

This chapter describes experiments undertaken to gain insight into the molecular mechanisms underlying these effects. Overall, Chapter 4 seeks to explore whether this lifespan extension activity could be linked to the activity of MTX as a JAK inhibitor, as we know that in patients, low doses of MTX exhibit this inhibitory effect, while high doses prove toxic (S. Thomas et al., 2015a). The toxicity at high doses is attributed to inhibition of the folate pathway, a well-documented mechanism in humans, thus folinic acid is administered as a rescue agent to counteract the effects of exposure to high-dose MTX (Cohen, 2013). Folinic acid rescue has been a known clinical strategy for over 50 years to mitigate against MTX-induced inhibition of DHFR/folate pathway, and hence toxicity, in humans (Hamed et al., 2022). However, it has not been shown that the toxicity of MTX is also caused by this same mechanism in model organisms such as *Drosophila*.

Thus, I investigated the influence of folinic acid on MTX-induced inhibition of fly eclosion. Firstly, I assessed the eclosion of embryos laid by w^{Dah} adult flies exposed to six different concentrations of MTX (0, 0.001, 0.01, 0.1, 1, and 4 µM) to assess its impact on their development. A total of 200 flies for each concentration were grown on MTX-containing food for two weeks, and the bottles were transferred to fresh bottles every 48 hours to ensure optimal culture conditions. Following this period, adult flies were allowed a 24-hour window for embryo deposition. Subsequently, adult flies were removed, and the bottles were monitored for five days from eclosion of the first adults. The primary outcome measured was the number of embryos that successfully eclosed each day during this observation period.

Based on the number of eclosed adults, dose-dependent inhibition of adult fly fertility was observed (Figure 25A), in agreement with the findings in Chapter 3 (Figure 15A). Having established that only the higher doses of MTX have a significant effect on *Drosophila* survival, this experiment was then repeated using flies exposed to high concentrations of MTX, 1 µM and 4 µM, with or without 0.1 µM folinic acid (Figure 25B). This experiment did not identify a significant difference in the outcome of this combinational exposure versus flies exposed to only MTX.

In a third experiment, the combinational regimen was changed to incorporate 1 μM folinic acid (10 times the initial concentration of 0.1 μM; Figure 25C). It was observed that 1 μM folinic acid significantly inhibited the toxic effects of MTX by significantly increasing the number of embryo eclosions in the groups exposed to 1 μM and 4 μM MTX ($p < 0.0001$ and $p < 0.0001$, respectively). This data suggests at high doses of folinic acid have a protective effect on MTX-induced inhibition of fertility. However, at low doses, the effect of folinic acid was not strong enough to affect adult female fertility. In addition, this data suggests that the fertility issues caused by high dose MTX are probably due to inhibition of the folate pathway/DHFR due to the protective effects observed during folic acid exposure, but indicate that low doses of MTX do not affect this pathway enough to alter adult fertility. As shown previously in Chapter 3 (Figure 15A), high-dose MTX significantly reduced the number of eggs laid, indicating that the decrease in eclosion observed is likely due, at least in part, to impaired fertility or egg-laying—rather than post-embryonic lethality.

In addition, these results show that administering 0.1 μM folinic acid did not significantly alter the toxic effects of MTX, whereas 1 μM folinic acid effectively reduced MTX-induced toxicity in flies. This suggests that MTX toxicity in *Drosophila* may indeed parallel the toxicity pathway observed in humans, and further indicates the inhibition of the folate pathway is a conserved mechanism across species. Moreover, the data indicted low concentrations of MTX do not affect the survival of flies and that the toxicity induced by high levels of MTX can be reversed by folinic acid.

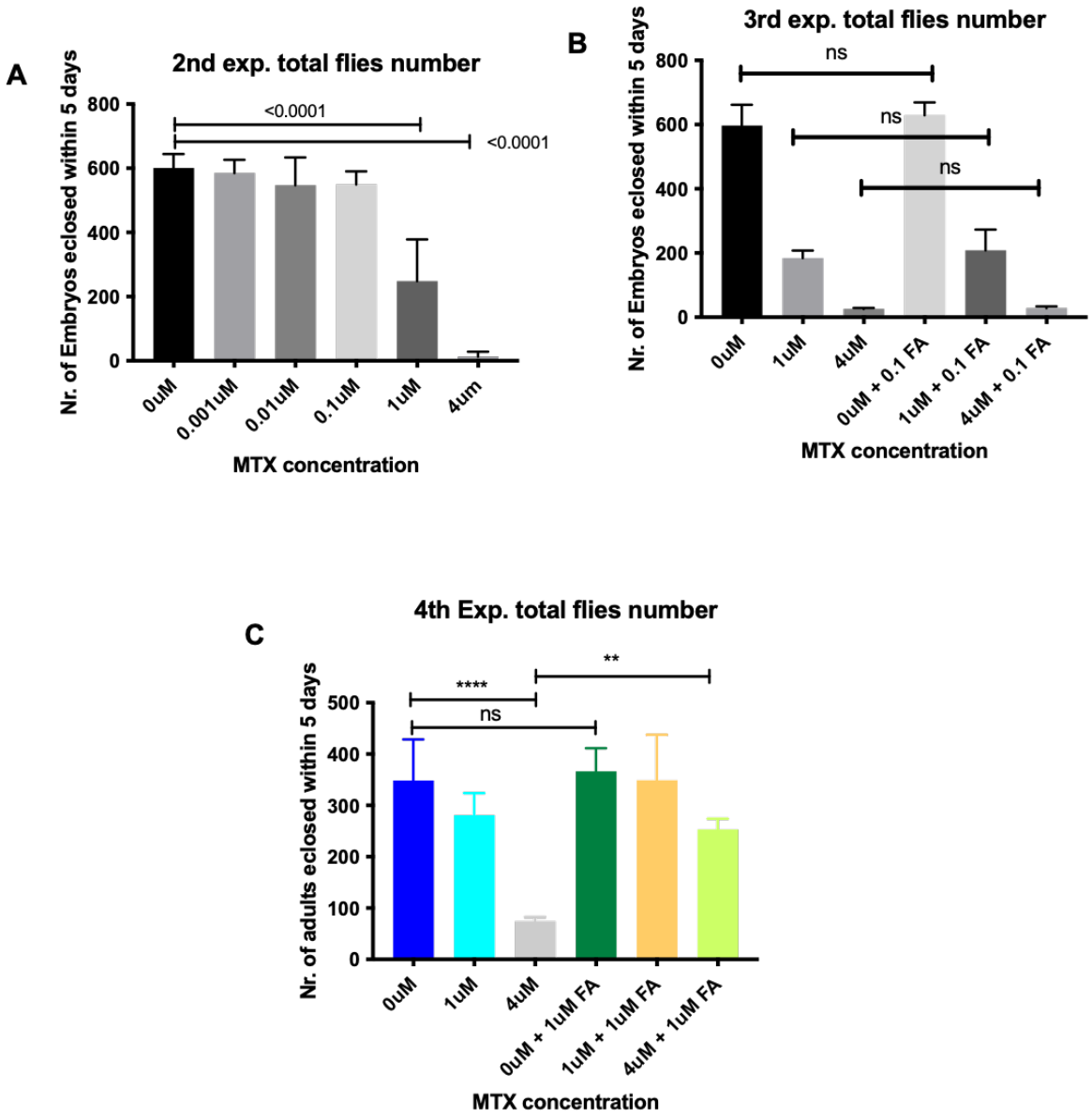
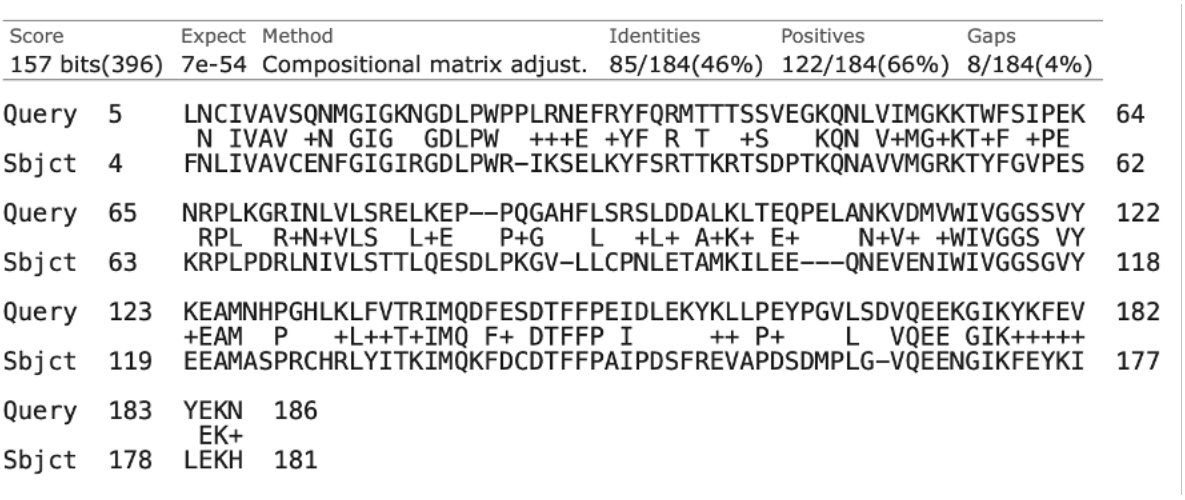


Figure 25. Effects of folinic acid on MTX-induced toxicity. Adult flies were fed with nutrients containing (A) MTX at different concentrations up to 4 µM, (B) different concentrations of MTX up to 4 µM and folinic acid at 0.1 µM, or (C) different concentrations of MTX up to 4 µM and folinic acid at 1 µM. Data is presented as mean ± SD; each experimental group consisted of 200 flies per vial, and the experiments were conducted in three independent replicates. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test to compare each MTX or MTX+folinic acid group to the untreated control (0 µM MTX) or to MTX-only groups, as appropriate; $p < 0.05$, $p < 0.01$, $p < 0.001$.

Humans and *Drosophila* diverged over 500 million years ago, with subsequent evolutionary changes leading to differences in DHFR between these species (Figure 26). This evolutionary distance suggests the possibility that MTX toxicity in *Drosophila* could operate through a distinct mechanism compared to humans. However, our results demonstrate that folinic acid can also rescue *Drosophila* from high-dose MTX toxicity. This observation implies that, despite the structural differences, MTX toxicity in *Drosophila* likely involves inhibition of DHFR, as in humans, indicating that species-specific protein differences do not interfere with the DHFR-MTX interaction.

Reflecting on these findings, future studies could incorporate folinic acid into lifespan assays to explore its potential impact on DHFR-associated pathways and lifespan. This approach could provide additional insights into the mechanisms of MTX toxicity and contribute to a deeper understanding of DHFR-related drug interactions.



Score	Expect	Method	Identities	Positives	Gaps
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Sbjct  4    FNLIVAVCENFGIGIRGDLPWR-IKSELKYFSRTTKRTSDPTKQNAVVMGRKTYFGVPES  62

Query  65   NRPLKGRINLVLSRELKEP--PQGAHFLSRSLDDALKLTEQPELANKVDMVWIVGGSSVY  122
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Sbjct  63   KRPLPDRLNIVLSTTLQESDLPKGV-LLCPNLETAMKILEE---QNEVENIWIVGGSGVY  118

Query  123  KEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEV  182
            +EAM  P   +L++T+IMQ F+ DTFFP I     ++ P+    L  VQEE GIK+++++
Sbjct  119  EEAMASPRCHRLYITKIMQKFDCDTFFPAIPDSFREVAPDSDMPLG-VQEENGIKFEYKI  177

Query  183  YEKN  186
            EK+
Sbjct  178  LEKH  181
```

Figure 26. Protein sequence alignment of dihydrofolate reductase (DHFR) between *Drosophila melanogaster* (P17719.3) [lower line labelled Sbjct] and Homo sapiens (P00374.2) [top line labelled Query]: Conserved amino acids are shown as single letter code between the two sequences. + indicates similar but different amino acids between the two sequences and an empty space indicates unconserved differences. Gaps are indicated in the sequences by - symbols. Overall identity and similarity (positives) are indicated in the header. Alignment was generated by Blastp via the National Library of Medicine (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.2.2 Reporter assay to detect JAK/STAT activity

It has previously been shown that MTX acts as an inhibitor of the JAK/STAT pathway in *Drosophila* cells (S. Thomas et al., 2015a). The JAK/STAT signalling pathway is a vital, evolutionarily conserved pathway that contributes to many biological processes, such as cell growth, differentiation, and immune responses. To determine whether the doses of MTX being investigated in this project are sufficient to inhibit JAK/STAT pathway activity *in vivo*, I used a previously described *in vivo* reporter assay to investigate the activation of this pathway (Alqarni and Zeidler, 2020). When the pathway is activated, the GFP is synthesized, and its presence can be detected through an ELISA-based assay using an anti-GFP antibody.

Given the apparent accuracy of the 10X STAT-GFP reporter in embryos and imaginal discs (Classen et al., 2009), I hypothesised that this system would also report global JAK/STAT pathway activity in whole adult flies and as such could detect the changes in pathway activity potentially caused by MTX in the flies' food. To test the activity of the reporter assay in whole adult flies — in which quantification of GFP fluorescence was impossible — I set out to develop an ELISA-based assay to directly quantify the amount of GFP protein present in adult flies carrying the 10X STAT-GFP reporter. Furthermore, flies expressing positive and negative controls of Act-GFP, as well as wild-type OreR flies that do not express GFP or empty vectors as blank controls were utilised. Stocks containing the 10X STAT-GFP reporter, which was constructed using a P-element vector backbone that randomly integrates into the genome, were obtained with insertions in both chromosome II and III (gift of Erika Bach).

The blank vectors were wells in the ELISA tray that did not have any protein added to them, to indicate the level of background in the system. As expected, these blanks did not show any signal for GFP when the anti-GFP ELISA was carried out. Similarly, the negative control of OreR showed very minimal readout, which is likely due to background at the highest concentration. Also, as expected, the Act-GFP genotype expressed GFP in a concentration-dependent manner (Figure 27A). This was as expected, as the Actin5c promoter is ubiquitously expressed in all cells of the fly (Jay et al., 2021).

To investigate the levels of JAK/STAT pathway activation *in vivo*, flies either homozygous (HOM) or heterozygous (HET) for 10X STAT-GFP were assayed (Figure 27A). The levels of GFP fluorescence detected for both the HOM and HET forms of STAT-GFP were similar from the highest concentration down to 0.8625 µg total protein where the HOM expression system exhibited a higher fluorescence in a concentration-dependent manner down to the lowest concentration of 0.216 µg total protein. The reason for this is that the concentration ranges up to 0.8625 µg still appeared to be out of the linear range of the signal for the curve. Both homozygotes and heterozygotes showed unexpectedly high basal levels of GFP expression. This observation suggests possible non-specific activation of the reporter system. The high basal levels could be masking the subtle changes expected in JAK/STAT pathway activation and explain why the flies exposed to low concentrations exhibited high fluorescence.

As the read-out for both the HOM and HET appeared to be out of the linear range, another experiment was conducted using a lower starting concentration, which may be closer to the physiological linear range where a difference in fluorescence can be observed. Female flies of two different genotypes were tested. One heterozygous for both the *stat92E* mutant allele and the *10X STAT-GFP* reporter (Het; $stat92E^{06346}$/+), and another heterozygous for both the reporter and the constitutively active JAK allele hop^{Tuml} (Het; hop^{Tuml}/+). If the reporter system was functioning correctly, flies carrying the hop^{Tuml} allele would be expected to exhibit significantly elevated GFP expression due to constitutive activation of the JAK/STAT pathway. Both strains were tested for reporter-driven GFP expression as explained above. The HOM and HET constructs from the previous experiment were also included as controls, with 18 µg total protein the highest concentration tested this time.

As expected, the HOM and HET constructs exhibited similar levels of GFP expression as in the previous experiment (Figure 27A). Higher concentrations showed similar GFP readout down to 1.125 µg total protein still indicating that this concentration range is out of the linear range for fluorescence quantification (Figure 27B). However, the linear range for the ELISA assay that allows accurate quantification of GFP fluorescence was determined to be between 0.216 µg/mL and 0.8625 µg/mL total protein. Concentrations above this range led to saturation, as evidenced by minimal changes in signal in Figure 27A. Unfortunately, both the *Het; STAT/- and the Het; Tum/+* showed similar readouts at all

concentrations, with concentration-dependent decreases in GFP readouts. This is likely because the reporter is non-functioning in the adult fly or due to swamping from non-JAK/STAT-related expression of GFP amplifying the readout for GFP.

Despite these findings, a notable concern arose regarding the unexpectedly high levels of GFP expression observed in the 10X STAT-GFP reporters compared to Act-GFP. This difference was surprising, given that the JAK/STAT pathway is active only in specific subsets of cells, as indicated by prior studies into *unpaired* expression (a pathway ligand) and pathway signalling in *Drosophila* (Zeidler et al., 1999). The higher GFP expression levels observed in the reporters, compared to the ubiquitously expressed *actin*-driven GFP, appear inconsistent and suggest potential issues with non-specific activation of GFP expression — possibly due to enhancer-trapping effects of the P-element insertion driving high levels of inappropriate, JAK/STAT pathway-independent GFP expression.

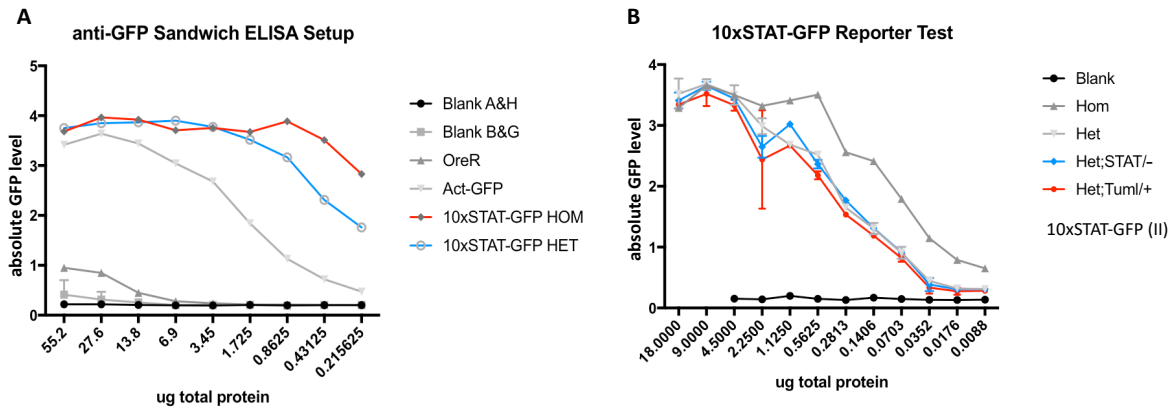

Figure 27. Levels of GFP expressed by 10X STAT-GFP reporters in adult *Drosophila*. Extracts from adult flies expressing different vectors containing HOM or HET *STAT-GFP* constructs were analysed for JAK/STAT activity using an anti-GFP ELISA. (A) Assay plate containing serially diluted total protein at 55.2 µg total protein and (B) assay plate containing serially diluted total protein from 18 µg. Data are presented as mean ± SD for n = 1 in two different experiments.

The results of the above experiment showed that JAK/STAT signalling was active in the adult *Drosophila*, but the readout at high concentrations of loaded total protein in the anti-GFP ELISA were similar for both HOM and HET. However, manipulation of the JAK/STAT pathway activity (in the form of heterozygosity of loss- and gain-of-function pathway alleles) did not produce detectable changes in reporter activity (Figure 27B). In addition, the overall levels of GFP expressed by even one copy of the reporter are considerably higher than those driven ubiquitously by the Actin5C promoter directly expressing EGFP (Figure 27A). Given that adult flies carrying the reporter showed considerably higher levels of abdominal fluorescence than the Actin-GFP control, it is hypothesised that the reporter is being expressed at very high levels in some adult tissues in a JAK/STAT pathway-independent manner which is masking any JAK/STAT pathway-dependent expression that may be occurring at lower levels. Given that the *10X STAT-GFP* series of reporters were integrated into the genome randomly by P-element transposition (Spradling et al., 1995), it is possible that other independent transformants do not suffer from this artefact.

In an attempt to identify an alternative *in vivo* JAK/STAT pathway reporter, another group of *Drosophilae* with different constructs was used. Firstly, reporters expressing the same construct as that used above were used. However, this construct contains a gene for a destabilised form of GFP (dGFP) downstream of the promoter in a different genomic position with a loss-of-function STAT (*10X STAT-dGFP/STAT* $^{-}$), one with w^{1118} mutation (*10X STAT-dGFP/*w^{1118}), a positive control containing the constitutively active form of JAK (*10X STAT-dGFP/*Hop^{Tuml}). These constructs were inserted into either chromosome II (Figure 28A and C) or III (Figure 28B and D). The "destabilised form of GFP" refers to a variant of GFP that has been engineered to have a shorter half-life within the cell (Spradling et al., 1995). GFP, in its natural form, is quite stable and can persist for long periods. However, for certain experimental purposes, such as studying rapid changes in protein expression or dynamic cellular processes, it is useful to reduce the stability of the fluorescent protein to allow for faster degradation. In the destabilized form, GFP is tagged with a specific degradation signal, known as a degron, that targets the protein for rapid proteolysis and thus provides a more accurate readout of real-time changes in gene expression (Corish and Tyler-Smith, 1999). This alternative *STAT-GFP* reporter line was

obtained from Erika Bach's lab and has been extensively used for JAK/STAT signalling studies in *Drosophila* (Spradling et al., 1995). In addition, *Act-GFP* and OreR were used as positive and negative controls, respectively.

As shown in Figure 28A, the reporters expressing the destabilised GFP variants expressed lower levels of GFP and the actin-GFP positive control. However, comparison of the dose-dependent decreases in GFP readout for the *dGFP/STAT*$^{-}$, *-dGFP/w*^{1118}, and *dGFP/hop*^{Tuml} genotypes indicated no detectable differences in GFP expression, likely due to the out-of-range concentration resulting in faster saturation. Thus, a quantifiable difference in the activities of the reporters could not be evaluated. The experiment in Figure 28C was repeated (as shown in Figure 27B) with the chromosome II construct, and a similar result was obtained.

Overall, as a similar level of GFP expression was observed for all of the reporter constructs (Figure 28C and D), this this approach would not be sensitive enough to identify changes in JAK/STAT pathway activity that might be caused by low doses of MTX. One potential explanation for the fact that GFP levels appear not to change following manipulation of the JAK/STAT pathway is that the stocks used no longer carried the mutations originally associated with them. While the stocks were checked when originally generated and added to the stock collection, they have likely not been checked recently. Many of the stocks used have been routinely used in the Zeidler lab for many years. For example, the *hop*$^{Tum-l}$ mutation was first identified in 1985 and published by the Perrimon lab. Similarly, loss of function alleles in *stat92E* were first identified and characterized in the 1990's and have been widely used in Zeidler lab research over the past 20 years (Chen et al., 2002). However, the individual stocks have not been specifically tested for this experiment, so it is possible that something may have changed with the stocks over time. In addition, a schematic of the *in vivo* STAT activity reporter construct in *Drosophila melanogaster* is shown in Figure 29.

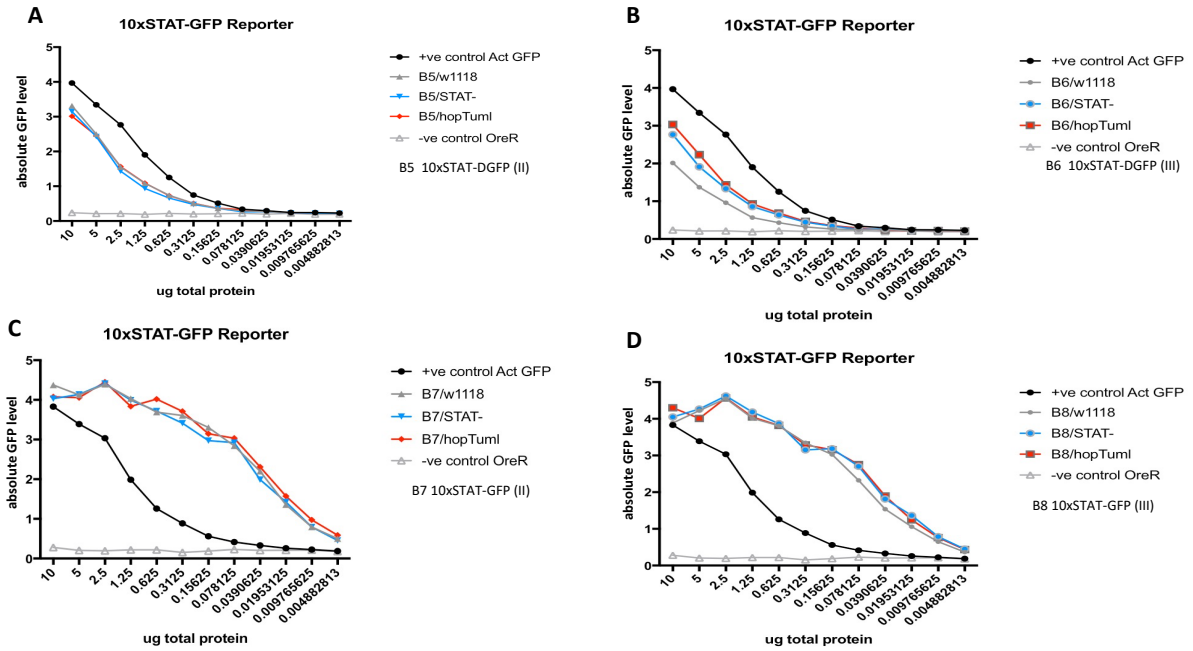

Figure 28. Assessment of different 10X STAT-GFP reporters in adult *Drosophila.* (A) *STAT-dGFP* on chromosome II, (B) *STAT-dGFP* on chromosome III, (C) *STAT-GFP* on chromosome II, and (D) *STAT-GFP* on chromosome III. Each panel represents an independent ELISA experiment testing a distinct STAT-GFP reporter line (total of four independent experiments). A summary of *10xSTAT-GFP* reporter lines and genotypes has been provided in Appendix D. These graphs present absolute GFP concentrations (ng per µg total protein) obtained directly from a single experiment, calculated using a GFP standard curve. No relative normalization was applied. The values were normalized only to total protein content within the same sample, ensuring that differences reflect actual measured concentrations. The calculation procedure has been described in more detail in Appendix F for clarity.

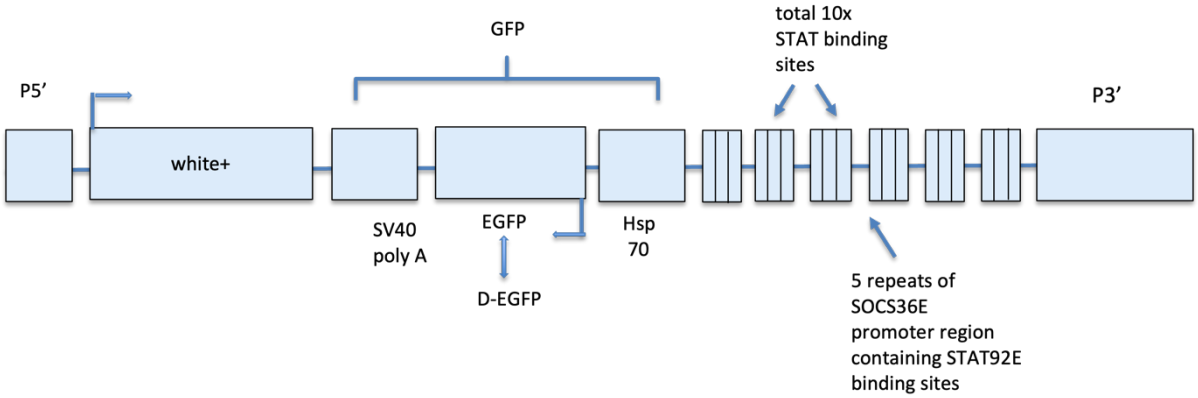

Figure 29. Schematic of the *in vivo* STAT activity reporter construct in *Drosophila melanogaster*. The reporter construct integrates into the genome via P-element-mediated transformation and includes a white^{+} selectable marker, which confers red eye colour in transgenic flies. STAT-responsive enhancer elements are located upstream of an EGFP reporter gene, driven by an hsp70 minimal promoter and terminated with an SV40 polyadenylation sequence to ensure proper transcriptional termination. This system allows for GFP expression in response to JAK/STAT pathway activation in vivo. Multiple independent transgenic lines were generated with insertions located on chromosome II (10XSTAT-GFP II) and chromosome III (10XSTAT-GFP III), enabling comparative studies of reporter activity (Bach et al., 2007).

However, due to the extremely high levels of GFP expression in these flies, we became suspicious that something was not functioning correctly. Nevertheless, further experiments were conducted to assess whether the reporters remained responsive to JAK/STAT pathway activity, despite the irregularities observed. To investigate, we compared *act-GFP*, *10X STAT GFP*, and *OreR* in whole flies at the same time under the same fluorescent microscope (Figure 30). These images clearly showed that most GFP expression in the *10xSTAT-GFP* reporter was very strongly detected within the thorax. Notably, there is no known role for STAT pathway activity in adult fly muscles, especially not at such high levels; however, it possible there is an unknown role for the STAT pathway in fly muscle cells.

It is likely that this reporter is trapping other enhancers because it is randomly integrated into the genome. As a result, the observed GFP expression is JAK/STAT pathway-independent background activity from the reporter itself; thus, the reporter is not responding specifically to JAK/STAT pathway

signalling. Several other STAT reporters were assessed, but none of these stocks produced logical results. Some showed levels of expression that were far too high and unchanging, while others had more reasonable levels but still showed no significant change. It is possible the alleles were incorrect; however, ultimately, it was concluded that this approach was not viable within the timeframe of the study.

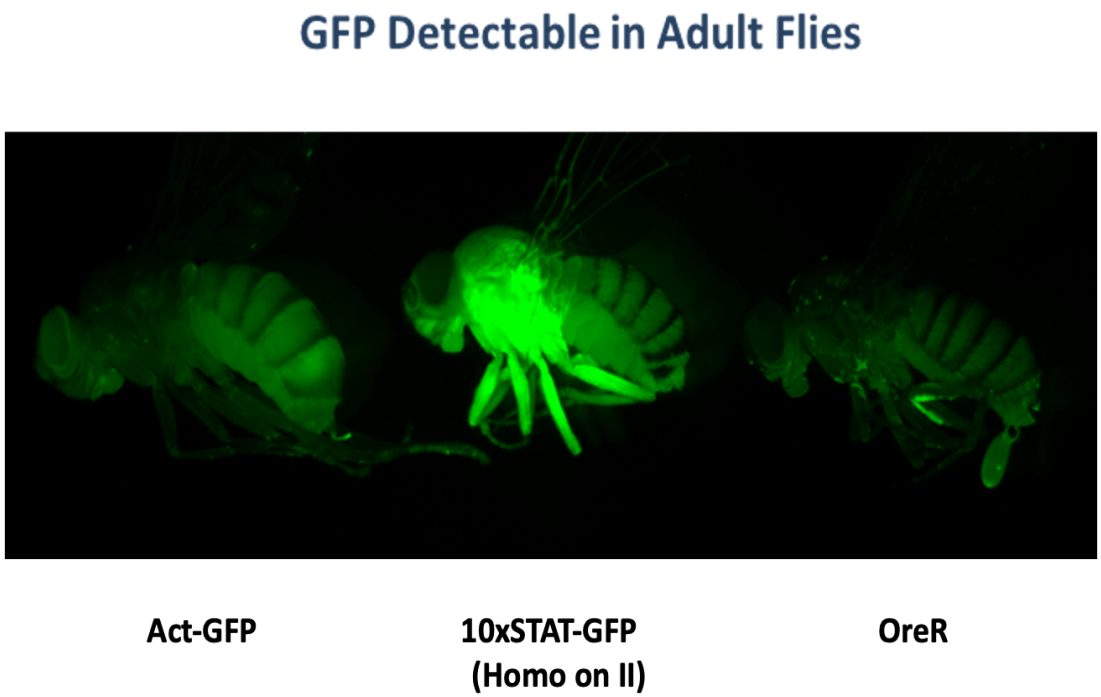

Figure 30. GFP fluorescence in adult *Drosophila melanogaster* observed under a dissecting fluorescence microscope. The image shows GFP expression in three adult flies with different genotypes. From left to right: a fly expressing actin-GFP, a fly with 10xSTAT-GFP, and a wild type fly without GFP expression. The fluorescent green signal indicates the presence and localization of GFP in each genotype. The low levels of green fluorescence in the OreR stock are likely due to background autofluorescence.

4.2.3 Effects of low-dose MTX on JAK/STAT activity

Given the failure of the *in vivo 10X STAT-GFP* reporters to reliably report changes in pathway activity caused by loss of a copy of *stat92E* or heterozygosity for the gain-of-function hop^{Tuml} allele, it seemed unlikely that the subtle change in pathway activity which I anticipated to be caused by low doses of MTX would be detectable using this approach at a 'whole fly' level. Despite this setback, it is clear that the *10X STAT-GFP* reporters can report pathway activity in diploid imaginal tissue during larval development (Bach et al., 2003). In addition, these reporters have previously been shown to work in the adult midgut (Jiang and Edgar, 2011), a tissue likely to be directly exposed to low doses of MTX taken up with food. While not able to report on pathway activity throughout the animal, an indication of pathway inhibition by MTX in the gut would provide support for a mechanism mediated by the modulation of the JAK/STAT pathway.

Therefore, flies carrying the *10X STAT-GFP* reporter integrated into either chromosome II and chromosome III were exposed to different doses of MTX up to 4 μM (Figure 31A and B). GFP expression in the gut of the cells was evaluated by means of a microscopic technique. The first time I undertook this experiment, the results showed that there was no significant change in JAK/STAT pathway reporter activity following feeding with low doses of MTX. This was the case for both the chromosome II and III constructs (Figure 31A and B). However, the wide variation observed between the technical repeats suggested this result may be due to technical errors.

Having identified and eliminated several points where variability occurred in the initial experiments, I repeated this experiment, which demonstrated dose-dependent inhibition of JAK/STAT activity up to an MTX concentration of 0.1 μM. Interestingly, these reductions were significant when compared with the control unexposed *Drosophila* (Figure 31C). By contrast, there was a steady increase for flies exposed to MTX concentrations of 1 and 4 μM but not up to the level of GFP without MTX exposure. This is potentially consistent with these higher MTX concentrations causing cellular/tissue damage, as it has previously been shown that damage to the gut caused by *Pseudomonas entomophilia*, a gram-negative bacteria reported to kill enterocytes, is sufficient to activate JAK/STAT signalling (Jiang and

Edgar, 2011). In my initial experiments, the data was variable, and my dissection techniques were inconsistent, leading to unclear results. To address this, I practiced dissection and optimized the process by reducing the time between dissections, so that all guts were processed within a shorter window before lysis to ensure the samples remained viable.

These initial attempts, while not ideal, demonstrated that GFP could be detected in individual guts. With improved techniques, I repeated the experiments using three guts per sample, twice over. To address technical variability between independent ELISA experiments and enable robust statistical analysis, the data presented in Figure 31C required normalization. The term "relative GFP" refers to GFP expression levels that have been normalized relative to the 0 μM MTX control group within each experiment. Specifically, for each MTX concentration tested, the GFP values were calculated as a proportion of the mean GFP value of the untreated control (0 μM MTX) from the same experiment using the formula: Relative GFP = (GFP value at X μM MTX) / (Mean GFP value at 0 μM MTX). This normalization approach was essential because the initial experiments (Figures 31A and 31B) exhibited considerable variability in absolute GFP values between technical replicates, likely arising from differences in protein extraction efficiency, minor variations in ELISA plate conditions, and natural biological variation in baseline JAK/STAT activity. By standardizing each experiment to its own control baseline, this method minimized technical variability while preserving the biological signal, enabling the combination of data from two independent experiments to improve statistical power and provide more reliable conclusions about MTX effects on JAK/STAT signalling. The calculation procedure has been described in more detail in Appendix F for clarity.

The findings support the hypothesis that low-dose MTX inhibits JAK/STAT signalling. At the low doses of MTX associated with lifespan extension (Chapter 3, Figure 19), we observed a decrease in JAK/STAT pathway activity. In contrast, at high doses, where toxicity occurs, JAK/STAT activity increased slightly. This may be due to MTX damaging the gut, and triggering stem cell proliferation and JAK/STAT-dependent activity—as is the case following *Pseudomonas entomophilia* infection.

However, the key observation is that the doses that extended lifespan correlated with decreased JAK/STAT pathway activity *in vivo*, as shown by reporter expression in the gut.

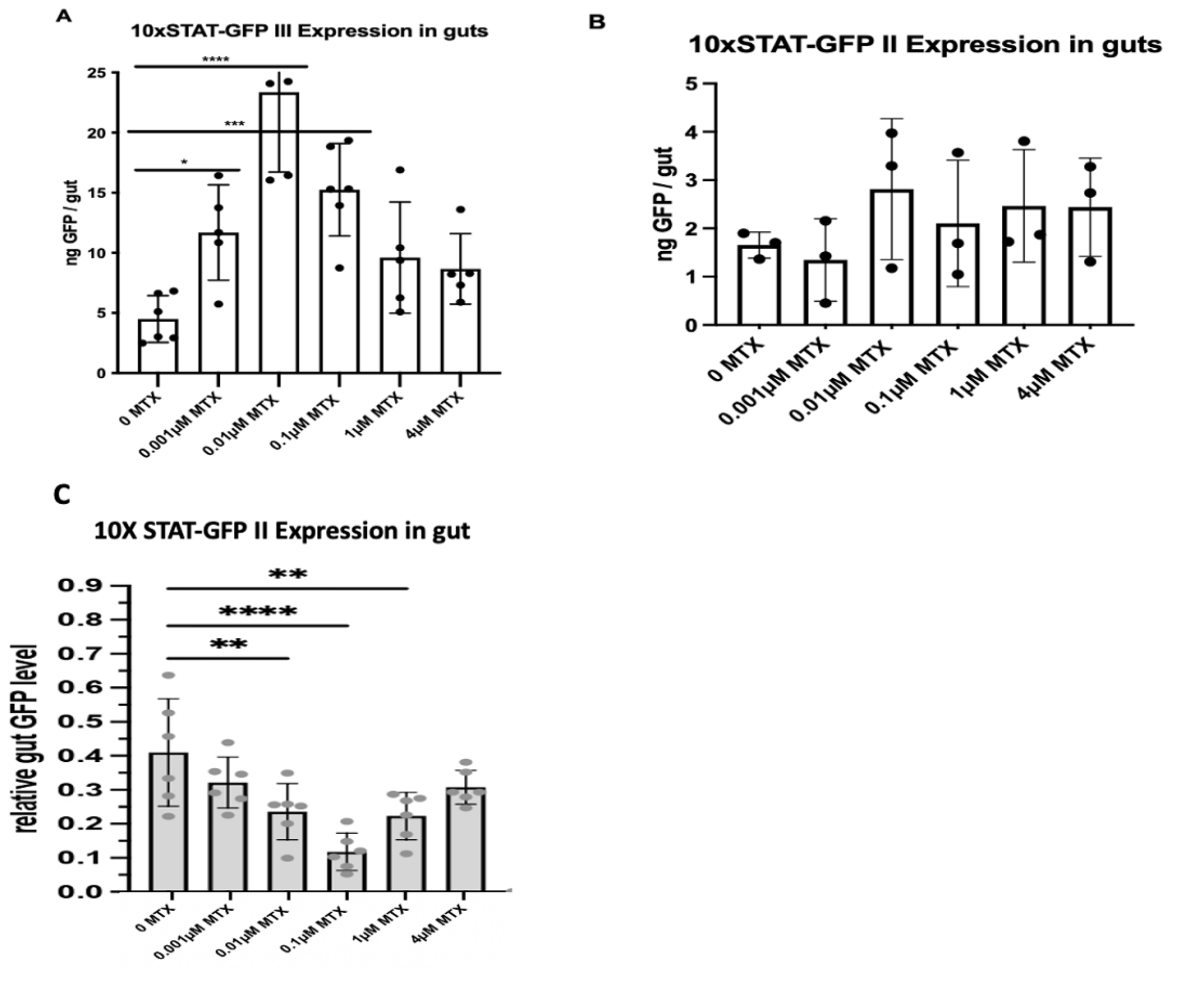

Figure 31. Inhibition of JAK/STAT signalling by low-dose MTX: Adult *Drosophila* expressing a *STAT-GFP* reporter in (A) chromosome III, (B) chromosome II, or (C) a repeat of *STAT-GFP* inserted in chromosome II were exposed to up to 4 µM MTX. The experiment was performed as three independent sandwich ELISA assays. In Panels A and B, three dissected guts were analysed per MTX concentration. Panel C shows combined data from two independent experiments using the chromosome II STAT-GFP reporter to improve statistical power. Panel C shows relative GFP levels normalized to the 0 µM MTX control group. Here, 'relative GFP' = (background-subtracted A_{450} for the sample) ÷ (mean background-subtracted A_{450} for the 0 µM MTX control on the same plate). Data are presented as mean ± SD. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test; *p <0.05, **p<0.01 and ***p<0.001.

In order to precisely quantify the levels of GFP in the adult *Drosophila* gut, an experiment was initiated wherein adult female *Drosophila* genetically engineered to express the *10X STAT-GFP* on chromosomes II and III were administered varying concentrations of MTX (0 as control, 0.001, and 0.1 units). When I first received the stock and noticed irregularities in the expected readouts, I decided to check whether the reporter was expressed in the expected pattern in the gut, as previously described. To do this, I dissected samples at different concentrations using two different reporters.

Unfortunately, due to constraints imposed by time limitations and the COVID-19 lockdown, it was not feasible to complete the quantification of GFP levels. However, I was able to capture visual data through the use of the confocal microscope, as shown in Figure 32. The images shown in Figure 32 were used to obtain a qualitative assessment of GFP expression in the gut, and provide a visual confirmation of the effects of varied concentrations of MTX on GFP levels in the adult *Drosophila* gut quantified in Figure 30. No quantitative data was directly extracted from these images. These images serve as a preliminary dataset that can be further analysed once conditions permit.

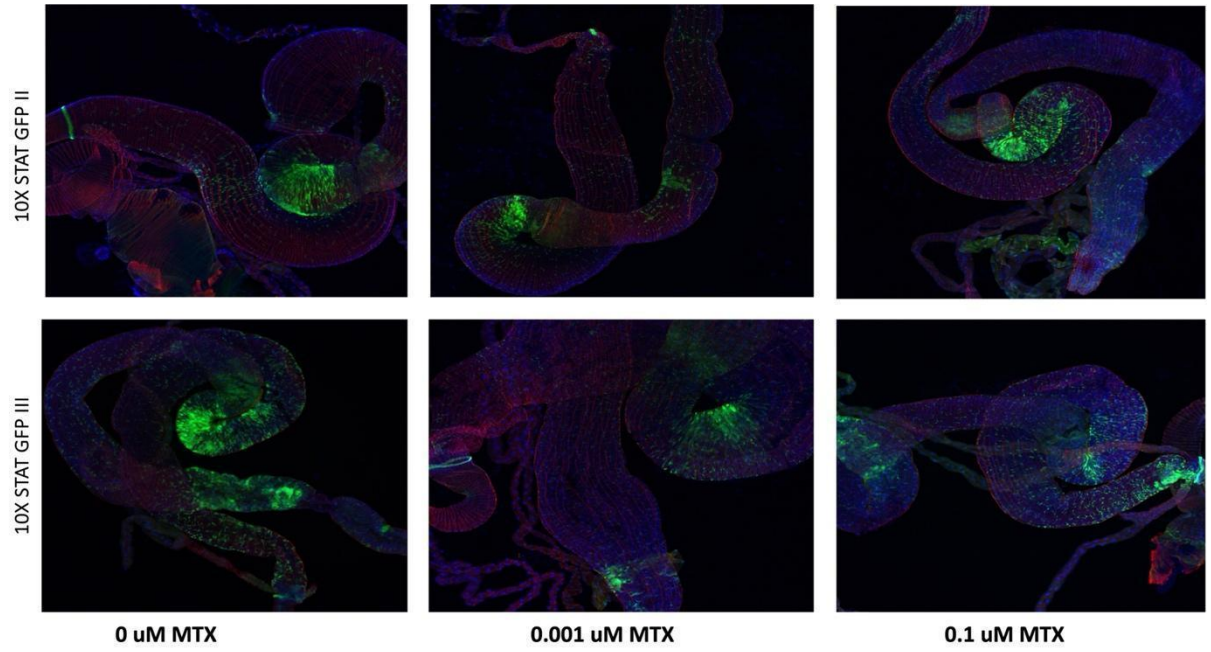

Figure 32: Confocal images of GFP in adult female gut: Levels of GFP in the gut of adult *Drosophila* with *10X STAT-GFP* on chromosomes II and III. These flies were fed varying concentrations of MTX (0 as control, 0.001, and 0.1 μM).

4.3 Discussion

DHFR is central to the folate pathway which is required for nucleotide production, and hence, DNA repair and synthesis. The DHFR enzyme is directly targeted by MTX (Robinson et al., 2020) which results in a decrease in the folate pool and subsequently leads to the indirect inhibition of other folate pathway enzymes such as thymidylate synthase and aminoimidazole carboxamide ribonucleotide transformylase (Baggott et al., 1993). Due to its potent inhibition of the folate pathway, MTX can cause toxic and teratogenic side effects (Hyoun et al., 2012). Even though the invertebrate system of *Drosophila* differs from that of mammals, the morphological abnormalities that appear in mammals following MTX administration are remarkably similar in *Drosophila*. In humans, exposure to MTX during pregnancy has been associated with a range of structural abnormalities that affect various organs and systems, such as the central nervous system, limbs, heart, and craniofacial region (Corona-Rivera et al., 2010). Similarly, MTX exposure can cause developmental defects that affect the body plan and

organ formation in *Drosophila*. These defects can manifest as an abnormal head shape, malformation of the sensory bristles, alterations in wing morphology, and defects in leg development (Affleck et al., 2006). Consequently, I aimed to investigate the mechanisms that underpin the regulation of healthspan and lifespan at low doses of MTX, such as DNA damage and repair, as well as compounds that can mitigate these toxic effects while improving the clinical effects of MTX using the *D. melanogaster* model. I chose to use this model as an alternative to sacrificing mammalian embryos for toxicological testing. Using this model organism, I evaluated mortality, female fecundity, and development, and studied selective pathways activated by MTX in *D. melanogaster*.

Exposure of *D. melanogaster* to up to 0.1 μM of MTX did not affect fly eclosure; however, exposure to between 1 and 4 μM of MTX resulted in a significant reduction in eclosure, which may be linked to the significant induction of DNA damage and serrated wings that were reported in Chapter Three. Importantly, low doses of MTX did not have an impact on embryo production or survival, suggesting that low doses of MTX did not cause toxicity via inhibition of the folate pathway.

Similar findings have been reported in previous studies, as MTX concentrations of > 5 ppm were shown to induce physiological effects in *D. melanogaster,* such as reduced egg production, flaccid ovaries, and underdeveloped follicles (Affleck et al., 2006). Interestingly, 5 ppm of MTX, with a molecular mass of 454 g/mol, represents a concentration of 11 μM; this value corresponds to the highest drug concentration I used, where I also saw reduced production of viable embryos. Another study showed that MTX at a concentration of 400 μg/Kg nutrients induced significant changes in wing morphology (Antosyuk and Suvorova, 2020). Assuming a food density of 1.2 g/mL, this would correspond to around 1 μM MTX Interestingly, I observed wing notches in flies exposed to ≥ 1 μM MTX (Chapter 3), further supporting our findings.

While the toxicity of MTX on humans using *Drosophila* as a model organism can provide insights into the mechanisms underlying MTX-induced toxicity, the fly model can also be used to investigate combinational therapy that could potentially provide benefits to cancer patients. I investigated the role of folinic acid in suppression of MTX-induced toxicity. Folinic acid, also known as leucovorin, is a

form of folate that can be used to counteract the toxic effects of MTX. Folinic acid is a reduced form of folic acid that can bypass the inhibition of DHFR caused by MTX, as it enters the folate metabolic pathway downstream of DHFR and can restore nucleotide synthesis and prevent the toxic effects of MTX. Folinic acid can also enhance the efficacy of MTX by increasing the intracellular concentration of reduced folates, which are necessary for the formation of the MTX-DHFR complex (van Ede et al., 2001). Studies in animal models, including *Drosophila*, have shown that folinic acid can mitigate the toxic effects of MTX. Treatment of the HEL leukaemia cell line with folinic acid after low dose MTX was shown to suppress STAT phosphorylation, which may have an impact on survival rates, although folinic acid directly inhibits JAK/STAT activity at the dose used (Thomas et al., 2015). Folinic acid has also been used in combination with MTX in clinical settings to reduce the toxic side effects of MTX therapy (Shea et al., 2013). In mice, the administration of folinic acid has been found to decrease the germ cell toxicity caused by MTX, as evidenced by reduced abnormalities in sperm head morphology, reduced seminiferous tubule damage, suppressed DNA damage in sperm, and an increase in sperm counts (Padmanabhan et al., 2009).

The results from this study showed that 1 μM folinic acid in combinational treatment with 4 μM MTX significantly mitigated MTX-induced inhibition of *Drosophila* survival, resulting in an increased number of developed flies. This is particularly a potentially positive finding, as it demonstrates the potential of folinic acid to mitigate the toxic side effects of MTX. As stated above, folinic acid is a reduced form of folic acid that can bypass the DHFR inhibition caused by MTX. Folinic acid enters the folate metabolic pathway downstream of DHFR, and can lead to restoration of nucleotide synthesis and prevent the toxic effects of MTX. Folinic acid can also enhance the efficacy of MTX by increasing the intracellular concentration of reduced folates, which are necessary for the formation of the MTX-DHFR complex. This may be the reason why 0.1 μM of folinic acid did inhibit MTX-induced toxicity, likely because the concentration was too low to produce the amount of folate that is required to supplement the deficiency in the folate metabolic pathway. Toxicity associated with high-dose methotrexate (MTX) in humans can be effectively reversed by folinic acid treatment, which is commonly used clinically in cases of MTX overdose or after acute MTX chemotherapy treatments (Hamed et al., 2022). F~~olinic acid~~

~~works by bypassing the inhibition of dihydrofolate reductase (DHFR), the enzyme targeted by MTX, thereby rescuing patients from toxicity.~~

Thomas et al. (2015) showed that MTX inhibits the JAK/STAT pathway, which is involved in cytokine signalling and inflammation. Their study found that MTX inhibits the JAK/STAT pathway in human cells and in *Drosophila,* but does not affect other phosphorylation-dependent pathways. Interestingly, another study also found that MTX inhibits T cell proliferation without affecting the expression of inflammatory cytokines in patients with HIV, an effect that could be reversed with folinic acid (Freeman et al., 2022), suggesting that the activity of MTX is mediated via the DHFR pathway.

Thus, I probed the activation of the JAK/STAT pathway by MTX (Figures 28 and 31). A dose-dependent reduction activation of JAK/STAT pathway was observed. However, all of the genetic mutants trialled, including a constitutively active mutant (HopTumI), the homozygous for STAT (HOM), and heterozygous $STAT^{+}/STAT^{-}$ (HET), produced the same readout for JAK/STAT pathway activation. This was unexpected since the HET only has one functional copy of the STAT transcription factor, which is supposed to result in half the output of the HOM construct. As such, it was proposed that this may be due to the inactivity of the reporter in the adult fly due to the swamping effect of other JAK/STAT activators being expressed in the flies. The constructs seem to be working as the controls produced outputs that are observable. To further confirm this result, a similar expression system conjugated to destabilised GFP was used. While a concentration-dependent reduction in JAK/STAT activity was observed, the level of activity was less than that for GFP constructs, which can be explained by the more rapid degradation of dGFP. Similarly, there was no difference in the activities of JAK/STAT in any the constructs, confirming the previous result. However, the Het; STAT/- and the Het; Tum/+ were indifferent in output which highly suggests swamping, since the whole adult fly was grounded for the ELISA analysis. This homogenate is composed of many different cell types, some with very high basal JAK/STAT activity and others with moderate activity which may overpower the overall signal, especially if the construct is not successfully expressed in all the cells.

Due to the possibility of a swamping effect from other factors that can induce activation of JAK/STAT signalling in the homogenate of whole adult fly, activation of the JAK/STAT pathway in the gut of the fly was examined using a Sandwich ELISA. While the first set of experiments did not show any influence of MTX on inhibition of the pathway, it was suggested this might be due to technical error in the first round of experiments. Indeed, a repeat of the experiment showed, for the first time, that low doses of MTX (0.001 μM to 0.1 μM) induced significant, dose-dependent inhibition of JAK/STAT signalling in the gut of *Drosophila*. This is in agreement with the findings of Thomas et al. (2015), who showed that MTX inhibits JAK/STAT signalling in human cell lines and *Drosophila*. Although, a steady increase in JAK/STAT signalling was observed at 1 and 4 μM MTX, the reason for the sudden increase in JAK/STAT signalling at higher concentrations is unclear. This effect may be due to MTX-induced stress promoting MTX-induced apoptosis. The JAK/STAT signalling pathway is a well-known mediator of cellular stress responses and has been implicated in MTX-induced apoptosis in various cell types, including cancer cells (Hu et al., 2021). MTX treatment has been shown to inhibit JAK/STAT signalling through inhibition of JAK/STAT phosphorylation in cancer cells, which in turn can induce apoptosis through a variety of mechanisms, including the upregulation of pro-apoptotic genes, the downregulation of anti-apoptotic genes, and the activation of caspase-mediated apoptotic pathways (Hu et al., 2021). In addition to its role in apoptosis, JAK/STAT signalling has also been implicated in the cellular response to MTX-induced stress. This pathway is known to regulate a variety of stress-response genes, including those involved in DNA repair, cell cycle regulation, and immune function, and may play a role in protecting cells from the toxic effects of MTX (Hu et al., 2021).

Taken together, this Chapter indicates that folinic acid in combination with low-dose MTX may provide an avenue to mitigate the side effects associated with MTX therapy in cancer. The novelty of this work lies in the demonstration that folinic acid can rescue MTX-induced toxicity in *Drosophila melanogaster*, particularly at higher MTX concentrations.Interestingly, the findings show that the high-dose toxicity of MTX likely operates via the same mechanism in *Drosophila* as in humans, despite the slight differences in dihydrofolate reductase between the two species. This is particularly remarkable given the 500 million years of evolutionary divergence, as one might expect significant

changes; however, our results reveal this mechanism remains conserved. Additionally, another key finding supports the hypothesis that low doses of MTX decrease JAK/STAT pathway activity *in vivo*, consistent with its known effects in cell-based studies. These results suggest a potential new therapeutic approach for minimizing MTX-related side effects while maintaining the efficacy of MTX in cancer treatment. Further investigation is needed to determine whether folinic acid has any direct or indirect effect on JAK/STAT pathway activity.

Furthermore, folinic acid in combination with low-dose MTX could act synergistically to prevent inflammation in cancer. One major limitation of this study was the issues related the method used to investigate JAK/STAT signalling, which was deficient in quantifying possible marginal changes in JAK/STAT activity. If time permitted, other techniques such as gene cloning and qPCR could be used to investigate genes that are modulated upon activation or deactivation of the JAK/STAT pathway. Furthermore, other *in vitro* or *in vivo* reporter systems such as the luciferase reporter system could be employed. These systems offer an advantage of real-time quantification of gene expression, which may possibly circumvent the swamping issue experienced with the ELISA.

4.4 Conclusion

The results from this chapter show that folinic acid mitigates the toxicity of MTX in adult *Drosophila*. Moreover, using an *in vivo STAT-GFP* reporter, it was shown that there is JAK/STAT activity in adult *Drosophila*. However, our finding shows the possibility of swamping effects that precluded the use of the reporter system to quantify GFP expression and subsequent JAK/STAT pathway activation. Investigation of JAK/STAT signalling within the gut of the adult fly showed low-dose MTX (≤ 0.01 μM) caused a dose-dependent and significant inhibition of JAK/STAT signalling. Although higher doses above 1 μM MTX resulted in moderate increases in JAK/STAT signalling, this is believed to be due to the stress induced by higher concentrations of MTX.

Chapter 5: DNA Sequencing Indicates Low-Dose MTX May Not Induce Mutations in *Drosophila melanogaster*

5.1 Introduction

In this chapter, I present the experiments conducted to evaluate the potential genotoxic effects of exposure to low-dose methotrexate (MTX) on genomic stability in *Drosophila melanogaster*. The study, designed and developed by Dr. Zeidler and Adel Alqarni (AA), involved AA conducting DNA extractions and genetic analysis. Data generation and analysis were performed by Novogen. Bioinformatic analysis of the sequencing data was assisted by Dr. Natalia Bulgakova and Dr. Martin Zeidler. This chapter was written by AA.

Genomic stability is fundamental for the preservation of genetic information across generations. Maintenance of an intact DNA structure is crucial for ensuring proper cellular function, and any disturbance in this equilibrium can have far-reaching consequences. One significant perturbation in genomic stability arises from exposure to genotoxic agents, which induce DNA damage (Yousefzadeh et al., 2021). MTX, a widely used chemotherapy drug, is known for its anti-folate properties and its efficacy in treating various forms of cancer (Koźmiński et al., 2020). However, as with many chemotherapeutic agents, the therapeutic benefits of MTX are often accompanied by genotoxic effects, including DNA damage (Alqarni and Zeidler, 2020).

Understanding the extent and mechanisms that lead to MTX-induced DNA damage is not only pivotal to comprehend the potential risks associated with its therapeutic use, but could also shed light on the broader aspects of genotoxicity and heredity. Investigating the transgenerational consequences of MTX-induced DNA damage in a model organism like *Drosophila melanogaster* provides a unique opportunity to explore the hereditary transmission of genomic instability. Due to its well-characterised genetics and relatively short generation time, *Drosophila* offers a valuable platform for deciphering how DNA damage induced by MTX exposure in one generation may impact the genomic integrity of subsequent generations.

The central hypothesis of this chapter is that low dose MTX can cause DNA damage. Although high doses of MTX (as shown in the *mwh*- LOH clone and *white*+ eye clone experiments shown in Chapter 3) induce significant DNA damage, the impact of low doses of MTX remains unclear. Previous data suggested that low doses of MTX might even decrease the clone frequency (Figures 16 &17). This effect could be due to the previously observed inhibition of JAK by MTX (Thomas et al., 2015), which may lead to STAT:HP1 dimer formation, and decrease levels of DNA damage *in vivo* (X.-J. Yan et al., 2011). As such, determining the actual effect of low levels of MTX on DNA damage rates would represent a key insight into what occurs in patients taking low-dose MTX for indications such as rheumatoid arthritis, Crohn's disease, and psoriasis. Thus, this chapter aims to evaluate the genotoxic effects of low-dose MTX in *Drosophila* and to investigate whether these mutations are passed down across generations.

Given the low frequency of spontaneous mutations likely to occur in untreated controls and in flies treated with low doses of MTX (Figures 16&17), assays such as the *mwh*- and *white*+ clonal approaches described previously are unlikely to be viable options. However, recent advances in sequencing technologies now provide an alternative. One of the simplest ways to investigate spontaneous mutations is to directly sequence the DNA to identify nucleotide changes and In-Dels using next-generation sequencing (NGS) technologies, approaches that are now available at a relatively low cost. As such, in the last part of this thesis, I investigated changes to the genomic DNA of flies that were either untreated (as controls) or treated with low doses of MTX. In *Drosophila*, this approach can easily be implemented because of the genetics of the system and the use of balancer chromosomes, which allows individual chromosomes to be identified and allows us to compare the X chromosome from a single male to the same chromosome carried two generations later by his grandsons (Figure 33). Therefore, it is possible to observe DNA damage that has occurred within two generations, by comparing the granddad's X chromosome with that inherited by the grandsons.

Moreover, if females carrying the paternal X-chromosome of interest are treated with low doses of MTX during development, any changes caused by MTX in the germline of these females will be passed on to their sons. This design allows us to investigate the potential transgenerational effects of MTX

exposure. By comparing MTX-treated flies to a negative control group, which has not been exposed to any drug, we can assess changes in the frequency, distribution, and nature of DNA alterations. The negative control will provide insights into the natural rate of spontaneous mutations in the genome. Additionally, a positive control group will be treated with high doses of MTX, which is expected to substantially increase DNA damage, as shown in previous *in vivo* experiments (Figures 16&17). It is hypothesised that flies exposed to low doses of MTX will exhibit a lower rate of genomic mutations, a finding that would be consistent with the results observed in Chapter 3, in that low MTX doses were shown to extend lifespan, improve climbing ability, and enhance healthspan.

5.2 Results

To evaluate and quantify the frequency (and possibly also the nature) of MTX-induced mutations, we developed an experimental approach in which a single X-chromosome present in a single w^{Dah} male fly (subsequently termed Grandad [GD]) was isogenically propagated to his grandsons via an intermediate generation treated with varying concentrations of MTX (Figure 33). This intermediate generation expresses the homozygous viable and fertile balancer chromosome FM7a [$In(1)sc^{8}$ +In(1)dl-49+In(1)15E-20A, y^{31d} w^{a} v^{Of} B] (Lattao et al., 2011), a balancer chromosome that contains the dominant Bar (B) mutation and multiple inversions which prevent meiotic recombination in the female germline (Lattao et al., 2011). Herein, this approach was harnessed to follow a single (and therefore isogenic) X chromosome.

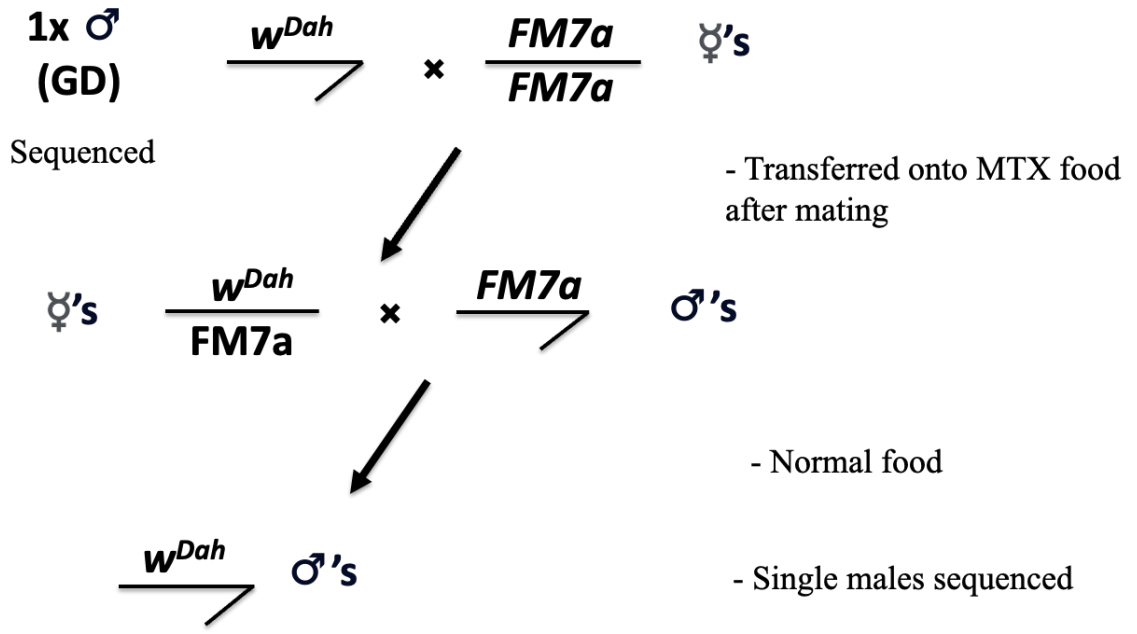

Figure 33: Scheme of the Sequencing Project: The experimental setup uses the FM7 balancer chromosome to suppress meiotic recombination and ensure accurate tracking of the X chromosome across generations. The FM7 balancer contains dominant markers that allow for easy visualization and identification of the X chromosome of interest. This design prevents recombination with the black X chromosome to ensure the chromosomal segment under study remains intact. MTX concentrations of 10 nM and 100 nM were selected, based on prior findings that concentrations above 1 µM cause significant DNA damage, to investigate whether these low MTX concentrations induce subtler genotoxic effects. By comparing the X chromosome in the initial generation (X) to the X* chromosome in subsequent generations, the rate of genetic differences and mutations induced could be quantified. A total of 1 granddad fly (GD) and 1 grandson fly per MTX condition (0, 10 nM, and 100 nM) were sequenced.

5.2.1 Mapping Statistics with Reference Genome

The average depth for each sample represents the average coverage of reads at each position across the genome. For example, sample GD has an average depth of 13.86x, while sample F5 has 29.14x, the control sample with no MTX has 46.07x, 10 nM MTX sample has 48.43x, and 100 nM MTX sample has 41.86x. This sequencing depth indicates how detailed the data is and how many times the genome has been covered, which is important for downstream analyses.

5.2.2 control assessment of sequencing data across multiple samples

The QC results supplied by Novogene demonstrated high sequencing quality across all samples, with effective rates above 97%, indicating strong data retention post-cleaning. The error rate was consistently low, at 0.03%, which indicates the precision and reliability of the sequencing process. The quality scores were robust, with Q20 values above 95.9% and Q30 scores exceeding 89%. These high Q scores indicate a very low error probability (less than 1 in 100 bases for Q20, and less than 1 in 1000 bases for Q30), indicating high precision and the accuracy and robustness of the data. Additionally, the GC content was stable around 41%, suggesting no significant biases or contamination. Overall, these metrics indicate that the sequencing data is of good quality and suitable for further genomic analyses (Figure 34).

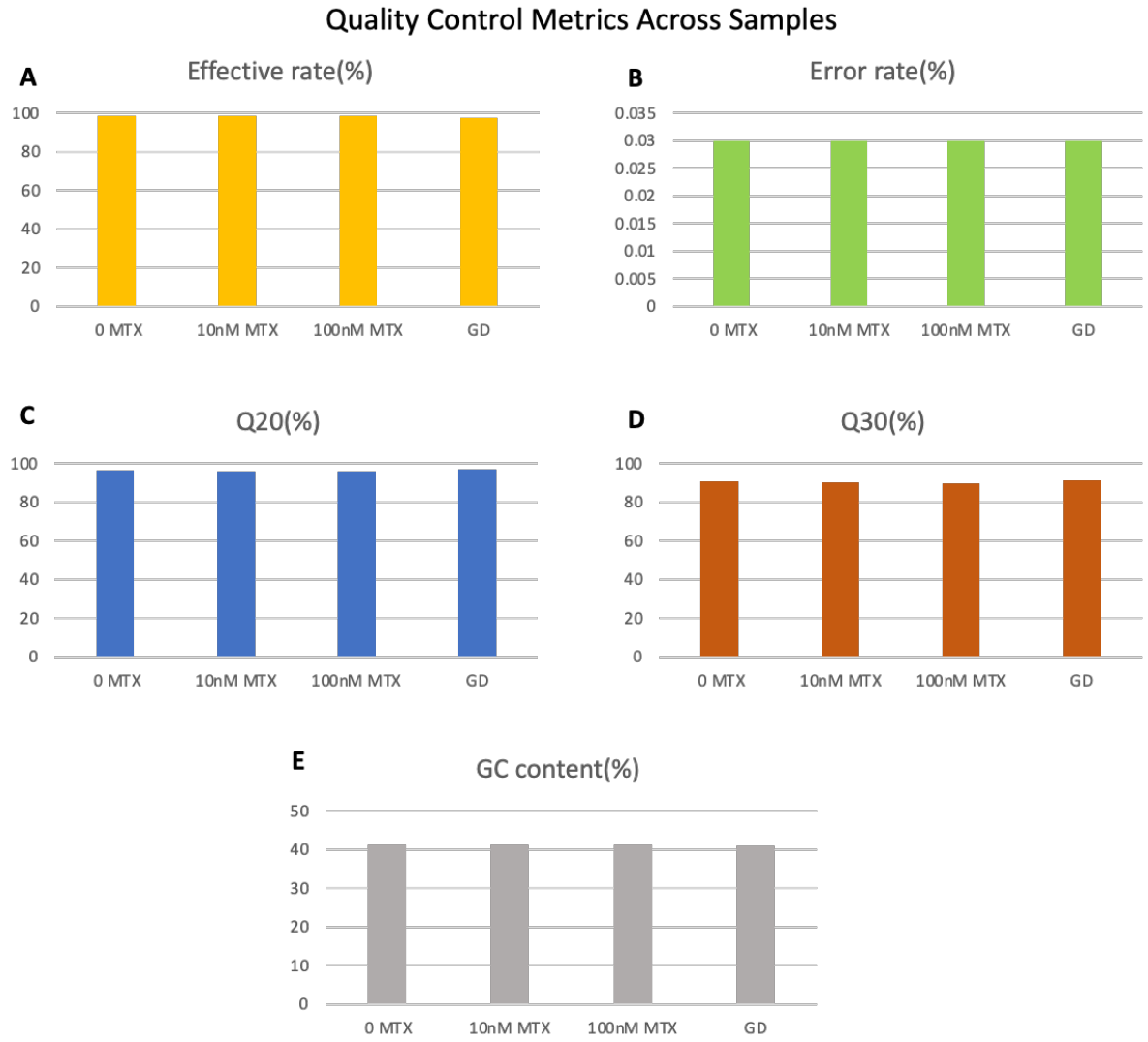

Figure 34. Quality control (QC) metrics for the sequencing data across samples: GD (granddad), 0, 10 nM MTX, and 100 nM MTX. Two groups of flies were examined: a control group not exposed to MTX and two experimental groups exposed to low doses of MTX (10 nM and 100 nM). Genomic DNA samples were collected from both the granddad (GD; initial generation) and each group of grandsons (subsequent generation) for DNA sequencing. (A) Effective rate (%), indicating the proportion of retained bases after cleaning; (B) Error rate (%), showing the percentage of erroneous bases; (C) Q20 (%), representing bases with a quality score ≥20 (99% accuracy); (D) Q30 (%), showing bases with a quality score ≥30 (99.9% accuracy); and (E) GC content (%), indicating the proportion of guanine and cytosine bases in the sequence. High effective rates, low error rates, and consistent Q20/Q30 values reflect the high accuracy and quality of sequencing data, while stable GC content indicates minimal bias.

A

Nr SNP differences v GD				
Sample	Differences to GD	Similarities to GD	% Different	% of Xchr
0 v GD	1226	47584	2.51	0.0056
10nM v GD	1221	47589	2.50	0.0056
100nM v GD	1272	47549	2.61	0.0058

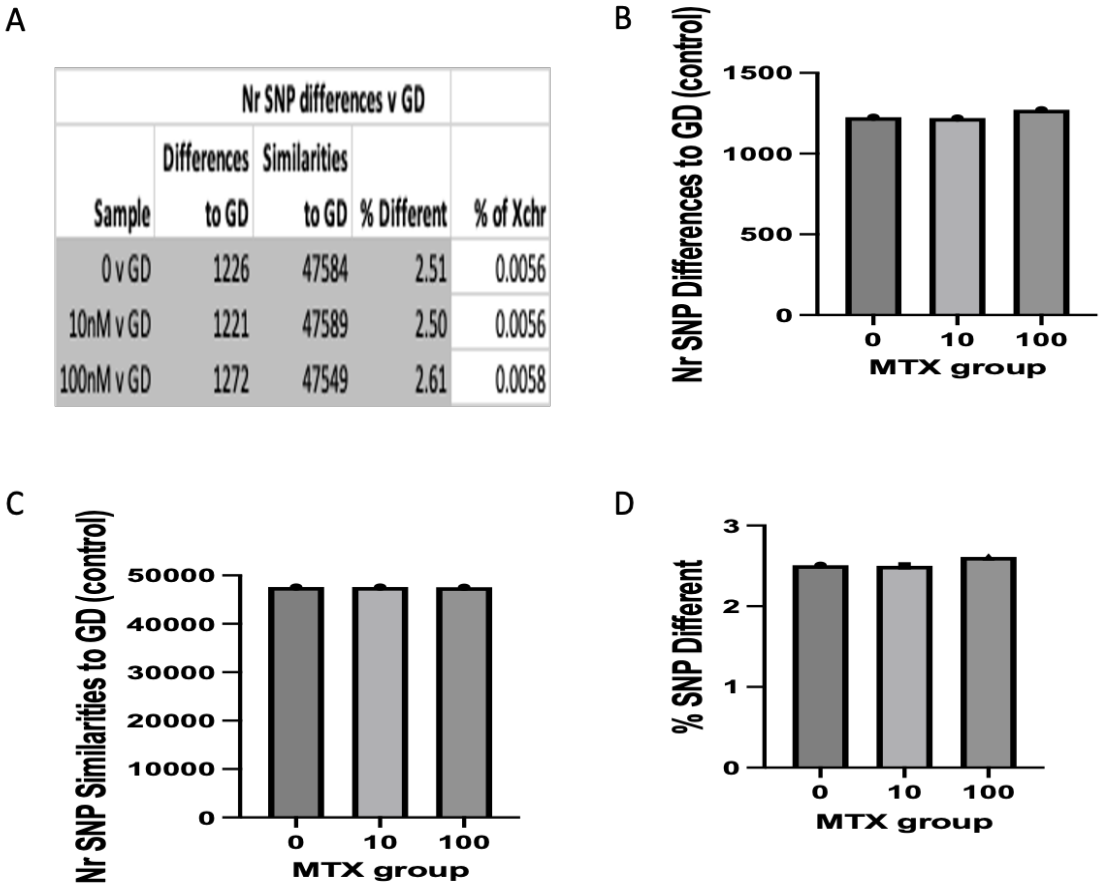

Figure 35. Transgenerational analysis of SNPs on X-chromosome DNA in response to MTX exposure. The potential induction and inheritance of mutations caused by MTX exposure over two generations in *Drosophila*. Two groups of flies were examined: a control group not exposed to MTX and two experimental groups exposed to low doses of MTX (10 nM and 100 nM). Genomic DNA samples were collected from both the granddad (GD; initial generation) and each group of grandsons (subsequent generation) for DNA sequencing. (A) Summary table, (B) the number of different SNPs in both generations, (C) the number of similar SNPs in both generations, and the (D) percentage of different SNPs in both generations. Data is represented as mean ± SD. A total of one granddad fly (GD) and one grandson fly per MTX condition (0, 10 nM, and 100 nM) were sequenced.

Novogene conducted sequencing on all DNA samples, retaining only the reads mapping to the X chromosome of *Drosophila melanogaster*. By focusing on the X chromosome, we were able to closely track the potential impact of MTX exposure specifically on this critical genetic region, which is essential to understand how such treatments might affect hereditary traits. These reads were compared to the

RefSeq, a reference sequence generated from a different strain many years ago. Variants were identified and classified into three categories: SNPs (single nucleotide polymorphisms or base changes), small insertions and deletions (In-Dels), and exonic variants, which are changes that theoretically alter protein sequences.

Most of the differences observed between the newly sequenced data and the RefSeq are likely attributable to variations in genetic background and genetic drift that have occurred since the RefSeq was generated. These differences are common to both the GD and grandsons. Thus, the analysis focused on differences between the grandsons exposed to varied concentrations MTX and the RefSeq, rather than the differences between GD and the RefSeq. These include positions where the GD is the same as RefSeq, but the grandsons have a different sequence. Considering the natural genetic variability and evolution over time, the discrepancies between the RefSeq and the new sequences also highlight the challenges of using a long-standing reference genome for accurate, up-to-date genetic comparisons.

5.2.3 Data analysis and definition of key terms

We focused on the following variations during the analysis:

Single Nucleotide Polymorphisms (SNPs): SNPs, point mutations at a single nucleotide position, are a common type of genetic variation. In our analysis, the SNP profiles of the grandsons and GD were compared to evaluate potential mutations induced by MTX exposure. Comparisons of SNPs is crucial to understand whether MTX exposure at low concentrations might induce subtle but potentially inheritable genetic changes that could accumulate over generations, and potentially influence phenotypic traits.

Insertion-Deletion Mutations (In-Dels): In-Dels are small additions or deletions of nucleotide sequences within the genome. These mutations were analysed in both the intronic (non-coding) and exonic (coding) regions of the X chromosome to assess the possible functional impacts of MTX exposure. By examining both coding and non-coding regions, we aimed to capture a comprehensive

view of how MTX might influence not only the structure of genes, but also regulatory regions that could affect gene expression and cellular function.

The analysis of the X-chromosome DNA revealed that SNP profiles were consistent between the GD and all groups of grandsons, regardless of MTX exposure. Similarly, the number of In-Dels was comparable across all control and treatment groups.

In addition, assessment of In-Dels in the intronic and exonic genomic locations showed that the number of In-Dels in all MTX-treated groups and control groups of grandsons were the same as the GD (Figures 36&37). Furthermore, analysis of the similarity of the In-Dels showed that all the groups exhibited similar In-Dels as the grandfather, in a similar trend to the findings for the SNPs.

This indicates that low-dose MTX exposure ($\leq$100 nM) did not significantly alter the mutation patterns or rates in the X chromosome across generations. This is suggestive of the same mutations passed down from the grandfather to the grandsons.

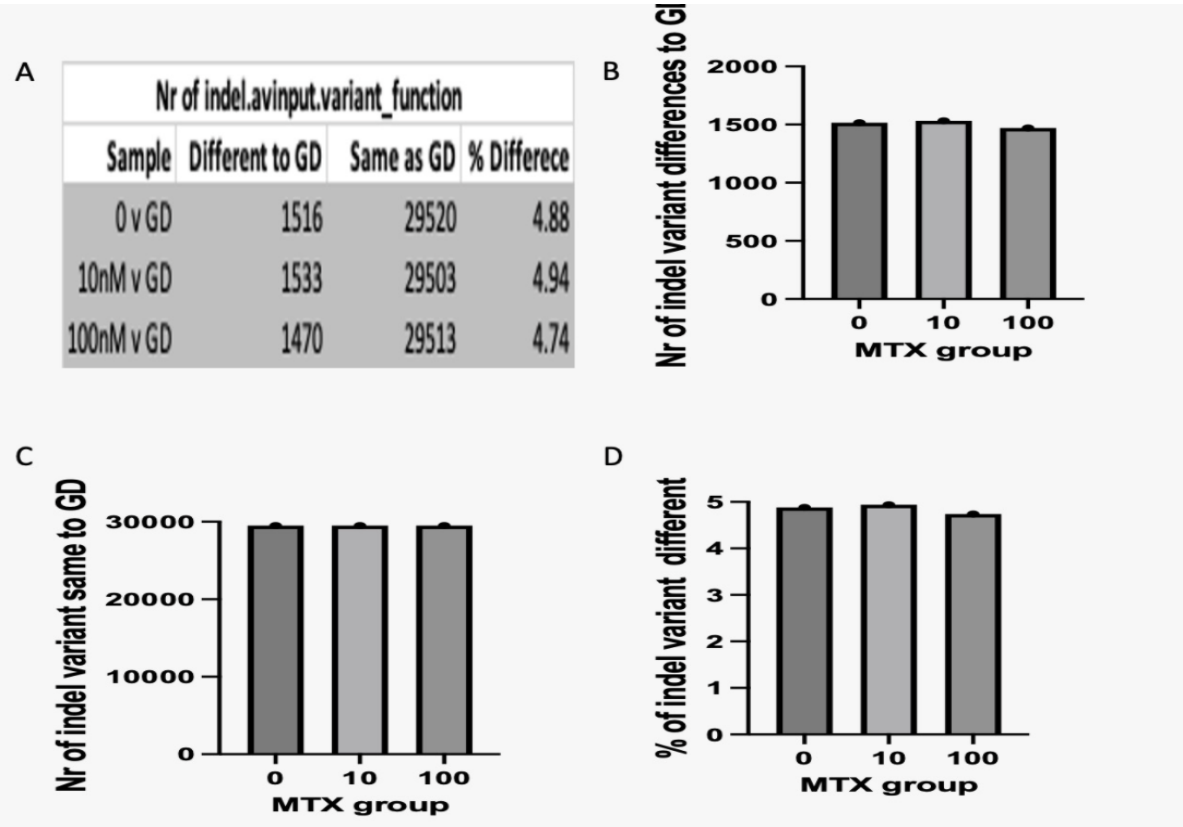

Figure 36. Transgenerational analysis of In-Dels in intronic regions of X-chromosome DNA in response to MTX exposure. An unexposed control group and flies exposed to low doses of MTX (10 nM and 100 nM) were assessed for In-Del DNA mutations. Genomic DNA samples were collected from the granddad (GD) and three grandsons for DNA sequencing. (A) Summary table, (B) the number of different In-Dels in both generations, (C) the number of similar In-Dels in both generations, (D) the percentage of different In-Dels variants in both generations. Data is represented as mean ± SD. A total of one granddad fly (GD) and one grandson fly per MTX condition (0, 10 nM, and 100 nM) were sequenced.

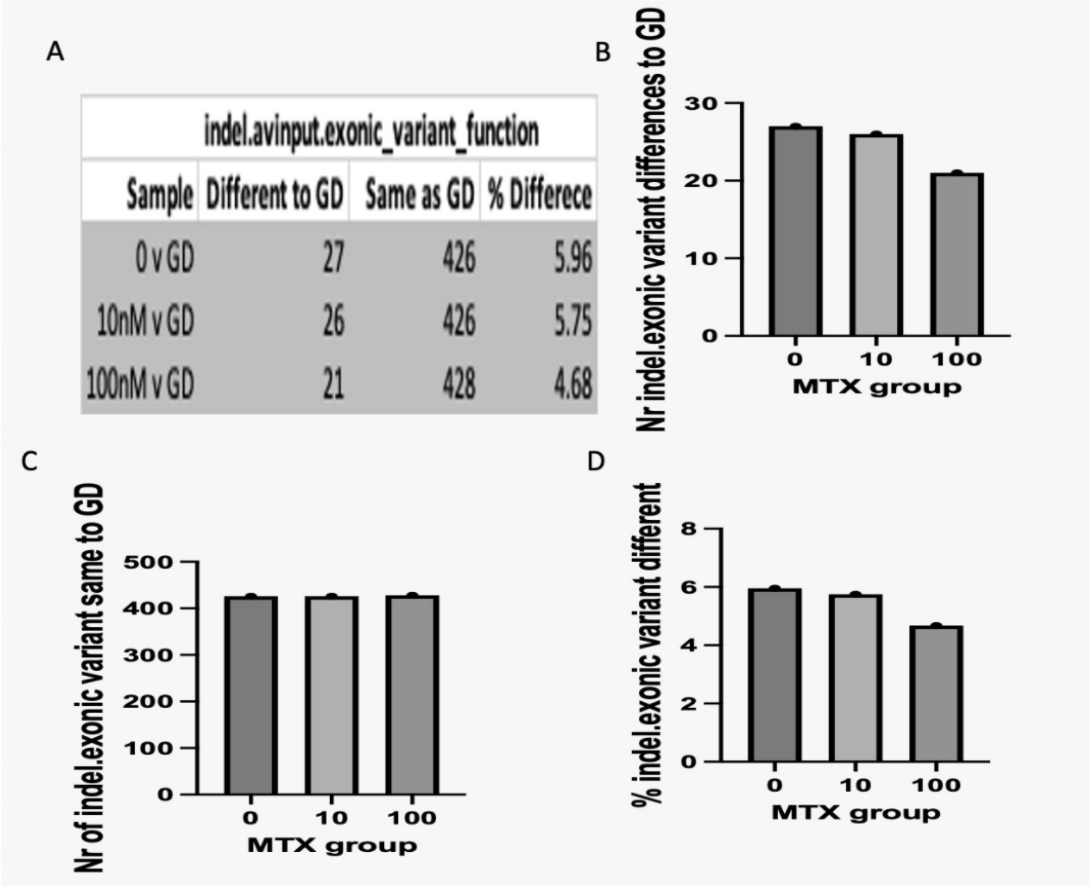

Figure 37. Transgenerational analysis of In-Dels in exonic regions of X-chromosome DNA in response to MTX exposure. An unexposed control group and flies exposed to low doses of MTX (10 nM and 100 nM) were assessed for In-Del DNA mutations. Genomic DNA samples were collected from the granddad (GD) and three grandsons for DNA sequencing. (A) Summary table, (B) the number of different In-Dels in both generations, (C) the number of similar In-Dels in both generations, (D) the percentage of different In-Dels variants in both generations. Data is represented as mean ± SD. A total of one granddad fly (GD) and one grandson fly per MTX condition (0, 10 nM, and 100 nM) were sequenced.

5.3 Discussion

The initial experiments reported in Chapter 3 showed that high concentrations of MTX (>1 μM) induced high levels of DNA damage and significantly affected the fertility of female flies and the development of larvae, and negatively impacted the climbing ability of adult male and female flies. Conversely, low concentrations of MTX (typically below 1 μM) did not affect any of these biological processes. Therefore, I conducted a DNA sequencing experiment to try to detect MTX-induced mutations and

examine the mutation rate between two generations in files exposed to low concentrations of 10 nM and 100 nM. This investigation into MTX-induced mutations across generations employed a balancer chromosome as a genetic tool, has yielded intriguing insights into the hereditary transmission of mutations in *Drosophila* to enable the precise tracking of specific chromosomal regions, specifically the X chromosome, and thereby investigate the potential inheritance patterns of genetic mutations induced by low-dose MTX in this chromosome.

In an attempt to assess whether MTX exposure induces inheritable mutations, I carried out an experiment comprising control flies untreated with MTX and exposed groups exposed to 10 nM and 100 nM MTX. The aim was to evaluate the occurrence of mutations that might spontaneously emerge and be passed down from the granddad (GD) to the grandsons. Sequencing of the X-chromosome DNA revealed a striking similarity in the grandsons' SNP profiles compared to the GD, in a manner that was not MTX-dependent. These findings support the results presented in Chapter 3, in that a MTX concentration ≤100 nM did not induce DNA damage or influence eclosion or wing serration.

One noteworthy implication of this data, within the constraints of this preliminary study's experimental design and sample size, it appears that exposure to MTX at relatively low concentrations did not lead to an observable increase in inherited mutations in the X chromosome DNA. This finding indicates the complexity of genotoxic mechanisms and the need for further investigation to ascertain whether low-dose MTX may sensitize the flies to radiation-induced DNA damage.

However, it is important to note that this study has limitations, including the relatively small sample size and the focus on a specific genomic region, i.e., the X chromosome. Further research with larger sample sizes and a broader genomic scope is needed to comprehensively elucidate the transgenerational effects of MTX exposure. Nonetheless, the findings presented here provide a starting point for future investigations into MTX-induced DNA damage and its possible transmission to subsequent generations, which may have broader implications for our understanding of genotoxicity and inherited mutations in other biological systems.

Chapters Three showed that MTX concentrations above 1 μM result in DNA damage and also significantly negatively influenced biological processes in *Drosophila,* such as formation of eye clones, wing serration, and egg eclosion. These findings are in agreement with studies that show that high concentrations of MTX >1 μM exert toxic side effects such as hepatotoxicity, neurotoxicity, and nephrotoxicity (Affleck et al., 2006). To further investigate the effect of high-dose MTX on the development of heritable mutations, a follow-up experiment could be conducted using a combination of low and high doses, with 1 μM MTX serving as a positive control. This approach would also involve unexposed negative control GD for comparison. Analysing the outcomes of such an experiment could provide valuable insights into the mutagenic potential of high-dose MTX and its impact on heritable genetic changes. Furthermore, by comparing the mutation rates between groups exposed to different dose of MTX, we could assess whether high doses induce a higher frequency of genetic alterations that are transmitted across generations. This would also help determine if there is a threshold dose at which MTX exposure significantly alters genomic integrity, which could have implications for its safe therapeutic use in humans. Additionally, this experiment could reveal potential dose-dependent effects, and shed light on the broader genetic consequences of MTX exposure, especially at clinically relevant concentrations.

5.4 Conclusion

This research analysed the effects of low-dose methotrexate (MTX) on genomic stability in *Drosophila melanogaster* throughout two generations by assessing both SNPs and insertion-deletion (In-Dels) mutations in the X chromosome. Analysis of X chromosome inheritance was followed through balancer chromosomes to enable accurate measurements of the mutation rate. Exposure to MTX concentrations of 10 nM and 100 nM resulted in no substantial increase of SNP or In-Del mutations compared with untreated controls. No detectable DNA damage or heritable genomic changes were detected, as the granddads (GD) and their groups of low-dose MTX-treated grandsons exhibited similar patterns of mutations. These findings support the results in Chapter 3 of the minimal effects of low-dose MTX in fertility, larval development, and climbing ability.

These experimental outcomes indicate low-dose MTX treatment poses no risk to genomic integrity; however, further research using larger sample sizes and broader analysis of genetic material is required to confirm whether MTX affects genomic stability. In addition, further assessments should investigate the cumulative effects of low-dose MTX among people with genetic predispositions and the *Drosophila* genetic safety assessments must be extended to different organisms. Overall, this research demonstrates low-dose MTX may be safe for non-cancer medical applications and paves the way for future studies of propagation of genetic damage and intergenerational genetic inheritance.

Chapter 6: General Discussion and Conclusion

6.1 Chapter 3: Low-Dose Methotrexate May Prevent DNA Damage and Increase Lifespan and Climbing in *Drosophila melanogaster*

The multifaceted effects of methotrexate (MTX), especially at varying doses, have been a subject of extensive research over the years. The findings presented in this chapter align with a growing body of evidence that underscores the differential impacts of MTX based on its dosage. At high concentrations, MTX has been shown to induce DNA damage, inhibit fecundity, and adversely affect both larval development and adult lifespan (Affleck et al., 2006). This is consistent with the known roles of MTX in cellular production of dNTPs (Taylor et al., 1982). However, the narrative changes significantly when the dose is reduced. Low-dose MTX, as evidenced by the present study and corroborated by others, does not induce DNA damage or impact DNA repair pathways (Bedoui et al., 2019). This is a significant revelation, especially considering the widespread use of low-dose MTX in treating chronic inflammatory diseases like rheumatoid arthritis (Alqarni and Zeidler, 2020a). The absence of DNA damage and alterations in DNA repair pathways after low-dose MTX treatment suggests that such therapy may be less toxic compared to high-dose treatments (Egan and Sandborn, 1996). This is further supported by studies that have shown that low-dose MTX treatment in elderly patients appears to be safe and well-tolerated (Hirshberg et al., 2000).

Furthermore, the potential lifespan-extending effects of low-dose MTX in *Drosophila melanogaster* might hint at broader implications for other organisms, including humans (Seong et al., 2011). The underlying mechanisms, such as the MTX effect on reducing the rate of DNA synthesis and slowing down DNA damage due to ageing, have been previously explored (Bitto et al., 2016). The JAK/STAT pathway, which has been implicated in the regulation of various cellular processes, including the proliferation and survival of immune cells, might play a pivotal role in these observed effects (Tzeng et al., 2021).

The differential effects of MTX on male and female *Drosophila* in terms of locomotion further add a layer of complexity to the drug's impact. While the exact reasons for enhanced locomotion in male flies post MTX exposure remain elusive, it's plausible that pathways activated by low-dose MTX, such as the JAK/STAT pathway, might be involved (Affleck et al., 2006).

In the broader context of cancer treatment, the combination of MTX with radiation has been explored as a potential therapeutic strategy (Condit et al., 1964). MTX's ability to sensitize cancer cells to radiation, enhancing the effectiveness of radiation therapy, has been documented in various cancer types(Condit et al., 1964). However, the potential toxic effects of MTX, especially at high doses, have been a concern, limiting its broader application in cancer treatment (Paxton, 1979). In conclusion, the findings from this chapter, juxtaposed with existing literature, emphasize the nuanced effects of MTX based on its dosage. The potential benefits of low-dose MTX, especially in terms of reduced toxicity and potential lifespan extension, warrant further exploration and could pave the way for novel therapeutic applications.

6.2 Chapter 4: Folinic Acid Bypasses the Toxic Effect of MTX on Fly Eclosion, and Low-Dose MTX May Inhibit JAK/STAT Activity in the *Drosophila* Gut

The intricate interplay between methotrexate (MTX) and the folate pathway, as well as its downstream effects on DNA synthesis and repair, has been a focal point of research for decades. MTX's potent inhibition of the folate pathway, primarily via its target DHFR, culminates in a cascade of effects that can lead to toxic and teratogenic outcomes (Wang et al., 2022). The parallels drawn between the morphological abnormalities observed in mammals and *Drosophila* post-MTX exposure underscore the drug's profound impact across species, highlighting its potential as a model for understanding MTX's molecular mechanisms (Affleck et al., 2006). The findings presented in this chapter shed light on the nuanced effects of MTX at varying concentrations. While low doses of MTX (up to 0.1 μM) appeared with reduced DNA damage, with no discernible impact on fly eclosure or embryo production,

concentrations between 1 and 4 μM manifested in significant reductions in eclosure, DNA damage, and wing morphological aberrations (Affleck et al., 2006). This dose-dependent response aligns with previous studies, further solidifying the understanding of MTX's physiological effects in *Drosophila*.

A particularly intriguing aspect of this research is the potential therapeutic application of folinic acid in mitigating MTX-induced toxicity. Folinic acid's ability to bypass DHFR inhibition and restore nucleotide synthesis offers a promising avenue for reducing the adverse effects of MTX, especially in cancer therapy (Shea et al., 2013). The observed mitigation of MTX toxicity by folinic acid, especially at a concentration of 1 μM, underscores its potential clinical significance. This is further bolstered by the drug's capability to enhance MTX's efficacy, possibly by augmenting intracellular concentrations of reduced folates essential for MTX-DHFR complex formation (Jolivet and Chabner, 1983).

The modulation of the JAK/STAT pathway by MTX, especially its inhibition at low doses, provides insights into the drug's broader cellular impacts. The observed dose-dependent inhibition of JAK/STAT signalling, coupled with the pathway's known roles in cellular stress responses and apoptosis, suggests that MTX might exert some of its effects through this pathway (S. Thomas et al., 2015b). The resurgence of JAK/STAT activity at higher MTX concentrations might be indicative of a cellular stress response, potentially preceding MTX-induced apoptosis (Thomas et al., 2015). However, the study was not without its limitations. The employed methods for investigating JAK/STAT signalling, particularly the GFP reporter system, might have masked marginal changes in pathway activity. Future research could benefit from more sensitive techniques, such as qPCR or luciferase reporter systems, to elucidate the finer nuances of MTX's effects on the JAK/STAT pathway. In conclusion, this chapter offers a comprehensive understanding of MTX's molecular mechanisms, especially its interaction with the folate pathway and the JAK/STAT signalling pathway. The potential therapeutic implications of folinic acid in mitigating MTX's side effects, combined with the insights into MTX's dose-dependent effects, pave the way for more targeted and effective therapeutic strategies in the future

6.3 Chapter 5: DNA Sequencing Indicates Low-Dose MTX May Not Induce Mutations in *Drosophila melanogaster*

The intricate relationship between MTX exposure and its potential genotoxic effects in *Drosophila melanogaster* has been a focal point of this research. Initial findings, as detailed in Chapter 3, highlighted the detrimental impacts of high MTX concentrations on DNA integrity, eclosion rates, and adult fly climbing and life span. However, the absence of observable adverse effects at concentrations below 1μM presented an intriguing avenue for further exploration, particularly in the context of heritable mutations (Ali et al., 2014). The subsequent investigation into the potential transgenerational effects of MTX on NGS experiment, leveraging the precision of balancer chromosomes, provided invaluable insights. Specifically, sequencing has been done to examine the mutation rate between two generations which are single grandad and his grandsons. The study's focus on the X chromosome, a critical genomic region, revealed that low MTX concentrations (10 nM and 100 nM) did not induce heritable mutations. This observation aligns with the earlier findings from Chapter 3, where MTX concentrations ≤100 nM did not manifest in DNA damage or any discernible alterations in biological processes such as eclosion, red clones, or wing serration. While these results might suggest that low MTX concentrations do not pose a significant genotoxic threat, it's imperative to approach these findings with caution. The study's design, with its emphasis on the X chromosome and a limited sample size, might not capture the entirety of MTX's potential genotoxic landscape. Furthermore, the absence of observable heritable mutations at these concentrations does not preclude the possibility of MTX sensitizing flies to other genotoxic agents, such as radiation. The established knowledge that MTX concentrations exceeding 1 μM can induce DNA damage and adversely affect various biological processes in *Drosophila* underscores the drug's dose-dependent genotoxic potential (Ali et al., 2014). The decision to extend the study to include this higher concentration, serving as a positive control, is commendable. However, the pending analysis of this data set leaves a gap in our understanding. Future research endeavours should prioritize this analysis to provide a more comprehensive picture of MTX's genotoxic effects across a broader concentration spectrum. In conclusion, while the current study offers

valuable insights into the potential hereditary impacts of MTX exposure in *Drosophila*, it also underscores the need for more extensive investigations. The nuanced interplay between MTX concentrations, DNA damage, and heritable mutations demands a multifaceted research approach, encompassing broader genomic regions and larger sample sizes, to truly unravel the complexities of MTX-induced genotoxicity.

6.4 Conclusion

The comprehensive exploration of MTX effects, spanning from its molecular interactions with the folate pathway to its potential genotoxic implications in *Drosophila melanogaster*, has provided a multifaceted understanding of this drug's impact. At the heart of these findings lies the recurring theme of dose-dependent effects. High concentrations of MTX consistently demonstrate adverse outcomes, including DNA damage, reduced fecundity, and compromised climbing and life span in adult flies. In stark contrast, low MTX concentrations, particularly those below 1 μM, appear to be with relatively minimal or no observable DNA damage or heritable mutations. This differential response underscores the drug's complex interaction with cellular pathways, notably the folate pathway and the JAK/STAT signalling pathway. The utilization of balancer chromosomes as a genetic tool has been instrumental in tracking hereditary transmission of mutations, particularly in the X chromosome. While the absence of heritable mutations at low MTX concentrations is reassuring, it also raises pertinent questions about the broader genomic implications and the potential for MTX to sensitize organisms to other genotoxic agents. The findings also emphasize the potential therapeutic value of folinic acid, which can mitigate MTX-induced toxicity and enhance its efficacy. However, as with any scientific endeavour, this research is not without its limitations. The focus on specific genomic regions, such as the X chromosome, and the constraints of the sample size, may not capture the full spectrum of MTX's genotoxic potential. Furthermore, the pending analysis of data from higher MTX concentrations leaves certain questions unanswered. In synthesizing the insights from these discussions, it becomes evident that while significant strides have been made in understanding MTX's multifaceted effects, there remains a vast landscape of uncharted territory. Future research endeavours, equipped with broader

genomic analyses and larger sample sizes, will be pivotal in unravelling the intricacies of MTX-induced genotoxicity. As the scientific community continues to probe the depths of MTX's impact, these findings serve as both a foundation and a beacon, guiding the path towards a more comprehensive understanding of this drug's role in biology and medicine.

6.5 Future work

The findings of this study open up several avenues for future research, particularly in understanding the differential effects of low and high doses of MTX on DNA damage, fecundity, and lifespan. The intriguing observation that low-dose MTX enhanced the lifespan of *Drosophila* warrants further investigation. Longitudinal studies could be designed to examine the long-term effects of low-dose MTX on aging and age-related diseases, potentially extending these findings to mammalian models or even clinical trials.

Furthermore, the observed sex differences in the effects of MTX on locomotion in *Drosophila* could be explored in greater detail, possibly examining hormonal influences and their interaction with MTX treatment. Understanding these sex-specific effects could have implications for personalized medicine approaches in the treatment of diseases like cancer and rheumatoid arthritis. Overall, the results of this study lay the groundwork for a more nuanced understanding of MTX's effects, offering the potential for safer and more effective therapeutic strategies.

In addition, the study provides a foundation for further inquiries into MTX-induced toxicity and the mitigating role of folinic acid, highlighting the need for more refined methods to investigate JAK/STAT signalling. Future research could benefit from employing advanced techniques like gene cloning and qPCR for real-time quantification of gene expression, and other reporter systems to circumvent the 'swamping effect' experienced with ELISA. Additionally, the dose-dependent effects of MTX and the potential mitigating role of folinic acid warrant further exploration, including studies designed to examine the synergistic effects of low-dose MTX and folinic acid in cancer-related inflammation.

Finally, more experiments needed to look at fly gut health following exposure to MTX – given what has already been published regarding JAK/STAT, guts and longevity.

The preliminary findings of NGS study open several avenues for future research. Immediate attention should be given to analysing the pending high-dose MTX experiment as positive control which I did in the second experiment and is still on progress to be analysed, as it could provide critical insights into the development of heritable mutations; a larger sample size could offer a more comprehensive understanding of MTX-induced mutations. Additionally, it would be valuable to investigate whether low-dose MTX sensitizes flies to radiation-induced DNA damage. Future work could also explore MTX's impact on other physiological and developmental aspects of *Drosophila*. These future studies would not only deepen our understanding of MTX's effects but also have broader implications for genetics, toxicology, and medicine.

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6.7 List of appendices

$$C_1V_1 = C_2V_2$$

C_1= 50 µM C_2= 1 µM
V_1= ml/L V_2= 200 ml/L

$$V_1 = \frac{0 \times 10^{-6} \times 200}{(50 \times 10^{-3})} = 0$$

$$V_1 = \frac{0.1 \times 10^{-6} \times 200}{(50 \times 10^{-3})} = 4 \times 10^{-4} = 0.0004 \text{ ml} = 0.4 \text{ µl}$$

$$V_1 = \frac{0.001 \times 10^{-6} \times 200}{(50 \times 10^{-3})} = 4 \times 10^{-6} = 0.000004 \text{ ml} = 0.004 \text{ µl}$$

$$V_1 = \frac{1 \times 10^{-6} \times 200}{(50 \times 10^{-3})} = 4 \times 10^{-3} = 0.004 \text{ ml} = 4 \text{ µl}$$

$$V_1 = \frac{0.01 \times 10^{-6} \times 200}{(50 \times 10^{-3})} = 4 \times 10^{-5} = 0.00004 \text{ ml} = 0.04 \text{ µl}$$

$$V_1 = \frac{4 \times 10^{-6} \times 200}{(50 \times 10^{-3})} = 16 \times 10^{-3} = 0.016 \text{ ml} = 16 \text{ µl}$$

Appendix A: MTX Drug Concentration and Fly Food Preparation Equations. This figure displays the equations used to prepare fly food containing varying concentrations of MTX, ranging from 0 nM to 4000 nM.

Appendix B: List of antibodies and reagents required for the sandwich ELISA

Capture Antibody	Abnova goat anti-GFP antibody, PAB10341
Primary Antibody	Rabbit anti-GFP, abcam ab290.
Secondary Antibody	Anti-Rabbit HRP : Supplier: Thermo Fisher Scientific (Life Technologies), Cat no: A16106
10mg tablet o-phenylenediamine	Supplier: SLS (Scientific Laboratory Supplies), Catalogue No: P8287-50TAB, Mfr. Part No: P8287-50TAB cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail : Roche #11836170001
Costar 96 well high-binding EIA plate	Supplier: Fisher Scientific Ltd, Catalogue No: 10544522, Mfr. Part No: 3590

Appendix C: List of buffers required for the sandwich ELISA

Buffers	Components
ELISA lysis buffer	1mM $MgCL_2$, 0.1% (w/v) BSA, 0.5% Triton-X 100 supplemented with cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail.
ELISA wash buffer	PBS, 0.2% (w/v) BSA, 0.5% Triton-X 100).
HRP assay buffer	51mM Na_2HPO_4, 27mM citric acid, pH 5.0 store filtered (0.2μm).
HRP developing solution	0.012% H_2O_2, 0.4mg/ml o-phenylenediamine in HRP assay buffer (prepared fresh for each assay).
Stop Solution	2M H_2SO_4

Appendix D: Summary of 10xSTAT-GFP Reporter Lines and Genotypes. (Bach et al., 2007).

Line Code	**Reporter Type**	**Chromosome**	**Genotypes Used**	**Description**
B5	*10xSTAT-dGFP*	II	*w1118, STAT^–/+, hopTumL/+*	STAT-responsive destabilised GFP (dGFP) reporter inserted on chromosome II.
B6	*10xSTAT-dGFP*	III	*w1118, STAT^–/+, hopTumL/+*	STAT-responsive destabilised GFP (dGFP) reporter inserted on chromosome III.
B7	*10xSTAT-GFP*	II	*w1118, STAT^–/+, hopTumL/+*	STAT-responsive stable GFP reporter inserted on chromosome II.
B8	*10xSTAT-GFP*	III	*w1118, STAT^–/+, hopTumL/+*	STAT-responsive stable GFP reporter inserted on chromosome III.
—	Positive Control	—	*Act-GFP*	Ubiquitously expressed GFP driven by Actin5C promoter.

—	Negative Control	—	*OreR*	Wild-type control flies not expressing GFP.

Appendix E: Median Survival table for the lifespan graphs shown in Figure 19.

	Experiment one		**Experiment Two**	
	Number of flies per group	**Median duration of survival (days)**	**Number of flies per group**	**Median duration of survival (days)**
Control	200	53	400	48
0.001 μM MTX	200	55	400	48
0.01 μM MTX	200	55	400	48
0.1 μM MTX	200	55	400	48
1 μM MTX	200	51	-	-
4 μM MTX	200	44	-	-
10 μM MTX	200	46	-	-

Appendix F: Calculation of relative and absolute GFP from ELISA

1. **Background subtraction**

   Blank wells (n=4): A_450 = 0.045, 0.047, 0.044, 0.046 → $\overline{A_{450}^{\text{blank}}} = 0.0455$.

   Sample well: A_450(sample) = 0.312 → $A_{450}^{\text{corr}} = 0.312 - 0.0455 = 0.2665$.

2. **Relative GFP (Fig. 31C style)**

   0 µM MTX control (n=3) on same plate after background subtraction: 0.251, 0.259, 0.246 → mean = 0.252.

   Relative GFP (ratio) = 0.2665 / 0.252 = **1.058**; as % control = **105.8 %**.

3. **Load-normalized signal (optional)**

   Loaded protein = 0.432 µg → $A_{450}^{\text{load-norm}} = 0.2665/0.432 = 0.617\,(\text{A\_450 per µg})$.

4. **Absolute GFP using a standard curve (optional)**

   Standards (0–10 ng GFP) gave: $A_{450}^{\text{corr}} = 0.020 \times \text{ng} + 0.010$ (R^2=0.997) in-range.

   Estimated ng GFP = (0.2665 − 0.010) / 0.020 = **12.825 ng** (note: if above standard range, dilute and re-run).

   Abundance = 12.825 ng / 0.432 µg protein = **29.7 ng GFP per µg protein**.