Evaluating the Effect of Embryonic Exposure to Antiretroviral Therapy (ART) on the development of Type-2 Diabetes Mellitus (T2DM) in a Zebrafish Model

by

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Preface

The work and experiments presented in this dissertation were conducted at the School of Life Sciences in the Discipline of Biochemistry, at the Zebrafish Research Unit of the University of KwaZulu-Natal, Pietermaritzburg, from October 2019 to February 2024 under the supervision of Professor C.U. Niesler

The study described here is original work by the author and have not been submitted to another university in any form. Where the use of another author's work has been made, it has been appropriately acknowledged.

Mlondi Shezi (13 August 2024)

As the supervisor, I agree to the submission of this dissertation by the candidate.

Professor C.U. Niesler (26 August 2024)

DECLARATION 1 - PLAGIARISM

I, ……Mlondi Shezi……., declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a. Their words have been re-written but the general information attributed to them has been referenced

b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1: Shezi M, Snyman C, Niesler CU. "*Establishing a zebrafish larvae model to investigate the effect of tenofovir, lamivudine and dolutegravir on T2DM development*", 16th International Zebrafish Conference (IZFC), 2021

Publication 2: Shezi M, Snyman C, Niesler CU. "*Candidate Gene Expression in Adult Zebrafish Models of Type 2 Diabetes Mellitus*" Zebrafish, in revision

Signed: 

CMC Feb 2012

Acknowledgements

To God be the glory, you have carried me this far and never abandoned me. I thank you LORD.

Prof Niesler, thank you for everything, you kept saying we getting there and we finally did, can't thank you enough,

Celia, still wish you were here many thanks to you too.

Abstract

The use of combination antiretroviral therapy (cART) has significantly increased the quality of life and lifespan of people living with HIV (PLWH), however this new lease on life is threatened by a high risk of developing metabolic disorders such hyperglycemia and type 2 diabetes mellitus (T2DM). TLD, a fixed-dose combination antiretroviral drug comprising tenofovir (300 mg), lamivudine (300 mg) and dolutegravir (50 mg) is a first-line treatment regimen for all PLWH in South African and other mid-to-low income countries, as recommended by the World Health Organisation (WHO). In adults, TLD is however increasingly associated with adverse side effects such as hyperglycemia, bodyweight gain and T2DM. The mechanisms underlying these effects are not well understood and indicate the need to develop tools and models to better understand the development of cART-related T2DM. Of critical importance, TLD is also widely used for viral suppression in HIV-positive pregnant women as it crosses the placenta, conferring some level of protection in the developing fetus and thereby reducing the risk of mother-to-child transmission. However, whether *in utero* exposure to TLD predisposes the embryo to developing metabolic diseases such as T2DM later in life are not known. Studies on the short- and long-term effects of TLD exposure *in utero* are therefore required. In this context, the zebrafish (*Danio rerio*) is seen as an ideal animal model for many human diseases and several zebrafish models of T2DM exist.

The aim of this study was therefore to utilise zebrafish to increase our understanding of the effects of embryonic TLD exposure on the development of T2DM in adult organisms, with post-natal bodyweight, fasting blood glucose, and the relative qPCR expression of hormones and enzymes central to glucose metabolism as indicator endpoints. In order to establish morphological (bodyweight), glycemic (fasting blood glucose) and relative qPCR genetic changes likely to occur in non-obese and obese diabetic zebrafish, we first developed adult zebrafish models of T2DM using glucose-immersion and overfeeding approaches. The mean bodyweight of the overfed group (0.73 g ± 0.05 g) was significantly higher than that of the control group (0.57 g ± 0.1 g), while there was no significant bodyweight change for the glucose model. The fasting blood glucose levels of the overfed (3.5 ± 0.9 mmol/L) and glucose-immersed (4.0 ± 0.7 mmol/L) zebrafish models were significantly higher than the controls indicating the progression to insulin resistance and T2DM. Both models revealed a lack of insulin and preproinsulin expression in some fish within these treatment groups relative to controls when assessed with qPCR (i.e. no target amplification).

The toxicity of TLD and its individual components *in vitro* (on HEK293 cells) and *in vivo* (on one-day-old dechorionated embryos), and thereby the appropriate TLD concentration for

treatment, were then determined. TLD did not induce toxicity in HEK293 cells even when used at the highest concentration (of 100 µM Tenofovir, 100 µM Lamivudine and 50 µM Dolutegravir), but did slow the rate of cell growth. TLD was also not toxic to zebrafish embryos after five days of exposure, but did slow their development by delaying the formation of the swim bladder and therefore the ability of developing embryos to maintain an upright posture. One-day-old dechorionated zebrafish embryos were then exposed to TLD for 5 days, followed by the evaluation of bodyweight and fasting blood glucose changes in adult zebrafish at 4 months. The bodyweight (0.26 g ± 0.12 g) and fasting blood glucose (3.5 ± 1.92 mmol/L) in response to TLD treatment was higher, but not significantly different to the controls (0.17 g ± 0.04 g and 2.0 ± 0.62 mmol/L) respectively. The relative expression of insulin (*zins*), preproinsulin (*ppins*) and phosphoenolpyruvate carboxykinase 1 & 2 (*pck1 & pck2*) (all referred to as candidate genes) was then assessed using qPCR. The qPCR expression of insulin (ΔCt 2.02 ± 1.0) and preproinsulin (ΔCt 1.87 ± 0.9) of pooled samples at 4 months after TLD treatment was variable but still higher than controls (ΔCt 1.0 ± 0.0). The expression of all the selected candidate genes at 5 days and at 1-month post treatment was not significantly different to controls.

In conclusion, to study the implication of embryonic TLD exposure on the development of T2DM in adult organisms, we first established T2DM models in zebrafish in order to understand and establish the changes (physiological and genetic) reflective of a diabetic state. We demonstrate that changes in relative qPCR gene expression in adult zebrafish was variable in both models, with some fish displaying elevated levels while others lost the ability to express insulin and its precursor preproinsulin. Furthermore, our study suggests that exposure to TLD during embryonic development may contribute to weight gain and hyperglycemia (i.e. insulin resistance) later in life, even long after the drug is removed. The genetic effects of TLD were not clearly visible, however it is possible that dietary challenge in the adult fish is required to demonstrate the extent of the effect. These preliminary findings provide insight on the implications of embryonic TLD exposure in the context of the development of metabolic diseases such as T2DM in adults.

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Chapter 1

Brief literature review

1. Introduction

South Africa had an estimated 4.6 million people aged 20-79 years living with diabetes in 2019, the second highest prevalence in the IDF African region (Grundlingh *et al.*, 2022). Approximately 2.4 million people living with diabetes in South African are undiagnosed (i.e. 52.4% of the estimated diabetic adult population; and South Africa has the highest number of diabetes-related deaths in woman in Sub-Saran Africa and the second highest overall in the region (Sifunda *et al.*, 2023). It is estimated that approximately 90 000 people died from diabetes in the country in 2019 and almost 3 500 people had impaired glucose tolerance (Saeedi *et al.*, 2019).

South Africa has the largest number of people living with HIV (PLWH) in the world (Gizamba *et al.*, 2023); efforts are being made to control the spread of HIV in the country and ensure timely effective treatment (Zuma *et al.*, 2022). There is compounding evidence indicating that the use of antiretroviral therapy (ART), also known as combination antiretroviral therapy (cART) may contribute to severe weight gain and obesity in HIV patients (Hill *et al.*, 2019). As the country with the largest antiretroviral drug program in the world, as a result of its HIV and AIDS burden, South African is also potentially likely to be plagued by increasing incidences of antiretroviral (ARV) drug-induced T2DM amongst other non-communicable diseases (NCDs) (Gizamba *et al.*, 2023). ART has not only increased the lifespan of PLWH, but has also reversed the HIV-associated muscle wasting or weight loss (S. Kumar and Samaras, 2018). Weight gain, which was previously a positive indicator of a person's return to good health, is now a risk factor for metabolic disease (Tate *et al.*, 2012; Yuh *et al.*, 2015; S. Kumar and Samaras, 2018). Several studies have reported an increasing prevalence and incidence of metabolic complications, impaired glucose intolerance, type 2 diabetes (T2DM) and other NCDs in PLWH (S. Kumar and Samaras, 2018)

In a global context, diabetes is one of the fastest growing non-communicable diseases and was among the top ten leading causes of death in 2019 (WHO, 2020_a). Diabetes-related deaths have increased by 70% from 2000 to 2019, with deaths in adult males rising by 80% (WHO, 2020_b) worldwide. The International Diabetes Federation (IDF) reported in 2019 that 8.3% of the adult population (aged 20-79 years were living with diabetes globally (Figure 1.1).

Of these, over four-million are estimated to have died from diabetes-related complications, with 400 000 more deaths in women than in men (Grundlingh *et al.*, 2022).

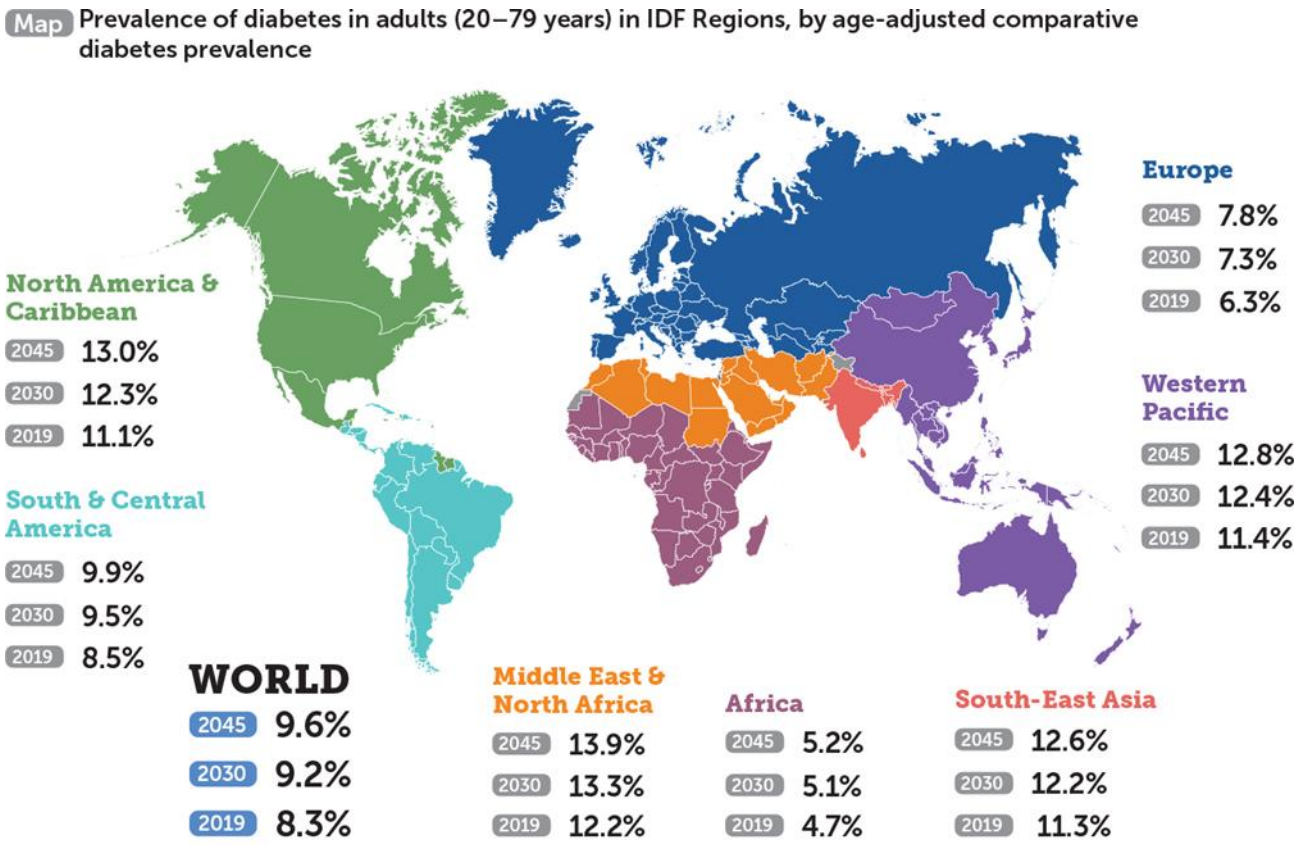


Figure 6.1 Global diabetes prevalence in adults aged 20-79 years.
The global and regional prevalence of T2DM in the adult population. Adopted from The IDF diabetes atlas 9th edition 2019. (https://diabetesatlas.org/atlas/ninth-edition/).

South Africa and other Sub-Saharan counties have adopted a single pill fix-dose combination drug comprising 100 mg tenofovir, 100 mg lamivudine and 50 mg dolutegravir (TLD), as a first-line regimen for all PLWH (Fokam *et al.*, 2023). Dolutegravir replaced efavirenz in this combination drug because of its superior viral suppression, high genetic barrier to resistance, milder side effects and lower risk of drug-to-drug interactions (Bessong *et al.*, 2021). Because dolutegravir is effective at lower doses it is the ideal candidate to combine with other drugs (Fokam *et al.*, 2023). However, some side effects of TLD have been reported among adults living with HIV, including bodyweight gain, hyperglycemia (Bahamdain, 2022) and insulin resistance, consistent with T2DM (Namara *et al.*, 2022).

2. Diabetes mellitus

Diabetes mellitus is a metabolic disease characterized by elevated blood glucose levels referred to as hyperglycemia (Kalra and Agrawal, 2013). Two main types of diabetes (type 1 and type 2) are very common and non-gender specific; while a third type (gestational diabetes) is only prevalent in pregnant women (IDF, 2021). Type 1 Diabetes Mellitus (T1DM) often initiates in childhood and is mostly prevalent in children and adolescents; it is characterized by absolute insulin deficiency (Figure 1.2) as a result of pancreatic β-cell destruction usually caused by the individual's own immune system (Katsarou *et al.*, 2017). Type 2 Diabetes Mellitus (T2DM) is the most common type of diabetes accounting for over 90% of all diabetes cases; it is marked by insufficient insulin secretion (Figure 1.2) by pancreatic β-cells combined with impaired insulin action on insulin sensitive tissue, a condition known as insulin resistance (WHO, 2019). The lack of insulin secretion and sensitivity or both, result in hyperglycemia and glucose intolerance (Sicree *et al.*, 2010).

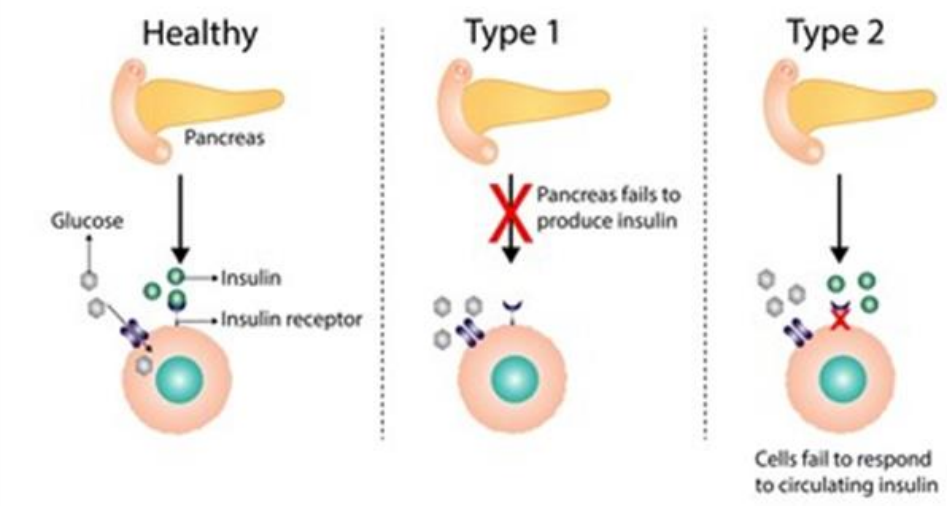


Figure 1.2 Simplified illustration on characteristics of type1 and type 2 diabetes mellitus.
In a healthy individual, the pancreas secretes sufficient insulin which is able to bind to the insulin receptor and stimulate glucose uptake in insulin sensitive target cells. However, in patients with type 1 diabetes, the pancreas is unable to produce insulin, while in type 2 diabetes, the target cell is unable to respond to insulin, a condition called insulin resistance. Ultimately, both forms of diabetes result in chronically elevated plasma glucose levels; in the case of T2DM this may lead to pancreas exhaustion long term. Adopted from: (https://www.shutterstock.com/image-vector/main-types-diabetes-mellitus-either-pancreas-273644213)

3. Clinical diagnosis of diabetes

The clinical diagnosis of diabetes mainly centers on the determination of blood glucose levels, as a result, the three main diagnostic approaches are: the fasting plasma glucose test, the oral glucose tolerance test and the glycated hemoglobin test (Olokoba *et al.*, 2012). The fasting plasma glucose test is performed after 8-12 hours of fasting; fasting blood glucose levels of between 70 to 100 mg/dL (3.9 mmol/L to 5.6 mmol/L) are considered normal, while levels between 100 to 125 mg/dL (5.6 to 6.9 mmol/L) are considered pre-diabetic, and levels above 126 mg/dL (≥7.0 mmol/L) reflect the diabetic state (Olokoba *et al.*, 2012) (Figure 1.3). The oral glucose tolerance test measures the plasma glucose levels before and 2 hours after the ingestion of 75 mg of glucose; a person with plasma glucose levels above 200 mg/dL (11.1 mmol/L) 2 hours after glucose ingestion is considered diabetic. The glycated hemoglobin (Hb) A1C test gives blood glucose measurements as an average, obtained from the test conducted within a 2 to 3 month period; a diagnosis above 6.5% (48 mmol/mol) is considered an indication of diabetes (Reed *et al.*, 2021) (Figure 1.3). Laboratories have also adopted these methods for animal models; in particular the fasting plasma glucose and oral glucose tolerance tests because they are relatively easy to perform (Kottaisamy *et al.*, 2021).

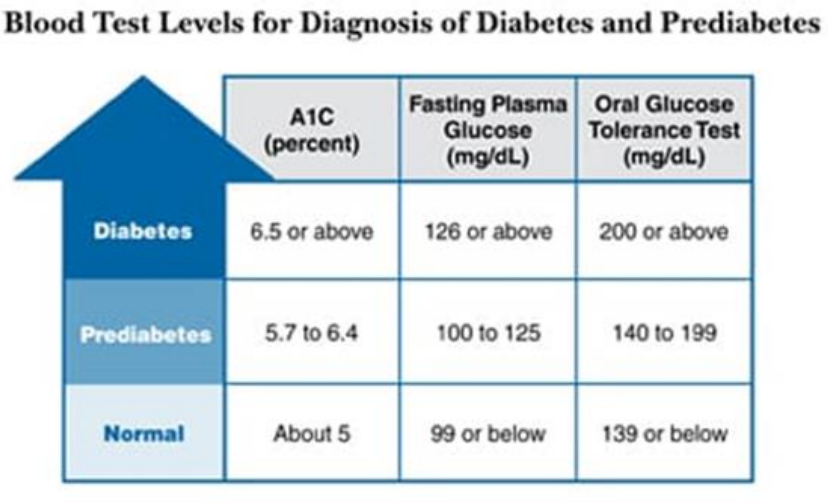


Figure 1.3 Plasma glucose levels for the diagnosis of diabetes.
Adopted from: (https://www.fightdiabetes.com/diagnosis-of-type-2-diabetes/)

4. Insulin regulation of glucose

Insulin is a hormone produced by β-cells of the pancreas to regulate blood glucose levels; it is synthesized as pre-proinsulin (an immature form of insulin) which is processed in the

endoplasmic reticulum (ER) to proinsulin (Fu *et al.*, 2013). Proinsulin is transported to the Golgi apparatus where the C-peptide is cleaved off and mature insulin is stored awaiting secretion (Fu *et al.*, 2013; Galicia-Garcia *et al.*, 2020). Glucose is the main trigger of insulin secretion however, amino acids and fats can also trigger insulin release to a lesser degree (Fu *et al.*, 2013). In the event of rising plasma glucose levels, the appropriate amount of insulin is released (in healthy individuals) to facilitate the catabolism of glucose to pyruvate through glycolysis. To initiate this process, insulin binds to the insulin receptor (IR) on the surface of insulin sensitive cells and tissue (such as liver, muscle and adipose); this binding recruits the insulin receptor substrate (IRS/ IRS-1) which activates phosphoinositide 3-kinases (PI3K) and, subsequently, AKT leading to the translocation of the glucose transporters, GLUT1 and GLUT4 to the cell surface facilitating glucose uptake (Figure 1.4). This insulin-mediated glucose uptake is essential for maintaining glucose homeostasis and removing rising blood glucose levels after a meal (Piero *et al.*, 2015). The insulin released is directly proportional to the amount of glucose consumed; however, a prolonged state of excessive glucose consumption eventually leads to insulin insensitivity (insulin resistance) as the cells require a higher dose of insulin to facilitate glucose uptake (Shulman, 2000). Insulin resistance over-burdens the pancreas to produce more insulin which eventually exhausts the capacity of the organ leading to impaired insulin secretion (Piero *et al.*, 2015).

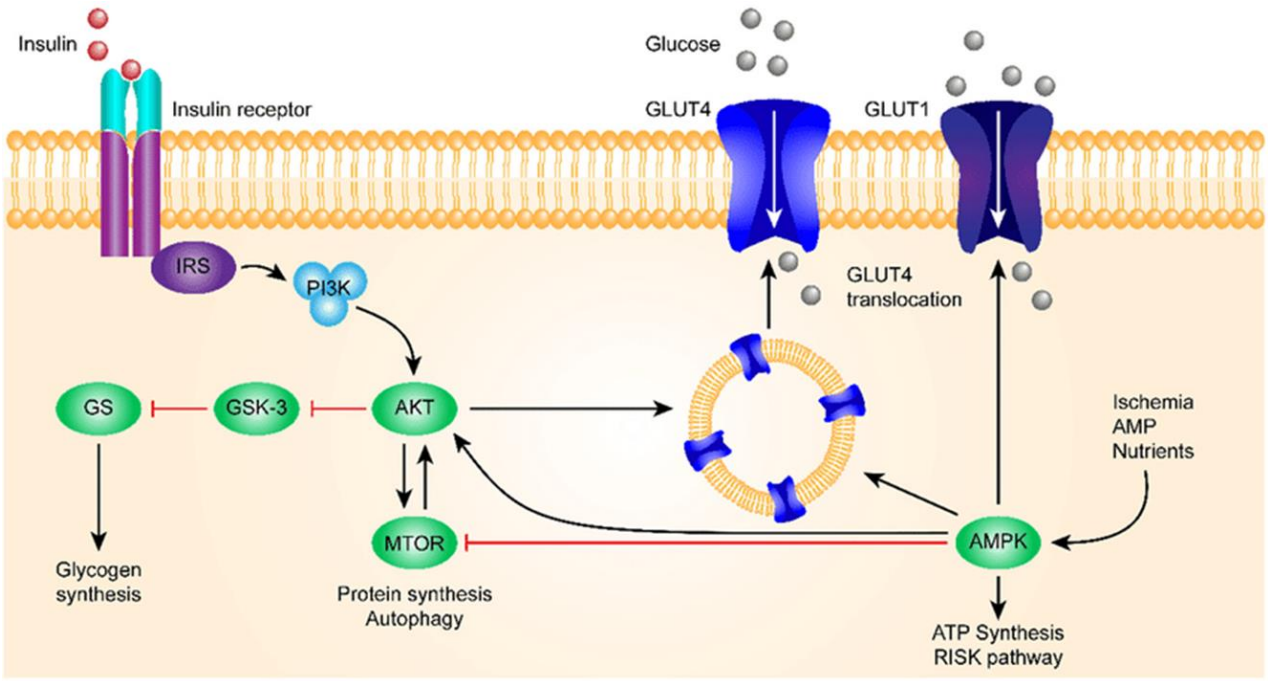


Figure 1.4 A simplified illustration of insulin signalling.
Showing how the binding of insulin to insulin-sensitive cells leads to glucose uptake.
Adopted from (Arneth *et al.*, 2019).

Insulin does not only control blood glucose uptake, but also regulates endogenous glucose production in the liver through a process called gluconeogenesis; this involves the activity of the metabolic enzyme phosphoenolpyruvate carboxykinase (PEPCK or PCK) (Shao *et al.*, 2005). PEPCK catalyzes a rate-limiting step in the process of gluconeogenesis, which produces glucose in hepatic cells during a state of low blood glucose to maintain glucose homeostasis (Figure 1.5). Therefore, in a non-diabetic state insulin and glucagon regulate plasma glucose levels by facilitating the removal or replenishment of glucose in the blood stream to maintain levels within a narrow physiological range (Figure 1.5). Insulin (and glucose to some extent) inhibit glucagon and the transcription of PEPCK preventing its gene expression (Gabbay *et al.*, 1996); this prevents glucose production in the liver when plasma glucose levels are high.

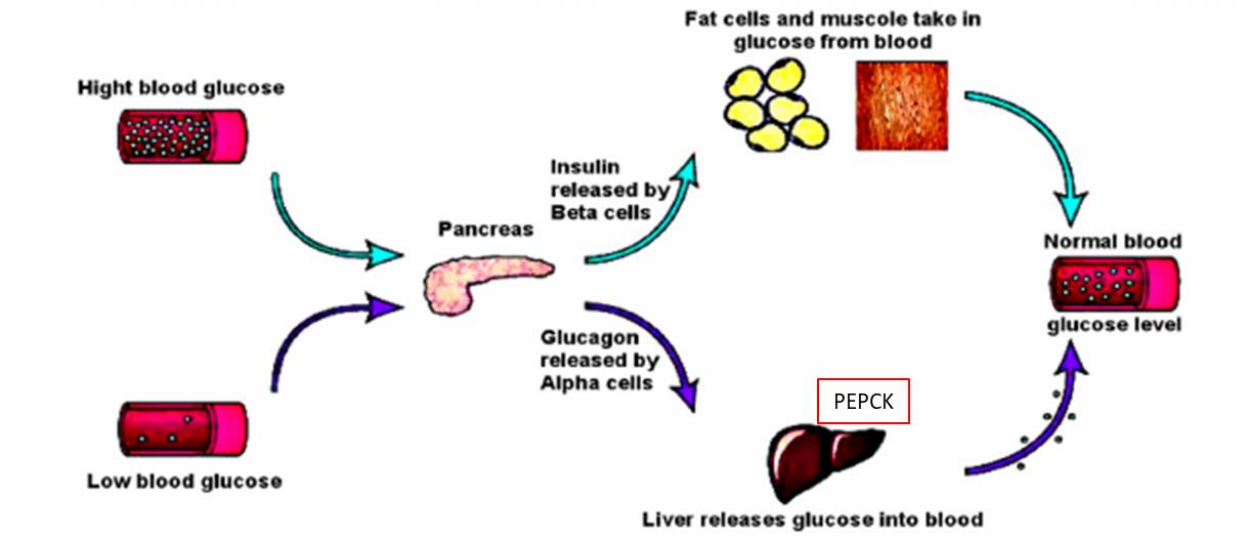


Figure 1.5 A simplified diagram of the insulin regulation of glucose.
Showing how insulin and glucagon normalise plasma glucose levels through the uptake and replenishment of glucose in the blood stream.
Adopted from: (https://www.shutterstock.com/search/blood-glucose-regulation)

5. T2DM and gene expression alterations

A number of changes occur in a diabetic state including, the downregulation of insulin receptor (IR) expression in muscle cells which leads to impaired insulin function (Wang *et al.*, 2019). The expression of GLUT4, the major glucose transporter in skeletal muscles, liver, adipose tissue and other peripheral tissue also becomes suppressed in a diabetic state preventing the

cellular entry of glucose leading to hyperglycemia and insulin resistance (Alam *et al.*, 2016). The reduced mRNA expression of the metabolic enzyme hexokinase II has also been reported in glucose sensitive tissues of diabetic patients (Vestergaard *et al.*, 1995). Glucose increases insulin gene expression by eliciting insulin and preproinsulin mRNA expression, stabilization and translation (Melloul *et al.*, 2002). Long-term glucose stimulation increases insulin biosynthesis (i.e. the processing of the precursor preproinsulin protein to the mature active insulin hormone) and insulin & preproinsulin mRNA. However, preproinsulin mRNA levels are reduced in rodent models of T2DM (Giddings *et al.*, 1982); T2DM is marked by hyperglycemia (or glucotoxicity) which contributes to the worsening of pancreatic β-cell function in part by inhibiting insulin gene expression (V. Poitout *et al.*, 2006). Hyperglycemia initially increases insulin gene expression, however sustained hyperglycemia over a long period of time, and high fatty acid levels in the blood stream of T2DM patients, ultimately impairs insulin gene expression (Vincent Poitout *et al.*, 2004), enhancing insulin resistance. Interestingly, while it may be counterintuitive, the metabolic enzyme PCK (or PEPCK) has been found to be over-expressed in a diabetic state (Shao *et al.*, 2005). This is attributed to the fact that chronically high plasma glucose levels reduce the potency of glucose and insulin to inhibit the expression and activation of PCK; therefore, in T2DM patients, the liver continues to produce glucose regardless of the amount already present in the blood stream. There are two isoforms of phosphoenolpyruvate carboxykinase, PCK1 (or PCK-C) the cytosolic form and PCK2 (or PCK-M) the mitochondrial form, encoded by separate genes (Yu *et al.*, 2021). The gene expression of PCK1 is inducible by diet (i.e. fasting or low blood glucose levels), hormones and diabetes, while PCK2 is mainly constitutively expressed (Hanson and Owen, 2013).

6. Risk factors of T2DM

The risk factors of diabetes and pre-diabetes are many and include obesity, particularly visceral-abdominal adipose tissue accumulation, and thus most people with T2DM are overweight or obese (Kahn, 2003). A positive family history of diabetes is accompanied by a host of genetic predisposition markers (e.g. *TCF7L2, KCNQ1*, whose mutation affects insulin secretion) and has been shown to be a positive predictor of T2DM (Annis *et al.*, 2005). Age, lack of exercise and an unhealthy diet are also all positively associated with T2DM prevalence (Kyrou *et al.*, 2020). The use of certain medication, such as antiretroviral drugs may lead to drug-induced diabetes or hyperglycemia and insulin resistance in patients (Latek *et al.*, 2019).

HIV itself, is also known to contribute to an increased risk of T2DM. HIV is associated with chronic inflammation as a result of the ongoing active immune response, which leads to a release of inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor alpha

(TNFα) (Vigouroux *et al.*, 2003; M. Kumar *et al.*, 2023). Elevated levels of TNFα mRNA and protein have been demonstrated in models of obesity and insulin resistance in animals and humans (Hotamisligil, 2000). These factors play a role in poor glucose tolerance and the development of insulin resistance (M. Kumar *et al.*, 2023). The treatment of insulin sensitive cells with TNFα has been shown to alter the catalytic activity of the insulin receptor (IR) subsequently reducing IR phosphorylation and inhibiting the activation and recruitment of the insulin receptor substrate (IRS-1) (Hotamisligil, 2000). Furthermore, HIV-associated alteration in fat distribution, referred to as lipodystrophy is also linked to the development of T2DM in PLWH due to increased free fatty acid circulation (Shetty and Kumari, 2021).

7. Animal models of T2DM

Animal models of T2DM are an integral part of research into this disease; as a result, a number of animal models have been developed. The role of these models is irreplaceable in scientific research as they have helped us understand the functioning of individual tissues, organs and organ systems in the context of this metabolic disease (Pandey *et al.*, 2023). Animal models of impaired glucose metabolism are important resources for studying T2DM development because they allow researchers a unique opportunity to investigate real-time changes that occur in the on-set of, and during, the diseased state. Creating a model of T2DM that is reflective of the human condition is very challenging, partly because of the complexities of diabetes and thus methods of induction are quite varied. However, one can classify these animal models of diabetes according to one of two main approaches, i.e. physiological induction or genetic manipulation (Kottaisamy *et al.*, 2021). Physiological induction is the most common approach to eliciting diabetes or diabetic symptoms, and involves the use of chemical reagents or compounds that promote hyperglycemia. Genetic manipulation on the other hand relies on the alteration of one or genes or mutations using gene editing technologies or transgenic means (Cao *et al.*, 2023).

Mouse and rat models of diabetes are common and have made a huge contribution in the understanding of obesity, inflammation, insulin resistance, hyperglycemia and other T2DM related conditions (Kottaisamy *et al.*, 2021). However, the use of more robust models that allow high throughput screening of compounds in small volumes as well as the ability to monitor developmental toxicity and side effects of treatment is necessary. Zebrafish (Danio rerio) is an important research tool in the field of biomedical science and its use as a model organism in the advancement of biomedical research has rapidly increased over the past two decades (Meyers, 2018; Teame *et al.*, 2019). The interest in zebrafish stems from its wide

application in approaches to understanding cellular, molecular and genetic mechanisms underlying vertebrate developmental processes and human disease (Eisen, 2020). Zebrafish are small bodied freshwater fish, 3 to 5 cm in length and originally found in flood plains, ponds and steams of the Ganges river in South Asia (Meyers, 2018). Today, Danio rerio's are commonly found in aquaria and pet shops across the world and have adopted the name "zebrafish" because of their horizontal stripy pigmentation. Multiple features make zebrafish an ideal model organism for biomedical research including a fully sequenced genome, the production of transparent, external embryos that develop rapidly with clearly defined stages (allowing the observation of developmental processes from the first moment of fertilization to hatching), amenability to genetic manipulation (allowing the investigation of genetic diseases) and finally the ability to rapidly produce many embryos (classically 50 – 300) in a single spawn, all year round (Kimmel *et al.*, 1995; Eisen, 2020). This allows experimental repetition for stronger, rigorous statistical data and high-throughput screening of therapeutic compounds. Zebrafish can be produced and housed relatively inexpensively with limited space in high numbers.

Zebrafish embryonic development is rapid and reaches completion by day 4-5 post fertilisation; that is, all the internal organs are formed by day 5 (Figure 1.6 A). However, the embryo still needs to develop external features (e.g. fins and pigmentation) as it progresses to the juvenile and adult stages (Figure 1.6 B). After spawning, the embryo is fertilised externally and the first cell forms within 0.1-0.7 hours post fertilisation (hpf) (Kimmel *et al.*, 1995); the individual cell cleaves multiple times to many cells forming a dome like structure during the epiboly stage of development (at 4-10 hpf). Segmentation occurs at 10-24 hpf, where somite pairs form in sequence, the tail develops, along with some of the primary organs. From 24-72 hpf functionally differentiated cells form the nervous, muscular and circulatory systems and earl pigmentation begins (Kimmel *et al.*, 1995). By 3 to 5 days' post fertilization (dpf), the embryo hatches from the chorion, develops a swim bladder and starts active movements. The yolk is completely depleted by day 6 to 7, forcing the growing embryo to start active feeding, and develop to juvenile and adult stages by 1 to 3 months (Figure 1.6 B).

The zebrafish (*Danio rerio*) has gained prominence as an ideal animal model for human diseases including diabetes (Kinkel and Prince, 2009; Sharchil *et al.*, 2022). Zebrafish metabolism and organ physiology are similar to mammals; zebrafish share 70% of their genetic make-up with that of humans, the majority of these genes are associated with human diseases (Kinkel and Prince, 2009; Howe *et al.*, 2013). A number of zebrafish models of diabetes have been developed using either larvae or adult zebrafish (van de Venter *et al.*, 2020). The zebrafish embryo also has many advantageous features as a drug toxicity

screening tool such as rapid development, small size (able to fit in a 96-well plate), ease of drug administration in small amounts, transparent embryos allowing observation of morphological changes during treatment, ease of maintenance and has high metabolic and genetic homology with mammals (Gorgulho *et al.*, 2018).

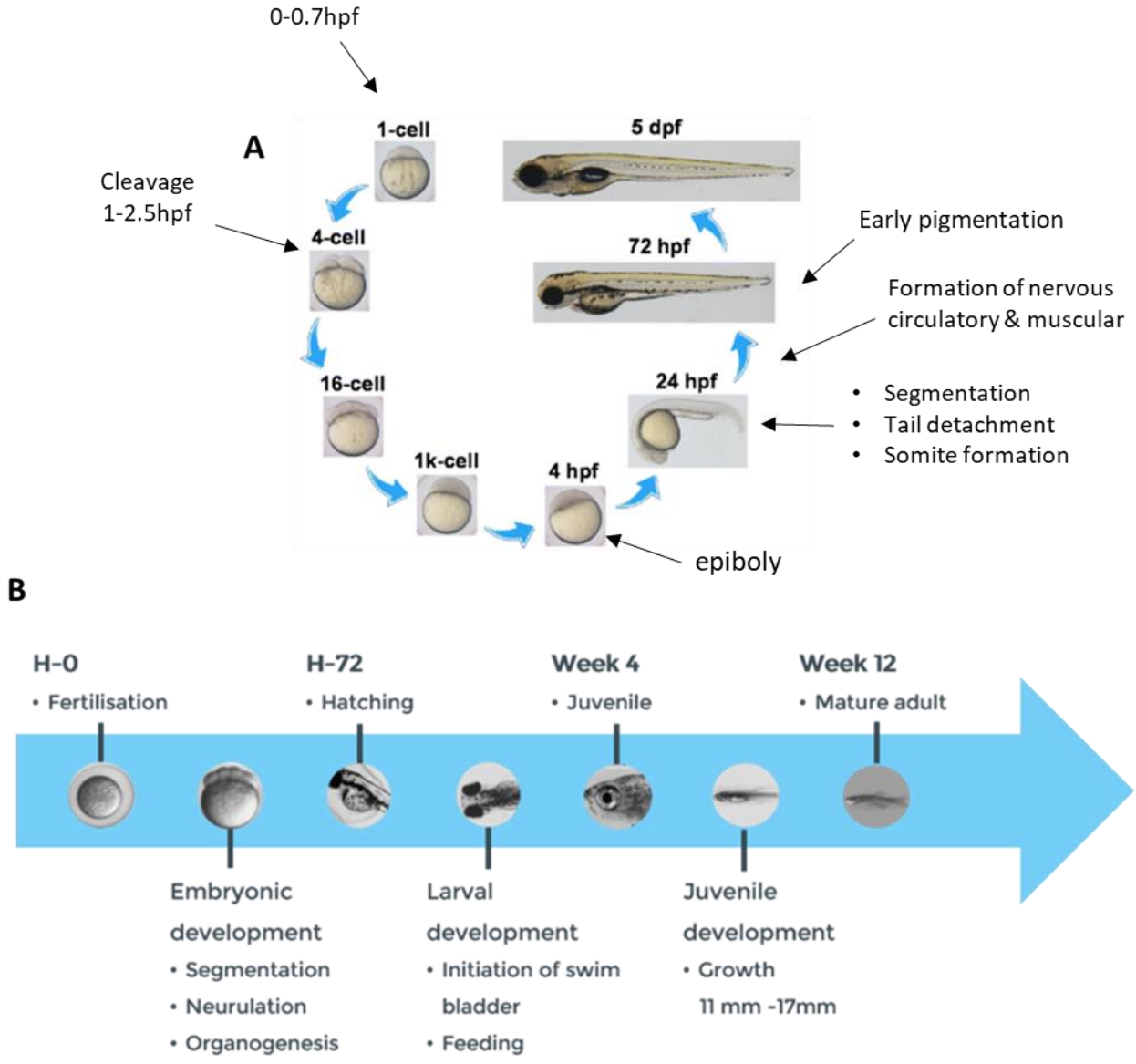


Figure 1.6 Zebrafish developmental stages A) early embryonic and B) progression to mature adult.
Early embryonic stages **(A**) from 24-hour post fertilization to 5-days post fertilization are crucial for toxicity screening studies. **B)** zebrafish rapid progression to different mature form important for experimental investigations that require developmental monitoring.
Adopted from: (https://www.sciencephoto.com/media/1215991/view/zebrafish-embryo-stages-illustration)

Zebrafish are routinely used to determine the toxicity of chemical compounds; antiretroviral drug compounds such as cabotegravir (Zizioli *et al.*, 2023) and dolutegravir (Zizioli *et al.*, 2024) have been tested for their effect on neurodevelopment in zebrafish. Dolutegravir has been shown to lead to developmental toxicity of zebrafish embryos, an effect that can be rescued through the addition of folate (Cabrera *et al.*, 2019). In mice, DTG has been shown to cause brain toxicity which contribute to neuropsychiatric adverse effect (Huang *et al.*, 2024). Antiretroviral drugs have been shown to cause metabolic disorders and insulin resistance in rats (Hruz *et al.*, 2002). These ARV side effects, especially T2DM are not clearly understood and require further investigation. To our knowledge there is no published data investigating the link between ARVs and T2DM in zebrafish; this further underscores the novelty of our study.

The zebrafish is a promising and versatile model to study diabetes, therefore a number of T2DM models have been developed in zebrafish. They differ depending on the approach used to induce diabetes, as well as on the stage of zebrafish development i.e. the zebrafish embryo or adult zebrafish (Table 1).

As in humans, hyperglycemia is the main way of confirming T2DM in zebrafish. In zebrafish and most animal models, T2DM is induced through diet, genetic means or the use of chemical reagents or compounds (e.g. glucose, Bisphenol S or Bisphenol F) (Salehpour *et al.*, 2021). The choice of inducer and zebrafish stage of development depends on the research outcomes, as well as the time taken to induction of diabetic symptoms. All these models are complete with hyperglycemia, insulin resistance, impaired insulin response and other T2DM endpoints, however, this study will focus on non-genetic approaches as these are the most likely to affect humans.

Glucose-based induction is favorable because it is an easy and relatively cheap way of inducing hyperglycemia in zebrafish. Different protocols of glucose-induction have been developed based on the concentration of glucose used, time of induction and the life stage of zebrafish (Salehpour *et al.*, 2021). The approach of inducing T2DM is zebrafish using glucose was primarily introduced by Gleeson *et al* (2007) who alternatively exposed zebrafish to 2% and 0% glucose for 30 days. This approach leads to diabetic retinopathy and high fasting blood glucose (74 ± 8.5 mg/dL and 89 ± 10.6 mg/dL for control and experimental group respectively) (Table 1). The fasting blood glucose of the experimental group increase to three times the control group in this study.

Table 1. Non-genetic zebrafish models of T2DM

Induction approach	Stage of development	Diabetic symptom / investigated genes	References
Alternate immersion in 2 and 0% glucose solutions	Adult (1-3 years) induced in 1 month	Retinopathy No gene expression	(Gleeson *et al.*, 2007)
Stepwise immersion in 1, 2 and 3% glucose solutions	Young fish (4-11 months) induced for 60 days	Insulin resistance; anti-diabetic drug responsiveness; no gene expression	(Connaughton *et al.*, 2016)
Stepwise immersion in 50, 100- and 200-mM glucose solutions	Young fish (4-11 months) induced for 10 days	Insulin resistance; anti-diabetic drug responsiveness; hyperglycemia	(Mohammadi *et al*., 2020)
Chronic immersion in 110 mM glucose solution	Adults (2-3 months) induced in 14 days	Persistent hyperglycemia; anti-diabetic drug responsiveness; elevated glycated proteins; impaired insulin response; decreased IR mRNA expression	(Capiotti *et al.*, 2014)
Immersion in 130 mM glucose solution	Embryo induced in 3 to 6 days	Retinopathy No gene expression	(Jung *et al.*, 2016)
Bisphenol F model of hyperglycemia	Embryo induced in 2 days	Insulin resistance; anti diabetic drug responsiveness; no change in glucagon mRNA	(Zhao, Wang*, et al.*, 2018)
Bisphenol S model of hyperglycemia	Adult (9 months) induced in 28 days	Hyperglycemia; no change in insulin mRNA; increased PCK1 mRNA; decreased PCK2 mRNA	(Zhao, Jiang*, et al.*, 2018)
High fat diet containing 1% egg yolk	Adults induced in 10 weeks	Insulin resistance	(Meng *et al.*, 2017)
Diet induced obesity model (DIO)	Adults (4-6 months) induced in 6 to 8 weeks	Insulin resistance; persistent hyperglycemia; glucose intolerance; no gene expression	(Liqing Zang *et al.*, 2017)

A model by Connaughton *et al* (2016) further investigated the Gleeson method and showed that it was stage-dependent. Connaughton showed that alternate immersion of zebrafish in 2% and 0% glucose solutions was more effective when applied in older fish (1-3 years of age) compared to younger fish (4-11 months old). This study demonstrated that younger fish adapt quicker that older fish and are able to lower their fasting blood glucose to normal levels during glucose treatment (Connaughton *et al.*, 2016). To counter this, Connaughton *et al* (2016) introduced a stepwise immersion protocol in solutions of increasing glucose concentration. This modified approach included initially exposing the young zebrafish to a 1% glucose solution for 2 weeks, followed by an incubation in a 2% glucose solution for another 2 weeks and finally a further incubation in a 3% glucose solution for 30 days. As a result of this modification the young zebrafish group sustained hyperglycemia for the entire duration of treatment (Connaughton *et al.*, 2016). Another approach by Mohammadi *et al* (2020) further demonstrated this idea in a much shorter period by incubating zebrafish for 4 days in a 50 mM glucose solution followed by a 3-day incubation in a 100 mM glucose solution and finally another 3-day incubation in a 200 mM glucose solution (Mohammadi *et al.*, 2020).

Chronic immersion in glucose solutions is another approach of eliciting hyperglycemia in zebrafish. Capiotti *et al*., (2014) introduced this approach by chronically exposing adult zebrafish (2-3 months) to concentrations of 55, 110 or 166 mM glucose equivalent to 1%, 2% and 3% glucose for 14 days. This study showed that chronic immersion at 110 mM was effective at inducing hyperglycemia with lower mortality compared to exposure to 166 mM (Capiotti *et al.*, 2014). This approach demonstrated fasting blood glucose levels four to five times higher than the control group and the elicited hyperglycemia lasted for up to 7 days after removal from the glucose solution. The larval immersion of zebrafish embryos in glucose solution has also been modeled to mimic fetus conditions in diabetic environments. The exposure at 3 dpf in a 130 mM glucose solution for 4 days effectively elevated larval glucose and induced diabetic retinopathy up to adulthood (Jung *et al.*, 2016).

When exposed to bisphenol F for 2 days, zebrafish embryos displayed increased levels of insulin mRNA and protein, and decreased mRNA expression of the insulin receptor substrate (IRS) at concentrations of 10 and 100 µg/L (Zhao, Wang*, et al.*, 2018). Bisphenol S, on the other hand caused significantly high fasting blood glucose levels in adult male zebrafish after 28 days of exposure at 1 and 10 µg/L (Zhao, Jiang*, et al.*, 2018). These studies suggest that bisphenol F and S are diabetic agents similar to glucose.

An unhealthy diet or over-eating is one of the risk factors of T2DM and it is no coincidence that almost 90% of T2DM patients are overweight or obese (Leitner *et al.*, 2017). Overfeeding

has been utilized to investigate the pathophysiology of obesity in relation to diabetes in a non-mammalian model (Liqing Zang *et al.*, 2017). This study utilized a high protein / high fat diet of Otohime B2 to induce hyperglycemia, insulin resistance and obesity in adult zebrafish within 6 to 8 weeks; this study demonstrated an over production of insulin in the overfed group however insulin gene expression was not investigated. In another study by Meng *et a*l (2017), zebrafish were fed a high fat diet of 1% egg yolk and brine shrimp for 10 weeks. This approach lead to obesity, hyperglycemia and insulin resistance. Additionally, they showed that the insulin receptor substrate 2 (IRS2) and glucose transporter 2 (GLUT2) were significantly reduced in insulin sensitive tissues such as the liver and muscle (Meng *et al.*, 2017).

8. Antiretroviral therapy association with diabetes

Human immunodeficiency virus (HIV) patients using antiretroviral treatment (ART) are up to 4-12 times likely to develop metabolic disorders associated with diabetes mellitus (Kalra and Agrawal, 2013). Reports have indicated the development of type 2 diabetes in HIV patients using specific classes of ARVs (Ismail *et al.*, 2009). This is correlated with other factors including a positive family history of diabetes, obesity and the virus itself (Kalra and Agrawal, 2013). However, the HIV infection might not be an important factor since HIV protease inhibitors themselves have been shown to cause insulin resistance in experimental animal models (Hruz *et al.*, 2002) and in health HIV-seronegative males (Noor *et al.*, 2001). This indicates a connection between HIV protease inhibitors (PIs) and insulin resistance, which could lead to diabetes (Ismail *et al.*, 2009). The HIV protease inhibitor class of ARVs interfere with insulin signaling and lipid metabolic pathways of the host, leading to insulin resistance, glucose intolerance, lipodystrophy, progressive insulin-producing β-cell dysfunction and type 2 diabetes.

The mechanism of the HIV protease inhibitors causing insulin resistance and type 2 diabetes is not well understood. Studies suggest that HIV protease inhibitors interact with GLUT-4, a major insulin-stimulated glucose transporter in insulin sensitive cells of the liver, muscle and adipose tissue.(Murata *et al.*, 2000; Hruz *et al.*, 2002) This reversible interaction is thought to block glucose uptake which leads to hyperglycemia and insulin resistance. Some HIV protease inhibitors (PIs) interfere with the signaling of insulin by influencing the downregulation of the insulin receptor (IR), phosphorylation events leading to the activation of key enzymes and suppression of GLUT-4 expression or translocation to the cell membrane of insulin sensitive cells (Rudich *et al.*, 2001; Ben-Romano *et al.*, 2004). Furthermore, HIV protease inhibitors alter the expression of regulatory proteins, which is linked with the metabolic dysfunction

observed in HIV patients using PIs (Ismail *et al.*, 2009). function of tyrosine kinases of the insulin signaling pathway (Ben-Romano *et al.*, 2004; Ismail *et al.*, 2009).

TLD consists of three drug compounds, tenofovir, lamivudine and dolutegravir. Tenofovir and lamivudine are nucleotide reverse-transcriptase inhibitors (NtRTIs) and nucleoside reverse-transcriptase inhibitors (NRTIs) respectively; a class of ARVs that compete for natural nucleosides to inhibit the reverse-transcriptase of the HI-virus (Holec *et al.*, 2017). Common side effect of this class of inhibitors include renal tubular dysfunction and mitochondrial dysfunction; however, these tend to be compound specific (Mashingaidze-Mano *et al.*, 2020). Dolutegravir, an integrase strand transfer inhibitor (INSTIs), blocks the integration of HIV DNA into host CD4 cell DNA (Park *et al.*, 2015); the most common side effects of this ARV class include weight gain, depression and recently hyperglycemia (Mastan and Kumar, 2010).

9. Aims and objectives

The aim of the study was to investigate whether the exposure of zebrafish embryos to ART would predispose the fish to developing T2DM as adult organisms. In order to achieve this, we first established models of T2DM in zebrafish adults to map out biological markers and features of T2DM. We then determined the toxicity profile of TLD and established appropriate doses for *in vivo* application. Finally, we exposed embryos to the determined doses of TLD and monitored their effect on bodyweight, plasma glucose and gene expression in zebrafish at the embryonic, juvenile and adult stages of development. Observed changes could be compared to the features reflected in the T2DM models, in order to draw appropriate conclusions.

Chapter 2

An Adult Zebrafish Model for Type 2 Diabetes Mellitus

1. Introduction

It is reported that approximately 10.5% (536.6 million people) of the world population was living with diabetes in 2021, this figure is estimated to rise to 783.2 million by 2045 (IDF, 2021). More than 90% of these diabetes cases are classified as type 2 diabetes mellitus (T2DM). In the onset of T2DM, cells of the muscle, liver and adipose tissue become insensitive to insulin (also called insulin resistance) triggering beta-cells of the pancreas to produce more insulin; over time pancreatic beta-cells become exhausted or damaged ultimately leading to reduced insulin production and chronic high blood glucose levels (hyperglycemia) (Kahn, 2003). Sustained hyperglycemia is used as clinical evidence of diabetes for diagnostic purposes. Multiple factors influence an individual's diabetic predisposition including age, bodyweight, family genetics, diet and medication amongst others (Kahn, 2003). T2DM heredity is more complex but involves variations in a cluster of genes (mostly associated with pancreatic β-cell function and insulin action) that make individuals more susceptible to developing the disease (Mambiya *et al.*, 2019). Weight-gain, or more specifically visceral-abdominal adipose tissue accumulation, is a major contributor to insulin insensitivity; most T2DM patients are therefore overweight or obese (Kahn, 2003; McFarlane, 2009). Bodyweight reduction has been shown to improve glycemic control and reduce the risk of cardiovascular complications in diabetic patients (Hermansen and Mortensen, 2007). Although there has been positive advancement in anti-diabetic medication, most options are not accompanied by a reduction in weight; some medication (sulfonylureas, insulin, and thiazolidinediones) may even promote weight-gain (McFarlane, 2009; Leitner *et al.*, 2017).

An emerging challenge is the growing influence of medication such as anti-retroviral therapy (ART) on bodyweight (Brennan *et al.*, 2023) as well as the contribution of ART to the development of insulin resistance and non-communicable diseases such as T2DM (McLaughlin *et al.*, 2017), (Lin *et al.*, 2018), (Hailu *et al.*, 2021). This is of particular relevance in Sub-Saharan Africa which accounts for 67% of the 38.4 million global population of people living with HIV (Moyo *et al.*, 2023). Dolutegravir (DTG), an integrase inhibitor, has been shown to increase bodyweight (Ruderman *et al.*, 2021) as well as fasting blood glucose in HIV patients after 3-6 months of therapy (Bahamdain, 2022). A case-controlled study showed that, out of 204 patients on a DTG-based regiment in Uganda, 54 (26.5%) individuals were

hyperglycemic, consistent with prediabetes, while 150 (73.5%) had hyperglycemia indicative of T2DM (Namara *et al.*, 2022). A South African cohort study showed that a group of patients using DTG for 12 months had a higher bodyweight than the group using the reverse transcriptase inhibitor, Efavirenz (Brennan *et al.*, 2023). Other antiretroviral drugs (such as the protease inhibitors Indinavir and Ritonavir) have also been shown to cause significant hyperglycaemia and insulin resistance in rat and rabbit models (Mastan and Kumar, 2010). To better understand the contributing mechanisms of ARTs in the development of hyperglycemia, insulin resistance, and T2DM, further studies are required.

Animal models of impaired glucose metabolism are an important tool for studying T2DM development as they provide a unique opportunity to investigate real-time changes that occur in the on-set of, and during, the diseased state. Various models of T2DM exist, including diet-induced (Surwit *et al.*, 1988), chemical-induced (Dufrane *et al.*, 2006), and genetic knock-outs or transgenic manipulated models (Haskell *et al.*, 2002), (Durham and Truett, 2006). Many of these models utilise rodents (McInerney *et al.*, 2004) or large mammals such as pigs and primates (Dufrane *et al.*, 2006).

A number of zebrafish models of diabetes have been developed using either larvae or adult zebrafish and involve either diet-based approaches, glucose-induction, chemical or genetic approaches or a combination of these (L. Zang *et al.*, 2018; Salehpour *et al.*, 2021; Chen and Liu, 2022). Genetic methods are more precise in targeting specific candidate genes and therefore produce better diabetic outcomes compared to non-genetic methods (Kahn, 2003). However, non-genetic methods such as glucose-, diet- or chemical-induction are more cost-effective, easier to implement, require less technical expertise and would be more relevant as most human T2DM is driven by non-genetic factors such as lifestyle and medication rather than single gene modifications (Kahn, 2003; Galaviz *et al.*, 2018). Based on previous studies, symptoms of T2DM can be induced in adult zebrafish with chronic immersion in a 2 - 4% glucose solution within a period of 2 to 8 weeks of induction (McCarthy *et al.*, 2021), while diet-induced models take 6 to 10 weeks (Liqing Zang *et al.*, 2017; Salehpour *et al.*, 2021).

The glucose induction model of adult zebrafish is usually associated with hyperglycemia, insulin resistance and diabetic retinopathy similar to that seen in diabetic humans (Gleeson et al., 2007). The immersion of zebrafish in a glucose solutions is the most commonly utilised method of inducing hyperglycemia and T2DM in zebrafish (Salehpour *et al.*, 2021); immersion protocols differ depending on zebrafish life-stage, glucose concentration and period of exposure. Embryo models are more adaptive to changes in glucose concentration in their surrounding environment and thus often require a stepwise increase (1%) in glucose

concentration up to 3% (Connaughton *et al.*, 2016); Older zebrafish are more susceptible to chronic immersion in fixed glucose concentrations, but exposure to concentrations above 2% for extended periods is lethal (Carnovali *et al.*, 2016). The exposure period is dependent on the glucose concentration and therefore tends to be shorter when used above 2% (Connaughton *et al.*, 2016).

Overfeeding as approach to developing a model of obesity and T2DM do exist in zebrafish and vary in the type of feed used. Fish overfed with *Artemia,* display an increased bodyweight and plasma triglyceride levels after 8-weeks (Oka *et al.*, 2010), while overfeeding a high-fat-diet comprising brine shrimp and 1% egg yolk for 10 weeks can also induce T2DM accompanied with weight-gain and insulin resistance in zebrafish (Meng *et al.*, 2017). Recently, a zebrafish diet-induced obesity (DIO) model was established by Zang *et al*., 2017 using a commercially available fish food known as Otohime; their study demonstrated that overfeeding with Otohime B2 (a high protein, high fat containing food) induced weight-gain, high fasting blood glucose, impaired glucose tolerance and early stages of insulin resistance in zebrafish, similar to that seen in humans. The advantage of this approach is that significant weight gain and elevated fasting blood glucose levels can be obtained in adult zebrafish within 6 weeks of overfeeding; overfeeding can be automated using automatic feeders, making it less labour intensive.

A useful way of monitoring the progression of T2DM in animal models is the investigation and characterisation of disease genetic markers. The candidate genes (insulin, pre-proinsulin and phosphoenolpyruvate carboxykinase 1 & 2) used in this study were selected because they play a critical role in the maintenance of blood glucose homeostasis and their mRNA expression is sensitive to glycaemic concentrations and are affected by T2DM leading to their overexpression and suppression as consequence of disease severity.

In order to determine whether the exposure of zebrafish embryos to ART would predispose the fish to developing T2DM as adult organisms, we first needed to establish and characterise this NCD in the adult model itself. We used glucose-induction and overfeeding protocols to achieve this; these approaches represent non-obese (glucose) and obese (overfed) models of T2DM using zebrafish (Connaughton *et al.*, 2016; L. Zang *et al.*, 2018). Once established, the relative qPCR expression of selected candidate genes could then be assessed in order to establish a baseline of gene expression changes (ΔCt and relative fold changes) that are associated with the diabetic state. Zebrafish bodyweight changes and fasting blood glucose levels, as well as relative mRNA expression of hormones such as insulin and its precursor, preproinsulin as well as the metabolic enzymes phosphoenolpyruvate carboxykinase 1 & 2

were established. This represents the first time these zebrafish models have been developed in South Africa and therefore also serves as a starting point for other local researchers.

2. Methods and materials

2.1. Animals

All animal procedures were approved by the Animal Research Ethics Committee (AREC; protocol reference number AREC/00005213/2023) of the University of KwaZulu-Natal (UKZN). Zebrafish were maintained in the UKZN Zebrafish Research Facility (ZRF) at 28.5°C in 2L tanks of the IWAKI system (Iwaki aquatic systems and services; USA) at shoaling densities of 6-8 fish per tank containing reverse osmosis (RO) water) under a blue-light cycle of 14-hour light and 10-hour dark period. The fish were feed brine-shrimp (hatched in the laboratory), tropical fish-flakes (TetraMin, Tropical Flakes, Virginia, USA) or Otohime-B2 (Marubeni Nisshin Feed, Tokyo, Japan) three times a day during week days and once a day on weekends

2.2. The zebrafish glucose model.

The glucose-induction protocol was performed according to Capiotti *et al.*, (2014) with modifications; initially adult fish (a mix of males and females at 4-6 months) were exposed to 110 mM (2%) glucose for 14 days. However, when the fasting blood glucose was assessed at 14 days, the fish were not hyperglycemic, as was the case in the referenced study (Capiotti *et al.*, 2014). Fasting blood glucose level assessments were subsequently made at day 20, 30 and 40 during treatment, and zebrafish were found to became diabetic at 40 days. As a result, mixed healthy male and female zebrafish wild-type (AB strain) were then assigned to tank water in the presence or absence of 2% glucose at densities of 6-7 fish per 2.5 litres (Figure 2.1) and fish exposed to these conditions for 40 days with water and glucose solutions changed every second day. At day 40, the fish were removed from the glucose solution, placed in reverse osmosis water and fasted for 12-hours after which they were anesthetised on ice (fish were placed into an ice bath at 18-12°C for 2-5 minutes, excess water was removed with paper towel) and weighed before they were euthanized (by decapitation while anesthetised), and blood drawn (following decapitation the glucometer strip was directly connected to the wound) to determine fasting blood glucose using a glucometer (Accu-Chek Instant, Roche, Germany). Given that this was our first attempt at establishing the model, we felt it appropriate to first use whole carcases as a starting point to assess whole body genetic changes due to treatment. Whole carcases were prepared in Trizol (Cat no.15596026, Life Technologies,

USA), and utilised to isolate total RNA using the Invitrogen PureLink RNA Extraction Mini Kit (Cat no. 12183018A, Life Technologies, California, USA) followed by cDNA synthesis using the Invitrogen SuperScript (IV) first-strand synthesis system (cat no. 18091050, Thermo-fisher Scientific, Massachusetts, USA) for qPCR.

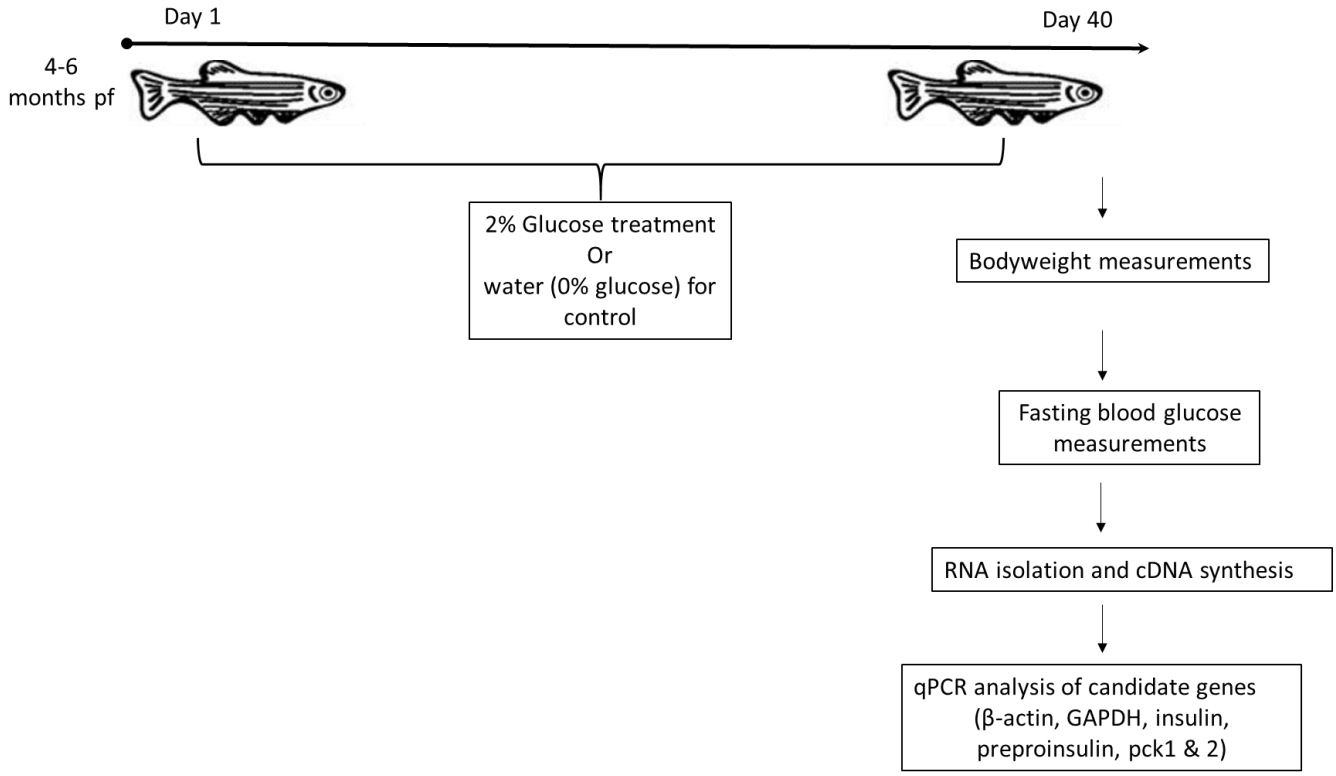


Figure 2.1 Experimental approach for the zebrafish 2% glucose T2DM model.
Adult zebrafish (4-6 months post-fertilisation (pf), male and female) were continuously exposed to 0% (control group) or 2% (experimental group) glucose solution for 40 days. Bodyweight, fasting blood glucose measurements and RNA isolation were performed on all carcases following euthanasia. Total RNA was transcribed to cDNA and used for qPCR analysis.

2.3. The zebrafish overfed (DIO) model

Healthy adult male and female zebrafish wild-type (AB strain) were assigned to either an overfeeding or a control group at densities of 6-7 fish per 2.5 litres (Figure 2.2). So-called DIO fish were hand-fed 120 mg commercially available fish food (Otohime B2; Marubeni Nisshin Feed, Tokyo, Japan) per fish per day; this was divided into six daily feedings spaced out every hour and half to two hours. The control group was fed a single 20 mg per fish per day. Otohime B2 contains 11% fats, 51% protein, 3% fiber, 2.3% calcium and 6.5% moisture. The fish were weighed at week 6, 7, 8, 9 and 10 during the experiment prior to feeding. At week 10 the fish were fasted for 12-hours, anesthetised on ice, and euthanized after which blood was drawn for fasting blood glucose measurements using a glucometer. To assess whole body genetic changes due to treatment, carcases were then prepared in Trizol, and utilised to isolate total

RNA using the Invitrogen PureLink RNA Extraction Mini Kit followed by cDNA synthesis for qPCR.

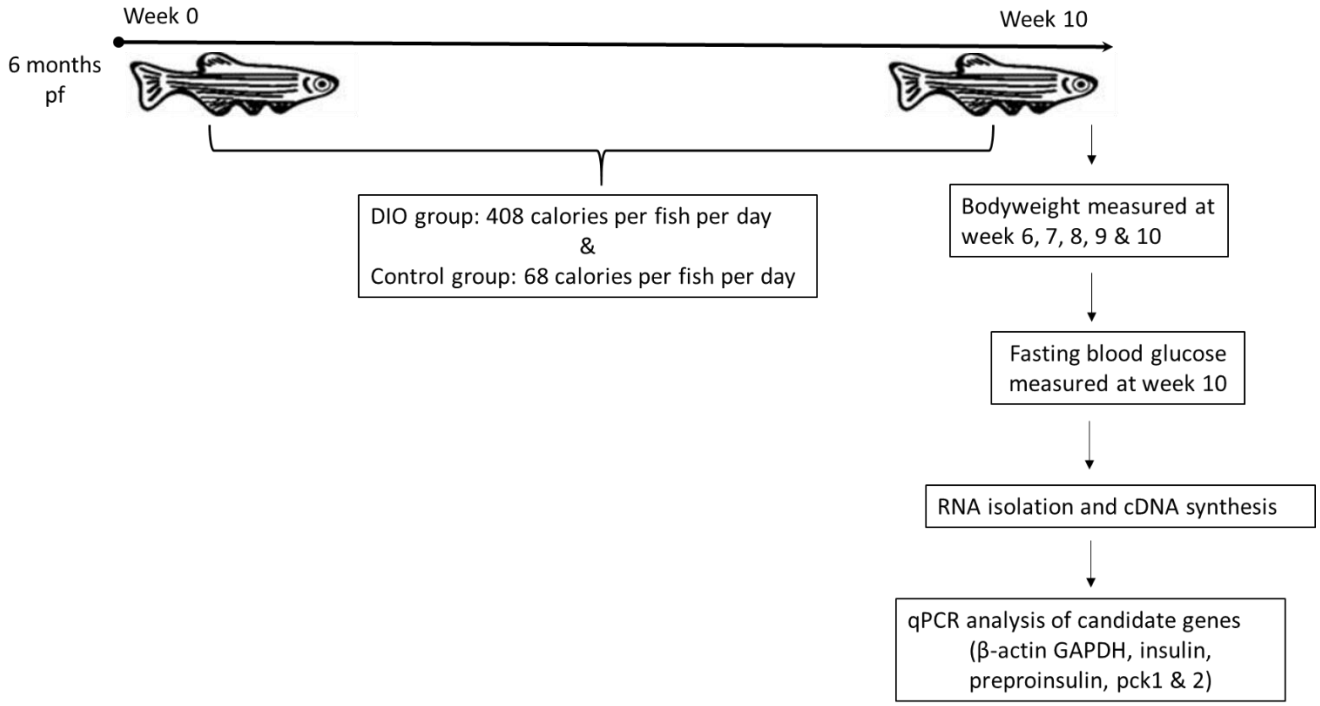


Figure 2.2 Experimental procedure for the zebrafish diet-induced obesity (DIO) and T2DM model. Adult zebrafish (6 months' post-fertilisation (pf), male and female) were fed 68 calories (control group) or 408 calories (experimental group) of Otohime per fish per day for 10 weeks. Bodyweight was determined at week 6, 7, 8, 9 and 10; fasting blood glucose measurements and RNA isolation were only performed at week 10 on all carcases following euthanasia. Total RNA was converted to cDNA and used for qPCR analysis.

2.4. qPCR analysis

The expression levels of GAPDH (*gapdh*); beta-actin (*β-actin*); zebrafish insulin (*zins*); zebrafish preproinsulin (*ppins*) and phosphoenolpyruvate carboxykinase 1 & 2 (*pck1* and *pck2*) mRNA were assessed; using the PowerUp SYBR Green master mix (Cat no. A25742; Applied-Biosystems), the signal was analysed using the Quant-Studio design and analysis software (Applied-Biosystems, Massachusetts, USA). The primer sequences used; *β-actin* and *ppins* (Elo *et al.*, 2007), *gapdh* (Myhre and Pilgrim, 2010), *zins* (Adeyemo *et al.*, 2019), *pck1* and *pck2* (Furukawa *et al.*, 2015), are listed in Table 2.1; all primers were obtained from (Inqaba Biotechnical Industries, Pretoria, South Africa) and achieved efficiencies of between 90% to 100%. The specificity of each PCR reaction was checked with a melting curve analysis.

Table 2. Zebrafish primers used for qPCR analysis

Gene name	Target abbreviation	Forward primer (5' – 3')	Reverse primer (5' – 3')
Beta-actin	*β-actin*	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC
Glyceraldehyde-3-phosphate dehydrogenase	*gapdh*	GTGTAGGCGTGGACTGTGGT	TGGGAGTCAACCAGGACAAATA
Insulin	*zins*	TGGTCGATGCCCTTTATCTGG	AGATGCTGCAGGGTTTGTGG
Preproinsulin	*ppins*	AGTGTAAGCACTAACCCAGGCACA	TGCAAAGTCAGCCACCTCAGTTTC
Phosphoenolpyruvate carboxykinase 1	*pck1*	CAGTAAACACGGCTGAAGACAC	CGGTTTTGATGCACTTGAGA
Phosphoenolpyruvate carboxykinase 2	*pck2*	TCTGGCAGAAGGAAACACA	TCAATCCCTCACTCTCTCCTC

Relative gene expression levels or fold changes were determined by using the ($2^{-\Delta\Delta Ct}$), equation below; β-actin was used as the reference gene for this study.

$$Ct = Ct\ (target\ gene) - Ct\ (reference\ gene)$$

$$\Delta\Delta Ct = \Delta Ct\ (test\ sample) - \Delta Ct\ (control)$$

$$Fold\ change = 2^{(-\Delta\Delta Ct)}$$

2.5. Statistical analysis

All results are represented as means with their standard deviation (SD). Data was analysed using the Student's t-test or one-way ANOVA with multiple comparisons depending on the number of comparisons. A *P*-value of less than 0.05 was considered significant.

3. Results

3.1 *Effect of 2% glucose exposure on weight and fasting blood glucose*

To model a glucose-induced diabetic state, zebrafish were exposed to a 2% glucose solution for a period of 40 days. Studies have shown that incubation of adult zebrafish in glucose solutions ranging from 2-4% glucose lead to hyperglycemia and varying forms of impaired insulin responses; however, chronic exposure to glucose solution at concentrations above 2% increases mortality (Salehpour *et al.*, 2021). Although some fish in the experimental group had a slightly higher bodyweight, there was no significant change in bodyweight of the experimental group when compared to the control group (Figure 2.3). At day 40, the fasting blood glucose of the experimental group (4.0 ± 0.7 mmol/L) was significantly higher (P <0.01) than that of the control group (2.3 ± 0.2 mmol/L), indicating that long-term exposure of zebrafish to 2% glucose solution induced hyperglycemia.

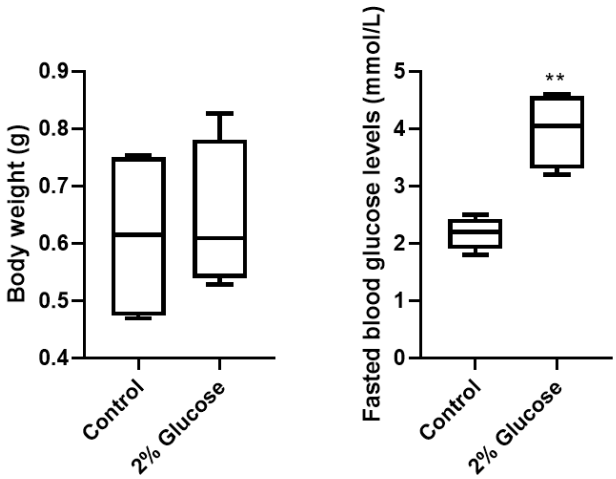


Figure 2.3 Bodyweight and fasting blood glucose levels of control and glucose exposed zebrafish. Adult zebrafish were continuously exposed to 2% glucose solution or tank water (control) for 40 days. Control group (n=6) and experimental group (n=7) were weighed and fasting blood glucose measured at day 40. Data analysed using Student's t-test; values are means± SD against control; ***P* <0.01. Any data that lacks a * is not statistically significant.

3.2 *Effect of overfeeding on weight and fasting blood glucose*

The diet-induced model of T2DM was carried out by overfeeding 408 calories of Otohime-feed per fish per day, control fish received 68 calories per fish per day (Liqing Zang *et al.*, 2017). At 6 weeks of overfeeding the bodyweight of the DIO group (0.73 g ± 0.05 g) was significantly (P <0.01) increased compared to the control group (0.57 g ± 0.1 g) (Figure 2.4). Subsequently, from week 7 to 10, the bodyweight of the overfed group remained significantly higher than that

of the control group: week 7 (0.72 g ± 0.1 g vs 0.55 g ± 0.15 g; p<0.01), week 8 (0.70 g ± 0.1 g vs 0.54 g ± 0.1 g; p<0.01), week 9 (0.70 g ± 0.05 g vs. 0.54 g ± 0.1 g; p<0.01) and week 10 (0.75 g ± 0.05 g vs 0.61 g ± 0.1 g; p<0.05). It is evident that the fish in the overfed group gained significant body mass compared to the control group from 6 to 10 weeks of overfeeding; even though there was more variation in the bodyweight of individual fish in the overfed group (Figure 2.4a). The bodyweight gain of the overfed group was clearly evident at week 10 compared to the control group (Figure 2.4b).

At week 10, the difference in fasting blood glucose in control fish (2.1 ± 0.2 mmol/L) versus those overfed DIO (3.5 ± 0.9 mmol/L) was also significant (Figure 2.4c; P <0.005), demonstrating that long-term overfeeding of a high protein, high-fat diet induced obesity and hyperglycemia in zebrafish compared to those fed a normal diet.

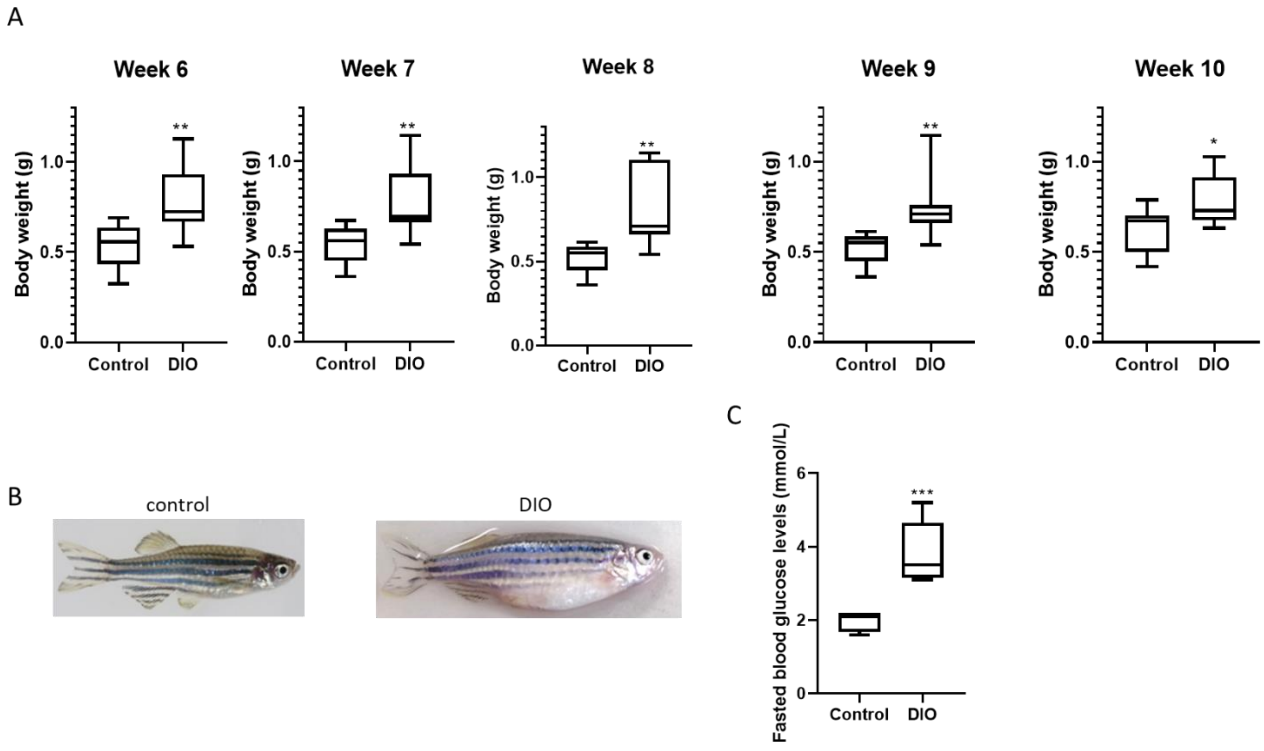


Figure 2.4 Bodyweight change and fasting blood glucose of normal-fed and overfed (DIO) zebrafish. Adult zebrafish were overfed (DIO; 408 calories per fish per day; n=7) or normal-fed (control; 68 calories per fish per day respectively; n=6) for 10 weeks. **(A)** The control and overfed groups were weighed weekly during the period of treatment (week 6 – 10 shown); **(B**) visual difference in bodyweight and **(C)** fasting blood glucose measurements at week 10. Data was analysed using the Student's t-test; values are means± SD against control; **P* <0.05, ***P* <0.01, ****P* <0.005.

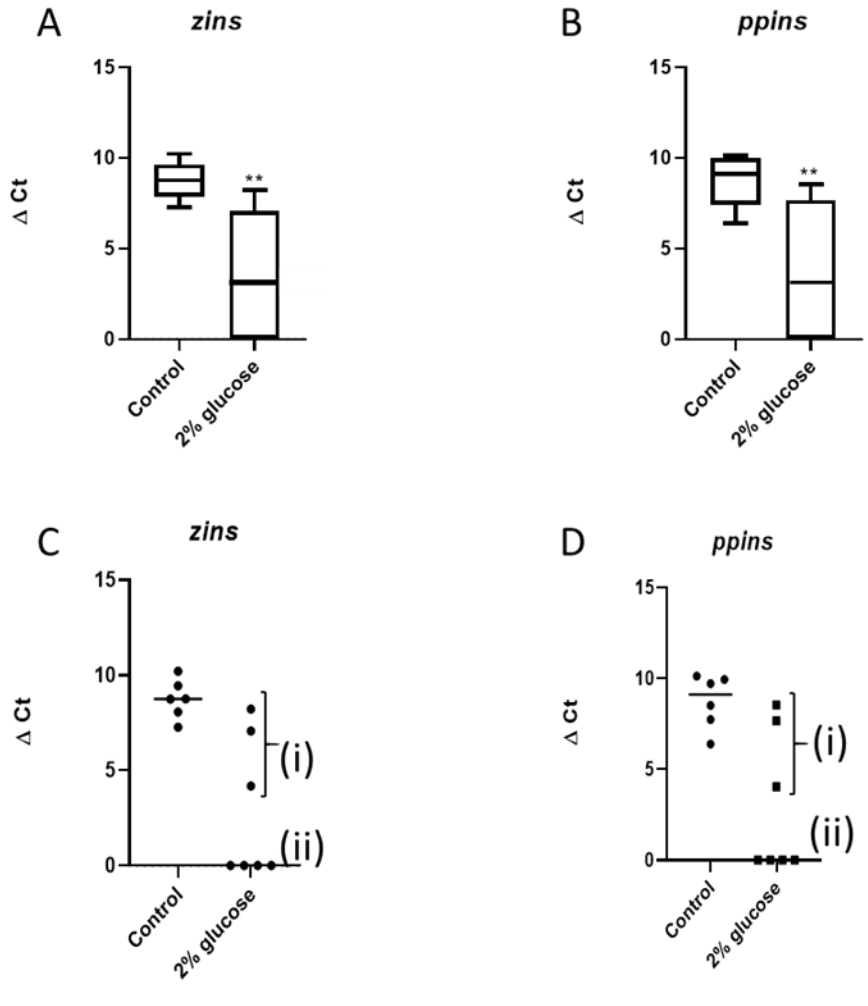
3.3 *Response of zebrafish candidate genes to glucose exposure*

a. Insulin and preproinsulin

Hyperglycemia is one of the early stages of insulin resistance and a diabetic state. We therefore analysed the expression profile of two related hormones, insulin (zins) and its precursor preproinsulin (ppins), essential for the maintenance of blood glucose homeostasis (Röder *et al.*, 2016). We specifically started by determining the delta-Ct under control and experimental conditions, which allowed us to compare the mRNA expression between these conditions. Mean delta-Ct (ΔCt) values were significantly different (P <0.01) in the control vs experimental group respectively for insulin (ΔCt 8.7 ± 1.0 vs ΔCt 2.8 ± 3.6) and preproinsulin (ΔCt 8.7 ± 1.5 vs ΔCt 2.9 ± 3.9), (Figure 2.5 A and B).

The delta-Ct values for zins and ppins expression in individual animals were observed to fall into two groups for each candidate gene reflecting either an increase in mRNA expression or an absolute loss of gene expression; these were subsequently referred to as group (i) and (ii) respectively (Figure 2.5 C). The delta-Ct of zins-group (i) (ΔCt 4.8 ± 1.9 to ΔCt 8.6 ± 1.9) and ppins-group (i) (ΔCt 4.6 ± 1.7 to ΔCt 8.7 ± 1.7) were lower than the control mean (ΔCt 9.1 ± 0.1) (Figure 2.5 C and D), reflecting an increase in the relative mRNA expression of insulin and preproinsulin in this group when compared with the control; this was confirmed by a higher relative fold-change in zins-group (i) and ppins-group (i) (4.6 and 4.0 higher than control respectively; Figure 2.5 E). The delta-Ct of zins-group (ii) and ppins-group (ii) was zero due to lack of transcript expression; fold changes in this group also indicated lack of insulin and preproinsulin mRNA expression.

Interestingly, the delta-Ct of zins-group (ii) and ppins-group (ii) was zero, suggesting a complete lack of transcript expression; fold changes in this group reflected this lack of insulin and preproinsulin mRNA expression, with values of 0 for both groups (Figure 2.5E).



E

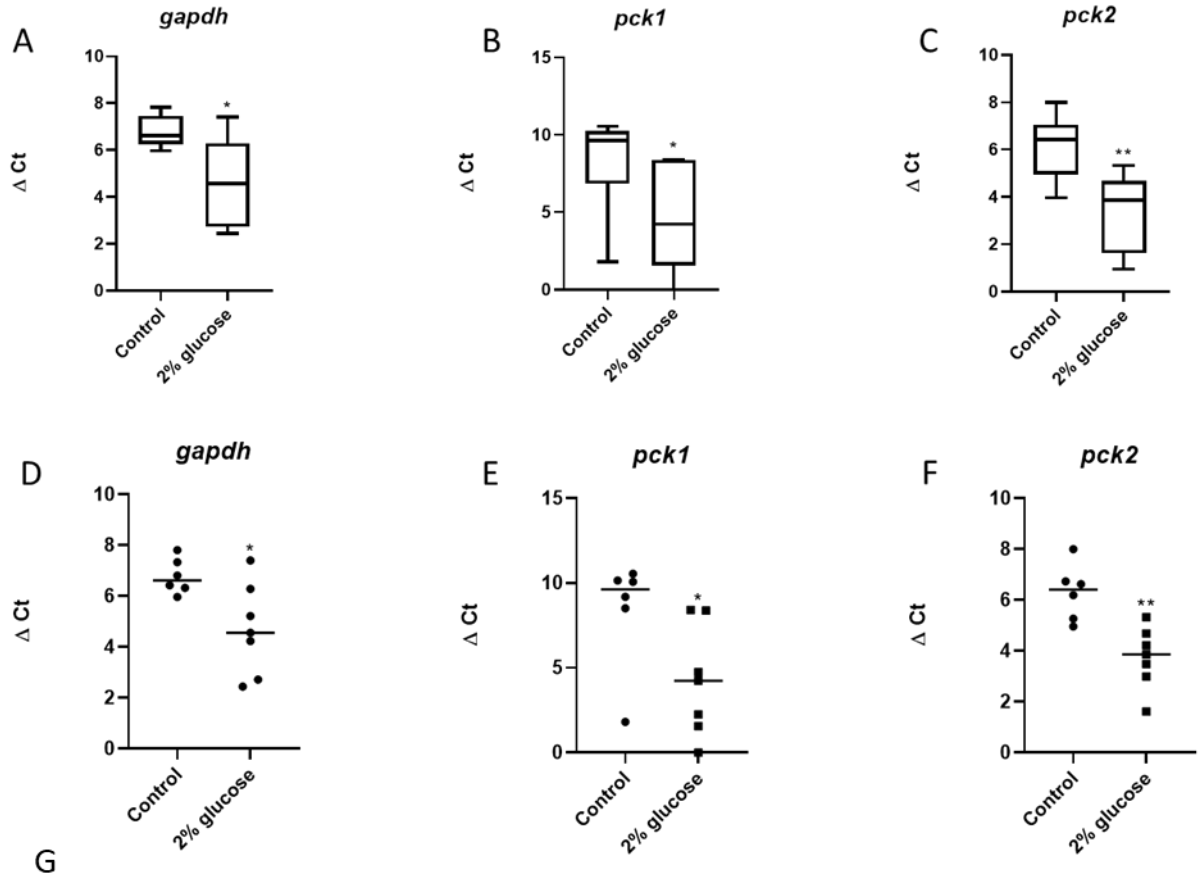
FOLD DIFF	ZINS Group (i)	ZINS Group (ii)	PPINS Group (i)	PPINS Group (ii)
2% Glucose	>1 (4.6)	<1 (0.0)	>1 (4.0)	<1 (0.0)

Figure 2.5 Difference in ΔCt values and fold change of hormones essential for glucose metabolism in zebrafish.
Adult zebrafish were continuously exposed to 2% glucose solution or tank water (control) for 40 days. Total RNA isolated from control group (n=6) and experimental group (n=7) generated the cDNA used for qPCR analysis of zebrafish insulin (*zins*) and preproinsulin (*ppins*). Delta-Ct values (ΔCt) were either take as a mean of the population (**A** and **B**) or were divided into group (i) & (ii) within the population (**C** and **D**) and a fold change was determined for each group (**E**). Data was analysed using one-way ANOVA; values are means± SD against control; **P<0.01.

b. GAPDH and phosphoenolpyruvate carboxykinase 1 & 2

The relative mRNA expression of metabolic enzymes GAPDH and phosphoenolpyruvate carboxykinase 1 & 2 (*pck1* & *pck2*) was then evaluated. The delta-Ct values were more variable in the experimental group compared to the control population for the expression of *gapdh*, *pck1* and *pck2* (Figure 2.6); the average delta- Ct value for *gapdh* (ΔCt 4.5 ± 1.8), *pck1*

(ΔCt 4.8 ± 2.6) and *pck2* (ΔCt 4.9 ± 2.1) were significantly (P< 0.05) below the control values (ΔCt 7.7 ± 0.6) (Figure 2.6 A, B and C). Each individual member in the experimental population expressed *gapdh*, *pck1* and *pck2* (Figure 2.6 D, E and F) meaning the up regulation of GAPDH, PCK1 and PCK2 in a high glucose environment was 4.6, 17.1 and 6.0 fold higher than control respectively (Figure 2.6 G), suggesting an increase in expression of these enzymes.



G

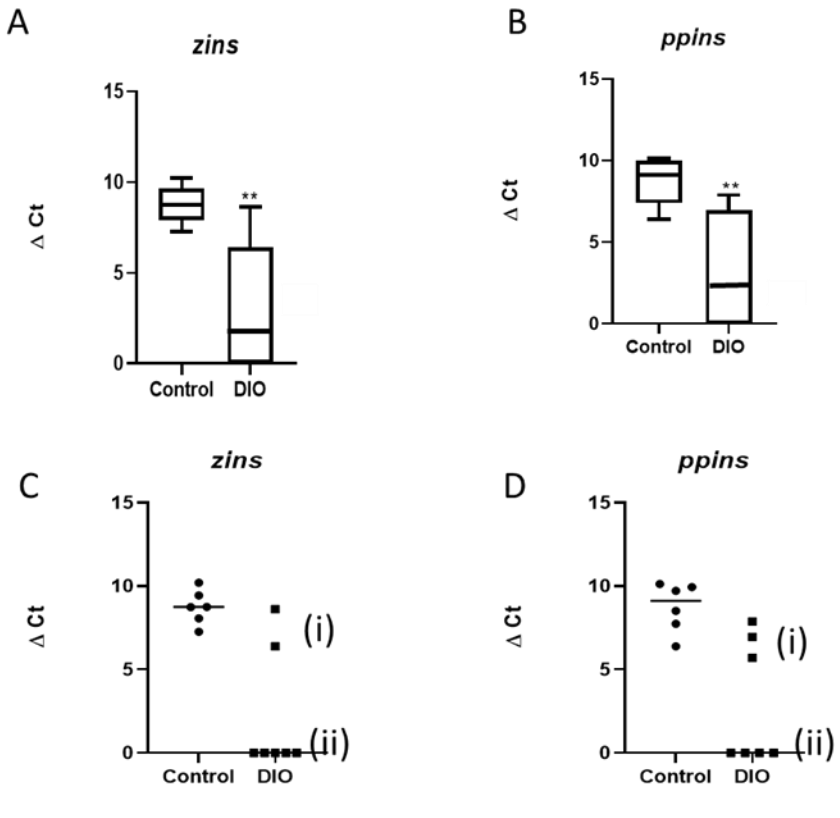
FOLD DIFF	GAPDH	PCK1	PCK2
2% Glucose	>1 (4.6)	>1 (17.1)	>1 (6.0)

Figure 2.6 Difference in ΔCt values and fold change of enzymes essential for glucose metabolism in zebrafish.
Adult zebrafish were continuously exposed to 2% glucose solution or tank water (control) for 40 days. Total RNA isolated from control group (n=6) and experimental group (n=7) generated the cDNA used for qPCR analysis of GAPDH (*gapdh*) and phosphoenolpyruvate carboxykinase 1 & 2 (*pck1* & *pck2*). Delta-Ct values (ΔCt) were plotted to indicate mean of the population (**A**, **B** and **C**) or individual expression (**D**, **E** and **F**) of each gene. Mean delta-Ct was used to determine fold changes (**G**). Data was analysed using one-way ANOVA; values are means± SD against control; *P<0.05, **P<0.01.

3.4 *Effect of overfeeding on candidate gene expression*

a. Insulin and preproinsulin

The zebrafish weight-gain and high fasting blood glucose after 10 weeks of overfeeding prompted us to investigate the relative qPCR expression of insulin and preproinsulin as they play an essential role in glucose homeostasis. The mean delta-Ct of the experimental population was significantly (P< 0.01) lower than that of the control population for the expression of insulin (ΔCt 2.1 ± 3.7 vs ΔCt 8.7 ± 1.0) and preproinsulin (ΔCt 2.9 ± 3.7 vs ΔCt 8.7 ± 1.5) respectively, Figure 2.7 A and B. The analysis of delta-Ct for insulin and preproinsulin expression revealed two groups (i and ii) reflecting increased gene expression or absolute loss of expression in the experimental population respectively. The delta-Ct of *zins*-group (i) (ranging from ΔCt 6.4 to 8.7) and *ppins*-group (i) (ranging from ΔCt 5.7 to 7.9) were below the mean delta-Ct of control (ΔCt 9.3 ± 0.8) (Figure 2.7 C and D) suggesting that there was increased insulin and preproinsulin mRNA in this overfed group compared to the control group. This was reflected by the fold changes that were 2.3 and 3.7 for insulin and preproinsulin respectively (Figure 2.7 E). The delta-Ct in the *zins*-group (ii) and *ppins*-group (ii) was zero indicating a total lack of transcript expression; fold changes in this group reflected lack of insulin and preproinsulin mRNA (Figure 2.7 E), similar to the glucose model in Section 3.3.



E

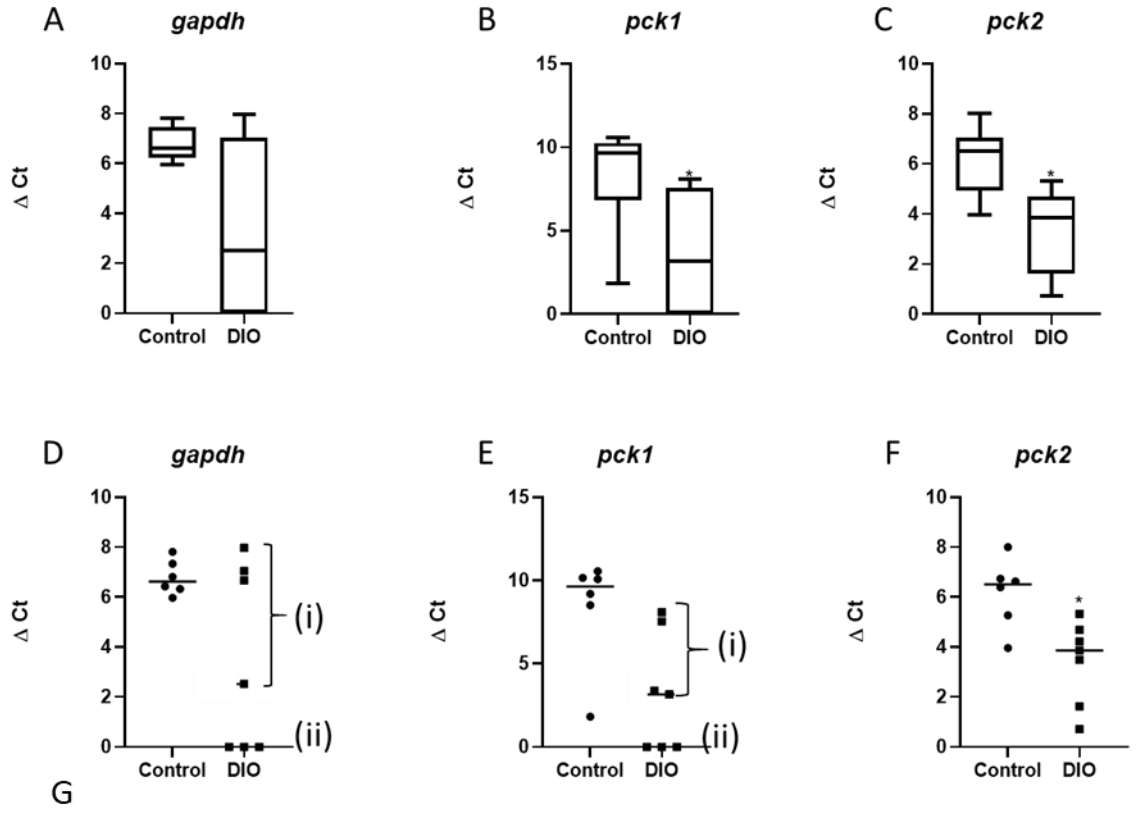
FOLD DIFF	ZINS Group (i)	ZINS Group (ii)	PPINS Group (i)	PPINS Group (ii)
DIO	>1 (2.3)	<1 (0.0)	>1 (3.7)	<1 (0.0)

Figure 2.7 Difference in ΔCt values and fold change of hormones essential for glucose metabolism in zebrafish.
Adult zebrafish were overfed (DIO; 408 calories per fish per day; n=7) or normal-fed (control; 68 calories per fish per day respectively; n=6) for 10 weeks. Total RNA isolated from control group and experimental group generated the cDNA used for qPCR analysis of zebrafish insulin (*zins*) and preproinsulin (*ppins*). Delta-Ct values (ΔCt) were either take as a mean of the population (**A** and **B**) or were divided into group (i) & (ii) within the population (**C** and **D**) and a fold change was determined for each group (**E**). Data was analysed using one-way ANOVA; values are means± SD against control; **P<0.01.

b. GAPDH and phosphoenolpyruvate carboxykinase 1 & 2

The relative qPCR expression of GAPDH and phosphoenolpyruvate carboxykinase 1 & 2 after 10 weeks of overfeeding was also evaluated. The population mean delta-Ct of the overfed group for the relative expression of *gapdh* (ΔCt 3.4 ± 3.6) was lower than the control group (ΔCt 6.8 ± 0.7), but not significantly so (Figure 2.8 A); while the delta-Ct for *pck1* and *pck2* were significantly (P< 0.05) lower than the control (ΔCt 3.2 ± 3.5 vs ΔCt 9.6 ± 3.2) and (ΔCt 3.4 ± 1.7 vs Ct 6.2 ± 1.4) respectively, (Figure 2.8 B and C).

The experimental group contained zebrafish that did not express *gapdh* or *pck1* mRNA at all; these are indicated as group (ii), had a delta-Ct and a fold-change value of zero (Figure 2.8 G). The mean delta-Ct of zebrafish within this group able to express GAPDH, PCK1 and PCK2 mRNA was ΔCt 6.0 ± 2.7; ΔCt 5.5 ± 1.5 and ΔCt 3.4 ± 1.7 respectively, and all were lower than control values ΔCt 6.5 ± 0.8; ΔCt 9.9 ± 0.5 and ΔCt 6.3 ± 1.2 (Figure 2.8 D, E and F). This suggested an increased GAPDH, PCK1 and PCK2 mRNA expression in this group confirmed by respective fold changes at 1.6; 6.9 and 6.0 higher than respective controls.



G

FOLD DIFF	GAPDH Group (i)	GAPDH Group (ii)	PCK 1 Group (i)	PCK1 Group (ii)	PCK2
DIO	>1 (1.6)	<1 (0.0)	>1 (6.9)	<1 (0.0)	>1 (6.0)

Figure 2.8 Difference in ΔCt values and fold change of enzymes essential for glucose metabolism in zebrafish.
Adult zebrafish were overfed (DIO; 408 calories per fish per day; n=7) or normal-fed (control; 68 calories per fish per day respectively; n=6) for 10 weeks. Total RNA isolated from control group and experimental group generated the cDNA used for qPCR analysis of GAPDH (*gapdh*) and phosphoenolpyruvate carboxykinase 1 & 2 (*pck1* & *pck2*). Delta-Ct values (ΔCt) were plotted to indicate mean of the population (**A**, **B** and **C**) or individual expression (**D**, **E** and **F**) of each gene; the delta-ct of each group (i) or (ii) were used to determine fold changes. Data was analysed using one-way ANOVA; values are means± SD against control; *P<0.05. Any data that lacks a * is not statistically significant.

4. Discussion

The adult zebrafish model of T2DM-related hyperglycemia was successfully established using two different approaches: continuous 2% glucose exposure and overfeeding in excess of 340 calories per fish per day in this study (Gleeson *et al.*, 2007; L. Zang *et al.*, 2018). Hyperglycemia is a major biochemical diagnostic feature of the two main forms of diabetes, type 1 or type 2 diabetes. Type1 (or insulin dependent) diabetes is an autoimmune condition caused by the destruction of insulin producing pancreatic β-cells, while type 2 (or non-insulin dependent) diabetes, caused by insulin insensitivity (Capiotti *et al.*, 2014). Type 1 diabetes patients would therefore produce no insulin because the pancreas is incapable; while in type 2 patients the pancreas would produce more and more to overcome insulin resistance, and once exhausted, they would then cease to produce this hormone (Cerf, 2013).

4.1 Glucose solution immersion method

Glucose exposure did not induce significant bodyweight changes, but did significantly increase fasting blood glucose levels; which indicated hyperglycemia in the treated group consistent with other studies (Carnovali *et al.*, 2016). The normal average fasting blood glucose levels in zebrafish was previously shown to be 3.1 ± 0.2 mmol/L (Cao *et al.*, 2023) ours was slightly lower at 2.3 ± 0.2 mmol/L, but still comparable; in the study by Cao et al (Cao *et al.*, 2023), the fasting blood glucose levels were (4.1 ± 0.5 mmol/L), this is similar to the levels observed in our study (4.0 ± 0.7 mmol/L). Another previous study exposing zebrafish to a 2% glucose solution for 14 days was able to achieve fasting blood glucose levels 4-5 times higher (13.52 ± 1.29 mmol/L) than that of the control group (2.99 ± 0.18 mmol/L) (Capiotti *et al.*, 2014). The fasting blood glucose levels observed in our study were two-fold higher in the glucose-treated group compared to the control group which is within acceptable levels for a zebrafish T2DM model the difference to the Capiotti study could in part be attributed to the low fasting blood glucose level of the control group (baseline) in our study.

4.2 Overfeeding method

The similar metabolic pathways of carbohydrate and dietary fat processing of zebrafish to that of humans permits the use of diet to induce T2DM in zebrafish. Feeding a high fat diet (brine shrimp, plus 1% egg yolk) to adult zebrafish for 10 weeks has been shown to cause obesity and increased fasting blood glucose levels including impaired insulin function (Meng *et al.*, 2017). Another study demonstrated obesity, hyperglycemia and insulin resistance in zebrafish by automated overfeeding a high protein, high fat diet (Otohime B2) for eight weeks (Liqing Zang *et al.*, 2017). We induced significant bodyweight gain and hyperglycemia in the treated group (3.5 ± 0.9 mmol/L) compared to control (2.0 ± 0.2 mmol/L) by overfeeding Otohime B2

for 10 weeks; this was comparable to a previous study by Zang *et al* which, by week 8 of overfeeding with Otohime, obtained an average fasting blood glucose level of 4.5 ± 0.8 mmol/L for the treatment group compared with 2.8 ± 0.1 mmol/L for control. The overfeeding approach is almost always associated with weight-gain or adipose tissue accumulation which contribute to insulin insensitivity and hyperglycemia (Kahn, 2003). It would be interesting to measure fasting blood glucose at earlier time points as the significant weight gain was already seen at 6 weeks; it is likely that fasting glucose was upregulated prior to 10 weeks. This would translate to a faster induction period of hyperglycemia, which would be beneficial.

4.3 Expression of candidate genes

Impaired glucose processing as a result of insulin resistance is one of the major determining factors of T2DM. To determine whether the hyperglycemia observed after the glucose treatment and overfeeding correlated with, or were accompanied by, reduced insulin responsiveness (resistance) or a lack of insulin production, we sought to quantify the mRNA expression levels of insulin and its precursor preproinsulin, hormones essential for carbohydrate metabolism (Röder *et al.*, 2016). Our approach involved utilising the whole carcass rather than specific specialised organs to determine the mRNA expression levels of insulin and preproinsulin. Mean delta-Ct values were significantly different in the control vs experimental group for insulin and preproinsulin respectively. On closer inspection we could identify two groups based on their gene expression response. Group 1, with moderate increases in insulin and preproinsulin mRNA expression and group 2, with decreased expression of both genes or in fact no expression at all; these were observed in both the glucose and diet (DIO) models. It is possible that the sustained hyperglycaemic burden of glucose and overfeeding reduced the function or productivity of insulin-producing cells leading to pancreatic exhaustion and ultimately lack of insulin expression in some individuals of this study. The delta-Ct values for both insulin and preproinsulin suggest that the ultimate down-regulation of insulin and preproinsulin mRNA expression may be a final response to continuous glucose exposure of zebrafish in our model. Analysis at both earlier and later timepoints through the inclusion of repeated blood draws may shed light on this. Published studies using zebrafish models of T2DM have demonstrated hyperglycemia to be a result of insulin insensitivity (resistance) and not necessarily lack of insulin production; this was shown in one study through fluorescence tagging of insulin (Liqing Zang *et al.*, 2017) and in another by measuring insulin mRNA expressing levels in specific target organs such as the liver and muscle (Meng *et al.*, 2017). A further adaptation in future studies would therefore be the analysis of mRNA in target organs, rather than the entire zebrafish as a whole. Finally, it would be interesting to analyse the expression of pancreatic genes, such as PDX1 to

determine organ function in our model and gain some insight as to the state of the pancreas itself.

The role of the metabolic enzyme phosphoenolpyruvate carboxykinase as a potential blood glucose indicator in a diabetic state in zebrafish has only been investigated in larvae; and its expression has been suggested as a potential indicator of blood glucose levels (Elo *et al.*, 2007). This means in an individual with normal blood glucose levels PCK would be supressed in the presence of insulin in the bloodstream and upregulated in the presence of glucagon. In the current study the mRNA expression of PCK 1 and 2 increased seventeen- and six-fold respectively in glucose-exposed zebrafish, while the increase was seven- and six-fold respectively in the overfed group (DIO). Uptake of external glucose is involuntary in zebrafish as a result of osmotic pressure regulation as the water exchanges between the fish and its environment (Cao *et al.*, 2023); while food is taken in through digestion. It is possible that the involuntary uptake of glucose or chronic hyperglycemia was more detrimental to insulin sensitive tissue than overfeeding, leading to severe insulin insensitivity and impaired regulation PCK or its upregulation. Studies have shown that prolonged hyperglycemia reduces the inhibitory effects of glucose and insulin on PCK, leading to its upregulation and subsequently more glucose production via gluconeogenesis (Shao *et al.*, 2005); this phenomenon has however not been observed with overfeeding.

While these are proven methods of inducing hyperglycemia, the limitation of this study is that one cannot ascertain that the fish are hyperglycaemic until they are sacrificed. Although a method for repeated bi-monthly blood draws from live animals has been reported, this method was not successfully implemented in the current study, and will require optimization for future research projects. The use of whole carcases for RNA isolation to determine gene expression of *zins, ppins pck1 and pck2* was also a limitation, as it did not provide target tissue-specific information. In future, insulin sensitive tissues such as the liver, pancreas and muscle should be isolated to understand gene expression changes in these specific target tissues. Another limitation is that although we assessed insulin levels through relative mRNA expression, we did not analyse protein expression itself. Additionally, zebrafish *β-actin* and *gapdh* are prone to variation during experimental treatment and development respectively; therefore their use as internal controls was a challenge(McCurley and Callard, 2008). Eight zebrafish housekeeping genes are routinely used in zebrafish studies; these include beta actin (*b-actin1)*, tubulin alpha 1 (*tuba1*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), glucose-6-phosphate dehydrogenase (*g6pd*), TATAbox binding protein (*tbp*), beta-2-microglobulin (*b2m*), elongation factor 1 alpha (*elfa*), and 18s ribosomal RNA (*18s*)) (McCurley and Callard, 2008). All have been found to have a degree of variability following treatment of

zebrafish. As was the case in the McCurley and Callard study, in our study; *β-actin* appeared less variable than gapdh, and was therefore utilised as the internal reference gene for control purposes.

In summary, using two different approaches, we established zebrafish models that were clearly hyperglycaemic, indicative of a diabetic state. We have evaluated three specific diabetic factors to validate our models; and these include bodyweight gain, fasting blood hyperglycemia and gene expression levels of metabolic hormones and enzymes. We sought to establish and evaluate genetic, physical and glycemic changes that occur in zebrafish during a diabetic state as a result of exposure to glucose and overfeeding. These evaluations would then allow us to establish a baseline that could be used to compare changes that occur in zebrafish in response to ARV exposure; this is presented in the following chapters. The model described may also be useful to local scientists and could be applied and modified by researchers studying diabetes in zebrafish.

Chapter 3

Tenofovir, Lamivudine and Dolutegravir (TLD) *in vitro* and *in vivo* toxicity testing

1. Introduction

Non-communicable disease (NCDs) kill an estimated 41 million people each year and this is equivalent to about 71% of all global deaths (Samodien *et al.*, 2021). The use of combination antiretroviral therapy (cART) (consisting of two or more drugs incombination) has been shown to increase the risk of metabolic disorders predisposing to type 2 diabetes (T2DM) and other NCDs (van Wijk and Cabezas, 2012). South Africa already has a high incidence of NCDs and the largest antiretroviral therapy (ART) program in the world (Gizamba *et al.*, 2023). As the burden of NCDs rises in the general global population, the burden in the population of people living with HIV (PLWH) is compounded by ART (Hadavandsiri *et al.*, 2023). The fixed-dose antiretroviral therapy combination of tenofovir (300 mg), lamivudine (300 mg) and dolutegravir (50 mg) (TLD), is a preferred first line regimen in Sub-Saharan Africa, for all HIV-positive patients (Fokam *et al.*, 2023). One of the main side-effects of TLD when used by HIV-positive woman during pregnancy is the potential risk of infant neural tube defect (NTD) attributed to dolutegravir (Zash *et al.*, 2019). Tenofovir on the other hand is associated with renal tubular dysfunction in HIV-positive children (Mashingaidze-Mano *et al.*, 2020). The use of animal models is important for understanding mechanisms of these drug side effects or drug-induced NCDs. Because the fixed-dose TLD formulation is clinically prescribed for adolescents and adults weighing at least 30kg (Bangalee *et al.*, 2022), tolerable doses need to be established for specific animal models before experimentation.

To determine the most appropriate dose of TLD, and its individual components, in animal models, concentration and toxicity profiles need to be evaluated in *in vitro* first before *in vivo* experimentation. Toxicity is an essential part of drug testing as it is the leading cause of mortality during animal experimentation (Morello *et al.*, 2018). Thus, drug toxicity screening tools are required for rapid screening of already available drug compounds, and one such tool is the zebrafish embryos (up to 5 days-post-fertilisation -dpf). The zebrafish embryo has many advantageous features as a drug toxicity screening tool such as rapid development, small size (able to fit in a 96-well plate), ease of drug administration in small amounts, transparent embryos allowing observation of morphological changes during treatment, ease of maintenance and high metabolic and genetic homology with mammals (Gorgulho *et al.*, 2018).

In this chapter we therefore investigated the toxicity of TLD and its individual compounds at different concentrations using *in vitro* (HEK293 cells) and *in vivo* (zebrafish embryo) models. Compound toxicity *in vitro* was assessed through MTS cell viability testing after 72 hours of exposure, while *in vivo* toxicity was monitored via the assessment of morphological changes that occur during exposure, including but not limited to heart edema, spinal curvature, embryo coagulation and defects in somite development. The studies were carried out in response to a range of concentrations of tenofovir, lamivudine and dolutegravir, individually and in combination.

2. Methods and materials

2.1. ARV Drug preparation

Tenofovir Disoproxil Fumarate (TDF) (Cat. no. SML1794, Sigma-Aldrich, St. Louis, USA) and Lamivudine (Cat. no. L1295, Sigma-Aldrich, St. Louis, USA), ≥98% (HPLC) powder were dissolved in Dimethyl sulfoxide (DMSO, Cat. no. D2650, Sigma-Aldrich, St. Louis, USA) to 100 mM stocks. Concentrations of 100 µM, 50 µM, 25 µM and 10 µM, were prepared from stocks diluted in 10% Dulbecco's Modified Eagle's Media (DMEM, Cat. no D5648, Capital Lab Supplies, South Africa) or E3 / embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4). The ARV drug compounds were diluted such that the maximum concentration of DMSO per test was ≤0.1% (v/v). Dolutegravir was dissolved in DMSO to a 20 mM stock solution diluted to 100 µM, 50 µM, 25 µM and 10 µM before treatment.

2.2. Overall approach to toxicity studies

Toxicity was carried out *in vitro* (on HEK 293 cells) and *in vivo* (on 24 hpf dechorionated zebrafish embryos) (Figure 2.1; see Section 2.3 and 2.5 for more details). The cells were incubated with individual TLD components (tenofovir, lamivudine and dolutegravir) at concentrations of 10, 25, 50 and 100 µM for 72 hours at 37°C. After exposure HEK 293 cells were tested for viability by using the MTS assay, where the absorbance values were converted to percentage viability relative to control. Additionally, HEK 293 cell images were take using an Olympus CKX41 light microscope to confirm live morphology. The highest non-lethal concentrations of the TLD drug components were subsequently used for the *in vivo* toxicity study. The embryos were dechorionated (1 mg/ml pronase E dissolved in RO water; Cat no. 9036-06-0, Merck, USA) to ensure maximum exposure of the embryo to the administered compounds. Dechorionated zebrafish embryos at 24 hpf were exposed to 100 µM tenofovir,

100 µM lamivudine and 100 or 50 µM dolutegravir for 72 hours (Figure 3.1). After the assessment of morphological toxicity of individual compounds, a TLD formulation was then tested on cells and embryos.

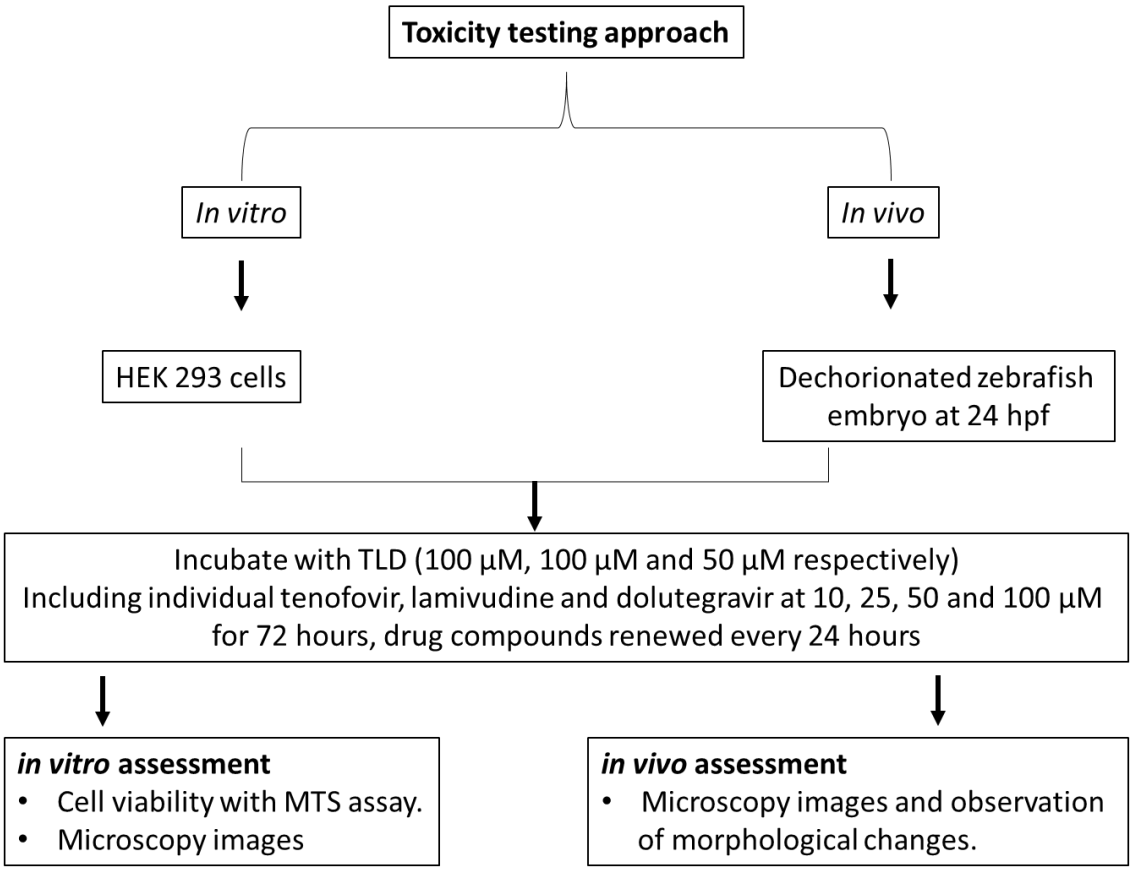


Figure3.1 Experimental approach for the *in vitro* and *in vivo* TLD toxicity testing.
The *in vitro* and *in vivo* toxicity testing was carried out by treating HEK-293 cells and 24 hpf dechorionated embryos respectively with (0-100 µM) TLD components and TLD combination for 72 hours. *In vitro* toxicity was assessed by indirectly measuring cell viability using the MTS assay, while *in vivo* toxicity was assessed visually by monitoring morphological changes due to treatment.

2.3. Cell culture and in vitro toxicity testing

Human embryonic kidney 293 cells (kindly provided by Prof. Raymond Hewer, School of Life Sciences, University of Kwazulu-Natal), were cultured in 10% DMEM growth-media (supplemented with 10% fetal bovine serum (FBS)) at 37°C with 5% CO2. Confluent HEK 293 cells were scrapped off, spun-down and re-suspended in fresh growth-media to determine cell viability using the TC20 automated cell counter (Bio-Rad, California, USA). Cells (100 µl) with viability ≥80% were seeded into 96-well cell culture plates at 2 x10^5 cells/ml (i.e. final number of 2 x10^4 cells/well) and incubated for 4-hours at 37°C with 5% CO2. Adherent cells were then treated with 100 µM, 50 µM, 25 µM and 10 µM of tenofovir, lamivudine and dolutegravir in triplicate at 37°C, 5% CO2 for 72 hours; media and ARVs were replaced each day. Controls

included DMEM growth-media in the absence and presence of 0.1% to 0.45% (v/v) DMSO. Effects of drug exposure were assessed through cell viability using the MTS assay (Section 2.4). Representative images of cells in the experimental group were taken with an Olympus CKX41 light microscope. Once individual toxicity was completed, the TLD combination could then be assessed using the same approach; concentrations were informed from the individual dose responses.

2.4. MTS assay

Following the treatment of HEK 293 cells with antiretroviral compounds for 72 hours, the Cell-Titer 96® Aqueous One Solution Reagent (Cat. no. G3580, Promega, Wisconsin, USA) was added into cells in the 96-well plate and incubated for 4 hours at 37°C with 5% CO^2. The 96-well cell culture plate was placed into the SpetraMax® ABS Plus ELISA plate reader (Molecular Devices, California, USA) and agitated (using the "shake" function) before measuring the absorbance at 490 nm.

2.5. In vivo toxicity testing

Spawned zebrafish embryos were incubated at 28.5°C for 24 hours, dechorionated using pronase E (Cat no. 9036-06-0, Merck, USA) by incubating the chorionated embryos in a 1 mg/ml pronase solution for 40 minutes, rinsed off with E3 media and incubated with tenofovir (100 µM), lamivudine (100 µM) and dolutegravir (50 µM or100) or the TLD combination (concentrations of tenofovir, lamivudine and dolutegravir determined from the previous toxicity study) for 72 hours with drug treatment renewed every 24 hours; 0.45% (v/v) DMSO was used as a control. Zebrafish embryos were observed under an Olympus CKX41 light microscope after treatment and images taken to identify morphological changes in treatment group compared to control.

2.6. Data and statistical analysis

The absorbance values indicate the mean with standard deviation (SD) from three individual experiments (n=3), each done in triplicates. The absorbance was converted to percent viability based on the growth media control. Data for cell viability was analysed with one-way ANOVA (using GraphPad prism 8.3.0). A value of $P < 0.05$ was considered significant. The zebrafish experiment was repeated at least three times (n=3); only representative images shown.

3. Results

3.1. Tenofovir, lamivudine and dolutegravir toxicity in vitro

To test the individual toxicity of the TLD components *in vitro*, HEK 293 cells were cultured in the presence of different concentrations (10 µM to 100 µM) of the drug compounds for 72 hours; growth media and 0.45% (v/v) DMSO were used as controls. The cell structure and appearance of HEK 293s was not negatively affected by exposure to 0.45% (v/v) DMSO, 100 µM tenofovir, 100 µM lamivudine and 100 µM dolutegravir individually (Figure 3.2 A-D).

There was no significant difference in the percentage viable cells when comparing growth media and DMSO exposed cultures (Figure 3.2 E-F); at concentrations of 0.1% to 0.45% (v/v) DMSO, this organic solvent did not exert a significant negative affect on HEK 293 cell viability. The exposure to tenofovir at 10 µM did not affect cell growth when compared to the DMSO control, while at higher concentrations (50 µM and 100 µM) tenofovir did reduce the mean cell viability to 75% at 100 µM, however, this effect was not significant (Figure 3.2 E). HEK 293 cell viability was not significantly affected by lamivudine or dolutegravir exposure even at the highest concentration (100 µM) used in this study with the cultures in these treatment groups reflecting 86% ± 0.8 and 98% ± 0.1 viability, respectively (Figure 3.2 F and G). It was apparent though that although DMSO, lamivudine and dolutegravir did not significantly affect the growth of HEK 293s, tenofovir at 100 µM did slow down the cell growth rate (Figure 3.2 E-G), and this explains the perceived reduction in cell viability observed. However, the number of cells after 72 hours of exposure was still higher than the initial number of the cells plated.

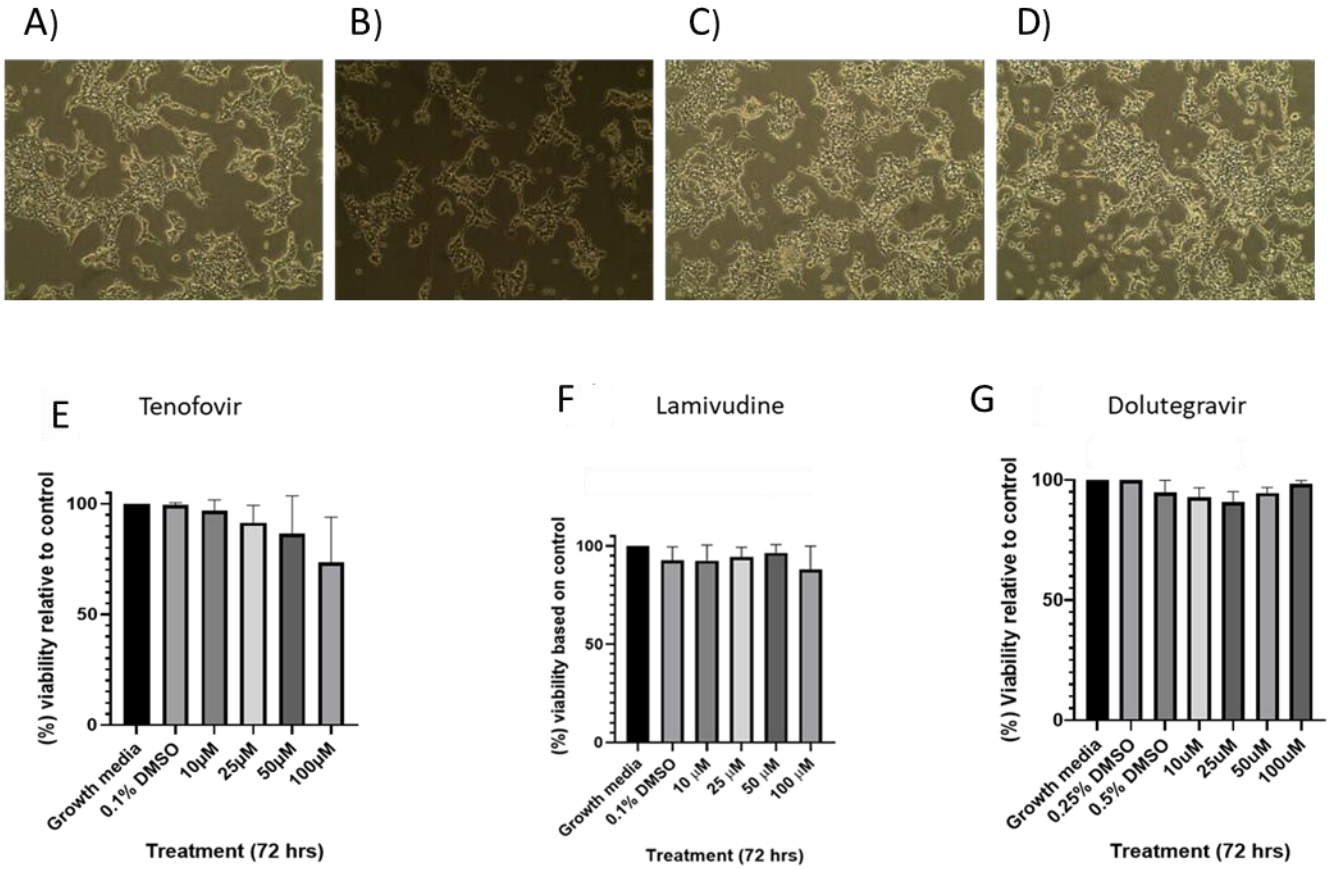


Figure 3.2. Cytotoxicity of tenofovir, lamivudine and dolutegravir on HEK-293 cells.
Cells were incubated in the presence or absence of ARVs for 72 hours. Microscope images of (**A**) 0.45% (v/v) DMSO (control), (**B**) 100 µM tenofovir, (**C**) 100 µM lamivudine or (**D**) 50 µM dolutegravir at 40x magnification were taken to monitor changes in cellular structure and cell proliferation. The MTS assay was used to indirectly measure cell viability in response to 10 µM, 25 µM 50 µM and 100 µM of **E**) tenofovir, **F**) lamivudine and **G**) dolutegravir. Growth media and DMSO controls were also included. Absorbance measurements were expressed as percentage viability relative to the growth media control. Data was analysed using one-way ANOVA; values are means± SD (n=3). Any data that lacks a * is not statistically significant.

3.2. Tenofovir, lamivudine and dolutegravir toxicity in vivo

Following the *in vitro* profiling of the individual TLD drug components indicating the survival of HEK 293 cells at the highest concentration (100 µM) used in this study; the same concentration was then used to assess the toxicity of tenofovir, lamivudine and dolutegravir *in vivo* on dechorionated zebrafish embryos. We first showed that DMSO exposure at 0.45% (v/v) was not toxic to zebrafish embryos after 24 hours and 72 hours of treatment; embryonic morphology and development was similar to embryos in E3 media alone (Figure 3.3 A).

We then chose 0.45% (v/v) DMSO, the drug diluent in this study as our subsequent control for further studies. The exposure of zebrafish embryos to the highest concentration of tenofovir (100 µM) and lamivudine (100 µM) did not lead to death or drug-related physical changes in zebrafish morphology after 24 hours and 72 hours of exposure (Figure 3.3 B ii) and iii)). In

contrast, the treatment of zebrafish embryos with the highest concentration of dolutegravir (100 µM) used in this study, although not toxic after 24 hours, was observed to be lethal to the embryos after 72 hours of exposure (Figure 3.3 B, v)). As a result, the concentration of dolutegravir used in subsequent studies was reduced to 50 µM as this concentration was not observed to be lethal or toxic to zebrafish embryos after 72 hours of exposure (Figure 3.3 B, iv)).

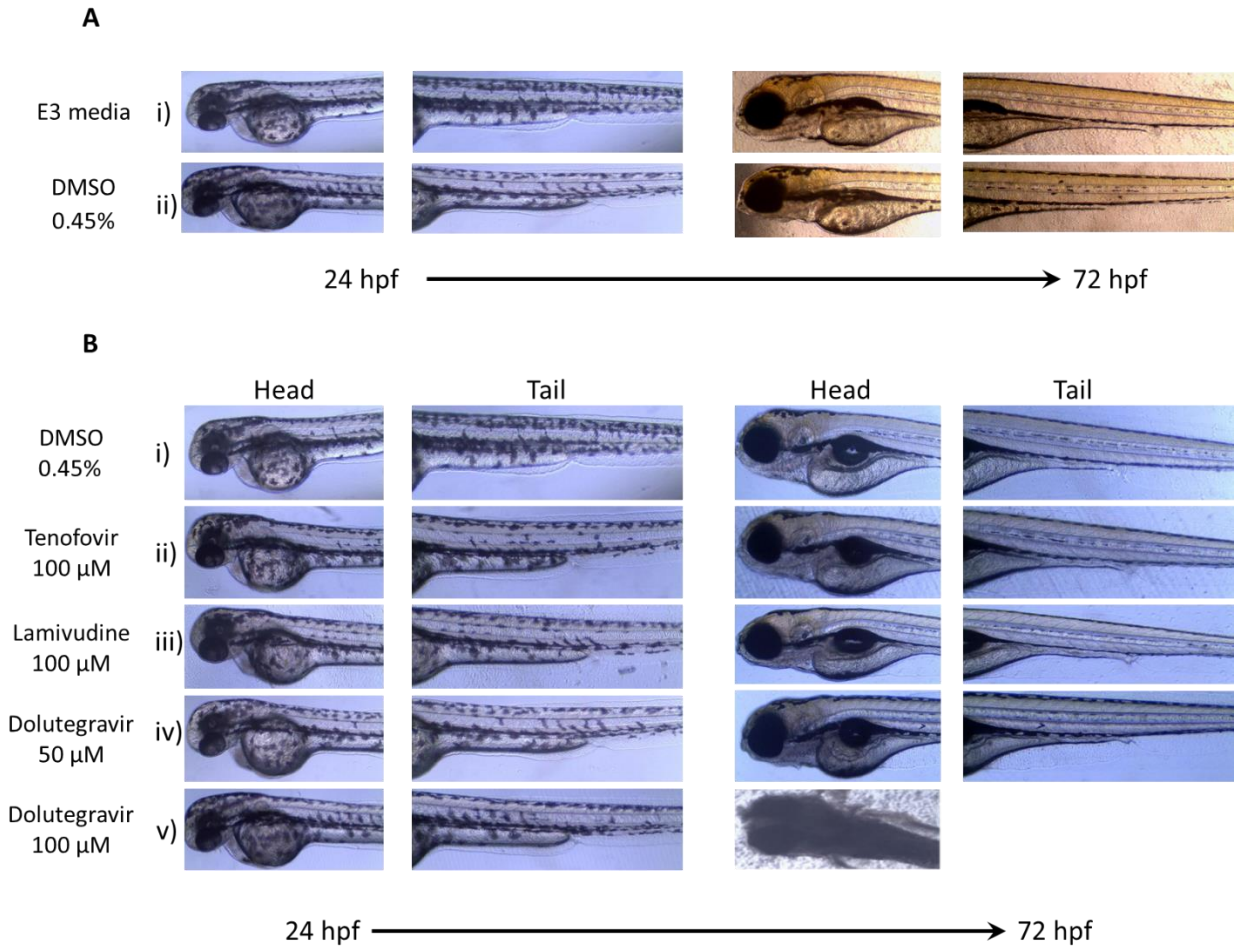


Figure 3.3. In vivo toxicity of tenofovir, lamivudine and dolutegravir on zebrafish embryos.
Zebrafish embryos were incubated at 28.5°C after spawning and dechorionated at 24 hours post fertilisation (hpf). (**A**) controls, **i)** E3 media vs**. ii)** 0.45% (v/v) DMSO were compared. (**B**) experiment groups **(ii**) tenofovir 100 µM; **iii**) lamivudine 100 µM, **iv**) dolutegravir 50 µM and **v**) dolutegravir 100 µM) were compared to **i**) DMSO control. During treatment microscope images of zebrafish embryos were taken for morphological observations 24 hours after treatment and at the end of the 72-hour treatment; the experiment was repeated at least three times (n=3).

3.3. TLD combination toxicity analysis in vitro and in vivo

After establishing the appropriate non-lethal or non-toxic concentrations of the individual TLD drug components in HEK 293 cells and zebrafish embryos, we then used this information to formulate our version of a fixed-dose TLD drug consisting of 100 µM tenofovir, 100 µM

lamivudine and 50 µM dolutegravir. This TLD formulation was then tested on cells *in vitro* and zebrafish embryos *in vivo* to assess toxicity in the final fixed-dose combination format.

After treatment, the percentage viable cells cultured in the growth media or and DMSO was similar at 97% ± 0.1 viability; while the percentage viable cells in response to TLD was significantly (*P*< 0.01) decreased compared to control at 78% ± 0.2 viability (Figure 3.4 A). This was further supported by microscopy images which showed more cells in the DMSO control (Figure 3.4 B) compared to the treated TLD experimental group (Figure 3.4 C). This effect was as result of slow growth rate in the TLD-treated cultures similar to that observed following tenofovir treatment of HEK 293s. Finally, exposing zebrafish embryos to TLD for 24 hours and 72 hours did not lead to toxicity or any observed form of morphological changes when compared with the DMSO control group (Figure 3.4 D).

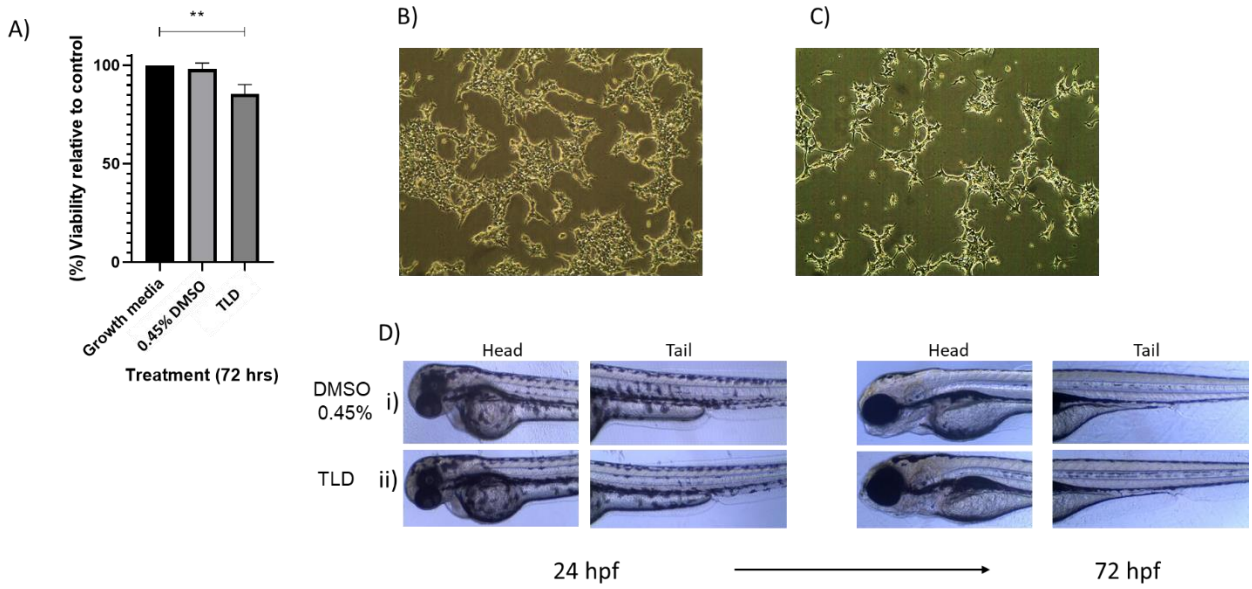


Figure 3.4. *In vitro* and *in vivo* toxicity of TLD after 27-hours of exposure.
HEK 293 cells were incubated with growth media, DMSO or TLD for 72 hours and cell viability was determined using the MTS assay (**A**). Microscope images of the DMSO (**B**) and TLD (**C**) treated cells were taken at 72 hours after treatment. (**D**) *In vivo* toxicity was tested on zebrafish embryos after 24 hours and 72 hours of **i)** 0.45% DMSO or **ii)** TLD exposure, morphological changes were evaluated using a light microscope after treatment. Data was analysed using one-way ANOVA, the experiment was repeated at least three times (n=3); values are means± SD against control; ***P*<0.01. Any data that lacks a * is not statistically significant.

3.4. TLD slows development of zebrafish embryos

Since toxic effects of drugs are not always observable after a shorter period of exposure, we extended the exposure period, to 120 hours after treatment. Although there were no noticeable differences in DMSO-treated zebrafish embryos compared to TLD-treated embryos after 72

hours, after 120 hours of treatment, the TLD-exposed embryos were delayed in their development; this was highlighted by the lack of swim bladder formation and the inability to remain upright (Figure 3.5). Once the drug is removed however, the growth rate or embryo development rate returns to normal, and the juveniles were able to remain upright. The effect was therefore reversible.

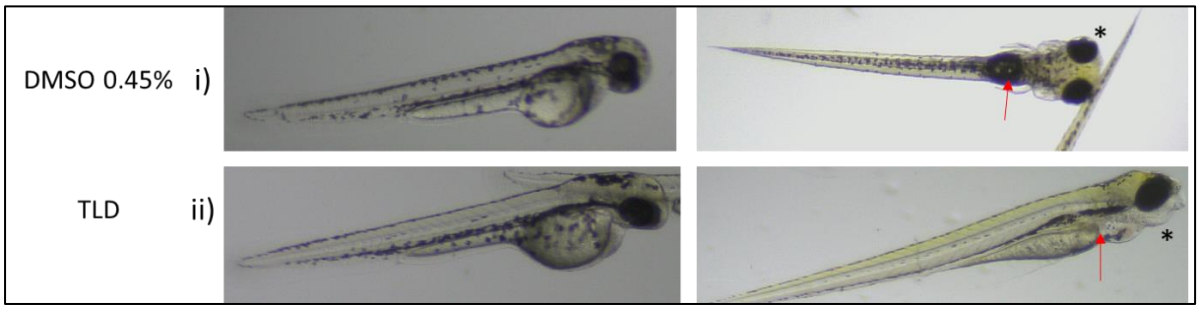


Figure 3.5. *In vivo* toxicity of TLD following 120 hours of exposure.
Dechorionated zebrafish embryos at 24 hours post fertilisation were treated with 0.45% (v/v) DMSO (**i**) or TLD (100 µM tenofovir, 100 µM lamivudine and 50 µM dolutegravir) (**ii**) for 120 hours. The embryos were monitored using a light microscope and images taken in the beginning and end of treatment period. The experiment was repeated at least three times (n=3) in triplicates. Left panels indicate the initiation of treatment at 24 hpf; right panels indicate zebrafish after 120 hours of treatment. The red arrow indicates the swim bladder. * indicate head position/ fish posture, meaning the larvae is upright when two eyes can be observed from the above and cannot keep an upright posture when only one eye can be observed from above.

4. Discussion

The zebrafish embryo has long been established as a useful tool for testing the toxicity of chemical compounds, including antiretroviral drugs (Zizioli *et al.*, 2023). Zebrafish embryo toxicity models differ in protocol design, and these differences relate to the initial time of exposure (6 to 24 hours post fertilisation), the duration of exposure (48 to 144 hours), whether to dechorionated embryos are used or not, and lastly static exposure vs drug renewal during treatment (Achenbach *et al.*, 2020). In our study we first investigated the toxic effects of the combination antiretroviral drugs TLD (tenofovir, lamivudine and dolutegravir) *in vitro* on HEK 293 cells and then *in vivo* on dechorionated zebrafish embryos. To ensure full drug exposure of specified doses in this study, the embryos were dechorionated before treatment and this informed our initial exposure time as dechorionation after 24 hpf reduces mortality. Our exposure period was informed by the need to treat whilst the yolk is being utilised by the embryos to avoid feeding during treatment; as a result, the treatment period was restricted to 3 to 5 days post-fertilisation. To ensure that the drug compounds were constantly available throughout the duration of treatment, the ARVs were replaced every 24 hours.

As expected lamivudine was found to be non-toxic to both HEK 293 cells and zebrafish embryos. This is consistent with previous studies which have found lamivudine to be less toxic or to have the least developmental side-effects compared to its TLD counterparts (Olaniyan *et al.*, 2015). Tenofovir on the other hand negatively affected HEK 293 cell proliferation which limited the number of cells able to convert tetrazolium to its insoluble form formazan of the MTS assay. Tenofovir is known to cause renal tubular dysfunction in kidneys during treatment (Mashingaidze-Mano *et al.*, 2020) and thus the decreased growth of Human Embryonic Kidney cells may be expected. Dolutegravir was not found to be toxic in HEK 293 cell at the highest concentration used in this study, but was lethal at this high concentration to zebrafish embryos; it is not clear why this is the case, however dolutegravir at half the highest concentration was not toxic. Dolutegravir has been shown to cause neural tube defects on zebrafish embryos during development (Cabrera *et al.*, 2019); this effect delays growth and was shown to be lethal at high concentrations in the absence of folate supplementation. This effect would explain the observed dolutegravir toxicity at high concentrations in this study.

Clinical drug formulations prescribed to humans are often administered based on bodyweight (Daimari *et al.*, 2018). The TLD prescribed formulation in humans of >30 kg in weight is 300 mg tenofovir, 300 mg lamivudine and 50 mg dolutegravir. These doses are too high for zebrafish embryos, therefore we tested the toxicity of a range of TLD individual components, as informed by the literature (Cabrera *et al.*, 2019). Exposure to TLD at a 100 µM tenofovir, 100 µM lamivudine and 50 µM dolutegravir formulation reduced the growth rate of HEK 293 cells in this study following 72 hours incubation and delayed the development of zebrafish embryos following 120 hours of exposure; the effect on embryo development was found to be reversible once the compounds were removed.

This study was not without limitations. The treatment period for zebrafish embryos had to be limited to 120 hours to avoid feeding during treatment. The highest concentration of drugs used in this study was limited to the stock amount of drug compound able to dissolved in DMSO, and dilutions thereof that are devoid of an effect of the DMSO alone. The drug compounds had to be replaced every 24 hours of treatment, making this approach labour intensive. Zebrafish embryos can be hyperactive during early stages of development; therefore, microscopic imaging was also a challenge; reducing the temperature of the treatment solution might reduce activity to allow for better imaging. In summary, we have assessed the in vitro and in vivo toxicity profile of Tenofovir, Lamivudine and Dolutegravir and determined appropriate concentrations of TLD to be used for subsequent studies related to NCDs.

Chapter 4

T2DM predisposition of zebrafish young and adults after embryo exposure to TLD

1. Introduction

Approximately 1.4 million women and girls living with HIV become pregnant every year, and about 1.2 million of them use antiretroviral medication to prevent mother-to-child transmission (Siemieniuk *et al.*, 2017). Sub-Saharan Africa is home to 91% of all pregnant HIV-positive women; this region includes South Africa which has the highest number of HIV-infected people in the world (Wedi *et al.*, 2016). As a result, in South Africa one in three pregnant women is HIV-positive and more than 95% of HIV-positive women receive prophylactic treatment (Santosa *et al.*, 2019). Although access to HIV-prophylaxis medication has increased in an effort to reduce new HIV-infections in both infants and adults, studies indicate that awareness of pre-exposure prophylaxis (Prep) is lacking in sexually active adult South Africans (Haffejee *et al.*, 2023); within this cohort, HIV-positive women are found to be generally more informed about Prep. Combination antiretroviral therapy (cART) is the most effective way of reducing the risk of mother-to-child transmission and is more effective when initiated before the third trimester of pregnancy up until birth or the cessation of breastfeeding (Forbes *et al.*, 2012). The risk of mother-to-child transmission without cART viral suppression is high, ranging from 15%-30% (Zipursky and Loutfy, 2020); while the use of cART lowers the transmission rate to less than 5 per 1000 births, i.e.0.5% (Forbes *et al.*, 2012).

The antiretroviral therapy combination of tenofovir, lamivudine and dolutegravir (TLD) is the preferred first line regimen in Sub-Saharan Africa, including South Africa, because of its superior efficacy compared to efavirenz, high genetic barrier to resistance and mild side-effects (Fokam *et al.*, 2023). However, the potential risk of developing neural tube defects (NTD) following dolutegravir exposure during pregnancy has been documented (Zash *et al.*, 2019); women are therefore advised to supplement with folic acid (folate) to prevent or minimise NTD. Animal studies have also demonstrated the neural tube defect in zebrafish embryos following exposure to dolutegravir, which was rescued by folate supplementation (Cabrera *et al.*, 2019). Given that the dolutegravir-related risk of infant neural tube defect is considered low, at 0.2%, the world health organisation has recommended TLD (a dolutegravir containing drug) as the preferred first and second line regiment for all people living with HIV (PLWH) including pregnant women and women of child bearing age (Zipursky and Loutfy, 2020; Romo *et al.*, 2022).

Dolutegravir is known to be associated with bodyweight gain (Hill *et al.*, 2019), hyperglycemia, prediabetes and new-onset diabetes upon initiation (McLaughlin *et al.*, 2017; Bahamdain, 2022). Little is known however about potential side-effects, and their mechanism, of dolutegravir-containing antiretroviral therapy (i.e. TLD). The implications of indirect *in utero* infant exposure to TLD during pregnancy and its role in increasing the risk of the unborn child developing insulin resistance and diabetes later in life (juvenile stage and adulthood) are unclear.

In this study we aimed to investigate this by using zebrafish as a model organism to mimic the conditions of *in utero* exposure of infants to TLD during pregnancy. Zebrafish embryonic development takes place over 5 to 7 days, with the fish reaching the juvenile stage at about 30 dpf and adulthood at about 4 to 6 months of development. We exposed zebrafish embryos to TLD for the first 5 days of development, a period resembling human *in utero* development from the first-trimester to birth. In the context of a zebrafish embryo, birth is taken as the moment of hatching from the chorion, which occurs within 5 days of development. The effect of *in utero* TLD exposure was then investigated at the embryonic stage post hatching (day 5), juvenile stage (day 30) and in adulthood (4-6 -months) by assessing the changes in bodyweight, and fasted blood glucose (where possible) as well as the relative mRNA expression of metabolic hormones and enzymes of the experimental groups compared to control groups. A glucose “positive control” was also included with every TLD experiment.

2. Methods and materials

2.1. Animals

Zebrafish were kept in tanks of the IWAKI system in reverse osmosis water (Iwaki Aquatic Systems and Services; USA) at 28.5°C under a blue-light cycle of 14-hour light and 10-hour dark period at the Zebrafish Research Facility of UKZN; fish were fed twice daily and once on weekends. Spawned embryos were incubated at 28.5°C for 24 hours, dechorionated (using pronase E; Cat no. 9036-06-0, Merck, USA) and treated with either glucose (Cat no. 50-99-7, Merck, USA), or a combination of tenofovir (Cat no. SML1794-10MG, Sigma-Aldrich, USA), lamivudine (Cat no. SML1295-10MG, Sigma-Aldrich, USA) and dolutegravir (Kindly provided by Prof. Carine Smith, Department of Medicine, University of Stellenbosch) (TLD) or DMSO (Cat no. D2650, Sigma-Aldrich, USA).

2.2. Short, mid and long-term effects of 3% glucose and TLD treatment

Dechorionated embryos at 1-day post fertilisation (dpf) were incubated for 5 days in a 3% (w/v) glucose solution (prepared with RO water) (Figure 4.1 and 4.2 A) with incubation in reverse osmosis (RO) water as control), or in a solution containing 100 µM Tenofovir, 100 µM Lamivudine and 50 µM Dolutegravir (TLD) (prepared in E3 media) (Figure 4.1 and 4.2 B) with incubation in a 0.45% (v/v) DMSO solution (prepared in E3 media) representing the highest concentration used in this study as control. Half of the embryos were then processed for qPCR immediately after treatment (Figure 4.1a), while the rest were further incubated in RO-water and fed brine-shrimp from 6 dpf until fish flakes were introduced at 20 dpf to 30 dpf (Figure 4.1b) or to 4 months post fertilisation (Figure 4.2). During the monitoring period (i.e. 30 dpf and 4 months after treatment; including control groups) the fish were fed three times a day; there were no treatment side-effects observed during the monitoring period. The 4-month time point was chosen because it emphatically reflects adulthood compared to 2 months of development.

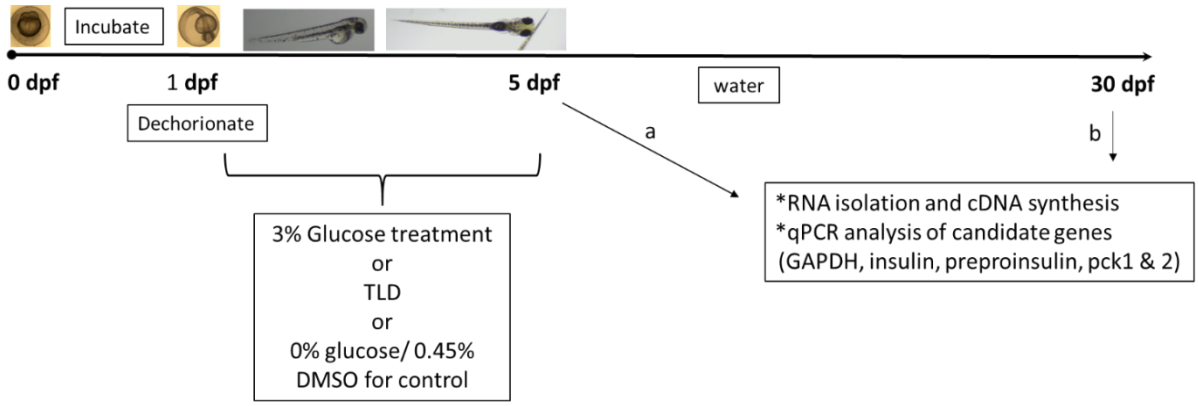


Figure 4.1 Experimental procedure for assessing *in utero* effects of glucose and TLD exposure on young zebrafish .
Embryos (10-20 per treatment) were incubated for one day in RO-water and then dechorionated at 1-day post fertilisation (dpf), treated in the presence of 3% (w/v) glucose or TLD (combination of 100µM tenofovir, 100µM lamivudine and 50µM dolutegravir) for 5-days; incubation in RO-water and 0.45% (v/v) DMSO solution were used as controls respectively. After the 5-day treatment, half of the embryos were processed for qPCR (**a**) n=3; the other half were incubated in RO-water and monitored to 30 dpf **(b**) where total RNA was isolated for qPCR analysis of candidate genes of hormones and enzymes associated with glucose metabolism. Embryos (5-10) were pooled per treatment for RNA isolation and the experiment repeated at least three times (n=3).

After 5 days (short-term), and at 30 dpf (mid-term) monitoring (Figure 4.1). To assess whole body genetic changes due to treatment whole fish carcases were prepared in Trizol (Cat no.15596026, Life technologies, USA), and utilised to isolate total RNA using the PureLink

RNA Extraction Mini Kit (Cat no. 12183018A, Life Technologies, California, USA) followed by cDNA synthesis using the Invitrogen SuperScript (IV) first-strand synthesis system (Cat no. 18091050, Thermo-fisher Scientific, Massachusetts, USA) for qPCR. After 4 months (long-term) monitoring (Figure 4.2), zebrafish were fasted for 12 hours, anesthetised in ice and weighed before blood was drawn for fasting blood glucose measurements with a glucometer (Accu-Chek Instant, Roche, Germany). Whole carcase total-RNA was isolated and qPCR performed as described above.

2.3. qPCR analysis

The expression levels of GAPDH (*gapdh*); beta-actin (*β-actin*); zebrafish insulin (*zins*); zebrafish preproinsulin (*ppins*) and phosphoenolpyruvate carboxykinase 1 & 2 (*pck1* and *pck2*) mRNA were assessed; using the PowerUp SYBR Green master mix (Cat no. A25742; Applied-Biosystems, Massachusetts, USA), the signal was analysed using the Quant-Studio design and analysis software (Applied-Biosystems, Massachusetts, USA). The primer sequences used are listed in Chapter 1, Table 1 and appendix 1; all primers achieved efficiencies of between 90 to 100%. The specificity of each PCR reaction was checked with a melting curve analysis. Relative gene expression levels or fold changes were determined by using the ($2^{-\Delta\Delta Ct}$) equation below; β-actin was used as the reference gene for this study.

$$\Delta Ct = Ct\ (target\ gene) - Ct\ (reference\ gene)$$

$$\Delta\Delta Ct = \Delta Ct\ (test\ sample) - \Delta Ct\ (control)$$

$$Fold\ change = 2^{(-\Delta\Delta Ct)}$$

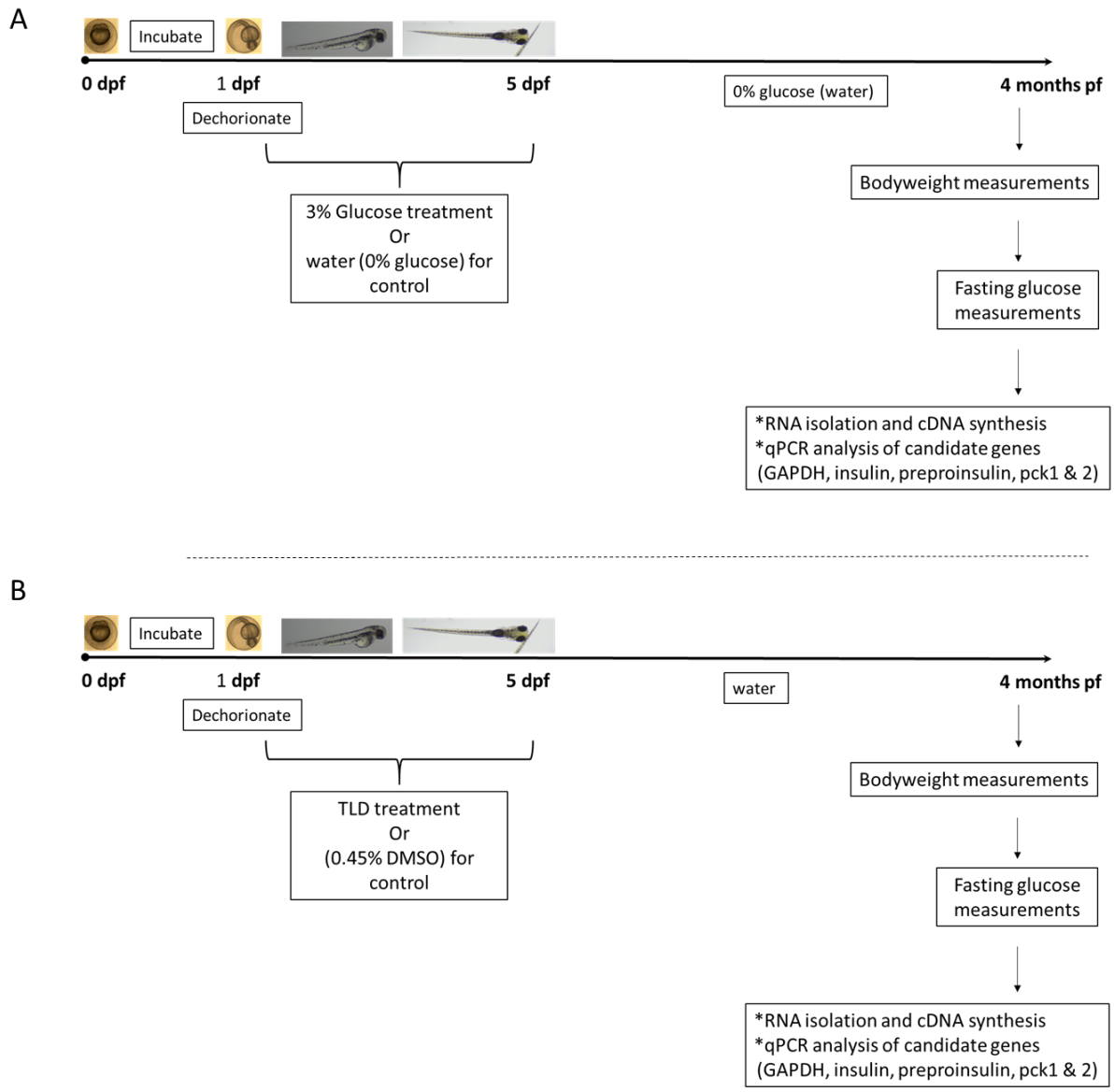


Figure 4.2 Experimental procedure for assessing *in utero* effects of glucose (A) and TLD (B) exposure on zebrafish adults.
Dechorionated embryos at 1 dpf were grown in the presence of 3% (w/v) glucose solution (**A**) or TLD (**B**) for 5-days, respective controls were incubated in OR-water or a 0.45% (v/v) DMSO solution; after 5 days, embryos were then incubated in RO-water and allowed to grow to 4-months post fertilisation (pf). The fish were then fasted for 12-hours, euthanised, weighed and fasting blood glucose levels were determined. Total RNA was isolated from whole carcase for qPCR analysis of candidate genes of hormones and enzymes associated with glucose metabolism.

2.4. Statistical analysis

All results are represented as means with their standard deviation (SD), statistical analysis was done using Graph-Pad prism (version 8.3.0). Data was analysed using the Student's t-test or one-way ANOVA with multiple comparisons depending on the number of comparisons. A P-value of less than 0.05 was considered significant.

3. Results

We first evaluated the implication of *in utero* TLD exposure on adult zebrafish (referred to as "long-term"); then analysed earlier stages, specifically zebrafish juvenile stages, and finally looked at immediate effects during gestation. To determine whether embryonic TLD exposure could potentially predispose zebrafish to T2DM later in life, embryos were incubated in TLD for the first 5-days of their development and allowed to develop to fully grown adults (4-months post fertilisation). Bodyweight and fasted blood glucose measurements were determined for adult zebrafish including controls; bodyweight and fasting blood glucose were not measured for larvae and juveniles due to their small size.

The gene expression profile of hormones and enzymes associated with glucose metabolism was evaluated by using qPCR to analyse the mRNA expression levels of GAPDH, insulin, preproinsulin and phosphoenolpyruvate carboxykinase 1 & 2. Based on our T2DM models (Chapter 2) and other studies (Jung *et al.*, 2016), glucose is an effective inducer of T2DM symptoms and was thus used in this study as a "positive control" in parallel with TLD exposure. The glucose-induction method was chosen rather than the overfeeding approach because embryos were incapable of actively feeding during the period of treatment.

3.1 *Effects of early (embryonic) glucose and TLD exposure on later adult zebrafish bodyweight and fasting blood glucose*

a. Glucose exposure (positive control)

To develop a positive control, zebrafish embryos at 24-hour post fertilisation were chronically exposed to a 3% glucose solution for 5 days, while the larvae in the control group were exposed to water. The bodyweight and fasting blood glucose levels of zebrafish were measured at the end of the 4-month monitoring period. Although not significant, the mean bodyweight of the glucose experiment group (0.63 g ± 0.047 g) was, as expected, higher than that of the control group (0.54 g ± 0.11 g) (Figure 4.3 A). Furthermore, the range of the control

group bodyweight (0.39 g to 0.70 g) was wider compared to that in the glucose experiment group (0.56 g to 0.67 g). Similarly, although there was no significant difference between the mean fasting blood glucose levels of the experimental group (2.5 ± 0.54 mmol/L) compared to the control group (2.6 ± 0.52 mmol/L), analysis of the range clearly reveals a shift from 1.1-2.8 mmol/L (control) to 2.4-4.2 mmol/L (experimental group) (Figure 4.3 B).

b. TLD exposure

The long-term effects of embryonic TLD exposure were determined by incubating dechorionated embryos 24-hour post fertilisation with TLD for 5 days, while embryos in the control group were incubate in a 0.45% (v/v) DMSO solution; after the incubation period, the embryos were returned to system water and bodyweight and fasting blood glucose then subsequently determined at 4-months. Bodyweight analysis of the TLD experimental group revealed a higher (0.26 g ± 0.12 g) mean bodyweight compared to control group (0.17 g ± 0.04 g); however, the effect was not significant (Figure 4.3 C). Furthermore, the range of the TLD experimental group bodyweight (0.1 g to 0.5 g) was wider compared to that of the control group (0.1 g to 0.2 g). The mean fasting blood glucose of the TLD experimental group (3.5 ± 1.92 mmol/L) was also higher than that of the control group (2.0 ± 0.62 mmol/L) at 4 months' post fertilisation following 5 days of TLD early exposure and analysis of the range indicated a clear shift from 1.2-3.0 mmol/L (control) to 1.4-6.0 mmol/L (experimental group); again (Figure 4.3 D). Although these effects were not significant, it does emphasize that early exposure may have long-lasting effects on physiological aspects.

3.2 *Effects of early (embryonic) glucose and TLD exposure on expression of metabolic hormones and enzymes in adult zebrafish*

a. Glucose exposure

We then analysed the expression of insulin (*zins*), preproinsulin (*ppins*) and phosphoenolpyruvate carboxykinase 1 & 2 (*pck1* and *pck2*) in control (water) and glucose-exposed zebrafish. The mRNA expression of metabolic hormones, although not significantly different, was increased in adult zebrafish compared to the control group such that the mean fold changes of *zins*, *ppins* were percentage 2.8 ± 4.8; 1.5 ± 5.4 respectively (Figure 4.3 E). The mRNA expression of the enzymes *pck1* and *pck2* following *in utero* glucose exposure were however relatively unchanged with mean fold changes of 0.8 ± 0.5 and 1.0 ± 0.2 respectively (Figure 4.3 E). The range of fold changes in the glucose experimental group was

1.0 to 12.8 and 1.0 to 17.5 for insulin and preproinsulin respectively, suggesting an effect of early glucose exposure later in their adult life.

b. TLD exposure

To further analyse the effects of embryonic TLD (experiment) and DMSO (control) treatment in adult zebrafish we evaluated changes in mRNA expression of *zins*, *ppins pck1* and *pck2*. The mean fold changes of insulin (2.0 ± 1.0) and preproinsulin (1.8 ± 1.1) mRNA expression in zebrafish exposed to TLD were significantly ($P<0.05$) different to that of the DMSO control group (1.0 ± 0.0) (Figure 4.3 F). In the experimental group, the expression of *pck1* and *pck2* was lower than control (1.0 ± 0.0) with fold changes of 0.6 ± 0.4 and 0.8 ± 0.2 respectively, however the effect was not significant.

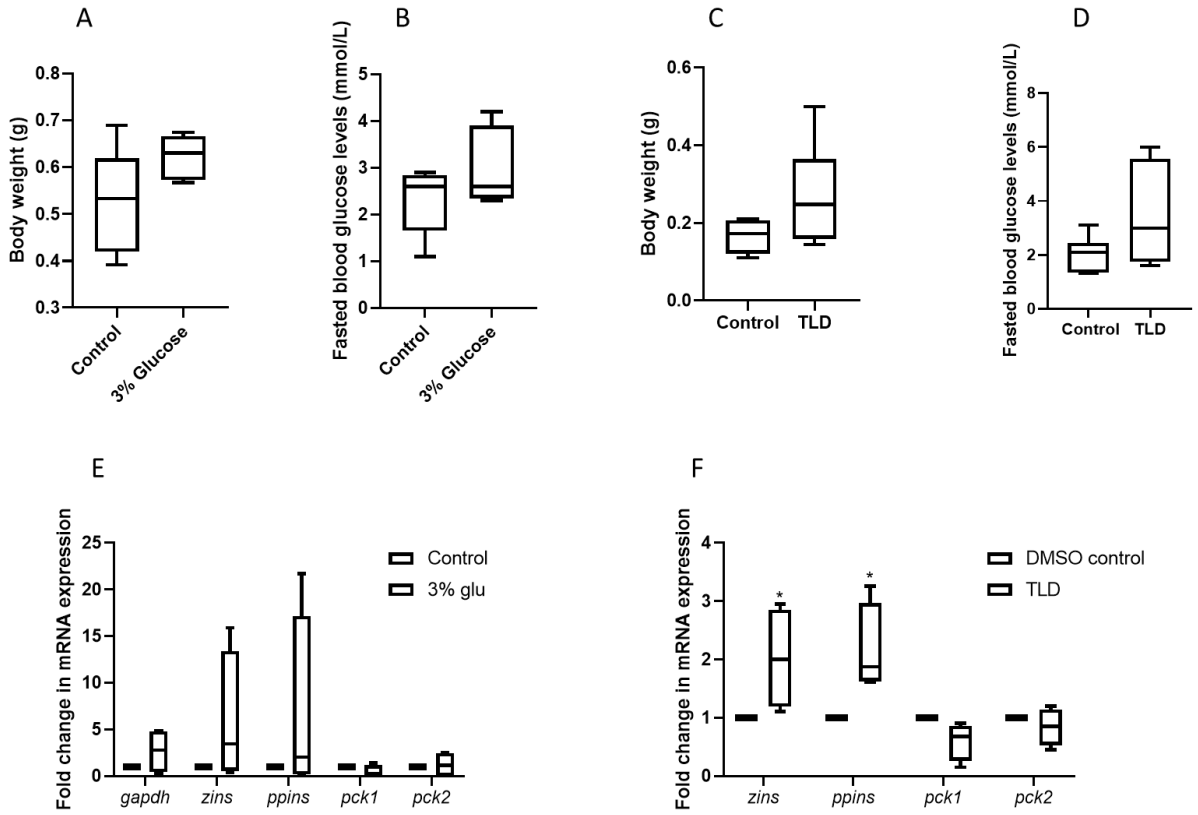


Figure 4.3 Implications of early zebrafish embryo exposure to TLD and glucose later at 4-months of age. The bodyweight measurements, fasting blood glucose levels and candidate gene expression of zebrafish were determined at the end of the 4-month monitoring period following 5 days of glucose (**A, B & E**) and TLD **(C, D & F**) exposure respectively. Dechorionated embryos were incubated in a 3% (w/v) glucose solution or in a TLD solution; with control groups incubated in RO-water (**A, B & E**) or 0.45% (v/v) DMSO solution (**C, D & F**) respectively. For bodyweight and fasting blood glucose measurements, the data was analysed using the Student's t-test; n=6 individuals in the population for both control and experimental groups; values are means± SD against control. For qPCR analysis of candidate genes: GAPDH (*gapdh*), insulin (*zins*), preproinsulin (*ppins*) and phosphoenolpyruvate carboxykinase 1 & 2 (*pck1 & pck2*), pooled total RNA was analysed (n=3) using one-way ANOVA; values are means± SD against controls; *P<0.05. RO-water (reverse osmosis water) *TLD solution (combination of 100µM tenofovir, 100µM lamivudine and 50µM dolutegravir). Any data that lacks a * is not statistically significant.

3.3 *Effects of early (embryonic) glucose and TLD exposure on expression of metabolic hormones and enzymes in juvenile zebrafish*

a. Glucose exposure

To determine whether similar changes in gene expression were already evident at the juvenile stage of development following *in utero* glucose treatment, the expression of *zins*, *ppins*, *pck1* and *pck2* were evaluated at 30 dpf. The relative qPCR expression of insulin (0.7 ± 0.3) and preproinsulin (0.6 ± 0.2) mRNA in the glucose experimental group were significantly ($P < 0.05$) lower than the control group (1.0 ± 0.0) (Figure 4.4). The expression of glucose metabolic enzymes phosphoenolpyruvate carboxykinase 1 & 2 (0.5 ± 0.3 and 0.54 ± 0.3, respectively) in the glucose experimental group were also significantly ($P < 0.01$) lower than of the control group (1.0 ± 0.0) (Figure 3.4).

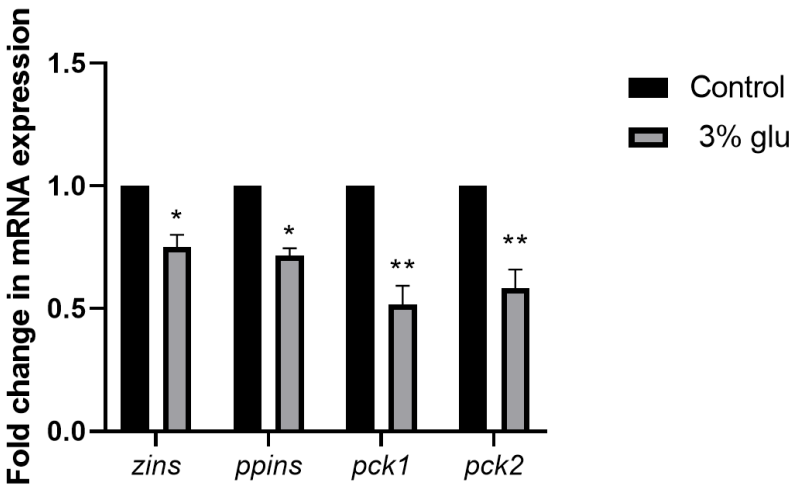


Figure 4.4 Expression of glucose-related metabolic genes in juvenile zebrafish following glucose treatment.
Following incubation of embryos in a 3% (w/v) glucose solution and RO-water (control) for 5 days. The embryos were allowed to develop to 30-days post fertilisation in RO-water. Total RNA was isolated from control group and experiment group for qPCR analysis of insulin (*zins*), preproinsulin (*ppins*) and phosphoenolpyruvate carboxykinase 1 & 2 (*pck1 & 2*). Data was analysed using one-way ANOVA, the experiment was repeated at least three times (n=3); values are means± SD against control; **P*<0.05, ***P*<0.01.

b. TLD exposure

In contrast to the effect of glucose on candidate gene expression in the juvenile stage, the expression of insulin, preproinsulin and phosphoenolpyruvate carboxykinase 1 was not significantly different to control following TLD exposure; mean fold changes at day 30 were 1.3 ± 0.8 vs. 1.0 ± 0.5; 1.0 ± 0. 2 vs. 1.0 ± 0.1 and 1.1 ± 0.1 vs.1.0 ± 0.0 for insulin, preproinsulin

and phosphoenolpyruvate carboxykinase 1 respectively (Figure 4.5 A, B, and C). Interestingly the expression of phosphoenolpyruvate carboxykinase 2 was lower in the TLD experimental group (0.75 ± 0.1) when compared to the control group (1.0 ± 0.0); however, this effect was not significant (Figure 4.5 D).

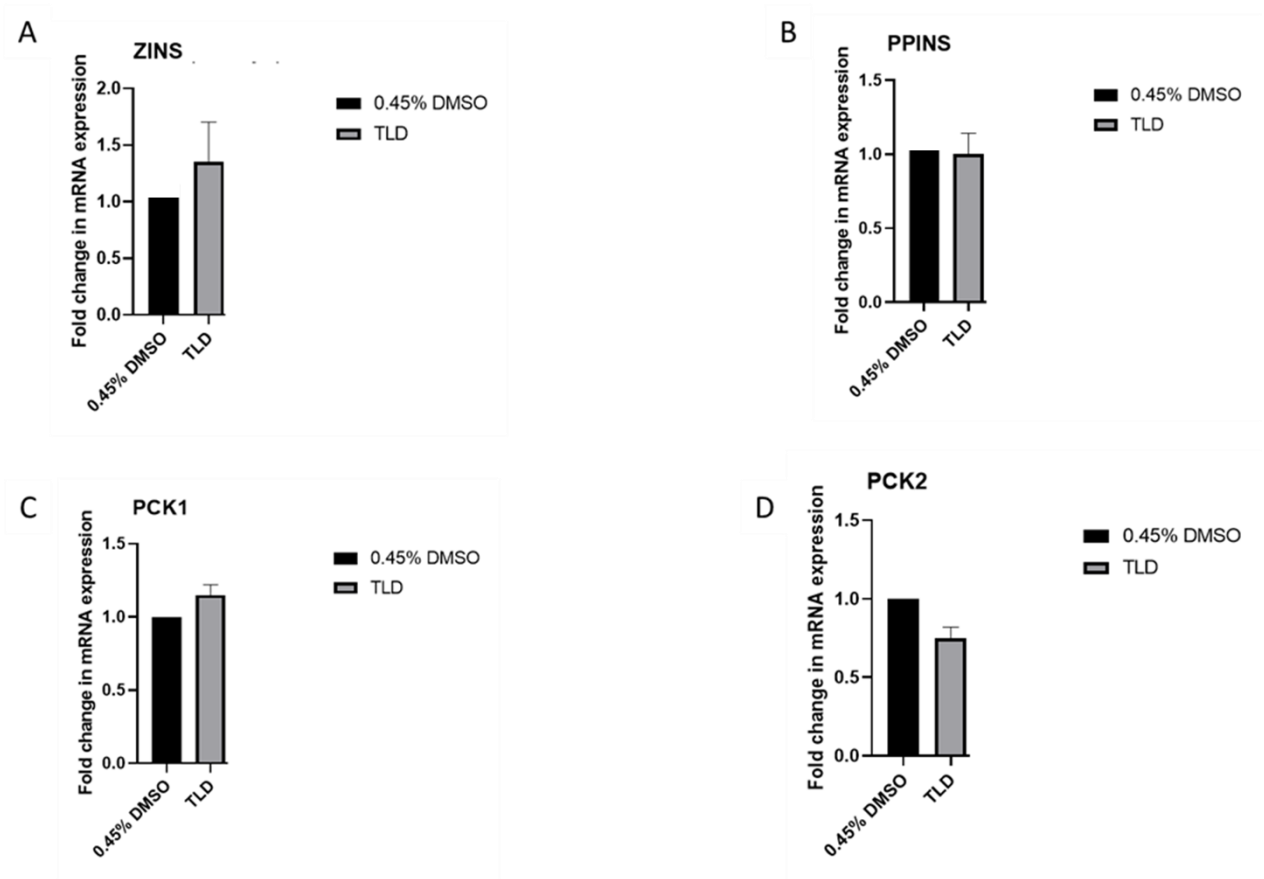


Figure 4.5 Expression of glucose-related metabolic genes in juvenile zebrafish after TLD treatment Figure Following incubation of embryos in TLD (combination of 100µM tenofovir, 100µM lamivudine and 50µM dolutegravir) experiment group or a 0.45% (v/v) DMSO solutions (control group) for 5 days. The embryos were allowed to develop to 30-days post fertilisation in RO-water. Total RNA was isolated from control group and experiment group for qPCR analysis of (**A**) insulin (*zins*), (**B**) preproinsulin (*ppins*) and (**C** and **D**) phosphoenolpyruvate carboxykinase 1 & 2 (*pck1 & 2*). Data was analysed using Student's t-test the experiment was repeated at least three times (n=3); values are means± SD against control. Any data that lacks a * is not statistically significant.

3.4 *Effects of early (embryonic) TLD exposure on expression of metabolic hormones and enzymes in zebrafish larva/ embryos.*

To assess the effect of TLD on the expression of insulin, preproinsulin and phosphoenolpyruvate carboxykinase 1 & 2 during gestation, hatched zebrafish embryos were processed for qPCR analysis immediately after exposure (day 5). E3 media was used to

evaluate expression in the presence of DMSO (the solvent control). The was no significant difference in expression of *zins*, *ppins*, *pck1* and *pck2* for zebrafish embryos exposed to E3 media vs. DMSO as indicated by mean fold changes of (1.0 ± 0.0 vs. 0.8 ± 0.2; 1.0 ± 0.0 vs. 1.0 ± 0.3; 1.0 ± 0.0 vs. 1.0 ± 0.3 and 1.0 ± 0.0 vs. 1.0 ± 0.1) respectively (Figure 4.6). The mRNA expression of *zins* (1.0 ± 1.2), *ppins* (0.8 ± 1.2), *pck1* (1.1 ± 0.3) and *pck2* (0.8 ± 0.1) by zebrafish embryos immediately after TLD exposure were all not significantly different when compared with the DMSO control group.

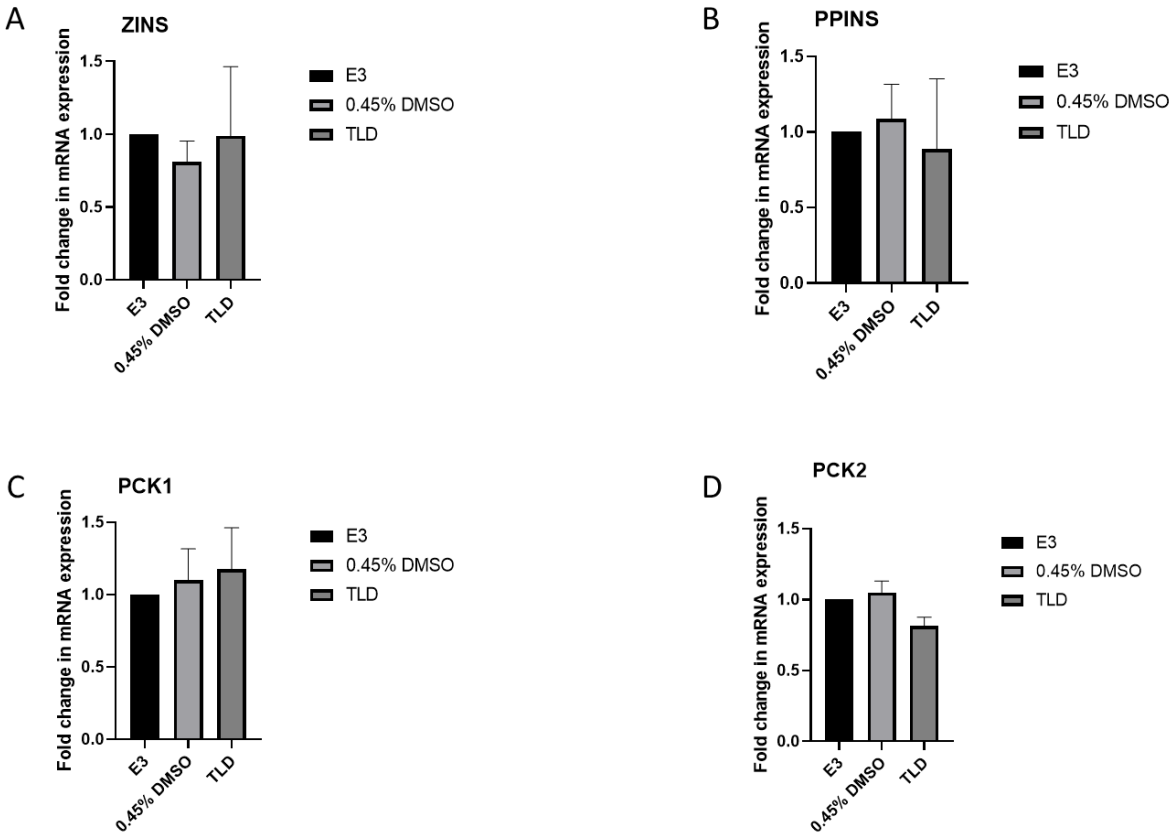


Figure 4.6 Expression levels of glucose related metabolic genes in zebrafish embryos after TLD treatment. Following incubation of embryos in TLD (combination of 100µM tenofovir, 100µM lamivudine and 50µM dolutegravir) experiment group or a 0.45% (v/v) DMSO solutions (control group) for 5 days, E3-media was used for comparison. The embryos were immediately processed for total RNA isolation from control group and experiment group for qPCR analysis of (**A**) insulin (*zins*), (**B**) preproinsulin (*ppins*) and (**C** and **D**) phosphoenolpyruvate carboxykinase 1 & 2 (*pck1 & 2*). Data was analysed using Student's t-test the experiment was repeated at least three times (n=3); values are means± SD against control. Any data that lacks a * is not statistically significant.

4. Discussion

The identification of neural tube defects in children of HIV-positive women following dolutegravir *in utero* prophylaxis has raised questions about other potential side effects of indirect *in utero* infant exposure to combination antiretroviral therapy; this was initiated by a study in Botswana reporting increased risk of neural tube defects in infants born of HIV-positive women who were receiving dolutegravir during pregnancy (Zash *et al.*, 2019). This risk has since been shown to be low, and dolutegravir containing cART regimen (TLD) is now fully endorsed by WHO for pregnant women (Zipursky and Loutfy, 2020). However, there is limited data on the potential of TLD exposure *in utero* to contribute to the development of metabolic diseases such as T2DM later in life. TLD is associated with bodyweight gain, hyperglycemia and diabetes; as a result HIV-positive patients initiating TLD are advised to know their diabetic status and monitor their bodyweight (Brennan *et al.*, 2023).

In our study, the bodyweight changes in adult zebrafish following embryonic glucose and TLD exposure were not significant, however the means of the experimental groups were higher that controls suggesting that exposure to either glucose or TLD during embryonic development could potentially promote bodyweight gain in zebrafish later in life. It would be of interest to analyse the combination of TLD and glucose in this context. The associated TLD adult bodyweight gain reported in other studies (Brennan *et al.*, 2023), is typically greater in females (Venter *et al.*, 2019). In our study the fish were fed in a similar manner regardless of treatment groups, which rules out nutrition as the only contributor to the observed weight gain and suggests a role for TLD in mediating this effect; Our fish were a mix of males and females, and therefore we cannot comment on a gender-specific difference. However, given that fish in the glucose and TLD groups had bodyweights higher than control in adulthood, one can infer that both glucose and TLD treatment played a role in mediating this weight gain.

Hyperglycemia or elevated fasting blood glucose is one of the main indicators of insulin resistance and diabetes in patients and animal models. We hypothesised that early (embryonic) glucose and TLD exposure might predispose zebrafish to hyperglycaemia and T2DM in adulthood, even after normal feeding for 4 months. In our study there was no significant difference in fasting blood glucose levels of the glucose experimental group compared to control in adulthood after treatment. This could be attributed to the short period of glucose exposure (5 days) of our study in an attempt to mimic *in utero* development; and potentially the fact that zebrafish larvae tend to be more adaptive to glucose exposure as indicated in other studies (Connaughton *et al.*, 2016). The mean fasting blood glucose levels of the zebrafish exposed to TLD on the other hand was higher than that of the control group

even though this effect was not significant. These results are in line with previously published literature, which suggest that dolutegravir (present in the TLD formulation) increases plasma glucose levels in patients to levels consistent with diabetes (Bahamdain, 2022).

Our approach sought to get a snapshot of the changes in mRNA expression of essential metabolic hormones and enzymes that are as a consequence of *in utero* TLD exposure at specific stages of zebrafish development. We observed significant expression of insulin and its precursor preproinsulin in adult zebrafish following *in utero* TLD treatment. This finding suggests that the embryonic exposure of TLD does predispose zebrafish to insulin resistance and potential T2DM later in life and is supported by elevated levels of fasting blood glucose of this treatment group. However, the low and non-significant levels of *pck1* and *pck2* expression suggest that the observed insulin resistance was not severe enough to promote glucose production in the liver via gluconeogenesis. The snapshot approach was utilised in an attempt to obtain more information on the onset of genetic changes following TLD exposure, however it did not allow us to pinpoint exactly when the increase in insulin and preproinsulin occurred during treatment, however it was not observed at the juvenile and embryonic stages and must therefore have started after these stages. This was also true for our glucose treatment control, where different insulin (*zins*) and preproinsulin (*ppins*) responses were observed at 30 days compared to 4 months. At 30 days, the *zins* and *ppins mRNA expression levels* were significantly lower compared to the control group, while at 4 months an increase was observed (however this was not significant). This could be related to physiological changes that occur at different developmental stages or the fact that younger zebrafish are more resilient than their older counterparts to changes in their rounding environment (Connaughton *et al.*, 2016). TLD treatment could lead to differences in the eating pattern of fish causing then to eat more, however this would be difficult to control and confirm. In future, it would be interesting to challenge the TLD-treated embryo's with either a glucose- or DIO-approach in order to determine whether life-style changes later in life promote T2DM in adults previously exposed to TLD in utero.

Our approach was not without its own limitations; The treatment period had to be restricted to resemble *in utero* exposure and this short exposure potentially affected our glucose "positive control", thus the use of other short acting reagents such as Bisphenol F could have been more effective. No side-effects of using RO water instead of E3 media for the preparation of the glucose solution were observed during the early stages of zebrafish embryo development; however, we cannot rule out the potential benefits of E3 media enrichment over RO water during early developmental stages. Targeting specific zebrafish developmental stages limits observations and endpoint determinations to those specific stages. The small size of zebrafish

limits blood draws in the early stages of development which would prevent blood glucose determination, thus using glucose calorimetric kits on treated zebrafish carcases might be beneficial. In summary, we have developed a model to assess T2DM predisposition as a result of *in utero* exposure to TLD. This model evaluated bodyweight gain, fasting blood glucose and the gene expression profile of essential metabolic hormones and enzymes. It was utilised to mimic indirect *in utero* TLD exposure during pregnancy, but can be applied by other studies seeking to understand *in utero* exposure of other compounds and drugs and their impact later in life.

Chapter 5

General discussion and recommendations for further research

The prevalence of non-communicable diseases (NCDs) is high amongst people living with HIV (PLWH) using antiviral therapy (Gizamba *et al.*, 2023), and complexities of the onset of metabolic syndrome, weight gain, insulin resistance and subsequent type 2 diabetes mellitus (T2DM) as a result of ARVs remains poorly understood. Studying the association of T2DM with antiretroviral therapy necessitates the use of animal models. In this thesis, we established zebrafish models which were used for 1) toxicity screening and evaluating appropriate ARV compound concentrations and 2) testing the effect of TLD *in utero* exposure on the initiation of T2DM at different stages of zebrafish development. This is the first reported establishment of a T2DM model in zebrafish in South Africa, and the first such study to evaluate the possibility of a link between early ARV exposure and T2DM in adulthood using a zebrafish model. The study also highlights the versatility of the zebrafish (*Danio rerio*) as a tool for drug screening and disease modelling.

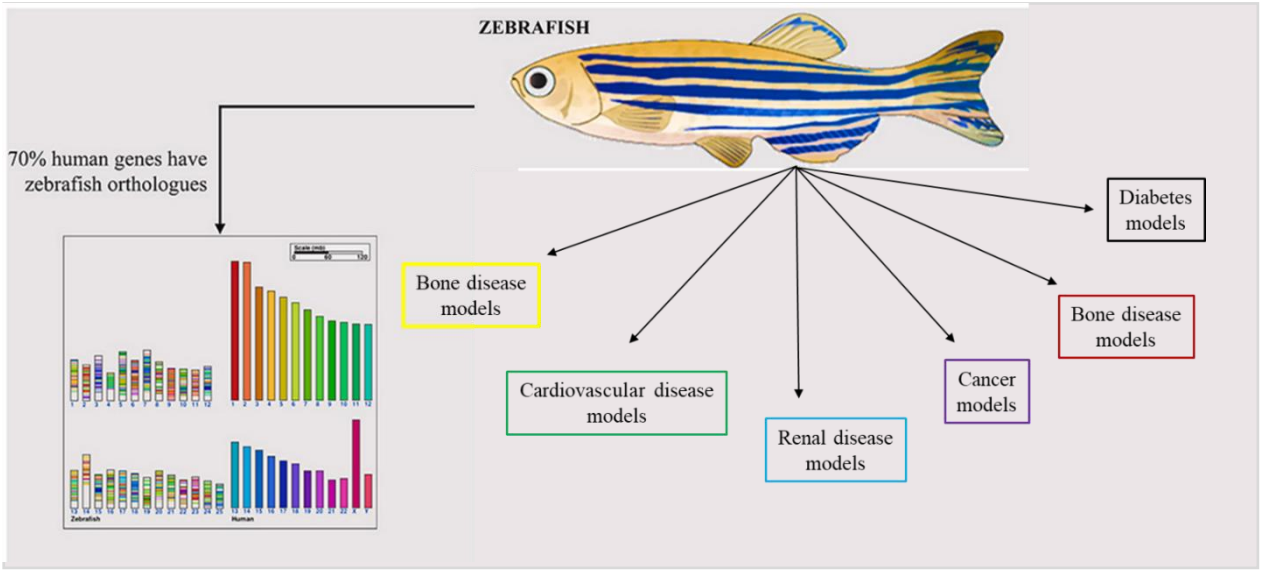


Figure 5.1 Schematic of zebrafish human disease models.
In addition to diabetes, zebrafish models of various human diseases have been developed, including blood, bone, cardiovascular and renal diseases as well as cancer. Figure compiled from (Mazumder Adhish and I. Manjubala, 2023).

The zebrafish is a powerful model with increasing usefulness in translational research of acquired and genetic human disease. Zebrafish and humans have 70% gene homology, and zebrafish has been widely utilised to investigate human disorders and diseases to better understand their complexities (M. Adhish and I. Manjubala, 2023). Models of bone disease, cardiovascular disease, renal diseases, cancer, blood disease and diabetes have all been developed in zebrafish and shown to be translatable (M. Adhish and I. Manjubala, 2023) (Figure 5.1). The imaging of live skeletal developmental processes in zebrafish models has facilitated studies on human bone development and repair, in this way, radiography of zebrafish skeletal defects led to a better understanding of the role of collagen in bone development through stressing the endoplasmic reticulum in osteoblasts and fibroblast (Fisher *et al.*, 2003). A zebrafish *atp6v1h* mutant model of osteoporosis, utilised to study bone mineralisation and spinal curvature, helped identify a new bone formation pathway in humans, paving the way for new therapies for patient care (Zhang *et al.*, 2017). Zebrafish cancer models have been effective at studying molecular mechanisms leading to cancer formation and progression. Mutant *pt53* zebrafish models of the human tumor suppressor gene have given more insight into the development of malignancy in peripheral nerve sheath tumors (PNST) (Berghmans *et al.*, 2005). These studies highlight a high degree of systems homology between zebrafish and humans including the relevance of zebrafish models and the translation of results to human disorders.

The zebrafish embryo has gained a lot of support as a model for developmental toxicity screening of chemical compounds and therapeutic drugs (Miyawaki, 2020). The small size of a zebrafish embryo is important as it allows testing to be conducted in small volumes which is ideal for compounds in low supply. Studies have shown that the zebrafish embryo absorbs chemical compounds dissolved in its water environment through the mouth, gills and skin (Miyawaki, 2020); as a result the combination antiretroviral therapy drug (TLD) used in this study was administered via the tank water that the embryo was exposed to. The subsequent screening of TLD on zebrafish embryos showed us that dolutegravir (D) is toxic at 100 µM which was the highest concentration tested in this study. This is consistent with a previous zebrafish study showing embryo toxicity of 100 µM dolutegravir treatment rescued by the addition of folate (Cabrera *et al.*, 2019). Dolutegravir at 50 µM was not found to be toxic. Tenofovir (T) and lamivudine (L), on the other hand could be used at the higher concentration of 100 µM, as they were not found to be toxic in either *in vitro* or *in vivo* assays. These concentrations were then used when investigating the TLD related T2DM in zebrafish.

In order to understand whether *in utero* TLD exposure predisposes zebrafish embryos to T2DM later in life, the NCD first had to be simulated in adult zebrafish so that the biological

markers and features of the disease could be mapped out. We used glucose and overfeeding as our approaches to induce T2DM in adult zebrafish and therefore establish a T2DM model in our new zebrafish research facility. Since glycaemia is one of the main ways to determine the diabetic status (Sacks *et al.*, 2023), our T2DM models were confirmed using fasting blood glucose measurements; bodyweight assessment and quantification of mRNA expression of essential metabolic hormones and enzymes were subsequently also carried out. We observed that following our protocol, both approaches resulted in hyperglycemia in our zebrafish cohort, as well as significant overexpression of insulin and preproinsulin mRNA indicative of insulin resistance. Previous studies have established that the chronic exposure of adult zebrafish to a 2% glucose solution (Capiotti *et al.*, 2014) or overfeeding calorie dense fish food (Liqing Zang *et al.*, 2017) leads to hyperglycemia and insulin resistance. Our study adopted the glucose immersion protocol established by Capiotti *et al.*, (2014) which demostrated decreased mRNA levels of insulin receptor (IR) in zebrafish muscle, hyperglycemia and glycation of proteins in zebrafish eyes following chronic exposure in a 110 mM (2%) glucose solution for 14 days. In our study, the fasting blood glucose levels of the treatment group were similar to that of the control group at the 13-day timepoint. As a result, treatment was extended to 40 days, at which point hyperglycemia was observed. The difference in time taken to establish hyperglycemia may be related to osmoregulation and fish size since our fish were smaller than those in the Capiotti *et al.*, (2014) study. Adult zebrafish glucose studies rarely investigate the expression of the insulin gene as a measure of insulin resistance, and rely rather on characteristics such as chronic hyperglycemia (Connaughton *et al.*, 2016), diabetic retinopathy (Gleeson *et al.*, 2007; Carnovali *et al.*, 2016) and insulin receptor gene expression (Capiotti *et al.*, 2014). Therefore, our results that demonstrate a lack of insulin and preproinsulin expression following glucose exposure or overfeeding in this model are novel.

A significant increase in bodyweight was observed in the DIO model, but not the glucose model; this was not surprising as overfeeding has been shown to increase bodyweight in zebrafish (Liqing Zang *et al.*, 2017), while there has been no significant weight change in zebrafish due to glucose treatment (Connaughton *et al.*, 2016). The overexpression of phosphoenolpyruvate carboxykinase 1 & 2 were significant only in the glucose-induced model. These characteristics and gene expression markers of T2DM in adult zebrafish were comparable to finding of other published studies (Shao *et al.*, 2005; Liqing Zang *et al.*, 2017).

The neural-tube defects during gestation as a result of *in utero* exposure to TLD is well reported (Cabrera *et al.*, 2019; Zash *et al.*, 2019). In our study, the inclusion of 50 µM dolutegravir, although not lethal, was found to slow the development of zebrafish embryos by delaying swim-bladder formation, therefore interfering with the ability of the zebrafish to

maintain an upright posture. We suspect that this phenomenon could be associated with neural-tube defects, however this requires further investigation. It would have been interesting to determine whether supplementation with folate was able to rescue this phenotype. However, our focus was on determining the possibility of a link between in utero TLD exposure and predisposition of zebrafish to T2DM in adulthood.

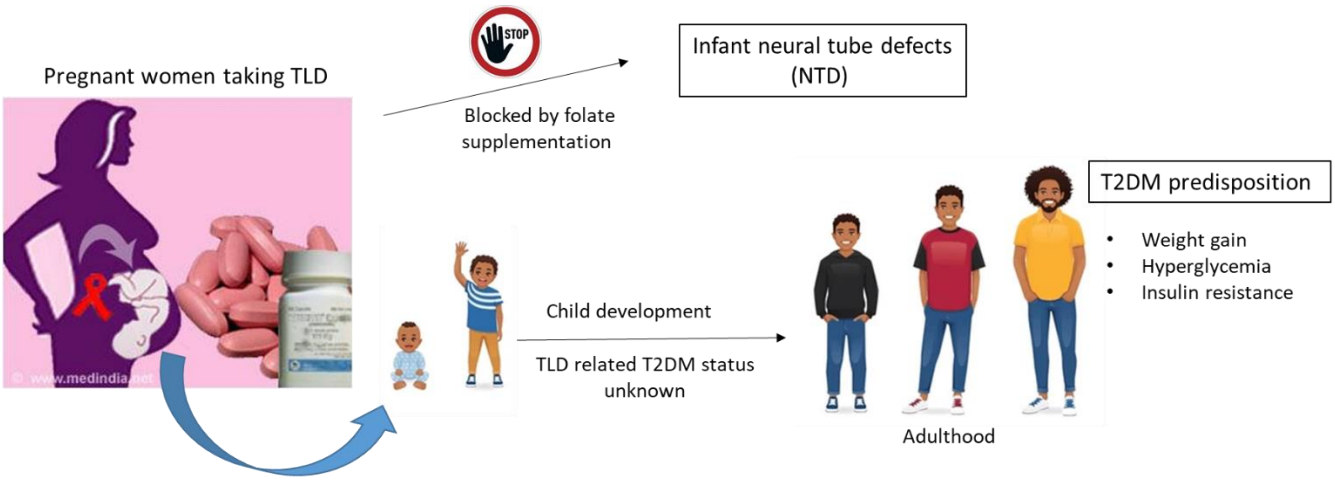


Figure 5.2 Schematic indicating hypothesis on the pathophysiology of TLD-related T2DM.
T2DM side effects of TLD are not know during development of children who were exposed early and what happens to their T2DM status in adulthood.

Pregnant women taking TLD could potentially predispose their unborn babies to T2DM in adulthood (Figure 5.2). We hypothesised that early embryonic TLD exposure could potentially predispose adult zebrafish to T2DM. The effect of TLD and its association to T2DM is well reported in adult HIV positive patients, however there is a lack of information on the effect of TLD on children born to pregnant women on these ARVs. Our study suggests that early embryonic zebrafish exposure to TLD does not initially affect the expression of selected diabetic genes (insulin, preproinsulin and phosphoenolpyruvate carboxykinase) in the embryonic and juvenile stages of zebrafish development. However, in the adult stages (4 months) the TLD-exposed fish had higher bodyweight, fasting blood glucose levels and increased expression of insulin and preproinsulin, suggesting embryonic exposure to TLD can have long-term effects in adulthood.

With our initial exposure time, exposure period and concentrations determined, we firstly assessed and analysed adult zebrafish 4 months after exposure, followed by analysis of juvenile (1-month) and embryonic (5 day) fish. The bodyweight and fasting blood glucose of *in utero* TLD-exposed adult zebrafish were both higher than control, but not significantly so.

Insulin and preproinsulin mRNA on the other hand were significantly overexpressed when compared with control. These finding suggested that early TLD exposure during gestation may predispose zebrafish to insulin resistance, which could eventually lead to T2DM; this may depend on the dietary conditions of the adult zebrafish. It would be interesting to challenge the adult zebrafish to the DIO or glucose treatment approach to determine how the fish that were exposed to TLD *in utero* react more overtly with clearer signs of T2DM, when compared with control conditions. Analysis of earlier stages of development did not give a clear indication of the time of onset of the observed insulin resistance. It may be necessary to include additional time points between 1 and 4 months of age in order to determine this.

Although the zebrafish used is this study were euthanised for blood draws and whole carcases used for RNA isolation and cDNA synthesis for qPCR analysis after treatment, repeated blood draws would be more beneficial for future work as it allows tracking changes that occur from the same fish overtime. A protocol for achieving this was published by (L. Zang *et al.*, 2015); this method, , which will be established in the UKZN Zebrafish Research Facility, requires significant expertise. Using this approach, blood can be sampled from adult zebrafish at 2 months of age, and repeated every 2 weeks, allowing the researcher to track the glucose levels in the same cohort of fish over a few months. In the juvenile population, glucose measurements could be carried out using commercially available ELISA kits. This will be included in future Longer treatment periods with TLD would be ideal to achieve a clearer induction of T2DM as a result of treatment, this is supported by the fact that the antiretroviral therapy related T2DM usually occurs 6-month or longer after treatment initiation in adult PLWH although there are some exceptions; our study treatment period was constrained by the need to simulate gestation period. Furthermore, exposing adult zebrafish to appropriate therapeutic TLD concentrations would be challenging if the drug is to be dissolved in fish water as this would require large amounts of the drug compounds, unless administering the dissolved drug compound to zebrafish orally is considered.

Future work in the context of this study should include: 1) separating the zebrafish according to sex to better understand physical (especially bodyweight) and genetic changes that occur in male vs. female zebrafish as a result of TLD treatment, 2) analysing gene expression changes of target zebrafish organs such as the pancreas and liver as well as muscle and adipose tissue if possible, 3) the introduction of repeated blood draws to help track changes in fasting blood glucose levels of individual fish during treatment, allowing better correlation with bodyweight and possibly genetic changes for each individual fish.

Finally, the zebrafish models of T2DM developed in this project could be useful for investigations into relationship between antiviral therapy in PLWH and the development of this NCD following adult exposure to cART. Taken together, we have established relevant zebrafish models of T2DM which includes data on candidate gene expression, and established that in utero TLD exposure can potentially predispose adult zebrafish to this NCD. Further work is required to extend the research and better understand the significance of these findings.

Appendix A: Additional methods (qPCR, agarose gels, primers)

1. RNA isolation

Zebrafish embryo RNA isolation was done as previously described by Peterson and Freeman (2009) with modifications guided by the instructions of the Pure-Link RNA min kit (Cat. number: 12183018A) manufacturer (Thermo Fisher Scientific). All surfaces and pipettes were decontaminated with a decontaminating reagent (i.e. RNAse Zap™ (Cat. no: AM9780) from Invitrogen™.

Fertilised zebrafish embryos were collected and thoroughly washed with tap water to reduce clumping. Clean fertilised embryos (i.e. 10-50) were transferred to 10 ml embryo media (5mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2$, 0.33mM $MgSO_4$) in a 95-mm Petri dish and incubated at 28.5°C. Peterson and Freeman (2009) recommend using 50 zebrafish embryos to attain sufficient amounts of RNA, we used 10, 20, 30, 50 embryos and tissue samples to optimise the protocol for this study. The embryos were checked for contamination daily, this included embryo media change, removal of dead embryos and floating embryo chorions after hatching. Also, muscle and pancreatic tissue from euthanised (with tricaine) adult zebrafish (~6 months) was surgically removed with forceps under a stereomicroscope, weighed and treated as described below. Zebrafish embryos (at 4 days post fertilisation (dpf)), muscle (0.015 g) and pancreas (0.018g) were transferred to 1.5 ml RNA/DNA-free or autoclaved eppendorf tubes and slightly vortexed to easily remove excess embryo media with pipetting. The embryos and tissue samples were homogenised in 250 µl Trizol reagent by pipetting up and down 20-times. A further 750 µl Trizol was added (to 1 ml) and the homogenate was incubated at room temperature (RT) for 5 min. At this stage the homogenate was either flash frozen in liquid nitrogen at -80°C or gently mixed via a rocking motion with 0.2 ml chloroform for at least 15 sec, and incubated at RT for 2-3 min. To separate RNA from proteins and DNA, the mixture was centrifuged at 12,000 x g for 15 min at 4°C. The colorless upper phase containing RNA was carefully pipetted and stored at 4°C overnight or transferred to new tubes containing 70% ethanol (diluted in RNase-free water). The content of the tubes was vortexed and 700 µl of the mix was transferred to spin columns and centrifuged at 12,000 x g for 15 sec at RT. This was repeated until the entire content of the tubes was cycled through the column. The column was washed once with 700 µl buffer-I and twice with 500 µl buffer-II (containing ethanol), centrifuged at 12,000 x g, RT for 15 sec. Excess buffer was removed by centrifuging the RNA bound column at 12,000 x g, RT for 2 min. Bound RNA was eluted with 30 µl RNase-free water, incubated at RT for 1 min and centrifuged at 12,000 x g for 2 min at RT. 1 µl was used

to determine the purity and concentration with the NanoDrop spectrophotometer and the rest of the isolated RNA was stored at -80°C until further use.

2. cDNA synthesis

Zebrafish embryo cDNA was synthesised from embryo RNA using the Invitrogen SuperScript First-Strand reserve transcription kit (Cat.no:18091050) (Thermo Fisher Scientific). The instructions from the manufacturer recommend using 10 pg - 5 µg total RNA (in a volume up to 11 µl) for cDNA synthesis. For this study, 1 µl total RNA isolated from 10-50 zebrafish embryos and weighed tissue samples was made up to 11 µl with RNase-free water and placed on ice (the concentrations of total RNA used for each sample are indicated in Table 2 below). The RNA was mixed with 1 µl random hexamers (50 µM) and 1 µl dNTP mix (10 mM), incubated at 65°C for 5 min and cooled on ice for 1 min. As the RNA/primer reaction cooled, 7 µl cDNA superscript reverse transcriptase mix (4 µl 5x-buffer; 1 µl DTT (100 mM); 1 µl recombinant RNase inhibitor and 1 µl superscript reverse transcriptase (200 U)) was added and the reaction (20 µl total volume) was incubated in a thermocycler (Thermo Fisher Scientific) at 23°C for 10 min, 55°C for 10 min and 80°C for 10 min. To remove RNA from synthesised cDNA 1 µl *E. coli* RNase-H was added and the reaction was incubated at 37°C for 20 min. After RNA digestion the cDNA was subsequently used for PCR.

3. Polymerase chain reaction (PCR**)**

Zebrafish embryo cDNA was diluted 1:9 with the primer mix (0.25 µl forward and 0.25 µl reverse primers-i.e. 0.25 µM final concentration); 5 µl *Taq* x2 Mater Mix (BioLabs) and 3.5 µl RNase-free water); 1 µl cDNA to a final reaction volume of 10 µl. Specific primers used are listed in Table 1, below. The PCR reaction mix was placed into a thermocycler programmed to: 94°C initial denaturation for 30 sec; 35X cycles of 94°C (30 sec) denaturation, 50°C (30 sec) annealing (unless stated otherwise in Table AA1), 68°C (30 sec) elongation and a final 94°C (30 sec) denaturation step. The PCR product (10 µl) was then loaded, run and analysed on agarose gels.

Table AA2. Zebrafish primers used for qPCR analysis

Gene name	Target abbreviation	Forward primer (5' – 3')	Reverse primer (5' – 3')
Beta-actin	*β-actin*	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC
Glyceraldehyde-3-phosphate dehydrogenase	*gapdh*	GTGTAGGCGTGGACTGTGGT	TGGGAGTCAACCAGGACAAATA
Insulin	*zins*	TGGTCGATGCCCTTTATCTGG	AGATGCTGCAGGGTTTGTGG
Preproinsulin	*ppins*	AGTGTAAGCACTAACCCAGGCACA	TGCAAAGTCAGCCACCTCAGTTTC
Phosphoenolpyruvate carboxykinase 1	*pck1*	CAGTAAACACGGCTGAAGACAC	CGGTTTTGATGCACTTGAGA
Phosphoenolpyruvate carboxykinase 2	*pck2*	TCTGGCAGAAGGAAACACA	TCAATCCCTCACTCTCTCCTC

4. Agarose gel electrophoresis

Agarose gels were used to assess the quality of the extracted zebrafish RNA and PCR products following cDNA amplification. Agarose gels were prepared in TBE buffer (Tris-base (0.1M) (Merck), Boric acid (0.1M) (BDH) and EDTA (2.0 mM) (Sigma) dissolved in uItrapure water). The miniGES agarose system was assembled to generate gel of 7 x 6.5 cm dimensions. TBE buffer (30 ml) was used to prepare the agarose gels (1 - 3% (w/v)). This solution was heated until the agarose was completely dissolved. Once cooled (to about 45°C) the agarose solution was poured into the miniGES cassette with a comb and allowed to set. RNA samples were prepared by mixing 1 µl RNA with 4 µl formamide-bromophenol blue and loaded on the gels (4 µl per well). The PCR product (cDNA) was loaded as is (9 µl per well). The gels were run at 80 V for 45-60 min in TBE buffer; soaked in ethidium bromide solution (0.025 mM), viewed under UV light and images captured using a gel imaging system.

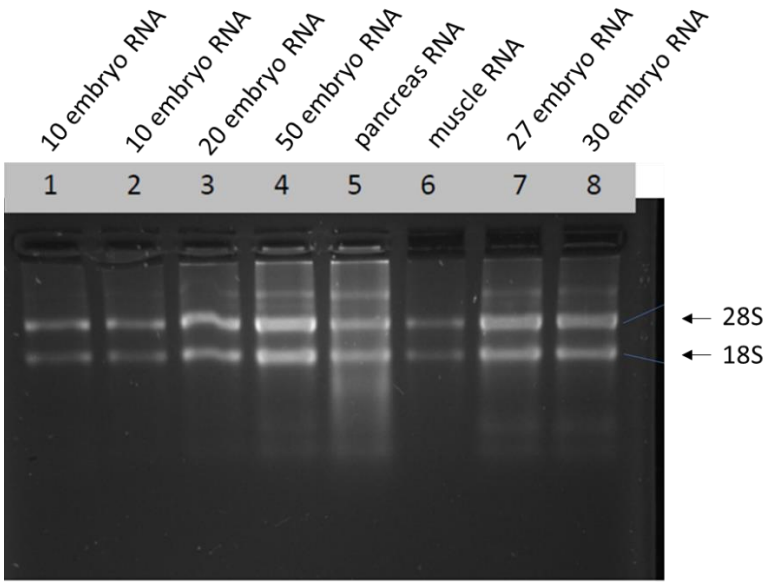


Figure A1. Ribosomal 28S and 18S RNA analysis from zebrafish embryos.
Zebrafish RNA was isolated from 4 dpf embryos and adult zebrafish tissue samples with Trizol. 1 µl RNA + 4 µl formamide-bromophenol blue was loaded and run on a 1%(w/v) agarose gel soaked in ethidium bromide. The gel was viewed under UV light for imaging. 0.018 g and 0.015 g of pancreatic and skeletal muscle tissue was used respectively.

5. cDNA polymerase chain reaction products

Markers associated with type 2 diabetes in humans have a zebrafish equivalent. These include insulin (ZINS and PPINS), pancreatic and duodenal homeobox 1 (PDX1) and phosphoenolpyruvate carboxykinase (PEPCK), which were amplified by specific primers in zebrafish as indicated by Figure 2. The primers amplified specific cDNA regions (ZINS (~250 bp); PPINS (~250 bp); PEPCK (~650 bp) and GAPDH (~250 bp)) that were not amplified in the absence of the zebrafish embryo cDNA templet (Figure 2a). The PCR products are between 100-1000 bp. There was no observable difference between PCR products from the different numbers of embryos as a starting material i.e. the PCR product from 10 embryos is comparable to that of 50 embryos (Figure 2 b and c). This suggests there was enough cDNA in the 10-embryo sample not to affect amplification. Therefore, embryo numbers can be reduced to 10 per treatment.

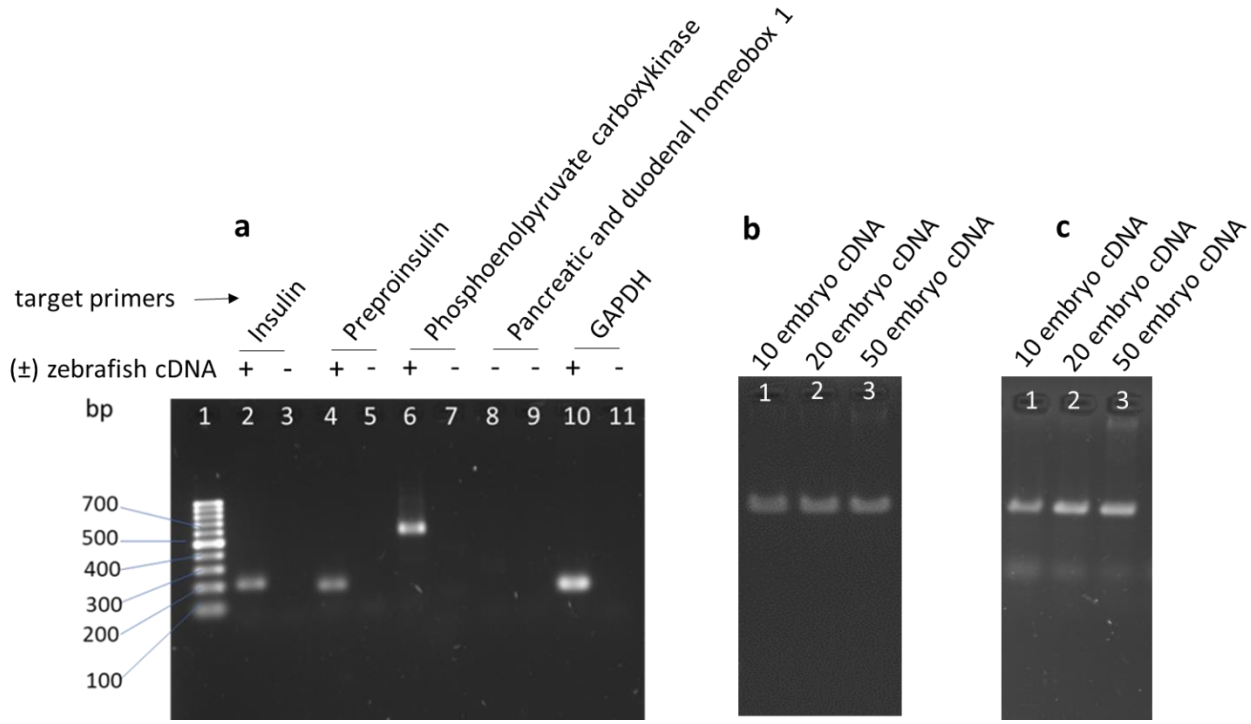


Figure A2. PCR products of cDNA amplification.
Zebrafish RNA was converted to cDNA and duplicated with PCR. a**)** RNA extracted from 20 embryos was used and PCR performed in the presence or absence of zebrafish cDNA to indicate primer specificity. **b)** GAPDH cDNA and **c)** PDX1 cDNA obtained with RNA from 10, 20 and 50 embryos indicating that the same amount of PCR product is attained in all samples if total RNA is within 10 pg – 5 µg.

Appendix B: Experimental methodology, husbandry and techniques used during zebrafish TLD treatment.

1. The IWAKI system

Male and female zebrafish were housed in the IWAKI system (Iwaki Aquatic Systems and Services; USA) at 28.5°C in 2.5L and 4.5L tanks at shoaling densities of 6-8 and 10-16 fish per tank respectively, containing reverse osmosis (RO) water under a blue-light cycle of 14-hour light and 10-hour dark period (Figure AB.1). The IWAKI system is an aquatic recirculation system that moves dirty/used water from the tanks (containing fish) through a series of filters (mechanical, biological and chemical) to reduce or remove waste in its gas, liquid and solid forms. Once chemical and biological waste is removed, the system uses ultra-violet light to sterilize the water before cycling it back to the fish tanks. The system repeats this process 12 times a day. During this cycling process, sensors in the water reservoir monitor the water levels as well as temperature, pH and conductivity, which are all automatically adjusted by the controller (Table AB.1).

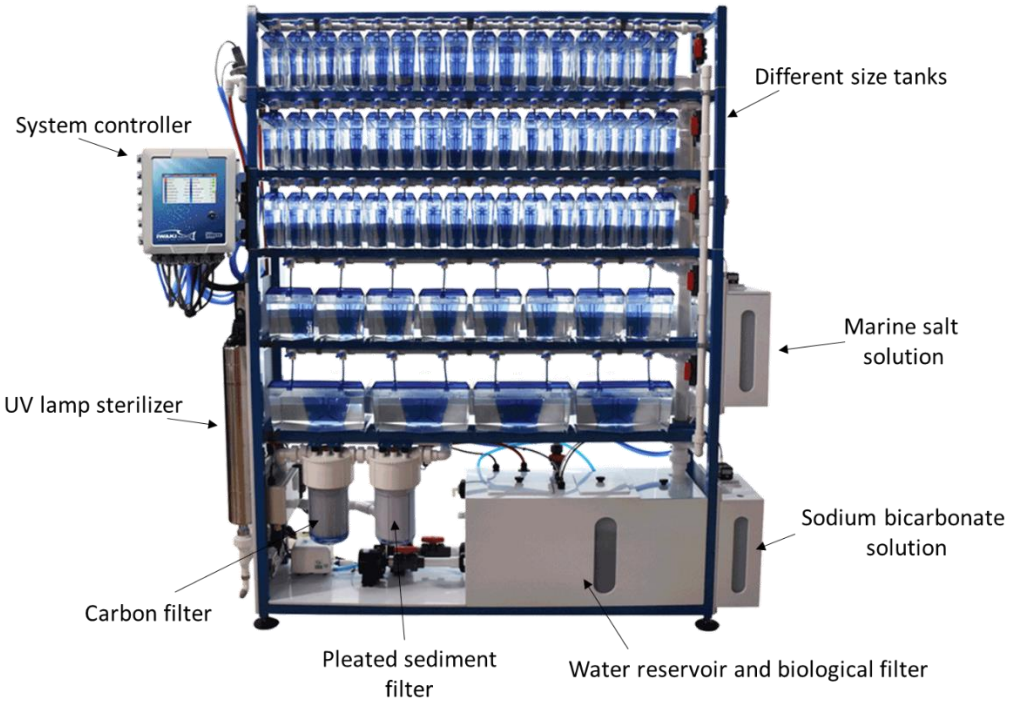


Figure AB.1. The IWAKI system used in our facility to house zebrafish.
A picture of the system used for all the zebrafish experiments in this study

Table AB1. Parameters of the zebrafish system used in this study

Water parameter	IWAKI optimum range
Temperature	26 to 28.5°C
Conductivity	200 -3000 µS
pH	7 to 8.5

The IWAKI system in our research facility does not have an automated feeder; as a result, fish were fed manually every day, three times a day on week days and once a day on weekends. Fish feed was either brine-shrimp (hatched in the laboratory), tropical fish-flakes (TetraMin, Virginia, USA) or Otohime-B2 (Marubeni Nisshin Feed, Tokyo, Japan). Sufficient food was provided (considering fish numbers) such that the fish would completely consume it within 10 minutes to minimise any leftover food that would spoil water quality.

2. <u>Spawning or breeding of embryos</u>

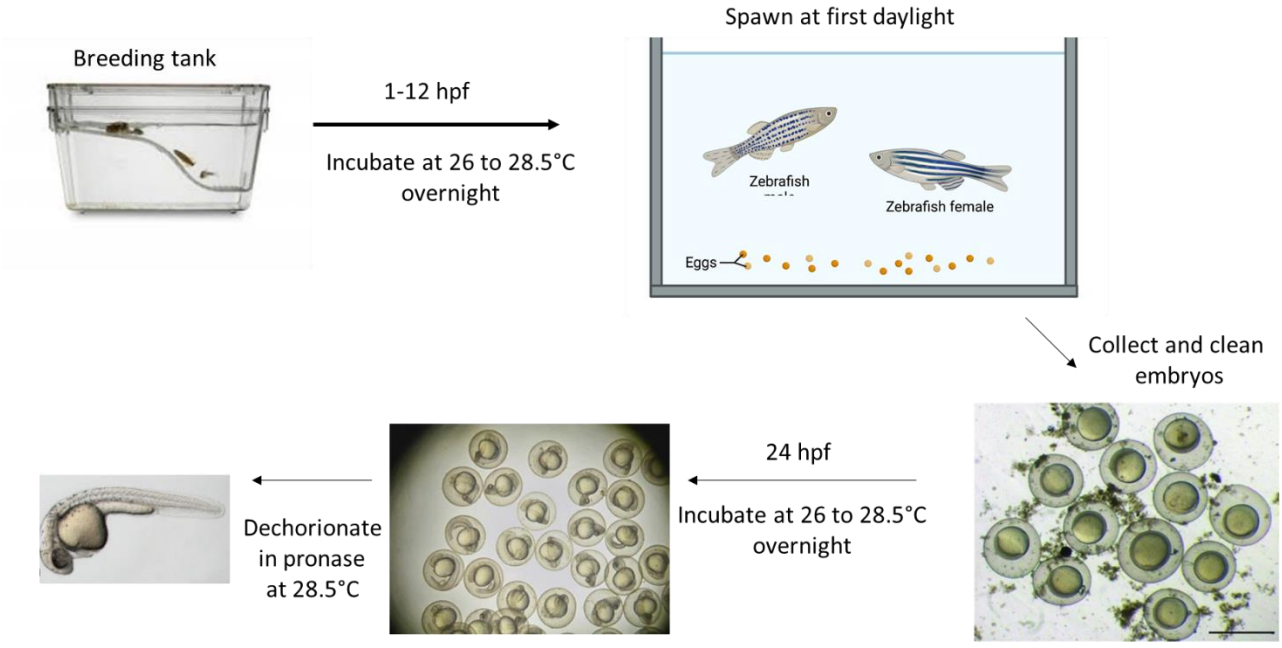


Figure AB.2. Illustration of zebrafish spawning and embryo processing before experimentation.
All animal procedures were approved by the Animal Research Ethics Committee.

To prepare for spawning, zebrafish pairs (2 females and 2 males) 4 to 8 months old were placed into a breeding tank (fitted with porous sloping surface inset which allows embryos to

fall through to the bottom of the tank) and left overnight a 28.5°C. Zebrafish pairs initiate breeding at the onset of light the following morning. After 1 hour of spawning the fish would be returned to the IWAKI system and fertilised embryos would be collected and cleaned (which involves washing in a stream of water at least 3 times). Clean fertilised embryos would then be placed in E3 media in a Petri dish or a cell-culture plate and incubated at 28.5°C overnight. The following day embryos would be dechorionated by placing them in a 50 ml centrifuge tube containing 20 ml pronase E solution (1 mg/ml; Cat no. 9036-06-0, Merck, USA) for 40 minutes at 28.5°C. The embryos would be gently removed from the pronase solution and placed in a second centrifuge tube containing E3 media, where they would be gently pipetted up and down using a 3 ml plastic dropper pipette to break the chorion. At this point the dechorionated embryos would sink to the bottom of the tube while the empty chorion shells would float allowing then to be easily removed by pipetting. The dechorionated embryos would then be rinsed in E3 media and used as needed.

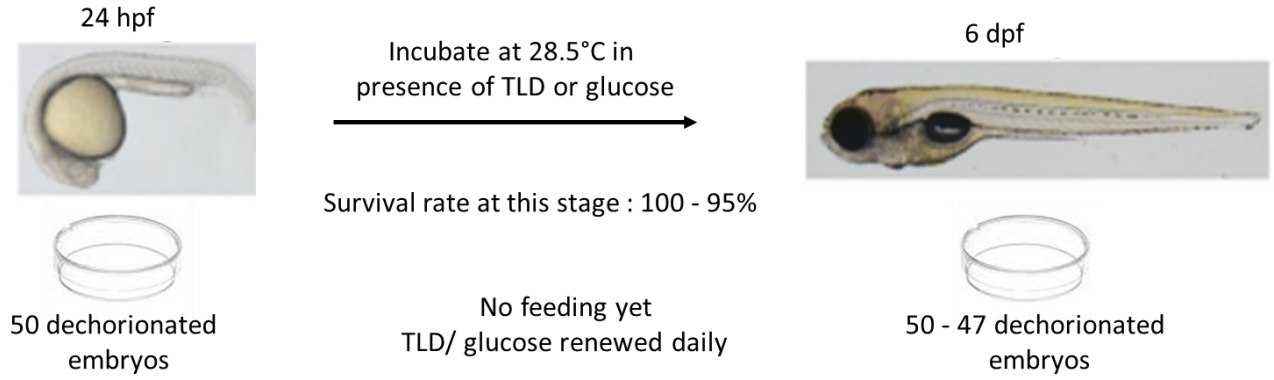


Figure AB.3. Survival rate and maintenance of embryos at 0-5 days during treatment at 1-6dpf
All animal procedures were approved by the Animal Research Ethics Committee

During the duration of TLD or glucose treatment (5 days) the embryos were not fed as the yolk still meets all the embryo's nutritional requirements. The embryo survival rate remains high because the introduction of feed at this stage in a petri dish spoils the water if not all the food is consumed; embryos usually do not feed during this stage of development. After 5 days of TLD/ glucose treatment, embryos would be removed from the treatment solutions and rinsed in RO water where they would remain for the rest of their development until feeding begins at 6dpf (and continues for the rest of zebrafish development).

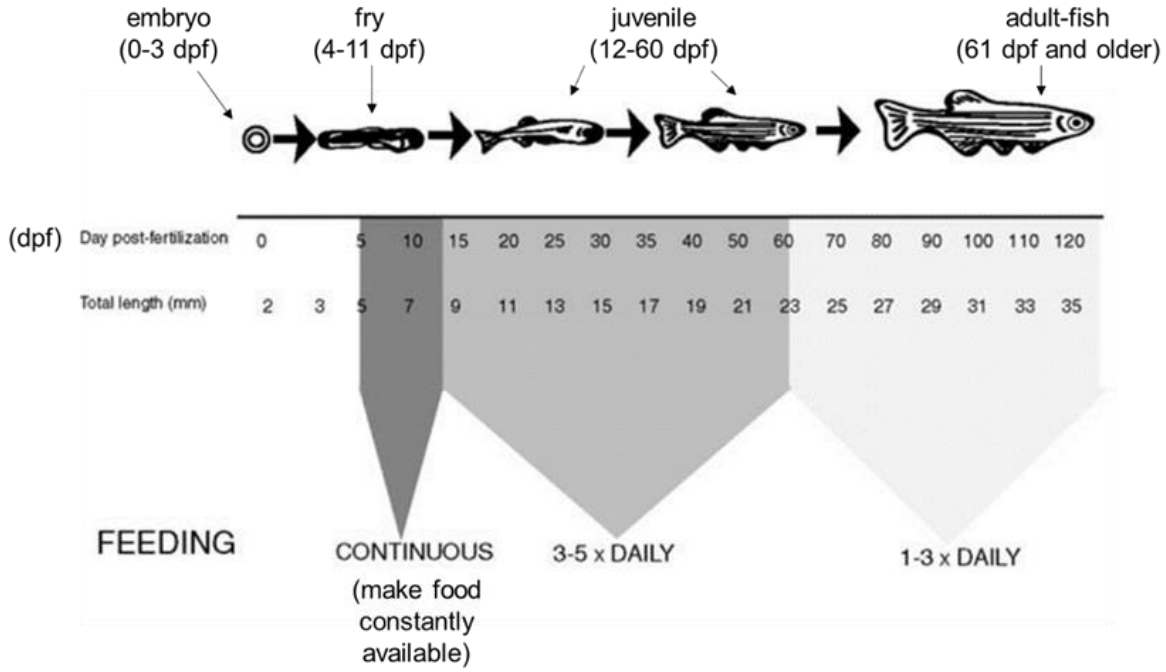


Figure AB.4. Feeding frequency for zebrafish during development.
Zebrafish were fed according to this cart during development, except when following the overfeeding protocol. Adopted from Lawrence (2011).

The critical phase of zebrafish development is from 6 – 20 dpf.This is because the early juvenile zebrafish (also referred to as fry) require more food, but the constraint is that they are housed in a petri dish with a limited volume of water, which spoils easily as the feed starts to decay. The survival rate at this point is between 45-60% depending on the level of care i.e. regular cleaning and removal of unfinished feed and waste (but it can also be noted that sometime zebrafish fry died even with the strictest level of care). The type of feed used at this critical phase would be fry food and paramecia, brine shrimp would not be suitable at this stage.

Table AB2. Different types of feed used on growing embryos

Feed type	Reasoning for use
Fry feed	Good enough but spoils and becomes sticky for fry
Paramecia	Perfect at this stage, small enough and doesn't spoil water
Brine shrimp	Too big for 6-20dpf fry

Raising 30 dpf juvenile zebrafish to adulthood (4 month) becomes easier after the critical phase has passed, where fish would be fed brine shrimp and fish flakes 3 times a day and the survival rate approaches 100%. At this stage the treated fish and controls would be taken back to the IWAKI system until they reach the desired adulthood stage of 4-months post fertilisation.

3. Anaesthetization, weighing and blood collection

At the end of the monitoring period, at 4-months post fertilisation, treated zebrafish were placed in reverse osmosis water, removed from the IWAKI system and fasted for 12-hours in separate standalone tanks. The fish were then anesthetised on ice (i.e. fish were placed into an ice bath at 18-12°C for 2-5 minutes, removed, excess water was removed with paper towel) and fish were weighed before they were euthanized (by decapitation using a sterile scalpel blades), and blood drawn (following decapitation the glucometer strip was directly connected to the wound) to determine fasting blood glucose using a glucometer (Accu-Chek Instant, Roche, Germany).

4. General methods

***Artemia nauplii* (brine shrimp) culture**

- Dissolve two tablespoons of nonionised salt or sea salt in 1-liter dechlorinated water.
- Use an inverted 2-liter (coke) bottle (with its base removed) as a hatching basin.
- Add 1 level tablespoon of brine shrimp eggs
- Use a tank heater to maintain the temperature at 28°C and an air-stone to supply oxygen (air pump).
- Hatch in constant light (lamp) for 24-48 hours.
- Collect hatched *nauplii*, rinse to remove salt and use as fish feed.
- Move the remaining *nauplii* to fresh saltwater in a clean fish tank.
- Grow newly hatched *nauplii* at 28°C in the presence of light
- Feed 5-10 drops of baker's yeast (dissolved in dechlorinated water) once daily.

Feeding fish.

- Pipette 10-50 ml of culture (i.e. about 10-100 *nauplii*/mL depending on culture concentration).
- Rinse on a fine mesh strainer or brine shrimp net (available in pet shops) with tap water to remove salt and feed to tanks.
- Excess brine shrimp can be stored overnight at 4°C for subsequent feeding (granted the shrimp have not disintegrated.

***Paramecia caudatum* culture**.

- Use a clean 2-liter container (2-liter is best for high fry numbers).
- Add 1.5 liters of dechlorinated water.
- Use a heater to maintain the water at 27-28.5°C.
- Add an air-stone for oxygen supply.
- Add a pinch of commercial fish flakes (and keep feeding every 2nd to 3rd day).
- Leave in permanent light (use a lamp if needed) for 7-12 days.
- Use a microscope to check for the presence of paramecia (a stereomicroscope works best).
- Before feeding to fry, strain paramecia water through a fine-mesh sieve and a 100 µm cell culture filter.
- The volume depends on feeding needs (i.e. the number of fry) and the concentration of the paramecia culture.
- Replace the volume of paramecia water removed with fresh dechlorinated water.

Appendix C: Conference abstract

Title: Establishing a zebrafish larvae model to investigate the effect of TLD and its individual drug components on T2DM markers.

Topic: Disease Models & Mechanisms; Toxicology

Abstract

Purpose and motivation

The rising incidence of obesity and type 2 diabetes mellitus (T2DM) in HIV-positive patients on antiretroviral treatment (ART) is a global concern and even more so for South Africa (SA). SA has the highest HIV prevalence in the world with the majority of this population on ART, and T2DM is the fifth leading cause of death in the country. The preferred first-line ART regimen in SA consists of a combination of tenofovir, lamivudine and dolutegravir (TLD), but has been associated with significant body-weight gain and clinical obesity and could potentially lead to T2DM. The underlying mechanisms on a genetic level of ART leading to body-weight gain, obesity, changes in glucose metabolism and T2DM are unclear and the effects of TLD and its individual components during pregnancy or early development of T2DM are not known. This is essential since tenofovir has the ability to cross the placenta. In addition, the question of whether early exposure to TLD and its individual drugs predisposes an individual to T2DM in adulthood has not yet been answered/investigated. The zebrafish and its larvae have become a valuable resource in biomedical research due mostly to its more than 70% genetic similarity to humans and the possibility to extrapolate observed effects to humans.

Aim

A zebrafish model is currently being developed at the recently-established UKZN zebrafish facility to mimic exposure during pregnancy and early development, as well as to investigate implications after long term ART exposure. The purpose of this study is to clarify the connection between ART and T2DM, focusing on the morphological and genetic effects of TLD and its individual components on the zebrafish larvae model.

Methods

TLD and its individual drug components were first tested *in vitro* on HEK 293 cells to determine cytotoxicity. The cells were treated with 10-100uM of the drug in DMSO as a diluent for 3 days and viability was determined via the MTS assay.

For *in vivo* studies, zebrafish embryos dechorionated at 24 hpf were exposed to TLD and its individual drug components at 10-100uM for 3, 5 and 6 days. Larvae were kept in E3 medium at 28°. The medium containing the drug(s) was changed every 24 hrs and images taken on the first and last day of exposure. Total RNA was isolated on the last day of exposure and converted to cDNA for qPCR analysis to correlate changes in mRNA targets (insulin, preproinsulin, PDX1, ZRANB-3, PCK 1 & 2 and GAPDH) associated with glucose metabolism.

Results

TLD and its individual drugs were not toxic on HEK 293 cells at the concentration levels used in this study, however 100uM TLD and 100uM tenofovir significantly reduced the cells' growth rate. The zebrafish larvae model was not affected by DMSO concentration of up to 7%. There were no visible morphological differences for tenofovir and lamivudine treated embryos at 3, 5 and 6 days of exposure compared to controls (E3 media and DMSO). Dolutegravir appears to be toxic after 48 hrs of exposure at 100uM and potentially reduces the size of embryos. The levels of GAPDH mRNA expression seem to slightly change in zebrafish larvae at 3, 5 and 6 days of treatment.

Conclusion

The zebrafish larvae model is showing promise as a valuable resource when investigating genetic and morphological changes associated with ART during early development. Our preliminary results have shown indications of morphological changes due to the exposure to antiretroviral drugs. The use of a second housekeeping gene might be required in addition to GAPDH. More work is required to further validate the model including the extension of the exposure period in order to better illustrate the impact on gene expression.

References

Achenbach, J. C., Leggiadro, C., Sperker, S. A., Woodland, C. and Ellis, L. D. (2020). Comparison of the Zebrafish Embryo Toxicity Assay and the General and Behavioral Embryo Toxicity Assay as New Approach Methods for Chemical Screening. *Toxics, 8*(4). doi:10.3390/toxics8040126

Adeyemo, A. A., Zaghloul, N. A., Chen, G., Doumatey, A. P., Leitch, C. C., Hostelley, T. L., Nesmith, J. E., Zhou, J., Bentley, A. R., Shriner, D., Fasanmade, O., Okafor, G., Eghan, B., Jr., Agyenim-Boateng, K., Chandrasekharappa, S., Adeleye, J., Balogun, W., Owusu, S., Amoah, A., Acheampong, J., Johnson, T., Oli, J., Adebamowo, C., Collins, F., Dunston, G. and Rotimi, C. N. (2019). ZRANB3 is an African-specific type 2 diabetes locus associated with beta-cell mass and insulin response. *Nat Commun, 10*(1), 3195. doi:10.1038/s41467-019-10967-7

Adhish, M. and Manjubala, I. (2023). Effectiveness of zebrafish models in understanding human diseases-A review of models. *Heliyon, 9*(3), e14557. doi:10.1016/j.heliyon.2023.e14557

Adhish, M. and Manjubala, I. (2023). Effectiveness of zebrafish models in understanding human diseases—A review of models. *Heliyon, 9*(3), e14557. doi:https://doi.org/10.1016/j.heliyon.2023.e14557

Alam, F., Islam, M. A., Khalil, M. I. and Gan, S. H. (2016). Metabolic Control of Type 2 Diabetes by Targeting the GLUT4 Glucose Transporter: Intervention Approaches. *Curr Pharm Des, 22*(20), 3034-3049. doi:10.2174/1381612822666160307145801

Annis, A. M., Caulder, M. S., Cook, M. L. and Duquette, D. (2005). Family history, diabetes, and other demographic and risk factors among participants of the National Health and Nutrition Examination Survey 1999-2002. *Prev Chronic Dis, 2*(2), A19.

Arneth, B., Arneth, R. and Shams, M. (2019). Metabolomics of Type 1 and Type 2 Diabetes. *Int J Mol Sci, 20*(10). doi:10.3390/ijms20102467

Bahamdain, F. (2022). Effect of Dolutegravir on Plasma Glucose Among Human Immunodeficiency Virus Patients in a Community Health Center Setting. *Cureus, 14*(10), e30556. doi:10.7759/cureus.30556

Bangalee, A., Hanky, S. and Bangalee, V. (2022). Dolutegravir as first-line antiretroviral therapy in South Africa: Beware the one-size-fits-all approach. *SAMJ: South African Medical Journal, 112*, 787-790. Retrieved from http://www.scielo.org.za/scielo.php?script=sci_arttext&pid=S0256-95742022001200004&nrm=iso

Ben-Romano, R., Rudich, A., Tirosh, A., Potashnik, R., Sasaoka, T., Riesenberg, K., Schlaeffer, F. and Bashan, N. (2004). Nelfinavir-induced insulin resistance is associated with impaired plasma membrane recruitment of the PI 3-kinase effectors Akt/PKB and PKC-zeta. *Diabetologia, 47*(6), 1107-1117. doi:10.1007/s00125-004-1408-5

Berghmans, S., Murphey, R. D., Wienholds, E., Neuberg, D., Kutok, J. L., Fletcher, C. D., Morris, J. P., Liu, T. X., Schulte-Merker, S., Kanki, J. P., Plasterk, R., Zon, L. I. and

Look, A. T. (2005). tp53 mutant zebrafish develop malignant peripheral nerve sheath tumors. *Proc Natl Acad Sci U S A, 102*(2), 407-412. doi:10.1073/pnas.0406252102

Bessong, P. O., Matume, N. D. and Tebit, D. M. (2021). Potential challenges to sustained viral load suppression in the HIV treatment programme in South Africa: a narrative overview. *AIDS Res Ther, 18*(1), 1. doi:10.1186/s12981-020-00324-w

Brennan, A. T., Nattey, C., Kileel, E. M., Rosen, S., Maskew, M., Stokes, A. C., Fox, M. P. and Venter, W. D. F. (2023). Change in body weight and risk of hypertension after switching from efavirenz to dolutegravir in adults living with HIV: evidence from routine care in Johannesburg, South Africa. *EClinicalMedicine, 57*, 101836. doi:10.1016/j.eclinm.2023.101836

Cabrera, R. M., Souder, J. P., Steele, J. W., Yeo, L., Tukeman, G., Gorelick, D. A. and Finnell, R. H. (2019). The antagonism of folate receptor by dolutegravir: developmental toxicity reduction by supplemental folic acid. *AIDS, 33*(13), 1967-1976. doi:10.1097/qad.0000000000002289

Cao, Y., Chen, Q., Liu, Y., Jin, L. and Peng, R. (2023). Research Progress on the Construction and Application of a Diabetic Zebrafish Model. *Int J Mol Sci, 24*(6). doi:10.3390/ijms24065195

Capiotti, K. M., Antonioli, R., Jr., Kist, L. W., Bogo, M. R., Bonan, C. D. and Da Silva, R. S. (2014). Persistent impaired glucose metabolism in a zebrafish hyperglycemia model. *Comp Biochem Physiol B Biochem Mol Biol, 171*, 58-65. doi:10.1016/j.cbpb.2014.03.005

Carnovali, M., Luzi, L., Banfi, G. and Mariotti, M. (2016). Chronic hyperglycemia affects bone metabolism in adult zebrafish scale model. *Endocrine, 54*(3), 808-817. doi:10.1007/s12020-016-1106-3

Cerf, M. E. (2013). Beta cell dysfunction and insulin resistance. *Front Endocrinol (Lausanne), 4*, 37. doi:10.3389/fendo.2013.00037

Chen, C. and Liu, D. (2022). Establishment of Zebrafish Models for Diabetes Mellitus and Its Microvascular Complications. *Journal of Vascular Research, 59*(4), 251-260. doi:10.1159/000522471

Connaughton, V. P., Baker, C., Fonde, L., Gerardi, E. and Slack, C. (2016). Alternate Immersion in an External Glucose Solution Differentially Affects Blood Sugar Values in Older Versus Younger Zebrafish Adults. *Zebrafish, 13*(2), 87-94. doi:10.1089/zeb.2015.1155

Daimari, R., Kwape, L. and Oyekunle, A. A. (2018). Suicidal overdose of dolutegravir: A case report. *Southern African Journal of HIV Medicine, 19*, 1-3. Retrieved from http://www.scielo.org.za/scielo.php?script=sci_arttext&pid=S2078-67512018000100017&nrm=iso

Dufrane, D., van Steenberghe, M., Guiot, Y., Goebbels, R. M., Saliez, A. and Gianello, P. (2006). Streptozotocin-induced diabetes in large animals (pigs/primates): role of GLUT2 transporter and beta-cell plasticity. *Transplantation, 81*(1), 36-45. doi:10.1097/01.tp.0000189712.74495.82

Durham, H. A. and Truett, G. E. (2006). Development of insulin resistance and hyperphagia in Zucker fatty rats. *Am J Physiol Regul Integr Comp Physiol, 290*(3), R652-658. doi:10.1152/ajpregu.00428.2004

Eisen, J. S. (2020). Chapter 1 - History of Zebrafish Research. In S. C. Cartner, J. S. Eisen, S. C. Farmer, K. J. Guillemin, M. L. Kent, andG. E. Sanders (Eds.), *The Zebrafish in Biomedical Research* (pp. 3-14): Academic Press.

Elo, B., Villano, C. M., Govorko, D. and White, L. A. (2007). Larval zebrafish as a model for glucose metabolism: expression of phosphoenolpyruvate carboxykinase as a marker for exposure to anti-diabetic compounds. *J Mol Endocrinol, 38*(4), 433-440. doi:10.1677/jme-06-0037

Fisher, S., Jagadeeswaran, P. and Halpern, M. E. (2003). Radiographic analysis of zebrafish skeletal defects. *Dev Biol, 264*(1), 64-76. doi:10.1016/s0012-1606(03)00399-3

Fokam, J., Nka, A. D., Mamgue Dzukam, F. Y., Efakika Gabisa, J., Bouba, Y., Tommo Tchouaket, M. C., Ka'e, A. C., Ngoufack Jagni Semengue, E., Takou, D., Moudourou, S., Fainguem, N., Pabo, W., Nayang Mundo, R. A., Kengni Ngueko, A. M., Ambe Chenwi, C., Flore Yimga, J., Nnomo Zam, M. K., Simo Kamgaing, R., Tangimpundu, C., Kamgaing, N., Njom-Nlend, A. E., Ndombo Koki, P., Kesseng, D., Ndiang Tetang, S., Kembou, E., Ebiama Lifanda, L., Pamen, B., Ketchaji, A., Saounde Temgoua, E., Billong, S. C., Zoung-Kanyi Bissek, A. C., Hadja, H., Halle, E. G., Colizzi, V., Perno, C. F., Sosso, S. M. and Ndjolo, A. (2023). Viral suppression in the era of transition to dolutegravir-based therapy in Cameroon: Children at high risk of virological failure due to the lowly transition in pediatrics. *Medicine (Baltimore), 102*(20), e33737. doi:10.1097/md.0000000000033737

Forbes, J. C., Alimenti, A. M., Singer, J., Brophy, J. C., Bitnun, A., Samson, L. M., Money, D. M., Lee, T. C., Lapointe, N. D. and Read, S. E. (2012). A national review of vertical HIV transmission. *AIDS, 26*(6), 757-763. doi:10.1097/QAD.0b013e328350995c

Fu, Z., Gilbert, E. R. and Liu, D. (2013). Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. *Current diabetes reviews, 9*(1), 25-53. Retrieved from https://pubmed.ncbi.nlm.nih.gov/22974359

Furukawa, F., Tseng, Y. C., Liu, S. T., Chou, Y. L., Lin, C. C., Sung, P. H., Uchida, K., Lin, L. Y. and Hwang, P. P. (2015). Induction of Phosphoenolpyruvate Carboxykinase (PEPCK) during Acute Acidosis and Its Role in Acid Secretion by V-ATPase-Expressing Ionocytes. *Int J Biol Sci, 11*(6), 712-725. doi:10.7150/ijbs.11827

Gabbay, R. A., Sutherland, C., Gnudi, L., Kahn, B. B., O'Brien, R. M., Granner, D. K. and Flier, J. S. (1996). Insulin regulation of phosphoenolpyruvate carboxykinase gene expression does not require activation of the Ras/mitogen-activated protein kinase signaling pathway. *J Biol Chem, 271*(4), 1890-1897. doi:10.1074/jbc.271.4.1890

Galaviz, K. I., Narayan, K. M. V., Lobelo, F. and Weber, M. B. (2018). Lifestyle and the Prevention of Type 2 Diabetes: A Status Report. *Am J Lifestyle Med, 12*(1), 4-20. doi:10.1177/1559827615619159

Galicia-Garcia, U., Benito-Vicente, A., Jebari, S., Larrea-Sebal, A., Siddiqi, H., Uribe, K. B., Ostolaza, H. and Martín, C. (2020). Pathophysiology of Type 2 Diabetes Mellitus. *Int J Mol Sci, 21*(17). doi:10.3390/ijms21176275

Giddings, S. J., Chirgwin, J. and Permutt, M. A. (1982). Effects of glucose on proinsulin messenger RNA in rats in vivo. *Diabetes, 31*(7), 624-629. doi:10.2337/diab.31.7.624

Gizamba, J. M., Davies, J., Africa, C., Choo-Kang, C., Goedecke, J. H., Madlala, H., Lambert, E. V., Rae, D. E., Myer, L., Luke, A. and Dugas, L. R. (2023). Prevalence of obesity, hypertension and diabetes among people living with HIV in South Africa: a systematic review and meta-analysis. *BMC Infect Dis, 23*(1), 861. doi:10.1186/s12879-023-08736-5

Gleeson, M., Connaughton, V. and Arneson, L. S. (2007). Induction of hyperglycaemia in zebrafish (Danio rerio) leads to morphological changes in the retina. *Acta Diabetol, 44*(3), 157-163. doi:10.1007/s00592-007-0257-3

Gorgulho, R., Jacinto, R., Lopes, S. S., Pereira, S. A., Tranfield, E. M., Martins, G. G., Gualda, E. J., Derks, R. J. E., Correia, A. C., Steenvoorden, E., Pintado, P., Mayboroda, O. A., Monteiro, E. C. and Morello, J. (2018). Usefulness of zebrafish larvae to evaluate drug-induced functional and morphological renal tubular alterations. *Arch Toxicol, 92*(1), 411-423. doi:10.1007/s00204-017-2063-1

Grundlingh, N., Zewotir, T. T., Roberts, D. J. and Manda, S. (2022). Assessment of prevalence and risk factors of diabetes and pre-diabetes in South Africa. *Journal of Health, Population and Nutrition, 41*(1), 7. doi:10.1186/s41043-022-00281-2

Hadavandsiri, F., Shafaati, M., Mohammad Nejad, S., Ebrahimzadeh Mousavi, M., Najafi, A., Mirzaei, M., Narouee, S. and Akbarpour, S. (2023). Non-communicable disease comorbidities in HIV patients: diabetes, hypertension, heart disease, and obstructive sleep apnea as a neglected issue. *Sci Rep, 13*(1), 12730. doi:10.1038/s41598-023-39828-6

Haffejee, F., Fasanmi-Kana, O., Ally, F., Thandar, Y. and Basdav, J. (2023). Four years later: Do South Africans know what pre-exposure prophylaxis for HIV is? *AIDS Care, 35*(4), 466-473. doi:10.1080/09540121.2022.2032573

Hailu, W., Tesfaye, T. and Tadesse, A. (2021). Hyperglycemia After Dolutegravir-Based Antiretroviral Therapy. *Int Med Case Rep J, 14*, 503-507. doi:10.2147/imcrj.S323233

Hanson, R. W. and Owen, O. E. (2013). Gluconeogenesis. In W. J. Lennarz andM. D. Lane (Eds.), *Encyclopedia of Biological Chemistry (Second Edition)* (pp. 381-386). Waltham: Academic Press.

Haskell, B. D., Flurkey, K., Duffy, T. M., Sargent, E. E. and Leiter, E. H. (2002). The diabetes-prone NZO/HlLt strain. I. Immunophenotypic comparison to the related NZB/BINJ and NZW/LacJ strains. *Lab Invest, 82*(7), 833-842. doi:10.1097/01.lab.0000018915.53257.00

Hermansen, K. and Mortensen, L. S. (2007). Bodyweight changes associated with antihyperglycaemic agents in type 2 diabetes mellitus. *Drug Saf, 30*(12), 1127-1142. doi:10.2165/00002018-200730120-00005

Hill, A., Waters, L. and Pozniak, A. (2019). Are new antiretroviral treatments increasing the risks of clinical obesity? *Journal of virus eradication, 5*(1), 41-43. Retrieved from https://pubmed.ncbi.nlm.nih.gov/30800425

Holec, A. D., Mandal, S., Prathipati, P. K. and Destache, C. J. (2017). Nucleotide Reverse Transcriptase Inhibitors: A Thorough Review, Present Status and Future Perspective

as HIV Therapeutics. *Curr HIV Res, 15*(6), 411-421. doi:10.2174/1570162x15666171120110145

Hotamisligil, G. S. (2000). Molecular mechanisms of insulin resistance and the role of the adipocyte. *Int J Obes Relat Metab Disord, 24 Suppl 4*, S23-27. doi:10.1038/sj.ijo.0801497

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J. C., Koch, R., Rauch, G. J., White, S., Chow, W., Kilian, B., Quintais, L. T., Guerra-Assunção, J. A., Zhou, Y., Gu, Y., Yen, J., Vogel, J. H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S. F., Laird, G. K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliot, D., Threadgold, G., Harden, G., Ware, D., Begum, S., Mortimore, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Lloyd, C., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D., Collins, J., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley, C. M., Ersan-Ürün, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberländer, M., Rudolph-Geiger, S., Teucke, M., Lanz, C., Raddatz, G., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Schuster, S. C., Carter, N. P., Harrow, J., Ning, Z., Herrero, J., Searle, S. M., Enright, A., Geisler, R., Plasterk, R. H., Lee, C., Westerfield, M., de Jong, P. J., Zon, L. I., Postlethwait, J. H., Nüsslein-Volhard, C., Hubbard, T. J., Roest Crollius, H., Rogers, J. and Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature, 496*(7446), 498-503. doi:10.1038/nature12111

Hruz, P. W., Murata, H., Qiu, H. and Mueckler, M. (2002). Indinavir induces acute and reversible peripheral insulin resistance in rats. *Diabetes, 51*(4), 937-942. doi:10.2337/diabetes.51.4.937

Huang, C., Hoque, M. T., Qu, Q. R., Henderson, J. and Bendayan, R. (2024). Antiretroviral drug dolutegravir induces inflammation at the mouse brain barriers. *Faseb j, 38*(13), e23790. doi:10.1096/fj.202400558R

IDF. (2021). International Diabetes Federation (IDF) Diabetes Atlas 10th Edition. 141. Retrieved from http://www.diabetesatlas.org

International Diabetes Federation. (2019). IDF Diabetes Atlas, 9th edition. 176. Retrieved from https://www.diabetesatlas.org

Ismail, W. I. W., King, J. A. and Pillay, T. S. (2009). Insulin resistance induced by antiretroviral drugs: Current understanding of molecular mechanisms. *Journal of Endocrinology, Metabolism and Diabetes of South Africa, 14*(3), 129-132. doi:10.1080/22201009.2009.10872209

Jung, S. H., Kim, Y. S., Lee, Y. R. and Kim, J. S. (2016). High glucose-induced changes in hyaloid-retinal vessels during early ocular development of zebrafish: a short-term animal model of diabetic retinopathy. *Br J Pharmacol, 173*(1), 15-26. doi:10.1111/bph.13279

Kahn, S. E. (2003). The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia, 46*(1), 3-19. doi:10.1007/s00125-002-1009-0

Kalra, S. and Agrawal, N. (2013). Diabetes and HIV: current understanding and future perspectives. *Curr Diab Rep, 13*(3), 419-427. doi:10.1007/s11892-013-0369-9

Katsarou, A., Gudbjörnsdottir, S., Rawshani, A., Dabelea, D., Bonifacio, E., Anderson, B. J., Jacobsen, L. M., Schatz, D. A. and Lernmark, Å. (2017). Type 1 diabetes mellitus. *Nature Reviews Disease Primers, 3*(1), 17016. doi:10.1038/nrdp.2017.16

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn, 203*(3), 253-310. doi:10.1002/aja.1002030302

Kinkel, M. D. and Prince, V. E. (2009). On the diabetic menu: zebrafish as a model for pancreas development and function. *Bioessays, 31*(2), 139-152. doi:10.1002/bies.200800123

Kottaisamy, C. P. D., Raj, D. S., Prasanth Kumar, V. and Sankaran, U. (2021). Experimental animal models for diabetes and its related complications-a review. *Lab Anim Res, 37*(1), 23. doi:10.1186/s42826-021-00101-4

Kumar, M., Singh, H. and Chakole, S. (2023). Exploring the Relation Between Diabetes and HIV: A Narrative Review. *Cureus, 15*(8), e43909. doi:10.7759/cureus.43909

Kumar, S. and Samaras, K. (2018). The Impact of Weight Gain During HIV Treatment on Risk of Pre-diabetes, Diabetes Mellitus, Cardiovascular Disease, and Mortality. *Frontiers in Endocrinology, 9*(705). doi:10.3389/fendo.2018.00705

Kyrou, I., Tsigos, C., Mavrogianni, C., Cardon, G., Van Stappen, V., Latomme, J., Kivelä, J., Wikström, K., Tsochev, K., Nanasi, A., Semanova, C., Mateo-Gallego, R., Lamiquiz-Moneo, I., Dafoulas, G., Timpel, P., Schwarz, P. E. H., Iotova, V., Tankova, T., Makrilakis, K., Manios, Y., Manios, Y., Cardon, G., Lindström, J., Schwarz, P., Makrilakis, K., Annemans, L., Garamendi, I., Kontogianni, M., Androutsos, O., Mavrogianni, C., Tsoutsoulopoulou, K., Katsarou, C., Karaglani, E., Qira, I., Skoufas, E., Maragkopoulou, K., Tsiafitsa, A., Sotiropoulou, I., Tsolakos, M., Argyri, E., Nikolaou, M., Vampouli, E.-A., Filippou, C., Gatsiou, K., Dimitriadis, E., Laatikainen, T., Wikström, K., Kivelä, J., Valve, P., Levälahti, E., Virtanen, E., Willems, R., Panchyrz, I., Holland, M., Timpel, P., Liatis, S., Dafoulas, G., Lambrinou, C.-P., Giannopoulou, A., Tsirigoti, L., Fappa, E., Anastasiou, C., Zachari, K., Rabemananjara, L., de Sabata, M. S., Ko, W., Moreno, L., Civeira, F., Bueno, G., De Miguel-Etayo, P., Gonzalez-Gil, E. M., Mesana, M. I., Vicente-Rodriguez, G., Rodriguez, G., Baila-Rueda, L., Cenarro, A., Jarauta, E., Mateo-Gallego, R., Iotova, V., Tankova, T., Usheva, N., Tsochev, K., Chakarova, N., Galcheva, S., Dimova, R., Bocheva, Y., Radkova, Z., Marinova, V., Bazdarska, Y., Stefanova, T., Rurik, I., Ungvari, T., Jancsó, Z., Nánási, A., Kolozsvári, L., Semánova, C., Martinez, R., Tong, M., Joutsenniemi, K., Wendel-Mitoraj, K. and on behalf of the Feel4Diabetes-study, G. (2020). Sociodemographic and lifestyle-related risk factors for identifying vulnerable

groups for type 2 diabetes: a narrative review with emphasis on data from Europe. *BMC Endocrine Disorders, 20*(1), 134. doi:10.1186/s12902-019-0463-3

Latek, D., Rutkowska, E., Niewieczerzal, S. and Cielecka-Piontek, J. (2019). Drug-induced diabetes type 2: In silico study involving class B GPCRs. *PLoS One, 14*(1), e0208892. doi:10.1371/journal.pone.0208892

Leitner, D. R., Frühbeck, G., Yumuk, V., Schindler, K., Micic, D., Woodward, E. and Toplak, H. (2017). Obesity and Type 2 Diabetes: Two Diseases with a Need for Combined Treatment Strategies - EASO Can Lead the Way. *Obes Facts, 10*(5), 483-492. doi:10.1159/000480525

Lin, S. P., Wu, C. Y., Wang, C. B., Li, T. C., Ko, N. Y. and Shi, Z. Y. (2018). Risk of diabetes mellitus in HIV-infected patients receiving highly active antiretroviral therapy: A nationwide population-based study. *Medicine (Baltimore), 97*(36), e12268. doi:10.1097/md.0000000000012268

Mambiya, M., Shang, M., Wang, Y., Li, Q., Liu, S., Yang, L., Zhang, Q., Zhang, K., Liu, M., Nie, F., Zeng, F. and Liu, W. (2019). The Play of Genes and Non-genetic Factors on Type 2 Diabetes. *Frontiers in Public Health, 7*. doi:10.3389/fpubh.2019.00349

Mashingaidze-Mano, R., Bwakura-Dangarembizi, M. F., Maponga, C. C., Morse, G. D., Monera-Penduka, T. G., Mtisi, T. J., Mudzviti, T. and Mujuru, H. A. (2020). Proximal renal tubular function in HIV-infected children on tenofovir disoproxil fumarate for treatment of HIV infection at two tertiary hospitals in Harare, Zimbabwe. *PLoS One, 15*(7), e0235759. doi:10.1371/journal.pone.0235759

Mastan, S. K. and Kumar, K. E. (2010). Effect of antiretroviral drugs on the pharmacodynamics of gliclazide with respect to glucose-insulin homeostasis in animal models. *J Exp Pharmacol, 2*, 1-11.

McCarthy, E., Rowe, C. J., Crowley-Perry, M. and Connaughton, V. P. (2021). Alternate Immersion in Glucose to Produce Prolonged Hyperglycemia in Zebrafish. *J Vis Exp*(171). doi:10.3791/61935

McCurley, A. T. and Callard, G. V. (2008). Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. *BMC Molecular Biology, 9*(1), 102. doi:10.1186/1471-2199-9-102

McFarlane, S. I. (2009). Antidiabetic medications and weight gain: implications for the practicing physician. *Curr Diab Rep, 9*(3), 249-254. doi:10.1007/s11892-009-0040-7

McInerney, M. F., Najjar, S. M., Brickley, D., Lutzke, M., Abou-Rjaily, G. A., Reifsnyder, P., Haskell, B. D., Flurkey, K., Zhang, Y. J., Pietropaolo, S. L., Pietropaolo, M., Byers, J. P. and Leiter, E. H. (2004). Anti-insulin receptor autoantibodies are not required for type 2 diabetes pathogenesis in NZL/Lt mice, a New Zealand obese (NZO)-derived mouse strain. *Exp Diabesity Res, 5*(3), 177-185. doi:10.1080/15438600490478029

McLaughlin, M., Walsh, S. and Galvin, S. (2017). Dolutegravir-induced hyperglycaemia in a patient living with HIV. *Journal of Antimicrobial Chemotherapy, 73*(1), 258-260. doi:10.1093/jac/dkx365

Melloul, D., Marshak, S. and Cerasi, E. (2002). Regulation of insulin gene transcription. *Diabetologia, 45*(3), 309-326. doi:10.1007/s00125-001-0728-y

Meng, X. H., Chen, B. and Zhang, J. P. (2017). Intracellular Insulin and Impaired Autophagy in a Zebrafish model and a Cell Model of Type 2 diabetes. *Int J Biol Sci, 13*(8), 985-995. doi:10.7150/ijbs.19249

Meyers, J. R. (2018). Zebrafish: Development of a Vertebrate Model Organism. *Current Protocols Essential Laboratory Techniques, 16*(1), e19. doi:https://doi.org/10.1002/cpet.19

Miyawaki, I. (2020). Application of zebrafish to safety evaluation in drug discovery. *J Toxicol Pathol, 33*(4), 197-210. doi:10.1293/tox.2020-0021

Mohammadi, H., Manouchehri, H., Changizi, R., Bootorabi, F. and Khorramizadeh, M. R. (2020). Concurrent metformin and silibinin therapy in diabetes: assessments in zebrafish (Danio rerio) animal model. *J Diabetes Metab Disord, 19*(2), 1233-1244. doi:10.1007/s40200-020-00637-7

Morello, J., Derks, R. J. E., Lopes, S. S., Steenvoorden, E., Monteiro, E. C., Mayboroda, O. A. and Pereira, S. A. (2018). Zebrafish Larvae Are a Suitable Model to Investigate the Metabolic Phenotype of Drug-Induced Renal Tubular Injury. *Frontiers in Pharmacology, 9*. doi:10.3389/fphar.2018.01193

Moyo, E., Moyo, P., Murewanhema, G., Mhango, M., Chitungo, I. and Dzinamarira, T. (2023). Key populations and Sub-Saharan Africa's HIV response. *Front Public Health, 11*, 1079990. doi:10.3389/fpubh.2023.1079990

Murata, H., Hruz, P. W. and Mueckler, M. (2000). The mechanism of insulin resistance caused by HIV protease inhibitor therapy. *J Biol Chem, 275*(27), 20251-20254. doi:10.1074/jbc.C000228200

Myhre, J. L. and Pilgrim, D. B. (2010). Cellular differentiation in primary cell cultures from single zebrafish embryos as a model for the study of myogenesis. *Zebrafish, 7*(3), 255-266. doi:10.1089/zeb.2010.0665

Namara, D., Schwartz, J. I., Tusubira, A. K., McFarland, W., Birungi, C., Semitala, F. C. and Muddu, M. (2022). The risk of hyperglycemia associated with use of dolutegravir among adults living with HIV in Kampala, Uganda: A case-control study. *Int J STD AIDS, 33*(14), 1158-1164. doi:10.1177/09564624221129410

Noor, M. A., Lo, J. C., Mulligan, K., Schwarz, J. M., Halvorsen, R. A., Schambelan, M. and Grunfeld, C. (2001). Metabolic effects of indinavir in healthy HIV-seronegative men. *AIDS, 15*(7), F11-18. doi:10.1097/00002030-200105040-00001

Oka, T., Nishimura, Y., Zang, L., Hirano, M., Shimada, Y., Wang, Z., Umemoto, N., Kuroyanagi, J., Nishimura, N. and Tanaka, T. (2010). Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. *BMC Physiol, 10*, 21. doi:10.1186/1472-6793-10-21

Olaniyan, L. W., Maduagwu, E. N., Akintunde, O. W., Oluwayelu, O. O. and Brai, B. I. (2015). Lamivudine-Induced Liver Injury. *Open Access Maced J Med Sci, 3*(4), 545-550. doi:10.3889/oamjms.2015.110

Olokoba, A. B., Obateru, O. A. and Olokoba, L. B. (2012). Type 2 diabetes mellitus: a review of current trends. *Oman Med J, 27*(4), 269-273. doi:10.5001/omj.2012.68

Pandey, S., Chmelir, T. and Chottova Dvorakova, M. (2023). Animal Models in Diabetic Research—History, Presence, and Future Perspectives. *Biomedicines, 11*(10), 2852. Retrieved from https://www.mdpi.com/2227-9059/11/10/2852

Park, T. E., Mohamed, A., Kalabalik, J. and Sharma, R. (2015). Review of integrase strand transfer inhibitors for the treatment of human immunodeficiency virus infection. *Expert Rev Anti Infect Ther, 13*(10), 1195-1212. doi:10.1586/14787210.2015.1075393

Piero, M., Nzaro, G. and Njagi, J. (2015). Diabetes mellitus-a devastating metabolic disorder. *Asian journal of biomedical and pharmaceutical sciences, 5*(40), 1.

Poitout, V., Hagman, D., Stein, R., Artner, I., Robertson, R. P. and Harmon, J. S. (2006). Regulation of the insulin gene by glucose and fatty acids. *J Nutr, 136*(4), 873-876. doi:10.1093/jn/136.4.873

Poitout, V., Stein, R. and Rhodes, C. J. (2004). Insulin Gene Expression and Biosynthesis. In *International Textbook of Diabetes Mellitus*.

Reed, J., Bain, S. and Kanamarlapudi, V. (2021). A Review of Current Trends with Type 2 Diabetes Epidemiology, Aetiology, Pathogenesis, Treatments and Future Perspectives. *Diabetes, Metabolic Syndrome and Obesity, 14*(null), 3567-3602. doi:10.2147/DMSO.S319895

Röder, P. V., Wu, B., Liu, Y. and Han, W. (2016). Pancreatic regulation of glucose homeostasis. *Exp Mol Med, 48*(3), e219. doi:10.1038/emm.2016.6

Romo, M. L., Patel, R. C., Edwards, J. K., Humphrey, J. M., Musick, B. S., Bernard, C., Maina, M. W., Brazier, E., Castelnuovo, B., Penner, J., Wyka, K., Cardoso, S. W., Ly, P. S., Kunzekwenyika, C., Cortés, C. P., Panczak, R., Kelvin, E. A., Wools-Kaloustian, K. K. and Nash, D. (2022). Disparities in Dolutegravir Uptake Affecting Females of Reproductive Age With HIV in Low- and Middle-Income Countries After Initial Concerns About Teratogenicity : An Observational Study. *Ann Intern Med, 175*(1), 84-94. doi:10.7326/m21-3037

Ruderman, S. A., Crane, H. M., Nance, R. M., Whitney, B. M., Harding, B. N., Mayer, K. H., Moore, R. D., Eron, J. J., Geng, E., Mathews, W. C., Rodriguez, B., Willig, A. L., Burkholder, G. A., Lindström, S., Wood, B. R., Collier, A. C., Vannappagari, V., Henegar, C., Van Wyk, J., Curtis, L., Saag, M. S., Kitahata, M. M. and Delaney, J. A. C. (2021). Brief Report: Weight Gain Following ART Initiation in ART-Naïve People Living With HIV in the Current Treatment Era. *J Acquir Immune Defic Syndr, 86*(3), 339-343. doi:10.1097/qai.0000000000002556

Rudich, A., Vanounou, S., Riesenberg, K., Porat, M., Tirosh, A., Harman-Boehm, I., Greenberg, A. S., Schlaeffer, F. and Bashan, N. (2001). The HIV protease inhibitor nelfinavir induces insulin resistance and increases basal lipolysis in 3T3-L1 adipocytes. *Diabetes, 50*(6), 1425-1431. doi:10.2337/diabetes.50.6.1425

Sacks, D. B., Arnold, M., Bakris, G. L., Bruns, D. E., Horvath, A. R., Lernmark, Å., Metzger, B. E., Nathan, D. M. and Kirkman, M. S. (2023). Guidelines and Recommendations for Laboratory Analysis in the Diagnosis and Management of Diabetes Mellitus. *Clinical Chemistry, 69*(8), 808-868. doi:10.1093/clinchem/hvad080

Saeedi, P., Petersohn, I., Salpea, P., Malanda, B., Karuranga, S., Unwin, N., Colagiuri, S., Guariguata, L., Motala, A. A., Ogurtsova, K., Shaw, J. E., Bright, D. and Williams, R. (2019). Global and regional diabetes prevalence estimates for 2019 and projections

for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9(th) edition. *Diabetes Res Clin Pract, 157*, 107843. doi:10.1016/j.diabres.2019.107843

Salehpour, A., Rezaei, M., Khoradmehr, A., Tahamtani, Y. and Tamadon, A. (2021). Which Hyperglycemic Model of Zebrafish (Danio rerio) Suites My Type 2 Diabetes Mellitus Research? A Scoring System for Available Methods. *Frontiers in Cell and Developmental Biology, 9*. doi:10.3389/fcell.2021.652061

Samodien, E., Abrahams, Y., Muller, C., Louw, J. and Chellan, N. (2021). Non-communicable diseases – a catastrophe for South Africa. *South African Journal of Science, 117*(5/6). doi:10.17159/sajs.2021/8638

Santosa, W. B., Staines-Urias, E., Tshivuila-Matala, C. O. O., Norris, S. A. and Hemelaar, J. (2019). Perinatal outcomes associated with maternal HIV and antiretroviral therapy in pregnancies with accurate gestational age in South Africa. *AIDS, 33*(10), 1623-1633. doi:10.1097/qad.0000000000002222

Shao, J., Qiao, L., Janssen, R. C., Pagliassotti, M. and Friedman, J. E. (2005). Chronic hyperglycemia enhances PEPCK gene expression and hepatocellular glucose production via elevated liver activating protein/liver inhibitory protein ratio. *Diabetes, 54*(4), 976-984. doi:10.2337/diabetes.54.4.976

Sharchil, C., Vijay, A., Ramachandran, V., Bhagavatheeswaran, S., Devarajan, R., Koul, B., Yadav, D. and Balakrishnan, A. (2022). Zebrafish: A Model to Study and Understand the Diabetic Nephropathy and Other Microvascular Complications of Type 2 Diabetes Mellitus. *Veterinary Sciences, 9*(7), 312. Retrieved from https://www.mdpi.com/2306-7381/9/7/312

Shetty, S. S. and Kumari, S. (2021). Fatty acids and their role in type-2 diabetes (Review). *Exp Ther Med, 22*(1), 706. doi:10.3892/etm.2021.10138

Shulman, G. I. (2000). Cellular mechanisms of insulin resistance. *J Clin Invest, 106*(2), 171-176. doi:10.1172/jci10583

Sicree, R., Shaw, J., Zimmet, P. and Heart, B. (2010). The global burden. *Diabetes and impaired glucose tolerance Baker IDI Heart and Diabetes Institute*.

Siemieniuk, R. A. C., Lytvyn, L., Mah Ming, J., Mullen, R. M., Anam, F., Otieno, T., Guyatt, G. H., Taylor, G. P., Beltrán-Arroyave, C., Okwen, P. M., Nduati, R., Kinuthia, J., Luma, H. N., Kirpalani, H., Merglen, A., Lesi, O. A., Vandvik, P. O., Agoritsas, T. and Bewley, S. (2017). Antiretroviral therapy in pregnant women living with HIV: a clinical practice guideline. *Bmj, 358*, j3961. doi:10.1136/bmj.j3961

Sifunda, S., Mbewu, A. D., Mabaso, M., Manyaapelo, T., Sewpaul, R., Morgan, J. W., Harriman, N. W., Williams, D. R. and Reddy, S. P. (2023). Prevalence and Psychosocial Correlates of Diabetes Mellitus in South Africa: Results from the South African National Health and Nutrition Examination Survey (SANHANES-1). *Int J Environ Res Public Health, 20*(10). doi:10.3390/ijerph20105798

Surwit, R. S., Kuhn, C. M., Cochrane, C., McCubbin, J. A. and Feinglos, M. N. (1988). Diet-induced type II diabetes in C57BL/6J mice. *Diabetes, 37*(9), 1163-1167. doi:10.2337/diab.37.9.1163

Tate, T., Willig, A. L., Willig, J. H., Raper, J. L., Moneyham, L., Kempf, M. C., Saag, M. S. and Mugavero, M. J. (2012). HIV infection and obesity: where did all the wasting go? *Antivir Ther, 17*(7), 1281-1289. doi:10.3851/imp2348

Teame, T., Zhang, Z., Ran, C., Zhang, H., Yang, Y., Ding, Q., Xie, M., Gao, C., Ye, Y., Duan, M. and Zhou, Z. (2019). The use of zebrafish (Danio rerio) as biomedical models. *Anim Front, 9*(3), 68-77. doi:10.1093/af/vfz020

van de Venter, M., Didloff, J., Reddy, S., Swanepoel, B., Govender, S., Dambuza, N. S., Williams, S., Koekemoer, T. C. and Venables, L. (2020). Wild-Type Zebrafish (Danio rerio) Larvae as a Vertebrate Model for Diabetes and Comorbidities: A Review. *Animals (Basel), 11*(1). doi:10.3390/ani11010054

van Wijk, J. P. and Cabezas, M. C. (2012). Hypertriglyceridemia, Metabolic Syndrome, and Cardiovascular Disease in HIV-Infected Patients: Effects of Antiretroviral Therapy and Adipose Tissue Distribution. *Int J Vasc Med, 2012*, 201027. doi:10.1155/2012/201027

Venter, W. D. F., Moorhouse, M., Sokhela, S., Fairlie, L., Mashabane, N., Masenya, M., Serenata, C., Akpomiemie, G., Qavi, A., Chandiwana, N., Norris, S., Chersich, M., Clayden, P., Abrams, E., Arulappan, N., Vos, A., McCann, K., Simmons, B. and Hill, A. (2019). Dolutegravir plus Two Different Prodrugs of Tenofovir to Treat HIV. *N Engl J Med, 381*(9), 803-815. doi:10.1056/NEJMoa1902824

Vestergaard, H., Bjørbaek, C., Hansen, T., Larsen, F. S., Granner, D. K. and Pedersen, O. (1995). Impaired activity and gene expression of hexokinase II in muscle from non-insulin-dependent diabetes mellitus patients. *J Clin Invest, 96*(6), 2639-2645. doi:10.1172/jci118329

Vigouroux, C., Maachi, M., Nguyên, T. H., Coussieu, C., Gharakhanian, S., Funahashi, T., Matsuzawa, Y., Shimomura, I., Rozenbaum, W., Capeau, J. and Bastard, J. P. (2003). Serum adipocytokines are related to lipodystrophy and metabolic disorders in HIV-infected men under antiretroviral therapy. *AIDS, 17*(10), 1503-1511. doi:10.1097/00002030-200307040-00011

Wang, Y., Zhou, H., Palyha, O. and Mu, J. (2019). Restoration of insulin receptor improves diabetic phenotype in T2DM mice. *JCI Insight, 4*(15). doi:10.1172/jci.insight.124945

Wedi, C. O., Kirtley, S., Hopewell, S., Corrigan, R., Kennedy, S. H. and Hemelaar, J. (2016). Perinatal outcomes associated with maternal HIV infection: a systematic review and meta-analysis. *Lancet HIV, 3*(1), e33-48. doi:10.1016/s2352-3018(15)00207-6

WHO. (2019). Classification of diabetes mellitus. 1-40.

WHO. (2020_a). The top 10 causes of death. Retrieved from https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death

WHO. (2020_b). WHO reveals leading causes of death and disability worldwide: 2000-2019. Retrieved from https://www.who.int/news/item/09-12-2020-who-reveals-leading-causes-of-death-and-disability-worldwide-2000-2019

Yu, S., Meng, S., Xiang, M. and Ma, H. (2021). Phosphoenolpyruvate carboxykinase in cell metabolism: Roles and mechanisms beyond gluconeogenesis. *Molecular Metabolism, 53*, 101257. doi:https://doi.org/10.1016/j.molmet.2021.101257

Yuh, B., Tate, J., Butt, A. A., Crothers, K., Freiberg, M., Leaf, D., Logeais, M., Rimland, D., Rodriguez-Barradas, M. C., Ruser, C. and Justice, A. C. (2015). Weight change after antiretroviral therapy and mortality. *Clin Infect Dis, 60*(12), 1852-1859. doi:10.1093/cid/civ192

Zang, L., Maddison, L. A. and Chen, W. (2018). Zebrafish as a Model for Obesity and Diabetes. *Front Cell Dev Biol, 6*, 91. doi:10.3389/fcell.2018.00091

Zang, L., Shimada, Y. and Nishimura, N. (2017). Development of a novel zebrafish model for type 2 diabetes mellitus. *Scientific reports, 7*(1), 1-11.

Zang, L., Shimada, Y., Nishimura, Y., Tanaka, T. and Nishimura, N. (2015). Repeated Blood Collection for Blood Tests in Adult Zebrafish. *J Vis Exp*(102), e53272. doi:10.3791/53272

Zash, R., Holmes, L., Diseko, M., Jacobson, D. L., Brummel, S., Mayondi, G., Isaacson, A., Davey, S., Mabuta, J., Mmalane, M., Gaolathe, T., Essex, M., Lockman, S., Makhema, J. and Shapiro, R. L. (2019). Neural-Tube Defects and Antiretroviral Treatment Regimens in Botswana. *N Engl J Med, 381*(9), 827-840. doi:10.1056/NEJMoa1905230

Zhang, Y., Huang, H., Zhao, G., Yokoyama, T., Vega, H., Huang, Y., Sood, R., Bishop, K., Maduro, V., Accardi, J., Toro, C., Boerkoel, C. F., Lyons, K., Gahl, W. A., Duan, X., Malicdan, M. C. and Lin, S. (2017). ATP6V1H Deficiency Impairs Bone Development through Activation of MMP9 and MMP13. *PLoS Genet, 13*(2), e1006481. doi:10.1371/journal.pgen.1006481

Zhao, F., Jiang, G., Wei, P., Wang, H. and Ru, S. (2018). Bisphenol S exposure impairs glucose homeostasis in male zebrafish (Danio rerio). *Ecotoxicol Environ Saf, 147*, 794-802. doi:10.1016/j.ecoenv.2017.09.048

Zhao, F., Wang, H., Wei, P., Jiang, G., Wang, W., Zhang, X. and Ru, S. (2018). Impairment of bisphenol F on the glucose metabolism of zebrafish larvae. *Ecotoxicol Environ Saf, 165*, 386-392. doi:10.1016/j.ecoenv.2018.09.017

Zipursky, J. and Loutfy, M. (2020). Dolutegravir for pregnant women living with HIV. *Cmaj, 192*(9), E217-e218. doi:10.1503/cmaj.191227

Zizioli, D., Quiros-Roldan, E., Ferretti, S., Mignani, L., Tiecco, G., Monti, E., Castelli, F. and Zanella, I. (2024). Dolutegravir and Folic Acid Interaction during Neural System Development in Zebrafish Embryos. *Int J Mol Sci, 25*(9). doi:10.3390/ijms25094640

Zizioli, D., Zanella, I., Mignani, L., Degli Antoni, M., Castelli, F. and Quiros-Roldan, E. (2023). Cabotegravir Exposure of Zebrafish (Danio rerio) Embryos Impacts on Neurodevelopment and Behavior. *Int J Mol Sci, 24*(3). doi:10.3390/ijms24031994

Zuma, K., Simbayi, L., Zungu, N., Moyo, S., Marinda, E., Jooste, S., North, A., Nadol, P., Aynalem, G., Igumbor, E., Dietrich, C., Sigida, S., Chibi, B., Makola, L., Kondlo, L., Porter, S., Ramlagan, S. and On Behalf Of The Sabssm, V. S. G. C. (2022). The HIV Epidemic in South Africa: Key Findings from 2017 National Population-Based Survey. *Int J Environ Res Public Health, 19*(13). doi:10.3390/ijerph19138125