

## A Zebrafish model system for drug screening in diabetes

Master Degree Project in Systems Biology  
Two years Level, 45 ECTS  
Autumn term 2019  
Version 2

Bobby Madhukumar Mathews  
a16bobma@student.his.se

Supervisor:  
Natalie van Zuydam  
[natalie.vanzuydam@igp.uu.se](mailto:natalie.vanzuydam@igp.uu.se),  
Uppsala University

Examiner:  
Erik Gustafsson  
[erik.gustafsson@his.se](mailto:erik.gustafsson@his.se),  
School of Biosciences,  
University of Skövde,  
BOX 407  
541 28 Skövde  
Sweden

## Abstract

GWAS (Genome wide association studies) have aided in the discovery of various novel variants associated with diabetes. However, a detailed study is required to uncover the role of these genes and to determine how their dysfunction affects pathophysiology. Previous work in the lab has been successful in establishing zebrafish as an efficient model to characterise the effects of these candidate genes. Consequently, efforts have been also made to establish zebrafish as an efficient model system for drug screening as well. The current POP (Proof of principle) study aims to find whether treatment with tolbutamide drug in zebrafish carrying MODY (Maturity onset diabetes of the young) mutations has the similar effects in humans. The study employed zebrafish carrying five (*gck, hnf1a, hnf1ba, hnf1bb, pdx1*) CRISPR induced MODY orthologues. The zebrafish larvae were supplemented with tolbutamide drug from 5dpf till 10dpf (day post fertilisation). At 10dpf, larvae were screened for various glycaemic traits, whole body glucose and lipids as well body size. CRISPR-CAS9- induced mutations were quantified using paired end sequencing. The results showed that treatment with tolbutamide had a significant effect on the hyperglycaemic outcome induced by *hnf1bb, hnf1a*, and *pdx1* mutations which was in line with the known effects of the drug in humans. In conclusion, the POP study proved to be successful in leveraging zebrafish as an efficient model system for, *in vivo* characterisation of drugs and can likely help to identify novel targets for therapeutic interventions.

## Popular scientific abstract

The Second half of the 20th century saw diabetes emerging as a major public health threat to mostly developed countries, but recent studies have shown that its prevalence has been rising more rapidly in developing countries. According to the world health organisation the number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014, and is estimated to be the cause of 1.6 million deaths. Despite being the deadliest epidemics of all time, very little is known about the treatment causing mechanism and treatment aspects of diabetes. Change in lifestyle and hereditary factors or both are found to be the possible factors for the disease. Numerous efforts have been made to cure the disease for a long time, but the complex nature of diabetes development has always confounded researchers around the globe. Most of the prevailing treatments only help the patients to manage the symptoms to a certain extent, but a complete cure is still uncertain. However, recent advancements in genetic studies conducted on large population of patients and healthy individuals have helped researchers to elucidate the genetic factors involved in diabetes. Consequently, to validate these findings and to develop potential diagnostic tests and treatments, model organisms are an inevitable part of diabetic research. Until recent times mouse/mice models have been extensively used to model human diseases and are still a seemingly popular model organism to study diabetes. Besides murine models, zebrafish models have also gained popularity among researchers worldwide, owing to its cost effectiveness and high throughput. This project aimed to supplement the suitability of zebrafish as a pre-clinical model to study diabetes and to test the efficacy of an existing diabetic drug. The current study tried to induce diabetes in zebrafish through genetic modification and overfeeding with high cholesterol diet. The study supposed that the genetic modification and over feeding can induce metabolic imbalance in zebrafish larvae similar to what we see in diabetic patients. As expected, zebrafish showed anomalies in various organ functioning comparable to that of a diabetic patient. Additionally, the diabetic zebrafish provided an opportunity to compare the effect of an established drug (Tolbutamide) on diabetic zebrafish in comparison to its effect on diabetic patients. The study proved that the existing drugs for diabetes have the similar effect in zebrafish as we see in humans, thus proving zebrafish as an excellent pre-clinical model for future drug trials.

Diabetes is a complex disease, detangling all the genetic factors contributing to diabetes is a strenuous process and to completely rely on murine models seems to be impractical. However, in accordance with my research, zebrafish have proved to be a reliable model to study the genetic and environmental aspects associated with diabetes. Although it may not be an appropriate model to study the long-term effect of diabetes, but its cost effectiveness and high throughput could bring a huge impact in understanding the root causes leading to diabetes. Even though a complete cure from diabetes is skeptical in the near future, the current study encompassed a considerable effort to understand the aetiology of diabetes which can undoubtedly make a huge difference in the lives of millions of people worldwide.

## List of abbreviations

|                                |   |
|--------------------------------|---|
| <b>CIGAR</b>                   | Concise idiosyncratic gapped alignment report             |
| <b>CRISPR</b>                  | Clustered regularly interspaced short palindromic repeats |
| <b>GFP</b>                     | Green fluorescent protein                                 |
| <b>gRNA</b>                    | Guide- ribonucleic acid                                   |
| <b>GWAS</b>                    | Genome wide association study                             |
| <b>HDLc</b>                    | High density lipo-protein                                 |
| <b>LDLc</b>                    | Low density lipo-protein                                  |
| <b>MDH</b>                     | Monodansylpentane   |
| <b>MODY</b>                    | Maturity onset diabetes of the young                      |
| <b>NPY</b>                     | Nucleopeptide Y   |
| <b>PEAR</b>                    | Paired-end read merger                                    |
| <b>PPAR<math>\gamma</math></b> | Peroxisome proliferator- activated receptor gamma         |
| <b>SNV</b>                     | Single nucleotide variation                               |
| <b>STAR</b>                    | Spliced transcripts alignment to a reference              |
| <b>T2DM</b>                    | Type- 2 – diabetes mellitus                               |
| <b>VEP</b>                     | Variant effect predictor                                  |

## Table of contents

|  |    |
|--|----|
| INTRODUCTION .....   | 7  |
| AIM .....  | 11 |
| MATERIALS AND METHODS.....                                       | 12 |
| RESULTS .....  | 17 |
| DISCUSSION .....   | 21 |
| ETHICAL ASPECTS AND IMPACTS OF THE RESEARCH ON THE SOCIETY ..... | 24 |
| FUTURE PERSPECTIVES.....   | 25 |
| ACKNOWLEDGMENT .....   | 25 |
| REFERENCES.....  | 26 |
| APPENDIX .....   | 34 |

## INTRODUCTION

Diabetes mellitus (DM) is a complex metabolic disorder characterised by hyperglycaemia, and disturbances in carbohydrate, protein and lipid metabolism (Harris *et al.*, 1994). The global prevalence of people effected with DM is estimated to be 215 million and its incidence is expected to increase to 642 million by 2040 (IDF ATLAS, 2015). American Diabetes Association classifies diabetes mellitus into: type 1; type 2; other types; and gestational diabetes. Type 2 diabetes (T2D) accounts for 90% of all the diabetic cases (Wild *et al.*, 2004). T2D is a complex disease caused due to the interaction between various lifestyle and genetic factors (Cynthia *et al.*, 2009). Pathogenesis of T2DM is characterised by insulin resistance, reduction in insulin production and beta-cell dysfunction which often leads to complications such as diabetic neuropathy, retinopathy, and nephropathy (Li *et al.*, 2016). T2D is a highly heritable disease and it is estimated that there is a 40% lifetime risk to inherit the disease if one of the parents have diabetes and 70%, if both parents have diabetes (Willemsen *et al.*, 2017). Since, T2D is polygenic, the disease-causing pathways are not completely understood, it is often categorised as a heterogenous concoction of various pathogenic mechanisms that does not represent a single disease process (Udler *et al.*, 2018).

On the other hand, maturity onset diabetes of the young (MODY) is an autosomal dominant form of monogenic diabetic disorder that causes nonketotic hyperglycaemia in young adults. MODY is often characterised by altered pancreatic beta cell function, through reduced glucose stimulated insulin secretion (Tattersall and Fajans, 1975). Even though MODY comprises only 1-2% of all the diabetes cases, its global prevalence is hugely underestimated and is often misdiagnosed or misclassified as T2D due to its similar presentation of syndromes. Genetic variants in 13 known genes are found to cause MODY, through pancreatic beta-cell dysfunction or liver dysfunction which subsequently leads to high blood glucose levels. The three most common forms of MODY are caused by mutations in *HNF4A* (MODY 1), *GCK* (MODY 2), and *HNF1A* (MODY 3), which makes up majority of all MODY cases (Yamagata *et al.*, 1997). *HNF4A* and *HNF1A* encode transcription factors promoting the transcription of genes involved in pancreatic cell development and insulin production while *GCK* encode glucokinase, is the enzyme that catalyses the phosphorylation of glucose. Other MODY genes include *PDX1* (MODY 4), *HNF1B* (MODY 5), *NEUROD1* (MODY 6), *KLF11*, *CEL*, *PAX4*, *INS* (MODY 10), *BLK*, *ABCC8*, and *KCNJ11* (Stoffers *et al.*, 1997; Horikawa *et al.*, 1997; Malecki *et al.*, 1999; Neve *et al.*, 2005; Raeder *et al.*, 2006; Plengvidhya *et al.*, 2007).

Until 2007, methods used to study the genetic aspects of diabetes were confined to linkage analysis and candidate gene approaches. These studies were useful to study large familial genetic variants with large effects, such as in maturity onset diabetes of the young (MODY), but it was proved to be unsuccessful in understanding polygenic traits such as type 2 diabetes where the effects of individual genetic variants are modest. However, with the

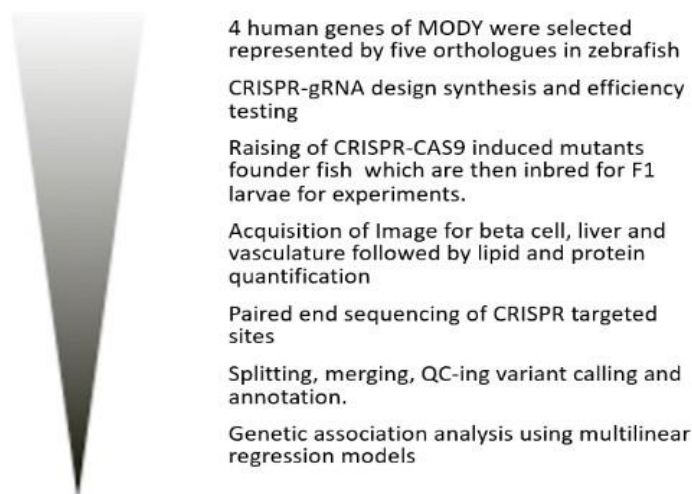
onset of the Human Genome and International HapMap Project to map common genetic variation, GWAS studies have emerged as an effective method to detect association of multiple genetic variants with small effects contributing to complex traits. The latest GWAS study conducted on 1 million subjects have identified close to 403 variants that are robustly associated with type 2 diabetes (Mahajan *et al.*, 2018). While causal variants at T2D loci have largely been identified for each locus, the genes represented by these variant associations remain unknown. Even though, the phenotypic outcomes of identified variants can be predicted through bio-informatic approaches, such as tissue-enrichment analysis and automated pathways, a detailed study is needed to uncover the role of these genes and determine how their dysfunction affects pathophysiology. Consequently, to validate the findings, these candidate genes need to be probed in a suitable model organism.

Zebrafish (*Danio rerio*) have been used to model glycaemic traits i.e. glucose and insulin production that are comparable to humans. These are likely to be a good model due to high similarities in organ physiology and metabolism between zebrafish and humans (Zang *et al.*, 2017). Studies have recently shown that approximately 70% of human genes have functional orthologues in zebrafish (Howe *et al.*, 2013), suggesting zebrafish as a suitable model system to study human pathogenesis (Howe *et al.*, 2013). Zebrafish reach sexual maturity by three months of age; thus, germline mutations can be easily and quickly transferred to the next generation from the founder lines. The larval zebrafish are small and transparent, which allows optical access to perform deep *in vivo* imaging (Prevedel *et al.*, 2014). Zebrafish strains with fluorescent protein expression have been widely used to study pancreatic development and glucose homeostasis (Kinkel and Prince, 2009), such as zebrafish line carrying fluorescently marked insulin have been used to measure beta cell mass and number (Li *et al.*, 2015). Similarly, live imaging in zebrafish has also emerged as a unique technique to investigate protein remodelling on lipid droplets using fluorescently labelled dye that can be easily combined with other fluorescent markers (Yang *et al.*, 2012). Since much of the biology in glucose homeostasis, from genes to organs are conserved from zebra fish to humans, live cell imaging in zebra fish coupled with genetic screens are likely to solve many outstanding questions in diabetes (Madison *et al.*, 2015).

The CRISPR/CRISPR-associated protein 9 (Cas9) system is the most rapidly developing genome editing tool. In theory, it can be used to easily target any genomic location of interest using a customizable short RNA guide (Mali *et al.*, 2013). Numerous studies have exploited the CRISPR/CAS9 to mutate genes in zebrafish to test the role of specific gene perturbations on zebrafish physiology (Hwang *et al.*, 2013; Jao *et al.*, 2013; Shah *et al.*, 2015; Varshney *et al.* 2015). Multiplexed bi-allelic genome editing was achieved simultaneously in CRISPR/CAS9 edited zebrafish models (Jao *et al.*, 2013) which enables to co-inject up to ten targets with Cas9 using a cloning free approach (Varshney *et al.*, 2018). Thus, zebrafish can successfully be used for mutant screening *in vivo* at high throughput and can very well be used to follow up the large number of candidate genes identified by GWAS studies.

Previous works in the lab has been successful in leveraging the potential of zebrafish in modelling T2D. Proof of principle (POP) studies done on maturity onset diabetes of the young (MODY) genes in zebrafish have established zebrafish as an effective model system for characterising causal genes associated with hyperglycaemic outcomes. POP studies on MODY genes was an ideal choice because each sub-type of MODY have a clear genetic basis and a reasonably distinct phenotypic presentation (Johansson *et al.*, 2012). Furthermore, studies have also shown strong evidences of several loci involved in the development of MODY associated with polygenic risk in T2D (Rees *et al.*, 2011). The POP study included five zebrafish orthologues (*gck*, *hnf1a*, *hnf1ba*, *hnf1bb*, *pdx1*) of human genes that were known to be causal for the major subtypes of MODY. The CRISPR/CAS9 induced mutation in the orthologues of already known MODY genes triggered diabetes related phenotypes, through protein dysfunction, that was in line with the known role of genes. i.e. effect on beta cell mass, liver fat, lipids, and circulating glucose. However, the CRISPR induced mutagenesis proved to be lethal for homozygous *Hnf1a*, *hnf1b*, *gck* larvae, as none of them very genetically viable.

Proof of principle study in MODY genes (Figure 1) confirmed that we can detect effects of CRISPR induced mutations in MODY genes on glycaemic traits in zebra fish. Hence, the POP study showed that if the casual transcript is known, the corresponding effect can be detected. Therefore, in a case where the causal gene is unknown and if it shows associations with glycaemic traits then that unknown gene can be characterised as likely to be causal.



**Figure 1.** Experimental pipeline describing the workflow from gene selection to trait association for the proof of principle experiment on MODY genes.

Progress in the development of anti-diabetic therapeutics is improving the prognosis of all types of diabetes but these only treat the symptoms and do not prevent diabetes. Tolbutamide are amongst the most common oral therapy drugs prescribed to stimulate insulin secretion in beta-cells (Poy *et al.*, 1982). They are also reported to improve diabetic control by decreasing the insulin resistance in type 2 diabetes mellitus (Poy *et al.*, 1982). Tolbutamide is also found to be effective in patients with MODY, by acting on adenosine triphosphate (ATP) sensitive potassium channels (Ahmet *et al.*, 2015). Lower doses of sulfonylureas (20-50 mg gliclazide daily) have become the preferred medication for the vast majority of *HNF1A* MODY patients (Raile *et al.*, 2015). Similar sulfonylureas efficiency has been described in *HNF4A* patients (Pearson *et al.*, 2005).

Metformin and Rosiglitazone are two among the most common drugs for the treatment of T2D. Even though metformin exerts its benefits by altering glucose metabolism and diabetic related complication, the mechanism underlying the mode of action is still unknown (Rena *et al.*, 2017). Besides treating diabetes, metformin has been found to be beneficial in treating diseases like ovary diseases fatty liver diseases and also for cardiovascular complications. Metformin is often administered in combination with sulfonylureas and are found to have beneficial effects on glycaemic control (Kasper *et al.*, 2019). Metformin is often administered to obese patients because of its effects on improving dyslipidaemia and can also act as a weight reducing agent in obese patients (Lin *et al.*, 2018). However, metformin is also associated with few side effects including its drastic weight loss in patients with diabetes and also in exerting anorectic effect on food intake mechanism (Paulisso *et al.*, 2001). Rosiglitazone is found to exert its effect through increasing insulin sensitivity, thereby reducing insulin resistance (Imura, 2007). Experiments on mouse models have proved the efficacy of rosiglitazone in preventing hyperglycaemia and in decreasing insulin secretion. (Bergeron *et al.*, 2006). Rosiglitazone was restricted from clinical use due to its potential risk to cause cardiovascular complications. Beside these side effects, numerous studies have also documented the appetite inducing effects of rosiglitazone in patients with diabetes (Justin *et al.*, 2017). However, rosiglitazone is now often considered as part of dual therapy and are usually administered in combination with either metformin or sulfonylureas.

Previous experiments in the lab have demonstrated the anti-hyperglycaemic effect of metformin and rosiglitazone treatment on zebrafish larvae. Administration of metformin and rosiglitazone showed significant effects on the anthropometric and glycaemic traits between the treated and the control group. The observed effects were in line with the known role of these drugs in diabetic patients. However, the anti-hyperglycaemic effect of the drugs still remains obscure, since the experiment results could not explain whether the anti-hyperglycaemic effects was the result of the drugs or if it's the influence of drug on feeding behaviour in larvae, which might had an indirect effect on the glycaemic outcome.

## **AIM**

The main objective of the thesis is to establish zebrafish larvae as a time and cost-effective model system for large scale drug screening. The current study aims to characterise the mechanism of action of tolbutamide drug on zebrafish carrying CRISPR induced MODY mutations. Furthermore, the current study also aims to characterise the regulatory effects of metformin and rosiglitazone on food intake in zebrafish larvae. If the study shows that treatment with these established drugs can exerts its anti-hyperglycaemic effects in zebrafish tissues and metabolites that are consistent with those observed in humans, then the experimental pipeline can be used as a pre-clinical screening step for future diabetic drugs and may prove useful for target specific small molecule screen.

## MATERIALS AND METHODS

### Gene selection and Mutagenesis

In order to validate zebrafish as an efficient model system, five MODY genes were targeted using a multiplexed CRISPR-Cas9 approach using the protocol described in (Varshney *et al.*,2016). Zebrafish orthologs to target human genes were identified using ensembl genome browser. Among the five genes selected - *pdx1*, *gck* and *hnf1a* have one ortholog each while *hnf1b* had two orthologs. Guide-RNAs (gRNAs) were designed using CHOPCHOP (Labun *et al.*,2016) and CRISPRScan (Moreno-Mateos *et al.*,2015) (Supplementary table: 13) based on high GC content (> 50%), location on early exon, targets not overlapping on zebrafish polymorphic sites and devoid of predicted off-targets. Guide RNAs were synthesised as described in protocol (Varshney *et al.*,2016) and the mutagenic efficiency of designed guide-RNAs was tested by micro-injecting each gRNAs along with Cas9 mRNA (Jao *et al.*, 2013) into separate embryos at single-cell stage.

### Zebrafish husbandry and microinjections

Zebrafish line (insulin+;lfabp+ixF3 ) with *GFP* labelled hepatocytes and *mCherry* labelled insulin were used to visualise the liver and beta cells respectively. The five gRNAs, designed for each ortholog, were co-injected with cas9 (final concentration 150ng/ml) into each fertilised embryo (total volume of 2nl each). The founder (F<sub>0</sub>) fish were in-crossed once they reach reproductive age (app. 3 months post fertilization), and the F<sub>1</sub> offspring were collected for experiments. Collected larvae were overfed twice a day with high cholesterol diet (4%) from 5 to 9 dpf and were grown in 1L tanks filled with 300 ml of 3% glucose water until day 10 dpf. Surviving larvae were collected on 10 dpf for optical screening and for other trait quantifications. Experiments were repeated in several batches to reach an optimum number of sample size to be screened approximately 384 larvae; which corresponds to the total number of wells in the sequencing plate.

### Tolbutamide drug intervention

To identify the effects of tolbutamide drug (MedChemExpress™) on zebrafish carrying CRISPR induced MODY genes in, F<sub>1</sub> offspring of the transgenic zebrafish (insulin+;lfabp+ixF3 ) line were employed. The larvae were divided per condition (treated vs untreated) and were overfed in glucose medium as described earlier. Tolbutamide drug was dissolved in DMSO to a concentration of 25 μM and was administered to larvae before the afternoon feed from 5 to 9 dpf. The larvae were sacrificed and optically screened at day 10 for diabetes-based traits (see imaging) followed by lipid and protein profiling.

### Food Intake

Wild type (AB strain) larvae were metabolically challenged by overfeeding them on high cholesterol diet (4% cholesterol) and growing them in high glucose (30%) medium from 5 to 7 dpf. The experiments were performed separately for each drug, where they were divided per condition (Treated vs Control). Drug was administered with larval feed dissolved in

diethyl ether to a concentration of 25  $\mu$ M. In order to visualise and quantify the food intake, the final feed on 7dpf was prepared by mixing a fluorescent tracer (FluroSpheres carboxylate 2.0 $\mu$ m, yellow-green (505/515) life technologies). The fluorescent labelled diet was prepared by mixing 75  $\mu$ l of fluorescent tracer mixed with 50 mg of food and 25 $\mu$ l of de-ionised water. The feed mixture was prepared the day before and was kept in darkness at room temperature to dry. The feed was crushed into fine powder the next day and were fed to the larvae in light for 60 minutes before imaging (Figure 3). The experiment was divided into batches on every day of imaging with approximately 1 hour time interval between consecutive batches. Multiple rounds of imaging were performed to reach the final sample size. To avoid bias, the experiment was single blinded as well as the time of imaging and conditions were alternated during imaging.

## Imaging

### Tolbutamide experiment (Drug Intervention)

Prior to imaging, 5 to 10 larvae were collected and were allowed to swim in 25 $\mu$ M MDH in PBS for 30 minutes, to enable visualising liver and vascular lipid deposition. After soaking, the larvae were anesthetized with tricaine (0.04 mg/ml) in a petri dish. Subsequently, larvae were aspirated one-by-one using a vertebrate Automated Screening Technology (VAST) BioImager (Union Biometrica Inc, Geel, Belgium), which was mounted on the stage of a Leica DM6000b Led automated upright fluorescence microscope (Micro Medic AB, Stockholm, Sweden). The VAST BioImager was programmed to position the larvae inside a borosilicate capillary, where they were detected by the systems camera. Twelve whole body images were captured, one image for every 30 degrees of rotation to quantify body length, dorsal, and lateral area and volume. Subsequently, VAST BioImager positioned the larvae to visualise the caudal vein and dorsal aorta within the field of view of microscope and triggered to start imaging. The researcher then manually focused the centre of vasculature through the MDH channel, followed by the acquisition of the MDH stained lipids in vasculature using the Leica HCX APO L 10X/0.20 W objective. Once the vasculature images were captured, researcher positioned the larvae to visualise the *GFP* labelled liver as well as the MDH stained lipid deposition on liver through L5 and Leica 405 filters respectively using HCX APO L 20X/0.50 W objective. Two images of larvae showing the MDH stained lipids in different orientation (180 degrees apart) were captured. Keeping the larva position unchanged, fluorescently labelled beta-cells were visualised through TXR filter using HCX APO 440 L 40X/0.80 W objectives.

### Metformin and Rosiglitazone (Food in-take)

Images of the food intake experiment were acquired through *GFP* channel using APO 5X/0.10 W objective which helped to visualise the fluorescently labelled food. For each channel, the fluorescence signal was recorded using a Leica DFC365 FX CCD camera. To avoid bias in the intensity of the images acquired, exposure levels were kept constant for all images. As soon as the optical screening was done, the larvae were collected in 96 well plate

for further processing, and the next larva was loaded for imaging. The whole imaging procedure takes 2 minutes per larva.

## **Quantification of morphological features in zebrafish larvae.**

### **Tolbutamide experiment (Drug Intervention)**

Bright-field images of the larvae by the VAST BioImager was used to quantify the body length, dorsal, lateral area and body volume. Images were pre-processed in ImageJ and were automatically segmented and quantified using a custom-made script in CellProfiler (Lamprecht *et al.*, 2007). The segmented images were manually annotated for the segmentation quality and larvae with suboptimal segmentation quality were excluded from further analysis. The larva was excluded if more than four images out of 12 images taken were annotated with poor quality. The script computes the dorsal and lateral area of the larva as number of pixels in the pre-processed image and the body length was estimated as the largest distance between two points on the larva outline touching a bounding box in the dorsal orientation.

Fluorescence signals from MDH (lipids), *mcherry* (beta-cells), *GFP* (liver) were quantified using a custom-made script in ImageJ, CellProfiler and ilastik. Prior to image analysis, beta-cell images were deconvoluted to remove any background noise. As an initial step in image analysis, maximal projection of each fluorescent channel was computed across all optical section in z using ImageJ, to yield a single image across multiple focal depths. Custom-made ImageJ macros were developed to quantify beta-cell volume and intensity from each deconvoluted beta-cell image. Custom-made CellProfiler scripts were used to quantify the liver fat deposition from MDH stained liver images and to detect the co-localisation of MDH stained lipid deposits in vasculature.

### **Metformin and Rosiglitazone (Food in-take)**

Food intake was quantified through the acquired images, firstly by converting the images into maximal projection of the acquired z-stacks using ImageJ, to yield a single image from multiple focal depths. An ilastik-based, lenient pixel classifier was used to detect the fluorescently labelled food and to separate from background noise from each converted image.

### **Lipid, glucose and protein extraction**

After imaging was completed, the larvae were euthanized by tricaine (MS-222, Sigma, Sweden). All excess liquid was removed from the well, and two 1.4mm zirconium bead (Diagnostics, NJ, USA) and 88µl of ice-cold PBS was added to each well containing larvae. Subsequently, the larvae were homogenised for 2 mins at 1000rpm (1600 MiniG-Automated homogenizer, Gammadata Instruments, Uppsala, Sweden) and centrifuges at 3500rpm for 5 mins at 4°C. After centrifugation, 12 µl of supernatant was transferred to a new 96-well plate for protein quantification, together with an additional 12.5µl of ice-cold

PBS per well. The remaining supernatant (~70 µl) from each well was transferred to Eppendorf tubes and were stored at -80°C, which are later to be sent for quantifying LDL, HDL, triglyceride, total cholesterol and glucose level. Protein content was quantified using Pierce bicinchonnic acid(BCA) protein assay kit(Thermo Fisher Scientific, Waltham, MA, USA) and a Varioscan LUX Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA). Total cholesterol, triglyceride, HDL, LDL and glucose levels were quantified using a fully automated Mindray Tm BS-380 analyser (Mindray Medical International Shenzhen, China) using direct LDLc (1E31), HDLc(3K33), triglyceride(7D74), cholesterol (7D62), and glucose (3L82) reagents from Abbott Laboratories( Abbott Park IL, USA).

### **DNA extraction, library preparation and paired-end sequencing**

DNA was extracted from the larvae that were part of the genetic intervention experiments. Extraction of DNA was done from the pellet remained after the lipid and protein profiling. 50 µl of lysis buffer containing proteinase K (dilute 1:100) was added to each well, followed by incubation at 55°C for 2 hours and incubation at 95°C for 10 minutes to deactivate proteinase K. The first PCR of the library preparation step was done by denaturation at 98°C for 30s; amplification for 35 cycles at 98°C for 10s, 62°C for 30s and 72°C for 30s; followed by a final extension at 72°C for 2 mins. Amplified PCR products were cleaned using magnetic beads (Mag-Bind PCR Clean-up Kit, Omega Bio-tek Inc. Norcross, GA). The purified products were used as templates for the second PCR, where Illumina Nextera DNA library sequences were incorporated to allow multiplexed sequencing of all CRISPR-targeted sites. The second PCR step was conducted by denaturation at 98°C for 30s, amplification for 25 cycles at 98°C for 10s, 66°C for 30s and 72°C for 30s, followed by a final extension at 72°C for 2 mins, followed by a purification step as mentioned above. Subsequently, all samples were pooled and sequenced in a single lane on a Miseq (300bp paired-end, illumine Incs., San Diego, CA) at the National Genomics Infrastructure, Sweden.

### **Post sequencing analysis**

A custom written bio-informatics pipeline was developed in collaboration with the National Bio-informatics Infrastructure Sweden to process the Miseq generated paired-end. fastq files per larva. First, a perl script was developed to de-multiplex the. fastq files by gene and well. Paired-end read merger (PEAR) (Zhang et al., 2014) was then used to merge paired-end reads, which was followed by the removal of low-quality reads using FastX (Pearson et al., 1997). Mapping of the reads to the wildtype zebrafish genome (Zv11) was done using Spliced Transcripts Alignment to a Reference or STAR (Doblin et al., 2013). SAMtools version 0.1.19 was used to convert files from SAM to BAM format which sorted the index BAM files and generated a summary of the coverage of mapped read on the reference sequence at a single bp resolution. Allele specific indels and SNVs (Single nucleotide variants) were called using a custom-written inhouse variant calling algorithm in R (Danio rerio Identification of Variants by Haplotype-DIVAH), that are reads with a length difference less than 170 bp compared to the reference sequence, and with an alignment

report string (Concise Idiosyncratic Gapped Alignment Report [CIGAR]) shorter than 50 characters. All variants within the 30 bp of the CRISPR targeted sites were functionally annotated using Ensembl's variant effect predictor (VEP). For each larva, a transcript specific dosage scores were calculated by retaining the variant with the highest predicted impact on protein function (no annotation=0; modifier=0.2; low=0.33; moderate=0.66; high=1), Transcript-specific dosage scores were calculated by summing up the allele specific scores for each target site in each larva.

## Statistical analysis

Statistical inference was made to analyse the effect of:

1) Treatment with metformin and rosiglitazone on food intake. To account the effects of metformin and rosiglitazone on food-intake, a multi-linear regression model on inverse normally transformed outcome (Food pellet size) was performed. The model was adjusted for treatment, time of imaging, batch and was included as covariates. The most parsimonious model was achieved through backward selection procedure where the final model only had the covariates which were statistically significant. These statistical analyses were done in R version 3.5.1. A *p* value of less than 0.05 was taken to be statistically significant.

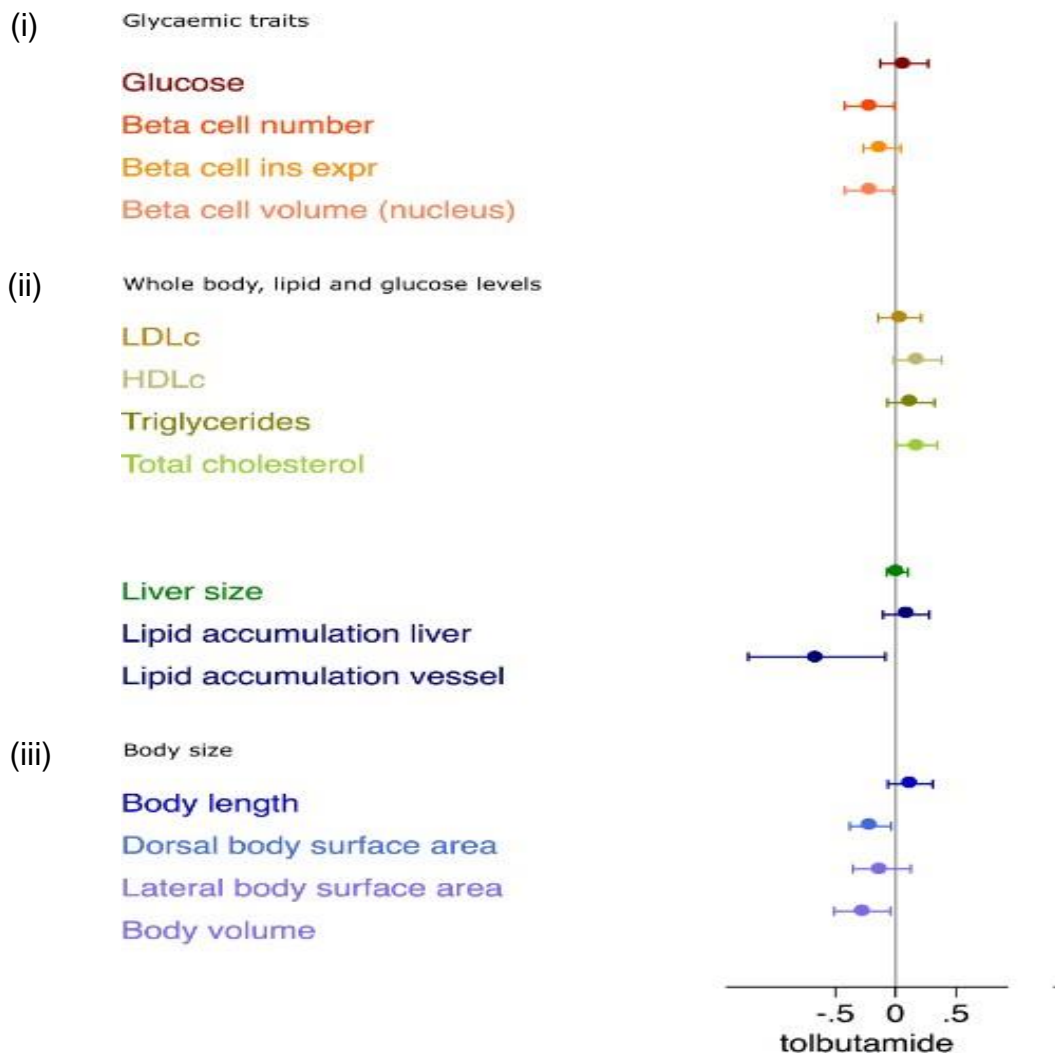
2) Treatment with tolbutamide (in the drug treatment intervention) on body size; early stage diabetic traits; and whole-body LDLc, HDLc, triglyceride, total cholesterol and glucose levels. This was accomplished by hierarchical linear regression models on inverse- normally transformed outcomes which can provide effect sizes and standard errors for the fixed factors, while providing the standard deviation of the outcome across random factors, for which the intercept – i.e. the value of non-expose larvae is allowed to vary. However, most of the image based hyperglycaemic traits showed negative binomial distribution. For such traits, the effects of drugs were examined using negative binomial regression.

All models were adjusted for the time of day the images were captured, batch, tank number and were included as covariables. Image based traits were adjusted for body length and dorsal body surface area and were also added as covariables to the model. For imaged based traits, LDLc, HDLc, triglyceride and glucose levels were added as additional covariables to size adjusted models, to find if they had any effect on drug treatment, and genetic interventions. For all the analysis, effect size, standard errors, significance level (> 0.05) and 95% confidence intervals were reported for the exposed compared with the unexposed group. All data management and statistical analysis were performed using Stata.

## RESULTS

### Treatment with tolbutamide abrogates the hyperglycaemic effects induced by CRISPR mediated MODY mutations in zebrafish larvae.

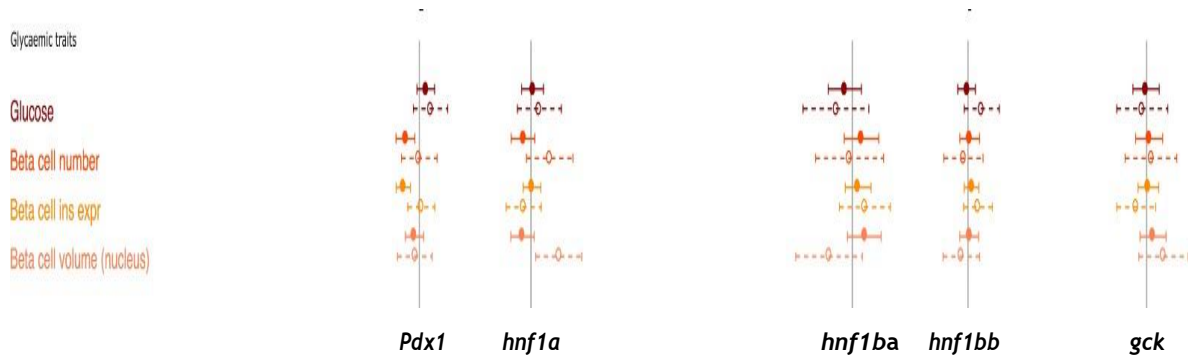
To examine whether the established anti-diabetic drug tolbutamide exert similar effect in zebrafish larvae, > 400 larvae carrying CRISPR induced MODY mutation on zebrafish orthologues of *gck*, *hnf1a*, *hnf1b*, *pdx1* genes were overfed on a cholesterol supplemented diet; with and without tolbutamide treatment; from 5dpf to 10dpf.



**Figure 2.** The effect of treatment with tolbutamide in 10dpf zebrafish on (i) glycaemic traits, (ii) whole body, lipid and glucose analysis, (iii) body size. For normally distributed traits, associations were examined using hierarchical linear models on inverse-normally transformed outcomes. For these traits effect sizes and 95% confidence intervals are expressed in standard deviation (SD) units. Full circles and filled lines represent the main effects of tolbutamide treatment on each of the traits.

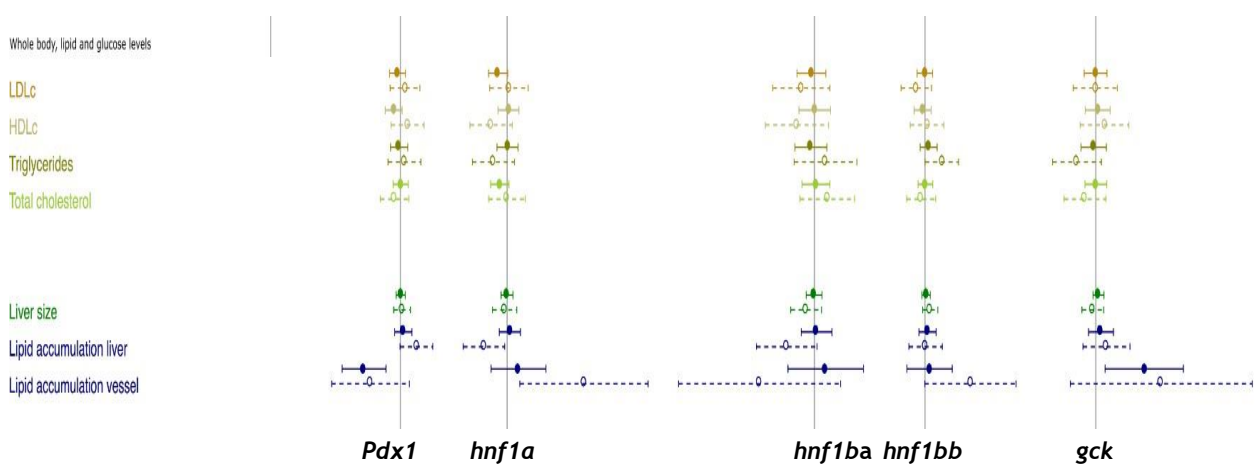
Compared with untreated larvae, five days of tolbutamide treatment resulted in larvae with lower body volume and dorsal surface area. (Figure 2(iii), supplementary table 10). Treatment with tolbutamide also resulted in a trend for higher HDLc, triglycerides, and

total cholesterol (Figure 2 (ii) supplementary table 6 and 8)). Besides that, treatment with tolbutamide resulted in lower vascular deposition of lipids as well (Figure 2 (ii)). However, the most significant effect of treatment with tolbutamide in zebrafish larvae was in lowering beta cell number and beta cell volume (Figure 2 (i)). Even though the beta cell number were reduced, insulin secretion did not show any significant trend (Figure 2 (i)).



**Figure 3:** Shows the effect of mutations in *pdx1*, *hnf1a*, *hnf1ba*, *hnf1bb*, *gck* and treatment with tolbutamide on glycaemic traits in 10dpf zebrafish larvae. For normally distributed traits, associations were examined using hierarchical linear models on inverse-normally transformed outcomes. For these traits effect sizes and 95% confidence intervals are expressed in standard deviation (SD) units. Full circles and filled lines show the main effect of mutations in the 5 genes, and open circles and the dotted lines represent the interaction of drug on each gene.

The current study examined the effect of mutation of each gene on the glycaemic traits and how treatment with tolbutamide exerts its effects on glycaemic outcomes induced by the CRISPR mutations i.e. drug vs gene interaction. Each additional mutated allele in *hnf1a* was associated with a lowering trend in beta cell volume and beta cell number ( $P>0.05$ ) (Figure 3, Supplementary table 3 and 4). However, the administration of tolbutamide was found to increase the beta cell number and volume in *hnf1a* mutants (Figure 3). Larvae with mutated *pdx1* alleles were characterised with lower beta cell number and insulin secretion ( $P>0.05$ ). However, treatment with tolbutamide resulted in improving insulin secretions and beta cell number in *pdx1* mutants (Figure 3).

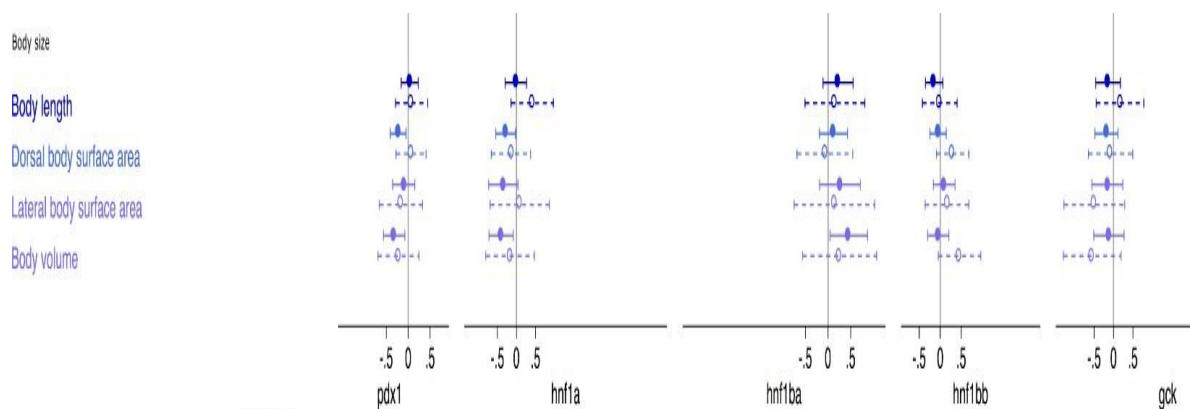


**Figure 4:** Shows the effect of mutations in *pdx1*, *hnf1a*, *hnf1ba*, *hnf1bb*, *gck* and treatment with tolbutamide on whole body lipid levels in 10dpf zebrafish larvae. For normally distributed traits, associations were examined using hierarchical

linear models on inverse-normally transformed outcomes. For these traits effect sizes and 95% confidence intervals are expressed in standard deviation (SD) units. Full circles and filled lines show the main effect of mutations in the 5 genes, and open circles and the dotted lines represent the interaction of drug on each gene.

When examined the influence of mutations in each gene separately, the study observed that larvae carrying *Hnf1a* mutation tend to have lower LDL and total cholesterol levels when compared with larvae free from CRISPR induced mutations in *Hnf1a* (Figure 4). On the other hand, larvae with *pdx1* mutations showed a significant decrease in vascular lipid accumulation.

Treatment with tolbutamide resulted in lowering liver lipid deposition and also in improving the LDL and cholesterol levels in *hnf1a* mutants (Figure 4). On the other hand, treatment with tolbutamide resulted in higher lipid accumulation in liver in *pdx1* mutants. However, treatment with tolbutamide on *hnf1bb* mutants showed a significant increase in vasculature lipid deposition as well as a trend in increasing triglyceride levels and body volume (Figure 4, Supplementary table 9 and 12).



**Figure 5:** Shows the effect of mutations in *pdx1*, *hnf1a*, *hnf1ba*, *hnf1bb*, *gck* and treatment with tolbutamide on anthropometric traits. For normally distributed traits, associations were examined using hierarchical linear models on inverse-normally transformed outcomes. For these traits effect sizes and 95% confidence intervals are expressed in standard deviation (SD) units. Full circles and filled lines show the main effect of mutations in the 5 genes, and open circles and the dotted lines represent the interaction of drug on each gene

Mutant larvae with *hnf1a* mutation showed a significant decrease in body volume ( $p > 0.05$ ). They were also characterised with lowering trend in lateral and dorsal surface area (Figure 5). Larvae with *pdx1* mutated alleles also showed a significant decrease in body volume. However, *hnf1ba* mutants showed a significant increase in body volume (Figure 5). Treatment with tolbutamide on *hnf1a* mutants resulted in a trend in increasing body length and also in abrogating the effect of *hnf1a* mutation on body volume, dorsal and lateral surface area (Figure 5). However, treatment with tolbutamide on *hnf1bb* mutants showed an increasing trend in body volume and dorsal surface area when compared with mutant larvae free from tolbutamide treatment (Figure 5).

## Metformin and Rosiglitazone have no effect on food intake.

To examine if supplementation of food with metformin or rosiglitazone affect food intake, the current study examined 192 larvae on a cholesterol supplemented diet with (n=91) and without (n=101) metformin supplementation (25 $\mu$ M), in 3% glucose from 5dpf until 7dpf. On 7dpf, food intake was measured by quantifying the amount of fluorescently labelled food individual zebra fish consumed (see methods). Treatment with metformin (25 $\mu$ M) was found to have no significant effect on food intake between the treated and untreated group ( $\beta=0.153$ , SE=0.11,  $p>0.05$ ) (Table 1).

Similarly, 166 larvae on a cholesterol supplemented diet were examined with (n=80) and without (n=86) rosiglitazone(25 $\mu$ M) supplementation from 5dpf until 7dpf in glucose rich medium. Food intake was measured with the aid of fluorescent labelled pellets (see methods). Treatment with rosiglitazone failed to show any significant effect on food intake in 7dpf zebrafish larvae ( $\beta=-0.08$ , SE=0.148,  $p>0.05$ ) (Table 2). These results indicates that the differences in glycaemic effects between treated and untreated zebrafish are not due to differences in food intake.

Table 1: The effect of rosiglitazone (25 $\mu$ M) on food intake in 7dpf zebrafish larvae \*

|  | EFFECT SIZE | SE      | P-VALUE | LCI       | UCI       |
|--|-------------|---------|---------|-----------|-----------|
| <b>ROSIGLITAZONE</b>                       | -0.0880     | 0.1487  | 0.55446 | -0.381725 | 0.205565  |
| <b>BATCH(date of imaging)</b>              | -0.4093     | 0.10324 | 0.00011 | -0.61319  | -0.205439 |
| <b>TIME OF DAY (In hours since 9.00am)</b> | -0.0006     | 0.00065 | 0.30653 | -0.00196  | 0.0006129 |
| <b>INTERCEPT</b>                           | -4.1230     | 4.7920  | 0.39085 | -13.58605 | 5.340008  |

Table 2: The effect of metformin (25 $\mu$ M) on food intake in 7dpf zebrafish larvae \*

|  | EFFECT SIZE | SE       | P-VALUE  | LCI       | UCI      |
|--|-------------|----------|----------|-----------|----------|
| <b>METFORMIN</b>                           | 0.15330     | 0.11681  | 0.19098  | -0.077122 | 0.383735 |
| <b>BATCH(date of imaging)</b>              | -0.40712    | 0.04744  | 3.47e-15 | -0.50071  | -0.3135  |
| <b>TIME OF DAY (In hours since 9.00am)</b> | 0.001964    | 0.000700 | 0.00557  | 0.00056   | 0.00334  |
| <b>INTERCEPT</b>                           | 1.743215    | 0.21131  | 2.72E-14 | 1.32635   | 2.16007  |

\*All outcomes were inverse-normally transformed before the analysis. Associations were examined using multi linear regression models and were adjusted for batch (Multiple batches of imaging were performed to reach the final sample size) and time of day images (In hours since 9.00am) were captured. Effects shown for rosiglitazone and metformin treatment are compared with unexposed controls. Lci and uci are lower and upper boundaries of the 95% confidence interval.

## DISCUSSION

### Treatment with tolbutamide abrogates the hyperglycaemic effect induced by CRISPR mediated MODY mutations in zebrafish larvae.

The tolbutamide intervention study developed and validated an experimental pipeline in zebrafish that is suitable to systematically characterise the drugs for diabetes and insulin resistance. The current study showed that treatment with tolbutamide can diminish the hyper-glycaemic effects induced by MODY orthologues in zebrafish larvae.

Treating 10dpf larvae with tolbutamide resulted in lower beta cell number and beta cell volume levels; and also resulted in leaner and smaller larvae (Figure 2). Taking together, these observations suggest that continuous exogenous supply of tolbutamide through medium might be toxic for the survival of beta cells. The evidence for tolbutamide in inducing beta cell apoptosis (Efanova *et al.*, 1998) have been demonstrated in previous experiments in mice. *In vitro* studies in human islet studies have also shown that prolonged use of tolbutamide can induce beta cell apoptosis (Maedler *et al.*, 2005). In line with these results from previous studies, the lowered beta cell volume and mass in zebrafish larvae treated with tolbutamide (25µm) confirms the toxic effect induced by tolbutamide drug.

The current study demonstrated the effects of *pdx1* mutants in lowering beta cell number and insulin secretion (Figure 3) which confirms and expands the earlier findings characterised in adult zebrafish *pdx1* mutants (Kimmel *et al.*, 2015) on insulin secretion and beta cell number. These findings stress the significant role of *pdx1* in beta cell development and its major role in insulin gene expression (Gao *et al.*, 2014). Besides the role in beta cell development *pdx1* gene is essential for regulating energy intake and nutrient disposal which can influence body size (Li *et al.*, 2005). The current results also show that the *pdx1* mutants have a significant decrease in body volume and area. (Figure 5). However, treatment with tolbutamide resulted in improving insulin secretion and beta cell number in *pdx1* mutants (Figure 3), thus the current results were in line with the earlier findings (Fujimoto *et al.*, 2009) on the role of tolbutamide in beta cell survival.

The current study reported the main effects of *hnf1a* mutants in lowering beta cell number and volume (Figure 3). These results indicate the fundamental role of *hnf1a* gene in the early development of pancreatic beta cells (Wang *et al.*, 2000). However, treatment with tolbutamide in *hnf1a* mutants led to an increase in beta cell number and volume without any upregulation in insulin secretion which in contrast to the known role of tolbutamide that stimulates insulin secretion without increasing the beta cell number (Jonkers *et al.*, 2001). Thus, in spite of the proven toxic effects of tolbutamide, the increase in beta cell number on *hnf1a* mutants remain plausible, as further downstream studies are needed.

In the current study, treatment with tolbutamide was found to decrease the lipid accumulation in *hnf1a* and *hnf1ba* mutants (Figure 4). These results confirmed the reported effect of tolbutamide in decreasing plasma lipoproteins by reducing hepatic production of

lipoprotein in diabetic subjects (Khovidhunkit *et al.*, 2014). However, in contrast to the expected results, tolbutamide treatment was found to increase lipid accumulation in liver in *pdx1* mutants (Figure 3). Thus, the contrasting outcomes of tolbutamide in zebrafish larvae in lipid levels are sufficiently sensitive to provide new insights into the action of tolbutamide drug and should be followed up in further experiments.

In conclusion, the current study highlighted the suitability of zebrafish larvae as a cost effective and comprehensive model system for large scale drug screening. The current study demonstrated that CRISPR induced MODY mutations can invoke hyperglycaemia in 10dpf zebrafish larvae and how tolbutamide drug can abrogate the hyperglycaemic outcomes induced by MODY mutations. Out of the five MODY orthologues *hnf1a* and *pdx1* zebrafish mutants showed the most promising results. Treatment with tolbutamide on *hnf1a* and *pdx1* mutants was found to abrogate the anti-hyperglycaemic effects in zebrafish tissues and metabolites (Figure 3.4,5) and were in line with the observed effects in humans. Hence, the current approach represents a cost effective, phenotype-based screening, whole animal model system which can be used to test the efficacy, toxicity and pharmacokinetics of various drugs in a cost-effective manner.

However, the study also encompasses few limitations to the experimental pipeline as well as in leveraging zebrafish as a model system. Zebrafish have revealed extreme differences between the phenotypes induced by mutation and knock downs (Rossi *et al.*, 2015). Hence, there is a large chance of genetic compensation effects to hinder the expected phenotypic outcomes. Furthermore, the doses of tolbutamide administered to the larvae also have a significant role in determining the expected glycaemic outcomes (Swada *et al.*, 2008). The current study also assumes that the beta cell apoptosis in treated larvae were induced through high concentration of tolbutamide (Figure 2 (i)). Hence, the tolbutamide concentration can limit the conclusions the study draws from the results. Thus, the current study recommends future downstream experiments using tolbutamide to lower the dose to perform experiments in zebrafish.

For all the analysis significance level ( $> 0.05$ ) and 95% confidence intervals were reported for the exposed compared with the unexposed group. When a critical significance level of 0.05 is used, the study expects a probability of 0.95 of coming to a correct conclusion for a true null hypothesis. If the same study was conducted 20 times, the probability that none will be significant is  $0.95^{20} = 0.36$ . This gives a probability of  $1 - 0.36 = 0.64$  of getting at least one significant result. Hence, it can conclude that even if the current study was repeated 20 times, the probability of getting a significant result among a mass of non-significant result is less than 1 and that too which can only happen by chance alone. Hence the necessity of testing for mass significance did not rise in the current study.

### **Metformin and Rosiglitazone have no effect on food intake**

The study demonstrated that rosiglitazone and metformin failed to induce any of their clinically proven effects on regulating food intake. Thus, in an adequacy powered drug intervention study, the results showed that the anti-hyperglycaemic effects exerted by rosiglitazone and metformin on zebrafish larvae were true to its anti-hyperglycaemic effects and not influenced by amount of food in taken.

Numerous studies have shown the anorectic effects of metformin in humans, rats and birds (Schnolig *et al.*, 2003, Lee *et al.*, 1998, Paolisso *et al.*, 1998), similarly, the orexigenic effects of rosiglitazone was also documented in various model organisms (Ryan *et al.*, 2011). However, the action of drugs on food regulatory mechanism is mediated through various factors. In humans and mouse models, food intake mechanism is mediated by neuropeptide y (NPY) levels through the central nervous system (Duan *et al.*, 2013). There, are numerous factors which control NPY levels, among them is leptin receptor which exerts its anorexigenic effect by suppressing NPY (Arora., 2008). Metformin demonstrates its anorectic effect through the inhibition of NPY by increasing leptin receptor expression (Aubert *et al.*, 2010), while rosiglitazone exerts its orexigenic effects by lowering the leptin receptor gene expression (Kim *et al.*, 2008). However, in teleost fish, studies show that there is no association between leptin levels and food intake. Leptin mRNA expression failed to show any variation between overfed and fasting teleost fish (Huisling *et al.*, 2006). Similar studies in zebrafish also showed that there is no significant change in leptin levels, when overfed (Oka *et al.*, 2010). Thus, in agreement with all these previously reported effects, the current study reports that neither metformin nor rosiglitazone can exert its effects on food intake in zebrafish as opposed to other model systems through leptin gene expression.

Apart from the neurological aspects, numerous clinical studies have hypothesised the drug toxicity induced by metformin and rosiglitazone may alter food intake mechanism in humans (Ellacott *et al.*, 2010, Kumar *et al.*, 2017). However, in the current study it remains plausible whether zebrafish were devoid of the toxic effects induced by these drugs in regulating food intake or if the concentration of drug (25µM) administered was lower than the minimum threshold needed to induce a toxic effect.

The potential role of the exogenous supply of glucose in medium should also be considered as a significant factor which might have hindered the proven effects of metformin and rosiglitazone in regulating food intake. Metformin acts as glucose reducing agent (Rena *et al.*, 2017). The anorectic effect of metformin largely depends upon its action on decreasing glucose levels (We-Shan *et al.*, 2012). Lowering of glucose levels stimulates NPY gene expression which facilitates metformin to exerts its anorectic effect through blocking the NPY expression (Chau-Van *et al.*, 2007). However, in the current study with constant exogenous supply of glucose (see methods) from the medium, zebrafish larvae might have been left unaffected by the glucose lowering effects of metformin and in turn might have not affected the NPY levels nor the food intake mechanism.

On the other hand, rosiglitazone is a PPAR $\gamma$  agonist that lowers blood glucose levels (Zhang *et al.*, 2014), Multiple studies have demonstrated the orexigenic effect of rosiglitazone through the expression of neuropeptide Y (NPY) mediated by lowered blood glucose levels (Matias *et al.*, 2015). Taking together these observations and in agreement to the known role of NPY in response to lowering of glucose, the current study speculates that the exogenous supply of glucose (see methods) was able to counter balance the glucose lowering effect of rosiglitazone, and in turn might have not affected the NPY levels nor the food intake mechanism.

In the current study, the constant supply of glucose through the medium is assumed to have mitigated the glucose lowering effects of metformin and rosiglitazone which might have limited the results in drawing conclusions. Thus, this study advises future research using metformin or rosiglitazone in zebrafish to perform experiments without glucose in medium. Furthermore, more conclusive evidence of the role of drugs in food intake could be obtained by altering the concentration of drugs administered and also through increasing the number of times of drug supplementation.

## **ETHICAL ASPECTS AND IMPACTS OF THE RESEARCH ON THE SOCIETY**

The study was performed as a part of proof of principle study to validate zebrafish as an effective model system to identify putatively causal genes contributing to T2D pathogenesis. All experiments were performed in 10-day old zebra fish larvae. Adult transgenic fish and CRISPR founders were raised and kept solely for breeding purpose. Adult fish were fed twice daily on rotifers and dry food (Sparcos, Olhao, Portugal), and were maintained in circulating and filtered water (Aquaneering Inc., San Diego, CA), in accordance with Swedish guidelines. Fine mesh nets were employed on all water outlets in the fish room to prevent any escape of fish to natural environment. To generate the required offspring, transgenic adult fish were in-crossed, and fertilized eggs were raised in an incubator at 28.5°C until 5 days post-fertilization (dpf). At 3dpf, embryos were optically screened for fluorescence in 96-well plates (EVOS FL Auto, Thermo Fisher Scientific, MA, USA), and embryos carrying the fluorescent transgene(s) were retained and placed back in the incubator. From 5 to 10dpf, zebrafish larvae were kept in 1L tanks filled with 300mL of water at a density of 30 larvae/tank. Larvae were fed twice daily until 9dpf. Waste products and debris were removed from the water by a regular glucose water change, followed by replenishing of the water level to 300ml. All procedures are performed in line with Swedish regulations, and all experiments have been approved by Uppsala Djurförsöksetiska nämnd, Uppsala, Sweden (Permit numbers C142/13 AND C14/16).

Diabetes is a complex disease that involves the interaction of genetics and environmental factors. In fact, the precise mechanism underlying the pathogenesis of diabetes are incompletely understood. However, many questions can be answered by the appropriate use of animal models in understanding the disease and in developing new therapeutic strategies. Zebrafish has been an attractive model to study the complications of diabetes

because of its similarity in lipid metabolism, glucose homeostasis and adipose biology to that of humans. In line with the advantages of zebrafish in diabetes modelling, the current study characterises the capabilities and limitations of using zebrafish in high throughput diabetic drug screening. The results will help in the implementation of zebrafish as a model for pre-clinical trials of new classes of drugs and repurposed drugs in the field of diabetes.

## **FUTURE PERSPECTIVES**

GWAS have identified a plethora of loci with insulin resistance and diabetic traits. Though the number of associated variants is increasing, there is no comparable trend in the functional characterisation of candidate genes. Due to tremendous advancements in imaging methods, variety of transgenic backgrounds, and high throughput, zebrafish can be employed as an effective model system to characterise the genes/variants and ideally the tissues, cell types and pathways through which these variants and genes exert their effect.

Concerning the findings made in this thesis project, the research found interesting associations and trends with anti-diabetic drugs and their clinical outcomes in zebrafish larvae. The study proved that the experimental pipeline can also be used to characterize mechanisms of action for other existing drugs, and may prove useful for target specific small molecule screen.

As for the tolbutamide and rosiglitazone drug, it would be very interesting to characterise the cardiovascular complexities associated with the administration of these drugs in zebrafish. Transcriptomic studies could also be conducted to identify differentially expressed genes involved in LDLc levels and atherosclerosis related traits along with the phenotypic screening of diabetic traits.

Further studies are also necessary to validate zebrafish as a model to study associations with metabolic or genetic interventions to various organ failures in diabetic conditions. Apart from liver and pancreatic beta cells, the organ changes including glomerulosclerosis in diabetic kidney, diabetic retinopathy, macular edema and other microvascular complications also need to be characterised.

Even though there is a lack of model for studying long term effects of diabetes, there have been numerous approaches to study diabetic complications using adult zebrafish. Ability of adult zebrafish induced with hyperglycaemia on impaired wound healing, changes in bone metabolism and the heritability of epigenetic changes has been extensively studied. Overall, the advent of zebrafish models offers particular advantages to model metabolic diseases and will foster knowledge to understand the aetiology of disease and provide new insights for disease treatment.

## **ACKNOWLEDGMENT**

I would like to express my sincere gratitude to my thesis supervisor Dr. Natalie Van Zuydam of department of Immunology, genetics and pathology, Uppsala University. She

was always graceful and patient throughout my thesis work and was never hesitant to answer any of my questions. Thank you for the great supervision and all the new skills I was able to learn from you, and making me a better scientist. Many thanks to Dr. Marcel den Hoed, who accepted me to the lab to whom I am deeply grateful for your belief in me and giving me the chance to do my master thesis.

All fellow IGP fellow lab members and friends at BMC; Tessa, Eugenia, Manoj, Bene, Endrina, Giota- thanks to all of you. Thank you for being always positive, supportive and all the skills you all taught me in the lab.

Last but not the least, to my whole family, for providing me time to time support and encouragement throughout the thesis. This accomplishment would not have been possible without them.

## REFERENCES

- Anik, A., Çatli, G., Abaci, A., & Böber, E. (2015). Maturity-onset diabetes of the young (MODY): An update. *Journal of Pediatric Endocrinology and Metabolism*.  
<https://doi.org/10.1515/jpem-2014-0384>
- Anubhuti, V., & Arora, S. (2008). Leptin and its metabolic interactions - An update. *Diabetes, Obesity and Metabolism*. <https://doi.org/10.1111/j.1463-1326.2008.00852.x>
- Aubert, G., Mansuy, V., Voirol, M. J., Pellerin, L., & Pralong, F. P. (2011). The anorexigenic effects of metformin involve increases in hypothalamic leptin receptor expression. *Metabolism: Clinical and Experimental*. <https://doi.org/10.1016/j.metabol.2010.02.007>
- Bergeron, R., Yao, J., Woods, J. W., Zycband, E. I., Liu, C., Li, Z., ... Doebber, T. W. (2006). Peroxisome proliferator-activated receptor (ppar)- $\alpha$  agonism prevents the onset of type 2 diabetes in Zucker diabetic fatty rats: A comparison with ppar $\gamma$  agonism. *Endocrinology*. <https://doi.org/10.1210/en.2005-1535>
- Bundhun, P. K., Janoo, G., Teeluck, A. R., & Huang, F. (2017). Adverse drug effects observed with vildagliptin versus pioglitazone or rosiglitazone in the treatment of patients with type 2 diabetes mellitus: A systematic review and meta-analysis of randomized controlled trials. *BMC Pharmacology and Toxicology*.  
<https://doi.org/10.1186/s40360-017-0175-0>
- Cermelli, S., Guo, Y., Gross, S. P., & Welte, M. A. (2006). The Lipid-Droplet Proteome Reveals that Droplets Are a Protein-Storage Depot. *Current Biology*.  
<https://doi.org/10.1016/j.cub.2006.07.062>
- Chen, J., Bardes, E. E., Aronow, B. J., & Jegga, A. G. (2009). ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic Acids Research*.  
<https://doi.org/10.1093/nar/gkp427>

- Defronzo, R. A., Barzilai, N., & Simonson, D. C. (1991). Mechanism of metformin action in obese and lean noninsulin-dependent diabetic subjects. *Journal of Clinical Endocrinology and Metabolism*. <https://doi.org/10.1210/jcem-73-6-1294>
- Duan, Y., Zhang, R., Zhang, M., Sun, L. J., Dong, S. Z., Wang, G., ... Zhao, Z. (2013). Metformin inhibits food intake and neuropeptide Y gene expression in the hypothalamus. *Neural Regeneration Research*. <https://doi.org/10.3969/j.issn.1673-5374.2013.25.009>
- Efanova, I. B., Zaitsev, S. V., Zhivotovsky, B., Köhler, M., Efendić, S., Orrenius, S., & Berggren, P. O. (1998). Glucose and tolbutamide induce apoptosis in pancreatic  $\beta$ -cells: A process dependent on intracellular  $\text{Ca}^{2+}$  concentration. *Journal of Biological Chemistry*. <https://doi.org/10.1074/jbc.273.50.33501>
- Ellacott, K. L. J., Morton, G. J., Woods, S. C., Tso, P., & Schwartz, M. W. (2010). Assessment of feeding behavior in laboratory mice. *Cell Metabolism*. <https://doi.org/10.1016/j.cmet.2010.06.001>
- Elo, B., Villano, C. M., Govorko, D., & 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. *Journal of Molecular Endocrinology*. <https://doi.org/10.1677/JME-06-0037>
- Fajans, S. S., Bell, G. I., & Polonsky, K. S. (2002). Molecular Mechanisms and Clinical Pathophysiology of Maturity-Onset Diabetes of the Young. *New England Journal of Medicine*. <https://doi.org/10.1056/nejmra002168>
- Friedman, J. M. (2009). Leptin at 14 y of age: An ongoing story. *American Journal of Clinical Nutrition*. <https://doi.org/10.3945/ajcn.2008.26788B>
- Fujimoto, K., & Polonsky, K. S. (2009). Pdx1 and other factors that regulate pancreatic  $\beta$ -cell survival. *Diabetes, Obesity and Metabolism*. <https://doi.org/10.1111/j.1463-1326.2009.01121.x>
- Gao, T., McKenna, B., Li, C., Reichert, M., Nguyen, J., Singh, T., ... Stanger, B. Z. (2014). Pdx1 maintains  $\beta$  cell identity and function by repressing an  $\alpha$  cell program. *Cell Metabolism*. <https://doi.org/10.1016/j.cmet.2013.12.002>
- Khovidhunkit, W., Kim, M. S., Memon, R. A., Shigenaga, J. K., Moser, A. H., Feingold, K. R., & Grunfeld, C. (2004). Effects of infection and inflammation on lipid and lipoprotein metabolism: Mechanisms and consequences to the host. *Journal of Lipid Research*. <https://doi.org/10.1194/jlr.R300019-JLR200>
- Harris, M. I., Flegal, K. M., Cowie, C. C., Eberhardt, M. S., Goldstein, D. E., Little, R. R., ... Byrd-Holt, D. D. (1998). Prevalence of diabetes, impaired fasting glucose, and impaired glucose tolerance in U.S. adults: The Third National Health and Nutrition Examination Survey, 1988-1994. *Diabetes Care*. <https://doi.org/10.2337/diacare.21.4.518>
- Heine, R. J. (1996). Role of sulfonylureas in non-insulin-dependent diabetes mellitus: Part II - "The cons." *Hormone and Metabolic Research*. <https://doi.org/10.1055/s-2007-979845>

- Herker, E., Harris, C., Hernandez, C., Carpentier, A., Kaehlcke, K., Rosenberg, A. R., ... Ott, M. (2017). a Dynamic Organelle. *Proceedings of the National Academy of Sciences*. <https://doi.org/10.1038/nrm1912>
- Horikawa, Y., Iwasaki, N., Hara, M., Furuta, H., Hinokio, Y., Cockburn, B. N., ... Bell, G. I. (1997). Mutation in hepatocyte nuclear factor-1 $\beta$  gene (TCF2) associated with MODY. *Nature Genetics*. <https://doi.org/10.1038/ng1297-384>
- Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., ... Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*. <https://doi.org/10.1038/nature12111>
- Huising, M. O., Geven, E. J. W., Kruiswijk, C. P., Nabuurs, S. B., Stolte, E. H., Spanings, F. A. T., ... Flik, G. (2006). Increased leptin expression in common carp (*Cyprinus carpio*) after food intake but not after fasting or feeding to satiation. *Endocrinology*. <https://doi.org/10.1210/en.2006-0824>
- Id, S., Count, W., & Count, C. E. R. (2016). *Project Proposal Draft 1*. <https://doi.org/10.1016/j.spinee.2013.06.060>
- Imura, H. (2002). A Novel Antidiabetic Drug, Troglitazone — Reason for Hope and Concern. *New England Journal of Medicine*. <https://doi.org/10.1056/nejm199803263381311>
- Jao, L.-E., Wentz, S. R., & Chen, W. (2013). Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proceedings of the National Academy of Sciences*. <https://doi.org/10.1073/pnas.1308335110>
- Johansson, S., Irgens, H., Chudasama, K. K., Molnes, J., Aerts, J., Roque, F. S., ... Njølstad, P. R. (2012). Exome sequencing and genetic testing for MODY. *PLoS ONE*. <https://doi.org/10.1371/journal.pone.0038050>
- Jonkers, F. C., Guiot, Y., Rahier, J., & Henquin, J. C. (2001). Tolbutamide stimulation of pancreatic  $\beta$ -cells involves both cell recruitment and increase in the individual Ca<sup>2+</sup> response. *British Journal of Pharmacology*. <https://doi.org/10.1038/sj.bjp.0704108>
- K, L., TG, M., JA, G., SB, T., & E, V. (2016). CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. *Nucleic Acids Research*. <https://doi.org/https://doi.org/10.1093/nar/gkw398>
- Kim, H. J., Kim, S. K., Shim, W. S., Lee, J. H., Hur, K. Y., Kang, E. S., ... Cha, B. S. (2008). Rosiglitazone improves insulin sensitivity with increased serum leptin levels in patients with type 2 diabetes mellitus. *Diabetes Research and Clinical Practice*. <https://doi.org/10.1016/j.diabres.2008.02.001>
- Kimmel, R. A., Dobler, S., Schmitner, N., Walsen, T., Freudenblum, J., & Meyer, D. (2015). Diabetic pdx1-mutant zebrafish show conserved responses to nutrient overload and anti-glycemic treatment. *Scientific Reports*. <https://doi.org/10.1038/srep14241>

- Kinkel, M. D., & Prince, V. E. (2009). On the diabetic menu: Zebrafish as a model for pancreas development and function. *BioEssays*. <https://doi.org/10.1002/bies.200800123>
- Lee, A., & Morley, J. E. (1998). Metformin decreases food consumption and induces weight loss in subjects with obesity with type II non-insulin-dependent diabetes. *Obesity Research*. <https://doi.org/10.1002/j.1550-8528.1998.tb00314.x>
- Li, Y., Cao, X., Li, L. X., Brubaker, P. L., Edlund, H., & Drucker, D. J. (2005).  $\beta$ -Cell Pdx1 expression is essential for the glucoregulatory, proliferative, and cytoprotective actions of glucagon-like peptide-1. *Diabetes*. <https://doi.org/10.2337/diabetes.54.2.482>
- Li, L., Jick, S., Breitenstein, S., & Michel, A. (2016). Prevalence of diabetes and diabetic nephropathy in a large U.S. commercially insured pediatric population, 2002-2013. *Diabetes Care*. <https://doi.org/10.2337/dc15-1710>
- Lin, S. H., Cheng, P. C., Tu, S. Te, Hsu, S. R., Cheng, Y. C., & Liu, Y. H. (2018). Effect of metformin monotherapy on serum lipid profile in statin-naïve individuals with newly diagnosed type 2 diabetes mellitus: A cohort study. *PeerJ*. <https://doi.org/10.7717/peerj.4578>
- Lucis, O. J. (1983). The status of metformin in Canada. *Canadian Medical Association Journal*.
- Lv, W. S., Wen, J. P., Li, L., Sun, R. X., Wang, J., Xian, Y. X., ... Gao, Y. Y. (2012). The effect of metformin on food intake and its potential role in hypothalamic regulation in obese diabetic rats. *Brain Research*. <https://doi.org/10.1016/j.brainres.2012.01.028>
- Madsen, K. S., Kähler, P., Kähler, L. K. A., Madsbad, S., Gnesin, F., Metzendorf, M. I., ... Hemmingsen, B. (2019). Metformin and second-or third-generation sulphonylurea combination therapy for adults with type 2 diabetes mellitus. *Cochrane Database of Systematic Reviews*. <https://doi.org/10.1002/14651858.CD012368.pub2>
- Maedler K, Carr RD, Bosco D, Zuellig RA, Berney T, Donath MY. Sulfonylurea induced beta-cell apoptosis in cultured human islets. *J Clin Endocrinol Metab* 2005;**90**:501–06.
- Mahajan, A., Go, M. J., Zhang, W., Below, J. E., Gaulton, K. J., Ferreira, T., ... Morris, A. P. (2014). Genome-wide trans-ancestry meta-analysis provides insight into the genetic architecture of type 2 diabetes susceptibility. *Nature Genetics*. <https://doi.org/10.1038/ng.2897>
- Malecki, M. T., Jhala, U. S., Antonellis, A., Fields, L., Doria, A., Orban, T., ... Krolewski, A. S. (1999). Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus. *Nature Genetics*. <https://doi.org/10.1038/15500>
- Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., ... Church, G. M. (2013). RNA-guided human genome engineering via Cas9. *Science*. <https://doi.org/10.1126/science.1232033>
- Matias, J. A., Gilbert, E. R., Denbow, D. M., & Cline, M. A. (2017). Effects of intracerebroventricular injection of rosiglitazone on appetite-associated parameters in

- chicks. *General and Comparative Endocrinology*.  
<https://doi.org/10.1016/j.ygcen.2015.12.021>
- Mercer, R. E., Chee, M. J. S., & Colmers, W. F. (2011). The role of NPY in hypothalamic mediated food intake. *Frontiers in Neuroendocrinology*.  
<https://doi.org/10.1016/j.yfrne.2011.06.001>
- Moreno-Mateos, M. A., Vejnár, C. E., Beaudoin, J. D., Fernández, J. P., Mis, E. K., Khokha, M. K., & Giraldez, A. J. (2015). CRISPRscan: Designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. *Nature Methods*. <https://doi.org/10.1038/nmeth.3543>
- Naito, Y., Hino, K., Bono, H., & Ui-Tei, K. (2015). CRISPRdirect: Software for designing CRISPR/Cas guide RNA with reduced off-target sites. *Bioinformatics*.  
<https://doi.org/10.1093/bioinformatics/btu743>
- Neve, B., Fernández-Zapico, M. E., Ashkenazi-Katalan, V., Dina, C., Hamid, Y. H., Joly, E., ... Froguel, P. (2005). From The Cover: Role of transcription factor KLF11 and its diabetes-associated gene variants in pancreatic beta cell function. *Proceedings of the National Academy of Sciences*. <https://doi.org/10.1073/pnas.0409177102>
- Nosadini, R., Avogaro, A., Trevisan, R., Valerio, A., Tessari, P., Duner, E., ... De Kreutzenberg, S. (1987). Effect of metformin on insulin-stimulated glucose turnover and insulin binding to receptors in type II diabetes. *Diabetes Care*.  
<https://doi.org/10.2337/diacare.10.1.62>
- Oka, T., Nishimura, Y., Zang, L., Hirano, M., Shimada, Y., Wang, Z., ... Tanaka, T. (2010). Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. *BMC Physiology*. <https://doi.org/10.1186/1472-6793-10-21>
- Paolisso, G., Amato, L., Eccellente, R., Gambardella, A., Tagliamonte, M. R., Varricchio, G., ... D'Onofrio, F. (1998). Effect of metformin on food intake in obese subjects. *European Journal of Clinical Investigation*. <https://doi.org/10.1046/j.1365-2362.1998.00304.x>
- Pearson, E. R., Pruhova, S., Tack, C. J., Johansen, A., Castleden, H. A. J., Lumb, P. J., ... Hattersley, A. T. (2005). Molecular genetics and phenotypic characteristics of MODY caused by hepatocyte nuclear factor 4 $\alpha$  mutations in a large European collection. *Diabetologia*. <https://doi.org/10.1007/s00125-005-1738-y>
- Pers, T. H., Karjalainen, J. M., Chan, Y., Westra, H. J., Wood, A. R., Yang, J., ... Franke, L. (2015). Biological interpretation of genome-wide association studies using predicted gene functions. *Nature Communications*. <https://doi.org/10.1038/ncomms6890>
- Plengvidhya, N., Kooptiwut, S., Songtawee, N., Doi, A., Furuta, H., Nishi, M., ... Banchuin, N. (2007). Brief report: PAX4 mutations in Thais with maturity onset diabetes of the young. *Journal of Clinical Endocrinology and Metabolism*.  
<https://doi.org/10.1210/jc.2006-1927>

- Prevedel, R., Yoon, Y. G., Hoffmann, M., Pak, N., Wetzstein, G., Kato, S., ... Vaziri, A. (2014). Simultaneous whole-animal 3D imaging of neuronal activity using light-field microscopy. *Nature Methods*. <https://doi.org/10.1038/nmeth.2964>
- Prykhozhij, S. V., Rajan, V., Gaston, D., & Berman, J. N. (2015). CRISPR multitargeter: A web tool to find common and unique CRISPR single guide RNA targets in a set of similar sequences. *PLoS ONE*. <https://doi.org/10.1371/journal.pone.0119372>
- Ræder, H., Johansson, S., Holm, P. I., Haldorsen, I. S., Mas, E., Sbarra, V., ... Njølstad, P. R. (2006). Mutations in the CEL VNTR cause a syndrome of diabetes and pancreatic exocrine dysfunction. *Nature Genetics*. <https://doi.org/10.1038/ng1708>
- Raile, K., Schober, E., Konrad, K., Thon, A., Grulich-Henn, J., Meissner, T., ... Holl, R. W. (2015). Treatment of young patients with HNF1A mutations (HNF1A-MODY). *Diabetic Medicine*. <https://doi.org/10.1111/dme.12662>
- Reaven, G. M., Johnston, P., Hollenbeck, C. B., Skowronski, R., Zhang, J. C., Goldfine, I. D., & Chen, Y. D. I. (1992). Combined metformin-sulfonylurea treatment of patients with noninsulin-dependent diabetes in fair to poor glycemic control. *Journal of Clinical Endocrinology and Metabolism*. <https://doi.org/10.1210/jcem.74.5.1569149>
- Rees, S. D., Hydrie, M. Z. I., O'Hare, J. P., Kumar, S., Shera, A. S., Basit, A., ... Kelly, M. A. (2011). Effects of 16 genetic variants on fasting glucose and type 2 diabetes in South Asians: ADCY5 and GLIS3 variants may predispose to type 2 diabetes. *PLoS ONE*. <https://doi.org/10.1371/journal.pone.0024710>
- Rena, G., Hardie, D. G., & Pearson, E. R. (2017). The mechanisms of action of metformin. *Diabetologia*. <https://doi.org/10.1007/s00125-017-4342-z>
- Ripsin, C. M., Kang, H., & Urban, R. J. (2009). Management of blood glucose in type 2 diabetes mellitus. *American Family Physician*.
- Rossi, A., Kontarakis, Z., Gerri, C., Nolte, H., Hölper, S., Krüger, M., & Stainier, D. Y. R. (2015). Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature*. <https://doi.org/10.1038/nature14580>
- Ryan, K. K., Li, B., Grayson, B. E., Matter, E. K., Woods, S. C., & Seeley, R. J. (2011). A role for central nervous system PPAR- $\gamma$  in the regulation of energy balance. *Nature Medicine*. <https://doi.org/10.1038/nm.2349>
- Sawada, F., Inoguchi, T., Tsubouchi, H., Sasaki, S., Fujii, M., Maeda, Y., ... Takayanagi, R. (2008). Differential effect of sulfonylureas on production of reactive oxygen species and apoptosis in cultured pancreatic  $\beta$ -cell line, MIN6. *Metabolism: Clinical and Experimental*. <https://doi.org/10.1016/j.metabol.2008.01.038>
- Schultes, B., Oltmanns, K. M., Kern, W., Fehm, H. L., Born, J., & Peters, A. (2003). Modulation of hunger by plasma glucose and metformin. *Journal of Clinical Endocrinology and Metabolism*. <https://doi.org/10.1210/jc.2002-021450>

- Shah, A. N., Davey, C. F., Whitebirch, A. C., Miller, A. C., & Moens, C. B. (2015). Rapid reverse genetic screening using CRISPR in zebrafish. *Nature Methods*. <https://doi.org/10.1038/nmeth.3360>
- Sidransky, E. (2006). Heterozygosity for a Mendelian disorder as a risk factor for complex disease. *Clinical Genetics*. <https://doi.org/10.1111/j.1399-0004.2006.00688.x>
- Staffers, D. A., Ferrer, J., Clarke, W. L., & Habener, J. F. (1997). Early-onset type-II diabetes mellitus (Mody4) linked to ipf1. *Nature Genetics*. <https://doi.org/10.1038/ng1097-138>
- Stemmer, M., Thumberger, T., Del Sol Keyer, M., Wittbrodt, J., & Mateo, J. L. (2015). CCTop: An intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. *PLoS ONE*. <https://doi.org/10.1371/journal.pone.0124633>
- Stoletov, K., Fang, L., Choi, S. H., Hartvigsen, K., Hansen, L. F., Hall, C., ... Miller, Y. I. (2009). Vascular lipid accumulation, lipoprotein oxidation, and macrophage lipid uptake in hypercholesterolemic zebrafish. *Circulation Research*. <https://doi.org/10.1161/CIRCRESAHA.108.189803>
- Tallapragada, D. S. P., Bhaskar, S., & Chandak, G. R. (2015). New insights from monogenic diabetes for “common” type 2 diabetes. *Frontiers in Genetics*. <https://doi.org/10.3389/fgene.2015.00251>
- Tattersall, R. B., & Fajans, S. S. (1975). A difference between the inheritance of classical juvenile onset and maturity onset type diabetes of young people. *Diabetes*. <https://doi.org/10.2337/diab.24.1.44>
- Tillil, H., & Kobberling, J. (1987). Age-corrected empirical genetic risk estimates for first-degree relatives of IDDM patients. *Diabetes*. <https://doi.org/10.2337/diab.36.1.93>
- Timsit, J., Bellanné-Chantelot, C., Dubois-Laforgue, D., & Velho, G. (2005). Diagnosis and management of maturity-onset diabetes of the young. *Treatments in Endocrinology*. <https://doi.org/10.2165/00024677-200504010-00002>
- Tranchevent, L. C., Barriot, R., Yu, S., Van Vooren, S., Van Loo, P., Coessens, B., ... Moreau, Y. (2008). ENDEAVOUR update: a web resource for gene prioritization in multiple species. *Nucleic Acids Research*. <https://doi.org/10.1093/nar/gkn325>
- Trevisan, R., Nosadini, R., Fioretto, P., Avogaro, A., Duner, E., Jori, E., ... Crepaldi, G. (1987). Ketone bodies increase glomerular filtration rate in normal man and in patients with Type 1 (insulin-dependent) diabetes mellitus. *Diabetologia*. <https://doi.org/10.1007/BF00270418>
- Turner, R. (1998). Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet*. [https://doi.org/10.1016/S0140-6736\(98\)07037-8](https://doi.org/10.1016/S0140-6736(98)07037-8)
- Udler, M. S., Kim, J., von Grotthuss, M., Bonàs-Guarch, S., Cole, J. B., Chiou, J., ... Florez, J. C. (2018). Type 2 diabetes genetic loci informed by multi-trait associations point to

- disease mechanisms and subtypes: A soft clustering analysis. *PLoS Medicine*.  
<https://doi.org/10.1371/journal.pmed.1002654>
- Varshney, G. K., Pei, W., Lafave, M. C., Idol, J., Xu, L., Gallardo, V., ... Burgess, S. M. (2015). High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. *Genome Research*. <https://doi.org/10.1101/gr.186379.114>
- Wang, H. (2000). Molecular targets of a human HNF1alpha mutation responsible for pancreatic beta-cell dysfunction. *The EMBO Journal*.  
<https://doi.org/10.1093/emboj/19.16.4257>
- Watanabe, K., Taskesen, E., Van Bochoven, A., & Posthuma, D. (2017). Functional mapping and annotation of genetic associations with FUMA. *Nature Communications*.  
<https://doi.org/10.1038/s41467-017-01261-5>
- Wild, S., Roglic, G., Green, A., Sicree, R., & King, H. (2004). Global Prevalence of Diabetes: Estimates for the year 2000 and projections for 2030. *Diabetes Care*.  
<https://doi.org/10.2337/diacare.27.5.1047>
- Willemsen, G., Ward, K. J., Bell, C. G., Christensen, K., Bowden, J., Dalgård, C., ... Spector, T. (2015). The Concordance and Heritability of Type 2 Diabetes in 34,166 Twin Pairs From International Twin Registers: The Discordant Twin (DISCOTWIN) Consortium. *Twin Research and Human Genetics*. <https://doi.org/10.1017/thg.2015.83>
- Xie, S., Shen, B., Zhang, C., Huang, X., & Zhang, Y. (2014). SgRNAs9: A software package for designing CRISPR sgRNA and evaluating potential off-target cleavage sites. *PLoS ONE*. <https://doi.org/10.1371/journal.pone.0100448>
- Yamagata, K., Furuta, H., Oda, N., Kaisaki, P. J., Menzel, S., Cox, N. J., ... Bell, G. I. (1996). Mutations in the hepatocyte nuclear factor-4alpha gene in maturity-onset diabetes of the young (MODY1). *Nature*. <https://doi.org/10.1038/384458a0>
- Yang, H. J., Hsu, C. L., Yang, J. Y., & Yang, W. Y. (2012). Monodansylpentane as a blue-fluorescent lipid-droplet marker for multi-color live-cell imaging. *PLoS ONE*.  
<https://doi.org/10.1371/journal.pone.0032693>
- Zang, L., Shimada, Y., & Nishimura, N. (2017). Development of a Novel Zebrafish Model for Type 2 Diabetes Mellitus. *Scientific Reports*. <https://doi.org/10.1038/s41598-017-01432-w>
- Zhang, W., Cline, M. A., & Gilbert, E. R. (2014). Hypothalamus-adipose tissue crosstalk: Neuropeptide y and the regulation of energy metabolism. *Nutrition and Metabolism*.  
<https://doi.org/10.1186/1743-7075-11-27>

## APPENDIX

### A: Tables

Table3: Showing the main effects of tolbutamide and of mutation of five genes on beta cell number (N= 279)

|  | <i>Effect size</i> | <i>SE</i> | <i>LCI</i> | <i>UCI</i> | <i>P-VALUE</i> |
|--|--------------------|-----------|------------|------------|----------------|
| <i>gck</i>   | 0.063              | 0.173     | -0.276     | 0.173      | 7.15E-01       |
| <i>hnf1a</i>   | -0.203             | 0.152     | -0.501     | 0.152      | 1.82E-01       |
| <i>hnf1ba</i>  | 0.197              | 0.189     | -0.173     | 0.189      | 2.97E-01       |
| <i>hnf1bb</i>  | 0.039              | 0.116     | -0.188     | 0.116      | 7.38E-01       |
| <i>pdx1</i>  | -0.309             | 0.106     | -0.517     | 0.106      | 3.62E-03       |
| <i>Tolbutamide treatment</i>                             | -0.216             | 0.105     | -0.421     | 0.105      | 3.98E-02       |
| <i>body length (in SD)</i>                               | 0.080              | 0.061     | -0.040     | 0.061      | 1.90E-01       |
| <i>length-normalised dorsal body surface area (inSD)</i> | 0.230              | 0.065     | 0.103      | 0.065      | 3.98E-04       |
| <i>time of day (in hours since 9AM)</i>                  | 0.063              | 0.020     | 0.024      | 0.020      | 1.35E-03       |
| <i>intercept_fixed</i>                                   | -0.223             | 0.199     | -0.613     | 0.199      | 2.64E-01       |
| <i>var_batch</i>   | 0.357              | 0.122     | 0.182      | 0.122      |                |
| <i>intercept_random</i>                                  | 0.860              | 0.037     | 0.790      | 0.037      |                |

Table4: Showing the main effects of tolbutamide and of mutation of five genes on beta cell volume (N= 334)

|   | <i>Effect size</i> | <i>SE</i> | <i>LCI</i> | <i>UCI</i> | <i>P-VALUE</i> |
|---|--------------------|-----------|------------|------------|----------------|
| <i>gck_D</i>                            | 0.157              | 0.166     | -0.169     | 0.483      | 3.45E-01       |
| <i>hnf1a_D</i>                          | -0.217             | 0.151     | -0.513     | 0.078      | 1.50E-01       |
| <i>hnf1ba_D</i>                         | 0.257              | 0.184     | -0.104     | 0.619      | 1.63E-01       |
| <i>hnf1bb_D</i>                         | 0.026              | 0.110     | -0.191     | 0.242      | 8.17E-01       |
| <i>pdx1_D</i>                           | -0.104             | 0.102     | -0.305     | 0.096      | 3.07E-01       |
| <i>tolbutamide</i>                      | -0.217             | 0.103     | -0.419     | -0.015     | 3.54E-02       |
| <i>time of day (in hours since 9AM)</i> | 0.120              | 0.019     | 0.083      | 0.158      | 3.31E-10       |
| <i>intercept_fixed</i>                  | -0.590             | 0.177     | -0.937     | -0.244     | 8.36E-04       |
| <i>var_batch</i>                        | 0.231              | 0.094     | 0.104      | 0.513      |                |
| <i>intercept_random</i>                 | 0.906              | 0.037     | 0.836      | 0.981      |                |

Table5: Showing the main effects of tolbutamide and of mutation of five genes on insulin secretion (N= 333)

|   | <i>Effect size</i> | <i>SE</i> | <i>LCI</i> | <i>UCI</i> | <i>P-VALUE</i> |
|---|--------------------|-----------|------------|------------|----------------|
| <i>gck_D</i>                            | 0.043              | 0.132     | -0.216     | 0.301      | 7.47E-01       |
| <i>hnf1a_D</i>                          | 0.027              | 0.114     | -0.197     | 0.250      | 8.16E-01       |
| <i>hnf1ba_D</i>                         | 0.125              | 0.141     | -0.151     | 0.402      | 3.75E-01       |
| <i>hnf1bb_D</i>                         | 0.082              | 0.086     | -0.087     | 0.251      | 3.40E-01       |
| <i>pdx1_D</i>                           | -0.354             | 0.081     | -0.513     | -0.196     | 1.21E-05       |
| <i>tolbutamide</i>                      | -0.112             | 0.079     | -0.267     | 0.044      | 1.59E-01       |
| <i>time of day (in hours since 9AM)</i> | -0.104             | 0.015     | -0.133     | -0.075     | 2.03E-12       |
| <i>intercept_fixed</i>                  | 0.831              | 0.228     | 0.383      | 1.279      | 2.77E-04       |

|                  |       |       |       |       |
|------------------|-------|-------|-------|-------|
| <i>var_batch</i> | 0.574 | 0.146 | 0.349 | 0.944 |
|------------------|-------|-------|-------|-------|

Table 6 : Showing the main effects of tolbutamide and of mutation of five genes on LDLc (N= 380)

|   | <i>Effectsize</i> | <i>SE</i> | <i>LCI</i> | <i>UCI</i> | <i>P-VALUE</i> |
|---|-------------------|-----------|------------|------------|----------------|
| <i>gck_D</i>                            | -0.007            | 0.153     | -0.307     | 0.293      | 9.63E-01       |
| <i>hnf1a_D</i>                          | -0.242            | 0.130     | -0.496     | 0.013      | 6.27E-02       |
| <i>hnf1ba_D</i>                         | -0.065            | 0.165     | -0.389     | 0.260      | 6.96E-01       |
| <i>hnf1bb_D</i>                         | -0.005            | 0.095     | -0.191     | 0.182      | 9.60E-01       |
| <i>pdx1_D</i>                           | -0.064            | 0.093     | -0.247     | 0.119      | 4.94E-01       |
| <i>tolbutamide</i>                      | 0.035             | 0.091     | -0.143     | 0.212      | 7.03E-01       |
| <i>time of day (in hours since 9AM)</i> | 0.027             | 0.017     | -0.006     | 0.060      | 1.05E-01       |
| <i>intercept_fixed</i>                  | -0.243            | 0.200     | -0.635     | 0.149      | 2.24E-01       |
| <i>var_batch</i>                        | 0.426             | 0.114     | 0.252      | 0.720      |                |
| <i>intercept_random</i>                 | 0.878             | 0.032     | 0.817      | 0.943      |                |

Table 7: Showing the main effects of tolbutamide and of mutation of five genes on HDLc (N= 380)

|   | <i>Effect size</i> | <i>SE</i> | <i>LCI</i> | <i>UCI</i> | <i>P-VALUE</i> |
|---|--------------------|-----------|------------|------------|----------------|
| <i>gck_D</i>                            | 0.049              | 0.166     | -0.277     | 0.375      | 7.69E-01       |
| <i>hnf1a_D</i>                          | 0.035              | 0.143     | -0.246     | 0.315      | 8.09E-01       |
| <i>hnf1ba_D</i>                         | 0.008              | 0.181     | -0.347     | 0.363      | 9.65E-01       |
| <i>hnf1bb_D</i>                         | -0.056             | 0.105     | -0.261     | 0.150      | 5.96E-01       |
| <i>pdx1_D</i>                           | -0.154             | 0.100     | -0.350     | 0.041      | 1.21E-01       |
| <i>tolbutamide</i>                      | 0.185              | 0.101     | -0.013     | 0.383      | 6.74E-02       |
| <i>time of day (in hours since 9AM)</i> | -0.009             | 0.018     | -0.044     | 0.027      | 6.30E-01       |
| <i>intercept_fixed</i>                  | 0.023              | 0.158     | -0.287     | 0.334      | 8.82E-01       |

|                         |       |       |       |       |
|-------------------------|-------|-------|-------|-------|
| <i>var_batch</i>        | 0.125 | 0.099 | 0.027 | 0.592 |
| <i>intercept_random</i> | 0.982 | 0.036 | 0.914 | 1.056 |

Table8: Showing the main effects of tolbutamide and of mutation of five genes on cholesterol (N= 380)

|   | <i>Effect size</i> | <i>SE</i> | <i>LCI</i> | <i>UCI</i> | <i>P-VALUE</i> |
|---|--------------------|-----------|------------|------------|----------------|
| <i>gck_D</i>                            | 0.000              | 0.146     | -0.286     | 0.287      | 9.99E-01       |
| <i>hnf1a_D</i>                          | -0.194             | 0.124     | -0.438     | 0.049      | 1.17E-01       |
| <i>hnf1ba_D</i>                         | 0.037              | 0.158     | -0.274     | 0.347      | 8.17E-01       |
| <i>hnf1bb_D</i>                         | 0.011              | 0.091     | -0.168     | 0.189      | 9.06E-01       |
| <i>pdx1_D</i>                           | 0.009              | 0.089     | -0.166     | 0.184      | 9.20E-01       |
| <i>tolbutamide</i>                      | 0.178              | 0.087     | 0.009      | 0.348      | 3.94E-02       |
| <i>time of day (in hours since 9AM)</i> | 0.043              | 0.016     | 0.011      | 0.074      | 7.75E-03       |
| <i>intercept_fixed</i>                  | -0.553             | 0.210     | -0.966     | -0.141     | 8.49E-03       |
| <i>var_batch</i>                        | 0.484              | 0.127     | 0.289      | 0.809      |                |
| <i>intercept_random</i>                 | 0.838              | 0.031     | 0.780      | 0.901      |                |

Table9: Showing the main effects of tolbutamide and of mutation of five genes on vascular lipid deposition (N= 246)

|                    | <i>Effect size</i> | <i>SE</i> | <i>LCI</i> | <i>UCI</i> | <i>P-VALUE</i> |
|--------------------|--------------------|-----------|------------|------------|----------------|
| <i>gck_D</i>       | 1.269              | 0.523     | 0.243      | 2.294      | 1.54E-02       |
| <i>hnf1a_D</i>     | 0.302              | 0.372     | -0.427     | 1.032      | 4.17E-01       |
| <i>hnf1ba_D</i>    | 0.256              | 0.431     | -0.588     | 1.100      | 5.52E-01       |
| <i>hnf1bb_D</i>    | 0.111              | 0.278     | -0.433     | 0.655      | 6.90E-01       |
| <i>pdx1_D</i>      | -0.841             | 0.258     | -1.347     | -0.336     | 1.11E-03       |
| <i>tolbutamide</i> | -0.655             | 0.291     | -1.225     | -0.085     | 2.43E-02       |

|   |        |       |        |       |          |
|---|--------|-------|--------|-------|----------|
| <i>time of day (in hours since 9AM)</i> | 0.430  | 0.153 | 0.130  | 0.730 | 4.91E-03 |
| <i>intercept_fixed</i>                  | 0.171  | 0.157 | -0.137 | 0.479 | 2.76E-01 |
| <i>var_batch</i>                        | -0.042 | 0.047 | -0.134 | 0.050 | 3.75E-01 |
| <i>intercept_random</i>                 | 5.042  | 0.401 | 4.256  | 5.829 | 3.25E-36 |

Table 10: Showing the main effects of tolbutamide and of mutation of five genes on length (**N= 326**)

|   | <i>Effectsize</i> | <i>SE</i> | <i>LCI</i> | <i>UCI</i> | <i>P-VALUE</i> |
|---|-------------------|-----------|------------|------------|----------------|
| <i>gck_D</i>                            | -0.185            | 0.152     | -0.483     | 0.113      | 2.24E-01       |
| <i>hnf1a_D</i>                          | -0.284            | 0.133     | -0.544     | -0.024     | 3.22E-02       |
| <i>hnf1ba_D</i>                         | 0.120             | 0.158     | -0.190     | 0.429      | 4.49E-01       |
| <i>hnf1bb_D</i>                         | -0.052            | 0.098     | -0.244     | 0.140      | 5.96E-01       |
| <i>pdx1_D</i>                           | -0.229            | 0.092     | -0.409     | -0.049     | 1.28E-02       |
| <i>tolbutamide</i>                      | -0.208            | 0.088     | -0.381     | -0.035     | 1.85E-02       |
| <i>time of day (in hours since 9AM)</i> | -0.015            | 0.016     | -0.047     | 0.017      | 3.62E-01       |
| <i>intercept_fixed</i>                  | 0.314             | 0.208     | -0.094     | 0.722      | 1.32E-01       |
| <i>var_batch</i>                        | 0.477             | 0.123     | 0.288      | 0.790      |                |
| <i>intercept_random</i>                 | 0.789             | 0.031     | 0.730      | 0.853      |                |

Table 11: Showing the main effects of tolbutamide and of mutation of five genes on lateral area (**N= 248**)

|                 | <i>Effect size</i> | <i>SE</i> | <i>LCI</i> | <i>UCI</i> | <i>P-VALUE</i> |
|-----------------|--------------------|-----------|------------|------------|----------------|
| <i>gck_D</i>    | -0.160             | 0.204     | -0.561     | 0.240      | 4.32E-01       |
| <i>hnf1a_D</i>  | -0.345             | 0.196     | -0.729     | 0.038      | 7.78E-02       |
| <i>hnf1ba_D</i> | 0.262              | 0.230     | -0.188     | 0.712      | 2.54E-01       |
| <i>hnf1bb_D</i> | 0.093              | 0.132     | -0.165     | 0.351      | 4.79E-01       |

|   |        |       |        |       |          |
|---|--------|-------|--------|-------|----------|
| <i>pdx1_D</i>                           | -0.102 | 0.129 | -0.355 | 0.152 | 4.31E-01 |
| <i>tolbutamide</i>                      | -0.114 | 0.122 | -0.353 | 0.125 | 3.51E-01 |
| <i>time of day (in hours since 9AM)</i> | 0.011  | 0.022 | -0.032 | 0.055 | 6.11E-01 |
| <i>intercept_fixed</i>                  | -0.008 | 0.206 | -0.412 | 0.396 | 9.69E-01 |
| <i>var_batch</i>                        | 0.261  | 0.097 | 0.127  | 0.539 |          |
| <i>intercept_random</i>                 | 0.950  | 0.043 | 0.868  | 1.038 |          |

Table 12: Showing the main effects of tolbutamide and of mutation of five genes on body volume (N= 232)

|   | <i>Effectsize</i> | <i>SE</i> | <i>LCI</i> | <i>UCI</i> | <i>P-VALUE</i> |
|---|-------------------|-----------|------------|------------|----------------|
| <i>gck_D</i>                            | -0.120            | 0.200     | -0.512     | 0.273      | 5.50E-01       |
| <i>hnf1a_D</i>                          | -0.404            | 0.162     | -0.722     | -0.087     | 1.26E-02       |
| <i>hnf1ba_D</i>                         | 0.459             | 0.211     | 0.045      | 0.873      | 2.98E-02       |
| <i>hnf1bb_D</i>                         | -0.050            | 0.129     | -0.303     | 0.204      | 7.01E-01       |
| <i>pdx1_D</i>                           | -0.320            | 0.124     | -0.563     | -0.077     | 9.82E-03       |
| <i>tolbutamide</i>                      | -0.277            | 0.120     | -0.511     | -0.042     | 2.07E-02       |
| <i>time of day (in hours since 9AM)</i> | -0.015            | 0.022     | -0.059     | 0.028      | 4.88E-01       |
| <i>intercept_fixed</i>                  | 0.435             | 0.205     | 0.034      | 0.837      | 3.34E-02       |
| <i>var_batch</i>                        | 0.291             | 0.100     | 0.149      | 0.570      |                |
| <i>intercept_random</i>                 | 0.897             | 0.042     | 0.818      | 0.984      |                |

Table 13: Orthologues of candidate genes in MODY associated locus

| Human Gene   | ENSG            | Zebrafish orthologue | ENSDARG                              | CHR | Target %Identity | Query Identity |
|--------------|-----------------|----------------------|--------------------------------------|-----|------------------|----------------|
| <b>HNF1A</b> | ENSG00000135100 | <b>Hnf1a</b>         | <a href="#">(ENSDARG00000009470)</a> | 8   | 51.96            | 45.61          |
| <b>HNF1B</b> | ENSG00000275410 | <b>Hnf1ba</b>        | <a href="#">(ENSDARG00000006615)</a> | 15  | 80.90            | 80.61          |
| <b>HNF1B</b> | ENSG00000275410 | <b>Hnf1bb</b>        | <a href="#">(ENSDARG00000022295)</a> | 21  | 67.86            | 64.81          |
| <b>PDX1</b>  | ENSG00000139515 | <b>Pdx1</b>          | <a href="#">(ENSDARG00000002779)</a> | 24  | 55.28            | 48.06          |
| <b>GCK</b>   | ENSG00000106633 | <b>gck</b>           | <a href="#">(ENSDARG00000068006)</a> | 8   | 78.99            | 80.69          |

Target %identity: percentage of the orthologous sequence matching the human sequence; Query %identity: percentage of the human sequence matching the sequence of the orthologue

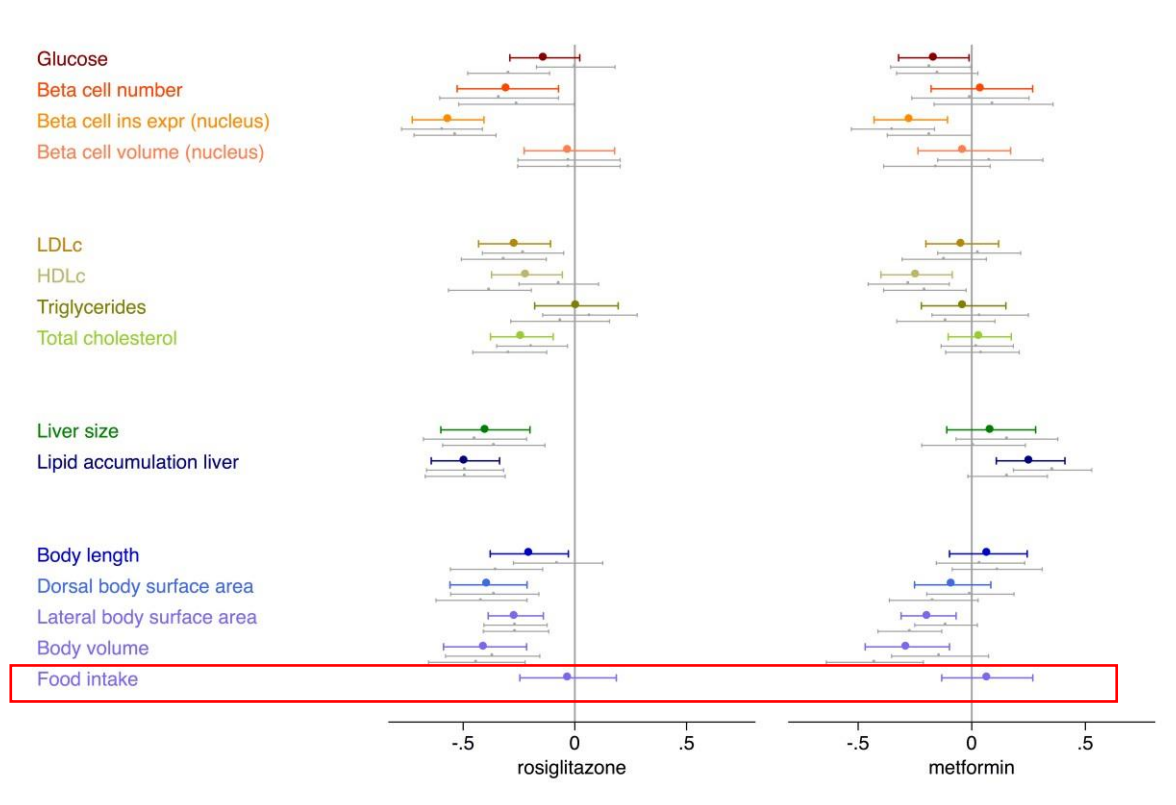
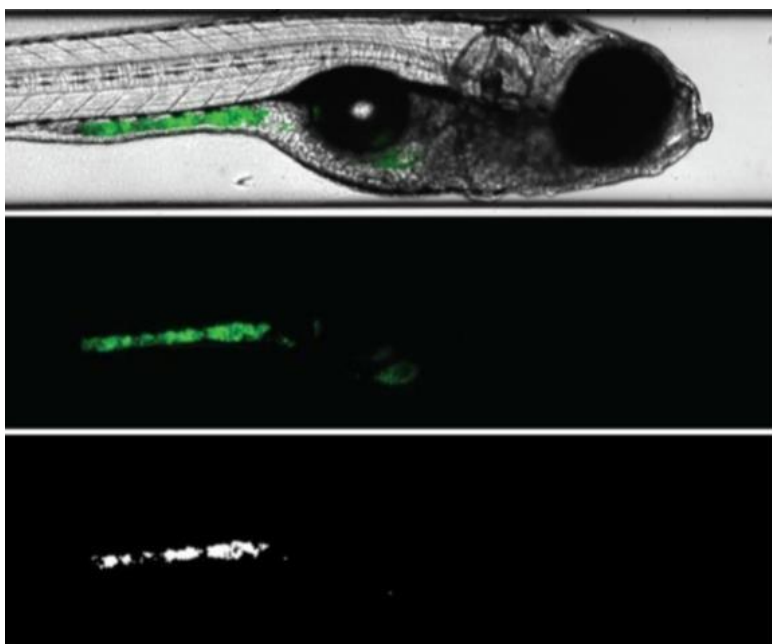


Figure 1. The effect of treatment with rosiglitazone and metformin on glycaemic traits including beta cell number and volume, insulin expression, whole-body lipid, glucose levels, Dorsal and lateral body surface area and body volume; For these traits effect sizes and 95% confidence intervals are expressed in standard deviation units (SD). For normally distributed traits, associations were examined using hierarchical linear models on inverse-normally transformed outcomes. Associations were adjusted for time of day and batch; and transgenic background. The red square portion shows the effect of treatment with metformin and rosiglitazone on food intake, assessed using multilinear regression models regression on , adjusted for time since feeding and batch (n=204). Dots and whiskers show effect size and 95% confidence interval.



**Figure 6.** Food intake as a function of dietary or drug treatment intervention. Mixing fluorescently labelled tracers in with standard dry food, standard dry food enriched with 4% extra cholesterol using diethyl ether, standard dry food treated with diethyl ether, and standard dry food enriched with 4% extra cholesterol using diethyl ether and further enriched with atorvastatin and ezetimibe allowed image-based quantification of food intake - i.e. surface area of fluorescence in the gastrointestinal tract - in eight-day-old zebrafish larvae