The molecular basis of metabolic syndrome: studies in zebrafish

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Chapter 5

Glucocorticoid modulation of metabolic changes induced by mycobacterial infection

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Abstract

People suffering from tuberculosis develop a state which is called wasting syndrome or cachexia and is characterized by dramatic weight loss and malnutrition. Glucocorticoid treatment may reduce the symptoms of wasting syndrome by inhibiting inflammatory signaling, but the metabolic effects of glucocorticoids in tuberculosis patients are still unclear. In the present study, we have investigated the effect of glucocorticoid treatment in a zebrafish model for tuberculosis, in which zebrafish larvae were infected with *Mycobacterium marinum*, a close relative of *M. tuberculosis*. Our results show that treatment with the glucocorticoid beclomethasone resulted in a higher bacterial burden and a slightly higher mortality, but that the infection-induced metabolic changes were attenuated. The increase in glucose levels was abolished and the decrease in muscle mass reduced by the beclomethasone treatment. Transcriptome analysis by RNA-sequencing was performed to study the molecular mechanisms underlying these changes, and the results of this analysis suggest that genes involved in glucose metabolism, insulin and leptin signaling play a crucial role in the observed metabolic changes resembling cachexia. Furthermore, these data demonstrated that during the mycobacterial infection the larvae are in a state of reduced sensitivity to glucocorticoids. Taken together, our data show that zebrafish larvae represent an interesting model system to investigate cachexia in human TB patients, which has provided novel insights into the molecular mechanisms underlying wasting syndrome and the possibilities for adjunctive glucocorticoid therapy to alleviate this metabolic state.

Introduction

Between a quarter and a third of the world's population, especially in low- and middle-income countries, is infected with the causative agent of tuberculosis (TB), *Mycobacterium tuberculosis*[1]. Most of these people develop a latent infection and do not show any symptoms[2,3], but approximately 5-10% of them develop clinically active TB[3,4]. Among those TB patients, the majority manifest a lung infection and around 20% show infection in other organs[3,5]. Currently, the standard treatment of TB consists of multiple antibiotic drugs, but for many years the bacterium has been developing resistance against mostly used antibiotics, and this resistance has made TB a major threat to global public health[6].

Patients with TB display a malnutrition state with significantly lower body weight, resulting from a depletion of both lean and fat tissue[7]. This process is called the wasting syndrome or cachexia and is a cardinal feature of TB. The dramatic weight loss and malnutrition have further consequences, including fatigue, delayed ambulation, higher risk for pulmonary embolism and secondary pulmonary infections, and represent the primary causes of death in TB patients[8,9]. A complex of different factors has been found to underlie this wasting syndrome. TB patients display a decreased appetite, increased glucose, fat and protein catabolism and a reduction in anabolic processes like protein synthesis[8]. Additionally, the disease has been associated with the development of impaired glucose tolerance and, as a

result, some patients can develop type 2 diabetes mellitus (T2DM) during the course of the infection[10,11]. Interestingly, both the weight loss and the altered glucose metabolism recover after successful TB treatment, although full recovery may not occur or take longer than the drug treatment[11,12,13]. The molecular mechanisms that cause these metabolic alterations have not been fully elucidated. In analogy with cachexia observed in other diseases, e.g. cancer, pro-inflammatory cytokines like TNF-α and IL-6 are often considered as important drivers of wasting in TB, possibly by increasing leptin levels[14]. However, in several studies the circulating TNF-α levels did not significantly correlate with body weight in TB patients[15,16,17], and leptin levels have been shown to be increased in TB patients in some studies[14,17,18], but decreased in others[19,20,21].

Glucocorticoids are a class of steroid hormones, which are mainly produced in response to stress. The main endogenous glucocorticoid in humans and fish is cortisol. After activation of the glucocorticoid receptor (GR), glucocorticoids suppress the immune response[22]. Synthetic glucocorticoids like prednisolone and beclomethasone are commonly used clinically as anti-inflammatory drugs in the treatment of many chronic immune-related diseases[23]. It has been shown that adjunctive glucocorticoid therapy improves the survival in subgroups of TB patients, suffering from tuberculous meningitis and pericarditis[24,25,26,27]. In pulmonary TB adjunctive glucocorticoid therapy is used to prevent complications, but the effect on survival is controversial[28]. The exact mechanism of action underlying these effects of glucocorticoids is unclear, which is particularly true for the effect of glucocorticoid therapy on cachexia. Since glucocorticoids have been shown to suppress TNF-α production in in hyper-inflammatory infection[29], it may be hypothesized that glucocorticoids reduce symptoms of wasting syndrome through inhibition of inflammatory signaling. However, in the absence of an infection, glucocorticoids are known to induce symptoms of the wasting syndrome rather than suppress them. They have been shown to induce an increase in blood glucose level, which can lead to diabetes mellitus[30], and are known to be important mediators of muscle wasting. Upregulation of forkhead box protein O1 (FOXO1) and deregulation of (myoblast determination protein 1) MyoD are the main mechanisms of glucocorticoid-induced muscle wasting[31]. Another process that is involved in the glucocorticoid-induced muscle atrophy is insulin resistance and increased calcium levels that enhance protein degradation[32]. Taken together, previous studies have shown that glucocorticoids regulate both the immune response and induce metabolic changes, but that little is known about their mechanism of action during TB and their impact on metabolism during the infection. In the present study, we have investigated the effect of glucocorticoids on the metabolic changes seen during TB infection.

As a model system to study effect of glucocorticoids, we have used the zebrafish (*Danio rerio*). The zebrafish is a powerful research model for studies on various metabolic diseases, such as diabetes and other metabolism-related diseases[33,34]. In addition, zebrafish larvae represent a versatile model to study mycobacterial infection[35]. In zebrafish, *Mycobacterium marinum,* a

close relative of *M. tuberculosis*, is able to cause a systemic and chronic infection, with containment of bacteria in granulomas that show strong structural similarity to those caused by *M. tuberculosis* in humans[29,36]. Since the progression of this infection shows strong similarities with human TB, it is an interesting model to study the metabolic changes associated with this mycobacterial infection. The zebrafish model is also used to study the molecular mechanisms of glucocorticoid action. Zebrafish express a Gr with high structural and functional similarity to its human equivalent[37], and this zebrafish Gr has also been shown to modulate metabolism and the immune system in a similar way to the human GR[38,39]. In recent studies, we have used the zebrafish model to investigate the inhibitory effect of glucocorticoids on the inflammatory response, in particular their effect on macrophages[39,40].

Our focus in the present study was on the effect of glucocorticoid treatment on the metabolic changes induced by *M. marinum* infection in zebrafish larvae. Our results show that treatment with the glucocorticoid beclomethasone attenuates the effect of the infection on glucose levels and muscle wasting. RNA-sequencing was performed to study possible molecular mechanisms underlying the effects of beclomethasone. Interestingly, the results of this analysis indicate that beclomethasone hardly changes gene transcription in infected larvae, suggesting that the infection induces a glucocorticoid-resistant state.

Materials and methods

Fish maintenance and handling

Zebrafish of the AB/TL line were maintained and handled according to the guidelines from the Zebrafish Model Organism Database (http://zfin.org), and in compliance with the directives of the local animal welfare committee of Leiden University. They were housed under a 14 hours light and 10 hours dark cycle at 28.5 °C. Fertilization was performed by natural spawning at the beginning of the light period. Embryos were grown at 28.5 °C in egg water (60 µg/ml Instant ocean sea salt, Sera Marin).

Bacterial preparation and zebrafish injections

M. marinum injections were performed as described by Benard et al.[35]. A *M. marinum* strain, containing an expression vector for the fluorescent protein Wasabi, was cultured as described in Benard et al.[35]. Two vials with 1 ml of the mycobacterial culture were centrifuged at 14680 rpm for 1 min. The pellets were washed three times with 1 ml phosphate-buffered saline (PBS). Suspensions were prepared based on the optical density at 600 nm, which corresponds to 1.0 x 10^8 colony forming units (cfu)/ml. The inoculums were suspended in 2% polyvinylpyrrolidone40 (PVP40, CalBiochem) to a concentration of 2.0 x 10^8 cfu/ml. Between 20 and 40 cfu were injected into the blood island of 28 hours post fertilization (hpf) embryos, which were kept under anesthesia in egg water containing 0.02% tricaine (3-aminobenzoic acid ethyl ester (Sigma). Non-infected (control) embryos were injected with a carrier solution

(PBS). The embryos were treated with beclomethasone (25 µM) or vehicle starting at 2 hours before the infection.

Measurement of mortality and bacterial burden

The infected larvae were monitored every 24 hours post infection to determine mortality rates. The larvae used for the survival assay were kept individually in 96 well plates. The larvae used for other assays were kept in petri dishes. We used the Complex Object Parametric Analyzer and Sorter (COPAS, Union Biometrica) system to determine the total level of green fluorescence, which was used as a measure for the bacterial burden. The following parameters were used: optical density threshold (extinction) = 390 mV (COPAS value: 20), minimum time of flight = 280 ms (COPAS value: 700), red photomultiplier tube (PMT) voltage = 500 V, green PMT voltage = 0 V, yellow PMT voltage = 0 V, fluorescent density threshold = 800. The fluorescence intensities of approximately 50 injected embryos were measured daily (every 24 hr) with the COPAS system until 4 days post infection (dpi). Embryos that were not successfully infected and did not show proper fluorescence were discarded from the experiment. Measurements were performed daily, and after each measurement the medium was refreshed. Data were pooled from three independent experiments. Data shown are means ± s.e.m.

Glucose measurement

Quantitative analysis of glucose levels was performed from whole body lysates using a glucose ELISA kit (Cayman Chemical, USA), according to the manufacturer's instructions. Fifteen zebrafish larvae per experimental group were sonicated in 30 µl Assay Buffer on ice. A total of 25 µl assay Enzyme Mix was added and the solution was incubated for 10 min at 37°C. Standard curves were generated using glucose standard solutions. Fluorescence was measured using a BioTek plate reader (excitation wavelength 514 nm) equipped with GEN 5 software (v.2.04, BioTek). Data shown are means ± s.e.m. of three independent experiments, which were performed in triplicate.

Measurement of muscle mass

A phalloidin staining was used to determine the muscle mass. Larvae were put into microcentrifuge tubes at 4 dpi, and fixed in 0.5 ml 4% paraformaldehyde. The tubes were kept at 4°C overnight. The next day, the fixative was removed and the larvae were rinsed three times for 5 min in 0.5 ml PBS/0.1%Tween20. After removing the PBS/ 0.1%Tween20, a solution of 0.5 ml PBS/2%TritonX-100 was added to each tube. The tubes were then gently rocked for 1.5 hour at room temperature. The liquid was removed and 19 µl of PBS/2%TritonX-100 was added to each tube, along with 1µl of AlexaFluor555Phalloidin. The tubes were gently rocked at 4°C overnight. The next day, the larvae were washed three times with 0.5 ml PBS. Fixed larvae were imaged using a Leica MZ16FA fluorescence stereomicroscope (Leica Microsystems). Quantification of the fluorescent signal was done

using dedicated bacterial pixel count software as previously described by Stoop et al.[41]. Three individual experiments were performed, which were done with at least ten replicates per experimental group. Data were pooled from three experiments, and data shown are means ± s.e.m.

RNA-sequencing (RNA-seq) analysis

In three independent experiments, pools of 20 embryos of four experimental groups (veh/non-inf, beclo/non-inf, veh/inf, beclo/inf) were collected at 4 dpi, snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted according to the manufacturer's instructions, using TRIzol (Life Technologies). A total of 2 µg of RNA was used to make RNA-seq libraries using the Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina, Inc., San Diego, CA, USA). The manufacturer's instructions were followed with the exception of two modifications. In the adapter ligation step, 1 µl adaptor was used (instead of 2.5 µl), and in the library size-selection step, the library fragments were isolated with a double Ampure XP purification with a 0.7× beads to library ratio (Beckman Coulter). The resulting mRNA-seq library was sequenced using an Illumina HiSeq2500 Instrument (Illumina Inc.), with a read length of 2×50 nucleotides. Image analysis and base-calling were done by the Illumina HCS version 2.0.12. Data analysis was performed using GeneTiles Software (www.genetiles.com, described in Veneman et al. [42]), and gene ontology and pathway analysis were performed using DAVID Functional Annotation Tool (https://david.ncifcrf.gov/). Finally a detailed gene ontology analysis was performed manually by gathering information from zfin.org, to select genes involved in metabolism.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). The survival rates (Fig.1A) were analyzed using a Log-rank Mantel-Cox test. The bacterial burden (Fig.1B) was analyzed using two-way ANOVA followed by Sidak's multiple comparisons. The glucose concentrations (Fig.2A) were analyzed by using two-way ANOVA followed by Tukey's post hoc test for multiple group comparisons. Data from the phalloidin staining (Fig.2B) were also analyzed by two-way ANOVA followed by Tukey's post hoc test for multiple group comparisons. RNA-seq data (Figs.3-6) were analyzed using GeneTiles software and DESeq2. Statistical significance was accepted at $p<0.05$.

Results

Beclomethasone increases the severity of *M. marinum* infection in zebrafish larvae

To study the effect of glucocorticoids on the course of the mycobacterial infection, wild type zebrafish embryos were injected with fluorescently (Wasabi-)labeled *M. marinum* strain at 28 hpf. The embryos were treated with beclomethasone or vehicle starting at 2 hours before the infection, and the survival was monitored during the next 4 days. We found that the mortality

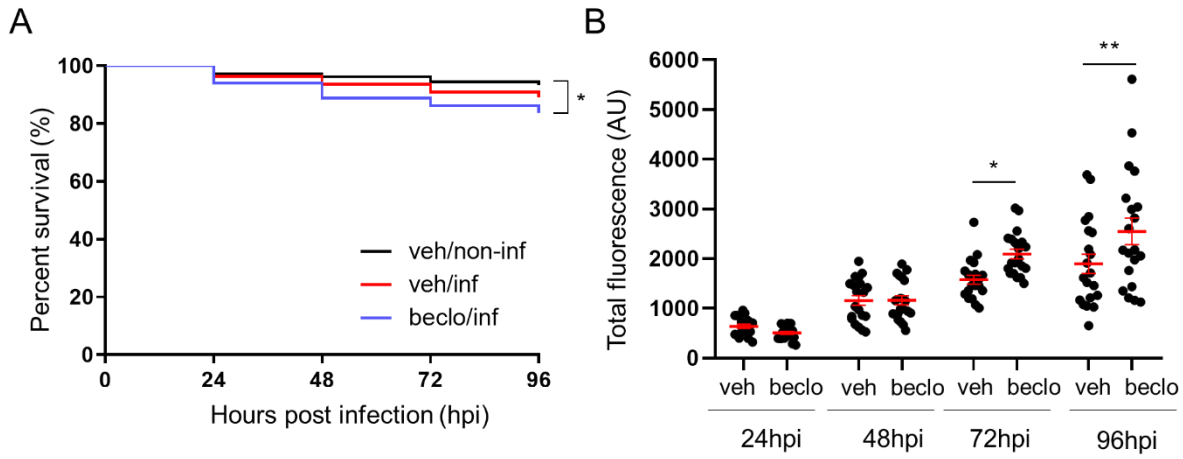
in the infected beclomethasone-treated (beclo/inf) group was significantly higher compared to survival in the non-infected control (veh/non-inf) group (reaching respectively ~17% and ~7% at 96 hpi). No difference was observed between the mortality in the infected vehicle-treated (veh/inf) group (which reached a mortality rate of ~11% at 96 hpi) and any of the other groups (Fig.1A).

In addition to their survival, the bacterial burden of the infected larvae was monitored during this period. To study the bacterial burden in the surviving larvae, we used COPAS flow cytometry to follow the progression of the infection. We found that at 1 and 2 dpi the average total fluorescence was not different between the vehicle- and beclomethasone-treated group, reaching levels of 614±42 Arbitrary Units (AU) and 525±31 AU at 1 dpi respectively, and 1257±97 AU and 1204±90 AU at 2 dpi (Fig.1B). A significant difference was noticeable at 3 dpi when the fluorescence was at 1630±89 AU in the vehicle-treated group and at 2070±97 AU in the beclomethasone-treated group. At 4 dpi the difference remained with a fluorescence level of 2086±194 AU in the vehicle-treated group and 2755±269 AU in the beclomethasone-treated group (Fig.1B).

Taken together, these data show that beclomethasone treatment significantly increases the severity of the *M. marinum* infection. It increases the mortality and the bacterial burden, although the effects are relatively small.

Beclomethasone abolishes infection-induced changes in glucose level and muscle mass

To study the effects of infection and beclomethasone treatment on the glucose metabolism during *M. marinum* infection, we measured the glucose levels in four different treatment groups: control-treated larvae (veh/non-inf), larvae that were treated with beclomethasone (beclo/non-inf), larvae that were infected (veh/inf), and larvae that received the combined treatment (beclo/inf). Embryos were infected at 28 hpf with the fluorescently labelled *M. marinum*. The glucose concentrations were measured at 1, 2, 3 and 4 dpi using an ELISA-based approach (Fig.2A). Both beclomethasone and infection enhanced glucose levels in the larvae at 2, 3 and 4 dpi, with glucose concentrations of 277±4 pmol/larva in the beclo/non-inf group and 291±37 pmol/larva in the veh/inf group at 4 dpi, compared to veh/non-inf control group. However, in the beclo/inf group, which received the combined treatment, the glucose level was significantly lower than the levels in both the beclo/non-inf and the veh/inf group at 2, 3 and 4 dpi, with a concentration of 213±22 pmol/larva at 4 dpi. Subsequently, to study muscle wasting, we used phalloidin staining, which labels actin fibers in the larvae, to visualize muscle tissue at 4 dpi[43,44] (Fig.2B). We found that beclomethasone treatment alone did not affect the muscle mass in the larvae, but that infection caused severe muscle loss, indicated by a decrease of ~55% in phalloidin staining in the veh/inf group (2548±532 AU), compared to the veh/non-inf group (6487±283 AU). Interestingly, the combined infection and beclomethasone treatment did not result in a change in muscle mass, indicated by a level of phalloidin staining in the beclo/inf group (4762±496 AU), which was not significantly different from the veh/non-



inf group. A representative images of the phalloidin staining of a larva from the veh/non-inf group is shown in Fig.2C.

Figure 1. The effect of beclomethasone treatment on *M. marinum* infection in zebrafish. (A) Survival curves of zebrafish upon *M. marinum* infection at 28 hpf. Survival was monitored between 24 and 120 dpi. Infected fish were either vehicle-treated (red line, veh/inf) or beclomethasone-treated (blue line, beclo/inf) and a vehicle-treated non-infected group was added as control (black line, veh/non-inf). At 120 hpi the percentage survival was approximately ~75% in the beclo/inf group and ~82% in the veh/inf group, compared to ~90% in the veh/non-inf group. A significant difference was observed between the beclo/inf and the veh/non-inf group. **(B)** The bacterial burden in larvae from the veh/inf and beclo/inf groups was determined between 1 and 4 dpi based on fluorescence intensities. In the beclo/inf larvae the bacterial burden at 3 and 4 dpi was significantly higher than in the veh/inf larvae. Data were pooled from three independent experiments. Data shown are means ± s.e.m. *P<0.05; **P<0.01

In summary, these results indicate that beclomethasone treatment abolishes both the infection-induced increase in glucose concentration and the loss in muscle mass due to the infection.

Transcriptome analysis by RNA-sequencing

To study the transcriptional changes underlying the observed effects of beclomethasone treatment during bacterial infection, we performed RNA-sequencing-based transcriptome analysis. RNA was isolated from whole larvae at 4 dpi, from the four experimental groups: veh/non-inf, beclo/non-inf, veh/inf and beclo/inf. In the analysis of the RNA-seq results, mRNA levels in the latter three groups were determined relative to the levels of the veh/non-inf group. Significance cutoffs of fold change<1.5 or >1.5, and p value<0.05 were used. Our data showed that 2542 genes were significantly regulated in the veh/inf group (Suppl.Table 1), 1407 in the beclo/ inf group (Suppl.Table 2), and 2319 in the beclo/ non-inf group (Suppl.Table 3) (see Venn diagrams in Fig.3).

About two times more genes were upregulated than downregulated in all studied groups (Figs.3B,C). Interestingly, the largest overlap (1284 genes) was observed between the clusters

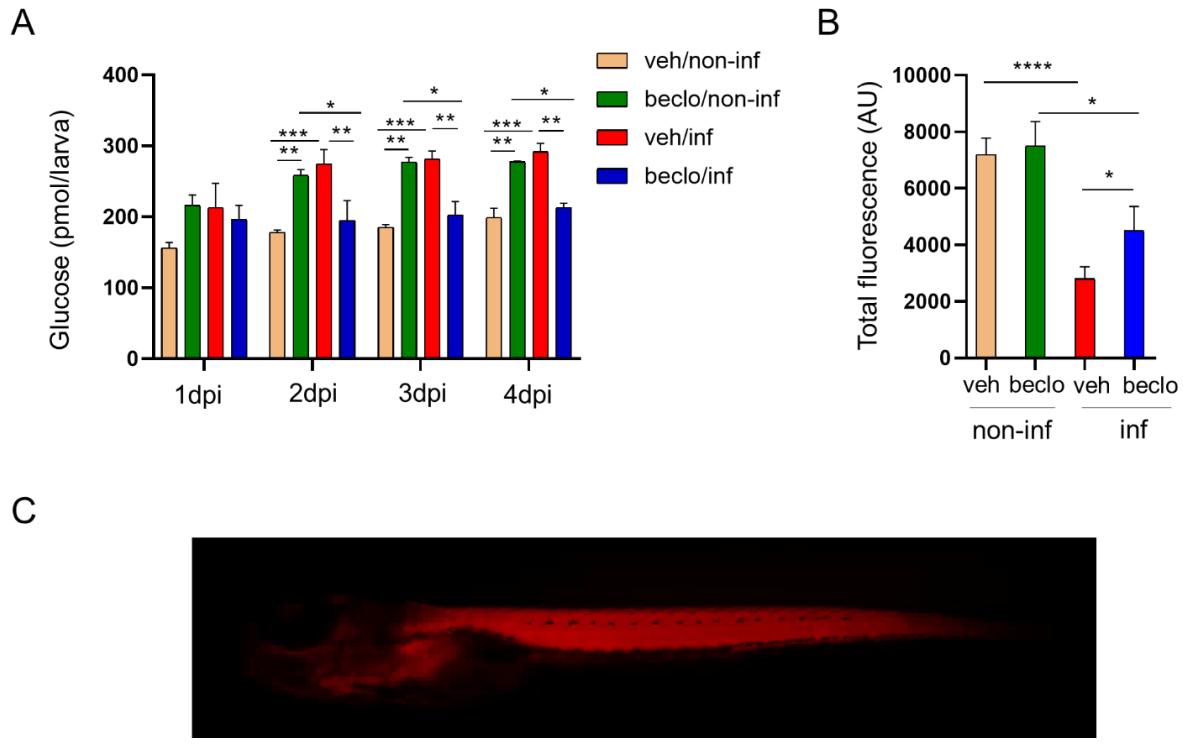


Figure 2. Effect of beclomethasone on the glucose level and muscle mass in *M. marinum* infected zebrafish. (A) Glucose levels in zebrafish larvae from the veh/non-inf, beclo/non-inf, veh/inf and beclo/inf groups at 4 dpi, determined by ELISA. Significant differences in glucose levels were observed between 2 and 4 dpi. The glucose level was increased in the beclo/non-inf and the veh/inf group compared to the veh/non-inf group. When beclomethasone treatment and infection were combined (beclo/inf treatment), the glucose levels were not different from those in the veh/non-inf group. **(B)** Muscle wasting in zebrafish larvae at 4 dpi, determined using phalloidin staining and quantitation of fluorescence in larvae. Larvae from the veh/inf group showed significantly lower muscle mass compared to the non-infected groups (veh/non-inf and beclo/non-inf). The combined beclo/inf treatment did not result in a significant change in muscle mass compared to the veh/non-inf group, and showed a significantly higher muscle mass compared to the veh/inf group. **(C)** Representative image of phalloidin staining of a larva at 4 dpi from the veh/non-inf group, captured using fluorescence microscopy. Data were pooled from three independent experiments, and data shown are means ± s.e.m. . *P<0.05; **P<0.01; *P<0.001; **P<0.0001.

of regulated genes in the veh/inf group and the beclo/inf group, which indicates that the effect of beclomethasone on transcription during an infection is limited. In addition, volcano plots of the gene regulation (p value plotted versus fold change for each individual gene) showed that the plots of the veh/inf group and the beclo/inf group were very similar (Figs.4A,B).

Furthermore, the plot for the beclo/non-inf group mainly showed lower fold changes for the upregulated genes (Fig.4C), and all plots showed more upregulated genes than downregulated ones (Figs.4A,B,C).

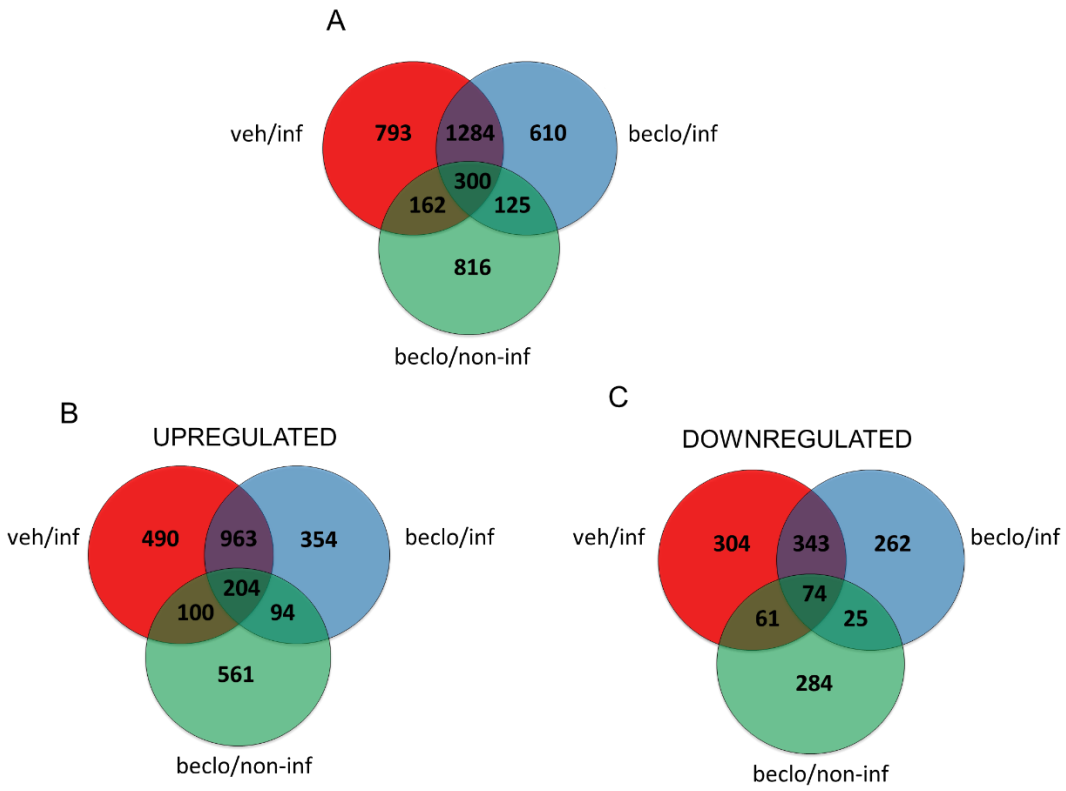


Figure 3. Venn diagrams showing numbers of regulated genes per treatment and overlaps between clusters of regulated genes. (A) Clusters of genes significantly regulated (compared to veh/non-inf treatment) by vehicle and infection (veh/inf), beclomethasone treatment (beclo/non-inf) and the combined infection and beclomethasone treatment (beclo/inf). The diagram shows that the largest number of genes was regulated by infection (veh/inf: 2542 genes). Combined treatment changed the expression of a relatively high number of genes as well (beclo/inf: 2319 genes), and the beclomethasone itself resulted in the lowest number of changes in gene expression (beclo/non-inf: 1407 genes). A large overlap (1284 genes) was observed between the veh/inf and the beclo/inf cluster. **(B)** Clusters of genes upregulated by the three different treatments (compared to veh/non-inf treatment). **(C)** Clusters of downregulated genes by the three different treatments (compared to veh/non-inf treatment).

To further investigate how beclomethasone treatment affects the transcriptome of *M. marinum* infected larvae, we compared the beclo/inf with the veh/inf group. A cluster of only 246 genes showed significantly different expression levels between these two groups (Suppl.Table 4), confirming the limited number of differently regulated genes between these two groups that we saw in the earlier analysis. This cluster should contain the genes that are responsible for the differences in glucose concentration and muscle mass that were observed between these two groups. Analysis of gene ontology (using the DAVID online functional annotation tool) showed enrichment of gene ontology groups related to processes involved in immune response ('defense response to virus', 'response to xenobiotic stimuli', 'humoral immune response' and 'chemotaxis'), as well as many general processes (such as, 'transport' and 'signal transduction' and 'potassium ion transmembrane transport') (Table 1).

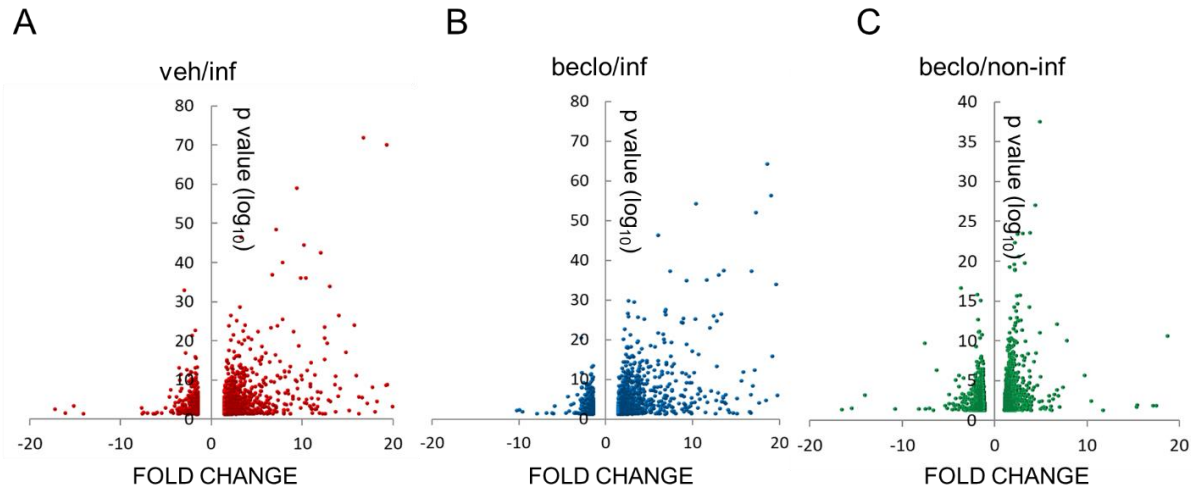


Figure 4. Volcano plots presenting the p-value as a function of the fold change for individual genes. (A) Gene regulation in the veh/inf group. **(B)** Gene regulation in the beclo/inf group. **(C)** Gene regulation in the beclo/non-inf group.

A look at significantly enriched KEGG pathways showed enrichment of four pathways, of which two pathways were connected to immunity: 'intestinal immune network' and 'cytokine-cytokine receptor interaction'. The other two significantly enriched pathways were 'retinol metabolism' and 'PPAR signaling pathway' (Table 2)(Suppl.Table 5).

A more detailed analysis of gene ontology showed that out of the 246 genes differentially expressed between the veh/inf and the beclo/inf group. Interestingly, 40 of these genes are directly or indirectly related to glucose metabolism and the insulin signaling pathway. Of these 40 genes, 25 were expressed at a lower level in the presence of beclomethasone (Suppl.Table 6). Some of these downregulated genes are known to be involved in glucose metabolism: *ptgs2a*[45,46], *slc27a1a*[47], *kcnj1a.4* and *kcnj1a.5*[48], *nfe2*[49,50] and *claudin*[51]. Downregulated genes that are known to be directly connected with insulin resistance and T2DM2 were *ghsrb* (which is also involved in leptin secretion and appetite stimulation[52]), *tent5c* (also connected with obesity[53]), *bcl6b*[54], *cxcr4a* (also an immune-related gene) and *serpine1*[55]. In this cluster of genes, we also distinguished two genes that are known to be involved in the PPAR signaling pathway, *slc27a1a* and *fabp7b*[56], one gene that regulates myoblast proliferation and differentiation, *odc1*, and *socs3a* which has been suggested to play a role in the development of leptin resistance[57] (Suppl.Table 6).

Only 15 genes were expressed at a higher level in the presence of beclomethasone (Suppl.Table 7), and some of these genes have been shown to be involved in the pathogenesis of T2DM: *arnt* and *ace2,* and *alpi.2* (known to act protectively against DM2)[58]. Other genes in

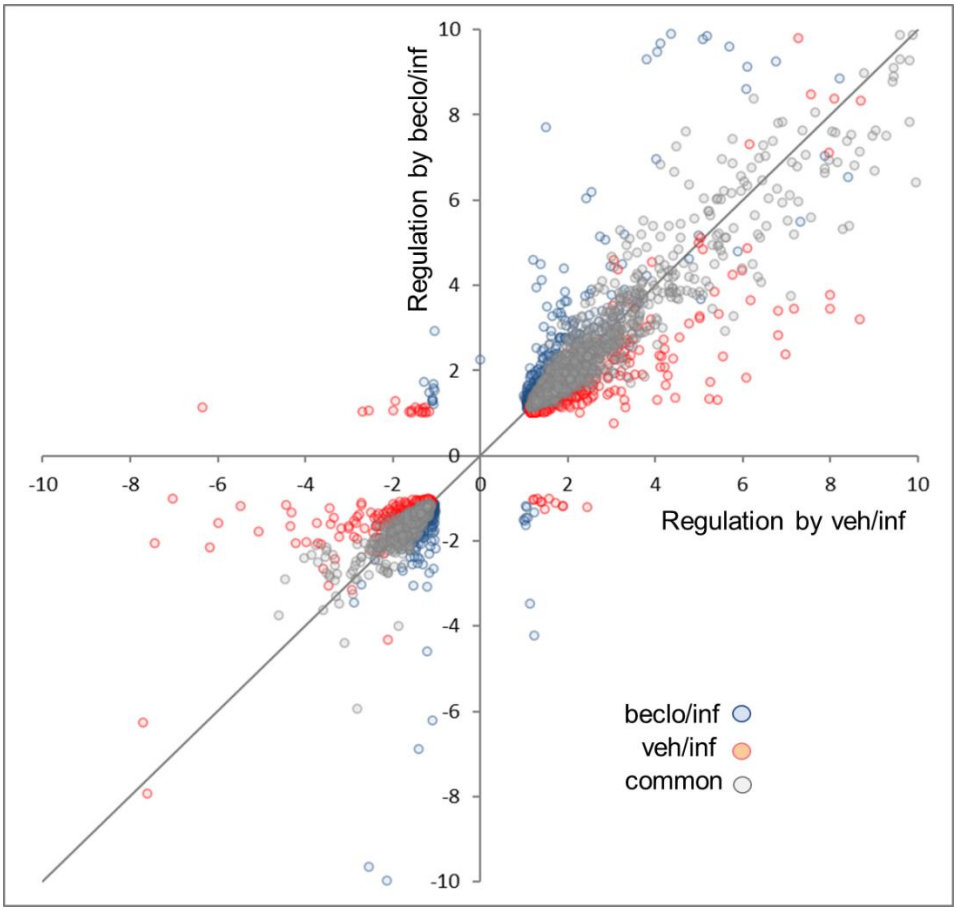


Figure 5. Scatter plot showing the effect of beclomethasone treatment on infection-regulated gene expression. For all genes showing significant regulation upon veh/inf treatment or the beclo/inf treatment, the fold change in the beclo/inf group was plotted as a function of the fold change due in the veh/inf group. Genes significantly regulated in the veh/inf group are indicated by red markers, genes significantly regulated in the beclo/inf group by blue markers, and genes significantly regulated by both treatments are indicated by gray markers. The diagonal line indicates the point at which beclomethasone treatment does not affect infection-induced changes. The plot shows that the vast majority of genes is similarly regulated in the absence and presence of beclomethasone, indicating that beclomethasone hardly affects the transcriptional response in the presence of an infection. Significantly regulated genes were selected by using a $p<0.05$ and $|FoldChange|>1.5$ cutoff.

this group were *grp,* that has been implicated in gastrointestinal and metabolic diseases[59], *serpinb1*, which is highly expressed in individuals with both diabetes and obesity[55], and the chitinase genes *chia.3* and *chia.1* which are known to be highly expressed in obesity and insulin resistance[60]. In addition, *ca4b* was found in this group, which lowers glucose concentration in the plasma[61], as well as *dgat1a* that plays a crucial role in the triglyceride and glucose metabolism[62] (Suppl.Table 7). Genes involved in lipid metabolism were *pdzk1*, involved in cholesterol metabolism[63], *faah2b* that participates in fat storage via the leptin signaling pathway[64], *asah2* which is essential for degradation of sphingolipids[65], and *igf1*[66] (Suppl.Table 7). Other upregulated genes were *rgs9b*, which is involved in *leptin a* transcription[67], and *abcc6a* of which insufficiency leads to mineralization of cardiovascular, ocular and dermal tissues[68] (Suppl.Table 7).

Discussion

In the present study, we have investigated whether glucocorticoid treatment alters the metabolic changes during *Mycobacterium marinum* infection in zebrafish. Our data show that the infection decreases the muscle mass and increases the glucose levels in the larvae, reflecting the metabolic changes related to infection-induced cachexia. Treatment with the glucocorticoid beclomethasone attenuated the decrease in muscle mass and abolished the increase in glucose concentration. RNA-sequencing analysis was performed to unravel the molecular mechanisms underlying these metabolic changes. Our data showed that beclomethasone had a relatively minor effect on transcription during *Mycobacterium marinum* infection, suggesting that the infection induces a glucocorticoid-resistant state. Still, a number of immunity- and metabolism- related genes were identified of which

Table 1. Gene ontology for genes showing significantly different expression levels between the veh/inf and beclo/inf groups. Performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

TERM	GENES
Chemotaxis	4
G-protein coupled receptor signaling pathway	14
Defense response to virus	3
Transport	15
Intercellular signal transduction	7
Chitin metabolic process	2
Thrombin receptor signaling pathway	2
Potassium ion transport	4
Humoral immune response	2
Chitin catabolic process	2
Signal transduction	15
Potassium ion transmembrane transport	3
Response to xenobiotic stimulus	2
Metabolic process	7

Table 2. Pathway analysis for genes showing significantly different expression levels between the veh/inf and beclo/inf groups, performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

PATHWAY	GENES
Retinol metabolism	3
Intestinal immune network for IgA production	3
Cytokine-cytokine receptor interaction	4
PPAR signaling pathway	3

beclomethasone altered the expression level during the infection. These genes may reveal the molecular pathways responsible for the infection-induced metabolic changes.

Although *Mycobacterium marinum* infection in zebrafish larvae is a well-established model for studying the pathogenesis of TB, the metabolic changes during this infection have hardly been studied. In the present study, we demonstrate a decrease in muscle mass and increased glucose levels in infected larvae. These changes are highly reminiscent of the metabolic alterations observed in human TB patients, which include increased protein degradation and reduced protein anabolism resulting in muscle atrophy and increased gluconeogenesis possibly resulting from insulin resistance, which may lead to T2DM[69]. Previously, the zebrafish *Mycobacterium marinum* infection was used to study changes in lipid metabolism, which also resembled alterations observed in TB patients[70]. Thus, the zebrafish *Mycobacterium marinum* infection model system shows translational value for studies on the metabolic changes that are observed during TB.

When the infected larvae were treated with the synthetic glucocorticoid beclomethasone, the infection-induced increase in glucose concentration was abolished and the reduction in muscle mass was attenuated. Interestingly, in the absence of an infection beclomethasone increased the glucose levels. This is a well-established glucocorticoid effect[71], that has previously been demonstrated in zebrafish embryos as well[38]. In contrast, beclomethasone did not affect the muscle mass in the uninfected larvae. Apparently, beclomethasone and infection do not simply have opposite effects on glucose levels and muscle mass, but beclomethasone modulates the metabolic response of the larvae to the infection, in line with conditional, context-specific action of the GR described in many other studies[72]. The observed attenuation of the metabolic effects was not due to diminished levels of infection, since the bacterial burden in the beclomethasone-treated larvae was actually increased.

In order to unravel the molecular mechanisms that lead to the metabolic changes during the *Mycobacterium marinum* infection, a transcriptome analysis was performed using RNA-

sequencing. Even though this analysis revealed that the infection induced a state of reduced glucocorticoid sensitivity, beclomethasone did modulate the transcription of a number of genes during infection, and these changes most likely underlie the altered glucose levels and muscle mass and studying this relatively small number of genes with altered expression could reveal crucial pathways contributing to the wasting syndrome in TB. Gene ontology analysis revealed that this cluster contains many-immune related genes, but also metabolism-related genes were enriched in this cluster. Out of 40 metabolism-regulated genes in this cluster, 25 genes showed a lower expression level in the presence of beclomethasone. Most of these genes were involved in glucose metabolism, leptin signaling pathway, insulin resistance and the pathogenesis of T2DM. In the cluster of 15 genes that showed a higher expression level in the presence of beclomethasone, most genes were associated with other metabolic processes, responsible for fat storage and cholesterol metabolism. However, some of them are also participating in the insulin signaling pathway.

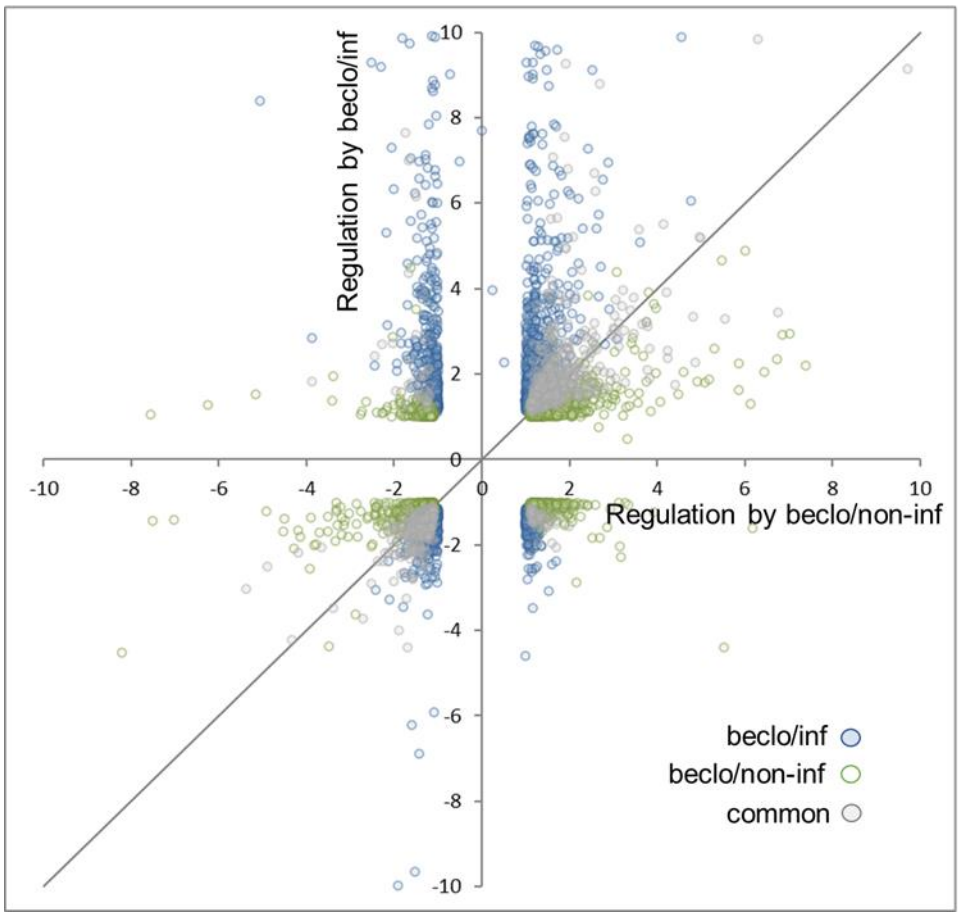
The glucocorticoid-resistant state induced by the *Mycobacterium marinum* infection was reflected by the RNA-sequencing results in two ways. First, the Venn diagrams (Fig.3) and scatter plot (Suppl.Fig.1) show that there was very little similarity between the gene regulation by beclomethasone in the absence of infection (beclo/non-inf) and the regulation by the combined beclomethasone/infection treatment (beclo/inf). These results indicate that most of the beclomethasone-induced gene regulation that is observed in the absence of infection does not occur in infected larvae. Second, when comparing the regulation in the beclo/inf and the veh/inf groups using the Venn diagrams (Fig.3) and scatter plot (Fig.5), we found that most of the gene regulation of highly similar between these groups, indicating that the regulation of the vast majority of genes is not affected by the beclomethasone administration. A direct comparison between these groups showed a cluster of only 246 genes regulated differently. These data show a striking contrast to previous work from our laboratory in which zebrafish larvae were subjected to tail fin amputation. In these experiments, beclomethasone attenuated almost the entire transcriptional response to the amputation, suggesting that the resistance to beclomethasone observed in the present study during *Mycobacterium marinum* infection, is specific to certain infectious and/or inflammatory conditions.

The observed glucocorticoid-resistant state has been associated with TB and other infectious and/or inflammatory conditions. Previously, it was shown in PBMCs from patients suffering from severe TB that the ratio between the expression level of the canonical GR and an alternative splice variant that may act as a dominant-negative inhibitor, GRβ, was reduced. This reduced ratio appeared to correlate with levels of pro-inflammatory cytokines, and could underlie a decreased sensitivity to glucocorticoids[732,74]. Bacterial lipopolysaccharide (LPS) has been shown to reduce glucocorticoid sensitivity in in various cell types[75,76,77], and a variety of bacterial toxins, like the anthrax lethal toxin have been shown to reduce GR function in a variety of cell types *in vitro* and *in vivo*[74]. Viral infections have been demonstrated to decrease

glucocorticoid sensitivity in lung epithelial cells[78,79,80], and the NF-κB, JNK[81] and TGF-β[82] pathways have been shown to be implicated, possibly resulting in posttranslational modification of the GR protein[79].

In summary, the results of this study show that *Mycobacterium marinum* infection in zebrafish larvae represents an interesting model system to investigate mechanisms underlying cachexia in human TB patients. We observed increased glucose levels and reduced muscle mass upon infection. These changes were abolished and attenuated respectively by treatment with the glucocorticoid beclomethasone, even though transcriptome analysis showed that the larvae were in a glucocorticoid-resistant state. The few transcriptional changes elicited by beclomethasone suggest that genes involved in glucose metabolism, insulin and leptin signaling play a crucial role in the observed metabolic changes resembling cachexia.

Supplementary materials



Supplemental Figure 1. Scatter plot showing the effect of infection on beclomethasone treatment. For all genes showing significant regulation upon beclo/non-inf treatment or the beclo/inf treatment, the fold change in the beclo/inf group was plotted as a function of the fold change due in the beclo/non-inf group. Genes significantly regulated beclo/non-inf group are indicated by green markers, genes significantly regulated in the beclo/inf group by blue markers, and genes significantly regulated in both conditions are indicated by gray markers. Significantly regulated genes were selected by using a $p<0.05$ and $|FoldChange|>1.5$ cutoff.

Supplemental Table 5. Enriched gene ontology groups in the cluster of significantly regulated genes between the veh/inf and beclo/inf groups, determined using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8.

ENRICHMENT SCORE: 2.84	COUNT
Transmembrane helix	63
Transmembrane	63
Membrane	66
Integral component of membrane	65
Membrane	64
ENRICHMENT SCORE: 1.65	**COUNT**
Chemokine receptor family	4
Chemokine receptor activity	4
Cxc chemokine receptor 4	3
Chemotaxis	4
G-protein coupled receptor signaling pathway	14
G-protein coupled receptor , rhodopsin like	11
Intestinal immune network for Iga production	3
Signal transduction activity	15
Cytokine-cytokine receptor interaction	4
ENRICHMENT SCORE: 1.11	**COUNT**
Metal ion binding	21
Zinc ion binding	12
Zinc finger	8
B30.2/spry domain	7
SPRY	6
BBOX	5

Supplemental Table 6. List of significantly downregulated genes between the veh/inf and beclo/inf groups, and their possible role in the development of metabolic disorders. The analysis of their possible roles was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8, and manually by using zfin.org and pubmed.ncbi.nlm.nih.gov.

GENE ID	NAME	PATHWAYS AND PROCESSES
ENSDARG00000004539	ptgs2a prostaglandin-endoperoxide synthase 2a	Prostaglandins signaling pathway Wasting, glucose metabolism, diabetes
ENSDARG00000006240	slc27a1a solute carrier family 27 (fatty acid transporter)	PPAR Signaling Pathway lipid uptake, metabolism, and transport in the Larval Zebrafish
ENSDARG00000090635 *ENSDARG00000089060*	kcnj1a.4 kcnj1a.5 potassium inwardly-rectifying channel, subfamily J, member 1a,	change in fasting glucose, onset diabetes glucose metabolism
ENSDARG00000009544	claudin b	glucose metabolism, diabetes
ENSDARG00000035198	gcnt4a glucosaminyl (N-acetyl) transferase 4, core 2, a	carbohydrate metabolism, lipid metabolism
ENSDARG00000056561	asb11 ankyrin repeat and SOCS box containing 11	pancreatic β-cells function, diabetes
ENSDARG00000056021	sostdc1b sclerostin domain containing 1b	Bmp/Wnt inhibitor signaling pathway diabetes type 1, enhances pancreatic islet function
ENSDARG00000057117	ghsrb growth hormone secretagogue receptor b	appetite leptin secretion diabetes type II
ENSDARG00000020606	nfe2 nuclear factor, erythroid 2	promote gluconeogenesis, onset and progression of type 2 diabetes mellitus inflammatory cytokines
ENSDARG00000017653	rgs13 regulator of G protein signaling 13	Inflammation, diabetes type I
ENSDARG00000010437	tent5c terminal nucleotidyltransferase 5C	Diabetes, obesity
ENSDARG00000056784	aire autoimmune regulator	type I diabetes, insulin autoimmunity
ENSDARG00000034650	fabp7b fatty acid binding protein 7, brain, b	PPAR signaling pathway diabetes type II, lipid metabolism

ENSDARG00000029866	slc6a14 solute carrier family 6 member 14	obesity
ENSDARG00000069335	bcl6b transcription repressor	diabetes type II
ENSDARG00000007377	odc1 ornithine decarboxylase	regulates myoblast proliferation and differentiation
ENSDARG00000056795	serpine 1	diabetes type II, obesity
ENSDARG00000025428	socs3a suppressor of cytokine signaling 3a	insulin resistance, leptin signaling
ENSDARG00000100564	sil1 SIL1 nucleotide exchange factor	glucose-stimulated insulin secretion
ENSDARG00000077130	bcl10 BCL10 immune signaling adaptor	diabetes type II, metabolic syndrome
ENSDARG00000015902	stat6 signal transducer and activator of transcription 6, interleukin-4 induced	beta cells, diabetes type I
ENSDARG00000088048	fgf18a fibroblast growth factor 18a	Obesity, diabetes type II
ENSDARG00000010169	myd88 MYD88 innate immune signal transduction adaptor	increases risk of diabetes, obesity
ENSDARG00000013855	slc12a3 solute carrier family 12 member 3	Diabetes, diabetic nephropathy

Supplemental Table 7. List of significantly upregulated genes between the veh/inf and beclo/inf groups, and their possible roles in the development of metabolic disorders. The analysis of their possible roles was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8, and manually by using zfin.org and pubmed.ncbi.nlm.nih.gov.

GENE ID	NAME	PATHWAYS AND PROCESSES
ENSDARG00000021855	ARNT Aryl hydrocarbon receptor nuclear translocator	diabetes type II, ARNT expression is reduced in diabetic human islets and β cell
ENSDARG00000045156	rgs9b regulator of G protein signaling 9b	leptin a transcription
ENSDARG00000022261	pdzk1 PDZ domain containing 1	cholesterol metabolism
ENSDARG00000016918	ace2 angiotensin I converting enzyme 2	diabetes type II, diabetic nephropathy
ENSDARG00000053774	alpi.2 alkaline phosphatase, intestinal, tandem duplicate 2	is protective against type 2 diabetes mellitus
ENSDARG00000054786	faah2b fatty acid amide hydrolase 2b	fat storage
ENSDARG00000094132	IGF-1 insulin-like growth factor 1	Low- and high-normal IGF-I levels are both related to insulin resistance.
ENSDARG00000043074	grp gastrin-releasing peptide	both gastrointestinal inflammatory states and classical chronic metabolic diseases such as diabetes
ENSDARG00000042293	ca4b carbonic anhydrase IV b	type I diabetes
ENSDARG00000103503	dgat1a diacylglycerol O-acyltransferase 1a	metabolic processes, conversion of diacylglycerol and fatty acyl CoA to triacylglycerol
ENSDARG00000009612	chia.3 chia.1 chitinase, acidic.3, acidic.1	increased in CHI3L1 levels in obesity, insulin resistance
ENSDARG00000016750	abcc6a ATP-binding cassette, sub-family C	diabetic vascular calcification, elastic fibre mineralisation and fragmentation
ENSDARG00000012829	asah2 N-acylsphingosine amidohydrolase 2	impaired glucose tolerance, lipids and amino acids metabolism
ENSDARG00000055416	serpinb1	Promotes Pancreatic β Cell Proliferation

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