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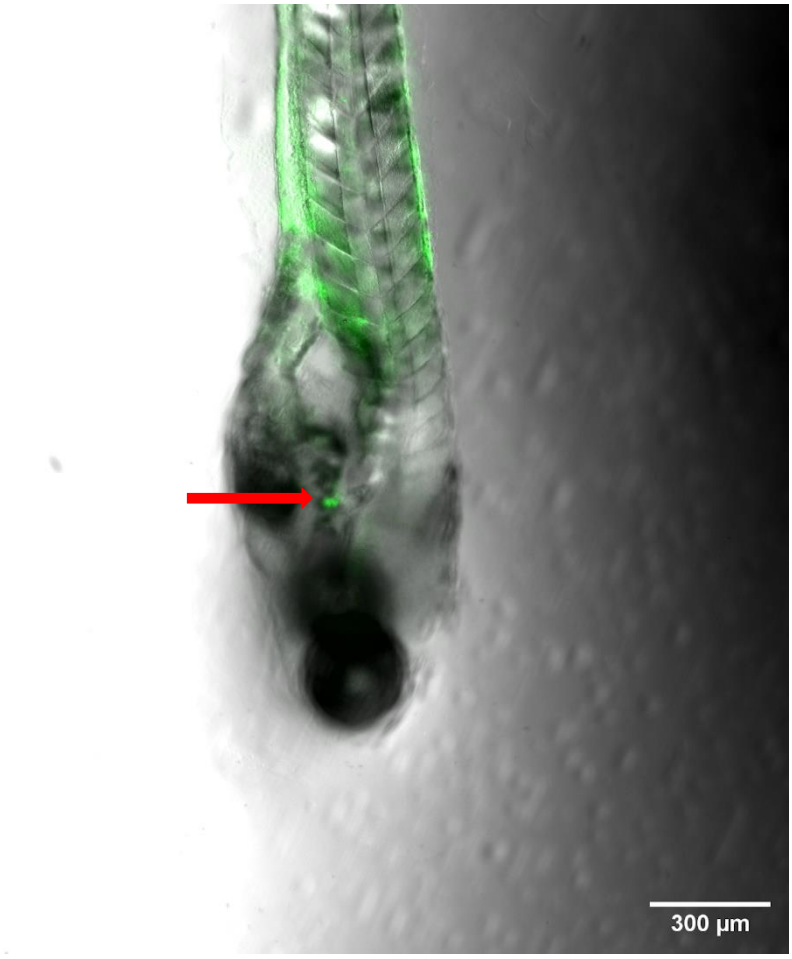
Insulin expression by beta cells affected after exocrine damage in a zebrafish model

Bachelor project
Developing and using fluorescence markers to unravel the effect of exocrine damage on the beta cells in the pancreas in zebrafish larvae



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Research Project Biomedical Sciences (IIb)

Abstract

Type 1 Diabetes (T1D) is an autoimmune disorder of the beta cells affecting insulin production. T1D has been quested for over 3000 years, but with new animal models like Zebrafish, we can give a better understanding of the mechanisms. It was found that in people with T1D the weight of the pancreas was decreased, which could be interpreted as the exocrine part also being affected in people with T1D. Our research focused on if Exocrine dysfunction has a direct effect on the beta cell insulin production. Furthermore the procedure for modelling T1D was optimised using different antibodies. We found that the only antibody staining that worked was for insulin. The model used for exocrine dysfunction are zebrafish expressing nitroreductase (NTR) on the exocrine cells, that when adding nifurpirinol (NFP) would cause exocrine dysfunction. Compared to a control group, the treated zebrafish that had exocrine dysfunction showed less insulin production from the beta cells and lower beta cell count (6.4 on average compared to 19.7 in the non treated group). These results support the idea that exocrine dysfunction may play an integral role in the cause or the maintenance of beta cell loss in T1D. Therefore, this may have impact on future research as a potential explanation for T1D beta cell loss.

Introduction

Autoimmune diseases have been on the rise in the last decade and seem to be rising for the foreseeable future. One of the most well-known autoimmune diseases is Type 1 Diabetes (T1D). T1D is an autoimmune disease resulting in beta cell destruction in the pancreas. Even though the cause of T1D has been quested over 3000 years, the exact autoimmune mechanism that initiates the destruction of the beta cells remains unclear (de Boer et al. 2020, Karamanou et al. 2016). For T1D Insulin has been the most common and one of the only few treatments for T1D (Nicolucci et al. 2008). Other treatments for T1D include islet of Langerhans and pancreas transplantation. Both treatments are more invasive and less common, because of how well insulin works for people with T1D. (American Diabetes Association. 2000).

A part in understanding type 1 diabetes starts with understanding the development of the disease. T1D can go through multiple stages before becoming symptomatic. The first stage being characterised by T1D–associated autoantibodies against Langerhans islets (Insel et al. 2015). In the second stage there is already glucose tolerance, but it is only in the third stage that it becomes symptomatic, presenting in different ways such as weight loss, fatigue and polydipsia.

The animal model used is the Zebrafish. Zebrafish are especially useful for in vivo models with a light microscope. They are small in size, with typically only one large Islet and 3-6 smaller islets that occupy the pancreas. Besides their size the zebrafish are transparent and the time it takes before they are sacrificed is only five days making them quick and easy to use for analyses. Furthermore, Zebrafish can be used as model for experiments on the pancreas, because of the genetic make up of their pancreas (Faraj et al 2022). Faraj et al (2022), tell us that 70% of the human genes have one obvious orthologue gene in zebrafish (Howe et al. 2013). In addition, from the 3,176 genes in the Online Mendelian Inheritance in Man database, 2,601 bear morbidity descriptions and related back to zebrafish orthologues. This means that 82% of morbid genes can be related back to zebrafish. Additionally, the pancreas of the zebrafish is also developed within three days, and the zebrafish develops in water making applications of drug easier(Kinkel & Prince 2009). The last advantage of using Zebrafish is that until 120 hours post-fertilization (hpf) they are not considered experimental animals. Therefore, up till five days the zebrafish are outside the legislations for experimental animals in the European Union (Faraj et al 2022).

The morphology of the pancreas of humans and zebrafish consist of both exocrine and endocrine gland. The zebrafish is simpler, generally showing only one islet of Langerhans before 120 hpf (Faraj et al 2022). The exocrine is the same in both mammalians and zebrafish, responsible for the secretion of digestive enzymes. In the endocrine cells the glucose homeostasis is maintained. There are different cells all releasing their specific hormone. The most known once are: Beta cells release insulin, alpha cells release glucagon. The exocrine part that contain acinar cells transport digestive enzymes towards the small intestine. (Yee et al. 2005).

The goal of the experiment is to find out the relationship between the exocrine pancreas damage and T1D. This experiment is based on experiments by for example Foster, et al (2020) where they looked specifically at T1D individuals. They showed that for T1D, the pancreas has a significantly smaller size compared to control groups. When looking at the morphology of the pancreas, 95% of the weight comes from the exocrine part. Acinar cells that are the functional part of the exocrine part, only amounting to two percent of this. In individuals with T1D, they

found that the volume of the exocrine part of the pancreas was decreased between 18 to 52% depending on the method (Foster et al. 2020). Another study looked at children between the ages of 2-8 years old who had T1D using ultrasound and measurements of faecal elastase-1 (FE-1) (Augustine et al. 2020). FE-1 was used to measure the function of the exocrine cells. Compared to control groups, children with T1D showed 30% lower FE-1 levels. 30% lower FE-1 levels mean that even in children with T1D the exocrine function is already compromised. However, in the majority of cases for children the FE-1, even though it is considerably lower, can still stay in the normal range. Because the exocrine part makes up 95% of the weight of the pancreas such a significant difference in weight must be largely associated with a decrease in exocrine part. Lastly, a study from Kondrashova et al. 2018 looked into preclinical stages of diabetes. Here they used stool samples to measure elastase and find out deviations in the exocrine functions. What they found was before the onset of diabetes when autoantibodies have already appeared the exocrine function decreases in the pre-clinical phase. Furthermore, they also mention that the exocrine part is affected by the inflammatory processes. Lastly that this could mean that the exocrine dysfunction could be a secondary effect or a causal effect.

The exact mechanism by which T1D originates is unknown, just as the mechanism by which the beta cells die. Previously, it was thought that autoimmune disorders were exclusively mediated by T-cells. (Citro et al 2021). However, the thinking of this has reshaped and in the killing of beta cells and exocrine cells, macrophages also play a role. They do this by activating cytokine pathways and killing the cells themselves.

The exact mechanism behind the decrease in exocrine function is unknown, however exocrine dysfunction resulting in beta cell stress has been hypothesised (Foster et al. 2020). Citro et al (2021), showed that exocrine damage is already shown after initiation of autoantibodies in the preclinical phase of T1D. This could mean that exocrine decrease can be a cause or consequence, understanding which one could be a imperative part in understanding T1D. Another newer study by Atkinson & Mirmira (2023), suggests evidence supports both notions and that both scenarios could be happening at the same time. (Roep et al. 2020).

The model used for this article will be the transgenic line zebrafish larvae a cross between +Tg(ins:GFP) and +Tg(elaA:PM-DEVD-mScarlet-NTR). These zebrafish express nitroreductase (NTR) specifically in their exocrine cells. In the experiments, nifurpirinol (NFP) simulates exocrine damage (Bergemann et al. 2018). NFP is converted then only by the NTR in the exocrine cells into a toxic compound, which then kills the exocrine cells. We use this model to analyse the effect of exocrine damage on the beta cells which will be done with whole mount immunostaining. With whole mounted immunostaining we will stain for insulin to get a view of the effect of NFP treatment on beta cells. Furthermore different antibodies will be stained in the paper one of them being l-plastin staining for the neutrophils and macrophages, because of their involvement in the killing of beta cells(Yang et al 2022). Furthermore, elastase, trypsin and amylase will be used for the staining of the exocrine part to see the effect of treatment on the cell count. Lastly, glucagon staining using whole mount immunostaining will be tried. Glucagon is there to determine if there is a potential effect on the alpha cells besides the beta cells after exocrine damage using NFP treatment.

The experiment is done to figure out the effect from exocrine dysfunction of the beta cell insulin secretion. We build the case that, targeting the exocrine part will result in Beta cell loss. The reasoning behind this is the significant decrease in exocrine volume in T1D individuals and inflammatory cells found in the exocrine part as well as the islets. (Rodriguez-Calvo et al. 2014, Atkinson & Mirmira 2023) Lastly, the method of analysing the zebrafish model for T1D was

optimised. Different staining methods will be mentioned for cell types relevant to the exocrine part, islets and inflammatory cells. We foresee that the implementation of these tools could help in the future for a better understanding of T1D and help in analysing the zebrafish model.

Materials & Methods

Zebrafish lines and husbandry For the first set of experiments, wild-type (AB) zebrafish (*Danio rerio*) were used. The fish were cultured in standard conditions: they were kept in a 14-10 hour light-dark cycle, and all procedures involving the fish were done according to the European and Dutch animal welfare legislation. The eggs were collected in a petri dish with E3 medium and kept at an optimal temperature of 28 ℃ (Urushibata et al. 2021). After 24 hours of incubation dead embryos, fish excrement and other impurities were removed from the medium. PTU was added to the medium and the embryos were kept at 28 ℃ for 72 hours.
Transgenic fish were handled in the same manner. These fish are a double transgenic line, crossed between +Tg(ins:GFP) fish and +Tg(elaA:PM-DEVD-mScarlet-NTR) fish, both developed in the BSCS department of the University Medical Centre Groningen (UMCG), creating a double transgenic line.
For the NFP treatment experiment, transgenic zebrafish were divided into two groups. NFP treated in which NFP (x1) was added to the medium at 4 dpf. The fish were kept for 12 hours at 28 ℃.

Fixing and immunostaining of zebrafish larvae after 12 hours all zebrafish larvae were euthanized using tricaine (MS-222) (10x). They were separated into 1.5 mL Eppendorf tubes. The larvae were centrifuged at 11000 rpm briefly to collect them at the bottom after which the medium was removed. 4% PFA in PBS solution was added to the tubes after which they were inverted 5 times to expose all the larvae to the solution. With the PFA solution the larvae were incubated at 4 ℃ for 24 hours. After 24 hours in 4% PFA the larvae were washed using PBS + 0.1% Tween 20 (PBS-T) at least twice for 5 min. A stepwise dehydration using methanol (MeOH) series followed in 25%, 50%, 75% and 100% methanol (dilution in PBS-T) with 5 min per step. The larvae were stored at -20 ℃ for a period of time between 1 and 3 days. When proceeding, larvae were stepwise rehydrated in 75%, 50%, 25% and 0% (PBS-T) methanol, with 5 min per step.

The PBS-T was removed from the larvae and they were incubated in 10 µg/mL proteinase K in PBS-T for 40 minutes or 1 hour. After an hour the proteinase K solution was removed and the larvae were fixed for 20 minutes in 4% PFA. The larvae were then washed using PBS-T twice for 5 minutes. The larvae were then incubated in a blocking solution (3% BSA in PBS-T, 0.1% donkey serum) for 2 hours. Afterwards, the larvae were incubated with primary antibody solution at 4℃ for at least 16 hours. Antibody dilutions are made with PBS-T/3% BSA. Supplementary Table 1 shows the primary antibodies and the secondary antibodies that were used for each experiment. After incubation of the first antibody the larvae were washed 10 times with PBS-T for 3 minutes per wash. All washes were done while gently rocking the tubes. The PBS-T was removed and the larvae were incubated with a secondary antibody solution at 4 ℃ for at least 16 hours. Dilutions were made with PBS-T/3%BSA mix. Furthermore, all samples were incubated with Dapi (Sigma, D8417-10 MG) in a dilution of 1:1000 (5 µg/ml).

Imaging After washing the larvae at least 6 times with PBS-T for 10-15 min, they were pipetted into WillCo-dishes®. Excess medium was discarded and 1% low-melting agarose gel (diluted in PBS) was added. The larvae were put on their left side and pushed into the medium to visualise

the pancreas on the right side. For microscopy a Leica Sp8 confocal microscope (using LasX software) and a Zeiss LCM 780 confocal microscope (using Zeiss ZEN lite software) were used. Objectives used: HC PL APO CS2 40x/1.30 OIL (Leica Sp8), HC PL APO CS2 63x/1.40 OIL (Leica Sp8), HC PL FLUOTAR 5x/0.15 DRY (Leica Sp8), EC Plan-Neofluar 40x/1.30 Oil DIC M27 (Zeiss LCM 780). Magnifications are mentioned in the respective figures. Images were analysed with ImageJ (Fiji) 2.14.0.

Quantification and statistical analysis

Images of the control (DMSO-incubated) vs. the exocrine damage (NFP-treated) model were compared in LasX Office. Insulin-producing cells were counted for each sample (control n=3, treated n=5). These numbers and the means were compared to each other using an unpaired two-sample t-test. The standard deviations were calculated and the p-value was considered significant at <0.05.

Supplementary table 1. Antibodies used for immunofluorescence stainings.

Antigen	**Target**	**Primary antibody**	**Secondary antibody**
Insulin	Beta cells	Anti-insulin (rabbit) 2:200 Abcam - AB210560	Alexa Fluor 594 1:250 (donkey, anti-rabbit) Invitrogen - A21207
Elastase	Exocrine cells	Anti-elastase (mouse) 1:200 Novus biologicals - lot 23436-2p170313	*Wild-type (AB) zebrafish experiments* Alexa Fluor 488 1:250 (donkey, anti-mouse) Invitrogen - A21202 *Transgenic zebrafish experiments* Dylight 649 1:250 (donkey, anti-rabbit) JacksonIR 711-495-152
L-plastin	Leukocytes	Anti-L-plastin (mouse) 4:200 Santa Cruz - sc-133218	Alexa Fluor 488 1:250 (donkey, anti-mouse)
Trypsin	Exocrine cells	Anti-trypsin (sheep) 15:200 ThermoFisher - PA5-47544	Alexa Fluor 647 1:250 (donkey, anti-sheep)
Glucagon	Alpha cells	Anti-glucagon (mouse) 1:200 Santa Cruz sc-57171	Alexa Fluor 488 1:250 (donkey, anti-mouse) Invitrogen A21202
Amylase	Exocrine cells	Anti-α-amylase (rabbit) 2:200 Sigma A8273-IVL	Dylight 649 1:250 (donkey, anti-rabbit) JacksonIR 711-495-152

Results

Methanol necessary for staining in zebrafish

Immunostaining zebrafish

The aim for the results is to find out if different antibodies and methods for whole mount immunostaining work and if beta cells are effected by exocrine dysfunction. Whole mount immunostaining will be used for every experiment, but the antibody staining differs. In figure 1 there is already the pancreas shown at the place of the red arrow, showing it is located between the yolk and the swim bladder.

For the first experiment wild type (AB) fish were used in which the goal is to analyse the beta cells in the pancreas. Transparency of the zebrafish done by adding PTU when incubated makes them easy to analyse using whole mount immunostaining (Gore et al. 2018). For the first experiment using wild type fish methanol dehydration step was compared to no methanol step as an aim to figure out if methanol is needed in whole mount immunostaining. In figure 2 single stainings of insulin and elastase were done. The results shown that for no methanol only insulin in red showed signal, and no elastase which should have lighten up in green. The same results for the methanol dehydration step. The difference between the no methanol and methanol insulin, was that the insulin staining for the fixation step was shown in the area of the ear where no DAPI is shown. While for the methanol dehydration step, insulin was in the area of the pancreas, also showing the location of the pancreas also in figure 1.

For the next part, in figure 3 it was a follow up experiment, but now without the methanol step using the transgenic fish line which is crossed partly with Tg(ins:GFP) fish to label the insulin in the beta cells. The results show no signal for insulin single staining, which should have shown in red. For the insulin + elastase double staining, for both their was also no signal, while elastase should have stained the exocrine part in green. Furthermore single staining of glucagon and amylase was down to find out if it is possible to stain the alpha and exocrine part respectively, However, both stainings should have shown in red, but no further signal. GFP signal staining for the beta cells, because of the transgenic line showed up in all stainings. This worked as a control to locate the beta cells and make sure the signals were not there.

Because the no methanol procedure did not show signal for the antibodies, for the next whole mount immunostaining the methanol dehydration step was used again. This experiment checked insulin staining using the transgenic line again. Compared to the first experiment elastase secondary antibody changed from Alexa fluor 488 to Dylight 649. Separate whole mount immunostainings were done with first single staining of insulin in red and elastase in green showing in figure 4. Elastase should stain the exocrine cells, however no signal was found in the area of the pancreas. Insulin did show signal compared to elastase, for the beta cells. From a different larvae a double staining of insulin + elastase was done. Insulin showed again signal in red, however no signal in green for the exocrine cells from the elastase was shown. GFP even though it is a transgenic line also didn't show signal as it was washed away in the methanol dehydration step,

Lastly, for the whole mount immunostaining again using the methanol dehydration step and using wild type fish other antibodies where tested and depicted in figure 5. In this experiment, trypsin was tested as an alternative staining for the exocrine part, because elastase showed no signal before. L-plastin was also stained, because it stains leukocytes like neutrophils and macrophages important in beta cell loss. In the same experiment insulin was stained again

which showed signal in red for the beta cells. Furthermore the control for it showed only DAPI signal and furthermore no other signal as it should have. Trypsin staining in green should have stained the exocrine part, however only background signal was found not indicating a specific structure around the area of the pancreas.

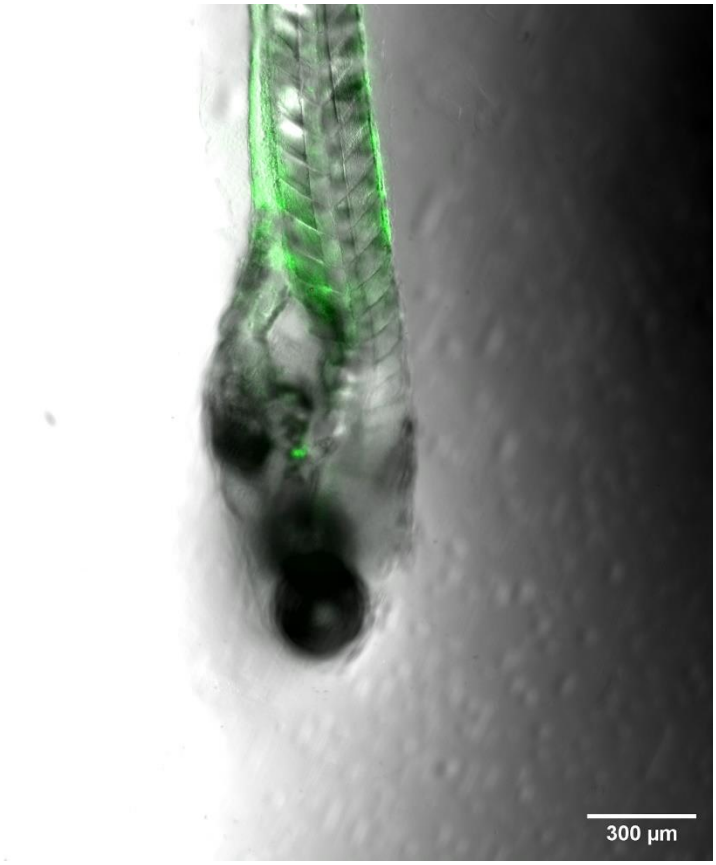


Figure 1. Brightfield image of a zebrafish larva (5x/0.15 DRY), 5 dpf, merged with GFP to show the beta cells of the pancreas. These are pointed out by the red arrow.

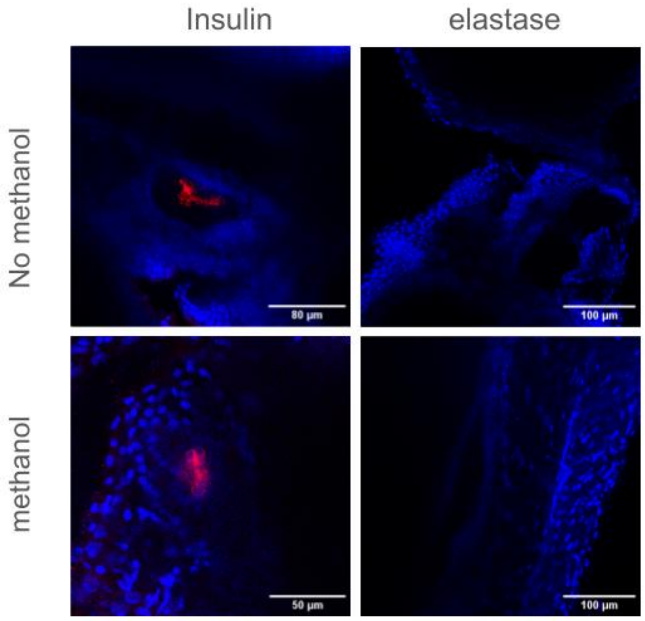
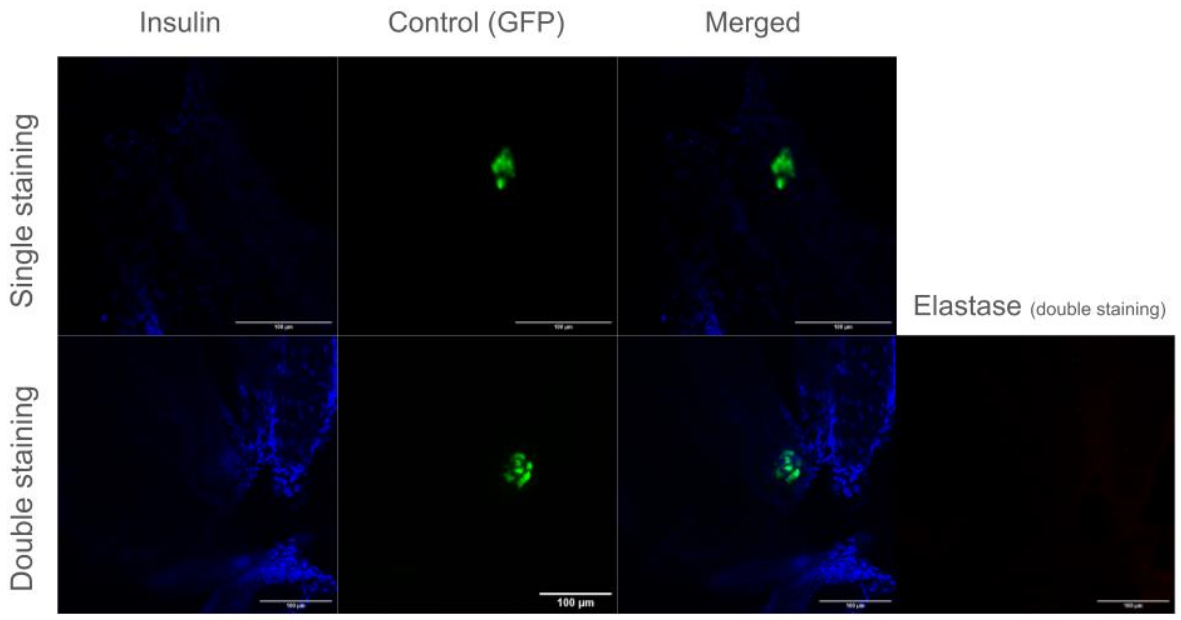


Figure 2. Insulin and DAPI staining in wild-type (AB) zebrafish larvae. The stainings labelled *fixation* regard the skipping of methanol dehydration and the bottom pictures did undergo methanol dehydration steps. Insulin signal was detected, but no elastase (green) for their respective stainings Scale bars, 100 µm for both elastase stainings, 80 µm for insulin no methanol and 50 µm for insulin methanol staining.

A



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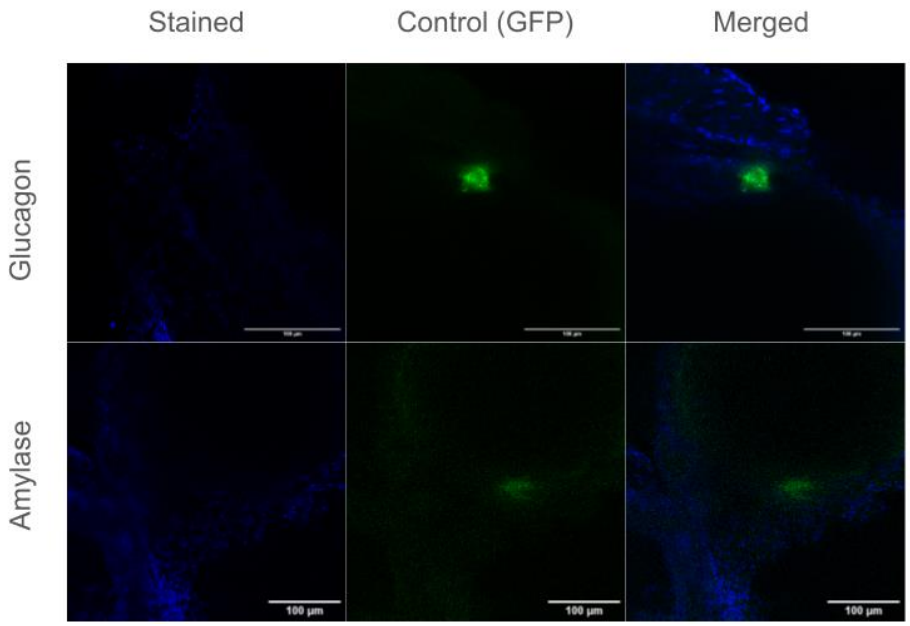


Figure 3. Insulin (red), glucagon (red), amylase (red) and DAPI (blue) were stained in transgenic line zebrafish larvae. Additionally, a double staining of insulin and elastase was performed. As control, GFP (green) is excited in the beta cells. There were no signals from the antibody stainings, possibly because the larvae were not stored in methanol. Only DAPI and GFP showing them both in the merged pictures as well. Scale bars, 100 µm for all stainings.

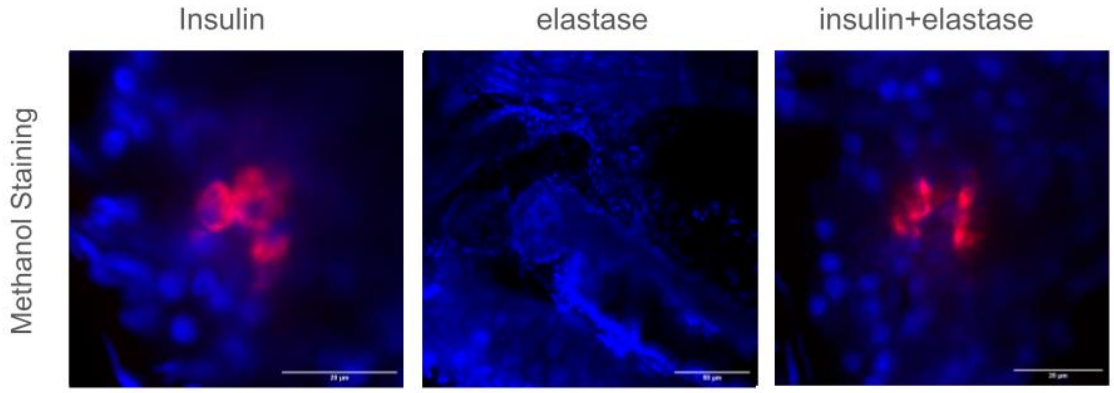


Figure 4. Single and double staining: insulin (red), elastase (not visible: channel 638-759 nm) and DAPI (blue) transgenic line zebrafish larvae using methanol dehydration step. The insulin signal in double and single staining shows the beta cells and no elastase signal was found. Samples for each staining were taken from different larvae. Scale bars, 20 µm for the insulin and insulin + elastase staining, 80 µm for elastase staining.

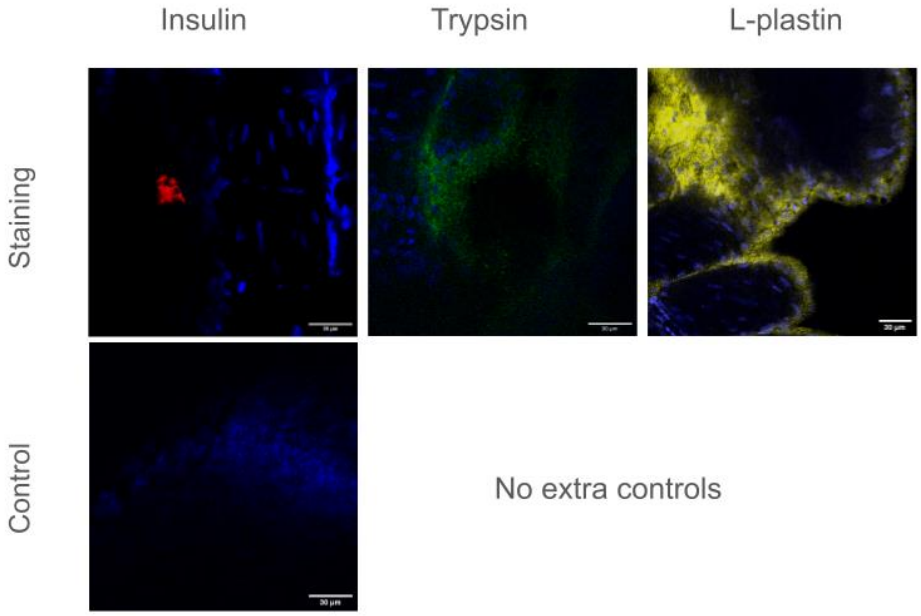


Figure 5. Single staining Insulin, trypsin, L-plastin and DAPI staining in AB-type zebrafish larvae. All stainings showed signals and the control only showed DAPI signals. The trypsin and insulin pictures were made around the area of the pancreas while the L-plastin was made more towards the posterior end of the yolk sac. Scale bars, 30 µm for all stainings.

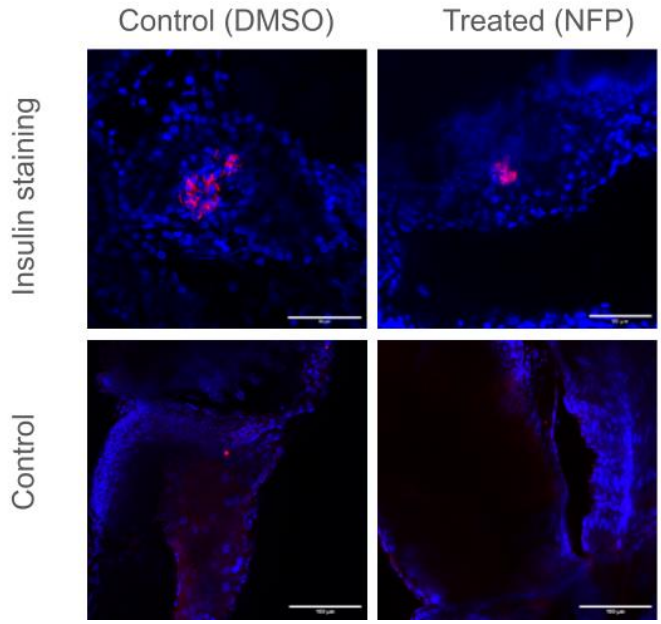
Exocrine dysfunction affect on beta cell insulin secretion.

For the final parts of the experiment the aim was to find out the effect of exocrine dysfunction on the beta cell insulin secretion. For this experiment transgenic fish larvae are used again. They are a cross between +Tg(ins:GFP) fish and +Tg(elaA:PM-DEVD-mScarlet-NTR) fish. Furthrmore these larvae express NTR, which when adding NFP would cause exocrine death. These NTR expressing fish are used to stimulate exocrine dysfunction to find out the effect on beta cell insulin production. Furthermore based on the previous experiment the only staining that will be used is insulin and the methanol dehydration step is included. Insulin is used as it was the only consistent antibody staining that worked staining for the beta cells. Next to insulin the methanol dehydration step is used, because with only fixation no reliable signal for exocrine or beta cells have been shown in the previous antibodies.

When dividing the larvae into groups of treated fish, treated with the NFP for 12 hours which causes exocrine dysfunction, and the untreated group. The untreated group was maintained in DMSO as a healthy control to the NFP treated group. When looking at the staining of both fish with insulin in figure 6a, there are both treated and untreated group signals displaying the beta cells. Both control groups got rabbit serum instead of anti-insulin antibody in the first antibody staining, which showed no signal for Insulin or beta cells, but only DAPI. When analysing the colour area of the staining the untreated group (DMSO) showed a wider area of signal for the beta cells compared to the treated group. The NFP treated group showed fewer and less dispersed signals on average compared to the treated group.

To figure out the exact effect of exocrine dysfunction after 12 hours of NFP the beta cells were counted from the insulin staining which showed cell structures for the beta cells. In figure 6b, the number of cells are depicted on the Y-axes with on the X-axes the untreated (DMSO) is compared to the treated (NFP) group. For the untreated group, 3 samples were taken with The first fish having 20 beta cells the second one 28 and the third one 11. For the treated fish 5 samples were taken, giving for the first fish 6 beta cells, the second 7, the third 9, the fourth 4 and the fifth 6. This gives an average of 19.7 beta cells for the untreated group, compared to 6.4 in the treated group. The average beta cells are also depicted figure 6b, which shows a standard deviation of 1.62 for the treated group and 6.94 for the treated group. Doing an unpaired T-test for the means of the groups gave a two tailed P-value gave of 0.0122 staying in the 95% confidence interval. This means that the difference in beta cell number between the groups is statistically significant. Lastly, for the controls no beta cells were found our counted meaning the experiment was successfully performed.

A



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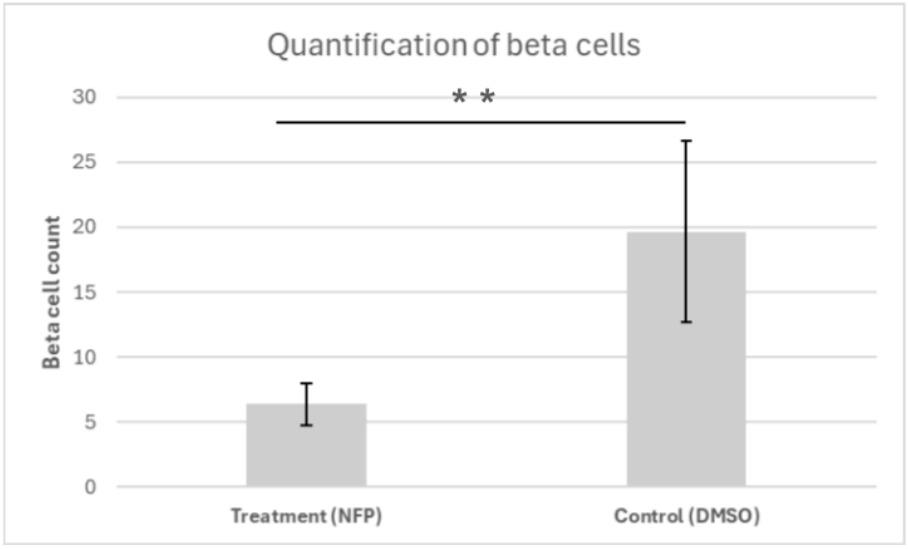

Figure 6a. Single staining of insulin was done in the top two pictures, comparing larvae being treated with NFP for 12 hours to larvae not being treated with NFP, but held in DMSO. The treated and DMSO larvae, both showed signals for insulin in the staining. In the bottom 2 pictures, a control group was used which was given the rabbit serum and showed no other signal except for DAPI.

Figure 6b. Shows a bar graph in which the mean of the beta cell count of the treated group (NFP for 12 hourr, 6.4) is compared to the control group (DMSO, 19.7). The standard deviations are shown: treatment group (NFP)=1.624807681, control group (DMSO) = 6.944222219). P-value is significant ($p = 0.0122$), $p < 0.01$ = ** .

Discussion

The aim of this study was to assess and optimise the staining for the pancreas of zebrafish larvae, so to analyse the effects of exocrine damage on the beta cells. The second main aim was to find the effect of exocrine dysfunction on beta cell insulin secretion. We first optimised the staining for larvae, through different types of experiments. First, it was important to find out if the methanol dehydration step was necessary and if we could stain the exocrine part, endocrine part and possible immune cells using antibodies. These cells were important for staining as they might give insight in the final experiment, treated versus untreated, as they can be related to T1D. The second part looked at the aim of exocrine dysfunction effect on beta cells. For the exocrine dysfunction NFP was added for 12 hours showing to affect the beta cells.

Antibody staining.

The protocol used for staining zebrafish is based on the protocol of Hammond-Weinberger & ZeRuth (2020). In the protocol it was debatable and unclear if the methanol dehydration step was necessary for the staining or only there for the long term storage of the larvae. It was found that the methanol dehydration step is necessary for the staining of different antibodies such as insulin. The reason for why methanol dehydration step might be necessary in immunostaining is, because it can also work as a fixative and permeabilise some cells (Thermo Fisher Scientific - IE. n.d.). From this it can be taken that methanol dehydration step is necessary to permeabilize the cells so the antibodies can enter them and stain. In figure 2 and 3, stainings for insulin, elastase, glucagon and amylase were performed without the methanol step. Insulin and elastase showed no signal for their target structures without the use of methanol. Furthermore, in figure 3, glucagon and amylase both stainings did not show result. Therefore, methanol dehydration is important to permeabilize the cells for the antibodies to stain. This concludes that no methanol does not permeabilize the cells enough to allow staining from different antibodies (Jamur & Oliver 2010).

Next single and double staining of insulin and elastase in separate larvae was done. This was to stain beta cells and exocrine cells, to get a good model for the final experiment. A different secondary antibody, Dylight 649 was used to stain elastase as the previous was unsuccessful. Insulin showed clear staining in both single and double staining for the beta cells, however elastase showed no signal. Next to elastase, trypsin staining for the exocrine part was also done. Trypsin should have stained the exocrine part of the pancreas, but fluorescent was found in the whole larvae. In different experiments trypsin is mostly found when staining in the pancreas and around the mouth, nose and gills (Kim et al. 2009). However, trypsin showed no signal in structures, suggesting the signal is most likely background noise. No control was used for the trypsin staining, therefore it is not possible to verify if the staining was background. Further research should be done with control samples and different concentrations. L-plastin is an actin-bundler that is calcium dependent and mostly stains leukocytes like neutrophils and macrophages (Linehan et al. 2022). Macrophages are important in T1D, because they can attract neutrophils and other chemokines (Yang et al 2022). Neutrophils have been found to cause ER stress in beta cells and kill them, making macrophages important to stain as they are an activator for this. Moreover, in line with other research of T1D and inflammatory cells, they found immune cells are in high concentration in the exocrine part of people with T1D(Rodriguez-Calvo, 2014). This adds on the importance of visualizing immune cells. It was found that macrophages High concentration of l-plastin are supposed to be found in the area of the tail (Linehan et al. 2022). However, l-plastin showed signals not representing structures for the

pancreas, but lit up most of the larvae, with the highest concentration being above the end of the yolk. This suggests that l-plastin staining did not work as the yolk is known to be auto fluorescent (Høgset et al. 2020).

To conclude this part, methanol dehydration step is necessary for the staining of zebrafish larvae. Moreover, because insulin was the only successful staining, the other antibodies should be tried to optimise for better visualisation of the pancreas as a T1D model. Suggested options for optimisation are changing the dilution of the antibodies. Next to this, the permeability can be increased by leaving for example the larvae longer in proteinase K or more tween in PBS. Lastly there should also be looked into different primary antibodies for the multiple targets.

Exocrine dysfunction effects beta cell insulin secretion

The results from the previous antibody stainings were going to be used to get the most optimal protocol for the final experiment. However, only insulin worked all the time with the methanol step and, therefore insulin will be stained to visualize the beta cells. The hypothesis for the last experiment was that with exocrine dysfunction, beta cell insulin secretion would be affected. When giving larvae, which express NTR on their exocrine cells, NFP exocrine dysfunction is stimulated, because NFP is converted by NTR to a toxic component killing the exocrine cells. The effect of NFP on the NTR larvae caused a decrease in beta cells compared to the DMSO control group. In figure 6b the average of the beta cell count is shown comparing the treatment (NFP) to the control (DMSO) group. This shows a clear difference of an average 19.4 for DMSO to 6.4 in the NFP larvae. To put it in other words there is less insulin being produced by the beta cells after exocrine dysfunction. Lower insulin production after exocrine dysfunction shows similarities with T1D in which studies for the preclinical phase, there is already less insulin produced and exocrine dysfunction (Koskinen et al. 2016). This suggests that upon exocrine dysfunction, the beta cells are secreting less insulin. The beta cell dysfunction could be caused by the ER stress, because in a article by Sahin et al. 2021, they found that upon exocrine damage the ER stress was increased in beta cells.

This research can implicate that in people with T1D, exocrine dysfunction is a possible cause of beta cell death. It was already shown that the weight of the exocrine part was decreased in people with T1D (Foster, et al. 2020). The weight decrease meant that exocrine cells were affected in T1D. Next to this in the preclinical phase of T1D when autoantibodies have appeared, exocrine function has already declined (Kondrashova et al. 2018). This correlates with our findings that exocrine dysfunction does play a role in beta cell insulin production reduction. As explained before ER stress was also found to increase in beta cells after exocrine dysfunction (Sahin et al. 2021). It is still unknown if the effect on the exocrine cells is a secondary effect of the beta cell death or if beta cell death was secondary to exocrine dysfunction (Foster, et al. 2020). Our findings show that exocrine cells can have a direct effect on beta cell insulin secretion. These results can be interpreted that exocrine cells are a part of the pathway in T1D for beta cell destruction. To sum up, previous findings show a decrease in weight and the correlation with ER stress increase in beta cells after exocrine dysfunction. From this, it can be suggested that Exocrine cell dysfunction in people with T1D causes beta cell death through inducing ER stress.

Lastly, this study is limited by the small sample size taken, only 3 and 5 for the treated and untreated group respectively. Therefore, these experiments should be replicated to confirm the results obtained. Furthermore we were also unable to visualize the exocrine damage. This means that even though we saw an effect on the beta cells we weren't able to validate that the

exocrine damage was happening, because the staining for it did not work. To finalize future studies should be conducted looking into the exact mechanism behind the exocrine dysfunction and how it possibly induces ER stress in the beta cells.

In conclusion, the data presented in this study favours the result that exocrine dysfunction causes a decrease in beta cell insulin secretion. Even though most stainings need further research and optimisation the insulin staining using the methanol step worked. The data found can help build the foundation for finding the mechanism behind T1D. For that to happen further research should be done to characterize what is exactly happening with the exocrine part in T1D. To come back to the zebrafish, the larvae made a good animal model that was quick to reproduce, fast to analyse, and was able to simulate endocrine dysfunction to give new information on T1D.

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