

# Improved regeneration and *de novo* bone formation in a diabetic zebrafish model treated with Paricalcitol and Cinacalcet

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## Abstract

Bone changes related to diabetes have been well established, but few strategies have been developed to prevent this growing health problem. In our work we propose to investigate the effects of calcitriol as well as of a vitamin D analog (paricalcitol) and a calcimimetic (cinacalcet), in fin regeneration and de novo mineralization in a zebrafish model of diabetes. Following exposure of diabetic transgenic Tg(*ins:nfsb*-mCherry) zebrafish to calcitriol, paricalcitol and cinacalcet, caudal fins were amputated to assess their effects on tissue regeneration. Caudal fin mineralized and regenerated areas were quantified by in vivo alizarin red staining. Quantitative real time PCR was performed using RNA from the vertebral column. Diabetic fish treated with cinacalcet and paricalcitol presented increased regenerated and mineralized areas when compared to non-treated diabetic group, while no significant increase was observed in non-diabetic fish treated with both drugs. Gene expression analysis showed an upregulation for *runt-related transcription factor 2b (runx2b)*, *bone gamma-carboxyglutamic acid-containing protein (bglap)*, *insulin a (insa)* and *insulin b (insb)* and a trend of increase for *sp7 transcription factor (sp7)* in diabetic groups treated with cinacalcet and paricalcitol. Expression of *insra* and *vdra* was upregulated in *both* diabetic and non-diabetic fish treated with cinacalcet. In non-diabetic fish treated with paricalcitol and cinacalcet a similar increase in gene expression could be observed but not so pronounced. The increased mineralization and regeneration in diabetic zebrafish treated with cinacalcet and paricalcitol can be explained by increased osteoblastic differentiation and increased insulin expression indicating pro-osteogenic potential of both drugs.

Keywords: Diabetes; bone; zebrafish; mineralization; vitamin D analogs; calcimimetics.

## Introduction

Prevalence of diabetes mellitus worldwide was estimated to be of 135 million in 1995 and is predicted to be of 300 million in the year 2025<sup>1</sup> leading to an increase in patients living with the risk of developing diabetes-related complications<sup>2</sup>. Diabetes has been strongly associated with development of bone fractures, that begins in childhood and extends throughout life, leading to morbidity and mortality<sup>3</sup>. Calcitriol (1,25-dihydroxyvitamin D3 - VitD) deficiency is common in chronic kidney disease (CKD) patients, leading to secondary hyperparathyroidism (SH)<sup>4</sup> and particularly in those diabetic patients that also undergo dialysis, there is a clear correlation with low 25-hydroxyvitamin D low serum<sup>5</sup>. Parathyroid hormone (PTH) stimulates bone resorption directly by activating *PTH* receptors in mesenchymal cells of the osteoblast lineage and indirectly by increasing differentiation and function of osteoclasts<sup>6</sup>. Bone loss and increased fracture risk are among the complications presented by CKD patients with SH<sup>7,8</sup> and are also seen in rodents<sup>9</sup>. VitD, the vitamin D analog paricalcitol<sup>10</sup> and the calcimimetic cinacalcet<sup>11</sup> have been used for the treatment of SH in CKD patients with beneficial effects in lowering PTH values and also in increasing bone mass<sup>7,8</sup>. Paricalcitol, by selectively activating the *vitamin D receptor (VDR)*<sup>12,13</sup>, and cinacalcet, by activating the *calcium sensing receptor (CaSR)*<sup>12,14</sup> in parathyroid, have been shown to be more efficient and fast in normalizing PTH levels and in reducing circulating bone turnover markers than VitD in patients with SH<sup>15,16</sup>. In addition, *VDR* and *CaSR* activation have been associated with increased insulin expression<sup>17,18</sup> and beneficial effects were reported under diabetic conditions<sup>19,20</sup>. Nevertheless, little is known on whether upregulation of insulin can occur in extrapancreatic tissues. In humans, extrapancreatic expression of insulin was first identified in brain<sup>21</sup> and then in thymus<sup>22</sup>, as a way for the immune system to recognize insulin, avoiding autoimmunity and  $\beta$ -cell destruction. Later Kojima et al (2003, 2004)<sup>23,24</sup> showed the presence of cells positive for insulin RNA in the liver, adipose tissue and bone marrow in several diabetic mice models, but not in nondiabetic mice. However, this insulin expressing cells had no impact on reducing hyperglycemia in diabetic mice. Later, Kojima et al.<sup>25</sup> demonstrated that beside not having any impact in regulating glucose levels, this Proins/TNF- $\alpha$ -expressing cells had their origin in bone marrow and then migrated to several parts of the body, initiating diabetic neuropathy<sup>26</sup>.

Cunha et al<sup>27</sup>, in their experiments with diabetic mice treated with streptozotocin, observed beneficial effects in the secretion of insulin by the tear film of the eye and showed that insulin was locally synthesized in the lachrymal gland<sup>27</sup>. With the current body of evidence, it is difficult to determine if extrapancreatic expression of insulin has any effect in glucose homeostasis, or if it serves other undetermined functions.

Zebrafish have been well established as a model for human diseases, spanning a wide range of human pathologies including genetic disorders and physiological processes that are known to be highly conserved throughout vertebrate evolution<sup>28</sup>. Recently it was demonstrated, under type 1 diabetic conditions, that fin regeneration was impaired in zebrafish with reduced cell proliferation and increased cell apoptosis<sup>29</sup>. We conducted experimental trials to understand if VitD, paricalcitol and cinacalcet, could have beneficial effects on caudal fin regeneration and bone mineralization in adult zebrafish and in operculum development of larvae under type 1 diabetic conditions. To test this hypothesis we used the transgenic Tg(*ins:nfsb*-mCherry) zebrafish that after being exposed to the pro-drug metronidazole becomes hyperglycemic and hypoinsulinemic due to beta-cell ablation, leading to a transient state of diabetes of 10 to 15 days prior to beta-cell regeneration<sup>30,31</sup>. To understand if vitamin D analogs and calcimimetics could have positive effects on bone development and mineralization, during a transient diabetes type I state, fin regeneration and bone mineralization were assessed and expression of bone and vitamin D metabolism markers evaluated.

## Methods

**Zebrafish strains and maintenance:** The transgenic Tg(*ins:nfsb*-mCherry) zebrafish line used in our experiments was kindly given by the Laboratory of Molecular Biology and Genetic Engineering, GIGA Research, Liege, Belgium. Transgenic zebrafish were maintained in a recirculating water system (Tecniplast, Buguggiate, Italy). All manipulations were performed by licensed researchers and conducted in accordance with principles and procedures following the guidelines from the Federation of Laboratory Animal Science Associations (FELASA) and in accordance with the EU and national regulations. The Tg(*ins:nfsb*-mCherry)

line generated by Pisharat et al.<sup>30</sup> with a Tübingen AB background, contains a construct in which the *nfsB* gene of *E. coli* and the fluorescent protein mCherry are inserted downstream to the promoter region of the *insa* gene. That bacterial gene encodes a nitroreductase (NTR) enzyme, that converts pro-drugs such as metronidazole (MET; Sigma-Aldrich, St. Louis, USA) to cytotoxins. By observation of loss mCherry fluorescence after MET treatment it is possible to visualize MET dependent  $\beta$ -cell ablation.

## Procedures

**Diabetes induction:** Larvae at 15 days post fertilization and male and female adults with 1 year old from the Tg(*ins:nfsB*-mCherry) zebrafish line were anesthetized with tricaine methanesulfonate (Sigma-Aldrich, St. Louis, USA)<sup>32</sup> and exposed to MET either by bath or through intraperitoneal (IP) injection at the concentration of 0,05M dissolved in citrate buffer (0,05M). Corresponding control groups were left untreated under the same housing conditions. Additionally, we have exposed non-transgenic siblings from a cross between a heterozygous transgenic and a wild type zebrafish, to discard potentially side effects of MET treatment in caudal fin regeneration. **Paricalcitol, cinacalcet and VitD treatments:** 72 hours post treatment (hpt) with MET, transgenic zebrafish were screened for loss of fluorescence due to  $\beta$ -cell ablation, as observed by Pisharath et al.<sup>30</sup>. To understand if treatments could induce bone alterations in diabetic conditions, 240 larvae and 145 adults were divided into experimental groups in triplicates and exposed by immersion (larvae) or IP injection (adults) of VitD (0,001 $\mu$ g/ml) (calcitriol, Sigma-Aldrich), paricalcitol (0,001 $\mu$ g/ml) (zemplar®, Abbott Laboratories, Illinois, USA) and cinacalcet (0,05 $\mu$ g/ml) (mimpara®, Amgen Europe B.V., Breda, Netherlands), mimicking the concentrations used in clinical practice. For the control groups of non-diabetic and diabetic fish we used a vehicle solution (citrate buffer 0,05M). Since cinacalcet was found to be lethal to larvae at the concentration used for adults, we have performed treatments with dilutions of the initial concentration of cinacalcet by 1:10 (0.005  $\mu$ g/ml), 1:50 (0.001  $\mu$ g/ml) and 1:100 (0.0005  $\mu$ g/ml) and included three additional groups of diabetic and non-diabetic larvae treated with cinacalcet. **Fin amputation:** After the IP injections with treatments or vehicle, the caudal fins of treated adults (n=8) were amputated two segments below the ray bifurcation. Both larvae and adults were maintained under treatment for 120 hours at 28.5°C and fed twice a

day with *Artemia nauplii* (EG strain, Inve, Belgium). **Staining of mineralized tissue:** Larvae (n=15) were fixed with PFA 4% for 1 hour, washed in PBS and stained in 0.01% alizarin red for 30 min. Adults were submitted to live staining in alizarin red (n=8) at a concentration of 0.01% for 15 min prior to observation. Adult regenerated caudal fins and larval opercula were photographed under fluorescence (546nm) in a stereomicroscope (Leica MZ9.5, Leica, Wetzlar, Germany) for identification of the calcified regions. **Quantification of operculum mineralized area in larvae:** Mineralized area of opercula stained by alizarin red were measured using image J software. Results were normalized by dividing operculum area (OA) by total area of the head (HA) **Quantification of regenerated and mineralized area of adult fin:** Regenerated area was determined by dividing regenerated area (REG) by stump width (STU) and mineralized area was determined by dividing mineralized area (MIN) by mean ray width (MRW) and divided by REG/STU. All quantifications were done using image J software. **Bone histology and histomorphometry:** 3 calcified regenerated fins from each of the different groups were transferred to 70% EtOH and processed for dehydration and infiltration on a routine overnight processing schedule. Samples were then embedded in paraffin and sections with 6  $\mu$ m prepared in a microtome. Before staining, sections were deparaffinized in xylene and dehydrated in an increasing gradient of EtOH. Sections were stained by von Kossa's as described elsewhere<sup>33</sup>. To determine fin area and thickness, the second, third and fourth hemiray of each fin were measured. Area was assessed by measuring total area of both hemirays and thickness was assessed by four longitudinal measurements of each hemiray. A detailed time course of the different procedures from metronidazole treatment to amputation and data acquisition is shown in supplementary figure 1.

**Glucose tolerance test:** To confirm that ablation of  $\beta$ -cells in *Tg(ins:nfsb-mCherry)* zebrafish led to an increase of glucose blood concentrations, we administrated a solution of glucose at a concentration of 0,1 M or vehicle by IP injection, to two groups of adult zebrafish after 72 hpt with MET. Blood glucose was monitored at 30, 60, 90, 120, 150 and 180 minutes after IP injection with glucose. The glucose levels were measured in 3 *Tg(ins:nfsb-mCherry)* zebrafish treated with MET and with vehicle at each time point. 6  $\mu$ l of blood were collected from the caudal aorta, diluted in 2  $\mu$ l of 2% heparin and rapidly transferred to a blood glucose meter

Glucocard™ MX (Arkray A. Menarini Diagnostics, Florence, Italy). All these procedures were repeated four times with 6 to 7 specimens by group. **Total RNA isolation:** Vertebral columns from 6 adults of each group were isolated and pooled in 2 groups (n=3/each) for RNA purification. The samples were placed in 1ml of Isol-RNA Lysis Reagent (5 PRIME, Hilden, Deutschland) and total RNA was purified according to manufacturer's protocol. RNA quantity and integrity were verified using Experion™ RNA Analysis Kit (BIO-RAD, Hercules, CA, USA). **Quantitative real-time polymerase chain reaction (qPCR):** Reverse transcription of 1 µg of total RNA was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Semi quantitative qPCR was performed using iQ™ SYBR® Green Supermix (Life Technologies), on an CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA), conducted for 45 cycles of 5 s at 95°C and 15 s at 60°C each. The primer sequences of *elongation factor 1alpha (efl-alpha)*, *18S ribosomal rna (18S)*, *runt-related transcription factor 2 b (runx2b)*, *bone gamma-carboxyglutamic acid-containing protein (bglap)*, *sp7 transcription factor (sp7)*, *parathyroid hormone receptor a (pthra)*, *vitamin D receptor a (vdra)*, *insulin a (insa)*, *insulin b (insb)* and *insulin receptor a (insra)* are listed in supplementary Table 1. All gene expression data were normalized against the mean of the gene expression levels of housekeeping genes *efl-alfa* and *18S*. To confirm extrapancreatic expression of *insa* and *insb* genes observed by qPCR, a RT-PCR reaction was performed and the identity of the amplicons confirmed by sequencing. Gene expression results are the mean of two different experiments. **Statistical analysis:** All statistical analyses were performed using Stata Statistical Software and data was evaluated using the one-way ANOVA followed by Bonferroni multiple comparisons test with  $p < 0.05$  considered statistically significant. Results are presented as means  $\pm$  standard deviation of the mean (SD).

## Results

As previously described, Tg(*ins:nfsb*-mCherry) presented loss of fluorescence in the region of the pancreas after 72 hpi with MET, confirming  $\beta$ -cell ablation (supplementary figure 2). To confirm that Tg(*ins:nfsb*-mCherry) exposed to MET could lead to a state of onset of diabetes, a glucose tolerance test was performed to

measure how well animals are able to break down glucose, or sugar. We found that glucose concentrations in the plasma of fish treated with MET were significantly higher than in fish treated with vehicle only, with these differences being highly significant at 90, 120, 150 and 180 minutes after IP injection (Figure 1).

An analysis of the mineralized area of the operculum (Figure 2.A, B) of larvae from the D group revealed a significant reduction when compared to non-diabetic samples. However, the mineralized area of the operculum was significantly increased when diabetic larvae were treated with paricalcitol, cinacalcet and VitD compared to untreated diabetic larvae. Non-diabetic larvae treated with the three different treatments showed a tendency for an increase in mineralization compared to untreated non-diabetic larvae, although not significantly different due to individual variability (Figure 2.C).

Following  $\beta$ -cell ablation and regeneration we have quantified the regenerated and mineralized areas (Figure 3.A). Diabetic zebrafish adults had a statistically significant impairment of fin regeneration when compared to non-diabetics. Furthermore, the diabetic and non-diabetic groups treated with paricalcitol and cinacalcet presented a significant increase in regenerated area when compared to diabetic fish, but a not significant increase in regeneration when compared to non-diabetics (figure 3.B). In non-diabetic treated groups, the increase in regenerated area was not significant. The regenerated area of the diabetic and non-diabetic zebrafish groups treated with VitD did not present any significant differences compared to respective control groups (Figure 3.B). Wild type (WT) fish treated with MET showed no differences to non-diabetics or to control WT fish exposed to vehicle.

Quantification of the mineralized area showed that groups treated with paricalcitol and cinacalcet had no differences when compared to untreated non-diabetic fish while the diabetic fish showed a significantly reduced mineralization when compared to non-diabetic and with diabetic paricalcitol and cinacalcet treated groups, while VitD treated groups showed no differences relative to the other treated groups (figure 3.C). Non-transgenic siblings treated with MET or vehicle (WT, WT+MET) showed no alterations both in regenerated and mineralized areas when compared to the ND group (figure 3.B and 3.C).

### Wound Repair and Regeneration



Histology of rays confirmed previous results, showing increased ray area and thickness (figure 4.A) in the regenerated caudal fins of diabetic fish treated with paricalcitol and cinacalcet when compared to untreated diabetic fish, while non-diabetic treated groups showed no significant increase (figure 4.B and 4.C).

Analysis of gene expression levels showed no significant differences relative to the expression of *pthra* when comparing non-diabetic and diabetic fish (figure 5.A). The *vdra* expression, showed a statistically significant increase in the cinacalcet treated groups compared to all other groups (figure 5.B). *insra* expression was found to be significantly down regulated in all diabetic groups ( $p < 0,5$ ) compared to non-diabetic, but the diabetic fish treated with cinacalcet showed a lower reduction in expression, with statistically higher values compared to diabetic, paricalcitol and VitD treated fish, while nondiabetic cinacalcet treated group showed increased expression compared to all other groups (figure 5.C). *sp7* expression showed a trend of reduction in all diabetic fish treated groups compared to non-diabetics, but significant downregulation was only found in diabetic compared to non-diabetic groups, no differences being observed among non-diabetic groups (figure 5.D). Regarding *runx2b*, a significant upregulation could be observed in groups treated with paricalcitol and cinacalcet (figure 5.E). Expression of *bglap* was downregulated in diabetic compared to non-diabetic fish while the paricalcitol and cinacalcet diabetic groups showed significant differences compared to untreated diabetic group. In non-diabetic fish, treated versus control groups were not significantly different. Gene expression of *insa* and *insb* were found to be upregulated ( $p < 0.001$ ) in both diabetic and non-diabetic paricalcitol and cinacalcet treated fish when compared to the other groups (figures 4.G and 4.H).

To demonstrate the extrapancreatic expression of both *insa* and *insb* in vertebral column, we performed an RT-PCR using cDNA of liver/pancreas, muscle, kidney, column and cleithrum/operculum from wild type zebrafish, using the same primers used for qPCR. We observed amplification of *ins* genes in all tissues analyzed. This result was further confirmed by sequencing the PCR amplicons which were confirmed to correspond to *insulin a* and *b* isoforms respectively (figure 6).

## Discussion

This study demonstrated that zebrafish is a suitable model for the study of bone pathologies related to diabetes. Ablation of  $\beta$ -cell, by exposing Tg(*ins:nfsb*-mCherry) zebrafish to MET, led to loss of mCherry fluorescence in the  $\beta$ -cells at 72 hpi, as already described by previous authors<sup>30,31</sup>, and to blood glucose increase, although differences were not as accentuated as previously reported<sup>30,31</sup>. Beta-cell ablation was confirmed by loss of mCherry fluorescence after 72 hours of treatment with MET. Transgenic fish that did not present a total loss of fluorescence were removed from further experimental procedures. The injection of glucose in diabetic fish led to an increase of the plasma glucose levels significantly higher than in non-diabetic controls. The levels of blood glucose started to decrease after 120 min post treatment, although they remained significantly higher in diabetic fish while in non-diabetics the levels returned to pre-treatment levels. The reduction of glucose levels in plasma observed in diabetic fish can be explained by elimination of excess plasma glucose in urine, as previously reported for other species, where after injection with levels from 6.4mM to 25mM glucose in tilapia the excretion of excess glucose in the urine was visible after few hours. It was reported that treated fish had a drastic increase in urine glucose levels that were the double of the values measured in plasma<sup>34</sup>. It has also been shown in rainbow trout that there is a renal regulation of glucose levels in the plasma with the observation that high concentrations of glucose in plasma lead to glycosuria and to elimination in the urine<sup>35</sup>. Moreover, it is known that small fish are considered to have lower glucose tolerance than large fish<sup>36</sup>.

Induction of diabetes in zebrafish caused an impairment in operculum mineralization and bone growth in 15 day larvae, similar to growth retardation observed in diabetes type 1 patients<sup>37</sup> and diabetes mice models<sup>38</sup>. Treatment with paricalcitol was more efficient than with VitD in promoting an increase in the mineralized area. The concentrations of Cinacalcet in our zebrafish treatments were the same as used in clinical therapies, but were found to be toxic in larvae with a rate of mortality of 100% after 24 hours. However, lower concentrations showed reduced lethality and induced an increase in mineralized area. Regenerated fin areas of adults showed an increase in the diabetic groups treated with paricalcitol and cinacalcet when compared to diabetic group treated with vehicle, while diabetic fish treated with VitD did not present such a marked increase. There are

some evidences that vitamin D analogs can have positive regenerative effects after vascular injury, as previously reported for healthy humans, diabetic mice models and conditional knockout of the *vitamin D receptor* mice<sup>39</sup>.

In our work, paricalcitol had no effect in *vdra* expression, but we could detect an upregulation in the group treated with cinacalcet, in agreement with previous *in vitro* studies with rat parathyroid glands, demonstrating that class II calcimimetics induce a stimulatory affect in *Vdra* expression<sup>40</sup>. Additionally, gene expression results for both *insa* and *insb*, which were found to be overexpressed in bone in diabetics treated with paricalcitol or cinacalcet, could help explain this increase in caudal fin regeneration. Vitamin D analogs and calcimimetics have been shown to induce insulin expression and  $\beta$ -cell proliferation and survival<sup>41-43</sup>, and our data suggest that this upregulation of insulin also occurs in bone cells. In the Tg(*ins:nfsb-mCherry*) zebrafish, it has been described that total pancreas regeneration occurs in 15 days after diabetes induction, but we do not know if this process occurred in a shorter period of time in the paricalcitol and cinacalcet treated groups, which could favor insulin signaling and glucose metabolism. In the cinacalcet treated group we could observe a significant increase in *insr* suggesting increased insulin signaling. In fact, VitD may have an important role in the treatment of diabetes as identified by Del Pino-Montes et al (2004)<sup>44</sup>, who showed that 55% of diabetic rats treated with calcitriol recovered from diabetes.

Insulin expression has also been found to be increased in several tissues under diabetic conditions in both humans and mice<sup>45</sup>, but in our study we could not observe such an increase in the non-treated diabetic group, at least in the vertebral column. It has been demonstrated that insulin can be almost ubiquitously expressed in human<sup>46</sup>, mice<sup>45</sup> and zebrafish<sup>47</sup> although at extremely low levels when compared to pancreatic insulin. Although not well understood, the function of extrapancreatic expression of insulin was associated in some works to local needs of glucose regulation, specially under diabetic conditions<sup>46,48</sup>, but in other reports this phenomenon was related to the development of pathologic conditions<sup>29</sup>. Our results demonstrated that paricalcitol and cinacalcet can upregulate extrapancreatic expression of insulin, including in bony tissues, like demonstrated in the vertebral column of adult zebrafish.

In humans and animal models of diabetes, hyperglycemia leads to accelerated accumulation of advanced glycation end products (AGEs)<sup>49</sup>, promoting an inflammatory response and increased apoptosis of cells expressing the receptor of AGEs such as osteoblasts<sup>50</sup>. In the non-treated diabetic group we could see impairment in osteoblastic activity, since *sp7*, *runx2b* and *bglap* expression were found to be downregulated compared to non-diabetic fish. In the diabetic groups treated with paricalcitol or cinacalcet, where an increase in mineralized area of the regenerated fin was observed, an upregulation of *runx2b* suggests an increase in the process of osteoblastic differentiation, contributing to the process of mineralization. This is in accordance with studies in humans, indicating that VitD effects on osteoblast differentiation are mostly stimulatory and associated with increased *RUNX2* expression<sup>51</sup>. Additionally, *in vitro* studies with mesenchymal stem cells from human amniotic fluid have correlated calcimimetics with osteogenic differentiation and upregulation of bone markers including *RUNX2*<sup>52</sup>. The principal objective of paricalcitol, cinacalcet and VitD in clinical treatment for SH is to reduce parathyroid hormone secretion. We could not observe reduced expression in *pthra* in all treated groups so no conclusions can be made relatively to the *pth* regulation of osteoclastic differentiation and bone resorption<sup>53</sup>. In fact, *pthra* results seems to support the idea that *pth* pathway was not altered in the treated groups because the VitD treated group did not present such a marked increase in mineralized area as observed in the other two treated groups, while having the same results for *pthra*. The fact that VitD acts more slowly in exerting its effects, at least when compared to paricalcitol<sup>54</sup>, can be one of the possible explanations for our results. Different pathways related to *pth* signaling, calcium metabolism or VitD induced osteoblastogenesis can be involved in the increase in bone mineralized and regenerated areas observed in the caudal fin of zebrafish under diabetic conditions treated with paricalcitol and cinacalcet. Upregulation of insulin and increased osteoblastic differentiation induced by upregulation of *runx2b* can help explain our results. Both paricalcitol and cinacalcet were shown to have positive effects in promoting mineral deposition, counteracting bone loss related to diabetes, and may constitute an alternative therapy for prevention of bone related disorders observed in type I diabetes patients.

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## Contribution Statement

F.R.C., P.J.G., A.R.F. participated in the experimental work and writing of the manuscript; M.L.C. and P.J.G. elaborated experimental planning and revised the results and the manuscript.

## Conflict of interest

No conflict of interests.

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## Figure legends

**Figure 1.** Transgenic zebrafish Tg(*ins:nfsb-mCherry*) 72 hours post induction of diabetes have increased blood glucose concentrations at 30, 60, 90, 120, 150, 180 min. after intra-peritoneal injection with glucose \* $p < 0,05$ ; \*\* $p < 0,001$ .

**Figure 2. A.** Measurements of operculum mineralized areas; **B.** Size of the operculum was obtained by dividing the operculum area by total head area; **C.** 15 day old diabetic larvae have reduced operculum mineralized area when compared to non-diabetic, but treatment with paricalcitol and cinacalcet at 0.005  $\mu\text{g/ml}$ , 0.001  $\mu\text{g/ml}$  and 0.0005  $\mu\text{g/ml}$  were found to increase the mineralized area compared to diabetic larvae. Bars with different superscript letters indicate significant differences ( $p < 0.05$ ). Operculum area (OA), total area of the head (HA). **ND** non-diabetic; **D** diabetic; **D CC 1:10**, **D CC 1:50**, **D CC 1:100**, **ND CC 1:10**, **ND CC 1:50**, **ND CC 1:100** diabetic and non-diabetic groups treated with cinacalcet at concentrations 10, 50 and 100 times lower than the used in clinical practice; **D PCT** and **ND PCT** diabetic and non-diabetic treated with paricalcitol; **D VitD** and **ND VitD** diabetic and non-diabetic treated with vitamin D.

**Figure 3. A.** Measurements of regenerated (top row) and mineralized (bottom row) areas in non-diabetic (ND) and diabetic (D) zebrafish adults treated with vitamin D (VitD), paricalcitol (PCT) and cinacalcet (CC) and in control wild type AB (WT) zebrafish or treated with metronidazole (MET); **B.** Diabetic group had decreased fin regenerated area compared to non-diabetic and to diabetic treated with paricalcitol and cinacalcet; **C.** Diabetic group had decreased fin mineralized area compared to non-diabetic and to diabetic treated with paricalcitol and cinacalcet. Bars with different superscript letters indicate significant differences ( $p < 0.05$ ). Regenerated area (REG), stump width (STU), mineralized area (MIN), mean ray width (MRW).

**Figure 4. A.** Histological sections of regenerated caudal fin rays stained by von Kossa. **B.** Diabetic group alone or treated with VitD presents reduced ray area compared to all other groups. **C.** Diabetic group alone presents reduced thickness compared to all other groups except diabetic treated with VitD.

**Figure 5.** RNA gene expression from the vertebral column of diabetic or diabetic with treatments is altered in zebrafish; **A.** Diabetic and non-diabetic treated groups showed no significant differences in *pthra* expression; **B.** Diabetic and non-diabetic treated with cinacalcet have an increase in *vdra* expression compared to all the other groups; **C.** Diabetic and non-diabetic groups treated with cinacalcet showed increased expression of *insra*; **D.** The diabetic fish showed a downregulation of *sp7* compared to non-diabetics. The treated groups showed no differences relatively to untreated groups; **E.** Both diabetic and non-diabetic groups treated with paricalcitol and cinacalcet showed an increase in the expression of *runx2b*; **F.** Diabetic group showed reduced expression of *bglap* compared to non-diabetic fish. All the treated groups with paricalcitol and cinacalcet showed significant

upregulation relatively to the untreated diabetic group; **G.** and **H.** Diabetic and non-diabetic groups treated with paricalcitol and cinacalcet showed an increase in the expression of both *insa* and *insb* gene. Bars with different superscript letters indicate significant differences ( $p < 0.05$ ).

**Figure 6.** Zebrafish presented extrapancreatic expression of both *insa* and *insb*. Expression of *insa* and *insb* could be detected in pancreas/liver, muscle, kidney, and bones from vertebral column and cleithrum/operculum.

### Supplementary figure legends

**Supplementary Figure 1.** Detailed timeline of diabetes induction and treatment with experimental drugs during caudal fin regeneration.

**Supplementary Figure 2.** Confirmation of beta-cell ablation. **A.** Metronidazole exposed Tg(*ins:nsfb*-mCherry) zebrafish lose mCherry fluorescence after 72 hours of treatment. **B.** Vehicle exposed Tg(*ins:nsfb*-mCherry) zebrafish maintain mCherry fluorescence after treatment.

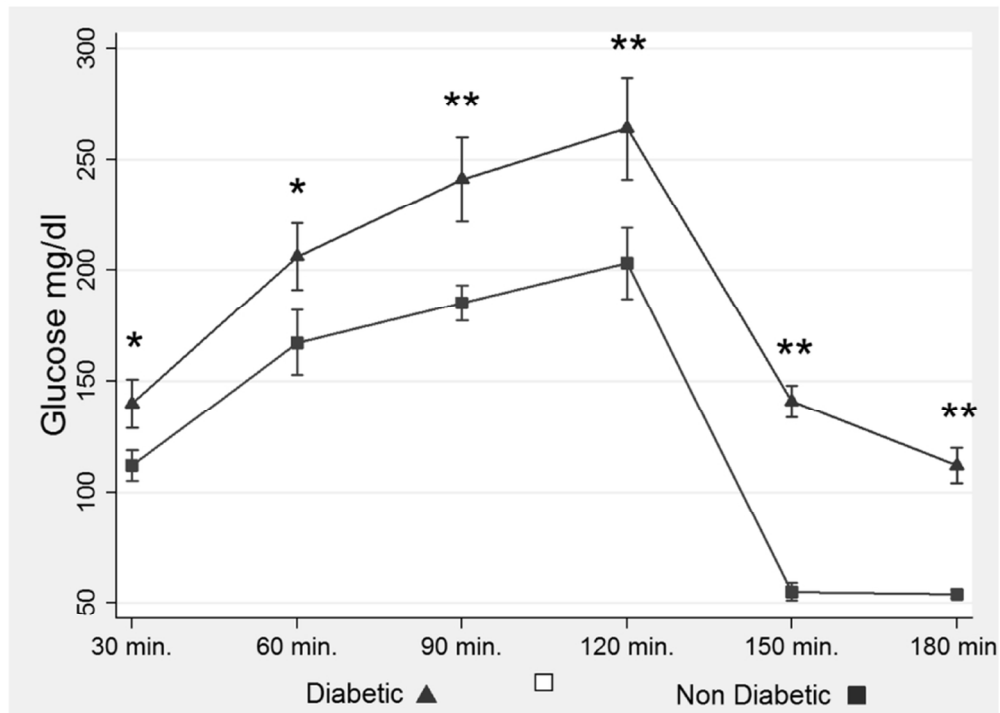


Figure 1. Transgenic zebrafish Tg(ins:nfsb-mcherry) 72 hours post induction of diabetes have increased blood glucose concentrations at 30, 60, 90, 120, 150, 180 min. after intra-peritoneal injection with glucose  
\*p<0,05; \*\*p<0,001.

Figure 1  
100x71mm (300 x 300 DPI)

Accepted

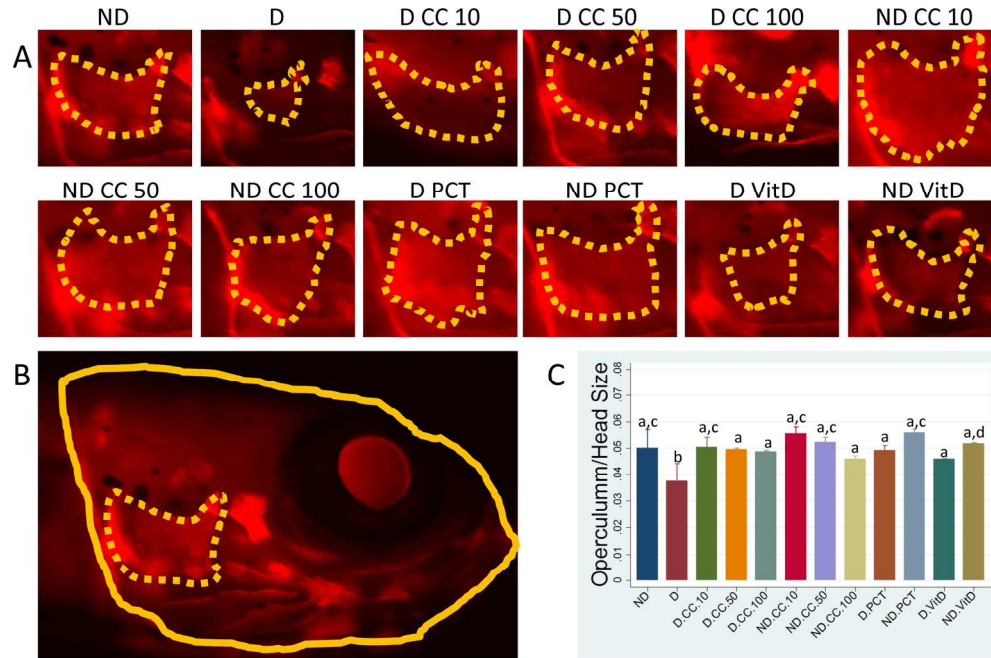


Figure 2. A. Measurements of operculum mineralized areas; B. Size of the operculum was obtained by dividing the operculum area by total head area; C. 15 day old diabetic larvae have reduced operculum mineralized area when compared to non-diabetic, but treatment with paricalcitol and cinacalcet at 0,005  $\mu\text{g/ml}$ , 0,001  $\mu\text{g/ml}$  and 0,0005  $\mu\text{g/ml}$  were found to increase the mineralized area compared to diabetic larvae. Bars with different superscript letters indicate significant differences ( $p < 0,05$ ). Operculum area (OA), total area of the head (HA). ND non-diabetic; D diabetic; D CC 1:10, D CC 1:50, D CC 1:100, ND CC 1:10, ND CC 1:50, ND CC 1:100 diabetic and non-diabetic groups treated with cinacalcet at concentrations 10, 50, 100 smaller than the used in clinical practice; D PCT and ND PCT diabetic and non-diabetic treated with paricalcitol; D VitD and ND VitD diabetic and non-diabetic treated with vitamin D.

Figure 2

176x116mm (300 x 300 DPI)

Accel

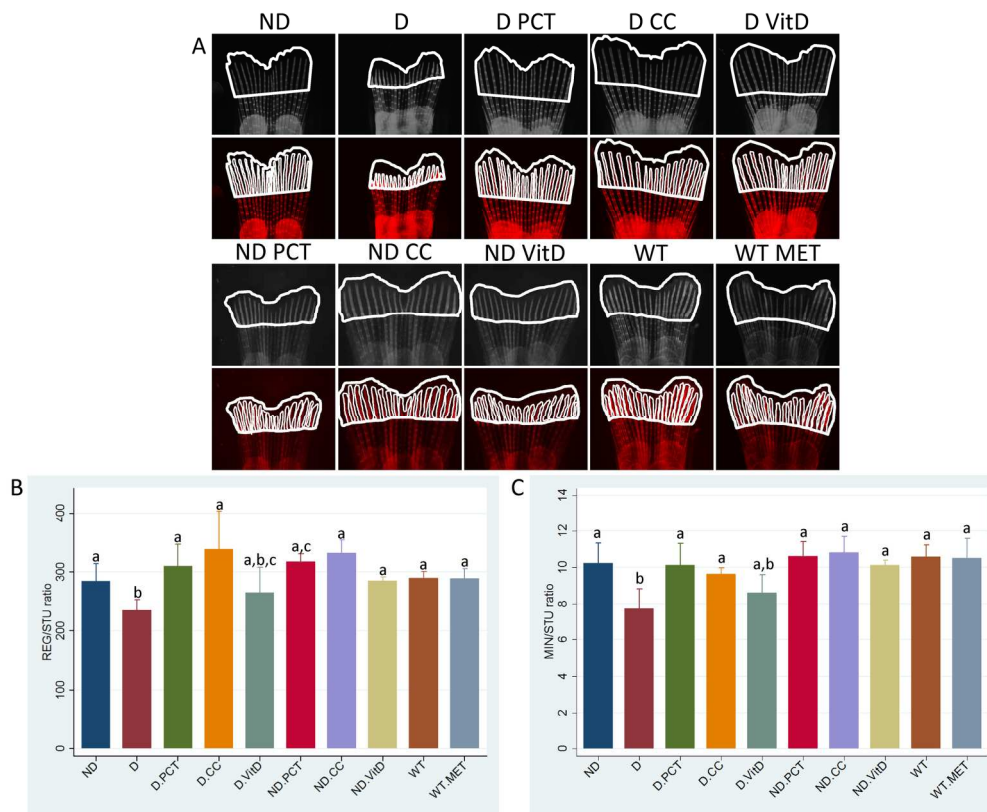


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Figure 3

179x146mm (300 x 300 DPI)

ACCE

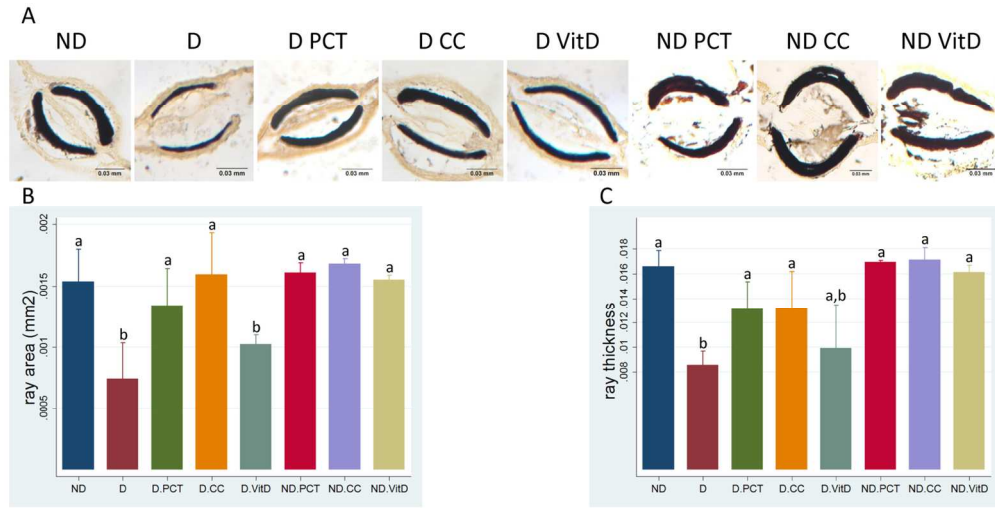


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Figure 4

139x70mm (300 x 300 DPI)

Accepted

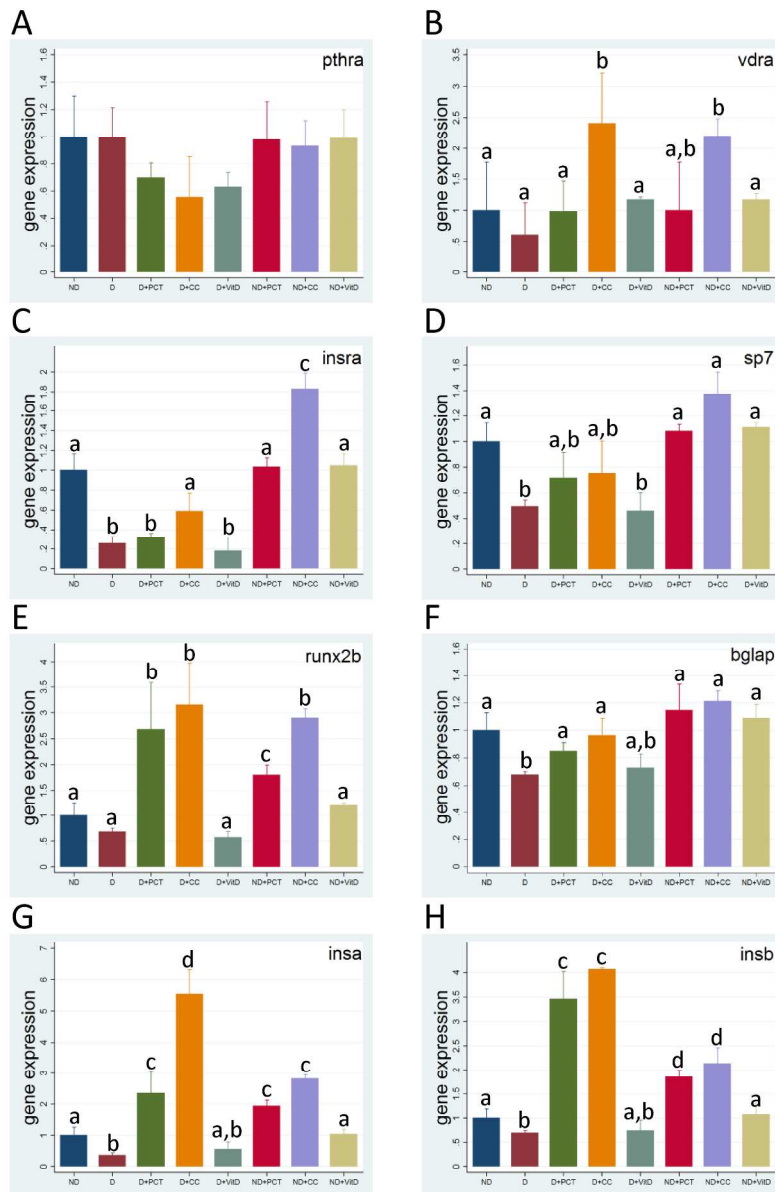


Figure 5. RNA gene expression from the vertebral column of diabetic or diabetic with treatments is altered in zebrafish; A. Diabetic and non-diabetic treated groups showed no significant differences in *pthra* expression; B. Diabetic and non-diabetic treated with cinacalcet have an increase in *vdra* expression compared to all the other groups; C. Diabetic and non-diabetic groups treated with cinacalcet showed increased expression of *insra*; D. The diabetic fish showed a downregulation of *sp7* compared to non-diabetics. The treated groups showed no differences relatively to untreated groups; E. Both diabetic and non-diabetic groups treated with paricalcitol and cinacalcet showed an increase in the expression of *runx2b*; F. Diabetic group showed reduced expression of *bglap* compared to non-diabetic fish. All the treated groups with paricalcitol and cinacalcet showed significant upregulation relatively to the untreated diabetic group; G. and H. Diabetic and non-diabetic groups treated with paricalcitol and cinacalcet showed an increase in the expression of both *insa* and *insb* gene. Bars with different superscript letters indicate significant differences ( $p < 0,05$ ).

Figure 5  
201x305mm (300 x 300 DPI)





Figure 6. Zebrafish presented extrapancreatic expression of both *insa* and *insb*. Expression of *insa* and *insb* could be detected in pancreas/liver, muscle, kidney, and bones from vertebral column and cleithrum/operculum.

Figure 6  
40x9mm (300 x 300 DPI)

Accepted A

Table 1- List of primers. Sequences presented in 5' to 3' orientation.

ef1a	Forward	AGCCCCTCCTGGCTTTCACCC
ef1a	Reverse	TGGGACGAAGGCAACACTGGC
18s	Forward	ACCACCCACAGAATCGAGAAA
18s	Reverse	GCCTGCGGCTTAATTTGACT
runx2	Forward	GCACGGAGAGGGACTGACGG
runx2	Reverse	AGGGCCACCACCTTAAACGC
oc	Forward	CCAACTCCGCATCAGACTCCGCATCA
oc	Reverse	AGCAACACTCCGCTTCAGCAGCACAT
osx	Forward	GTTTCCCAGGACCCTTCGCT
osx	Reverse	GCAATCGCAAGAAGACCTCC
pth1ra	Forward	GTTTCGTCTATGGTCTGGTTCG
pth1ra	Reverse	GATTGCTCGCTCACATTTTC
vdr	Forward	GTCCAACCAGTCCTTCAGTCT
vdr	Reverse	AGTGTGACCCGCCTTAGTG
insb	Forward	CTCTGCTCACTCAGGAAAAGG
insb	Reverse	GGATGGAGAAGACTGCGAT
insa	Forward	CATTCTCGCCTCTGCTTC
insa	Reverse	TGCCTGGGTTAGTGCTTACA
insra	Forward	TCTACAGCGAGGAAAACAAGC
insra	Reverse	AGAGATAAGATGCGTCCGTTTT