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# Characterization of bone pathologies in the ins2$^{+/akita}$ mouse, a new model for diabetes insulin-dependent: contribution for a better understanding of the disease in humans

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Tese para a obtenção do grau de Doutor em Ciências Biomédicas

**Trabalho efetuado sob a orientação de:**

Professora Doutora Leonor Cancela

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Declaração de Autoria do trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Os trabalhos consultados encontram-se citados no texto e estão devidamente anotados na listagem de referências.

---

Para a Ana, Violeta e João

**Navegar é preciso, viver não é preciso**

Cneu Pompeu

# AGRADECIMENTOS

A conclusão deste trabalho só foi possível graças a várias pessoas e ao qual eu queria agradecer do fundo do meu coração.

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# ABSTRACT

In the past 30 years developed and developing countries faced significant lifestyle changes that made the incidence of diabetes mellitus to reach pandemic proportion worldwide. This increase, seen in both types of the disease, made diabetes mellitus one of the leading causes of morbidity and mortality of our times. Bone is one t of several organs that are affected by diabetes mellitus, being morbidity caused by bone fractures highly correlated to diabetic bone. Alterations in the constituents of bone, microarchitecture changes and bone loss have been pointed as the main reasons for its fragility. This work aimed to contribute to identify the molecular players or the functional changes affecting bone and caused by diabetes mellitus. In chapter 2, using as animal model of type 1 diabetes mellitus the *Ins2*$^{+/akita}$ mouse, we identified bone growth retardation related to growth plate impairment. These changes were associated to reduced expression of *Igf1* and increased expression of cartilage degradation enzymes like *Adams-5*. We also identified severe microarchitecture changes caused by reduced bone formation and resorption that could be explained by leptin deficiency and/or decreased insulin signaling. In chapter 3, we concluded that paricalcitol (vitamin D analog) and cinacalcet (calcimimetic), two drugs used for the treatment of secondary hyperthyroidism, have beneficial effects in fin regeneration and mineralization in a zebrafish model of diabetes. These results could be explained by the downregulation of *pthr* suggesting reduced signaling of parathyroid hormone, that is a potent activator of bone remodeling, and increased expression of *runx2*, indicating increased osteoblast differentiation. Increased expression of the two zebrafish insulin genes, *insa* and *insb*, could be observed, suggesting that both drugs promote an increase in insulin signaling. In chapter 4, we suggest that in humans, the insulin paralog gene, *INS-IGF2*, does not have a redundant function with the insulin gene, in contrast with what is seen in mice and probably in zebrafish. We could also conclude that extrapancreatic expression of insulin is present in human, mouse and zebrafish. In humans

this expression results mainly from expression of the ancestral gene while in mice and zebrafish it is due to expression of the insulin paralogues.

# RESUMO

Alterações significativas no estilo de vida nos países desenvolvidos e em desenvolvimento, durante as ultimas décadas, fizeram com que a incidência da diabetes mellitus tenha tomado proporções semelhantes às de uma pandemia. Este aumento, observado em ambos os tipos da doença, fez com que diabetes mellitus se tenha tornado uma das principais causas de morbidade e mortalidade dos nossos tempos. O osso está entre os vários órgãos que são afetados pela diabetes mellitus, estando a morbidade causada por fraturas ósseas, altamente correlacionada com esta doença. Perda óssea, alterações nos constituintes e na microarquitetura do osso, têm sido apontadas como os principais causas para a fragilidade óssea destes pacientes. Neste trabalho propusemo-nos identificar intervenientes moleculares que estão envolvidos nas alterações ósseas despoletadas pela diabetes mellitus. No capítulo 2, usando como modelo animal de diabetes mellitus tipo 1 o ratinho *Ins2*$^{+/akita}$, procurámos identificar alterações histomorfométricas na estrutura do osso e cartilagem e identificar genes com a expressão alterada através de PCR em tempo real quantitativo. Pudemos observar diminuição no crescimento ósseo causado por uma redução no número de condrócitos proliferativos e aumento no número de condrócitos hipertróficos na placa de crescimento. Estas alterações foram acompanhadas por uma redução na expressão de fatores de crescimento como a *Igf1* e aumento da expressão de enzimas responsáveis pela degradação da cartilagem como *Adams-5*. Igualmente, pudemos observar alterações profundas na microarquitetura do osso do ratinho *Ins2* $^{+/akita}$, que sugerem reduzida formação e reabsorção óssea. O facto de a diabetes mellitus tipo 1 estar associada a um estado de hipoleptinémia e hipoinsulinémia faz com que ambas as hormonas, leptina e insulina, sejam as principais candidatas a explicar as alterações observadas, visto que ambas desempenham um papel ativador da função dos osteoblastos e osteoclastos. No capítulo 3, tentamos perceber se a vitamina D, análogos da vitamina D e calcimiméticos, podem ter um papel benéfico na

regeneração e mineralização óssea na diabetes mellitus tipo 1. Foi possível concluir que os medicamentos paricalcitol (análogo da vitamina D) e cinacalcet (calcimimético), usados para o tratamento do hipertiroidismo secundário, têm efeitos benéficos na regeneração e mineralização da barbatana caudal num modelo de diabetes mellitus tipo 1 de peixe-zebra. Estes resultados poderão ser explicados pela diminuição da expressão da *pthr*, que sugere uma redução da hormona paratiróide circulante, que é um activador da remodelação óssea, e um aumento da expressão de *runx2*, que sugere maior diferenciação dos osteoblastos, assim como um aumento da expressão de ambos os genes *insa* e *insb* da insulina no osso, sugerindo que estes medicamentos poderão por um lado aumentar a expressão extrapancreática no osso e por outro a expressão pancreática, aumentando assim a sua sinalização nas células alvo. No capítulo 4 concluímos que nos humanos, o gene parálogo da insulina, o *INS-IGF2,* não tem uma função redundante em contaste com o que foi observado nos ratinhos e, provavelmente, no peixe-zebra. Foi também possível concluir que a expressão extrapancreática da insulina ocorre em humanos, ratinho e peixe-zebra e que nos humanos este fenómeno é devido principalmente à expressão do gene ancestral, enquanto no ratinho e no peixe-zebra parece ser devido à expressão dos parálogos respectivos.

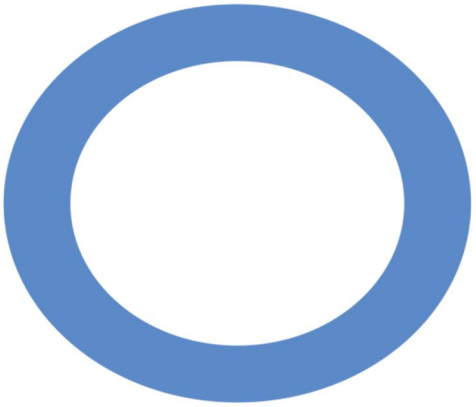
# TABLE OF CONTENTS

# TABLE OF FIGURES

# ABBREVIATION LIST

DM diabetes mellitus
*INS* Insulin
*Igf1 Insulin growth factor 1*
*INS-IGF2-insulin-insulin growth factor 2*
*pthr parathyroid hormone receptor*
*runx2 runt-related transcription factor 2*
*insa insulin a*
*insb insulin b*
T1DM Type 1 diabetes mellitus
T2DM Type 2 diabetes mellitus
HPA hypothalamic pituitary adrenal
ACTH adrenocorticotropic hormone
MP mesenchymal progenitors cells
HSCs haematopoietic stem cells
BMD bone mineral density
Gla-OCN carboxylated osteocalcin
Glu-OCN undercarboxylated osteocalcin
Opg osteoprotegerin
GF1 Insulin growth factor 1
GH Growth hormone
AGEs advanced glycation end products
FFA free fatty acids s
*OSX osterix*
*RANKL* receptor activator of nuclear factor (NF)-kB (RANK) by RANK ligand
AGEs Advanced glycation end products
KO knockout
BV/TV Bone volume/Total volume
*Adrb2* β2-adrenergic receptor
qRT-PCR Quantitative real-time polymerase chain reaction
VitD Calcitriol; 1,25-dihydroxyvitamin D3 –
MET Metranidazole
STZ Streptozotocin

# CHAPTER 1.
# GENERAL INTRODUCTION



*Diabetes Blue circle symbol. Copyright permission from International Diabetes Federation*

# CHAPTER 1. GENERAL INTRODUCTION

## 1.1. Type 1 Diabetes Mellitus

### 1.1.1. Prevalence

Diabetes mellitus (DM) is the most rapidly growing chronic disease of our time. In developed and developing countries is predicted to occur a decrease in life expectancy for the first time in 200 years because of DM [1]. In 2011 there were 366 million people with DM and expected to rise to 552 million by 2030 [2] with a estimation of being the 7th leading cause of death in 2030 [3]. Population growth, aging, urbanization, and growing prevalence of obesity and physical inactivity are between the principal causes. Originally expected to occur in type 2 diabetes mellitus (T2DM), the increase in childhood diabetes has been of both type 1 and 2 DM [4]

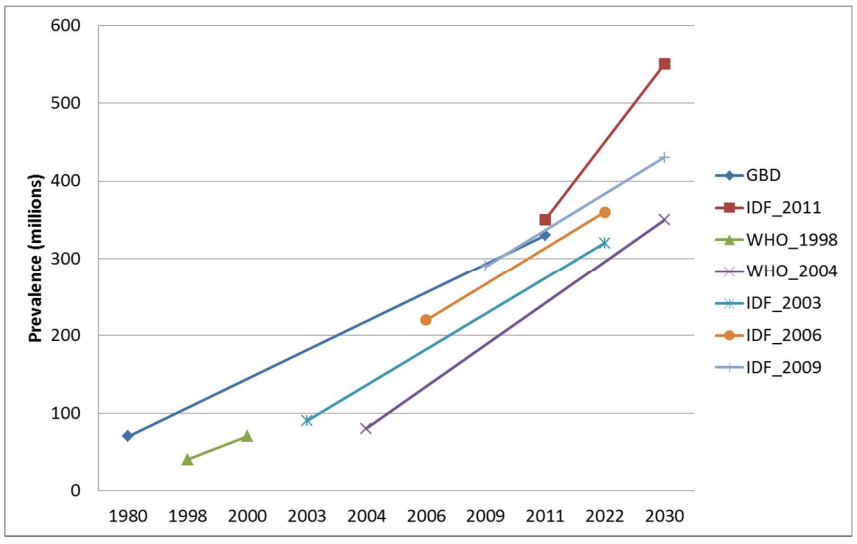



**Figure 1.** .Estimates and projections of global diabetes prevalence. All projections from different studies suggest growing prevalence of diabetes over the next decades. GBD, Global Burden Diabetes, IDF, International Diabetes Federation, WHO, World Health Organization. Adapted from Whiting et al., 2011.

Type 1 diabetes mellitus (T1DM) accounts for 10% of all DM cases [2] with an increasing incidence of 2-5% a year [5]. Although the reasons for this increase are still unknown, genetic susceptibility together with environmental factors suggest that a multifactorial process might be involved [6]. Children with T1DM have in 90–95% of incidences a susceptibility in the human leukocyte antigen complex in chromosome 6 [7], but only 5% of the individuals that carry this susceptibility actually develop the disease [8] supporting the idea that other factors are needed to trigger T1DM.

### 1.1.2. Causes

T1DM is divided in two sub-groups, the most common is type 1a that is linked to an autoimmune-mediated destruction of beta cells, while in type 1b are included rare forms of the disease associated to genetic defects in beta-cell function, with a sub-classification according to the gene involved.

Type 1a of DM is characterized by a disorder of glucose homeostasis by susceptibility to ketoacidosis. Besides the genetically heterogeneous autoimmune predispositions, environmental triggering hypothesis have been made like hygiene, viral infections, vitamin D deficiency, breast milk and cow's milk [6]. At the present time, the hygiene hypothesis seems to be the best candidate to explain onset of the disease. Strachan [9] proposed that exposure to a variety of infectious agents during early childhood might be protective. Beside this, authors suggest that the reason for the increase in the incidence of the disease probably is multifactorial [6].

Type 1a DM results in a dysregulation to self-tolerance proteins. During thymus development, T-cells that do not express highly self-reactive T cell receptors mature and leave the thymus while highly autoreactive T cells are deleted in healthy individuals. But in T1DM these highly autoreactive T cells are not eliminated and escape to the periphery participating in the pathological immune response to self-antigens.

Type 1b DM, also called monogenic diabetes of infancy, is a broad definition of diabetes, permanent or transient, during the first years of life with onset caused by a single gene defect [10]. Candidate gene defects are homozygous or heterozygous mutations in potassium inwardly-rectifying channel, subfamily J, member 11 (KCNJ11), Glucokinase (GCK), ATP-binding cassette transporter sub-family C member 8 (ABCC8) and insulin genes [11]. It is characterized by patients without pancreatic autoantibodies, implying that these β-cells malfunction is caused by a mutation and not from autoimmune response.

### 1.1.3. Pathophysiology

Abnormalities in β-cell functions leads to reduced insulin secretion that starts before the onset of symptoms. Low insulin activity results in increased glucose production by the liver and reduced glucose uptake by insulin sensitive tissues. As glucose accumulates in plasma, renal tubules are incapable of reabsorbing all the glucose, producing an osmotic diuresis. As insulin sensitive cells are deprived from glucose, fat and muscle breakdown is initiated to use free fatty acids and amino acids as fuels for glucose production by the liver, leading to weight loss. Accelerated fatty acids breakdown leads to overproduction of ketone bodies, leading to ketoacidosis and together with hyperglycemia and acidosis creates a fatal condition, if insulin therapy is not initiated [12].

Hypoinsulinemia has been associated to the dysregulation of several other hormones that also mediate the onset of T1DM. Lack of insulin signaling in adipocytes leads to low secretion of leptin, leading to increased hypothalamic pituitary adrenal (HPA) axis activity that centrally will increase glucagon, adrenocorticotropic hormone (ACTH) and corticosterone plasma concentration [13,14].

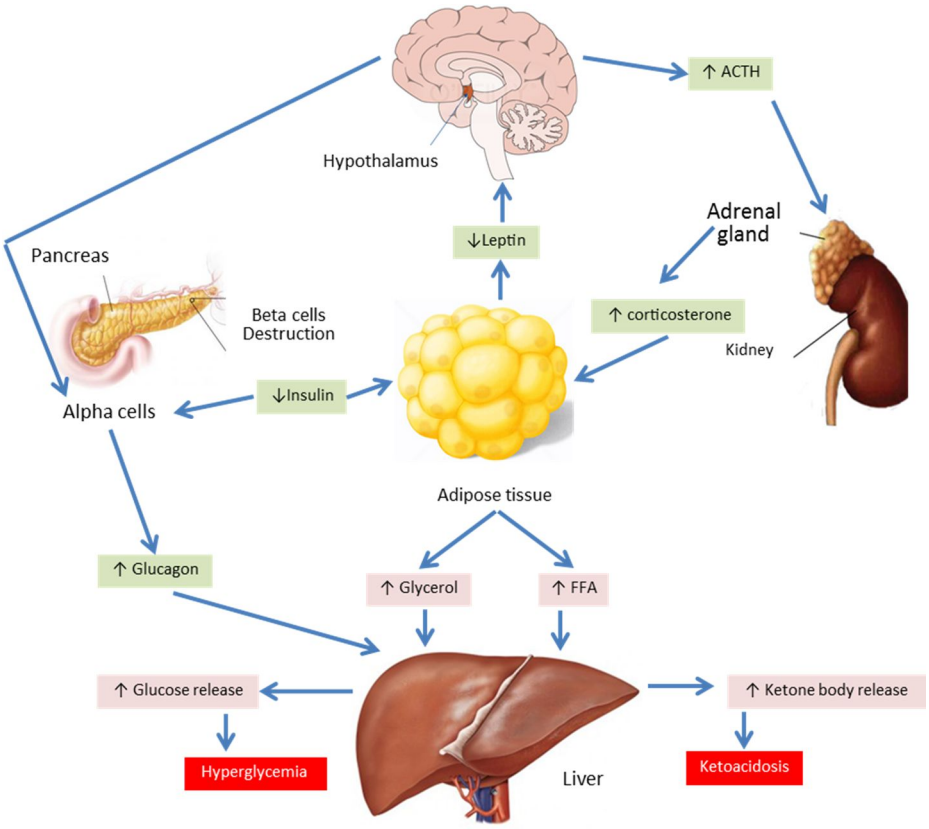



**Figure 2.** T1DM leads to hyperglycemia and ketoacidosis. Hypoinsulinemia decreases plasma leptin concentration by decreasing its secretion from adipose tissue. Low insulin and leptin secretion increase glucagon production by α-cells, signaling glucose release by the liver. Low leptin increases hypothalamus-pituitary-adrenal (HPA) axis activity and Adrenocorticotropic hormone (ACTH) release from the pituitary, increasing cortisol secretion from the adrenal glands and induce lipolysis of adipose tissue triglycerides. The increased release of free fatty acids (FFAs) and glycerol from adipose tissue into the bloodstream increases the delivery of glycerol and fatty acids substrates to the liver. Incomplete oxidation of fatty acids in the liver produces ketone bodies and increases hepatic acetyl-CoA, contributing to hyperglycemia by increasing the conversion of pyruvate to glucose. Adapted from Mittendorfer & Klein., 2014.

In is work , Perry et al. [15] could see that leptin treatment reduced glucagon production, and that gluconeogenesis and fat breakdown in poorly controlled T1DM rats was caused by hypoleptinemia induced activity of the HPA and more importantly, that changes could be dissociated from insulin concentrations.

## 1.2. Bone

### 1.2.1. Formation

Bone homeostasis is achieved by balanced activity of bone forming cells, the osteoblasts, and bone resorption cells, the osteoclasts. Osteoblasts are responsible for the secretion of a group of extracellular proteins that will give rise to the extracellular bone matrix. This includes osteocalcin, alkaline phosphatase and a large amount of type I collagen. First a non-mineralized matrix is formed, constituted by type 1 collagen, named osteoid and later mineralized through the accumulation of calcium phosphate in the form of hydroxyapatite, leading to the formation of the major mineral constituent of bone [16].

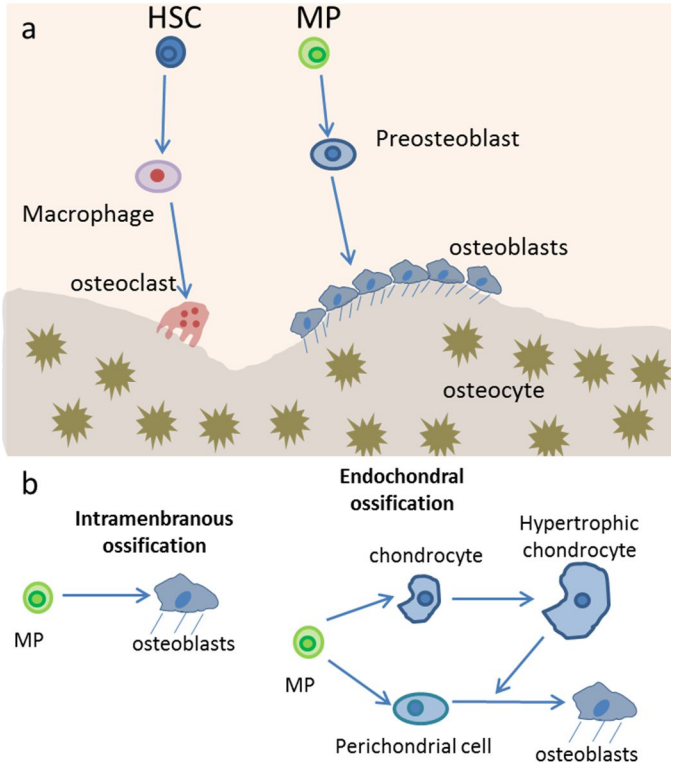



**Figure 3.** Bone homeostasis is accomplished by osteoclasts and osteoblasts. **A)** Osteoblasts arise from mesenchymal progenitors (MP) through preosteoblasts and then becomes another type of osteoblast lineage cell, the osteocytes, after being trapped in the bone matrix. Osteoclasts are multinucleated resorptive cells derived macrophages, which are in turn progenies of hematopoietic stem cells (HSCs). **B)** Osteoblasts differentiate from MP through intramembranous or endochondral ossification. In intramembranous ossification, MP condenses and differentiates into osteoblasts. In endochondral ossification, they condense and differentiate into perichondrial cells and chondrocytes, the latter undergo hypertrophy, which triggers the differentiation of perichondrial cells to osteoblasts. Adapted from Long, 2014.

Osteoblasts are generated from mesenchymal cells through two distinct processes: intramembranous or endochondral ossification. During intramembranous ossification, mesenchymal progenitors condense and directly differentiate into osteoblasts. But in endochondral ossification, mesenchymal progenitors condense to form chondrocytes and perichondrial cells. Chondrocytes proliferate and undergo hypertrophy, and only then do osteoblasts differentiate from the perichondrial cells [17].

### 1.2.2. Transcription factors involved in osteoblast differentiation

Osteoblasts differentiation from mesenchymal stem cells initiates by the action of SRY (Sex Determining Region Y)-Box 9 (SOX9) and although SOX9 is indispensable for chondrogenesis, it also marks the mesenchymal progenitors that give rise to all osteoblasts. However, SOX9 is not expressed by mature osteoblasts.

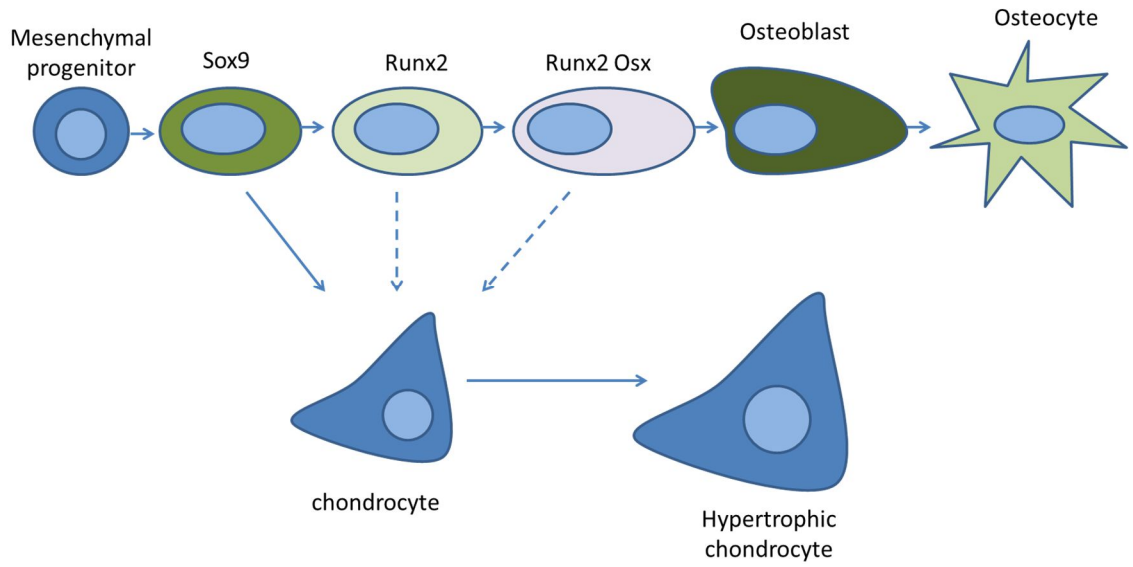



**Figure 4.** The different stages of osteoblast lineage cell differentiation. Mesenchymal progenitors that give rise to osteoblasts and chondrocytes are initially marked by the transcription factor SOX9. This is followed by the expression of RUNX2 and then OSX, leading to the development of osteoblasts. Some osteoblasts become osteocytes by being entombed in the bone matrix. SOX9 cells are bipotential and can also differentiate into chondrocytes. Dashed arrows represent that during development osteoblasts can switch fates under these conditions. . Adapted from Long, 2014.

During the process of differentiation, preosteoblasts are characterized by the expression of runt-related transcription factor 2 (RUNX2) and, in a more advanced stage of differentiation, both RUNX2 and osterix (OSX), while mature osteoblasts are characterized by the expression of osteocalcin [16]. Then a part of osteoblasts become osteocytes being trapped within the bone matrix and the rest either go into apoptosis or become inactive bone lining cells [18].

### 1.2.3. Remodeling

Once formed, bone undergoes remodeling that involves resorption and the formation of new bone; this occurs in micro scale throughout the skeleton. Bone remodeling is the predominant metabolic process regulating bone structure and function during adult life [19]. The cell type responsible for this process is the osteoclast, a tissue-specific macrophage polykaryon created by the differentiation of haematopoietic stem cells (HSCs) lineage at or near the bone surface. Mature osteoclasts activity is triggered by activation of receptor activator of nuclear factor (NF)-kB (RANK) by RANK ligand (RANKL) expressed by osteoblasts [20]. After its activation, osteoclasts undergo internal structural changes that prepare it to resorb bone, such as the rearrangements of the actin cytoskeleton and formation of a tight junction between the bone surface and basal membrane to form a sealed compartment. This external vacuole is then acidified by the export of hydrogen. Secretion continues with the export of the lytic enzymes TRAP and pro-CATK into a resorption pit. Through this process the osteoclast erodes the underlying bone. Degradation products, collagen fragments and solubilized calcium, phosphate and undercarboxylated osteocalcin are processed within the osteoclast and released into the circulation [16].

## 1.3. Bone Health in Type 1 Diabetes Mellitus

### 1.3.1. T1DM and bone loss

It is well established that T1DM is linked to a decrease of bone mineral density (BMD) and bone loss in humans [21–26], mice [27–32] and also in fin regeneration in zebrafish [33]. Different factors have been identified as responsible for this outcome. T1DM reduces osteoblast activity, as determined by serum osteocalcin levels which were found to be decreased in DMT1 mice models [31,34,35]. mRNA levels were also found to be reduced and associated with a reduced rate of mineral apposition [31,36–38]. In addition, several studies have demonstrated an increase in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive dying osteoblasts in diabetic compared to control [39,40]. So, enhanced osteoblast death caused by T1DM contributes to a reduction in osteoblast number and activity. In DMT1 mice models, osteoclast activity does not appear to be altered or decreased [36,41] in the majority of DMT1 studies, with only a few studies showing increased osteoclast markers [42], but this was proposed to be linked to the dose of streptozotocin used to induce diabetes in this model.

### 1.3.2. Insulin signaling and IGF1

Insulin plays an important role in bone growth due to its anabolic properties. Mice with 3–6 weeks, with insulin receptor (IR) deletion in osteoblasts revealed reduced bone volume and osteoblast number [43]. Also it has been proposed that insulin signaling in osteoblasts is necessary for whole-body glucose homeostasis, because insulin signaling in osteoblasts increase bone resorption by osteoclasts. With the increase in bone resorption, osteocalcin in its undercarboxylated form is released into the blood stream and acts as a hormone, signaling insulin expression in β-cells and other sensitive tissues [44,45]. Furthermore, animal models and cell studies showed that

osteocalcin can increase cell proliferation, stimulate insulin expression and secretion by pancreatic cells and enhance energy expenditure [43,46,47].

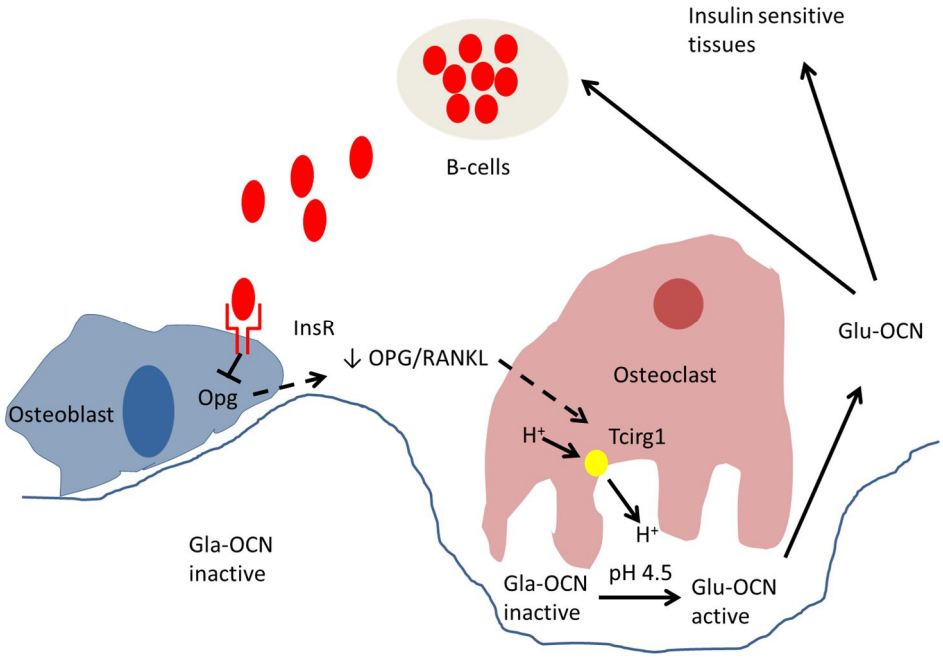



**Figure 5.** Model of action of insulin in bone resorption and osteocalcin regulation of insulin expression. Insulin signaling in osteoblasts decreases osteoprotegerin (Opg) expression. The decrease in the OPG/RANKL ratio results in an increased expression of Tcirg1 in osteoclasts. Tcirg1 encodes for a proton ($H^+$) pump subunit and contributes to acidification of the resorption lacunae. The acidic pH of approximately 4,5 generated during bone resorption is a sufficient mean to decarboxylate and activate the osteocalcin molecules (Gla-OCN) stored in the bone extracellular matrix. The undercarboxylated active osteocalcin (Glu-OCN) promotes insulin sensitivity in peripheral organs and stimulates insulin secretion by the β-cells of the pancreas. Adapted from Clemens and Karsenty, 2011.

*Insulin growth factor 1 (GF1)* is crucial for postnatal skeletal growth. Although it is mostly known for its effect on growth plate cartilage, it also regulates various aspects of osteoblast lineage cells metabolism [48]. *IGF1* is regulated centrally by growth hormone (GH) and mainly produced by the liver. The direct effects of IGF1 on bone could be seen when *IGF1 receptor (IGF1R)* knockout (KO) mice specifically in osteoblasts treated with GH failed to increase osteoblast number and osteoblast-specific deletion or overexpression of *IGF1R* reduced or increased bone formation [49,50].

### 1.3.3. Advanced glycation end products (AGEs)

Hyperglycemia increases nonenzymatic protein glycation, leading to the formation of a variety of chemically modified proteins known as advanced glycation end products (AGEs). Type 1 collagen is a target for AGEs leading to an increase in non-enzymatic collagen glycation [34], by forming covalent cross-links with collagen molecules through enzymatic reactions. AGEs are associated with reduced osteocalcin levels, inhibiting osteoblastic cells function [51], increasing cell clumping and decreasing cellular spreading [52]. AGEs increase with age and are accumulative and irreversible. Diabetic bone also exhibit increase in AGEs receptor (RAGE) causing AGEs increase [53]. In turn RAGE activation initiates inflammatory signals that stimulate a generation of reactive oxygen species and inflammation mechanisms which enhance AGE formation [54].

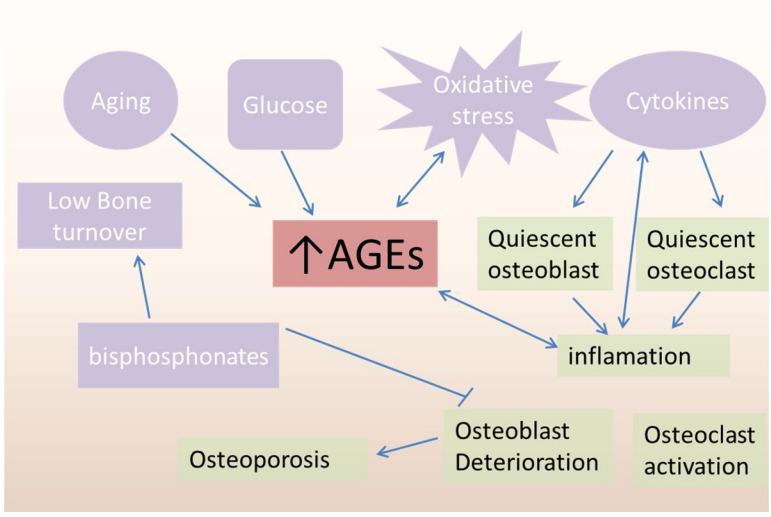



**Figure 6.** Principal causes for accumulation of AGEs in bone. Oxidative stress, high glucose, aging processes, and low bone turnover contribute to an increased formation and accumulation of AGEs in bone. Together with an increase of certain pro-inflammatory cytokines, AGEs induce both the activation of osteoclastogenesis and osteoblast dysfunction; leading to an accelerated development of osteoporosis. Adapted from Sanguineti et al. 2014.

#### 1.3.3.1. Inflammation

Bone inflammation has been linked to T1DM, principally because of AGEs or by autoimmunity reaction in T1DM.

It is currently assumed that both types 1 and 2 diabetes are associated with inflammation [37,40,55]. RAGE cell expression leads to activation of inflammation and tissue damage, upregulating inflammatory mediators like tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-6, and C-reactive protein (CRP) [54]. These suggest that RAGE activation contributes to perpetuation of AGEs and pro-inflammatory ligands synthesis, generating a microenvironment favorable for ligands production and suppressing protective mechanisms [56]. Diabetic mice models have demonstrated elevated TNF-α mRNA expression in bone marrow and is linked to early osteoblast death [37,57]. Treatment with TNF-α inhibitors reduced diabetes-induced increase in osteoblast apoptosis [58,59], suggesting a role for inflammation within the bone marrow in inducing osteoblast death.

### 1.3.4. Bone marrow adiposity

Both osteoblasts and adipocytes derive from MSC, this evidence has led to the assumption that lineage selection, adipocyte over osteoblast, could be involved in the diminishing of BMD due to an increase in bone marrow adiposity . Although an increase in marrow adiposity has been observed in some T1DM mice models [31,37], this relation is not seen in the vertebrae where there is a loss of bone but no increase in bone marrow adipocytes [60]. Supporting this idea are the results obtained from treating bone marrow adipocytes, either with PPARγ antagonist [61] or with leptin [60], where, in both cases, it was possible to prevent marrow adiposity but in neither of the treatments bone loss was prevented. This finding suggests that T1DM bone marrow adiposity increase in some T1DM mice models alone couldn´t be the cause for a decrease in bone volume [27].

### 1.3.5 Leptin and T1DM

The regulation of bone by leptin has been one of the major findings in the field of bone metabolism in the last fifteen years. Leptin is an adipocyte-derived hormone that is traditionally associated with controlling appetite and reproduction. But it was proposed that leptin signaling, through its receptors in the hypothalamus, stimulates noradrenaline secretion, that binds to β2-adrenergic receptors (Adrb2) on osteoblasts, inhibiting bone formation and increasing RANKL secretion, thus leading to increase bone resorption by osteoblasts [62]. After this findings several works have focused in understanding how leptin regulates bone and to explain the phenotype of the leptin KO ob/ob mice that have increased trabecular bone volume, but decreased cortical bone mass [63–65]. The most important matter of debate at the present moment, is if leptin regulates bone centrally or peripherally or by both ways. After the work of Ducy et al. [66], Steppan et al. [67] could see increase in bone mineral density after peripheral administration of leptin in ob/ob mice (although leptin can cross the blood-brain barrier and could have acted centrally) and similar results could be seen by Burguera et al. [68].

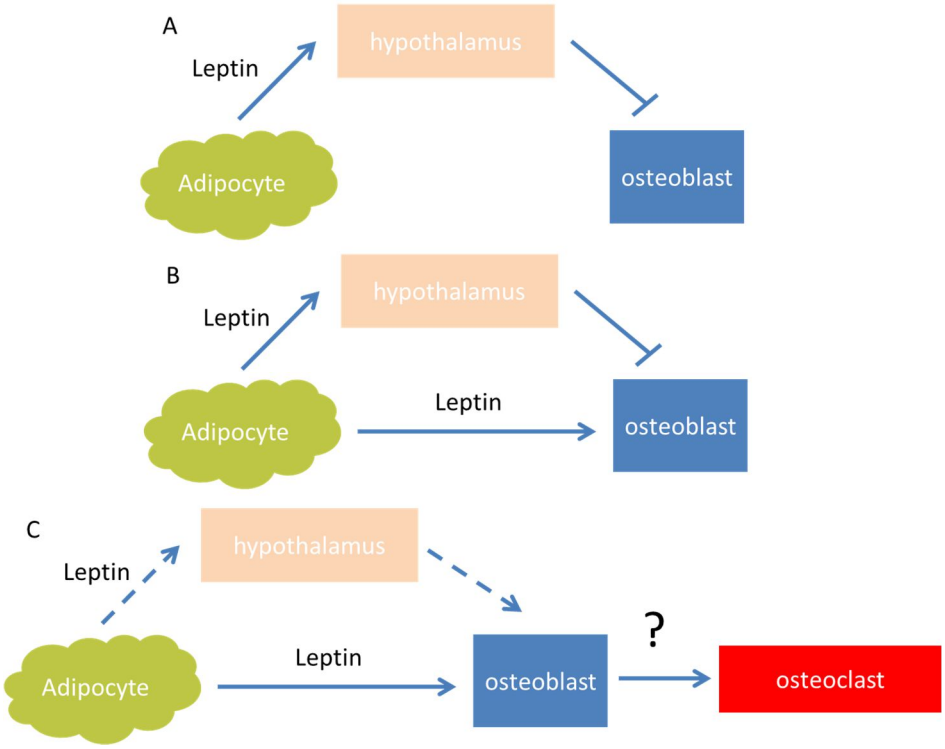



**Figure 7.** Models proposed for action of leptin on bone. A) Ducy's model, leptin indirectly inhibits bone formation through an hypothalamic relay. B) Burguera/Hamrick model, skeletal effects of leptin result from anabolic actions of peripheral leptin and antiosteogenic actions of hypothalamic leptin. C) Turner model, leptin directly increases bone growth, osteoblast number and function, as well as osteoclast function. These physiological actions of the hormone are largely mediated through peripheral leptin signaling. Solid arrows represent the major route of action of action. The question mark indicates that mediation of the leptin-dependent increase in osteoclast activity by the osteoblast is speculative. Adapted from Turner et al., 2013.

Later, Hamrick et al. [63] reported that, consistent with observations from the Karsenty group, ob/ob mice exhibit a high bone mass in the lumbar vertebrae, but a contrastingly low trabecular and cortical bone mass in the femur, proposing a concurrence of both axial catabolic and appendicular anabolic effects of leptin on the skeleton. Finally, Turner et al. [65] was extremely polemic about the work of Ducy et al. [66] saying that the central regulation of leptin is not the principal pathway for the regulation of bone and the direct signaling of leptin through the leptin receptors in both osteoblasts and osteoclasts enhance bone formation and bone resorption respectively.

Independently from how leptin acts in bone, it is widely assumed that low levels of leptin secretion induce changes in bone homeostasis. Low controlled T1DM patients also develop hypoleptinemia, another important factor in the diabetic bone phenotype.

## 1.4. T1DM animal models used in our study

### 1.4.1. Ins2$^{+/akita}$ mouse

Mice have two different genes for insulin, *Ins1* and *Ins2,* the latter being the ortholog for human *INS* gene. *Ins2*$^{+/akita}$ mouse, first generated in 1997 by Yoshioka and colleagues [69], have only one allele (*Ins2)* mutated. The mutant allele is characterized by a transition from G to A at nucleotide 1907 disrupting an Fnu4HI site in exon 3. This mutation changes the seventh amino acid in the A chain of mature insulin, from Cys96 (TGC), to Tyr (TAC).

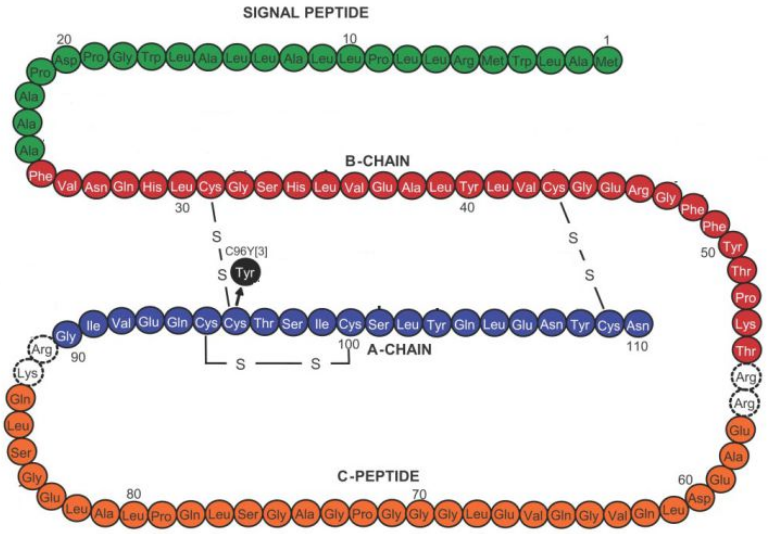



**Figure 8.** Diabetes of the *Ins2*$^{+/akita}$ mouse is caused by a single locus mutation, and is inherited in an autosomal dominant manner. A G⇒A mutation changed amino acid Cys96 (TGC) to Tyr (TAC). Cys96 corresponds to the seventh amino acid in the A chain (A7) of mature insulin and forms one of the three intramolecular disulfide bonds with Cys31 located at B7 [70]. Adapted from Støy J et al.

This cysteine residue normally forms an intramolecular disulfide bond with cysteine at B7. Disruption of the disulfide bonds is expected to induce a drastic conformational change of the molecule. Normal and mutant *Ins2* alleles are transcribed similarly in pancreatic islets of heterozygous mice, although immunofluorescence and immunoblot analyses detected reduced levels of insulin and proinsulin [69], rendering $\beta$ cells incapable of insulin secretion in a dominant negative way [71].

Two of the proteins playing a major role in regulation of *Ins2*, *protein disulfide isomerase* (*PDI*) and *binding immunoglobulin protein* (*BiP*), are overexpressed in the islets of *Ins2*$^{+/akita}$ mice. Both proteins reside in the ER, where disulfide formation and folding of secreted protein occur. Disulfide formation can be formed in the wrong temporal order and even randomly in some proteins. In this case, lack of cysteine at A7 in the mutant proinsulin will leave cysteine at B7 free and thereby increase the possibility that later, cysteine forms incorrect disulfide bonds with other molecules. This would affect the disulfide bond formation of coexisting wild-type proinsulin and result in an aggregation in *Ins2*$^{+/akita}$ mice .

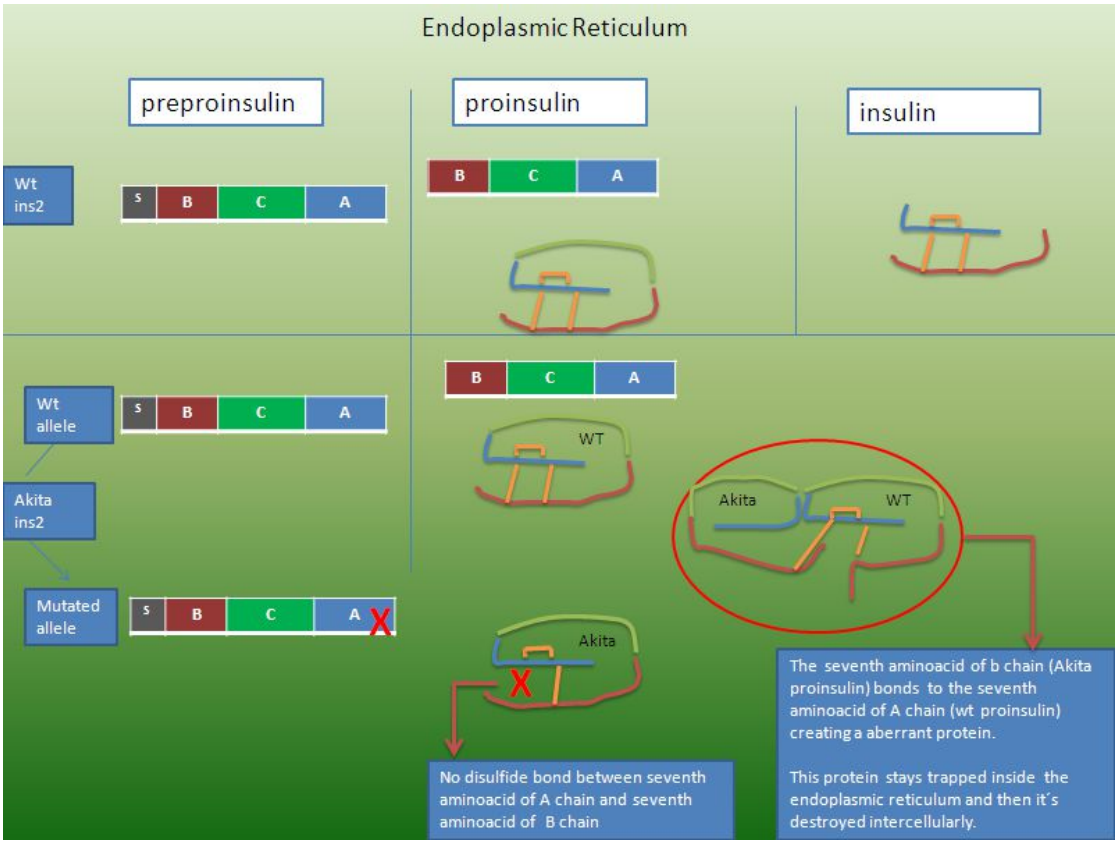



**Figure 9.** Pathway of preproinsulin to insulin in WT and *Ins2*$^{+/akita}$ mice. In *Ins2*$^{+/akita}$ mouse, mutant proinsulin will acquire a deficient structure, becoming unable to exit the ER. The seventh amino acid of the B chain will create a disulfide bond, with the seventh amino acid of the A chain of proinsulin non mutated allele, forming an aberrant structure. This protein will not be expressed and it will be eliminated.

This form of T1DM, modeled by *Ins2*$^{+/akita}$ mouse [69,71,72], is also seen in humans [7-9] and is part of the sub-group Type 1b DM or permanent neonatal diabetes mellitus (PNDM).

Symptoms in heterozygous *Ins2*$^{+/akita}$ mutant mice include hyperglycemia, hypoinsulinemia, hypoleptinemia, polydipsia, and polyuria, beginning around 3-4 weeks of age but no obesity, infertility, or immunological alterations have been described to date in these mice. Histological signs of glomerulosclerosis have also been described in 20-wk-old *Ins2*$^{+/akita}$ mice and renal function impairment after age 30 weeks [73]. But although this model develops bone loss, with a clear decrease in bone mineral density, no histological characterization of its bone structure/morphology or any *in vitro* work using *Ins2*$^{+/akita}$ at adult and aging time points is available.

### 1.**4.2. Transgenic zebrafish Tg(ins:Eco.NfsB-mCherry)**

With the objective of generating a zebrafish model of β-cell regeneration, Pisharath and colleagues [74] inserted an Escherichia coli gene downstream from the promoter region of *insa* gene called *nfsB*. This bacterial gene encodes a nitroreductase (NTR) enzyme, which can convert prodrugs such as metronidazole (Met) to cytotoxins. By fusing *nfsB* to a mCherry florescent protein, it is possible to visualize *insa* expressing cells and ablation of β-cells 3 days after an intraperitoneal (IP) injection with Met.

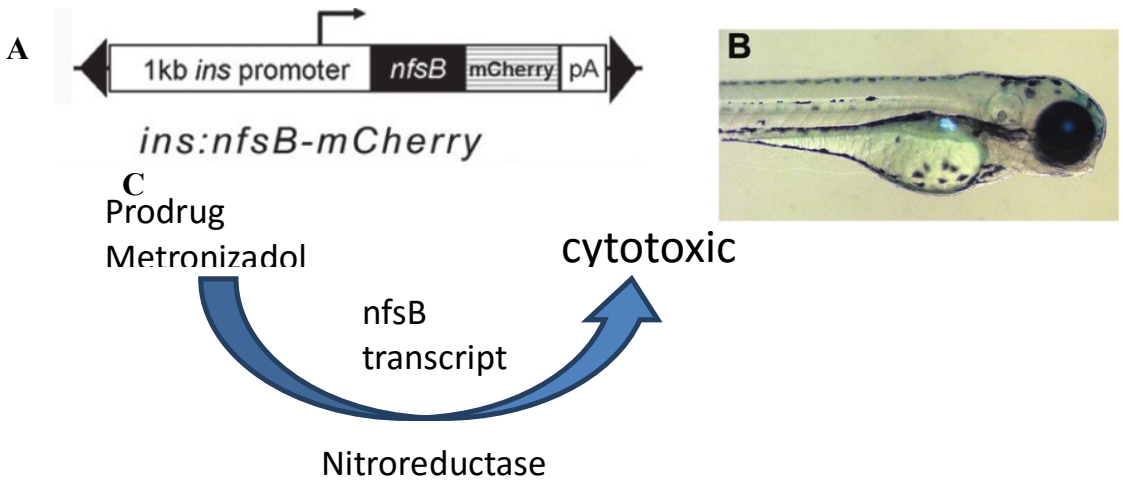



**Figure 10.** Transgenic zebrafish Tg(*ins:Eco.NfsB*-mCherry) is a suitable model to study DM; **A** This model as a e.coli gene *nfsB* and a mCherry florescent protein downstream of the promoter region of the insa gene; **B** Transgenic larvae with florescent insa expressing cells, mainly the pancreas; C nitroreductase enzyme from *nfsB* gene catalysis Met generating a cytotoxic compound induced ablation in insa expressing cells. Adapted from Pisharath et al., 2007.

After this period, increased blood glucose can be detected and progressive regeneration of β-cells leads to normalization of glycaemia after two weeks of Met exposure [75]. Also it was observed that after 3 day exposure to Met, blood glucose readings were higher than in the streptozotocin (STZ) induced diabetic WT zebrafish, suggesting that β-cell ablation may be more complete in the Tg(ins:Eco.NfsB-mCherry), making this model more suitable for the study of diabetes [76].

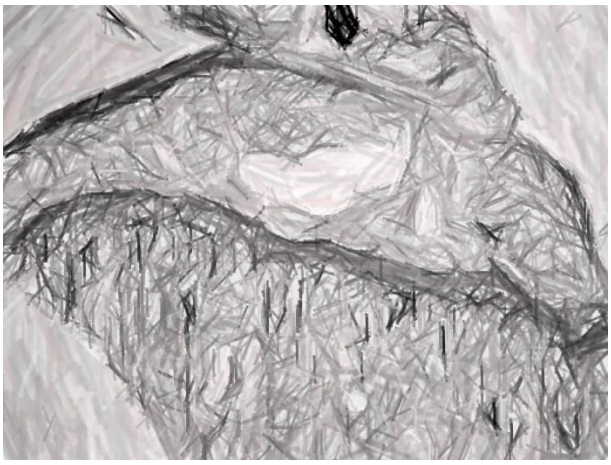
Also it was observed that β-cell ablation was achieved without affecting the neighboring α and δ cells [74]. During the window period of induced hypoinsulinemia, different protocols can be applied to study the effects of DM, also the hyperglycemic state can be prolonged if continuous exposure to Met is applied.

## 1.5. Objectives of this work

- In chapter two we proposed to identify histological and microarchitecture alterations in the long bones of diabetic mice model *Ins2*$^{+/akita}$ and provide some evidence towards identifying the molecular players regulating these changes.
- In chapter three we aimed to test the effects of vitamin D, vitamin D analogs and calcimimetics in fin regeneration and mineralization under diabetic conditions in a transgenic zebrafish model of diabetes.
- In chapter four we focused in understanding the role of paralogs genes of insulin in human, mouse and zebrafish, especially if they can also regulate glucose as the ancestral gene. Also in this chapter we proposed to investigate if extrapancreatic expression of both INS and INS-IGF2 can occur, mimicking pancreatic INS action.

# CHAPTER 2.

# ALTERED BONE ARCHITECTURE IN THE TYPE 1 DIABETES MOUSE MODEL INS2$^{+/AKITA}$



# CHAPTER 2. ALTERED BONE ARCHITECTURE IN THE TYPE 1 DIABETES MOUSE MODEL *INS2*$^{+/AKITA}$

**Filipe R. Carvalho**
Sofia M. Calado
Gabriela A. Silva
Gabriela S. Diogo
Joana Silva
Rui L. Reis
M. Leonor Cancela
Paulo J. Gavaia

This chapter is part of a research paper submitted for publication to BONE

**Author´s contribution:** Study design MLC and PJG. Study conduct: FRC and PJG. Data collections: FRC, PJG, SMC, GAS, GSD, JS, RLR. Data analyses: FRC and PJG. Data interpretation: FRC and PJG. Drafting manuscript: FRC. Revising manuscript content: PJG and MLC. Approving final version of manuscript: all authors.

## 2.1. Abstract

Type 1 diabetes mellitus (T1DM) is related to several cartilage and bone alterations including growth retardation, increased fracture risk and bone loss. Accumulation of advanced glycation end products (AGEs), diminished insulin signaling, imbalance in calcitropic hormone levels, and leptin deficiency has been identified as possible causes for these alterations. To determine the effect of long term diabetes on bone we used adult and aging *Ins2*$^{+/akita}$ mice that develops T1DM of 5 weeks after birth. Both *Ins2*$^{+/akita}$ and WT mice at 4, 6 and 12 months were used to assess bone parameters such as femur length, growth plate thickness and number of mature and pre-apoptotic chondrocytes. In addition, bone microarchitecture of the cortical and trabecular regions was measured by microCT and gene expression of *Adamst-5, ColII, Igf1, Runx2* and *Oc* was quantified by qRT-PCR. *Ins2*$^{+/akita}$ mice

showed a decreased longitudinal growth of the femur that was related to decreased growth plate thickness, reduced number of chondrocytes and to a higher number of pre-apoptotic cells. These changes were associated with increased expression of *Adamst-5*, suggesting higher cartilage degradation, and with low expression of *Igf1* and *ColII* that influence cartilage matrix formation. *Ins2*$^{+/akita}$ bone changes were characterized by low cortical bone area (Ct.Ar) but higher trabecular bone volume (BV/TV) and to reduced gene expression of *Oc* and *Runx2*. We suggest that *Ins2*$^{+/akita}$ mice bone phenotype is caused by reduced bone formation and even more reduced bone resorption due to leptin and insulin deficiency.

Keywords: bone; cartilage; diabetes; insulin; leptin.

## 2.2. Introduction

The global prevalence of type 1 diabetes mellitus (T1DM) has been increasing at a rate of 2-5% a year [5] leading to an increase in diabetes related pathologies, including bone disorders. T1DM was previously shown to induce alterations in cartilage [27,28,31,77,78] and bone loss [27,28,30,35,37,60,79,80], associated to different factors like increased glucose serum concentration and reduced insulin secretion by the β-cells, inflammation and altered gene expression. Advanced glycation end products (AGEs) are proteins or lipids that are formed in hyperglycemic environments. Since their cumulative effects increase with age AGEs represent a key player in vascular disease associated to diabetes [81,82]. In addition to affecting the vascular system, AGEs are involved in an increase of inflammatory activity and associated to a decreased bone formation due to osteoblastic apoptosis and decreased osteoblast proliferation [29,51,52,83] or increased osteoclastic activation [56], and chondrocyte apoptosis in cartilage [29,84]. The receptor for AGEs (RAGE) seems to be the molecular intervenient that activates the pathways leading to oxidative stress and inflammation

[54,85] including in bone since osteoblasts, osteoclasts and chondrocytes express RAGE [53,86]. Hypoinsulinemia present in T1DM can also affect bone metabolism since insulin signaling in osteoblasts was found to regulate bone resorption by activating osteoclasts activity [47], releasing undercarboxylated osteocalcin to the blood stream, which in turn affects glucose homeostasis by signaling insulin secretion in β-cells and others insulin sensitive tissues [43]. This relationship between bone and insulin was demonstrated when *Ob-IR* mice, lacking the insulin receptor (IR) only in osteoblasts, became glucose intolerant [47]. T1DM patients and mice models face a rapid weight loss during the onset of the disease [27,28,78,80,87], that persists if not treated, creating a state similar to the observed by accelerated fastening, that result in loss of fat and proteins. Weight loss has been associated with low bone mass [88], but interestingly only a decrease in fat mass was found to be correlated with decreased bone mineral density (BMD) and not lean mass or total body weight [89–91]. This close relationship between fat and bone seems to be explained by the fact that adipocytes secrete leptin. Accordingly, both the leptin knockout (KO) mice *ob/ob* and the leptin receptor KO *db/db* mice have impaired bone formation, exhibiting a normal or decreased cortical bone volume (BV/TV) although presenting a higher trabecular BV/TV [65,66]. It was then postulated that leptin binding to its receptors in the hypothalamus increases the expression of noradrenaline activating β2-adrenergic receptors pathway in osteoblasts, inhibiting bone formation and increasing the expression of RANKL, promoting the differentiation and proliferation of osteoclasts [62,92,93]. These findings were then supported by the results of β2-adrenergic receptor KO mice (*Adrb2*$^{-/-}$), that exhibit an increase in trabecular bone at the age of 6 months [92]. It has been proposed that peripherally leptin promotes osteoblast proliferation through leptin receptor signaling [94,95] and, more recently, Turner and colleagues [65]

proposed that peripheral leptin induces bone formation and resorption and that it represents the main route of action of leptin in bone. Food intake is correlated with high levels of leptin and fasting periods are associated with low levels of leptin as previously observed in fasting mice and in anorexia nervosa [96,97]. Devlin et al. [96], performing experiments with mice under caloric restriction (CR) from 3 to 12 weeks of age, not only correlated leptin levels with CR, but also with low cancellous BV/TV and low cortical area, assuming that CR in juvenile mice under a fast period of growth lead to bone loss. But unexpected results were observed in the 6 months mice after a period of CR of 10 weeks [98], that presented low cortical mass, but higher trabecular BV/TV in the vertebra and unchanged trabecular BV/TV in the femur. In our study we hypothesized that inflammation together with leptin and insulin deficiency could be the principal causes involved in cartilage and bone phenotype of *Ins2*$^{+/akita}$.

## 2.3. Materials and Methods

### 2.3.1. Mouse models

Wild type C57BL/6 and *Ins2*$^{+/akita}$ (C57BL/6 background) mice at 4, 6 and 12 months were used to perform the experimental procedures. Diabetes was monitored by blood glucose measurements using a Glucose Assay Kit (Free Style Precision, Abbott Laboratories, Illinois, USA) and only Ins2$^{+/akita}$ mice with glucose values >300 mg/dl were used in this experiment. All animal manipulations were conducted in accordance with principles and procedures followed the guidelines from the Federation of Laboratory Animal Science Associations (FELASA). Age matched male wildtype mice were purchased from Jackson Laboratory (Bar Harbor, Maine). All mice were kept on a light/dark (12h/12h) cycle at 23ºC, and received food (standard lab chow) and water ad libitum.

### 2.3.2. Total RNA isolation

Left femur and tibia were isolated, cleaned from adhering tissues then snap-frozen in liquid nitrogen. Frozen bones were crushed using a mortar and pestle and RNA extracted with the Isol-RNA Lysis Reagent 5 PRIME® (Hilden, Deutschland) according to manufacturer's protocol. RNA integrity was verified using Experion™ RNA Analysis Kit (BIO-RAD, Hercules, CA, USA).

### 2.3.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Reverse transcription was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Using iQ™ SYBR® Green Supermix (Life Technologies), qRT-PCR was performed on an CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA) 45 cycles 15 sec. for annealing and 30 sec. for amplification. All gene expression data were normalized against *hypoxanthine phosphoribosyltransferase* 1 (*Hrpt1*).

### 2.3.4. Bone histology and histomorphometry

The right tibias were fixed in 4% paraformaldehyde, (pH 7.4 in PBS), and decalcified in 10% EDTA/TRIS-HCl (pH 7.4) for 15 days, then 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 µm prepared in a microtome. Before staining, sections were deparaffinized in Xylene and dehydrated in an increasing gradient of EtOH. Sections were stained with safranin O, fast green and Mayer´s hematoxylin [99].

### 2.3.5. Growth plate measurements

For measurement of the growth plate (GP) thickness, 8 to 10 measurements were performed, separated by 0,05 mm of distance per GP. 5 animals for each group were used and 3 consecutive histological sections per tibia were analyzed. Proliferative chondrocytes were identified according to its morphology and position in the growth plate.

### 2.3.6. Immunohistochemistry

Sections were processed as described above. After deparaffination and hydration, we performed heat mediated epitope retrieval for 25 min in tris-EDTA pH9 buffer and hyaluronidase (Sigma H3506) treatment for 30 min. Sections were then incubated for 30 min. with blocking buffer (Goat serum and BSA) to block unspecific binding sites. Sections were incubated overnight at 4 °C with rabbit polyclonal Anti-Caspase-3 diluted in PBS 1:100 (ab13847, ABCAM, Cambridge, UK). Slides were then incubated in 0.3% $H_2O_2$ in PBS with 0.1% Triton X-100 for 15 min. The secondary antibody, anti-rabbit IgG – peroxidase (Sigma Aldrich) was applied diluted 1:100 in PBS and incubated for 90 min at room temperature. The signal was detected by incubating the sections for 4 min in a 0.1% 3,3′-diaminobenzidine (DAB) substrate and 0.02% hydrogen peroxide solution. The sections were counterstained with Mayer's hematoxylin.

### 2.3.7. Detection of apoptosis

Total number of Caspase 3 positive proliferative and hypertrophic cells was counted and hematoxylin stained cells were used as negative control for pre-apoptotic cells. From each animal tested at least 3 sections from each femur separated by 25 µm, were counted and the percentage of apoptotic cells in growth plate calculated. Cells were counted using the cell counter plug-in of imagej software.

### 2.3.8. Micro-computed Tomography (CT) analysis

Three femurs from each time point and genotype were scanned using a Bruker microCT Skyscan™ CT 1072 scanner (Kontich, Belgium) with an accelerated voltage of 50 kV and a current source of 197 uA at 5.1μm of pixel size. Measurements in the trabecular region were made in the distal epiphysis of the femur defined at 0.255mm under the growth plate extending 1mm (200 layers) toward to diaphysis, and excluding the outer cortical shell. Quantitative parameters were obtaining by the Skyscan™ CT-analyzer software for each region of interest. The thickness of the cortical bone was measured through the images of DataViewer software taken from the distal femoral methaphysis and all measurements were done using the ImageJ software.

### 2.3.9. Statistical analysis

All statistical analyses were performed using Stata Statistical Software data analysis 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 ± standard deviation of the mean (SD).

## 2.4. Results

### 2.4.1. T1DM reduces femur length and body weight in *Ins2*$^{+/akita}$

Diabetes in *Ins2*$^{+/akita}$ mice caused a reduction in femur length and body weight at all 3 analyzed age stages. Reduction in femur length were found to be of 5.35%, 5.34% and 3.8%, at 4, 6, and 12 months respectively (figure 10.B), the differences being statistically significant with $p<0.001$. This reduction was also observed in body weight of *Ins2*$^{+/akita}$, with differences of 27.06%, 26.16% and 26.92%, at 4, 6, and 12 months respectively, with $p<0.05$ (figure 10.C).

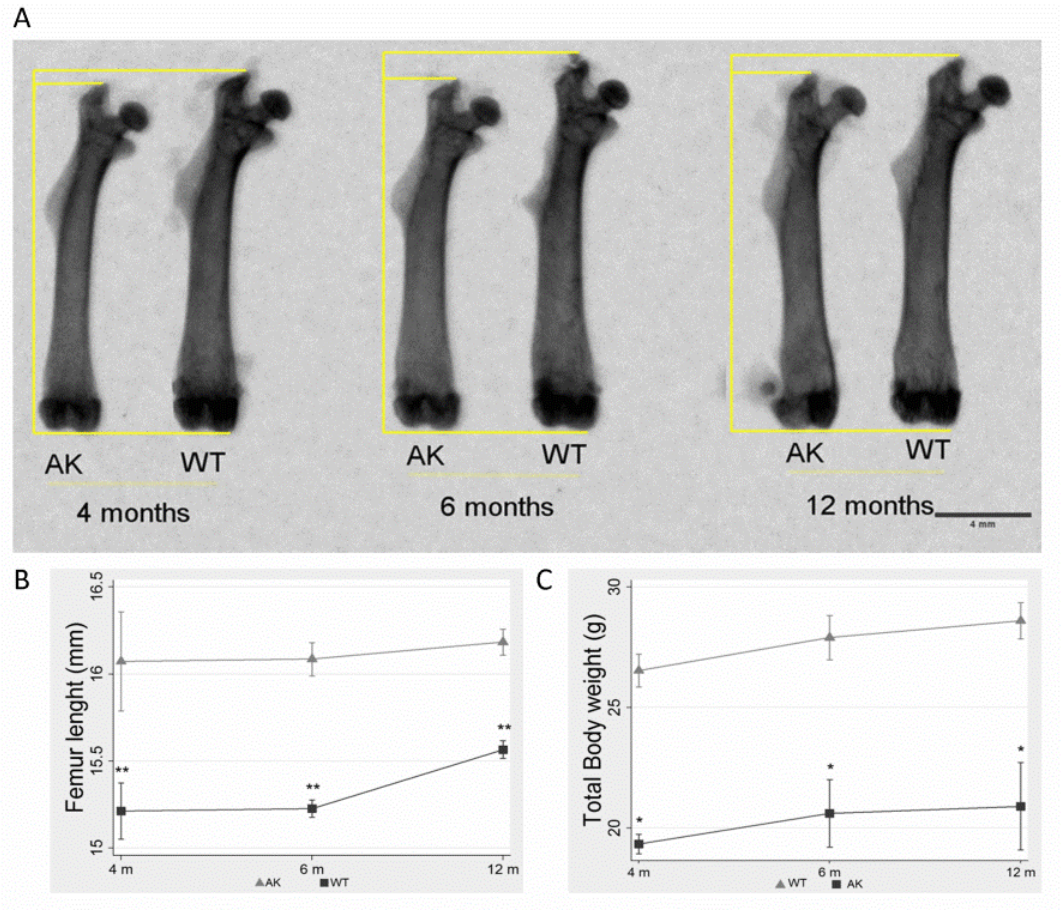



**Figure 11** **A)** X-ray analysis of *Ins2*$^{+/akita}$ and WT mice femurs at 4, 6 and 12 months; **B)** *Ins2*$^{+/akita}$ Femur length is highly significant smaller than WT at 4, 6 and 12 months, demonstrating that type 1 diabetes mellitus is related to growth retardation. is li **C** All body weight of *Ins2*$^{+/akita}$ is constantly significantly reduced than WT at 4, 6 and 12 months, caused by the impaired glucose homeostasis leading to activation of alternative pathways degrading fatty acids and proteins from soft tissues leading to weight lost. *p<0.05, **p<0.001.

### 2.4.2. Growth plate thickness is reduced in *Ins2*$^{+/akita}$

Potential alterations in growth plate structure will impair longitudinal bone growth so we investigated if growth plate of *Ins2*$^{+/akita}$ could be affected. Growth plate measurements showed that at 4 and 6 months the thickness was reduced, but at 12 months of age only a trend for reduction was observed (figure 11.B). Taking into account that it is during the proliferative phase of chondrocytes that bone lengthening occurs, we counted the total number of proliferative chondrocytes and significant differences were observed at 4 and 6 months in *Ins2*$^{+/akita}$ compared to WT, showing a reduction in the number of proliferative chondrocytes (figure 11.C). No differences

were observed at 12 months, a result which is in agreement with growth plate thickness measurements. To determine if the number of hypertrophic chondrocytes was altered, we performed an immunohistochemical detection of caspase 3 with the objective of identifying pre-apoptotic cells. The total number of chondrocytes in growth plate was determined by counting the cells under the microscope and significant differences were observed at 4 months, with the group of *Ins2*$^{+/akita}$ having a higher percentage of pre-apoptotic cells (figure 12.B).

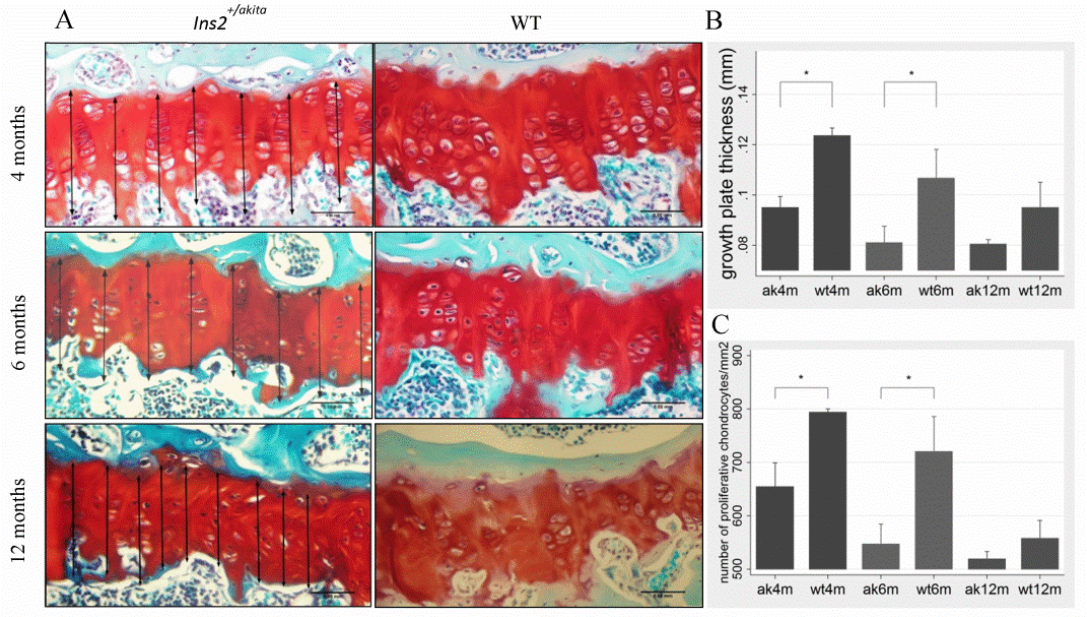



Figure 12. **A)** Growth plate thickness of *Ins2*$^{+/akita}$ mice and WT. Reduced number of proliferative chondrocytes leaded to thinner growth plates and to longitudinal bone growth, black arrows represent average of growth plate thickness of WT; **B)** and **C)** growth plate thickness and number of proliferative chondrocytes are significantly reduced at 4 and 6 months in *Ins2*$^{+/akita}$ but not at 12 months.

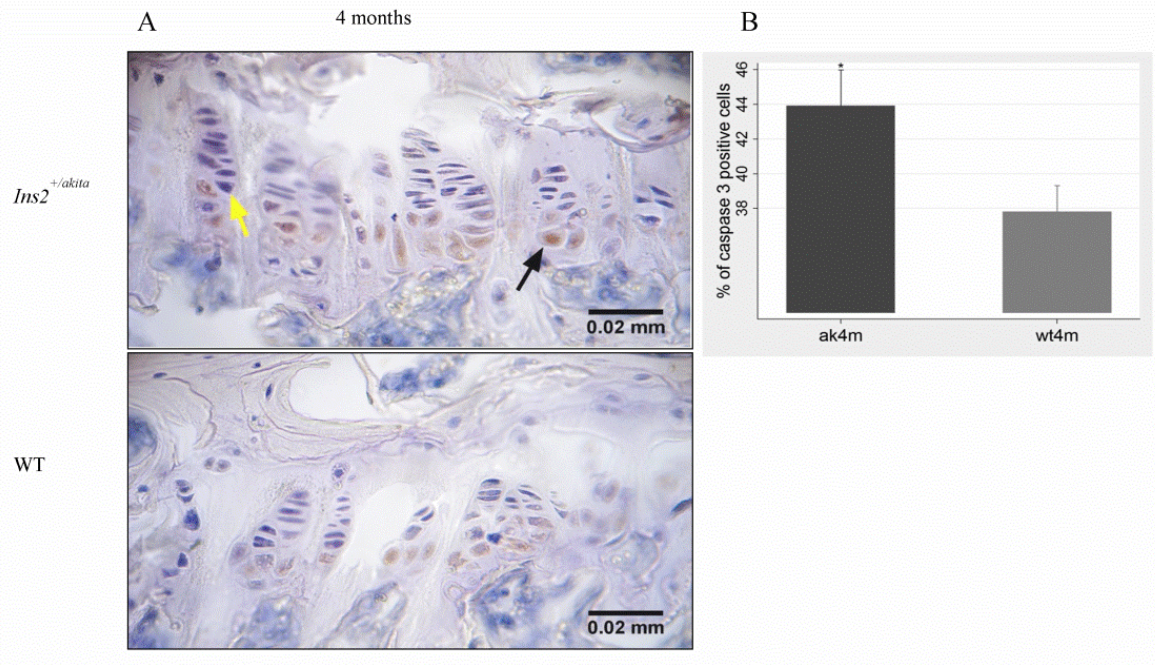



**Figure 13**. A) Evaluation of pre-apoptotic cells by Immunohistochemistry in growth plate, black arrow caspase 3 positive cells, yellow arrow negative cells; B) *Ins2*$^{+/akita}$ showed increased number of pre-apoptotic cells in growth plate than WT significant differences *p<0.05.

### 2.4.3. *Ins2*$^{+/akita}$ have reduced cortical area and higher trabecular bone volume at 4, 6 at 12 months

Total area (Tt.Ar) of cortical bone in *Ins2*$^{+/akita}$ was found to be reduced by 32% at 4 months, 16% at 6 months and 25% at 12 months of age. These differences were significant for all ages analyzed ($p<0.05$) (Table 1). The reduction in cortical bone was principally due to a substantial decrease in cortical area (Ct.Ar), of 53% at 4 months, 35% at 6 months and 25% at 12 months, with all results highly significant compared to WT controls ($p<0,001$) (Table 1 and Figure 13.B). This decline in cortical bone observed in the diabetic mice was confirmed by a decrease in the cortical area fraction (Ct.Ar/Tt.Ar), cortical thickness (Ct.Th) and periosteal perimeter (Ps.Pm) ($p<0.05$) (Table 1). Although not significantly different, the marrow area (Ma.Ar), showed a reduction in area at all ages. A significant reduction was found in the endocortical perimeter (Ec.Pm) ($p<0.05$) at 12 months (Table 1). Cortical bone parameters were

relatively constant from 4 to 12 months in *Ins2*$^{+/akita}$. Diabetic mice did not show any signs of recovery or aggravation of the low cortical area with aging.

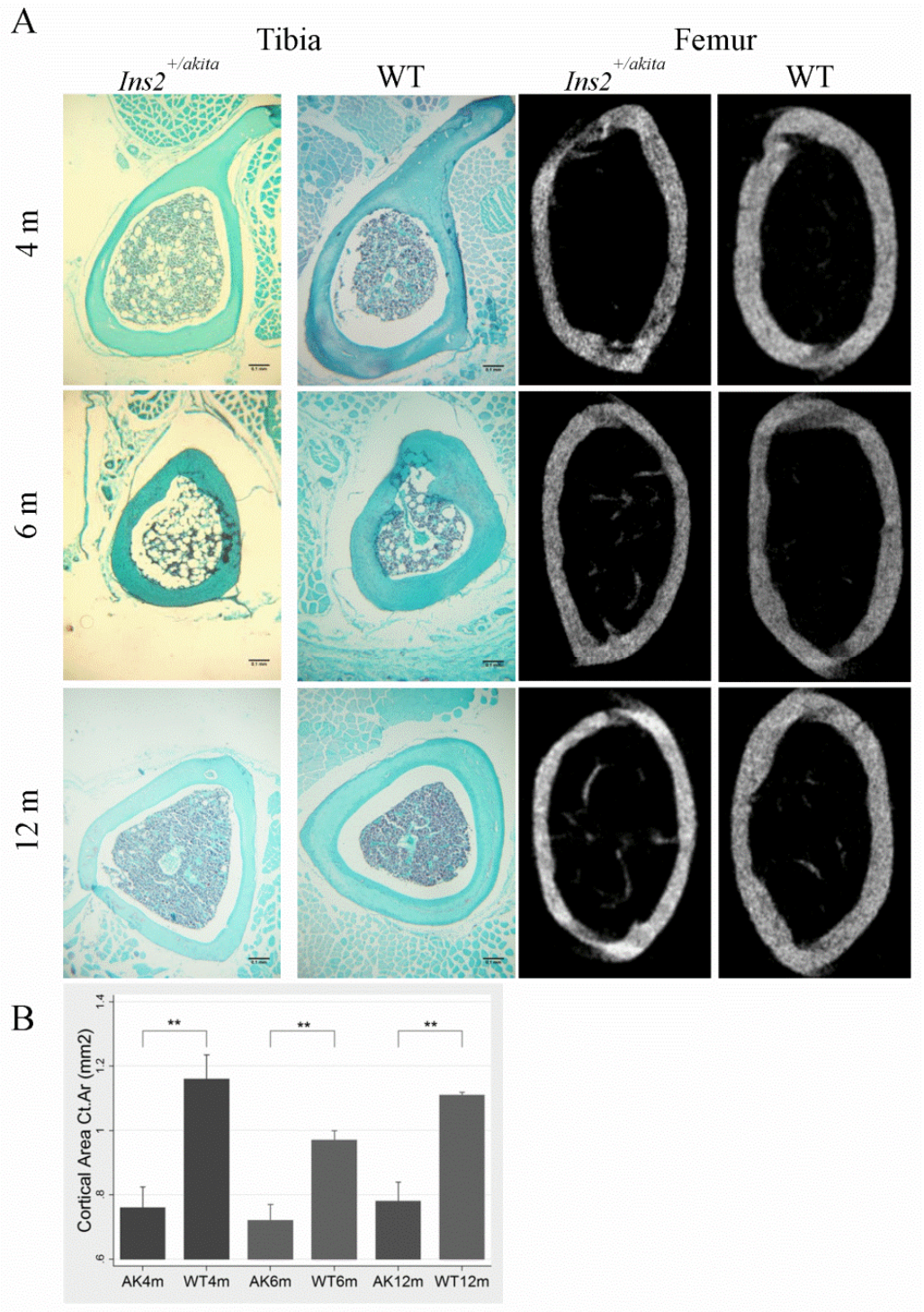



**Figure 14. A)** Histological sections of the cortical proximal diaphyseal, mid and distal diaphyseal shaft of the tibia and microCT images of the cortical diaphyseal distal shaft of the femur of *Ins2*$^{+/akita}$ and WT mice of 4, 6 and 12 months; Diabetes induced cortical bone loss and significant differences could be found in Tt.Ar, Ct.Ar, Ct.Ar/Tt.Ar, Ct.Th, Ps.Pm. bone volume reduction occurred by the decline periosteum perimeter suggesting reduced bone formation; **B)** *Ins2*$^{+/akita}$ mice has highly significant reduce cortical bone area when compared to WT mice at 4, 6 and 12 months,; **p<0.001.

Trabecular bone parameters showed opposite results from those found in cortical bone. Differences in bone volume relative to trabecular volume (BV/TV) in *Ins2*$^{+/akita}$ were found to be highly significant at 4 months ($p<0.001$) with an increase of 45%, an increase of 46% ($p<0,05$) at 6 months, and an increase of 30% at 12 months (Table 1 and Figure 14.B).

**Table 1.** Femur morphometry data of trabecular distal region and of metaphysis distal cortical region.

| | 4 months | | 6 months | | 12 months | |
|---|---|---|---|---|---|---|
| **Trabecular** | Akita n = 3 | WT n = 3 | Akita n = 3 | WT n = 3 | Akita n = 3 | WT n = 3 |
| **BV/TV (%)** | 33,13* ± 3,53 | 18,34 ± 0,772 | 14,86* ± 0,370 | 8,01 ± 3,420 | 10,18 ± 1,334 | 7,13 ± 1,984 |
| **BS/TV ($mm^2/mm^3$)** | 0,022** ± 0,001 | 0,013 ± 0,000 | 0,012** ± 0,001 | 0,006 ± 0,002 | 0,008* ± 0,001 | 0,005 ± 0,001 |
| **BS/BV ($mm^2/mm^3$)** | 0,068 ± 0,004 | 0,073 ± 0,003 | 0,082 ± 0,002 | 0,082 ± 0,010 | 0,083 ± 0,006 | 0,073 ± 0,005 |
| **SMI** | 1,82* ± 0,182 | 2,27 ± 0,070 | 2,48 ± 0,107 | 2,69 ± 0,227 | 2,26 ± 0,033 | 2,65 ± 0,231 |
| **Tb.N (1/mm)** | 0,0060** ± 0,0005 | 0,0033 ± 0,0001 | 0,0031* ± 0,0002 | 0,0015 ± 0,0006 | 0,0021 ± 0,0002 | 0,0012 ± 0,0003 |
| **Tb.Th (mm)** | 55,29 ± 1,724 | 55,94 ± 2,391 | 48,11 ± 1,304 | 52,93 ± 5,632 | 47,91* ± 3,041 | 59,40 ± 1,020 |
| **Tb.Sp (mm)** | 95,79** ± 5,847 | 160,58 ± 7,572 | 148,40** ± 1,251 | 234,20 ± 21,043 | 200,52** ± 3,991 | 272,14 ± 23,111 |
| **Cortical** | | | | | | |
| **Tt.Ar ($mm^2$)** | 1,91** ± 0,180 | 2,52 ± 0,242 | 1,97 ± 0,04 | 2,30 ± 0,06 | 2,08* ± 0,144 | 2,60 ± 0,135 |
| **Ct.Ar ($mm^2$)** | 0,76** ± 0,065 | 1,16 ± 0,076 | 0,72** ± 0,05 | 0,97 ± 0,03 | 0,78** ± 0,060 | 1,11 ± 0,009 |
| **Ma.Ar (mm2)** | 1,14 ± 0,12 | 1,36 ± 0,18 | 1,24 ± 0,01 | 1,33 ± 0,08 | 1,30 ± 0,11 | 1,49 ± 0,14 |
| **Ct.Ar/Tt.Ar (%)** | 0,40* ± 0,015 | 0,46 ± 0,020 | 0,37** ± 0,02 | 0,44 ± 0,02 | 0,37* ± 0,020 | 0,43 ± 0,025 |
| **Ct.Th (mm)** | 0,15** ± 0,014 | 0,20 ± 0,010 | 0,13** ± 0,01 | 0,17 ± 0,01 | 0,15* ± 0,005 | 0,18 ± 0,013 |
| **Ps.Pm (mm)** | 5,14* ± 0,323 | 6,04 ± 0,394 | 5,32* ± 0,07 | 6,03 ± 0,09 | 5,36* ± 0,212 | 6,23 ± 0,292 |
| **Ec.Pm (mm)** | 4,07 ± 0,243 | 4,55 ± 0,299 | 4,33 ± 0,01 | 4,75 ± 0,10 | 4,31* ± 0,177 | 5,05 ± 0,527 |

BV/TV Bone volume %; BS/TV Bone surface density Ratio $mm^2/mm^3$; BS/BV Specific bone surface $mm^2/mm^3$; SMI Structure model index; Tb.N Trabecular number 1/mm; Tb.Th Trabecular thickness mm; Tb.Sp Trabecular separation mm; Tt.Ar Total area inside the periosteal envelope $mm^2$; Ct.Ar Cortical bone area mm; Ma.Ar Medullary area $mm^2$; Ct.Ar/Tt.Ar Cortical area fraction %; Ct.Th Average cortical thickness mm; Ps.Pm Periosteal perimeter mm; Ec.Pm Endocortical perimeter mm *$p<0.05$, **$p<0.001$.

Differences were also observed on the increased bone surface relative to trabecular volume (BS/TV) at 4 ($p<0,001$), 6 ($p<0.001$) and 12 months ($p<0.05$). Higher BV/TV values in *Ins2*$^{+/akita}$ were due to a significant increase in the number of trabeculae (Tb.N), that was of 45% at 4 months ($p<0.001$), 52 % at 6 months ($p<0.05$) and 43% at 12 months, and not due to the size of the trabeculae, since no differences were observed in the specific bone surface (BS/BV) or in the trabecular thickness

(Tb.Th) (Table 1). In *Ins2*$^{+/akita}$ the high Tb.N led to a highly significant ($p<0.001$) reduction in trabecular separation (Tb.Sp) parameters in all 3 time points analyzed (Table 1).

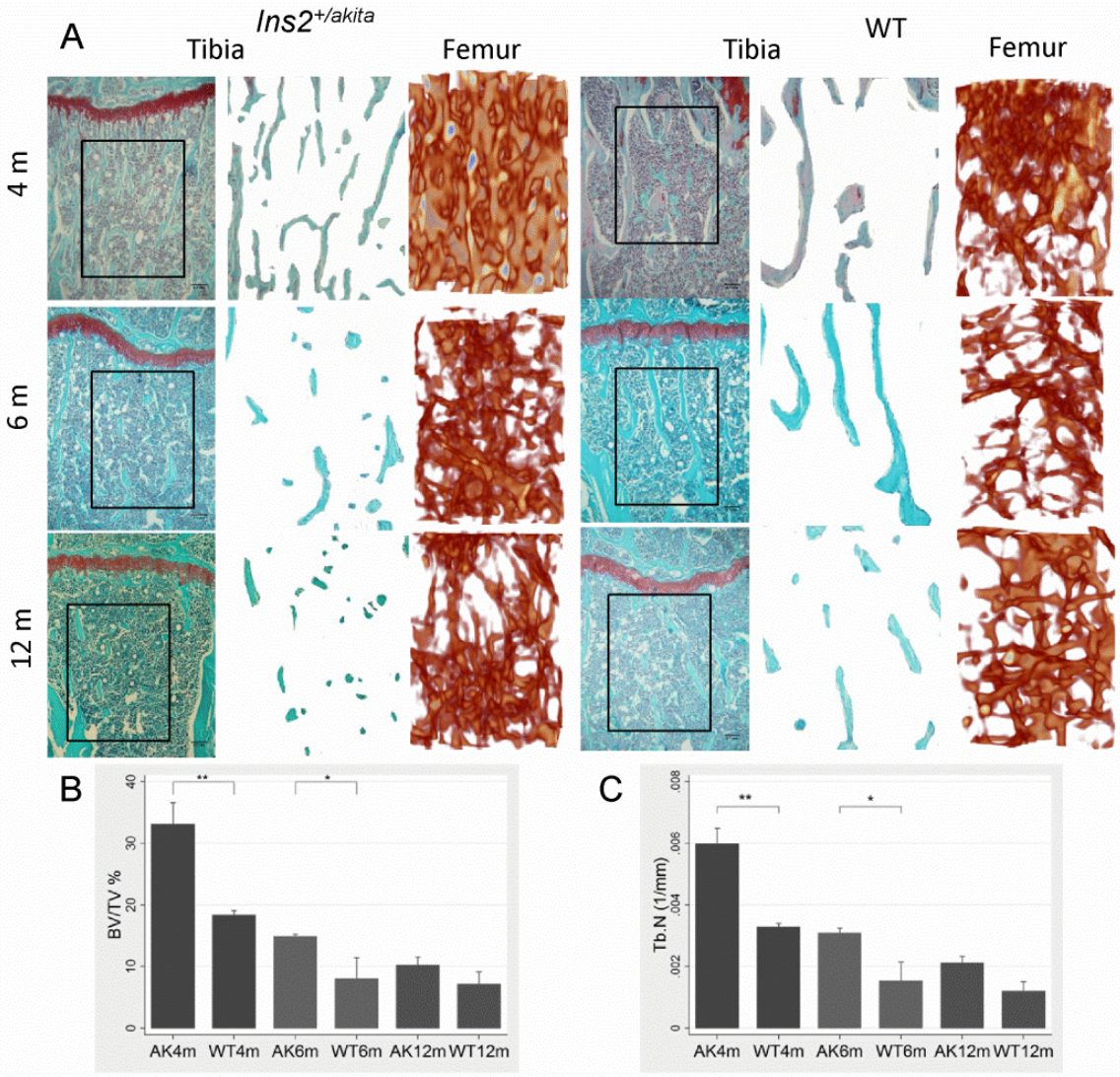



**Figure 15. A)** Histological sections of the proximal mid-epiphysis of the tibia and MicroCT images of the distal mid-epiphysis of femur of *Ins2*$^{+/akita}$ and WT mice of 4, 6 and 12 months and significant differences could be seen in BV/TV, BS/TV, Tb.N and Tb.Sp bone parameters. The higher bone volume observed in *Ins2*$^{+/akita}$ trabeculae is explained by the increase in trabeculae number and not trabeculae thickness or size. These results also suggest reduced bone resorption; **B)** *Ins2*$^{+/akita}$ mice has significant increase in trabecular bone volume (BV/TV) when compared to WT mice at 4, 6 and a trend of increase at 12 months *$p<0.05$, **$p<0.001$.

### 2.4.3. Expression of cartilage and bone marker genes is altered in *Ins2*$^{+/akita}$

To determine the mechanisms leading to alterations in the cartilage in *Ins2*$^{+/akita}$ we examined the expression levels of *Adamst-5*, that cleaves proteoglycans, and *col2*,

the most abundant protein in cartilage. *Adamst-5* was found to be overexpressed at all time points in *Ins2*$^{+/akita}$ being highly expressed at 4 and 12 months ($p<0.001$) and also significantly upregulated at 6 months ($p<0.05$) (Figure 16.A). *Col2* expression was found to be downregulated at both 4 and 6 months ($p<0.05$) compared to WT (Figure 16.B). To determine if growth factors were also affected, *Igf1* gene expression levels were found to be downregulated at 6 months ($p<0.05$) and highly downregulated at 4 at 12 months ($p<0.001$) (Figure 16.C). Expression levels of *Runx2*, one of the main transcription factor involved in osteoblast differentiation, was found to be significantly reduced at 4 and 6 months ($p<0.05$) in *Ins2*$^{+/akita}$ (Figure 16.E) while the marker of mature osteoblasts osteocalcin (OC or BGP) was signidficantly upregulated at 4 months but not at later stages.

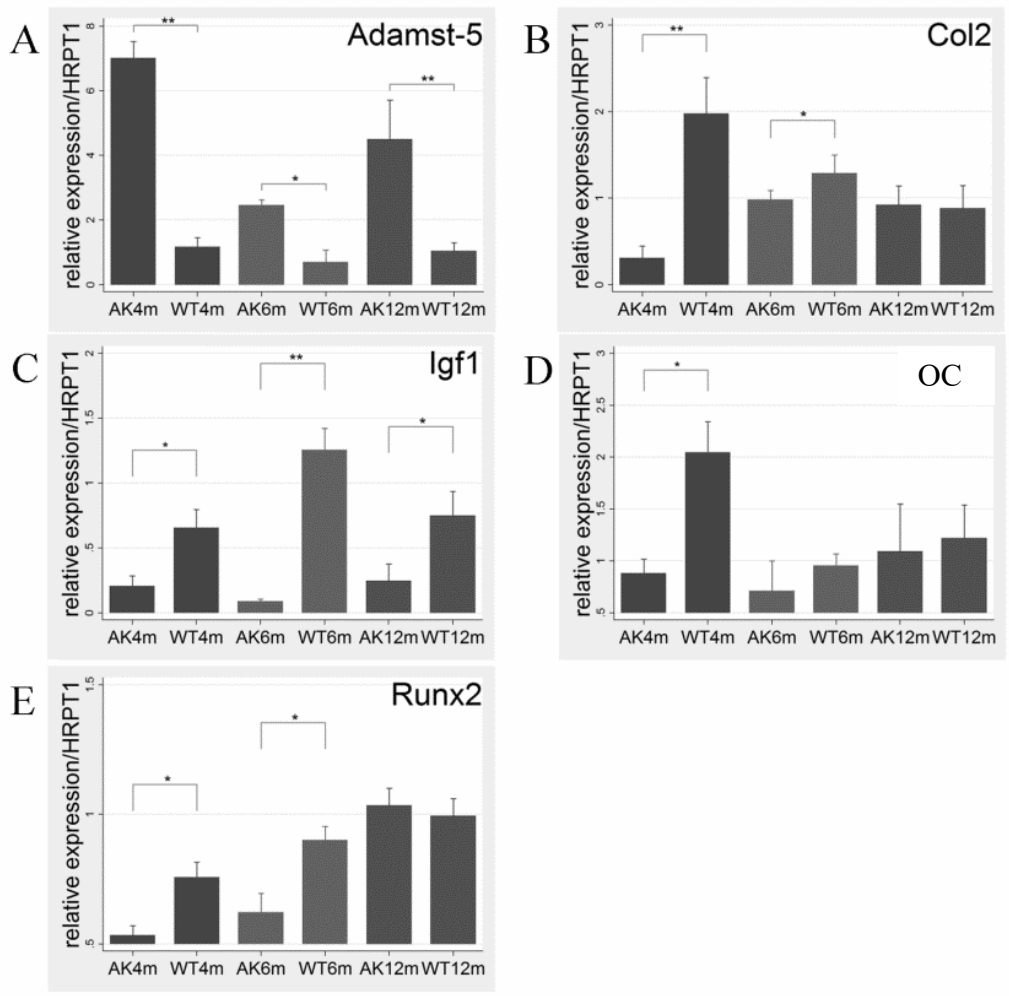



**Figure 16.** Diabetes in *Ins2*$^{+/akita}$ induced changes in mRNA gene expression in cartilage and bone; **A**) Adamst-5 expression is increased at all ages in *Ins2*$^{+/akita}$ suggesting higher cartilage degradation; **B)** Col2 expression is reduced at 4 and 6 months in *Ins2*$^{+/akita}$ being in accordance with the reduced cartilage matrix area of growth plate; **C**. Igf1 expression is reduced at all ages in *Ins2*$^{+/akita}$ explaining in part the growth retardation phenotype; **D)** and **E)** Oc and Runx2 expression is reduced at 4 months and at 4 and 6 months *Ins2*$^{+/akita}$ respectively, suggesting reduced bone growth. *p<0.05, **p<0.001.

## 2.1.5. Discussion

T1DM is related to bone growth retardation in puberty [100,101] and increased risk of fracture throughout life, leading to increased morbidity and mortality [102]. Increased bone porosity and smaller cortical area are the principal causes for the observed decrease in biomechanical properties, as previously reported for type 2 diabetic postmenopausal women [103]. In the present study, growth retardation could

also be observed in *Ins2*$^{+/akita}$ mice, reflected by a decreased length of the femurs when compared to WT mice at all time points. Similar results were found in *Ins2*$^{+/akita}$ at 10 weeks [27], in streptozotocin induced diabetic mice and rats [27,28,80], in CR mice [96,98], in *ob/ob* and leptin receptor *db/db* mutants [62,63,65]. In our work, impaired bone growth in length could be explained by a reduction in growth plate thickness of *Ins2*$^{+/akita}$ mice at 4 and 6 months and increased number of pre-apoptotic chondrocytes in growth plate at 4 months that reflect a lower metabolic activity of the cartilage and translates in reduced bone growth. Similar results were observed in diabetic rodents and in CR mice [27,79,96] demonstrating a direct relation between diabetes and impairment of long bone growth. Reduction in growth plate thickness could also be explained by a decrease in the number of proliferative chondrocytes and downregulation of *Col2*, also observed in *Ins2*$^{+/akita}$ at 10 weeks [27]. Increased bone inflammation has been associated with osteoblast death, that have been related to bone marrow inflammatory events [57] and specially by increasing pro-apoptotic and pro-inflammatory cytokines in bone environment [37]. One of those well-defined cytokines is *tumor necrosis factor α (TNFα)* [104,105] which has been associated with upregulation of metalloproteinases including *Adamst-5* [106]. Accordingly, this enzyme was found to be highly expressed in our study, likely contributing to higher cartilage degradation. Our results also showed low expression levels of *Igf-1* in *Ins2*$^{+/akita}$ at all time points. Reduced circulating Igf-1 concentrations have been associated with reduced linear growth [107], cartilage degradation, chondrocytic and osteoblastic proliferation [108]. Serum Igf-1 was also found to be reduced in CR mice [96,98] caused by impaired growth hormone signaling [109]. These results suggest that a decreased Igf signaling might be involved in the reduction of bone quality parameters observed in diabetics. Diabetes has been associated to leptin deficiency [38,110,111], and leptin treated mice were shown to have

induced chondrocyte proliferation and increased growth plate [65,95]. In diabetes intracellular glucose starvation mimics starvation periods and, not surprisingly, the results of growth retardation showed by *Ins2*$^{+/akita}$ resembles the CR mice phenotype. Our results on the microarchitecture of the distal femur, showing low cortical and high trabecular bone also resembles the results observed in CR mice [98], *Adrb2*$^{-/-}$ [112] and *ob/ob* [66] at 6 months, *Adrb2*$^{-/-}$ mice at 4 months [64] and in *ob/ob* at 3 and 6 months [65,113] in the vertebrae. Reduced insulin signaling in adipocytes and weight loss in diabetes leads to low expression of leptin [60] and what *Ins2*$^{+/akita}$ as in common that could explain this similarities with previous models is leptin deficiency. To explain the mosaic phenotype, Hamrick et al. (2008) [98] suggested that under caloric restriction there is a leptin deficiency and increased neuropeptide Y signaling leading to reduced cortical bone. Baldock et al [114] reported an increase in cortical bone volume in *Y2 receptor KO* mice but double *Y2 receptor* and *leptin KO* mice had a cortical bone volume similar to the presented by leptin KO mice, meaning that cortical bone growth in leptin deficient mouse models cannot be explained only by this pathway. In the trabecular region, neither Coe et al. [27], with *Ins2*$^{+/akita}$ mice at 10 weeks, nor Devlin et al. [96] using CR mice at 12 weeks, found higher trabecular bone volume, but instead there was a reduction of trabecular bone observed in those reports. These results are consistent with the fact that high trabecular bone volume could only be observed in *Adrb2*$^{-/-}$ mice at 6 months (58) and, more recently, at 4 months [64,92]. In our study we could detect this increase in *Ins2*$^{+/akita}$ starting at 4 months. It was shown by Ducy et al. [66] that both *ob/ob* and *db/db* KO mice at 6 months had increased trabecular BV/TV both in vertebrae. To explain the high trabecular volume and low cortical bone volume, conflicting theories have been presented, emphasizing in one side the neuroendocrine role of leptin [113,115] in inhibiting bone formation and stimulating bone resorption or,

on the other hand, suggesting a higher importance of the stimulatory effect of leptin in bone peripherally [68]. Turner et al. [65] have proposed that leptin can influence bone by acting centrally and peripherally and in both cases leptin induces bone formation and resorption, concluding that regulation was predominantly made by direct signaling on both osteoblastic and osteoclastic lineages. Lower osteocalcin and even lower cross-linked C-telopeptide serum levels in leptin KO *ob/ob* and in leptin receptor KO *db*/*db* mice was associated with low bone formation and low bone resorption. These conclusions led to the assumption that increased bone volume in the trabecular bone of the vertebrae was due to reduced bone formation but even further reduced bone resorption. Turner and collaborators [65] also raised the theory that the regulation of osteoclasts by osteoblasts is merely speculative, questioning the β2-adrenergic receptor pathway by which osteoclasts are indirectly regulated. This hypothesis immediately raises the question: if leptin signaling in the hypothalamus activating the β2-adrenergic pathway in osteoblasts is not the primary route of action in increasing bone resorption by osteoclasts, so why mutant *Adrb2*$^{-/-}$ present such a pronounced bone phenotype? It was expected that a direct action of leptin in bone cells could stabilize normal bone formation and resorption, or maybe this pathway is only active later in life as mentioned above. Again Turner and collaborators [65] proposed an interesting model to explain the reduced cortical bone and increased trabecular bone phenotype. Since leptin acts over chondrocytes, osteoblasts and osteoclasts to enhance their number and/activity, changes in bone mass and architecture are dependent on local prevalence of osteoblasts and osteoclasts. So, in the periosteum of the cortical bone, where we have an increased presence of osteoblasts and reduced presence of osteoclasts, it is expected a reduced bone formation. In trabecular bone, the reduction in bone resorption can preserve trabecular number, providing a template for addition of new bone. This theory is in

agreement with our results, since trabecular bone surface (BS/TV) and trabecular number (Tb.N) in *Ins2*$^{+/akita}$ are always significantly increased at all time points, and expression of genes associated with bone formation (*Oc* and *Runx2*) showed to be downregulated, principally at 4 months, when we could observe more pronounced differences by morphometry in trabecular and cortical bone. To the best of our knowledge, this theory seems to be the best explanation to the local increases of trabecular bone and decreases in cortical bone mass. Other cause for the presumable reduced bone formation and resorption rate expressed by our results, is the fact that insulin signaling in osteoblasts have been associated to increased osteoblast and osteoclast activity promoting both bone formation and resorption [43]. Fulzele et al. [116] in their work with mice lacking insulin receptor specifically in osteoblasts, *Ob-IR,* could see reduced numbers of osteoblasts, low bone formation rate and low serum CTx. Although they presented signs of reduced bone formation and resorption, *Ob-IR* showed reduced BV/TV and Tb.N in the trabecular region at 3 and 6 weeks. But at 3 months *Ob-IR* presented only a trend of reduced BV/TV and Tb.N leading us to question what would be the trabecular phenotype of older *Ob-IR* mice. Nevertheless, *Ins2*$^{Akita}$ is insulin deficient and according to previous studies leptin deficient [117,118], two antiresorptive conditions, and this double disorder may explain why in our study diabetic mutants presented such marked differences, where in the trabecular region of the *Ins2*$^{+/akita}$ at 4 months bone volume was almost two times higher. Finally, we have for the first time identified a clear effect of diabetes in the microarchitecture of the long bones in the T1DM *Ins2*$^{+/Akita}$ model, with a mosaic pattern most likely caused by presumable leptin deficiency, lack of insulin signaling or both.

## 2.6. Acknowledgments

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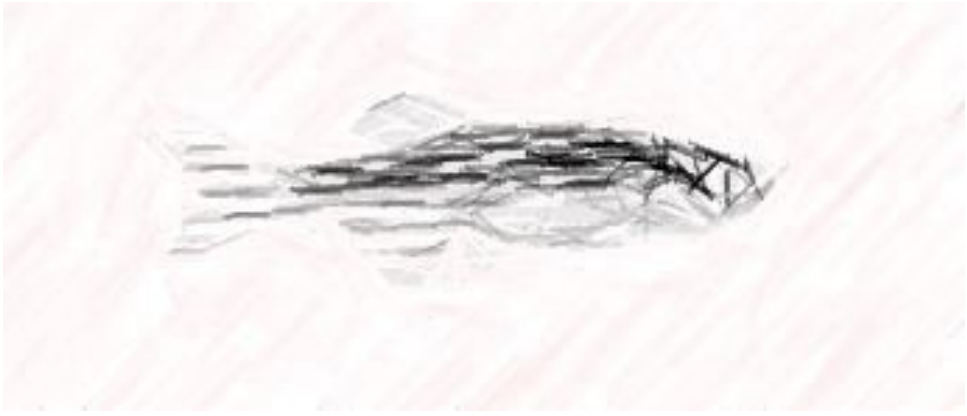
# CHAPTER 3.

# IMPROVED REGENERATION AND DE NOVO BONE FORMATION IN A DIABETES ZEBRAFISH MODEL TREATED WITH PARICALCITOL AND CINACALCET



# CHAPTER 3. IMPROVED REGENERATION AND DE NOVO BONE FORMATION IN A DIABETES ZEBRAFISH MODEL TREATED WITH PARICALCITOL AND CINACALCET

**Filipe R Carvalho**

Ana R Fernandes

Leonor Cancela

Paulo J Gavaia

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**Author´s contribution:** Study design PJG. Study conduct: FRC and PJG. Data collections: FRC, PJG, ARF. Data analyses: FRC and PJG. Data interpretation: FRC and PJG. Drafting manuscript: FRC. Revising manuscript content: PJG and MLC. Approving final version of manuscript: all authors.

## 3.1. Abstract

Bone changes related to diabetes have been well stablished, but few strategies have been developed to prevent this growing health problem. In our work we proposed to understand the effects calcitriol, a vitamin D analog and a calcimimetic in fin mineralization and regeneration in a zebrafish model of diabetes. We exposed diabetic transgenic Tg(*ins:nfsb*-mcherry) zebrafish to calcitriol, paricalcitol, cinacalcet and caudal fins were amputated to assess the effects on 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 for determining expression levels of *parathyroid hormone receptor (pthr), vitamin D receptor (vdr), insulin receptor a (insra) runt-related transcription factor 2 (runx2), osterix (osx),*

*osteocalcin (oc), insulin a (insa) and insulin b (insb)*. Increased regenerated and mineralized areas could be found in diabetic fish treated with cinacalcet and paricalcitol, when compared to non-treated diabetic group. Gene expression analysis suggested a trend of decreased *pthr* expression in all three treated groups, while *runx2, insa*, *insb* were found to be upregulated and a trend of increase was observed for *osx* and *oc* in diabetic groups treated with cinacalcet and paricalcitol. *insra* and *vdr* expression was found to be upregulated in diabetics treated with cinacalcet. Increased mineralization and regeneration in diabetic zebrafish treated with cinacalcet and paricalcitol can be explained by a decrease in circulating parathyroid hormone, reducing bone resorption and increased osteoblastic differentiation, indicating the pro-osteogenic potential of the drugs tested.

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

## 3.2. 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 [119] leading to an increase in patients living with the risk of developing diabetes-related complications [120]. Diabetes has been strongly associated with development of bone fractures, that begins in childhood and extends throughout life, leading to morbidity and mortality [121,122]. Calcitriol (1,25-dihydroxyvitamin D3 - VitD) deficiency is common in chronic kidney disease (CKD) patients, leading to secondary hyperparathyroidism (SH) [123] and particularly those with diabetes undergoing dialysis have been correlated to low 25-hydroxyvitamin D in the serum due to lack of suprarenal gland activity [122]. 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 [124]. Bone loss and increased fracture risk is

among the complications presented by CKD patients with SH [122,125,126] and also seen in rodent models [127,128]. VitD, the vitamin D analog paricalcitol [129] and the calcimimetic cinacalcet [130] have been used for the treatment of SH in CKD patients with beneficial effects in lowering PTH values and also in increasing bone mass [122,125,126]. Paricalcitol, by activating selectively *vitamin D receptor (VDR)* [131,132], and cinacalcet, activating *calcium sensing receptor* [131,133] in parathyroid, have been shown to be more efficient and fast in normalizing PTH levels and also in reducing circulating bone turnover markers than VitD [134,135]. Zebrafish models of human diseases have been well established, spanning a wide range of human pathologies including genetic disorders and physiological processes that are known to be highly conserved throughout vertebrate evolution [136]. Recently it was demonstrated, under type 1 diabetic conditions, that fin regeneration was impaired in zebrafish with reduced cell proliferation and increased cell apoptosis [33]. 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 exposed to the pro-drug metronidazole becomes hyperglycemic and hipoinsulinemic due to beta-cell ablation, leading to a transient state of diabetes during 10 to 15 days prior to beta-cell regeneration [74,76]. To understand if vitamin D analogs and calcimimetics could have positive effects on bone, during a diabetic window period, fin regeneration and bone mineralization areas were quantified and expression analysis of bone and vitamin D metabolism markers was investigated.

## 3.3. Methods

### 3.3.1. Zebrafish strains and maintenance

The trangenic Tg(*ins:nfsb*-mcherry) zebrafish line used in our experiment 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. [74] with a Tübingen AB background, contains a construct downstream to the promoter region of the *insa* gene of the gene *nfsB* of *E. coli* and the florescent protein mCherry. This bacterial gene encodes a nitroreductase (NTR) enzyme, that converts pro-drugs such as metranidazole (MET; Sigma-Aldrich, St. Louis, USA) to cytotoxins. By observation of loss mCherry florescence it is possible to visualize MET induced beta-cell ablation.

### 3.3.2. Procedures

Diabetes induction: Larvae of 15 days and Male and female adults with 1 year old Tg(*ins:nfsb*-mcherry) zebrafish were anesthetized with tricaine methanesulfonate (Sigma-Aldrich, St. Louis, USA) [137] and exposed to MET either by bath or through intraperitoneal (IP) injection at the concentration of 0,05M dissolved in citrate buffer (0,05M). Paricalcitol, cinacalcet and VitD treatments: 72 hours post treatment (hpt) with MET, transgenic zebrafish loss florescence due to beta-cell ablation, as observed by Pisharath et al. [74]. To understand if treatments could induce bone alterations in diabetic conditions, groups of 125 larvae and 125 adults were divided and exposed by immersion or IP injection, respectively, of VitD (0,001µg/ml) (calcitriol, Sigma-

Aldrich), cinacalcet (0,05µg/ml) (mimpara®, Amgen Europe B.V., Breda, Netherlands), paricalcitol (0,001µg/ml) (zemplar®, Abbott Laboratories, Illinois, USA) or vehicle (citrate buffer 0,05M) for the control groups of non-diabetic and diabetics. Fin amputation: After the IP injections with treatments or vehicle, the caudal fins of treated adults were amputated two segments bellow the bifurcation of the second ray. Both larvae and adults were maintained under treatment for 120 hours at 28,5ºC and fed twice a day with Artemia nauplii (EG strain, Inve, Belgium). Mineral staining: Larvae 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 at a concentration of 0,01% for 15 min prior to observation. Adult regenerated caudal fins and larval opercula were photographed under green florescent light under a microscope for identification of the calcified regions. Quantification 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 and divided by REG/STU. All quantifications were done using image J software. 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, 180 minutes after IP injection with glucose. The glucose levels were measured in 3 Tg(ins:nfsb-mcherry) zebrafish treated with MET and vehicle at each time point. 6 µl of blood were collected from the caudal aorta, diluted in 2 µl of 2% heparin and rapidly

transferred to a blood glucose meter Glucocard™ MX (Arkray A. Menarini Diagnostics, Florence, Italy). Total RNA isolation: Vertebral columns were isolated and 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 was 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 sec at 95ºC and 15 sec at 60ºC each. The primer sequences of *elongation factor 1alpha (ef1-alpha), 18S ribosomal rna (18S), runt-related transcription factor 2 (runx2), osteocalcin (oc), osterix (osx), parathyroid hormone receptor (pthr),vitamin D receptor (vdr),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 average of the gene expression levels of housekeeping genes *ef1-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.

## 3.4. Results

To confirm that Tg(*ins:nfsb*-mcherry) exposed to MET could leed to a state of diabetes type I, a glucose tolerance test was performed. We found that glucose concentrations in the plasma of fish treated with MET, had significantly higher

concentrations than fish treated with vehicle only, with this differences being highly significant at 90, 120 ,150 and 180 minutes after IP injection (Figure 16).

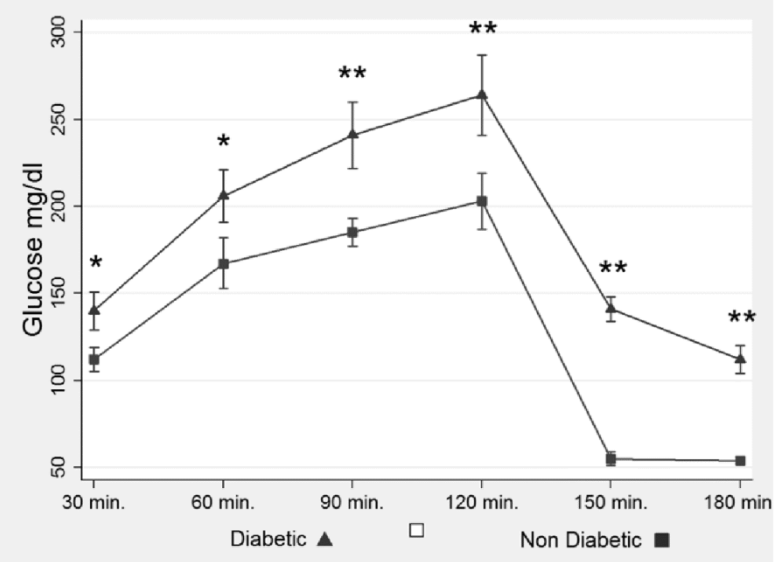


**Figure 17.** Transgenic zebrafish Tg(*ins:nfsb*-mcherry) 72 hours post induction of diabetes have increased blood glucose concentrations at 30, 60, 90, 120, 150, 180 min. after intra-peritoneal injection with glucose *p<0,05; **p<0,001.

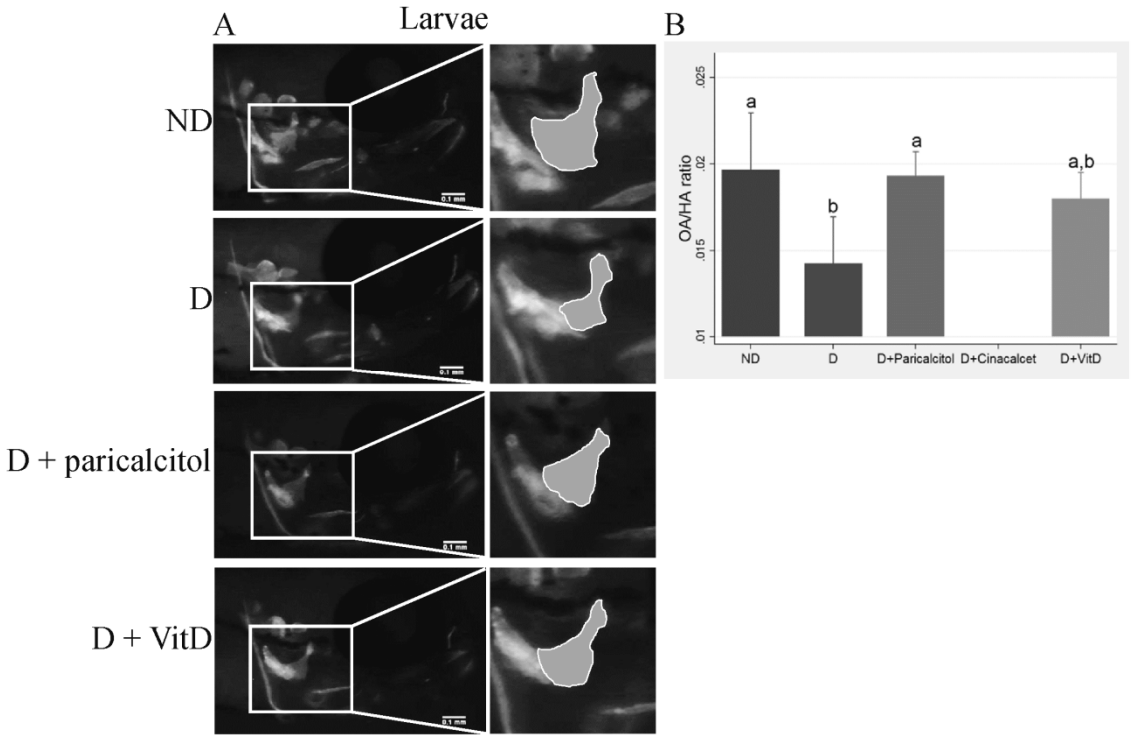


**Figure 18. A)** Paricalcitol induced operculum mineralization in diabetic larvae; **B)** 15 day old diabetic larvae have reduced operculum mineralized area when compared to non-diabetic, but treatment with paricalcitol increased the mineralized area compared to diabetic.

An analysis of the mineralized area of the operculum of larvae from the diabetic group revealed a significant reduction when compared to non-diabetic. However, the operculum mineralized area was significantly increased when diabetic were treated with paricalcitol compared to diabetic alone. Diabetic treated with VitD showed an increase in mineralization compared to untreated diabetic, although not significantly different due to individual variability. Cinacalcet concentrations used in these experiments showed to be lethal for diabetic larvae (figure 17.B). Beta-Cell ablation in adults led to a statistically significant impairment of fin regeneration in diabetic when compared to non-diabetic. Also, the treatment groups with paricalcitol and cinacalcet presented a significant increase in regenerated area when compared to diabetic and a trend of increased regeneration when compared to non-diabetic (figure 18.B).

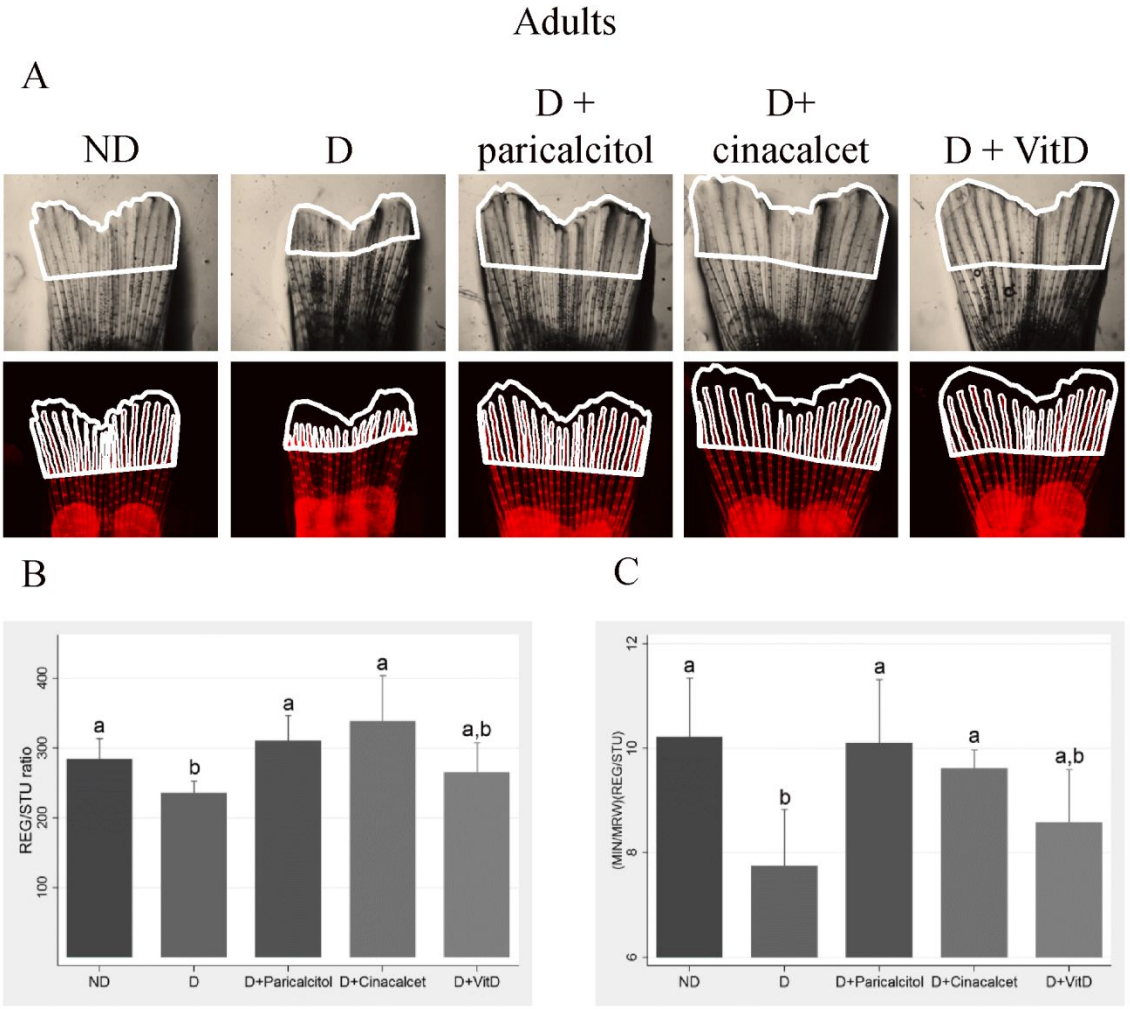


**Figure 19 A**. Treatment with paricalcitol and cinacalcet induced fin regeneration and bone mineralization in adult diabetic zebrafish; B. Diabetic group had decreased fin regenerated area compared to non-diabetic and to diabetic treated with paricalcitol and cinacalcet; C. Diabetic group had decreased fin mineralized area compared to non-diabetic and to diabetic treated with paricalcitol and cinacalcet.

The regenerated area of the diabetic group treated with VitD did not present any significant differences compared to the other groups. Quantification of the mineralized area showed that groups treated with paricalcitol and cinacalcet had no differences when compared to non-diabetic, while the diabetic fish showed a significantly reduced mineralization when compared to non-diabetic, paricalcitol and cinacalcet treated groups, while VitD group showed no differences relative the other groups (figure 18.C).

Analysis of gene expression showed that VitD, paricalcitol and cinacalcet treated groups presented a trend of reduction in the expression of *pthr* when compared to non-diabetic and to diabetic fish (figure 19A). The *vdr* expression, showed a statistically

significant increase in the cinacalcet group compared to all other groups (figure 19.B). *insra* expression was found to be significantly down regulated in all groups ($p<0,5$) compared to non-diabetic, but the cinacalcet group showed a lower reduction in expression, with statistically higher values compared to diabetic, paricalcitol and VitD treated fish (figure 19.C). *osx* expression showed a trend of reduction in all treated groups compared to non-diabetic but only significant downregulation was found in diabetic compared to non-diabetic (figure 19.D). Regarding *runx2* a significant upregulation could be observed in groups treated with paricalcitol and cinacalcet ($p<0,05$) (figure 19.E). Expression of *oc* was found to be downregulated in diabetic compared to non-diabetic while the remaining groups showed no significant differences. Gene expression of *insa* and *insb* was found to be upregulated ($p<0,001$) in both paricalcitol and cinacalcet treated fish compared to the other groups (figures 19.G and 19.H).

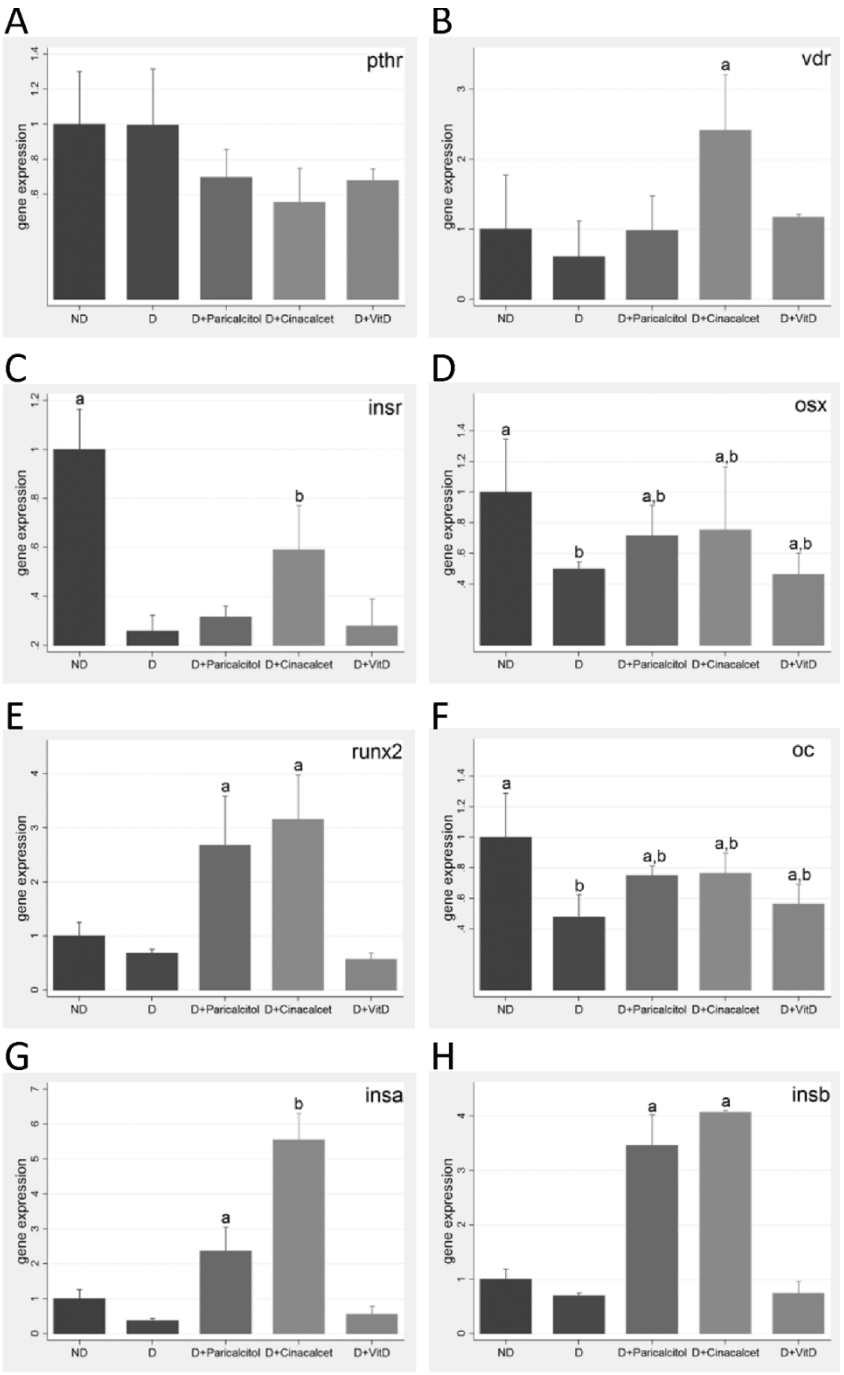


**Figure 20.** RNA gene expression from the vertebral column of diabetic or diabetic with treatments is altered in zebrafish; **A.** Diabetic treated groups presented a trend of reduction in *pthr* expression; **B.** Diabetic treated with cinacalcet have an increase in *vdr* expression compared to all the other groups; **C.** All diabetic groups presented a reduction in *insr* expression with the exception of the group treated with cinacalcet; **D.** Differences could be observed between non-diabetic and diabetic in *osx* expression, treated groups show no differences between untreated groups ; **E.** Diabetic groups treated with paricalcitol and cinacalcet showed an increase in the expression of *runx2*; **F.** Diabetic group showed reduced expression of *oc* compared to non-diabetic, and treated groups show no differences relating untreated groups; **G.** and **H.** Diabetic groups treated with paricalcitol and cinacalcet showed an increase in the expression of both *insa* and *insb* gene.

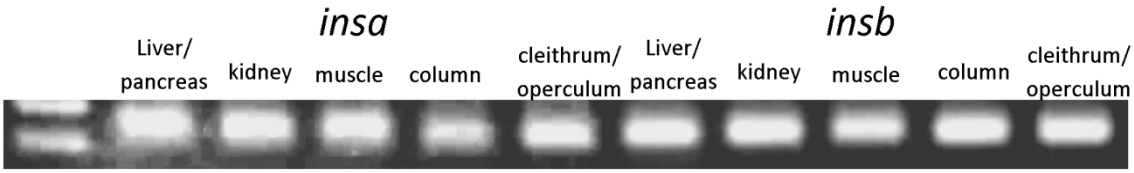


**Figure 21.** Zebrafish can have extrapancreatic expression of both *insa* and *insb*. Expression of *insa* and *insb* could be detected in pancreas/liver, muscle, kidney, and bones from vertebral column and cleithrum/operculum.

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, using the same primers used for qPCR. We observed amplification of *ins* genes in all tissues analyzed (figure 20). This result was further confirmed by sequencing of the PCR amplicons that proved to be correspondent to *insulin a* and *b* isoforms respectively.

## 3.5. 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 described by previous authors [74,76], and caused a significant increase in blood glucose , although differences were not so accentuated as previously reported [74,76]. 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 [138] and diabetes mice models [139]. Treatment with paricalcitol led to an increase in the mineralized area more efficiently than VitD. The concentrations used of Cinacalcet, same as used in clinic therapies, were found to be toxic in larvae as the rate of mortality was of 100% after 24 hours. Regenerated fin area of adults showed an increase in the diabetic groups treated with paricalcitol and cinacalcet when compared to diabetic group treated with vehicle. Diabetics from the VitD treated group did not

present such a marked increase. There are some evidences that vitamin D analogs can have positive regenerative effects after vascular injury, like previously been reported for healthy humans, diabetic mice models and conditional knockout of the *vitamin D receptor* mice [140]. In our work, paricalcitol had no effect in *vdr* 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 *Vdr* expression [141]. Additionally, gene expression analysis showed that both *insa* and *insb* were overexpressed in bone of diabetics treated with paricalcitol or cinacalcet. Other studies using analogs and calcimimetics have also shown to induce insulin expression and improve beta-cell proliferation and survival [142–145]. This can explain the fact that the regeneration process of the beta-cells occurred in a shorter period of time in the paricalcitol and cinacalcet treated groups by favoring insulin signaling and glucose metabolism. In the cinacalcet treated group we could observe a significant increase in *insr* suggesting an 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) [146], who showed that 55% of diabetic rats treated with calcitriol recover from diabetes. Insulin expression have also been found to be increased in several tissues under diabetic conditions in both humans and mice [147], 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 [148], mice [147,149] and zebrafish [150] although at extremely low levels when compared to pancreatic insulin. Although not well understood, the function of extrapancreatic expression of insulin in some works was associated to local needs of glucose regulation specially under diabetic conditions [148,151] but in other reports this phenomenon was related to the

development of pathologic conditions [152]. Our results demonstrated that paricalcitol and cinacalcet can upregulate extrapancreatic expression of insulin including in bony tissues, like demonstrated for the vertebral column. In humans and animal models of diabetes, hyperglycemia leads to accelerated accumulation of advanced glycation end products (AGEs) [56], promoting an inflammatory response and increased apoptosis of cells expressing the receptor of AGEs such as osteoblasts [52,106]. In the non-treated diabetic group we could see impairment in osteoblastic activity, since *osx, runx2* and *oc* expression was found to be downregulated compared to non-diabetics. In the diabetic groups treated with paricalcitol or cinacalcet, where an increase in mineralized area of the regenerated fin was observed, an upregulation of *runx2* suggest an increase in the process of osteoblastic differentiation, contributing to the process of mineralization, in accordance with studies in humans, indicating that VitD effects on osteoblast differentiation are mostly stimulatory and associated with increased *RUNX2* expression [153,154]. 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* [155]. The principal objective of paricalcitol, cinacalcet and VitD in clinical treatment for SH, is to reduce parathyroid hormone secretion. We could observe a trend of reduced expression in *pthr* for all diabetic treated groups, suggesting reduced circulating parathyroid hormone, leading to a decrease in osteoclastic differentiation and bone resorption [156]. Nevertheless, VitD treatment alone could not explain our results, since the VitD treated group did not present such marked increase in mineralized area as the other two groups. This may be explained by the fact that VitD acts more slowly, at least when compared to paricalcitol [157]. 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 *runx2* and *osx* can 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 diabetes type I patients.

### 3.6. Acknowledgments

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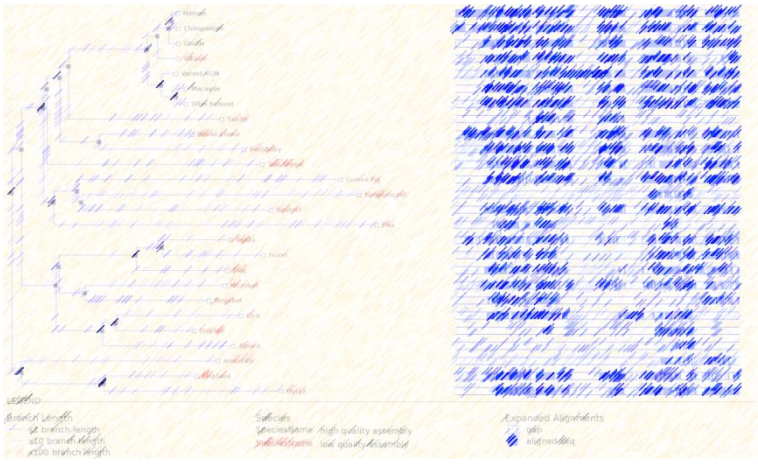
# CHAPTER 4.
# CAN EXTRAPANCREATIC EXPRESSION OF INSULIN AND PARALOGS REGULATE GLUCOSE HOMEOSTASIS?



# CHAPTER 4. CAN EXTRAPANCREATIC EXPRESSION OF INSULIN AND PARALOGS REGULATE GLUCOSE HOMEOSTASIS?

## 4.1. Abstract

To overcome type 1 Diabetes Mellitus all strategies should be taken seriously as this multifactorial disease has shown to be complex. In this work, we used data from human, mouse and zebrafish to try to understand (I) if insulin *(INS)* paralog, *INS-IGF2* can be a regulator of glucose in humans, and (II) if extrapancreatic expression of *INS* and *INS-IGF2* can also play a role in the regulation of glucose levels. Phylogenetic analysis revealed two insulin genes occurring in mammals *(INS, INS-IGF2)* and in fish (*insa* and *insb*). Mice and all Murinae are an exception among mammals, since they lack *INS-IGF2*, having *ins1* as paralogue of *ins2*, the ortholog of *INS*. *Ins1*, as well as *Ins2* can regulate glucose since both proteins are highly similar and with a redundant function, as confirmed by *in vivo* experiments that showed that both knockout *Ins1* or *Ins2* are normoglycemic. In zebrafish, the *insb* paralogue also suggest cofunctionalization with the ancestral gene, although identity between proteins is not as high as observed in mouse. In human, *INS-IGF2* lacks motif 3 of the insulin signature, and part of the predicted protein structure does not present any known domain, which makes the prediction of a possible function for this gene inconclusive. Extrapancreatic expression of insulin has been observed in diabetic mice with contradictory effects, but available gene expression data in repository data banks also suggests that extrapancreatic expression of insulin occurs in human, mouse and zebrafish under basal condition. Weather the role of extrapancreatic insulin is to regulate glucose homeostasis

remains unknown. Further investigation should be made as this can represent a complementary strategy in diabetes treatment.

## 4.2. Introduction

Since the discovery of insulin in 1921 [158], patients with type 1 Diabetes Mellitus (T1DM) passed from a fatal prognostic to chronic patients, thanks to insulin replacement therapy. Although this treatment is the most widely peptide or protein based medicine used in the world [159], it was shown not to be totally efficient in treating all insulin-dependent diabetics, leading to morbidity later in life. The increasing worldwide prevalence of type 1 (T1DM) [4] and type 2 [2] Diabetes makes urgent the need for new strategies that can help stopping this uncontrolled pandemic. In humans the pre-pro-insulin is a protein with 110 amino acids (aa) encoded by the insulin gene. This peptide is composed by a signal peptide (1-24 aa), B chain (24-54 aa), C chain (57-87 aa) and A chain (90-110 aa). After removal of the signal peptide (proinsulin) and the C chain, insulin reaches is final conformation with the A and B chains, and is ready to travel, from β-cells throughout the body as a hormone [159]. Insulin has been characterized as responsible for decreasing blood glucose concentration, increases cell permeability for monosaccharides, amino acids and fatty acids, accelerates glycolysis, pentose phosphate cycle and glycogen synthesis in liver, and together with IGF1 and IGF2 promotes embryonic growth [160]. Mouse has been the most widely animal model used for the study of diabetes [27,31,38,161], but recently, protocols for the induction of diabetes and transgenic lines in *zebrafish* have been developed [74,162]. The canonical gene of insulin in mouse is *Ins2* and in zebrafish is *insa* while in human it is *INS* (Human insulin gene) and all sharing a high percentage of identity. In these three species we can find a paralog for insulin gene, being in mouse *Ins1,* in zebrafish *insb* and in human *INS-IGF2*. In human it has been identified a paralog of the insulin gene,

*INS-IGF2* [163], which is a conjoined gene sharing *INS* gene in the 5' region and the *IGF2* gene in the 3' region. *INS-IGF2* is a parental imprinting gene that was first identified as being expressed both in the eye and pancreas [163] and a possible autoantigen like *INS* in T1DM [164]. In mouse, the two genes encoding for insulin, *Ins1* and *Ins2,* are thought to have duplicated because of an infection in the genetic material by a RNA virus originating *Ins1* (retrogene). Investigations done with mouse *Ins1* or *Ins2* knockouts (KO) revealed that *Ins1* alone can regulate glucose homeostasis, although in some cases it cannot prevent type 1 diabetes mellitus (T1DM). Duvillie et al. [165] showed that double *Ins1* and *Ins2* KO mice died prematurely, but mice lacking just one insulin gene were viable and fertile [165]. Leroux et al. [166], working with the same mutant lines observed, not only, that both *Ins1* and *Ins2* KO mice were normoglycemic, but that *Ins2* KO mice had a dramatic increase in the expression of *ins1* accompanied with an increase in β-cell mass [166]. Babaya et al. [167], showed that *Ins2* KO nonobese diabetic (NOD) male mice, with just one *ins1* allele, $NOD^{ins1+/-,ins2-/-}$, developed diabetes at around 10 weeks of age, but males with both *ins1* alleles $NOD^{ins1+/+,ins2-/-}$ and females from the two mutant lines only presented signs of diabetes later in life [167].

In zebrafish the existence of two insulin genes (*insa* and *insb*) is likely due to the genome duplication event that occurred in the stem lineage of teleost fishes [150]. Papasani et al. [168], identified the second insulin gene in zebrafish and observed expression of *insb* both in pancreas and brain and suggested that it may regulate both autocrine/paracrine and endocrine systems via both *insulin receptor a* (*insra*) and *insulin receptor b insrb* (14).

In human extrapancreatic expression of insulin was first identified in brain [169] and then in thymus [170], has a way to the immune system recognize insulin, avoiding

autoimmunity and β-cell destruction. Later Kojima et al showed the presence of cells that stained positive for insulin RNA in the liver, adipose tissue and bone marrow in several diabetic mice models but not in nondiabetic mice [171,172]. However, this insulin expressing cells had no impact on hyperglycemia in diabetic mice. Later, Kojima et al. [173] demonstrated, that beside not having any impact in regulating glucose, this Proins/TNF-α-expressing cells had their origin in bone marrow and then migrated to several parts of the body, initiating diabetic neuropathy [152]. Cunha et al [174], 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 locally synthesized in the lachrymal gland [174]. Using *in silico* analyses we proposed to understand; 1) if INS-IGF2 can be a regulator of glucose homeostasis and 2) if extrapancreatic expression of both INS and INS-IGF2 can occur mimicking pancreatic INS action.

## 4.3. Methods

### 4.3.1. Phylogenetic tree construction

To better understand the evolution of insulin genes we conducted a phylogenetic analysis of *INS, INS-IGF2, Ins1, Ins2, insa and insb* together from available sequences human, chimpanzee, orangutan, gibbon, marmoset, otolemur, tree shrew, mouse lemur, squirrel, lesser jerboa, chinese hamster, rat, mouse, ferret, cat, panda, dolphin, cow, elephant, manatee, walrus, microbat, guine pig, hyrax, marmoset, coelacanth, frog, anole lizard, green sea turtle, duck, chicken, turkey, rock dove, collared flycatcher, zebra finch, ground tit, medium ground finch, white throated sparrow, tilapia, japonese pufferfish, green spotted puffer, zebrafish, medaka, platyfish, greater amberjack, stickleback and ciona intestinalis. Collection of protein sequences from insulin and paralogues was obtained in Ensembl Genome Browser (EGB) [175] and protein

database of National Center of Biotechnology Information (NCBI) [176]. We collected 32 INS, 11 INS-IGF2, 2 ins1, 2 ins2, 7 insa and 5 insb protein sequences from the different species analyzed (Table 2). For multiple sequence alignment we used the algorithm Multiple Sequence Comparison by Log-Expectation (MUSCLE) [177]. Based on the sequence alignment results, the phylogenetic tree reconstruction was made using the Maximum-likelihood statistical method. Test of phylogeny was done using the bootstrap method and the number of replications was 1000. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [178]. The insulin like 1 precursor of 275 amino acids sequence of ciona intestinalis was used as an outgroup in the phylogenetic tree.

#### 4.3.2. Graphical representation of conserved motifs

To determine percentage of identity between paralogs we aligned both proteins sequences for human, mouse and zebrafish species using algorithm MUSCLE and presenting it graphically using CLC Genomics Workbench v6.5 (CLC bio, Aaarhus, Denmark). Also, we applied *INS, INS-IGF2, Ins1, Ins2 insa* and *insb* to InterProScan database [179] to identify insulin signatures and motifs present in protein sequences.

#### 4.3.3. Transcripts and gene expression

To understand the pattern of expression of insulin genes in different tissues we gathered information available in data bases that provide transcriptome and microarray results from different experiments. Tissue gene expression pattern from *insa* (Dr.75811), *insb* (Dr.87912) and *Ins2* (Mm.4946) were obtained from UniGene data base [180] and *INS* [181], *INS-IGF2* [181] and *Ins1* [182] from Expression Atlas [183]. Tissue distribution extrapancreatic expression of insulin were presented as transcript per million. Graphic presentation was made using CIRCOS Circular Genome Data Visualization [184].

## 4.4. Results

Insulin is an important regulator of life and not surprisingly, is conserved throughout all animalia kingdom including animals from subphylum tunicata like *Ciona intestinalis*. Our phylogenetic analyze (Figure 21) demonstrates that insulin paralogues INS-IGF2, ins1 and insb have different origins, INS-IGF2 is an exclusive gene of Mammals except for murinae (mouse and rat) that do not have INS-IGF2 gene, but instead have ins1 gene. On the other and chinese hamster, that belongs to the same order (rodentia) of mouse and rat possess INS-IGF2 gene.

Table 2 – Insulin proteins, from the different species used for phylogenetic reconstruction

| Sl. No. | Common name | Scientific name | Protein identification | Length aa |
|---|---|---|---|---|
| **INS** | Human | Homo sapiens | NP_001172027.1 | 110 |
| **Ins** | chimpanzee | Pan troglodytes | NP_001008996.1 | 110 |
| **Ins** | Gorilla | Gorilla Gorilla | XP_004050475 | 110 |
| **Ins** | Marmoset | Callithrix jacchus | XP_002755713 | 108 |
| **Ins** | northern greater galago | Otolemur | XP_003798420 | 110 |
| **Ins** | Tree Shree | Tupaia chinensis | XP_006141129 | 110 |
| **Ins** | mouse lemur | Microcebus murinus | XP_012599453.1 | 110 |
| **Ins** | Squirrel | Ictidomys tridecemlineatus | NP_001269184 | 110 |
| **Ins** | lesser jerboa | Jaculus jaculus | XP_004654275.1 | 110 |
| **Ins** | Chinese hamster | Cricetulus griseus | XP_007625431.1 | 110 |
| **Ins2** | Rat | Rattus norvegicus | AAA41439 | 110 |
| **Ins2** | Mouse | Mus musculus | NP_001172013.1 | 110 |
| **Ins1** | Rat | Rattus norvegicus | AAA41440.1 | 110 |
| **Ins1** | Mouse | Mus musculus | NP_032412.3 | 108 |
| **Ins** | Ferret | Mustela putorius furo | XP_004759728.2 | 109 |
| **Ins** | Cat | Felis catus | NP_001009272.1 | 110 |
| **Ins** | Panda | Ailuropoda melanoleuca | XP_002920166.1 | 109 |
| **Ins** | Dolphin | Tursiops truncates | XP_004317908 | 110 |
| **Ins** | Cow | Bos Taurus | NP_001172055 | 105 |
| **Ins** | Elephant | Loxodonta Africana | XP_003422420 | 105 |
| **Ins** | Manatee | Trichechus manatus latirostris | XP_004389446.1 | 106 |
| **Ins** | Coelacanth | Latimeria chalumnae | XP_006008147.1 | 107 |
| **Ins** | Walrus | Odobenus rosmarus | XP_004403859.1 | 110 |
| **Ins** | Guinea Pig | Cavia porcellus | XP_013006103.1 | 110 |
| **Ins** | microbat | Myotis lucifugus | G1P146_MYOLU | 105 |
| **Ins** | coelacanth | Latimeria chalumnae | XP_006008147.1 | 107 |
| **Ins** | frog | xenopus laevis | NP_001079350.1 | 106 |
| **Ins** | Anole Lizard | Anolis carolinensis | G1KJA0_ANOCA | 107 |
| **Ins** | Chinese softshell turtle | Pelodiscus sinensis | K7G107_PELSI | 108 |
| **Ins** | Duck | Anas platyrhynchos | R0L6M4_ANAPL | 107 |
| **Ins** | Chicken | Gallus gallus | NP_990553.1 | 107 |
| **Ins** | turkey | Meleagris gallopavo | XP_003206326.1 | 107 |
| **Ins** | Rock dove | Columba livia | XP_005499300.1 | 107 |
| **Ins** | collared flycatcher | Ficedula albicollis | XP_005046861.1 | 107 |

| | | | | |
|---|---|---|---|---|
| **Ins** | Zebra finch | Taeniopygia guttata | H0ZG98_TAEGU | 107 |
| **Ins** | Ground tit | Pseudopodoces humilis | XP_005522396.1 | 107 |
| **Ins** | Medium ground finch | Geospiza fortis | XP_005427262.1 | 107 |
| **Ins** | White-throated sparrow | Zonotrichia albicollis | XP_005486624.1 | 107 |
| **Insa** | Tilapia | Oreochromis niloticus | I3IUZ1_ORENI | 123 |
| **Insa** | Japanese pufferfish | Takifugu rubripes | XP_003978300.1 | 120 |
| **Insa** | Spotted green pufferfish | Tetraodon nigroviridis | Q4T1L5_TETNG | 89 |
| **Insa** | Medaka | Oryzias latipes | H2MW49_ORYLA | 115 |
| **Insa** | Southern platyfish | Xiphophorus maculatus | M4AFY5_XIPMA | 117 |
| **Insa** | greater amberjack | Seriola dumerili | BAE96120.1 | 116 |
| **Insa** | stickleback | Gasterosteus aculeatus | ENSGACG00000001771 | 117 |
| **Insb** | Tilapia | Oreochromis niloticus | I3KM26_ORENI | 111 |
| **Insb** | Japanese pufferfish | Takifugu rubripes | H2RZ45_TAKRU | 110 |
| **Insb** | Zebrafish | Danio rerio | NP_001034153.1 | 107 |
| **Insb** | Medaka | Oryzias latipes | H2MXM6_ORYLA | 113 |
| **INS-IGF2** | Gibbon | hylobates lar | G1RSR9 | 222 |
| **INS-IGF2** | Rock hyrax | Procavia capensis | ENSPCAT00000013013 | 206 |
| **INS-IGF2** | Chinese hamster | Cricetulus griseus | EGW08477.1 | 248 |
| **INS-IGF2** | Human | Homo sapiens | ABD93453.1 | 200 |
| **INS-IGF2** | Orangutan | Pongo pygmaeus | H2NCF9_PONAB | 199 |
| **INS-IGF2** | Marmoset | callithrix jacchus | F7FC22_CALJA | 194 |
| **INS-IGF2** | Elephant | Loxodonta africana | G3TLG9_LOXAF | 202 |
| **INS-IGF2** | Manatee | Trichechus manatus | XP_004389487.1 | 339 |
| **INS-IGF2** | Ferret | Mustela putorius | M3Y2P9_MUSPF | 192 |
| **INS-IGF2** | Walrus | Odobenus rosmarus | XP_004403860.1 | 199 |
| **INS-IGF2** | Panda | Ailuropoda melanoleuca | D2HDS9_AILME | 212 |
| **insulin-like 2** | Ciona intestinalis | Ciona intestinalis | NP_001123345.1 | 130 |

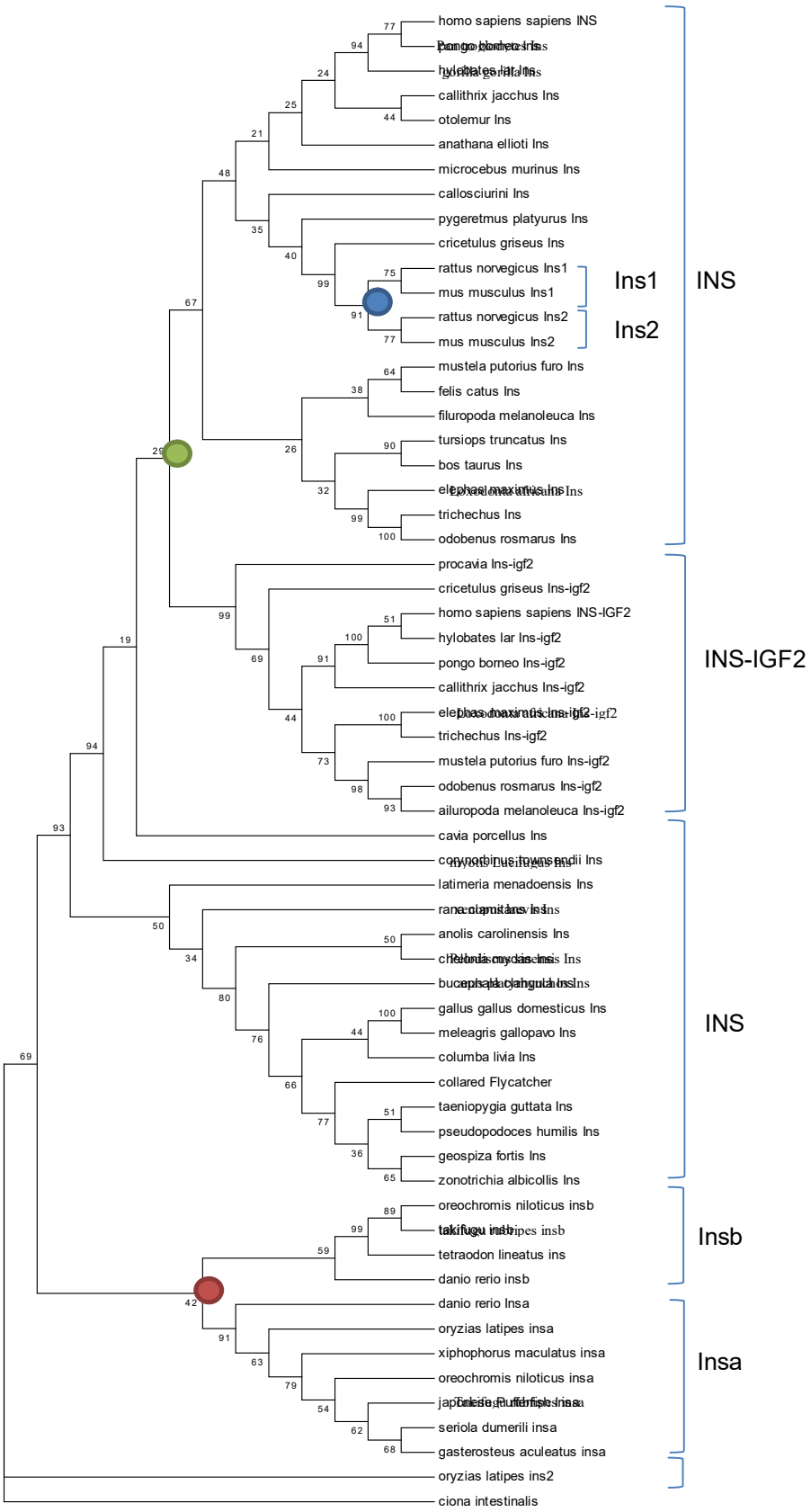



**Figure 22.** Phylogenetic tree of insulin, INS INS-IGF2, Ins2, Ins1, insa and insb proteins Sequences were obtained in Ensembl Genome Browser [175], aligned using MUSCLE [177] and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [193]. For outgroup it was used insulin like 1 precursor protein sequence from *Ciona intestinalis*. Green circle INS, INS-IGF2 duplication, Blue circle Ins1, Ins2 duplication, red circle insa, insb duplication. Numbers at the branches represented the bootstrap support values.

The Insb paralogue seems to be exclusive of osteichthyes, and probably all species from this superclass have a second insulin gene, as we have identified possible regions for insb in the genome of stickleback and platyfish (data not shown). Transcript analyses (Figure 22) demonstrate that the regulatory elements of INS in human and ins2 in mouse are well conserved, since for each human transcript there is a similar mouse transcript (NP_000198.1 similar to NM_001185083.1, NP_001172026.1 similar to NM_008387.4, NP_001172027.1 similar to NM_001185084.1) except for ins2-006. Other transcripts are annotated in EGB [175], for ins2 (ins2-003, ins2-004 and ins2-008), that predict protein structures smaller than the canonical sequence and this may need of further investigation to confirm it´s evidence. In zebrafish insa, presents only one known transcript, constituted of 3 exons like its human and mouse orthologues. Insb presents 3 different transcripts one with also 3 exons (NP_001034153.1) and two transcripts with 5 exons and a different coding DNA sequence, with the predicted protein being of more 49 aminoacids.

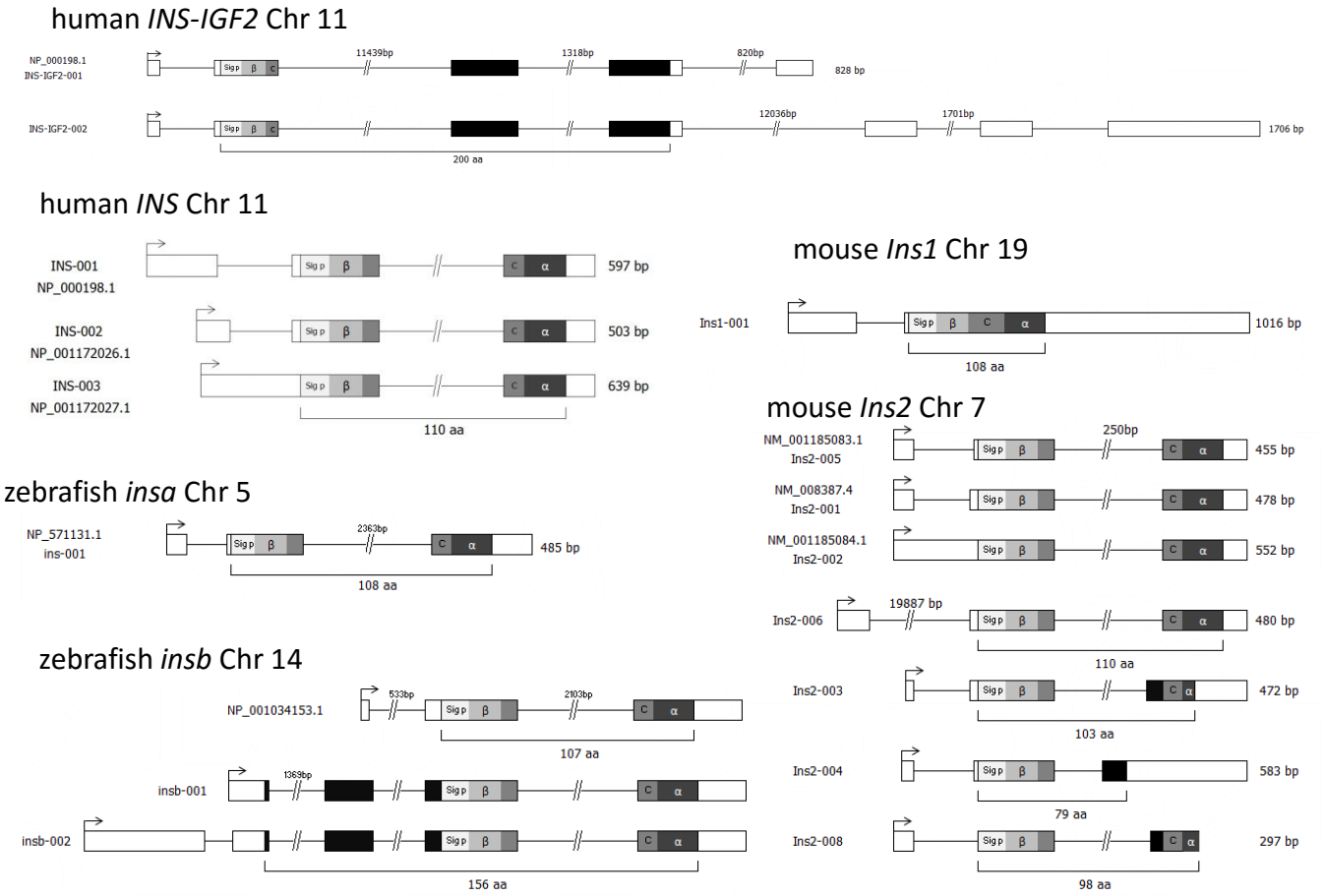



**Figure 23.** Transcripts for INS and INS-IGF2 in Human, ins2 and ins1 in mouse, insa and insb in zebrafish.

Two transcripts have been identified for INS-IGF2 were the two first exons share the same open reading frame (ORF) and coding DNA sequence (CDS) of INS transcripts, two other exons with CDS that encode for some unknown protein domain (confirmed by InterProScan [179], data not shown).

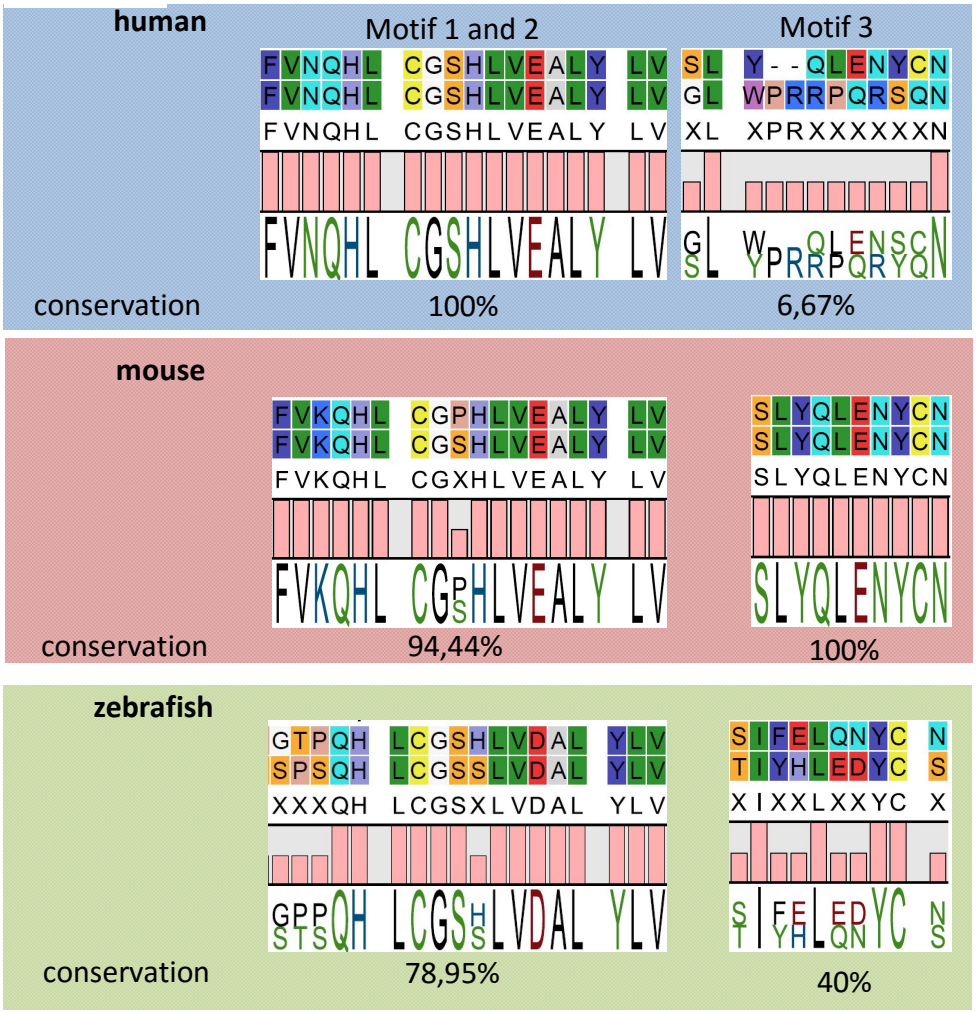



**Figure 24.** Mouse and zebrafish insulin paralogs are highly similar, but Human paralog INS-IGF2 don´t have the third motif of insulin protein

Only one copy of ins1 is identified and this transcript presents a large size and structure similarities with of ins2, but without the second intron. Moreover, insa gene has only one known transcript and its structure is highly conserved compared with homologs INS and ins2, being identicaly constituted by 3 exons and with a similar size and structure. Insulin paralog insb of zebrafish presents 3 different transcripts, although the one annotated in NCBI (NP_001034153.1) seems to be a shorter versions of the insb-001 and insb-002 present in EGB [175] and recently in our lab we have confirmed the existence of this two predicted transcripts by PCR amplification and sequencing. After, we aligned protein sequences (Figure 23) of human, mouse and zebrafish paralogs to understand the degree of conservation of the new insulin genes with ancestral genes. In human motif 1 and 2 of INS and INS-IGF2 are 100% identical as both proteins are translated from the same CDS, but INS-IGF2 lacks motif 3 signature from INS confirmed by InterProScan motif prediction [179]. On the other hand, mice paralogs have all three motif regions very well conserved, with an homology of motif 1 and 2 of 94,44% and motif 3 100%. Although not as conserved as mouse paralogs, in zebrafish, insb, seems to be reasonably conserved, as InterProScan [179] predicts the existence of the 3 insulin motifs, being the identity of insa and insb of 78,95% in motif 1 and 2 and of 40% in motif 3. In the past few years there has been a growing body of evidences showing that insulin can be expressed in several tissues beside pancreas. Here we show (Figure 24) data from transcriptome and microarray obtained in the repository data bases UniGene [180] and Expression atlas [183] demonstrating that in human, mouse and zebrafish there are indications of insulin expression in different tissues.

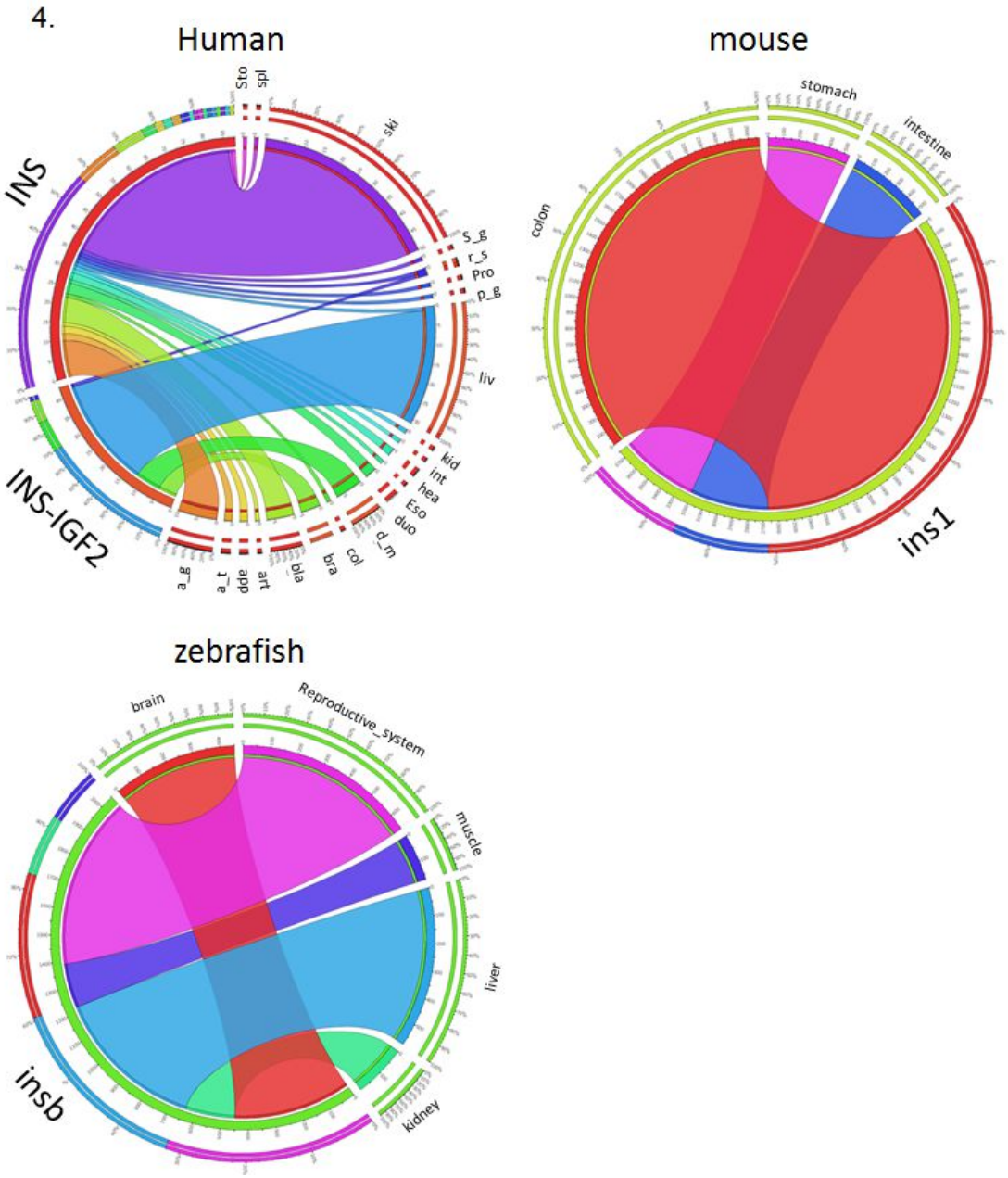



**Figure 25**. Gene expression data in different tissues, available in data banks, suggests that in all 3 species insulin is expressed outside the pancreas. In Human INS is expressed artery (art), kidney (kid), esophagus (eso), adipose tissue (a_t), adrenal gland (a_g), appendix (app), bladder (bla), muscle (mus), colon (col), duodenum (duo), heart (hea), liver (liv), prostate (pro), salivary gland (s_g), skin (ski), intestine (int), spleen (spl), stomach (sto), pituitary gland (p_g), reproductive system (r_s). INS-IGF2 was found in liver (liv), brain (bra), dura mater (d_m) and reproductive system (r_s). In mouse ins1 expression was found in colon (col), stomach (sto) and intestine (int), but no expression of ins2 was detected [182]. In zebrafish obtained data suggests that insa expression is restricted to pancreas while insb is expressed in brain, reproductive system, muscle, liver and kidney.

Human sequence analysis [181] showed that INS can be expressed in artery, kidney, esophagus adipose tissue, adrenal gland, appendix, bladder, muscle, colon, duodenum, heart, liver, prostate, salivary gland, skin, intestine, spleen, stomach, pituitary gland, reproductive system. Also extrapancreatic expression of INS-IGF2 could be detected in liver, brain, dura mater and reproductive system [181]. In our review of mouse sequences we could found evidences of expression in colon, stomach and intestine for the ins1 gene but no extrapancreatic expression of ins2 [182]. Similar results could be found in zebrafish as insa expression seems to be restricted to pancreas while insb was found in brain, reproductive system, muscle, liver and kidney.

## 4.5. Discussion

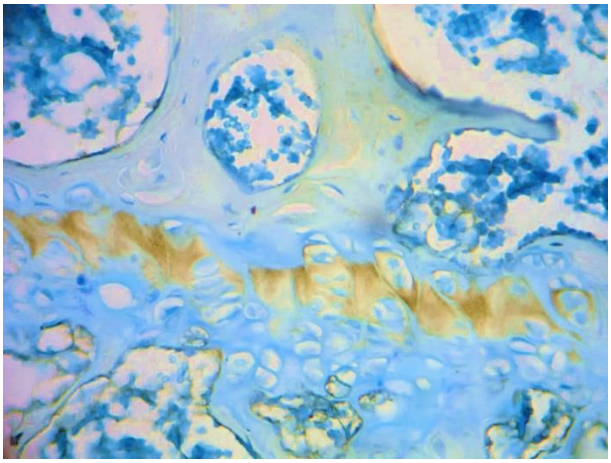
Strategies to overcome T1DM have been diverse, from vaccines [185], stem cells and pancreas transplantation [186,187], drug [188] and antibody therapy [189], with promising results in finding a possible cure. Other possible solutions, like extrapancreatic expression of insulin, have taken a marginal place although it could have some therapeutic potential in specific cases. The first question that we tried to answer was if INS-IGF2 could mimic the role of its ancestral. INS-IGF2 does not have all the conserved motifs of INS and although this may suggest other functions for this gene, data bases like STRING [190] recognize a protein interaction with INSR. INS, INS-IGF2 duplication event in mammals preceded the ins1 and ins2 duplication. It is possible that the conservation of ins1 led to the elimination of INS-IGF2 in Murinae, probably because it presents a protein structure extremely similar to ins2 and can, with accuracy, mimic ancestral gene function better than INS-IGF2. The cofunctionalization of ins1 and ins2 could be demonstrated in KO mice lacking ins2. These mutants did not present signs of diabetes and could stay normoglycemic by overexpressing ins1and increasing β-cell mass [166]. Finally, ins1 replaced INS-IGF2 in Murinae genomes,

because it was more efficient in performing the role ins2. Nevertheless, part of the INS-IGF2 protein sequence that is different from the INS, lacks any known domain that could also suggest neofunctiolization for this gene, although this has not yet been described. Additionally, a recent published work [191] observed that after trying to detect INS-IGF2 by both Western blotting and immunohistochemistry in a human beta cell line they only detected cross reaction to native proinsulin, concluding that there is no evidence of a mature protein from INS-IGF2. Further investigations have to be performed to understand if INS-IGF2 can have some biological function or if it just a pseudogene. Evidences of extrapancreatic expression of insulin are becoming the rule rather than the exception. Large scale genome-wide association studies like The Genotype-Tissue Expression (GTEx) project [181], are enabling a global view of the gene expression in Human tissues. In this work the researchers could detect INS expression in twenty different tissues and INS-IGF2 in four. Transcriptome analyses in mouse and zebrafish suggest that extrapancreatic expression of insulin is achieved by ins1 and insb[180,182]. Although we look at this data with enthusiasm, we have the perfect conscience that the levels of expression, in some tissues, are very low and that proofs of protein secretion are needed. Evidences of extrapancreatic and extrathymic translation of insulin have been demonstrated in mice [173]. Although in this case these insulin producing cells had origin in bone marrow and migrated to different parts of the body, and were associated with the onset of diabetic neuropathy. Other studies could see advantages in the secretion of insulin by tear film cells, under diabetic conditions [174]. Recently in our lab, we could detect both insa and insb expression in kidney, muscle and bone in WT zebrafish under non diabetic conditions. Also, in a diabetic zebrafish model of diabetes, we could detect increased expression of both genes in bone after treating them with a vitamin D analog and a calcimimetic [192], demonstrating that not

only pancreatic INS expression is sensitive to some types of treatments [142,144], but also that this drugs can have the same effect in other cell types including bone. In this work we have gathered evidence that in mice, and probably in zebrafish, insulin paralogues can regulate glucose homeostasis, but in Human the answer is not clear. Extrapancreatic expression of insulin occurs in the three studied species and, at least in zebrafish, it is sensitive to drug therapy. If confirmed, the therapeutic potential of this event would be of considerable relevance for the treatment of diabetes, as this could represent a simple, reliable and safe way for self-production of insulin.

# CHAPTER 5.

# GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES



# CHAPTER 5. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Bone pathologies related to DM are rapidly becoming one of the principal causes associated to morbidity and tend to increase as both types of DM keep on growing worldwide [2]. During the onset and progression, T1DM can affect several different organs and tissues including bone. The fact that T1DM can alter metabolism, induce inflammation, increase apoptosis and growth retardation, as made a challenging task to determine the true causes of bone changes.

In chapter 1we believe to have contributed with novel evidences on how T1DM affects bone. Our most important finding is that a possible leptin deficiency can be an important cause that modulates bone changes in diabetes. We believe that previously authors have skipped this phenotype since the studies using diabetic mice models were not performed beyond 3 months. In fact, previous studies with hipoleptinemic mice revealed that this microarchitecture changes could only be detected starting at 4 months. But probably reduced leptin signaling in bone cells is not the only explanation for our results since the lack of insulin signaling in bone cells also reduces bone resorption and formation, leading us to propose that in our study hipoinsulinemia as contributed to the reduced bone formation phenotype. Additionally, our results suggest that there are increased signs of inflammation in the bone environment, as detected by an increase of enzymes involved in cartilage degradation that could explain the growth retardation of our diabetic mice. It was observed that leptin deficient mice also presented growth retardation and that chondrocytes express the leptin receptor and once again leptin deficiency could be the explanation for impaired growth. To the best of our knowledge, treatment of diabetic mice with leptin was tested with no effects on bone volume [38],

besides this, future works in this field should reconsider leptin treatment as a way to revert this bone phenotype.

In chapter 3 we report an increase in fin regeneration and mineralization in a zebrafish model of diabetes after treating this transgenic fish with paricalcitol and cinacalcet. We could validate this model as suitable for the study of bone pathologies related to diabetes and a response to vitamin D metabolism was achieved with benefits for preventing bone growth retardation. Indeed, non-treated diabetic group had impaired fin regeneration and mineralization with increased blood glucose levels. There are other protocols for the induction of diabetes in zebrafish, particularly the STZ method, but with more side effects than the one used in our studies.

Beside paricalcitol and cinacalcet, we also tested VitD3 but the results only showed a trend of increase of bone formation. The treatments tested are currently used as therapeutic solutions to treat SH, and in these patients the results suggest that treatment with VitD can have the same positive results as paricalcitol and cinacalcet, but with more prolonged exposure periods. Also, if in our study the exposure and regeneration period had been longer, the VitD group probably could have had a more pronounced effect on fin mineralization and regeneration.

To better interpret our results we performed a gene expression analysis by qRT-PCR to a group of genes of interest and we could detect a trend of decrease in all three treated groups for *pthr* that could be related to a decrease in circulating *pth*, inhibiting bone resorption. But this result alone could not explain the observed increased regenerated and mineralized areas in the paricalcitol and cinacalcet groups, because the VitD group did not show the same increase. Other explanation could be the increased expression of *runx2* found in paricalcitol and cinacalcet groups that could have triggered an increase in osteoblast differentiation.

Finally we could detect *insa* and *insb* expression in RNA from muscle, kidney and bone supporting the conclusions in the previous chapter. But more interestingly, was the fact that both *insa* and *insb* were upregulated in the paricalcitol and cinacalcet treated groups. Despite these results we feel more comfortable to say that both paricalcitol and cinacalcet may have increased the expression of insulin in the pancreas, as it was previously described that activation of VitD receptor and calcium sensing receptor induce insulin expression in the pancreas.

Future studies should focus in understanding if both drugs actually favor osteoblastic differentiation and if upregulation of insulin could actually explain these results.

In chapter 4 we focused on two possibilities: i. Does the *INS-IGF2* gene have the capacity to mimic the action of its paralogue *INS* and; ii. Does extrapancreatic INS expression occurs and if it has any function in regulating glucose.

Our *in silico* analysis showed that *INS-IGF2* do not have all the motifs that characterize *INS* transcripts. Murinae have two redundant insulin genes, that we believe that the rise of *ins1* favored the loss of *Ins-Igf2* in this subfamily suggesting that both genes could have the same function, but hardly *INS-IGF2* in humans can have a redundant function with *INS*. The zebrafish genome also contains two different insulin genes with all the motifs. This suggests co-functionalization, like the observed in mice.

Extrapancreatic expression of insulin is a proven fact and in our work we have gathered data that supports this statement. In mouse and zebrafish this expression is mainly made by paralogues while in human is made by *INS*. Although this could have therapeutic potential, little is known on how this process is regulated. Some researchers have linked extrapancreatic expression of insulin to diabetes with consequences in

development of other diseases, but others have observed beneficial effects in local secretion of insulin by different organs.

Future works in this field should first understand if extrapancreatic insulin is translated and secreted, and if this can be potentiated, favoring overall body metabolism in diabetes.

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# APPENDIX I

Table 1. Primers for qRT-PCR used in chapter 2

mouse

| | | |
|---|---|---|
| Oc | Forward | AAGCAGGAGGGCAATAAGGTAGTGAACA |
| Oc | Reverse | GAGTTTGGCTTTAGGGCAGCACAGGTC |
| Runx2 | Forward | AGGGAGAGGACAACAGAAGAGAA |
| Runx2 | Reverse | TCAAAGTAAAGTGGGACTGCCTAC |
| Adamst5 | Forward | TCAGCCACCATCACAGAA |
| Adamst5 | Reverse | CCAGGGCACACCGAGTA |
| Hrrpt1 | Forward | GGTGGATATGCCCTTGACTATAATGA |
| Hrrpt1 | Reverse | CAACATCAACAGGACTCCTCCTATT |
| ColII | Forward | CCAACACCGCTAACG |
| ColII | Reverse | GGTCTTGCCCCACTTAC |
| Igf1 | Forward | AAAGTGGTCCTGGCGTGGGTAGATT |
| Igf1 | Reverse | TCTACAACATCCATGCATTTTCGGC |

# APPENDIX 2

Table1. Primers used for qRT-PCR in chapter 3

zebrafish

| | | |
|---|---|---|
| ef1a | Forward | AGC CCC TCC TGG CTT TCA CCC |
| ef1a | Reverse | TGG GAC GAA GGC AAC ACT GGC |
| 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 | GTTTCGTCTATGGTCTGGTCG |
| pth1ra | Reverse | GATTGCTCGCTCACATTTTC |
| Vdr | Forward | GTCCAACCAGTCCTTCAGTCT |
| Vdr | Reverse | AGTGTGACCCGCCTTAGTG |
| Insb | Forward | CTCTGCTCACTCAGGAAAAGG |
| Insb | Reverse | GGATGGAGAAGACTGCGAT |
| Insa | Forward | CATTCCTCGCCTCTGCTTC |
| Insa | Reverse | TGCCTGGGTTAGTGCTTACA |
| Insra | Forward | TCTACAGCGAGGAAAACAAGC |
| Insra | Reverse | AGAGATAAGATGCGTCCGTTTT |

# APPENDIX 3

List of protein sequences used for phylogenetic tree reconstruction in chapter 4:

#Human
>homo sapiens sapiens_INS
MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTP
KTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLE
NYCN
>homo sapiens sapiens_INS-IGF2
MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTP
KTRREAEDLQASALSLSSSTSTWPEGLDATARAPPALVVTANIGQAGGSSSRQF
RQRALGTSDSPVLFIHCPGAAGTAQGLEYRGRRVTTELVWEEVDSSPQPQGSES
LPAQPPAQPAPQPEPQQAREPSPEVSCCGLWPRRPQRSQN
#chimpanzee
> Pan troglodytes
MALWMRLLPLLVLLALWGPDPASAFVNQHLCGSHLVEALYLVCGERGFFYTP
KTRREAEDLQVGQVELGG
GPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN
>gorilla gorilla gorilla Ins
MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTP
KTRREAED
LQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN
>gorilla gorilla gorilla INS-IGF2
PSRTGCIRRGHQAGHCPSAMALWMRLLPLLALLALWGPDPAPAFVNQHLCGS
HLVEALYLVCGERGFFYTPKTRREAEDPQASALSPSSSTSTWPEGLDATARAPP
ALVATANFGQVGGSSSRQFRQRALGTSDSPVLFIHCPGAAGTARGLEYRGRRV
TTELVWEEVDSSPQPQGSESLPAQPPAQPAPQPEPQQARAAGEPSPEVSCCGLW
PRRPQRSQN
#Marmoset
>Callithrix jacchus
MAPWMPLLPLLALLALWGPEPAPAFVNQHLCGPHLVEALYLVCGERGFFYAP
KTRREAEDLQVGQVELGGGSITGSLPALEGPLQKRGVVDQCCTSICSLYQLQNY
CN
#Marmoset_
>Callithrix jacchus_INS-IGF2
MAPWMPLLPLLALLALWGPEPAPAFVNQHLCGPHLVEALYLVCGERGFFYAP
KTRREAEDLQASALSPSSSSTSTRLEGLNATPALVATAHIGQASGAPSWQLRQT

ALGTSDPVLFTHCPGAVGTQQRLESGGEGATAPLQEVDSSPQPRGSEPLPAHPA
QPAPQPEPEQARPESSPEVSCCGLWPRRAQHSQN
#Ferret
>Mustela putorius furo
MALWMRILPLLALLALWAPAPTAAFVNQHLCGSHLVEALYLVCGERGFFYTP
KARREAEDLQARDSELGGAPGAGGLALGLEGALQKRGIVEQCCTSICSLYQLE
NYCN
>Mustela putorius furo_INS-IGF2
MPLPASAVSIPRSPSPAMALWMRILPLLALLALWAPAPTAAFVNQHLCGSHLVE
ALYLVCGERGFFYTPKARREAEDLQASASSPASTSSTRLEGLGTTARAPPALAA
TANIGPTSGSPAGQLQQRGLGSGDSPVLFIHRPGTSGTTQRLEYRGRRVTAELPE
EEVAPQPQGPTSHPAEPPAQPAPQPEPKAARATREPSPELRCCGLWPRQSGRAQ
N
#Elephant
> Loxodonta africana Ins
MALWTRLLPLLALLAVGAPPPARAFVNQHLCGSHLVEALYLVCGERGFFYTPK
TRREVEDTQVGEVELGTGLQPFPAEAPKQKRGIVEQCCTGVCSLYQLENYCN
> Loxodonta africana INS-IGF2
MALWTRLLPLLALLAVGAPPPARAFVNQHLCGSHLVEALYLVCGERGFFYTPK
TRREVEDTQASSTSTSSSPSTRLEGLGTTARAPPALAATANIGPTSGSSAGQFQE
RALGTSDSPVLIIHRPGAAGTAQRLEYRGRRVTADLVEEEVGTRPQHQGPPAGP
AHSAYPLAQPALQPARASRDPSPEVSCCGLWPQRSQRSQN
#Orangutan
>Pongo borneo
MALWMRLLPLLALLALWGPDPAQAFVNQHLCGSHLVEALYLVCGERGFFYTP
KTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLE
NYCN
>Pongo borneo_INS-IGF2
MALGMRLLPLLALLALWGPDPAQAFVIFRLCGSHLVEALYLLFGERGFFYTPKT
RREAESALSPSSSTSTWPEGLDATARAPPALVATANIGQAGRSSSRQFRQRALG
TSDSPVLFIHCPGAAGTAQGLGYRGRRVTAELVWEEVDSSPQPQGSESLPAQPP
AQPAPEPEPQQARVAREPSPEVSCCGLWPRRPQRSQN
#Manatee
>Trichechus
MALWTRLLPLLALLAVGAPPPARAFVNQHLCGSHLVEALYLVCGERGFFYTPK
TRRELEDPQVGKVEPGAGGRQPFPAEVPREKRGIVEQCCTGVCSLYQLENYCN
>Trichechus_INS-IGF2
MALWTRLLPLLALLAVGAPPPARAFVNQHLCGSHLVEALYLVCGERGFFYTPK
TRRELEDPQASSTSTSSSPSTRLEDLGTTARTPPALAATANIGPTSGSSTGQFQQR
DLGTSNSPVLIIHRPGAAGTAQRLEYRGRRVTAELVQEELGTRPQHQGPPAHPA
CLAHPPAQPAPQPEPQQARASRDPSPEVSCCGLWPQRSQRSQN
#Walrus
>Odobenus rosmarus

MALWTRLLPLLALLAVGAPPPARAFVNQHLCGSHLVEALYLVCGERGFFYTPK
TRRELEDPQVGKVEPGAGGRQPFPAEVPREKRGIVEQCCTGVCSLYQLENYCN
>Odobenus rosmarus_INS-IGF2
MALWTHLLPLLALLALWAPAPSRAFVNQHLCGSHLVEALYLVCGERGFFYTPK
ARREVEDPQASASSPSSSSTSTRLEGLGTAARAPPTLAATANIGPTSGSSAGQLQ
QRGLGTSDSPVLFIHRPGTSGTTQRLEYRGRRVTAELLEEEVDPKPQGPASHPAE
TPAQATPQPEPQAAREPSREVSCCGLWPRWSPRAQN
#Panda
>Ailuropoda melanoleuca
MALWTRLLPLLALLAVWAPVPARTFVNQHLCGSHLVEALYLVCGERGFFYTP
KARREVEDLPAGDAELDRVPGADPQPRALAGALQRRGIVEQCCTSICSLYQLEN
YCN
>Ailuropoda melanoleuca_INS-IGF2
MALWTRLLPLLALLAVWAPVPARTFVNQHLCGSHLVEALYLVCGERGFFYTP
KARREVEDLPASASSPSSSSTSTRLEGLGTTARAPPTLAATANTGPTSGSSARQF
RQRDLGTSDSPVLFIHRPGTSGTTQRLEYRGRRVTGELLEEEVDPKPQSPASHPA
EPPAQVAHQPEPQLEPQTQSEPKPARTTREPSPEVSCCGLWPRRSPHAQN
>Hyrax
#Procavia_INS-IGF2
MALWTRLLPLLALLAVGPPPPARAFVNQHLCGSHLVEALYLVCGERGFFYTPK
ARREVEDPQAPPPSTSPSASTRLQGLDLTARAPPALSETDNTSPTGGPPAGQFQE
TAFGTSDSPVLTIHHPGAAGTPQQLEFRGQSVTTGLVEEEEASTRPQHQSPPPLP
ALPPAQPAPQPKLQQVRFSRDPSPEVRCCGLWPWRRSWHLQN
#Chinese_hamster
>Cricetulus griseus
MALWMRLLPLLALLALWEPNPAQAFVNQHLCGSHLVEALYLVCGERGFFYTP
KSRRGVEDPQVTQLELGGGPGAGDLQTLALEVAQQKRGIVDQCCTSICSLYQL
ENYCN
>Cricetulus griseus_INS-IGF2
MALWMRLLPLLALLALWEPNPAQAFVNQHLCGSHLVEALYLVCGERGFFYTP
KSRRGVEDPQASASSPSTSSTSTRLERLGTTARAPPALEEVADPGPASRSSPKQF
RKRIFGTSEPPVLFIHRPGASGTTKRLEYRGRVVTTELQVIVQEEDPEPQSRGPAS
PGDEPLAQPPKPLPRPEPQRPEPSPEARCCGLWPRRSQRSQN
#Rat
>Rattus norvegicus_INS1
MALWMRFLPLLALLVLWEPKPAQAFVKQHLCGPHLVEALYLVCGERGFFYTP
KSRREVEDPQVPQLELGGGPEAGDLQTLALEVARQKRGIVDQCCTSICSLYQLE
NYCN
>Rattus norvegicus_INS2
MALWIRFLPLLALLILWEPRPAQAFVKQHLCGSHLVEALYLVCGERGFFYTPMS
RREVEDPQVAQLELGGGPGAGDLQTLALEVARQKRGIVDQCCTSICSLYQLEN
YCN
#mouse
#mus musculus_INS1

MALLVHFLPLLALLALWEPKPTQAFVKQHLCGPHLVEALYLVCGERGFFYTPK
SRREVEDPQVEQLELGGSPGDLQTLALEVARQKRGIVDQCCTSICSLYQLENYC
N
>mus musculus_INS2
MALWMRFLPLLALLFLWESHPTQAFVKQHLCGSHLVEALYLVCGERGFFYTP
MSRREVEDPQVAQLELGGGPGAGDLQTLALEVAQQKRGIVDQCCTSICSLYQL
ENYCN
#Squirrel
>Callosciurini
MALWTRLLPLLALLALLGPDPAQAFVNQHLCGSHLVEALYLVCGERGFFYTPK
SRREVEEQQGGQVELGGGPGAGLPQPLALEMALQKRGIVEQCCTSICSLYQLEN
YCN
#Tree_shrew
>Anathana ellioti
MALWTCFLPLLTLLALWGPEPAPAFVNQHLCGSHLVEALYLVCGERGFFYTPK
TRREVEDSQGQVELRGPAGSLQPLALEVPPQKRGIVEQCCTSICSLYQLENYCN
#Dolphin
>Tursiops truncatus
MVLWLRLGPLLALLALWAPAPARAFVNQHLCGSHLVEALYLVCGERGFFYTP
KARREVEGPQVGAVELGGGPGAGGLQPPALEGPPQKRGIVEQCCTSICSLYQLE
NYCN
#Otolemur
>Otolemur agyisymbanus
MAVWMRLLPLLALLALWGPEPAPAFVNQHLCGSHLVEALYLVCGERGFFYTP
KARRDTEDPQVGQVGLGGSPITGDLQSLALDVPPQKRGIVEQCCTSICSLYQLE
NYCN
#Lesser_E_jerboa
>Pygeretmus platyurus
MTLWTRLLPLLALLALWGADPAQAFVNQHLCGSHLVEALYLVCGERGFFYTP
KSRREVEDPQAGQLELGGGPGAGGPQSLALELAPQKRGIVDQCCTSICSLYQLE
NYCN
#Mouse_lemur
>Microcebus murinus
MALWTRLLPLLALLALWGPEPAPAFVNQHLCGSHLVEALYLVCGERGFFYTPK
SRREVEDAQAGQVGPDGGLGAGGLQALALEGAPQKRGIVEQCCTSICSLYQLE
NYCN
#Microbat
>Corynorhinus townsendii
MALWTRLLPLLALLALWAPAPAQAFNHEHLCGEDLVDIMTIICGDQGFKNPKA
ARELPDPQEGEVDMGAGGPKALTVEELLQNTDIVEVCCTNICSFYDMETYCN
#Cow
>Bos taurus
MALWTRLAPLLALLALWAPAPARAFVNQHLCGSHLVEALYLVCGERGFFYTP
KARREVEGPQVGALELAGGPGAGGLEGPPQKRGIVEQCCASVCSLYQLENYCN

#Cat
>Felis catus
MAPWTRLLPLLALLSLWIPAPTRAFVNQHLCGSHLVEALYLVCGERGFFYTPK
ARREAEDLQGKDAELGEAPGAGGLQPSALEAPLQKRGIVEQCCASVCSLYQLE
HYCN
#Guine_pig
>Cavia porcellus
MALWMHLLTVLALLALWGPNTGQAFVSRHLCGSNLVETLYSVCQDDGFFYIP
KDRRELEDPQVEQTELGMGLGAGGLQPLALEMALQKRGIVDQCCTGTCTRHQ
LQSYCN
#Chicken
>Gallus gallus domesticus
MALWIRSLPLLALLVFSGPGTSYAAANQHLCGSHLVEALYLVCGERGFFYSPK
ARRDVEQPLVSSPLRGEAGVLPFQQEEYEKVKRGIVEQCCHNTCSLYQLENYC
N
#Ficedula albicollis
>Collared_Flycatcher
MALWLRSLPLLALLALSSPGSSQAAVSQHLCGSHLVEALYLVCGERGFFYQPK
ARRDVEQPLAYQQRCLSGPLHGELGELPFQQEEFEKVKRGIVEQCCHNTCSLY
QLENYCN
#Rock_Dove
>Columba livia
MALWIRSLPLMALLALSGPGTSHAAANQHLCGSHLVEALYLVCGDRGFFYSPK
ARRDIEQPLVSGPLHGEIGELPFQQEEFEKVKRGIVEQCCHNTCSLYQLENYCN
#Turkey
>Meleagris gallopavo
MSLWIRSLPLLALLVFSGPGTSYAAANQHLCGSHLVEALYLVCGERGFFYSPKA
RRDVEQPLVSSPLRGEAGVLPFQQEEYEKVKRGIVEQCCHNTCSLYQLENYCN
#Duck
>Bucephala clangula
MALWIRSLPLLALLALSGPGISHAAANQHLCGSHLVEALYLVCGERGFFYSPKT
RRDVEQPLVNGPLHGEVGELPFQHEEYQKVKRGIVEQCCENPCSLYQLENYCN
#Zebra_finch
>Taeniopygia guttata
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ARRDVEQPLVSGPLHGELGELPFQQEEFETVKRGIVEQCCHNTCSLYQLENYCN
#Budgerigar
>Melopsittacus undulatus
MALWILSLPLLALLALSGPGTSHAAATQHLCGSHLVEALYLVCGERGFFYSPKA
RRDVEQPLVSGPLHGEVGELPFRPEEFQKVKRGIVEQCCHNTCSLYQLENYCN
#Ground_Tit
>Pseudopodoces humilis
MALWIQSLPLLALLAFSGPGSSHAAVNQHLCGSHLVEALYLVCGERGFFYQPK
ARRDVEQPLVSGPLHGELGELPFQQEEFEKVKRGIVEQCCHNTCSLYQLENYCN

#Medium_Ground_Finch
>Geospiza fortis
MALWIRSLPLLALLALSGPGSSHAAVNQHLCGSHLVEALYLVCGERGFFYQPK
ARRDAEQPLVSGPLHGELGELPFQQEEFEKVKRGIVEQCCHNTCSLYQLENYCN
#White_Throated_Sparrow
>Zonotrichia albicollis
MALWIRSLPLLALLALSGPGSSHAAVNQHLCGSHLVEALYLVCGERGFFYQPK
ARRDAEQPLMSGPLHGELGELPFQQEEFEKVKRGIVEQCCHNTCSLYQLENYC
N
#Anole_lizard
>Anolis carolinensis
MTLWISSLPLLVLIAVSAPTISYALPNQHLCGSHLVEALYLVCGDRGFYYSPKTR
RNIEQPLASGSLQNEVETLPFQPQDFQKVKRGIVQQCCENTCSLYELENYCN
# Chinese softshell turtle
> Pelodiscus sinensis
MALWIRSLPLLALLALSGPPISHAAANQHLCGSHLVEALYLVCGERGFFYSPKA
RRDLEQPLVRQRGAPQNEVELPFQQQEFQQAKRGIVEQCCHNTCSLYQLENYC
N#Frog
>Xenopus laevis
MALWMQCLPLVLVLLFSTPNTEALANQHLCGSHLVEALYLVCGDRGFFYYPKI
KRDIEQAMVNGPQDNELDGMQLQPQEYQKMKRGIVEQCCHSTCSLFQLESYC
N
>danio rerio_Insa
MAVWLQAGALLVLLVVSSVSTNPGTPQHLCGSHLVDALYLVCGPTGFFYNPK
RDVEPLLGFLPPKSAQETEVADFAFKDHAELIRKRGIVEQCCHKPCSIFELQNYC
N
>danio rerio_Insb
MGTFVFPLMPSRAMGQAAGRYISSTIQAQSWPLFTDSAHSGKGCACPQSVSTM
VLLLQASVLILLLASLPGSQSSPSQHLCGSSLVDALYLVCGPRGFFYTNRGRDLE
TLLALLSNLAGYEAADADPLKEKVMKMKRGIVEQCCHRPCTIYHLEDYCS
#Tilapia
>Oreochromis niloticus_insa
MCFCVQLFSSMAALWLQTFSLLVLMMVSWPGSQAVGGPQHLCGSHLVDALY
LVCGDRGFFYNPRRDVDPLLGFLPPKAGGAVVQGGENEVTFKDQMEMMVKR
GIVEECCHKPCTIFDLQNYCN
>Oreochromis niloticus_insb
MARVSWAVSMLLLLMLCSPGGSSVPLKHLCGSHLVDALYFVCGERGFFYNPSR
THKRDVEHLLGFLSKRARQDQRLWRALSGRDEPKVKRGIVEQCCHKPCSIYHL
EGYCD
#Japonese_Pufferfish
>Takifugu__insa
MAALWLQSVSLLLLMVVSSPGSQAMAPPQHLCGSHLVDALYLVCGDRGFFYN
PKRDVDSMMGILPPKAGGAAGVDNEVAEYAFKDQMEMMVKRGIVEQCCLRP
CNLLDLQNYCN

>Takifugu__insb
MARLWEVSALLLLVLSSPGVSPFPAQHLCGSHLVDALYIVCGERGFFADPDRR
HKRDVEDLLGFLSNRARRQQRLWKVLSGHNEPKVKRGIVEQCCHKPCSIHHLQ
RYCD
#Medaka
>Oryzias latipes_insa
MAALWLQTFSLLFLLIVSCPGSQAIAPQHLCGSHLVEALYLVCGDRGFFYTPKR
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>Oryzias latipes_ins2
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#Platyfish
>Xiphophorus maculatus-insa
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#Greater_Amberjack
>Seriola dumerili
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#Green_Spotted_Puffer
#Tetraodon
>Tetraodon lineatus
MTHMGCRVWNRRMCLWVSSVPIQHLCGSQLVDTLYFICGERGFYADGNHPHK
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#Coelacanth
>Latimeria menadoensis
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YCN
#Stickleback
>Gasterosteus aculeatus
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>ciona intestinalis
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RGYQLQQDQSCMD