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PREVENTION OF GROWTH FAILURE CAUSED BY GLUCOCORTICOIDS AND INFLAMMATION

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Prevention of growth failure caused by glucocorticoids and inflammation
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To Niclas, Eddie and Stella

ABSTRACT (ENGLISH)

Longitudinal bone growth is a complex process that takes place in the growth plate, and normal growth is dependent on a precise balance of several endocrine and paracrine factors. Growth impairment is common in children with inflammatory diseases and is associated with elevated levels of pro-inflammatory cytokines and long-term glucocorticoid treatment. To date, no treatments are available to treat growth impairment caused by glucocorticoids and there is a need to find new strategies to counteract the negative side effects of glucocorticoids on bone growth.

The aim of this thesis was to study bone growth impairment induced by glucocorticoids and inflammation and the underlying molecular mechanisms. The aim was also to address whether the mitochondrial derived peptide humanin potentially could rescue from glucocorticoid-induced bone growth impairment and apoptosis in the growth plate, without interfering with the anti-inflammatory effect of glucocorticoids.

In **study I** the effect of glucocorticoids and humanin on bone growth was assessed in several different experimental models. We discovered that the synthetic humanin analogue, HNG, completely rescued from dexamethasone-induced bone growth impairment and that humanin over-expressing mice were resistant to glucocorticoid-induced growth impairment. In addition, our results indicate that humanin is a novel regulator of Hedgehog signaling.

In **study II** we assessed whether HNG could rescue from glucocorticoid-induced apoptosis in the growth plate in a model of chronic inflammation. We showed that HNG treatment suppressed apoptosis in both growth plate and articular cartilage. Importantly, we found that HNG did not interfere with the anti-inflammatory effect of dexamethasone.

In **study III** we investigated the effect of dexamethasone on bone growth and chondrogenesis in a disease model of chronic inflammation. By using the transgenic TNF over-expressing mouse model (tgTNF) we found that chronic inflammation by itself suppressed bone growth and that dexamethasone treatment further suppressed bone growth despite its anti-inflammatory actions. We also showed that Indian hedgehog and humanin expression levels were suppressed in the growth plate of the tgTNF mice, suggesting a new mechanism for inflammation induced growth impairment.

ABSTRAKT (SVENSKA)

Längdtillväxt är en komplex process som sker i tillväxtplattan. Brosket i tillväxplattan är uppbyggt av kondrocyter i olika stadier av differentiering. För att längdtillväxten skall ske på ett normalt sätt är dessa celler strikt reglerade av olika endokrina- och parakrina faktorer. Försämrad längdtillväxt ses ofta vid kroniska inflammatoriska sjukdomar där vanligt förekommande långvarig kortisonbehandling bidrar till den försämrade tillväxten. I dagsläget finns ingen effektiv behandling som kan motverka denna typ av tillväxthämning. Det finns därför ett behov av att utveckla nya metoder och läkemedel för att undvika de negativa bieffekterna som glukokortikoider och inflammation har på längdtillväxten.

I avhandlingsarbetet har det övergripande målet varit att studera effekterna av glukokortikoiden dexametason på längdtillväxten och kondrogenesen i tillväxtplattan samt att studera den mitokondriella peptiden humanin och dess potential att förhindra tillväxthämmning till följd av glukokortikoidbehandling. Vi har även studerat en modell av kronisk inflammation där den proinflammatoriska cytokinen TNF är överuttryckt och längdtillväxten hämmad för att kartlägga hur behandling med en glukokortikoid påverkar tillväxten i denna sjukdomsmodell.

I **studie I** har vi påvisat att humanin kan hindra glukokortikoid-inducerad tillväxthämmning i växande normala möss. Vi såg även att längdtillväxten och kondrogenesen i humanin-överuttryckande möss inte påverkades negativt av dexametasonbehandling. Våra resultat visar att humanin reglerar Hedgehog-signaleringskaskaden vilket kan vara en ny mekanism för hur humanin verkar på kondrocyterna i tillväxtplattan.

I **studie II** har vi påvisat att humanin hämmar apoptos i tillväxtplattan som uppstår till följd av glukokortikoidbehandling i en djurmodell med kronisk inflammation. I denna studie visade vi även att humanin i kombination med dexametason inte påverkar den anti-inflammatoriska effekten.

I **studie III** har vi påvisat att längdtillväxten hämmas i möss med överuttryck av cytokinen TNF och att dexametason, trots dess anti-inflammatoriska effekt, ytterligare hämmar tillväxten i dessa möss. Vi såg även att uttrycket av Indian hedgehog och humanin i tillväxtplattan var nedreglerat i möss med överttryck av TNF vilket kan ge insikt i en ny molekylär mekanism för hur tillväxthämning uppstår till följd av kronisk inflammation.

LIST OF SCIENTIFIC PAPERS

I. Humanin is a novel regulator of Hedgehog signaling and prevents glucocorticoid-induced bone growth impairment

Farasat Zaman, Yunhan Zhao, **Bettina Celvin**, Hemal Mehta, Junxiang Wan, Dionisios Chrysis, Claes Ohlsson, Bengt Fadeel, Pinchas Cohen and Lars Sävendahl

Published in *FASEB J* (2019) 33(4): 4962-74

II. Humanin prevents undesired apoptosis of chondrocytes without interfering with the anti-inflammatory effect of dexamethasone in collagen-induced arthritis

Bettina Celvin, Farasat Zaman, Cecilia Aulin and Lars Sävendahl

Published in *Clin Exp Rheum* (2019) Epub ahead of print (PMID: 31172921)

III. Dexamethasone suppresses bone growth and chondrogenesis in TNF-overexpressing mice

Bettina Celvin, Yunhan Zhao, Maria Denis, Niki Karagianni, Cecilia Aulin, Farasat Zaman and Lars Sävendahl

Manuscript

PAPERS NOT INCLUDED IN THE THESIS

Recent research on the growth plate: Impact of inflammatory cytokines on longitudinal bone growth

Bettina Sederquist, Paola Fernandez-Vojvodich, Farasat Zaman and Lars Sävendahl

Published in *J Mol Endocrinol* (2014) 53(1): T35-T44

CONTENTS

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bid	BH3 interacting-domain death agonist
CIA	Collagen induced arthritis
Dexa	Dexamethasone
ELISA	Enzyme-linked immunosorbent assay
GC	Glucocorticoid
GH	Growth hormone
GHR	Growth hormone receptor
GR	Glucocorticoid receptor
CRH	Corticotropin-releasing hormone
gp130	Glycoprotein 130
Hh	Hedgehog signaling
HNtg	Transgenic humanin over-expressing mice
HNG	[Gly^{14}]-Humanin
HPA	Hypothalamic-pituitary-adrenal axis
IGF-1	Insulin-like growth factor 1
IGF-2	Insulin-like growth factor 2
IGFBP-3	Insulin-like growth factor- binding protein 3
IL-1β	Interleukin-1β
IL-6	Interleukin-6
Ihh	Indian hedgehog
IHC	Immunohistochemistry
Ip	Intraperitoneal injection
JIA	Juvenile idiopathic arthritis
MDP	Mitochondrial derived peptide
MOTS-c	Mitochondrial open reading frame of the 12S rRNA-c
MSC	Mesenchymal stem cells
NF-κB	Nuclear factor kappa light-chain-enhancer of activated B cells

ORF	Open reading frame
PCNA	Proliferating cell nuclear antigen
PTHrP	Parathyroid hormone-related peptide
Sc	Subcutaneous injection
SOCS	Suppressor of cytokine signaling
tgTNF	Human TNF-overexpressing transgenic mouse line 197
TNF	Tumor necrosis factor-α
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling
UC	Ulcerous colitis

1 INTRODUCTION

1.1 LONGITUDINAL BONE GROWTH

Longitudinal bone growth is a complex series of events highly dependent on the proper balance between several crucial factors including hormones, growth factors and nutrition, which all influence the final height of an individual. The growth potential is genetically determined but environmental factors, poor psychosocial situation, and a variety of chronic diseases may also influence the bone growth and may result in short stature if not treated. Therefore, bone growth in general is a very good indicator of good health.

Postnatal longitudinal bone growth in humans can be divided into three phases; infancy, childhood and pubertal according to the ICP-model established by Karlberg in 1989 [1]. During infancy and childhood, longitudinal bone growth is stimulated to a large extent by growth hormone (GH) and nutrition while sex steroids are the main stimulators of growth during puberty. At the end of puberty, sex steroids stimulate growth plate closure and final adult height is achieved [2].

1.1.1 Growth plate structure

Longitudinal bone growth is a result of a mechanism called endochondral ossification, where bone is formed after a cartilaginous template has been generated. This process occurs at the growth plate, a thin layer of cartilage situated at both ends between the diaphysis and the epiphysis of all long bones [3]. The growth plate consists of chondrocytes at different levels of differentiation, distributed within three distinct zones; the resting, proliferative and hypertrophic zone [4] (Figure 1).

The chondrocytes within these three zones have different characteristics. Chondrocytes in the resting zone are not very active metabolically, but these stem-like cells serve as an important reservoir for the growth plate and studies have shown that the resting zone alone can give rise to the entire growth plate [5]. When they divide they give rise to daughter cell columns in the proliferative zone. The chondrocytes of the proliferative zone, on the other hand, proliferate actively and play a major role in the matrix production. These cells are larger in size and form columns parallel to the long axis of the bone. Further maturation of these chondrocytes leads to hypertrophy. Chondrocytes in the hypertrophic zone increase in size and produce collagen type X, alkaline phosphatase and matrix metalloproteinases. Later, the terminally differentiated chondrocytes die by apoptosis and the growth plate is invaded by blood vessels and bone cell precursors, which gradually remodel the hypertrophic zone cartilage into bone [4].

Longitudinal bone growth is therefore a result of proliferation, enlargement of hypertrophic chondrocytes and production of extracellular matrix proteins in the proliferative and hypertrophic zones.

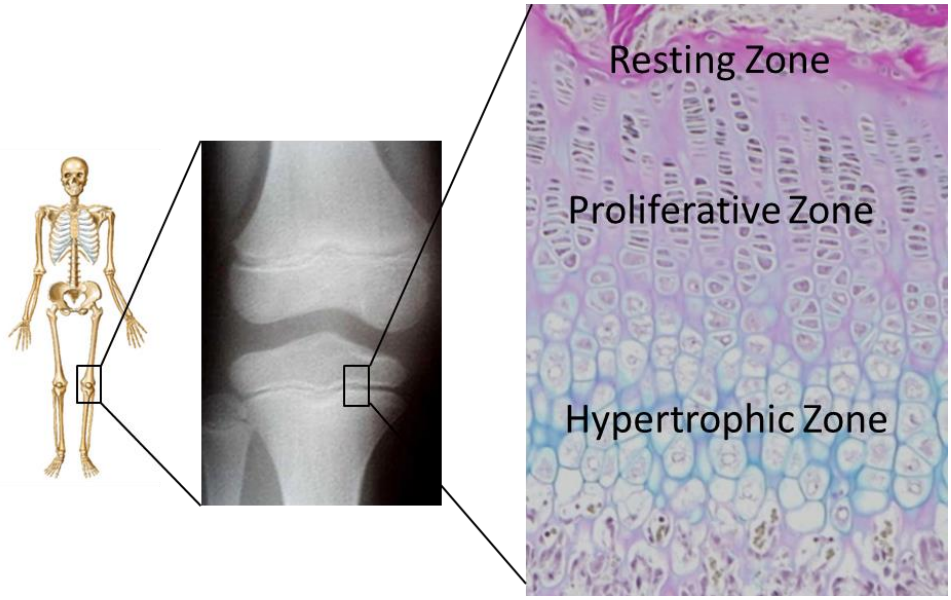

Figure 1. Structure of the growth plate cartilage. The growth plate is located in each end of the long bones and is divided into three distinct zones; the resting, proliferative and hypertrophic zone.

1.1.2 Regulation of longitudinal bone growth

Hormones are the major regulators in the process of longitudinal bone growth and most central and most studied is the growth hormone/insulin-like growth factor 1 (GH/IGF-1) axis together with thyroid hormones and sex steroids [6]. Several local factors are also involved in the regulation of bone growth, including the Indian Hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP) signaling as well as bone morphogenic proteins, fibroblast growth factors and vascular endothelial growth factors. How these hormonal and local factors interact with each other to regulate bone growth is still unclear.

1.1.2.1 GH/IGF-1 axis

GH is a peptide hormone secreted by the pituitary gland and is mainly promoted by GH-releasing hormone (GHRH) and inhibited by somatostatin from the hypothalamus. GH is known to stimulate bone growth indirectly by affecting circulating IGF-1 levels but also by stimulating local production of IGF-1 in the growth plate [2]. However, according to the original "somatomedin hypothesis", GH (somatomedin) only has indirect effects on bone growth by stimulating the production of IGF-1 in the liver [7]. Later, this hypothesis was

modified because new findings showed that GH also has direct effects on longitudinal bone growth independent of IGF-1 [8-10].

The action of GH is mediated by its binding to the GH receptor (GHR), which is highly expressed in many tissues such as liver, heart, kidneys, pancreas, cartilage and skeletal muscle where it stimulates the synthesis of IGF-1 [11]. The GHR can also be detected in the chondrocytes of all zones of the growth plate, indicating that GH has a direct growth promoting effect in the growth plate [12]. Further evidence for direct actions of GH has been shown in studies where local GH injection into the tibia growth plate of rats accelerated bone growth compared to the untreated unilateral bone [9]. GH is capable of stimulating proliferation by direct action on the resting chondrocytes in the growth plate [13].

The local action of GH involves recruitment of resting chondrocytes into the proliferative state and stimulation of local IGF-1 production, which stimulates proliferation of proliferative chondrocytes. Suppressor of cytokine signaling (SOCS) 2 has been suggested to locally regulate GH in the growth plate as SOCS2 knockout mice display an overgrowth that is associated with increased GH/IGF-1 signaling. In addition, local GH signaling is increased in chondrocytes and metatarsals from SOCS2 knockout mice [14,15].

IGF-1 is essential for bone growth during both embryonic and postnatal life; whereas null mutation in the IGF-1 gene is associated with severe growth retardation [16]. However, liver-specific IGF-1-deficient mice (LID) display normal bone length and body size despite a 25% reduction in circulating IGF-1 [17]. IGF-1, IGF-2 and IGF-binding protein 2 (IGFBP-2) have all been shown to be expressed in the growth plate of pre-pubertal mice, predominantly in the proliferative and pre-hypertrophic zones [18]. Patients with Laron syndrome have very low levels of circulating IGF-1 as a result of mutations in the GH receptor and abnormal GH signaling. These patients display extreme short stature and osteoporosis [19]. Similarly, bone growth is severely suppressed in IGF-1 and IGF-1R knock-out supporting the fact that IGF-1 is essential for normal regulation of bone growth [20].

1.1.2.2 Ihh/PTHrP signaling

The Hedgehog (Hh) signaling pathway is known to play a key role in bone growth regulation [21]. Among the three Hh ligands; Sonic (Shh), Indian (Ihh), and Desert (Dhh), only Shh and Ihh contribute to growth plate function. The Ihh ligand is expressed and secreted by pre-hypertrophic and early hypertrophic cells in the growth plate [22]. Binding of Ihh to its receptor Patched-1 (Ptc-1) releases the membrane protein Smoothend (Smo), which activates the Hh signaling pathway and allows the activated transcription factor Gli to enter the nucleus

and enhance the transcription level of downstream target genes [22]. Recently, it has been proposed that Ihh signaling actually takes place in the primary cilia, sensory organelles protruding from the cell surface, which are important for mechano-biological signal transduction in chondrocytes [23].

Knock-out studies have reported that about 50% of Ihh-null embryos die early in development and those who developed to term die at birth, displaying abnormal chondrocyte proliferation and absence of mature osteoblasts [24]. Furthermore, studies have shown that Ihh over-expression alone is sufficient to increase chondrocyte proliferation [25]. Recent studies have reported that the Ihh inhibitor Vismodegib, which is used in the treatment of medulloblastoma, causes early growth plate fusion and growth impairment in treated children [26]. Vismodegib inhibit Hh signaling via Smo by suppressing Gli1 [27,28]. Altogether, these findings confirm the important role of Ihh in the growth plate and its regulation.

1.2 BONE GROWTH IMPAIRMENT

Short stature is generally defined as a standing height more than 2 standard deviations below the population mean (or below the 2.5^{th} percentile) for each sex. Many factors act individually or in combination to suppress bone growth by direct actions on the growth plate or indirectly by altering the GH/IGF-1 axis. In this thesis, the main focus has been on the impact of glucocorticoids (GCs) and inflammation on longitudinal bone growth and these factors will be described in more detail below. Figure 3 illustrates some of the important factors involved in the growth impairment frequently observed in children with chronic inflammatory diseases.

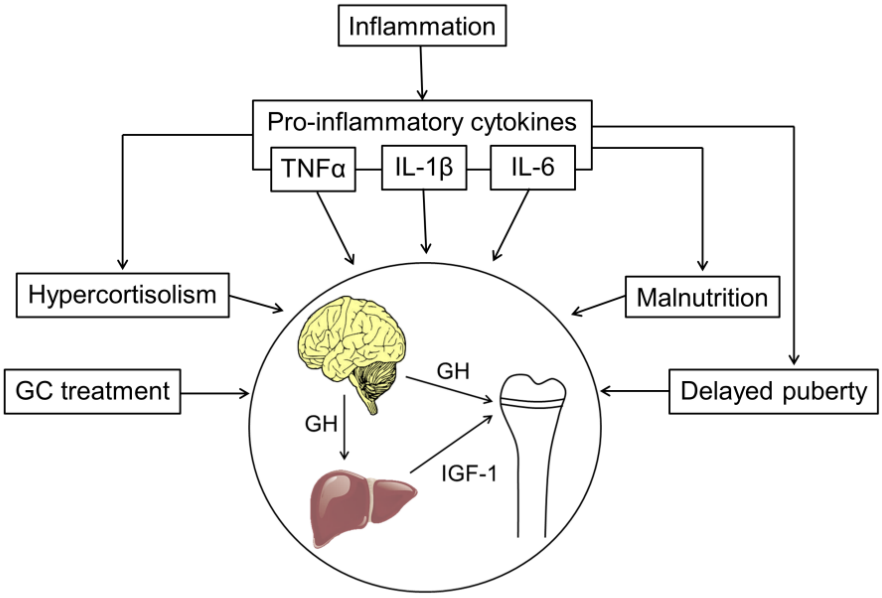

Figure 2. Several factors are contributing to growth impairment in conditions of chronic inflammation.

Source: Reprinted with permission from BioScientifica Limited. Sederquist et al. Recent research on the growth plate: Impact of inflammatory cytokines on longitudinal bone growth. J Mol Endocrinol (2014) 53(1): T35-T44.

1.3 GLUCOCORTICOIDS AND BONE GROWTH

1.3.1 Endogenous glucocorticoids

Endogenous GCs are produced in the adrenal cortex and belong to the group of lipophilic steroid hormones that play a key role in lipid metabolism and adaptation to stress. Cortisol is the endogenously produced GC in humans and its synthesis and release is regulated by the hypothalamic-pituitary-adrenal (HPA) axis. Environmental and physiological stress triggers the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) by the hypothalamus. CRH stimulates the synthesis and secretion of adrenocorticotropic hormone (ACTH) in the anterior pituitary gland which in turn stimulate the production of GCs in the adrenal cortex [29]. Feedback mechanisms ensure a tight control on the cortisol production and release.

Because of their lipophilic structure, GCs can freely diffuse through the cell membrane and enter the cell. GCs bind to the glucocorticoid receptor (GR), which in its unbound and inactive form, resides in the cytosol in a complex containing heat shock protein 90 and 70, and other factors that enhance GRs affinity for its ligand. When GCs bind to the GR, it undergoes conformational change, which in turn leads to dissociation from the protein

complex and translocation to the nucleus. In the nucleus GR interacts with DNA by binding to glucocorticoid response elements (GRE) and eventually activating (transactivation) or repressing (transrepression) specific sets of immune genes [30]. These genes code for cytokines, chemokines, inflammatory enzymes and receptors, and adhesion molecules that play a role in migration of cells towards sites of inflammation. Additionally, the GRs are capable of directly binding to specific transcription factors such as nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB).

These genomic actions of GCs are considered to be the classical GC signaling pathway; however, it has been shown that GCs in addition have non-genomic actions independent on DNA interaction. These effects are rapid and are only observed following high-dose GC treatment [31]. Studies have revealed that GCs bind to membrane bound receptors which act through second messenger signaling, for example Ca^{2+} and cAMP, which in turn activates different protein kinase pathways [32].

1.3.2 Glucocorticoid treatment

Hench and colleagues were the first ones to introduce GCs to clinical practice in 1949 and these potent anti-inflammatory agents have been widely used ever since [33]. GCs such as dexamethasone (Dexa) and prednisolone are commonly used due to their high therapeutic efficacy as anti-inflammatory or immunosuppressant agents. However, long-term GC treatment is associated with severe side-effects including osteoporosis and bone growth impairment [34]. There are also individual differences in GC sensitivity which may be due to genetic factors.

GC-induced growth impairment in children may be due to a combination of several factors and the degree of growth failure seems to be influenced by the duration of therapy, dosing and the specific agent. Nevertheless, clinical studies clearly show that longitudinal bone growth is negatively affected by GC treatment in children with chronic inflammatory diseases like juvenile idiopathic arthritis (JIA) and asthma [35]. Furthermore, a new study reports that early GC withdrawal improves long-term longitudinal bone growth and bone mineral density in children with renal transplantation, emphasizing the adverse effects of GCs on bone health in different conditions [36]. The growth suppressing effects of GCs are complex and multifactorial and currently, the underlying mechanisms of the growth suppressing effects of GCs are not fully understood. However, GCs are known to suppress bone growth through both systemic and local mechanisms.

1.3.3 Systemic growth suppressive effects of glucocorticoids

Levels of circulating endogenous GCs are known to increase significantly during early onset of inflammation [37]. By inhibiting GH secretion and suppressing GH receptors in the liver, GCs indirectly inhibit IGF-1 production, resulting in impaired bone growth [38-40]. IGF-1 levels have been shown to be decreased after short-term GC treatment, an effect which contributes to the GC-induced growth retardation in pre-pubertal mice [18].

1.3.4 Local growth suppressive effects of glucocorticoids

Evidence of direct action of GCs on the growth plate was reported by Baron et al. in a study where local injection of Dexa suppressed tibia length compared to the contralateral untreated bone [41]. GCs directly target the growth plate cartilage by inhibiting chondrocyte proliferation and mineralization as well as increasing apoptosis [41-43]. Dexa and IGF-1 have opposite effects, where Dexa suppresses proliferation and IGF-1 stimulates proliferation of growth plate chondrocytes. Interestingly, it has been shown that the number of GCR decrease in the growth plate after some time of GC treatment, making the chondrocytes less sensitive to GCs [43].

Studies in young growing rats show that treatment with high dose GC induces undesired cell death in growth plate chondrocytes resulting in growth retardation [44]. The observed GC-induced apoptosis was found to be regulated by activation of the caspase cascade, including caspase-8 and 9 as well as suppression of the Akt-phosphatidylinositol 3-kinase (PI3K) signaling pathway [45-48]. Additionally, GC treatment has been reported to regulate the Bcl-2 family proteins such as Bcl-2 associated X protein (Bax), BH3 interacting-domain death agonist (Bid) and Bcl-2 homologous antagonist/killer (Bak) to activate apoptosis in proliferative chondrocytes [47]. Interestingly, young mice lacking the pro-apoptotic protein Bax are resistant to GC-induced bone growth impairment, suggesting an important role for the apoptotic machinery in the regulation of longitudinal bone growth and chondrogenesis [48]. A new study reports that fibroblast growth factor 23 (FGF23), which is involved in the regulation of phosphate homeostasis and bone growth, is upregulated in bone and plasma upon GC exposure [36]. Elevated FGF23 plasma levels are known to cause phosphate wasting, growth retardation, and impaired bone mineralization [49]. These results therefore suggest a novel mechanism which is partly responsible for the GC-induced growth suppression observed in pediatric kidney transplantation patients treated with GCs.

The growth pattern known as catch-up growth is defined as the acceleration in growth in response to recovery from a disease or starvation and was first described by Prader et al. in

1963 [50]. Partial catch-up growth has been reported in children after withdrawal of GC treatment [51,52]. Similar results were also obtained in a study with postnatal rat metatarsal bones where partial catch-up growth was demonstrated after GC withdrawal [53]. However, a study in patients with cystic fibrosis reported permanent growth impairment due to GC treatment, suggesting a dose- and duration-dependent window for catch-up growth to occur [52]. Despite these clinical observations, the molecular mechanisms for catch-up growth are still poorly understood.

1.4 INFLAMMATION AND BONE GROWTH

Chronic inflammatory disorders like juvenile idiopathic arthritis (JIA), Crohn's disease (CD) and ulcerative colitis (UC) are usually accompanied with growth retardation in young children. Several factors, such as pro-inflammatory cytokines, GC therapy and malnutrition, act individually or in combination and contribute to the growth retardation frequently observed in these children. Since they are acting both through systemic and local effects, it is difficult to distinguish if one factor is more significant than the other [54].

Nutrition is an important regulator of bone growth where malnutrition is associated with chronic inflammation. The importance of proper nutritional status has been confirmed by studies in mice where food restriction resulted in deceased IGF-1 levels and suppressed GH receptor expression in the growth plate [55,56]. Malnutrition does contribute to growth retardation as shown in rat models of colitis, but only to about 60%, where the remaining growth impairment is due to the inflammatory condition itself [57]. The contribution of inflammation to growth impairment is further implicated in chronic inflammatory conditions such as JIA, where growth retardation has been reported historically, before GCs were commonly used as treatment as well as in JIA patients never treated with GCs [58,59]. These findings indicate that other factors than GC treatment contributes to the growth retardation seen in children with chronic inflammatory conditions.

1.4.1 Systemic growth suppressive effect of cytokines

Elevated levels of pro-inflammatory cytokines in conditions of chronic inflammation are known to act both individually and in combination to cause bone growth impairment [60]. Cytokines like TNF, IL-1β and IL-6 are secreted signaling molecules and are the most abundant cytokines up-regulated in inflammatory conditions like JIA [60].

I has been reported that the pro-inflammatory cytokines TNF, IL-1β and IL-6 reduce the effect of IGF-1 in growth plate chondrocytes, an effect which was linked to reduced phosphorylation of signaling molecules of the PI3K and MAPK/ERK1/2 signaling pathways

[61]. Furthermore, *in vivo* studies in transgenic mice overexpressing TNF or IL-6 both show growth retardation confirming the systemic growth suppressive effects of cytokines [62,63]. The IL-6 overexpressing mice display a growth reduction of 50-70% compared to non-transgenic littermates, an effect associated with decreases in IGF-1 and IGFBP-1 levels [62]. Similar findings have been reported in patients with JIA as elevated levels of IL-6 negatively affect the levels of IGF-1 and IGFBP-3 [64].

1.4.2 Local growth suppressing effects of cytokines

To date, there have not been many reports about the effects of pro-inflammatory cytokines in the growth plate. Best described are TNF, IL-1β and IL-6, which have shown varying effects on the growth plate chondrocytes.

Mårtensson *et al*. reported that TNF can suppress longitudinal bone growth by directly targeting the growth plate cartilage in a model of cultured fetal rat metatarsal bones [65]. When metatarsal bones were exposed to a high concentration of TNF, they grew significantly slower compared to control bones. The observed growth suppression was associated with decreased chondrocyte proliferation and hypertrophy as well as increased apoptosis. Similar findings have been reported by others in the ATDC5 chondrocyte cell line where TNF was found to reduce mRNA expression of aggrecan, collagen II and collagen X, further emphasizing its negative impact on the growth plate cartilage [66].

It has been reported that TNF is endogenously produced throughout the growth plate and plays a role in the normal regulation of longitudinal bone growth. Interestingly, treatment with etanercept, a soluble TNF receptor, improved bone growth as seen in cultured fetal rat metatarsal bones [67]. These results indicate that the negative growth suppressive effects of TNF only occur at very high concentrations.

Mice overexpressing TNF have been reported to have decreased total body length [63,68], decreased bone formation as well as numbers of osteoblasts [69]. Similarly, studies in the human TNF-overexpressing transgenic mouse line 197 (tgTNF) showed decreased trabecular bone in vertebrae and mice were susceptible to develop spontaneous disc herniation [70].

Similarly to the effects of TNF, IL-1β has been reported to suppress longitudinal bone growth in cultured fetal rat metatarsal bones by direct action on the growth plate [71]. The growth suppressive effect was associated with decreased chondrocyte proliferation, hypertrophy, and increased apoptosis and treatment with the IL-1 antagonist, anakinra, improved longitudinal bone growth [72]. However, unlike TNF and IL-1β, IL-6 did not have any effect on growth

plate chondrocytes or bone growth in the experimental model of metatarsal bones [71]. Later studies in ATDC5 chondrocytes showed, however, that IL-6 inhibits differentiation and decrease the expression of type II collagen, aggrecan, and type X collagen. In addition, another study in fetal rat metatarsal bones reported growth suppression when treated with IL-6 in combination with its soluble receptor IL-6 Rα, suggesting a direct effect on the growth plate chondrocytes [73].

1.5 GROWTH PROMOTING THERAPIES

GH is the main therapeutic approach to treat patients with short stature and has been used in the clinic since 1985, when synthetic GH was first produced [74]. Treatment of GC-induced growth impairment with GH has proved to increase linear bone growth [75,76]. However, at higher doses of GCs GH treatment was insufficient [77]. GH treatment in children with severe JIA improved height velocity and lean body mass indicating that GH could be a potential treatment to improve growth in these patients [75]. Another study reported that GH treatment is the most efficient in JIA patients with moderate disease activity [76]. However, the long-term safety of GH treatment has been questioned and it has not been approved as a treatment for GC-induced growth impairment. Therefore new strategies are needed to treat growth impairment induced by GCs.

The introduction of anti-TNF therapeutics has revolutionized the management of autoimmune diseases and is an alternative to GC treatment with less adverse effects on bone growth and bone mineral density [78]. However, many patients do not respond to anti-TNF and require other forms of treatment to treat the primary disorder. Etanercept is a recombinant fusion protein that binds to TNF and inhibits its interaction with TNF receptors on the cell surface, thereby diminishing TNF-driven inflammation that plays a key role in many arthritis patients [78]. Due to the anti-inflammatory effect as well as decreased exposure to GC treatment, patients with JIA have proved to improved bone growth after initiation of etanercept [79].

Current challenges with GC treatment include the well-known adverse effects on bone growth and bone metabolism. Efforts have been made to minimize as many as possible of these side effects by optimizing GR-associated beneficial effects. A new class of pharmacological compounds such as GR agonists and modulators (SEGRAMs) has been intensively investigated during the past years [80]. AL-438 is an example of a non-steroidal GR ligand which shows reduced adverse effects on osteoblasts and bone growth but full anti-

inflammatory effect *in vitro* [81]. More studies are needed to clarify the anti-inflammatory potential for these new compounds *in vivo* and whether they affect longitudinal bone growth.

1.5.1 Mitochondrial derived peptides

Mitochondrial derived peptides (MDPs) belong to a group of highly interesting, newly found signaling molecules that have turned out to be actively involved in the regulation of biological processes such as metabolism. The MDPs are encoded from short open reading frame (sORF) within the mitochondrial genome and after the discovery of the first MPD, humanin, the interest in this new class of molecules have increased and their positive effects have been studied in many different fields. Recently, another mitochondrial peptide, MOTS-c peptide (mitochondrial open reading frame of the 12S rRNA-c), was discovered and it has been shown to reduce obesity and insulin resistance in treated mice [82]. Six additional peptides have also been identified in the same region as humanin and were named small humanin like peptides (SHLPs). Out of these six peptides SHLP2 and SHLP3 in particular, seem to have similar protective and anti-apoptotic effects as humanin [83]. Thus, mitochondria play a critical role in metabolism by producing potent mitochondrial peptides and opens up a new field of research and possibilities to discover new drug targets.

In this thesis we have payed particular attention to humanin and it will be described in more detail below.

1.5.1.1 Humanin

Humanin is a 24-amino acid peptide, encoded within the ORF of the 16S ribosomal RNA of the mitochondrial genome. The peptide was first identified in 2001 when isolated from a cDNA library of surviving neurons from an Alzheimer disease patient, and was described as a potent neurosurvival factor protecting neurons against amyloid-beta toxicity [84,85]. Simultaneously, the synthetic humanin analogue, humanin-Gly14 (HNG), was identified. In HNG, serine is replaced with glycine at position 14 in the amino-acid sequence and this analogue showed to be 1000 times more potent that the original peptide [84-86]. HNG has been shown to have a promising therapeutic potential in Alzheimer disease [85], diabetes [87], myocardial ischemia [88] and stroke [89]. Even though humanin has shown promising therapeutic effect in several different conditions, this has so far only been confirmed in experimental models and not in humans.

Humanin is both an intracellular and secreted protein, which is thought to act trough binding to different cell surface receptors leading to initiation of intracellular signaling cascades eventually providing cytoprotection [90]. The identified receptors and associated signaling

cascades are illustrated in Figure 4. Humanin was first found to bind to formylpeptide receptor-like-1 (FPRL1), a seven-transmembrane G-protein-coupled receptor [91]. This subsequently activates the downstream signaling pathway of ERK1/2. Later, humanin was also found to bind to a trimeric receptor consisting of ciliary neurotrophic factor receptor (CNTFR), the cytokine receptor WSX-1, and the transmembrane glycoprotein gp130 (CNTFR/WSX-1/gp130) [92]. By binding to the gp130 receptor, humanin is capable to activate the canonical downstream signaling cascades of STAT3, Akt and ERK1/2 [93].

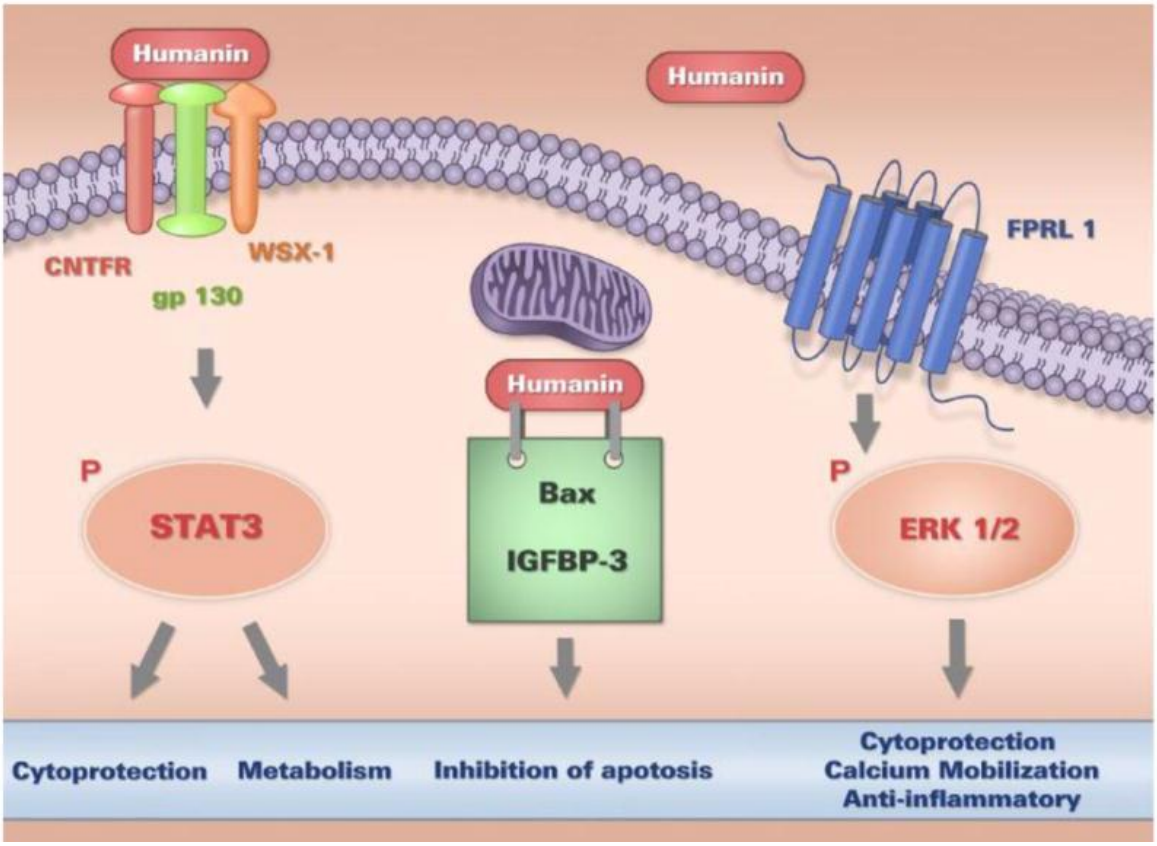

Figure 4. Interaction of humanin with different receptors and activation of downstream signaling pathways.

Source: Reprinted with permission from Elsevier. Lee et al. Humanin: a harbinger of mitochondrial-derived peptides? Trends Endocrinol Metab (2014) 24(5): 222-8.

Humanin has shown anti-apoptotic effects both *in vitro* and *in vivo*. It can bind to IGFBP-3 with high affinity and protect against IGFBP-3 induced apoptosis [94]. In addition, humanin has been reported to bind with the pro-apoptotic protein Bax and thereby prevent its translocation from cytosol to mitochondria and suppress cytochrome c release [95].

Furthermore, humanin is known to exert anti-inflammatory effects by suppressing cytokine levels such as TNF and IL-6 [92].

Interestingly, GH-transgenic mice, characterized by displaying elevated levels of circulating GH and IGF-1, increased body size and reduction in lifespan [96], have been reported to have a 70% reduction of plasma humanin levels [97]. In contrast, Ames dwarf mice were reported to have a 40% increase in circulating humanin levels [97]. Similarly, an interesting study in patients with Laron syndrome, displaying extreme short stature and low levels of IGF-1, showed an 80% increase in plasma humanin levels compared to normal matched relatives [97]. These results clearly suggest that GH and IGF-1 are important regulators of humanin levels. Remarkably, humanin has been shown to exert an anti-cancer effect by itself by reducing the growth of cancer xenografts in SCID mice [98]. This finding may provide an explanation to the fact that patients with Laron syndrome, who have elevated levels of circulating humanin, have a decreased cancer risk.

Humanin expression is age-dependent. Along with lower humanin levels in the hypothalamus, skeletal muscle, and cortex of older rodents, the circulating levels of humanin were found to decline with age in both humans and mice. These findings suggest that humanin plays a key role in the aging process [99].

In this thesis, we have used HNG as well as humanin overexpressing mice (HNtg) to investigate the potential of this peptide to prevent GC-induced bone growth impairment.

2 AIM OF THE THESIS

The overall aim of this thesis was, by different approaches, to study bone growth impairment induced by GCs and inflammation.

The specific aims of the projects were:

- To study the protective effects of humanin on GC-induced bone growth impairment in different model systems *in vitro* and *in vivo.*

- To study the potential for humanin to rescue from GC-induced apoptosis in the growth plate.

- To investigate if HNG treatment interferes with the anti-inflammatory effect of GCs in an animal model of arthritis.

- To study the effects of GCs and TNF on bone growth and chondrogenesis in an animal model of TNF overexpression.

3 MATERIALS AND METHODS

The main model systems and methodologies used for this thesis are discussed in the following section. More detailed information about methodologies can be found in the separate papers.

3.1 MODEL SYSTEMS

There are several different models available to study longitudinal bone growth, and they all have advantages and drawbacks. However, it is crucial to choose the model that best suits the current investigation. It is also important to be aware of the differences between rodent models and humans when interpreting results. We have made an effort to choose *in vitro* models in order to minimize the use of animals. However, one disadvantage of the *in vitro* approach is that the observed responses may not reflect the complex interplay of systemic and local factors that are involved *in vivo*.

3.1.1 Cell lines

The human clonal chondrocytic cell line HCS-2/8 (chondrosarcoma-derived) was used for mechanistic studies to investigate Dexa-induced apoptosis and the potential for HNG to rescue from this effect (study I). The HCS-2/8 cell line is well characterized and widely used and has capacity to go through both proliferative and differentiated/hypertrophic phases. The RCJ3.1C5.18 rat chondrocytic cell line was also used to study Dexa-induced apoptosis (study II) and cultured as previously described [100].

Human macrophages were used to study if HNG may interfere with the desired anti-inflammatory effect of Dexa. Briefly, mononuclear cells were isolated from healthy adult blood donors and re-suspended in 24-well tissue-culture plates in RPMI-1640 medium. Monocytes were separated by adhesion to tissue-culture plastic for 1 hour at 37 °C. Human monocyte-derived macrophages were then stimulated with 50 ng/ml macrophage colony-stimulating factor (R&DSystems, Minneapolis, MN, USA) for additional 3 days in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. TNF levels in culture media were measured with ELISA.

3.1.2 Organ cultures

Fetal (embryonic day 20) rat metatarsal bones have been used to study the effects of different compounds on longitudinal bone growth [71]. The bones were dissected out and were thereafter cultured in serum-free medium that was changed every 2-3 days and cultured for

up to 12 days. Microscopic images were taken several times throughout the experiment allowing us to follow and measure bone growth. The increase in length is expressed as percent increase from day 0.

3.1.3 Animal models

All experimental procedures were performed with approval from the Stockholm North Ethical Committee in Sweden, University of Southern California in Los Angeles, CA, in the USA and the General Directorate of Rural Economy and Veterinary, region of Attica, in Greece.

3.1.3.1 Normal mice

In order to study longitudinal bone growth, young rapidly growing mice were used. To study GC-induced bone growth impairment, young FVB female mice were treated with Dexa (2 mg/kg) and/or HNG (100 μg/kg), starting at 4 weeks of age and given by intraperitoneal (ip) or subcutaneous (sc) injection for 28 consecutive days. To exclude that the obtained results were strain- or sex related, C57BL/6 male mice were also treated with the same dose of Dexa.

To mimic systemic inflammation, 4-week-old female C57BL/6 mice were challenged with lipopolysaccharide (LPS) (30 mg/kg). Animals received ip injections of HNG (100 μg/kg) and Dexa (2.5 mg/kg), 30 min before the injection of LPS. Three hours after injecting LPS, all mice were euthanized, blood was collected and serum IL-6 levels were measured.

3.1.3.2 Humanin over-expressing transgenic mice

Cytomegalovirus (CMV)-driven expression of humanin in C57BL/6 mice transgenic mice (HNtg) resulted into stable high expression of native humanin levels globally. A construct that includes the HN-ORF driven by a CMV promoter was injected into the pronucleus of fertilized B6D2F1 mice ova (Transgenic and Knockout Rodent Core Facility, USC, USA). Mice harboring the humanin transgene were viable and fertile. To obtain a congenic line, the transgene was backcrossed into the C57BL/6 strain. The overexpression of humanin was confirmed with ELISA.

Four-week-old wild-type and HNtg male mice were treated with Dexa (2.5 mg/kg body weight/day, sc) or saline for 28 days. At the end point, plasma, femur and tibia were collected for further analysis.

3.1.3.3 Collagen-II induced arthritis

The most commonly used animal model for rheumatoid arthritis is collagen-II induced arthritis (CIA). CIA in mice shares many features with the human rheumatoid arthritis, including symmetric joint involvement, synovitis as well as bone and cartilage destruction. Susceptibility to CIA in mice is associated with the expression of certain class II major histocompatibility molecules, which in mice is referred to as the H-2 complex. In mice, strains expressing H-2, such as DBA/1, are therefore highly susceptible to CIA [101]. In our study, we used DBA/1 female mice, with a weight of 18-20 g (approximately 8 weeks old). Under isoflurane anesthesia, mice were injected subcutaneously at the base of the tail with type II collagen (CII) prepared from bovine nasal cartilage emulsified in Freund's complete adjuvant. Each mouse received 100 μg of CII and 300 μg of Mycobacterium tuberculosis in 100 μL of emulsion. On day 28 after the first injection, the mice received a booster injection of 100 μg CII in 100 μL Freund's incomplete adjuvant. Mice that did not develop any signs of arthritis within 14 days after the booster injection were excluded from the study. The study design is illustrated in Figure 5.

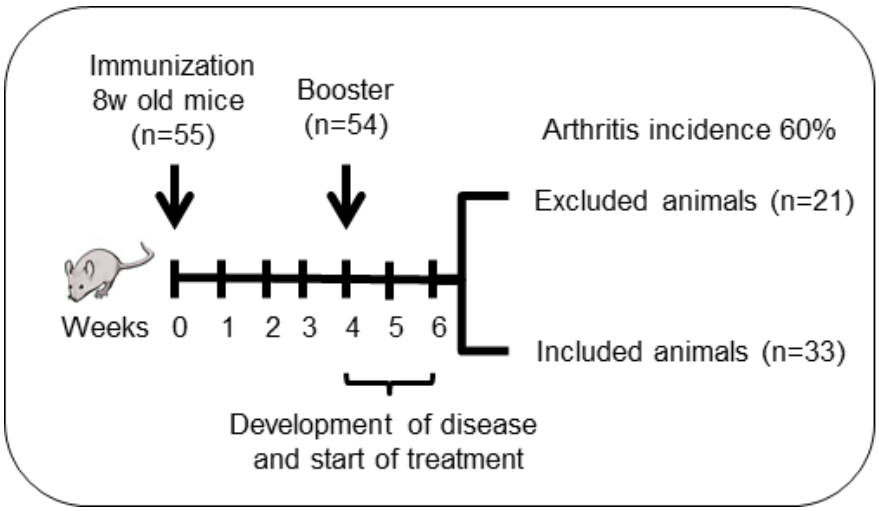

Figure 5. Experimental design including time points of induction of CIA, treatment and number of animals included.

3.1.3.4 Clinical evaluation of arthritis

The mice were observed daily for the presence of arthritis including signs of erythema and swelling of the joints by a well-established protocol described previously (16). Briefly, the interphalangeal joints of the digits, the metacarpophalangeal joints and wrist in the forepaws

and the metatarsophalangeal joints and ankle joints in the hind-paws were each considered as one category of joints. Individual paws were scored on a scale of 0–3 as follows: 0 = no signs of arthritis, 1 = one type of joint affected, 2 = two types of joints affected, and 3 = the entire paw affected. Thus, the maximal score for each animal is 12, but mice reaching a total score of 9 were sacrificed due to ethical restrictions; therefore, score 9 represents the highest possible score allowed in this study. Clinical evaluation of arthritis was performed by three observers blinded to the identity of the animals.

3.1.3.5 TNF transgenic models (tgTNF)

The tg197 mouse stain, developed and described by Keffer et al., has been used in this thesis work [102]. Briefly, this animal model is characterized by over-expression of human TNF leading to the development of chronic, erosive and symmetrical polyarthritis resembling human rheumatoid arthritis. In the following text, we refer to this specific mouse strain as tgTNF.

Starting from four weeks of age, the tgTNF animals received daily subcutaneous injections with Dexa (3 mg/kg) or saline for 4 consecutive weeks. After 4 weeks of treatment the animals were sacrificed by CO_2, blood collected by heart punctuation and serum stored at -80°C until analyzed. Hind limbs were dissected out for histopathological analysis. To assess longitudinal bone growth, femur length was measured by digital caliper after sacrifice. Femur from all animals were dissected and fixed in 4% formaldehyde for 24 hours followed by decalcification in EDTA buffer for 3–4 weeks before dehydration and paraffin embedding. Serial sections (5 μm thick) were stained with hematoxylin-eosin.

3.1.4 Human growth plate biopsies

We had the advantage of verifying our results obtained from cells and rodents in biopsies of intact human growth plate cartilages. The human tissue collection was approved by the local ethical committee (Stockholm North Ethical Committee, Sweden). According to this approval, informed consent was obtained from each subject and their parents, which was documented in the original hospital records.

The biopsies were obtained from pubertal children (13-15 years old) undergoing epiphyseal surgery due to constitutional extreme tall statute or leg length difference. The growth plate cartilage from both femur and tibia was removed with a special biopsy needle and was immediately placed in sterile PBS on ice. Under sterile conditions, the biopsies were cut into thin slices under a microscope and were cultured in separate wells with serum-free medium

for 24 hours. The biopsies were then fixated with formaldehyde and decalcified with EDTA before being embedded in paraffin and sectioned for immunohistochemistry.

3.2 ANALYSIS OF BONE GROWTH AND STRUCTURE

3.2.1 X-ray

X-ray has been used for analyzing longitudinal bone growth in mice. The mice were lightly anesthetized with isoflurane and X-ray images were taken once a week from the start of the experiment. The femur length was measured using computerized software (Sectra Image Display System 5). The total increase in percentage was calculated by dividing the length at time point 0 with the end point measurement. To measure longitudinal bone growth, femur X-ray images were captured before the start of treatment and thereafter every week throughout the study.

3.2.2 Quantitative histology of the growth plate

Growth plate histology was analyzed in femur sections stained with Alcian blue/van Gieson. Images were taken under light microscopy and further analyzed by the Image J software. Specifically, total growth plate area, total growth plate height and the height of the specific zones were analyzed.

3.3 PROTEIN EXPRESSION

3.3.1 Immunohistochemistry

Immunohistochemistry was used for detecting and quantifying the expression of different proteins in formalin-fixed tissue sections of the growth plate. Briefly, this method utilizes specific antibodies to detect and localize target proteins in tissue sections. Detailed description of each protein can be found in the individual papers.

Antibodies against the following proteins were used: Ihh, humanin, Bax, caspase-3, PCNA and collagen X.

Sections were incubated with anti-caspase 3 antibody (sc-1226; Santa Cruz Biotechnology, Dallas, TX, USA), anti-proliferating cell nuclear antigen (PCNA) antibody (ab-18197; Abcam, Cambridge, United Kingdom), anti-humanin antibody (NB100-56877; Novus biologicals), anti-collagen X antibody (ab-58632 Abcam, Cambridge, United Kingdom) and anti-Ihh antibody (sc-1196; Santa Cruz Biotechnology).

Proliferating cell nuclear antigen (PCNA) is an intranuclear peptide which is a marker of cell proliferation and was used in our studies to assess proliferation in the growth plate. PCNA expression was analysed in serial sections of femur growth plates (n=5 bones per group) as described previously (17). Briefly, after deparaffinization and rehydration, antigen retrieval was performed in sodium citrate buffer (10 mM pH 6.0). The sections were blocked with 3% bovine serum albumin (BSA) before incubation overnight at 4°C with the primary rabbit anti-PCNA antibody (1:100, Abcam 18197). Sections were incubated for 1 hour at RT with secondary goat anti-rabbit IgG-HRP antibody (1:300, Santa Cruz sc-2004) followed by incubation with an avidin-peroxidase complex (Vectastain ABC-kit PK-6100) and visualized with 3,3' diaminobenzidine (DAB) (Dako K3468) development for 3 minutes. Thereafter sections were counterstained with Alcian blue, dehydrated and coverslipped. Images were captured by a Nikon Eclipse E800 light microscope connected to a digital camera (Hamamatsu C4742-95, Hamamatsu City, Japan) with a digital color camera system (Olympus DP70). Using the Image J software, the number of PCNA positive cells was determined by one observer blinded to the treatment.

3.3.2 ELISA

Circulating humanin was measured from wild-type control mice and HNtg mice plasma by an in-house sandwich ELISA, as previously described by Chin et al. [103]. Prior to assay, plasma was extracted with 90% acetonitrile and 10% 1N HCl. Briefly, 200 μl of extraction reagent was added to 100 μl of plasma, gently mixed and incubated at room temperature. The supernatant was removed and dried by SpeedVac after being centrifuged. The dried extracts were reconstituted with PBS and then used for ELISA. Synthetic HN was used as a standard within range 0.1 ng/ml to 50 ng/ml. 96-well microtiter plates were coated with capture antibody in 50 mM sodium bicarbonate buffer on a shaker. The plates were washed and blocked with Superblock buffer. Standards, controls or extracted samples and pretitered detection antibody were added to the appropriate wells and incubated overnight. The absorbance was read at 490 nm on a plate spectrophotometer followed by streptavidin-HRP and OPD steps.

3.3.3 Western Blot

Western immunoblotting was performed as described previously by us [47]. The primary antibodies against humanin C-14 (1:100), and Bax (1:1000), were from Santa Cruz Biotechnology. The antibody against Bid (P955957) was purchased from Cell signaling technology. To quantify Western blots Image J software was used.

3.4 CELL DEATH ANALYSES

3.4.1 TUNEL assay

Apoptotic cell death by DNA fragmentation in chondrocytes in growth plate cartilage from humans and mice was studied by terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated UTP nick end labeling (TUNEL). Serial sections of growth plate were stained with a TUNEL kit (EMD Millipore, Billerica, MA, USA and QIA33, TdT-FragEL, Calbiochem), according to the manufacturer's instructions. Images covering the whole growth plate were analyzed under a fluorescence microscope (a Nikon E800 fitted with an Olympus DP70 camera). All TUNEL positive cells (Alexa-546, Invitrogen) were counted and divided by the total number of cells (DAPI stained) to achieve the percentage of TUNEL positive cells. For the digital cell counting, the Image J software was used.

3.4.2 Analyses of histone-associated DNA fragmentation and mitochondrial potential

To investigate cell death in cultured HCS-2/8 cells, cytoplasmic histone-associated DNA fragments (mono- and oligonucleasomes) were quantified using a Cell Death ELISA kit as previously described [45]. Mitochondrial membrane potential was measured in Dexa and HNG-treated chondrocytes as previously reported [48].

3.5 STATISTICAL ANALYSES

Statistical significance of differences between two groups was evaluated by 2-tailed Student's *t* test with 95% confidence intervals. For differences between several groups, parametric one-way analysis of variance (ANOVA) was applied followed by Holm-Sidak test, or the non-parametric Kruskal- Wallis ANOVA on Ranks followed by Dunn's multiple comparison test.

Statistical analysis of the clinical score in study II is based on area under the curve for each individual animal. The values are median clinical score and the error bars represent interquartile range. For the histological scoring, a mean value was calculated for each animal.

All statistical analyses were performed using the SigmaPlot software (Systat Software Inc., IL, USA). All values are shown as means ± SD and ***$p<0.001$, **$p<0.01$ and *$p<0.05$ were considered statistically significant.

4 RESULTS AND DISCUSSION

4.1 HUMANIN IS A NOVEL REGULATOR OF HEDGEHOG SIGNALING AND PREVENTS GLUCOCORTICOID-INDUCED BONE GROWTH IMPAIRMENT (PAPER I)

Bone growth impairment in children is a common side effect of long-term and high-dose GC treatment and therefore it is desirable to find new treatment strategies to overcome this condition without interfering with the anti-inflammatory effect of GCs. In this study we sought to assess the potential for the mitochondrial derived peptide humanin, to rescue from Dexa-induced bone growth impairment without interfering with the anti-inflammatory effect. We also looked into the underlying molecular mechanisms and payed special attention to Hh-signaling, an important regulator of bone growth.

We could show that Dexa treatment in young wild-type mice significantly reduced bone growth whereas systemic administration of HNG treatment was able to rescue from this effect when administered in combination with Dexa. We had the advantage of studying the novel humanin over-expressing mouse model (HNtg) and we reported, for the first time, that HNtg mice treated with Dexa are resistant to Dexa-induced bone growth retardation (Figure 6).

Dexa treatment in wild-type mice was accompanied with suppression of total growth plate height and a decreased height of the resting + proliferative zone. HNG was able to restore this loss of growth plate height in Dexa treated wild-type mice. Interestingly, we observed that growth plate height was not affected by Dexa treatment in the HNtg mice. Mechanistic studies revealed that Dexa treatment suppressed chondrocyte proliferation in the growth plates of wild-type mice whereas co-treatment with HNG was able to rescue from this effect. Similar results were also obtained in cell cultures of chondrocytes and *ex vivo* cultured rat metatarsal bones. In the HNtg mice, on the other hand, chondrocyte proliferation was not affected upon Dexa treatment.

We further found that HNG suppressed GC-induced chondrocyte apoptosis in the growth plate both *in vivo* and in cultured fetal metatarsal bones. Similar results were obtained from experiments performed in cultured human growth plate cartilage, where Dexa treatment increased the apoptosis level but HNG was able to counteract this effect. Anti-apoptotic effects of humanin has previously been reported in different cell types, including pancreatic β-cells, germ cells and neurons, but this is the first study to report anti-apoptotic effects of

HNG in chondrocytes of human growth plate. We also showed for the first time that endogenous humanin is expressed in human growth plate cartilage (Figure 7).

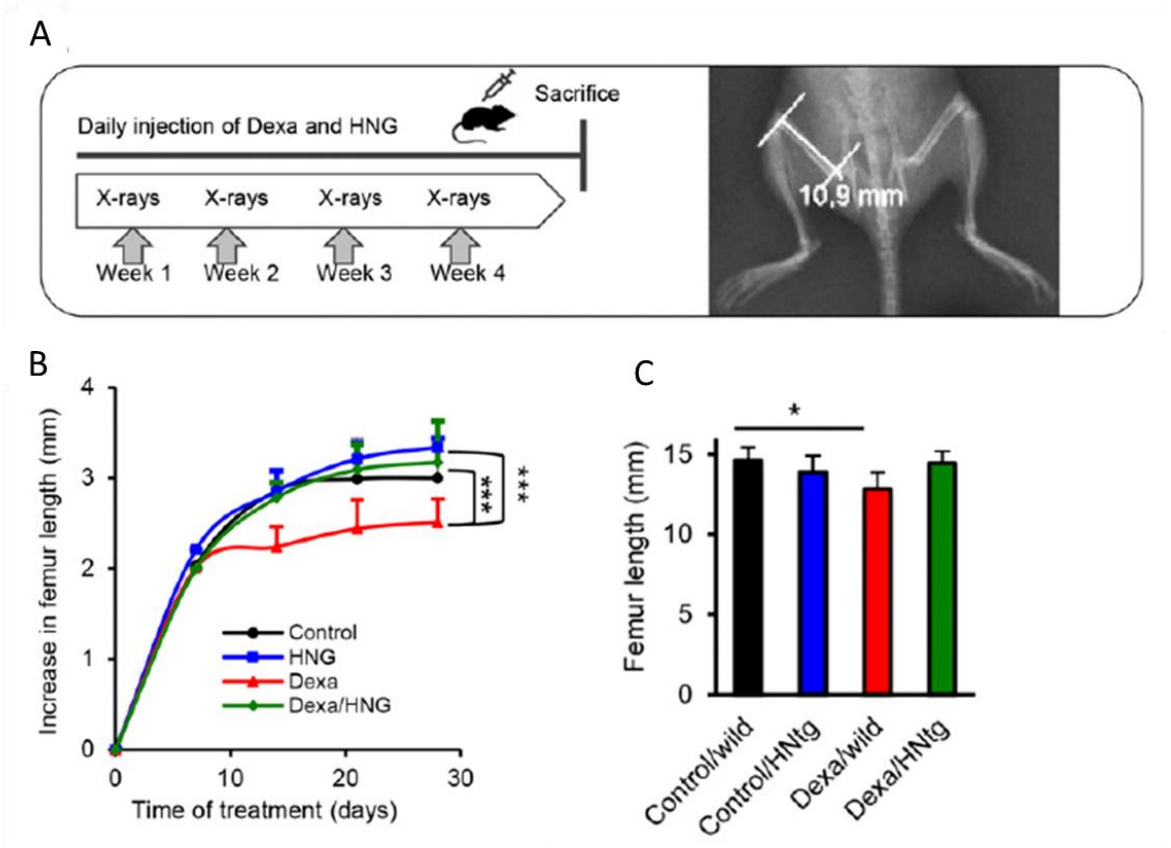

Figure 6. Humanin prevents from GC-induced bone growth impairment *in vivo*. A) Schematic representation of experimental procedure. B) Longitudinal bone growth measured by weekly X-rays. C) Four week- old male wild-type and HNtg (C57BL6 background) mice were treated with/without Dexa (2.5 mg/kg body weight/d) for 28 consecutive days femur bone length was measured by digital caliper.

Previous studies have suggested that the anti-apoptotic effect of HNG is mediated through the binding of the pro-apoptotic protein Bax [95]. It has also been shown that GC-induced apoptosis in the growth plate is Bax-mediated and that Bax knock-out mice are resistant to GC-induced apoptosis [48]. Based on these findings we hypothesized that humanin may protect from GC-induced apoptosis in the growth plate. Indeed, in this study we found increased Bax expression in the growth plate upon Dexa treatment whereas HNG treatment was able to suppress Bax levels. This correlates well with the previous findings and strengthens the evidence for humanin to be a potent Bax-mediated inhibitor of apoptosis.

We further found that Ihh expression was suppressed in the growth plates of Dexa treated wild-type mice and that HNG was capable of restoring Ihh levels in the growth plate. Hh-signaling is known to be essential for chondrocyte proliferation and differentiation and

studies have reported that suppression of Ihh expression in chondrocytes cause growth plate fusion and impaired bone growth [26,104]. Ihh signaling has been proposed to take place in the primary cilia of the chondrocytes and the length of the primary cilium is proposed to modulate cilia function. One example is that in the degenerative disease of osteoarthritis where increase in cilia length in articular chondrocytes is observed [105]. Elongation of the primary cilia in articular chondrocytes has also been reported upon LiCl treatment and was associated with inhibition of Hh signaling [106]. Interestingly, Dexa also seems to induce elongation of the primary cilia, however, this has so far not been studied in chondrocytes [107]. We further found that Dexa treatment in the HNtg mice did not suppress Ihh expression, suggesting that humanin promotes the survival of chondrocytes. This finding is highly interesting and provides a potential new mechanism for how humanin can prevent from bone growth impairment. Taken together, these findings suggest that the growth suppressive effects of Dexa may be due to suppression of Ihh signaling by affecting the size of the primary cilia. However, this needs to be clarified in future studies as well as the regulatory mechanisms for humanin.

Importantly, we showed that HNG did not interfere with the anti-inflammatory effect of Dexa and that HNG by itself had an anti-inflammatory effect by lowering the levels of TNF and IL-1β in mice with LPS-induced inflammation. These findings are in line with previous studies that have shown reduced levels of pro-inflammatory cytokines upon HNG treatment [108,109]. Although our study showed a direct action of HNG on the immune cells, the exact mechanism for how humanin exerts its anti-inflammatory effect is still unknown.

In this study, we had the advantage of using several different model systems to test our hypothesis, which strengthens the results obtained. More specifically, we used the HNtg animal model in order to study the protective role of humanin on GC-induced bone growth impairment, and confirmed our findings in rodent tissues and in rare biopsies of human growth plate cartilage.

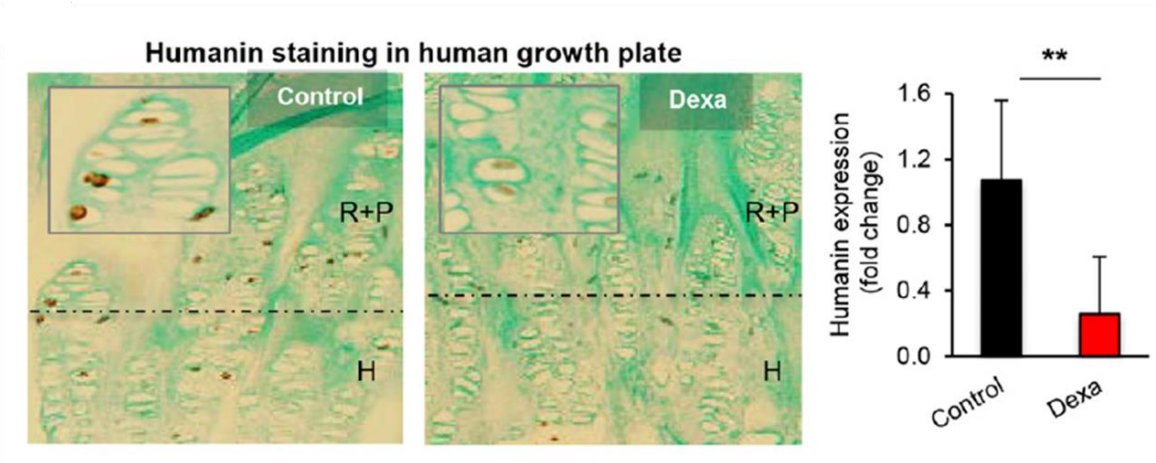

Figure 7. Humanin expression and protective effects of humanin in cultured human growth plate cartilage. A) Studies of endogenous humanin levels in human growth plate cartilage treated with Dexa (1 mM) for 24 hours shown in resting+proliferative zone and hypertrophic zone. B) Quantitative analysis of humanin expression (fold change).

4.2 HUMANIN PREVENTS UNDESIRED APOPTOSIS OF CHONDROCYTES WITHOUT INTERFERING WITH THE ANTI-INFLAMMATORY EFFECT OF DEXAMETHASONE IN COLLAGEN-INDUCED ARTHRITIS (PAPER II)

There is a need for new treatment strategies to prevent bone growth impairment in children with chronic inflammatory disorders that are treated with GCs. In study I, we reported that humanin does not interfere with the anti-inflammatory effect of Dexa, using the LPS-induced inflammation model. In study II, we investigated if HNG can prevent GC-induced apoptosis in the growth plate in a disease model of chronic inflammation and, as a second step, if HNG may interfere with the anti-inflammatory effect of Dexa. To test our hypothesis, we used the well-established CIA model in DBA/1 mice, which is the most widely used mouse model of rheumatoid arthritis sharing many features with the human disease.

From this study, we reported that HNG treatment suppressed GC-induced apoptosis in the growth plates of CIA mice (Figure 8). These findings were also verified in *in vitro* cultures of chondrocytes where HNG was able to suppress GC-induced apoptosis. These findings correlate well with our previous findings that HNG can protect from GC-induced apoptosis in the growth plate of healthy wild-type mice [110]. However, this is the first study to demonstrate the deleterious effect of Dexa on growth plate cartilage in a disease model with chronic inflammation.

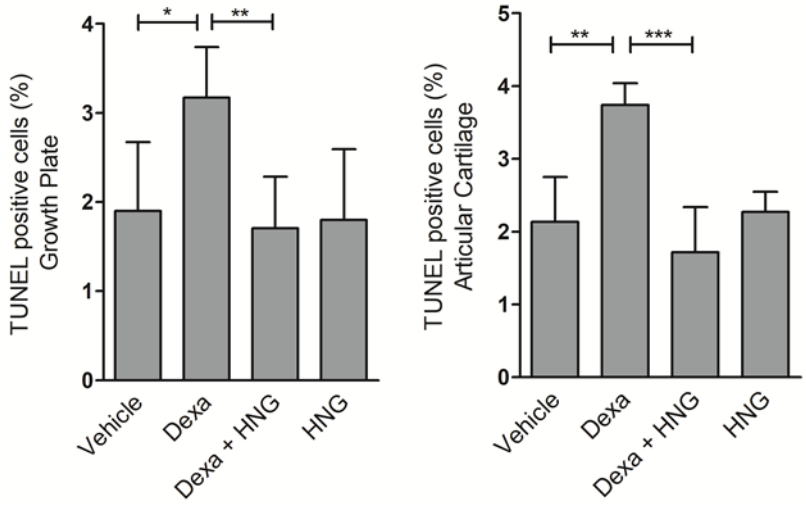

Figure 8. Effect of HNG on GC-induced apoptosis in femur growth plate and articular cartilage from CIA mice. Quantification of TUNEL positive cells in the growth plate and articular cartilage.

Importantly, we showed that HNG does not interfere with the anti-inflammatory effect of Dexa. These results are in line with our previous studies performed with normal mice where HNG protect from Dexa induced apoptosis in the growth plate. Interestingly, we observed a protective effect of HNG not only in the growth plate but also in the articular cartilage, which is a new area of research.

Chronic inflammation, which results in up-regulation of pro-inflammatory cytokines, has a negative effect on the growth plate cartilage. However, it has not been clear to what extent GC treatment negatively affects chondrogenesis in the growth plate in a model of chronic inflammation as most studies have been performed in healthy wild-type mice. The CIA model of arthritis is well-characterized but to date, no studies have been performed on the growth plate cartilage. In this study, we were not able to examine longitudinal bone growth due to the age of mice at disease induction, which is one of the limiting factors of the study. At the time point for disease onset, the growth had reached a plateau and therefore this model was not suitable for analyzing bone growth. Interestingly, we show that Dexa treatment; despite effectively suppressing arthritis score and local inflammation, induced apoptosis in the growth plate chondrocytes. This suggests that Dexa may have growth suppressing effects in conditions of chronic inflammation but this needed to be clarified in another model allowing studies in younger animals.

In previous studies by us and others, humanin has been reported to have anti-inflammatory effects both *in vitro* and *in vivo* [109,110]. In this study, we were not able to detect an anti-inflammatory effect of humanin. The CIA mouse model displays acute and local inflammation and it is possible that the humanin dose used was too low to suppress inflammation. As humanin was administered systemically it is also possible that humanin was not able to penetrate into the joint and reach the location of the inflammation. Therefore, it would be important to study the effects of humanin in a different model of inflammation to verify the anti-inflammatory effects observed in earlier studies.

In conclusion, this study showed evidence that GCs, in a model of chronic inflammation, induce apoptosis in the growth plate cartilage and humanin has the potential to rescue from this effect. These data adds to the understanding of how humanin can be used in combination with GC to protect from GC-induced bone growth impairment.

4.3 DEXAMETHASONE SUPPRESSES BONE GROWTH AND CHONDROGENESIS IN TNF OVER-EXPRESSING MICE (PAPER III)

The reason for the observed growth impairment in young children is multifactorial but it is known that high levels of pro-inflammatory cytokines and long-term use of GCs are involved. The primary objective of study III was to elucidate the effect of Dexa on bone growth and chondrogenesis in a condition of chronic inflammation. To be able to address both bone growth and chronic inflammation, we used the human TNF over-expressing transgenic mouse model (tgTNF). Since tgTNF mice spontaneously develop chronic inflammation and arthritis at a young age this was a prerequisite for studying the longitudinal bone growth.

Study III demonstrated that femur bone length and total growth plate height was significantly impaired in young tgTNF mice and that Dexa treatment, despite its anti-inflammatory effect, increased this negative effect (Figure 9). Our findings, with regard to the decreased bone growth, conform to other studies in the tgTNF mouse model where total body length was reported to be decreased [111] and bone formation reduced [69]. The additive growth suppressive effect of Dexa is a novel finding even though a similar effect has been shown in non-inflamed wild-type mice.

The pro-inflammatory cytokine TNF, which is also called cachexin, is known to cause loss of appetite and a significant loss of body weight due to an altered metabolism [112]. In this study, we did not observe any loss of body weight in the tgTNF animals supporting the fact that the growth suppressing effects were most likely due to direct effects of TNF on the growth plate and not due to decreased food intake.

The growth suppressing effect observed in the tgTNF mice was associated with increased apoptosis level and decreased chondrocyte hypertrophy in the growth plate. Dexa treatment did not further suppress chondrocyte proliferation or hypertrophy, nor did the treatment increase chondrocyte apoptosis in this animal model. It is well known that Dexa has deleterious effects on the growth plate cartilage and longitudinal bone growth by suppressing proliferation and hypertrophy of chondrocytes while increasing apoptosis in the growth plate of non-diseased wild-type mice [110]. Thus, our results suggest that Dexa treatment in the tgTNF mice, despite successfully reducing inflammation, is not capable of restoring chondrogenesis in these mice.

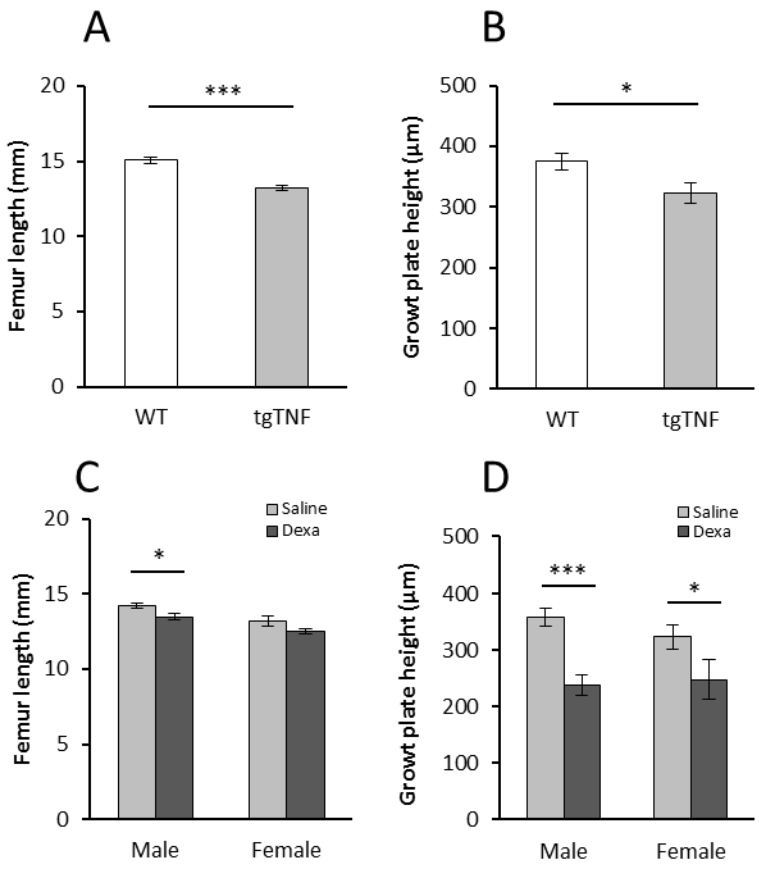

Figure 9. Femur bone length and total growth plate height in female wild-type (WT) and transgenic TNF-over expressing mice (tgTNF) treated with saline or Dexa (A-B). Femur bone length and total growth plate height in male and female tgTNF mice (C-D).

Interestingly, we observed that Ihh expression was significantly decreased in the growth plate cartilage of the tgTNF mice compared to wild-type mice. In a previous study by Tevlin *et al*. [113], elevated levels of TNF in diabetic mice directly suppressed Ihh expression in mouse skeletal stem cells, indicating a link between TNF and Ihh-signaling. These data suggest that TNF overexpression contributes to the suppression of Ihh expression in the growth plate, which may explain the impaired differentiation in the growth plates of tgTNF animals.

Furthermore, we observed that circulating humanin levels as well as local humanin expression in the growth plate were suppressed in tgTNF mice when compared to wild-type mice. Suppression of humanin in the tgTNF model suggests that cytokines like TNF are capable of suppressing pro-survival signals which may then sensitize the chondrocytes to cellular toxicity. Furthermore, in study II, we showed that systemic treatment with the humanin analogue, HNG, blocked undesired apoptosis in the growth plate in wild-type mice with collagen-II induced arthritis [114]. Taken together, the data suggests that exogenous

treatment with a potent humanin analogue could be a feasible strategy, not only to restore humanin levels in conditions of chronic inflammation, but also to prevent undesired inhibitory effects of Dexa and thereby prevent cellular toxicity and impaired chondrogenesis in the growth plate.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The underlying cause of growth suppression is complex and multifactorial. However growth retardation in children with chronic inflammation and GC treatment remains a clinical problem. The work in this thesis has focused particularly on the growth suppressive effects of GCs and inflammatory cytokines and how these effects potentially could be prevented by the mitochondrial peptide humanin.

Specifically we have demonstrated that:

- Systemic humanin treatment can protect from GC-induced bone growth impairment in young mice

- Humanin over-expressing mice are resistant to GC-induced bone growth impairment

- GC-induced bone growth impairment is associated with suppression of Ihh in growth plate chondrocytes and humanin treatment is able to restore the Ihh levels in wild-type mice

- Humanin is expressed in the human growth plate and the expression level is decreased when the tissue is exposed to Dexa *ex vivo*

- Humanin protects from GC-induced chondrocyte apoptosis in growth plates of mice with collagen-II induced arthritis

- Humanin does not interfere with the anti-inflammatory effect of Dexa and has potentially an anti-inflammatory effect on its own

- Femur bone growth and growth plate chondrogenesis are suppressed in TNF over-expressing mice, effects that are even more pronounced under Dexa treatment

- Expression levels of humanin and Ihh are suppressed in growth plates of TNF over-expressing mice

The unwanted outcomes of GC treatment create a dilemma for clinicians because improvement in the primary inflammatory disease is achieved only by accepting substantial adverse effects that are often difficult to prevent or treat. Efforts have been made and different strategies have been implemented to prevent and treat GC-induced growth impairment in children. Recombinant human GH has been reported to improve growth in some children treated with GCs, although the effect is moderate and the cost high. However, it is important to emphasize that GH treatment has not been approved as a treatment for GC-induced growth impairment. Anti-cytokine treatments have shown less adverse effects on bone growth and bone mineral density; however, many patients do not respond to the treatment.

This thesis presents studies of a novel strategy to prevent GC-induced bone growth impairment, namely co-administration with humanin, a mitochondrial-derived peptide. Our data clearly show that humanin plays an important role in the growth plate cartilage. In combination with Dexa, humanin protects from GC-induced bone growth impairment which is associated with increased apoptosis and decreased proliferation. In study I, we report that humanin is a novel regulator of Hh signaling. Dexa treatment suppressed Ihh expression in the mouse growth plate and humanin was able to restore the expression levels. In the HNtg mouse model, Ihh expression was not affected upon Dexa treatment. These results are very interesting and indicate that humanin is involved in the regulation of Ihh; however, more studies are needed to clarify the exact underlying mechanisms. One possibility is that humanin interacts with the primary cilia, which has been reported to be the location for Ihh signaling in chondrocytes. Elongation of the primary cilia is associated with impairment in Ihh signaling and it would be important to investigate the possible links between humanin and structural changes of the primary cilia.

Furthermore, we report that humanin does not interfere with the anti-inflammatory effect of Dexa, both in experiments with LPS induced inflammation (study I) and in a disease model of chronic inflammation (study II). These results suggest that a combination treatment with GC and humanin could provide a feasible approach to treat growth impairment. It is important to further investigate the anti-inflammatory effects of humanin applying different *in vivo* models of chronic inflammation.

In study II, we observed that humanin was able to suppress GC-induced apoptosis not only in the growth plate but also in the articular cartilage. High doses of GCs, usually given as intra-articular injections to treat arthritis, are known to have deleterious and toxic effect on the chondrocytes of the articular cartilage. Our results suggest that humanin treatment may have

the potential to rescue from this effect. However, further studies are needed to investigate the effect of humanin on articular cartilage and its potential to rescue from cartilage degradation. In addition to the adverse effects of GCs on chondrocytes, GCs are well known to have negative effects on osteoblasts resulting in osteoporosis in many of the treated patients. Based on our data, it can be speculated that humanin may also have protective effects on osteoblasts but this remains to be verified in future experimental studies.

In study III, we found that serum humanin levels and the local growth plate expression of humanin were both decreased in the tgTNF mouse model. Altogether, our data suggest that inflammation with elevated TNF levels and GC treatment both suppress endogenous humanin making growth plate chondrocytes more sensitive to undergo apoptosis. Further *in vivo* studies are needed to clarify if humanin at different doses has the potential to rescue from growth impairment induced by chronic inflammation and concomitant GC treatment.

In conclusion, our results confirmed both *in vivo* and *in vitro*, suggest that humanin has the potential to rescue from GC-induced bone growth impairment without interfering with the desired anti-inflammatory effect of GCs. Humanin act mainly by suppressing apoptosis but also by regulating Ihh expression at the growth plate level. Local humanin expression in the growth plate and circulating humanin levels were both decreased in the tgTNF mouse model suggesting that exogenous humanin treatment could potentially rescue the bone growth. Our findings, along with further advances in our understanding of the biology of bone growth, the pharmacology of GCs and mechanisms of humanin action, will hopefully lead to the development of new strategies to prevent growth impairment in children with chronic inflammatory disorders.

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