**CHARLES UNIVERSITY**
**Second Faculty of Medicine**

Summary of the Dissertation

# Novel insights into the pathophysiology of growth retardation and other endocrine conditions: Lessons learned from consanguineous and non-consanguineous families

Nové pohledy na patofyziologii růstové retardace a dalších endokrinních poruch: Poznatky z konsanguinních a non-konsanguinních rodin

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# TABLE OF CONTENTS

# 1. Abstract

In rare diseases, parallel studies of consanguineous and non-consanguineous populations facilitate elucidating not only genetic origin of individual conditions, but also their pathophysiology and disease mechanisms. The aim of the dissertation is to compare findings in three paediatric endocrine conditions – congenital hyperinsulinism, monogenic diabetes, and short stature – conducted in a unique cohort of children from a highly consanguineous region of Sulaimani, Kurdistan, Iraq (consanguinity rate 44%) with a non-consanguineous cohort from Prague, Czech Republic. With informed consent, DNA of all probands were primarily analyzed by NGS methods followed by variant selection and verification by a bioinformatic pipeline. In consanguineous individuals, the genetic cause of congenital hyperinsulinism was elucidated in 100%, monogenic diabetes in 74% and short stature in 65% of patients. Homozygous variants were the most prevalent, with the spectrum of causative genes and thus disease mechanisms differing considerably from non-consanguineous individuals. In studies of non-consanguineous patients with growth hormone deficiency and those born small for gestational age, the rate of positive findings were 29% and 42% respectively with largely prevailing monoallelic (dominant) genetic conditions. In addition, this research produced the first ever papers describing large cohorts of children from consanguineous populations with diabetes and short stature. A statistical significance of consanguinity and the occurrence of syndromic diabetes was described. This research highlights the fundamental contribution of studies in consanguineous families to novel insights into disease origin and mechanisms.

**Keywords**

Consanguinity, endocrine disorders, endocrine genetics, next generation sequencing, short stature, monogenic diabetes, congenital hyperinsulinism

## 2. Background

Genetic disorders, though diverse in nature, share a common thread of complexity and impact, especially in paediatric populations. Over 5% of live births are affected by genetic disorders recognizable by 25 years of age (Baird, 1988). This prevalence underscores the critical need for ongoing research and improved understanding of these conditions. The realm of paediatric endocrinology is particularly affected, as many conditions, though rare, can significantly impact a child's growth and development, metabolism, and overall well-being. Despite being widely described in literature, the precise pathophysiological, genetic and molecular mechanisms of many paediatric endocrine conditions including congenital hyperinsulinism, monogenic diabetes and short stature are still being elucidated.

Recent decades have witnessed remarkable advancements in genetic testing. These developments have enabled more accurate diagnosis and understanding of complex genetic disorders. In the past decade, Next-Generation Sequencing (NGS) has emerged as the gold standard for genetic examinations in patients with complex phenotypes (Roy, 2018). The choice of the library, representing the portion of the human genome under examination, is a critical consideration, with options including Whole Genome Sequencing (WGS), Whole Exome Sequencing (WES), and custom targeted panels (t-NGS). WGS provides a comprehensive analysis of the entire genome, detecting various mutation types, while WES focuses on the protein-coding region. T-NGS is suitable for specific diagnoses based on a known list of genes. The American College of Medical Genetics and Genomics (ACMG) guidelines serve as the gold standard for variant classification, enabling a comprehensive understanding of variants from benign to pathogenic (Richards et al, 2015). Thereafter, potentially pathogenic variants are confirmed by Sanger sequencing (Sanger, 1977).

## 2.1. Consanguinity

"Consanguinity", otherwise called "inbreeding" in population genetics refers to non-random mating where humans mate with others who are more genetically similar, rather than mating at random in the population. This practice, rooted in demographic, cultural, and religious traditions, is more prevalent in certain regions, particularly Asia, Africa, and the Middle-East (Bittles, 2001). Despite increased awareness of potential health consequences, over 20% of the global population, with more than 8.5% of all children, are estimated to have consanguineous parents (Shawky et al, 2013).

In a comprehensive genetic study in Egypt, recessive disorders were predominantly found in consanguineous families (78.8%). Child deaths and stillbirths were more common in children from consanguineous parents compared to non-consanguineous families as well (Shawky et al, 2013). The increased frequency of recessive genetic conditions in offspring from consanguineous parents is attributed to a higher frequency of homozygous genotypes, allowing less common alleles to manifest. This challenges the Hardy–Weinberg equilibrium due to non-random mating (Antonarakis, 2019). Consanguineous families, with their high homozygosity, offer optimal conditions for discovering novel pathophysiological mechanisms, gene variants and even novel genes in monogenic conditions (**Amaratunga et al, 2022**).

Conversely, the study of non-consanguineous families allows for the exploration of genetic heterogeneity and specific environmental influences in studied conditions. Together, these approaches contribute to a more comprehensive understanding of genetic determinants and shared mechanisms underlying paediatric endocrine conditions.

## 2.2. Congenital Hyperinsulinism (CHI)

CHI is a genetically heterogeneous condition characterized by uncontrolled insulin secretion from pancreatic β-cells. The incidence of CHI has been estimated to be around 1 in 2500 in communities with a higher rate of consanguinity, this is significantly higher than the 1 in 50,000 incidence in non-consanguineous populations (Matthew, 1988). The mode of inheritance and genetic causes of CHI differ between high- and low-consanguinity areas, with recessive transmission prevailing in consanguineous regions. This inheritance pattern can impact disease severity, distinguishing between diffuse and focal lesions (Snider et al, 2013). The pathophysiology of CHI primarily involves genetic defects in β-cell function, with over 15 genes implicated, including *ABCC8* and *KCNJ11*, which encode proteins in the ATP-sensitive K+ channel crucial for insulin regulation (Shah et al, 2017). Loss of channel activity leads to continuous insulin release, causing hyperinsulinism.

Candidate gene methods (using Sanger sequencing) can be applied in a majority of cases, as 85% of all cases of CHI in non-consanguineous populations are due to pathogenic variants of either *ABCC8*, *KCNJ11* or *HNF1A* (Rozenkova et al, 2015). In consanguineous populations, a targeted gene panel or WES may be employed. Diazoxide is the first-line treatment for focal CHI, while severe cases may require pancreatectomy (Aynsley-Green et al, 2000). Genetic testing is crucial for confirming the diagnosis, tailoring therapy, providing genetic counselling, and explaining fatal outcomes in previous offspring (**Amaratunga et al, 2019**). In developed countries with low rates of consanguinity, tailored treatment based on genetic confirmation has significantly improved outcomes, ensuring normal development and quality of life for children with CHI.

## 2.3. Diabetes mellitus (DM)

DM is a complex condition caused by an absolute or relative insulin deficiency, with or without insulin resistance, resulting in hyperglycemia (Patterson C, et al, 2017). Type 1 DM is the most common paediatric diabetes subtype globally, even in consanguineous families (**Amaratunga et al, 2023**). The other types of diabetes are much less common in children, these types include type 2 DM and monogenic diabetes (Pacaud et al, 2016). Monogenic diabetes comprises of neonatal diabetes mellitus (NDM), syndromic diabetes, autoimmune monogenic diabetes, and Maturity Onset Diabetes of the Young (MODY). The genetic causes of monogenic diabetes are mainly associated with mechanisms around the pancreas, pancreatic β-cell and insulin production and action, with distinct prevalence patterns in regions with varying rates of consanguinity (**Amaratunga et al, 2023**). Neonatal diabetes (occurring within the first six months) is often due to activating variants in genes such as *ABCC8* and *KCNJ11*; however, consanguinity influences the genetic landscape, with different genetic causes prevailing in consanguineous regions compared to non-consanguineous ones (De Franco et al, 2015).

Syndromic diabetes is typically characterized by the negativity of β-cell autoantibodies and presence of additional non-diabetic (usually extra-pancreatic) phenotypic features. It is rarer in non-consanguineous populations as it is commonly caused by recessive variants (**Amaratunga et al, 2023**). Autoimmune monogenic diabetes represents an overlap between classical T1DM and syndromic monogenic diabetes. Specific monogenic syndromes, like autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome and the X-linked syndrome of immune polyendocrinology on the X-chromosome (IPEX) are rare but have higher incidence rates in populations with consanguinity

as well (Björses et al, 1996). Maturity Onset Diabetes of the Young (MODY) is the most common subtype of monogenic diabetes in non-consanguineous populations, with genes like *GCK*, *HNF1A*, and *HNF4A* being major contributors (Dusatkova et al, 2022). Timely diagnosis is crucial for appropriate management, with some MODY cases responding well to low-dose sulfonylurea treatment (Dusatkova et al, 2022). Overall, understanding the genetic underpinnings of diabetes in both consanguineous and non-consanguineous families is essential for accurate diagnosis and effective treatment strategies.

### 2.4. Short stature

Statuary growth in humans is a complex interplay of genetic and environmental factors, with approximately 80% of individual adult height determined by genetic factors. Short stature is a common focus in paediatric endocrine clinics, defined as height below -2 standard deviations (SD) for a given age, sex, and population (Jee et al, 2017). It can result from various factors such as genetic variants, endocrine diseases, chronic non-endocrine conditions, and nutritional deficiencies. Recent research has elucidated major pathways involved in longitudinal bone growth, including the GH-IGF-1 axis, growth plate regulation, and fundamental intracellular processes (Baron, 2015).

The GH-IGF-1 axis is crucial for human growth, where GH stimulates IGF-1 production in the liver, promoting tissue and organ growth. Disturbances in transcription factors during pituitary gland development can lead to growth hormone deficiency (GHD), occasionally associated with deficiencies in other pituitary hormones. Isolated GHD, with an incidence of 1 in 4,000 to 1 in 10,000 live births, involves genes such as *GH1* and *GHRHR* (Baron, 2015). The discovery of genetic causes, such as variants in the *POU1F1* and *PROP1* genes, has been aided by studying consanguineous families (**Amaratunga et, al 2023**).

Extracellular matrix cartilage protein defects, including genes like *ACAN*, *COL1A2*, *COL2A1*, *COL11A1*, *FBN1*, and *MATN3*, can cause milder short stature in heterozygous (dominant) form in non-consanguineous families and severe dwarfism in homozygous (recessive) form in consanguineous families. Paracrine factors like FGFs and CNP play roles in growth plate regulation, while defects in intracellular pathways, as seen in RASopathies, transcriptional factor genes like *SHOX*, and DNA repair genes like *PCNT*, contribute to a spectrum of growth failure disorders, ranging from syndromic short stature to severe skeletal dysplasia and primordial dwarfism (Jee et al, 2017).

## 3. Hypotheses and aims of the dissertation project

**Project Hypothesis**

In children from consanguineous families with apparent phenotypes, it may be possible to find novel variants of known causative genes, or even novel genes (due to a higher risk of recessive mutations), thereby elucidating novel mechanisms and pathophysiological pathways causing endocrine conditions in children. In addition, the spectrum of causative genes will be varied when comparing consanguineous and non-consanguineous populations.

**Research aims and objectives**

1) Use NGS methods to identify the genetic causes of short stature, monogenic diabetes and congenital hyperinsulinism in children from a highly consanguineous region
2) Use NGS methods to identify the genetic causes of short stature in offspring of non-consanguineous families from a region with a low rate of consanguinity
3) Compare causative genes among consanguineous and non-consanguineous populations
4) Shed light on pathophysiological mechanisms of growth retardation, monogenic diabetes and congenital hyperinsulinism
5) Analyse paediatric diabetes subtypes in a selected consanguineous population and evaluate the effect of consanguinity on the prevalence of specific diabetes subtypes
6) Potentially discover novel gene(s) associated with studied diseases by NGS methods
7) Help elucidate phenotypic features with the aim of contributing to human phenotype ontology that would help identify patients with similar conditions
8) Improve diagnosis and treatment of paediatric patients with endocrine conditions.

# 4. Patients and Methods

Patients in the consanguineous cohort were selected from the endocrine clinic at the Dr. Jamal Ahmad Rashed hospital, Sulaimani in Kurdistan, Iraq. The region has a consanguinity rate of 44%. The patients from the other cohort came from a non-consanguineous region and were seen at the Pediatric Clinic, 2nd Faculty of Medicine, Charles University in Prague.

## 4.1. Congenital Hyperinsulinism

We examined three children with CHI from Kurdish consanguineous families who were diagnosed on the 6th day, 3rd week and 3rd year of life respectively with recurrent hypoglycaemia often combined with convulsions and unconsciousness. DNA was analyzed primarily using methods of direct Sanger sequencing. The complete coding regions and intron-exon boundaries of genes *ABCC8* and *KCNJ11* were analyzed as two of the most common causes of CHI.

## 4.2. Monogenic diabetes

Data from 754 patients registered at the diabetic clinic in Iraq, were obtained. Consanguineous and non-consanguineous patients with neonatal diabetes and syndromic diabetes were offered genetic testing. Finally, DNA from nineteen children from 17 families (12 with neonatal diabetes and 7 with syndromic diabetes, including two sibling pairs) was available for genetic testing with informed consent.

Genomic DNA was extracted from peripheral blood and analysed using WES. Detected variants were filtered using software Variant Annotation and Filter Tool. Copy number variants subanalysis from raw WES data was done using program

Decon (Fowler, 2022). Prioritized variants were then further evaluated using the American College of Medical Genetics and Genomics (ACMG) standards and guidelines (Richards et al, 2015). All the variants with potential clinical significance were confirmed using Sanger sequencing.

### 4.3. Short stature

Out of 1124 children examined with short stature of uncertain aetiology at the endocrine clinic, Dr. Jamal Ahmad Rashed hospital, sixty-eight children fulfilled our inclusion criteria (offspring of consanguineous families with body height for given age and sex ≤ -2.25SD). Thereafter, 51 probands (30 females) with short stature from consanguineous families were enrolled into the primary study with informed consent. Their DNA was analyzed by WES methods described above.

For second part of the short stature study, a cohort of children born SGA with persistent short stature were selected from over 800 children treated with GH at the centre in Prague between May 2008 and December 2018. Finally, 176 children with SGA-SS (birth weight or length <−2 SD and body height <−2.5 SD after 3 years of life) agreed to genetic testing to elucidate the genetic background of short stature in SGA.

Another part of the non- consanguineous study cohort was selected from a similar pool of children treated with GH in Prague. Out of these, 52 had primary GHD and vertically transmitted short stature defined as height SDS ≤-2 SD in both the child and his/her shorter parent and agreed to genetic testing. All patients were analysed using WES / t-NGS methods described above.

# 5. Results

## 5.1. Congenital Hyperinsulinism (CHI)

We found three novel causative homozygous variants in by $K_{ATP}$ channel genes - p. Trp514Ter in the *ABCC8* gene, and p. Met1Val and p. Tyr26Ter in the *KCNJ11* gene in the three children examined. There was a notable history of unexplained neonatal deaths in siblings within two of these families who apparently suffered from the same condition that was not recognized and not properly managed. This was published in **Amaratunga et al, 2020.**

## 5.2. Monogenic diabetes

A total of 269/735 patients followed at the single centre (36%) were offspring of consanguineous families. An overwhelming majority of 714/754 (95%) patients had clinically defined Type 1 DM (35% of them were born to consanguineous parents) whereas only 8/754 (1%) had Type 2 DM. Fourteen (1.9%) had neonatal diabetes (50% consanguineous), 7/754 (0.9%) syndromic diabetes (100% consanguineous), and 11/754 (1.5%) clinically defined MODY.

We found that syndromic diabetes is strongly associated with consanguinity. The genetic cause was elucidated in 10/12 neonatal diabetes patients (homozygous variants in *GLIS3*, *PTF1A*, *ZNF808* and heterozygous variants in *ABCC8* and *INS*) and 4/7 syndromic diabetes patients (homozygous variants in *INSR*, *SLC29A3*, *WFS1*. In addition, a patient referred as syndromic diabetes was diagnosed with mucolipidosis gamma with his diabetes probably being type 2. This was published in **Amaratunga et al, 2022.**

## 5.3. Short stature

### *5.3.1. Consanguineous cohort from Kurdistan, Iraq*

A pathogenic cause of short stature was elucidated in 33/51 (65%) children. Pathogenic or likely pathogenic variants (17 novel) were found in genes involved in the GH-IGF-1 axis (*GHR*, *SOX3*), the thyroid axis (*TSHR*), the growth plate extracellular matrix (*COL1A2, COL10A1, CCDC8, FLNA, FN1, MMP13, LTBP3, PCNT*), the regulation/function of chondrocytes (*DYM, NPR2, PTPN11, CTSK, SHOX*), DNA/RNA replication (*LIG4, GZF1, DNAJC21*), transport (*SLC34A3, SLC7A7*) , enzyme coding (*CYP27B1, GALNS, GNPTG*) and ciliogenesis genes (*CFAP410*). In addition, three children had Silver-Russell and DiGeorge syndromes.

### *5.3.2 Non-consanguineous cohort from Prague, Czech Republic*

The genetic aetiology was elucidated in 74/176 (42%) children born SGA with persistent short stature. Of these, 12/74 (16%) had pathogenic variants affecting pituitary development (*LHX4, OTX2, PROKR2, PTCH1, POU1F1*), the GH-IGF-1 or IGF-2 axis (*GHSR, IGFALS, IGF1R, STAT3, HMGA2*), 2/74 (3%) the thyroid axis (*TRHR, THRA*), 17/74 (23%) the cartilaginous matrix (*ACAN*, collagen genes, *FLNB, MATN3*), and 7/74 (9%) the paracrine chondrocyte regulation (*FGFR3, FGFR2, NPR2*). In 12/74 (16%) we found variants affecting fundamental intracellular/intranuclear processes (*CDC42, KMT2D, LMNA, NSD1, PTPN11, SRCAP, SON, SOS1, SOX9, TLK2*). *SHOX* deficiency was found in 7/74 (9%), Silver-Russell syndrome in 12/74 (16%) (11p15, UPD7), and miscellaneous chromosomal aberrations in 5/74 (7%) children. This was published in **Toni et al, 2022.**

In patients primary GHD and vertically transmitted short stature, causative variant in a gene that affects growth was discovered in 15/52 (29%) children. Of them, only 2 (13%) had a genetic variant affecting GH secretion or function (*GHSR* and *OTX2*). Interestingly, in 10 (67%) children we discovered a primary growth plate disorder (*ACAN, COL1A2, COL11A1, COL2A1, EXT2, FGFR3, NF1, NPR2, PTPN11*), in one (7%) a genetic variant impairing IGF-1 action (*IGFALS*) and in two (12%) a variant in miscellaneous genes (*SALL4, MBTPS2*). This was published in **Plachy et al, 2022.**

## 6. Discussion

The results mentioned above offer a profound insight into the world of genetic research, with a primary focus on paediatric endocrine conditions in both consanguineous and non-consanguineous families. The primary test centre in Kurdistan, Iraq was chosen due to high consanguinity rate of the region (44%). This is a region with a high percentage of neonatal deaths as well. There was a notable history of unexplained neonatal deaths in siblings within two of the three families examined with CHI. By elucidating the genetic diagnosis, we shed light on the potential cause of their deceased siblings' conditions, emphasizing the importance of early CHI diagnosis when encountering neonates with unexplained seizures or recurrent hypoglycaemia.

Our unique single centre study on monogenic diabetes from the same centre confirms that even in a highly consanguineous population, clinically defined type 1 diabetes represents the prevailing paediatric diabetes subtype. We found that syndromic diabetes is strongly associated with consanguinity. The rates of pathogenic findings were high with 83% of genes causing neonatal diabetes and 57% of syndromic diabetes being confirmed. Causative genes (*PTF1A, SLC29A3, INSR, WFS1, INS, ABCC8, GLIS3*) were markedly different when comparing with non-consanguineous and other consanguineous populations as well with recessive variants being more common in the consanguineous group. One participant initially diagnosed with syndromic diabetes was subsequently found to have mucolipidosis gamma and potentially concurrent type 2 diabetes. These findings underline the complexity of diagnosing syndromic diabetes in consanguineous communities, suggesting the potential need for revised diagnostic criteria that consider additional phenotypic features, such as short stature and hepatosplenomegaly which were recurring features in our cohort.

In short stature cohort from Kurdistan, there was a very high rate of positive findings with 65% of the probands having a pathogenic cause of short stature, with genetic variants spanning an array of crucial growth-related genes. Notably, 52% of causative genes were genes influencing the growth plate (chondrocytes and the extracellular matrix) with only 4% being genes of the GH-IGF1 axis. These findings underscore the power of WES in identifying causative genes in predominantly consanguineous populations as the rate of positive results would be reduced using common t-NGS panels. Moreover, it emphasizes the importance of the timely diagnosis of syndromic short stature for proactive screening for potential concomitant conditions and the management of these complex conditions.

When comparing findings with the non-consanguineous cohorts from Prague, the main similarities were the high percentage of variants being in genes affecting the growth plate. This further strength the concept of the growth plate playing an equally important role as the GH-IGF1 axis in growth regulation. The stark difference however was in the percentage of recessive or biallelic variants with 75% of variants in the consanguineous cohort being recessive.

## 7. Conclusion

Our results demonstrate how consanguineous families, which constitute a significant portion of the global population, have played a pivotal role in the discovery of crucial pathophysiological mechanisms via novel genes, particularly in the realm of paediatric endocrinology.

The conditions studied were chosen due to their positions on the gradient of pathophysiological complexity and represent three levels of intricacy, with short stature being the most complex and congenital hyperinsulinism the most straightforward. In all conditions, it was made clear how the genetic background, and therefore the pathophysiological mechanism are varied between consanguineous and non-consanguineous populations. In CHI, a pathogenic variant was found in all patients explaining premature neonatal deaths in two families.

In monogenic diabetes, we published the first-ever single centre study from a consanguineous region looking at paediatric diabetes subtypes and the prevalence of consanguinity. The study revealed that consanguinity was significantly associated with the presence of syndromic short stature and found that T1DM was the most common paediatric diabetes subtype even in consanguineous families.

In the realm of short stature, patients with short stature of unknown aetiology from consanguineous families had a very high rate of positive findings via WES. These results highlighted the importance of considering different genetic testing methods in such populations. This dissertation provided comparison of the genetic findings in non-consanguineous families with findings from a GHD and SGA cohort. Interesting, all results further strength the concept, that genes affecting the growth plate (chondrocytes and the extracellular matrix) play a crucial role in growth regulation.

Moreover, these studies highlight the complex interplay between genetics and endocrine function, providing valuable insights into disease pathophysiology in both consanguineous and non-consanguineous families. Such research can help pave the way for targeted interventions and personalized medical management, ultimately enhancing our capacity to address these disorders and improve patient outcomes.

## 8. Summary

As part of this dissertation project, genetic testing was done in individuals with three paediatric endocrine conditions (congenital hyperinsulinism, monogenic diabetes and short stature). We are the first study group to study large cohorts of patients from a region with a consanguinity rate as high as 44%. We studied diabetic children from single centre serving a population of 2.33 million people and found that T1DM was the most common diabetes subtype. Types of monogenic diabetes such as syndromic and neonatal diabetes were more prevalent than in a non-consanguineous population. We found that syndromic diabetes was significantly associated to consanguinity and described how the diagnoses of syndromic diabetes is more complex in consanguineous families due to the potential of multiple recessive conditions. Overall, the causative genes of syndromic and neonatal diabetes (*PTF1A, SLC29A3, INSR, WFS1, INS, ABCC8, GLIS3*) were highly varied when comparing with patients from non-consanguineous populations.

In congenital hyperinsulinism, we elucidated the genetic cause in three children from three families, thereby explaining the probable cause of unexplained neonatal deaths in these families. Recessive forms of CHI are more common in such consanguineous families and cause more severe disease (diffuse CHI), thereby timely diagnosis is crucial. The final analysed diagnosis was short stature, we analysed a large cohort of 51 consanguineous children from the same single centre. Two children had Silver-Russell and one had DiGeorge syndromes. Pathogenic variants were found in another 30 individuals in genes involved in the GH-IGF-1 axis (*GHR, SOX3*), the thyroid axis (TSHR), the growth plate extracellular matrix (*CCDC8, CTSK, COL1A2, COL10A1, FLNA, FN1, LTBP3, MMP13, PCNT*), the regulation/function of chondrocytes (*DYM, NPR2, SHOX*), signal transduction (*PTPN11*), DNA/RNA replication (*DNAJC21, GZF1, LIG4*), transmembrane

transport (*SLC34A3, SLC7A7*) , enzyme coding (*CYP27B1, GALNS, GNPTG*) and ciliogenesis (*CFAP410*). The rate of positive variants was high (65%) compared to other analysed cohorts from a centre with low consanguinity (29% / 42%). As expected there was a very high proportion of homozygous variants in the consanguineous region. Interesting, results across populations further strengthen the concept, that genes influencing the growth plate play a crucial role in growth regulation.

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