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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

Dissertation Thesis

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Prague, 2023

Declaration

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A.T.S.D Amaratunga

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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 pediatric 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

LIST OF ABBREVIATIONS

aCGH	Array Comparative Genomic Hybridization
ACMG	American College of Medical Genetics and Genomics
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
B	Benign
CNV	Copy Number Variants
DM	Diabetes mellitus
ER	Endoplasmic reticulum
G6P	Glucose-6-phosphate
GH	Growth hormone
CHI	Congenital hyperinsulinism
LB	Likely benign
LP	Likely pathogenic
MLPA	Multiplex-ligation dependent probe amplification
NGS	Next Generation Sequencing
P	Pathogenic
PCR	Polymerase chain reaction
SGA	Small for gestational age
SGA-SS	Small for gestational age without catch-up growth
SNV	Single Nucleotide Variant
T1DM	Type 1 diabetes mellitus
tNGS	Targeted Next Generation Sequencing
VUS	Variant of uncertain significance
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing

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1. Introduction

Five percent of all live births have genetic disorders recognizable until 25 years of age (Baird, 1988). These genetic disorders and their mechanisms of disease are being increasingly elucidated in the human population; this is partly impacted by the progress of genetic analysis methods in the recent past. Even so, the most paediatric endocrine conditions are classified as "rare disorders" with a prevalence lower than 1 in 2000 live-born children (Danese et al, 2018). Nevertheless, due to their total count (several hundreds of different conditions) and their serious impact on a child`s growth, development, metabolism, water-and-electrolyte balance, and general well-being, they represent a substantial part of paediatric morbidity and mortality.

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. With increasing advancements in genetic testing technology and the development of tools such as next generation sequencing (NGS), elucidation of the aetiology of complex disorders is more accessible now than ever before. Therefore, genetic testing is becoming an increasingly more common part of a comprehensive diagnostic workup.

The decision on carrying out genetic examination is not only based on potential therapeutic benefit for the patient and their family, but on the feasibility, availability, cost-effectiveness as well (Eggermann et al, 2020). The latter is highly influenced by the country or region. Prior to the indication of genetic testing, comprehensive medical history should be obtained; this should include the patients' clinical data, laboratory tests, and family data including the presence of consanguinity in the family.

The knowledge on the genetic cause of disease allows precise and individualized clinical management, faster diagnosis without the need of invasive diagnostic tests in some cases, prediction of long-term outcome and genetic counselling in the family. Even though many gene variants causing short stature, monogenic diabetes and short stature have already been described, positive genetic findings in every additional patient continue to contribute in a significant way toward elucidating the specific pathophysiological mechanisms of disease in complex phenotypes and clarifying genotype – phenotype relationships. Consanguineous

families have especially played a role in the discovery of novel genes (**publication in 7.1**). Thus, genetics trigger novel advancements in understanding physiological principles and mechanisms.

This dissertation embarks on a journey to explore novel insights into the pathophysiology of these conditions, drawing lessons from both consanguineous and non-consanguineous families. It will feature seven articles published in impacted journals and one manuscript ready for submission. By examining these two distinct population types, this project aimed to contribute to our understanding of the pathophysiology of complex paediatric endocrine conditions, shed light on diverse genetic determinants and potential shared mechanisms that underlie growth disorders, monogenic diabetes and congenital hyperinsulinism, with the ultimate goal of advancing diagnostic and therapeutic strategies for affected individuals.

1.1 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. Consanguineous matings are linked to demographic, cultural and religious practises, which served many purposes from ancient times. They are more common in some areas around the world than others, especially in Asia, Africa and the Middle-East (Bittles, 2001). Currently, despite increased awareness on the possible health consequences for children of consanguineous families, it has been estimated that over 20% of the world population live in communities having a preference to such marriages, and that over 8.5% of all children worldwide have consanguineous parents (Shawky et al, 2013).

In a large genetic study done in Egypt, recessive disorders were found mainly among families with consanguinity (78.8%). Consanguinity was present in 100% of cases of mental retardation and in 92.6% of patients with limb anomalies. Child deaths and stillbirths were more prevalent among children from consanguineous parents when compared to non-consanguineous families as well (Shawky et al, 2013). The higher frequency of recessive genetic conditions in descendants of consanguineous parents than those of unrelated parents can be attributed to the increased frequency of homozygous genotypes in children from such marriages allows less common alleles to manifest as homozygous. For instance, first cousins are predicted to share 12.5% (1/8) of their genes. Therefore, their progeny will be

homozygous at 6.25% (1/16) of gene loci. This means that they will have identical gene copies from each parent at these sites in their genome, called “runs of homozygosity” (Bennett et al, 2002). This shows how consanguinity represents a substantial genetic burden for offspring.

DNA analysis can help calculate the frequency of carriers of pathogenic autosomal recessive variants (for certain disorders in specific populations). This frequency can be estimated by the Hardy–Weinberg equation as well. This equation is $p^2 + 2pq + q^2$, where p^2 is normal allele frequency (AA), 2pq is the frequency of carriers (Aa) and q^2 is the frequency of affected individuals (aa). However, consanguinity means that there is non-random mating, which contradicts the main assumption of the Hardy–Weinberg equilibrium (Antonarakis, 2019). If a variant with a heterozygosity frequency of 1 in 100 is considered, there is a 1 in 10,000 chance that both unrelated spouses are carriers (1/100 x 1/100 = 1/10000). However, if they are first cousins, the chance of both being carriers of this pathogenic variant is 12.5 times higher, thereby the chance is 1 in 800 (1/100 x 1/8= 1/800). In families with multiple consanguinity, this risk would be even higher (Antonarakis, 2019).

Due to the high homozygosity in the genome, consanguineous families provide the best chances for novel gene discovery when a monogenic condition is suspected, even in rare and complex disorders. In the conditions that are the focus of this dissertation, many novel genes have been described for the first time thanks to consanguinity. The discovery of each new gene came with new pathophysiological insights helping us to understand the condition further (**publication in 7.1**).

In contrast, the study of non-consanguineous families allows for the exploration of genetic heterogeneity and specific environmental influences in studied conditions.

1.2 Next Generation Sequencing (NGS): Targeted panels (t-NGS), Whole Exome Sequencing (WES) and Whole Genome Sequencing (WGS)

Within the past decade, NGS has become a golden standard when genetically examining patients with complex phenotypes. However, a vital factor is the selection of the library to be adopted. The library refers to the extent of the human genome, which is examined, some

types of libraries are custom targeted panels (t-NGS), Whole Exome Sequencing (WES) or Whole Exome Sequencing (WGS).

WGS looks at the entire genome and makes it possible to analyse all possible mutation types – single nucleotide variants (SNVs), insertions or deletions (indels), structural variants and copy number variants (CNVs). WES is focused on the detection of SNVs and indels in the 1% protein-coding region in the human genome (Persani et al, 2018). T-NGS is a suitable, affordable tool to analyse patients with a specific diagnosis, which is caused by a group of known genes, at a greater quantity if a suitable gene panel has been created and tested. Due to the smaller tested region and thereby, lower number of possible variants, WES and target NGS remain more cost-effective and easier to interpret at the current level of knowledge.

The data obtained by these NGS methods need to be analysed by a process called a bioinformatic pipeline. This pipeline encompasses steps such as sequence generation, sequence alignment, variant calling, variant filtering and variant annotation (Roy, 2018). Technical advancements of the Internet have facilitated the storage and rapid exchange of this data, allowing more efficient analysis, consulting of variants and international collaboration. Other developments as the complete sequencing of the human genome was followed by the creation of expansive databases such as the 1000 genome project, and the ExAc and GnomAD databases. These databases contain WES and WGS data from thousands of patients, therefore helping in the "variant filtering" process or analysis of possibly causative variants.

In-silico analysis tools and prediction programs of variant analysis such as MutationTaster, SIFT and Polyphen-2 are useful in variant filtering and annotation as well, however, it is recommended to use many for the sake of comparison (Roy, 2018). Many online databases such as those containing information on genes and their functions (OMIM), modes of transmission of disorders and their clinical features are available as well. Newly developed bioinformatic analysis software are capable of gathering all of the above mentioned information about a single variant, and give an overview to the viewer to enable faster filtering and annotation of variants. The gold standard in variant classification are the American College of Medical Genetics and Genomics (ACMG) guidelines which aids the

classification of possible variants in a range from benign to pathogenic (Richards et al, 2015).

Sanger sequencing is then used to analysis the segregation of variants in the family, thereby differentiating between autosomal recessive, dominant and de-novo variants (Sanger, 1977). Sanger sequencing can be used in the candidate gene approach as well, using Polymerase Chain Reaction (PCR) analysis of a few specific genes known to cause the condition.

2. Congenital hyperinsulinism (CHI)

CHI is a heterogeneous genetic condition caused by a primary genetic defect of the pancreatic β-cell resulting in uncontrolled insulin secretion. 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). Similar to some other conditions, it has been clearly shown that not only the incidence and genetic causes of CHI, but also the mode of inheritance differs between high- and low-consanguineous areas, with recessive (biallelic) transmission prevailing in high-consanguineous, and monoallelic (dominant / uniparental isodisomy) transmission prevailing in low-consanguineous areas (**publication in 7.2**). The mode of inheritance may directly impact the disease severity (diffuse versus focal lesions) as well, with biallelic or recessive mutations commonly causing diffuse CHI (Snider et al, 2013).

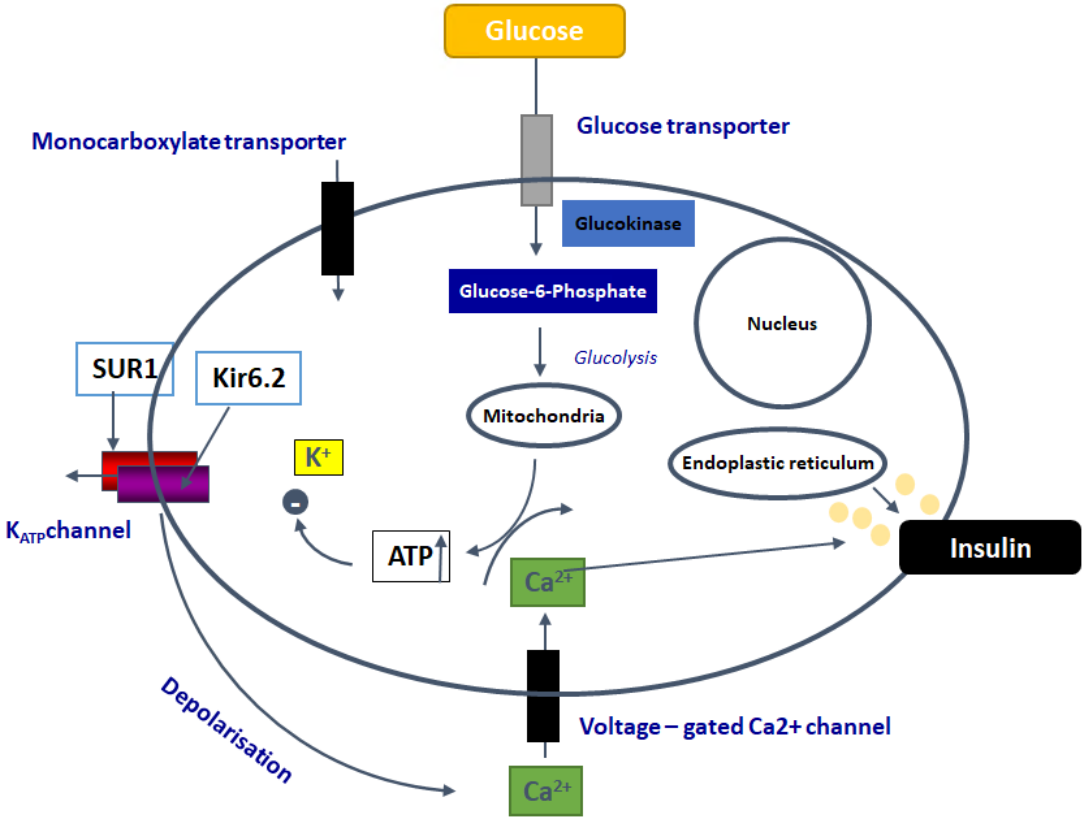

Fig.1. Functional components of pancreatic β-cell as the basis for understanding the molecular (patho)-physiology of congenital hyperinsulinism

The process begins with the uptake of glucose, which is phosphorylated to glucose-6-phosphate (G6P). Elevated G6P levels lead to increased ATP production, closing K_{ATP} channels. This closure results in membrane depolarization, activating voltage-gated Ca^{2+} channels. The influx of calcium triggers insulin release. Mutations in any one of these parameters could cause CHI.

The pancreatic β-cell is one of the most sophisticated cells in the human body – because each beta-cell works independently with no upstream regulation. It has the capability to detect the current blood glucose level, to produce and store insulin in large amounts, and to release insulin to the bloodstream to maintain, or to achieve, blood glucose levels within the normal range, without the risk of hypoglycaemia (Shah et al, 2017). The pathophysiology of CHI is based around genetic defects of β-cell function in a vast majority of cases, with a limited number of genes involved. At this time, pathogenic variants causing CHI have been found in over 15 genes but the most common genes are *ABCC8* and *KCNJ11*, each encoding the SUR1 and Kir6.2 proteins of the ATP-sensitive K^+ (K_{ATP}) channel in the pancreatic β-cell

respectively (Fig.1). This channel plays a pivotal role in the regulation of insulin secretion. Loss of K_{ATP} channel activity due to such gene variants lead to persistent membrane depolarization and continuous insulin release, regardless of the glucose level, thereby causing hyperinsulinism (Shah et al, 2017). In addition, some genetic syndromes such as Beckwith-Wiedemann, Kabuki and Turner syndromes are associated with hyperinsulinism as well (Stanley, 2016).

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*, a hepatocyte nuclear transcription factor (Rozenkova et al, 2015). In addition, a t-NGS or WES can be used in patients who are negative for initial testing or patients coming from consanguineous areas where the most common genes are not clearly mapped out.

Diazoxide treatment represents the first-line option in children with focal types of CHI (Aynsley-Green et al, 2000). Based on histological form of CHI and non-responsiveness to pharmacological treatment, partial, subtotal, or near-total pancreatectomy might be necessary (Cretolle et al, 2002). In certain areas of the world, there is still a high risk of sudden death at neonatal age, epilepsy, and severe psychomotor delay due to repeated unrecognized hypoglycemia. However, in more developed countries in the Western world, tailored treatment of patients with genetically confirmed CHI has tremendously improved their perspective and these children have normal development and quality of life. Genetic testing in children with CHI is therefore crucial for confirming diagnosis and tailoring therapy; it may play an important role in genetic counselling, or to explain fatal outcomes in previous offspring as well (**publication in 7.2**).

3. Diabetes Mellitus (DM)

DM is a complex condition that is caused by an absolute or relative insulin deficiency, with or without insulin resistance, which results in hyperglycemia (Patterson C, et al, 2017). Type 1 diabetes mellitus (T1DM) is the most common form of paediatric diabetes worldwide, even in among consanguineous families (**publication in 7.3**). T1DM is a polygenic disease which is heritable (30–70% identical twin concordance and a 1–9% risk in children with a

diabetic parent) but has a significant environmental component as well (candidate factors being viral infections, diet in the first few months of life, vitamin D levels, and gut microbiota) (Pociot, 2016). Two HLA haplotypes have been discovered which are linked to antigen presentation and 50% disease heritability (Pociot, 2016). There are other haplotypes which reduce the risk of T1DM but the mechanisms in which these haplotypes function is currently unknown. Previous studies have shown that the prevalence of T1DM is not influenced by consanguinity, however there is a higher risk of its development if there is a history of diabetes in first-cousin parents (Albishi et al, 2022). The other types of diabetes are much less common in children, these types include type 2 diabetes mellitus (T2DM) and monogenic diabetes (Pacaud et al, 2016).

3.1 Monogenic diabetes

Monogenic diabetes can be divided into 4 partially overlapping subtypes, which are neonatal diabetes mellitus (NDM), syndromic diabetes, autoimmune monogenic diabetes and Maturity Onset Diabetes of the Young (MODY). The genetic causes of monogenic diabetes are mostly based on pathophysiological pathways in and around the pancreatic β-cell, or rarely on impaired insulin action (Fig. 2). The prevalence of monogenic diabetes in the UK paediatric population (low rate of consanguinity) was found to be 2.5% in a cohort of 808 children with diabetes with a positive urinary C-peptide creatinine ratio (UCPCR) and negative islet cell-antibodies (GAD and IA-2) (Shepherd et al, 2016). A similar study carried out in Turkey showed a prevalence of 6% but the incidence in other countries where consanguinity is prevalent is not known (Haliloğlu, 2018). However, the genetic aetiology of monogenic diabetes has been shown to significantly differ between areas of low and high rates of consanguinity (**publication in 7.3**).

3.1.1. Neonatal diabetes mellitus (NDM)

NDM, which is diabetes with onset within the first 6 months of age, is clearly defined by the age, whereas other subtypes of monogenic diabetes may remain underdiagnosed or misdiagnosed due to unspecific phenotypic features, especially in resource-limited countries where testing of pancreatic autoantibodies is rarely done. The most common cause of NDM represents activating variants in the genes *ABCC8* and *KCNJ11* encoding ATP-sensitive potassium channel at the membrane of the pancreatic β-cells.

In a study showing the different structure of genetic causes of permanent neonatal diabetes mellitus (PNDM) in consanguineous and non-consanguineous populations, it was shown that in consanguineous regions, the most common gene causing neonatal diabetes was *EIF2AK3* causing Wolcott-Rallison syndromes and genes *KCNJ11* and *ABCC8* accounted only for 12% of cases, whereas in the non-consanguineous areas – these genes prevailed (46%) (De Franco et al, 2015).

3.1.2. Syndromic diabetes

Syndromic diabetes is typically characterized by the negativity of beta-cell autoantibodies and additional non-diabetic (usually extra-pancreatic) phenotypic features. Consanguinity shows statistically significant association with syndromic diabetes (**publication in 7.3**). It is rarer in non-consanguineous populations as it is commonly caused by recessive variants.

Therefore, many forms of syndromic diabetes have been discovered thanks to research and genetic testing done on consanguineous families or in populations with prevalent consanguinity. Such was the observance of children with diabetes mellitus, diabetes insipidus and optic atrophy in consanguineous families with healthy parents and other healthy siblings, suggesting a condition with autosomal recessive inheritance (Fraser, 1977). This syndrome was named Wolfram syndrome (or DIDMOAD) caused by homozygous pathogenic variants and the causative gene was later mapped to the *WFS1* gene that encodes protein wolframin (Strom et al, 1998). In non-consanguineous pedigrees, syndromic diabetes can have different (more rare) disease mechanism – e.g. maternally inherited mitochondrial diabetes presenting with sensorineural hearing loss or myopathy, encephalopathy, lactic acidosis and stroke (Goto et al, 1990). The early diagnosis of syndromic forms of diabetes is crucial because of accurate management of possible comorbidities that might be present or further develop in time and assessment of correct treatment. For example, patients with mitochondrial diabetes should avoid metformin interfering with mitochondrial functions.

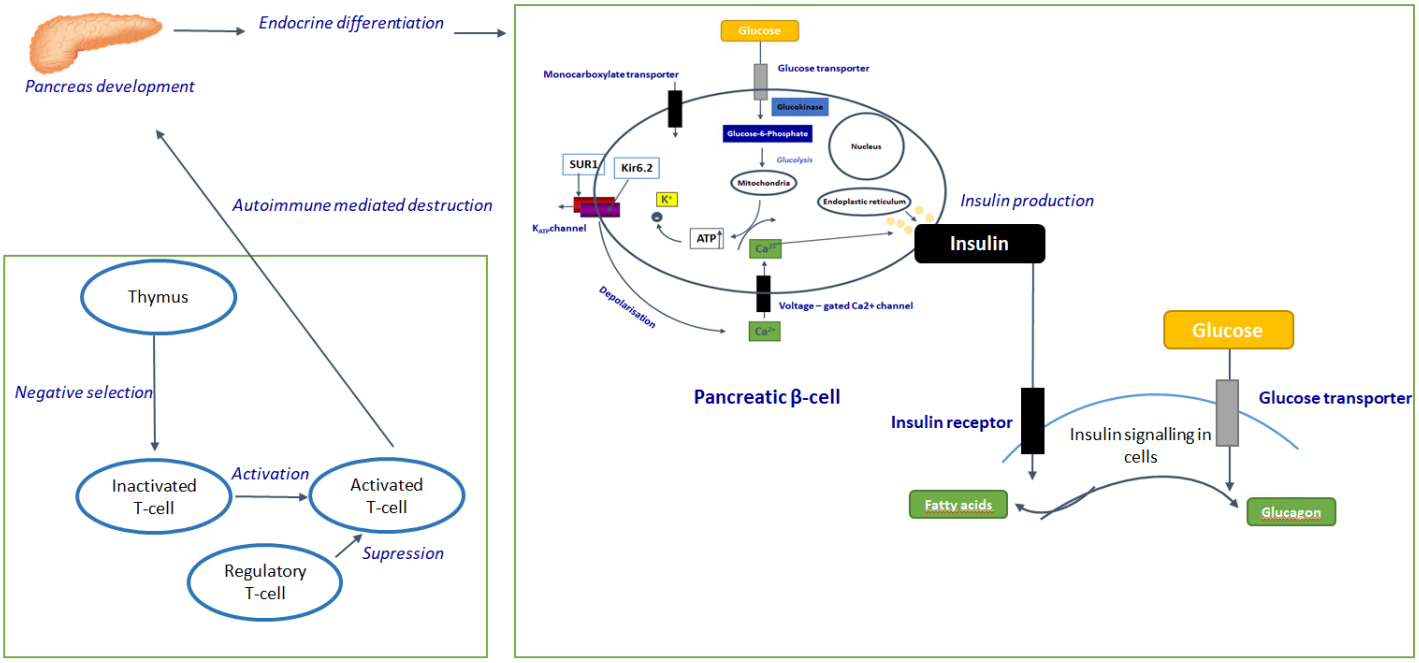

Fig. 2. Overview of pathways essential for the molecular (patho)-physiology of monogenic diabetes

The figure shows the stages of pancreatic development including pancreatic endocrine differenciation and intricate functioning of the β-cell. The β-cell has close relations with all cells in the body (via insulin) with regard the glucose uptake and storage. In addition, this entire axis can be disrupted by mechanisms of autoimmune mediated destruction as shown above. Monogenic diabetes can result from variants in genes of all stages of the pancreatic beta-cell development and function, insulin action, and genes affecting immune tolerance as well, leading to autoimmune mediated destruction of β-cells.

3.1.3. Autoimmune monogenic diabetes

Autoimmune monogenic diabetes represents an overlap between classical T1DM and syndromic monogenic diabetes which typically manifests at an early age. Two monogenic syndromes of polyglandular autoimmunity that may include monogenic autoimmune diabetes as one of disease components were described over 20 years ago: autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome caused by variants in the *AIRE* gene and the X-linked syndrome of immune polyendocrinology on the X-chromosome (IPEX) caused by variants in the *FOXP3* gene (Nagamine et al, 1997, Bennett, 2001). These are rare diseases but for example, APECED syndrome with an overall incidence of about 1:100,000 has been detected at higher rates among certain populations

such as the Finnish (1:25000), Sardinian (1:14000) and Iranian Jewish (1:6500 - 1:9000) as a result of founder mutations (Björses et al, 1996, Zlotogora, 1992). The comparatively higher frequency among the Iranian Jewish population can be attributed to prevalent consanguinity in this population.

3.1.4. Maturity Onset Diabetes of the Young (MODY)

MODY can be defined as non-insulin-dependent diabetes presenting at childhood or early adulthood caused by heterozygous variants in the genes related to pancreatic beta cell development or function. It represents the most common subtype of monogenic diabetes in non-consanguineous populations. Pathogenic variants in three genes (*GCK*, *HNF1A*, *HNF4A*) accounts for more than 80% of cases (Dusatkova et al, 2022). Persistent mild hyperglycemia, caused by loss of function variants in the *GCK* gene, does not require treatment outside pregnancy (Stride, 2014). Patients having causal variants in the genes *HNF1A* and *HNF4A* are more sensitive to low-dose sulfonylurea treatment compared to insulin, even more sensitive than patients with Type-2 diabetes (Dusatkova et al, 2022). Timely diagnosis of these conditions are important to provide adequate treatment.

4. Short stature

Statuary growth in humans is regulated by genetic and environmental factors. Genetic factors determine ~80% of individual adult height whereas environmental factors are responsible for the additional ~20% (Silventoinen et al, 2003). Environmental factors influencing height include nutrition, general health and well-being, and may reflect social-economic status of the family, of the community and of the whole society, respectively. Since genetic factors play such a key role, this is an area of extensive research interest especially over the last decade.

Short stature is a commonly investigated condition in paediatric endocrine clinics (Fig.3). It is clinically defined as height <-2 standard deviations (SD) for the mean height of given age, sex and population (Jee et al, 2017, Fig.3). It can be caused by a variety of factors such as genetic variants, endocrine diseases, chronic non-endocrine conditions (e.g. renal disease) and nutritional deficiencies (Baron, 2015).

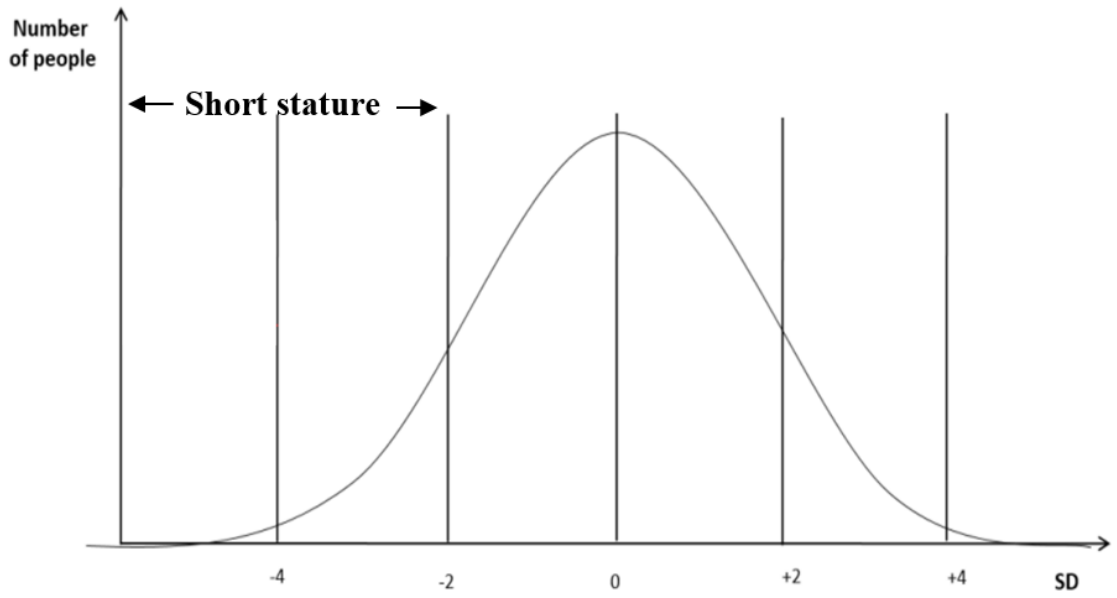

Fig. 3. Gaussian Curve of Height Distribution

Recent strides in the understanding of the regulation of human growth highlight that a few major pathways regulate longitudinal bone growth. Namely, the GH-IGF-1 axis, the intricate regulation of the growth plate involving the paracrine and autocrine regulation of chondrocytes and the extracellular cartilaginous matrix, as well as fundamental intracellular processes such as signal transduction, DNA expression, transcription, replication, and repair in all body cells (Dauber, 2014). Short stature can arise from any disturbance or malfunction within these critical components (Fig.4).

Short stature can manifest as the primary and sole phenotype, referred to as isolated or 'non-syndromic' short stature. Alternatively, it may present alongside various clinical abnormalities such as craniofacial dysmorphism, skeletal disproportionality, overt skeletal dysplasia, developmental delay, and/or internal organ anomalies, categorizing it as 'syndromic' short stature. Noteworthy examples of well-known causes of syndromic short stature include conditions like Noonan, Turner, and Prader-Willi syndromes.

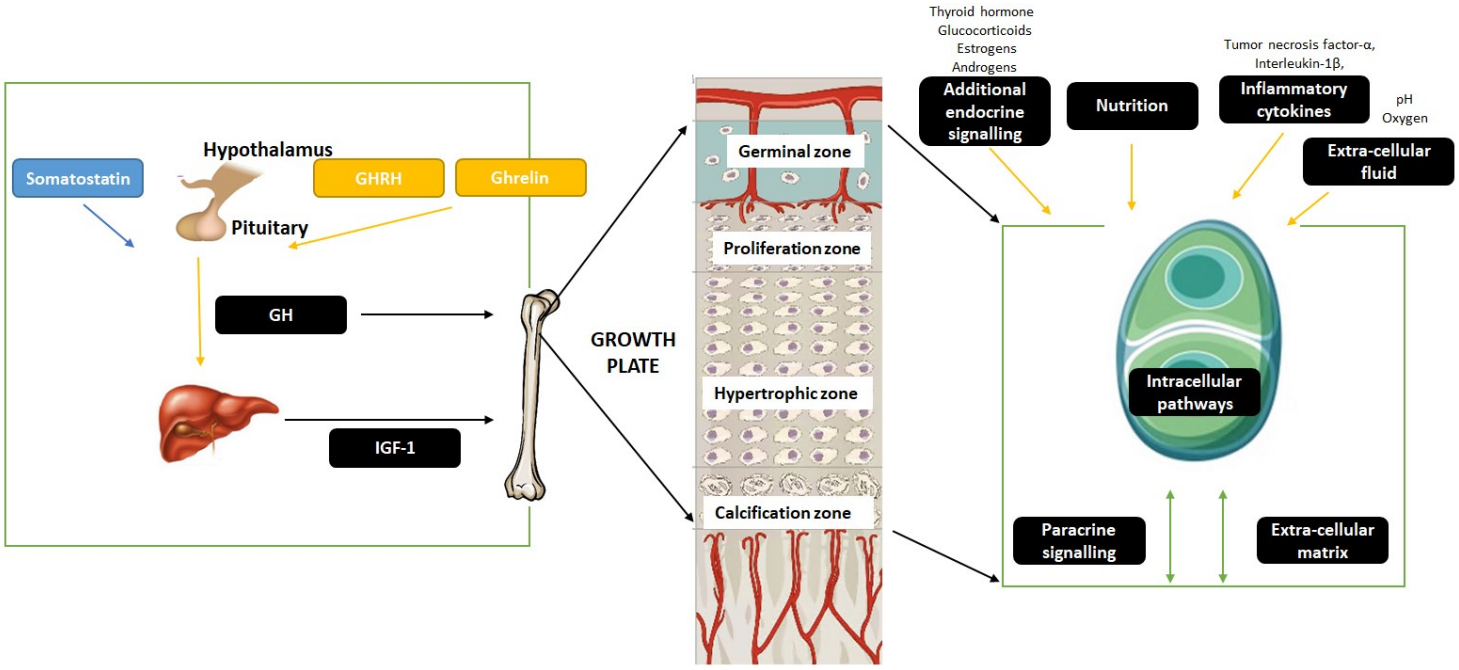

Fig.4. Novel Model of Growth Regulation (Diagram partially adapted from Baron, 2015)

GH is secreted by the pituitary gland, which stimulates the liver to produce IGF-1. This, in turn, promotes the growth and development of various tissues, particularly in the growth plates of long bones, by influencing cell division, proliferation, and differentiation. Chondrocytes in the growth plate play a vital role in longitudinal bone growth, secreting an extracellular matrix rich in collagens and proteoglycans, regulated by many factors as shown in the figure

1.1.GH-IGF-1 axis

The GH-IGF-1 axis plays a pivotal role in regulating human growth. GH is secreted by the pituitary gland and stimulates the liver to produce IGF-1. IGF-1, in turn, promotes the growth and development of various tissues and organs by stimulating cell division, proliferation, and differentiation, particularly in the growth plates of long bones. This intricate hormonal cascade is crucial for the normal growth and development of individuals.

The development of the pituitary gland occurs in parallel with the development of the midbrain and facial structures through the interplay of a network of transcription factors (Dauber, 2014). Disturbances in these transcription factors lead to impaired pituitary morphogenesis and/or function. Deficiency of growth hormone is usually associated with a

deficiency of other pituitary hormones (combined pituitary hormone deficiency, or overt panhypopituitarism in some cases) and disorders of midline structures (e.g. hypotelorism, cyclopia, cleft palate, thinning of the corpus callosum or ectopic neurohypophysis) (Mullis, 2007).

During differentiation of the anterior lobe of the pituitary gland, five cell lineages gradually develop cells that subsequently produce growth hormone, thyroid stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), gonadotropins (FSH and LH) and prolactin. Pituitary differentiation disorders are characterised by a combined deficiency of hormones pituitary gland without the presence of associated midline structure anomalies (Mullis, 2007). The most common genetic cause of this condition in Western Europe are variants in gene *POU1F1*, previously known as *PIT1*. In 1992, two sisters from a consanguineous family with cretinism were reported as the first documented cases of this genetic defect (**publication in 7.1**). Their condition encompassed deficiencies in TSH, GH, and prolactin. This breakthrough marked the first instance of a transcript activator defect causing deficiencies in multiple target genes. Interestingly, in Eastern Europe, the most prevalent cause of impaired differentiation of pituitary cell lines are *PROP1* (prophet of PIT1) gene defects, due to founder effect (Dusatkova et al, 2015).

Isolated growth hormone deficiency (IGHD) is a distinct condition with an incidence ranging from 1 in 4,000 to 1 in 10,000 live births. Children affected by IGHD typically exhibit proportionate short stature, characterized by reduced growth velocity, low IGF-1 levels, and an inadequate response during GH provocative tests (Phillips, 1981). The primary genes associated with IGHD are the growth hormone gene (*GH1*) and the growth hormone releasing hormone receptor (*GHRHR*). Interestingly, there have been no reported cases of GH deficiency resulting from mutations in the gene encoding growth hormone-releasing hormone (*GHRH*) itself, emphasizing the multifaceted genetic factors influencing this endocrine disorder (**publication in 7.1**).

GHD is diagnosed by a combination of auxological, biochemical and radiological data. Children with clinically suspected GHD will undergo targeted testing to assess GH secretion. GH is secreted from the anterior lobe of the pituitary gland in a pulsatile manner. Therefore, a random GH value cannot therefore be used to diagnose GHD (Murray, 2016). Instead IGF-

1, whose serum concentrations are significantly more stable compared to GH, are used. Serum IGF-1 concentrations <-2 SD compared to the mean for age and sex strongly support the diagnosis of GHD (Growth Hormone Research Society 2000). Thereafter, a GH stimulation test is carried out. This is the gold standard for the diagnosis of GHD. These tests are based on the administration of a substance that causes marked stimulation of GH secretion in the pituitary gland. Thus, the maximum capacity of GH secretion is assessed (maximum stimulated GH concentration <10 ug/l in two different tests is diagnostic for GHD).

The development of our understanding of the GH-IGF1 axis and its genetic underpinnings dates back to 1966, when Dr. Laron observed a distinctive disorder characterized by severe growth retardation, obesity, and small genitalia in three siblings from a consanguineous Jewish family (Laron et al, 1966). This condition was subsequently named Laron syndrome causing GH resistance (inability of GH to bind to its respective receptor). Tthe causative gene, the growth hormone receptor gene (*GHR*), was discovered through gene linkage methods in two consanguineous families hailing from the Mediterranean region (**publication in 7.1**).

Impaired IGF-1 production caused by biallelic mutations in the IGF-1 gene is another known cause of short stature. Because IGF-1 secretion during intrauterine development is independent to the action of GH, children with primary GHD or growth hormone resistance have normal birth parameters and their growth disorder typically begins postnatally. In extremely rare cases of primary impairment of IGF-1 production due to *IGF1* gene defects, affected children are born small for gestational age (SGA). IGF-1 also plays an essential role in the development of the central nervous system: therefore, these children have microcephaly, psychomotor retardation and hearing impairment as well (Dauber et al. 2014).

Variants in the gene coding for the IGF-1 receptor (*IGF1R*) also lead to milder growth impairment, which is distinguished from the previous disorder by high serum IGF-1 concentrations (Baron et al, 2015).

1.2. Extracellular matrix cartilage protein defects

Chondrocytes in the growth plate secrete an extracellular matrix rich in collagens and proteoglycans. This is important for the structure and function of the growth plate. Therefore, the *ACAN* gene, which encodes the protein fraction of the aggrecan proteoglycan, is one of the genes that cause growth disruption. Other causes of short stature with impaired extracellular matrix structure include collagen regulating genes such as *COL1A2*, *COL2A1*, *COL11A1*, fibrillin genes such as *FBN1* and matrilin genes such as *MATN3* (Jee at al, 2017). These gene mutations always cause milder forms of short stature in heterozygous form (seen in non-consanguineous families) and more severe forms in homozygous / recessive form (seen in consanguineous families. Consanguineous families have helped extend the disease phenotype and genotype-phenotype relationship such as in the case gene *MATN3* where a novel form of autosomal recessive spondylo-epi-metaphyseal dysplasia was described in a large consanguineous family with 5 affected individuals (Borochowitz et al, 2004).

1.3.Disorders of paracrine regulation

Paracrine factors in the growth place coordinate changes in chondrocyte morphology, proliferation, differentiation and matrix assembly. Fibroblast growth factors (FGFs) play an important role in paracrine regulation (Jee et al, 2017). Pathogenic variants in the *FGFR3* gene affect bone growth and lead to a wide range of phenotypes – from hypochondroplasia to achondroplasia to the most severe form, thanatophoric dysplasia, a perinatally lethal form of skeletal dysplasia. Another important paracrine regulator is type C natriuretic peptide (CNP), which binds to the NPR2 receptor. Homozygous inactivating mutations in the *NPR2* gene cause severe acromesomelic Maroteaux dysplasia whereas heterozygous inactivating mutations cause milder forms of isolated short stature (Bartels et al, 2004).

1.4.Defects in intracellular pathways

Defects in intracellular signalling cause a wide heterogenic spectrum of growth failure disorders. Among them are “RASopathies” which include Coffin-Lowry syndrome, Noonan syndrome, Costello syndrome, LEOPARD syndrome, and others. RASopathies are caused by defects in the Ras / MAPK signalling cascade which plays a role in information transfer

between the growth factor membrane receptor and the cell nucleus (Lebl, et al, 2020). In the cell nucleus, it regulates the transcription of several genes responsible for cell proliferation, migration, differentiation and apoptosis. Abnormal activation of this cascade leads to the above-mentioned syndromes (Lebl, et al, 2020). Genes such as *PTPN11*, *KRAS*, *SOS1*, *BRAF* and others have been published as causing RASopathies and in 60% cases the inheritance is de novo heterozygous (Jee et al, 2017).

Mutations in transcriptional factor genes which are involved in transcription repression can cause short stature as well. Short stature homeobox-containing gene (*SHOX*) mutations influence a transcription factor important for growth plate chondrocyte function. These mutations cause severe skeletal dysplasia in homozygous form, these patients characteristically have severe short stature, mesomelia and the Madelung deformity, whereas heterozygous mutations cause a milder form of skeletal dysplasia or with idiopathic short stature (Shears, et al, 1998).

1.5.DNA repair defects

Impairment of DNA repair can result in forms of very severe short stature such as primordial dwarfism. One example is the gene *PCNT* which encodes pericentrin, a centrosomal protein. This gene was discovered as causing microcephalic osteodysplastic primordial dwarfism type II (MOPD II) in 2008 by linkage analysis studies in 3 consanguineous families (Rauch et al, 2008). This is a very rare autosomal recessive disorder which causes patients to have a birth weight typically less than 1500 g at term and an adult height of less than 100 cm.

5. Hypotheses and aims of the dissertation project

5.1. 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.

5.2. 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

6. Methods

6.1. Data collection including cohort selection (see 7.2 – 7.8 for further information)

Patients in the primary 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 Paediatric Clinic, 2nd Faculty of Medicine, Charles University in Prague.

6.1.1. Congenital hyperinsulinism

We examined three children with CHI who were diagnosed on the 6th day, 3rd week and 3rd year of life respectively with recurrent hypoglycaemia often combined with convulsions and unconsciousness. There was a notable history of unexplained neonatal deaths in siblings within two of these families.

6.1.2. Monogenic diabetes

Data from 754 patients registered at the diabetic clinic, Dr. Jamal Ahmad Rashed hospital, Sulaimani, Kurdistan, 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.

6.1.3. Short stature

6.1.3.1. Consanguineous study (further details can be found in 7.4)

Out of 1124 children examined with short stature of uncertain aetiology at the endocrine clinic, Dr. Jamal Ahmad Rashed hospital in Sulaimani, Kurdistan, Iraq between January 2018 and February 2020. 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 study with informed consent.

6.1.3.2. Non-consanguineous studies (further details can be found in 7.5, 7.6 and 7.7)

For this first part of the study, a cohort of children born SGA with persistent short stature (SGA-SS) were selected from over 800 children treated with GH at our centre between May 2008 and December 2018. Extremely preterm children (gestational age <28 weeks) were excluded due to missing relevant normative values for their size at birth. After the excluding causes of secondary GHD and children with a life-minimum height >−2.5 SD), 256 children with SGA-SS (birth weight or length <−2 SD and body height <−2.5 SD after 3 years of life) remained for further evaluation. Out of them, 176/256 (69%) families agreed to genetic testing to elucidate the genetic background of short stature in SGA.

The second part of the study cohort was selected from a similar pool of children treated with GH at our centre until December 2021. Out of these, 70 had primary GHD and vertically transmitted short stature defined as height SDS ≤-2 SD in both the child and his/her shorter parent. Out of them, 52/70 (74%) families agreed to genetic testing.

6.2. Genetic examination

6.2.1. Whole Exome Sequencing (WES)

Genomic DNA was extracted from peripheral blood using the QIAmp DNA Blood Mini system (Qiagen, Hilden, Germany). DNA was analysed using whole-exome sequencing (WES). WES was performed using SureSelect Human All Exon Kit V6 + UTRs (Agilent Technologies, Santa Clara, CA), and the indexed products were sequenced by synthesis in an Illumina NextSeq 500 analyser (San Diego, CA). Our bioinformatic pipeline is based on the Genome Analysis Toolkit version 4.1.9.0 developed by Broad Institute (Van der Auwera, 2020). First step of the protocol is performed by the Burrows-Wheeler Aligner (Li, 2009) with Maximal Exact Match that maps the sequence reads from input FASTQ files to the human reference genome to produce a mapping file in SAM/BAM format sorted by coordinate. In order to call germline variants, the HaplotypeCaller tool is used (Van der Auwera, 2020). Two more tools are integrated into our pipeline: SavvyCNV that detects copy number variants using a bin size of 2 kbp (Laver, 2022) and SavvyHomozygosity that determines runs of homozygosity/autozygosity in the genome.

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 implemented in the VarSome software as pathogenic (P), likely pathogenic (LP), benign (B), likely benign (LB), or as variants of uncertain significance (VUS) (Richards et al, 2015). All the variants with potential clinical significance were confirmed using Sanger sequencing. In order to evaluate the segregation of genetic variants within families, Sanger sequencing was performed in both parents, and healthy/affective siblings (when available) with informed consent.

6.2.2. Additional testing methods – congenital hyperinsulinism

DNA was analyzed primarily using methods of direct Sanger sequencing by sequencer ABI 3130xl. The complete coding regions and intron-exon boundaries of genes *ABCC8* and *KCNJ11* were analyzed as two of the most common causes of CHI. All possibly pathogenic variants were thereafter evaluated according to the American College of Medical Genetics and Genomics standards and guidelines (Richards et al, 2015).

6.2.3. Additional testing methods – monogenic diabetes

All probands with neonatal diabetes without a detected causal variant using WES underwent Sanger sequencing of the *PTF1A* enhancer region and Multiplex-ligation dependent probe amplification (MLPA) in order to detect the aberrant methylation and/or gene dosage of chromosomal region 6q22, 6q24, and 11p15 linked to transient neonatal diabetes (ME033, MRC Holland, Amsterdam, Netherlands).

6.2.4. Additional testing methods – short stature

In probands without a detected causal variant using WES, we carried out MLPA (probe mixes ME030 and ME032 examining regions of 11p15, 7q32, 7p12 and 14q32, respectively and subsequent data analyses by software Coffalyser were performed according to manufacturer's instructions, MRC Holland, Amsterdam, The Netherlands). and arrayCGH testing to look for Silver-Russell Syndrome and micro-deletions /micro-duplications respectively.

6.3. Statistical analysis (further details in 7.3. and 7.4)

Statistical significance, where applicable, was calculated using a chi-square test or Fisher's two-sided exact test.

7. Publications with an accompanying discussion

7.1. Amaratunga SA, Tayeb TH, Dusatkova P, Pruhova S, Lebl J. Invaluable Role of Consanguinity in Providing Insight into Paediatric Endocrine Conditions: Lessons Learnt from Congenital Hyperinsulinism, Monogenic Diabetes, and Short Stature. Horm Res Paediatr. 2022;95(1):1-11

This review highlights the pivotal role of consanguineous families in gene discovery and advancing our understanding of pathophysiological mechanisms. It traces the historical journey of discoveries in paediatric endocrinology, such as the discovery of the first K_{ATP} channel genes (*ABCC8* and *KCNJ11*) responsible for CHI in the 1990s, to more recent breakthroughs like mutations in *YIPF5* causing monogenic diabetes by disrupting endoplasmic reticulum (ER)-to-Golgi trafficking in beta cells and increasing ER stress.

These positive genetic findings in children from consanguineous backgrounds have been instrumental in unravelling novel genes and disease mechanisms, significantly advancing our knowledge of disease pathophysiology. The review's central aim is to shed light on the insights gained from consanguineous pedigrees through three fundamental endocrine conditions, each representing varying levels of pathophysiological complexity: from the relatively straightforward CHI to monogenic diabetes, which, despite diverse causes, manifests with uniform biochemical parameters (hyperglycemia and glycosuria), up to the intricate domain of genetic regulation in human growth, one of the most complex developmental phenomena.

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Mini Review Article

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Invaluable Role of Consanguinity in Providing Insight into Paediatric Endocrine Conditions: Lessons Learnt from Congenital Hyperinsulinism, Monogenic Diabetes, and Short Stature

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Novel genes · Genetic testing · Consanguineous families · Paediatric endocrinology · Endocrine genes

Abstract
Consanguineous families have often played a role in the discovery of novel genes, especially in paediatric endocrinology. At this time, it has been estimated that over 8.5% of all children worldwide have consanguineous parents. Consanguinity is linked to demographic, cultural, and religious practises and is more common in some areas around the world than others. In children with endocrine conditions from consanguineous families, there is a greater probability that a single-gene condition with autosomal recessive inheritance is causative. From 1966 and the first description of Laron syndrome, through the discovery of the first K_{ATP} channel genes *ABCC8* and *KCNJ11* causing congenital hyperinsulinism (CHI) in the 1990s, to recent discoveries of mutations in *YIPF5* as the first cause of monogenic diabetes due to the disruption of the endoplasmic reticulum (ER)-to-Golgi trafficking in the β-cell and increased ER stress; positive genetic findings in children from consanguinity have been important in elucidating novel genes and mechanisms of disease, thereby expanding knowledge into disease pathophysiology. The aim of this narrative review was to shed light on the lessons learned from consanguineous pedigrees with the help of 3 fundamental endocrine conditions that represent an evolving spectrum of pathophysiological complexity – from CHI, a typically single-cell condition, to monogenic diabetes which presents with uniform biochemical parameters (hyperglycaemia and glycosuria), despite varying aetiologies, up to the genetic regulation of human growth – the most complex developmental phenomenon.

Introduction

Five per cent of all live births have genetic disorders that are recognizable until 25 years of age [1]. These genetic disorders are being increasingly diagnosed in the human population due to developments in genetic testing technology and tools such as next generation sequencing (NGS).

The knowledge on the genetic cause of disease allows precise and individualized clinical management, faster diagnosis without the need of invasive diagnostic tests (in

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some cases), prediction of long-term outcome and family planning. The decision on carrying out genetic examination is not only based on the potential therapeutic benefit, but on the feasibility, availability, and cost-effectiveness as well [2]. Prior to the indication of genetic testing, a comprehensive medical history should be obtained; this should include the presence of consanguinity in the family.

Due to the impact that paediatric endocrine disorders can have on a child's metabolism, electrolyte balance, and growth and development, they represent a substantial part of paediatric morbidity and mortality. However, multiple conditions remain genetically and/or pathophysiologically unexplained.

Positive genetic findings in patients continue to contribute in a significant way towards elucidating the specific pathophysiological mechanisms of disease in complex phenotypes and clarifying genotype – phenotype relationships. Consanguineous families have especially played a role in the discovery of novel genes.

Consanguinity

"Consanguinity" or "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. Consanguinity is linked to demographic, cultural, and religious practises [3]. These practises served many purposes from ancient times, and are more common in some areas around the world than others, especially in Asia, Africa, and the Middle-East [3]. In modern times, despite increased awareness on the possible health consequences for children of consanguineous families, it has been estimated that over 20% of the world population live in communities which prefer such marriages and that over 8.5% of all children worldwide have consanguineous parents [4].

In a large genetic study performed in Egypt, recessive disorders were found mainly among families with consanguinity (78.8%). Consanguinity was present in 100% of cases of mental retardation and in 92.6% of patients with limb anomalies. Child deaths and stillbirths were more prevalent among children from consanguineous parents when compared to non-consanguineous families as well [4].

The higher frequency of recessive genetic conditions in descendants of consanguineous parents than those of unrelated parents can be attributed to less common alleles manifesting as homozygous. For example, first cousins are predicted to share 1/8th of their genes. Therefore, their progeny will be homozygous (or autozygous) at 1/16th of gene loci. In other words, this means that they will have identical gene copies from each parent at these sites in their genome; these are called "runs of homozygosity" [5]. Thereby, consanguinity represents a substantial genetic burden for the offspring.

If a variant with a heterozygosity frequency of 1 in 100 is considered, there is a 1 in 10,000 chance that both unrelated spouses are carriers ($1/100 \times 1/100 = 1/10{,}000$). However, if they are first cousins, the chance of both being carriers of this pathogenic variant is 12.5 times higher, thereby the chance is 1 in 800 ($1/100 \times 1/8 = 1/800$). In families with multiple consanguinity, this risk would be even higher [6].

Thus, when a rare and complex monogenic condition is suspected, consanguineous families provide the best chances for novel gene discovery. This has been proven over time in scientific research, even in research into paediatric endocrine disorders, which will be elaborated in the individual sections below.

Genetic Testing Methods

Karyotype testing was the only available method for identifying genetic defects until the 1970s. It was possible to identify chromosome aberrations and other structural abnormalities (major translocations, deletions, and inversions) using karyotyping [7]. In 1977, Sanger et al. [8] first described the basic concepts of DNA sequencing as a method to detect point mutations and with the growing popularity of polymerase chain reaction, and this method was widely used allowing amplification of a targeted region of DNA. Sanger sequencing still remains the gold standard for identification of mutations, using the "candidate gene approach" in situations where a specific gene is suspected based on the patient phenotype [7].

In the 1990s, methods of fluorescent in situ hybridization, array comparative genomic hybridization, and linkage analysis were developed. These techniques lead to the discovery of many novel genes, though limited to the detection of large copy number variants [7].

The successful sequencing of the entire human genome in 2001 led to a boom of genetic testing techniques including NGS over the last 2 decades [7]. NGS methods (the present standard when genetically examining patients with complex phenotypes) have unified all abovementioned methods and provide a way to detect all mutation types – single nucleotide variants, insertions or deletions,

Table 1. Overview of genes mentioned in the manuscript (in the order of mention) and the role of consanguinity in their discovery

Gene	Condition caused	First published	Method used for gene discovery	Role of consanguinity	Ref
ABCC8	CHI, neonatal diabetes	1995	FISH, direct Sanger sequencing	By testing affected individuals from 9 consanguineous families	[19]
KCNJ11	CHI, neonatal diabetes	1996	Direct Sanger sequencing	By testing 1 affected individual from a consanguineous family	[20]
PTF1A	Neonatal diabetes	2003	Genome-wide linkage analysis	By testing 3 affected individuals from a consanguineous family	[26]
YIPF5	Neonatal diabetes	2020	WGS	By testing 6 affected consanguineous children	[27]
WFS1	Syndromic/neonatal diabetes	1998	Genetic linkage analysis	One of 5 tested families was consanguineous	[29]
EIF2AK3	Syndromic/neonatal diabetes	2000	Genome-wide linkage analysis	By testing done in 2 affected consanguineous children	[30]
SLC19A2	Syndromic diabetes	1999	Positional cloning and direct Sanger sequencing	By testing in 6 families, 3 reported a history of consanguinity	[31]
IL2RA	Autoimmune monogenic diabetes	1997	RT-PCR and sequencing	By testing an affected child from a first-cousin marriage	[37]
LRBA	Autoimmune monogenic diabetes	2012	Genetic linkage analysis	By studying 5 affected individuals from 4 consanguineous families	[38]
GHR	Short stature	1989	Genetic linkage analysis	By studying affected children from 2 consanguineous families	[42]
GH1	Short stature	1981	Restriction endonuclease analysis	By studying 2 siblings from first-cousin parents	[46]
GHRHR	Short stature	1996	Direct Sanger sequencing	By testing several severe affected members of a consanguineous family	[47]
POU1F1	Short stature	1992	Direct Sanger sequencing	By testing an affected child from 1 consanguineous family	[50]
PAPPA2	Short stature	2016	WES	One of 2 tested affected families was consanguineous	[51]
PCNT	Short stature	2008	Genome-wide linkage analysis	By testing 4 affected children from 3 consanguineous families	[55]

RT, reverse transcriptase; PCR, Polymerase Chain Reaction; WGS, Whole Genome Sequencing; WES, Whole Exome Sequencing; FISH, fluorescent in situ hybridization.

structural variants, and copy number variants. However, a vital factor is the selection of the library to be adopted (due to cost and interpretation of results) [9]. The library refers to the extent of the human genome which is examined, and some types of libraries are custom-targeted panels (t-NGS), whole-exome sequencing (WES – which is focused on the 1% protein coding region of the genome), or whole-genome sequencing. T-NGS is a suitable, affordable tool to analyse many patients with a specific diagnosis (which is caused by a known group of genes) if a suitable gene panel has been created and tested [9].

The data obtained by NGS methods need to be analysed by a process called a bioinformatics pipeline [7]. Technical advancements have facilitated the storage and rapid exchange of these data, allowing more efficient analysis (using bioinformatics software capable of gathering comprehensive information about a single variant), consulting of variants, and international collaboration [7, 9]. The gold standard in variant classification is the American College of Medical Genetics and Genomics (ACMG) guidelines, which classifies possible variants in a range from benign to pathogenic [10]. Pathogenic variants are confirmed by Sanger sequencing [8].

Genetic testing methods used in consanguineous families are not largely different from methods used in non-consanguineous families. It is important to note the higher percentage of "runs of homozygosity" in consanguineous individuals and the importance of using homozygous

markers (homozygosity mapping) to discover the true genetic cause, especially when having many variants of unknown significance [11].

Genetic Causes of Specific Disorders: Insight into the Role of Consanguinity

In this review, we will focus on the 3 groups of paediatric endocrine conditions to highlight the contribution of consanguineous offspring to elucidate their genetic and pathophysiological background (Table 1). These conditions are representative of gradients of pathophysiological complexity, congenital hyperinsulinism (CHI) (a "single cell condition") being the most straightforward and short stature the most complex. We believe that highlighting the evolution of genetic knowledge in these 3 conditions will provide an overall picture, shared by almost all paediatric endocrine disorders. It is important to note that this review does not include all genes that were discovered with the help of consanguinity; the genes mentioned in detail under each condition were selected due to their prominence and frequency in literature.

Congenital Hyperinsulinism

CHI is a heterogeneous genetic condition caused by a primary genetic defect of the pancreatic β-cell resulting in uncontrolled insulin secretion. The incidence of CHI has been estimated to be around 1:2,500 in communities with a higher rate of consanguinity, whereas it is 1:50,000 in non-consanguineous populations [12]. Similar to some other conditions, it has been clearly shown that not only the incidence and genetic causes of CHI, but also the mode of inheritance differs crucially between high- and low-consanguineous areas, with recessive (biallelic) transmission prevailing in high-consanguineous, and monoallelic (dominant/uniparental isodisomy) transmission prevailing in low-consanguineous areas [13]. In CHI, the mode of inheritance directly impacts disease severity, for example, recessive biallelic variants mostly cause (more severe) diffuse CHI and paternally inherited monoallelic variants cause focal CHI, where complete surgical resection of the lesion can be curative [14].

The pancreatic β-cell has the capability to detect the current blood glucose concentration and to produce, store, and release insulin to the bloodstream to maintain a normal blood glucose concentration without the risk of hypoglycaemia [15]. The pathophysiology of CHI is mostly based around genetic defects of β-cell function (shown in Fig. 1). Genetic testing in children with CHI is crucial for confirming diagnosis and tailoring therapy; it may play an important role in genetic counselling, or to explain fatal outcomes in previous offspring [16].

The 2 most common genes known to cause CHI are *ABCC8* and *KCNJ11* (encoding the SUR1 and Kir6.2 subunits of the ATP-sensitive K+ [K_{ATP}] channel in the pancreatic β-cell). Pathogenic variants in these genes cause the most severe forms of CHI, due to the loss of K_{ATP} channel activity, persistent membrane depolarization, and continuous insulin release, regardless of the blood glucose concentration [17].

Linkage analysis studies done by Glasser et al. [18] in 1994 in 15 families lead to CHI being mapped to chromosome 11p14–15.1. This confirmed the existence of a Mendelian disease locus for the condition [18]. Twelve were Ashkenazi Jewish families (most came from areas where marriage between second and third cousins was common), and 2 were Arabic (with confirmed consanguinity). In 1995, Thomas et al. [19] mapped SUR to chromosome 11p15.1 by using fluorescent in situ hybridization. By testing affected individuals from 9 consanguineous families, he identified 2 SUR gene splice site mutations, which segregated with disease, thereby confirming the existence of the *ABCC8* gene. This signified the start of a deeper understanding into the pathophysiological relationship of the pancreatic β-cell function with K_{ATP} channel proteins in CHI.

In 1996, Tomas et al. [20] considered Kir6.2 as another candidate gene for CHI because of its close location to the SUR gene and its necessity for the functioning of the β-cell K_{ATP} channel. They found a homozygous missense variant in a child with CHI from a consanguineous family, confirming that variants in gene Kir6.2, currently called *KCNJ11*, cause CHI as well.

At the point of writing this article, pathogenic variants causing CHI have been found in over 15 genes including *GLUD1*, *GCK*, *HNF4A,* and *HNF1A* (shown in Fig. 1) [16]. In addition, some genetic syndromes in non-consanguineous populations such as Beckwith-Wiedemann, Kabuki, and Turner syndromes have been associated with hyperinsulinism as well [17, 21].

Diabetes Mellitus

Diabetes mellitus (DM) is a group of conditions that result from an absolute or relative insulin deficiency, with or without insulin resistance, which leads to hyperglycae-

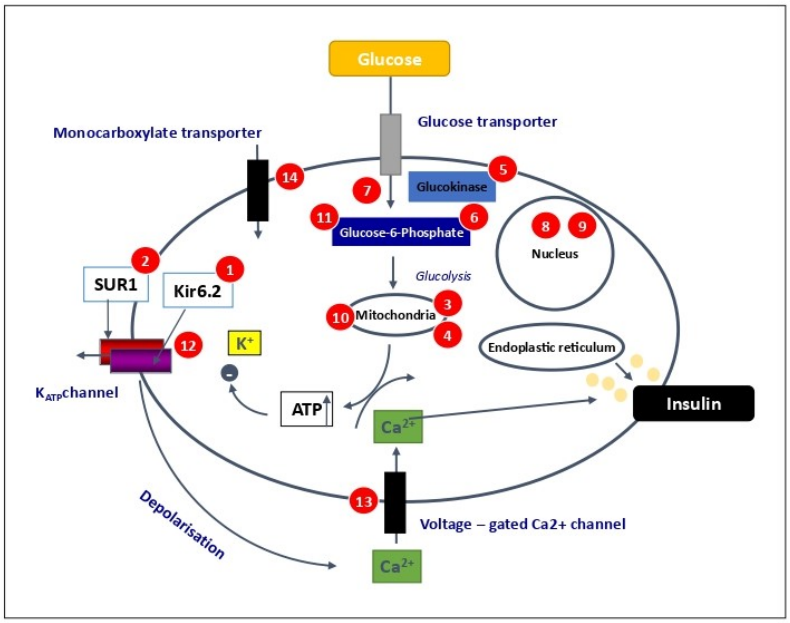

Fig. 1. Functional components of the pancreatic β-cell and the molecular pathophysiology of CHI. Cellular locations of proteins encoded by genes causative for CHI are assigned the following numbers: 1 – *KCNJ11*, 2 – *ABCC8*, 3 – *GLUD1*, 4 – *HADH1*, 5 – *GCK*, 6 – *PGM1*, 7 – *HK1*, 8 – *HNF4A*, 9 – *HNF1A*, 10 – *UCP2*, 11 – *PMM2*, 12 – *FOXA2*, 13 – *CACNA1D*, 14 – *SLC16A1*.

mia and glycosuria. In addition to type 1 DM and type 2 DM, which are polygenic diseases with significant environmental components, the less frequent monogenic forms of diabetes continue to help uncover various biological processes causing diabetes due to insufficient insulin production or defects in insulin action [22].

Monogenic diabetes comprises of partially overlapping subtypes, such as neonatal DM (NDM), syndromic diabetes, autoimmune monogenic diabetes, and maturity-onset diabetes of the young. Maturity-onset diabetes of the young follows a pattern of autosomal dominant inheritance while syndromic diabetes is almost exclusively recessive, and NDM and autoimmune monogenic diabetes show both forms of inheritance [23]. The prevalence of monogenic diabetes in the UK paediatric diabetic population was 2.5% in a 2016 study examining 808 patients with positive urinary C-peptide creatinine ratio and negative islet cell-antibodies [23]. A similar Italian study showed a prevalence of 6.5% [24].

However, even though such studies have not yet been carried out in a population with prevalent consanguinity, it has been clearly shown that (among children with permanent NDM) the spectrum of genetic aetiologies differs largely between areas with high and low rates of consanguinity (shown in Fig. 2). In consanguineous regions, the most common gene was *EIF2AK3* causing Wolcott-Rallison syndrome and genes *KCNJ11* and *ABCC8* accounted only for 12% of cases, whereas in the non-consanguineous areas – these 2 genes prevailed (46%). Predictably, there was a much higher incidence of recessive forms of permanent NDM (81% of recessive findings were from consanguineous offspring vs. 13% in non-consanguineous offspring) [25].

NDM (diabetes which develops before 6 months of age) is caused by single-gene mutations (in genes such as *ABCC8*, *KCNJ11*, *GCK*, *PDX1*, *INS*, *GATA6*, *NEUROG3*, etc.) which reduce pancreatic β cell number or impact its' function [24] (shown in Fig. 2, 3). Many genes such as *PTF1A* were discovered due to genetic testing on consanguineous families [26]. In 2020, Franco et al. [27] identified the first gene (*YIPF5*) to cause NDM by increased endoplasmic reticulum (ER) stress caused by the disruption of ER-to-Golgi trafficking in the β-cell. *YIPF5* was described in 6 consanguineous children with NDM, microcephaly, and epilepsy. This helped further highlight the overlap in genes (such as *CNOT1*) crucial for the development and functioning of both β cells and neurons [27].

Monogenic syndromic diabetes is typically characterized by the negativity of beta-cell autoantibodies and additional (typically non-autoimmune) phenotypic abnormalities. In 1977, children with diabetes insipidus, DM,

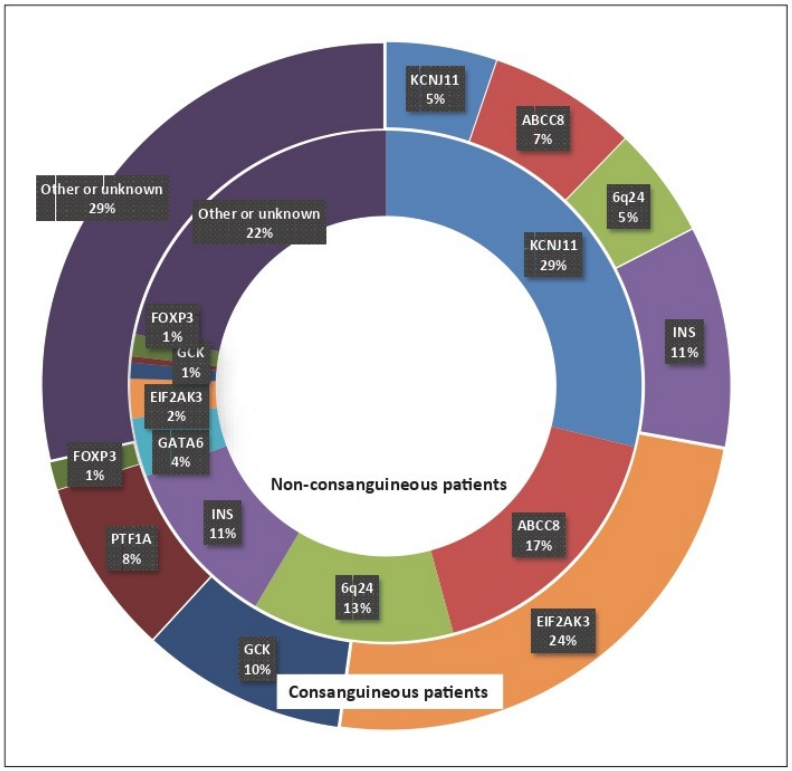

Fig. 2. Spectrum of genes causing permanent neonatal diabetes in patients born to non-consanguineous parents in comparison with children born to consanguineous parents (diagram adapted from reference [24]).

optic atrophy, and deafness (DIDMOAD, later called Wolfram syndrome) were observed, particularly in consanguineous families with healthy parents and siblings, suggesting a condition with autosomal recessive inheritance [28]. It was later mapped due to homozygous mutations in gene *WFS1*-encoding wolframin (an ER protein involved in the regulation of the response to ER stress) [29]. Testing in 2 consanguineous families led to gene discovery (*EIF2AK3* – an ER protein which is a key ER stress transducer) in previously mentioned Wolcott-Rallison syndrome which causes PNDM [30].

Another example is recessively inherited thiamine-responsive megaloblastic anaemia causing megaloblastic anaemia, DM, and sensorineural deafness. This was mapped to gene *SLC19A2* in 1999 by testing in 6 families, 3 reported a history of consanguinity; however, they were all shown to be linked to a gene region based on homozygous markers, suggesting linkage disequilibrium and the existence of a founder mutation [31].

Autoimmune monogenic diabetes represents an overlap between classical T1DM and syndromic monogenic diabetes, and typically manifests at an early age. Two monogenic syndromes of polyglandular autoimmunity were genetically elucidated more than 20 years ago: the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome and the X-linked (IPEX) syndrome caused by variants in the *AIRE* and *FOXP3* genes, respectively [32, 33]. These are rare diseases but, for example, the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome has been detected at high rates among certain populations such as the Finnish (1:25,000), Sardinian (1:14,000), and Iranian Jewish (1:6,500–1:9,000) as a result of founder mutations [34, 35]. The higher frequency in Iranian Jews can be additionally attributed to the presence of consanguinity in this population. Since then, multiple other genes responsible for autoimmune monogenic diabetes have been described (shown in Fig. 3) [36]. These genes can be divided further into genes causing autosomal recessive forms of autoimmune monogenic diabetes (*IL2RA* and *LRBA*), X-linked recessive forms (*FOXP3*), dominant forms (*CTLA4* and *STAT3*), and genes that can be inherited both ways (*AIRE*)

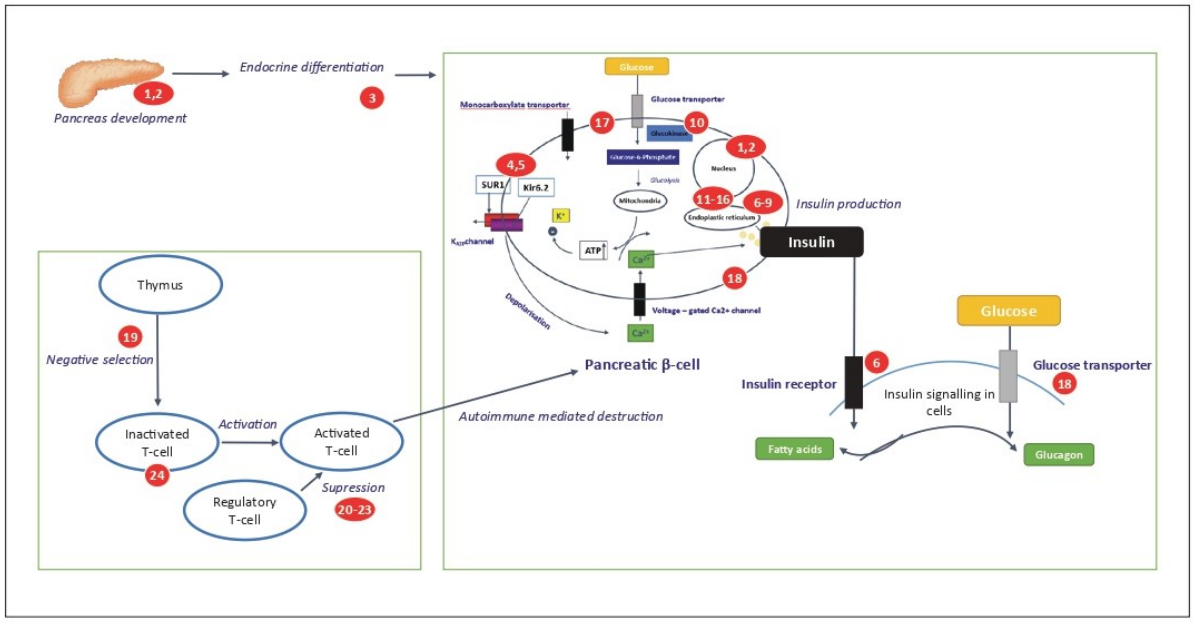

Fig. 3. Overview of the molecular pathophysiology of monogenic diabetes. Functional locations of proteins encoded by causative genes (mentioned in the manuscript) are assigned the following numbers: 1 – *PDX1*, 2 – *CNOT1* (pancreas development genes), 3 – *NEUROG3* (an endocrine differentiation gene), 4 – *ABCC8*, 5 – *KCNJ11* (K_{ATP} channel genes), 6 – *INS*, 7 – *EIF2AK3*, 8 – *WFS1*, 9 – *YIPF5* (insulin and ER genes), 10 – *GCK* (glucokinase gene), 11 – *HNF4A*, 12 – *HNF1A*, 13 – *HNF1B*, 14 - *PTF1A*, 15 – *FOXP3*, 16 – *GATA6* (transcription factor genes present in the nucleus), 17 – *SLC19A2*, 18 – *GLUT1* (transporter genes), 19 – *AIRE*, 20 – *FOXP3*, 21 – *IL2RA*, 22 – *LRBA*, 23 – *CTLA4*, 24 – *STAT3* (immune regulating genes).

(shown in Fig. 3) [36]. Consanguinity played a role in the discovery of *IL2RA* (found in a boy from a first-cousin marriage with an immunodeficiency syndrome) and *LRBA* (due to genetic linkage analysis carried out in consanguineous families with hypogammaglobulinemia) [37, 38].

Growth Regulation Disorders/Short Stature

Many different genes impact growth by various mechanisms. The hypothalamic-pituitary axis of growth hormone (GH) and insulin-like growth factor (IGF)-1 was long believed to be the main linear growth regulator in children. However, recent studies have shown that it is rather regulated by 2 major tiered pathways – the GH-IGF-1 axis and the complex regulation of growth plate chondrocytes and the extracellular cartilaginous matrix (shown in Fig. 4) [39, 40].

GH-IGF-1 Axis

Mutations in genes regulating steps in the entire GH-IGF-1 axis, ranging from GH deficiency (GHD), primary IGF-1 deficiency to IGF-1 resistance may cause short stature. The first genetic syndrome related to this axis was clinically described by Laron et al. [41] in 1966. It was characterized by severe growth retardation, obesity, and small genitalia in 3 siblings from a consanguineous Jewish family and was later named Laron syndrome [41]. It was characterized by high serum GH concentrations and low to undetectable IGF-1 concentrations. In patients with this rare syndrome, GH resistance was demonstrated by the administration of exogenous human GH for 7 days, which did not lead to IGF-1 increase [42]. Later, in 1984, Eshet et al. [43] proved that the mechanism of GH resistance is due to an inability of GH to bind to the respective receptor. Soon thereafter, in 1989, the GH receptor gene was described as causative by Amselem et al. [42], by using gene linkage methods in 2 consanguineous families from the Mediterranean region.

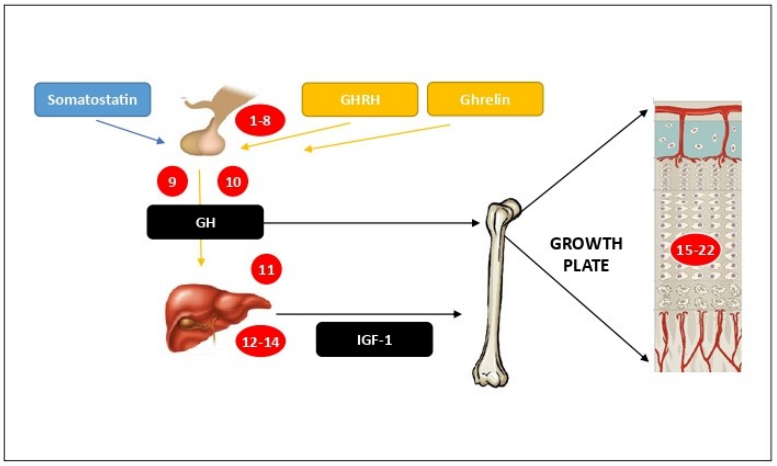

Fig. 4. Overview of the molecular pathophysiology of short stature due to genetic defects in the GH-IGF-1 axis and the growth plate. Functional locations of proteins encoded by causative genes (mentioned in the manuscript) are assigned the following numbers: 1 – *POU1F1*, 2 – *PROP1*, 3 – *GLI2*, 4 – *FGF8*, 5 – *HESX1*, 6 – *SOX2*, 7 – *SOX3*, 8 – *FOXA2* (pituitary transcription factors), 9 – *GH1*, 10 – *GHRHR*, 11 – *GHR*, 12 – *PAPPA2*, 13 – *IGF-1R*, 14 – *ALS* (other genes causing short stature by affecting the GH-IGF-1 axis), 15– *COL1A2*, 16 – *COL2A1*, 17 – *COL11A1*, 18 – *FBN1*, 19 – *MATN3* (extracellular matrix genes), 20 – *NPR2* (paracrine signalling gene), 21 – *SHOX* (gene involved in intracellular pathways), 22 – *PCNT* (gene involved in cell division and DNA replication/repair).

During this time, the genetic transmission of severe isolated GH deficiency (IGHD) was studied as well. This occurs with an incidence of between 1 in 4,000 and 1 in 10,000 live births [44]. Classical genetic causes of IGHD include mutations of the gene-encoding GH (*GH1*) and the GH releasing hormone receptor (*GHRHR*). Thus far, there have been no instances of GHD as a result of mutations in GH releasing hormone (*GHRH*) itself [44].

Historically, genetic forms of IGHD were classified into 4 types, depending on the inheritance pattern, as autosomal recessive (types IA and IB), dominant (type II), or X-linked (type III) [45]. This classification no longer applies due to expanding knowledge into the pathophysiology of GHD. Autosomal recessive IGHD type IA was first described in patients with homozygous *GH1* deletions, including 2 siblings from Italian first-cousin parents having IGHD due to a 7.6 kb deletion in the GH gene cluster [46]. Patients present with severe growth failure by 6 months of age with undetectable GH concentrations. Some of these children tend to develop antibodies on treatment, thereby resulting in poor response to GH therapy [46].

Subsequently, the first homozygous loss-of-function *GHRHR* mutation causing profound IGHD was described in 2 patients from a consanguineous Indian family with extreme short stature, frontal bossing, truncal obesity, and severe GHD. These siblings failed to respond to neither GH stimulation tests nor repetitive GHRH stimulation [47]. This was followed by the description of the "dwarfs of Sindh" a cluster of consanguineous patients from Sindh, Pakistan, with severe GHD and *GHRHR* mutations which results in the inability to transmit the GHRH signal [48].

Short stature can be caused by conditions causing combined pituitary hormone deficiencies as well. Pituitary development is governed by the activation of a cascade of transcription factors that orchestrate pituitary morphogenesis and differentiation. Thereby, defects in pituitary transcription factor genes such as *POU1F1*, *PROP1*, *GLI2*, *FGF8*, *HESX1, SOX2*, *SOX3*, *FOXA2,* and others been found to lead to varying degrees of combined pituitary hormone deficiencies [44, 49].

The first gene which was shown to cause multiple pituitary hormone deficiency in humans was *POU1F1* (originally *PIT1*). A biallelic pathogenic variant was described in 1992 in 2 sisters from a consanguineous family with a clinical presentation suggestive of cretinism. Hormonal workup showed profound deficiencies of thyroid-stimulating hormone, GH, and prolactin. These patients were the first described case of a defect of a transcriptional activator causing deficiency of multiple target genes [50].

Recently, 2 families (one of which was consanguineous) with children with progressive postnatal growth failure and markedly elevated serum concentrations of IGF-1, IGF-binding proteins (IGFBP)3, IGFBP5, acid labile subunit, and IGF-2 concentrations were reported. This was shown to be resulting from homozygous loss of function of the *PAPPA2* gene, which leads to low IGF-1 bioavailability. This indicates that PAPPA2 is a key regulator of IGF-1 bioavailability by regulating the proportion of IGF-1 that is bound to IGF-binding proteins [51].

Growth Plate Chondrocytes and Extracellular Matrix

Disorders of chondrocyte paracrine regulation (for example, *NPR2* defects), defects in intracellular pathways such as "RASopathies" or mutations in transcriptional factor genes (for example, *SHOX*) cause short stature as well [40, 44, 52, 53]. Homozygous inactivating mutations in *NPR2* cause severe acromesomelic Maroteaux dysplasia and was first described in 2004 via testing done in a predominantly consanguineous cohort [54].

Impairment of DNA repair can result in rare autosomal recessive forms of very severe short stature (such as microcephalic osteodysplastic primordial dwarfism type II – birth weight typically <1,500 g at term and an adult height of <100 cm). One example is the gene *PCNT* which encodes pericentrin (a centrosomal protein) first discovered by linkage analysis studies in 2 consanguineous families [55].

Extracellular matrix structure genes including collagen regulating genes such as *COL1A2*, *COL2A1*, *COL11A1*, fibrillin genes such as *FBN1* and matrilin genes such as *MATN3* impact growth as well [52, 56]. These gene mutations most often cause milder forms of short stature in heterozygous form and more severe forms in homozygous/recessive form. Consanguineous families have helped extend the disease phenotype and genotype-phenotype relationship such as in the case of gene *MATN3* where a novel form of autosomal recessive spondylo-epimetaphyseal dysplasia caused by homozygous *MATN3* mutations was described in a large consanguineous family with 5 affected individuals [57].

Conclusion

The presence of consanguinity in families with children with endocrine disease represents a substantial genetic burden for the offspring due to the higher probability of a single-gene condition with autosomal recessive inheritance. Not all pathogenic genes in the above-mentioned conditions were discovered with the help of related marriages; however, consanguineous families have been invaluable in elucidating novel genes and novel mechanisms of disease despite this genetic burden. Thus, it can be said that genetic examination in consanguineous families could trigger novel advancements in pathophysiological research and extend medical knowledge. Therefore, continued genetic testing (specially using methods such as whole-genome sequencing) in areas with prevalent consanguinity could further help shed light on conditions where the genetic background and pathophysiology are not fully known.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

J.L. designed this review. S.A.A. wrote the manuscript. T.H.T. and P.D. provided insight into genetic findings and testing in consanguineous areas. S.A.A. and J.L. prepared the figures. S.P. and J.L. revised the manuscript critically. All authors contributed to the discussion, reviewed or edited the manuscript, and approved the final version to be published.

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7.2. Amaratunga SA, Hussein Tayeb T, Rozenkova K, Kucerova P, Pruhova S, Lebl J. Congenital Hyperinsulinism Caused by Novel Homozygous KATP Channel Gene Variants May Be Linked to Unexplained Neonatal Deaths among Kurdish Consanguineous Families. Horm Res Paediatr. 2020;93(1):58-65

In this study, we investigated three unrelated children from consanguineous Kurdish families with repeated severe neonatal hypoglycemia resulting from CHI. One child was diagnosed very late (at 2.8 years of age). She had only frequent feeds and had severe psychomotor delay because of repeated hypoglycaemia from a young age. Two families had experienced multiple unexplained neonatal deaths with convulsions.

Genetic analysis using Sanger sequencing revealed the presence of three novel pathogenic homozygous variants in the *ABCC8* and *KCNJ11* genes, confirming that K_{ATP} channel gene variants were responsible for CHI in these cases. This retrospective diagnosis sheds 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. Prompt genetic testing can save the lives of affected neonates, and improve overall outcomes and quality of life, particularly in regions with higher rates of consanguinity.

Novel Insights from Clinical Practice / Case Report

HORMONE RESEARCH IN PÆDIATRICS

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Congenital Hyperinsulinism Caused by Novel Homozygous K_{ATP} Channel Gene Variants May Be Linked to Unexplained Neonatal Deaths among Kurdish Consanguineous Families

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Established Facts

- Congenital hyperinsulinism (CHI) is a potentially life-threatening cause of severe hypoglycemia in the neonatal and infant period.
- The incidence of CHI has been estimated to be around 1 in 50,000 in non-consanguineous populations and as high as 1 in 2,500 in areas with a higher rate of consanguinity.
- Certain countries with high rates of consanguinity have a much higher infant and neonatal mortality rate than the rest of the world.

Novel Insights

- Three novel homozygous variants are reported in genes *ABCC8* and *KCNJ11* causing CHI in three Kurdish consanguineous families. Two of these families have a notable history of unexplained neonatal deaths.
- A small but significant percentage of all unexplained neonatal deaths could be due to undiagnosed CHI. Therefore, especially in regions with a high prevalence of consanguinity, undiagnosed CHI can contribute to higher infant and neonatal mortality rates.

Keywords
Congenital hyperinsulinism · K_{ATP} channel variants · *ABCC8* · *KCNJ11* · Consanguinity

Abstract
Introduction: Neonatal hypoglycemia due to congenital hyperinsulinism (CHI) is a potentially life-threatening condition. Biallelic pathogenic variants in K_{ATP} channel subunit genes (*ABCC8*, *KCNJ11*), causing severe forms of CHI, are more prevalent in regions with a significant rate of consanguinity and may lead to unexplained neonatal deaths. We hypothesized that K_{ATP} channel gene variants are the cause of CHI in three unrelated children from consanguineous

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Kurdish families with histories of four unexplained neonatal deaths with convulsions. ***Cases:*** (1) A girl presented on the 6th day of life with recurrent hypoglycemic convulsions (blood glucose 2.05 mmol/L, insulin 58 mIU/L, C-peptide 2,242 pmol/L). (2) A girl with severe developmental delay was diagnosed with CHI at 3 years of age (blood glucose 2.78 mmol/L, insulin 8.1 mIU/L, C-peptide 761 pmol/L) despite a history of recurrent hypoglycemia since neonatal age. (3) A girl presented at 3 weeks of age with convulsions and unconsciousness (blood glucose 2.5 mmol/L, insulin 14.6 mIU/L, C-peptide 523 pmol/L). Coding regions of the *ABCC8* and *KCNJ11* genes were tested by Sanger sequencing. Potential variants were evaluated using the American College of Medical Genetics standards. Three novel causative homozygous variants were found – p.Trp514Ter in the *ABCC8* gene (Pt2), and p.Met1Val (Pt1) and p.Tyr26Ter (Pt3) in the *KCNJ11* gene. ***Conclusion:*** CHI caused by K_{ATP} channel variants was elucidated in three children, providing a highly probable retrospective diagnosis for their deceased siblings. Future lives can be saved by timely diagnosis of CHI when encountering a neonate with unexplained seizures or other signs of recurrent and/or persistent hypoglycemia.

Introduction

Severe hypoglycemia in the neonatal and infant period is a potentially life-threatening condition. One of the most severe causes of such hypoglycemia is congenital hyperinsulinism (CHI) [1]. CHI is a heterogeneous genetic condition caused by a primary genetic defect of the pancreatic β-cells resulting in uncontrolled insulin secretion [2, 3]. In severe cases, hypoglycemia can occur even right after a child has been fed [1]. Symptoms may be subtle and unspecific, and among them could be symptoms such as seizures, tremulousness, irritability, tachycardia, and hypothermia; therefore, obtaining a correct and timely diagnosis can be challenging [1, 4]. If not treated in time, neonates and infants could die. Repeated significant hypoglycemia interferes with brain development, leading to mental retardation and other long-term neurological sequelae [5, 6].

CHI is caused by variants in genes such as *ABCC8* [7], *KCNJ11* [8], *GLUD1* [9], *HADH1* [10], *GCK* [11], *PGM1* [12], *HK1* [13], *HNF4A* [14], *HNF1A* [15], *SLC16A1* [16], *PMM2* [17], *FOXA2* [18], *INSR* [19], and possibly *UCP2* [20, 21] and *CACNA1D* [22]. In addition, some genetic syndromes such as Beckwith-Wiedemann, Kabuki, and Turner syndromes are associated with hyperinsulinism [23, 24]. This makes a total of over 15 genes currently associated with CHI.

The most severe forms of CHI are caused by pathogenic variants in genes *ABCC8* and *KCNJ11*, each encoding the SUR1 and Kir6.2 proteins of the ATP-sensitive K^+ (K_{ATP}) channel in the pancreatic β-cell, respectively [23, 24]. The K_{ATP} channel plays a pivotal role in the regulation of insulin secretion. Loss of K_{ATP} channel activity due to *ABCC8* and *KCNJ11* gene variants leads to persistent membrane depolarization and continuous insulin release, regardless of glucose level, thereby causing hyperinsulinism [24]. These variants can either be autosomal recessive or dominant, where recessive biallelic variants usually cause diffuse CHI with a more significant clinical impact [25].

Pharmacological therapies include diazoxide (a K_{ATP} channel inhibitor), octreotide (a short-acting somatostatin analogue), and continuous subcutaneous glucagon in patients who are diazoxide unresponsive (commonly patients having SUR1 and Kir6.2 subunit variants) [26, 27]. Two long-acting somatostatin analogues are currently in use, octreotide long-acting release (octreotide-LAR/Sandostatin-LAR [28]) and Somatuline autogel (lanreotide) [29]. A novel therapeutic option is an inhibitor of the mammalian target of rapamycin (mTOR) signaling pathway sirolimus [30]. Nifedipine, a calcium channel blocker, has also been used with variable success [31].

When conservative medical therapy fails, it is possible to cure focal forms of CHI (distinguished by an ^{18}F-DOPA PET CT scan) with complete surgical resection, while the diffuse form requires a near total pancreatectomy [32, 33].

The incidence of CHI has been estimated to be around 1 in 50,000 in non-consanguineous populations [2, 34, 35]. However, a much higher frequency of 1 in 2,500 was estimated in communities with a higher rate of consanguinity [36].

Timely diagnosis of CHI in the Kurdish region of Iraq could be challenging due to the presence of a less effective health care system and a higher number of home deliveries. All of these factors contribute to the higher neonatal and infant death rates in the region. In 2017, neonatal (NMR) and infant (IMR) mortality rates in Iraq were 17 and 25 per 1,000 live births, respectively [37]. This is in contrast to European countries, which have an average NMR and IMR of 3 and 4, and the United States of America, which has a NMR and IMR of 4 and 6 per 1,000 live births, respectively [37].

We present three children from Kurdish consanguineous families, all diagnosed with CHI at a neonatal age. There is a notable history of unexplained neonatal deaths in two of these families.

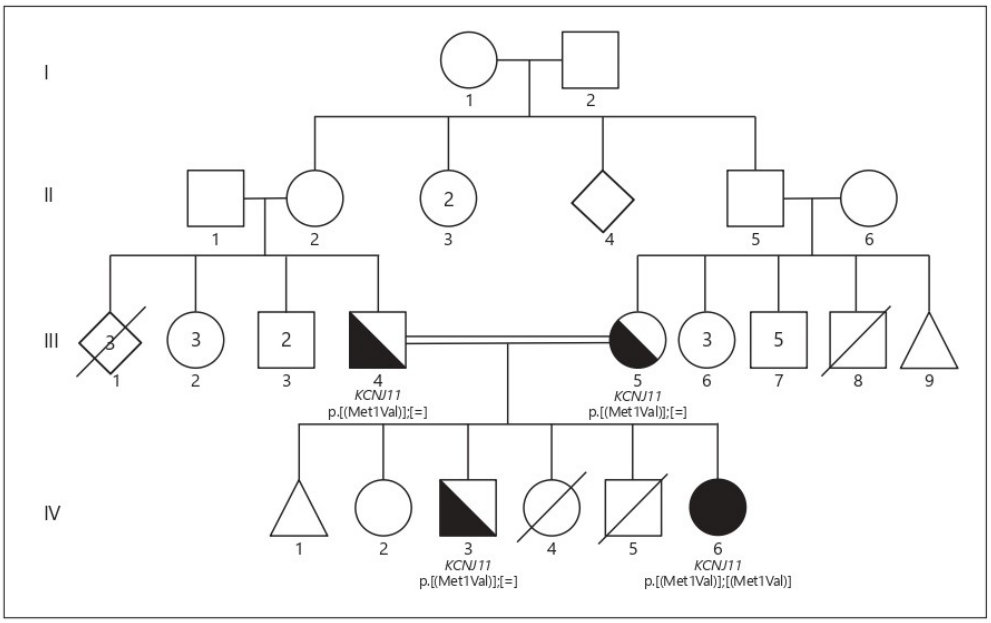

Fig. 1. Patient 1's family pedigree. II-2 unknown cause of death, III-1 three deaths with the cause and sex unknown, III-8 death at 4–5 months of age, IV-4 death at 3 days of age with convulsions, IV-5 death at 13 days of age with convulsions, IV-6 proband.

Table 1. Pathogenic gene variants in the 3 patients

	Gene	Region	Variant at protein level	Variant at cDNA level	Zygosity	Prediction programs		
						mutation taster	SIFT/ Provean	Polyphen2
1	*KCNJ11* REF SEQ NM_000525.3	Exon 1	p.Met1Val	c.1A>G	Homo	Disease causing	Damaging	Probably damaging
2	*ABCC8* REF SEQ NM_000352.4	Exon 10	p.Trp514Ter	c.1541G>A	Homo	Disease causing	Deleterious	–
3	*KCNJ11*	Exon 1	p.Tyr26Ter	c.78C>A	Homo	Disease causing	Neutral	–

Case Reports

Patient 1

A female infant was born full term, with a birth weight estimated above 4 kg (large for gestational age, standard deviation [SD] over +2.0 [38]) and unknown length, to healthy Kurdish parents who are first cousins (Fig. 1). During the neonatal period, she presented having recurrent convulsions due to hypoglycemia. She was suspected of having hyperinsulinism based on high random insulin level (basal insulin 205 mIU/L [normal range 2.6–24.9], C-peptide 4,701 pmol/L) and was therefore put on short-acting octreotide due to the lack of diazoxide in this region. At 4 months of age, when a partial pancreatectomy was being considered, she was referred to a pediatric endocrinologist, where blood glucose during critical sample was 2.05 mmol/L (37 mg/dL), with high insulin 58 mIU/L and C-peptide 2,242 pmol/L, and inappropriately low cortisol of 8.15 nmol/L (normal range 171–536) and ACTH of 4.65 ng/L (normal range 7.2–63.6). An ACTH stimulation test (Synacthen test with i.m. depot Synacthen) helped to exclude primary adrenal insufficiency with stimulated cortisol of 943 nmol/L.

The patients' and their respective family members' genomic DNA was extracted from peripheral blood (obtained with informed consent) using QIAmp DNA Blood Mini (Qiagen, Hilden, Germany). In some family members, DNA was isolated from saliva using Oragene DNA OG-500 (DNA Genotek, Ontario, Canada). DNA was analyzed using methods of direct Sanger sequencing by sequencer ABI 3130xl. The complete coding regions and intron-exon boundaries of genes *ABCC8* and *KCNJ11* were analyzed

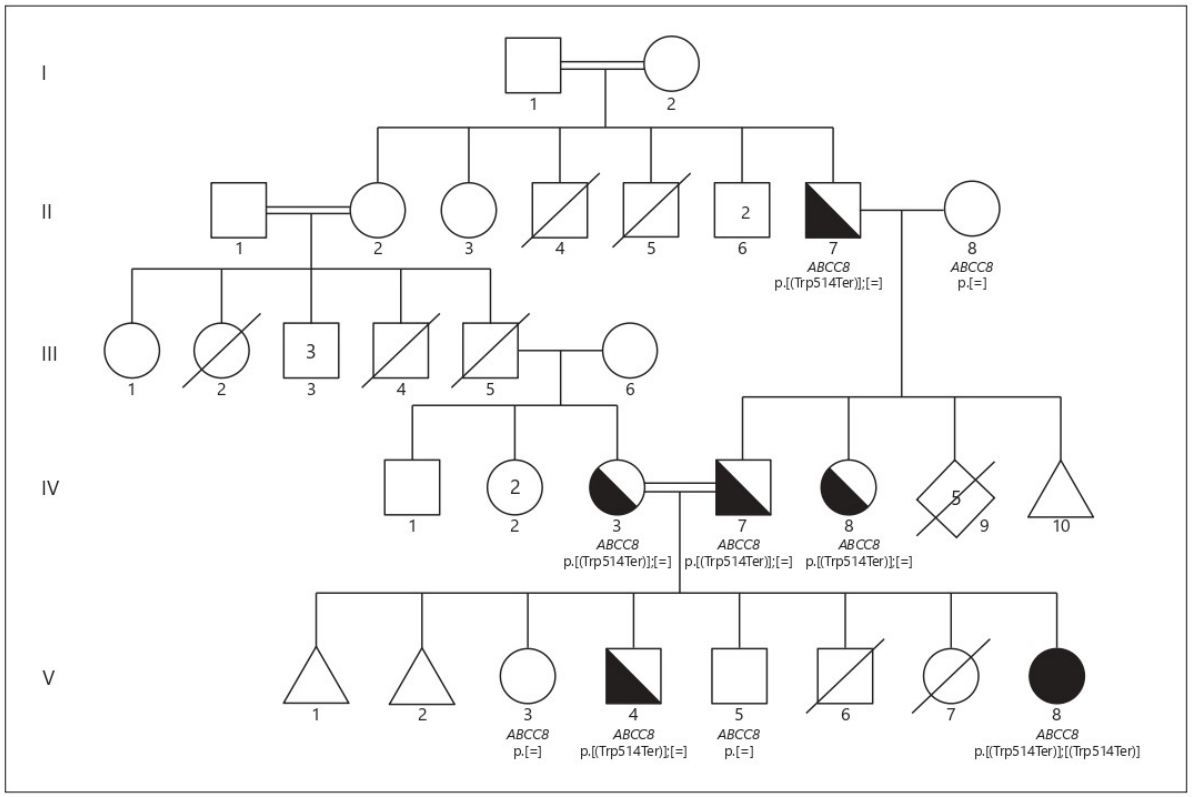

Fig. 2. Patient 2's family pedigree. I-1 and I-2 distant relatives, II-1 and II-2 distant relatives, II-2 diabetes mellitus, II-4 death at 5 years, II-5 death due to war, III-2 and III-4 death due to an accident, III-5 death due to war, IV-3 gestational diabetes mellitus, III-9 five male infants died of an unknown cause, V-6 death at 1 day of life with convulsions and cyanosis, V-7 death at 1 day of life with convulsions and cyanosis, V-8 proband.

as two of the most common causes of CHI. Results were verified using the program Mutation Surveyor. Each possibly pathogenic variant was then evaluated according to the American College of Medical Genetics and Genomics standards and guidelines.

A novel homozygous pathogenic variant p.Met1Val (c.1A>G) was found in gene *KCNJ11* (Table 1). Her parents and one healthy sibling tested heterozygous for the same variant (Fig. 1).

After genetic diagnosis, she was started on octreotide-LAR. Three doses were given (1.2 mg subcutaneously per month), after which hypoglycemia resolved. During this 3-month period, she was given short-acting octreotide 40 µg/day as well. Since her hypoglycemia was difficult to control and she showed a poor cortisol response during hypoglycemia, as a temporary solution, hydrocortisone 15 mg per surface area was added to the treatment regimen for a period of 1 month (three doses a day).

At the age of 13 months (length 75 cm [–0.4 SD], weight 11.3 kg [+1.5 SD]), her dose of long-acting octreotide was increased to 4.6 mg per month. Her HbA1c was 31 mmol/mol (DCCT – 5.0%), suggesting that she maintained her mean blood glucose level within the normal range. Her growth and psychomotor development are normal.

Notably, the patient has a history of two siblings, female and male, who died on the 3rd day and 13th day of life, respectively, with generalized seizures. The cause of death was not properly elucidated.

Patient 2

A female infant was born in a Kurdish consanguineous family (Fig. 2) to a mother with a history of gestational diabetes in all her pregnancies. She was born preterm, at the 35th week of gestation, with a birth weight of 3.3 kg (large for gestational age, +2.0 SD) [39].

From 4 days of age, she had recurrent symptomatic hypoglycemia but was only treated with frequent feeding and glucose, during which she had several severe hypoglycemic episodes. At the age of 3 years and 8 months, she was referred to a pediatric endocrinologist, where a critical sample was obtained with a blood glucose level of 2.8 mmol (50 mg/dL), inappropriately high insulin level of 8.1 mIU/L, and C-peptide level of 761 pmol/L. At the time of hypoglycemia, cortisol was appropriately elevated to 893 nmol/L; thereafter, the diagnosis of hyperinsulinemic hypoglycemia was

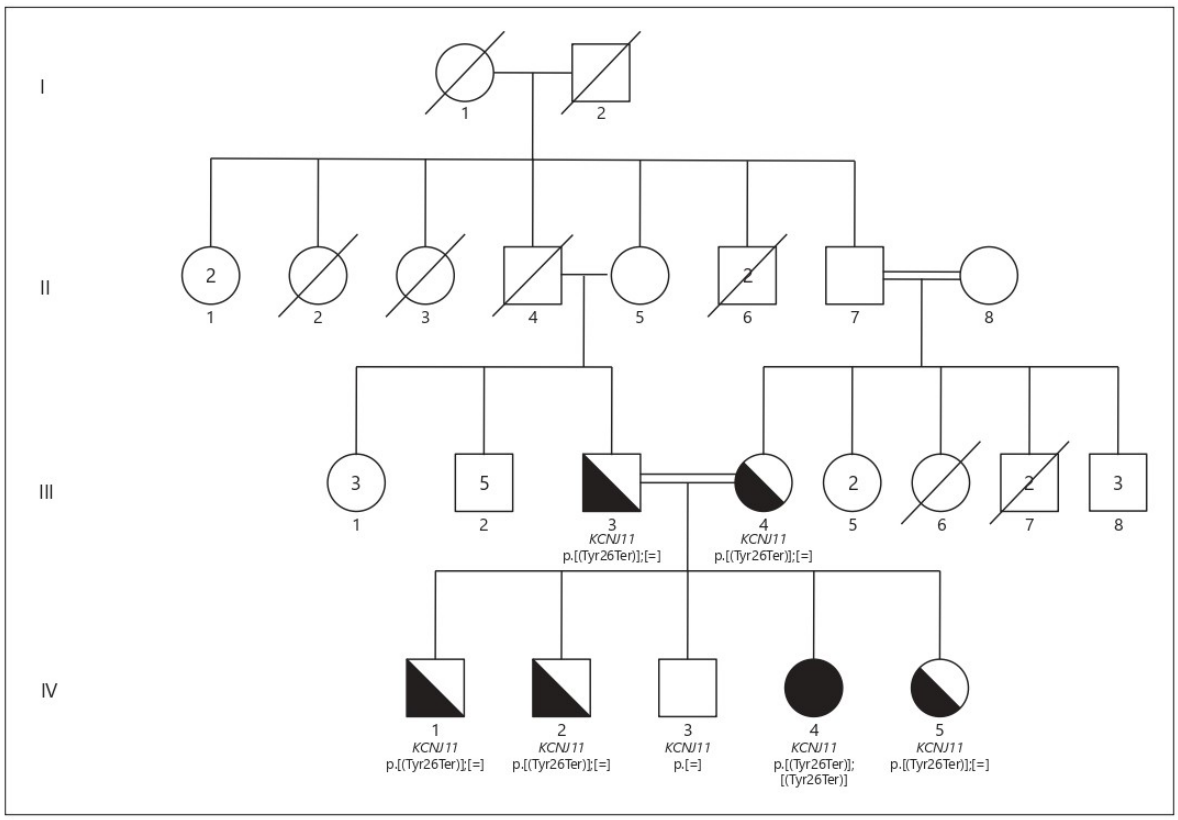

Fig. 3. Patient 3's family pedigree. II-2, -3, -4, -6 died in middle to old age for unknown reasons, II-7 and II-8 distant relatives, II-7 myocardial infarction, III-6 death at 1 year of age due to fever, III-7 unknown cause of death in 2 males, III-8 death at 5 years due to unknown cause, IV-1 8 years old, IV-2 5 years old, IV-3 3 years old, V-5 proband, 2.5 years old, IV-6 1 year old.

made. At this stage, she already had severe developmental delay. At her current age of 7 years, she does not speak and is unable to walk, partially due to muscle spasticity.

After her clinical diagnosis, she was put on corn starch and nifedipine 0.2 mg/kg/day, due to the lack of diazoxide in this region. Nevertheless, she continued to have further episodes of hypoglycemia (blood glucose around 2.7 mmol/L, 50 mg/dL), which improved with the introduction of uncooked starch to her diet.

Genetic testing using methods described above revealed that she is homozygous for a novel nonsense pathogenic variant in the *ABCC8* gene p.Trp514Ter (c.1541G>A) (Table 1). Her parents tested heterozygous for the same variant (Fig. 2). After genetic diagnosis and due to the uncertain effect of nifedipine in the management of CHI, her medication was then changed to octreotide-LAR (a lower dose of 2.5 mg, due to hypoglycemic events being in the range of 2.7–3.3 mmol/L, 50–60 mg/dL) and she is doing well clinically with no further hypoglycemic episodes.

It is important to note that this patient's healthy consanguineous parents have three other healthy children; however, they also have a history of two spontaneous abortions and two children who died 1 day after birth having cyanosis and convulsions (as described by their parents), these children were born at home and not taken to hospital even after they died. Their family history also includes five male infants, siblings of the patient's father, who died from an unknown cause.

Patient 3

A female baby was born full term (gestational age 39 weeks + 4 days, birth weight 3.0 kg – appropriate for gestational age, 0.0 SD) to healthy consanguineous Kurdish parents with three other healthy children (Fig. 3). She presented with hypoglycemia at 3 weeks of life causing convulsions and loss of consciousness. She was discharged from a local hospital on recommended frequent feeds, uncooked starch, and glucagon injections if needed, with no definitive diagnosis.

At 3 months of age, she was referred to the pediatric endocrinologist due to frequent episodes of symptomatic hypoglycemia and she was admitted to the hospital for further investigation. Af-

ter 4 h of fasting, her blood glucose came down to 2.5 mmol/L (45 mg/dL) and a critical sample was obtained. The insulin level (14.6 mIU/L) was not suppressed at the time of hypoglycemia, C-peptide was 523 pmol/L, cortisol 220 nmol/L, and ketone bodies were absent in urine. She was put on nifedipine 0.23 mg/kg/day, on which there was no improvement. When diazoxide was made available to this patient, her treatment was then changed to diazoxide with no noticeable improvement either.

Parents then took her to India, where an ^{18}F-DOPA PET CT scan was carried out revealing a diffuse form of CHI. She was put on a combined therapy of diazoxide and octreotide. Since she responded to her first dose of octreotide, at 6 months of age (back in Iraq), her treatment with diazoxide was tapered off (due to development of hirsutism and lack of response) and short-acting octreotide was given three times a day.

Genetic analysis revealed a novel homozygous nonsense pathogenic variant in the *KCNJ11* gene, p.Tyr26Ter (c.78C>A) (Table 1). Her parents tested heterozygous for the same variant and her healthy siblings tested heterozygous or negative (Fig. 3). After genetic diagnosis was confirmed, octreotide-LAR 2 mg per month was started. Short-acting octreotide was stopped 1 month after starting long-acting octreotide. Currently, at the age of 2.5 years, she is doing well with a dose of 3 mg per month of long-acting octreotide. Her HbA1c is 34 mmol/mol (DCCT – 5.3%).

Discussion

Consanguineous marriages are common in some countries including the Kurdish region of Iraq. The increased frequency of homozygous genotypes in children from such marriages allows less common alleles to manifest as homozygous; thus, descendants of consanguineous parents have a higher frequency of recessive genetic conditions than those of unrelated parents. If a single-molecule mechanism is the pathophysiological mechanism of the individual conditions, consanguineous families provide the best chances to successfully encounter novel genes and variants.

The cause of CHI varies among populations depending on the percentage of consanguinity. For example, in Europe, where consanguinity is quite rare, CHI is predominantly caused by autosomal dominant mode of inheritance, which leads to generally milder patient phenotypes [34]. In consanguineous populations, autosomal recessive variants prevail, causing more severe CHI [23, 24]. Monoallelic CHI is more likely to respond to diazoxide therapy, whereas recessive disease most often requires therapy with octreotide [25]. Timely identification of the genetic background helps clinicians decide on the type of treatment. However, genetic diagnosis is not often available in certain countries. This along with limited availability of diazoxide in such regions could be the cause of some CHI patients having to undergo a possibly unnecessary near total pancreatectomy.

In all three of our families, pathogenic novel homozygous variants were found in K_{ATP} channel genes. All identified variants were not listed in the GnomAD database and were described as pathogenic by the in silico prediction programs Mutation Taster, SIFT, and PolyPhen-2. The American College of Medical Genetics and Genomics standards and guidelines classified these variants as being pathogenic (Ia) (Table 1).

Variant 1 (p.Met1Val in gene *KCNJ11*) causes the loss of the initiating methionine and changes the Kozak sequence. It could be presumed that this protein is shortened or not coded at all. The two other variants (p.Trp514Ter in gene *ABCC8* and p.Tyr26Ter in gene *KCNJ11*) cause a stop signal leading to the premature termination of protein synthesis of the SUR1 protein from amino acid 514 and the Kir6.2 protein from amino acid 26, respectively, which results in a shorter protein with lost or changed function.

All three children come from heterozygous asymptomatic parents and have asymptomatic siblings who tested negative or heterozygous. The above factors strongly support the pathogenicity of these variants.

Most children with CHI, like in our three families, are born to healthy parents. Two of our families, notably, had a history of unexplained neonatal deaths. Family 1 reported two children dying on the 3rd and 13th day of life with convulsions (Fig. 1). Family 2's description of two newborns dying with convulsions and cyanosis, both on the first day of life, could be quite indicative of hypoglycemia (Fig. 2).

It is highly probable that the infant deaths in these families could be caused by undiagnosed hyperinsulinism and that these infants were also born homozygous for the same pathogenic variants as found in our patients, respectively. In addition, the family history of Family 2 (Fig. 2) included five male infants, siblings of the patient's father, who died from an unknown cause. However, the possibility of the same mutation causing these deaths was ruled out as the paternal grandmother tested negative for the same *ABCC8* variant. The cause of these deaths could only be speculated.

In Family 3, no infant deaths were reported (Fig. 3), all children born to the family were heterozygous or negative until the birth of our patient. However, if CHI had not been correctly considered at the time of presentation of hypoglycemia symptoms, this child may have had a worse outcome as well.

A prospective study from Iran revealed a mortality rate of 53.8% out of 14,000 hypoglycemic infants who were evaluated with regard to blood glucose level at the first 24 h of life over a 2-year period. Prematurity (61.5%) was the leading cause of death. A percentage of 1.9% of these deaths were reportedly due to CHI [40]. Whereas data on the IMR

in neonates due to hypoglycemia are otherwise not available, it could be speculated that even a higher proportion of neonatal deaths worldwide could be due to CHI.

All three patients are now successfully managed with monthly doses of long-acting octreotide; there are currently no episodes of hypoglycemia or lack of consciousness.

Due to the fact that pathogenic homogenous variants in K_{ATP} channel genes were found in all these families, diagnosing CHI, it is highly probable that these neonatal deaths, described with the presence of convulsions, could indeed have been due to hypoglycemia. Such undiagnosed cases, unfortunately, may be classified as unexplained neonatal deaths in such regions due to home births, not taking children to the hospital, lack of facilities and of genetic counseling. There being a lack of any other publication regarding CHI stemming from the Kurdistan region to our knowledge, our findings provide a glimpse into the possibly high incidence of CHI and neonatal deaths that could be prevented if diagnosed in time. This translates to the same necessity in all regions with prevalent consanguinity. These cases show the importance of considering hyperinsulinism as a possible diagnosis when encountering a child with recurrent and persistent hypoglycemia and/or seizures in order to save future lives.

Among the clear limitations of our study belong factors such as the lack of all complete clinical data from primary care physicians, unreliable birth size parameters due to home births, and limited reliability of laboratory results. A significant limitation is that the pathogenicity of the elucidated novel variants was assessed and deemed causative only by the clinical phenotype, segregation in the family, and prediction programs. Further functional assessment of these variants is not available. Genetic testing in the deceased siblings of these patients was not possible.

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Statement of Ethics

Our patients' parents gave informed consent for the genetic testing reported in this paper and for the publication of related data. This study was approved by the Ethics Committee at the 2nd Faculty of Medicine, Charles University in Prague. The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

J.L. and S.P. designed the study. S.A.A. did the genetic testing for most patients and family members and coordinated the study. S.A.A. and K.R. wrote the manuscript. T.H.T. referred the patients, provided their clinical information, and reviewed the manuscript. P.K. provided insight on variant analysis. All authors contributed to the discussion and reviewed or edited the manuscript.

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7.3. Amaratunga SA, Hussein Tayeb T, Muhamad Sediq RN, Hama Salih FK, Dusatkova P, Wakeling MN, De Franco E, Pruhova S, Lebl J. Paediatric diabetes subtypes in a consanguineous population: a single-centre cohort study from Kurdistan, Iraq. Diabetologia (2023)

The primary objectives of this single-centre study were to identify paediatric diabetes subtypes in a highly consanguineous region, assess consanguinity prevalence, and delve into the genetic basis of syndromic and neonatal diabetes cases. This is a unique study with the tested centre being the sole clinic responsible for paediatric diabetes care in the entire region of Sulaimani in Kurdistan, Iraq (population 2.33 million people, 256,000 children under 5 years of age).

To accomplish these objectives, data was collected on all registered patients (754 individuals aged up to 16 years). The majority of patients had clinically defined type 1 diabetes (Fig.5). We found that consanguinity was statistically associated with syndromic diabetes, unlike other diabetes subtypes. The genetic etiology was successfully determined in 83% of participants with neonatal diabetes, and 57% with syndromic diabetes. Notably, 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. In addition, identified causative genes differed significantly from those in non-consanguineous populations.

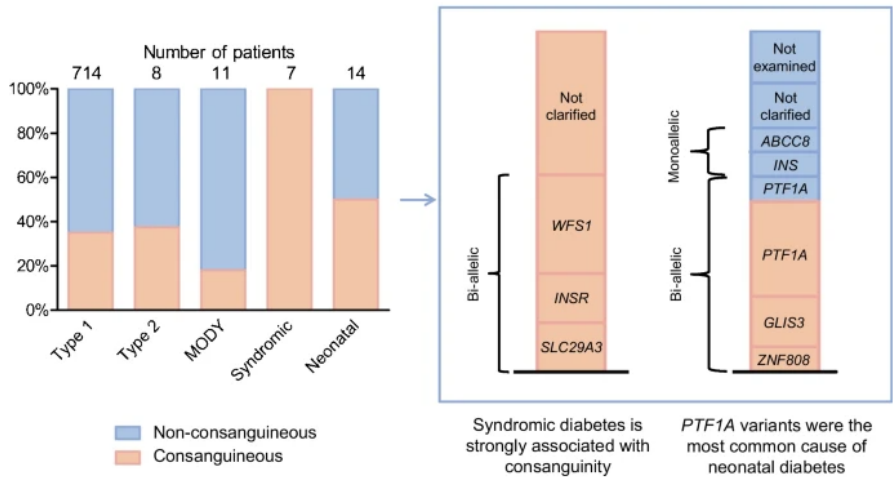

Fig. 5. Distribution of pediatric diabetes subtypes and genetic etiology of syndromic and neonatal diabetes in a single center in an area with high level of consanguinity

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ARTICLE

Paediatric diabetes subtypes in a consanguineous population: a single-centre cohort study from Kurdistan, Iraq

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Abstract

Aims/hypothesis Monogenic diabetes is estimated to account for 1–6% of paediatric diabetes cases in primarily non-consanguineous populations, while the incidence and genetic spectrum in consanguineous regions are insufficiently defined. In this single-centre study we aimed to evaluate diabetes subtypes, obtain the consanguinity rate and study the genetic background of individuals with syndromic and neonatal diabetes in a population with a high rate of consanguinity.

Methods Data collection was carried out cross-sectionally in November 2021 at the paediatric diabetic clinic, Dr Jamal Ahmad Rashed Hospital, in Sulaimani, Kurdistan, Iraq. At the time of data collection, 754 individuals with diabetes (381 boys) aged up to 16 years were registered. Relevant participant data was obtained from patient files. Consanguinity status was known in 735 (97.5%) participants. Furthermore, 12 families of children with neonatal diabetes and seven families of children with syndromic diabetes consented to genetic testing by next-generation sequencing. Prioritised variants were evaluated using the American College of Medical Genetics and Genomics guidelines and confirmed by Sanger sequencing.

Results A total of 269 of 735 participants (36.5%) with known consanguinity status were offspring of consanguineous families. An overwhelming majority of participants (714/754, 94.7%) had clinically defined type 1 diabetes (35% of them were born to consanguineous parents), whereas only eight (1.1%) had type 2 diabetes (38% consanguineous). Fourteen (1.9%) had neonatal diabetes (50% consanguineous), seven (0.9%) had syndromic diabetes (100% consanguineous) and 11 (1.5%) had clinically defined MODY (18% consanguineous). We found that consanguinity was significantly associated with syndromic diabetes (p=0.0023) but not with any other diabetes subtype. The genetic cause was elucidated in ten of 12 participants with neonatal diabetes who consented to genetic testing (homozygous variants in *GLIS3* [sibling pair], *PTF1A* and *ZNF808* and heterozygous variants in *ABCC8* and *INS*) and four of seven participants with syndromic diabetes (homozygous variants in *INSR*, *SLC29A3* and *WFS1* [sibling pair]). In addition, a participant referred as syndromic diabetes was diagnosed with mucolipidosis gamma and probably has type 2 diabetes.

Conclusions/interpretation This unique single-centre study confirms that, even in a highly consanguineous population, clinically defined type 1 diabetes is the prevailing paediatric diabetes subtype. Furthermore, a pathogenic cause of monogenic diabetes was identified in 83% of tested participants with neonatal diabetes and 57% of participants with syndromic diabetes, with most variants being homozygous. Causative genes in our consanguineous participants were markedly different from genes reported from non-consanguineous populations and also from those reported in other consanguineous populations. To correctly diagnose syndromic diabetes in consanguineous populations, it may be necessary to re-evaluate diagnostic criteria and include additional phenotypic features such as short stature and hepatosplenomegaly.

Keywords Consanguineous population · Consanguinity · Diabetes genes · Genetics · Monogenic diabetes · Neonatal diabetes · Paediatric diabetes · Syndromic diabetes

Abbreviations

ACMG	American College of Medical Genetics and Genomics
NGS	Next-generation sequencing
ROH	Runs of homozygosity
VUS	Variant of uncertain significance
WES	Whole-exome sequencing

Extended author information available on the last page of the article

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Research in context

What is already known about this subject?

- Offspring of consanguineous parents are at increased risk of developing autosomal recessive conditions, including neonatal and syndromic diabetes (monogenic diabetes)
- In neonatal diabetes, differences in genetic aetiology have been observed between consanguineous and non-consanguineous populations
- Monogenic diabetes has an incidence of 1–6% among paediatric diabetes cases in non-consanguineous populations

What is the key question?

- What is the distribution of diabetes subtypes, including genetic causes of neonatal and syndromic diabetes, among the paediatric population of a highly consanguineous region?

What are the new findings?

- Type 1 diabetes is the main paediatric diabetes subtype even in a population with a high consanguinity rate
- Consanguinity is strongly associated with syndromic diabetes but not with other diabetes subtypes. Phenotypic features such as short stature and hepatosplenomegaly may be important additional diagnostic criteria for syndromic diabetes
- The spectrum of causative genes in monogenic diabetes is unique, even between two consanguineous populations

How might this impact on clinical practice in the foreseeable future?

- When examining children with diabetes from consanguineous families, it is important to identify monogenic diabetes, including syndromic diabetes, using specific phenotypic features and genetic testing (if available), to improve diagnostic precision and allow timely recognition of additional disease components

Introduction

The majority of diabetes cases diagnosed in childhood and adolescence are of polygenic aetiology, with significant contributing environmental factors. This is the case for both type 1 diabetes, which is the predominant form, and type 2 diabetes, which occurs much less frequently but is now on the rise in some parts of the world due to an increase in the prevalence of childhood obesity [1]. Monogenic diabetes is more rare, with varied aetiology and multiple clinical forms [2].

Paediatric forms of monogenic diabetes can be divided into four distinct but partially overlapping types: MODY, which exhibits autosomal dominant transmission; neonatal diabetes, with clinical onset within the first 6 months of age; syndromic diabetes, characterised by additional non-diabetic (usually extra-pancreatic) phenotypic features; and monogenic autoimmune diabetes, which is associated with additional immune-mediated conditions.

Neonatal diabetes is clearly defined by the age at clinical onset, whereas other subtypes of monogenic diabetes may remain underdiagnosed or misdiagnosed, especially in resource-limited countries where testing of pancreatic autoantibodies is rarely carried out and cases of monogenic diabetes may be classified as type 1 or type 2 diabetes. Thus, with the exception of neonatal diabetes, the incidence of all subtypes of monogenic diabetes may be underestimated [3]. The fact that some genetic variants are phenotypically variable, with milder forms of diabetes appearing only later in adolescence or in adulthood, complicates the correct disease classification even further. Some syndromic forms of monogenic diabetes may present initially as insulin-dependent diabetes mellitus, while further features may be delayed for years, as is the case with Wolfram syndrome, or are inconsistent, such as in renal cyst and diabetes syndrome [4].

Monogenic diabetes has been estimated to account for 1–6% of all diabetes cases in European countries, which are primarily non-consanguineous [5–7]. An incidence of 6% has been reported from a large centre in Turkey but the incidence in other countries where consanguinity is prevalent is not known [8, 9]. However, the genetic aetiology of monogenic diabetes, specifically neonatal diabetes, differs significantly between areas with low and areas with high rates of consanguinity [10].

Our study centre is the sole clinic responsible for paediatric diabetes care in the entire region of Sulaimani in

Kurdistan, Iraq. According to unpublished 2021 statistics available from the regional authorities in Sulaimani, the population of this region was 2.33 million, with 256,000 being children under 5 years of age. There were 46,000 live births in the region in 2021 and, according to United Nations Children's Fund (UNICEF) data, the infant mortality rate in Iraq is 21 per 1000 live births [11]. The consanguinity rate in this region has not been documented to the authors' knowledge, but in geographically and/or ethnically related regions it has been reported as 39–44% [12–14].

Investigations such as basic biochemical and HbA_{1c} testing are available at the clinic, but antibody assessment is carried out only in private laboratories, at the individual's expense, and thus is available for only a few people. Furthermore, genetic testing is almost inaccessible. Taking all of these factors into account, it is very likely that monogenic diabetes in this region is underdiagnosed.

In this single-centre study, we aimed to evaluate the prevalence of clinically defined subtypes of paediatric diabetes, calculate the consanguinity rate and study the genetic background of individuals with neonatal and syndromic diabetes.

Methods

Data collection A total of 754 individuals were registered at the diabetic clinic at Dr Jamal Ahmad Rashed Hospital in the region of Sulaimani in Kurdistan, Iraq, in November 2021. This is the only clinic serving the region and therefore the study sample (which is derived from the paediatric population comprising mostly Kurdish people and some Arabic individuals [self reported]) is representative of the entire population of Kurdistan, Iraq. Data were collected from these individuals with their consent, with the use of information from hand-written medical records, in the form of a cross-sectional survey. Additional data were obtained during participant check-ups and direct telephone interviews with participants' families were carried out regarding consanguinity if this information was not available. The date of diagnosis, age at diagnosis, sex (determined during examination), type of clinically determined diabetes, insulin dosage, syndromic features (if any), anthropometric measurements, family history of diabetes and consanguinity status were recorded. Positive consanguinity was defined as children born to first, second or third cousin parents, as reported by the respective families.

The clinical determination of diabetes subtype was carried out as follows:

(1) Type 1 diabetes: insulin-dependent diabetes diagnosed at age >6 months with or without diabetic ketoacidosis at disease onset, without vertical transmission of diabetes in the family, dysmorphic features or concomitant conditions, except for coeliac disease and/or autoimmune thyroid disease.
(2) Type 2 diabetes: non-insulin-dependent diabetes diagnosed in children and adolescents with a BMI higher than that of peers of the same age with diabetes; features of insulin resistance; normal or high C-peptide levels; and testing negative for autoantibodies (when available).
(3) Neonatal diabetes: diabetes diagnosed up to 6 months of age.
(4) Syndromic diabetes: diabetes diagnosed after 6 months of age, testing negative for autoantibodies (when available) and accompanied by other significant features (dysmorphic phenotypic signs, short stature, hepato- and/or splenomegaly, known renal cysts or additional immunopathological conditions, excluding autoimmune thyroid disease and/or coeliac disease).
(5) MODY: apparent vertical transmission of diabetes in families, with diabetes diagnosed at age >6 months with no diabetic ketoacidosis at disease onset.

From the information collected, association of diabetes subtypes and consanguinity status was analysed using Fisher's two-sided exact test, with $p<0.05$ considered statistically significant.

Participants with neonatal diabetes or syndromic diabetes were offered genetic testing. Nineteen children from 17 families (12 with neonatal diabetes and seven with syndromic diabetes, including two sibling pairs) were sent for genetic testing with written parental consent. Peripheral blood for DNA extraction was collected from the children, both parents and, if possible, additional family members to allow for more extensive segregation analysis. Genetic analysis by next-generation sequencing (NGS) was carried out at the Laboratory of Molecular Genetics, Department of Paediatrics, Motol University Hospital and Charles University in Prague, Czech Republic.

Genetic examination Genomic DNA was extracted from peripheral blood using the QIAmp DNA Blood Mini system (Qiagen, Hilden, Germany). DNA was analysed using whole-exome sequencing (WES). WES was performed using the SureSelect Human All Exon V6+UTR kit (Agilent Technologies, Santa Clara, CA, USA) and the indexed products were sequenced by synthesis in an Illumina NextSeq 500 analyser (San Diego, CA, USA). Our bioinformatic pipeline includes evaluation of the most prevalent variants in mitochondrial DNA, as described previously [15]. Copy number variants subanalysis from raw WES data was carried out using the DECoN program [16]. Variant frequency was assessed using the Genome Aggregation Database (gnomAD) (total allele

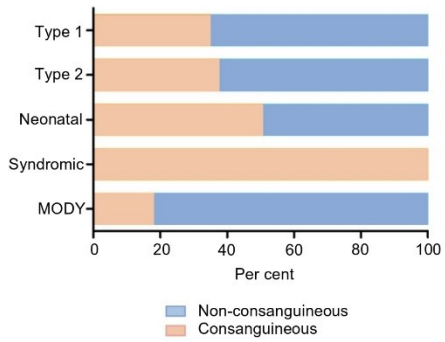

Fig. 1 Distribution of consanguinity within each diabetes subtype

frequency across all populations) [17]. Prioritised variants were then further evaluated using the American College of Medical Genetics and Genomics (ACMG) standards and guidelines [18]. All of the variants with potential clinical significance were confirmed using Sanger sequencing, as described previously [19]. To evaluate the segregation of genetic variants within families, Sanger sequencing was performed in both parents and healthy/affected siblings (when available), with written informed consent.

Probands with neonatal diabetes without a detected causal variant using WES underwent Sanger sequencing of the *PTF1A* enhancer region and methylation-specific multiplex ligation probe-dependent amplification in order to detect the aberrant methylation and/or gene dosage of chromosomal regions 6q22, 6q24 and 11p15 linked to transient neonatal diabetes (SALSA MS-MLPA Probemix ME033-A1 TNDM, MRC Holland, Amsterdam, the Netherlands). Furthermore, WES data from probands without a detected causal variant after the previous steps were further examined using the pipeline developed at the department of Clinical and Biomedical Sciences, University of Exeter Medical School, Exeter, UK. This pipeline has been previously described and is primarily used to search for variants in candidate genes [20].

Genome-wide runs of homozygosity (ROH) were analysed in all probands from FASTQ data using SavvyCNV to verify reported consanguinity. The discovered regions elude to the per cent of the autosome covered by homozygous regions at least 3 Mbp in size [21].

Ethics statement Participants' parents gave informed consent for the genetic testing reported in this paper and for the publication of related data and full-face images of participants. This study was approved by the Ethics Committee of Motol University Hospital and 2nd Faculty of Medicine, Charles University, Prague (approval no. EK-1263.1.1/19). The research was conducted ethically in accordance with the World Medical Association's Declaration of Helsinki.

Results

General participant data Data were obtained from a total of 754 children from 735 families (381 boys, 50.5%) of predominantly Kurdish origin. The consanguinity status was known for 735 participants; the remaining participants could not be contacted for further information. A total of 269 participants (36.5%) from 258 families were offspring of consanguineous families. Multiple children with diabetes from the same family all had the same diabetes subtype. Consanguinity was significantly associated with syndromic diabetes (p=0.0023) but not with other diabetes subtypes.

Type 1 diabetes, type 2 diabetes and MODY Of the 754 participants followed at the study centre, 714 (94.7%) were clinically classified as having type 1 diabetes. In total, 35% of these participants were from consanguineous families (Fig. 1). The median age of diabetes onset in type 1 diabetes was 7.7 years (IQR 4.2–10.2). All participants were treated using multiple daily insulin injections, and the median total daily insulin dose was 0.9 U/kg (IQR 0.73–1.3). The median BMI in this group was 16.6 kg/m^2 (IQR 14.4–20.5) at the age of the most recent check-up. BMI SD normative values are not available for this population; therefore, WHO normative values were used when applicable [22].

Eight children (1.1%) had clinically assigned type 2 diabetes, three of whom (38%) were born to consanguineous parents (Fig. 1). The median BMI in this group was 20.1 kg/m^2 (IQR 19.3–26.0) and median BMI SD was +1.8 SD (IQR +1.5 to +2.3) at the age of the most recent check-up (median 10.2 years, IQR 7.7–11.3). All participants in this group had acanthosis nigricans as a clinical sign of insulin resistance and were being treated with metformin, with three being on a combination of metformin and insulin.

Eleven participants (1.5%) had clinically defined MODY, two of whom (18%) were born to consanguineous parents (Fig. 1).

Neonatal diabetes A total of 14 participants (1.9%) had neonatal diabetes (50% consanguineous), with a mean age at diagnosis of 25 days (IQR 12.5–45) (Fig. 1).

Twelve of these participants were available for testing by WES, of whom ten had permanent neonatal diabetes. A pathogenic variant was found in 83% (10/12) of participants. In an additional proband, we found two novel compound heterozygous variants in the enhancer region of *PTF1A*. Even though one variant did not fulfil criteria for pathogenicity

Table 1 Relevant variants found in participants with neonatal and syndromic diabetes who underwent testing and participants' clinical characteristics

Participant ID	Type of diabetes	Age at diagnosis	Reported consanguinity	Clinical characteristics in addition to diabetes	Gene (reference sequence in hg19)	Variant at cDNA level	Variant at protein level	Zygosity	ACMG	ROH percentage via WES	Reference if published
11298	Neonatal (permanent)	11 days	Yes	Congenital hypothyroidism, facial features (flat nasal bridge, long philtrum, low-set ears)	*GLIS3* (NM_001042413)	chr9:3785092_3828441del		HOM	P	6.6	Novel
11299	Neonatal (permanent)	2 months	Yes	Congenital hypothyroidism, facial features (flat nasal bridge, long philtrum, low-set ears)	*GLIS3*	chr9:3785092_3828441del		HOM	P	7.5	Novel
11682	Neonatal (permanent)	5 months	Yes	Prolonged jaundice after birth	*ZNF808* (NM_001039886)	c.1805_1806del	p.Lys602Serfs*9	HOM	P	12.5	Novel
13186	Neonatal (transient)	2 months	No	–	*PTF1A* (NM_178161)	chr10:23508314T>C		Comp. HET	VUS	0.0	Novel
				–	*PTF1A*	chr10:23508363A>C			LP		Novel
13192	Neonatal (permanent)	50 days	No	–	*INS* (NM_000207)	c.94G>A	p.Gly32Ser	HET	P	0.8	[28]
14020	Neonatal (permanent)	10 days	No	–	*PTF1A*	chr10:23508437A>G		HOM	P	2.0	[29]
14105	Neonatal (transient)	13 days	No	–	*ABCC8 (NM_000352)*	c.3592C>T	p.Pro1198Ser	HET	LP	0.3	Novel
14109	Neonatal (permanent)	6 months	Yes	–	*PTF1A*	c.571C>A	p.Pro191Thr	HOM	P	7.0	[23]
14341	Neonatal (permanent)	12 days	Yes	Prolonged jaundice after birth, abdominal distension	*PTF1A*	c.571C>A	p.Pro191Thr	HOM	P	8.5	[23]
14346	Neonatal (permanent)	35 days	Yes	Abdominal distension, hypogammoglobulinaemia, anaemia	*PTF1A*	chr10:23508437A>G		HOM	P	3.8	[29]
14348	Neonatal (permanent)	18 days	Yes	–	*PTF1A*	chr10:23508437A>G		HOM	P	5.9	[29]
10479	Syndromic	12 years	Yes	Coarse facial features, hypertrichosis, hyperpigmentation, acanthosis nigricans, overcrowded teeth, short stature, hypogonadotropic hypogonadism, severe insulin resistance	*INSR* (NM_00020)	c.2810C>T	p.Thr937 Met	HOM	P	3.5	[30]
11231	Syndromic	7 years	Yes	Short stature, suspected diabetes insipidus	*WFS1* (NM_006005)	c.2589C>G	p.Ile863 Met	HOM	LP	13.5	Novel
11232	Syndromic	12 years	Yes	Short stature, mild optic atrophy	*WFS1*	c.2589C>G	p.Ile863 Met	HOM	LP	13.0	Novel
11279	Syndromic	8 years	Yes	Short stature, camptodactyly, hepatosplenomegaly, aortic regurgitation, myopia, lacrimal duct obstruction	*SLC29A3 (*NM_018344)	c.1041delC	p.Leu349Serfs*56	HOM	P	9.9	[31]
14117	Syndromic/type 2 diabetes	11 years	Yes	Joint stiffness, genu valgum, claw-hand deformity, mild intellectual disability	*GNPTG* (NM_032520)	c.494dupC	p.T165fs	HOM	P	3.5	[32]

Comp. HET, compound heterozygous variant; HOM, homozygous variant; HET, heterozygous variant; LP, likely pathogenic; P, pathogenic; ROH, runs of homozygosity/per cent of the autosome covered by homozygous regions at least 3 Mbp in size; VUS, variant of uncertain significance

according to ACMG guidelines, it is possible that these variants may be the cause of diabetes in this participant.

The clinical phenotypes of these participants are shown in Table 1. Seven consanguineous participants (including one sibling pair) had homozygous variants in the *GLIS3*, *ZNF808* or *PTF1A* genes. Two participants from non-consanguineous families had a heterozygous pathogenic variant in *ABCC8* (transient diabetes) or *INS* (de novo) and one had a homozygous variant in the *PTF1A* enhancer region (Table 1).

The sibling pair (11298 and 11299 in Table 1) had a large homozygous deletion in the *GLIS3* gene causing neonatal diabetes and congenital hypothyroidism. On examination, these participants had distinct facial features typical for this genetic variant (flat nasal bridge, long philtrum, low-set ears, eye protuberance). Growth parameters were normal.

The bi-allelic variant in *ZNF808* was found in a participant born preterm (32nd gestational week). He was diagnosed with neonatal diabetes at age 5 months (adjusted age). He had prolonged jaundice with conjugated hyperbilirubinaemia until the fourth month of life, with failure to thrive, and at 5 months of age he was hospitalised for diabetic ketoacidosis.

We observed a high percentage of pathogenic *PTF1A* variants among probands with genetically confirmed neonatal diabetes (50% of all pathogenic variants), including variants in the enhancer region. Two non-related participants from consanguineous families and one non-consanguineous participant were homozygous for the chr10:23508437A>G variant in the enhancer region (Table 1). Another participant with transient neonatal diabetes was compound heterozygous for two variants in the *PTF1A* enhancer region. One variant (chr10:23508363A>C) was classified as likely to be pathogenic while the other (chr10:23508314T>C) was classified as being a variant of uncertain significance (VUS); both have not been published previously. All participants who were positive for pathogenic *PTF1A* enhancer variants were diagnosed earlier than other children with neonatal diabetes, at 12–35 days of age.

Two non-related participants from consanguineous families had a homozygous hypomorphic coding variant in *PTF1A* (p.Pro191Thr). This variant has been published as causing pancreatic aplasia/hypoplasia with reduced exocrine function and normal neurological function [23]. One of these participants was diagnosed with diabetes at 12 days of age and the other was diagnosed at 6 months of age.

Syndromic diabetes Seven participants (0.9%) had syndromic diabetes, all of whom were from consanguineous families (Fig. 1). A (likely) pathogenic variant was found in four of seven (57%) participants (Table 1). All pathogenic variants were detected in the homozygous state (in *INSR*, *SLC29A3* and *WFS1* [in a sibling pair]). All participants had relatively distinct phenotypic features (Table 1, Fig. 2). The most apparent features were present in the participant with the *INSR* pathogenic variant, who had coarse facial features, severe hypertrichosis, acanthosis nigricans, a height of −3.2 SD at 13 years of age and a bone age delay of 4 years.

Pathogenic variants in *SLC29A3* cause histiocytosis–lymphadenopathy plus syndrome or H-syndrome. The participant with this pathogenic variant had a short stature at 12 years of age (−3.4 SD), autoantibody-negative diabetes, hepatosplenomegaly, camptodactyly and mild aortic regurgitation. She also had a sensorineural hearing impairment and myopia.

Two siblings who were diagnosed with diabetes at 7 and 12 years of age and who had a short stature had a homozygous mutation in *WFS1* causing Wolfram syndrome. The older sibling had partial optic atrophy with normal vision and the other had polyuria due to partial diabetes insipidus.

Discussion

Our retrospective single-centre study of 754 children with diabetes in a highly consanguineous population found that 95% had a clinical diagnosis of type 1 diabetes. To our knowledge, this is the first such study from a region with a high consanguinity rate of up to 44%. The other subtypes of diabetes were much less common: 1.9% had neonatal diabetes, 1.5% had clinically defined MODY, 1.1% had type 2 diabetes and 0.9% had syndromic diabetes. In a study from Turkey (20% consanguinity), 84% of participants were classified as having type 1 diabetes and 5.7% as having type 2 diabetes [8]. A more recent Turkish study focused on children with monogenic diabetes and found a prevalence of 3.1%, which was similar to that in a cohort from the UK (lower rate of consanguinity) [20]. Our study found the prevalence of monogenic diabetes to be 4.2% among all participants followed at the single centre. However, because of the lack of standard antibody testing in those with diabetes, a proportion of individuals with MODY subtypes (such as *HNF1A*/*HNF4A*-MODY) may be misdiagnosed as having type 1 diabetes. Therefore, the actual percentage of individuals with MODY in this population may be higher, which could lead to a greater overall prevalence of monogenic diabetes. The higher prevalence may be attributed to the presence of more recessive forms of diabetes due to the high percentage of consanguinity.

The rate of consanguinity in the study region of Sulaimani has not been formally documented, but a study investigating congenital heart disease in the same region reported 41% consanguinity among the study population [12]. In a 2010 study evaluating consanguinity in the region of

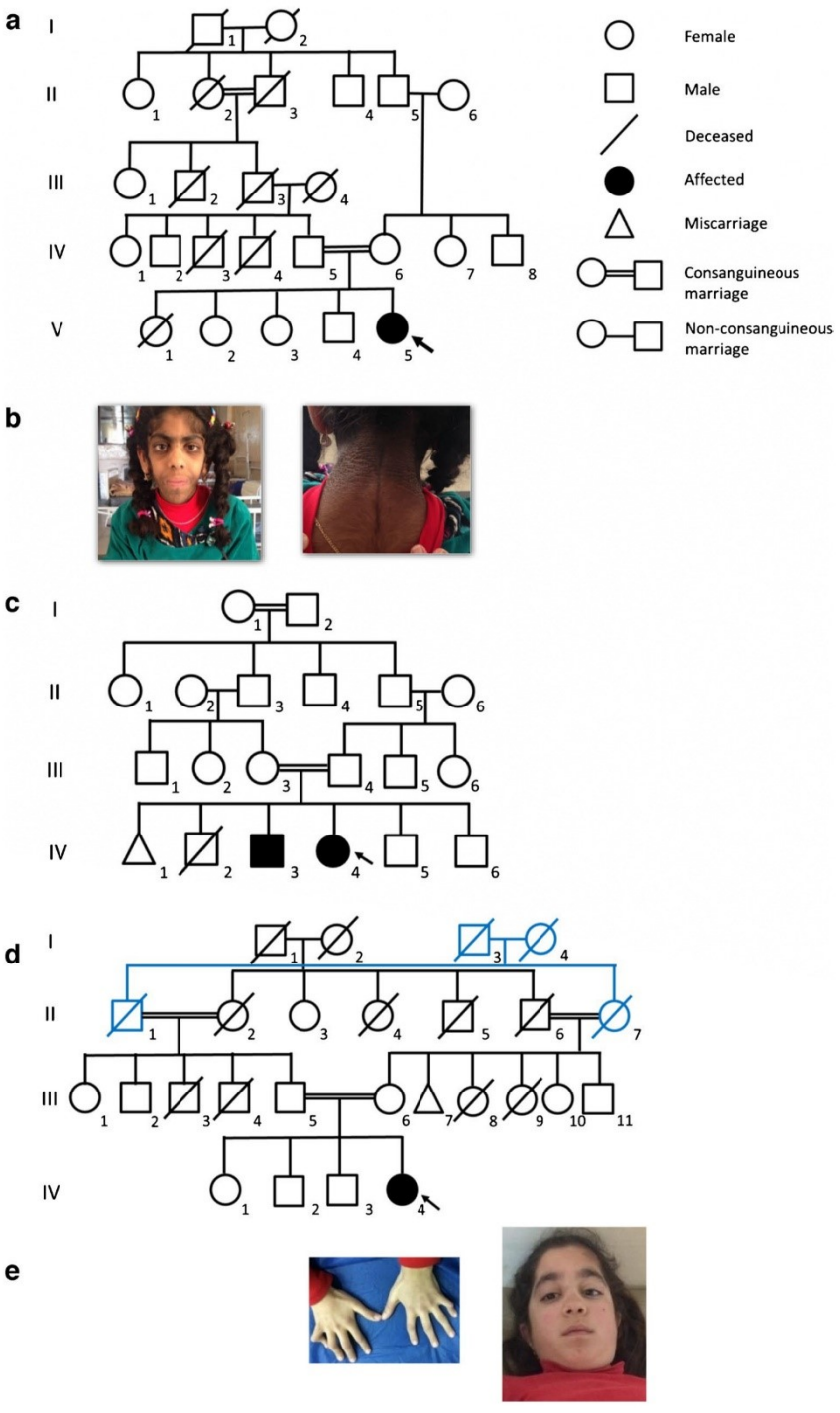

Fig. 2 Participant pedigrees and images of participants with syndromic diabetes. Full facial images are shown with parental permission. (**a**) Pedigree of the participant with the *INSR* variant (V-5); (**b**) dysmorphic facial features in V-5 from (**a**), including hypertrichosis and hyperpigmentation; (**c**) pedigree of the siblings with the *WFS1* variant (IV-3 and IV-4); (**d**) pedigree of the participant with the *SLC29A3* variant (IV-4); II-1 and II-7 are siblings and II-2 and II-6 are siblings; (**e**) mild facial dysmorphy and camptodactyly in IV-4 from (**d**)

Baghdad, Iraq, the rate was 44% [13]. There was a significant association between consanguinity and sociodemographic characteristics, such as differences in rates among urban and rural populations. Furthermore, in a study from north-west Iran, which is geographically close to Kurdistan, Iraq, the rate of consanguinity was 39.1% [14]. These data can be used to make an approximation of the possible rate of consanguinity in the region of Sulaimani.

The consanguinity rate among participants followed at our centre was 36.5%. This is close to the rate among the majority group of participants—those with type 1 diabetes (35%). Although previous studies have shown that the prevalence of type 1 diabetes is not influenced by consanguinity, there is a higher risk of development of type 1 diabetes if there is a history of diabetes in first cousin parents [24]. We confirmed that there was no statistically significant association between consanguinity and type 1 diabetes.

It would be expected that a child from a first cousin marriage would have 6% of the genome covered in ROH. Probands with positive consanguinity who underwent genetic testing (all from reported first cousin marriages) had 3.8–13.5% ROH (Table 1). The participants with no reported consanguinity had 0.0–2.0% ROH. However, because only coding regions were analysed by WES, there may be additional ROH in non-coding regions of the genome.

A genetic diagnosis was identified in 83% of participants with neonatal diabetes who were available for testing by WES (i.e. 10/12). Among these, 80% were homozygous for pathogenic variants causing the disease. There were only two participants with transient neonatal diabetes, with variants in *ABCC8* and *PTF1A* (VUS). In a study comparing genetic causes of neonatal diabetes among consanguineous and non-consanguineous populations, the most common cause of neonatal diabetes among participants born to consanguineous parents was recessive *EIF2AK3* gene variations causing Wolcott–Rallison syndrome, whereas in non-consanguineous populations pathogenic variants in the *KCNJ11* and *ABCC8* genes accounted for the majority of cases (46%). These genes (*KCNJ11* and *ABCC8*) accounted for only 12% of cases in the consanguineous group [10]. Moreover, there was a much higher incidence of recessive forms of neonatal diabetes in consanguineous regions, which we also found in our study.

The overall spectrum of monogenic diabetes genes found in our study population was different from what is found in non-consanguineous populations [10, 25]. We observed homozygous, causal variants in genes such as *PTF1A*, *GLIS3*, *INSR* and *SLC29A3*, which are uncommon in non-consanguineous populations. Consanguineous populations may differ in their genetic burden because of founder effects and the frequency of heterozygotes in potentially pathogenic genes. This is apparent in regard to the number of *PTF1A* variants in our study population. In comparison, the study from Turkey had a predominance of recessive variants in the *WFS1* and *SLC19A3* genes [20]. Therefore, it can be concluded that each consanguineous population is unique, which can allow specific insights into the genetics of conditions such as monogenic diabetes [26].

Most participants with syndromic diabetes in our cohort had relatively distinct phenotypic features suggestive of a monogenic condition, for example the participant with the *INSR* pathogenic variant who had features typical of Rabson–Mendenhall syndrome. At 63 days, she underwent a bilateral oophorectomy owing to the presence of bilateral cysts and the suspicion of a juvenile granulosa cell tumour. This led to hypergonadotropic hypogonadism with absent pubertal development. In our participant, surgery was carried out very early; however, individuals with *INSR* defects can present peripubertally with features resembling polycystic ovary syndrome or adrenache and, if genetic testing is carried out in a timely manner, invasive measures can be avoided.

In the participant with the *SLC29A3* variant causing H-syndrome, the genetic diagnosis was crucial for correct management. Initial evaluation of this participant was performed because of their short stature, hepatosplenomegaly and camptodactyly, which led to examination for mucopolysaccharidosis, revealing mildly decreased levels of alpha-iduronidase. The diagnosis of mucopolysaccharidosis type I was confirmed and the participant was on expensive enzyme therapy (with no effect) until genetic diagnosis.

One participant who was suspected of having syndromic diabetes had a pathogenic variant in the *GNPTG* gene, confirming a diagnosis of mucolipidosis gamma. With regard to his diabetes, we did not find a causal variant. He is currently being treated with metformin, so it is uncertain if his diabetes could be clinically classified as type 2 diabetes or if there is an impact from an unrecognised gene variant.

This raises an interesting point about the diagnosis and classification of individuals with syndromic diabetes, especially in consanguineous regions. It can be argued that the usual set criteria for syndromic diabetes can be misleading in some cases [20]. In addition, in consanguineous families, individuals may have a single gene condition causing the extra-pancreatic phenotype and concurrently develop type 1/type 2 diabetes. Another possibility is the presence of multiple causative homozygous variants causing two conditions, including monogenic diabetes.

Our results showed that consanguinity was significantly associated with syndromic diabetes (p=0.0023) but not with other diabetes subtypes. Therefore, genetic testing in individuals with a suspicion of syndromic diabetes from consanguineous regions is crucial. However, setting criteria for genetic testing in such individuals is restricted by factors such as limited antibody testing. Our testing criteria (see Methods) yielded a high percentage of positive results. We observed that the presence of short stature and hepatosplenomegaly were crucial in finding monogenic diabetes variants

in certain participants. A recent study also found that the presence of specific non-autoimmune extra-pancreatic features (deafness, anaemia and developmental delay) markedly improved the identification of autosomal recessive monogenic diabetes [20]. Consanguinity of parents was a helpful identifying factor as well [20]. Taken together, we suggest testing all individuals matching our selection criteria, with special emphasis on the presence of short stature, hepatosplenomegaly, deafness, anaemia and/or developmental delay.

Enabling genetic testing of people with diabetes in consanguineous populations is important in improving diagnostic criteria for monogenic diabetes [20]. In addition, studies in consanguineous populations have led to the ongoing discovery of novel genes and pathophysiological pathways [26]. One pathogenic variant among our cohort was in the *ZNF808* gene (Table 1), which was identified very recently as causing neonatal diabetes in consanguineous families [27].

Our study provides a new rare insight into the influence of consanguinity on diabetes subtypes in Kurdistan, Iraq, and on the spectrum of genes that are causative of monogenic diabetes (specifically neonatal and syndromic diabetes). The main limitations of our research are the lack of a computerised system for collecting and maintaining patient data, leading to possible transcription errors and missing data for some participants. Furthermore, a lack of records on family history of diabetes and a lack of or limited access to certain laboratory tests such as routine antibody testing and evaluation of C-peptide levels were limiting factors in calculating genetic risk scores. Subtypes were mostly defined clinically by the attending physicians. Genetic testing in those with a clinical suspicion of MODY was not carried out because of a lack of informed consent and available blood samples for genetic testing in many. Furthermore, we believe that MODY prevalence was underestimated because of the reasons mentioned above, the lack of a comprehensive family history of diabetes and the lack of preventive check-ups to identify cases of hyperglycaemia.

Conclusions Our single-centre study provides a unique insight into the prevalence and genetic causes of neonatal and syndromic diabetes in a highly consanguineous population. Our data confirm that, even in such populations, type 1 diabetes is the prevailing paediatric diabetes subtype. It was found that syndromic diabetes is strongly associated with consanguinity. The causative gene in monogenic diabetes was successfully elucidated in 83% of participants with neonatal diabetes and 57% of participants with syndromic diabetes. Homozygous variants made up 80% of all pathogenic variants identified. The spectrum of causative genes (*PTF1A*, *GLIS3*, *WFS1*, *INSR*, *SLC29A3*, *ZNF808*, *ABCC8*, *INS*) is markedly different from the monogenic diabetes genes seen in non-consanguineous cohorts, and also different from those seen in other consanguineous populations. In addition, we observed that phenotypic features such as short stature and hepatosplenomegaly may be important diagnostic criteria for syndromic diabetes in consanguineous populations, in whom diagnosis can be complicated due to the presence of concomitant conditions.

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Data availability The data that support the findings of this study are not openly available because of institutional ethics restrictions but are available from the corresponding author on reasonable request.

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Authors' relationships and activities The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

Contribution statement All authors contributed to the study concept and design. SAA performed the data analysis, including NGS variant analysis, and wrote the manuscript. THT, RNMS and FKHS, who are local Kurdish physicians, complied comprehensive patient data from the study centre. In addition, THT referred participants for genetic testing and provided further clinical information. PD was responsible for NGS and provided insight into the variant analysis. EDF and MNW carried out further NGS variant analysis in participants who had negative genetic results and contributed to overall data interpretation. All authors contributed to the discussion, reviewed or edited the manuscript and approved the final version for publication. JL is responsible for the integrity of the work as a whole.

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7.4. Amaratunga SA, et al. High yield of monogenic short stature among children from consanguineous families: a genetic testing algorithm based on a cohort from Kurdistan, Iraq (manuscript prepared for submission)

This research aims to contribute to the evolving field of short stature by focusing on a unique cohort of children coming from consanguineous families in Sulaimani, Iraq. A research of this nature in a high consanguineous population has not been previously carried out.

A total of 51 children with short stature of unknown etiology were included in the study. 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 (Fig.6). These findings underscore the power of WES in identifying causative genetic factors in predominantly consanguineous populations. If a gene panel of short stature genes was used the maximum rate of positive findings would be 33%. The research also highlights the notable diversity in the spectrum of causative genes when compared to non-consanguineous populations. 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.

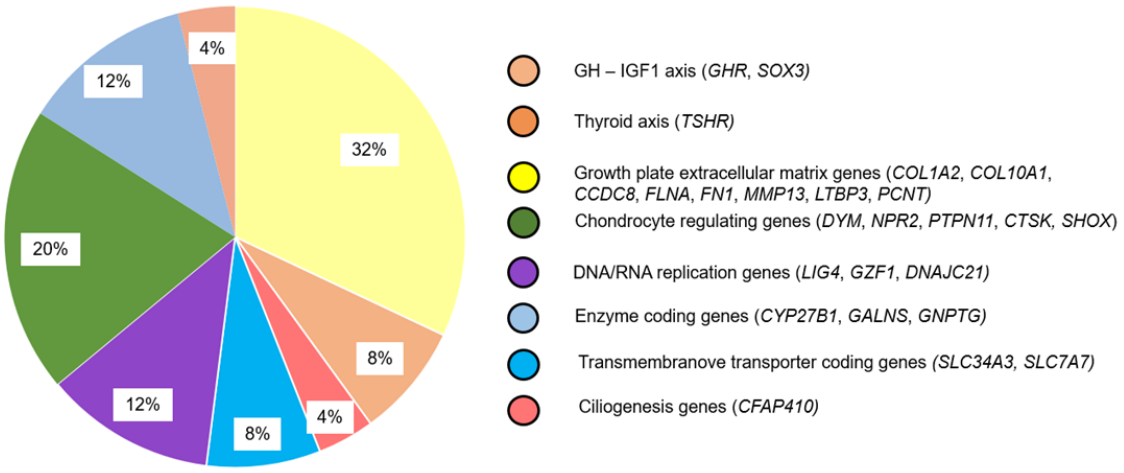

Fig.6. Distribution of pathogenic gene variants (with gene functions) found in the consanguineous short stature cohort

High yield of monogenic short stature among children from consanguineous families: a genetic testing algorithm based on a cohort from Kurdistan, Iraq

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Running Title: Genetic causes of short stature in a consanguineous population

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Abstract

Introduction: Linear growth is a complex process. Current knowledge sheds importance to the GH-IGF-1 axis, growth plate and fundamental intranuclear processes, however the genetics of short stature are not fully understood. This study aims to contribute by investigating a unique cohort of children from consanguineous families from Sulaimani, Iraq and inspire a genetic testing algorithm for similar populations.

Methods: Fifty-one children (30 females) from consanguineous families, with body height for given age and sex ≥ -2.25 SD, were tested. Their median height was -3.3 SD (IQR -4.2 SD to -3.0 SD) and median age 8 years (IQR 5-10). DNA was analyzed by Whole Exome Sequencing. Prioritized potentially pathogenic variants were evaluated by ACMG standards. Probands' without a detected causal variant further underwent MS-MLPA and arrayCGH testing. In addition, a comparative analysis was conducted by juxtaposing our findings with hypothetical results that could have been obtained through the utilization of different short stature panels.

Results*:* 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 (*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*). In addition, two patients had Silver-Russell syndrome and one had a large CNV (22q.11.21). Two variants of uncertain significance were found in potential candidate genes related to growth (*ZSWIM6* and *NISCH*) and one patient had UPD of chr1. If a short stature panel had been used on the above cohort the rate of positive results would have been reduced to 18-48%.

Conclusion*:* The genetic cause of short stature was elucidated in 65% of probands. WES yielded a much higher percentage of positive results than if a gene panel was used. The spectrum of causative genes varied when comparing to non-consanguineous populations. Many cases of syndromic short stature were diagnosed, enabling active screening for possible concomitant conditions and timely management.

Introduction

Linear growth is a multifaceted process influenced by the interaction of genetic, epigenetic, and environmental factors. Traditionally, the growth hormone (GH) and insulin-like growth factor (IGF)-1 axis was regarded as the main regulator of linear growth in children. However, over the last decade the understanding has shifted. It has been showed that growth is primarily overseen by several major hierarchical pathways: the GH-IGF-1 axis, the complex regulation of the growth plate including chondrocytes and the extracellular cartilaginous matrix, and fundamental intracellular processes of all body cells such signal transduction, and processes of DNA expression, transcription, replication, and repair [1, 2]. Any disruption or malfunction within these crucial components can give rise to the condition of short stature.

Short stature is a common diagnosis seen at the paediatric endocrinologist worldwide [1]. It is commonly defined as height below 2 standard deviations (SD) however, other cut-offs can be used in certain indications such as a -2.25 SD in the US in the case of idiopathic short stature [3, 4]. Short stature can be the major sign and a sole phenotype (isolated or "non-syndromic" short stature) or occur alongside other clinical abnormalities such as craniofacial dysmorphism, skeletal disproportionality or even overt skeletal dysplasia, developmental delay, and/or internal organ anomalies ("syndromic" short stature). Some of the well-known and relatively prevalent causes of syndromic short stature include Noonan, Turner and Prader-Willi syndromes [5]. Advances in next-generation sequencing (NGS) have led to the discovery of numerous genes associated with short stature in the recent past [3, 6]. There has been a significant impact of testing in consanguineous families on the success of gene discovery [7]. Nevertheless, majority of current studies on the genetics of short stature have been done in primarily non-consanguineous populations [8-12].

Numerous studies have shown that consanguineous families provide the best opportunity for the discovery of novel gene variants and pathogenic mechanisms. This is due to the increased occurrence of recessive genetic disorders in offspring of closely related parents compared to those of unrelated parents because of a higher chance of rare gene variants being expressed as homozygous [13]. For instance, when first cousins reproduce, they are expected to share one-eighth of their genetic material.

Thus, this study aims to contribute to current knowledge by investigating a unique cohort of children from consanguineous families from Sulaimani, Iraq.

Methods

Cohort selection

A total number of 1124 children with short stature were seen at the Pediatric Endocrinology outpatient clinic at the Dr. Jamal Ahmad Rashed hospital in the region of Sulaimani in Kurdistan between January 2018 and February 2020. This region has a reported consanguinity rate of 44% [14].

Out of them 68 children fulfilled our inclusion criteria (offspring of consanguineous families with body height for given age and sex ≤-2.25SD). Consanguinity was defined as children coming from 1st, 2nd or 3rd cousin matings. Thereafter, 51 probands (30 females) with short stature of unknown origin from unrelated consanguineous families were enrolled into the study with informed consent. All participants underwent physical examination including phenotype description and basic laboratory testing including the evaluation of GH secretion by IGF-1 and GH stimulation tests, when applicable.

The probands were categorized as follows: (1) Probands with short stature (with/ without GH deficiency) and no other phenotypic features – isolated short stature, (2) Probands with short stature (with/ without GH deficiency) and one or more phenotypic features such as – craniofacial dysmorphy including micro/macrocephaly, apparent statural dysproportionality or signs of overt skeletal dysplasia, scoliosis, kyphosis, dental anomalies, cardiac anomalies, developmental delay - syndromic short stature.

This study was approved by the Ethics Committee of Motol University Hospital and 2nd Faculty of Medicine, Charles University in Prague, approval number EK-1263.1.1/19.

Genetic examination

Genomic DNA was extracted from peripheral blood using the QIAmp DNA Blood Mini system (Qiagen, Hilden, Germany). DNA was analysed using whole-exome sequencing (WES). WES was performed using SureSelect Human All Exon Kit V6 + UTRs (Agilent Technologies, Santa Clara, CA), and the indexed products were sequenced by synthesis in an Illumina NextSeq 500 analyser (San Diego, CA). Detected variants were filtered using software Variant Annotation and Filter Tool [15]. Copy number variants subanalysis from raw WES data was done using program Decon [16]. Prioritized variants were then further evaluated using the American College of Medical Genetics and Genomics (ACMG) standards and guidelines implemented in the Franklin software as pathogenic (P), likely pathogenic (LP), benign (B), likely benign (LB), or as variants of uncertain significance (VUS) [17, 18]. All the variants with potential clinical significance were confirmed using Sanger sequencing as described previously [19]. In order to evaluate the segregation of genetic variants within families, Sanger sequencing was performed in both parents, and healthy/affective siblings (when available) with informed consent.

In probands without a detected causal variant using WES, we carried out Methylation-specific Multiplex-ligation dependent probe amplification (MS-MLPA, probe mixes ME030 and ME032, MRC Holland, Amsterdam, The Netherlands) examining regions of 11p15, 7q32, 7p12 and 14q32, respectively associated with Silver-Russell Syndrome.). Moreover, array comparative genomic hybridization (aCGH) testing was performed in order to reveal deletions and duplications across the human genome.

In addition, a comparative analysis was conducted by juxtaposing our findings with hypothetical results that could have been obtained through the utilization of different short stature panels, including our custom gene panel, which was developed primarily non-consanguineous populations [8, 10, 11]. Statistical significance of difference, where applicable, was calculated using a chi-square test.

Results

Cohort characteristics

The median height of the 51 probands was -3.3 SD (Interquartile Range, IQR -4.2 - -3.0 SD) and median age at diagnosis was 8 years (IQR 5-10). All probands were children of 1st cousin matings. Thirty-three probands were classified as syndromic short stature (33/51) whereas the remainder (18/51) had isolated short stature associated with no other phenotypic features (Table 1). Fifteen probands (30% of families) had an affected sibling who shared the same phenotype. GH deficiency was diagnosed in 12 probands.

Genetic results

A pathogenic cause of short stature was elucidated in 33/51 (65%) probands (Table 1). Thirty probands had a bi-allelic or monoallelic variant in a single gene causing monogenic short stature. Two probands had Silver-Russell syndrome and one had a large CNV variant (22q.11.21) typically known to cause DiGeorge syndrome.

Pathogenic or likely pathogenic variants verified by ACMG standards were found 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*) including genes involved in the regulation and function of chondrocytes (*DYM*, *NPR2*, *SHOX*). In addition, we found variants in genes involving key cell functions as signal transduction (*PTPN11*), DNA or RNA replication (*DNAJC21*, *GZF1*, *LIG4*) and transmembrane transport (*SLC34A3*, *SLC7A7*). Followed by enzyme coding genes (*CYP27B1*, *GALNS*, *GNPTG*) and one ciliogenic gene (*CFAP410*) (Table 1).

Seventeen variants are novel and have not been previously published. Majority of variants (24) are homozygous, while 6 were monoallelic (*NPR2*, *COL1A2*, *FN1*, *SHOX)*, including de-novo variants (*FLNA*, *PTPN11*). Three variants of uncertain significance (VUS) were found in two candidate genes related to growth (*ZSWIM6* and *NISCH*). and one patient had uniparental isodisomy (UPD) of chromosome 1.

Among the patients with syndromic short stature, the rate of a positive finding was 88% whereas in the isolated short stature group it was only 22%. Therefore the presence of other phenotypic features was statistically significant to finding a genetic cause.

Comparison with gene panels

Patient data was analysed using three gene panels primarily used for non-consanguineous populations and yielded only 12, 22, and 33% positive results respectively depending on the genes present in each panel.

Discussion

Using NGS methods, a pathogenic gene variant causing short stature was found in 33/51 probands from Sulaimani, Kurdistan, Iraq. To our knowledge, this is the first ever study analysing the genetic background of short stature in a population of children with short stature in a highly consanguineous region. Genetic testing was done in a step-wise manner. Majority of variants (30/51; 59%) were found by the first step which was WES. The remaining diagnoses of Silver Russell and DiGeorge syndromes were found by MS-MLPA and aCGH methods carried out subsequently.

Overall, the spectrum of genes found in this highly consanguineous population was highly varied from genes found in short stature cohorts from non-consanguineous regions where the most common genes variants are of autosomal dominant inheritance [8, 9, 20]. Most variants in our cohort were bi-allelic (47%) and in genes such as *CFAP410*, *CTSK*, *CYP27B1*, *GNPTG* and *PCNT which are not usually found in non-consanguineous populations*. Studies on short stature genetics from consanguineous populations are extraordinary, they are mostly limited to single case reports or small series of preselected subjects. Nevertheless, from available data, it could be speculated that the common causative genes are varied even between consanguineous populations [21, 22].

Only two individuals from the cohort who were diagnosed as having GH deficiency had variants in the GH-IGF1 axis, thereby having true genetic GH deficiency whereas the others had variants in other genes not linked to GH. This supports the current shift in the paradigm of growth showing the important of mechanisms other than the GH-IGF1 axis in growth regulation and a tendency of over-diagnosing GH deficiency by currently used diagnostic procedures [23].

Participants with syndromic short stature had the highest rate of positive genetic diagnosis (30/33; 91%). The most common syndromic features were microcephaly, developmental delay and intellectual impairment. Therefore, the presence of short stature below -2.25 SD and other phenotypic features such as those mentioned above, could be a strong indicator for physicians when considering a genetic testing algorithm for patients from consanguineous families. However, it is important to mention that in consanguineous families, due to the increase percentage of shared genome, concomitant genetic conditions could be present. This could complicate the genetic confirmation of syndromic short stature as diverse clinical and phenotypic features could be attributed to multiple gene conditions in a single patient.

Identical CNV variants in two genes were seen in 5 unrelated probands (three with a variant in *SLC7A7* and two with *DYM*, causing lysinuric protein intolerance and Smith-McCort dysplasia respectively), suggesting a founder effect in this population. In addition, we found two potential novel candidate genes for short stature among our cohort (*ZSWIM6* and *NISCH*). These patients had no other gene variants via MLPA or arrayCGH. *ZSWIM6* has been previously published as causing short stature as part of acromelic frontonasal dysostosis and neurodevelopmental disorder syndromes but its specific mechanism with relation to growth failure is unknown [24]. *NISCH* is not yet known to cause disease in humans, however it has been shown to influence cell-signaling cascades triggering to cell survival, growth and migration and found to stunt growth in mice [25]. All the above methods factors support the pathogenicity of the variant however further investigation is necessary to prove causality with short stature. One patient had a UPD of chromosome 1, which has currently been a topic of debate with regard to its association with short stature [26, 27].

It was found that if a short stature panel was used on this cohort of consanguineous patients only 6-17/51 patients (11-33%) would obtained a positive result. This is a significant reduction of the positivity rate if only WES (59%). That brings up an important point about the use of universal gene panels and their reliability in all populations. Most short stature panels are developed for non-consanguineous populations where mono-allelic / heterozygous variants are most probable. Within our consanguineous cohort from Sulaimani, Iraq, 73% of variants were homozygous and over half were in genes not present in common gene panels. This highlights the necessity for a tailored approach to genetic testing, particularly in populations with a high rate of consanguinity. Our study contributes to a more

comprehensive understanding of the genetic landscape of short stature in this specific population of Kurdistan, Iraq and provides a starting point for the customization in genetic testing strategies (in-house panels for specific populations) to ensure accurate and effective diagnoses.

The main limitations of our study were limited access to detailed laboratory results and limited standardization in certain laboratories in Sulaimani. Response to GH treatment could not be systematically evaluated due to intermittent availability of treatment for most patients. In addition, whole genome sequencing or trio analysis was not carried out.

Conclusion

In a unique cohort from a primarily consanguineous area, the genetic cause of short stature was elucidated in 65% of individuals with short stature using of WES, MS-MLPA or aCGH. If a gene panel was used, this yield would be reduced to 11 - 33%. When compared to regions with less consanguinity and other regions with high consanguinity, the range of genes responsible for short stature varied. As anticipated, the majority of identified genetic variations were of a recessive nature. Interestingly, several instances of syndromic short stature were diagnosed, even though they had initially been referred to as idiopathic short stature, often with mild or unrecorded phenotypic characteristics. This enabled proactive screening for potential concurrent conditions and timely intervention.

Table 1: Patient characteristics and their detected pathogenic variants. Abbreviations GH: growth hormone, HOM: homozygous variant, HEM: hemizygous variant, HET: heterozygous variant, ACMG: American College of Medical Genetics classification, LP: Likely Pathogenic, P: Pathogenic, SD: Standard Deviation, VUS: Variant of Uncertain Significance, NA: not applicable

Patient No.	Gender	Age at first examination (years)	Height (cm)	SD	Additional clinical characteristics	Gene	Nucleotide variant*	Protein variant *	Zygosity	ACMG
Patients with variants in genes in the GH-IGF-1 axis										
11263	F	10.3	120.5	-2.9	None	*GHR*	c.1807_1808delinsA	p.Ser603fs	HOM	LP
11183	M	10.9	110	-3.1	GH deficiency, Glucose-6-phosphate dehydrogenase deficiency	*SOX3*	c.740_741insTGCCGCGGCCGCAGCCGCTGC	p.Ala242_Ala248dup	HEM	LP
						G6PD	c.563C>T	p.Ser188Phe	HEM	P
Patients with variants in genes in the thyroid axis										
12533	F	4.5	91	-3.1	Hypothyroidism, Scoliosis	*TSHR*	c.1555C>T	p.Arg519Cys	HOM	LP
Patients with variants in genes in the growth plate extracellular matrix										
12559	F	8.1	91.5	-7.4	Blue sclera, Scoliosis, Tibial bowing, Short deformed femur	*COL1A2*	c.1406G>C	p.Gly469Arg	HET	LP
13218	M	2.5	80	-3.2	Short curved lower limbs	*COL10A1*	c.1954C>T	p.Leu652Phe	HOM	LP
12590	F	5.1	96.5	-2.7	Frontal bossing, triangular facies	*CCDC8*	c.963delA	p.Ala323fs	HOM	P
11251	F	6.9	103.8	-3.4	Cleft pallet and rocker bottom feet	*FLNA*	c.7462_7463insGGCACCT	p.Leu2488fs	HET	P
11289	F	6.75	97	-4.9	Madelung deformity, disporportional short stature	*FN1*	c.6167C>T	p.Pro2056Leu	HET	LP
11242	F	6.3	100.5	-3.4	Metaphyseal dysplasia, bow legs	*MMP13*	c.696C>G	p.His232Gln	HOM	LP
11824	M	9.1	115.9	-3	Triangular face, absent upper teeth, amelogenesis imperfekta	*LTBP3*	c.2223delC	p.Cys742fs	HOM	LP
12507	M	0.8	46	-15.5	IUGR, developmental delay, microcephaly, severe growth failure	*PCNT*	c.78_81del	p.Leu26fs	HOM	LP
Patients with variants in genes involved in the regulation or function of chondrocytes										
11293	M	7.9	109	-3.3	Prominent nose, micrognathia, osteosclerosis	*CTSK*	c.894G>A	p.Trp298Ter	HOM	P

Patients with variants in genes involved in the regulation or function of chondrocytes										
11293	M	7.9	109	-3.3	Prominent nose, micrognathia, osteosclerosis	*CTSK*	c.894G>A	p.Trp298Ter	HOM	P
11152	F	10.7	107	-5.3	Microcephaly, scoliosis, developmental delay, waddling gait	*DYM*	arr[GRCh37] 18q21.1(46617017x2,46621125_46623932x1,46639625x2)		HOM	P
12579	M	6.2	81	-6.8	Microcephaly, coarse facies, short neck, severe developmental delay, skeletal dysplasia, genua valgum	*DYM*	arr[GRCh37] 18q21.1(46617017x2,46621125_46623932x1,46639625x2)		HOM	P
11316	M	4.9	92.4	-3.4	Gastrostomy inserted for palatopharyngeal incoordination	*NPR2*	c.2720C>T	p.Thr907Met	HET	P
11986	M	12.7	135.5	-2.4	Triangular face, ptosis, pectus excavatum	*PTPN11*	c.1403C>T	p.Thr468Met	HET	P
11218	F	10.2	118	-3.3	Disporportionate short stature	*SHOX*	arr[GRCh37] Xp22.33(550508x2,578734_607199x1,611306x2)		HET	P

Patients with variants in genes involved in transport processes									
M	12.2	116	-4.8	Microcephaly, ptosis, cleft lip, mental retardation	*SLC34A3*	c.1058G>T	p.Arg353Leu	HOM	LP
F	13.6	138	-3.2	Congenital blindness of the left eye, significant hepatosplenomegaly, anemia	*SLC7A7*	g.23242443-23249258del		HOM	P
F	2.6	81	-2.5	Splenomegaly, mild hepatomegaly, anemia, leukopenia, thrombocytopenia	*SLC7A7*	g.23242443-23249258del		HOM	P
F	9.3	104.5	-5.2	Hepatosplenomegaly,	*SLC7A7*	g.23242443-23249258del		HOM	P
Patients with variants in enzyme coding genes									
M	9.1	114	-3.4	Frontal bossing, rickets, widened, epiphyses, bowing of legs, hypocalcemia, hypophosphatemia, increased serum parathyroid hormone (PTH)	*CYP27B1*	c.1325_1326insCCCACCC	p.Pro442fs	HOM	P
F	13.2	133	-3.6	Coarse facies, joint stiffness, joint pain, scoliosis	*GNPTG*	c.499dupC	p.Leu167fs	HOM	P
M	5.0	91	-3.8	Kyphosis, pectus excavatum, widened metaphyses	*GALNS*	c.410T>C	p.Ile137Thr	HOM	LP
Patients with variants in miscellaneous genes / hypomethylation / microdeletion syndromes									
M	14.2	137	-3.2	Thoracic deformation, mild mitral valve regurgitation	*CFAP410*	c.64A>T	p.Lys22Ter	HOM	LP
M	2.1	75	-3.8	Mild facial dysmorphy, chronic diarrhea, congenital heart disease	*22q11*	del22q11.21		HET	P
F	10.0	117.8	-3.2	Triangular face, Micrognathia, Thin lips	*11p15*	g.1975970-1976580 |lom		NA	P
F	6.3	101.5	-3.2	Triangular face, hemihypertrophy	*11p15*	g.1975970-1976580 |lom		NA	P

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SGA-SS, affecting 10-15 % of SGA children, remains enigmatic in terms of its underlying mechanisms. This study examines the genetic cause of short stature among children who were born small for gestational age (SGA) and failed to catch up in growth (SGA-SS) from a single center in Prague, Czech Republic (a non-consanguineous population).

Within a cohort of 820 patients who received GH treatment, 256 individuals were classified as SGA-SS based on stringent criteria. Among these, 176 patients had available DNA data from both the child and their parents, and were included in the study.

The research revealed a high diagnostic yield, with 42% of the children having an elucidated genetic aetiology. This study uncovers the complex genetic landscape underlying SGA-SS, emphasizing the pivotal role of the growth plate and implicating various axes, signalling pathways, and intracellular regulatory mechanisms. These findings provide fresh insights into the genetic factors contributing to SGA-SS in a non-consanguineous cohort, shedding light on its intricate pathophysiology.

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The Genetic Landscape of Children Born Small for Gestational Age with Persistent Short Stature

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Keywords
Small for gestational age · Short stature · Genetics · GH-IGF-1 axis · Growth plate

Abstract
Introduction: Among children born small for gestational age, 10–15% fail to catch up and remain short (SGA-SS). The underlying mechanisms are mostly unknown. We aimed to decipher genetic aetiologies of SGA-SS within a large single-centre cohort. ***Methods:*** Out of 820 patients treated with growth hormone (GH), 256 were classified as SGA-SS (birth length and/or birth weight <−2 SD for gestational age and life-minimum height <−2.5 SD). Those with the DNA triplet available (child and both parents) were included in the study (176/256). Targeted testing (karyotype/FISH/MLPA/specific Sanger sequencing) was performed if a specific genetic disorder was clinically suggestive. All remaining patients underwent MS-MLPA to identify Silver-Russell syndrome, and those with unknown genetic aetiology were subsequently examined using whole-exome sequencing or targeted panel of 398 growth-related genes. Genetic variants were classified using ACMG guidelines. ***Results:*** The genetic aetiology was elucidated in 74/176 (42%) children. Of these, 12/74 (16%) had pathogenic or likely pathogenic (P/LP) gene 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*, various collagens, *FLNB*, *MATN3*), and 7/74 (9%) the paracrine chondrocyte regulation (*FGFR3*, *FGFR2*, *NPR2*). In 12/74 (16%), we revealed P/LP 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. ***Conclusions:*** The high diagnostic yield sheds a new light on the genetic landscape of SGA-SS, with a central role for the growth plate with substantial contributions from the GH-IGF-1 and thyroid axes and intracellular regulation and signalling.

Based on the abstract that was granted the Henning-Andersen Prize at the 60th ESPE Annual Meeting, Rome, September 2022.

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Introduction

Approximately 5% of children are born small for gestational age (SGA) – with a birth weight and/or length below −2 SD compared to normative values for their gestational age [1]. The aetiology of SGA is heterogeneous (environmental, maternal, placental, and endogenous factors, including defined gene variants [2]). Up to 90% of SGA children develop catch-up growth during the first 2 years of life, while the remaining fail to catch up and are referred to as 'small for gestational age – short stature' (SGA-SS). These children are known to remain small throughout childhood and reach a substantially reduced adult body height [3, 4]. They are therefore indicated for treatment with growth hormone (GH) [1, 5, 6]. Nevertheless, the response to GH administration is variable among individual SGA-SS children, which may reflect the heterogeneous aetiology of their growth failure [7, 8].

In SGA-SS, several genetic mechanisms should be taken into consideration: imprinting disorders and abnormal methylation patterns such as Silver-Russell syndrome (SRS), Temple syndrome, IMAGe syndrome, and others [9–11]. In addition, a long list of single gene conditions has been associated with the regulation of human growth and thus impact on final height, albeit not necessarily associated with prenatal growth restriction [12, 13]. Some of these genes regulate the structural development of the cerebral midline and pituitary and functional components of the GH-IGF-1 axis (hormones, their receptors, and post-receptor signalisation). Moreover, new genes have been discovered which code for important growth plate paracrine factors, proteins of cartilage extracellular matrix, components of intracellular regulating cascades, and proteins involved in fundamental intranuclear processes [2].

The elucidation of the genetic background of SGA-SS was initiated no more than 2 decades ago [2]. In some cases, a child might present with typical features, leading to targeted genetic testing. A typical example is the genetic diagnosis of SRS in individuals fulfilling the Netchine-Harbison clinical criteria [9]. However, most SGA-SS children present with no apparent syndromic features; therefore, genetic diagnosis is challenging.

New possibilities of genetic testing such as next-generation sequencing (NGS) allowed new advancements in discovering the genetic aetiology of short stature within the past decade [14]. Knowledge of the genetic basis of growth disorders in these children not only helps in better understanding the pathophysiology of growth but may have important consequences for their treatment and follow-up as well. The aim of this study was to decipher genetic aetiologies among a large single-centre cohort of SGA-SS children treated with GH and to stratify them according to molecular mechanisms leading to combined pre- and postnatal growth failure.

Patients

The study cohort was selected from 820 children treated with GH in our centre between May 2008 and December 2018 using a stepwise selection process as displayed in Figure 1. Other causes of growth failure were considered and appropriately evaluated before starting GH therapy. Extremely preterm children (gestational age <28 weeks) were excluded due to missing relevant normative values for their size at birth. After exclusion of children treated with GH for other causes (chronic kidney disease, acquired GH deficiency (GHD), Turner syndrome, Prader-Willi syndrome, and primary GHD born either appropriate for gestational age or SGA but with life-minimum height >−2.5 SD), 256 children with SGA-SS (birth weight or length <−2 SD and body height <−2.5 SD after 3 years of life) remained for further evaluation. Out of them, 176/256 (69%) families agreed to genetic testing; therefore, the child and both of his/her parents were enrolled in the study (Fig. 1). The clinical assessment of all children included measurements of weight (using an electronic scale) and height (mean of three measurements using a calibrated stadiometre to the nearest 1 mm). These results were converted to the SDS using age- and sex-specific normative values [15]. The height of the parents was either obtained during the patients' visit using the same method or referred from their medical records. Birth parameters were obtained from medical records.

The cohort included 93 males and 83 females. The median birth term was 39 weeks (interquartile range [IQR] 37; 40), median birth weight was 2,485 g (IQR 2,108; 2,788), and median birth length was 45 cm (IQR 43; 47). The median age at initiation of GH therapy was 4.95 years (IQR 3.13; 7.18), and the median life-minimum height SDS was −3.04 (IQR −3.49; −2.72).

All SGA-SS children underwent long-term GH treatment with a dosage of 35 μg/kg/day as suggested in the consensus from Clayton et al. [5]. If the child was also found to have GHD, the dose was in the range of 25–35 μg/kg/day in accordance with summary of product characteristics, and in the case of SHOX deficiency, 50 μg/kg/day as recommended in previous studies [16].

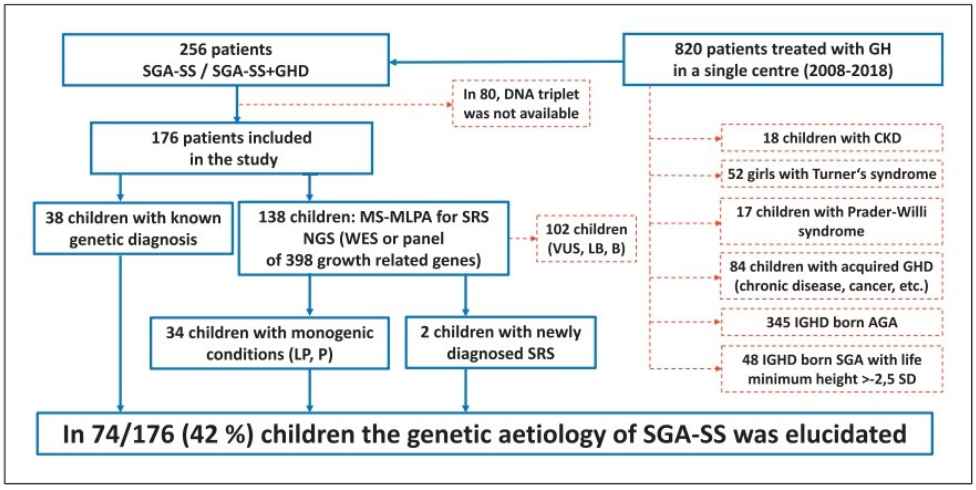

Fig. 1. Flowchart of the study. GH, growth hormone; CKD, chronic kidney disease; GHD, growth hormone deficit; IGHD, idiopathic growth hormone deficit; AGA, appropriate for gestational age; MS-MLPA, methylation-specific multiplex ligation-dependent probe amplification; WES, whole-exome sequencing; SRS, Silver-Russell syndrome; VUS, variant of uncertain significance; LB, likely benign; B, benign; LP, likely pathogenic; P, pathogenic.

Methods

Genetic Testing

Genetic Testing Prior to the Study

All children with a clinical suspicion of a specific genetic disorder underwent genetic examination with an appropriate method (karyotype, FISH, MLPA, targeted Sanger sequencing) prior to the study. The remaining children were examined for SRS. After its' exclusion, patients were examined by NGS methods.

Examination of SRS

Methylation-specific multiplex ligation-dependent Probe amplification (MS-MLPA) was done in all patients. MS-MLPA (probe mixes ME030 and ME032 examining regions of 11p15, 7q32, 7p12, and 14q32, respectively) and subsequent data analyses by software Coffalyser were performed according to the manufacturer's instructions (MRC Holland, Amsterdam, The Netherlands).

Targeted NGS

Genomic DNA was extracted from peripheral blood using QIAmp DNA Blood Mini (Qiagen, Hilden, Germany) or from saliva (collected into Oragene OG-500) according to the manufacturer's instructions (DNA Genotek, Ontario, Canada). DNA of patients without a verified genetic cause of their growth failure was analysed using a custom-targeted NGS panel of 398 genes with a known or potential association with growth [17] using SureSelect Custom Kit (Agilent Technologies, Santa Clara, CA, USA), and the indexed products were sequenced by synthesis on an Illumina MiSeq platform (San Diego, CA, USA) with ×100 average coverage. Altogether 6 DNA samples from probands underwent the whole-exome sequencing using SureSelect Human All Exon v6+UTR Kit (Agilent Technologies). The indexed products were sequenced by synthesis on an Illumina MiSeq or NextSeq platform (San Diego, CA, USA) with ×100 average coverage. Obtained sequences were annotated and mapped to reference genome followed by variant calling as described previously [17]. Detected variants were filtered using software Variant Annotation and Filter Tool [18] with filter settings described previously [17].

Evaluation of Genetic Findings

Confirmation of all variants of interest in the patient and segregation analyses in available family members were performed by direct Sanger sequencing [19]. Subsequently, variants were scored according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines [20] implemented in the VarSome software [21] as pathogenic (P), likely pathogenic (LP), benign (B), likely benign (LB), or as variants of uncertain significance (VUS). Consideration of co-segregation in the pathogenicity classification of variants (criterion PP1 in the ACMG guidelines) was applied based on recommendations by Jarvik and Browning [22].

Ethics Statement

This study protocol was reviewed and approved by the Institutional Ethics Committees of the 2nd Faculty of Medicine, Charles University in Prague, and University Hospital Motol, Czech Republic (date of approval: June 30, 2017; not numbered). Written informed consent was obtained from the parents/legal guardians of the patients for publication of the details of their medical cases and any accompanying images.

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Table 1. Clinical findings in children born small for gestational age with persistent short stature (SGA-SS) with elucidated genetic diagnosis

Patient No.	Gender	GW	BW, g	BW (SDS)	BL, cm	BL (SDS)	Father's height (SDS)	Mother's_ height (SDS)	Age at start of GH therapy, years	Height (SDS) at start of GH therapy	Height (SDS) after 1 year of GH therapy	Height (SDS) after 3 years of GH therapy	Final height (SDS)	IGF-1 (SDS) prior to therapy	BA-CA, years	Primary diagnosis leading to GH treatment	Brain MRI	Dysmorphic features
1	F	37	1,860	−2.8	42	−3.8	−1.3	−2.7	6.3	−3.2	−2.0	−1.6	N/A	1.09	−1.2	GHD+SGA	Normal	No
2	M	42	2,890	−2.1	50	−1.4	0.4	−3.9	4.1	−3.1	−2.4	−1.9	N/A	−2.01	−0.4	GHD+SGA	Normal	No
3	M	40	2,840	−1.9	47	−2.4	−1.7	−0.7	7.7	−2.7	−2.3	−1.6	−2.6	0.11	N/A	GHD+SGA	Normal	No
4	F	36	1,690	−3.0	37	−6.4	0.0	−0.5	3.2	−4.2	−3.3	−3.2	N/A	N/A	N/A	SGA	N/A	Yes
5	F	39	2,210	−2.9	44	−3.4	−1.5	0.1	3.5	−2.7	−2.0	−1.4	N/A	1.27	−1.5	SGA	N/A	No
6	M	32	1,260	−1.9	38	−3.9	−1.6	−2.7	7.0	−3	−2.2	−1.7	N/A	0.02	−0.3	SGA	N/A	No
7	F	39	2,480	−2.2	45	−2.8	−0.7	−0.4	7.4	−4.3	−4.0	−3.4	−2.8	−1.73	N/A	SGA	N/A	No
8	M	37	2,060	−2.5	44	−2.8	0.3	0.6	2.3	−3.8	−3.8	−2.7	N/A	−5.37	N/A	GHD+SGA	Bilateral anophthalmia, agenesis of optic nerves, and chiasm	Yes
9	M	33	1,750	−1.1	41	−2.7	−0.6	0.4	1.1	−5.0	−3.1	−1.9	N/A	−4.40	−1.1	GHD+SGA	N/A	No
10	F	40	2,350	−2.9	48	−1.6	0.4	−1.2	13.5	−3.3	−2.3	−1.1	−1.0	−4.78	−1.4	GHD+SGA	Rathke cleft cyst	No
11	M	30	1,120	−1.3	36	−4.0	0.4	0.4	2.8	−2.6	−1.9	−0.7	N/A	−1.84	−1.8	GHD+SGA	Normal	No
12	M	35	2,200	−1.1	44	−2.1	−0.03	−1.2	6.3	−2.5	N/A	N/A	N/A	−2.74	−0.8	SGA	N/A	No
13	M	40	2,750	−2.1	48	−1.9	−0.7	−0.8	7.1	−3.1	−2.6	−2.2	N/A	−1.78	−2.3	GHD+SGA	N/A	No
14	M	40	2,900	−1.8	47	−2.4	−2.9	−2.0	11.6	−3.7	−3.9	−2.9	−2.6	−3.22	−3.3	SGA	N/A	No
15	F	39	2,850	−1.6	46	−2.2	−1.7	−2.9	5.7	−3.0	−2.7	−2.5	N/A	0.74	1.9	SGA	N/A	No
16	M	38	2,670	−0.6	45	−2.7	0.7	−1.6	3.6	−2.5	−1.5	−1.0	−0.5	0.91	N/A	SGA	N/A	No
17	F	40	2,960	−1.3	46	−2.6	0.7	−1.2	4.4	−3.1	−2.4	−1.9	−3.6	1.19	0.1	SGA	N/A	No
18	M	40	2,920	−1.8	45	−3.4	−3.6	−0.8	7.4	−3.3	−2.7	−2.1	−2.0	−3.10	0.5	SGA	Normal	No
19	F	33	1,350	−2.1	38	−4.3	−2.4	−2.3	3.3	−2.7	−2.6	−2.1	−1.5	−1.51	0.2	SGA	N/A	No
20	M	39	2,450	−2.4	46	−2.55	−2.3	−2.7	12.6	−2.7	−2.3	−2.7	−3.1	0.73	−0.3	SGA	N/A	No
21	F	40	2,350	−2.9	46	−2.7	−1.0	−1.5	5.4	−3	−2.8	−1.9	N/A	−1.35	−0.3	GHD+SGA	Normal	Yes
22	M	40	2,520	−2.6	49	−1.4	−2.0	0.0	4.3	−2.5	−2.0	−1.9	−2.3	−0.64	−0.9	GHD+SGA	N/A	No
23	F	39	2,270	−2.7	42	−4.4	−1.5	−3.4	5.7	−2.8	−2.1	−0.8	−2.7	0.49	−0.4	SGA	N/A	No
24	M	39	2,450	−2.5	45	−3.1	−2.9	−1.3	12.0	−3.4	−2.7	−2.1	−2.2	−1.51	−1.6	SGA	N/A	No
25	F	40	2,500	−2.5	43	−4.3	−2.2	−0.4	7.0	−3.1	−2.4	−2.1	N/A	0.93	−2.5	SGA	N/A	No
26	M	39	2,660	−2.0	47	−2.1	−3.2	−1.5	6.0	−3.7	−3.2	−2.8	N/A	−3.16	N/A	SGA	Cyst of cisterna magna	No
27	M	40	2,750	−2.1	48	−1.9	−1.3	−1.5	9.2	−2.7	−2.2	−1.4	N/A	0.34	−1.4	SGA	N/A	No
28	M	40	2,400	−2.9	48	−1.9	−1.5	−1.2	10.2	−2.8	−2.0	−0.6	−1.5	−1.26	N/A	GHD+SGA	N/A	Yes
29	F	40	3,000	−1.3	45	−3.2	0.8	−1.5	6.4	−2.8	−2.2	−1.5	−2.1	−0.15	−0.2	SGA	Normal	No
30	M	40	3,200	−1.0	47	−2.4	−3.2	0.8	1.5	−2.5	−1.8	−1.5	N/A	−0.27	N/A	SGA	N/A	No
31	M	40	2,650	−2.3	47	−2.4	0.4	−2.3	9.1	−2.5	−1.6	−0.8	N/A	0.09	0.1	SGA	N/A	No
32	F	41	3,190	−1.0	47	−2.4	−1.2	−1.2	7.1	−2.5	−1.6	0.1	N/A	−0.90	−2.1	GHD+SGA	Pituitary microadenoma	No
33	F	40	2,600	−2.3	45	−3.2	−1.1	−1.2	6.0	−2.9	−2.3	−1.4	N/A	0.32	N/A	SGA	Normal	No
34	F	41	2,880	−1.8	47	−2.4	0.8	−2.4	4.0	−2.9	−2.9	−1.9	N/A	0.15	N/A	GHD+SGA	Normal	No
35	M	39	2,710	−1.9	46	−2.6	N/A	−1.8	3.3	−2.8	−2.4	−2.2	N/A	−2.01	−0.4	GHD+SGA	Normal	No
36	F	41	2,800	−2.0	48	−1.8	−2.4	−2.3	3.1	−3.6	−3.0	−2.5	N/A	−6.04	N/A	SGA	N/A	No
37	F	40	2,630	−2.2	47	−2.1	−2.0	−1.3	3.4	−3.5	−2.7	−2.5	−2.7	−1.65	−0.3	SGA	N/A	No
38	M	38	2,460	−2	44	−3.2	−1.5	−3.7	4.5	−3.5	−2.9	−2.5	N/A	−2.48	N/A	GHD+SGA	Normal	No
39	M	39	2,490	−2.4	48	−1.6	−2.9	−1.2	1.5	−2.9	−2.9	−2.5	N/A	−2.50	−0.8	SGA	Normal	Yes
40	M	40	2,460	−2.8	49	−1.4	−0.5	0.4	2.2	−2.5	−1.1	−0.9	N/A	−0.45	0.0	GHD+SGA	Small pituitary	Yes
41	M	40	3,270	−0.9	46	−2.9	−0.46	0.43	1.9	−4	−3.0	−4.8	N/A	−1.37	−0.5	GHD+SGA	Small pituitary	Yes
42	F	39	2,444	−2.3	44	−3.4	−1.4	0.2	3.9	−2.9	−2.3	−2.5	N/A	0.88	N/A	SGA	N/A	Yes

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Table 1 (continued)

Patient No.	Gender	GW	BW, g	BW (SDS)	BL, cm	BL (SDS)	Father's height (SDS)	Mother's_ height (SDS)	Age at start of GH therapy, years	Height (SDS) at start of GH therapy	Height (SDS) after 1 year of GH therapy	Height (SDS) after 3 years of GH therapy	Final height (SDS)	IGF-1 (SDS) prior to therapy	BA-CA, years	Primary diagnosis leading to GH treatment	Brain MRI	Dysmorphic features
43	F	35	2,050	−1.3	42	−2.9	0.8	−1.5	2.3	−3.9	−3.3	−2.9	N/A	0.12	N/A	SGA	N/A	Yes
44	F	42	2,800	−2.0	46	−3.1	−1.6	−1.5	5.2	−3.3	−3.0	−2.5	−3.0	−0.42	−0.8	GHD+SGA	Normal	No
45	M	38	2,390	−2.2	N/A	N/A	−0.7	−0.4	2.1	−4.3	−3.5	−3.1	N/A	<-2.5	N/A	GHD+SGA	Pachygyria, heterotopia of grey matter, thinning of corpus callosum	Yes
46	F	39	2,500	−2.2	46	−2.3	−2.3	−1.0	2.9	−3.4	−2.9	−2.0	−1.9	−1.26	N/A	GHD+SGA	Normal	No
47	M	40	3,350	−0.7	47	−2.4	−1.0	−1.6	4.2	−3.0	−2.2	−1.7	N/A	−2.85	N/A	GHD+SGA	Normal	No
48	F	42	2,820	−2.0	48	−1.9	1.7	0.1	3.1	−3.7	−3.0	−3.1	−3.7	0.15	−1.9	SGA	N/A	Yes
49	F	38	2,480	−1.8	45	−2.4	−1.2	−0.8	1.6	−3.4	−2.8	−2.0	N/A	−0.37	N/A	GHD+SGA	Small pituitary, thin pituitary stalk	Yes
50	M	38	2,900	−1.0	45	−2.7	−1.5	−1.9	3.0	−3.6	−2.9	−2.4	N/A	−1.60	N/A	SGA	N/A	No
51	F	40	2,790	−1.8	46	−2.7	0.8	−3.1	9.8	−3.0	−2.3	−1.8	−2.6	N/A	−2.2	SHOX	N/A	Yes
52	M	40	3,200	−1.0	47	−2.4	−3.2	−2.0	11.5	−2.9	−2.3	−2.1	−3.0	0.48	1.1	SHOX	N/A	Yes
53	F	34	2,130	−0.5	43	−1.9	−1.7	−0.4	8.4	−2.8	−2.3	−1.7	N/A	0.67	−1.2	SHOX	N/A	Yes
54	F	40	3,400	−0.3	44	−3.8	−2.9	−1.6	1.7	−2.5	−2.3	-1.8	N/A	0.45	N/A	SHOX	N/A	Yes
55	M	30	1,280	−0.8	38	−2.7	−1.9	−2.4	4.1	−3.8	−3.1	−2.2	N/A	−2.47	N/A	SHOX	N/A	Yes
56	M	38	2,860	−1.1	43	−3.7	0.8	−3.5	5.4	−3.6	−3.7	−2.8	−3.8	−1.14	−0.8	SHOX	N/A	Yes
57	F	40	2,500	−2.5	46	−2.7	−2.6	−2.0	7.5	−3.1	−2.7	−1.7	N/A	N/A	N/A	SHOX	N/A	Yes
58	F	37	2,090	−2.3	45	−2.1	−0.3	−0.7	2.9	−3.3	−2.8	−1.7	N/A	N/A	N/A	SGA	N/A	Yes
59	F	40	2,190	−3.3	42	−4.9	−0.6	−1.2	1.2	−3.5	−2.8	−2.8	N/A	0.53	N/A	SGA	N/A	Yes
60	M	34	1,345	−2.7	37	−5.5	0.0	0.7	2.2	−2.6	−2.3	−0.9	N/A	−2.72	−1.1	GHD+SGA	N/A	No
61	M	30	840	−2.2	N/A	N/A	−1.5	0.1	1.0	−5.3	−4.9	−4.2	N/A	−1.89	N/A	SGA	N/A	Yes
62	F	38	1,750	−3.6	41	−4.5	0.0	1.4	0.8	−2.5	−1.6	−0.9	N/A	2.30	N/A	SGA	N/A	Yes
63	M	35	1,700	−2.3	38	−5.4	−1.0	−1.2	3.3	−3.4	−3.3	−3.2	N/A	−1.17	−1.1	SGA	N/A	No
64	F	38	1,700	−3.8	42	−3.9	−1.6	0.6	1.4	−4.2	−3.4	−2.9	N/A	0.71	−0.2	SGA	N/A	Yes
65	F	37	2,100	−2.2	45	−2.1	1.4	0.0	1.5	−3.2	−3.0	−2.0	N/A	−2.44	N/A	SGA	N/A	Yes
66	M	35	1,900	−1.8	42	−3.2	N/A	N/A	1.0	−4.8	−3.7	−2.9	N/A	−2.50	N/A	SGA	N/A	Yes
67	M	30	775	−2.4	N/A	N/A	1.4	1.4	1.5	−4.2	−3.3	−2.6	−1.9	1.09	N/A	SGA	N/A	Yes
68	F	38	1,670	−3.8	39	−5.5	−0.6	0.4	2.3	−4.3	−3.5	−2.4	N/A	0.93	N/A	SGA	N/A	Yes
69	M	40	2,150	−3.5	43	−4.5	−0.7	−0.5	1.1	−3.0	−2.2	−1.7	N/A	0.02	N/A	SGA	N/A	Yes
70	F	33	1,210	−2.6	37	−4.9	−1.5	−0.5	4.1	−4.8	−2.3	−3.6	−4.7	0.88	N/A	SGA	Normal	Yes
71	M	33	1,645	−1.4	38	−4.4	−1.5	−1.8	2.8	−3.5	−3.4	−3.0	N/A	0.93	0.2	GHD+SGA	Normal	Yes
72	F	42	2,950	−1.7	46	−3.1	0.5	1.1	7.5	−3.3	−1.8	−1.8	−1.6	0.23	−0.3	GHD+SGA	Normal	Yes
73	F	40	3,350	−0.4	47	−2.1	0.0	−1.5	12.6	−4.1	−4.1	−3.3	−2.8	−1.51	−3.0	SGA	Normal	Yes
74	M	41	2,720	−2.4	48	−2.2	−0.5	0.0	1.7	−3.7	−3.2	−3.1	−3.8	−2.45	−1.7	GHD+SGA	Absent posterior pituitary, frontal atrophy	Yes

BA-CA, difference between bone age and chronological age; BL (cm), birth length (cm); BW (g), birth weight (grams); GW, gestational week; GH, growth hormone; GHD, growth hormone deficiency; MRI, magnetic resonance imaging; N/A, not available; SDS, standard deviation score.

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Table 2. Genetic results in children born small for gestational age with persistent short stature (SGA-SS)

Patient No.	Gene	Transcript/ karyotype variant	Reference	Protein variant	Reference	Zygosity	Classification*	Previously published variant	MAF
1[a]	*GHSR*	c.526G>A	NM_198407.2	p.Gly176Arg	NP_940799.1	Het	LP	[17]	–
2[a]	*HMGA2*	c.233C>T	NM_003483.6	p.Arg75Trp	NP_003474.1	Het	LP	[17]	–
3	*IGF1R*	c.2215C>T	NM_000875.5	p.Arg739Trp	NP_000866.1	Het	LP	[41]	–
4	*IGF1R*	del15q26.3				Het	P	[42]	–
5	*IGF1R*	c.807C>G	NM_000875.5	p.Tyr269Ter	NP_000866.1	Het	P	Novel	–
6[a]	*IGFALS*	c.589C>T	NM_004970.3	p.Arg197Cys	NP_004961.1	Het	LP	[17]	–
7	*LHX4*	del1q25.1q25.3				Het	P	Novel	–
8[a]	*OTX2*	del14q22q23				Het	P	[43]	–
9	*POU1F1*	c.437T>G	NM_000306.4	p.Leu146Ter	NP_000297.1	Het	P	Novel	–
10	*PROKR2*	c.254G>A	NM_144773.4	p.Arg85His	NP_658986.1	Het	P	[44]	0.00119
11	*PTCH1*	c.3912G>T	NM_000264.5	p.Arg1304Ser	NP_000255.2	Het	LP	Novel	0.0000179
12[a]	*STAT3*	c.2144C>T	NM_139276.3	p.Pro715Leu	NP_644805.1	Het	P	[45]	0.0000088
13	*THRA*	c.725T>C	NM_003250.6	p.Leu242Pro	NP_003241.2	Het	P	Novel	–
14[a]	*TRHR*	c.392T>C	NM_003301.7	p.Ile131Thr	NP_003292.1	Hom	P	[46]	0.0000176
15[a]	*ACAN*	c.916A>T	NM_001135.4	p.Ser306Cys	NP_001126.3	Het	P	[47]	–
16	*ACAN*	c.7162T>A	NM_001135.4	p.Glu2388Lys	NP_001126.3	Het	LP	Novel	0.0000355
17	*ACAN*	c.4927delC	NM_001135.4	p.Pro1643fs	NP_001126.3	Het	LP	Novel	–
18[a]	*ACAN*	c.1425delA	NM_001135.4	p.Val478fs	NP_001126.3	Het	P	[47]	–
19[a]	*COL11A1*	c.475A>G	NM_001854.4	p.Ile159Val	NP_001845.3	Het	LP	[24]	–
20[a]	*COL11A1*	c.1543C>G	NM_001854.4	p.Gln515Glu	NP_001845.3	Het	LP	[17]	–
21	*COL1A1*	c.4369G>A	NM_000088.4	p.Asp1457Asn	NP_000079.2	Het	LP	Novel	0.0000353
22[a]	*COL1A2*	c.577G>A	NM_000089.4	p.Gly193Ser	NP_000080.2	Het	P	[17]	0.000008794
23[a]	*COL2A1*	c.410G>A	NM_001844.5	p.Arg137His	NP_001835.3	Het	LP	[48]	0.0001009
24[a]	*COL2A1*	c.3106C>G	NM_001844.5	p.Arg1036Gly	NP_001835.3	Het	LP	[17]	0.00006200
25[a]	*COL2A1*	c.3106C>G	NM_001844.5	p.Arg1036Gly	NP_001835.3	Het	LP	[17]	0.00006200
26[a]	*COL2A1*	c.2129C>T	NM_001844.5	p.Pro710Leu	NP_001835.3	Het	LP	[17]	0.000007776
27	*COL9A1*	c.876+2T>A	NM_001851.6			Het	P	[49]	0.0001403
28	*COL9A2*	c.1918C>T	NM_001852.4	p.Arg640Ter	NP_001843.1	Het	P	[49]	0.00005501
29	*FLNB*	c.4388G>A	NM_001457.4	p.Arg1463Gln	NP_001448.2	Het	LP	Novel	0.00002760
30	*FLNB*	c.1599C>T	NM_001457.4	p.Pro250Leu	NP_001448.2	Het	P	Novel	–
31	*MATN3*	c.671G>A	NM_002381.5	p.Arg224Gln	NP_002372.1	Het	LP	Novel	0.0001716
32	*FGFR2*	c.28C>G	NM_000141.5	p.Leu10Val	NP_000132.3	Het	LP	Novel	0.00002323
33	*FGFR3*	c.1633C>T	NM_000142.5	p.Arg545Cys	NP_000133.1	Het	LP	Novel	0.000008856
34[a]	*FGFR3*	c.251C>T	NM_000142.5	p.Ser84Leu	NP_000133.1	Het	LP	[50]	–
35	*NPR2*	c.2864G>C	NM_003995.4	p.Arg955Thr	NP_003986.2	Het	LP	Novel	–
36[a]	*NPR2*	c.1670G>A	NM_003995.4	p.Arg557His	NP_003986.2	Het	LP	[23]	0.00001759
37[a]	*NPR2*	c.1673T>C	NM_003995.4	p.Ile558Thr	NP_003986.2	Het	P	[23]	0.00001759
38[a]	*NPR2*	c.1808G>C	NM_003995.4	p.Ser603Thr	NP_003986.2	Het	LP	[23]	–
39	*CDC42*	c.191A>G	NM_001791.4	p.Thr64Cys	NP_001782.1	Het	P	[51]	–
40	*KMT2D*	c.1967delT	NM_003482.4	p.Leu656fs	NP_003473.3	Het	P	[52]	–
41[a]	*LMNA*	c.433G>A	NM_005572.4	p.Glu145Lys	NP_005563.1	Het	P	[53]	–
42	*NSD1*	dup5q35.2q35.3				Het	P	[54]	–
43	*PTPN11*	c.236A>G	NM_002834.5	p.Gln79Arg	NP_002825.3	Het	P	[55]	–
44	*PTPN11*	c.802G>A	NM_002834.5	p.Gly268Ser	NP_002825.3	Het	P	[56]	0

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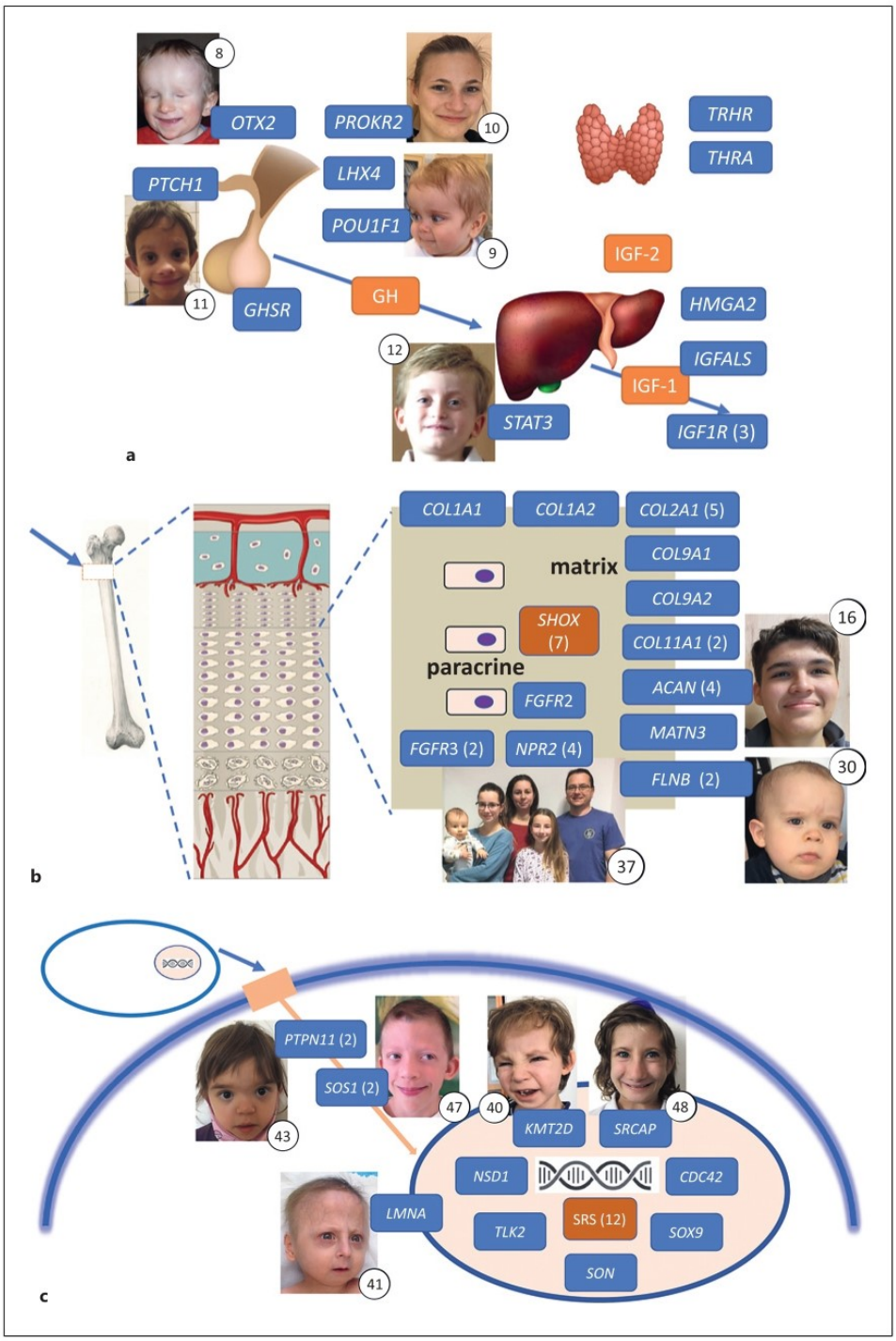
Table 2 (continued)

Patient No.	Gene	Transcript/ karyotype variant	Reference	Protein variant	Reference	Zygosity	Classification*	Previously published variant	MAF
45	*SON*	c.2680C>T	NM_138927.4	p.Gln894Ter	NP_620305.3	Het	P	Novel	-
46	*SOS1*	c.2105A>G	NM_005633.4	p.Tyr702Cys	NP_005624.2	Het	LP	[57]	0.00001555
47	*SOS1*	c.755T>C	NM_005633.4	p.Ile252Thr	NP_005624.2	Het	LP	[58]	0.00007039
48	*SRCAP*	c.7330C>T	NM_006662.3	p.Arg2444Ter	NP_006653.2	Het	P	[59]	–
49	*TLK2*	c.968+1G>A	NM_006852.6			Het	P	[60]	–
50	*SOX9*	c.673G>A	NM_000346.4	p.Gly225Ser	NP_000337.1	Het	LP	Novel	0.00005492
51	*SHOX*	delXp22.3				Het	P	[61]	–
52	*PPP2R3B*	dupXp22.33				Het	P	Novel	–
53	*SHOX*	delXp22.3				Het	P	[62]	–
54	*SHOX*	delXp22.3				Het	P	[61]	–
55	*SHOX*	c.-10delG	NM_000451.4			Het	P	Novel	–
56	*PPP2R3B*	dupXp22.33				Het	P	Novel	–
57	*SHOX*	delXp22.3				Het	P	[61]	–
58	–	UPD7				–	P	[62]	–
59	–	Hypometh 11p15				–	P	[63]	–
60	–	UPD7				–	P	[62]	–
61	–	UPD7				–	P	[62]	–
62	–	Hypometh 11p15				–	P	[63]	–
63	–	Hypometh 11p15				–	P	[63]	–
64	–	Hypometh 11p15				–	P	[63]	–
65	–	UPD7				–	P	[62]	–
66	–	UPD7				–	P	[62]	–
67	–	Hypometh 11p15				–	P	[63]	–
68	–	Hypometh 11p15				–	P	[63]	–
69	–	Hypometh 11p15				–	P	[63]	–
70	–	del17q24.2				Het	P	N/A	–
71	–	del1p31.1p31.3				Het	P	[64]	–
72	–	del22q11.2				Het	P	N/A	–
73	–	del6q24.3q25.1				Het	P	[65]	–
74	–	dupXq				–	P	N/A	–

Het, heterozygous; Hom, homozygous; hypometh 11p15, hypomethylation 11p15; LP, likely pathogenic; MAF, minor allele frequency in the European, non-Finnish population in the gnomAD database; N/A, not available; P, pathogenic; UPD7, uniparental disomy of chromosome 7. *Based on American College of Medical Genetics and Genomics (ACMG) standards and guidelines [20] implemented to the VarSome software [20] (on the date December 16, 2021). [a]Probands who carried these variants have been reported previously in our studies.



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(For legend see next page.)

Results

In total, the genetic diagnosis was elucidated in 74/176 (42%) children (Fig. 1). We confirmed pathogenic or likely pathogenic (P/LP) gene variants affecting pituitary development or GH secretion (*LHX4*, *OTX2*, *PROKR2*, *PTCH1*, *POU1F*, *GHSR*) and/or the GH-IGF-1 axis and IGF-2 axis (*IGFALS*, *IGF1R*, *STAT3*, *HMGA2*) in 12/74 (16%) patients. Two out of 74 children (3%) had P/LP gene variants affecting the thyroid axis (*TRHR*, THRA). P/LP gene variants affecting the growth plate were revealed in 31/74 (42%). Of these, 17/74 children had P/LP variants in genes responsible for components of the cartilaginous matrix (*ACAN* [in four], *COL1A1*, *COL1A2*, *COL2A1*, *COL9A1*, *COL9A2*, *COL11A1*, *FLNB*, *MATN3*), 7/74 had impaired paracrine regulation of chondrocytes (*FGFR3*, *FGFR2*, *NPR2*), and 7/74 had *SHOX* gene defects. In 12/74 children (16%), we revealed P/LP variants in genes involved in fundamental intracellular and intranuclear processes (*CDC42*, *KMT2D*, *LMNA*, *NSD1*, *PTPN11*, *SRCAP*, *SON*, *SOS1*, *SOX9*, *TLK2*). SRS was diagnosed in 12/74 (16%) (11p15, UPD7), and miscellaneous chromosomal aberrations were identified in 5/74 (7%) children.

Overall, in our cohort, 40 out of 74 patients (54%) had positive genetic findings and no dysmorphic features. Part of these results were published in our previous reports on children from families with vertical transmission of short stature ("familiar short stature") [17] and/or in a paper summarising the effect of GH therapy in children with pathogenic *NPR2* variants [23] and non-syndromic collagenopathies [24]. The principal clinical and growth data are summarised in Table 1. All the genetic findings are presented in Table 2. The single-gene conditions (and SRS) and their significance at three levels of growth regulation are displayed in Figure 2a–c.

Discussion

In our study, we examined a unique large single-centre cohort of SGA-SS children by NGS methods. We elucidated the genetic cause of growth disorder in 42% (74/176) of them. The results demonstrate a multifarious genetic landscape of SGA-SS and further contributed to the understanding of its aetiology.

Advances in genetic diagnostics led to better knowledge regarding the mechanisms causing short stature. Depending on the study cohort, NGS methods elucidated the aetiology of a growth disorder in 14.5–52% of cases [17, 26–30]. Two of these studies focused on SGA-SS children. Freire et al. identified monogenic SGA-SS in 8/55 (15%) children with no apparent syndromic features. The aetiologies of their short stature were mostly primary growth plate disorders accompanied by the disruption of the RAS/MAPK signalling pathway [29]. Li et al. [26] demonstrated that including syndromic SGA-SS children can substantially increase the detection rate of genetic variants elucidating the aetiology of SGA-SS (17% in non-syndromic SGA-SS vs. 31% in syndromic SGA-SS). In our study, we have managed to find the genetic cause of SGA-SS in a higher number of children (42%), even in the case of non-syndromic SGA-SS (54%).

The aetiology of SGA-SS in our study cohort was rather heterogeneous. Not surprisingly, 42% of children with genetic aetiology elucidated carried a causal variant in the gene that is essential for correct growth plate function. This finding is in line with previous studies [26, 29] and corresponds with the new paradigm with the growth plate playing a key role in short stature pathogenesis [31]. As expected, other relatively frequent genetic diagnoses in our SGA-SS study cohort were causal variants in genes affecting fundamental intracellular

Fig. 2. Three levels of the genetic growth regulation in children born small for gestational age with persistent short stature (SGA-SS). The numbers in brackets in blue boxes show the numbers of patients identified with P/LP variants of the entire gene (if more than one). The numbers in closed circles refer to patient numbering in Table 1. **a** Genes involved in cerebral midline and pituitary development, and in the GH-IGF-1 and thyroid axes. **b** Genes encoding growth plate matrix components and elements of chondrocyte paracrine regulation. **c** Genes involved in intracellular signalling, in the stability of nuclear membrane, and in the fundamental intranuclear processes. A brief description of selected patients' facial phenotypes: (8) *OTX2:* bilateral anophthalmia; (9) *POU1F1:* depressed nasal bridge and frontal bossing; (10) *PROKR2:* no apparent facial dysmorphism; (11) *PTCH1:* mild orbital hypotelorism, midface hypoplasia, and anteverted ears; (12) *STAT3:* no specific facial signs in a boy with severe immune dysregulation leading to early onset diabetes, hypothyroidism, cytopenia and lymphoproliferation, and short stature due to defective STAT3 signalling; (16) *ACAN:* mild facial dysmorphism similar to previously published cases; (30) *FLNB:* facial phenotype of atelosteogenesis type I – prominent forehead, depressed nasal bridge with a grooved tip, and micrognathia; (37) *NPR2:* the father and two daughters with vertical transmission of an *NPR2* pathogenic variant, no facial phenotype; (40) *KMT2D:* Kabuki syndrome resembling the makeup in traditional Japanese theatre; (41) *LMNA:* gradually developing phenotype of Hutchinson-Gilford progeria syndrome; (43) *PTPN11* and (47) *SOS1:* facial signs of Noonan syndrome; (48) *SRCAP*: typical face of Floating-Harbour syndrome. Image adapted from [25].

processes including RASopathies which corresponds with the results of previous studies as well [26, 29].

Another condition typically associated with SGA-SS is SRS [9]. Not surprisingly, SRS was another frequent diagnosis in our study cohort (16% of cases with genetic aetiology elucidated). Importantly, SRS is diagnosed clinically using the Netchine-Harbison scoring system (NHS). Genetic examination may consequently provide useful confirmation of the clinical diagnosis [9]. In our study, we took a different approach – genetic examination of SRS was performed in all SGA-SS children. Surprisingly, we have genetically diagnosed SRS in 2 children who do not fulfil NHS criteria. Genetic examination of SRS can therefore be considered in all SGA-SS children, regardless of the presence of its typical clinical features.

In our study, we also had several less expected findings. GH is essential for normal growth, and children with GHD may have severe short stature [32]. However, GH is generally considered to affect mainly the postnatal phase of growth, and children with GHD should therefore be born with normal birth parameters [32]. In contrast with this concept, we have found causative genetic variants in genes affecting pituitary development or directly influencing GH production in 6/74 (8%) of SGA-SS children with genetic diagnosis in our study cohort. Some of them might have different causes of prenatal growth failure (e.g., patient no. 1 with LP *GHSR* variant whose mother suffered from HELLP syndrome during pregnancy); however, in other children, we found no additional explanation of prenatal growth impairment. On the other hand, other studies also have discovered genetic findings typical for GHD in SGA children [2]. We can speculate that GH might play a role in prenatal growth in some children or genetic variants found might affect growth on other levels besides affecting GH.

Another interesting result was the pathogenic variant found in the gene *THRA* encoding thyroid hormone receptor type A, which has also been previously associated with short stature [33]. In one patient, we diagnosed a homozygous variant in the gene *TRHR*, leading to central hypothyroidism. Since its first observation in 1997 [34], short stature has been recognised as one of the consistent features of central congenital hypothyroidism due to *TRHR* defects and their bi-allelic pathogenic variants. However, perinatal data were not displayed in patients published so far. Thus, we are adding the *TRHR* gene as a novel causative gene for SGA. These two findings in the thyroid axis increase the diagnostic yield by additional 3% and clearly show that pre- and postnatal growth is affected by the thyroid axis far beyond the classical hypothyroidism. The other variants found in our study were genes involved in the regulation of growth plates and other fundamental processes of intracellular signalling.

The clinical response to GH treatment has not been systematically studied in the sub-cohorts of SGA-SS with defined genetic aetiology, with the exception of children with SRS [35]. Thus, the currently available reports have their origin in retrospective analysis of children genetically diagnosed at a late stage of their therapy. The currently available data are scarce, as summarised in the latest consensus [36]. In our cohort, the long-term growth data are available only in a minority of children; therefore, we present short-time growth data following 1 year and 3 years of GH administration and, when available, final height SDS. Continuing the observation of the study cohort might bring important new data on the impact on the individual of the new genetic finding.

Some genetic studies in short stature tend to suffer from selection bias as the study population originates mostly from tertiary centres [26, 37, 38]. This questions the extrapolation of the genetic spectrum of growth disorders to the general population. The strength of our study is its population-based principle: all newborns in the Czech Republic have their birth weight and length measured and carefully recorded together with their gestational week. All children subsequently undergo regular, mandatory body height examinations that enable the identification of growth failure, an early referral to a paediatric endocrinologist, and the start of GH treatment if indicated. Our centre provides GH to about 30% of children in our country [39]; their selection depends mainly on their residence. Thus, our study should be relatively free of selection bias with the exception of those who neglected regular body height checkups (which is rare) or refused either GH therapy or genetic testing.

Our study had some limitations as well. Chromosomal analysis including microarrays was performed only in children whose phenotype led to the initial referral to the department of clinical genetics. Moreover, non-coding variants (with the exception of the disruption in exon-intron boundaries), epigenetic, and somatic changes were not captured by NGS. Finally, our study lacks the functional studies to help evaluate the pathogenicity of the discovered variants.

To conclude, our study elucidated the genetic aetiology of 42% of SGA-SS children from a genetically relatively homogenous, non-consanguineous population. The results demonstrate a complex aetiology of short stature affecting all the three key levels of growth regulation including the endocrine system, growth plate function,

and fundamental processes of intracellular regulation and signalling. A conclusive genetic finding not only provides a clear explanation of the growth disorder but also enables focussing on possible associated hidden comorbidities and genetic consulting [40]. In our opinion, routine genetic testing may therefore become a standard of diagnostic care in resource-rich countries for all SGA-SS children after other causes of growth failure are ruled out.

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We are grateful to the children and their parents for consenting to display the portrait photographs without covering their eyes, in favour to support the general medical knowledge and understanding.

Statement of Ethics

This study protocol was reviewed and approved by the Institutional Ethics Committees of the 2nd Faculty of Medicine, Charles University in Prague, and University Hospital Motol, Czech Republic (date of approval: June 30, 2017; not numbered). Written informed consent was obtained from the parents/legal guardians of the patients for publication of the details of their medical cases and any accompanying images. The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Written informed consent was obtained from the participants' parents/legal guardians to participate in the study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Prof. Dr. Jan Lebl and Dr. Ledjona Toni designed the study. Dr. Ledjona Toni, Dr. Lukas Plachy, and Prof. Dr. Jan Lebl wrote the manuscript. Dr. Petra Dusatkova did the NGS data analysis and coordinated the study. Prof. Dr. Zdenek Sumnik, Dr. Stanislava Kolouskova, Dr. Marta Snajderova, Dr. Barbora Obermannova, Dr. Stepanka Pruhova, and Prof. Dr. Jan Lebl referred patients and provided their clinical information. Dr. Petra Dusatkova, Dr. Lenka Elblova, and Dr. Shenali Anne Amaratunga provided insight on variant analysis. All authors contributed to the discussion and reviewed or edited the manuscript.

Data Availability Statement

The datasets presented in this article are not readily available for ethical and legal reasons relating to the participants' privacy rights. The raw sequencing data are available upon reasonable request to the corresponding author.

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This study delves into the intricate diagnostic landscape of GHD in children from a non-consanguineous population. GHD is a diagnosis often shrouded in controversy, particularly due to the limited specificity of GH stimulation tests.

The cohort encompassed 52 children diagnosed with primary GHD and familial short stature. The diagnosis of GHD was established based on multiple criteria, including growth data indicative of GHD, IGF-1 levels below age and sex-specific standards, and peak GH concentrations below a defined threshold in two stimulation tests.

The results of this study (pathogenic variants found in 15/52 participants, 29%) revealed an intriguing discrepancy between clinical diagnoses of GH deficiency and genetic findings. While a minor subset of children did indeed possess genetic variants influencing GH secretion or function, a significant proportion had variants causing primary growth plate disorders, impairing IGF-1 action, or miscellaneous genetic variants. These findings raise questions about the reliability of conventional methods for diagnosing GH deficiency and emphasize the intricate and multifactorial nature of growth disorders in these children. This research highlights the complexities of diagnosing GHD and underscores the importance of considering broader genetic factors in understanding short stature aetiology.

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Isolated growth hormone deficiency in children with vertically transmitted short stature: What do the genes tell us?

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Introduction: The growth hormone deficiency (GHD) diagnosis is controversial especially due to low specificity of growth hormone (GH) stimulation tests. It is therefore believed that children diagnosed with GHD form a heterogeneous group with growth disorder frequently independent on GH function. No study evaluating the complex etiology of growth failure in children with diagnosed GHD has been performed thus far.

Aims: To discover genetic etiology of short stature in children with diagnosed GHD from families with short stature.

Methods: Fifty-two children diagnosed with primary GHD and vertically transmitted short stature (height SDS in the child and his/her shorter parent <-2 SD) were included to our study. The GHD diagnosis was based on growth data suggestive of GHD, absence of substantial disproportionality (sitting height to total height ratio <-2 SD or >+2 SD), IGF-1 levels <0 for age and sex specific SD and peak GH concentration <10 ug/L in two stimulation tests. All children were examined using next-generation sequencing methods, and the genetic variants were subsequently evaluated by American College of Medical Genetics standards and guidelines.

Results: The age of children at enrollment into the study was 11 years (median, IQR 9-14 years), their height prior to GH treatment was -3.0 SD (-3.6 to -2.8 SD), IGF-1 concentration -1.4 SD (-2.0 to -1.1 SD), and maximal stimulated GH 6.3 ug/L (4.8-7.6 ug/L). No child had multiple pituitary hormone deficiency or a

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midbrain region pathology. 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* [2x]), in one (7%) a genetic variant impairing IGF-1 action (*IGFALS*) and in two (12%) a variant in miscellaneous genes (*SALL4*, *MBTPS2*).

Conclusions: In children with vertically transmitted short stature, genetic results frequently did not correspond with the clinical diagnosis of GH deficiency. These results underline the doubtful reliability of methods standardly used to diagnose GH deficiency.

KEYWORDS

short stature, growth hormone, growth hormone deficiency, genetics, next-generation sequencing

Introduction

The correct production, secretion, and function of growth hormone (GH) is important for the physiological growth and optimal functioning of the human organism (1, 2). For people with growth hormone deficiency (GHD), treatment with recombinant GH is essential to achieve normal adult height and, in cases of severe GHD, prevent repeated episodes of hypoglycaemia or other possible consequences of impaired metabolic GH function (1, 3). Precise diagnosis of individuals with GHD allowing early GH treatment is therefore crucial (2).

The diagnosis of GHD is complex combining auxological, laboratory and radiological examination. Growth hormone stimulation tests are performed for the confirmation of the diagnosis (3, 4). However, these tests are known to have low specificity, potentially causing false positive results (5–8). Consequently, children diagnosed with GHD likely form a rather heterogeneous group with different etiology of growth disorder frequently independent of GH production or function (9). However, no studies evaluating the complex genetic etiology of growth failure in children that have been clinically classified as having GHD have been performed so far.

Importantly, modern genetic methods including next-generation sequencing (NGS) have shown their potential to discover the causes of growth disorders on a molecular basis (9, 10). The boom in genetic diagnostics in the last two decades has led to a substantial progress in understanding the etiology of short stature (9, 11). In our study, we aimed to search for genetic background of short stature in children diagnosed as GHD from families with short stature.

Materials and methods

Patients

Inclusion criteria

According to the records database, 747 children are currently treated with GH in our center. After excluding children with Prader-Willi syndrome, Turner syndrome, and those with known secondary cause of their growth disorder (e.g., chronic kidney disease, secondary GHD caused by intracranial tumor, surgery and/or irradiation), 528 patients remained for further evaluation. Within this group, 419 individuals were diagnosed with primary GHD. Out of these, 70 had vertically transmitted short stature defined as a height SDS ≤-2 SD in both the child and his/her shorter parent and therefore, were chosen for the study. A total of 52 study participants/their legal guardians signed written informed consent before genetic examination and were included in the study. The study was approved by the institutional Ethics Committees of the 2nd Faculty of Medicine (approval number EK-753.3.5/21) of Charles University in Prague, Czech Republic.

Clinical evaluation

The heights and body proportionality (sitting height to total height [SHH] ratio) of all participants were obtained during anthropometric measurements. Data regarding birth parameters were obtained from the medical records. The parents' heights were measured to the nearest 1 mm and the heights of other relatives were obtained from the parents. All the data was standardized according to recent normative values (12–14).

Bone age was evaluated using the Tanner-Whitehouse method (15).

Diagnostics of growth hormone deficiency

Growth hormone deficiency was diagnosed using current guidelines (3, 4). In all children with auxological data suggestive of GHD (i.e., current height <-3 SD below the mean, current height <-1.5 SD below the midparental height and/or current height <-2 SD below the mean combined with a decrease in height >0.5 SD over one year in a child older than 2 years), IGF-1 levels <0 SD (reference ranges standardized for sex and age) and no substantial disproportionality (SHH ratio <-2 SD or >+2 SD) GH stimulation tests were performed. Children with a maximum GH concentration <10 ug/L in both the clonidine and insulin hypoglycemia tests were classified as having GHD. Sex-steroid priming was performed in children aged 9 years and older.

Genetic testing

Genomic DNA was extracted from peripheral blood (QIAamp Blood Mini Kit, Quiagen, Hilden, Germany) in all children included in the study. Firstly, some children underwent basic genetic testing. Turner syndrome and SHOX haploinsufficiency were examined in all girls by fluorescence *in situ* hybridization (FISH). In children with a clinical suspicion of a specific genetic disorder, targeted genetic testing was performed. Children with no genetic cause of short stature elucidated at this point were subsequently examined using the NGS methods: whole-exome sequencing (WES) or custom-targeted NGS panel containing 398 genes associated with growth (Supplementary Table 1). All variants from the NGS were confirmed by Sanger sequencing as we described previously (16). The method of genetic examination we described in detail in previous studies (11, 17, 18).

All variants with potential clinical importance were evaluated by American College of Medical Genetics and Genomics (ACMG) standards and guidelines (19). For variant evaluation, we also used the ACMG criteria implemented into the VarSome software (20) and Franklin software (https://franklin.genoox.com version date 2nd November 2022) that score each ACMG rule as very strong, strong, moderate, or supporting based on ACMG recommendations and its more up-to-date modifications. In some cases, the strength of the rules was modified according to extended investigation of various databases and clinical evaluation of the patient. To evaluate the segregation of genetic variants with short stature in the families, DNA and height data of other relatives was obtained. The guidelines formulated by Jarvik et al. were followed (21) and applied to co-segregation in the pathogenicity classification. At the end, all genetic variants were classified as pathogenic (P), likely pathogenic (LP), benign (B), likely benign (LB) or as variants of uncertain significance (VUS).

Results

In total, 52 children with a primary GHD diagnosis and vertically transmitted short stature were enrolled to the study. Their age at study enrolment was 11 years (median; IQR 9-14 years), their pretreatment height was -3.0 SD (-3.6 to -2.8 SD), their shorter parent's height was -2.6 SD (-2.9 to -2.2 SD), their IGF-1 concentration prior to the GH treatment was -1.4 SD (-2.0 to -1.1 SD),their stimulated GH concentration maximum was 6.3 ug/L (4.8-7.6 µg/L), and their bone age was delayed by 1.1 years (0.3-1.7 years). Seventeen children had mild GHD with stimulated GH concentrations of 7.0-9.9 µg/L, 34 children had stimulated GH concentrations 3.0-6.9 µg/L and only one child had severe GHD with stimulated GH concentration <3.0 µg/L. No child had multiple pituitary hormone deficiency or a midbrain region pathology on magnetic resonance imaging. The birth length and birth weight of the children in the study cohort was -1.8 SD (median; IQR -2.4 to -1.2 SD) and -1.3 SD (-2.0 to -0.7 SD), respectively. Twenty-two children were born small for gestational age (SGA) (9 for both birth length and weight, 11 only for birth length, and 2 only for birth weight). The children have been treated with GH for a median 5.0 years (3.5-6.0 years), with a median dose of 32 µg/kg/day (30-34 µg/kg/day) during the first year of treatment.

A monogenic cause of growth failure was elucidated in 15 (29%) of 52 children with diagnosed primary GHD and vertically transmitted short stature who were enrolled to the study. Of them, only 2 (13%) had a genetic variant affecting GH secretion or function (*GHSR* and *OTX2*). Interestingly, 10/15 (67%) children had a genetically proven primary growth plate disorder (4 had extracellular matrix protein defect [genes *ACAN*, *COL1A2*, *COL11A2*, and *COL2A1*], 2 had impaired paracrine regulation of the growth plate [genes *NPR2*, and *FGFR3*], and 4 had a disorder affecting fundamental intracellular processes of the growth plate [*PTPN11* gene in 2 patients, and *EXT2* and *NF1* genes each in a single patient]. Among the remaining children, 1/15 (7%) had altered IGF function (gene *IGFALS*), and 2/15 (13%) had mutations in miscellaneous genes (*MBTPS2* and *SALL4*). Specific genetic variants and phenotypes of the children are summarized in Table 1, data evaluating children with genetically confirmed GHD and those with genetically diagnosed primary growth plate disorder are summarized in Table 2.

Discussion

In our study, we evaluated the complex genetic etiology of growth disorders in children with diagnosed primary GH deficiency from families with short stature. Interestingly, GHD was genetically confirmed as a cause of growth failure only in a minority of children (13% of children with genetic etiology discovered, 4% of the whole study cohort). On the other hand, modern genetic examination

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TABLE 1 A table evaluating clinical characteristics and genetic examination results in children with proven monogenic aetiology of their short stature.

Patient	Sex	Age at last follow-up (years)	GH treatment initiation age (years)	GH dose in the first year of GH treatment (μg/kg/day)	Shorter parent's height (SD)	Birth weight (SD)	Birth length (SD)	Height SDS prior to GH treatment	Height SDS after 1 year of GH treatment	Height SDS after 3 years of GH treatment	Growth velocity prior to GH treatment (cm/year)	Growth velocity in the first year of GH treatment (cm/year)	BA prior to GH treatment (difference to CA, years)	IGF1 prior to GH treatment (SD)	Stimulated GH maximum (ug/l)	SHH ratio (SD)	Additional phenotypic features	Genetic examination method	Gene	Variant type	Transcript variant	Protein variant	Classification	ACMG criteria
Impaired growth hormone production																								
1	F	10	7	31	-2.8	-2.6	-3.1	-3.2	-2.1	-1.4	4.2	10.6	-0.6	-2.1	7.2	0.1	Mother HELLP syndrome in pregnancy	WES	GHSR	M/n	c.526G>A	p.Gly176Arg	LP	PM2(m), PP3(m), PP5(m)
2	M	10	6	28	-2.8	-1.2	-1.1	-4.3	-3.0	-3.4	4.5	12.1	0.0	<-3	1.7	0.4	Psychomotor retardation	NGS panel	OTX2	M/n	c.106delC	p.Arg36fs	P	PVS1(vs), PM2(m), PP3(sp)
Alteration of IGF function																								
3	M	11	7	34	-2.8	-1.6	-1.7	-3.5	-2.6	-1.6	5.3	9.8	-0.2	-1.8	6.1	0.6	–	WES	IGFALS	M/n	c.589C>T	p.Arg197Cys	LP	PP3(st), PM2(m)
Primary growth plate disorder																								
Extracellular matrix																								
4	M	16	7	38	-5.6	-1.6	-3.3	-3.7	-2.9	-2.2	3.7	8.6	1.7	-1.6	3.2	0.9	–	Sanger	ACAN	M/n	c.1635del	p.Val546Trp*14	P	PVS1(vs), PM2(m), PP1(st)
5	M	13	3	35	-2.0	-2.5	-1.5	-3.2	-2.3	-1.9	3.8	9.9	-2.0	-0.9	6.4	-0.2	Frequent long bone fractures, vertebral compression fractures	NGS panel	COL1A2	M/n	c.577G>A	p.Gly193Ser	P	PM1(m), PM6(m), PS2(sp), PP3(st), PP4(sp), PP5(s)
6	M	16	10	30	-2.8	-1.0	-0.7	-3.4	-2.7	-2.1	3.8	7.8	-1.5	-1.2	8.9	0.7	–	NGS panel	COL11A2	M/n	c.3700C>T	p.Arg1234Cys	LP	PM2(m), PP3(st), PP1(sp)
7	M	16	12	30	-2.9	-2.4	-3.0	-3.4	-2.9	-2.2	6.1	8.7	-1.5	-1.6	8.4	1.2	–	WES	COL2A1	M/n	c.3106C>G	p.Arg1036Gly	LP	PM1(sp), PP3(st), PM2(sp), PP5(sp)
Paracrine growth plate regulation																								
8	F	5	4	35	-2.4	-1.6	-2.4	-3.0	-2.5	NA	3.7	6.7	NA	-2.1	6.9	0.3	–	NGS panel	FGFR3	M/n	c.250C>T	p.Ser84Leu	LP	PM2(m), PP1(sp), PP2(sp), PP3(st)
9	M	8	7	32	-2.6	0.9	-1.7	-2.9	-2.2	NA	5.6	10.3	-1.7	-1.6	6.7	0.3	–	NGS panel	NPR2	M/n	c.613C>T	p.Arg205*	P	PVS1(vs), PM2(m), PP3(sp)
Fundamental intracellular processes of the growth plate																								
10	M	12	8	30	-2.9	-2.4	-3.4	-2.7	-2.0	-1.3	4.8	9.0	0.3	-1.0	3.9	0.3	–	WES	EXT2	M/n	c.2004C>G	p.Asn668Lys	LP	PM2(m), PP3(st), PP1(sp)
11	M	16	11	33	-3.0	1.0	-1.2	-3.0	-2.9	-2.6	2.5	9.2	0.5	-0.5	6.2	0.7	Vitiligo	Sanger	PTPN11	M/n	c.211T>A	p.Phe71Ile	LP	PM1(m), PM2(m), PM5(m), PP2(sp), PP3(sp)
12	M	12	7	33	-2.6	NA	NA	-2.7	-1.9	-1.2	4.8	9.3	-1.6	-1.6	6.8	1.3	–	Sanger	PTPN11	M/n	c.1403C>T	p.Thr468Met	P	PS3(st), PM1(sp), PM2(m), PM5(m), PP2(sp), PP3(sp), PP5(st)
13	F	12	8	33	-3.9	1.0	0.1	-2.3	-1.7	-1.0	4.8	8.7	-0.7	-1.1	1.2	NA	Café-au-lait spots, hamartomas in the brain	Sanger	NF1	M/n	c.4267A>G	p.Lys1423Glu	P	PS3(st), PM1(m), PM2(m), PM5(m), PP2(sp), PP3(sp), PP5(vs)
Genes with miscellaneous function																								
14	M	10	3	34	-2.7	-1.1	-1.8	-4.4	-4.0	-3.6	3.0	7.2	NA	-1.1	6.6	0.9	Immunodeficiency, Hypertension, cerebral scarring	WES	MBTPS2	M/-	c.1336T>C	p.Ser446Pro	LP	PM1(sp), PM2(m), PP3(m), PP5(sp)
15	M	13	7	34	-3.6	-1.8	-1.4	-2.8	-2.1	-1.0	6.3	8.7	NA	-1.0	4.6	-0.3	Radial ray defect, right kidney dystopia	WES	SALL4	M/n	c.1717C>T	p.Arg573*	P	PVS1(vs), PM2(m), PP1(sp), PP4(sp), PP5(sp)

ACMG, American College of Medical Genetics guidelines; BA, bone age; CA, calendar age; F, female; GH, growth hormone; LP, likely pathogenic; M, male; (m), moderate strength of the criterion used; M/-, hemizygous; M/M, homozygous; M/n, heterozygous; NA, not available; NGS, next-generation sequencing; P, pathogenic; SD, standard deviation; (sp), supporting strength of the criterion used; (st), strong strength of the criterion used; SHH, sitting height to height; (vs), very strong strength of the criterion used; WES, whole exome sequencing.

TABLE 2 Table evaluating clinical data in children with genetically confirmed GH deficiency and those with genetically diagnosed primary growth plate disorder.

	Patient 1 with genetically confirmed GHD	Patient 2 with genetically confirmed GHD	Patients with genetically diagnosed primary growth plate disorder
Gene found	*GHSR*	*OTX2*	NA
Shorter parent's height (SD)	-2.8	-2.8	-2.8 (-2.9 to -2.4)
IGF-1 prior to GH treatment (SD)	-2.1	<-3.0	-1.4 (-1.6 to -1.1)
Maximal stimulated GH concentration (ug/L)	7.2	1.7	5.4 (4.0 to 6.4)
Height prior GH treatment (SD)	-3.2	-6.3	-3.1 (-3.4 to -2.7)
Height after one year of GH treatment (SD)	-2.1	-5.0	-2.5 (-2.9 to -2.0)
Height after 3 years of GH treatment (SD)	-1.4	-3.4	-2.0 (-2.2 to -1.3)
Growth velocity prior to GH treatment (cm/year)	4,2	4,5	3.9 (3.7 to 4.6)
Growth velocity in the first year of GH treatment (cm/year)	10,6	12,1	8.9 (8.6 to 9.3)

The values in children with genetically diagnosed primary growth plate disorder are expressed as medians and interquartile ranges. GH, growth hormone; NA, not applicable; SD, standard deviation.

methods frequently discovered other mechanisms causing growth disorders independent of GH production, secretion or function, further broadening the doubts about the GHD diagnostics.

Studies evaluating the genetic etiology in children classified as GHD are scarce. Depending on the cohort, causative genetic variants were found in approximately 11% of children with primary isolated GHD (22). In the remaining majority of children with diagnosed IGHD, specific causes of their short stature are unknown. These children are traditionally labelled with a descriptive diagnosis of an "idiopathic growth hormone deficiency" (9) and the etiology of their growth disorder remains to be elucidated. In our study, we found genetic causes of GHD in even smaller proportion of children (2/52; 4%). However, unlike in the previous studies, we focused not only on genes causing GHD, but on other possible genetic causes of short stature as well. Using this strategy, we discovered the genetic etiology of growth failure in an additional 25% (13/52) of children. Surprisingly, 67% (10/15) of them had a primary growth plate disorder.

The discrepancy between the original clinical diagnosis of GHD and the genetic finding in most cases raises a question of which method is more accurate. Importantly, current diagnostics of GHD faces many difficulties and is considered as one of the most controversial issues in pediatric endocrinology (5). Growth hormone stimulation tests currently used as golden standard of the GHD diagnosis (4) are not physiological and have a very low specificity and reproducibility. Furthermore, they are affected by pubertal development, obesity, or other characteristics of the examined individual (3, 5). Unfortunately, there is no method reliably proving GHD to validate GH stimulation tests (5). A recent study by Bright et al. calculated an extremely low probability (2.8%) of a true-positive GH stimulation test in a child with short stature (23). Due to all these reasons, it is believed that most children with diagnosed GHD have a non-pituitary etiology of their short stature and are erroneously labelled as GHD (9). The results of our study support this presumption and offers the first insight into the possible non-pituitary causes of growth disorders in such children.

On the other hand, in the absence of a validation method for GHD diagnostics (5), there is no way to prove the diagnosis of GHD is incorrect. We must therefore admit the possibility that GHD might contribute to the patients' short stature in additional to the cause discovered by genetic testing. To correctly interpret the results of our study, we need to consider the possibility of false positive genetic results especially if the likely pathogenic variants are considered causative. However, the probability of a likely pathogenic variant to be truly pathogenic is >90% (19) and are therefore more trustworthy than the methods traditionally used to diagnose GHD.

Our study had several strengths. Firstly, we examined a homogenous population of patients from a single center of an economically stable country with high quality health care, low consanguinity rate, and negligible social causes of growth failure such as malnutrition. Secondly, all GH stimulation tests were

performed by experienced investigators using a defined protocol and the results were analyzed in a single laboratory using the same methodology. However, our study had several limitations as well. Firstly, functional studies were not performed in our study. However, according to ACMG standards and guidelines, the causality of genetic variants can be proven also by other methods (19). In our study evaluating children with vertically transmitted short stature, the segregation of genetic variants in short people within the families was an important factor. Secondly, protein non-coding variants (in the exception of disruption in the exon-intron boundaries) were not captured by the NGS. Thirdly, copy number variants were not evaluated in the current study. Fourthly, although our NGS panel included a large number of genes associated to growth disorders (398), causative variants in the genes not present in the panel could have been missed. Moreover, children with known genetic cause of their short stature prior to the study were not examined using NGS. Finally, only children with vertically transmitted short stature were included in our study. To generalize these results to all children with diagnosed GHD, further studies are warranted.

Conclusion

In children with vertically transmitted short stature, genetic results frequently did not correspond with the clinical diagnosis of GH deficiency. These results underline the doubtful reliability of methods standardly used to diagnose GH deficiency.

Data availability statement

The datasets presented in this article are not readily available for ethical and legal reasons relating to the participants' privacy rights. The raw sequencing data are available upon reasonable request to the corresponding author (lukas.plachy@fnmotol.cz).

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committees of the 2nd Faculty of Medicine (approval number EK-753.3.5/21) of Charles University in Prague, Czech Republic. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

LuP organized the study, contributed to the study design of the study, helped to obtain clinical data, contributed to the interpretation of the genetic results, contributed to the results interpretation, wrote the manuscript. SA contributed to the study design of the study, contributed to the results interpretation, contributed to the interpretation of the genetic results, contributed to the final version of the manuscript. PD contributed to the study design of the study, performed the genetic examination, supervised the interpretation of genetic results, contributed to the results interpretation, contributed to the final version of the manuscript. KM, DZ contributed to the study design of the study, performed antropometric measurements, contributed to the results interpretation, contributed to the final version of the manuscript. VN, LeP, BO, MS, SK, ZS, JL contributed to the study design of the study, helped to obtain clinical data, contributed to the results interpretation, contributed to the final version of the manuscript SP. Supervised the whole study, contributed to the study design of the study, helped to obtain clinical data, contributed to the genetic results interpretation, contributed to the results interpretation, contributed to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1102968/full#supplementary-material

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This case report centres on a non-consanguineous family with a vertically transmitted pathogenic variant in the *SALL4* gene, a crucial transcription factor implicated in early embryonic development. This genetic variant led to radial hypoplasia and kidney dystopia, affecting multiple generations within the family. In the proband, this *SALL4* variant also coincided with an additional condition: GHD.

The proband was born small for gestational age (SGA) at birth and exhibited bilateral asymmetrical radial ray malformation, including radial hypoplasia, ulnar flexure, and bilateral thumb aplasia. However, he did not present with other characteristic anomalies associated with *SALL4* variants, such as cardiac malformations, clubfoot, ocular coloboma, or Duane anomaly. Notably, he also had a dental anomaly - a central mandibular ectopic canine, suggestive of a mild developmental midline anomaly. He was diagnosed with isolated GH deficiency and the subsequent GH therapy resulted in catch-up growth.

Furthermore, the proband's successful response to GH therapy highlights the potential for targeted interventions in such cases. This case is unique in demonstrating the coexistence of a *SALL4* variant-induced upper limb defect and isolated GHD without major apparent cerebral or facial midline anomalies.

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SALL4 Phenotype in Four Generations of One Family: An Interplay of the Upper Limb, Kidneys, and the Pituitary

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Established Facts

- *SALL4* encodes a transcription factor that plays an essential role in early embryonic cellular differentiation of the epiblast and primitive endoderm.
- Monoallelic pathogenic variants of the *SALL4* gene cause Okihiro/Duane-radial ray syndrome, Holt-Oram syndrome, or acro-renal-ocular syndrome with partially overlapping phenotypes. Common features include limb malformations, eye disorders, heart, or kidney defects.
- In murine models, *SALL4* is expressed among others in the midbrain, rostral edge of the forebrain, and the pituitary. In humans, just a single previous observation revealed a *SALL4* pathogenic variant in a boy with growth hormone deficiency (GHD), empty sella, and maxillary central incisor.

Novel Insights

- Monoallelic pathogenic *SALL4* variants may cause GHD without apparent cerebral or facial midline dysmorphia. The phenotype is variable even among several affected family members, but all share a similar radial ray hypoplasia.
- *SALL4* extends the spectrum of candidate genes for "syndromic" pituitary hormone deficiency with extra-pituitary dysmorphic features.

Keywords
SALL4 gene · Radial ray malformation · Growth hormone deficiency · Growth hormone therapy

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Abstract

Introduction: The *SALL4* gene encodes a transcription factor that is essential for early embryonic cellular differentiation of the epiblast and primitive endoderm. It is required for the development of neural tissue, kidney, heart, and limbs. Pathogenic *SALL4* variants cause Duane-radial ray syndrome (Okihiro syndrome), acro-renal-ocular syndrome, and Holt-Oram syndrome. We report a family with vertical transmission of a *SALL4* pathogenic variant leading to radial hypoplasia and kidney dystopia in several generations with additional growth hormone deficiency (GHD) in the proband. ***Case Presentation:*** Our male proband was born at the 39th week of gestation. He was born small for gestational age (SGA; birth weight 2,550 g, −2.2 SDS; length 47 cm, −2.0 SDS). He had bilateral asymmetrical radial ray malformation (consisting of radial hypoplasia, ulnar flexure, and bilateral aplasia of the thumb) and pelvic kidney dystopia, but no cardiac malformations, clubfoot, ocular coloboma, or Duane anomaly. He was examined for progressive short stature at the age of 3.9 years, where his IGF-1 was 68 µg/L (−1.0 SD), and growth hormone (GH) after stimulation 6.2 µg/L. Other pituitary hormones were normal. A brain CT revealed normal morphology of the cerebral midline and the pituitary. He had a dental anomaly – a central mandibular ectopic canine. MRI could not be done due to the presence of metal after multiple corrective plastic surgeries of his hands. His mother's and father's heights are 152.3 cm (−2.4 SD) and 177.8 cm (−0.4 SD), respectively. His father has a milder malformation of the forearm. The affected paternal grandfather (height 164 cm; −2.3 SD) has a radial ray defect with missing opposition of the thumb. The family reports a similar phenotype of radial dysplasia in the paternal grandfather's mother. The proband started GH therapy at age 6.5 years when his height was 109 cm (−2.8 SDS) and he experienced catch-up growth as expected in GHD. Puberty started spontaneously at the age of 12.5 years. At age 13, his height was 158.7 cm (−0.2 SDS). Whole-exome sequencing revealed a nonsense variant in the *SALL4* gene c.1717C>T (p.Arg573Ter) in the proband, his father, and paternal grandfather. ***Conclusion:*** This is the first observation of a patient with a congenital upper limb defect due to a pathogenic *SALL4* variant who has isolated GHD with no apparent cerebral or facial midline anomaly and has been successfully treated with growth hormone.

Introduction

Embryonic morphogenesis and differentiation is orchestrated by a network of transcriptional factors. SALL4 (sal-like protein 4) is a very early-stage transcription factor with eight zinc finger motifs [1]. It is encoded by the *SALL4* gene that is localized on chromosome 20q13.2. Pathogenic variants in *SALL4* have been reported to cause autosomal dominant Duane-radial ray syndrome (DRRS; also known as Okihiro syndrome) [2, 3], Holt-Oram syndrome (HOS) [4], and acro-renal-ocular syndrome (AROS) [2, 5]. These syndromes have a partially overlapping phenotype including limb malformation, eye disorders (Duane anomaly), and abnormal heart and kidney development [1, 3, 4].

The SALL4 protein plays a unique role in early embryonic cellular differentiation of the epiblast and primitive endoderm. In murine models, *SALL4* is expressed early and required for the caudalization and spinal cord differentiation of neural tissue. It is expressed in a large number of body structures such as the midbrain, rostral edge of the forebrain, pituitary gland, maxillary arch, liver, somites, genital tubercle, limb buds, tail, and the left ventricular myocardium [6–9]. After birth, *SALL4* expression is down-regulated and is finally absent in most adult mouse tissues. Later in life, human *SALL4* may be re-expressed in various human cancers similar to other transcription factors [6].

SALL4 is of interest to pediatric endocrinologists due to its role in the morphogenesis and differentiation of midbrain structures. Deciphering gene interactions during early embryogenesis can shed some light on the potential impact of *SALL4.* It has been found that the sonic hedgehog (SHH) pathway and the *SOX2* gene are candidate partners of *SALL4* in midbrain morphogenesis and differentiation [10].

In mice, *SALL4* interacts with *GLI3* in the proper development of the upper limb skeletal elements [11] via the SHH pathway [12]. The SHH pathway is essential to maintain the apical ectodermal ridge, it acts as a signaling center for formatting the proximal-distal upper limb axis [13] but is well known for its key role in brain development as well. Defects in genes encoding for its components in mice and humans (*SHH, GLI2, PATCH, GLI3,* and others) lead to a spectrum of phenotypes ranging from holoprosencephaly to isolated pituitary hormone deficiencies [14].

Another gene that interacts with *SALL4* in early embryogenesis is *SOX2* which, in some cases, causes anophthalmia and hypopituitarism syndrome and septo-optic dysplasia, with optic nerve hypoplasia resembling the Duane anomaly caused by *SALL4* [11]. In a few previous case studies, cranial midline defects, microcephaly, and short stature have been described in individuals with pathogenic variants in *SALL4* but just a single case was reported in detail at two different occasions [2, 15].

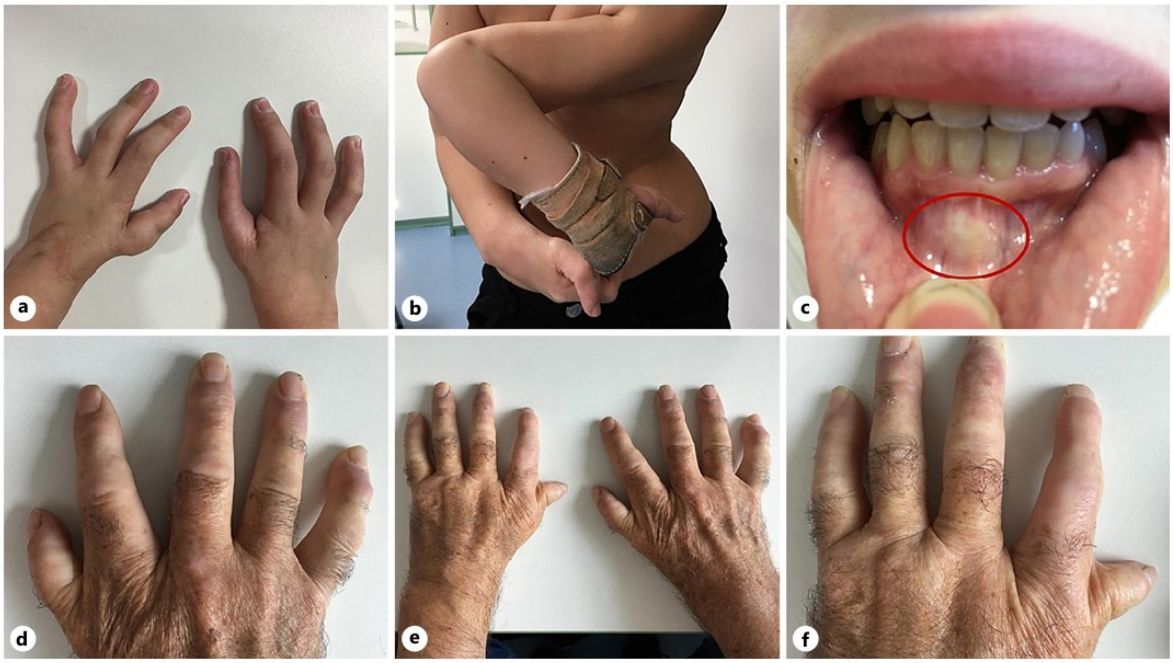

Fig. 1. Phenotypic variability in the proband and in his paternal grandfather. **a** Proband – condition after surgery – centralization of the wrist and thumb opposition modeling. **b** Proband – joint hypermobility due to radial ray hypoplasia. **c** Proband – ectopic placement of the left lower canine. **d–f** Paternal grandfather's hands – radial defect with missing opposition of the thumb.

Case Presentation

We report on a family with congenital malformation of the upper limbs in four generations. The male proband came to our attention at 3.9 years due to growth retardation. He was born from the 3rd normal pregnancy at the 39th week of gestation and was small for gestational age (SGA; birth weight 2,550 g, −2.2 SDS; length 47 cm, −2 SDS) [16]. His two maternal half siblings are healthy. His birth was spontaneous and early postnatal adaptation was normal with only mild neonatal jaundice. His neuropsychological development was normal. He had bilateral asymmetrical radial ray malformation (more pronounced on the left side) consisting of radial hypoplasia, ulnar flexure, and bilateral aplasia of the thumb. At an age of 2 months, the deformity was partially corrected by using a positioning orthosis to achieve the axial position of the wrist. He did not have clubfoot. Later, he underwent 12 operations in total, aiming to centralize the wrist and model thumb opposition (Fig. 1a, b).

His mother's and father's heights are 152.3 cm (−2.4 SDS) and 177.8 cm (−0.4 SDS), respectively. Both parents have normal body proportionality. The father's radial ray malformation of the forearm is milder than that of the proband. The paternal grandfather is affected as well, his height is 164 cm (−2.3 SDS), and he has a radial defect with missing opposition of the thumb (Fig. 1d–f). His arm-span was significantly shortened (−3.6 SDS) [17] and that the bones on the left side were more affected. Furthermore, the father and paternal grandfather have pelvic dystopia of kidney. The family reports that the phenotype of radial dysplasia was apparent in the paternal grandfather's mother as well, and her height was about 160 cm (−1.2 SDS).

At the age of 3.9 years, the proband's height was 92.6 cm (−2.8 SDS) (Fig. 2). His nutritional intake was adequate. His serum IGF-1 level was 68 μg/L (−1.0 SD), and the peak growth hormone (GH) level in two stimulation tests (insulin and clonidine) was 6.2 μg/L at maximum. Levels of other pituitary hormones were normal. Further abnormalities include pelvic dystopia of his right kidney and persistent primary nocturnal enuresis. He had no cardiac malformations, metabolic abnormalities, ocular coloboma, or Duane anomaly. Anthropometry indicated that the proband's arm-span was significantly shortened (−6.6 SDS) [17] and that the bones on the left side were more affected (similar to grandfather's). The difference in length between both upper limbs was about 2 cm, a difference in circumference was found as well. The length of the left ulna was 8.5 cm (−8.4 SDS), and the length of the radius was 4.5 cm (−12.5 SDS), respectively (Fig. 3a), clearly showing the predominant abnormality of the radial ray.

Although isolated growth hormone deficiency (GHD) was detected, the parents initially refused treatment. The boy started regular GH therapy later, at an age of 6.5 years, when his height

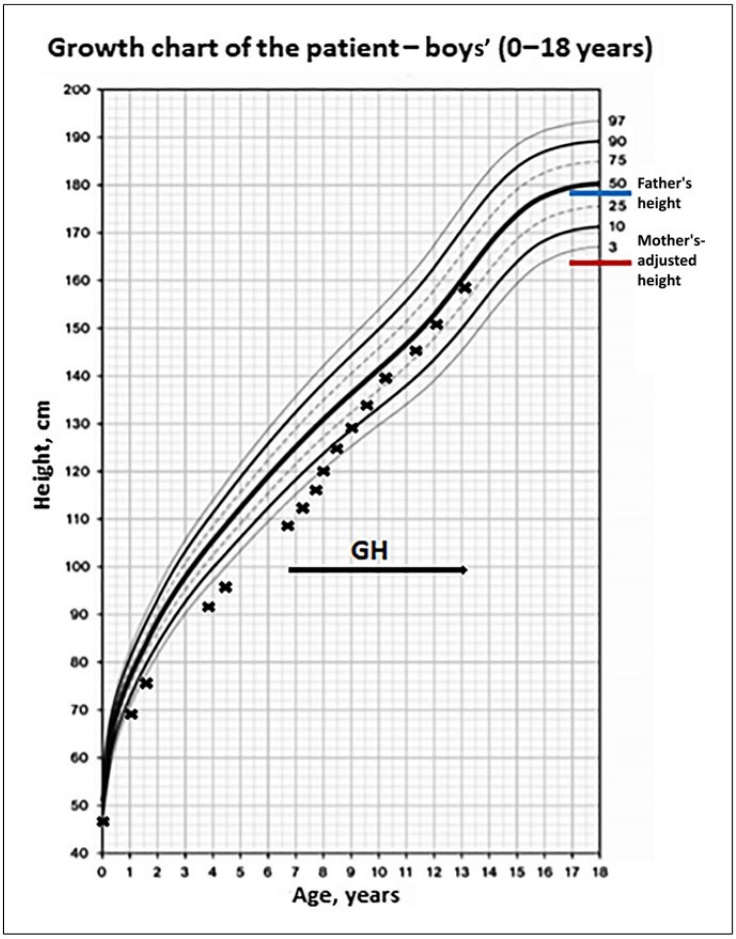

Fig. 2. Growth chart of the patient. GH – initiation of growth hormone therapy. Mother's-adjusted height was 165 cm, and father's height was 178 cm.

was 109 cm (−2.8 SDS). Between 6.5 and 11 years, he experienced catch-up growth typical for a child with GHD (Fig. 2). After the age of 11 years, his height was stabilized at about −0.6 SDS in accordance to the mid-parent height with a target height of 171.5 cm. Puberty started spontaneously at the age 12.5 years. At age 13 his height was 158.7 cm (−0.2 SDS).

By the age of 13.3 years, radiographs showed that length of the left ulna and radius were 16.5 cm (−5.8 SDS) and 10.5 cm (−9.7 SDS), respectively, which implies that the difference between the individual bones was similar, but the deviation from the norm in both bones had diminished (Fig. 3e, f). On the other hand, concavity of the radius had become more pronounced. Anthropometry did not reveal progress within the disproportion of the upper limbs –87.5% of his present height. The right upper limb length was 61.7 cm (−2.1 SDS) – about 8 cm shorter compared to the norm, and the left upper limb length was 56.4 cm (−3.4 SDS) – 13.5 cm shorter compared to the norm, respectively.

A detailed morphologic evaluation at the age of 13.3 showed a medium-sized mesocephalic cranium, hypertelorism, epicanthic folds, a smaller orbit, and low-set earlobes. Furthermore, the patient was found to have a central mandibular ectopic canine, which was extracted thereafter (Fig. 1c). This was the only sign of a midline developmental defect. Brain MRI could not be done safely, due to the presence of metallic material from the corrective surgeries on the left thumb. Therefore, a brain CT scan was done at the age of 13.9 years, showing normal morphology of the brain midline structures and the pituitary gland. Unfortunately, further examinations including an MRI or CT of the brain of any other family members were not possible.

Due to the suggestive dominant transmission of a developmental abnormality, we carried out whole-exome sequencing (WES). Genomic DNA was extracted from peripheral blood using the QIAmp DNA Blood Mini system (Qiagen, Hilden, Germany). WES was performed using SureSelect Human All Exon Kit V6 + UTRs (Agilent Technologies, Santa Clara, CA, USA). The library was

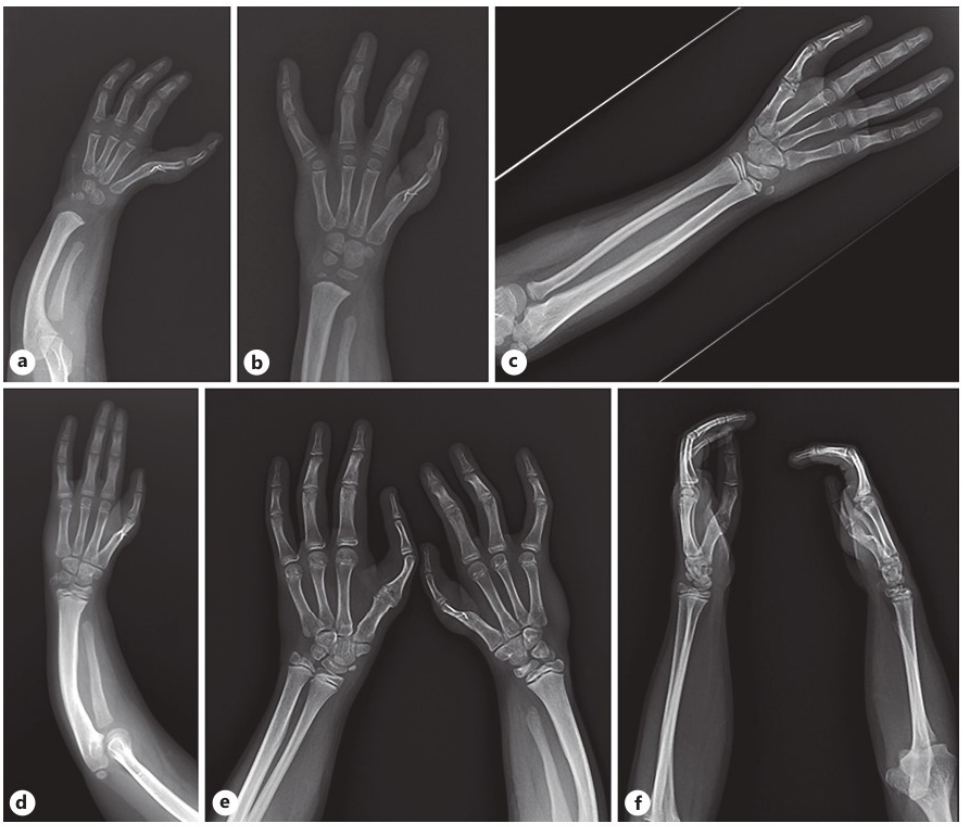

Fig. 3. Radiographs: growth, development, state of hands, and forearms during surgeries across the years. **a** 3.9 years – left hand. **b** 7.8 years – left hand. **c** 11.8 years – right hand. **d** 11.8 years – Left hand. **e**, **f** 13.3 years – both hands.

prepared according to manufacturer's instructions, and the indexed products were sequenced in an Illumina NextSeq 500 analyzer (San Diego, CA) with ×100 average coverage. The obtained sequences were aligned to the reference human genome (hg19 build) using BWA tool [18]. After subsequent bioinformatic steps, the detected and annotated variants were filtered using VarAFT software [19], mainly based on frequency in population databases gnomAD, ExAC, 1000G, in silico prediction tools (SIFT, PolyPhen2, MutationTaster, CADD, DANN), and protein function, expression, and interaction. All variants with potential clinical importance obtained from WES as well as segregation within the respective families were analyzed using Sanger sequencing as described previously [20]. Selected variants were finally evaluated using the American College of Medical Genetics and Genomics standards and guidelines [21]. This process revealed an already published [22] nonsense heterozygous variant in the *SALL4* gene c.1717C>T (p.Arg573Ter, NM_020436) in the proband. His father and paternal grandfather had the same variant (Fig. 4). The variant is not present in the gnomAD database or in other databases like ExAC, 1000G, and dbSNP. The other gene variants in our proband are shown in the online supplementary Table 1 (for all online suppl. material, see https://doi.org/10.1159/000531996).

With the aim to elucidate segregation of GHD within the family tree, we performed laboratory testing of the proband's mother and paternal grandfather to determine the level of IGF-1. The results were as follows: mother's IGF-1 is 153 μg/L (−0.3 SDS) – she has apparently no GHD; on the other hand, the paternal grandfather's IGF-1 level is 69 μg/L (−1.9 SDS), which is highly supportive of GHD.

Discussion

We report four generations of a family having radial ray malformation and pelvic dystopia of the kidney, including short stature in our proband and his paternal

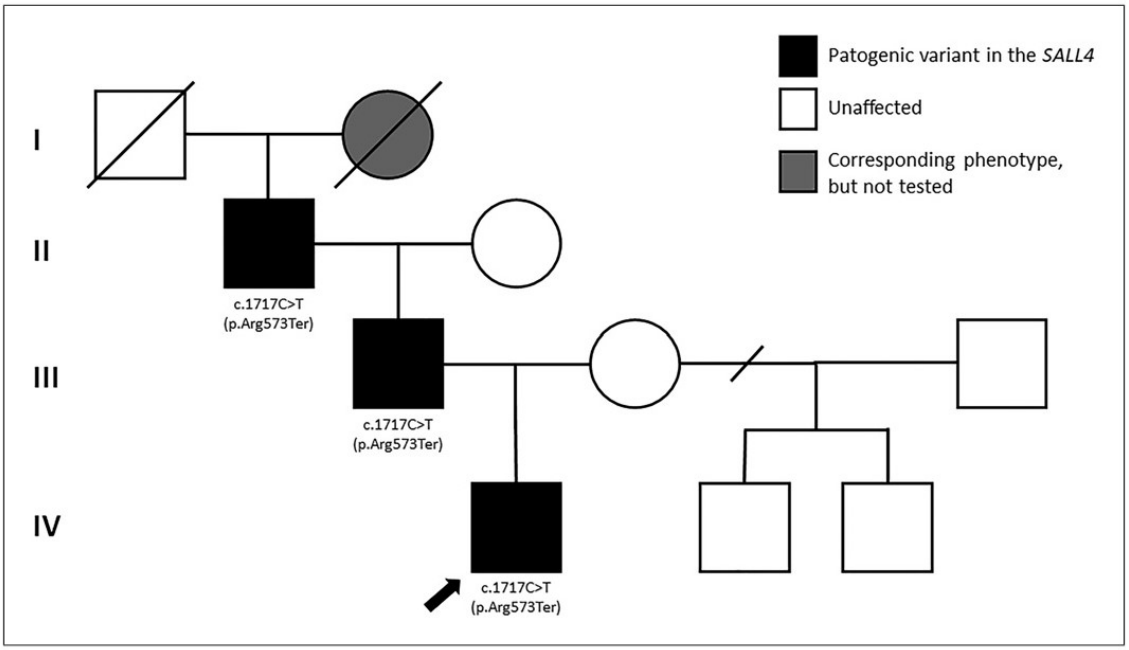

Fig. 4. Pedigree of the patient showing affected and unaffected family members.

grandfather. Three of them have a confirmed pathogenic variant in *SALL4,* which highlights the wide variability of expression even within a single family. Several partly overlapping phenotypes have been associated with pathogenic variants in the *SALL4* gene up to now – Okihiro syndrome/DRRS, HOS, and AROS [1, 4, 5]. Members of our family do not have the Duane anomaly – a congenital disorder of eye movement defined by limited or absent abduction and/or adduction of the eye [23]. The most significant phenotype of all family members can be found in the proband whose features are most consistent with the diagnosis of Okihiro syndrome. None of these syndromes have been reported in association with cranial midline defects, holoprosencephaly, abnormalities of midbrain development, and impaired pituitary function yet. However, there is one more indication supporting the hypothesis of the brain midline structures disorder by central ectopic canine. Unfortunately, we have no further information about the phenotype of the patient with same nonsense heterozygous variant in the *SALL4* gene for comparison [22]. Interestingly, GHD has been described in relation to the Cat-Eye syndrome, which is characterized by Duane retraction syndrome. However, in our family, there is no Duane anomaly, and the genetic background was different [24]. However, GHD was diagnosed in one member of a previously described Italian family with a *SALL4* pathogenic variant [15] with an empty sella and microcephaly.

Similarly, to our family, in other affected family members, the phenotype was milder and did not include empty sella and pituitary insufficiency. One of the potential explanations may be the phenomenon of nonsense-mediated mRNA decay [25]. Its main function is to reduce errors in gene expression by eliminating mRNA transcripts that contain premature stop codons. On the other hand, several mechanisms escaping nonsense-mediated mRNA decay leading, in turn, to truncated toxic proteins have been described [26]. Decreased penetrance of protein truncating variants even within a single family has been repeatedly described, i.e., for protein-truncating variants in the *RFX6* gene causing monogenic diabetes [27] or frameshift variant in the *APDS2* gene causing the SHORT syndrome, characterized by short stature, joint hyperextensibility, ocular depression, Rieger anomaly, and delayed tooth eruption [28]. The reasons of incomplete penetrance could be presence of rare or common genetic modifiers, epigenetic

changes, environmental factors, age, gender, different strength of expression of normal allele, or combination of these factors [29].

A handful of studies revealed genes that regulate transcription and can lead to anterior pituitary deficiency with or without additional dysmorphic signs [30]. We propose that SALL4 may enrich the choice of candidate genes responsible for "syndromic" hypopituitarism in individuals with specific phenotypic features and growth failure.

Statement of Ethics

All tested individuals and the proband's parents gave written informed consent for the genetic testing reported in this paper and for the publication of data related to their family study, of the photographs, and radiographs. This study was approved by the Ethics Committee at the 2nd Faculty of Medicine, Charles University in Prague (date of approval: June 30, 2017; not numbered). The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

The idea of this review was created by Aneta Kodytková and Jan Lebl. Manuscript was written by Aneta Kodytková, Jan Lebl, and Shenali Anne Amaratunga. Genetic background and analysis were supervised and performed by Petra Dušátková and Štěpánka Průhová a Lukáš Plachý. Evaluation of laboratory results and growth hormone therapy was done by Jan Lebl and Stanislava Kolouškova. Anthropometric examination was performed by Daniela Zemková and Klára Maratová. Figure preparation was done by Aneta Kodytková. Jan Lebl and Štěpánka Průhová were supervising the study. Each author had an important role in the study by acquiring data, drafting the article, interpreting the result, revising the manuscript, creating its final version, and approved it for submission.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

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7.8. Kodytková A, Dusatkova P, Amaratunga SA, Plachy L, Pruhova S, Lebl J. Integrative role of the SALL4 gene: From thalidomide embryopathy to genetic defects of the upper limb, internal organs, cerebral midline, and pituitary. Horm Res Paediatr (2023)

This review delves into the aftermath of the thalidomide disaster, which resulted in severe congenital malformations in thousands of children in the late 1950s and early 1960s. Despite numerous proposed mechanisms, it has only recently been confirmed that thalidomide and its derivative, 5-hydroxythalidomide (5HT), in conjunction with the cereblon protein, disrupt early embryonic transcriptional regulation. Specifically, 5HT triggers the selective degradation of *SALL4*, a crucial transcription factor in early embryogenesis. Notably, genetic syndromes arising from pathogenic variants in the SALL4 gene exhibit striking similarities to thalidomide embryopathy, with congenital malformations ranging from limb defects to heart, kidney, ear, eye, and potentially cerebral midline and pituitary abnormalities.

SALL4's role extends beyond embryogenesis, as it interacts with other transcriptional regulators, including TBX5, and modulates the sonic hedgehog signaling pathway. This review further explores the implications of *SALL4* pathogenic variants, occasionally leading to cranial midline defects, microcephaly, and short stature due to growth hormone deficiency (**shown in 7.7**). These findings position *SALL4* as a potential candidate gene contributing to monogenic syndromic pituitary insufficiency especially in non-consanguineous families.

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Integrative Role of the *SALL4* Gene: From Thalidomide Embryopathy to Genetic Defects of the Upper Limb, Internal Organs, Cerebral Midline, and Pituitary

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Keywords
Thalidomide · *SALL4* · Embryogenesis · Limb development · Pituitary development

Abstract
Background: The thalidomide disaster resulted in tremendous congenital malformations in more than 10,000 children in the late 1950s and early 1960s. ***Summary:*** Although numerous putative mechanisms were proposed to explain thalidomide teratogenicity, it was confirmed only recently that thalidomide, rather its derivative 5-hydroxythalidomide (5HT) in a complex with the cereblon protein, interferes with early embryonic transcriptional regulation. 5HT induces selective degradation of SALL4, a principal transcriptional factor of early embryogenesis. Genetic syndromes caused by pathogenic variants of the *SALL4* gene phenocopy thalidomide embryopathy with congenital malformations ranging from phocomelia, reduced radial ray, to defects of the heart, kidneys, ear, eye, and possibly cerebral midline and pituitary. *SALL4* interacts with *TBX5* and a handful of other transcriptional regulators and downregulates the Sonic hedgehog signaling pathway. Cranial midline defects, microcephaly, and short stature due to growth hormone deficiency have been occasionally reported in children carrying *SALL4* pathogenic variants associated with generalized stunting of growth rather than just the loss of height attributable to the shortening of leg bones in many children with thalidomide embryopathy. ***Key Messages:*** Thus, *SALL4* joins the candidate gene list for monogenic syndromic pituitary insufficiency. In this review, we summarize the journey from the thalidomide disaster through the functions of the *SALL4* gene to its link to the hormonal regulation of growth.

Introduction

Thalidomide (alpha-phthalimido-glutarimide; $C_{13}H_{10}N_2O_4$), marketed between 1957 and 1962 in Europe and elsewhere under brand names Contergan, Thalomid, and some others [1], caused the largest preventable man-made medical disaster ever within almost 5 years of promoted and uncontrolled use. Recommended as a sedative drug to manage fatigue and nausea, it became a popular relief from first trimester morning sickness. The increasing miscarriage rate and the birth of over 10,000 seriously malformed babies with an infant mortality as

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high as 40%, obviously due to inborn defects of internal organs, finally led to the withdrawal of thalidomide from medical use for several decades and the development of novel concepts of drug testing and pharmacovigilance.

The mechanism of thalidomide action was a challenging research field for several decades. However, thalidomide derivatives have been shown just recently to interfere with the early embryonic transcriptional regulation.

Thalidomide Embryopathy: The Unfortunate History of a Medical Disaster

The combination of congenital malformations of many tissues and organs due to thalidomide exposure, including limb teratogenicity (represented typically as amelia and phocomelia predominantly of upper limbs) was collectively named thalidomide embryopathy [2]. The most critical time for teratogenicity is between days 34–50 after the start of the mother's last menstrual period. Individual embryonal structures are apparently vulnerable for just part of that time slot with changing potential severity in terms of ongoing embryonic development. Nevertheless, the risk of a teratogenic hit was very high if exposed to thalidomide within the critical period, and only a few embryos known to be exposed remained unaffected. Thus, the teratogenic potential of thalidomide is apparently mostly independent of individual maternal and/or embryonic factors [3].

Thalidomide teratogenicity had the potential to affect almost any tissue and organ. The external signs ranged from very severe amelia or phocomelia to a milder reduction of the radial part of the forearm, with the thumbs being the most sensitive skeletal structure within the whole potential exposition window. Thumbs can be absent, hypoplastic, triphalangeal, and/or not in the opposing position ("fingerised"). The second major group of defects involves the ears, eyes, eye muscles, tear glands, and innervation of the face. Internal organs affected in individual cases include the heart, kidneys and urinary tract, intestine (duodenal atresia, anorectal atresia), and/or the genital tract.

Children with thalidomide embryopathy were often short and grew inadequately, resembling generalized stunting of growth rather than just the loss of height attributable to the shortening of leg bones. This was already noticed in the 1960–1970s during early descriptions of the thalidomide syndrome but was not elucidated in detail due to limited routine investigations [1, 3]. Survivors of the thalidomide disaster had additional significant handicaps in addition to congenital anomalies, including precocious arthritis and arthrosis, and early coronary heart disease.

Putative Mechanisms of Thalidomide Teratogenic Action

The research of thalidomide action led to the yield of over 2,000 scientific papers by the year 2000 which proposed several mechanisms, including direct inhibition of angiogenesis or the generation of reactive oxygen stress leading to cell death [3, 4]. Later, experimental studies pointed out the tendency of thalidomide to bind to cereblon – a protein encoded by the *CRBN* gene. The thalidomide-cereblon complex may compromise the capacity of cereblon to form an E3 ubiquitin ligase complex. Insufficient ubiquitination by currently unclear mechanisms reduces the levels of fibroblast growth factors 8 (FGF8) and 10 (FGF10); out of these, predominantly, FGF8 is essential for limb formation and auditory vesicles [5]. However, many unanswered questions remained as pathogenic variants of *CRBN* caused only autosomal recessive intellectual disability, probably due to the dysregulation of brain potassium channels, but did not cause dysmorphic features [6].

Donovan with co-workers [7] were the first to collect evidence of the role of SALL4 in thalidomide teratogenicity. They have shown that thalidomide and its analogs, in a complex with cereblon, disrupt the network of developmental transcriptional factors with multiple zinc finger domains. SALL4 (sal-like protein 4) plays a key role among them. Encoded by *SALL4* and localized on chromosome 20q13.13-13.2., it is a very early-stage transcription factor with eight Cys2-His2 (C2H2) zinc finger motifs [8, 9].

Embryonic cellular differentiation and morphogenesis are orchestrated by a complex network of transcriptional regulation. Early differentiation of the mammalian embryo and its specialized organs spans from the blastocyst, which consists of two distinct tissue lineages – the pluripotent inner cell mass (ICM) and the trophectoderm [9]. Cells of the ICM give rise to the epiblast and the extraembryonic endoderm, while trophectoderm cells have restricted potential and give rise to the trophoblast cell layers of the placenta [10]. In murine models, *Sall4* is expressed as early as the two-cell stage and, later, in majority of cells at embryonic stages, 8 and 16 cells (Fig. 1a, b). Its expression persists to the blastocyst, where the Sall4 RNA and Sall4 protein are found in some cells of the trophectoderm and ICM [9] (Fig. 1c). Early *Sall4* expression is required for caudalization and, importantly, for spinal cord differentiation of neural tissue; therefore, *Sall4* is expressed in a large variety of body structures such as the midbrain, rostral edge of the forebrain, pituitary gland, maxillary arch, liver, somites, genital tubercle, limb buds, tail, and the left ventricular myocardium [9, 11–13]

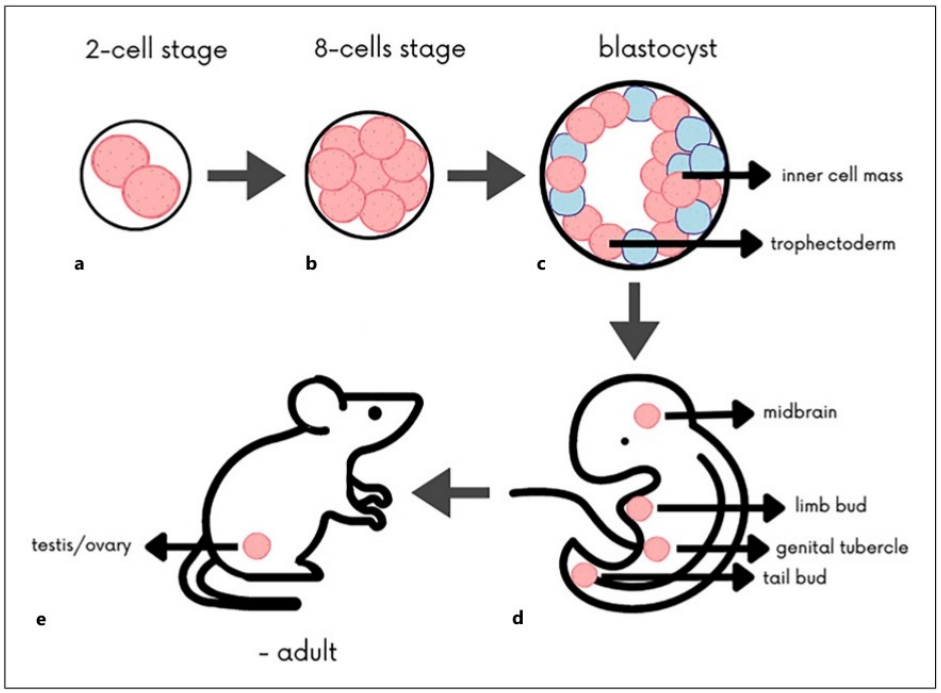

Fig. 1. *Sall4* gene expression in murine models (pink cells represent *Sall4* expression) – foremost, Sall4 protein is reported at the 2-cell stage (**a**), then the 8-cell stage mouse embryo (**b**), and in late blastocyst, where Sall4 protein is present in the inner cell mass (ICM) and in the trophectoderm (**c**). **d** In the late embryo, *Sall4* is expressed in the midbrain, limb bud, genital tubercle, and the tail bud. **e** In adults, *Sall4* expression is reduced to the germ cells (Inspired and edited by Tatetsu H, Kong NR, Chong G et al. 2016).

(Fig. 1d). Postnatally, *Sall4* expression is down-regulated and restricted to germ cells (Fig. 1e), undifferentiated spermatogonia, and oocytes. Later in life, *Sall4* may be reexpressed in various cancers [11].

Thalidomide is mainly modified using 5-hydroxylation. The enzyme-induced proteasomal degradation of the *SALL4* protein is induced mainly by 5-hydroxythalidomide (5 HT), a thalidomide derivative generated by the enzymatic activity of cytochrome P450 isozymes, through the interaction with cereblon. 5 HT induces *SALL4* degradation more than thalidomide alone and is said to mediate thalidomide teratogenicity [14]. *SALL4* is a plausible candidate target of thalidomide teratogenicity not only due to its sensitivity to degradation by thalidomide and its derivatives [15–17], but because it leads to genetic syndromes (caused by heterozygous *SALL4* mutations in humans and by *Sall4* defects in the murine model) phenocopying thalidomide embryopathy, with a spectrum of congenital malformations such as reduced radial ray, phocomelia, and heart, ear, and eye defects [12, 18, 19].

Interestingly, unlike *SALL4*/*Sall4* genetic variants, thalidomide induces the degradation of SALL4 exclusively in humans, primates, and rabbits, but not in rodents or fish, due to a different structure of the gene product across species. Thus, this explains the species-specific risk of thalidomide embryopathy [7, 20]. *SALL4* degradation by thalidomide and its analogs offers a relevant explanation for its anticancer action by *SALL4* overexpressing in tumor cell lines. This has remained speculative for years after its controlled reintroduction into human medicine as an anticancer drug [21, 22].

Interaction Partners and Pathways of *Sall4* in Murine Models

The action of *Sall4* in various cells is exerted in interaction with its protein partners and downstream targets (Fig. 2). Transcriptional factors including

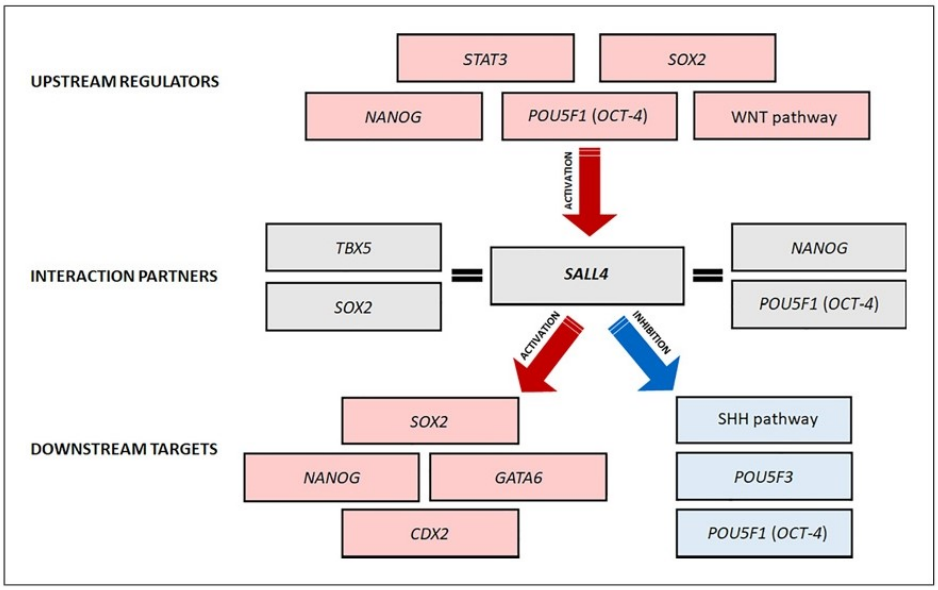

Fig. 2. Key regulating and target genes of *SALL4* in early embryogenesis and development (red boxes and arrows indicate activation, while blue boxes and arrow indicate inhibition).

Pou5f1 (*Oct-4*), *Sox2,* and *Nanog* are crucial for pluripotency and often interact with *Sall4* to define its molecular function in early embryogenesis. Wherein each of the four factors can regulate its own expression as well as that of others [23–25], *Sall4* knockout mice fail to maintain a pluripotent ICM [10], thereby null embryos lack *Pou5f1* expression in the ICM, increase *Cdx2* expression, and replace epiblast with trophectoderm [25, 26]. Murine *Sall4* has been identified as an upstream activator for *Gata6* – a transcription factor of lineage-defining extraembryonic primitive endoderm and an antagonist to *Nanog* that is thought to specify epiblast [24]. Furthermore, *Sall4* represses *Pou5f3*, a stem cell factor, to release cells from an undifferentiated state [27].

In addition to gene interactions, *Sall4* co-regulates some key cellular signaling pathways as well. *Sall4* and *Gli3* cooperate toward the proper development of the anterior-proximal skeletal elements and function upstream in the Shh-dependent posterior skeletal element development via the Sonic hedgehog (Shh) pathway [18, 23]. During embryogenesis, the initiation of upper limb outgrowth is triggered by wingless-type (Wnt) signaling factors, fibroblast growth factors (FGF), and the expression of *Tbx5* (T-box) [28]. In mouse models, a complex interplay between the *Sall4* and T-box genes has been found in the patterning and morphogenesis of the limbs and heart, where *Sall4* is regulated by *Tbx5*, and both *Tbx5* and *Sall4* interact positively and negatively to modulate transcription [12]. Interaction with the Shh pathway and other genes may be suggestive of the role of *Sall4* in pituitary development and function [10].

Role of *SALL4* in the Morphogenesis of the Limbs, Cerebral Midline, and the Pituitary in Human Embryos

The SALL4 protein plays a unique role in human embryogenesis and development – separately or through interactions with numerous factors and epigenetic complexes [8], e.g., with *TBX5* [12]. The *TBX5* gene, localized on chromosome 12q24.21, belongs to the T-box family of transcription factors. The *Brachyury* gene, founder of the T-box family, is known to play an important role in early vertebrate development [29]. The T-box family, containing many genes, has a common sequence-specific DNA-binding motif (the T-box) and a transcriptional activator or repressor domain. Those genes tend to be expressed in specific organs or cell types, especially during embryogenesis, and they are generally required for the development of those tissues [29, 30].

A recent study showed that *TBX5* regulates the *SALL4* gene and interacts with the SALL4 protein to regulate heart and upper limb development and establish thumb morphogenesis. Pathogenic *SALL4* and *TBX5* variants cause autosomal dominant Duane-radial ray syndrome (DRRS; also known as Okihiro syndrome) [31, 32] and Holt-Oram syndrome (HOS) [33].

SALL4 and *TBX5* interactions lead to similar limb phenotypes within DRRS and HOS [12]. DRRS is characterized by the Duane eye anomaly and radial ray malformations of the limbs. The Duane anomaly is a congenital disorder of eye movement defined by the limited or absent abduction and/or adduction of the eye [34]. Radial ray malformations in DRRS range from triphalangeal thumbs, preaxial polydactyly, hypoplasia, or aplasia of the thumbs, up to the hypoplasia or aplasia of the radii, and the shortening and radial deviation of the forearms. HOS, an autosomal dominant disorder, is characterized by radial ray malformations and congenital cardiac defects [35]. Although a major locus for HOS was identified in *TBX5*, only approximately 30% of these cases clinically identified as HOS were found to have a pathogenic variant in this locus [36]. In addition, the truncating mutations as well as larger deletions of *SALL4* have been reported to cause acro-renal-ocular syndrome (AROS), which is presented with radial ray malformations and Duane anomaly, along with other features such as ocular coloboma and renal abnormalities [37, 38]. Other less common features belonging to DRRS and AROS include hearing loss, ear malformations, epicanthal folds, and very rarely, heart defects – atrial and ventricular septal defects [31].

SALL4 affects the development of the upper limb partly by interaction with the *TBX5* gene but through the Sonic hedgehog (SHH) pathway as well. *SALL4* enters this pathway by interacting with the *GLI3* gene [18]. The upper limb bud emerges as a bulge of lateral plate mesoderm, covered by an outer layer of ectoderm. *TBX5* leads to the development of two signaling centers in the limb bud that specify the orientation of developing limb growth [39]. The first center, the apical ectodermal ridge (AER), is a signaling center for proximal-distal upper limb axis formation [40]. The development and differentiation along the antero-posterior (radial-ulnar) axis are controlled by the zone of polarizing activity (ZPA) in the posterior limb (ulnar) mesoderm. ZPA guides radio-ulnar limb differentiation through feedback loops that include SHH and FGF triggered by TBX5 [18, 41]. Disruption of the AER leads to transverse deficiencies. Duplication of the ZPA leads to a mirror hand, while the loss of ZPA results in ulnar longitudinal deficiencies. Because of the interdependence of AER and ZPA functions, ulnar longitudinal deficiencies may lead to malformations of radial-sided structures as well. Conversely, persistence of ZPA with diminution of AER function leads to radial longitudinal deficiencies [42].

Thus, the SHH pathway is essential to maintaining the AER. In parallel, it is known to be a major regulator of the cerebral midline and pituitary as well. Abnormal SHH signaling leads to phenotypes ranging from holoprosencephaly through milder midline anomalies up to non-syndromic functional pituitary insufficiency presenting either as isolated growth hormone deficiency or combined pituitary hormone deficiency [18, 19]. Some previous observations confirmed occasional findings of cranial midline defect, microcephaly, and growth disorder in mice and humans with pathogenic variants in *SALL4*, probably due to the interaction with the SHH pathway [32, 37].

In addition to SHH, another potential network partner affecting midbrain development is *SOX2* which interacts with *SALL4* in early embryogenesis. *SOX2* can be causal in anophthalmia and hypopituitarism syndromes or in septo-optic dysplasia. Some components of the phenotypic spectrum of septo-optic dysplasia are also characteristic of the Duane anomaly due to haploinsufficient *SALL4* [33].

Varied phenotypic severity even within one family was documented in an Italian family with autosomal dominant transmission of a *SALL4* pathogenic variant [36, 39]. The index patient had severe pre- and postnatal growth retardation with confirmed isolated growth hormone deficiency and an empty sella, a single maxillary central incisor, thenar dysplasia, polydactyly, and type III Duane anomaly. The phenotype in his father and paternal grandfather was milder; both had bilateral hypoplasia of thenar eminencies and proximal insertion of the thumbs, clearly suggesting a radial ray dysplasia. An additional 4 patients with radial ray dysplasia due to *SALL4* pathogenic variants but with missing phenotypic data on growth were reported within a series of targeted sequencing of individuals with congenital limb malformations [40]. We are currently reporting on 4 generations of one family with a *SALL4* pathogenic variant which sheds more light on the phenotypic interplay between the forearm, kidney, and pituitary, with varied severity among generations [43] (submitted). The growth hormone-deficient proband was successfully treated with growth hormone.

Conclusion

The thalidomide disaster, an unfortunate episode of 20th-century medical history, sheds new insights into the understanding of the recent genetic findings in children

with congenital malformations, including syndromic short stature due to cerebral midline defects and, tentatively, pituitary insufficiency.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

The idea of this review was created by Aneta Kodytková and Jan Lebl. The manuscript was written by Aneta Kodytková, Jan Lebl, and Shenali Anne Amaratunga. Genetic background and analysis were supervised and performed by Petra Dušátková, Štěpánka Průhová, and Lukáš Plachý. Figure preparation was done by Aneta Kodytková. Each author had an important role in the study by acquiring and validating information, revising the manuscript, creating its final version, and approving it for submission.

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8. Discussion

This project provides a rare and unique insight into endocrine disease in consanguineous families looking into the genetic aetiology of three endocrine conditions, chosen based on their position on the gradient of pathophysiological complexity, the relatively straightforward CHI, monogenic diabetes, which manifests with uniform biochemical parameters despite diverse causes and short stature or growth failure, one of the most complex developmental phenomena. Genetic findings were analyzed in non-consanguineous families as well to allow for comparison.

The primary test centre in Kurdistan, Iraq was chosen due to the high consanguinity rate of the region (44%) and previous collaboration. This region has 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 (publication in 7.2.). 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 especially in consanguineous families.

Our unique single centre study (publication in 7.3.) on monogenic diabetes from the same centre using data from 754 paediatric diabetes patients confirmed 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 the primary test centre in Iraq (publication in 7.4.), there was a very high rate of positive findings with 65% of the probands having a pathogenic cause of short stature. Notably, 52% of causative genes were genes influencing the growth plate including chondrocytes and the extracellular matrix (*CCDC8*, *CTSK*, *COL1A2*, *COL10A1*, *DYM*, *FLNA*, *FN1*, *LTBP3*, *MMP13*, *NPR2*, *PCNT*, *SHOX*) with only 4% being genes of the GH-IGF1 axis (*GHR*, *SOX3*). The rate of positive variants was higher than other analyzed cohorts with SGA and GHD from the centre in Prague with a low rate of consanguinity (29% /42%). In addition, 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 (maximum rate of positive findings would be 33%). Moreover, these findings emphasize the importance of the timely diagnosis of syndromic short stature for proactive screening of potential concomitant conditions and the management of these complex conditions.

Interesting, results across consanguineous / non-consanguineous populations' further strength the concept, that genes influencing the growth plate play a crucial role in growth regulation (publications in 7.5. and 7.6.). The stark difference, however, was in the percentage of recessive or biallelic variants with 75% of variants in the consanguineous cohort being recessive. Even among non-consanguineous families, there can be new important pathophysiological insights such as GHD being caused by a heterozygous variant in *SALL4*, making the gene a potential candidate gene monogenic syndromic pituitary insufficiency (publications in 7.7. and 7.8.).

Relationship of the work to the tested hypothesis

Main scientific 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.

In our study, we chose three diagnoses namely congenital hyperinsulinism, monogenic diabetes and short stature based on their representation of three levels of pathophysiological

complexity. In each diagnosis, we found a pathogenic variant in over 50% of patients with most variants being recessive. We successfully compared incidence and causative genes between consanguineous and non-consanguineous populations and described the differences among populations. These findings helped strengthen novel mechanisms and gave insight into pathophysiological pathways in each diagnosis.

9. Conclusion

The publications presented in this dissertation collectively 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. These studies 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.

As part of her PhD studies, the PhD student coordinated the examination of the entire consanguineous cohort, participated in the molecular genetic examination of all patients, analyzed data obtained by NGS methods and statistically processed and evaluated the results of the project. She wrote all research papers where the PhD student is the first author. In the other studies from non-consanguineous cohorts, she participated in the molecular genetic examination by analysing NGS data, and provided insight into each research paper.

The conditions that are included in this dissertation 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. There was a very high rate of positive findings in the consanguineous cohort with biallelic variants being most common as hypothesized. 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, all variants with homozygous as expected in consanguineous 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 provided insights into the complex diagnosis of this condition. The rates of pathogenic findings were high with 83% of genes causing neonatal diabetes and 57% of syndromic diabetes being confirmed.

Majority of new insights were in the field of short stature research. A unique study looking at the largest cohort of patients with short stature of unknown aetiology from consanguineous families had a very high rate of positive findings via WES (65%). If a panel of genes for short stature was used the rate of positive results would have been reduced. These results highlighted the importance of considering different genetic testing methods in such populations. 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.

This dissertation provided comparison of the genetic findings in non-consanguineous families with findings from a GHD and SGA cohort. It was shown how causative genes among both populations are varied with the mechanism of inheritance differing as well. All results further strength the concept, that genes affecting the growth plate (chondrocytes and the extracellular matrix) play a crucial role 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.

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.

10. Summary

As part of this dissertation project, genetic testing was done in individuals with three pediatric 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 analyzed diagnosis was short stature, we analyzed 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 analyzed 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 strength the concept, that genes influencing the growth plate play a crucial role in growth regulation.

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