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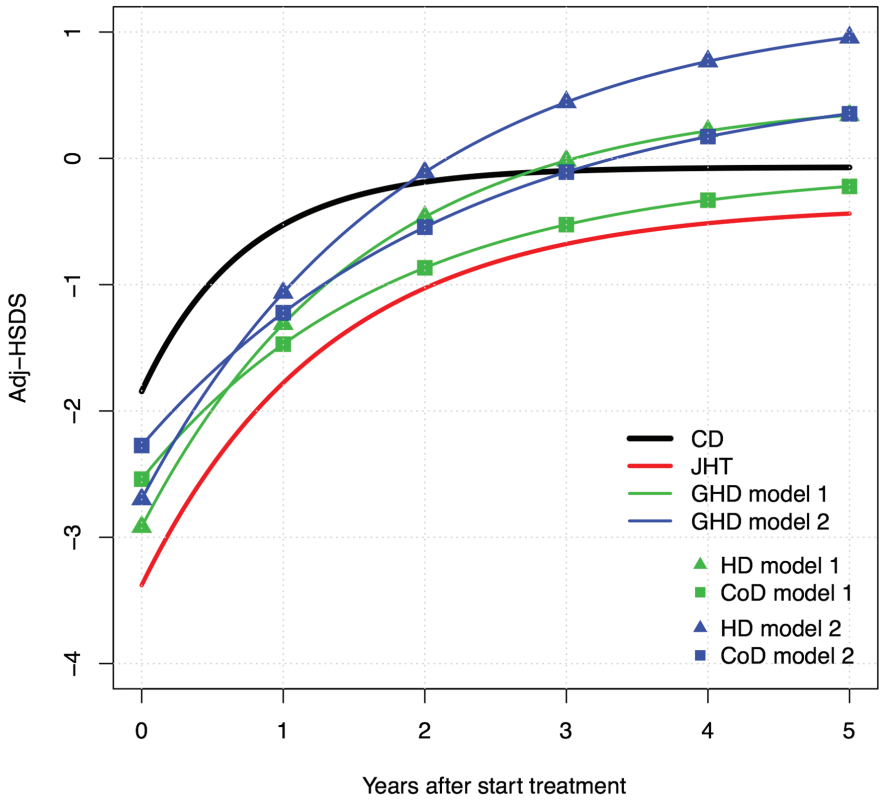

Modelled mean adjusted-height standard deviation score of children with juvenile hypothyroidism, growth hormone deficiency (CoD and high dose, models 1 and 2) in comparison with the catch-up growth model for celiac disease

Catch-up Growth in Prepubertal Children Treated for Juvenile Hypothyroidism and Growth Hormone Deficiency can be Modelled with a Monomolecular Function

Wit J.M. et al.

Page: 15-22

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Review

Original Articles

Case Reports

CONTENTS

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CONTENTS

REVIEW

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The Clinical Spectrum of Resistance to Thyroid Hormone Alpha in Children and Adults

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Abstract

Resistance to thyroid hormone alpha occurs due to pathogenic, heterozygous variants in *THRA*. The entity was first described in 2012 and to date only a small number of patients with varying severity have been reported. In this review, we summarize and interpret the heterogeneous clinical and laboratory features of all published cases, including ours. Many symptoms and findings are similar to those seen in primary hypothyroidism. However, thyroid-stimulating hormone levels are normal. Free triiodothyronine (T3) levels are in the upper half of normal range or frankly high and free thyroxine (T4) levels are low or in the lower half of normal range. Alterations in free T3 and free T4 may not be remarkable, particularly in adults, possibly contributing to underdiagnosis. In such patients, low reverse T3 levels, normo- or macrocytic anemia or, particularly in children, mildly elevated creatine kinase levels would warrant *THRA* sequencing. Treatment with L-thyroxine results in improvement of some clinical findings.

Keywords: Constipation, developmental delay, growth failure, central hypothyroidism, autism spectrum disorder, LT4, impaired sensitivity to thyroid hormone

Introduction

The thyroid gland has important roles in energy homeostasis, skeletal growth, cardiac and gastrointestinal function, and maturation of the central nervous system (1). Thyrotropin-releasing hormone (TRH) produced by the hypothalamus stimulates the pituitary gland to release thyroid-stimulating hormone (TSH), which results in synthesis and secretion of thyroid hormones (TH) from the thyroid. The term TH comprises T4 (thyroxine, a prohormone and the predominant product of thyroid) and T3 (tri-iodothyronine, the bioactive hormone). A negative-feedback mechanism provides balance between TH levels and TRH-TSH production (2).

TH enter cells via a number of membrane transporters, including tissue specific entities such as monocarboxylate transporter 8 (MCT8) in the central nervous system (3). Intracellular deiodinase enzymes regulate TH concentrations and convert T4 to T3 and various metabolites (4). T3 binds nuclear receptor proteins and regulates target gene transcription. In the absence of T3, receptor-protein complexes repress basal gene transcription (5). There are two types of TH receptor (TR): alpha (TRα) and beta (TRβ). These receptors are highly homologous and encoded by the genes *THRA* (chromosome 17) and *THRB* (chromosome 3), respectively. TRα has two isoforms produced with alternative splicing. TRα1 is mainly expressed in the central nervous system, bone, myocardium, skeletal muscle and gastrointestinal tract, while TRα2 is expressed in various tissues but has no binding site for T3 and thus its function is enigmatic (6,7). TRβ1 is predominantly expressed in liver, kidney, thyroid gland, brain, pituitary, and inner ear. TRβ2 expression is limited to the hypothalamus, pituitary gland, inner ear and retina, and plays the main role in the hypothalamic-pituitary-thyroid (HPT) axis (6-8).

Variants in TR genes cause particular forms of resistance to TH (RTH) (9). The first instance of this disease spectrum was reported by Refetoff et al (10) in 1967. However, demonstration of the underlying genetic defect in *THRB* took more than two decades (11). Pathogenic variants in *THRB* result in RTH beta (RTHβ, dominant OMIM #614450 and recessive OMIM #274300). The incidence of RTHβ is reported to be approximately 1/40000 and is characterized

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by goiter, tachycardia, hyperactivity, failure to thrive and cognitive impairments with high serum TH levels, but normal or mildly elevated TSH (12-14). The first case of TH resistance in TRα (RTHα, OMIM # 614450) due to a pathogenic, heterozygous variant in *THRA*, was published in 2012 by Bochukova et al (15). To date, 40 cases (13 adults, 27 children) from 28 different families with 25 different variants in *THRA* gene have been published (Tables 1, 2) (15-32).

The main symptoms and findings of RTHα include varying degrees of constipation, developmental delay, growth failure, and anemia, which are associated with the tissues where TRα is the main TR and are common to both primary hypothyroidism and RTHα. In the former, there is inadequate TH to induce TRα while reduced activity of TRα is the mechanism in the latter (33). Furthermore, there are interesting additional features in some of the cases with RTHα including skin tags (18,19,25), epilepsy (18,23), and the individual clinical picture or laboratory findings becoming less remarkable with age (17,24). The disease is thought to be underdiagnosed, given that serum TH levels are not distinctive as is seen in RTHβ, and TSH is not elevated since TRβ is intact (33,34).

Genetics

To date, 25 different variants in *THRA* have been published (Tables 1, 2). Six variants were inherited from an affected parent. Three of the 25 variants were frameshifts, which affected four cases more severely (16,18,24). Three distinct variants resulted in a premature stop codon (21,28,31). However, most of the variants in *THRA* were missense mutations (15,19-26,29,30,32). All of the RTHα patients were heterozygous for the variant, showing that mutant TRα had a dominant-negative effect on the wild-type receptor, in a similar fashion to RTHβ (33). It should be noted that some of the variants have not been functionally characterized (20,21,26-29,31). In addition, one of the variants (c.1044G>T) found among subjects with autism spectrum disorder was a synonymous substitution (26).

The reported cases showed that there was a genotype-phenotype correlation in patients with RTHα. The most severe cases tended to have frameshift variants, but missense variants usually caused a milder phenotype (18,21,24). In addition, patients with the same variants in *THRA* can present with different clinical phenotypes, suggesting that additional factors, possibly cofactor proteins, affect TH activity (35).

It was reported that, in the presence of high T3 levels, mutant TRα can exhibit some degree of transcriptional activity, in a similar fashion to the wild-type receptor. This finding suggests that increased circulating T3 levels might have some benefit in ameliorating the dominant-negative activity of mutant TRα, although it is not clear whether high levels of T3 are a result of a compensatory mechanism (19,23,24). With the exception of one case with a mutation in both TRα1 and 2, who presented with severe atypical malformations (22), similar clinical features have been observed due to variants affecting either TRα1 alone or TRα1/2 (33).

Pathophysiology

The mutant TRα behaves as a dominant-negative repressor of T3 target gene expression in RTHα and also inhibits the function of wild-type TR (15). TRα and TRβ act via transcriptional repressors, such as nuclear receptor corepressor-1 (NCoR1), in the absence of T3. This effect results in modification of histone deacetylase (HDAC) enzymes into a co-repressor complex, which suppresses basal T3 target gene transcription with remodeling of chromatin (36). When T3 binds to its receptors, a structural change is initiated, which results in disruption of TR and NCoR1. Furthermore, modification of nuclear receptor coactivators initiate the expression of T3 target genes (37,38).

If TRα is mutant, it cannot release NCoR1 as a response to T3. Consequently, T3 target gene transcription remains suppressed because of the inhibition of wild-type TR through constant HDAC-induced chromatin remodeling. In the light of this molecular information, RTHα demonstrates clinical features with reduced T3 action in related tissues. In addition, a dominant-negative potential of the mutant TRα determines the severity of disease (38).

Clinical Features

The first experimental study of TRα was reported in 1997, 15 years before the first human cases were reported, showing that a TRα knock-out mouse had postnatal growth arrest with delayed maturation in small intestine and bones (39).

Data regarding physical features of patients with RTHα are generally limited and heterogeneous in the published reports. No descriptive data were given for seven children who were shown to have *THRA* variants during genetic analyses for autism spectrum disorder (20,26). The clinical features and underlying mechanisms, mainly derived from animal studies, are summarized in Table 3.

Appearance

Patients with RTHα are usually born after an uneventful pregnancy (33). In severe cases, macroglossia, coarse facial features, and umbilical hernia have been noted in early

Table 1. Genetic and laboratory findings in reported children with *THRA* variants (n = 27). Except for two subjects, all of the patients with available data had at least one symptom or sign associated with hypothyroidism. Laboratory data were obtained before LT4 use in all subjects except patients 22 and 26, who were receiving LT4 treatment. When available, the data were given as exact values [high (H), normal (N), or low (L)] and relevant reference ranges in the original reports were given as footnotes

Case	Variant	Type	Amino acid	Age (years)	LT4-naive	fT3	TT3	rT3	fT4	TT4	TSH	CK	Hgb	Ref[#]
1	632A > G	Missense	D211G	1.5	Yes	N/A	3.6 (H)[b1]	0.09 (L)[c1]	9 (L)[d1]	110 (N)[e1]	4.4 (N)[f1]	N/A (N)[N/A]	6.2 (N)[h1]	23
2	776T > C	Missense	M259T	12	Yes	6.6 (H)[a1]	N/A	N/A	10.8 (L)[d2]	N/A	1.6 (N)[f2]	N/A	11.2 (L)[h2]	32
3	787G > T	Missense	A263S	2.6	Yes	7.28 (N)[a2]	3.65 (H)[b2]	0.31 (N)[c2]	16.4 (N)[d3]	85 (N)[e2]	2.1 (N)[f3]	236 (H)[g1]	11.6 (N)[h3]	24
4[#]	787G > T	Missense	A263S	7.4	Yes	7.96 (H)[a2]	3.46 (H)[b2]	0.27 (N)[c2]	17.6 (N)[d3]	98 (N)[e2]	1.4 (N)[f3]	218 (H)[g1]	10.8 (L)[h4]	24
5	787G > T	Missense	A263S	8.8	Yes	6.65 (N)[a2]	2.96 (H)[b2]	0.24 (N)[c2]	16.1 (N)[d3]	112 (N)[e2]	2.59 (N)[f3]	240 (H)[g1]	11.8 (N)[h4]	24
6[#]	787G > T	Missense	A263S	17	Yes	6.65 (N)[a2]	2.53 (H)[b2]	0.19 (L)[c2]	14.4 (N)[d3]	89 (N)[e2]	2.03 (N)[f3]	115 (N)[g1]	10.9 (L)[h5]	24
7	788C > T	Missense	A263V	17	Yes	7.6 (H)[a1]	N/A	< 0.07 (L)[c3]	10 (N)[d4]	N/A	3.6 (N)[f4]	136 (N)[g2]	N/A (L)[N/A]	25
8	817A > G	Missense	T273A	2	Yes	9.6 (N)[a3]	N/A	N/A	6.8 (L)[d5]	N/A	2.09 (N)[f5]	N/A	9.3 (N/A)[N/A]	32
9	821T > C	Missense	L274P	11	Yes	N/A	N/A	N/A	9 (L)[d6]	N/A	2.4 (N)[f6]	N/A	N/A	25
10	871G > A	Missense	G291S	4	Yes	5.04 (H)[a4]	N/A	N/A	0.93 (N)[d7]	N/A	3.89 (N)[f4]	396 (H)[g3]	10.4 (L)[N/A]	29
11	1044G > T	Synonymous	A348A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	26
12	1053C > G	Missense	H351Q	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	26
13	1099C > A	Missense	L367M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	26
14	1138del.4nt	Frameshift	C380fs387X	1.3	Yes	12.4 (H)[a2]	2.76 (H)[b2]	N/A	5.1 (L)[d3]	53 (L)[e2]	1.4 (N)[f3]	N/A	8.9 (L)[h3]	24
15	1144G > C	Missense	A382P	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	26
16	1150C > T	Missense	R384C	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	20
17	1151G > A	Missense	R384H	0.9	Yes	8.0 (H)[a2]	5.2 (H)[b2]	0.31 (N)[c2]	13.9 (N)[d3]	107 (N)[e2]	1.89 (N)[f3]	268 (H)[g1]	8.6 (L)[h3]	24
18	1176C > A	Nonsense	C392X	2	Yes	5.18 (H)[a5]	N/A	N/A	0.78 (N)[d8]	N/A	2.775 (N)[f7]	N/A (H)[N/A]	N/A (L)[N/A]	21
19	1176C > A	Nonsense	C392X	4	Yes	5.13 (N)[a6]	11.96 (N)[b3]	N/A	70 (N)[d9]	0.73 (N)[e3]	4.98 (N)[f8]	N/A	96 (L)[h6]	31
20	1183G > T	Nonsense	E395X	2	Yes	5.23 (H)[a7]	2.18 (N)[b4]	N/A	0.91 (L)[d10]	77.8 (N)[e4]	1.38 (N)[f9]	982 (H)[g4]	86 (L)[h7]	27, 28
21	Insert 1nt	Frameshift	F397fs406X	5	Yes	N/A	N/A (H)[N/A]	N/A (L)[N/A]	N/A (N)[N/A]	N/A (N)[N/A]	N/A (N)[N/A]	N/A	11.5 (L)[N/A]	16, 17
22	1193C > G	Missense	P398R	8	No	5.62 (N)[a8]	N/A	N/A	9.05 (L)[d11]	N/A	0.45 (N)[f7]	N/A (H)[N/A]	N/A (L)[N/A]	21
23	1202T > C	Missense	F401S	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	26
24	1207G > A	Missense	E403K	6	Yes	6.94 (N)[a9]	N/A	N/A	13.35 (N) [d12]	N/A	1.89 (N)[f7]	N/A (H)[N/A]	N/A (L)[N/A]	21
25	1207G > T	Nonsense	E403X	6	Yes	0.4 (N)[a10]	155 (N)[b5]	0.07 (L)[c4]	0.5 (L)[d13]	3.3 (L)[e5]	1.04 (N)[f10]	N/A	N/A	15
26	1207G > T	Nonsense	E403X	2.5	No	7.14 (H)[a11]	N/A	N/A	1.6 (H)[d14]	N/A	0.004 (L)[f7]	N/A (H)[N/A]	N/A (L)[N/A]	21
27	1213T > C	Missense	F405L	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	26

H: high, N: normal, L: low, N/A: not available

[#]Asymptomatic, Reference ranges: fT3: free T3; [a1] 3.5-6.5 pmol/L, [a2] 3.8-7.6 pmol/L, [a3] 3.6-10.4 pmol/L, [a4] 2.3-4.2 pg/mL, [a5] 1.45-3.5 pg/mL, [a6] 1.78-5.6 ng/dL, [a7] 2.75-4.68 pg/mL, [a8] 3.88-8.02 pmol/L, [a9] 3.93-7.7 pmol/L, [a10] 0.3-0.5 ng/dL, [a11] 1.5-4 pg/mL. TT3: total T3; [b1] 1.3-2.7 nmol/L, [b2] 1.4-2.5 nmol/L, [b3] 7.0-22.0 ng/L, [b4] 0.99-2.27pg/mL, [b5] 130-221 ng/dL. rT3: reverse T3 (nmol/L); [c1] 0.11-0.44, [c2] 0.22-0.52, [c3] 0.12-0.36, [c4] 0.21-0.37. fT4: free T4; [d1] 10.0-23.0 pmol/L, [d2] 11.5-22.7 pmol/L, [d3] 11.0-25.0 pmol/L, [d4] 10-19.8 pmol/L, [d5] 7.5-21 pmol/L, [d6] 10.0-18.7 pmol/L, [d7] 0.89-1.76 ng/dL, [d8] 0.7-0.9 ng/dL, [d9] 50-230 pg/L, [d10] 1.2-1.73 ng/dL, [d11] 12.5-21.5 pmol/L, [d12] 12.6-21.5 pmol/L, [d13] 0.8-1.7 ng/dL, [d14] 0.6-1.4 ng/dL, TT4: total T4; [e1] 70-150 nmol/L, [e2] 58-128 nmol/L, [e3] 0.45-1.54 µg/L, [e4] 51.8-122.5 ng/mL, [e5] 7.4-12.1 µg/dL. TSH: thyroid-stimulating hormone (mIU/L); [f1] 0.5-5.0, [f2] 0.51-4.9, [f3] 0.4-4.3, [f4] 0.35-5.5, [f5] 0.7-6.4, [f6] 0.4-5.5, [f7] 0.4-6.0, [f8] 0.25-7.31, [f9] 0.38-7.31, [f10] 0.8-6.2. CK: creatine kinase (IU/L); [g1] 30-168, [g2] 47-163, [g3] 41-277, [g4] 25-225. Hgb: hemoglobin; [h1] 6-9 mmol/L, [h2] 12-16 g/dL, [h3] > 11 g/dL, [h4] > 11.5 g/dL, [h5] > 12 g/dL, [h6] 115-150 g/L, [h7] 110-140 g/L

Table 2. Genetic and laboratory findings in reported adults with resistance to thyroid hormone alpha (n = 13). All of the patients had at least one symptom or sign associated with hypothyroidism. The data of patients 6, 7, 8, and 10 were obtained after discontinuation of L-thyroxine (LT4), which was used for many years. Remaining laboratory data were obtained before LT4 use. When available, the data were given as exact values [high (H), normal (N), or low (L)] and relevant reference ranges in the original reports were given as footnotes

Case	Variant	Type	Amino acid	Age (years)	LT4-naive	fT3	TT3	rT3	fT4	TT4	TSH	CK	Hgb	Ref
1	632A > G	Missense	D211G	N/A	Yes	N/A	2.25 (N)^{b1}	0.12 (N)^{c1}	10.1 (N)^{d1}	85 (N)^{e1}	1.6 (N)^{f1}	N/A	7.3 (L)^{h1}	23
2	767T > C	Missense	M256T	19	Yes	N/A	2.9 (H)^{b2}	0.18 (L)^{c2}	10.6 (L)^{d2}	67 (N)^{e2}	1.83 (N)^{f2}	N/A	N/A	30
3	787G > T	Missense	A263S	31	Yes	5.94 (N)^{a1}	2.51 (H)^{b2}	0.27 (N)^{c3}	16.1 (N)^{d2}	87 (N)^{e2}	0.95 (N)^{f2}	87 (N)^{g1}	9.6 (L)^{h2}	24
4	787G > T	Missense	A263S	35	Yes	6.16 (N)^{a1}	3.21 (H)^{b2}	0.28 (N)^{c3}	15.6 (N)^{d2}	131 (H)^{e2}	2.44 (N)^{f2}	125 (N)^{g1}	10.5 (L)^{h2}	24
5	787G > T	Missense	A263S	55	Yes	5.96 (N)^{a1}	2.57 (H)^{b2}	0.28 (N)^{c3}	13.6 (N)^{d2}	98 (N)^{e2}	1.58 (N)^{f2}	125 (N)^{g1}	13.5 (N)^{h3}	24
6	788C > T	Missense	A263V	60	No	4.4 (N)^{a2}	1.3 (N)^{b3}	< 50 (L)^{c4}	9.4 (L)^{d5}	60 (L)^{e3}	4.6 (N)^{f3}	364 (H)^{g2}	120 (N)^{h4}	19
7	788C > T	Missense	A263V	30	No	6.4 (N)^{a2}	1.7 (N)^{b3}	50 (L)^{c4}	10.5 (N)^{d3}	76.6 (N)^{e3}	4.8 (N)^{f3}	385 (H)^{g2}	129 (L)^{h5}	19
8	788C > T	Missense	A263V	26	No	6.8 (H)^{a2}	2.1 (N)^{b3}	< 50 (L)^{c4}	9.7 (L)^{d3}	66.3 (L)^{e3}	3.2 (N)^{f3}	184 (N)^{g2}	125 (L)^{h5}	19
9	1075A > T	Missense	N359Y	25	Yes	0.4 (N)^{a3}	N/A	0.17 (N)^{c5}	0.8 (N)^{d4}	N/A	0.343 (L)^{f4}	55 (N)^{g3}	10.8 (L)^{h6}	22
10	c1144delG	Frameshift	A382PfsX7	45	No	4.9 (N)^{a2}	1.7 (N)^{b3}	10 (L)^{c6}	10 (N)^{d3}	85 (N)^{e3}	5.8 (H)^{f3}	387 (H)^{g2}	12.7 (N)^{h7}	18
11	1151G > A	Missense	R384H	35	Yes	6.3 (N)^{a1}	3.32 (H)^{b2}	0.2 (L)^{c3}	13.6 (N)^{d2}	80 (N)^{e2}	2.51 (N)^{f2}	125 (N)^{g1}	11.2 (L)^{h4}	24
12	Insert 1nt	Frameshift	F397fs406X	41	Yes	N/A	N/A (H)$^{N/A}$	N/A (L)$^{N/A}$	N/A (N)$^{N/A}$	N/A (L)$^{N/A}$	N/A (N)$^{N/A}$	N/A	10 (L)$^{N/A}$	16, 17
13	1207G > A	Missense	E403K	39	Yes	2.2 (N)^{a4}	N/A	N/A	76 (N)^{d5}	N/A	2.4 (N)^{f5}	N /A	N /A	21

H: high, N: normal, L: low, N/A: not available. Reference ranges: fT3: free T3; ^{a1} 3.8-7.6 pmol/L, ^{a2} 3.5-6.5 pmol/L, ^{a3} 0.2-0.4 ng/dL, ^{a4} 1.33-3.05 pg/mL. TT3: total T3 (nmol/L); ^{b1} 1.3-2.7, ^{b2} 1.4-2.5, ^{b3} 0.9-2.8. rT3: reverse T3; ^{c1} 0.11-0.44 nmol/L, ^{c2} 0.22-0.54 nmol/L, ^{c3} 0.22-0.52 nmol/L, ^{c4} 80-250 ng/L, ^{c5} 0.14-0.54 ng/mL, ^{c6} 11.0-32.0 ng/dL. fT4: free T4; ^{d1} 10.0-23.0 pmol/L, ^{d2} 11.0-25.0 pmol/L, ^{d3} 10.0-19.8 pmol/L, ^{d4} 0.7-1.2 ng/dL, ^{d5} 58-154 ng/dL. TT4: total T4 (nmol/L); ^{e1} 70-150, ^{e2} 58-128, ^{e3} 69-141. TSH: thyroid-stimulating hormone (mIU/L); ^{f1} 0.5-5.0, ^{f2} 0.4-4.3, ^{f3} 0.35-5.5, ^{f4} 0.4-3.6, ^{f5} 0.46-0. CK: creatine kinase (IU/L); ^{g1} 30-168, ^{g2} 26-192, ^{g3} 20-180, ^{g4} 25-225. Hgb: hemoglobin; ^{h1} 8.5-10.5 mmol/L, ^{h2} > 12 g/dL, ^{h3} > 13 g/dL, ^{h4} 115-160 g/L, ^{h5} 130-170 g/L, ^{h6} 12-16 g/dL, ^{h7} 11.5-16 g/dL

infancy (18,24,32). However, there were also two children with no suggestive symptoms or clinical findings associated with hypothyroidism, who were diagnosed by family screening (24).

Coarse face including macroglossia, flattened large nose, thick lips, deep voice, and hoarse cry are the common features in nearly one third of the patients with RTHα (15,16,18,19,21-25,28-32). In addition, micrognathia and/or hypertelorism were reported in several cases (21,22).

Rough and dry or thickened skin, reflecting hypothyroidism, has been reported particularly in children in contrast to adult cases (16,21,28,31). In mice with mutant TRα, tissue iodothyronine deiodinase (DIO) 3 levels were reduced (40). In addition, topical inhibition of DIO3 enzyme was demonstrated to increase keratinocyte proliferation in animal models (40,41). Therefore, dermal symptoms in TRα patients are thought to be related to a similar mechanism. Skin tags were present in 21% of cases with RTHα; seven among 33 cases with available data (18,19,24,25). Bilateral inguinal hernia and umbilical hernia were reported in two children (25,29).

Skeletal Findings

Skeletal manifestations such as growth retardation, patent cranial sutures, epiphyseal dysgenesis, and delayed dental eruption have been demonstrated in mice with mutant TRα1 receptor (42,43). In addition, mice with *THRA* variant presented with decreased endochondral and intramembranous ossification, with retarded closure of skull sutures (44). Delayed ossification in these animal models caused impaired bone remodeling and thus short stature with skeletal deformities. However, bone strength was normal, which may explain why pathologic fractures are not seen in humans with RTHα (43). Further molecular studies demonstrated that mutant TRα caused reduced transcription of target genes including growth hormone receptor, insulin-like growth factor-1 (IGF-1) or its receptor and fibroblast growth factor receptor-1 or -3. Moreover, decreased signaling in post-receptor pathways in osteoblasts or chondrocytes was reported (45-50).

Short stature is one of the most common clinical findings in children with RTHα (12 among 20 children with available data, 60%). Ten of the 12 short children did not receive L-thyroxine (LT4) therapy before diagnosis and the lowest height standard deviation (SD) score was -3.1 (15,16,21,23-25,28,29). A previously untreated, three years and 11 months old Chinese female was reported with a height of 85.5 cm but the SD score was not provided (31). All of the remaining eight children with normal height had missense variants. Six of them (85.7%) had a height SD score between

Table 3. Summary of clinical features and underlying mechanism for resistance to thyroid hormone alpha. Pathophysiological mechanisms were observed from animal models, except for hematological findings

Affected system	Pathophysiology	Clinical features
Skin	- Reduced DIO3 levels - Increased keratinocyte proliferation	- Coarse face - Macroglossia - Thickened skin - Skin tags
Skeletal	- Delayed ossification - Impaired bone remodeling - Reduced transcription of target genes such as growth hormone receptor, IGF-1 or its receptor and fibroblast growth factor receptor-1 or 3	- Short stature - Wormian bones - Cranial hyperostosis - Macrocephalia - Skeletal deformities - Delayed bone age - Delayed tooth eruption
Neurological and cognitive	- Impaired neuronal migration, synaptogenesis, maturation and myelination - Deficient differentiation of oligodendrocytes or glial cells - Abnormal evolution of GABAergic neurons	- Delayed milestones - Impaired cognitive functions - Motor incoordination - Slow movements - Dyspraxia - Speech delay - Dysarthric speech - Seizures - Anxiety - Autism spectrum disease
Gastrointestinal	- Shortened villi, increased differentiation in crypt cells and decreased stem cell proliferation - Decreased peristaltism	- Constipation
Cardiovascular	- Impaired cardiac myoblast differentiation - Weak cardiac contractions	- Bradycardia - Cardiomyopathy - Pericardial effusion
Metabolic	- Impaired facultative thermogenesis - Hyperphagia	- Obesity - Low metabolic rate - Hyperlipidemia
Hematological	- Compromised fetal and adult erythropoiesis - Slowed down differentiation of progenitor cells - Increased serum IL-8 levels	- Normocytic or macrocytic anemia

IGF-1: insulin-like growth factor-1, IL-8: interleukin-8

-1.66 and 0 and none of them had received any treatment. Half of the 12 adult cases with available data had normal height, the tallest being 186 cm. All of them had missense variants and three had received LT4 starting from childhood (16,18,19,21-24).

Wormian bones in skull sutures were present in 10 among 31 cases with available data (32%) (15,24,25). Various other skeletal deformities, including delayed bone age, genu valgum, coxa valga, short tubular hand bones, late closure of fontanelles, and femoral epiphyseal dysgenesis were also reported (15,19,21,22,24,25,28,29,31). Mesomelic shortening of upper and lower limbs cause increased sitting/total height ratio (21,24,25). Skull radiography showed cranial hyperostosis in some patients (18,19,24). Espiard et al (22) reported a 27 years-old case with RTHα, who had severe deformities resembling cleidocranial dysplasia (clavicular agenesis, humero-radial synostosis, syndactyly of toes, agenesis of the 12th ribs and scoliosis). However, these findings were atypical for RTHα and have not been reported in any other case to date. Bone mineral density was reported to be normal in three adult patients (19).

Normally, tooth eruption is expected to occur before 13 months of age (51). Delayed tooth eruption was detected in eight among 18 children with available data (44%) (15,24,25,29).

Bochukova et al (15) reported a mild hypermobility and ligamentous laxity in ankles and knees. Although muscle tone was decreased in some cases with RTHα, their muscle strength was almost normal (15).

Neuromotor Development

T3 and its receptors play a major role in neuronal migration, synaptogenesis, maturation, myelination and differentiation of oligodendrocytes or glial cells (52). That is why TRα knockout animals showed a severe delay in postnatal development and locomotor dysfunction (53). TRα disruption had significant effects on cerebellar formation and hippocampal functions and TRα mutant mouse models had reduced brain mass (54-56). Wilcoxon et al (57) demonstrated behavioral inhibition and decreased learning and memory function in mice lacking all isoforms of TRα.

In infants with RTHα, delayed milestones for motor and speech abilities are the most common symptoms, noted in 34 among 40 cases (85%) (15,16,18-21,23-26,28-32). Reduced IQ, notable impairments in cognitive functions, slow motion movements, evident motor discoordination including dyspraxia, ataxia, and broad or unstable gait are some of the clinical findings on neurological examination

(15,18,19,24,28,32). Remarkably, two cases with the A263V variant were able to attend university without LT4 treatment (Demir-unpublished observation of Patient 3.III.1 in reference 24,25). The first patient had no symptoms and was detected during family screening (24). The second case had mild delay in motor and mental development during childhood and received little teaching support (25). Axial hypotonia and slow motor development can also be seen (23). Clumsiness due to motor discoordination and difficulty with fine motor abilities has been reported in some patients, who were incapable of writing or drawing (15,18,28). Speech delay and dysarthric or slow speech are significant disabilities and are seen in the majority of cases (15,16,18,19,21,23,24,28). Macrocephalia is also a common clinical finding (23 among 33 cases with available data, 70%) (15,16,18-25,29-31).

Furthermore, Demir et al (24) reported a 35-year-old adult case, whose developmental delay during childhood was more remarkable compared to her affected son. As an adult, she presented with an attenuated clinical picture including mild intellectual deficit, no cardiac problems, and normal thyroid function tests, despite not being treated. Similar observations were also made in a mouse model with a heterozygous TRα1 variant at the same position (53,58). These mice showed severe but transient impairment of postnatal development and growth. The mechanisms underlying the amelioration of deficits caused by these TRα1 variants with age are unknown.

Seizures after stimulation with light or audio and abnormal evolution of GABAergic neurons in TRα1 mutant mice correlated with epilepsy in human cases (42,59,60). To date, three cases with RTHα were reported to be suffering from epileptic seizures in childhood (18,23,32).

A notable anxiety in unfamiliar environments and reduced cognitive functions were observed in TRα1 mutant animal models (59). Another study demonstrated that TRα1 mutant mice developed depressive and anxiety behaviors (61). Kalikiri et al (26) investigated 30 children diagnosed as autism spectrum disorder and found *THRA* variants in six of them. Unfortunately, no additional clinical data regarding these children were provided. Coexistence of autism spectrum disorder and RTHα was reported in two more patients, suggesting that RTHα should be excluded in patients with autism spectrum disorder (20,31).

Constipation

TRα is the dominant TR in the intestinal tract (6,7). In a study with TRα1 mutant mice models, shortened villi, increased differentiation in crypt cells and decreased stem cell proliferation were observed (62). Independent of age, constipation is one of the most common clinical symptoms in human cases, being reported in 26 among 31 cases with available data (84%) (15,16,18,19,21,23-25,28,29,31,32). The atypical patient reported by Espiard et al (22), was the only patient to develop chronic diarrhea, at the age of 12. Abdominal radiographs showed dilated bowels. Decreased peristalsis was also observed by colonic manometry in several cases with RTHα (15,18).

Cardiovascular System

TRα1 is expressed in myocardium and it was suggested to be responsible for cardiac myoblast differentiation in experimental studies (63). Mutant TRα1 mice models showed symptoms in the cardiovascular system associated with hypothyroidism, such as bradycardia or weak cardiac contractions (64). Makino et al (65) found that the predominant TR in mouse coronary smooth muscle cells was TRα, and suggested that coronary vascular tone was regulated by TRα. However, cardiac pathologies or symptoms do not seem to be common in humans with RTHα. Although most of the patients had normal heart rate or blood pressure, some cases were reported to have bradycardia (15,18,19). At the time of writing, three cases with cardiomyopathy and one case with pericardial effusion have been reported (21,24).

Metabolic Problems and Fertility

TRα null or mutant mice had lower core body temperature due to impaired facultative thermogenesis (66). Although most of the animal models with mutant TRα were thin, several studies described obesity (58). In the same study, it was also reported that the TRα1 R384C mutant mice were hyperphagic but resistant to obesity (58). It was suggested that hypermetabolism, mediated centrally through apo-TRα1 resulted in reduced adipose tissue and lower body weight (67). However, eight among 33 humans diagnosed as RTHα with available data (24%) were obese and six of them were adults (15,18,23,24). Low resting energy expenditure (metabolic rate) was also reported in some patients with RTHα (15,18,19,22). In addition, total cholesterol and low-density lipoprotein (LDL) levels were high in several patients (16,18,19).

As RTHα can be seen in children of affected adults, it suggests that fertility might be unaffected in either gender. Regular pregnancies after spontaneous conception were reported, in even moderately affected and untreated female RTHα cases (24). Only one patient had late-onset of puberty and menarche at 16 years-old, with normal gonadotropin and estrogen levels (18).

Laboratory

Unfortunately, relevant measurements were inconsistently reported in the published cases and so data is incomplete for all the case reports. In addition, while the majority of available data in the literature were presented as exact values with their reference ranges, some reports included only categorized data (Tables 1, 2).

Thyroid Function Tests

Thyroid function tests of individuals suspected of having RTHα should be cautiously interpreted since the literature data were derived from cases with varying severity of RTHα and from different age groups. Abnormal TH levels are more likely to be found in severe cases and in children. Since the TH and TSH levels seem to differ if there has been previous LT4 use, we chose to evaluate the data from the cases who had not received LT4 previously (LT4-naive) separately from the patients who were analyzed after discontinuation of LT4 treatment.

Individuals Who had not Receive Any Thyroid Hormone

A normal neonatal congenital hypothyroidism screening result [total T4 62 nmol/L (-1.3 SD), TSH 1 mIU/L] was reported in a case with RTHα, who also had an uneventful neonatal period (23).

TSH levels were all normal in affected children. Among the adult patients, an atypical case with severe malformations was the only one with abnormal TSH (0.343 mIU/L, normal range 0.4-3.6) (Figure 1) (22).

Differences of TH levels among treatment-naive children and adults are also shown in Figure 1. All of the free T3 (fT3) and the majority of total T3 levels were in the upper half of normal range or frankly elevated. Elevated fT3 levels were found only in treatment-naive children but not in such adult cases. All of the free T4 (fT4) and the majority of total T4 levels were below the reference range or in the lower half of the normal range. Low fT4 concentrations were more frequently present among children. In adult patients, fT4 levels were all normal, except for one case (30).

Both fT4 and TSH were normal in 61% (11 among 18) of children and 78% (7 among 9) of adults. Normal fT3, fT4 and TSH were noted in 33% (5 among 15) and 83% (5 among 6) of children and adults, respectively (Figure 2). In such cases, a high T3/T4 ratio or low or low-normal reverse T3 (rT3) level, resulting in an increased T3/rT3 ratio can be suggestive of RTHα (33). These abnormalities in RTHα patients may be the result of changes of DIO1 and DIO3 levels in tissues, as the expression of both are regulated by TRα. In a study, TRα1 mutant mice had raised hepatic DIO1 levels, which converts T4 to T3 (42). Therefore, this

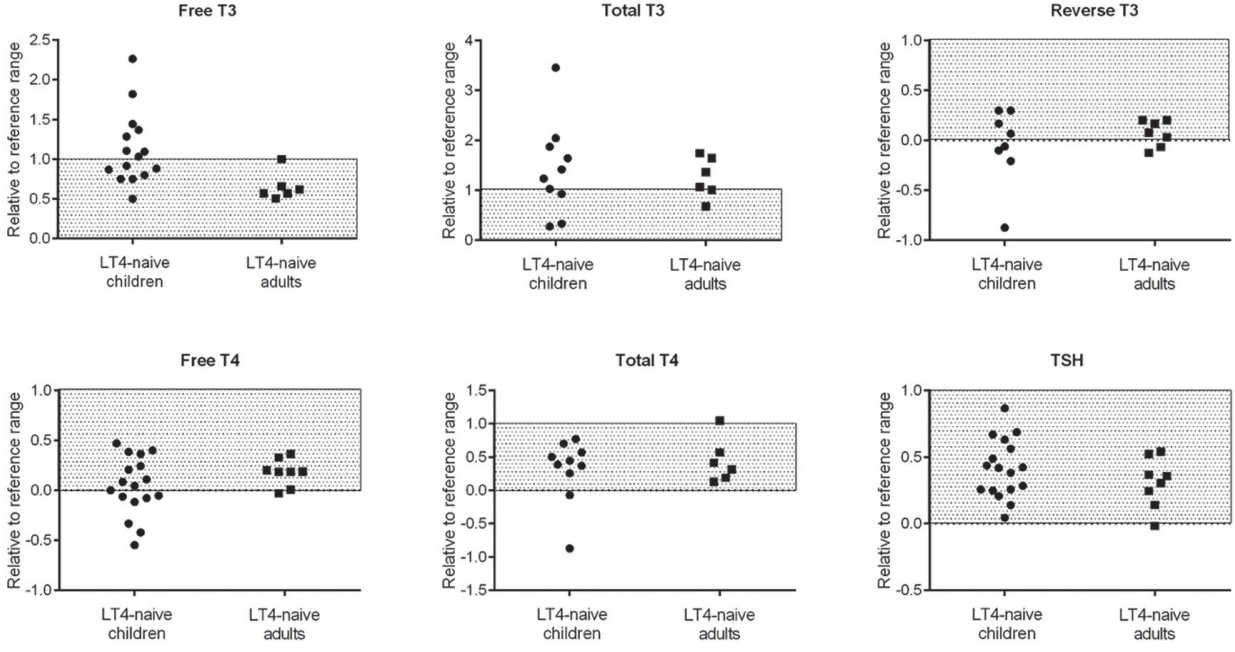

Figure 1. Thyroid function test results in previously untreated children and adults [derived from all available data in Table 1 (Cases 1-10, 14, 17-20, 24, and 25) and Table 2 (Cases 1-5, 9, 11, and 13)]. All of the data (x) was expressed relative to the relevant reference range with the following formula: (x - lower limit of normal range) / (upper limit of normal range - lower limit of normal range). Grey shaded areas indicated the normal range

LT4: L-thyroxine

finding was related to high T3 levels and an increased T3/T4 ratio in RTHα. In addition, decreased DIO3 levels in tissues may result in low rT3 levels, causing reduced inner-ring deiodination of T4 to rT3 (40).

Individuals Who Discontinued Treatment

After cessation of LT4 treatment, mildly elevated TSH may be seen, as was reported in one adult and one child with RTHα (17,18). The child, in whom TSH rose at the age of 11 after discontinuation of LT4, had normal pretreatment TSH levels at 5 and 6 years of age (17). In contrast, TSH remained in the normal range in three adult patients and an adolescent case (19,25) after LT4 cessation. Off thyroxine treatment, patients had marginally low or low-normal fT4. A wide range of free or total T3 data (varying from the lower half of the normal range to elevated levels) was reported. Nevertheless, rT3 levels were all low (17-19,25).

Individuals Receiving Thyroid Hormone

Under LT4 treatment, fT3 and fT4 levels increased in patients with RTHα, while TSH was suppressed, a similar pattern to that found during the treatment of central hypothyroidism (15,17-19,23,24,29). One patient with atypical phenotype was treated with liothyronine, which caused a rise in fT3 level, suppressed TSH level, and markedly reduced fT4 concentration (22).

Anemia

The relationship between anemia and hypothyroidism is well-known (68). Animal models lacking TRα demonstrated compromised erythropoiesis (69,70). In a study by van Gucht et al (71) of progenitor cells derived from RTHα patients, it was shown that these cells differentiated more slowly than controls. In humans, 23 among 30 cases with available data (77%) had anemia, and it has been one of the most common findings in humans with RTHα (16,18,19,21-25,28,29,31,32). The rate of anemia was similar between treatment-naive children (80%) and adults (86%) (Figure 2). In the reports where exact values were included, hemoglobin levels ranged between 8.6-10.9 g/dL and 9.6-12.9 g/dL in children and adults, respectively. In the majority, anemia was normocytic and normochromic; macrocytic anemia was described in three cases (13%) (15,18,22).

An increase in serum levels of interleukin-8 (IL-8), a pro-inflammatory cytokine, was shown in RTHα patients. However, neutrophil or macrophage functions, which are partly mediated by IL-8, were found to be normal in those cases (72).

Other Biochemical Findings

Both thyroglobulin and urinary iodine levels are expected to be in the normal range (34). Similar to primary hypothyroidism, high total cholesterol and LDL levels, and low or low-normal levels of IGF-1 can be found in RTHα (33,34).

In primary hypothyroidism, creatinine kinase (CK) can also be elevated (73). Human data demonstrate that CK might be a promising biomarker for diagnosis of RTHα, particularly in children. Eight among 11 treatment-naive children (73%) with available data had elevated CK levels (range; 218-981 U/L; 1.3-4.36 times upper limit of normal), while all of the treatment-naive adults with available data (n = 5) had normal CK levels (Figures 2 and 3) (15,16,22-25,28,29,31). In contrast, elevated CK levels were noted in three of four adult patients (364-387 U/L; 1.90-2.02 times upper limit of normal) and in the two children (196-213 U/L; 1.03-1.31 times upper limit of normal) who were assessed after discontinuation of LT4 (17-19,25).

Recently, Boumaza et al (74) reported that biofluids (urine and plasma samples) of TRα-mutant mice showed distinct metabolomic profiles from controls, including increased urinary levels of hippurate and decreased urinary levels of isovalerylglycine, dimethylamine, trimethylamine, and choline. They suggested that easily accessible nuclear magnetic resonance-based metabolic fingerprints of biofluids could be used to diagnose RTHα in humans (74).

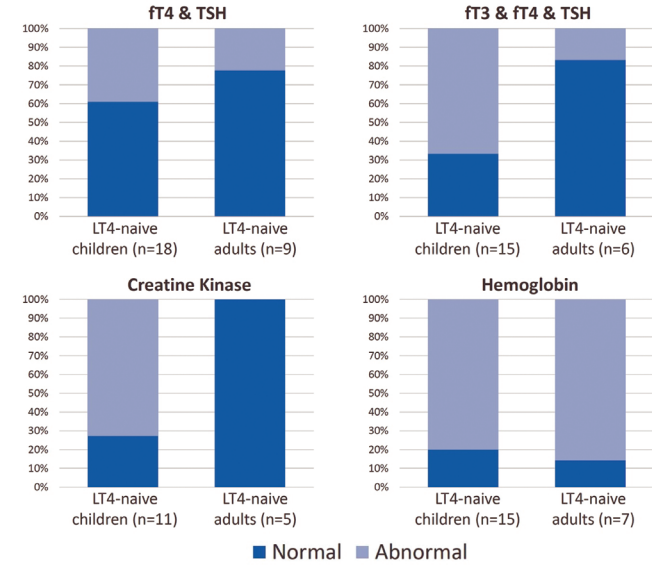

Figure 2. Classification of thyroid hormone profiles and peripheral indicators of hypothyroidism belonging to previously untreated children and adults [derived from all available data in Table 1 (Cases 1-10, 14, 17-21, 24, and 25) and Table 2 (Cases 1-5, 9, and 11-13)]

LT4: L-thyroxine, fT3: free T3, fT4: free T4, TSH: thyroid-stimulating hormone

Differential Diagnosis

RTHα should come to mind when various clinical features indicate hypothyroidism but TSH is normal and free T4 is low or in lower half of normal range in patients who have not received LT4 treatment (Figure 4). Parental medical history should be investigated thoroughly for similar clues due to autosomal dominant inheritance. More common conditions including non-thyroidal illness, recovery from thyrotoxicosis, or technical assay problems, may result in similar biochemical features (75). However, they are not associated with clinical features of RTHα.

Central hypothyroidism should be ruled out when free T4 is low and TSH is low, normal, or slightly elevated. The presence of hypothalamic-pituitary disease, hypo- or hyper-secretion of other pituitary hormones or genetic findings would indicate an etiology of central hypothyroidism (75). On the other hand, if T3 levels are elevated or close to the upper limit, the probability of central hypothyroidism is low.

Laboratory findings including elevated/normal T3, reduced rT3, normal or low T4, and normal/elevated TSH are also found in MCT8 deficiency (Allan Herndon Dudley syndrome). However, clinical and laboratory signs of peripheral thyrotoxicosis are present in this disease in addition to cerebral hypothyroidism (76-79). Furthermore, MCT8 deficiency is inherited in an X-linked manner (80). Thus, the mothers of affected patients, all of whom would be expected to be male, are asymptomatic carriers. However, an affected parent can be found in case of RTHα (16,21,23-25,77,78).

Additional clues for RTHα in LT4-naive children and adults are free or total T3 in the upper half of the normal range or above the upper limit, along with at least one of normocytic/macrocytic anemia *or* mildly elevated CK *or* low rT3. Among the subjects with available data, the algorithm in Figure 4 is valid for 15 of 16 children (94%) and for six of eight adults (75%) (15-17,21,22-25,27-32). When the data of four additional adult cases, whose assessments were available after discontinuation of LT4, are also included, the algorithm should be modified regarding T3 and TSH data,

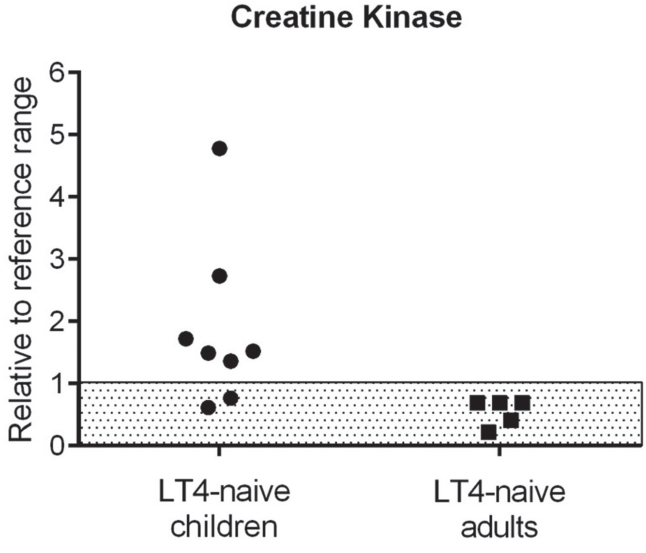

Figure 3. Numerical values of creatine kinase levels obtained from previously untreated children and adults with resistance to thyroid hormone alpha [derived from all available data in Table 1 (Cases 3-7, 10, 17, and 20) and Table 2 (Cases 3-5, 9, and 11)]. All of the data (x) was expressed relative to the relevant reference range with the following formula: (x - lower limit of normal range) / (upper limit of normal range - lower limit of normal range). Grey shaded area indicated the normal range

LT4: L-thyroxine

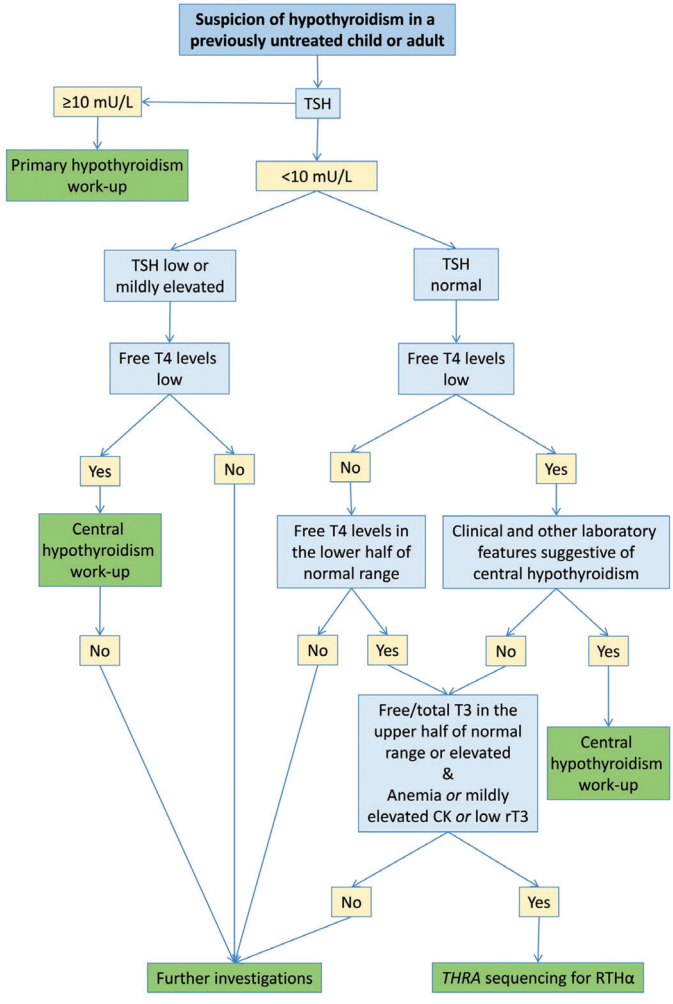

Figure 4. Algorithm for the differential diagnosis of hypothyroidism in previously untreated children and adults with particular emphasis on resistance to thyroid hormone alpha

TSH: thyroid-stimulating hormone, RTHα: resistance to thyroid hormone alpha, CK: creatine kinase, rT3: reverse T3

given that fT3 levels may also be in the lower half of the normal range and TSH levels can be mildly elevated. In these subjects, after exclusion of central hypothyroidism, presence of either normocytic or macrocytic anemia *or* mildly elevated CK values *or* low rT3 levels would be an additional clue leading to *THRA* sequencing. This approach is valid for 10 of 12 adult patients with available data (83%) (16-19,22-24,30). Both approaches require confirmation of these specificities in future studies.

Treatment and Outcomes

There is only limited data about the treatment of RTHα and thus long-term follow-up data is required. LT4 treatment has been the first choice to date, in order to overcome the resistance in TRα with higher dosage. T4 and rT3 levels come into the normal range with this treatment and T3 level remains high. Since the feedback mechanism of the HPT axis is intact, LT4 treatment causes TSH suppression in RTHα patients (15,17-19,23,24,29).

In animal models with mutant TRα, increasing serum TH levels alleviated locomotor and behavioral irregularities (59). Therefore, LT4 supplementation to raise circulating TH levels was suggested to be beneficial in RTHα. Bassett et al (43) reported that prolonged T4 treatment advanced bone rigidity and strength in TRα mutant mice. However, it did not exert any effect on skeletal development, linear growth or mineralization of bones (43). Vennström et al (58) suggested that high doses of T3, given in the appropriate developmental time period, should improve the abnormalities depending on the specific mutation present in TRα. They also showed that metabolic symptoms of mice with mutant TRα, were well treated by T3. Regarding this, Espiard et al (22) reported that their case with an atypical phenotype received liothyronine treatment and a notable cardiac and metabolic response was observed. Nevertheless, other parameters did not change significantly, suggesting that the variant in this case only exhibited limited resistance to T3.

Van Mullem et al (17) reported the results of two RTHα patients (a daughter and her father, with the same variant), who were treated with LT4 for over five years. They showed that some clinical features, such as constipation or nerve conductance, were improved. However, fine motor abilities or cognitive functions did not benefit from treatment (17). On the other hand, most of the LT4 treated patients had better motor coordination, alertness, school performance, concentration or motivation (19,25,29,31). However, limited benefit on linear growth has been reported (15,17,23). Hypotonia was ameliorated and accelerated neuromotor development was observed in children (23,31,32). Thus, if the treatment was started at an early age, the benefits for development and growth would be more distinguishable. As described in the report by van Mullem et al. (17), constipation improved with LT4 treatment in most of the other RTHα cases (15,19,25,29).

With the peripheral effects of LT4 treatment, increases in sex hormone binding globulin (SHBG) or IGF-1 levels can be seen, as previously reported in RTHα patients. In addition to this, CK or cholesterol levels were reduced in these cases, reflecting the improved tissue response to TH (15,17-19,23-25). It has also been shown that when LT4 treatment was interrupted, all these indicators turned back to pretreatment levels (17). Korkmaz et al (29) reported a decrease in SHBG levels and found IGF-1 levels unaltered after LT4 treatment in a patient with RTHα, although the TSH level was suppressed and CK levels were decreased. Moran et al (18) reported a progressive rise in bone turnover markers after LT4 treatment in a case with RTHα. Growth hormone was added to LT4 therapy, due to low-normal IGF-1 levels in an affected child, but sufficient improvement in linear growth was not observed (17).

Anemia seems to be unresponsive to LT4 treatment, as described in most of the RTHα cases (18,19,25,29). Although van Gucht et al (71) showed that human erythroid progenitors responded to T3 exposure in an experimental study, they hypothesized that mutant TRα may play a role in the earlier stages of erythropoiesis, which they could not examine in their research. In addition, LT4 treatment had a limited effect on cardiac function in several cases with RTHα (18,19). Increase in heart rate was observed in one patient after LT4 treatment (22).

Patients who had frameshift variants in *THRA*, including the carboxy-terminal part of TRα1, had varying responses to LT4 treatment. Like their severity of clinical presentation, this situation was also associated with the specific location of the variant or the degree to which this molecular region was affected (17,18,24). In patients with frameshift variants skeletal abnormalities did not respond to LT4 treatment (17,18,24).

Since LT4 administration to RTHα patients will excessively stimulate TRβ in TRβ-dominant tissues, development of TRα1-selective thyromimetics would be ideal (33,81). Alternative investigations targeted HDAC activity or interaction with the co-repressor complex to inhibit the dominant-negative effect of wild-type analogue of mutant TRα1. It was shown in a murine study that a mutation in NCoR can disrupt its co-action with TRα1 and reverses the effects of mutant TRα (82). An HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), was used to relieve the repression in target genes and phenotypic features improved in TRα1 mutant

mice (81,83,84). However, Freudenthal et al (38) showed that SAHA was unlikely to treat skeletal abnormalities and had no effect on bone structure or strength in TRα mutant mouse models. These authors suggested that alternative co-repressors, in addition to NCoR, may interact with TRα in skeletal cells (36,38).

Conclusion

The diagnosis of RTHα is not straightforward since TH levels might not be helpful and the entity is not widely known. As published data is limited concerning RTHα, absence of phenotypic features or laboratory findings would not exclude RTHα. Currently, only fT4 and TSH levels are recommended for evaluation of growth failure in children (85). However, these tests can be normal in a subject with RTHα and astute clinicians should do further investigations in such a case when the clinical picture is similar to hypothyroidism. In addition, RTHα should be kept in mind in patients diagnosed with apparent central hypothyroidism, particularly when the exact etiology cannot be determined.

Ethics

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: İbrahim Mert Erbaş, Korcan Demir, Concept: İbrahim Mert Erbaş, Korcan Demir, Design: İbrahim Mert Erbaş, Korcan Demir, Data Collection or Processing: İbrahim Mert Erbaş, Korcan Demir, Analysis or Interpretation: İbrahim Mert Erbaş, Korcan Demir, Literature Search: İbrahim Mert Erbaş, Korcan Demir, Writing: İbrahim Mert Erbaş, Korcan Demir.

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Catch-up Growth in Prepubertal Children Treated for Juvenile Hypothyroidism and Growth Hormone Deficiency can be Modelled with a Monomolecular Function

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What is already known on this topic?

Catch-up growth (CUG) occurs if a growth disorder can be adequately treated. In prepubertal children with coeliac disease treated with a gluten-free diet, height standard deviation score during CUG after start of treatment can be modelled with a monomolecular function.

What this study adds?

CUG in most children treated for juvenile hypothyroidism or growth hormone deficiency can be modelled with a monomolecular function. Theoretically, this method may be superior to current outcome parameters to objectify the influence of clinical factors on CUG in growth hormone treated children with growth hormone deficiency.

Abstract

Objective: We hypothesized that modelling catch-up growth (CUG) as developed for coeliac disease (CD), might also fit CUG in adequately treated children with juvenile hypothyroidism (JHT) or growth hormone deficiency (GHD).
Methods: We used a monomolecular function for all available prepubertal data on height standard deviation score (HSDS) minus target height SDS (adjHSDS) in children with JHT (n = 20) and GHD (n = 18) on a conventional (CoD) or high GH dose (HD), based either on a national height reference with an age cut-off of 10 (girls) and 12 (boys) years (model 1) or prepubertal height reference values, if age (0) was ≥3, with no upper age limit (model 2).
Results: The models could be fitted in 83-90 % of cases; in other cases the HSDS decreased after several measurements, which violated the assumption of an irreversible growth process. In JHT, the rate constant (k) and adjHSDS (0) were lower than in CD (p = 0.02), but adjHSDS (end) was similar. In GHD (model 1), k was lower than for CD (p = 0.004) but similar to JHT, while adjHSDS (0) and adjHSDS (end) were similar to CD and JHT. Thus, the shape of CUG is similar for children with JHT and GHD, while children with CD had less growth deficit at start and a faster CUG. The differences in CUG parameters between GH dose subgroups did not reach statistical significance.
Conclusion: Modelling CUG of prepubertal children with JHT and GHD can be used for assessing the adequacy of CUG and the influence of clinical treatment modalities on its speed and magnitude.
Keywords: Growth, catch-up growth, coeliac disease, growth hormone deficiency, hypothyroidism

Introduction

One of the most fascinating phenomena in the field of regulation of linear growth is catch-up growth (CUG). CUG is a physiological condition of temporary overgrowth, first described by Prader et al (1). In a review paper, the classical form of CUG [type A according to Tanner (2)] was defined as "a height velocity above the statistical limits of normality for

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age and/or maturity during a defined period of time, following a transient period of growth inhibition" (3). Based on previous studies, CUG usually takes 3-4 years, the duration being dependent on the initial height deficit. The effect of CUG is to take the child toward or right onto his original pre-retardation growth curve (3). On average, this growth curve would be expected to come close to the gender-adjusted midparental height [target height (TH)] standard deviation score (SDS) because, based on twin studies, the genetic influence on adult height (AH) is estimated at approximately 80 % (4).

In conditions where the cause of growth failure can be completely compensated or cured [such as hypothyroidism, coeliac disease (CD) and successful operation of an ACTH-secreting pituitary adenoma], one would expect a classical type A CUG. In fact, this has been observed in cohorts and case reports of children with these conditions, including juvenile hypothyroidism (JHT) (5,6). In prepubertal children with JHT (6) CUG is usually complete, but not so in adolescents, probably because of bone age advancement due to simultaneous occurrence of puberty (5). In children with a virtually certain diagnosis of GHD, the growth response to an adequate substitution dosage of GH is expected to have a similar shape and duration as CUG in other forms of secondary growth disorders (7). However, so far in GHD the growth response has usually been expressed as yearly height velocities [cm/year or delta height SDS (HSDS)] (8,9,10), which do not offer an impression of the full pattern of CUG.

We reasoned that, in theory, a mathematical model of the whole phase of CUG would be better than yearly height velocities to assess the adequacy of CUG and to analyse the influence of baseline and treatment-related variables on the growth response to GH treatment in prepubertal GHD children. For prepubertal children with CD, our group (11) reported that HSDS during CUG can be modelled by a monomolecular function: A*(1-B*EXP(-k*t))-5, with t = time in years (0 = start of therapy), A-5 = HSDS(end), A*(1-B)-5 = HSDS(0), B = integration constant, and k as rate constant.

For this study we hypothesized that: 1) the monomolecular function developed for CD gives a good fit for CUG in L-thyroxine treated prepubertal children with JHT; 2) the same model can be used for prepubertal children with GHD; and 3) in children with GHD the model can be used to analyse the influence of GH dose on CUG.

Methods

For this study we used two sets of published data on CUG. The first set was derived from the publication on a retrospective study in German children with JHT (6). For the present analysis we used the individual data as reported in the publication. The second set was derived from a previous publication on a prospective, multicentre, dose-response study in Dutch children with GHD (12). For the present analysis we used the raw data (courtesy Dr. T.C.J.Sas).

All available prepubertal HSDS data were collected, and adjusted for TH (TH, the sex-adjusted mid-parental height). HSDS minus TH SDS, was abbreviated as adjHSDS. From children with JHT (n = 20), prepubertal data on yearly adjHSDS for three years were used as reported in the paper (6), and the difference between adjHSDS and adjusted AHSDS (adjAHSDS) was calculated (n = 11). HSDS was expressed using the 1966 UK reference data (13,14) and TH was calculated as the sex-adjusted arithmetical mean of parental heights transformed into SDS (13,14). For our analysis, we used an age cut-off of 10 and 12 years for girls and boys, respectively, in order to prevent distorting effects of increasing percentages of pubertal children on mean and SD of height for age in the general population from these ages.

From children with GHD participating in a GH dose-response study (12), all prepubertal data on adjHSDS were used. In this previous study the long-term effect of a conventional dose of GH (0.67 mg/m^2 body surface per day, n = 10, CoD) was compared with a high dose (HD) (1.33 mg/m^2 body surface per day, n = 9, HD). These dosages are approximately equivalent to 24 and 48 ug/kg/day. From the anonymous database containing all data on age, height and pubertal stage the relevant data were selected (courtesy Dr. T.C.J.Sas). As mentioned in this paper (12), the protocol was approved by the medical ethics committees of all participating centres, and all parents gave their written informed consent for the study (coordinating centre: University Medical Centre Rotterdam, registration number: 87.74). For this group, the TH was defined as ½ x (height father + height mother + or - 13) + 4.5 for boys and girls, respectively, because the secular trend over 30 years in the Netherlands was estimated at 4.5 cm between 1965 and 1997 (15).

Statistical Analysis

For these groups, we took two approaches. First, we used cross-sectional Dutch references (15) with an age cut-off of 10 and 12 years for girls and boys, respectively (model 1), similarly to the approach for JHT. Second, in order to maximize the number of data points and statistical power, we used the IC component of the Infancy-Childhood-Puberty model of longitudinal growth (16) with no age cut-off (model 2).

We modelled all available prepubertal adjHSDS data with a mixed-effects model using a monomolecular function of adjHSDS over time: A*(1-B*EXP(-k*t))-5, with t = time in

years (0 = start of therapy), A-5 = adjHSDS(end), A*(1-B)-5 = adjHSDS(0), B = integration constant and k as rate constant.

In mathematical terms, this is described as follows:

Let *n* be the number of children, *t* the time in years (0 = start of treatment) and *y* the adjHSDS of the *i*-th child at time *t* with $i = 1,\ldots,n$. According to the monomolecular growth function the *y* of the *i*-th child can be modelled by the non-linear mixed-effects procedure as:

$$y_i(t) = HSDS_i(t) - THSDS_i = A(1 - B\exp\{-kt\}) - 5 + \varepsilon_{it}$$

with $A = A_0 + A_{i0}$, $B = B_0 + B_{i0}$, $k = k_0 + k_{i0}$, with A_0, B_0,k_0 fixed effects and A_{i0}, B_{i0}, k_{i0} random effects.

The measurement errors ε_{it} are assumed to be independent across individuals and to be normally distributed with mean zero and a common variance. We assume that the random effects have a multivariate normal distribution with mean vector zero and are independent of the measurement errors.

Since by definition CUG has an upward pattern, the model that was chosen for CUG in CD did not allow for a decreasing HSDS (11). In some of our patients a slight downward pattern was noted at the end of CUG. Patients in whom this downward trend was >0.15 SD were excluded from further analysis because a decreasing HSDS (after several measurements showing an increasing HSDS) would violate the assumption of an irreversible growth process.

Modelled CUG was compared between groups (JHT, GHD and CD). To investigate the influence of GH dose on CUG, linear regression analyses were performed to test the difference of the parameters of the monomolecular function between dose groups.

Results

Juvenile Hypothyroidism

In 18 out of 20 cases (90%) of JHT, adjHSDS could be modelled properly. Figure 1 shows the results versus age, and for 11 cases also adjAHSDS. In the 10 out of 11 cases in whom data were available on HSDS after three years of start therapy, mean adjAHSDS was identical to adjHSDS after three years of start therapy, but with a remarkably wide range (-2.6 to 1.9). Thus, in some patients adjAHSDS was substantially lower than adjHSDS after three years, while in others CUG apparently continued after pubertal onset.

Figure 2 shows the individual modelled curves of CUG versus time after start of medication, as well as the average CUG curve. Results of the model and the derived adjHSDS at start [adjHSDS(0)] and end of CUG [adjHSDS(end)] are shown in Table 1.

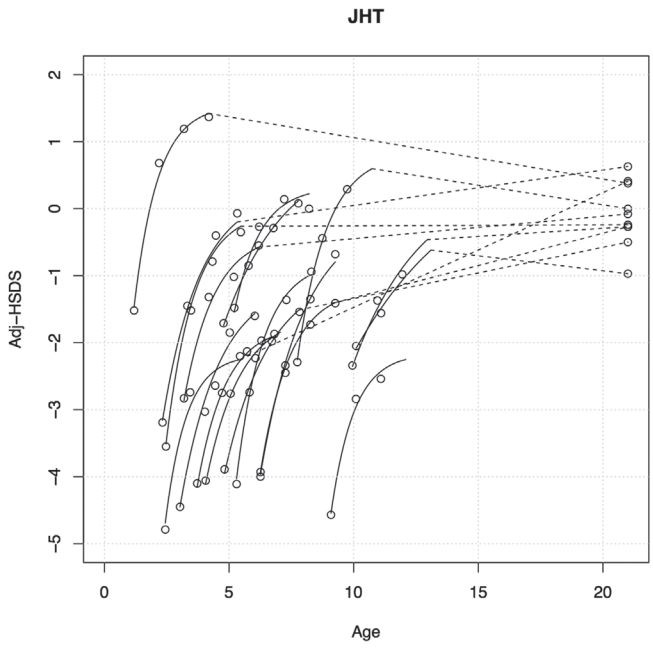

Figure 1. Modelled curves (uninterrupted lines) and raw data (open circles) describing catch-up growth [adjusted height standard deviation score (HSDS) versus age] for each child with juvenile hypothyroidism before reaching puberty, as well as adjusted adult HSDS. Stippled lines connect the last measurement before onset of puberty with adjusted adult HSDS

JHT: juvenile hypothyroidism, Adj-HSDS: adjusted-height standard deviation score

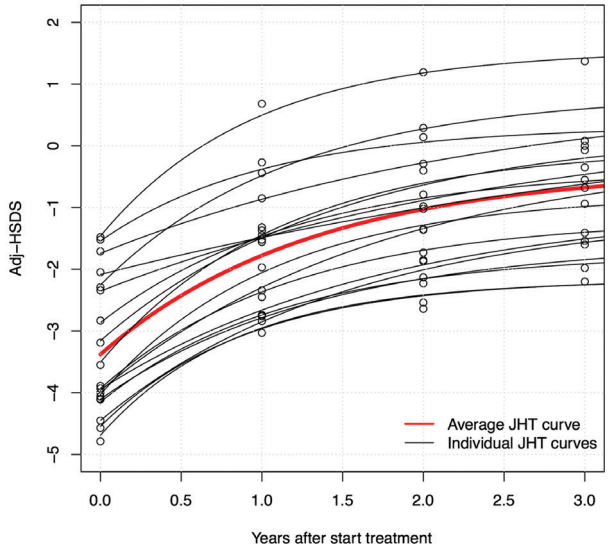

Figure 2. Modelled individual curves and raw data describing catch-up growth of prepubertal children with juvenile hypothyroidism during three years, as well as the average curve

JHT: juvenile hypothyroidism, Adj-HSDS: adjusted-height standard deviation score

The univariate correlations between the rate constant k versus age and adjHSDS(0) were -0.45 (p = 0.06) and -0.31 (p = 0.21), respectively. Although these correlations are not statistically significant, this implies that the rate constant becomes smaller when age or adjHSDS at start are higher, as illustrated in Suppl Figure 1.

Growth Hormone Deficiency

In 15 out of 18 cases (83%) with GHD adjHSDS could be modelled properly (Figure 3). Figure 4 shows the individual modelled curves of CUG versus time after start of medication, as well as the average CUG curve, in both dose groups (panels A and B). Results of the derived adjHSDS(0) and adjHSDS(end) of CUG for both models are shown in Table 1. There was a tendency toward a faster CUG (k) and higher adjHSDS(end) in the HD group compared to CoD, but the difference did not reach statistical significance (p = 0.626 and 0.293 in models 1 and 2, respectively). After correction for adjHSDS at start, the difference was 0.04 (p = 0.772, model 1) and 0.07 (p = 0.228, model 2). The difference between adjHSDS(end) in the CoD and HD groups was 0.56 (p = 0.326) and after adjustment for age and adjHSDS at start 0.68 (p = 0.108) using model 1. For model 2, these were 0.54 (p = 0.428) and after adjustment for age and adjHSDS at start 0.67 (p = 0.189), suggesting that model 2 may be more sensitive to detect effects of clinical parameters than model 1.

Comparison Between Diagnostic Groups

The modelled mean adjHSDS of JHT, GHD (models 1 and 2) and CD is shown in Figure 5. Compared with CD, in JHT adjHSDS at start was lower (p = 0.0002) as well as k (p = 0.02), also after adjustment for adjHSDS(0) (p = 0.003), but adjHSDS after three years was equal (Table 1). In GHD patients, using model 1, k was lower than for CD but similar to JHT; adjHSDS(0) and adjHSDS(end) were similar to CD and JHT.

Table 2 shows the predicted growth parameters of the monomolecular function given adjHSDS at start. This information could be useful in predicting the growth trajectory at start of treatment and monitoring specific treatment cases. For example, if a child with JHT has an adjHSDS of -3 at start of treatment, the predicted growth trajectory could be described by: adjHSDS (t) = 4.99*(1-0.60*EXP(-0.72*t))-5, with t in years. The predicted adjHSDS two years after start treatment (t = 2) is then expected to be -0.72.

Table 1. Monomolecular models for celiac disease, juvenile hypothyroidism and growth hormone (GH) deficiency treated with conventional (CoD) or high GH dose^

Diagnosis	n	Age at start (year) Mean (SD)	Model 1	k Mean (SD)	AdjHSDS at start Mean (SD)	AdjHSDS at end Mean (SD)	k: Diff with CD (p)	AdjHSDS at start Diff with CD (p)	AdjHSDS at end Diff with CD (p)	k: Diff with JHT (p)	AdjHSDS at start Diff with JHT (p)	AdjHSDS at end Diff with JHT (p)
CD	16		AdjHSDS = 4.93*(1-0.36*EXP(-1.36*t))-5	1.36 (0.86)	-1.8 (0.9)	-0.1 (0.8)				↑ (0.02)	↑ (0.0002)	- (0.364)
JHT	18	5.1 (2.7)	AdjHSDS = 4.63*(1-0.65*EXP(-0.76*t))-5	0.76 (0.26)	-3.3 (1.1)	-0.4 (1.1)	↓ (0.02)	↓ (0.0002)	- (0.364)			
GHD	15	6.7 (3.4)	AdjHSDS = 5.14*(1-0.55*EXP(-0.60*t))-5	0.60 (0.25)	-2.6 (1.4)	0.1 (0.9)	↓ (0.004)	- (0.072)	- (0.507)	- (0.084)	- (0.156)	- (0.157)
GHD (CoD)	9	7.3 (3.7)	AdjHSDS = 4.92*(1-0.50*EXP(-0.57*t))-5	0.57 (0.28)	-2.5 (1.0)	-0.1 (0.6)	↓ (0.003)	- (0.123)	- (0.957)	- (0.119)	- (0.086)	- (0.403)
GHD (HD)	6	5.7 (2.9)	AdjHSDS = 5.48*(1-0.62*EXP(-0.64*t))-5	0.64 (0.22)	-2.8 (1.9)	0.5 (1.2)	↓ (0.006)	- (0.274)	- (0.336)	- (0.282)	- (0.600)	- (0.161)
			Model 2*									
GHD	15	6.7 (3.4)	AdjHSDS = 5.90*(1-0.57*EXP(-0.48*t))-5	0.48 (0.16)	-2.4 (1.4)	0.9 (1.0)						
GHD (CoD)	9	7.3 (3.7)	AdjHSDS = 5.68*(1-0.52*EXP(-0.44*t))-5	0.44 (0.13)	-2.3 (1.0)	0.7 (0.7)						
GHD (HD)	6	5.7 (2.9)	AdjHSDS = 6.22*(1-0.63*EXP(-0.54*t))-5	0.54 (0.19)	-2.6 (2.0)	1.2 (1.4)						

*HSDS for GHD children in model 2 is based on prepubertal height references from three years onwards
^No significant differences in AdjHSDS at start and end, and k between CoD and HD in models 1 and 2.
AdjHSDS = HSDS-THSDS
If there was no statistically significant difference between diagnostic groups, this is indicated as "-"; ↑ signifies "increased" and ↓ signifies "decreased" in comparison with the respective other diagnostic groups.
CD: celiac disease, CoD: conventional dose, JHT: juvenile hypothyroidism, GHD: growth hormone deficiency, HD: high dose, SD: standard deviation, HSDS: height standard deviation score, AdjHSDS: adjusted HSDS

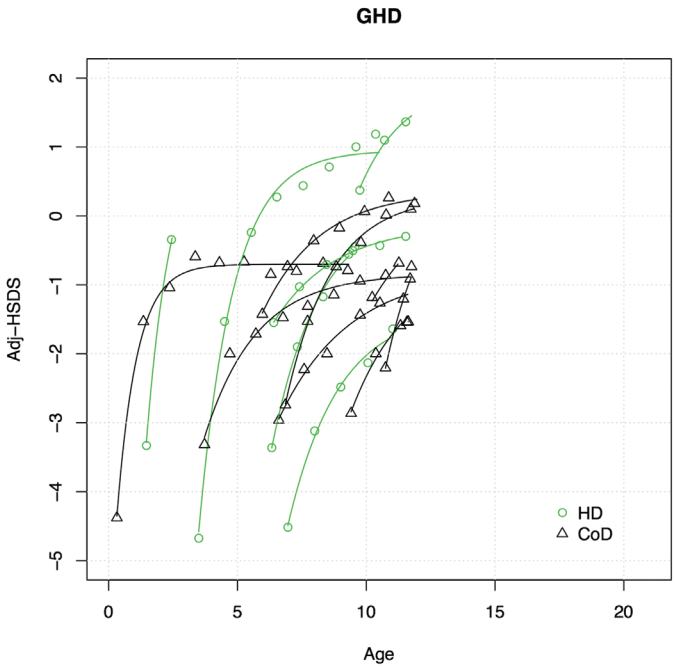

Figure 3. Modelled curves and raw data describing catch-up growth for each child with growth hormone deficiency before reaching puberty

Adj-HSDS: adjusted-height standard deviation score, GHD: growth hormone deficiency, HD: high dose, CoD: Conventional dose

Discussion

The shape of CUG in most children treated for JHT and GHD, when expressed as HSDS or adjHSDS, can be described using the same monomolecular model as we reported for CD (11). The average curves of CUG in JHT and GHD were similar, and differed from the model for CD in the sense that the rate constant was lower (thus a less fast CUG) and the end of CUG was reached later, which is probably related to more initial height deficit than in patients with CD. Advantages of modelling CUG in comparison to yearly indicators of growth include that a full picture is obtained of CUG by using all available growth data rather than data at full years. An additional advantage of this procedure is that measuring errors are smoothed out. A potential adaptation of our approach is to analyse the effect of various predictors (before and during treatment) on the whole phase of CUG in children with GHD. One could, for example, envisage that this technique might be more sensitive to detect additional predictors of the growth response than the ones discovered in the studies by Ranke et al (8) and Ranke and Lindberg (17) using first and second year height velocity. Regarding the effect of variables affecting the growth response to GH during treatment, we recently demonstrated the usefulness of this approach by reporting on the effect of various degrees of non-adherence on CUG in GHD patients included in a large database (18).

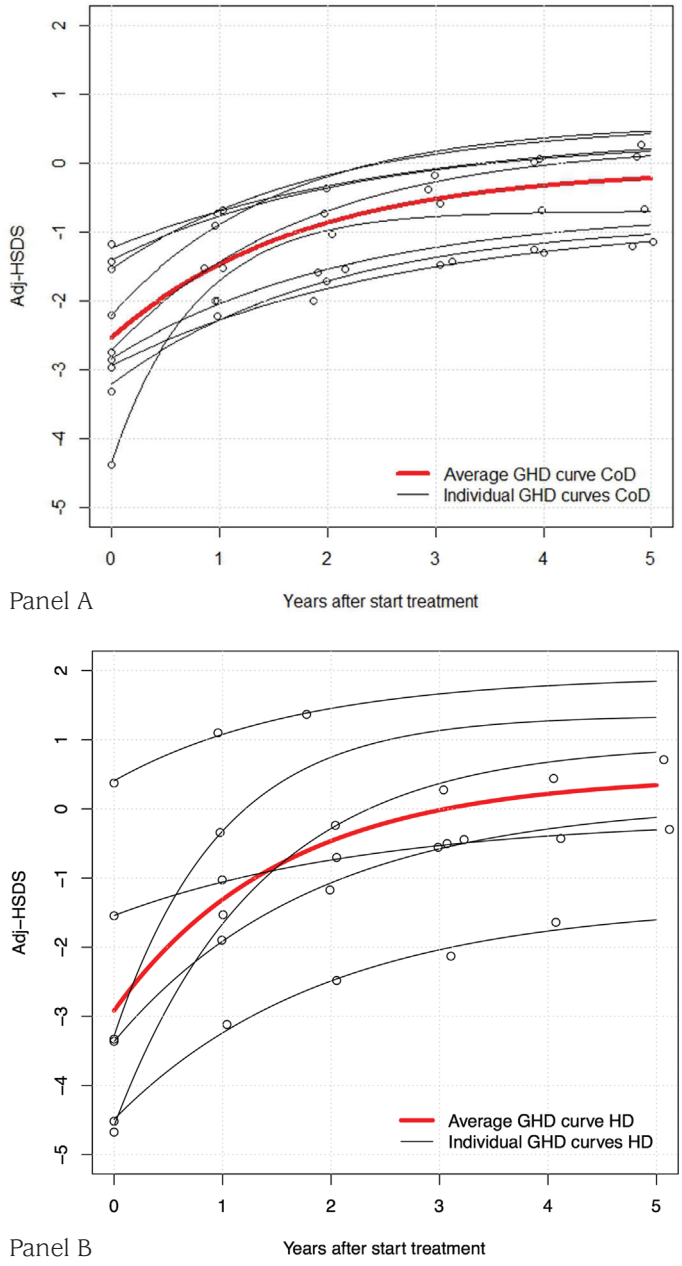

Figure 4. Modelled individual curves and raw data describing catch-up growth of prepubertal children with growth hormone deficiency during three years, as well as the average curve. Panel A: conventional growth hormone dose. Panel B: high growth hormone dose

Adj-HSDS: adjusted-height standard deviation score, GHD: growth hormone deficiency, HD: high dose, CoD: Conventional dose

CUG occurs after the initiation of appropriate treatment of growth impairment due to various conditions, including endocrine disorders (hypothyroidism, Cushing syndrome, GHD), gastrointestinal diseases (CD), and psychosocial disturbances (psychosocial deprivation and starvation with psychosocial deprivation). In prepubertal children, the

Table 2. Predicted growth parameters of the monomolecular function given adjusted height standard deviation score at start

	JHT	GHD
AdjHSDS at start	A; B; k	A; B; k (Model 1)
-4	4.03; 0.75; 0.81	4.74; 0.79; 0.74
-3.5	4.54; 0.67; 0.77	4.86; 0.69; 0.68
-3	4.99; 0.60; 0.72	5.02; 0.60; 0.63
-2.5	5.40; 0.54; 0.69	5.21; 0.52; 0.58
-2	5.78; 0.48; 0.65	5.41; 0.45; 0.54
-1.5	6.13; 0.43; 0.61	5.61; 0.38; 0.51
-1	6.47; 0.39; 0.58	5.81; 0.31; 0.47

AdjHSDS: adjusted height standard deviation score, JHT: juvenile hypothyroidism, GHD: growth hormone deficiency

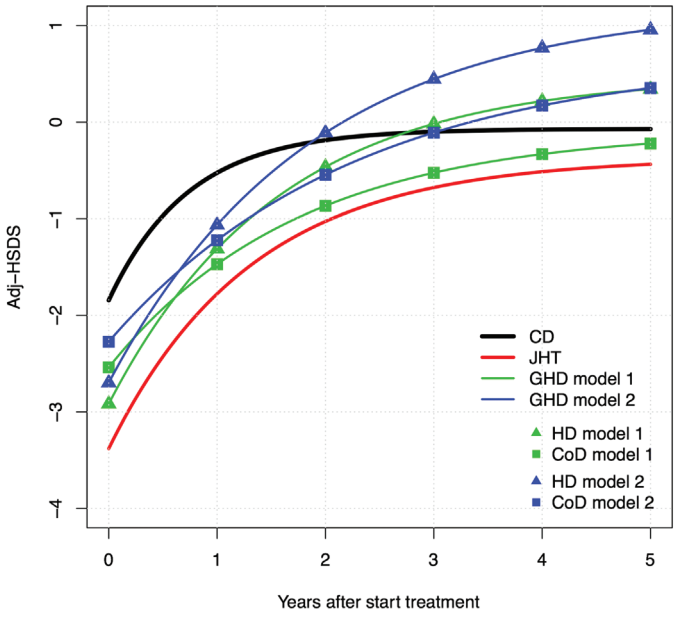

Figure 5. Modelled mean adjusted-height standard deviation score of children with juvenile hypothyroidism, growth hormone deficiency (CoD and high dose, models 1 and 2) in comparison with the catch-up growth model for celiac disease

Adj-HSDS: adjusted-height standard deviation score, CD: celiac disease, HD: high dose, GHD: growth hormone deficiency, JHT: juvenile hypothyroidism, CoD: Conventional dose

phase of CUG is followed by a maintenance phase, in which HSDS remains stable, as shown for GHD (7). In adolescence, linear growth (thus also CUG) is strongly influenced by the timing of puberty, so that during this phase CUG cannot be analysed separately. Therefore, during this phase the effect of a certain treatment on growth is usually expressed as total pubertal height gain (19).

Tanner (2) distinguished three types of CUG: type A, B and C. Type A is the classical pattern of CUG, which takes the child back onto his original pre-insult centile or SDS position within a few years. Types B and C are characterized by a normalization of height velocity for bone age (type B) or age (type C), in combination with a delayed maturation, which in the end may result in a normal AH. More recently, we proposed an intermediate type AB, in which CUG initially does not result in complete normalization of HSDS, but still leads to a normal AH because of delayed maturation (20). Such a pattern was particularly evident in patients with GHD on a relatively low GH dose, as illustrated by several individual curves of patients participating in the dose-response study (12), as shown in Figures 3 and 4. It therefore appears that there is a difference between CUG in GHD versus conditions where the cause can be removed completely (e.g. hypothyroidism, removal of tumour, etc): in GHD CUG is dependent on GH dose, and in each individual child it is impossible to know what the GH dose should be to mimic the "natural" GH secretion during the various phases of CUG observed in children with other conditions.

The mechanism responsible for CUG is still elusive. For the neuroendocrine hypothesis proposed by Tanner in 1963 (21) the experimental evidence has not been convincing so far. In line with the growth plate hypothesis of Baron et al (22), based on earlier work of Williams et al (23,24), Emons et al (25) observed that CUG in infants with CD showed a pattern of normal growth velocity for height age, a pattern described by Tanner as type B CUG (2). However, we believe that this hypothesis cannot fully explain the very fast initial height velocity (much faster than normal for bone age) that can be observed in older children with CD (26) as well as in some children with JHT and GHD (illustrated by some individual curves in the present study). More recently, additional pathophysiologic mechanisms have been proposed, for example regarding a possible role of ghrelin (27), sirtuins, fibroblast growth factor 21, and specific miRNAs and histone deacetylases, reviewed in (28).

According to the current definition of CUG (3), we previously proposed that the growth response to GH in non-GHD patients should not be called "CUG", but should rather be termed "therapy-induced growth enhancement" (29). It would be interesting to analyse to what extent the mathematical model of CUG that we developed for CD, JHT and GHD applies to the growth pattern of GH-treated children with non-GHD conditions, such as children born small-for-gestational age with failure to catch-up spontaneously after birth, Turner syndrome and idiopathic short stature.

Study Limitations

We acknowledge that the number of patients in both patient groups is limited, and that the restriction that CUG can only

be properly studied in prepubertal children further limits the number of data that could be used for the analysis. We tried to alleviate this restriction by using a prepubertal growth reference (model 2), whereby the number of measurements could be maximized. This indeed led to lower p-values in the comparison between GH dosage groups, but we assume that the considerable variation in CUG curves between individuals and the relatively small patient groups precluded reaching statistical significance. Therefore, this report should be considered rather as a proof of principle than as a definitive study. Further, the variation in the pattern of CUG in both diagnostic groups is striking. In particular, the apparent "overshoot" of CUG in some patients with JHT in contrast to insufficient CUG before puberty in others is difficult to explain. Similarly contrasting CUG patterns were seen in children with GHD, and in these children our data suggest that an overshoot of prepubertal CUG was seen more often in children treated with the high GH dose than on a conventional dose, as reported previously (12). However, the low number of patients with sufficient prepubertal data and the uncertainty about AH in the GH dose-response study precludes a firm conclusion.

Conclusion

CUG of prepubertal children with CD, JHT or GHD can be modelled with a monomolecular function. This can be used for assessing the adequacy of CUG and the influence of pretreatment variables, GH dose and adherence on the growth response to GH in prepubertal GHD children.

Ethics

Ethics Committee Approval: Raw data were used of the Dutch multicenter dose-response study on growth hormone deficiency. Approved by the medical Ethics Committee of the Academic Hospital Rotterdam (now called University Medical Center Rotterdam), registration number 87.74

Informed Consent: All parents gave their written informed consent for the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Jan M. Wit, Design: Jan M. Wit, Data Collection or Processing: Theo C. J. Sas, Michael B. Ranke, Jan M. Wit, Analysis or Interpretation: Paula van Dommelen, Jan M. Wit, Michael B. Ranke, Literature Search: Jan M. Wit, Writing: Jan M. Wit, Paula van Dommelen, Michael B. Ranke.

Financial Disclosure: None of the authors have accepted any reimbursement or fee which may have an effect on our results or conclusions of the study.

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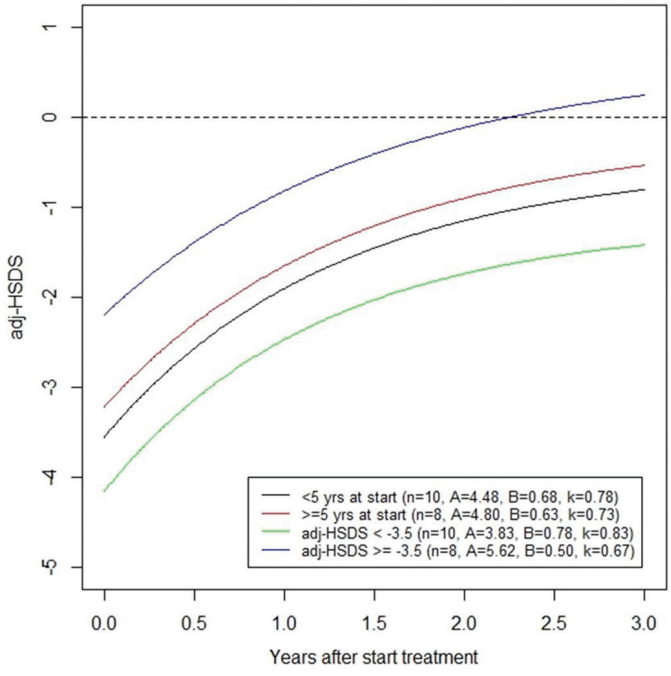

Suppl Figure 1. Modelled mean adjusted-height standard deviation score (adj-HSDS) of children with juvenile hypothyroidism in the first 3 years of L-thyroxine therapy, according to age at start (< 5 or ≥5 years) and to adj-HSDS at start of therapy (< -3.5 or ≥-3.5). Values for the parameters A, B and k are indicated

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Quality of Life and Psychological Well-being in Children and Adolescents with Disorders of Sex Development

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What is already known on this topic?

Articles about quality of life (QoL) in disorders of sex development (DSD) patients who were evaluated with qualitative and quantitative tools in developing and developed countries were reviewed. A broad spectrum of QoL emerged with results better, worse or similar for QoL compared with the unaffected population. No study from Turkey evaluating the QoL of children and adolescents with DSD was found.

What this study adds?

In our study, there was no significant difference between 46,XX DSD and 46,XY DSD groups for both child and parent using the total Pediatric Quality of Life Inventory (PedsQL) scores. In the subscale scores, the PedsQL Physical Functionality Score of affected children was significantly lower in the 46,XX DSD group than in the 46,XY DSD group (p = 0.01). PedsQL School Functionality Score, reported by children and adolescents, was significantly lower in the group with a psychiatric diagnosis compared to the group without a psychiatric diagnosis. In addition, the PedsQL Total Score, PedsQL Emotional Functionality Score, PedsQL Social Functionality Score, and PedsQL School Functionality Score reported by parents in the group with psychiatric diagnosis were significantly lower than the group without psychiatric diagnosis. These results suggest that psychiatric disorders in patients with DSD are the most important factor affecting the QoL.

Abstract

Objective: The aim of this study was to assess the quality of life (QoL) and psychological well-being in child and adolescent with disorders of sex development (DSD).

Methods: Sixty-two cases, aged 2-18 years, who were followed by a multidisciplinary DSD team were included. All participants and their parents were requested the complete the Pediatric Quality Of Life Inventory (PedsQL) and the Strengths and Difficulties Questionnaire. The psychiatric diagnoses of the patients were evaluated according to Schedule for Affective Disorders and Schizophrenia for School-Age Children/Present and Lifetime Turkish Version.

Results: There was no significant difference between the 46,XX DSD and 46,XY DSD groups for both child and parent in Total PedsQL score. In the subscale scores, the PedsQL Physical Functionality Score reported by children was significantly lower for the 46,XX DSD group than for the 46,XY DSD group (p = 0.01). There was a psychiatric diagnosis in 25.8% of cases. The PedsQL School Functionality Score reported by children in the group with psychiatric diagnosis was significantly lower than the group without psychiatric diagnosis (p = 0.018). In the group with psychiatric diagnosis, the PedsQL Total Score and the subscale scores (Emotional Functionality Score, Social Functionality Score, School Functionality) reported by parents were significantly lower than in parents of the group without psychiatric diagnosis.

Conclusion: This study emphasized that psychiatric disorders in DSD patients negatively affect the QoL. Psychiatric support and counseling from a multidisciplinary team are very important for families affected by DSD.

Keywords: Disorder of sex development, quality of life, psychiatric disorder, child and adolescent

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Introduction

Rare congenital conditions that are characterized by incompatibility of chromosomal, gonadal, and phenotypic gender characteristics are classified as disorders of sex development (DSD) (1). The incidence of DSDs is approximately 1 in 4500-5500 (2). Studies to date have focused on psychosexual outcomes, such as gender dysphoria, sexual function status and satisfaction of surgical outcomes in individuals with DSD but very few have assessed general well-being or social participation (3). Long-term psychological, physical and social consequences of young people with DSD are uncertain (4).

Health-related quality of life (HRQOL) has a multidimensional structure that includes various core states, including physical functionality and symptoms, psychological and emotional state and social functionality, that reflect subjective assessments of the patient and his/her family (5). HRQOL measures are increasingly used to determine the impact of medical interventions on compliance and psychosocial well-being (6,7). Important information to guide sex assignment in newborns with indeterminate genital organs is the QoL of these patients in adulthood. The rareness of occurrence of most DSD conditions complicates long-term follow-up of affected patients during adulthood. In the study of Amaral et al (8), articles concerning the QoL in DSD patients who were evaluated with both qualitative and/or quantitative tools in developing and developed countries were reviewed. A broad spectrum of QoL emerged with results better, worse or similar for QoL compared with the unaffected population.

In addition, most of the patients' dissatisfaction was not associated with poor management of the disease or with the assigned gender. A better understanding of their condition, and co-operation between the family and medical team lead to increased satisfaction with treatment. The review of Amaral et al (8) showed that a talented, multidisciplinary team is necessary to deal with these patients throughout their diagnosis and life, and co-operation with patients and parents is crucial.

There is no study from our country evaluating the QoL of children and adolescents with DSD, to the best of our knowledge. In our study, it was aimed to evaluate the HRQOL in children and adolescents with DSD and their parents, in order to better understand future health interventions and approaches.

Methods

In this one-year study 62 cases aged 2-18 years who were followed by multidisciplinary DSD team and were referred to Ege University Department of Child and Adolescent Mental Health and Diseases were included. All participants and their parents were requested to complete the Pediatric Quality of Life Inventory (PedsQL). The Strengths and Difficulties Questionnaire (SDQ), related to emotional and behavioral problems, was completed by parents and teachers of 4-17 year-old cases and, in addition, in patients aged above 11 years old the SDQ was also self-completed. The psychiatric diagnoses of the patients were evaluated according to Schedule for Affective Disorders and Schizophrenia for School-Age Children/Present and Lifetime Turkish Version (K-SADS-PL-T) and Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5) diagnostic criteria.

The classification of the medical diagnosis of patients with DSD was made according to the Lawson Wilkins Pediatric Endocrine Society and the European Society for Pediatric Endocrinology Consensus Statement (9).

In order to make statistical comparisons between groups, endocrine diagnoses were grouped into four groups: 46,XX DSD Group, 46,XY DSD Group, Syndromic Group and Chromosomal Disorder Group. However, since there were not enough cases in the Syndromic and Chromosomal Disorder groups they were removed during the statistical evaluation and the comparisons between the scales were made between the 46,XY DSD and 46,XX DSD groups.

Socio-demographic data, including age, gender, school, mother's and father's education were recorded in the case data form prepared by the authors. Following a full description of the study and study procedure, patients who could give informed consent and all parents were asked to provide written consent. The study was approved by Ege University Medical Research Ethics Committee (19-10.1T/56, 16.10.2019).

Tools

K-SADS-PL-T: A semi-structured interview form, K-SADS-PL-T was developed by Kaufman et al (10) (1997) in order to determine the past and present psychopathologies of children and adolescents according to DSM-5 (11) diagnostic criteria. The validity and reliability study for the Turkish language version was conducted by Gökler et al (12) (2004). In K-SADS-PL-T, the presence and severity of symptoms are determined by combining the views of the child/adolescent, parents and clinician. During the study period two clinicians confirmed the psychiatric diagnoses according to DSM-5 diagnostic criteria (13).

PedsQL: HRQOL was assessed using the PedsQL which contains 23 items in four subscales, including physical (eight items), emotional (five items), social (five items) and school

(five items) functioning. There are four different forms of the scale for the 2-4, 5-7, 8-12 and 13-18 age groups. Children rated how often the item has been a problem for them in the past one month using a five-point response-scale format (0 = never a problem, 1 = almost never a problem, 2 = sometimes a problem, 3 = often a problem, 4 = almost always a problem). The scores ranged from 0 to 100, with higher scores indicating better HRQOL. The total PedsQL score was computed as the sum of all items divided by the number of items answered. For the PedsQL internal consistency (Cronbach alfa = 0.70-0.89) and clinical reliability are high (14). The reliability and validity of the Turkish version of PedsQL in adolescents (for the 8-12 and 13-18 age groups) were reported by Cakin Memik et al (15) and Memik et al (16) while the versions for 2-7 year olds was validated by Üneri. (17)

SDQ: This scale is used in screening emotional and behavioral problems in children. It was developed by Goodman (18) in 1997 and it contains 25 questions. These questions are under five subtitles, each of five questions; Emotional Problems, Attention Deficit and Hyperactivity, Behavioral Problems, Peer Problems and Social Behaviors. This questionnaire has a parent and teacher form for ages 4-17 and an adolescent's self-filled forms for ages 11-17. The Turkish validity and reliability study of this questionnaire for both the parent and adolescent forms was conducted (19,20). However, the Turkish validity and reliability has never been confirmed, to the best of our knowledge.

Statistical Analysis

Statistical analysis was done using SPSS, version 22 (IBM Inc., Armonk, NY, USA. The normality assumption of quantitative data was assessed in each group by Shapiro-Wilk test. The statistical significance was investigated using t-test for numerical variables, Mann-Whitney U test for non-normal distributions, cross table, Pearson chi-square test and Fisher's exact test for categorical variables. A $p < 0.05$ was considered statistically significant. Variable correlation was evaluated by Pearson correlation, if normal distribution was detected, and Spearman correlation if non-parametric.

Results

The average age of 62 cases participating in the study was 9.70 ± 4.18 years. Thirty-six (58.1%) of the cases were raised in the female sex and 26 (41.9%) in the male sex. Socio-demographic characteristics are summarized in Table 1.

Endocrine diagnoses of the cases were: 46,XX DSD in 30.6% (n = 19), 46,XY DSD in 67.7% (n = 42) and chromosome disorders in 1.6% (n = 1). Endocrine diagnoses are summarized in Table 2.

There was a psychiatric diagnosis in 16 (25.8%) cases, and there was no psychiatric diagnosis in the remaining 46 (74.2%). The mean age of patients with a psychiatric diagnosis was 11 ± 4.02 years, and the mean age of those without a psychiatric diagnosis was 9.26 ± 4.17 years. The most common psychiatric diagnosis was attention deficit and hyperactivity disorder (ADHD) (= 13, 21.0%). The other psychiatric diagnoses were depressive disorder (n = 2,

Table 1. Sociodemographic characteristics of patients and their parents

Mean age (years)		**9.70 (± 4.18)**
Gender of rearing	**n (percent)**	
Female	36 (58.1%)	
Male	26 (41.9%)	
Education	**N**	
Not yet in school	19 (30.6%)	
Kindergarten	2 (3.2%)	
Elementary education	34 (54.9%)	
High school	7 (11.3%)	
Age of mother (years)		**34.83 (± 6.72)**
Education level of mother	**N**	
Primary school	39 (62.9%)	
High school	10 (16.1%)	
University	7 (11.3%)	
Illiterate	6 (9.7%)	
Mental illness in mother	**N**	
Yes	3 (4.8%)	
No	59 (95.2%)	
Physical illness in mother	**N**	
Yes	8 (12.9%)	
No	54 (87.1%)	
Age of father (years)		**38.16 (± 7.01)**
Education level of father	**N**	
Primary school	27 (43.6%)	
High school	25 (40.3%)	
University	9 (14.5%)	
Illiterate	1 (1.6%)	
Mental illness in father	**N**	
Yes	2 (3.2%)	
No	60 (96.8%)	
Physical illness in father	**N**	
Yes	6 (9.7%)	
No	56 (90.3%)	
Consanguinity between parents	**N**	
	22 (35.5%)	
Yes	40 (64.5%)	
No		

Table 2. Endocrine diagnoses

Karyotype	Endocrine diagnoses	n (%)	Gender of rearing F/M
46,XY DSD	CGD	5 (8.1)	5 F
	PGD	5 (8.1)	5 M
	ASD (*LHRH* gene, SLOS, StAR, CYP11A1, HSD3B2, HSD17B3, POR, SRD5A2)	17 (27.4)	11 F/6 M
	CAIS	3 (4.8)	2 F/1 M
	PAIS	2 (3.2)	2 M
	Persistent Müllerian duct syndrome	3 (4.8)	3 M
	No diagnosis 46,XY DSD	3 (4.8)	2 F/1 M
	46,XY syndromic	4 (6.5)	4 M
46,XX DSD	Ovotesticular DSD	1 (1.6)	1 M
	CAH	15 (24.2)	13 F/2 M
	Virilizing tumor luteoma in mother	1 (1.6)	1 F
	No diagnosis 46,XX DSD	2 (3.2)	1 F/1 M
Sex chromosome disorders	45,XO/46,XY (mixed gonadal dysgenesis)	1 (1.6)	1 F

DSD: disorders of sex development, F: female, M: male, CAH: congenital adrenal hyperplasia, CGD: complete gonadal dysgenesis, PGD: partial gonadal dysgenesis, ASD: androgen synthesis defects, CAIS: complete androgen insensitivity syndrome, PAIS: partial androgen insensitivity syndrome

3.2%), mental retardation (n=2, 3.2%), anxiety disorder (n=3, 4.8%), autism (n=1, 1.6%), and specific learning disability (n=1, 1.6%).

PedsQL and SDQ Scores: Scale score comparisons were made between 46,XY DSD and 46,XX DSD groups.

Patient diagnoses in the 46,XX DSD group included CAH (n=15, 24.2%), virilizing tumor lutheoma in mother (n=1, 1.6%), 46,XX DSD with no diagnosis (n=2, 3.2%) and ovotesticular 46,XX DSD (n=1, 1.6%) diagnoses were included.

Diagnoses in the 46,XY DSD group were partial gonadal dysgenesis (n=5, 8.1%), complete gonadal dysgenesis (n=5, 8.1%), androgen synthesis defects (n=17, 27.4%), partial androgen insensitivity syndrome (n=2, 3.2%), complete androgen insensitivity syndrome (n=3, 4.8%), persistent Müllerian duct syndrome (n=3, 4.8%) and 46,XY with no diagnosis (n=3, 4.8%) were included.

There was no significant difference between 46,XX DSD and 46,XY DSD groups in both child and parent total PedsQL scores. In the subscale scores, the PedsQL Physical Functionality Score (PFS) reported by children was significantly lower in the 46,XX DSD group than in the 46,XY DSD group ($p=0.01$).

When the relationship between the sex in which the child was raised and the QoL scores was evaluated, no significant difference was found in both child and parent scores ($p>0.05$).

There was mental illness in the family in 8.1% (n=5) of the cases. There was no significant relationship between mental illness in the family and QoL scores of the children and parents ($p>0.05$).

Forty-nine (79.0%) patients had surgical intervention and 13 (21.0%) had no surgical intervention. Surgical interventions included examination, corrective operation and gonadectomy.

The QoL PFS reported by children was significantly higher in patients with surgical intervention than those without ($p=0.039$).

In our study 72.6% (n=45) of the cases were prepubertal and 27.4% (n=17) were pubertal. No case was sexually active. When looking at the QoL scores of the cases according to their pubertal status, no significant difference was found between prepubertal and pubertal cases in terms of QoL scores in the scales completed by children. PedsQL Emotional Functionality Score ($p=0.029$) and PedsQL School Functionality scores ($p=0.003$), among the QoL subscale scores completed by the parents, were found to be significantly lower in pubertal cases than in prepubertal cases.

Table 3 and Table 4 show the relationship between children's and parent's PedsQL scores and socio-demographic characteristics, endocrine groups, pubertal status and surgical intervention.

No significant correlation was found between endocrine diagnosis age (3.23 ± 4.30 years), disease duration calculated from age of diagnosis (6.79 ± 4.19 years) and PedsQL scores ($p>0.05$, Spearman correlation test).

When the relationship between patient age and QoL was examined, no significant correlation was found between age and QoL scores completed by the children. In the parental scores, PedsQL total score ($p=0.012$) and PedsQL school functionality ($r=0.657$, $p<0.05$) scores decreased with increasing age and there was a significant negative correlation between them (Spearman correlation test).

Hormone replacement therapy was used in 29% (n=18) of the cases. No significant difference was found between those using hormone replacement therapy and those not

Table 3. Relationship between children's Pediatric Quality of Life Inventory scores and sociodemographic characteristics, endocrine groups, pubertal status and surgical intervention

Sociodemographic characteristics	PedsQL subscale				
	Median (minimum-maximum)				
	Physical functionality	Emotional functionality	Social funcitonality	School funcitonality	Total PedsQL score
Gender of rearing					
Female	76.56 (34.38-100)	82.5 (30-100)	97.5 (45-100)	77.5 (10-100)	81.52 (35.86-97.83)
Male	84.37 (68.75-100)	80 (30-100)	95 (60-100)	77.5 (45-100)	82.06 (57.61-96.74)
p value	0.062	0.837	0.955	0.530	0.561
Mother's education					
< 8 years	84.37 (34.38-100)	87.5 (30-100)	95 (45-100)	80 (10-95)	81.52 (35.87-96.74)
> 8 years	75 (50-100)	80 (55-100)	100 (60-100)	75 (15-100)	80.43 (35.86-97.83)
p value	0.079	0.233	0.853	0.276	0.306
Father's education					
< 8 years	84.37 (34.38-100)	90 (30-100)	95 (45-100)	80 (10-100)	83.69 (35.87-96.74)
> 8 years	78.12 (50-100)	80 (30-100)	97.5 (45-100)	75 (15-95)	79.89 (35.86-97.83)
p value	0.213	0.105	0.546	0.272	0.298
Mental illnes in the family					
Yes	79.16 (50-100)	86.2 (55-100)	91.25 (65-100)	67.5 (15-90)	79.88 (35.86-97.83)
No	79.72 (34.38-100)	80.8 (30-100)	88.6 (45-100)	72.32 (10-100)	80.1 (35.87-96.74)
p value	0.831	0.365	0.464	0.673	0.268
Endocrine groups					
46,XX DSD	68.7 (50-100)	80 (55-100)	92.5 (45-100)	80 (15-90)	78.80 (35.86-97)
46,XY DSD	85.93 (34.38-100)	82.50 (30-100)	100 (45-100)	77.50 (10-100)	81.52 (35.87-95.65)
p value	**0.018**	0.476	0.381	0.932	0.509
Pubertal status					
Prepubertal	79.5 (56.25-100)	82 (30-100)	89.51 (55-100)	75 (45-100)	81.23 (57.61-96.74)
Pubertal	79.96 (34.38-100)	80 (30-100)	87.64 (45-100)	66.47 (10-90)	78.06 (35.86-97.83)
p value	0.482	0.966	0.655	0.410	0.657
Surgical intervention					
Yes	84.37 (34.38-100)	85 (30-100)	100 (45-100)	80 (10-95)	82.60 (35.87-97.83)
No	75 (50-93.75)	80 (55-100)	85 (55-100)	75 (15-100)	78.26 (35.86-95.65)
p value	**0.039**	0.391	0.097	0.945	0.193

PedsQL: Pediatric Quality of Life Inventory, DSD: disorders of sex development

using hormone replacement therapy in both child and parent QoL scores ($p > 0.05$, Mann-Whitney U Test).

Considering the QoL scores between the two most common endocrine diagnostic groups, CAH and ASD, the PedsQL PFS filled by children was significantly lower in patients with CAH than those with ASD ($p = 0.017$).

When the SDQ scores were analyzed, the SDQ behavioral total difficulty score reported by children was found to be significantly higher in the 46,XY DSD group than the 46,XX DSD group ($p = 0.002$).

SDQ Behavioral Total Score reported by the patients was found to be significantly lower in CAH than ASD ($p = 0.027$). In SDQ, filled by parents, Behavioral Total Score was found to be significantly higher in CAH than in ASD ($p = 0.010$). According to the SDQ scale filled by teachers, Emotional Total Score was found to be significantly higher in CAH than ASD ($p = 0.042$).

The Relationship Between Psychiatric Diagnosis, PedsQL and SDQ Scores

PedsQL School Functionality Score reported by children and adolescents in the group with psychiatric diagnosis was significantly lower than the group without psychiatric diagnosis ($p = 0.018$). Parents of the group with psychiatric diagnosis reported the PedsQL Total Score, PedsQL Emotional Functionality Score, PedsQL Social Functionality Score, Peds QL School Functionality to be significantly lower

Table 4. Relationship between parent's Pediatric Quality of Life Inventory scores and sociodemographic characteristics, endocrine groups, pubertal status and surgical intervention

Socio-demographic characteristics	PedsQL subscale				
	Median (minimum-maximum)				
	Physical functionality	Emotional functionality	Social funcitonality	School funcitonality	Total PedsQL score
Gender of rearing					
Female	78.12 (31.25-100)	85 (30-100)	100 (10-100)	80 (35-100)	83.69 (44.57-98.91)
Male	71.87 (21.88-100)	80 (30-100)	85 (20-100)	80 (30-100)	76.08 (51.09-100)
p value	0.158	0.590	0.087	0.815	0.090
Mother's education					
< 8 years	78.12 (21.88-100)	85 (30-100)	100 (10-100)	80 (40-100)	82.60 (44.57-98.91)
> 8 years	78.12 (31.25-100)	80 (30-100)	85 (20-100)	80 (30-100)	78.26 (46.73-100)
p value	0.792	0.464	0.142	0.324	0.613
Father's education					
< 8 years	71.87 (21.88-100)	85 (30-100)	90 (10-100)	80 (30-100)	78.26 (44.57-98.91)
> 8 years	78.12 (31.25-100)	85 (30-100)	100 (20-100)	80 (35-100)	81.52 (46.73-100)
p value	0.043	0.754	0.183	0.802	0.311
Mental illnes in the family					
Yes	75 (31.25-100)	71 (45-95)	68 (20-100)	59 (30-90)	71.30 (46.73-96.74)
No	74.21 (21.88-100)	80.7 (30-100)	86.14 (10-100)	17.8 (40-100)	13.71 (44.57-100)
p value	0.683	0.291	0.784	0.092	0.475
Endocrine groups					
46,XX DSD	78.12 (31.25-100)	85 (30-100)	95 (20-100)	80 (35-100)	79.89 (46.73-96.74)
46,XY DSD	78.12 (21.88-100)	80 (30-100)	100 (10-100)	80 (30-100)	82.06 (44.57-100)
p value	0.587	0.751	0.401	0.758	0.896
Pubertal status					
Prepubertal	74.92 (21.88-100)	83 (30-100)	86.77 (20-100)	81.71 (30-100)	81.11 (51.09-100)
Pubertal	72.61 (31.25-100)	71.8 (35-100)	79.1 (10-100)	65 (35-95)	78.06 (35.86-97.83)
p value	0.735	**0.029**	0.540	**0.003**	0.074
Surgical intervention					
Yes	76.56 (21.88-100)	85 (30-100)	95 (10-100)	80 (40-100)	80.43 (44.57-80.43)
No	81.25 (31.25-100)	80 (30-100)	85 (20-100)	85 (30-100)	84.78 (46.73-97.83)
p value	0.327	0.721	0.330	0.932	0.885

PedsQL: Pediatric Quality of Life Inventory, DSD: disorders of sex development

than parents of the group that did not have a psychiatric diagnosis ($p = 0.002$, $p = 0.005$, $p = 0.001$, and $p = 0.001$, respectively).

In the group with psychiatric diagnosis, SDQ total difficulty score filled by teachers and parents was significantly higher than the group without psychiatric diagnosis (teacher $p = 0.001$, parent $p = 0.029$). Table 5 shows the relationship between psychiatric diagnosis, PedsQL and SDQ scores.

Psychiatric disorders were diagnosed in 22.2% (n = 10) of prepubertal cases and in 35.3% (n = 6) of pubertal cases. There was no significant difference between pubertal status and presence of psychiatric diagnosis ($p > 0.05$, chi-square test).

Discussion

Most studies on DSD focus on children's sex development and psychosocial well-being, sexual orientation and adult life. In the literature, the results of QoL in children and adolescents with DSD were different (21). In one study, psychological well-being and QoL were not impaired in prepubertal children with DSD (22). In our study, no significant difference was found in terms of QoL scores between prepubertal and pubertal cases in the scales completed by children. However, PedsQL Emotional Functionality Score and PedsQL School Functionality scores, which are the scores of QoL subscale completed by

Tablo 5. Relationship between psychiatric diagnosis, Pediatric Quality of Life Inventory and the Strengths and Difficulties Questionnaire scores

PedsQL Subscales	Without psychiatric diagnosis			With psychiatric diagnosis			
Children	Median	Minimum	Maximum	Median	Minimum	Maximum	p
Physical functionality	**82.81**	34.38	100.00	**78.12**	50.00	100.00	0.160
Emotional functionality	**85.00**	55.00	100.00	**80.00**	30.00	100.00	0.385
Social functionality	**100.00**	45.00	100.00	**90.00**	45.00	100.00	0.056
School functionality	**80.00**	45.00	100.00	**65.00**	10.00	95.00	**0.018**
Psychosocial functionality	**86.66**	58.33	98.33	**73.33**	28.33	96.67	0.090
Total PedsQL score	**83.69**	58.70	97.83	**77.17**	35.86	96.74	0.056
Parent	Median	Minimum	Maximum	Median	Minimum	Maximum	p
Physical functionality	**78.12**	21.88	100.00	**78.12**	21.88	100.00	0.912
Emotional functionality	**90.00**	30.00	100.00	**65.00**	30.00	100.00	**0.005**
Social functionality	**100.00**	35.00	100.00	**80.00**	10.00	100.00	**0.001**
School functionality	**85.00**	40.00	100.00	**60.00**	30.00	100.00	**0.001**
Psychosocial functionality	**90.00**	45.00	100.00	**68.33**	36.11	100.00	**0.000**
Total PedsQL score	**84.78**	51.09	100.00	**66.30**	44.57	100.00	**0.002**
SDQ	With psychiatric diagnosis			Without psychiatric diagnosis			
Children	Median	Minimum	Maximum	Median	Minimum	Maximum	p
Emotional symptoms	**2**	0	5	3	0	7	0.145
Hyperactivity/inattention	**3**	2	7	3	0	7	0.502
Conduct problems	**2**	0	3	2	0	5	0.469
Peer relationship problems	**2**	1	5	2	0	7	0.274
Pro-social behavior	**9.5**	8	10	9	7	10	0.614
Total difficulty score	**11**	6	19	9	2	16	0.096
Parent	Median	Minimum	Maximum	Median	Minimum	Maximum	p
Emotional symptoms	**1**	0	7	2	0	6	0.305
Hyperactivity/inattention	**5**	0	9	3	0	7	**0.010**
Conduct problems	**1**	0	8	1	0	5	0.231
Peer relationship problems	**2**	0	6	2	0	6	0.936
Pro-social behavior	**8**	4	10	8	3	10	0.252
Total difficulty score	**10**	1	24	7	1	21	**0.029**
Teacher	Median	Minimum	Maximum	Median	Minimum	Maximum	p
Emotional symptoms	**2**	0	6	**1**	0	5	0.101
Hyperactivity/inattention	**6**	0	9	**2**	0	7	**0.002**
Conduct problems	**1**	0	9	**0**	0	4	0.051
Peer relationship problems	**4**	0	7	**1**	0	6	**0.004**
Pro-social behavior	**8**	0	10	**9**	4	10	0.180
Total difficulty score	**13.5**	0	29	**5**	0	16	**0.001**

SDQ: The Strengths and Difficulties Questionnaire, PedsQL: Pediatric Quality of Life Inventory

the parents, were found to be significantly lower in pubertal cases compared to prepubertal cases. In another study, it was reported that there is an increased risk for emotional problems in children and adolescents with DSD (23). In the results of studies with adults with DSD, although some studies have reported psychological, functional and sexual disorders (24,25,26), others have not confirmed severe restrictions or psychological problems (27,28).

Psychosexual outcomes and QoL in DSD have been most extensively studied in CAH, which accounts for about half of DSD cases. According to Kuhnle et al (29), long-term effects on general HRQOL are not expected in CAH. Johannsen et al (25), have identified lower QoL and more psychiatric symptoms in adult Danish women with CAH. Nordenskjöld et al (24) reported that both mutation type and surgical procedure affected long-term QoL for women with CAH. In a study involving both male and female CAH patients, both reported much lower scores in QoL than in the general population. The authors concluded that in both groups, poor hormone replacement therapy, obesity and

sexual dysfunction may be responsible for impaired QoL (30). Children with 46,XY DSD are less extensively studied. Physical well-being is reported in most cases to not be different from the general population. However, condition-specific effects on gender identity or self-perception have been described in adolescents (21).

Most of the DSD QoL studies in the literature focused on CAH and female raised and adult cases (24,25). As far as we know, our study is the first study evaluating QoL in Turkey by comparing both 46,XX and 46,XY DSD. In their study conducted in Finland, Jaaskelainen and Voutilainen (31) found that QoL scores in cases with CAH (16 women and 16 men) were better than the control group.

In their cohort studies between female social gender and male social gender DSD patients in Brazil, Amaral et al (8), found that the adult QoL in DSD patients was good in both genders. However, they found that male social gender DSD patients with either 46,XX or 46,XY had better scores in the psychological domain than female social gender DSD patients.

When looking at QoL studies in children and adolescents with DSD, in their studies on 60 adolescents aged 13-16, Kleinemeier et al (21) found that general psychological well-being was not affected. In their study, Jürgensen (22) reported that children aged 8 to 12 years with DSD had significantly lower scores in self-completed HRQOL than those without DSD and notable deficits were reported in self-esteem, physical health and school functionality dimensions. Comparison of HRQOL between the diagnostic endocrine groups revealed no significant group differences.

In our study PedsQL PFS filled by children was found to be significantly lower in patients with CAH than those with ASD. Considering that all cases with CAH have XX karyotype and all cases with ASD have XY karyotype, this finding may be attributed to gender difference. In addition, it was observed that the QoL Physical Functioning subscale scores completed by the children were significantly lower in the 46,XX DSD group compared to the 46,XY DSD group. When these results are taken together, it suggests that QoL of cases with 46,XX, DSD in our country who were raised in the female gender was lower. This finding is different from the findings of studies conducted in other countries (8,31,21,22), in which both genders and endocrine groups were compared. It is important in terms of being the first data specific to Turkey.

In research conducted in The Netherlands, the scores of QoL reported by the parents were not impaired in any dimension (32). Similarly, in our study, no significant difference was found between the two endocrine groups in the parental total and subscale PedsQL scores. The fact that information about the QoL was obtained from both cases and parents is a strength of our study.

In our study, when the relationship between patient age and QoL was examined, no significant correlation was found between age and QoL scores completed by the children. However in the parental scores, it was found that PedsQL total score and PedsQL school functionality scores decreased with increasing age and there was a significant negative correlation between them. In the research of Jürgensen (22), the differences between the scores of children and parents, especially in terms of self-esteem, psychological and physical well-being, have been previously described for other chronic diseases (33,34). This study and the other similar studies emphasize the need for self-reporting in volunteer children who can report on themselves.

Jürgensen (22) reported that variables, such as gender identity/gender dysphoria, gender role behavior, genital surgery status of the child, number and timing of surgery or diagnosis subgroups, and knowledge of the child about the current diagnosis were not associated with decreased QoL. In our study, it was found that variables such as endocrine diagnosis age, disease duration, education status of the mother, did not affect the QoL. The PedsQL PFS reported by parents increased significantly as the education level of the father increased. It is thought that this situation may be related with the increase in understanding and coping skills as the education level increases.

In the study of Crawford et al (35), lower HRQOL was reported in patients with DSD who underwent surgery. In the research of Jürgensen (22) no relation was found between genital surgery and HRQOL. However, in our study, the PedsQL PFS filled by children was significantly higher in those with surgical intervention than those without.

Ege University Faculty of Medicine DSD multidisciplinary team consists of pediatric endocrinology, pediatric surgery, genetics, and child and adolescent psychiatry specialists. The team meets every month and discusses the patients followed up with the diagnosis of DSD and organizes follow-up and treatment. Multidisciplinary team meetings are held to ensure that the intervention in DSD cases is performed at the most appropriate time and condition. It was thought that the higher PedsQL PFS in our patients who underwent surgery might be related to this. In the studies of Migeon et al (28) thirty-nine, 46,XY DSD case were evaluated for long-term medical and surgical results using questionnaire and semi-structured interview. They concluded that most of the participants were satisfied with their body image and that there was no difference in satisfaction with their sexual

functions among men and women. The authors concluded that the assignment to either sex would lead to a successful long-term outcome in most 46,XY individuals with severe genital uncertainty.

In our study, a psychiatric diagnosis was assigned to 16 (25.8%) cases. Children and adolescents with DSD are at risk, due to the difficult processes they have experienced from birth, so psychiatric evaluation is required. According to a research by Özbaran et al (36), ADHD, depression and anxiety disorder were found in DSD as psychiatric diagnoses. In the study of Jürgensen (22) it was reported that the mental health of adolescents with DSD was not affected, compared to adolescents in the control group. In previous studies of CAH patients, it has been reported that anxiety disorder and ADHD are frequently seen in these cases (37). Studies on stress and QoL levels of CAH patients show that these patients are under emotional stress that can cause depression and anxiety disorders (25). In our study the most common psychiatric diagnosis was ADHD (n=13, 21.0%). This rate is higher than the prevalence of ADHD (3.4%) reported by Polanczyk et al (38) in a meta-analysis (38). In a study assessing the prevalence of childhood psychopathology in Turkey, mental disorder prevalence was 17.1% and ADHD prevalence was 12.4% (39). The values in our study were higher for both disorders (respectively, 25.8%, 21%).

The incidence of psychiatric disorders varies according to the age group and psychiatric diagnosis. While the rates of depression and some anxiety disorders (social phobia, panic disorder) increase with adolescence, the rates of some disorders such as ADHD and separation anxiety disorder decrease (40). In our study, the mean age of patients with psychiatric disorders was 11 (±4.02) years, and the most common diagnosis was ADHD and depressive disorder.

In the research of Şan et al (41) investigating a Turkish cohort, physical health total score, psychosocial health total score and scale total score filled by parents and children were found to be statistically significantly lower in the ADHD group compared to the control group. In other studies, it has been reported that the areas of psychosocial, academic and family functionality are the most affected in children diagnosed with ADHD (42).

In our study the PedsQL School Functionality Score, which was completed by the children and adolescents was significantly lower in the group with a psychiatric diagnosis, the most common being ADHD, compared to the group without a psychiatric diagnosis. In addition, PedsQL Total Score, PedsQL Emotional Functionality Score, PedsQL Social Functionality Score, and PedsQL School Functionality Score filled by parents in the group with psychiatric diagnosis were significantly lower than the group without psychiatric diagnosis.

In the research of Sawyer et al (43), it was reported that children with psychiatric disorder had much worse HRQOL than children without psychiatric disorder in many areas and also a worse HRQL than children with physical disorders.

Study Limitations

One of the strengths of our study is that we evaluate psychiatric disorders through a semi-structured interview. The fact that information about the QoL was obtained from both cases and parents is another strength of our study. Our study is important to emphasize that psychiatric diseases in DSD patients negatively affect the QoL. The biggest limitation of our study is that the sample size is relatively small and there is no control group. Our other limitation is that the teacher form of the SDQ questionnaire has no Turkish validity and reliability. A further limitation was that clinical severity of psychiatric illness and the presence of side effects related to drugs were not evaluated.

Conclusion

These results suggest that psychiatric disorders in patients with DSD are the most important factor affecting QoL. To the best of our knowledge there is no other study reporting the effect of psychiatric disorder on QoL in DSD patients in Turkey. Therefore, our study is important to highlight that psychiatric illnesses in DSD patients negatively affect the QoL. Consequently, the importance of psychiatric support and consultancy of a multidisciplinary team is very clear, especially for children and families. Further studies will be important to demonstrate whether multidisciplinary team collaboration and psychiatric support have a positive effect on HRQOL in individuals with DSD.

Our results also suggest that, in Turkey, the QoL is lower in patients with 46,XX DSD who were raised in the female gender. This finding is different from the findings of studies conducted in other countries in which both genders were compared. As in many countries, it is a fact that women in our country are exposed to more risk factors than men, starting from intrauterine life, during childhood, adolescence, adulthood and old age. Future studies with larger samples and control groups will shed light on this issue.

Ethics

Ethics Committee Approval: The study was approved by Ege University Medical Research Ethics Committee (19-10.1T/56, 16.10.2019).

Informed Consent: Patients who could give informed consent and all parents were asked to provide written consent.

Peer-review: Externally peer-reviewed

Authorship Contributions

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ORIGINAL ARTICLE

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Identification of Three Novel and One Known Mutation in the *WFS1* Gene in Four Unrelated Turkish Families: The Role of Homozygosity Mapping in the Early Diagnosis

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What is already known on this topic?

Wolfram syndrome 1 (WS1) is a clinically heterogeneous disease with variable manifestations and progression pattern depending on the underlying molecular genetic aetiology. Patients may present with incomplete phenotype, but the disease has a progressive nature with a negative impact of poor glycaemic control. Identification of molecular genetic aetiology provides early diagnostic confirmation and thereby an opportunity to detect and manage the subtle symptoms more appropriately.

What this study adds?

Our study expands the mutation database of WFS1 with three novel variants and provides further insights into the genotype and phenotype association. We used homozygosity mapping as an adjunctive tool, which contributed to early detection of molecular genetic etiology in cases that presented with incomplete WS1 phenotype.

Abstract

Objective: Bi-allelic mutations in the *wolframin* gene *(WFS1)* cause Wolfram syndrome 1 (WS1 or DIDMOAD) characterized by non-autoimmune diabetes mellitus, optic atrophy, diabetes insipidus, sensorineural deafness, urinary tract abnormalities, and neuropsychiatric disorders. Patients presenting with an incomplete phenotype of WS1 were evaluated using homozygosity mapping and subsequent whole-exome sequencing.

Methods: Four unrelated consanguineous Turkish families, including seven affected children, and their unaffected parents and siblings were evaluated. Homozygosity mapping was performed, followed by whole-exome sequencing of *WFS1*. Mutations were classified according to results of "*in silico*" analyses, protein prediction, and functional consequences.

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Results: Homozygosity mapping confirmed shared homozygous regions on chromosome 4 (chr4p16.1) between the affected individuals, that was absent in their unaffected siblings. Exome sequencing identified three novel (c.1215T > A, c.554G > A, c.1525_1540dup) and one known (c.1522_1523delTA) mutations in *WFS1*. All mutations were predicted to cause stop codon leading to early termination of protein synthesis and complete loss-of-function. All patients were found to be homozygous for the change, with parents and other unaffected siblings being carriers.
Conclusion: Our study expands the mutation spectrum of *WSF1* mutations with three novel mutations. Homozygosity mapping may provide enrichment for molecular genetic analysis and early diagnosis of WS1 patients with incomplete phenotype, particularly in consanguineous pedigrees.
Keywords: Wolfram syndrome, WFS1, diabetes mellitus, diabetes insipidus, optic atrophy, sensorineural deafness

Introduction

Wolfram syndrome (WS), first described in 1938 by Wolfram and Wagener (1) in four siblings, is an autosomal recessive disorder characterized by early-onset diabetes mellitus (DM), progressive neurodegeneration, endocrine dysfunction, and psychiatric disorders (2). WS1 is also known as a syndrome with the acronym DIDMOAD, which describes the frequent clinical features of the disease; diabetes insipidus (DI) and DM with optic atrophy (OA) and deafness. As WS1 is a progressive degenerative disease, additional clinical features including ataxia, urinary tract and renal disorders, and psychiatric disorders may present later in life (2).

WS1 is a rare cause of early-onset, non-autoimmune DM, which is the most common clinical feature, and usually occurs within the first decade of life (median age: 6 years). This is followed by progressive OA (median age of presentation at around 11 years), which first begins with colour and peripheral vision loss and can eventually lead to blindness over the next decade of life, as neurodegeneration progresses (3). All four clinical features described by DIDMOAD were observed in around 66% of patients in a review of 392 WS patients (4).

Biallelic loss-of-function mutations in wolframin endoplasmic reticulum (ER) transmembrane glycoprotein gene (*WFS1*), located at chromosomal position 4p16.1, accounts for the molecular genetic aetiology of WS1 (5). *WFS1* encodes for the protein wolframin, which is expressed ubiquitously, while the steady-state levels vary significantly among organs (6). It is highly expressed in brain neurons, pancreas, heart and muscle, and lower expression is observed within the liver and skeletal muscle, while the lowest expression is in kidney and spleen (6). Within the pancreas, wolframin has a higher expression in the islet cells than the pancreatic exocrine cells (5). Wolframin, a transmembrane glycoprotein composed of a cytoplasmic N-terminal domain, a central nine-transmembrane domain, and a luminal C-terminus, is predominantly localized in the ER (7). It is involved in the regulation of ER-stress, which is critically important in establishing intracellular homeostasis, integrity and survival of the cell (8,9). Wolframin is primarily involved in the unfolded protein response (UPR), which transduces the stimulus for increased unfolded proteins and maintains the balance between anti-apoptotic and pro-apoptotic processes (9,10). The UPR regulates ER-stress by eliminating misfolded proteins or attenuating protein translation (11,12). Loss-of-function in wolframin causes decreased UPR activity, and thereby a chronic ER-stress mediated apoptosis and cell death (8,9). This eventually causes both neurodegeneration and loss of beta-cell mass (13). Wolframin also plays an essential role in the stimulus-response coupling mechanism, which regulates beta-cell insulin synthesis and secretion (13,14).

WS1 is a clinically heterogeneous disease with variable presentation as well as progression pattern depending on the underlying molecular genetic aetiology (4,15,16). Such diversity can make diagnosing WS1 difficult, especially in the context of an outbred population, multiple genes may need to be explored and sequenced. However, in consanguineous families, homozygosity mapping can prove to be an efficient tool to localize causative genes for recessive traits. It allows targeting of specific chromosomal regions of DNA that are shared only by affected individuals, thereby facilitating the process of finding candidate genes and detecting mutations, when used alongside exome sequencing.

In the present study, evaluation was performed of seven affected individuals and their apparently healthy relatives from four unrelated, consanguineous Turkish families with a variable clinical phenotype which was found to be due to three novel and one previously described *WFS1* mutation.

Methods

Patients

In this study, seven affected patients (six males and one female) were evaluated who had presented with rare forms of DM and sensorineural deafness (SND) together with their apparently healthy parents and siblings so that a total of 21 individuals from four unrelated families were included.

All patients are from first-degree consanguineous parents. Homozygosity mapping was performed in all patients and their unaffected siblings, from all families. Specific chromosomal regions were identified which were shared by the patients alone, and absent in their unaffected siblings. In these regions candidate genes were then identified, and exome sequencing data was used to detect mutations in the region of interest.

Family 1

Family 1 consists of three male children, two affected with DM and SND, and one unaffected. Parents were first cousins (Figure 1A).

Patient 1 was born at term with a birth weight of 2.6 kg. At the age of seven years, he presented with polyuria and polydipsia. Blood glucose level was 340 mg/dL (18.9 mmol/L) at the time of diagnosis with no diabetic ketoacidosis (DKA). Diabetes autoantibodies [islet cell antibodies (ICA), insulin autoantibodies (IAA), and GAD65] were negative. He was started on insulin therapy. He also developed SND when he was about 13 years old. A DNA sample was collected due to non-autoimmune, early-onset DM and SND. Although he had a history of decreased visual acuity observed at the age of eight years, the diagnosis of OA was considered only after reassessment due to genetically proven WS1 diagnosis (Table 1). During the follow-up, he developed all clinical features of WS1, as displayed in Table 1.

Patient 2 was born at term with a birth weight of 2.8 kg. At the age of 11 years, he presented with polyuria and polydipsia. Blood glucose level was 301 mg/dL (16.7 mmol/L) with no DKA at the time of diagnosis. Diabetes autoantibodies (ICA, IAA, and GAD65) were negative. He also had a history of decreased visual acuity, which was first observed at the age of eight years. Blood sample for DNA was collected due to his early onset, non-autoimmune diabetes and medical history of his elder brother. A diagnosis of OA was also considered when a full ophthalmological evaluation was performed after the results of DNA analysis. These two patients from family 1, unfortunately, did not attend their regular follow-up visits. At the latest follow-up visit, when he was 16 years old, his audiological evaluation also revealed the diagnosis of SND, although the patient was not suffering from a hearing problem (Figure 1A). The clinical features of WS1 and the age of onset for symptoms in this patient are displayed in Table 1.

Family 2

Family 2 is a large consanguineous family with two affected male siblings (Figure 1B). **Patient 3 (P3)** was born at term with a birth weight of 2.9 kg. At the age of two years, he was diagnosed with DM. At presentation, his blood glucose level was 270 mg/dL (15 mmol/L), and diabetes autoantibodies (ICA, IAA, and GAD65) were negative. He was started on insulin therapy. He also had a history of SND, which was noticed at the age of two years. He had no other features of WS1 at the time of the DNA sampling (at the age of 17 years), while he developed central DI at the age of 20 years. He also had mild-moderate mental retardation and emotional instability (Table 1).

Patient 4 (P4) was born at term with a birth weight of 2.7 kg. At the age of five years, he was diagnosed with DM. The blood glucose level at admission was 306 mg/dL (17 mmol/L), and diabetes autoantibodies (ICA, IAA, and GAD65) were negative. Insulin therapy was commenced. He also had a history of SND detected at the age of two years. At his latest follow-up visit at the age of 20 years he had no other feature of WS1, but a mild developmental delay was observed (Table 1).

Family 3

Patient 5 (P5) is a female patient born to first-cousin parents (Figure 1C). She had two unaffected male siblings and a history of one sister and one first-cousin with DM and SND who both died with unknown aetiology. She was born at term with a birth weight of 2.5 kg. She had SND, which was first noticed at the age of two years and required hearing aid at the age of five years-old. She presented with polyuria and polydipsia at the age of six years. At presentation, she had a blood glucose level of 360 mg/dL (20 mmol/L) with no DKA. The diabetes autoantibodies (ICA, IAA, and GAD65), were negative. A diagnosis of DM was considered, and insulin therapy commenced. She developed decreased visual acuity, which was first noticed at the age of 10 years, and a diagnosis of OA was considered at the age of 13 years. She developed central DI at the age of 16 and had all the cardinal features of WS1 at her latest follow-up visit at 18 years-old although there were no renal and psychiatric disorders (Table 1).

Family 4

Family 4 is a first-degree consanguineous family with two affected male (patients 5 and 6) and one unaffected female sibling (Figure 1D).

Patient 6 (P6) was born at term with a birth weight of 2.7 kg. At the age of five years, he presented with polyuria and polydipsia. His fasting blood glucose level was 340 mg/dL (18.9 mmol/L), and diabetes autoantibodies (ICA, IAA, and GAD65) were negative. A diagnosis of DM was considered, and insulin therapy commenced. He subsequently developed visual and hearing deficits around the age of seven years.

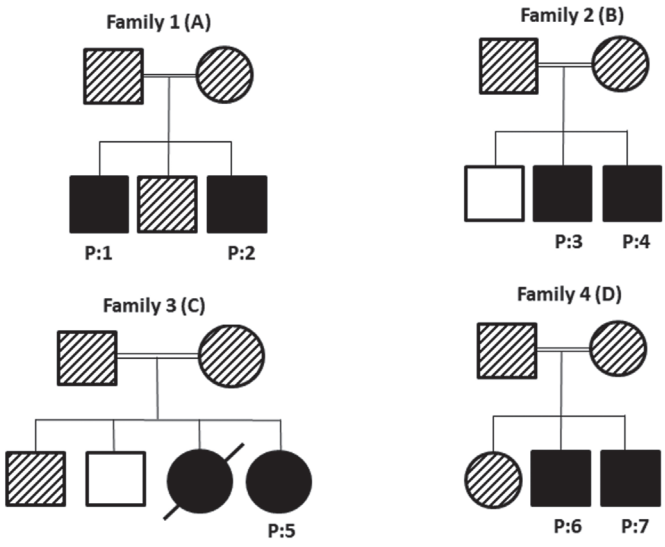

Figure 1. A, B, C, D) Family pedigrees of Wolfram syndrome patients. Black-filled boxes refer to homozygous and clinically affected members, while black shaded boxes indicate heterozygosity and completely empty boxes refer to mutation-negative unaffected family members (A female sibling of P5 did not undergo mutation analysis, but she had clinical features of WS1 similar to P5. It was therefore thought that she presumably had an identical mutation and is displayed with black-filled box)

His developmental milestones were achieved appropriately for age. The younger brother (P7), was born at term with a birth weight of 2.9 kg. At the age of six years, he presented with polyuria and polydipsia, and a fasting blood glucose level of 234 mg/dL (13 mmol/L). Diabetes autoantibodies (ICA, IAA, and GAD65) were also negative. He had no visual or hearing loss at the time of this study (Table 1).

The ethical approval was granted by University College of London (UCL), Institute of Child Health, Great Ormond Street Hospital for Children (R&D number: 12CM47). Informed consent was obtained from all patients or their legal guardians and unaffected family members.

Molecular Genetics Analysis

Families were originally recruited alongside a cohort of other families in a study to identify rare causes of DM and SND. Not all patients had evidence of OA at the time of the presentation, and therefore WS1 was not considered initially. Genomic DNA was isolated through standard techniques at the UCL Genomics centre. DNA samples from these patients were sent for homozygosity mapping at the UCL Genomics centre, as all patients belonged to consanguineous parents. The Illumina microarray platform was used for genotyping, following the Infinium HD Ultra Assay protocol (Rev B, 2010, Illumina Inc, San Diego, USA). Results were generated using the Illumina Genomestudio software, and copy number variation and loss of heterozygosity data was generated (cnvPartition v3.1.6, Illumina). The minimum homozygous region size was 1 Mb, with a minimum of 50 consecutive SNPs. Further, to identify rare variants possibly explaining early-onset DM and SND, exome sequencing was performed out of UCL. Primer 3 software was used to design the primers for the *WFS1* gene. The sequencing reaction was conducted using the BigDye Terminator V1.1 Cycle Sequencing kit (Applied BioSystems, Foster City, CA, USA). The sequences were compared to a reference sequence using the Sequencher® 5.3 software. The variants were classified based on the 2015 American College of Medical Genetics and Genomics and Association for Molecular Pathology guidelines using InterVar (17). The variants were also classified concerning effects on protein synthesis, genotypic classification and functional consequences using the classifications described by de Heredia et al (4) and Rohayem et al (16) (Table 2).

Results

Homozygosity mapping results demonstrated shared homozygous regions on chromosome 4 (chr4p16.1) between the affected individuals, and these were absent from their unaffected siblings. The search was targeted in this region. The 4p16.1 locus contains 81 genes of which 51 are protein-coding. Although 23 of these genes are defined in the Online Mendelian Inheritance in Man database (OMIM), only four genes (*WFS1, HMX1, SLC2A9, DRD5*) are associated with a phenotype in the OMIM morbid list (Figure 2). We performed whole-exome sequencing that identified *WFS1* gene mutations, which was confirmed by Sanger sequencing (Table 2). In total, we analyzed samples from 21 individuals, including affected subjects and their apparently unaffected family members.

A novel nonsense c.1215T > A (p.Tyr405Ter) variant was detected in exon 8 of the *WFS1* gene in family 1 (P1 and P2) (Table 2). This variant has not been listed in mutation databases (HGMD, Clinvar), sequence variant databases (Exome Variant Server, dbSNP, EXAC and 1000genome) or not published elsewhere in the literature search including Google and PubMed databases. The pathogenicity and classification of the variant according to various classifications are displayed in Table 2.

In family 2 (P3 and P4), we identified another novel nonsense variant in exon 5 of *WFS1*, where the base pair change c.554G > A, leads to early termination of the protein chain (p.Trp185Ter), leading to synthesis of a truncated protein (Table 2). The pathogenicity and classification of

Table 1. Age of onset for clinical characteristics for the WS1 features in seven cases with homozygous *WFS1* mutations

Patient (Sex)/ family number	Age of the mutation analysis	Age at the latest F/up visit	DM	SND	OA	DI	Neuro-psychiatric disorders and other symptoms
P1 (M) (Family 1)	15 years	19 years	7 years-old	13 years-old (HA) (Presumably earlier as the symptoms were present earlier)	15 years-old (Age of onset for symptoms was reported at 8-9 years)	13 years	Mild mental retardation, emotional disability Renal USG normal Developed dysphagia at the age of 20 and declared died with an aspiration problem during feeding presumably developed due to problem in swallowing
P2 (M) (Family 1)	12 years	18 years	11 years-old	16 years-old (HA) (Presumably earlier as the symptoms were present earlier)	13-years (Age of onset for symptoms was reported at 8-9 years)	Absent at the latest fw/up visit	Has mild anxiety and depressive mood which observed at the age of 16 years-old. Renal USG and urine protein excretion normal.
P3 (M) (Family 2)	17 years	22 years-old	2 years-old	2 years-old (HA)	Absent	20 years-old	Mild-moderate mental retardation, Has severe emotional disability since 15 years-old
P4 (M) (Family 2)	15 years	20 years-old	5 years-old	2 years-old (HA)	Absent	Absent	Mild mental retardation No psychiatric symptoms observed Renal USG and ECO: normal
P5 (F) (Family 3)	13 years	18 years-old	7 years-old	2 years-old (HA)	10 years-old	16 years-old	Absent
P6 (M) (Family 3)	10 years	10 years	5 years-old	7 years (HA)	7 years	Absent	Absent
P7 (M) (Family 4)	7 years	7 years	5 years-old	Absent	Absent	Absent	Absent

M: male, F: female, SND: sensorineural deafness, DM: diabetes mellitus, OA: optic atrophy, DI: diabetes insipidus, HA: hearing aid, USG: ultrasonography, ECO: echocardiography

the variant according to various classifications are displayed in Table 2. We first presented this mutation at European Society For Paediatric Endocrinology 2014 meeting (18), but shortly after our report, another group published the same variant in a Jordanian family (19).

In family 3 (P5), a known frameshift/nonsense mutation (c.1522-1523delTA, p.Y508X) was identified in the *WFS1* gene. This mutation has been reported only once before, in 2006, in two Turkish male siblings with features of WS1 and suicidal behaviour (20). The pathogenicity and classification of the variant according to various classifications are displayed in Table 2.

In family 4 (P6 and P7), a novel C.1525_1540dup15 duplication mutation was identified. This variant has not been listed in mutation databases (HGMD, Clinvar), sequence variant databases (Exome Variant Server, dbSNP, EXAC and 1000 genome) and not published elsewhere in the literature search including Google and PubMed databases. The pathogenicity and classification of the variant according to various classifications are displayed in Table 2.

Discussion

In the present study, we evaluated the clinical characteristics, underlying molecular genetics and follow-up of seven patients with WS and their 14 unaffected relatives from four unrelated, consanguineous Turkish families. As the patients' phenotypes were incomplete for WS1 diagnosis, homozygosity mapping was used to enrich the molecular genetic analysis and identified three novel variants, and one previously reported mutation in another Turkish family from the same geographical location (20).

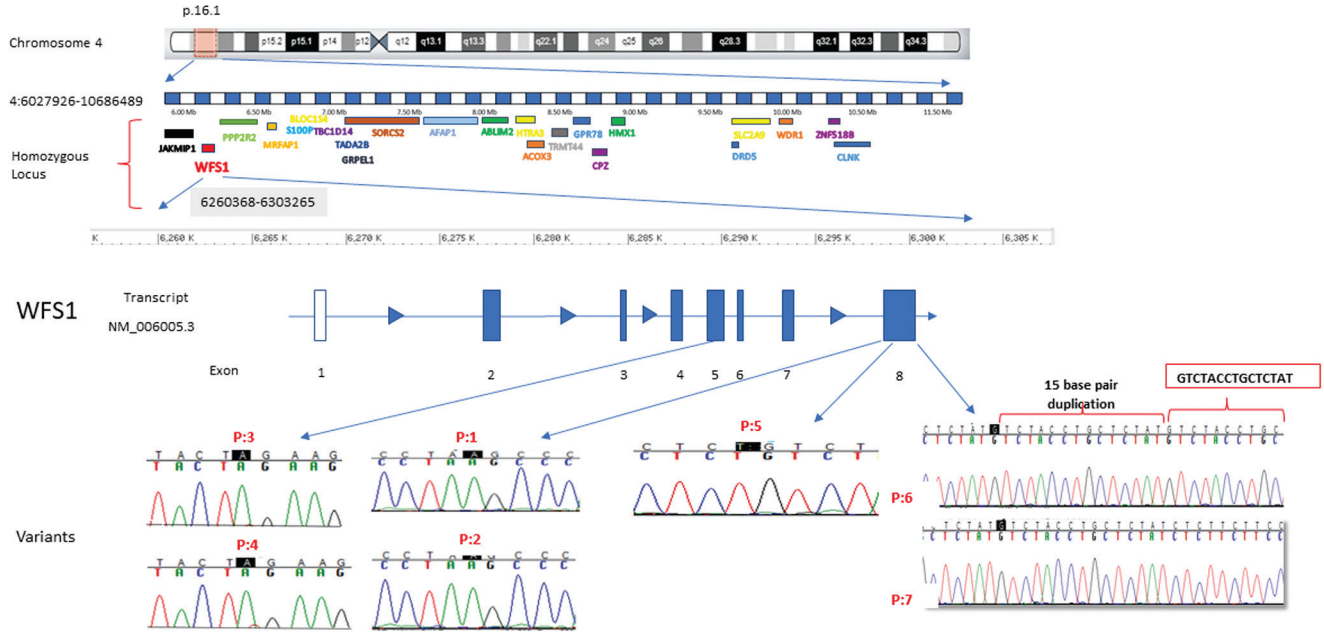

Figure 2. Homozygosity mapping showed a shared region between affected members where the *WFS1* gene was located in the same region. Electropherogram of the WFS1 analysis for patients 1-7

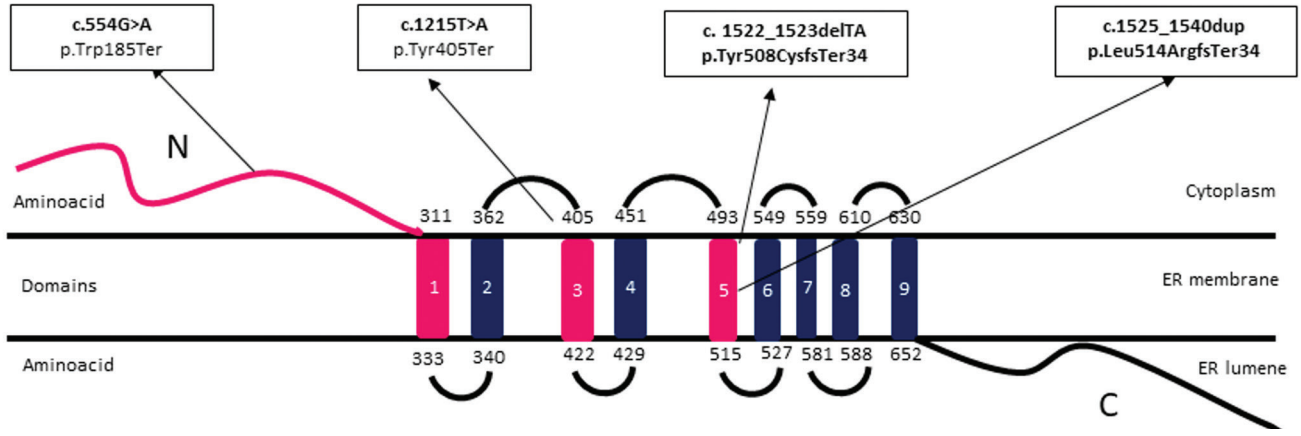

Figure 3. A motif of *WFS1* showing the NH2-terminal, nine transmembrane and the -COOH terminal domains and location of mutations detected in the present report

WFS1 gene is intolerant to the loss-of-function mutations - the score of probability for being loss-of-function intolerant is 1.0 (21). Functional studies and protein analysis of fibroblast cell lines of WS1 patients have shown that nonsense, splicing site and frameshift mutations of *WFS1* cause nonsense transcripts that are unstable *in vivo* and rapidly degraded by nonsense-mediated mRNA decay (6,22,23). Missense variants have been shown to cause a WS1 phenotype by affecting post-transcriptional modifications, protein stability and regulation of the degradation of wolframin transcripts (12). Besides, missense mutations are predicted to cause reduced half-time and low steady-state level of the wolframin, and thus are suggested to have a dosage-sensitive effect (6,22,23).

To date more than 330 variants have been described in the *WFS1* gene (The Human Gene Mutation Database website: http://www.hgmd.cf.ac.uk/ac/index.php, latest access 22nd May 2020). Of these around 230 have been reported to be associated with a WS1 phenotype. The common type of mutations include missense, nonsense, frameshift, splice-site mutations, in-frame deletions/insertions or duplications. The majority of *WFS1* mutations have been detected in exon-8, which accounts for about 86 % of variants detected (24). Mutations detected in our case series were nonsense (n = 2), deletion (n = 1) and duplication (n = 1) mutations leading to a stop codon, thereby, early termination of the protein wolframin.

The novel nonsense c.1215T > A (p.Tyr405Ter) variant is located on the third transmembrane domain of the *WFS1* gene (Figure 3), and results in a premature stop codon and early termination of the protein chain (PVS1) (Table 2). This nonsense mutation is predicted to lead to nonsense-mediated mRNA decay and complete depletion of wolframin protein, and therefore complete loss-of-function. Homozygous affected members and heterozygous/wildtype variants in the unaffected family members showed a phenotype and genotype co-segregation.

The novel nonsense c.554G > A (p.Trp185Ter) variant leads to early termination of the protein chain and thereby synthesis of a truncated protein (Table 2). This is an "N terminal" stop-gain variant that causes complete loss-of-function due to premature termination of protein synthesis and rapid degradation of the truncated transcripts (Table 2 and Figure 3). Although this mutation is predicted to severely affect wolframin expression leading to complete loss-of-function, the clinical phenotype in our cases was relatively mild compared to patients from the Jordanian family (19). The age of onset for DM was similar and between 2-5 years-old in both families. However, in our patients SND was observed earlier (two years vs five years). The most striking discrepancy was the development of OA and DI, which were detected around the ages of four years (DI) and five years (OA) in Jordanian patients. However, none of our cases developed OA until their latest follow-up visit at the age of 20 and 22 years. None of our cases developed urinary tract abnormalities which were observed in one of the Jordanian patients (hydronephrosis and gall bladder stones). Patients from both families had a moderate intellectual disability.

Table 2. Mutations characteristics, results of *"in silico"* analyses, protein prediction, functional consequences and individuals with particular mutations

NM_006005.3	**Family 1**	**Family 2**	**Family 3**	**Family 4**
Mutation	c.1215T > A	*c.554G > A	c.1522_1523delTA	c.1525_1540dup
Exon	8	5	8	8
Protein change	p.Tyr405Ter	p.Trp185Ter	p.Tyr508CysfsTer34	p.Leu514ArgfsTer34
Mutation type	NS	NS	NS/FS	Dup/FS
Location	Transmembrane domain (3rd segment)	N-terminal domain	Transmembrane domain (5th segment)	Transmembrane domain (5th segment)
Zygosity	Homozygous	Homozygous	Homozygous	Homozygous
***In silico* analyses**				
Mutation taster	Disease-causing	Disease-causing	Disease-causing	Disease-causing
DANN score	0.9825	0.9971	NA	NA
Eigen score	Benign	Pathogenic		
FATHMM-MKL (coding prediction)	Neutral	Damaging		
GERP (conservation prediction)	Not-conserved	Conserved		
ACMG classification	Pathogenic	Pathogenic	Pathogenic	Pathogenic
	PVS1 PM1 PM2 BP4	PVS1 PM1 PM2 PP3	PVS1 PM1 PM2	PVS1 PM1 PM2
Mutation effect [Rohayem et al (16)]	Group 1 (Complete loss of function)	Group 1 (Complete loss of function)	Group 1 (Complete loss of function)	Group 1 (Complete loss of function)
Mutation type [de Heredia et al (4)]	Type 3	Type 1	Type 3	Type 3
Genotypic class [de Heredia et al (4)]	C	A1	C	C
Phenotype-genotype co-segregation	Both parents and unaffected sibling are heterozygous carriers	Parents are heterozygous carriers for the mutation, an unaffected sibling is homozygous for the normal allele.	Parents and one unaffected sibling are heterozygous carriers, an unaffected sibling is homozygous for the normal allele.	Both parents and unaffected sibling are heterozygous carriers of the mutation

DANN: Deleterious Annotation of Genetic Variants, GERP: Genomic Evolutionary Rate Profiling, ACMG: American College of Medical Genetics and Genomics, NS: nonsense, FS: frameshift, Dup: duplication, HM: homozygous.
*We first described this mutation (presented at European Society For Paediatric Endocrinology annual meeting 2014) (18), but was later published by another group in 2016 (19)

The previously published frameshift/nonsense, c.1522-1523delTA (p.Y508X) mutation causes deletion of two base-pairs (TA) in exon-8 of *WFS1 (*Figure 3). It causes disturbance in the normal reading frame which result in early termination of the amino acid sequence and synthesis of a truncated protein. This mutation has been reported only once before, in 2006, in two Turkish male siblings with WS1 features and suicidal behaviour (20). The previously reported two siblings were from the same city as our case, while both families were not related. The age of DM in the first report and our case were similar, while our case developed hearing loss at an earlier age of two years, suggesting congenital SND. Besides, our case developed other clinical features at a later age compared to the first cases and still has not yet developed psychiatric complications nor urinary tract problems.

The novel C.1525_1540dup15 mutation duplicates 15-base pairs in the five amino acids from V509_Y513 (Figure 3). This mutation causes production of a stop codon and changes the reading frame and thereby termination of protein synthesis at the 34th amino acid in the sequence. Sanger sequencing confirmed that two affected siblings had a homozygous mutation, whereas their unaffected family members were heterozygote carriers.

WS1 is a clinically heterogeneous disorder with variable age of onset for clinical features. DM and OA are the most common presenting features with a frequency of 98.2% and 82.1%, respectively (4). The disease has a progressive nature, and other components of DIDMOAD develop within a variable timeframe depending on mutation characteristics. Therefore, mutation analysis of the *WFS1* gene in patients with any two DIDMOAD symptoms is warranted for early detection of WS1 patients (4,15,16). In our case series, the age of onset for DM was similar to previous reports and consistent with the mutations characteristics, while OA was detected at a lower rate and with variable timing. Besides, at the time of the genetic analysis, OA was not confirmed in the majority of cases (only two out of seven). SND was the second most prominent feature, which was detected in five out of seven patients at the time of the genetic analysis. Therefore homozygosity mapping was performed, which provides enrichment for detection of mutations in *WFS1*. Homozygosity mapping has previously been reported as contributing to the detection of novel mutations, as well as a new coding region responsible from the WS phenotype (25). Mutations in this second locus cause a distinct WS phenotype with upper gastrointestinal tract bleeding and the absence of DI (WS2) (25).

Age of presentation for deafness can be quite variable ranging from severe congenital deafness to late-onset mild and progressive hearing loss (4,26). The age of presentation in three out of six patients who developed SND was two years. Heterozygous mutations in *WFS1* are also associated with a dominant form of hearing loss, known as low-frequency sensorineural hearing loss (27). Although we were not able to perform audiological evaluation for family members who were carrying a heterozygous mutation, none had declared symptoms of hearing loss. Nevertheless, diagnostic evaluation based on parents or patients' declaration or examination without using standardized method may result in underdiagnosis of hearing loss, which suggests a need for using standardized audiological methods for the evaluation of patients presenting with WS1 symptoms (26).

Urinary tract abnormalities, including upper urinary tract dilatation (hydroureteronephrosis), recurrent urinary tract infections, urinary incontinence due to atonic bladder, and end-stage renal failure are common features of WS1 (2,28). The rate of urinary system problems has been reported in up to 90% with a median age of 20 years and three peaks observed at the age of 13, 21 and 33 years (2). Indeed, two patients with the c.554G>A and c. 1522-1523delTA mutations have been previously reported to have urinary tract problems (19,20). Nevertheless, until their latest follow-up visits at the age of 22 years, none of our patients with identical mutations developed urinary tract problem.

Mutations that cause earlier presentation of DM are suggested to also cause accelerated neurodegeneration (4,16). Besides, glucotoxicity is also associated with an increased risk of neurodegenerative disorders in WS1 patients (16). Good glycaemic control (HbA1c<7.5%) has been shown to correlate with a lower rate of DI, deafness, and neurological and psychiatric symptoms (16). In our case series, early-onset DM and SND were seen in the majority of cases. However, other neurological features of WS1 developed later over wide range of ages. Notably, two patients with the earliest presentation for DM and SND had not developed OA at the ages of 20 and 22 years. One of these patients had also not yet developed other features of WS1 except for DM and SND, while the other developed DM, DI and SND in addition to psychiatric symptoms (Table 1).

WS1 patients present with a wide variety of clinical symptoms and signs due to the clinically heterogenous nature of the disease. Furthermore, the varying ages of presentation of symptoms, and the different rates of progression, makes WS1 a very difficult syndrome to diagnose in the early stages, especially when only one symptom may be apparent, consequently leading to delay in the treatment and management. With the use of homozygosity mapping, we were able to narrow down specific regions of the human

genome, which were shared only by affected individuals of different families, allowing us to fast track the search for the causative gene.

Study Limitations

A limitation of the present study was the unavailability of functional analyses of the novel variants identified in our cases.

Conclusion

In conclusion, our study expands the mutation spectrum of WSF1 with three novel nonsense variants in three unrelated consanguineous families, confirming variable phenotypical expression and heterogeneity in presenting features as well as the progressive nature of the disease. The prominent WS1 features in our cases were early onset SND, lower rate and delay in development of OA, and lack of urinary tract problems. Homozygosity mapping proved to be a useful tool for enrichment of molecular genetic analysis in the early diagnosis of WS1 patients with an incomplete phenotype, particularly those with no OA, or belonging to consanguineous pedigrees.

Ethics

Ethics Committee Approval: Ethical approval for the study was granted by the University College of London, Institute of Child Health, Great Ormond Street Hospital for Children (R&D number: 12CM47).

Informed Consent: Informed consent was obtained from all patients or their legal guardians, and all unaffected family members who participated to the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

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ORIGINAL ARTICLE

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Very High Incidence of Type 1 Diabetes Among Children Aged Under 15 Years in Tlemcen, Northwest Algeria (2015-2018)

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What is already known on this topic?

Algeria ranked among the top 10 countries with highest number of children with type 1 diabetes (T1D) in 2019.

What this study adds?

This study is the first to report the incidence of T1D in children under 15 years in the region of Tlemcen in Northwest Algeria. The incidence of T1D in children under 15 years was 38.5/100,000 during 2015-2018 in the region of Tlemcen.

Abstract

Objective: In Algeria, there is a lack of epidemiological data concerning childhood type 1 diabetes (T1D). The International Diabetes Federation estimated in 2019 that Algeria ranked 7th among countries with the highest prevalence of T1D. This study aimed to determine the incidence of T1D in children < 15 years, living in Tlemcen in Northwest Algeria.

Methods: A retrospective study using data from children (< 15 years) who have been diagnosed with T1D in Tlemcen between 2015 and 2018, using the two-source capture–recapture method to estimate the completeness of ascertainment (%). Total average incidences, by sex, by onset age group, and by season of onset were calculated *per* 100,000 and *per* year.

Results: During the study period, 437 new cases of T1D were registered, among them, 233 boys and 204 girls, with a sex ratio of 1.14. The average annual incidence rate of childhood T1D was 38.5/100,000 with a 95% confidence interval (CI): 35.20-41.79; boys: 40.51, 95% CI: 38.16-42.85; girls: 36.49, 95% CI: 34.17-38.80. Overall incidence rates in 2015, 2016, 2017 and 2018 were respectively 36.6 (95% CI: 33.72-39.48), 38.7 (95% CI: 35.43-41.97), 39.3 (95% CI: 35.97-42.62) and 39.5 (95% CI: 36.12-42.87)/100,000. Newly diagnosed children were more likely to present in winter and autumn. Ketoacidosis at diagnosis was diagnosed in 29.2%.

Conclusion: The mean incidence of childhood T1D in Tlemcen was 38.5/100,000, this incidence is in the "extremely high" category of the World Health Organization DiaMond project classification of diabetes giving this region a very high risk.

Keywords: Type 1 diabetes, children, incidence, Tlemcen, Northwest Algeria

Introduction

Type 1 diabetes (T1D) or insulin-dependent diabetes is the most common endocrine and metabolic disorder in children and represents 80-90% of diabetes in children and adolescents (1,2). Since 1950, the incidence of diabetes in children has increased substantially around the world. The World Health Organization (WHO) Multinational Project for Childhood Diabetes (WHO DiaMond Project) estimates an annual average increase at around 3% (3).

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The incidence of T1D varies widely between countries, and even between regions of the same country; there is a geographical disparity in the epidemiological trends of childhood diabetes worldwide (4). The highest incidence rates were recorded in Finland, in Sweden and in Sardinia while East Asian and American Indians populations have the lowest rates (5). This geographic heterogeneity in incidence trends is due to factors such as variability in genetic predisposition and environmental factors for autoimmune destruction of beta-pancreatic cells (6,7). The role of environmental triggers in the development of childhood diabetes has been suggested because of the marked seasonal variation in the onset of childhood diabetes (8).

According to estimates in the 8th Edition of the International Diabetes Federation Diabetes Atlas, Algeria ranks seventh in the world among countries with the highest estimated number of prevalent children aged under 15 years with T1D (n = 20,100) (9). Algeria is also the country in the Middle East and North Africa (MENA) Region with the highest number of new cases (incidence) of T1D in this age group with 3,100 children in 2019 (10).

In Algeria there is a lack of available scientific data on the incidence and prevalence of T1D in children, with only three functional regional registries for T1D in children under 15 years of age (11). In Algiers (in north-central Algeria) the incidence of T1D among children under 15 rose from 22.3 per 100,000 in 2010 to 29.0 per 100,000 in 2015 (12). In Oran (northwest region) the incidence of T1D in children under 15 rose from 4.7 per 100,000 in the period 1979-1988 to 24.46 per 100,000 in the period 2010-2014 with an annual increase in incidence of 5.04 (13,14). In Constantine in the North East of Algeria the incidence of T1D in children under 15 increased from 9.57 per 100,000 between 1990-1994 to 17.44 per 100,000 in 2003 (15). However, in the region of Tlemcen in the North-West of Algeria, no epidemiological data on T1D in children are available.

The objective of this study was to assess the incidence of T1D in children under 15 years from the region of Tlemcen, in northwestern Algeria between January 1, 2015 and December 31, 2018.

Methods

Study Design and Data Collection

This retrospective study was conducted in Tlemcen, one of the largest cities in northwestern Algeria. This region, bordering on Morocco, is defined by a diverse geography, a Mediterranean climate and an Arab Muslim sociodemographic structure. According to the 2014 census, the population numbers 1,032,065 inhabitants and the population of children under 15 years was estimated at 267,597 children (male: 136,084; female: 131,513), accounting for 25.93% of the total population. There are five pediatric units in Tlemcen: pediatric department of the Mother and Child Specialized Hospital at Tlemcen's Teaching Hospital, and pediatric departments of four Public Hospitals (PH), PH of Maghnia, PH of Ghazaouet, PH of Remchi and PH of Sebdou.

The mid-year estimates of the children population under 15 years were obtained from the annual statistical census data of the office of the Ministry of the Interior and from the regional Statistical Office of Tlemcen.

The diagnosis of T1D was made by the pediatric physician, according to the accepted criteria of the American Diabetes Association (16). The date of diagnosis of diabetes was accepted as the day of the first insulin injection. The months of diagnosis of T1D were sorted by seasons to examine the possibility of seasonality in the onset of childhood diabetes. Diabetic ketoacidosis at the time of diagnosis was observed and recorded as defined by the ISPAD Clinical Practice Consensus Guidelines (17).

All children under 15 years of age living in the region of Tlemcen for at least six months prior to diagnosis, and presenting as newly diagnosed T1D for the period from from January 1, 2015 to December 31, 2018, were included. We excluded children with another type of diabetes (type 2 diabetes mellitus, neonatal diabetes, maturity onset diabetes of the young, and diabetes caused by other conditions).

The main source data on children diagnosed with T1D were based on the registers and the hospital records of the pediatric department of the Mother and Child Specialized Hospital at Tlemcen's Teaching Hospital, and derived from the hospital records of pediatric departments of the four PH of Tlemcen. In the region of Tlemcen, all children under the age of 15 years, newly diagnosed with T1D are referred to these five pediatric units, as they are the only clinical institutions authorized to write a report for the initiation of insulin treatment and for follow-up.

The secondary independent data source of ascertainment was based on the Algerian social security system (Algerian national Health Insurance, ANHA). In Algeria, every child with T1D receives free treatment and diabetes is one of the chronic conditions which benefits from full coverage by the Algerian State (ANHA).

To measure case ascertainment and confirm the completeness of the recording, the capture-recapture method was used (18). This method would be expected to identify

all new cases of children with T1D by capturing them in the first source and recapturing them in the second source in order to minimize the probability of underestimating the real number of new cases and to adjust accordingly the incidence of childhood T1D in the region.

The authors believe that a full census of all children under 15 years, newly diagnosed with T1D during the study period in the region of Tlemcen, was performed for this study.

This study was approved by the University Ethics and Deontology Council of the University of Tlemcen, Tlemcen, Algeria (approval number: CEDUT/DZ/019/117). Informed consent was obtained from the parents of children.

Statistical Analysis

The average annual incidence rates were calculated by dividing the newly diagnosed cases of T1D in children aged under 15 years in a specific year, by population at risk aged under 15 years residing in Tlemcen in that year, and is expressed per 100,000 persons per year. Total average incidences were calculated by sex, by three pediatric age groups (0-4, 5-9 and 10-14 years) and by the season of the year at diagnosis.

The 95% confidence intervals (CI) of the annual incidence rates were calculated based on Poisson distribution. Independent chi-squared test was used to compare the rates between years, sexes and age groups, a p value (p) < 0.05 was considered significant. Poisson regressions were used to analyze the changes in diabetes incidences with age, sex, season at diagnosis and year period. Statistical analysis was performed using the software R [R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria] (x64 3.3.2).

Results

Overall ascertainment with capture-recapture method using the two independent sources was estimated to be 96% complete for the study period.

During the period from January 1, 2015 to December 31, 2018, 437 new cases of T1D in children under 15 were registered in the region of Tlemcen consisting of 233 (53.32%) boys and 204 (46.68%) girls, with a male/female sex ratio of 1.14. Children were classified into three age groups: 29.06% of children diagnosed were under the age of five years, 34.78% aged between 5-9 years, and 36.16% of children aged 10-14 years (Figure 1).

The overall mean age at onset of T1D in this population was 7.51 ± 4.12 years (95% CI: 6.56-8.35), with no significant difference between boys 7.46 ± 4.14 years (95% CI: 6.40-8.62) and girls 7.56 ± 4.11 years (95% CI: 6.70-8.21) ($p > 0.05$).

The average annual incidence rate of T1D among children in these four years was 38.5 new cases per 100,000 persons under 15 years old (95% CI: 35.20-41.79) (boys: 40.51, 95% CI: 38.16- 42.85, girls: 36.49, 95% CI: 34.17-38.80). The difference in the incidence rate between boys and girls was only statistically significantly different in 2015 ($p = 0.00064$), while for the other years of the study, there was no significant preferential difference between boys and girls ($p > 0.05$). The incidence rates in 2015, 2016, 2017 and 2018 were respectively 36.6 (95% CI: 33.72-39.48), 38.7 (95% CI: 35.43-41.97), 39.3 (95% CI: 35.97-42.62) and 39.5 (95% CI: 36.12-42.87) per 100,000 respectively without significant difference between these four years of study. The number of cases and annual incidence rates by sex are presented in Table 1.

The incidence of T1D was lower in children of 0-4 years old years (31.11 per 100,000, 95% CI: 29.12-33.09) and higher in the 5-9 and 10-14 years age groups, with a peak of 44.78 per 100,000, 95% CI: 42.96-46.59) between 5-9 years, these differences between age groups of onset of childhood diabetes were not statistically significant ($p > 0.05$). The annual incidence rates by sex and by age groups are presented in Table 2 and Table 3.

Poisson regression results show that the 5-9 years old group had 1.43 times risk, and the 10-14 years old group had 1.31 times risk compared to the 0-4 years old group ($p < 0.05$).

The study of seasonality in the diagnosis of T1D showed that most cases in the region of Tlemcen were diagnosed in autumn (25.06%) and winter (28.25%), the cooler and rainier seasons of the year but fewer in spring and summer

Figure 1. Number of new cases (males and females) of type 1 diabetes by age groups in the 2015-2018 period

(24.45% and 22.24%, respectively), the warmer seasons of the year, but the seasonal variation were not statistically significant ($p > 0.05$). This trend in onset seasonality was present in both sexes and in the three age classes. November was the month with the highest number of newly diagnosed children (9.83%) and June was the month with the lowest number of new cases (5.72%).

A total of 138 children (29.2%) had ketoacidosis at diagnosis. Diabetic ketoacidosis (DKA) was more common (53.62%, 74/138) in girls, but no significant difference between the two sexes. Regarding the frequency of DKA by age group, the difference between the frequencies of the different age groups was not statistically significant.

Discussion

This study is the first to produce a reliable estimate of the incidence of T1D in children under 15 years old in Tlemcen. During the study of 2015 to 2018 inclusive, the incidence of T1D in children was estimated at 38.5 per 100,000 children under 15 years per year. Our results show that the region of

Table 1. Number of cases and annual incidence of type 1 diabetes among children under 15 years age per 100,000 persons per year (95% confidence interval) by sex in Tlemcen between 2015 and 2018

Year	Total		Boys		Girls	
	Number of cases	Incidence rate (CI)	Number of cases	Incidence rate (CI)	Number of cases	Incidence rate (CI)
2015	99	36.60 (33.72-39.48)	62	45.02 (42.71-47.32)	37	27.80 (25.53-30.06)
2016	108	38.70 (35.43-41.97)	58	40.89 (38.55-43.22)	50	36.47 (34.17-38.76)
2017	113	39.30 (35.97-42.62)	53	36.28 (33.90-38.65)	60	42.49 (40.15-44.82)
2018	117	39.50 (36.12-42.87)	60	39.87 (37.46-42.27)	57	39.19 (36.82-41.55)
2015-2018	437	38.50 (35.20-41.79)	233	40.51 (38.16-42.85)	204	36.49 (34.17-38.80)

CI: confidence interval

Table 2. Annual incidence of type 1 diabetes among children under 15 years age per 100,000 persons per year (95% confidence interval) by age groups in Tlemcen between 2015 and 2018

Year	Age group (years)		
	0-4	5-9	10-14
2015	41.91 (39.93-43.88)	34.45 (32.66-36.23)	32.71 (30.82-34.59)
2016	23.82 (21.85-25.78)	64.50 (62.7-66.29)	31.76 (29.83-33.68)
2017	25.05 (23.05-27.04)	46.39 (44.55-48.22)	48.31 (46.36-50.25)
2018	33.68 (31.65-35.7)	33.78 (31.93-35.62)	50.89 (48.92-52.85)
2015-2018	31.11 (29.12-33.09)	44.78 (42.96-46.59)	40.92 (38.97-42.86)

Table 3. Annual incidence of type 1 diabetes among children under 15 years age per 100,000 persons per year (95% confidence interval) by sex and by age groups in Tlemcen between 2015 and 2018

Year	Boys			Girls		
	Age group (years)			Age group (years)		
	0-4	5-9	10-14	0-4	5-9	10-14
2015	54.37 (52.97-55.76)	43.35 (41.92-44.77)	36.53 (35.18-37.87)	29.06 (27.69-30.42)	25.15 (23.9-26.39)	27.94 (26.6-29.27)
2016	21.51 (20.09-22.92)	67.81 (66.52-69.09)	37.55 (36.18-38.91)	26.20 (24.81-27.58)	61.05 (59.79-62.3)	25.79 (24.45-27.09)
2017	20.27 (18.81-21.72)	34.05 (32.74-35.35)	54.69 (53.3-56.06)	29.35 (27.95-30.75)	59.27 (57.99-60.54)	41.74 (40.37-43.07)
2018	33.17 (31.71-34.62)	39.67 (36.85-42.48)	47.19 (45.79-48.59)	34.20 (32.77-35.62)	27.62 (26.32-28.91)	54.70 (53.31-56.08)
2015-2018	32.33 (30.9-33.75)	46.22 (44.93-47.5)	43.99 (42.61-45.72)	29.70 (28.36-31.03)	43.27 (42.0-44.53)	37.54 (36.17-38.9)

Tlemcen presents a very high risk of T1D in children under 15 years.

In Algeria there are only a few reports of the epidemiological profile of T1D in children aged under 15 years. Nevertheless, the incidence in our childhood population is comparable to that of other studies conducted in Algeria. In 2016, in the capital Algiers, the incidence of T1D was 29.35 per 100,000 children under 15 years (19). In the region of Oran, the incidence of T1D in children under 15 was 31.12 per 100,000 in the period 2013-2017 (20). All these studies from Algeria report a T1D incidence in children in the "extremely high category" (incidence rate >20 per 100,000 persons per year) of the WHO DiaMond project classification for diabetes (21).

There is a clear difference in the incidence of childhood T1D in these different regions of Algeria. Similar differences in the incidences of T1D in children between regions of the same country are well documented (21,22,23).

During this study period, the incidence of childhood T1D ranged from 36.60 per 100,000 in 2015 to 39.50 per 100,000 in 2018, but due to the short period of our study, we cannot reliably estimate the rate of increase in the incidence of T1D in children under 15 years old in the region.

Worldwide, after the Nordic countries (Finland, Sweden, and Norway), some countries with an Arab population (Kuwait and Saudi Arabia) have the highest rates of T1D (9). In Africa, epidemiological data are incomplete and many countries have no studies on the incidence of T1D in children. The incidence of childhood diabetes in Tlemcen is clearly higher than in neighboring North African countries, notably Tunisia (7.7 per 100,000) during 1990-1999 (3), Libya (7.8 per 100,000) during 1991-2000 (24), Sudan (10.1 per 100,000) in 1990 (25), and Egypt (3.1 per 100,000) in 2011 (26). It is also higher than in some other MENA countries, notably Qatar (31.83 per 100,000) in 2016 (27), Iraq (8.0 per 100,000) in 2016 (28) and in Turkey (10.8 per 100,000) during 2011-2013 (23), but it was lower than that of eastern Saudi Arabia (52.93 per 100,000) in 2007 (29) and than that of Kuwait (40.9 per 100,000) during 2011-2013 (30). It should be noted that the studies carried out in most of the North African countries mentioned are relatively old. The high rate of incidence of T1D in our population in Tlemcen, compared to neighboring countries, is presumably due to as yet undetermined genetic or environmental factors, although the period between the older North African studies and the present study will account for some of the difference in incidence as there is a general global increase of 3% per annum.

In comparison with the Mediterranean countries, the incidence of diabetes in our pediatric population is higher that of Spain (22.84 per 100,000) during 2013-2016 (31), France (19.1 per 100,000) in 2015 (32), Italy (25.2 per 100,000) during 2009-2013 (33), Montenegro (18.8 per 100,000) in 2011 (34), Croatia (17.23 par 100,000) during 2004-2012 (35), and Cyprus 11.4/100,000 during 2011-2016 (36) but it remains lower than that Sardinia (51.0 per 100,000) during 2007-2009 (37).

In our study, the mean age of diagnosis of type diabetes was 7.51, which was high compared to Saudi Arabia (7.0 years) (38), but lower than in Kuwait, Spain and Turkey (respectively 8.1, 8.3, and 9.1 years) (30,31,39). It is notable that in this study, approximately one-third of children were diagnosed below 5 years of age, which would lower the average age of diagnosis of T1D in our pediatric population. Recent data from several regions of the world have also shown a large increase in the incidence of T1D in the youngest age-group (0-4) years (3,40). The incidence of childhood diabetes differs by age groups and is often reported to peak during the pubertal period. Moreover, an increase in the incidence of diabetes with age to puberty has been reported in several regions in the world (3). In this period of study, the highest incidence rates in Tlemcen were observed in the age groups of 5-9 years and 10-14 years. In this population, the incidence increases with age and peaks between 5-9 years, which is similar to studies conducted in Kuwait (30), in Italy (33), and in Finland (41). While Saudi Arabia (38), Turkey (23), Spain (31), Croatia (35) and some regions of Algeria (12,14,15), have described peak incidence of T1D in the 10-14 age group.

The incidence of childhood diabetes may differ by gender. In our population in Tlemcen, the number of incident cases of T1D is slightly higher in boys than in girls, but the incidence rates were only statistically different in 2015 between boys and girls. However, no significant difference in the incidence of childhood diabetes between boys and girls was observed in Algiers (19) and in several countries of the world (30,31,33,41). In contrast, a female predominance is observed in Saudi Arabia (38), and a male excess has been observed in Hungary (42) and in Finland (43).

The seasonality of the onset of childhood diabetes has been confirmed by the Eurodiab study, and the existence of a winter peak in the onset of childhood diabetes has been described in different regions of Europe (44). During this 4-year period, we noted a predominance of winter peak without significant seasonal variation in the onset of T1D in the region of Tlemcen. Similar findings were reported in other regions of Algeria (12,20) and in other countries (26,27), where more cases of childhood T1D occurs in the

winter season. In contrast, higher incidences were observed in the spring season in Diyarbakir in the Southeast region of Turkey (39). This seasonal variation is supportive of the hypothesis of a viral trigger for childhood diabetes (8), principally the hypothesis of the triggering infection being due to enterovirus (45).

Worldwide, the DKA frequency at diagnosis of T1D varies from 12.8% to 80% (46). However, the frequency of DKA at diagnosis of T1D in our study was 29.2%. Recently in 2016 in the capital Algiers, 17.6% of children aged 0-14 years had DKA (19). Compared to previous studies from other countries, the frequency of DKA in our diabetic children (29.2%) was higher than in Spain (17.8%) (47) and in France (14.8%) (48), but was lower than that reported in Kuwait (33.6%) (49), in Saudi Arabia (40%) (29) and in Turkey (65.9%) (39).

Study Limitations

However, this study presented some limitations. First, it was possible that some cases of monogenic diabetes in children was misclassified because genetic testing for monogenic causes were not routinely practiced in all new children diagnosed with diabetes before nine months of age. Second, due to the short duration of the study, we cannot reliably describe trends of childhood diabetes in this region. Third, we cannot explain the causes of the very high incidence of childhood diabetes in our population because the data on genetic susceptibility factors and environmental triggers are limited.

Conclusion

The incidence of childhood T1D in Tlemcen in Northwest Algeria was 38.5 per 100,000. This incidence is in the "extremely high" category of the WHO project classification for diabetes giving the region a very high risk. Other large-scale epidemiological studies at the national level should be conducted to determine the incidence of childhood diabetes mellitus in Algeria. In addition, further studies on genetic and environmental risk factors for T1D are needed to better explain the high incidence of T1D in children in Algeria.

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Ethics

Ethics Committee Approval: This study was approved by the University Ethics and Deontology Council of the University of Tlemcen, Tlemcen, Algeria (approval number: CEDUT/DZ/019/117).

Informed Consent: Informed consent was obtained from the parents of children.

Peer-review: Externally peer-reviewed.

Authorship Contribution

Concept: Sarra Khater, Ammaria Aouar, Salih Bendedouche, Design: Sarra Khater, Ammaria Aouar, Salih Bendedouche, Data Collection or Processing: Sarra Khater, Nawel Bensmain, Abdellatif Moussouni, Houari Hamdaoui, Zakarya Moqaddem, Analysis or Interpretation: Sarra Khater, Nafissa Chabni, Nawel Bensmain, Abdellatif Moussouni, Literature Search: Sarra Khater, Houari Hamdaoui, Zakarya Moqaddem, Writing: Sarra Khater, Ammaria Aouar, Salih Bendedouche.

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ORIGINAL ARTICLE

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A New Cause of Obesity Syndrome Associated with a Mutation in the Carboxypeptidase Gene Detected in Three Siblings with Obesity, Intellectual Disability and Hypogonadotropic Hypogonadism

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What is already known on this topic?

Various genetic factors play a role in childhood obesity. Mutations in the carboxypeptidase E (*CPE*) gene lead to the inactivation of the *CPE* enzyme, resulting in obesity, age-dependent infertility, hyperglycaemia, disorders of bone metabolism, inflammatory bowel disease and neurological abnormalities in mouse models.

What this study adds?

This study represents a potential disease-causing mutation in *CPE* which is not linked to a specific Mendelian syndrome in the Online Mendelian Inheritance in Man database. Together with only one previously reported case, this study confirms *CPE* as a novel form of Mendelian obesity syndrome.

Abstract

Objective: Carboxypeptidase E (CPE) plays a critical role in the biosynthesis of peptide hormones and neuropeptides in the endocrine system and central nervous system. *CPE* knockout mice models exhibit disorders such as diabetes, hyperproinsulinaemia, low bone mineral density and neurodevelopmental disorders. Only one patient is described with morbid obesity, intellectual disability, abnormal glucose homeostasis and hypogonadotropic hypogonadism, which was associated with a homozygous frameshift deletion in *CPE*.
Methods: Herein are described three siblings with obesity, intellectual disability and hypogonadotropic hypogonadism. Whole exome sequencing (WES) was performed in the index case. Candidate variants were prioritised and segregation of the variant, consistent with the phenotype of the index case, was assessed by Sanger sequencing in affected siblings and parents.
Results: WES analysis revealed a homozygous nonsense c.405C>A (p.Y135*) mutation in *CPE*. Validation and segregation analysis confirmed the homozygous mutation in the index case and his affected siblings. The parents were phenotypically normal heterozygous mutation carriers.
Conclusion: This study provides additional evidence of the association between a homozygous nonsense mutation in *CPE* and a clinical phenotype consisting of obesity, intellectual disability and hypogonadotropic hypogonadism, which may be considered as a new monogenic obesity syndrome.
Keywords: Obesity, hypogonadotropic hypogonadism, carboxypeptidase, carboxypeptidase E

Introduction

Obesity and intellectual disability are found in many genetic disorders, ranging from monogenic disorders to microdeletion syndromes. This suggests that multiple genes are involved or share common pathways in obesity and intellectual disability. Carboxypeptidase E (CPE), which is enriched in mature secretory vesicles, was the

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first identified secretory pathway carboxypeptidase (1). It plays a major role in the biosynthesis of peptide hormones and neuropeptides in endocrine and neuroendocrine cells (2). CPE also serves as a regulated, secretory pathway-sorting receptor for many peptides, including proinsulin, proenkephalin, proopiomelanocortin (POMC) and brain derived neurotrophic factor (NF) (BDNF) (3). Appetite–controlling neuropeptides, such as POMC must be cleaved into several biologically active peptides, including α–melanocyte stimulating hormone and β–endorphin to inhibit food intake. This cleavage is mediated by prohormone convertases such as PC1, PC2, CPE, and peptidyl α–amidating monooxygenase suggesting that inactivated PC1 and CPE play a role in obesity (4). The *CPE* gene is not linked to a specific human syndrome. However, some mouse model studies indicate its role in pathological conditions. Peptidomic studies of mouse brain regions from knockout mice lacking *Cpe* (*Cpe*$^{fat/fat}$) reveal lower levels of most secretory pathway peptides, indicating a major role of CPE in peptide biosynthesis (5). In addition to its role in neuronal development, CPE is also neuroprotective (6,7). *Cpe*$^{fat/fat}$ mouse models, with an inactive CPE enzyme due to point mutations in *Cpe*, have obesity, age-dependent infertility, hyperglycaemia, disorders of bone metabolism, inflammatory bowel disease and neurological abnormalities (3,8,9). *CPE* is located on chromosome 4q32.3 and has nine exons. To date, only one mutation, a frameshift homozygous mutation (c.76_98del; p.E26RfsX68) within *CPE*, has been described in a patient with morbid obesity, type 2 diabetes mellitus, intellectual disability and hypogonadotropic hypogonadism; this is not listed as a specific Mendelian syndrome in the Online Mendelian Inheritance in Man (OMIM) database (10).

Clinical and genetic heterogeneity in obesity and intellectual disability represents a major diagnostic challenge. Although the presence of distinct clinical features may help in identifying a specific cause in some cases, most patients remain undiagnosed. Many genes have been linked to underlying Mendelian aetiology; however, the diagnostic power of genome sequencing remains limited, ranging from 8-70 % of cases (11). Whole exome sequencing (WES) is a powerful tool identifying many single gene disorders, including genetic causes for patients with syndromic obesity. Here we present WES results of three Turkish siblings, born to consanguineous parents, having obesity, intellectual disability, and hypogonadotropic hypogonadism, revealing a homozygous nonsense mutation in *CPE*. These cases support the previously reported syndromic case with a truncating mutation, which suggests a new monogenic obesity syndrome. Therefore, *CPE* deficiency should be considered in such cases.

Methods

Cases

The index case was a 15-year-old boy (Case 1), who has an affected 19-year-old sister (Case 2) and an affected three-year-old brother (Case 3). Consanguineous marriage was confirmed between their parents as shown in the pedigree (Figure 1). Peripheral blood samples were collected from the patient and his siblings for routine haematological and biochemical examination and DNA sequencing analysis. Informed consent was obtained from the parents for genetic analysis and for publishing their photographs in this study.

Case 1

The index case was referred to paediatric endocrinology with obesity, hypothyroidism, micropenis, undescended testes and intellectual disability at the age of 11 years. He was the second child from a first cousin marriage. He was born on the 38th week of gestation by caesarean section. At birth, he weighed 3200 g [-0.4 standard deviation score (SDS)] and measured 50 cm in length (0 SDS). Medical history revealed delayed developmental milestones. He had significant hypotonicity at infancy. He achieved head control at 1-year-old and could walk at five-year-old. He had marked speech delay and could say a few words only. Excess weight gain was reported after four years of age due to increased appetite. He used a ventilation tube due to recurrent otitis media. Hypothyroidism was diagnosed at nine years of age. Physical examination showed his weight was 62.8 kg (2.1 SDS), height was 139.5 cm (-0.78 SDS), body mass index (BMI) was 32.2 kg/m^2 (2.4 SDS) and target height was 170.2 cm (-0.96 SDS) (Figure 2). Dysmorphic examination showed that he had a round face, full cheeks, micrognathia, gynaecomastia and micropenis (Figure 3). His stretched penile length was 3.5 cm (< 3rd percentile) with cryptorchidism (suprascrotal gonads) of 1 mL volume. His laboratory findings were as follows: morning fasting

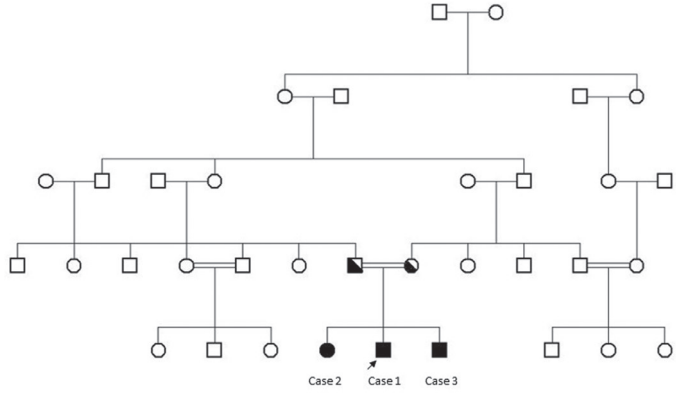

Figure 1. Pedigree of the family

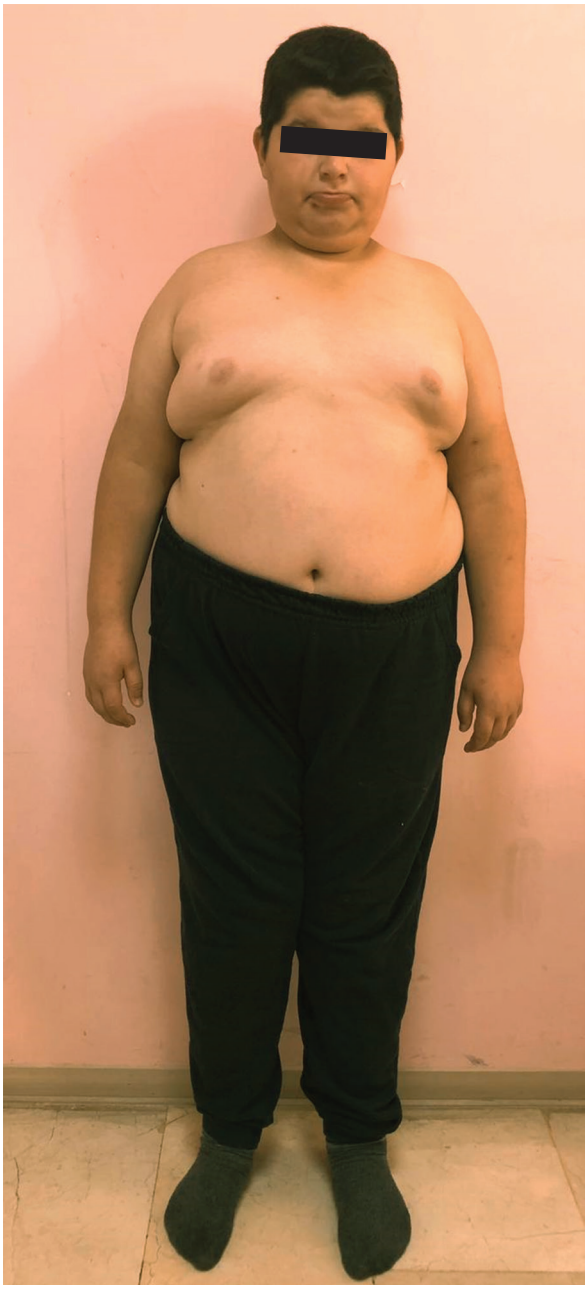

Figure 2. General appearance of the index case

glucose 80 mg/dL, insulin 66 uU/mL, HOMA-IR 11.8 (severe insulin resistance), thyroid stimulating hormone (TSH) 0.07 mU/L and free thyroxine (FT4) 0.59 ng/dL, suggesting central hypothyroidism (Table 1). Thyrotropin releasing hormone stimulation test revealed that the TSH response was 0.83, 0.85 and 0.41 mU/L at 30, 60 and 90 min respectively. A diagnosis of central hypothyroidism was made. Upon evaluating other pituitary hormone deficiencies, the following was reported: early morning (08:00 am) prolactin 6.9 ng/mL, follicle-stimulating hormone (FSH) 0.76 mIU/mL, LH 0.22 mIU/mL, total testosterone 0.1 ng/dL, adrenocorticotropic hormone (ACTH) 31.2 pg/mL and

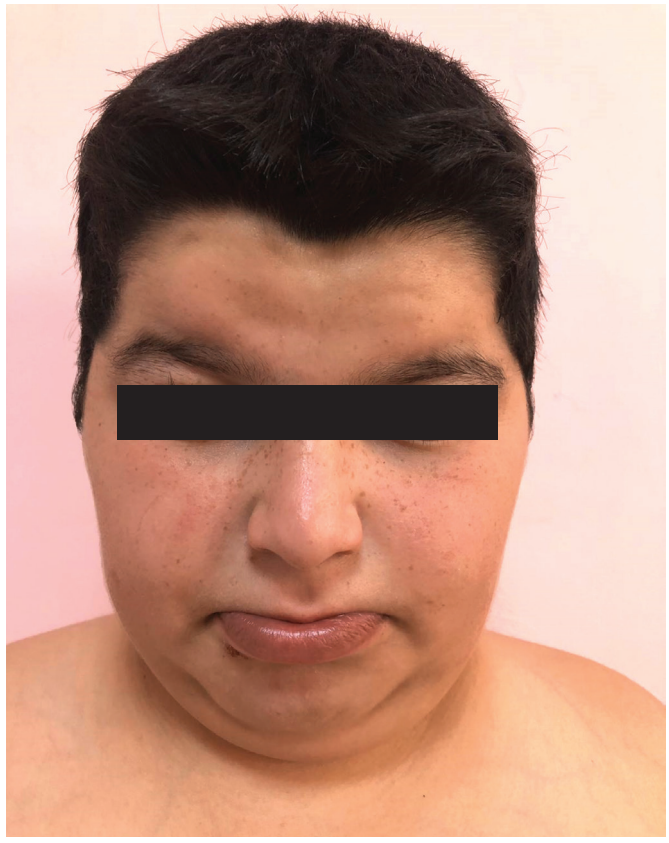

Figure 3. Facial appearance of the index case

cortisol 6.1 µg/dL. Corticotrophin releasing hormone (CRH) stimulation test showed a normal peak cortisol response of 22.1 µg/dL, which excluded central adrenal insufficiency. On his last visit at the age of 15 years, he weighed 96 kg (+2 SDS), was 156 cm (-2 SDS) tall and had a BMI of 39.4 kg/m^2 (+3.28 SDS). He remained prepubertal and had low levels of gonadotropins with micropenis and cryptorchidism. His initial diagnosis was hypogonadotropic hypogonadism and a luteinising hormone (LH) releasing hormone (LHRH) stimulation test was planned.

Cranial/hypophysial magnetic resonance imaging (MRI), abdominal ultrasonography, skeletal survey, echocardiography and eye examination were normal. Chromosomal analysis revealed a normal male karyotype (46,XY). Differential diagnosis fluorescence in situ hybridization analysis for Prader-Willi syndrome and chromosomal microarray analysis were found to be normal. According to the clinical and laboratory findings of the index case and similar clinical findings in his 19-year-old sister, with intellectual disability and obesity, and three-year-old brother, with global developmental delay and central hypothyroidism, it was thought that a differential diagnosis for obesity and mental retardation syndromes, with an autosomal recessive inheritance pattern, should be established.

Table 1. Clinical and laboratory findings of patients

Clinical and laboratory features	Case 1	Case 2	Case 3
Age (years)/sex	**15/male**	**19/female**	**3/male**
Pathologic findings	Obesity Infantile hypotonia Round face Full cheeks micrognathia Gynaecomastia Central hypothyroidism Hypogonadotropic hypogonadism Micropenis Undescended testis Intellectual disability Motor developmental delay ASD	Obesity Infantile hypotonia Round face Full cheeks Large ears micrognathia Puberty tarda Primary amenorrhea Intellectual disability Motor developmental delay	Infantile hypotonia Round face Full cheeks Large ears micrognathia Central hypothyroidism Intellectual disability Motor developmental delay
Glucose (mg/dL)	80 (70-100)	76 (70-100)	83 (70-100)
Insulin (uU/mL)	**66 (0-17)**	37.1 (0-17)	NA
TSH (mU/L)	**0.07 (0.9-5.5)**	2.63	**0.92 (0.9-5.5)**
FT4 (ng/dL)	**0.59 (0.95-2.0)**	**0.55 (0.93-1.7)**	**0.03 (0.95-2.0)**
ACTH (pg/mL)	31.2 (6-48)	N/A	23.2 (6-48)
Cortisol (µg/dL)	6.1 (3-21)	N/A	9.6 (3-21)
FSH (mIU/mL)	**0.76 (1.8-3.2)**	N/A	0.83 (0.26-3.0)
LH (mIU/mL)	0.22 (0.2-4.9)	8.68 (2.4-12.6)	0.12 (0.02-0.3)
Total testosterone (ng/dL)	**0.1 (18-150)**	-	< 12 (< 10)
Estradiol (pg/mL)	-	22.5	-
Prolactin (ng/mL)	6.9 (3.2-19)	22.5	20.4 (3.2-19)
IGF-1 (µg/L)	104 (139-395)	3.77 (4.7-23.3)	93.5 (15-129)
Leptin (ng/mL)	11.8 (3.3-18.3)	N/A	N/A
TRH test peak TSH (mU/L)	**0.85**	N/A	
CRH test peak cortisol (µg/dL)	22.8 (> 18)	N/A	
CRH test peak ACTH (pg/mL)	130		

ACTH: adrenocorticotropic hormone, ASD: atrial septal defect, CRH: corticotrophin releasing hormone, FT4: free thyroxine, FSH: follicle-stimulating hormone, IGF-1: insulin-like growth factor-1, LH: luteinising hormone, TSH: thyroid stimulating hormone, TRH: thyrotropin-releasing hormone, N/A: not available

Case 2

The proband's 19-year-old sister was delivered at full term by caesarean section with appropriate birth weight. Her developmental milestones were delayed. She started to walk at six-year-old, used a few words and could not form sentences. Her history revealed puberty tarda, with a primary amenorrhea. Physical examination showed that her weight and height were 78 kg (> 97th centile) and 160 cm (25-50th centile), respectively. She was obese with a BMI of 30.4 kg/m^2. She was Tanner stage 2. She had intellectual disability. Physical examination revealed full cheeks, micrognathia and round face as dysmorphic features (Figure 4). In the most recent laboratory examination, insulin resistance and central hypothyroidism were detected. Her LH value was within normal range, but she had insufficient estrogen values. Whether this is a late onset puberty, or a pause of puberty can't be differentiated. Further stimulation tests were planned.

Case 3

The proband's three-year-old brother was born at the 38th week of gestation via caesarean section with a weight of 3200 g. During the neonatal period, he was diagnosed with congenital hypothyroidism on the 15th day of life. He was hypotonic and at two months old he had a history of aspiration. Medical history revealed delayed developmental milestones; he could not walk or talk and had been sitting up for only six months. Physical examination showed that he weighed 18 kg (1.9 SDS), was 99 cm (1.5 SDS) tall and had a BMI of 16.32 kg/m^2 (0.26 SDS). On his genital examination bilateral testicles were palpable within the scrotum with a volume of 2 mL. Dysmorphic features included round

Figure 4. Facial appearance of affected sister (Case 2) of the index case

Figure 5. Facial appearance of affected brother (Case 3) of the index case

face, full cheeks, large ears and micrognathia (Figure 5). Thyroid function tests were FT4 0.03 ng/dL and TSH 0.92 mU/L, indicating central hypothyroidism. Other laboratory biochemical parameters were normal; FSH 0.83 mIU/mL, LH 0.12 mIU/mL, ACTH 23.2 pg/mL and cortisol 9.6 µg/d. Since he was pre-pubertal, it was impossible to detect whether he had hypogonadotropic hypogonadism. The pituitary MRI was normal. Echocardiography showed atrial septal defect and patent ductus arteriosus.

Whole Exome Sequencing

Next-generation sequencing was performed for WES analysis using the Ion S5™ Sequencer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The Ion AmpliSeq™ Exome RDY kit (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) was used, according to the manufacturer's protocol. The Ion reporter software v.5.2 (Thermo Fisher Scientific) was used to analyse the mutations (https://ionreporter.thermofisher.com/ir/). Under an assumed autosomal recessive mode of inheritance, all variants were assessed individually according to the clinical phenotype, minor allele frequency (MAF) score and pathogenicity scores were calculated using prediction programmes. Variants were filtered to retain non-synonymous changes with a MAF of <0.01 using combined datasets from the 1000 Genomes Project, the Exome Variant Server project and the Genome Aggregation Database (GnomAD). The potential functional impact of the disease candidate variants were assessed using SIFT (http://sift.jcvi.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), MutationTaster (http://www.mutationtaster.org/) and VarSome (https://varsome.com/). All genetic variants were screened for pathogenicity, mode of inheritance and clinical phenotypes. Finally, candidate pathogenic variants identified by WES were verified with Sanger sequencing.

Validation by Sanger Sequencing

Sanger sequencing was used to validate the novel *CPE* mutation using the 3130 genetic analyser (Applied Biosystems, Foster City, USA). Primers designed to amplify exon 2 of *CPE* (NM_001873.4) are as follows: forward, 5'-TGTAGGTATACAATATATTTGGCTCTG-3' and reverse, 5'-CCATCTGTAAGCTTTGTGCG-3'. The sequencing results were analysed using the Genomics Workbench 20.0 (QIAGEN) (https://digitalinsights.qiagen.com/). Sanger sequencing was also performed in the affected brother and sister and the parents to evaluate segregation in the family.

Results

WES analysis, performed due to a lack of preliminary diagnosis of the index case, revealed 52,465 unfiltered variants initially. An average coverage at $>140\times$ read depth for 96 % of the exome was attained. The variants were filtered out according to zygosity, MAF, location and type of

mutation. Thereafter, 14 variants in seven genes were found to be potential disease-causing variants and classified as pathogenic or variants of unknown significance, according to the American College of Medical Genetics guidelines (Table 2). Initial analysis revealed 2 homozygous and hemizygous nonsense mutations in the *CPE* and *COL4A6* genes, respectively. After interpretation of these variants with the scope of clinical presentation of the case, homozygous nonsense (NM_001873.4):c.405C > A (p.Y135*) mutation in *CPE* gene (Chr4:166385639-C/A) was considered to be the candidate disease-causing variant (Figure 6). Mutations in *COL4A6* is associated with deafness which was not found in our patient. The *CPE* variant was not found in the Exome Aggregation Consortium, GnomAD exomes or genomes, and in our 100 in-house controls. The gene was not linked to a specific human disease in the OMIM database. The homozygous nonsense mutation showed high pathogenic scores in prediction software, such as VarSome, Mutation Taster and FATHMM-MKL, suggesting a strong causative role in the pathogenicity of the disease (Table 3) (12,13). After confirming the mutation by Sanger sequencing, the siblings and parents of the index case were also sequenced (Figure 7). The affected siblings were homozygous and the parents were heterozygous for the mutation.

Discussion

In this study, we present three affected siblings having the same homozygous c.405C > A (p.Y135*) mutation in *CPE*, which can be classified as a distinct syndromic obesity gene. CPE is a neuropeptide-processing enzyme, expressed abundantly in neural and endocrine tissues (14). CPE plays an important role during embryonic and postnatal brain development as a neuroprotective factor. CPE is also known as NF-α1 as it induces ERK and Akt signalling, similar to classic trophic factors, such as BDNF or nerve growth factor (13). CPE was thought to function intracellularly to process a precursor protein with neuroprotective activity besides its role as an extracellular neuroprotective trophic factor, independent of its enzymatic activity (15). A novel role for CPE in the development and branching of proximal dendrites, necessary for cortical neuron migration and dendritogenesis, has been proposed (6). CPE also has functions in prohormone sorting and vesicle transport in the endocrine and nervous system (7,14). The neuroprotective role of *Cpe* has been demonstrated in knockout mouse models showing severe neurodevelopmental delay, neurodegeneration and depression. Cases presented in this study showed severe neurodevelopmental delay that may be linked to a loss of enzymatic activity of CPE and may be linked to defective cortical neuron development.

The $Cpe^{fat/fat}$ mice exhibit a decrease in CPE levels and an increase in proinsulin levels, thereby confirming the role of CPE in insulin dysregulation (16,17). A spontaneous point mutation in *Cpe*, which diminishes its enzymatic activity, results in severe obesity; thus, the model carrying this mutation is called the Cpe^{fat}/Cpe^{fat} mouse model (18). In another mouse model, *Cpe* knockout mice, in which exons 4 and 5 are deleted, showed insulin resistant diabetes, obesity, infertility and neurological and behavioural abnormalities (19). The Cpe^{fat}/Cpe^{fat} mouse model exhibits elevated levels of hormones and neuropeptide precursors and decreased

Table 2. List of pathogenic variants after filtration strategy

Gene name	Zygosity	Mutation	ACMG criteria	Pathogenity
CPE	Homozygous	c.405C > A (p.Y135X)	PVS1, PM2, PP3	Pathogenic
COL4A6	Hemizygous	c.2680G > T (p.G894X	PVS1, PM2, PP3	Pathogenic
COL4A6	Hemizygous	c.2681G > T (p.G894V)	PM2, PP3	VUS
TNXB	Homozygous	c.7460G > A (p.R2487H)	PM1, PM2	VUS
DNAH8	Homozygous	c.6323C > T (p.S2108L)	PM1, PM2, PP3	VUS
MUC4	Homozygous	c.6344A > T (p.D2115V)	PM1, PM2, BP4	VUS
MUC4	Homozygous	c.6265A > G (p.I2089V)	PM2, BP4	VUS
MUC4	Homozygous	c.6206A > G (p.N2069S)	PM2, BP4	VUS
PRKCSH	Homozygous	c.1144C > T (p.R382W)	PM1,PM2, PP3	VUS
KIR2DL3	Homozygous	c.842A > G (p.E281G)	PM2, BP4	VUS
KIR2DL3	Homozygous	c.941G > C (p.R314T)	PM2, BP4	VUS
KIR2DL3	Homozygous	c.953G > C (p.R318P)	PM2, BP4	VUS
KIR2DL3	Homozygous	c.971T > C (p.V324A)	PM2, BP4	VUS
KIR2DL3	Homozygous	c.166C > G (p.Q56E)	PM2, BP4	VUS

ACMG: American College of Medical Genetics criteria: PVS: pathogenic very strong, PM: pathogenic moderate, PP: pathogenic supporting, BP: benign supporting, VUS: variant of unknown significance

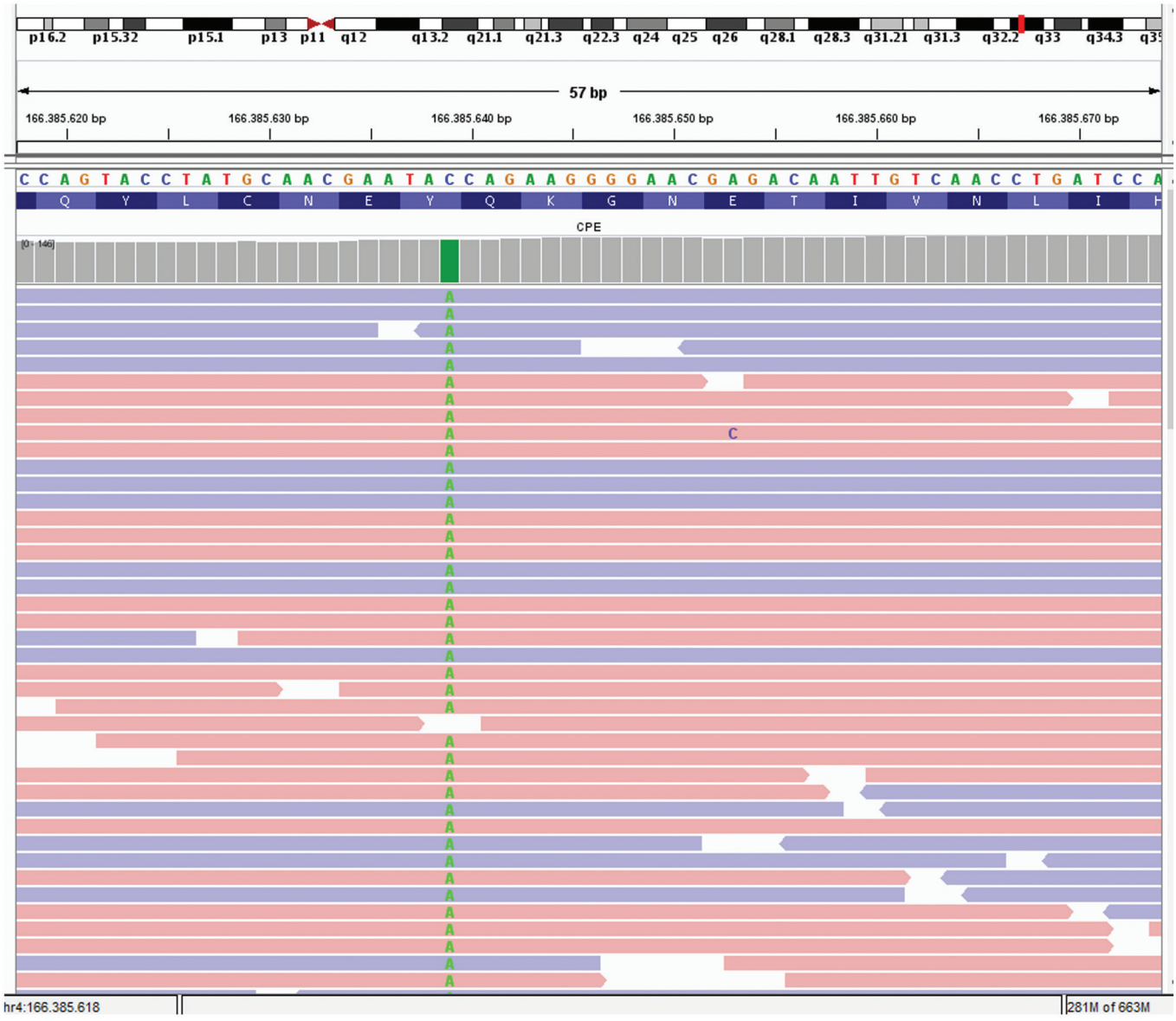

Figure 6. Next generation sequencing analysis of the proband showing homozygous c.405C > A (p.Y135*) mutation in *CPE* gene

Table 3. Results of prediction programs for the *CPE* mutation c.405C > A (p.Y135X)

Prediction program	Prediction (score)
Varsome	Pathogenic
DANN score	0.9967
Mutation taster	Disease causing (1)
FATHMM-MKL	Damaging (0.8675)

levels of bioactive peptides, suggesting that CPE has a role in the processing of prohormones and proneuropeptides (16,17,18,20). *Cpe*$^{fat/fat}$ mice with p.Ser202Pro mutation present with endocrinological abnormalities, such as obesity, diabetes and infertility due to the lack of enzyme activity and display a variety of behavioural abnormalities (21). In another study, a mutation in CPE-NFα1, consisting of three adenosine inserts, introduced nine amino acids, including two glutamines into the mutant protein, called CPE-QQ, and resulted in its accumulation in the endoplasmic reticulum (ER) and the subsequent degradation by proteasomes. Mice having this mutation show neurodegeneration in the hippocampus and prefrontal cortex, deficits in neurogenesis at the dentate gyrus and hyperphosphorylation of tau protein (22). A mutation in human *CPE-NFα1*, c.T980C (p.W235R), causes a loss of its enzyme activity and neurotoxic accumulation in the ER, which results in ER stress and cell death and finally, neurological disorders (23).

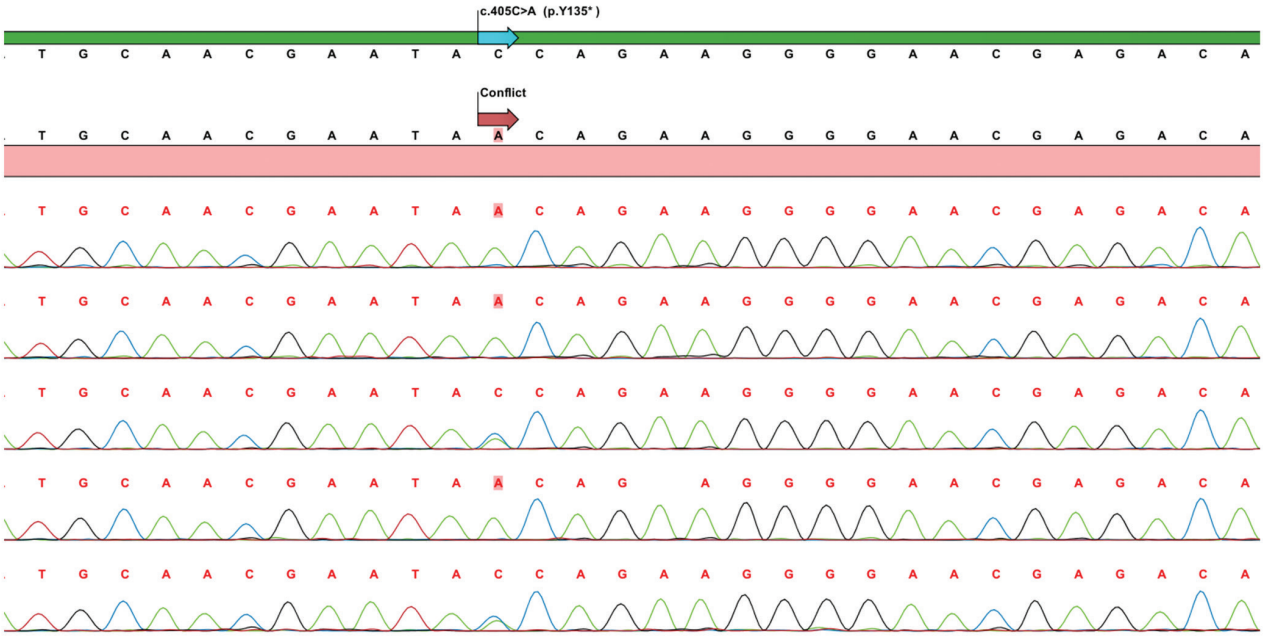

Figure 7. Validation and segregation analysis of the family by Sanger sequencing revealing c.405C > A (p.Y135*) (NM_001873) mutation. (Line 1: index case, Line 2: Case 2, Line 3: Mother, Line 4: Case 3, Line 5: Father)

Only one study has demonstrated a case with a null mutation having similar symptoms to the three siblings, including obesity, diabetes, hypogonadotropic hypogonadism and impaired intellectual ability (10). Together with the previous case, we may conclude that the nonsense mutation detected in our index case resulted in a nonsense-mediated mRNA decay and the complete absence of a functional CPE protein, which caused the syndromic features of the cases. Polymorphism studies revealed an association of *CPE* variants with BMI or non-insulin dependent type 2 diabetes mellitus in different cohorts (24,25). A missense c.847C > T (p.Arg283Trp) variant was detected in type 2 diabetes mellitus in Ashkenazi families, which was linked to hyperproinsulinaemia and diabetes (26). Both *Cpe* knock out mice models and polymorphism studies in humans support the role of CPE in endocrinological defects, such as obesity, hypothyroidism and hypogonadotropic hypogonadism, which are present in our cases.

CPE is not linked to a specific Mendelian syndrome in the OMIM database. During WES data interpretation, if only OMIM genes were analysed by analysing software programs, this variant may have been filtered out. It is necessary to search the databases to support a correlation between potential disease-causing variants and clinical findings in the index case. In our case, as this variant has a high score in the ACMG guidelines variant classification, we focused on this variant by validating in the index case and segregation in the family. The variant found in our family may improve the diagnostic yield in patients with this syndromic obesity.

Study Limitations

Our results should be supported with more cases with variations in *CPE* and obesity, intellectual disability and hypogonadotropic hypogonadism. Moreover, functional studies should be performed using *in vivo* models.

Conclusion

Obesity and intellectual disability have clinical and genetic heterogeneity. In this report we present three siblings having obesity, intellectual disability and hypogonadotropic hypogonadism with a novel mutation in *CPE*, which is not linked to a specific human Mendelian disease. Together with the findings from a previous case, *CPE* can be considered as a candidate gene for a new monogenic obesity syndrome.

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Ethics

Ethics Committee Approval: The study were approved by the Ege University of Local Ethics Committee (protocol number: 21-3T/8).

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally and internally peer-reviewed.

Authorship Contribution

Medical Practices: Asude Durmaz, Ayça Aykut, Durdugül Ayyıldız Emecen, Aysun Ata, Tahir Atik, Samim Özen, Esra Işık, Damla Gökşen, Özgür Çoğulu, Ferda Özkınay, Data Collection or Processing: Asude Durmaz, Ayça Aykut, Tahir Atik, Durdugül Ayyıldız Emecen, Aysun Ata, Samim Özen, Analysis or Interpretation: Asude Durmaz, Ayça Aykut, Literature Search: Asude Durmaz, Ayça Aykut, Durdugül Ayyıldız Emecen, Samim Özen, Writing: Asude Durmaz, Tahir Atik, Durdugül Ayyıldız Emecen, Samim Özen.

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ORIGINAL ARTICLE

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Transforming Growth Factor-β1 and Receptor for Advanced Glycation End Products Gene Expression and Protein Levels in Adolescents with Type 1 Diabetes Mellitus

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What is already known on this topic?

As the non-enzymatic glycation products rise under conditions of chronic hyperglycemia, advanced glycation end products (AGE) through interaction with its receptor (RAGE) activates a range of signaling pathways which play an important role in the pathogenesis of diabetic complications (nephropathy, retinopathy, neuropathy and atherosclerosis). Transforming growth factor-β1 (TGF-β1) as a multifunctional cytokine, exerts pleiotropic effects from differentiation and development to cell growth and immunity regulation. Induced by many factors, including hyperglycemia, TGF-β1 is an important mediator in the pathogenesis of diabetic nephropathy, mainly stimulating the production of extracellular matrix components.

What this study adds?

The decrease in TGF-β1 gene expression in peripheral blood mononuclear cells significantly independently correlated with type 1 diabetes (T1D) and might be used as a potential biomarker for early cardiovascular risk assessment in adolescents with T1D by predicting early elevation of urinary albumin excretion rate. Decrease in transmembrane full-length RAGE gene expression, increase in TGF-β1 and soluble sRAGE concentrations could serve as biomarkers independently associated only with T1D presence.

Abstract

Objective: Type 1 diabetes (T1D) mellitus is one of the most frequent autoimmune diseases in childhood. Chronic complications are the main causes of cardiovascular morbidity and mortality in T1D. Although interactions between advanced glycation end products (AGE) and their receptors (RAGE) and transforming growth factor-β1 (TGF-β1) are implicated in development and progression of diabetic micro- and macro-vascular complications, they also have important roles in immune system regulation.

Methods: Blood samples were obtained from 156 adolescents with T1D and 80 apparently healthy controls. T1D patients diagnosed with any other autoimmune disease and receiving any kind of drugs except insulin therapy were excluded from this study. Exclusion criteria for controls were positive family history of T1D and drugs/supplements application. TGF-β1 and transmembrane full-length RAGE (flRAGE) messenger ribonucleic acid (mRNA) levels in peripheral blood mononuclear cells (PBMC) were obtained by quantitative polymerase chain reaction (qPCR) method. Circulating levels of biochemical markers, TGF-β1 and soluble RAGE (sRAGE) levels were also determined.

Results: TGF-β1 and flRAGE mRNA levels were significantly higher in controls compared to patients ($p < 0.001$, for both). However, TGF-β1 and sRAGE levels were higher in patients than controls ($p < 0.001$, for both). There were significant independent associations of all mRNA and protein levels with T1D. TGF-β1 mRNA was the only marker independently negatively associated with urinary albumin excretion rate in T1D adolescents ($p = 0.005$).

Conclusion: Our results indicated gene expression downregulation of TGF-β1 and flRAGE in PBMC of T1D adolescents. TGF-β1 mRNA downregulation may be useful for predicting early elevation of urinary albumin excretion rate.

Keywords: Transforming growth factor-β1, receptor for advanced glycation end products, type 1 diabetes, urinary albumin excretion rate, quantitative polymerase chain reaction

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Introduction

Type 1 diabetes (T1D) mellitus is a chronic, T lymphocyte-mediated autoimmune disease leading to destruction of pancreatic Langerhans β-cells, endogenous insulin secretion decline and consequent hyperglycemia (1). Although, autoantibodies for β-islet cell constituents are mediate the ongoing autoimmune process and are useful in T1D diagnosis (2), other markers that reflect T lymphocyte activities can be used (3). Growing knowledge about gene expression changes in regulatory and effector immune cells, as new biomarkers, could provide novel insights into the pathogenesis of T1D (3,4). Autoreactive T cells, together with immune cells such as monocytes and other lymphocytes, which target islet β-cells, are capable of inducing T1D development.

Chronic hyperglycemia, either good or poorly regulated, causes the formation of advanced glycation end products (AGEs), which are a large group of irreversibly modified proteins (5). Contrary to early glycated end products (Schiff bases and Amadori products such as fructosamine) (5), AGEs express their effects by binding to transmembrane, full-length receptor for AGEs (flRAGE) (6). flRAGE on interacting with AGE elicits a loss of balance in oxidant and antioxidant production and gives rise to inflammatory and thrombogenic responses in endothelial cells (7). Accordingly, AGE-flRAGE interaction has been reported to induce vascular stiffening, angiogenesis, and extracellular matrix (ECM) accumulation (5,8). Through these mechanisms, flRAGE-AGE interaction exerts major effects in chronic micro- and macro-vascular complications (nephropathy, neuropathy, retinopathy, and atherosclerosis) development. Also, enhanced flRAGE expression interacting with AGE in T cells may induce their inflammatory functions, leading to β-cell injury during T1D progression (9). However, flRAGE activation may also suppress autoimmune responses by activation of regulatory T cells (Treg) (10).

Circulating RAGE isoforms, termed soluble RAGE (sRAGE), can bind AGEs and thus prevent their detrimental effects following flRAGE activation (11). In this manner, sRAGE exhibits a protective role. It has been reported that by blocking flRAGE, sRAGE exerted an anti-atherogenic effect through inhibiting cell migration and enlargement of atherosclerotic lesions (12). However, other studies demonstrated that sRAGE induced vascular permeability by increasing production of proinflammatory and chemo-attractant molecules, thus maintaining vascular inflammation and progression of atherosclerotic process (13).

As multifunctional cytokine, transforming growth factor-β1 (TGF-β1) stimulates production of ECM proteins including collagen 1 and 4, laminin, and fibronectin, thus participating in extracellular remodelling in peripheral organs leading to development of micro- and macro-vascular chronic complications (14,15). TGF-β1 is likely to be an essential factor in the development of diabetic nephropathy. On the other hand, TGF-β1 secreted by Tregs has a role in autoreactive T cells suppression, induction of immune tolerance and inhibition of proinflammatory cytokine production (14). These opposing effects of TGF-β1 make it an interesting biomarker for early evaluation of T1D and its chronic complications development.

The association of AGE-flRAGE and TGF-β1 exists during development of diabetes and diabetic nephropathy. TGF-β-activated kinase 1 stimulated by AGEs, plays an important role in innate immune responses and inflammation, activating main proinflammatory pathways (mitogen-activated protein kinases - MAPKs and nuclear factor-κB), macrophage polarization from M2 to M1, and inflammatory cytokine production (16,17). Moreover, AGEs stimulate fibrotic processes leading to the appearance of myofibroblasts and the accumulation of ECM components via the TGF-β1-independent Smad3 signaling pathway (18).

Accordingly, the first aim of this study was to investigate whether changes in TGF-β1 and flRAGE gene expression in peripheral blood mononuclear cells (PBMCs) and TGF-β1 and sRAGE serum protein levels were associated with the presence of T1D autoimmunity. Our second goal was to contribute to current knowledge by identifying whether the expression of these genes and protein levels could be related to suboptimal/poor glycemic control and future diabetic chronic complications, such as early elevation of urinary albumin excretion rate.

Methods

Adolescents with T1D and apparently healthy adolescents as controls were enrolled in the study. The groups were matched by age, pubertal stage, and body mass index (BMI). The participants were recruited from the Mother and Child Health Care Institute of Serbia "Dr. Vukan Čupić", Belgrade, during regular follow in the outpatient clinic. Diabetes was diagnosed according to Serbian national and international guidelines of good clinical practice for diagnosis and treatment of diabetes mellitus (19,20). Patients with T1D were treated with intensive insulin therapy given as a basal-bolus regimen. Good, suboptimal/poor glycemic control in T1D patients was defined according to a glycated hemoglobin A1c (HbA1c) concentration cut-off of 7.5% (21). Exclusion criteria for T1D patients were: medicaments usage (except insulin); presence of cystic fibrosis; presence

of other autoimmune diseases such as thyroiditis and celiac disease. Exclusion criteria for the control group were positive family history of T1D and use of drugs and supplements.

In all study participants the following evaluations were performed: demographic data collection, clinical data collection including age, body weight, body height, BMI, puberty staging, age at diabetes onset, diabetes duration, insulin dosage and laboratory tests included blood glucose, creatinine, HbA1c, and C-reactive protein (CRP), urinary albumin and glomerular filtration rate (GFR), and TGF-β1 and RAGE gene expression and protein levels.

Body weight was measured to the nearest 0.1 kg by a portable electronic scale (Tanita, Amsterdam, Netherlands). Body height was measured to the nearest 0.1 cm using a portable wall-mounted stadiometer. BMI was calculated as body weight (kilograms) divided by the squared height (meters). Puberty stages were stratified by clinical examination according to Tanner (22).

Blood samples from all study participants were collected into two serum and one ethylenediaminetetraacetic acid containing vacutainers (BD Vacutainer®, New Jersey, USA). After venepuncture, serum and plasma were immediately separated and stored at -80 °C prior analyses.

Glucose and creatinine were assayed in serum samples using routine laboratory methods. HbA1c level was determined by competitive turbidimetric inhibition immunoassay. CRP was measured using immunoturbidimetric method. All the analyses were performed on Roche/Hitachi c501 automated analyser (Roche, Mannheim, Germany). Urinary albumin was determined in the timed overnight sample using a nephelometer, the Siemens BN ProSpec® System (Siemens, Erlangen, Germany). Early elevation of albumin excretion rate cut-off was set as ≥7.5 μg/min because these values were shown to predict subsequent persistent microalbuminuria development later in life (23). Estimated glomerular filtration rate (eGFR) was assessed using the Schwartz equation. The TGF-β1 protein levels were determined in serum samples and sRAGE protein levels were measured in plasma, using enzyme-linked immunosorbent assays according to the manufacturer's manual (DuoSet, R&D systems, Wiesbaden, Germany).

PBMCs were isolated after plasma separation using the Ficoll-Paque® PLUS gradient-gel (GE Healthcare, Wisconsin, USA) according to the manufacturer's instructions. After isolation, but before freezing at -80 °C, PBMC were suspended in 1 mL of TRIzol™ reagent (Invitrogen Life Technologies, Foster City, USA).

Total ribonucleic acid (RNA) from PBMCs was isolated using a modified classic RNA isolation protocol, described by Chomczynski (24), which was optimized for the laboratory of the Department for Medical Biochemistry, University of Belgrade-Faculty of Pharmacy, explained in detail and published elsewhere (25).

Reverse transcription and quantitative real-time polymerase chain reaction (qPCR) experiments were performed on the 7500 real-time PCR System using Assays-on-Demand based on TaqManTM chemistry (Applied Biosystems, Foster City, USA).

The qPCR reactions were performed using TaqMan™ 5'-nuclease gene expression assays (Applied Biosystems, Foster City, USA) for TGF-β1 (Hs00998133_m1) and flRAGE (Hs00153957_m1) genes. The relative standard curve method was used for gene expression quantification. Relative gene expression levels were expressed as a ratio between target gene and constitutively expressed gene as housekeeping gene (β-actin) messenger RNA (mRNA) using the following equations:

Normalised TGF-β1 mRNA levels = TGF-β1 mRNA/β-actin mRNA

Normalised flRAGE mRNA levels = flRAGE mRNA/β-actin mRNA.

Negative controls for reverse transcription (no reverse transcriptase enzyme) and for qPCR (no complementary deoxyribonucleic acid) were included in the experiments.

The study was carried out in line with the principles of the Declaration of Helsinki and approved by the Ethics Committees of Mother and Child Health Care Institute of Serbia "Dr. Vukan Čupić" (protocol number: 8/8, date: April the 9th 2015) and University of Belgrade-Faculty of Pharmacy (protocol number: 2536/2, date: December 26th 2018). Written informed consent was obtained from all the participants and their parents.

Statistical Analysis

All statistical testing was performed using a statistical program, SPSS Statistics, version 22 (IBM Inc., Chicago, IL., USA). Distribution of continuous variables was tested by Kolmogorov-Smirnov test. Continuous normally distributed data are presented as arithmetic mean ± standard deviation. If normal distribution was not achieved after logarithmic transformation, skewed distributed data were presented as median (interquartile range).

Comparisons between the tested groups were made using Student's *t*-test for normally distributed data and by Mann-Whitney *U* and Kruskal-Wallis tests for skewed distributed

data. Categorical data were given as absolute frequencies and compared by chi-square test for contingency tables. Associations between clinical data were tested by Spearman's bivariate correlation analysis. In-depth possible associations of tested markers with T1D and urinary albumin excretion rate were assessed by univariate and multivariate binary logistic and ordinal regression analyses, respectively. Binary logistic regression analysis was used to find single predictors and models (mRNA and protein levels) as explanatory variables associated with T1D presence. Ordinal regression analysis was used as a statistical test to determine potential associations of single markers and models and T1D complication (early elevation of urinary albumin excretion rate). In multivariate ordinal regression analysis, there were no multicollinearity between independent variables (predictors) and all of them had an identical effect at each cumulative split of the ordinal dependent variable (urinary albumin excretion rate quartiles). Data from bivariate correlation are presented as correlation coefficient (ρ). Data from binary logistic and ordinal regression analyses are presented as odds ratio (OR) and 95% confidence interval (CI). Explained variations in T1D and urinary albumin excretion rate were assessed by Nagelkerke R^2. The statistically significant level was set at $p < 0.05$.

Results

Clinical and Laboratory Data in Tested Groups

156 adolescents with T1D and 80 controls were enrolled in the study. The median age of the T1D adolescents (49% females) was (12-16) years. The median T1D duration was 7 (5-8) years. In the T1D group, 33 (21%) had good glycemic control and 123 (79%) had suboptimal/poor glycemic control. In the control group of 80 participants (82% females) median age was 15 (13-17) years. General anthropometric and biochemical data of tested populations were presented in the Table 1. No statistically significant differences between T1D patients and the controls were found in terms of age and BMI. However, significantly more females than males were in the control group compared to the patient group ($p < 0.001$). Adolescents in the control group had significantly lower glucose, HbA1c and CRP than patients with T1D (Table 1).

Patients with T1D had significantly lower TGF-β1 mRNA and flRAGE mRNA levels (Figure 1A, 1C), but higher TGF-β1 and sRAGE protein concentrations than controls (Figure 1B, 1D).

In order to examine whether discrepant gender distribution in tested groups could affect obtained results, we first compared clinical markers between males and females of the control group. Then we compared only females and finally we compared only males between patient and control groups. There were no significant differences in any clinical marker between genders (66 females vs 14 males) of the control group. The same trends for examined markers were found between females from control group ($n = 66$) vs patient group ($n = 76$) and males from control group ($n = 14$) vs patient group ($n = 80$) as we already obtained when we analysed both genders joined in each examined group (Tables 1, 2, Figure 1). Briefly, glucose, HbA1c, CRP, TGF-β1, and sRAGE levels were significantly higher in patients compared to controls for each gender. TGF-β1 mRNA and flRAGE mRNAs were significantly lower in patients compared to controls for each gender. Accordingly, we performed further statistical analysis in the initially formed groups.

Binary Logistic Regression of mRNA and Protein Levels for Association with T1D

We further investigated whether TGF-β1 mRNA and flRAGE mRNA levels and TGF-β1, sRAGE and CRP levels were associated with T1D (Table 2). Significant ORs obtained in univariate binary logistic regression analysis were evident for all tested markers, indicating their significant associations with T1D. Nagelkerke R^2 showed that each predictor TGF-β1 mRNA, flRAGE mRNA, sRAGE protein, TGF-β1 protein and CRP, in univariate analysis could explain the variation in T1D development by 24.1%, 17.8%, 14%, 8.2% and 6.6%, respectively. These predictors were further adjusted for demographic and laboratory variables (gender, age and CRP), which were significantly different between tested groups or implicated in T1D development, to assess their possible independent associations with T1D. As presented by the Model 1 TGF-β1 mRNA and flRAGE mRNA levels were independently negatively associated with T1D ($OR = 0.284$, $p < 0.001$ and $OR = 0.396$, $p < 0.001$, respectively). On the other hand, CRP, TGF-β1 and sRAGE protein levels was independently positively associated with T1D ($OR = 1.438$, $p = 0.018$, $OR = 1.037$, $p = 0.002$ and $OR = 3.552$, $p < 0.001$, respectively) (Table 2).

Correlation Analyses of mRNA and Protein Levels with Other Clinical and Laboratory Markers in T1D Patients

Next, we conducted Spearman's correlation analysis to test bivariate associations between TGF-β1 mRNA, flRAGE mRNA, TGF-β1 and sRAGE protein levels with other markers in patients with T1D (Table 3). It was found that lower TGF-β1 mRNA levels were associated with older age of diabetes onset and higher urinary albumin excretion rate. Lower flRAGE mRNA levels were related to higher serum

Table 1. General characteristics and biochemical markers of two study groups

	T1D	Control group	p
Sex, N	Males n = 80 Females n = 76	Males n = 14 Females n = 66	< 0.001
Age, years	14 (12-16)	15 (13-17)	0.078
BMI, kg/m^2	20.62 (18.18-22.62)	20.50 (19.10-22.70)	0.064
Tanner stage, N			0.367
Stage 1	17	8	
Stage 2	13	6	
Stage 3	25	9	
Stage 4	26	8	
Stage 5	75	49	
Diabetes duration, years	7 (5-8)	/	/
Age at diabetes onset, years	7 (5-10)	/	/
Insulin dosage, U/kg/day	1.02 (0.86-1.21)	/	/
Glucose, mmol/L	10.72 (7.75-15.26)	4.81 (4.64-5.08)	< 0.001
HbA1c, %	7.80 (7.10-8.77)	5.00 (4.80-5.10)	< 0.001
Glycemic control, n			
Good/suboptimal and poor	33/123	/	/
Creatinine, μmol/L[a]	74.54 ± 13.47	76.18 ± 14.25	0.393
eGFR, mL/min/1.73 m²	79.78 (73.31-86.99)	78.00 (70.00-87.00)	0.305
Urinary albumin excretion rate, μg/min	4.20 (2.90-7.60)	/	/
CRP, mg/L	0.70 (0.30-1.70)	0.40 (0.20-0.90)	0.001

Data are presented as median (interquartile range) and compared by Mann-Whitney U test.
[a]Data are presented as arithmetic mean ± standard deviation and compared by Student's t-test.
Categorical variables are presented as absolute frequencies and compared by chi-squared test for contingency tables.
T1D: type 1 diabetes mellitus, BMI: body mass index, HbA1c: glycated hemoglobin A1c, CRP: C-reactive protein, eGFR: estimated glomerular filtration rate

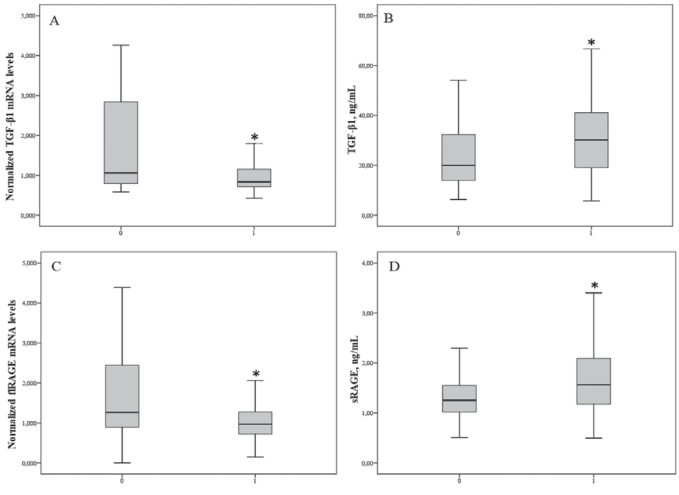

Figure 1. flRAGE and TGF-β1 normalised mRNA levels and protein concentrations between tested groups

Data are presented as median (interquartile range) and compared by Mann-Whitney U test.

**p < 0.001.*

0-Control group; 1- T1D.

T1D: type 1 diabetes mellitus, TGF-β1: transforming growth factor-β1, flRAGE: full-length receptor for advanced glycation end product, sRAGE: soluble receptor for advanced glycation end products, mRNA: messenger ribonucleic acid

creatinine levels and lower eGFR. sRAGE and TGF-β1 protein levels correlated positively with diabetes duration and negatively with eGFR. Also, TGF-β1 protein concentration correlated positively with age and creatinine level (Table 3). Furthermore, TGF-β1 mRNA and flRAGE mRNA levels were in mutual positive correlation, as were sRAGE and TGF-β1 protein concentrations. There were no significant correlations either between TGF-β1 mRNA and its protein levels or between flRAGE mRNA and sRAGE levels.

mRNA and Protein Levels According to Glycemic Control in T1D Patients

Additionally, we wanted to test whether good or suboptimal/poor metabolic control in T1D could influence TGF-β1 mRNA and flRAGE mRNA, and TGF-β1 and sRAGE protein levels, as well as CRP concentration. Consequently, we divided the T1D group into two subgroups (0 - HbA1c < 7.5%; 1 - HbA1c ≥7.5%). No significant differences in mRNA and protein levels, according to glycemic control were identified. Also, no significant correlations were evident between HbA1c and mRNA and protein levels in T1D patients (Table 3). However, when comparing CRP concentrations between the groups, adolescents with suboptimal/poor glycemic control (median: 0.85 mg/L, interquartile range: 0.40-2.30

mg/L) had significantly higher CRP concentration (p = 0.020) than those with good glycemic control (median: 0.50 mg/L, interquartile range: 0.30-0.75 mg/L).

mRNA and Protein Levels According to Urinary Albumin Excretion Rate Quartiles in T1D Patients

Our further intention was to determine whether TGF-β1 mRNA and flRAGE mRNA and TGF-β1 and sRAGE protein levels were associated with early elevation of urinary albumin excretion rate in T1D patients. To achieve this, urinary albumin excretion rate values were divided into quartiles. Each quartile consisted of 39 participants. Early elevation of albumin excretion defined as ≥7.5 μg/min corresponds to the fourth quartile. TGF-β1 mRNA levels were significantly different between quartiles (p = 0.025) being lower in the fourth than in the first quartile group (p = 0.005) (Figure 2A). There were no significant differences in other tested markers between urinary albumin excretion rate quartile groups (Figure 2B, 2C, 2D). Also, we were not able to determine significant differences in CRP concentration between quartile groups (p = 0.967) (data not presented in Figure 2).

Ordinal Regression Analysis of TGF-β1 mRNA Levels for Association of Early Elevation of Urinary Albumin Excretion Rate in T1D Patients

Due to the significantly high negative correlation between TGF-β1 mRNA and urinary albumin excretion rate in T1D patients (Table 3), our further intention was to determine whether an in-depth association between them existed. To achieve this, univariate and multivariate ordinal regression analysis was performed. TGF-β1 mRNA levels showed significant ORs for urinary albumin excretion rate in univariate analysis (OR = 0.278, 95% CI: 0.126-0.612, p = 0.001). Nagelkerke R^2 for TGF-β1 mRNA levels was 0.140. Multivariate analysis revealed an independent association of TGF-β1 mRNA levels with early elevation of urinary albumin excretion rate when tested with other clinical variables which might be implicated in its elevation. Those variables were age, diabetes duration, CRP and HbA1c. A decrease in TGF-β1 mRNA levels increased the probability of elevation of urinary albumin excretion rate (OR = 0.309, 95% CI: 0.136-0.698, p = 0.005). Nagelkerke R^2 of 0.162 indicated that multivariate regression model could explain 16.2% variation in urinary albumin excretion rate.

Discussion

The present study demonstrated that a decrease in TGF-β1 mRNA levels was independently associated with T1D and early elevation of urinary albumin excretion rate, while

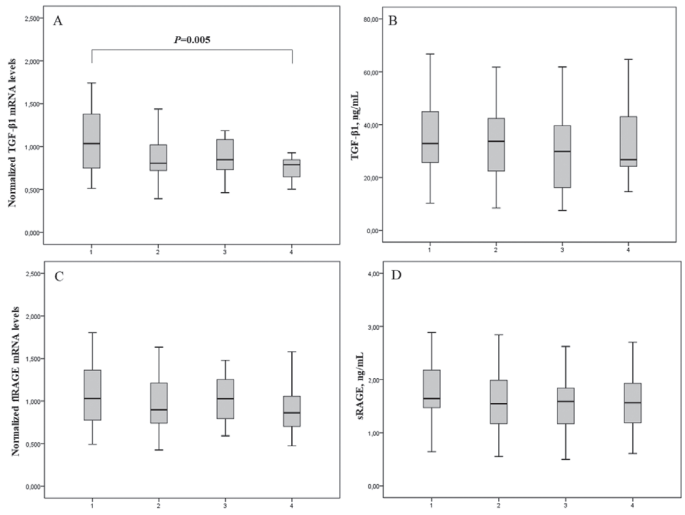

Figure 2. TGF-β1 and flRAGE mRNA levels, TGF-β1 and sRAGE protein concentration in adolescents with type 1 diabetes mellitus according to urinary albumin excretion rate quartiles

Data are presented as median (interquartile range) and compared by Kruskal-Wallis and Mann-Whitney U tests.

1) the first quartile (≤2.92 μg/min); 2) the second quartile group (2.93-4.24 μg/min); 3) the third quartile group (4.25-7.49 μg/min); 4) the fourth quartile group (≥7.5 μg/min). Each quartile consisted of 39 participants.

TGF-β1: transforming growth factor-β1, flRAGE: full-length receptor for advanced glycation end product, sRAGE: soluble receptor for advanced glycation end products, mRNA: messenger ribonucleic acid

Table 2. Univariate and multivariate binary logistic regression analysis for the associations of tested markers and type 1 diabetes mellitus development

Univariate	Unadjusted OR (95% CI)	p	Nagelkerke R^2
CRP, mg/L	1.421 (1.080-1.870)	0.012	0.066
TGF-β1 mRNA	0.347 (0.239-0.503)	< 0.001	0.241
TGF-β1, ng/mL	1.040 (1.018-1.063)	< 0.001	0.082
flRAGE mRNA	0.412 (0.291-0.585)	< 0.001	0.178
sRAGE, ng/mL	3.969 (2.177-7.237)	< 0.001	0.140
Multivariate	**Adjusted OR (95% CI)**	**p**	**Nagelkerke R^2**
CRP, mg/L[a]	1.438 (1.064-1.945)	0.018	0.213
TGF-β1 mRNA[b]	0.284 (0.176-0.457)	< 0.001	0.394
TGF-β1, ng/mL[b]	1.037 (1.013-1-062)	0.002	0.264
flRAGE mRNA[b]	0.396 (0.264-0.595)	< 0.001	0.323
sRAGE, ng/mL[b]	3.552 (1.857-6.792)	< 0.001	0.314

[a]Adjusted for gender (categorical variable), age (continuous variable).
[b]Adjusted for gender (categorical variable), age and CRP (continuous variables).
T1D: type 1 diabetes mellitus, OR: odds ratio, CI: confidence interval, CRP: C-reactive protein, TGF-β1: transforming growth factor-β1, flRAGE: full-length receptor for advanced glycation end product, sRAGE: soluble receptor for advanced glycation end products, mRNA: messenger ribonucleic acid

Table 3. Significant correlations between flRAGE and transforming growth factor-β1 mRNA and protein concentrations with demographic data and biochemical markers in adolescents with type 1 diabetes mellitus

	TGF-β1 mRNA	TGF-β1, ng/mL	flRAGE mRNA	sRAGE, ng/mL
Age, years	-0.087	0.164*	-0.112	0.041
Tanner stage, N	-0.006	-0.097	-0.106	-0.077
Diabetes duration, years	0.069	0.243**	-0.086	0.210**
Age at diabetes onset, years	-0.176*	0.114	-0.012	-0.111
BMI, kg/m^2	0.067	0.045	-0.089	-0.098
Insulin dosage, U/kg/day	0.058	0.139	-0.025	0.006
Glucose, mmol/L	0.066	0.113	0.047	0.024
HbA1c, %	-0.057	0.109	0.070	-0.058
Creatinine, μmol/L	-0.061	0.181*	-0.186*	0.138
eGFR, mL/min/1.73 m²	0.032	-0.177*	0.162*	-0.163*
Urinary albumin excretion rate, μg/min	-0.292**	-0.052	-0.133	-0.098
CRP, mg/L	-0.090	0.079	-0.088	-0.109
TGF-β1 mRNA	-	0.125	0.273**	-0.060
TGF-β1, ng/mL	0.125	-	-0.047	0.221**
flRAGE mRNA	0.273**	-0.047	-	-0.107
sRAGE, ng/mL	-0.060	0.221**	-0.107	-

Data are presented as correlation coefficient (ρ).
*$p < 0.05$, **$p < 0.01$
TGF-β1: transforming growth factor-β1, flRAGE: full-length receptor for advanced glycation end products, sRAGE: soluble receptor for advanced glycation end product, mRNA: messenger ribonucleic acid, BMI: body mass index, HbA1c: glycated hemoglobin A1c, eGFR: estimated glomerular filtration rate, CRP: C-reactive protein

CRP, TGF-β1 and sRAGE protein concentrations were independently associated with T1D only. In addition, adolescents with T1D expressed lower flRAGE mRNA levels than controls, which were independently associated with T1D, but not to early elevation of urinary albumin excretion rate. None of the tested markers were related to glycemic control in T1D adolescents.

As an autoimmune disease accompanied by chronic inflammation, T1D tends to develop in childhood (1). Regardless, vascular complications can be detected in adolescents after five-years duration of T1D, indicating faster development than in adults (26). Such complications are one of the most important causes of mortality in T1D patients. Although assessment of autoantibodies in blood is the gold standard for identification of patients with T1D or at-risk patients (27), new biomarkers are required to indicate and assess β-cells destruction and monitor T1D progression in clinical practice (4).

It is very difficult to obtain samples of body organs, e.g. pancreas, blood vessel endothelium or kidneys in adolescents. However, PBMC, lymphocytes and monocytes, can serve as surrogate cells for RNA isolation and gene expression determination (28). Gene expression profiles in peripheral blood immune cells may provide new insights into the T1D pathogenesis (4,28). Still, there are contradictory results and conflicting explanations concerning RAGE and TGF-β1 gene expression in PBMC in T1D patients published to date (3,29,30,31,32,33).

TGF-β1, as multifunctional cytokine, is produced by virtually all cells in humans, and has contradictory effects depending on the tissue being assessed (14,15). Demonstrated by Saxena et al (14), TGF-β1 plays a dual role during the development and progression of systemic autoimmune-inflammatory disease in mice. Reduced TGF-β1 synthesis by immune cells indicated autoimmune onset in early life. Also, TGF-β1 produced by Treg cells was shown to inhibit autoantibody synthesis. On the other hand, increased TGF-β1 synthesis in other tissues predisposes local fibrogenesis and likely leads to organ damage, for example kidney injury (14). Results from our study supported these findings. We found significantly lower TGF-β1 gene expression in PBMC from T1D patients compared to healthy adolescents ($p < 0.001$). On the other hand, TGF-β1 protein concentration in T1D patients was significantly higher than in controls ($p < 0.001$). This was expected because TGF-β1 production is enhanced in other peripheral organs in children with T1D (34). Furthermore, although they were not in mutual correlation, downregulation in TGF-β1 gene and increase in TGF-β1 protein levels were found to be independently associated with T1D development ($p < 0.001$

and p = 0.002, respectively). Adolescents with lower TGF-β1 mRNA levels were 71.6% more likely to exhibit T1D than those with higher levels. However, the odds of having T1D was 1.037 times greater in adolescents with higher TGF-β1 concentration.

TGF-β1 downregulation has been previously reported in PBMC of children with T1D, indicating depressed immunity in patients with long-term T1D (29). Tolerance against self-antigens can be maintained through activation of Treg cells that produce TGF-β1 (14). However, in our adolescents with T1D downregulation of TGF-β1, reflected as lower mRNA levels than in controls was evident. This apparently suggested maturation of T helper 1 lymphocytes, which could have been implicated in destruction of pancreatic β-cells (35). Nevertheless, dysregulation of TGF-β1 expression by Treg cells occurs even during the pre-diabetic stage (29). Children positive for islet cell antibodies had significantly lower TGF-β1 mRNA levels than controls (29). In our study, another confirmation of immune cell dysregulation was demonstrated by flRAGE mRNA levels, which were lower in T1D patients than in controls ($p < 0.001$). Membrane RAGE also modulates Treg cell function and suppresses the autoimmune response and the downregulation of membrane RAGE has been implicated T1D development (10,36). Decrease in flRAGE mRNA levels was found to be independently associated with T1D development ($p < 0.001$). Adolescents with lower flRAGE mRNA levels were 60.4% more likely to exhibit T1D than those with higher levels. Additionally, positive correlation between TGF-β1 mRNA and flRAGE mRNA levels ($p < 0.01$) was evident in the T1D, cohort suggesting potential interplay of their signalling pathways in T1D pathogenesis.

In contrast to our and other studies, Jin et al (33) published a study of gene expression profiles in human PBMC of T1D patients using microarray technology. They analysed 18 genes involved in inflammation and immunity, among which TGF-β1 showed higher expression in T1D patients than in controls. These authors suggested that increase in TGF-β1 gene expression may have a role in T1D through stimulation of proinflammatory cellular pathways in PBMC (33).

T1D as a chronic metabolic disease causes future micro- and macro-vascular complications (26). Disturbance in blood vessel integrity correlates with poor glycemic control and diabetes duration (37). Heier et al (37) demonstrated that after five years from diabetes onset, accelerated atherosclerosis was evident in children with T1D. Though microalbuminuria has been related to diabetic nephropathy development and progression (38), it has recently been recognized as an independent predictor for endothelial dysfunction and cardiovascular disease (CVD) (39).

In our T1D study participants, with an average diabetes duration of seven years, glycemic control as defined by HbA1c seemed not to have any influence on the main tested markers. TGF-β1 mRNA, flRAGE mRNA, TGF-β1 and sRAGE protein levels did not differ between T1D adolescents with good vs suboptimal/poor glycemic control. Also, there were no correlations between them and HbA1c. However, when urinary albumin excretion rate values were divided into quartiles, TGF-β1 mRNA levels were lower in the fourth compared to the first quartile group ($p = 0.005$). The fourth quartile corresponds to early elevation of urinary albumin excretion rate that could predict permanent micro- and later macro-albuminuria (23). Interestingly, our results indicated independent associations of lower TGF-β1 mRNA levels with elevated urinary albumin excretion rate in T1D adolescents ($p = 0.005$). Adolescents having T1D with lower TGF-β1 gene expression were 69.1% more likely to have elevated urinary albumin excretion rate than those with higher expression. In our group of T1D adolescents with average diabetes duration of seven years, TGF-β1 mRNA levels could be used as a potential biomarker for CVD risk assessment indicating not only dysregulation of immune response to autoantigens, but also predicting future cardiovascular complications. It was expected that TGF-β1 protein, a fibrogenic factor, would correlate with urinary albumin excretion rate (14). However, this was not supported by our results. Yet, TGF-β1 protein levels in blood correlated significantly positively with creatinine ($p < 0.05$) and negatively with eGFR ($p < 0.05$) linking TGF-β1 with potential future renal function decline in T1D patients.

Miura et al (30) demonstrated that lower cell surface RAGE expression in monocytes of children with T1D could be partly explained by enhanced ligand binding, indicating an imbalance in receptor function on monocytes making them more prone to modification in subcellular space. Moreover, patients with incipient or clinical diabetic nephropathy showed a significant decrease in monocyte RAGE mRNA levels compared to patients without nephropathy. Our results may support these findings. We did not determine AGE concentration in blood of our participants and were not able to explain lowering RAGE expression with AGE engagement as in the Miura et al (30) study, but a relationship between lowering flRAGE mRNA and future diabetic vascular complications was apparent in our study.

sRAGE, made by protein cleavage of the extracellular ligand binding domain of transmembrane RAGE, has been suggested to be a biomarker for vascular disease development (11,12,13). Our results indicated that higher sRAGE levels were independently associated with T1D ($p < 0.001$). The OR of having T1D was 3.552 times greater

in adolescents with higher sRAGE concentration. However, sRAGE was not related to urinary albumin excretion rate. It correlated significantly positively with diabetes duration ($p < 0.01$) and negatively with eGFR ($p < 0.05$). Not only mRNA levels, but TGF-β1 and sRAGE protein levels correlated significantly positively ($p < 0.01$), pointing out to one more evidence for TGF-β1 and sRAGE proteins probable mutual implication in T1D pathogenesis. These results could support sRAGE potent pro-atherogenic effect in addition to its immunomodulating properties (11).

As T1D is an immuno-inflammatory disease, CRP levels were expected to be increased in adolescents with T1D (37). According to our results, CRP levels were significantly higher in patients with T1D than in the control group and were also independently associated with T1D. These results are in line with the fact that not only an autoimmune process, but inflammation could be related to destruction of islet β-cells (40).

Changes in TGF-β1 mRNA and flRAGE mRNA, as well as in TGF-β1 and sRAGE protein levels were evident between patients and controls. Disturbances in TGF-β1 and flRAGE gene expression levels in PBMC are detectable in the pre-diabetic stage and persist during development of T1D (10,29,36,41). Therefore, mRNA measurement would be beneficial to perform at an early age when there is a suspected onset of diabetes, for example in patients with a positive family history. The same can account for TGF-β1 and sRAGE protein levels. Also, TGF-β1 mRNA levels should be determined at T1D diagnosis for possible diabetes complications assessment (37,38). However, due to possible effects of acute and chronic hyperglycemia (5,41,42,43) on these markers, multiple prospective measurements would be strongly recommended.

Study Limitations

This study has several limitations. Firstly, this research was carried out as a cross-sectional study, which demonstrated significant associations between tested markers and T1D presence and urinary albumin excretion rate, but was not able to assess causal relationships between them. However, our findings need to be confirmed in prospective studies to determine whether progressive downregulation of the TGF-β1 gene occurs as microalbuminuria worsens. Secondly, TGF-β1 and RAGE protein concentration determination in PBMC of patients and controls should be addressed in future studies, together with their mRNA levels, to demonstrate whether their protein levels were lower and correlated with lower mRNA levels in T1D and to confirm immunomodulatory dysfunction in those cells of T1D patients. Finally, although the presence of significantly more females than males in the control group did not skew the results and conclusions of this study, the inclusion of more males would have made our current findings more robust. Nevertheless, our current study might form the basis for future research.

Conclusion

In conclusion, T1D onset progresses in tandem with lower PBMC TGF-β1 mRNA and flRAGE mRNA levels, together with increased secretion of the proinflammatory cytokines TGF-β1 and sRAGE by other cells, as well as systemic low-grade inflammation. In addition, downregulation of the TGF-β1 gene might be used as a potential biomarker for early CVD risk assessment in adolescents with T1D, due to its independent significant negative association with urinary albumin excretion rate.

Ethics

Ethics Committee Approval: The study was carried out in line with the principles of the Declaration of Helsinki and approved by the Ethics Committees of Mother and Child Health Care Institute of Serbia "Dr. Vukan Čupić" (protocol number: 8/8, date: April the 9th 2015) and University of Belgrade-Faculty of Pharmacy (protocol number: 2536/2, date: December 26th 2018).

Informed Consent: Written informed consent was obtained from all the participants and their parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Experimental Work: Dragana Bojanin, Miron Sopić, Marija Mihajlović, Jelena Munjas, Aleksandra Stefanović, Surgical and Medical Practices: Dragana Bojanin, Tatjana Milenković, Vesna Spasojević-Kalimanovska, Concept: Ana Ninić, Tatjana Milienković, Jelena Vekić, Vesna Spasojević-Kalimanovska, Design: Ana Ninić, Tatjana Milienković, Jelena Vekić, Vesna Spasojević-Kalimanovska, Data Collection or Processing: Ana Ninić, Miron Sopić, Marija Mihajlović, Analysis or Interpretation: Ana Ninić, Jelena Vekić, Literature Search: Ana Ninić, Miron Sopić, Marija Mihajlović, Writing: Ana Ninić, Dragana Bojanin, Miron Sopić, Marija Mihajlović, Jelena Munjas, Tatjana Milenković, Aleksandra Stefanović, Jelena Vekić, Vesna Spasojević-Kalimanovska.

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Frequency of Celiac Disease and Spontaneous Normalization Rate of Celiac Serology in Children and Adolescent Patients with Type 1 Diabetes

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What is already known on this topic?

Celiac disease (CD) prevalence varies between 1% and 10% in children and adolescents with type 1 diabetes mellitus (T1DM). In previous reports in about half of the cases, CD was detected at the time of the diagnosis of T1DM. Recently, a few studies have shown normalization of celiac serology in patients with T1DM, even with no gluten-free dietary intervention.

What this study adds?

In our study, the majority (97.8%) of cases were diagnosed within the first five years of T1DM. In 23.3% of cases, positive celiac serology spontaneously resolved without a gluten-free diet (GFD). Therefore, considering all of the serologically positive individuals as CD and giving a GFD imposes an additional psychological burden for children and families. This implication would negatively affect the compliance to the T1DM management. The presence of symptoms and high anti-tissue transglutaminase IgA levels were shown to be highly predictive for biopsy-proven CD (BPCD).

Abstract

Objective: The prevalence of celiac disease (CD) varies between 1% and 10% in patients with type 1 diabetes mellitus (T1DM). This study aimed to determine the frequency of spontaneous recovery of celiac serology and the biopsy-proven CD (BPCD) frequency in patients with T1DM.

Methods: The data of 668 patients with available celiac serology tests from a total of 779 patients who were followed for the last 10 years with the diagnosis of T1DM were retrospectively evaluated.

Results: Positive serology was detected in 103 out of 668 (15.4%) patients. There was spontaneous normalization in 24 (23.3%), fluctuation in 11 (10.7%) and permanently positive serology in 68 (66%). In 46 out of 53 (86.8%) patients with positive serology and biopsy, CD diagnosis was confirmed by biopsy (BPCD). The frequency of BPCD was 6.9%, and the serology in 76.1% was positive at the time of diagnosis of T1DM. The weight, height and body mass index-standard deviation score at diagnosis were lower in patients with BPCD compared to the group without CD. An anti-tissue transglutaminase-IgA (anti-TTG-IgA) level of 11.8 times the upper limit of normal was the most sensitive (93%) and specific (90%) cut-off for BPCD (area under the curve: 0.95; 95% confidence interval: 0.912-1; $p < 0.001$).

Conclusion: In our cohort, the frequency of positive serology for CD was 15.4%, while the rate of BPCD was 6.9%. The majority (97.8%) of cases were diagnosed within the first five years of T1DM. In 23.3% of cases, positive anti-TTG-IgA spontaneously resolved without a gluten-free diet (GFD). Therefore, serological follow-up instead of immediate duodenal biopsy or GFD therapy, particularly for patients with asymptomatic and mild anti-TTG IgA level, is warranted.

Keywords: Celiac disease, children, spontaneous normalization, type 1 diabetes

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Introduction

Type 1 diabetes mellitus (T1DM), one of the most common chronic diseases in children, characterized by insulin deficiency due to autoimmune destruction of pancreatic beta cells. There is an increased risk of other autoimmune disorders in children with T1DM. The most common autoimmune diseases accompany to T1DM are autoimmune thyroiditis (AITD) and celiac disease (CD) (1). The prevalence of CD in the general population is estimated to be between 0.3% and 1% (2). However, due to increased genetic predisposition, CD prevalence varies between 1% and 10% in children and adolescents with T1DM (3,4,5,6). Since the majority of CD patients can be asymptomatic, screening for CD at the time of T1DM diagnosis is recommended by both American Diabetes Association and the International Society for Pediatric and Adolescent Diabetes (1,7). In seronegative cases at the first screening, if there are no CD symptoms, regular screening every 2-5 years is recommended. However, in patients with CD symptoms or history of CD in first-degree relatives more frequent screening is recommended (1,7). Testing of asymptomatic CD would provide a prompt diagnosis of CD and enable better metabolic control for T1DM patients (8). However, recently, some studies have shown normalization of celiac serology in patients with T1DM, even with no gluten-free dietary intervention. In the mentioned studies, spontaneous normalization developed in 20-35% of the cases (9,10,11). Therefore, considering all of the serologically positive individuals as CD and giving a gluten-free diet (GFD) imposes an additional psychological burden for children and families. This implication would also negatively affect the compliance to the T1DM management.

In the latest European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) guidelines, it was highlighted that the level of anti-tissue transglutaminase-IgA (anti-TTG IgA) should be at least 10 times higher than the upper limit of normal (ULN) for diagnosis of CD without duodenal biopsy. Rarely, although children with high TGA-IgA (10xULN) levels, they can have normal histopathology. For this reason, it is recommended by EPSGHAN that the diagnosis of CD without biopsy must be confirmed with a positive anti-endomysial antibody (EMA)-IgA test in a second blood sample (12).

The aim of present study was to determine the frequency of biopsy-proven CD (BPCD) and spontaneous resolution of high anti-TTG IgA levels in patients with T1DM. We also investigated the predictive factors for BPCD and spontaneous normalization of celiac serology.

Methods

The hospital files of 779 patients who have been followed for the last 10 years (2009-2019) with the diagnosis of T1DM at the Pediatric Endocrinology Clinic of Gazi Yaşargil Training and Research Hospital, University of Health Sciences Turkey were retrospectively analyzed. The age, gender, mean glycated hemoglobin (HbA1c) level, and anti-TTG IgA level status of patients with T1DM were recorded. Patients in whom anti-TTG IgA levels were not available were excluded. Anti-TTG IgA level was measured by enzyme-linked immunosorbent assay (Euroimmun kit, Euroimmun Analyzer I, Euroimmun Medizinische Labordiagnostika AG,-23560 Lübeck Germany). Samples were analyzed in a central laboratory where the same method was used for analysis of celiac serology. According to the method used in our laboratory; anti-TTG IgA level <12 IU/mL was considered as negative, 12-18 IU/mL as borderline, and >18 IU/mL as positive celiac serology. Initially positive anti-TTG IgA antibodies that persistently remained negative (<12 IU/mL) for six months was considered as spontaneous normalization of celiac serology (group 1). If anti-TTG IgA level was initially positive, temporarily resolved and then became positive again, this pattern was defined as fluctuation of celiac serology. Pathology reports of all cases who underwent endoscopic biopsy were examined. According to the biopsy results of the patients, those with Marsh scores 2 and 3 were accepted as BPCD (group 2). Those with anti-TTG IgA positive but Marsh score 0 and 1 were considered as potential CD (12).

Serological autoantibody titers were recorded as multiples of the ULN. The threshold value for the ULN was taken as 18 IU/mL. Three times lower than the ULN was considered mild, as ten times higher than the ULN was considered high for anti-TTG IgA level. Bodyweight standard deviation score (SDS), height SDS and body mass index (BMI) SDS values were extracted from the patient medical files. In addition, anthropometric measurements of patients with BPCD were assessed before and during a GFD.

The study was performed in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Gazi Yaşargil Training and Research Hospital (document number: 17.01.2020/411). Since the study was retrospective, informed consent was deemed unnecessary and not obtained from the parents of the patients.

Statistical Analysis

Statistical analyses were performed using SPSS for Windows, version 21 (IBM Corp., Armonk, NY, USA). For evaluation of the normality distribution of the data, the Shapiro-Wilk

test was used. Numerical variables were expressed as mean ± SD or median and interquartile range, categorical variables were expressed as number and per cent (%). For numerical comparisons, independent sample t-test or Mann-Whitney U tests were used subject to the normality distribution of data. Chi-square test was used to compare categorical variables. The repeated measure of weight-SDS, height-SDS and BMI-SDS values of the patients with BPCD at the time of the diagnosis and the last follow-up visit were compared with a paired-sample t-test. In the diagnosis of BPCD, a receiver operating characteristics (ROC) curve analysis was performed for anti-TTG IgA level recorded as multiples of the ULN. A $p < 0.05$ value was considered statistically significant.

Results

The study included 779 patients, 367 (47.1%) male and 412 (52.9%) female with T1DM. Of those 668 (85.75%) patients had at least one anti-TTG IgA test result (Figure 1). The mean age of diagnosis of T1DM was 8.75 ± 6.75 (range: 0.5-17.92) with a mean follow-up duration of 3.91 ± 4.17 (range: 0.17-16.92) years.

To exclude the false-negative anti-TTG IgA serology due to concomitant IgA deficiency, total serum IgA was measured in all patients undergoing CD serological testing and was within normal limits in all cases. Positive anti-TTG IgA was detected in 103 out of 668 (15.4%) patients. Spontaneous normalization was detected in 24 out of 103 (23.3%) patients within a median duration of nine (range: 3-24 months) months. In the spontaneous normalization group, median follow-up time after the disappearance of anti-TTG IgA antibody was 25.5 months (range: 6-105). There was a statistically significant difference between serum anti-TTG IgA levels of groups 1 and 2 at diagnosis (group 1, median 2 x ULN (range 1.1-11.5) and group 2, median 16.6 x ULN (range 4.1-123) ($p < 0.05$) (Table 1). In one of 24 patients who had spontaneous normalization, the anti-TTG IgA level was above 11 times the ULN. In two of 46 patients who had persistent antibody positivity, the anti-TTG IgA level was below 11 times the ULN.

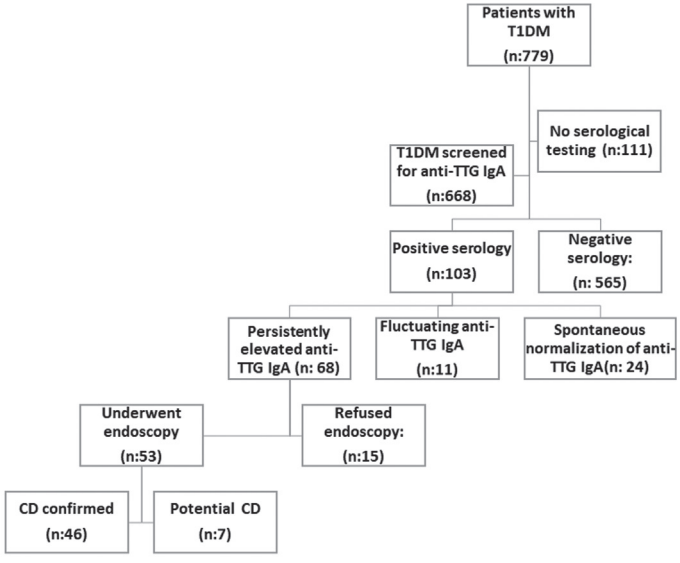

Figure 1. A flow diagram of the study participants

T1DM: type 1 diabetes mellitus, CD: celiac disease, anti-TTG IgA: anti-tissue transglutaminase-IgA

In 103 patients with positive celiac serology, fluctuating celiac serology was detected in 11 (10.7%) while celiac serology remained positive in 68 (66%). Autoantibodies became positive again after a median duration of five months (range 3-6 months) in the fluctuation group. The antibody levels of the groups, showing anti-TTG IgA levels of persistent, fluctuation and spontaneous normalization are summarized in Figure 2. An endoscopic biopsy was performed in 53 out of 68 (77.9%) patients who had permanently positive serology. The biopsy was not performed in 15 (22.1%) cases due to family refusal. Forty-six out of 53 (86.8%) patients who underwent biopsy were diagnosed with BPCD suggesting a frequency of BPCD of 6.9% (46/668).

Thirty-five out of 46 (76.1%) patients with BPCD were diagnosed at the time of the diagnosis of T1DM, 11 (21.7%) within following five years and one patient (2.2%) 8.5 years after T1DM diagnosis. Anthropometric measurements were repeated for patients with BPCD before on a GFD and while taking GFD as median of 2.66 years (range 0.25-14.3 years). In BPCD patients, weight ($p < 0.001$), height ($p = 0.02$) and BMI-SDS at the time of the diagnosis ($p = 0.01$) and height-SDS at the final follow-up visit (p 0.001) were found to be significantly lower than the patients who did not have BPCD. There was no statistically significant difference between the mean HbA1c levels of those with BPCD and celiac

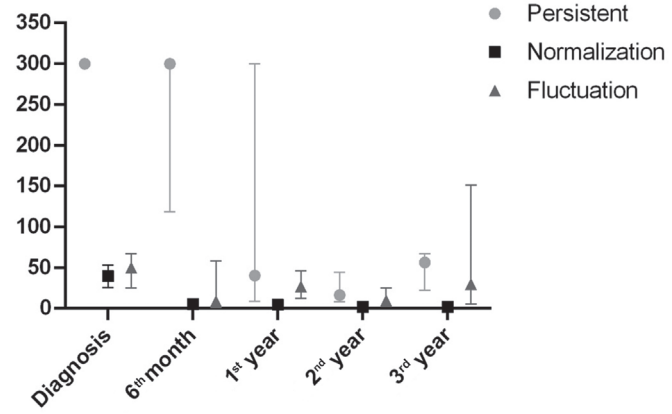

Figure 2. Trend of the anti-tissue transglutaminase-IgA levels in patients with persistent, fluctuation and spontaneous normalization group

negative patients (Table 2). In the ROC analysis, an anti-TTG IgA level that was 11.8 times higher than the ULN had the best sensitivity (93%) and specificity (90%) for BPCD (area under the curve: 0.95, 95% CI: 0.912-1, p < 0.001; Figure 3).

Anti-thyroid peroxidase and anti-thyroglobulin serology was examined in 562 of the patients with celiac serology (84.1%) and at least one antibody was positive in 69 cases. While BPCD was present in 8/69 (11.6%) patients with T1DM and positive thyroid autoantibody, it was present in 30/493 (6.1%) patients with negative thyroid autoantibody (p = 0.054; Table 3).

BPCD was found in 13/146 (8.9%) patients diagnosed with T1DM under the age of five, and in 33/489 (6.7%) patients over the age of five (p = 0.38; Table 3).

The rate of BPCD did not differ between girls at 6.4% and boys at 8.2% (p = 0.39; Table 3). There was no statistically significant difference between the weight, height and BMI-SDS values at the time of the diagnosis and the final follow-up visit of patients with BPCD (Table 4).

Discussion

In the present study, serological CD prevalence was 15.4%, and BPCD prevalence was 6.9%. In patients with T1DM, due to genetic predisposition, the frequency of CD and other autoimmune diseases is higher than the normal population (2). In an international comparative study of 52,721 children and adolescents with T1DM, the overall CD prevalence was reported as 3.5% with a frequency of 1.9% in the USA and 7.7% in Australia (13). This is similar to our

Table 1. Comparison of anthropometric and laboratory features of type 1 diabetes mellitus patients with biopsy-proven celiac disease (group 2) and spontaneously recovered positive celiac serology (group 1)

	Group 1 (n = 24)	Group 2 (n = 46)	p value
Age of diagnosis (year)	9.07 ± 4.16	8.12 ± 4.58	0.40†
Latest age (year)	13.33 (2.25-22)	14.04 (4.25-19.5)	0.683*
Duration of T1DM (year)	2.83 (0.92-9.75)	3.12 (0.25-16.33)	0.569*
Anti-TTG-IgA** (ULN)	2 (1.32-2.58)	16.6 (16.6-16.6)	< 0.001*
Mean HbA1c (%)	8.8 ± 1.78	9.45 ± 2.06	0.24†
Weight at diagnosis (SDS)	-1.04 ± 0.91	-1.21 ± 1.37	0.63†
Height at diagnosis (SDS)	-0.58 ± 1.00	-0.98 ± 1.29	0.29†
BMI at diagnosis (SDS)	-1.11 ± 1.03	-0.85 ± 1.37	0.50†
Latest weight (SDS)	-0.50 ± 1.14	-1.11 ± 1.40	0.10†
Latest height (SDS)	-0.55 ± 1.23	-1.46 ± 1.27	0.01†
Latest BMI (SDS)	-0.17 ± 0.87	-0.37 ± 1.27	0.53†

Anti-TTG-IgA: anti-tissue transglutaminase-IgA, BMI: body mass index, HbA1c: glycated haemoglobin, SDS: standard deviation score, T1DM: type 1 diabetes mellitus, ULN: upper limit of normal.
*Mann-Whitney U test, †Student's t-test; data are given as mean ± standard deviation or median (interquantile range 25th-75th percentile).
**Anti-TTG-IgA antibody titer reported as times the ULN.

Table 2. Comparison of anthropometric and laboratory features of type 1 diabetes mellitus patients with and without biopsy-proven celiac disease

	CD negative (n = 589)	CD positive (n = 46)	p value
Age of diagnosis (year)	9 (5.3-12)	7.58 (4.08-12.37)	0.410*
Latest age (year)	13.83 (9.83-16.5)	14.04 (9.81-16.04)	0.818*
Duration of T1DM (year)	4 (1.68-6.25)	3.12 (2.08-7.02)	0.754*
Mean HbA1c (%)	8.75 (7.8-10.35)	9.37 (8.1-10.77)	0.318*
Weight at diagnosis (SDS)	-0.41 ± 1.11	-1.21 ± 1.37	< 0.001†
Height at diagnosis (SDS)	-0.29 ± 1.21	-0.98 ± 1.29	0.02†
BMI at diagnosis (SDS)	-0.35 ± 1.16	-0.85 ± 1.37	0.01†
Latest weight (SDS)	-0.57 ± 1.15	-1.11 ± 1.40	0.05†
Latest height (SDS)	-0.80 ± 1.15	-1.46 ± 1.27	< 0.001†
Latest BMI (SDS)	-0.17 ± 1.07	-0.37 ± 1.27	0.25†

BMI: body mass index, HbA1c: glycated haemoglobin, SDS: standard deviation score, CD: celiac disease, T1DM: type 1 diabetes mellitus.
*Mann-Whitney U test, †Student's t-test; data are given as mean ± SD or median (interquantile range 25th–75th percentile).

Table 3. The frequency of biopsy-proven celiac diseaseaccording to age and presence of autoimmune thyroid disease accompanying type 1 diabetes mellitus

	BPCD n (%)	p value
Female	22 (6.4%)	0.39
Male	24 (8.2%)	
AITD	8 (11.6%)	0.05
No AITD	30 (6.1%)	
T1DM diagnosis age < 5 years	13 (8.9%)	0.38
T1DM diagnosis age > 5 years	33 (6.7%)	

BPCD: biopsy proven celiac disease, AITD: autoimmune thyroid disease, T1DM: type 1 diabetes mellitus

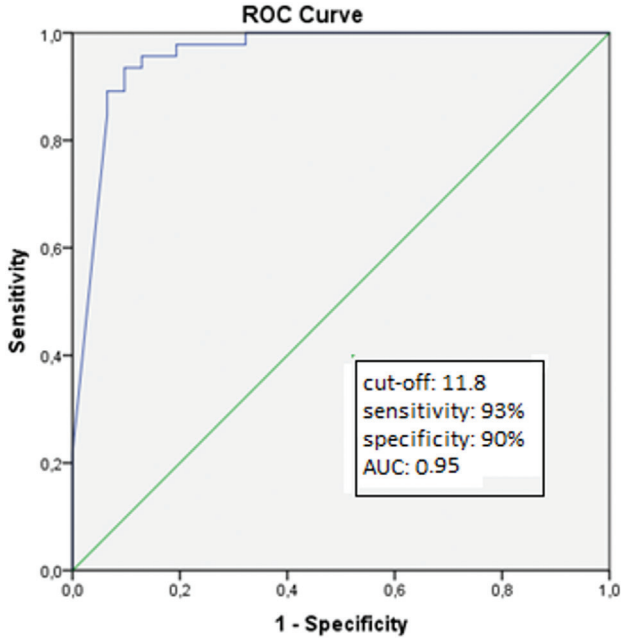

Figure 3. Receiver operating characteristics analysis of anti-tissue transglutaminase-IgA level for prediction of biopsy-proven celiac disease (sensitivity: 93%, specificity: 90%, area under the curve: 0.95, p < 0.001)

AUC: area under the curve, ROC: receiver operating characteristics

Table 4. Comparison of anthropometric features of type 1 diabetes mellitus patients at the time of the diagnosis of celiac disease and during gluten-free diet at follow up

	At diagnosis of CD	After gluten-free diet	p*
Weight SDS	-1.18 ± 1.31	-1.14 ± 1.35	0.82
Height SDS	-1.17 ± 1.33	-1.44 ± 1.32	0.051
BMI SDS	-0.74 ± 1.41	-0.42 ± 1.21	0.14

CD: celiac disease, BMI: body mass index, SDS: standard deviation score
*Paired sample t-test

study and previous studies conducted in Turkey, which have reported a CD prevalence in children with T1DM of between 3.5% and 7.8% (14,15,16,17).

Recently, some studies evaluating CD prevalence in patients with T1DM, have reported spontaneously normalizing celiac serology in up to 20-35% (9,10,11,18). The duration for a positive serology to become negative was about 1-2 years after diagnosis (10,11). Similarly, in our study in 23.3% of patients, positive celiac serology spontaneously recovered within a median duration of nine months (3-24 months), without GFD intervention. In a study involving 446 pediatric T1DM patients, the rate of spontaneous recovery of celiac serology was reported as 27.6%. Having a negative anti-EMA, and low anti-TTG IgA levels (2.3 ± 2.1 ULN) have been reported as predictive factors (10). In our study, all patients with spontaneously recovered celiac serology were asymptomatic, and median anti-TTG IgA levels were low in the majority. In only one case, the anti-TTG IgA level was 11.5 x ULN. In previous studies, spontaneous recovery of celiac serology in very high anti-TTG IgA levels has not been reported (9,10,11). Therefore, we suggest that serological follow-up might be a more appropriate strategy in patients with asymptomatic and mildly elevated anti-TTG IgA levels instead of performing an intestinal biopsy immediately (9,10,11,18).

In previous studies evaluating spontaneous normalization of celiac serology, there is limited data on re-positivity of celiac serology in patients with spontaneous normalization (9,10,11). In only one study, it was reported that autoantibodies re-appeared in three of 18 patients with spontaneous normalization (10). However, there was no data about the duration for re-appearance (10). In our study, the median follow-up time after anti-TTG IgA level was negative in the spontaneous normalization group was 25.5 months. In three of the 24 patients who with spontaneous normalization of CD serology, the follow-up period while remaining negative was less than one year, while in 21 patients the follow-up period was at least 15 months. Even though the duration of remaining negative was not short, this does not eliminate the possibility of reappearance of CD autoantibodies. Therefore, regular follow-up of celiac serology in patients with spontaneous normalization is warranted.

In the latest ESPGHAN guidelines, it was highlighted that the level of anti-TTG IgA should be at least 10 times higher than the ULN for diagnosis of CD without duodenal biopsy (12). Also, for a serology-based diagnosis without biopsy, human leukocyte antigen testing and the presence of symptoms are not mandatory criteria (12). In our study, the cut-off value of anti-TTG IgA was 11.8 x ULN and shown to have high sensitivity and specificity in predicting BPCD.

Most patients with T1DM and CD have little or no symptoms of malabsorption, and gastrointestinal complaints are usually

mild. Therefore, it is challenging to consider a diagnosis of CD in patients with T1DM based on clinical findings or routine laboratory tests. Serological examinations would help to detect subclinical disease (19). In our study, 54.3% of patients with BPCD had gastrointestinal symptoms (abdominal pain, diarrhoea, constipation, distention) or non-gastrointestinal system symptoms (short stature, weight loss, recurrent episodes of hypoglycemia). In a previous study, the presence of CD symptoms, younger age for onset of T1DM, anti-TTG IgA level higher than 7-8 x ULN, and positive anti-EMA were suggested to be predictive for BPCD (11). Similarly, the presence of gastrointestinal symptoms and high anti-TTG IgA levels was shown to be a reliable predictor for CD. In the same study, the endoscopic biopsy was performed in two cases with gastrointestinal symptoms and intermediate levels of anti-TTG IgA (9-16 U/mL), while the biopsy was compatible with CD (20). In our study, the presence of symptoms and high anti-TTG IgA levels were shown to be highly predictive for BPCD. Only in one asymptomatic patient with high anti-TTG IgA level (>10 x ULN), a biopsy was negative, which further emphasized the importance of the presence of CD symptoms.

The overall prevalence of CD is higher in females (21). Various studies in children and adolescents with T1DM reported variable sex distribution; a higher prevalence in girls (10,13,22,23), in boys (3,24) or no difference in boys and girls (9,18,25). In our study, there was no sex predominance of CD prevalence.

The frequency of CD is reported to be higher in patients with an earlier age of T1DM diagnosis (especially <5 years) (5,13,18,21). In contrast, other studies revealed no relationship between the age for diagnosis of T1DM and the frequency of CD (3,19,24,26,27). In our study, there was no statistically significant difference in the frequency of CD between the patients with age for diagnosis of T1DM <5 years and >5 years. In previous reports about half of the cases, the CD was detected at the time of the diagnosis of T1DM diagnosis (9,19), and most of the remaining cases were identified within the first five years following diagnosis of T1DM (9,18). In a review of nine longitudinal cohort studies of celiac screening in patients with T1DM between 5 and 18 years old, it was reported that 79% of celiac cases were diagnosed within the first five years following the diagnosis of T1DM. Therefore, screening for CD is recommended at diagnosis of T1DM and in the subsequent two and five years in case of asymptomatic and negative family history of CD. In the same review, it was mentioned that determination of the frequency of CD after five years of diabetes period is controversial due to a lack of data obtained from long-term follow-up. In some studies with long-term follow-up, 16% of CD cases were reported to be diagnosed between five and 10 years, and 5% after >10 years (28). Thus, CD should be considered at any time in T1DM patients with symptoms of CD (28). In our study, CD was detected in 76.1% of cases at the time of the diagnosis of T1DM, in 21.7% within five years and in 2.2% of the cases 8.5 years following the diagnosis of the T1DM. To the best of our knowledge, the rate of detection CD at the time of the diagnosis of T1DM (76.1%) is the highest ever reported in the literature.

The comorbidity of CD and T1DM in children has been reported to be associated with an increased risk of AITD (29,30,31). However, although studies evaluating CD prevalence in patients with both T1DM and AITD are scarce, the few studies conducted have revealed no difference (32,33). In our study, CD prevalence in patients with T1DM and AITD (11.6%) was higher than in patients with T1DM alone (6.1%), but the difference did not reach statistical significance.

There are controversial data regarding metabolic control and its association with T1DM and CD comorbidity. Some studies have reported no difference in metabolic control between children with T1DM only and children with concomitant T1DM and CD (34,35,36), while in some studies, HbA1c was lower in patients with T1DM and CD comorbidity (37). In the present study, we did not find a difference in HbA1c levels of T1DM patients with and without CD. However, it should be kept in mind that having a relatively acceptable HbA1c concentration does not eliminate the risk of developing diabetes complications. In addition, CD may increase glycemic variability and frequent hypoglycemia due to malabsorption which may result in a low HbA1c, thereby undersetimating the degree of glycemic control.

It has been shown that there was no difference in height and BMI SDS scores between children with a diagnosis of T1DM only and children with both T1DM and CD (19,37,38). However, some studies reported a lower height SDS in T1DM patients with CD (13,39). There are also studies indicating that GFD therapy does not affect height and BMI SDS (37,38,40), while some others reported a better height SDS after GFD (41). In our study, the height, weight and BMI SDS values of T1DM patients with CD were lower than those without CD. In addition, we did not find any difference between the weight, BMI and height SDS of the patients with CD before and after the GFD. This finding was in line with some previous reports (37,38). However, the lack of improvement in growth parameters may be attributed to non-compliance with GFD due to the low socioeconomic and cultural level of the region where our study was conducted.

Study Limitations

The main limitation of our study was that some individuals with positive TTG-IgA antibodies (n = 15) did not undergo duodenal biopsy. Another major limitation of the study is the retrospective nature of design. It was also a limitation that anti-EMA were not checked.

Conclusion

The frequency of BPCD in our patients with T1DM was 6.9%. Approximately three quarters of the cases were diagnosed at the time of diagnosis of T1DM and 97.8% were diagnosed within the first five years. High anti-TTG IgA titers, particularly in patients with CD symptoms, can be used as a valuable parameter to predict CD. However, spontaneous normalization of celiac serology suggested performing serological follow-up instead of immediate duodenal biopsy or GFD therapy, especially in patients with asymptomatic and mild anti-TTG IgA antibody levels. Having CD at the time of diagnosis of T1DM did not affect the metabolic control whilst being associated with poor growth parameters. Nevertheless, no improvement was seen in growth parameters which were attributed to non-compliance to GFD.

Ethics

Ethics Committee Approval: The study was performed in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Gazi Yaşargil Training and Research Hospital (document number: 17.01.2020/411).

Informed Consent: Since the study was retrospective, informed consent was deemed unnecessary and not obtained from the parents of the patients.

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Authorship Contributions

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Genotype and Phenotype Heterogeneity in Neonatal Diabetes: A Single Centre Experience in Turkey

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What is already known on this topic?

Neonatal diabetes mellitus is defined as diabetes diagnosed during the first six months of life. The most frequent mutations in Europe are reported to affect the ATP-dependent potassium channel genes. Genetic testing is essential for diagnosis and management.

What this study adds?

Neonatal diabetes mellitus can, in rare cases, arise after the age of six months. In our cohort, most of the patients had ATP-dependent potassium channel mutations similar to the literature. Genetic results in these patients lead to improved treatment with a transition to sulphonylurea therapy in those likely to benefit.

Abstract

Objective: Neonatal diabetes mellitus (NDM) may be transient or permanent, and the majority is caused by genetic mutations. Early diagnosis is essential to select the patients who will respond to oral treatment. In this investigation, we aimed to present the phenotype and genotype of our patients with NDM and share our experience in a single tertiary center.

Methods: A total of 16 NDM patients from 12 unrelated families are included in the study. The clinical presentation, age at diagnosis, perinatal and family history, consanguinity, gender, hemoglobin A1c, C-peptide, insulin, insulin autoantibodies, genetic mutations, and response to treatment are retrospectively evaluated.

Results: The median age at diagnosis of diabetes was five months (4 days-18 months) although six patients with a confirmed genetic diagnosis were diagnosed >6 months. Three patients had *KCNJ11* mutations, six had *ABCC8* mutations, three had *EIF2AK3* mutations, and one had a *de novo INS* mutation. All the permanent NDM patients with *KCNJ11* and *ABCC8* mutations were started on sulfonylurea treatment resulting in a significant increase in C-peptide level, better glycemic control, and discontinuation of insulin.

Conclusion: Although NDM is defined as diabetes diagnosed during the first six months of life, and a diagnosis of type 1 diabetes is more common between the ages of 6 and 24 months, in rare cases NDM may present as late as 12 or even 24 months of age. Molecular diagnosis in NDM is important for planning treatment and predicting prognosis. Therefore, genetic testing is essential in these patients.

Keywords: Neonatal diabetes, genetic, sulfonylurea, monogenic diabetes, potassium channel, syndromic neonatal diabetes

Introduction

Diabetes presenting in the first six months of life is classified as neonatal diabetes mellitus (NDM) (1,2,3,4). Its incidence in Europe is reported to be 1:90,000 (5). NDM may be transient or permanent with about 50-60% of NDM being transient (2,3). Although most cases remit within three months after diagnosis, about 50% of the patients relapse later in life, and most frequently during adolescence (5). Insulin treatment is usually required during the first few days following initial diagnosis, but it is life-long after relapse (3,4).

The incidence of permanent NDM in the Middle East is more than in Europe at 1:21,000 (6). To date, mutations in more

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than 25 genes have been reported to cause NDM (7,8). The most frequent mutations in Europe are reported to affect the pancreatic ATP-dependent potassium channel genes (*KCNJ11* and *ABCC8*), and most of them are spontaneous mutations (9). Early diagnosis is essential because NDM due to these mutations is responsive to sulphonylurea (SU) treatment, and early treatment improves neurocognitive development (10,11,12,13,14,15).

In this investigation, we present the genotypic and phenotypic characteristics of patients with NDM, followed at the pediatric endocrinology clinic of Bursa Uludağ University Hospital.

Methods

Patients

A total of 16 NDM patients from 12 unrelated families were included in the study. Clinical data were obtained from medical records, and a consent form for genetic analysis was filled out by all parents and participants. Patients diagnosed with diabetes below the age of 12 months and/or those with infantile diabetes with syndromic features and/or those with a family history of NDM were included in the study. The clinical presentation, age at diagnosis, perinatal and family history, consanguinity, gender, glycated hemoglobin (HbA1c), C-peptide, insulin, and insulin autoantibodies, genetic mutations, and response to treatment were retrospectively evaluated. Informed consent for genetic testing was obtained from the parents. The study was approved by the Ethical Committee of Bursa Uludağ University (approval number: 2020-8/23).

Laboratory Analysis

Serum glucose was analyzed by spectrophotometric methods (C16000 Architect System, Abbott, USA). C-peptide and insulin were assessed with chemiluminescent microparticle immunoassay (i2000 Architect System, Abbott, USA). HbA1c was measured by high-pressure liquid chromatography (Hb9210 Trinity Biotech Premier, USA). Glutamic acid decarboxylase antibody (GAD-65) and anti-insulin antibody were performed by enzyme immunoassay (DiaSarin ETI-MAX 3000, Italy). Pancreatic islet cell antibody was studied by indirect fluorescent antibody method.

Genetic Analysis

Analysis of all coding regions and exon/intron boundaries of the *KCNJ11, ABCC8, INS* and *EIF2AK3* genes was performed by Sanger sequencing. Genetic testing for all known genetic causes of NDM for eight of the patients was performed by the Exeter genomic laboratory, as previously described (16). The clinical significance of the variant was assessed using the Association for Clinical Genomic Science Best Practice Guidelines for Variant Classification 2019 (17).

Statistical Analysis

Descriptive analysis was performed using SPSS, version 21.0 (IBM Inc., Armonk, NY, USA). Data were expressed as median (minimum-maximum range) or mean ± standard deviation (range).

Results

The median age at diagnosis of diabetes for the whole cohort (n = 16) was five months (4 days to18 months), and the female to male ratio was 1.3:1. The mean glucose level at diagnosis was 475 ± 137 mg/dL. Nine patients presented with diabetic ketoacidosis (DKA), two patients with ketosis, and four with hyperglycemia. One patient was diagnosed elsewhere, and the initial presentation was not known (patient 12.15). The median HbA1c at the time of diagnosis was 10.2% (5.8-17.1%), and the median C-peptide was 0.085 ng/mL (0.01-1.22 ng/mL) (reference range 0.78-5.19 ng/mL). Eleven patients were born full-term, three of them with low birth weight (< 2,500 g), and five with a birth weight of 2,500-3,500 g. The gestational age and birth weight of four patients were not available. Multiple insulin regimens such as intermediate-acting (NPH), rapid-acting (insulin lispro) and short-acting insulin (regular), were started in 15/16 of the patients. Only one patient was treated with an insulin pump. Pancreatic imaging (sonographic examination) was performed in all of the patients, and none of them showed pancreatic abnormality. A genetic test was performed in 15 patients (Table 1).

A mutation in a gene known to cause NDM was identified in thirteen (86.7%) patients, but for two patients testing for all the known NDM genes did not detect a likely causative mutation. These patients without a mutation identified were diagnosed at the age of seven months and four days, respectively, and were both positive for anti-GAD antibodies (concentrations were 26.5 and 53.95 IU/mL, normal level < 5 IU/mL) (patients number 7.9 and 9.11 in Table 1). Although anti-GAD antibodies were positive, anti-insulin antibodies were in the normal range (concentrations were 0.2 and 2.5 IU/mL, normal level 0-10 IU/mL). Their birth weights were 3,700 g and 2,300 g, and they were not significantly different from the rest of the cohort.

Three unrelated patients had the *KCNJ11* mutations, six (including three from the same kinship) had *ABCC8* mutations, three had *EIF2AK3* mutations, and one had a *de novo INS* mutation.

Table 1. The age of diagnosis, genetic analysis, and treatment response of neonatal diabetes mellitus patients

Family number and patient number	Age at diagnosis (d/m/y)	Current age (year)	Sex	Consanguinity	HbA1c at diagnosis	C-peptide at diagnosis	Gene	Location
1.1	3 m	6.5	F	No	11.8	0.01	*KCNJ11*	Exon 1
2.2	2.5 m	6	F	No	N/A	N/A	*KCJN11*	Exon 1
3.3	40 d	6	F	No	10.5	0.07	*KCJN11*	Exon 1
4.4	4 m	5	M	No	9.9	0.75	*INS*	Exon 3
5.5	45 d	9	M	No	N/A	N/A	Unknown	-
6.6	18 m	23	F	Yes (1st degree cousins)	10.8	1.22	*ABCC8*	Exon 7
6.7	9 m	27	M	Yes (1st degree cousins)	N/A	N/A	*ABCC8*	Exon 7
6.8	18 m	36	M	Yes (1st degree cousins)	N/A	N/A	*ABCC8*	Exon 7
7.9	7 m	5.5	F	Yes (1st degree cousins)	6.7	0.02	No disease-causing variant identified (anti GAD65 ab positive)	-
8.10	12 d	8	F	No	6.5	0.06	ABCC8	Exon 28
9.11	4 d	Died	M	Yes (2nd degree cousins)	5.8	0.9	No disease-causing variant identified (anti GAD65 ab positive)	-
10.12	6 m	1	F	Yes (1st degree cousins)	17.1	0.01	*ABCC8*	Exon 29
10.13	8 m	3	F	Yes (1st degree cousins)	N/A	0.1	*ABCC8*	Exon 29
11.14	15 m	14.5	M	No	13.1	N/A	*EIF2AK3*	Exon 13
12.15	17 m	15.5	M	Yes (2nd degree cousins)	N/A	N/A	*EIF2AK3*	Exons 11-13
12.16	3.5 m	4.5	F	Yes (2nd degree cousins)	9.7	0.48	*EIF2AK3*	Exons 11-13

**Novel mutations.
N/A: not applicable, SU: sulphonylurea, M: male, F: female, m: month, d: day, y: year, HbA1c: hemoglobin A1c, NDM: neonatal diabetes mellitus

Patients with ATP-Dependent Potassium Channel Mutations

Patient 6.6 was diagnosed with ketosis at 18 months of age and was on insulin treatment until she was 17 years old when she was found to be homozygous for a previously reported *ABCC8* mutation classified as pathogenic (p.Glu382Lys) and switched to SU treatment. She had two cousins with diabetes on insulin treatment at 18 and 24 years of age who were also diagnosed during infancy (patients number 6.7

DNA-protein description	Variant classification according to ACMD guidelines	Consequence	Zygosity	NDM subtype	Treatment	SU response
c.175G > A p.Val59Met (p.V59M)	Pathogenic	Missense	Heterozygous	Permanent	SU	Yes
c.175G > A p.Val59Met (p.V59M)	Pathogenic	Missense	Heterozygous	Permanent	SU + insulin	Yes (has been added insulin treatment four years later)
c.175G > A p.Val59Met (p.V59M)	Pathogenic	Missense	Heterozygous	Permanent	SU	Yes
c.285C > G p.Cys95Trp (p.C95W) **	Likely pathogenic	Missense	Heterozygous	Permanent	Insulin	-
-	-	-	-	Permanent	SU	Yes
c.1144G > A p.Glu382Lys (p.E382K)	Pathogenic	Missense	Homozygous	Permanent	SU	Yes
c.1144G > A p.Glu382Lys (p.E382K)	Pathogenic	Missense	Homozygous	Permanent	SU	Yes
c.1144G > A p.Glu382Lys (p.E382K)	Pathogenic	Missense	Homozygous	Permanent	SU	Yes
-	-	-	-	Permanent	Insulin	-
c.3548G > A p.Arg1183Gln (p.R1183Q)	Likely pathogenic	Missense	Heterozygous	Transient	Insulin	-
-	-	-	-	Permanent	Insulin	-
c.692G > T p.Trp231Leu**	Likely pathogenic	Missense	Homozygous	Permanent	SU	Yes
c.692G > T p.Trp231Leu**	Likely pathogenic	Missense	Homozygous	Permanent	SU	Yes
p.Glu926Lys** p.Lys939Arg**	Likely pathogenic	Missense	Homozygous	Permanent	Insulin	-
c.1886_(c.2817+1_c.2818-1) del p.?	Pathogenic	Partial gene deletion	Homozygous	Permanent	Insulin	-
c.1886_(c.2817+1_c.2818-1) del p.?	Pathogenic	Partial gene deletion	Homozygous	Permanent	Insulin	-

and 6.8). These patients were also found to be homozygous for the *ABCC8* pathogenic variant and switched to SU. These three patients all responded well to oral treatment, and insulin was successfully discontinued.

One patient, diagnosed at twelve days of age with a previously reported *ABCC8* heterozygous mutation (p.Arg1183Gln), was off-treatment at four months of age, confirming transient NDM (patient 8.10).

Two sisters, diagnosed with NDM at six and eight months of age, were homozygous for the p.Trp231Leu mutation in the *ABCC8* gene (patients 10.12 and 10.13). Although, this variant was not previously reported in the literature and initially classified as a variant of uncertain significance, a trial switch from insulin treatment to SU was successful and the variant could therefore be re-classified as likely pathogenic.

Three unrelated patients were found to be heterozygous for the previously reported pathogenic *KCNJ11* p.Val59Met mutation. This variant has been previously reported in patients with iDEND (18,19). However, none of our patients was reported to have neurological features at the ages of seven, six and a half and six years.

All the permanent NDM patients with *KCNJ11* and *ABCC8* mutations were successfully transferred to SU treatment, resulting in a significant increase in C-peptide level after three months, better glycemic regulation, and discontinuation of insulin (Table 2). SU was started at a dose of 0.2 mg/kg/day, twice a day. Later, doses were adjusted with blood glucose levels. The doses of SU were in the range 0.2-1.2 mg/kg/day. Only one patient required a single dose of long-acting insulin four years after the diagnosis (patient 2.2).

Patients with Mutations in Other Genes

One patient with a novel heterozygous *de novo* mutation in the *INS* gene (p.Cys95Trp) was diagnosed at the age of four months. He remains insulin-treated (patient 4.4). One patient diagnosed at 15 months of age developed elevated levels of AST and ALT after one year, anemia, and leukopenia later during follow-up (patient 11.14). He also had congenital stenosis of the aorta and skeletal dysplasia, which became evident after infancy. Anti-GAD was negative, and Wolcott Rallison syndrome was confirmed by the detection of two homozygous *EIF2AK3* mutations. He is still on insulin and supportive therapy (for orthopedic complications and autoimmune hepatitis) at the age of 14.5 years. Similarly, another unrelated patient diagnosed at 15 months of age showed elevated transaminase levels, persistent hyperkalemia, thrombocytopenia, and skeletal dysplasia after two years and was also found to be homozygous for an *EIF2AK3* mutation (patient 12.15). His sister, diagnosed with NDM at four months of age, was homozygous for the same mutation (patient 12.16). Both patients are still on insulin and supportive treatment (for orthopedic and renal complications) at the age of 15.5 and 4.5 years.

Table 2. Values of C-peptide and hemoglobin A1c levels of neonatal diabetes mellitus patients with ATP-dependent potassium channel mutations before and after sulphonylurea treatment

Family and patient number	HbA1c at diagnosis and after SU treatment		C-peptide at diagnosis and after SU treatment	
1.1	11.8	5.9	0.01	2.31
2.2	N/A	6.8	N/A	2.89
3.3	10.5	6.3	0.07	1.11
5.5	N/A	7.1	N/A	1.12
6.6	10.8	9.3	1.22	5.8
6.7	N/A	8.1	N/A	3.18
6.8	N/A	7.4	N/A	2.5
10.12	17.1	9.6	0.01	3
10.13	N/A	8.1	0.1	3

N/A: not applicable.
SU: sulphonylurea, HbA1c: hemoglobin A1c, SU: sulphonylurea

Discussion

Although NDM is defined as diabetes diagnosed during the first six months of life, recent research has shown that, rarely, it may present as late as 12 or even 24 months of age (1-4) although between the ages of six and 24 months a diagnosis of type 1 diabetes is much more common. The median age of diagnosis in our study was five months (four days-18 months). The most striking finding in this investigation was the presentation of diabetes in a patient with genetically confirmed NDM at 18 months of age. This patient and his two cousins were found to have a homozygous pathogenic *ABCC8* mutation, and after many years on insulin treatment, they were successfully switched to SU therapy. NDM genes must therefore be considered when carefully collected family history suggests a possible genetic cause.

There was no statistical difference in terms of gender in our patients. Iafusco et al (20) similarly reported no gender difference in their cohort.

In a study reported by Russo et al (21), 75% of the patients with NDM diagnosed during the first six months of life had a mutation in *KCJN11*, *ABCC8*, or *INS* gene. This ratio dropped to 12% in patients diagnosed between 7-12 months. The same study also reported that the patients diagnosed with permanent NDM before six months of age but without mutations in *KCJN11*, *ABCC8*, or *INS* had higher birth weight than those with the mutations. In our smaller cohort, we did not observe a similar difference between patients with and without a causative mutation. Similarly to Besser et al (22), more than 50% of our patients with NDM had low birth weight despite term delivery, likely due to *in utero* hypoinsulinemia. Letourneau et al (23) reported that 66.2% of the patients with monogenic diabetes presented with DKA, similar to our patients, with 60% having DKA at the time of diagnosis.

Previous reports have suggested that autoantibodies are usually negative in NDM patients diagnosed before six months of age, except for maternal autoantibodies, which may have crossed the placenta (24) and patients with monogenic autoimmunity such as IPEX syndrome (25). GAD-65 were positive, but anti-insulin antibodies were negative, in two of our patients diagnosed at seven months and four days (the patients' number 7.9 and 9.11). These patients did not have mutations in the known NDM genes (including monogenic autoimmunity genes such as *FOXP3, IL2RA,* and *LRBA),* however more causal genes remain to be discovered and a monogenic etiology is therefore possible. Whilst the antibody positivity in the patient diagnosed at seven months suggests a diagnosis of type 1 diabetes is likely, further investigations are needed to define the genetic etiology of the patient diagnosed at four days, since antibody positivity is common in patients with NDM caused by monogenic autoimmunity (26).

Molecular diagnosis in NDM is important for planning treatment and predicting prognosis. Therefore, genetic testing is essential in these patients. Carmody et al (13) have discussed the pros and cons of trying SU treatment awaiting the results of genetic tests. The advantages are a neurologic improvement, shorter hospital stay, lower cost, easier than insulin injections, and safety. On the other hand, increased expectance and disappointment of the family in case of treatment failure, risk of hypoglycemia in transient NDM, unknown long-term risks, and lack of FDA approval for SU in infants are the disadvantages. One of our patients was started on SU and responded well before the genetic test result was obtained, and insulin was successfully discontinued (patient 5.5). After receiving the test results, all of the patients were switched to SU, and better glycemic control was achieved along with significant elevation in C-peptide. Other family members with diabetes were also tested and switched to SU, which markedly improved their quality of life. Bowman et al (15) published a cohort of 90 patients with *KCNJ11* mutations causing permanent NDM and followed for ten years. SU response was excellent in 93%, and neurologic development was improved by 47%. Similarly, we observed an excellent response to SU in 7/8 (87.5%) patients with mutations affecting the pancreatic potassium channel. Only one patient required the addition of long-acting insulin to the treatment.

Despite the importance of genetic diagnosis, it may not be possible in all patients as some etiological genes still remain to be discovered. De Franco et al (16) reported that a genetic mutation was detected in 82% of patients in an international cohort of 1200 probands. Similarly, we found a causative mutation in 87% of our patients.

The most common syndromic form of NDM in countries with high consanguinity rate is Wolcott Rallison syndrome (16). We had three patients with this syndrome, including two siblings born to consanguineous parents. All three patients had hepatic dysfunction and skeletal dysplasia, which are known features of the syndrome (27). They are on insulin treatment, and their diabetes is well controlled. Demirbilek et al (28) investigated the genetic profile of the patients with NDM in Southeastern Turkey and found that mutations in potassium channel were less common in consanguineous families, while syndromic diabetes was more common. In our cohort, potassium channel mutations were more common, similarly to what is reported from Western countries.

Study Limitations

There were some difficulties in obtaining complete data because of the retrospective nature of the study. The age range of the patients was wide, and some patients had antibody positivity, which rendered patient selection for the study difficult. The relatively small number of patients in this cohort is limited, and further, ideally prospective, research with larger numbers of patients is warranted.

Conclusion

The recognition of NDM has increased with the identification of new genetic causes and the wider availability of genetic testing. Early diagnosis is essential to identify the patients who may respond to SU treatment. NDM has been defined as diabetes diagnosed during the first six months of life, but it is now increasingly recognized that the presentation of NDM may be delayed. In rare cases, it may present as late as 12 or even 24 months of age. Therefore very careful investigation of family history is essential. However, most patients still present before six months of age, and rapid genetic diagnosis must be obtained to plan the treatment. Syndromic diabetes must be considered in those with additional findings.

Ethics

Ethics Committee Approval: The study was approved by the Ethical Committee of Bursa Uludağ University (approval number: 2020-8/23).

Informed Consent: Consent form for genetic analysis was filled out by all parents and participants.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Elif Sobu, Özgecan Demirbaş, Yasemin Denkboy Öngen, Concept: Yasemin

Denkboy Öngen, Erdal Eren, Ömer Tarım, Design: Elif Sobu, Özgecan Demirbaş, Data Collection or Processing: Elisa De Franco, Elif Sobu, Analysis or Interpretation: Yasemin Denkboy Öngen, Erdal Eren, Sian Ellard, Literature Search: Ömer Tarım, Sian Ellard, Elisa De Franco, Writing: Yasemin Denkboy Öngen, Erdal Eren, Elisa De Franco, Ömer Tarım.

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ORIGINAL ARTICLE

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Pediatric Primary Adrenal Insufficiency: A 21-year Single Center Experience

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What is already known on this topic?

Primary adrenal insufficiency (PAI) is characterized by deficient production of glucocorticoids and/or mineralocorticoids from the adrenal glands due to dysfunctional or unresponsive adrenal tissue. Congenital adrenal hyperplasia (CAH) is the most common and well-known etiology in childhood. Non-CAH etiologies are rare, and have varying rates of distribution across different populations. There is limited epidemiological and clinical information regarding non-CAH PAI.

What this study adds?

To the best of our knowledge, this is the first cohort study of PAI in children from Turkey. We were able to determine the etiology in 95.8% of PAI patients. Non-CAH etiologies accounted for 30% of PAI and are presented in detail, along with a literature review. The most common non-CAH etiology was adrenoleukodystrophy. A potential novel p.Q301* hemizygous frameshift mutation of the *DAX-1* gene was also identified in one patient.

Abstract

Objective: Primary adrenal insufficiency (PAI) is a rare but potentially life-threatening condition. In childhood, PAI is usually caused by monogenic diseases. Although congenital adrenal hyperplasia (CAH) is the most common cause of childhood PAI, numerous non-CAH genetic causes have also been identified.
Methods: Patients aged 0-18 years and diagnosed with PAI between 1998 and 2019 in a tertiary care hospital were retrospectively evaluated. After the etiologic distribution was determined, non-CAH PAI patients were evaluated in detail.
Results: Seventy-three PAI patients were identified. The most common etiology was CAH (69.9%, n=51). Non-CAH etiologies accounted for 30.1% (n=22) and included adrenoleukodystrophy (ALD; n=8), familial glucocorticoid deficiency (n=3), Triple A syndrome (n=5), autoimmune adrenalitis (n=1), adrenal hypoplasia congenital (n=1), IMAGe syndrome (n=1), and other unknown etiologies (n=3). The median age at the time of AI diagnosis for non-CAH etiologies was 3.52 (0.03-15.17) years. The most frequent symptoms/clinical findings at onset were hyperpigmentation of skin (81.8%), symptoms of hypoglycemia (40.9%), and weakness/fatigue (31.8%). Hypoglycemia (50.0%), hyponatremia (36.4%) and hyperkalemia (22.7%) were prominent biochemical findings. Diagnosis of specific etiologies were proven genetically in 13 of 22 patients. A novel p.Q301* hemizygous frameshift mutation of the *DAX1* gene was identified in one patient.
Conclusion: Etiology was determined in 86.3% of children with non-CAH PAI through specific clinical and laboratory findings with/without molecular analysis of candidate genes. ALD was the most common etiology. Currently, advanced molecular analysis can be utilized to establish a specific genetic diagnosis for PAI in patients who have no specific diagnostic features.
Keywords: Primary adrenal insufficiency, pediatric, etiology

Introduction

Adrenal insufficiency (AI) is a life-threatening condition characterized by deficient production of glucocorticoids (GC) and/or mineralocorticoids (MC) from the adrenal glands or reduced response to these steroids. If AI is caused by dysfunctional or unresponsive adrenocortical tissue, it is classified as primary AI (PAI). However, if it

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is caused by disordered function of the pituitary gland and/or hypothalamus, it is termed secondary AI (1). A diagnosis of PAI depends on low serum cortisol and high plasma adrenocorticotropic hormone (ACTH) as well as clinical findings, such as hyperpigmentation of the skin, hypoglycemia, salt wasting, hypotension, and other non-specific symptoms such as fatigue, weight loss, failure to thrive, depression, and convulsions (2,3).

Primary AI has a prevalence of 93-140 per million and an incidence of 4.7-6.2 per million in the white adult populations (4). It is thought to be less common in the pediatric population. In childhood, PAI is usually caused by hereditary or sporadic monogenic disease. Congenital adrenal hyperplasia (70-85%) is the most common cause, with an estimated prevalence of 1/10,000-18,000 (1,2,5,6). In several studies non-CAH etiologies generally accounted for 10-30% of childhood PAI and autoimmune AI was usually the most common (1,5,6). Other genetic etiologies of non-CAH are adrenal gland developmental disorders [X-linked adrenal hypoplasia congenital (AHC), steroidogenic factor-1 related and other syndromic causes], ACTH resistance including familial GC deficiency (FGD) and related conditions and Triple A syndrome (TAS), metabolic causes [cholesterol synthesis/metabolism defects, adrenoleukodystrophy (ALD), other defects of the peroxisome, lysosome, endoplasmic reticulum and mitochondria], GC resistance and aldosterone synthesis/action defects (7,8). Infections, infiltrative diseases, adrenal hemorrhage, bilateral adrenalectomy and some drugs are the non-genetic causes of PAI.

Non-CAH PAI cases are less common, so the exact frequencies of non-CAH etiologies are still unknown, with the exception of ALD which has an estimated prevalence of 1/17000 at birth and the literature contains limited clinical data regarding these rare subgroups (2,8,9). Sharing clinical information about these patients will raise awareness about the disease. Early diagnosis and appropriate treatment is essential for avoiding lethal outcomes in PAI patients.

The aim of this study was to review the etiologies, clinical presentations, laboratory findings, genetic analysis, treatments and follow up features of non-CAH PAI cases that were followed in a pediatric endocrinology department of a tertiary care hospital over a period of 21 years.

Methods

We retrospectively evaluated patients aged 0-18 years who had their diagnosis and follow up for PAI, between August 1998 and October 2019, at İnönü University Faculty of Medicine, Turgut Özal Medical Center, Department of Pediatric Endocrinology. After the etiologic distribution was determined, non-CAH PAI patients were evaluated in detail. Data was retrospectively extracted from patient records including date of birth, age at diagnosis, sex, clinical characteristics, comorbidities, laboratory results [serum glucose, electrolytes, plasma ACTH, serum cortisol, plasma renin, aldosterone, plasma very long chain fatty acids (pVLCFA), autoantibodies], imaging results (adrenal, central nervous system and other), mutational analysis results, AI etiologies and treatment information. All the information was obtained from clinical records and the hospital's electronic database, and reviewed by an endocrinologist. Written informed consent forms were filled out by the patients and/or their families so that medical data (including genetic analysis results) may be collected and reported for educational and/or scientific purposes.

Primary AI was diagnosed based on the coexistence of at least the first two of the following criteria: 1) clinical symptoms/signs were suggestive of PAI (recurrent hypoglycemia, hyperpigmentation of skin, hyponatremia with hyperkalemia); 2) plasma ACTH levels at 8 am being twice the upper limit of normal and a cortisol level of <138 nmol/L. If a patient had clinical symptoms and signs suggestive of PAI, but had a serum cortisol level >138 nmol/L at 8 am with high ACTH, a standard dose synacthen stimulation test was performed. Serum cortisol was recorded at 0, 30 and 60 minutes after 250 μg/m^2 intravenous ACTH. If the peak plasma cortisol level was under 500 nmol/L the patient was also diagnosed with PAI (10); 3) a positive genetic analysis report indicated one of the etiologies of PAI. After PAI diagnosis, CAH subtypes were evaluated by clinical and biochemical analysis initially, and a target gene sequence analysis was done in patients diagnosed with CAH. Only classical CAH patients were included in the study and non-classical cases were excluded. Non-CAH patients were then evaluated for autoimmune adrenalitis and ALD. The presence of 21-hydroxylase-antibody in serum was evaluated by enzyme immunoassay. Plasma VLCFA (pVLCFA) were analyzed with gas chromatography-mass spectrometry. Alacrima was confirmed by using Schirmer's test. Achalasia was diagnosed based on clinical symptoms and timed barium esophagogram. Brain magnetic resonance imaging (MRI) was conducted in patients who had neurological symptoms or high pVLCFA levels. If certain genetic tests were available, with the permission of the parents, specific genetic analysis was done for target genes in PAI patients. Patients with PAI, who had high pVLCFA levels with/without neurological symptoms/leukodystrophy on brain MRI or a family history of ALD, were diagnosed as ALD clinically and DNA sequencing analysis of the *ABCD1* gene was done. In patients with PAI, who were also diagnosed with

alacrima and/or achalasia, a DNA sequencing analysis of the *AAAS* gene was done to investigate TAS. Patients who had evident growth retardation and other dysmorphic features were evaluated for syndromic PAI etiologies. Patients with early onset PAI who had no specific clinical features were evaluated for *MC2R* and/or *DAX1* gene mutations using DNA sequencing. The genetic analyses were conducted in several different commercial genetic laboratories in Turkey (Detagen, Intergen and Düzen Genetic Laboratories).

This study was approved by the Ethical Committee of İnönü University (approval number: 2019/407), and was conducted in accordance with the World Medical Association Declaration of Helsinki.

Statistical Analysis

Data were analyzed by descriptive statistical methods. Qualitative variables were expressed as number (%). Continuous quantitative variables were expressed as mean and standard deviation if they conformed to a normal distribution and as median and range if they did not.

Results

Seventy-three patients were diagnosed with PAI in either an inpatient or outpatient setting over a 21-year period.

1. Congenital Adrenal Hyperplasia

CAH was the most common etiology and 51 (69.9%) patients had CAH. Twenty-four CAH patient had 21-hydroxylase deficiency (21-OHD) (47% of CAH), 19 patients had 11-beta-hydroxylase deficiency (11-OHD) (37.2% of CAH), six patients had 17-alpha-hydroxylase deficiency, one patient had 21-22 desmolase deficiency, one patient had steroidogenic acute regulatory protein deficiency. The CAH patients are not reviewed further.

2. Non-CAH Etiologies

Non-CAH etiologies accounted for 30.1% (n=22) of PAI: ALD n=8 (11%); FGD n=3 (4.1%); TAS n=5 (6.8%); and autoimmune adrenalitis in one patient, AHC in one patient and IMAGe syndrome in one patient. Etiology of PAI could not be clarified in three patients (4.1%) in whom CAH was excluded (Table 1). Median age at the diagnosis of AI was 3.52 (0.03-15.17) years and male/female ratio was 6.33 (19/3) for non-CAH patients (Table 2). Mean height SDS and weight SDS were normal on admission. The most frequent symptoms and clinical findings were hyperpigmentation of skin (81.8%), symptoms of hypoglycemia (40.9%) and weakness/fatigue (31.8%). The other symptoms and clinical findings were alacrima, adrenal crisis, achalasia, neonatal prolonged jaundice, learning disability, vomiting, headache, low school performance, mental retardation, walking disability, epilepsy, and polyneuropathy. Parental consanguinity was very frequent, and was present in 77.8% (14/18) of cases. Hypoglycemia (50.0%), hyponatremia (36.4%) and hyperkalemia (22.7%) were prominent biochemical findings. Mean plasma ACTH level was very high, while mean serum cortisol level was very low (Table 2). All non-CAH PAI patients were treated with oral hydrocortisone (HC); however, 27.3% of them also received oral fludrocortisone (FC) treatment. The mean follow up duration was 65.04 ± 36.23 months.

Adrenoleukodystrophy

Eight male patients from four different families were diagnosed with ALD (Table 3). Patient 1 (P1) and P2 were brothers and their family history revealed that four of their brothers had died from AI. P3 was a nephew, who had died of encephalitis when he was seven years old, and P4 was a distant relative of them. P5 and P6 were also brothers from a different family. The other two patients (P7, P8) were from different unrelated families.

Median age at AI diagnosis was 7.17 (2.89-15.17) years for ALD patients. All but one of them presented with hyperpigmentation of the skin. Other symptoms/findings were adrenal crisis, headache, vomiting and hypoglycemia symptoms. In laboratory analysis three patients had hyponatremia and two of them also had hyperkalemia. All patients had low serum cortisol (mean level 105.71 ± 66.79 nmol/L) and very high plasma ACTH levels. Four patients presented with neurological problems (Table 3). Plasma VLCFA levels were evaluated in four patients, and revealed high plasma C26 levels in three and high C26/C22 ratio in all of them. Four patients showed signs of white matter involvement on brain MRI, and one demonstrated a thin corpus callosum and hydrocephalus. Four patients were diagnosed based on clinical and laboratory findings as well as molecular analysis; they all had the same p. P543L (c.C1628T) mutation in the *ABCD1* gene. The other four patients' diagnoses were based on clinical and laboratory findings with/without family history. All patients were given HC at a median dose of 17.5 mg/m^2/day. Three patients had MC deficiency (P1 and P3 had mild hyponatremia and high plasma renin activity, P2 presented with severe hyponatremia and hyperkalemia), and needed FC at a dose of 0.05 mg/day, orally. One patient (P4) also had transient hyponatremia and high renin level but he did not need MC replacement. Three patients were prescribed Lorenzo's oil and lovastatin.

Triple A (Allgrove) Syndrome

Five patients (P9-13) (three males, two females) from three different families were diagnosed with TAS. P9 and P10 were siblings and P11 was their cousin, the other two patients were from different families without a consanguineous marriage. All patients were living in the same city in east Turkey. Three patients had a classical triad of achalasia, alacrima, AI; the other two had two symptoms from the triad (alacrima and AI). Four of the patients presented with hypoglycemia symptoms and convulsions, all patients had hyperpigmentation of skin, two patients had mild facial dysmorphism, (wide/depressed nasal root, down slanting palpebral fissures, short philtrum), one had mild mental retardation, nasal speech, thenar atrophy and short stature, and the other one had normocytic anemia and corpus callosum hypoplasia. The earliest symptom was alacrima for all patients, which was noticed in early infancy. Hyperpigmentation of skin was noticed at three-five years old. The median age at diagnosis of AI was 6 (3.19-7.83) years old. Transient hyponatremia was seen in one patient; hyperkalemia was not seen. Median serum cortisol level was 30.36 (13.8-218.04) nmol/L, plasma ACTH level was 275 (186.34-440) pmol/L. Molecular analysis of patients revealed the same homozygous mutation p.L356Vfs*8 (c.1066_1067delCT) in the *AAAS* gene. According to the Clinvar database, this mutation (variation ID: 264992) was

Table 1. Etiologies of primary adrenal insufficiency in different cohorts

Etiologies	Our cohort 2020	Simm et al (11) 2004	Perry et al (5) 2005	Hsieh and White (3) 2011	Tsai et al (12) 2016	Ventura et al (1) 2019	Wijaya et al (6) 2019
CAH	51 (69.9%)	Not reviewed	74 (71.8%)	35 (45.5%)	Not reviewed	35 (85.3%)	362 (83.4%)
21-hydroxylase deficiency	24		72	Subgroups were not reviewed			351
11-hydroxylase deficiency	19		-				3
3β-hydroxy steroid dehidrogenase	-		2				-
17α hydroxylase deficiency	6		-				5
21-22 desmolase	1		-				-
StAR deficiency	1		-				2
Non-CAH etiologies	22 (30.1%)	16	29 (28.2%)	42 (54.5%)	6	6 (14.6%)	49 (11.3%)*
ALD	8	5	4	3	-	1	22
Triple A	5	-	1	-	-	-	2
FGD	3	-	-	4	1	-	-
Autoimmune adrenalitis (all)	1	5	13	23	3	3	3
- Isolated	1		4	18	-		-
- APS	-		9	5	3		3
Congenital adrenal hypoplasia	1	5	1	2	-	-	20
IMAGe	1?	1	-	(1)?	-	-	-
Wolman disease	-	-	3	-	-	-	-
Zelweger disease	-	-	1	-	-	-	-
Steroidogenic factor 1 deficiency	-	-	-	-	-	-	1
Pearson disease	-	-	-	-	-	1	-
Bilateral adrenal hemorrhage	-	-	-	2	-	1	-
Bilateral adrenalectomy	-	-	-	5	-	-	1
Unknown etiology	3	-	6	3	2	-	23*
Total PAI	73	16	103	77	11	41	434

*Unknown etiologies classified as a separate group in this study, they were not included in non-CAH group.
CAH: congenital adrenal hyperplasia, PAI: primary adrenal insufficiency, ALD: adrenoleukodystrophy, FGD: familial glucocorticoid deficiency, APS: autoimmune polyendocrine syndromes

a previously reported pathogenic mutation (13). All patients were given HC at a median dose of 15 mg/m^2/day orally, but none of them needed FC. Surgery for achalasia was undertaken for P9 and P10. All patients were prescribed eye drops to prevent dry eyes.

Table 2. Non-congenital adrenal hyperplasia primary adrenal insufficiency patients' characteristics, treatment and follow up

Number of patients	22
Male/female	19/3
Age at diagnosis	3.52 (0.03-15.17)
Median (minimum-maximum) years	
Height SDS at diagnosis	-0.63 ± 1.56
Mean ± SD	
Weight SDS at diagnosis	-0.95 ± 1.61
Mean ± SD	
Symptoms and clinical findings	
Hyperpigmentation of skin	81.8% (18/22)
Hypoglycemia symptoms	40.9% (9/22)
Convulsions	27.3% (6/22)
Weakness/fatigue	31.8% (7/22)
Alacrima	22.7% (5/22)
Adrenal crisis	22.7% (5/22)
Achalasia	13.6% (3/22)
Neonatal prolonged jaundice	13.6% (3/22)
Learning disability	13.6% (3/22)
Vomiting	9.1% (2/22)
Headache	9.1% (2/22)
Low school performance	1/22
Mental retardation	1/22
Walking disability	1/22
Epilepsy	1/22
Polyneuropathy	1/22
Parental consanguinity	77.8% (14/18)
Laboratory findings	
Hypoglycemia	50.0% (11/22)
Hyponatremia	36.4% (8/22)
Hyperkalemia	22.7% (5/22)
ACTH (reference range < 10.56 pmol/L)	284.68 ± 69.95
Cortisol (reference range 138-580 nmol/L)	85.56 ± 87.49
Medical treatment given to patients	
Hydrocortisone (mg/m^2/day)	100% (22/22) (14.5 ± 2.65)
Fludrocortisone (mg/day)	27.3% (6/22) (0.05)
Follow up period (months)	65.04 ± 36.23

To convert ACTH pmol/L to pg/mL divide by 0.22. To convert cortisol nmol/L to µg/dL divide by 27.6.
SD: standard deviation, SDS: SD score, ACTH: adrenocorticotropic hormone

Familial Glucocorticoid Deficiency

Three patients (P14-16) (two males, one female) from three different families were diagnosed with FGD with genetic confirmation. The age at onset of AI was 1.5, 21 and 2.5 months old, respectively. Two of them presented with hyperpigmentation of skin and hypoglycemia. One patient (P14) also had pes equinovarus and posterior embriyotoxone of the eyes. All of them had a history of prolonged jaundice. Laboratory analysis revealed very low cortisol levels, very high plasma ACTH levels and normal plasma VLCFA levels for all patients. One patient had transient hyperthyrotropinemia. MRI of the adrenal glands was normal in all patients. Genetic analysis showed; P14 and P15 had a homozygous p.G116V (c.347 G > T) mutation in the *MC2R* gene; and P16 had a homozygous p.L225R (c.674T > G) mutation in the *MC2R* gene. All patients were given oral HC at a median dose of 12.5 mg/m^2/d, but none of them needed FC.

Congenital Adrenal Hypoplasia

A twenty-one months old boy (P17), diagnosed with PAI in another medical center at 16 days old was admitted to our hospital. He had salt wasting during AI onset. Medical history revealed that his brother had died due to AI at 10 months old. Biochemical results had shown that he had hyponatremia and hyperkalemia in the neonatal period. At 16 days old his serum cortisol level was low, plasma ACTH level was high, and serum gonadotropin levels were normal. CAH was excluded. He had normal external genitalia. MRI of the adrenal glands was normal. Brain MRI (T2A) showed bilateral hyperintense changes in occipital white matter. Genetic analysis through DNA sequencing revealed a p.Q301* (c.901C > T) hemizygous non-sense mutation in the *DAX1* gene (Figure 1). This variation has not been reported previously; however, it is predicted to result in a premature stop codon and was most likely pathogenic according to both Mutation Taster and ClinVar data (14,15). Genetic analysis of the mother was not performed. He was treated with HC 15 mg/m^2/day and FC 0.05 mg/day orally.

Isolated Autoimmune Adrenal Insufficiency

A thirteen-year old male patient (P18) presented with hyperpigmentation of skin, weakness and fatigue. He had no neurological signs. Laboratory analysis showed severe hyponatremia (Na = 117 meq/L) and mild hyperkalemia (K = 5.5 meq/L). Serum glucose was normal. Serum cortisol level was low, and plasma ACTH level was high. He was examined for autoimmune diseases; thyroid autoantibodies, and celiac antibodies were not detected. Plasma VLCFA test revealed a normal C26 level and high C26/C22 ratio.

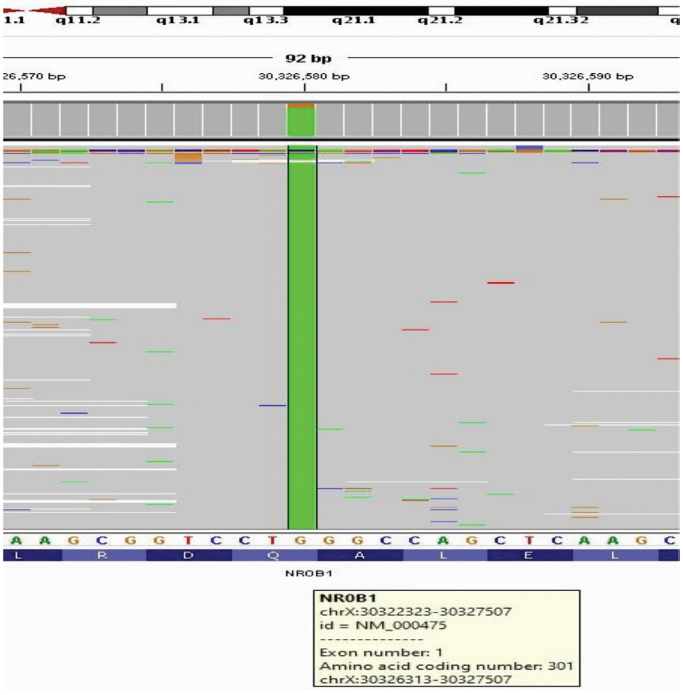

Figure 1. DNA sequence analysis of the patient with adrenal hypoplasia congenital

Mutational analysis of the *ABCD1* gene was normal. In his follow-up, serum 21-hydroxylase antibody was positive and he was diagnosed with isolated autoimmune AI. Left renal agenesis was also revealed during the evaluations. He was treated with HC 10-15 mg/m^2/day. In the follow up period (47 months), he became obese and demonstrated insulin resistance, type 2 diabetes mellitus, hypertension, hepatosteatosis and dyslipidemia but did not develop any other autoimmune disease.

Syndromic Primary Adrenal Insufficiency

One male patient (P19) was admitted to another center with adrenal crisis when he was 10 days old. He was diagnosed with PAI, treated with HC and FC, and subsequently referred to our hospital after one month of hospitalization in an intensive care unit. He was born at 41 weeks of gestation with a weight of 2400 g (small for gestational age). He had facial dysmorphic features (frontal bossing, deeply located eyes, hypertelorism, depressed nasal bridge), bilateral undescended testicles, and penile hypospadias. His karyotype was 46,XY, SRY locus (+). His testes exhibited normal hormonal functions. Diagnosis of CAH was excluded. Echocardiogram showed thin patent ductus arteriosus. Abdominal US revealed ectopic and fusioned kidneys. Brain and lumbar MRI revealed multiple hemorrhagic lesions in the brain and a 35x4 mm syringohydromyelia at the lumbar region. It was considered that he may have syndromic PAI due to IMAGe (intrauterine growth retardation-metaphyseal dysplasia-AHC-genital anomalies) syndrome, but genetic analysis has not been performed at the time of writing.

Unknown Etiology

A total of three male patients were diagnosed non-CAH PAI of unknown etiology.

One male patient (P20) presented with hyperpigmentation and an incidental mass in the right adrenal gland at 13.7 years old. He also had hereditary spherocytosis. He had no neurological/psychiatric complaints or findings. Other than hypoglycemia, biochemical analysis was normal and hormonal assays revealed only partial PAI. Basal level of serum cortisol was normal, but plasma ACTH levels were very high. A standard dose synacthen test resulted in a peak serum cortisol level of 331.08 nmol/L (low). He had no MC deficiency. Plasma VLCFA levels were normal. Adrenal antibodies could not be analyzed, but thyroid autoantibodies and tissue transglutaminase antibodies were negative. Adrenal MRI showed a 4.3x3.1 cm mass in the right adrenal gland. Following right adrenalectomy, the lesion pathology was revealed to be an adrenal hemorrhage. He was given HC replacement.

A two-year-old boy (P21) presented with weakness, hyperpigmentation of the skin, hypoglycemic symptoms including hypoglycemic convulsions. He had no other neurological symptoms or signs. Laboratory analysis showed apparent AI and normal plasma VLCFA levels. Adrenal antibodies were negative. Molecular analysis showed no mutation in the *DAX1* gene. He was treated with HC. The patient was thought to have FGD; however, molecular analysis has not been performed.

A seven-month old boy (P22) presented with hypoglycemia symptoms and hyperpigmentation of the skin. Medical history revealed that he was term with normal birthweight, he had adrenal crisis and hypoglycemic episodes in the newborn period and laboratory analysis showed hyponatremia, hyperkalemia, hypoglycemia, low serum dehydroepiandrosterone sulfate, and very high plasma ACTH. He was treated with HC and FC in another medical center. Physical examination in our hospital revealed that he had microcephaly, diplopia and motor mental retardation. Plasma VLCFA analysis showed a high C26 level, but C26/C22 ratio was normal. His brain MRI revealed cerebral atrophy, calcification in the basal ganglia, and corpus callosum atrophy. Further genetic analysis could not be performed. He was treated with HC and FC for two years.

Table 3. Etiologies and characteristics of non-congenital adrenal hyperplasia primary adrenal insufficiency patients

No	Sex	Age at onset AI (year)	Clinical presentation	Additional pathologies and imaging findings	Serum cortisol nmol/L	Serum ACTH pmol/L	Serum VLCFA	Etiologies	Gene mutation and variant	Treatment
1[α]	M	9.44	Hyperpigmentation of skin, weakness, learning disability	VSD, enuresis At 15 year: White matter involvement	121.44	> 275.0	High	ALD	*ABCD1* p.P543L mutation	HC, FC, Lorenzo's oil Lovastatin
2[α]	M	5.15	Hyperpigmentation of skin, adrenal crisis	Thin corpus callosum, mild hydrocephalus	< 27.6	> 275.0	-	ALD	*ABCD1* p.P543L mutation	HC, FC
3[α]	M	2.89	Hypoglycemia symptoms (sweating, confusion), vomiting	-	245.64	> 275.0	-	ALD	Not done	HC
4[α]	M	9.19	Hyperpigmentation of skin	White matter involvement	88.87	> 275.0	High	ALD	*ABCD1* p.P543L mutation	HC Lorenzo's oil Lovastatin
5[β]	M	3.65	Hyperpigmentation of skin, hypoglycemia symptoms	-	146.28	> 275.0	-	ALD	*DAX-1, ABCD1, MC2R* no mutation	HC, FC 5 years
6[β]	M	3.21	Hyperpigmentation of skin	Motor-mental retardation, epilepsy, stereotypic movements, cerebral atrophy, white matter involvement	67.62	> 275.0	High	ALD	*ABCD1, MC2R* no mutation	HC
7	M	15.17	Hyperpigmentation of skin, weakness, learning disability, headache, walking difficulty	White matter involvement	121.44	> 275.0	-	ALD	*ABCD1* p.P543L mutation	HC Lorenzo's oil Lovastatin
8	M	11.39	Hyperpigmentation of skin	Polyneuropathy, status epilepticus	< 27.6	> 275.0	High	ALD	Not done Lost the follow up	HC
9[#]	F	7.83	Hyperpigmentation of skin, hypoglycemia symptoms, convulsions, achalasia, alacrima, learning disability	Mental retardation, short stature, facial dysmorphism, thenar atrophy, nasal speech	30.36	> 275.0	-	Tripple A sydrome	*AAAS* p.L356Vfs*8 homozygous mut	HC Surgery for achalasia
10[#]	M	7.39	Hyperpigmentation of skin, achalasia, alacrima	-	41.4	> 275.0	-	Tripple A sydrome	*AAAS* p.L356Vfs*8 homozygous mut	HC Surgery for achalasia
11[#]	F	6.07	Hyperpigmentation of skin, hypoglycemia symptoms, convulsions, achalasia, alacrima, vomiting, headache	-	218.04	186.34	-	Tripple A sydrome	*AAAS* p.L356Vfs*8 homozygous mut	HC
12	M	3.39	Hypoglycemia symptoms, convulsions, weakness, fatigue, alacrima, hyperpigmentation of skin	Mild fasial dismorphism, normocytic anemia, posterior corpus callosum hypoplasia	< 13.8	> 440.0	-	Tripple A sydrome	*AAAS* p.L356Vfs*8 homozygous mut	HC

Table 3. Continuation

13	M	3.19	Hyperpigmentation of skin, hypoglycemia symptoms, convulsions, weakness, alacrima	-	< 13.8	> 440.0	N	Triple A syndrome	*AAAS* p.L356Vfs*8 homozygous mut	HC
14	F	0.13	Hyperpigmentation of skin, hypoglycemia, prolonged jaundice	Pes equinovarus, posterior embriotoxone	< 27.6	> 275.0	-	FGD	*MC2R* p.G116V homozygous mut	HC
15	M	1.78	Hyperpigmentation of skin, hypoglycemia symptoms, convulsions, prolonged jaundice	-	33.12	> 275.0	-	FGD	*MC2R* p.G116V homozygous mut9	HC
16	M	0.22	Prolonged jaundice	Transient hypertirotropinemia, ASD, hypomyelinization of periventricular white matter	< 27.6	> 275.0	-	FGD	*MC2R* p.L225R homozygous mut	HC
17	M	0.04	Adrenal crisis in newborn	White matter involvement	79.21	> 275.0	-	Congenital adrenal hypoplasia	*DAX-1* p.Q301* hemizygous mut (novel)	HC, FC
18	M	13.49	Hyperpigmentation of skin, weakness, fatigue, adrenal crisis	Obesity, metabolic syndrome, unilateral renal agenesis	< 27.6	> 275.0	C26:N C26/24 high	Autoimmune AI (isolated)	-	HC, Metformin, ACE inhibitor
19	M	0.03	Adrenal crisis in newborn	Facial dysmorphism, bilateral undescendent testes, penil hypospadias, renal ectopy, patent ductus arteriosus, growth and mental retardation	.	.	-	MIRAGe syndrome? Syndromic PAI	Genetic analyse for Wolf Hirschorn was normal	HC, FC
20	M	13.69	Hyperpigmentation of skin	Hereditary spherocytosis, unilateral surrenal hematoma, unilateral undescendent testis	328.68	120.34	N	UD	*DAX1* no mutation	HC
21	M	2.09	Hyperpigmentation of skin, hypoglycemia symptoms, convulsions, weakness	-	< 27.6	> 275.0	N	UD	*DAX-1, DMD* no mutation	HC
22	M	0.03	Adrenal crisis in newborn, hypoglycemia symptoms, hyperpigmentation of skin	Microcephaly, diplopia, cerebral and corpus callosum atrophy, calcification in basal ganglia	.	391.6	C26 high	UD	-	HC, FC (2 years)

[α]: relatives, [β]: siblings, [#]: relatives. M: male, F: female, N: normal levels. To convert cortisol nmol/L to µg/dL divide by 27.6. To convert ACTH pmol/L to pg/mL divide by 0.22.
HC: hydrocortisone, FC: fludrocortisone, VLCFA: very long chain fatty acids, ACTH: adrenocorticotropic hormone, ALD: adrenoleukodystrophy, AI: adrenal insufficiency

Discussion

Primary AI in childhood is a relatively rare but potentially life-threatening condition. Although PAI is mostly caused by monogenic diseases in children, it is often acquired in adults (2).

We reviewed 73 children with PAI over a period of 21 years in a single tertiary center in Turkey. Non-CAH PAI patients

were especially reviewed in detail and compared with the literature. To the best of our knowledge this is the first cohort of PAI in children from Turkey. In a previously conducted nationwide cohort study, Guran et al (16) reported clinical and molecular genetic characteristics of children with PAI of unknown etiology (patients with CAH, ALD, autoimmune AI or obvious syndromic PAI such as TAS were excluded) from Turkey.

We were able to determine the etiology in 95.8% (70/73) of all PAI patients and in 86.3% (19/22) of the non-CAH PAI patients in our cohort through clinical and laboratory findings, with some being confirmed genetically.

Although CAH is still the most common cause of childhood PAI at present, numerous non-CAH genetic causes have been identified in the last 25 years, but their prevalance in children with PAI is not yet clear (1,2,5,6). Among CAH etiologies, 21-OHD is the most common (90-95%) (5,6,17). In our cohort, CAH was also the most frequent etiology (69.9%) and 21-OHD was the most common type of CAH (47%). However, in contrast to the literature, 11-OHD (19 cases from 15 families) (37.2%) was also very common in our study. Diagnosis of 11-OHD was confirmed with genetic analysis in 18 of the cases. Racial characteristics and frequent consanguineous marriage in our region might have caused this difference. In a review of 273 Turkish patients with CAH, Kandemir and Yordam (18) reported that 11-OHD was the second most common cause of CAH and accounted for 13.5% of cases, a rate which was still high compared to other populations in which it is reported to be 5-8%.

Similar to the literature, non-CAH causes accounted for 30.1% of childhood PAI in our cohort (Table 1). In contrast, Hsieh and White (3) reported a higher rate (54.5%) of non-CAH etiologies within 77 pediatric PAI patients. Many studies from western countries reported that autoimmune etiologies were the most common cause and accounted for 30-55% of non-CAH PAI in children (1,3,5,11,12). In their Chinese cohort, Wijaya et al (6) reported that ALD (44.9%) and AHC (40.8%) were the most common etiologies in the non-CAH group while autoimmune etiologies only accounted for 6.1%. In our cohort, ALD was the most common etiology (36.3%, n=8), and autoimmune AI was rare (only one patient) which is similar to the study of Wijaya et al (6). This suggests that racial features affect the etiological distribution of AI.

Autoimmune PAI can be isolated or a component of autoimmune polyendocrine syndromes (APS). Detecting anti-adrenal antibodies in serum of patients with PAI leads to the diagnosis of autoimmune PAI. Mutations in the autoimmune regulator gen (*AIRE*), are responsible for APS-1 in which PAI is usually combined with hypoparathyroidism and mucocutaneus candidiasis. APS-2 typically combines PAI with autoimmune thyroid disorders and type 1 diabetes mellitus, and shows a complex inheritance pattern (HLA-DR3/DR4, CTLA-4) similar to isolated autoimmune PAI (2,7). P18, a male adolescent patient had positive serum anti 21-OH antibodies but he had no family history of autoimmunity nor other accompanying autoimmune disease over nearly four years of follow up, and so was ultimately diagnosed with isolated autoimmune PAI.

ALD is an X-linked hereditary metabolic disorder caused by mutations of the *ABCD1* gene, which encodes a peroxisomal transport protein necessary for VLCFA degradation (≥C22). Toxic accumulation of VLCFA in plasma and multiple tissues (white matter of the brain, spinal cord and adrenal cortex) is associated with a proinflammatory state and eventual cell death (19). In male patients, Addison only, cerebral ALD (CALD; childhood, adolescent, or adult onset), and adrenomyeloneuropathy phenotypes can be seen (9). Perry et al (5) reported four ALD patients in their study (two Addison only, one childhood and one adolescent CALD). In our cohort, three patients had Addison only phenotypes, three had childhood CALD, and two had adolescent CALD. Hyperpigmentation of skin was the most common symptom. All patients had either neurological symptoms/white matter involvement, elevated plasma VLCFA, or family history of ALD. ALD causes GC deficiency, and MC deficiency may also be detected. Three of eight ALD patients in our cohort needed MC treatment. Perry reported MC deficiency in one of four ALD patients, while Wijaya et al (6) reported zero cases of MC deficiency in 22 ALD patients (5). If ALD is suspected in a male with neurological symptoms (with or without typical brain MRI abnormalities) or Addison's disease, diagnosis is made based on elevated VLCFA levels in plasma; genetic confirmation is useful for genetic counselling (9). For molecular confirmation of ALD, a sequence analysis of the *ABCD1* gene is first performed, and if no pathogenic variant is found, it is followed by a gene-targeted deletion/duplication analysis. This is because the sequence analysis method has a reported diagnostic value of 97% when identifying mutations of the *ABCD1* gene, and the remaining 3% can be detected using deletion/duplication analysis by way of the multiplex ligation-dependent probe amplification method (20). In addition, mutations in regions, such as the promoter region, that play a role in regulating gene expression, may not fall within the sequenced region in sequencing analysis. Four of our patients had a p.P543L mutation in exon 6 of the *ABCD1* gene, which has been reported previously (21) while in two cases *ABCD1* sequencing analysis revealed no mutation, but subsequent

deletion/duplication analysis was not performed. Genotype-phenotype correlation or the trigger for cerebral disease has not been described. The only currently available standard therapy is hematopoietic stem cell transplantation, which should be performed in the early stage of demyelination in boys with CALD.

Triple A (Allgrove) syndrome (OMIM 231550), characterized by achalasia, alacrima, and AI as well as neurological (central, peripheral and autonomic nervous system) and dermatological problems, is caused by homozygous or compound heterozygous mutations in the gene encoding aladin (*AAAS*) on chromosome 12q13. Palmoplantar hyperkeratosis (PH), hypothenar atrophy, short stature, facial dysmorphism, deafness, mental retardation, and nasal speech can also be present (22,23). Even with the same *AAAS* gene mutation, patients have phenotypical heterogenity (23,24). Alacrima was the earliest and the most consistent finding in our five TAS patients; hyperpigmentation of the skin and hypoglycemia symptoms were also common, in accordance with the study of Polat et al (23) who reported a large Triple A cohort (23 patients from 14 families) from Turkey. Therefore, symptoms of alacrima and achalasia must be investigated in all non-CAH PAI patients as the etiology may be TAS. Although Polat et al (23) reported short stature and PH in more than half of the cases, our cohort contained only one patient with short stature and PH was not present at all. Since our study has a retrospective design, PH may have been overlooked or not noted. On the other hand PH was only present in patients with the p.R478* mutation in the aforementioned study. This finding may be specific to the mutation. Grant et al (25) reported fissured palms in half of 20 patients in whom molecular analysis was not conducted. All of our patients with TAS had the homozygous p.L356Vfs*8 mutation in the *AAAS* gene, which was previously reported in a nine year old Turkish boy who had presented with achalasia, alacrima, stimulated cortisol deficiency, pitosis, pallor of optic disc, anisocoria and dry skin (26).

Familial glucocorticoid deficiency (FGD) is characterized by isolated GC deficiency in early infancy or in childhood, and presents with hyperpigmentation and hypoglycemia symptoms. All three of our patients were younger than two years at presentation and had a prolonged jaundice or history of it. In addition two of them presented with hyperpigmentation of skin and hypoglycemia. Akın et al (27) also reported the case of a 17-day-old newborn diagnosed with FGD type 1 who presented with hyperbilirubinemia and hyperpigmentation. Therefore, hyperpigmentation, persistent hypoglycemia and prolonged jaundice should suggest the possibility of AI in infancy. Although MC requirement and transient hyponatremia has been occasionally reported (16,28), none of our FGD patients had MC deficiency. Mutations in the *MC2R* gene (encoding the ACTH receptor protein) and *MRAP* gene (encoding MC2R accessory protein) are well described causes (almost 50%) of FGD (7). Although other rare genetic defects are also reported as causes in FGD, the underlying cause is unknown in about 40% of cases (2,7,29). These genetic defects manifest as phenotypically indistinguishable. Molecular genetic analysis of our patients revealed two different mutations in the *MC2R* gene, which have been previously reported (16,27,30).

Mutations in the *NR0B1 (DAX1)* gene located on Xp21.3-p21.2 and deletions in Xp21 (contiguous gene deletion) lead to impaired development of the adrenal glands, hypothalamus, pituitary gland and gonads and cause AHC. AI typically begins in early infancy or in childhood, but rarely begins in adulthood (31). Patients can also have hypogonadotropic hypogonadism (HH), which is characterized by undescended testes, micropenis, delayed puberty or infertility, associated with low levels of gonadotropins. It was reported that AHC due to *DAX1* mutation is a relatively frequent cause of non-CAH PAI in Chinese children (6,32). Wijaya et al (6) reported 20 male AHC cases (19 had *NR0B1* gene mutation) among 49 children with PAI. Of these, five patients presented with a typical adrenal crisis, 10 with salt craving, three with generalized hyperpigmentation at onset, and six patients with HH during follow-up. In this study the age at onset of AHC was <3 months in 13 of 20 patients, and ≤ 2 years in 17 of 20 patients. Lin et al (33) reported *DAX1* mutations in 58% (37 of 64) of 46,XY phenotypic boys with AI (not caused by CAH, ALD, or autoimmune disease) and in all boys (eight of eight) with HH and a family history suggestive of AI in males. AI had begun in early infancy in 81% of patients in their study. Only one male patient was diagnosed with AHC in our cohort, who had presented with salt wasting in the neonatal period. He had normal external genitalia and gonadotropin levels. Molecular analysis revealed a novel p.Q301* hemizygous non-sense mutation in the *DAX1* gene.

In recent years, many syndromic diseases that can cause PAI have been identified (Table 1). IMAGe syndrome was primarily defined by a spectrum of intrauterine growth restriction, metaphyseal dysplasia, CAH and genital anomalies. Patients can also have dysmorphic craniofacial features, hypocalcemia, and scoliosis (34). This disease is caused by gain of function mutations in the cyclin dependent kinase inhibitor (*CDKN1C*) gene, which regulates prenatal and postnatal growth. MIRAGE syndrome, another known cause of syndromic PAI, is due to a heterozygous *SAMD9* gain of function mutations and is characterized by

myelodysplasia, infection, restriction of growth, adrenal hypoplasia, genital anomalies and enteropathy. Neurological findings, such as microcephaly, hydrocephalus, white matter abnormalities, and perivascular calcifications were also described (35). Our two patients with neonatal onset PAI had dysmorphic features. One of them (P19) had severe pre- and post-natal growth retardation, dysmorphic facial features and urogenital anomalies accompanying PAI and was clinically diagnosed as IMAGe syndrome. The other one (P22), in contrast to IMAGe and MIRAGE syndrome, had no growth retardation, so it was considered that the neurological findings of the patient could have been due to severe hypoglycemic episodes and electrolyte imbalances during the newborn period, or caused by a peroxisomal or undefined syndromic disorder.

According to both the literature and our study, in at least 80% of children with non-CAH PAI, the etiology can be determined by specific clinical and laboratory findings with or without molecular analysis of a candidate gene. For patients who do not exhibit specific clinical findings, predicting the exact etiology can be challenging. Nevertheless, establishing a specific genetic diagnosis in PAI is very valuable for a number of reasons: 1) providing clear information about disease spectrum, potential comorbidities and prognosis; 2) modifying treatments, such as requirement for MC replacement; 3) genetic counseling of affected individuals and their families, identifying presymptomatic children before onset of potentially life-threatening symptoms; and 4) increasing knowledge about the normal biology and pathomechanisms of PAI (2). Comprehensive diagnostic algorithms for PAI in children are available in the literature (5,7) For patients without a definite diagnosis despite referral to these algorithms, gene panel based next generation sequencing, whole exome sequencing, or array comparative genomic hybridization are now more widely available.

Study Limitations

One of the limitations of our study was its retrospective design. Another limitation was that diagnostic molecular analysis could not be performed for all patients due to the limit in resources, especially in the earlier years of the study period.

Conclusion

As PAI is a life-threatening condition, early recognition and proper treatment are crucial. Signs, such as hyperpigmentation of skin, recurrent hypoglycemic episodes with or without prolonged jaundice, chronic fatigue and hyponatremia with hyperkalemia could most likely suggest AI in children. During diagnostic studies, CAH must initially be excluded. Afterwards, specific clinical and laboratory features must be evaluated and proven with appropriate candidate gene analysis. Due to the many advantages, advanced molecular analysis should be considered for patients who have no specific diagnostic features.

Ethics

Ethics Committee Approval: This study was approved by the Ethical Committee of İnönü University (approval number: 2019/407), and was conducted in accordance with the World Medical Association Declaration of Helsinki.

Informed Consent: Written informed consent forms were filled out by the patients and/or their families so that medical data (including genetic analysis results) may be collected and reported for educational and/or scientific purposes.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Emine Çamtosun, İsmail Dündar, Ayşehan Akıncı, Leman Kayaş, Nurdan Çiftci, Concept: Emine Çamtosun, Ayşehan Akıncı, Design: Emine Çamtosun, Ayşehan Akıncı, Data Collection or Processing: Emine Çamtosun, İsmail Dündar, Ayşehan Akıncı, Analysis or Interpretation: Emine Çamtosun, Ayşehan Akıncı, Literature Search: Emine Çamtosun, Writing: Emine Çamtosun, Ayşehan Akıncı.

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CASE REPORT

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Homozygous Mutation in the Insulin Receptor Gene Associated with Mild Type A Insulin Resistance Syndrome: A Case Report

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What is already known on this topic?

Insulin receptor (INSR) mutations lead to heterogeneous disorders that may be as severe as Donohue syndrome or as mild as "type A insulin resistance syndrome". Patients with severe disorders usually harbor homozygous or compound heterozygous mutations whereas type A insulin resistance syndrome is usually associated with a heterozygous mutations. Homozygous *INSR* gene mutations may rarely be responsible for mild type insulin resistance syndrome.

What this study adds?

The case presented with mild type A insulin resistance syndrome, was due to a novel homozygous mutation in the *INSR* gene. The novel mutation was p.Leu260Arg in exon 3 of the *INSR* gene. This highlights that homozygous *INSR* mutations may also cause mild clinical forms.

Abstract

Insulin receptor (INSR) mutations lead to heterogeneous disorders that may be as severe as Donohue syndrome or as mild as "type A insulin resistance syndrome". Patients with severe disorders usually harbor homozygous or compound heterozygous mutations. In contrast, type A insulin resistance syndrome has been associated with heterozygous mutations; homozygous mutations are rarely responsible for this condition. We report a novel, homozygous mutation, p.Leu260Arg in exon 3, of the *INSR* gene in a female adolescent patient with type A insulin resistance syndrome together with clinical details of her medical follow-up. Different mutations in the *INSR* gene cause different phenotype and vary depending on the inheritance pattern. This report adds to the literature, increases understanding of the disease mechanism and aids in genetic counseling.
Keywords: Hirsutism, insulin resistance, insulin receptor gene

Introduction

A functional insulin receptor (INSR) is crucial for eliciting the intracellular molecular effects of insulin and *INSR* mutations lead to genetically severe insulin resistance. *INSR* is a transmembrane protein and a member of the receptor tyrosine kinase family. This receptor is a heterotetramer, consisting of two α and two β subunits. The α subunits are extracellular, whereas the β subunits extend from the extracellular side of the membrane, traverse the membrane and protrude into the cytoplasm, this latter region possesses the tyrosine kinase activity. Activation of *INSR* requires trans-autophosphorylation of one β subunit by the other β subunit. A single gene, *INSR*, encodes for both subunits, and is located on chromosome 19. Each allele of this gene encodes one αβ half-receptor and two of them form αββα heterotetrameric *INSR*. This phenomenon explains how heterozygous mutations may result in impaired β subunit tyrosine kinase activity (1,2).

INSR mutations lead to heterogeneous disorders that range in severity from Donohue syndrome (leprechaunism) and

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Rabson-Mendenhall syndrome to the mild "type A insulin resistance syndrome". Patients with severe disorders are usually homozygous or compound heterozygous for these mutations (3,4). In contrast, type A insulin resistance syndrome has been associated with heterozygous mutations; homozygous mutations are rarely responsible for this condition (5,6,7).

Type A insulin resistance syndrome manifests itself in the peripubertal period, as oligomenorrhea and hyperandrogenism with acanthosis nigricans. In this article, we report an adolescent with type A insulin resistance syndrome due to a novel homozygous mutation in the *INSR* gene and also report details of three years of medical follow-up.

Case Report

A 12-year-old girl was admitted to the pediatric endocrinology department for excess hair growing on her body. This complaint had become evident over the preceding year. The patient was born of non-consanguineous marriage and her parents were healthy. She was born with a normal weight and her past medical history was uneventful. She had a severe hirsutism (Modified Ferriman-Gallwey Score was 30), acneiform rash on her face and severe acanthosis nigricans was observed in the axilla, neck and antecubital area (Figure 1A, 1B). Her blood pressure was 110-70 mmHg. Her height was 156.5 cm (70th percentile), weight was 68.4 kg (99th percentile), and body mass index was 27.9 kg/m^2 (98th percentile) at the admission.

Her pubertal development was Tanner stage 4 and her bone age was 13.5 years old. There was no history of spontaneous menarche. Her laboratory examinations results are detailed in Table 1. Laboratory investigations revealed elevated fasting insulin level with normal fasting glucose. Glycohemoglobin (HbA1c) level and oral glucose tolerance test results showed that she was in a pre-diabetic state with marked hyperinsulinemia. Despite the severe hyperinsulinemia, she had normal triglyceride, high-density lipoprotein (HDL) cholesterol and sex hormone binding globulin (SHBG) level and there was no hepatosteatosis on ultrasonographic evaluation. Her gonadotropin levels revealed luteinizing hormone dominancy with increased

Table 1. Laboratory data of a patient

Parameter	Result (Normal)
Glucose	74 mg/dL
Insulin	217 mIU/mL (N < 20)
Glycohemoglobin	5.74 %
OGTT 120 minute glucose	162 mg/dL
OGTT 120 minute insulin	893.5 mIU/mL
Triglyceride	59 mg/dL
High-density lipoprotein-cholesterol	71 mg/dL
Total-cholesterol	144 mg/dL
ALT/AST	15/15 IU/L
FSH	6.5 mIU/mL
LH	14.4 mIU/mL
Total testosterone	190 ng/dL (N < 55)
DHEA-SO_4	104 mcg/dL (N < 350)
SHBG	92.07 nmol/L (N: 17-155)

OGTT: oral glucose tolerance test, ALT: alanine aminotransferase, AST: aspartate aminotransferase, FSH: follicle-stimulating hormone, LH: luteinizing hormone, DHEA-SO_4: dehydroepiandrosterone sulphate, SHBG: sex hormone binding globulin

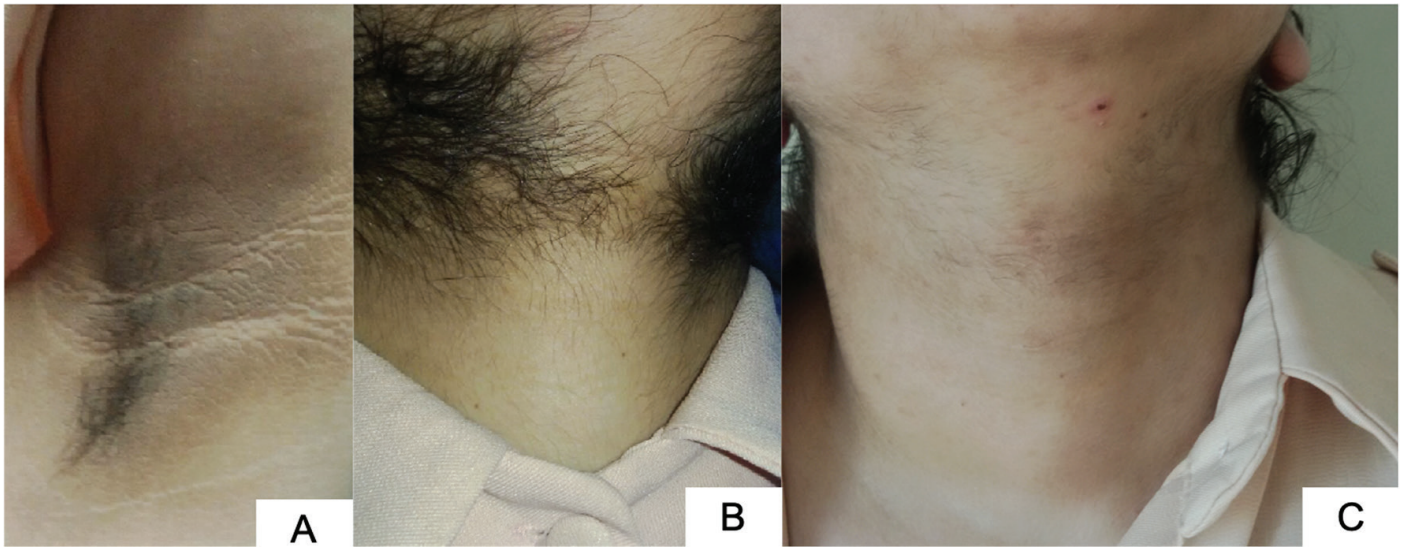

Figure 1. Clinical features of patient. A) severe acanthosis nigricans; B) hirsutism before treatment; and C) after treatment

testosterone level. There was a polycystic ovary appearance on ultrasonographic evaluation. Through the findings of clinical and laboratory examination, type A insulin resistance syndrome was considered and *INSR* mutation analyses were planned. DNA Sanger Sequence analyses of all coding exons of *INSR* showed that a novel, homozygous mutation, *NM_000208.4 c.779 T>G* (p.Leu260Arg) was present in exon 3 (Figure 2). Genetic analysis of the parents demonstrated both were carriers of the same mutation. There was no clinical or biochemical hyperandrogenism or disorder of glucose metabolism in her mother. Unfortunately, her father was not investigated due to extenuating circumstances.

First choice treatment was metformin with life style modification. It was planned to increase the dose gradually up to 2 gram/day. Unfortunately the patient was unable to comply with the life style modification consistently during whole therapy process. After one year of this therapy, oral contraceptive (OCP) (OCP; 2 mg cyproterone acetate plus 35 microgram ethinyl estradiol) were added because of increasing hirsutism. The clinical aim of this treatment was to suppress ovarian hyperandrogenism and to gain the additional benefit of the anti-androgenic potential of cyproterone acetate. After one year starting OCP her hirsutism score had markedly decreased (Figure 1C). Menarche also occurred after this treatment.

During her clinical follow-up basal-bolus insulin regimen was added into her therapy because of marked hyperglycemia, especially in the postprandial period, and a high HbA1c level (8.6%). Her HbA1c decreased to 7% after six months of basal-bolus insulin treatment. During the follow-up, bolus insulin was discontinued while retaining basal insulin and metformin therapy. The mean HbA1c was 7.4% at one-year follow-up. The patient and her mother provided informed consent.

Written consent form was obtained from the parents.

Discussion

If a patient presents in the adolescent period with hyperandrogenism and severe insulin resistance, and these findings are not explained by other reasons, such as obesity, then investigating clinicians should consider genetic insulin resistance syndromes. Mutations of *INSR* should be kept in mind in patients with severe insulin resistance but without metabolic dyslipidemia, low SHBG level and hepatosteatosis. Clinical and laboratory features of this patient were suggestive of *INSR* mutation.

Metabolic dyslipidemia (hypertriglyceridemia and low HDL-cholesterol levels) and steatohepatitis are closely associated with prevalent forms of insulin resistance (8). A key factor in the development of metabolic dyslipidemia and hepatic steatosis is postreceptor hepatic insulin resistance. Reduced liver fat synthesis plays a key role protection from dyslipidemia observed in patients with insulin receptoropathy (9). Absence of metabolic dyslipidemia and fatty liver in a patient with severe insulin resistance, as in this patient, is suggestive of a primary *INSR* mutation.

There are no obvious genotype–phenotype correlations for *INSR* mutations. It has been suggested that the homozygous mutations of the α-subunit cause more severe clinical features, whereas heterozygous β-subunit mutations lead to milder forms (1,3). However, this is not the case in all patients. Five patients type A insulin resistance patients with α-subunit mutations have been reported, similar to this case (5,6,7). Generally, patients with type A insulin resistance syndrome have been found to have heterozygous mutations. However, homozygous inheritance of mutations may rarely be responsible for this disease. Nakashima et al (7) previously described a Japanese patient, diagnosed as type A insulin resistance syndrome with a homozygous

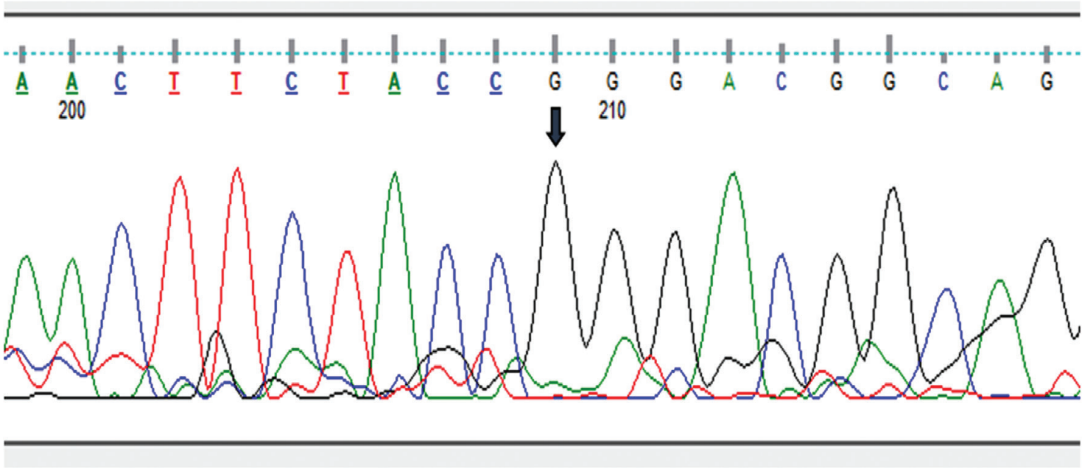

Figure 2. DNA sequencing chromatogram of the patient. The arrow indicates a homozygous *NM_000208.4 c.779 T>G* (*p.Leu260Arg*) mutation in exon 3 of the *INSR* gene

mutation. Later, the same mutation was detected in a patient from Morocco (6). Homozygous inheritance was reported in three of eight patients with type A resistance syndrome in another series (6). Interestingly all type A insulin resistance syndrome patients with α subunit mutations had a homozygous inheritance pattern (6). We detected a novel homozygous mutation in exon 3 at position 260 (p.Leu260Arg). A homozygous mutation that resulted in proline instead of leucine at the same codon was reported previously in a family. The homozygous form of this specific variant, (p.Leu260Pro), has been associated with leprechaunism (10). It was concluded that a mutation in this region disrupts the signaling from the insulin binding site on the α-subunit with the tyrosine kinase domain on the β subunit (10). It was reported that the heterozygous form of p.Leu260Pro was associated with a normal phenotype with mild insulin resistance (1). It has also been reported that different missense mutations in the same codon can cause variable phenotype (3).

Currently available therapies for genetic insulin resistance syndromes are nonspecific. Dietary changes and exercise, in addition to drug therapy (metformin with or without insulin), have been the mainstay with the clinical aim of reducing insulin resistance (8). Commonly, patients with mild *INSR* defects present peripubertally with oligomenorrhea and hyperandrogenism and acanthosis nigricans; misdiagnosis with polycystic ovary syndrome has occurred (11). At presentation, diabetic hyperglycemia has often yet to develop, as in our patient (8). Hirsutism treatment may be difficult in these cases, but a good response to cyproterone acetate treatment has been described (11).

Conclusion

In conclusion, we report a novel, homozygous mutation, p.Leu260Arg, in exon 3 of the *INSR* gene in a patient with type A insulin resistance syndrome. The unusual feature in this case is the homozygous inheritance pattern. As different mutations in the *INSR* gene cause different phenotypes, as do different inheritance patterns, this report is important for expanding understanding of the disease mechanisms at work and in aiding the genetic counseling process. It should be borne in mind that homozygous *INSR* mutations might, rarely, be associated with type A insulin resistance.

Ethics

Informed Consent: Informed consent was obtained from the patient and mother.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Bülent Hacıhamdioğlu, Kenan Delil, Data Collection and Processing: Bülent Hacıhamdioğlu, Kenan Delil, Elif Gülşah Baş, Analysis and Interpretations: Bülent Hacıhamdioğlu, Kenan Delil, Elif Gülşah Baş, Literature Search: Bülent Hacıhamdioğlu, Kenan Delil, Elif Gülşah Baş.

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CASE REPORT

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The Unusual Case of Fibroma of Tendon Sheath in a Young Girl with Turner Syndrome Undergoing Growth Hormone Treatment

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What is already known on this topic?

Fibroma of tendon sheath (FTS) is an uncommon mass that arises from the tendon sheath of extremities, particularly in children. Recombinant human growth hormone (rGH) treatment in patients with Turner syndrome is accepted worldwide because the syndromic short stature. The International Turner Syndrome Consensus Group does not recommend a specific cancer screening protocol.

What this study adds?

To our knowledge, there have been no reports of the co-occurrence of Turner syndrome and FTS in a young child during rGH treatment. The rGH treatment seems to affect the growth of tumor in this case because of early-onset and rapid growth compared with well-known characteristics of FTS in adults. When a hand mass occurs in Turner syndrome patients undergoing rGH treatment, it may be worth considering FTS as a possible diagnosis in order to not miss appropriate management.

Abstract

Fibroma of tendon sheath (FTS) is an uncommon mass that arises from the tendon sheath of extremities. The tumor typically affects adults between ages 20 and 50 years with a predominance in males. To date, growth hormone (GH) treatment is safe for children with Turner syndrome without risk factors and is accepted worldwide. This article reports the case of a nine-year-old female patient with Turner syndrome and FTS during GH treatment. She had been treated with daily subcutaneous GH to improve growth failure with a mean dose of 0.28 mg/kg/week and the level of insulin-like growth factor-1 was within the normal range. During the follow-up period, she complained about a mass in her hand, subsequently diagnosed as FTS. This report illustrates the clinical impact of Turner syndrome and GH treatments on the occurrence of this tumor through literature reviews. Further studies are needed to highlight the association between FTS and GH treatment, especially in Turner syndrome.

Keywords: Fibroma, tendons, Turner syndrome, growth hormone

Introduction

Turner syndrome patients show increased morbidity due to metabolic disease, thyroid and particularly to the well-known cardiac and aortic dissection risk. The risk of cancer in Turner syndrome patients has also been studied. To date, recombinant human growth hormone (rGH) treatment in patients with Turner syndrome is accepted worldwide because of the syndromic short stature. Apart from the beneficial effect of GH on stature, childhood GH therapy in Turner syndrome favorably affects the cardiovascular system via improvement in the lipid profile and a decreased prevalence of arterial hypertension (1). The long-term safety of GH was not associated with an increased risk of new malignancy, leukemia, non-leukemic extracranial tumors or recurrence of intracranial malignancy in patients without

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risk factors during rGH treatment (2). There was evidence of an increased risk of a second neoplasm in children previously treated for cancer. However, current experimental data supports the hypothesis that the GH/insulin-like growth factor-1 (IGF-1) status may facilitate carcinogenesis and influence cancer biology (3,4).

The first fibroma of tendon sheath (FTS) was described by Geschickter and Copeland (5) about 70 years ago. FTS is an uncommon, benign lesion arising from the tendon sheath of extremities, particularly the hands. The tumor typically affects adults between 20 and 50 years with a male to female ratio ranging from 1.5:1 to 3:1 (6). The clinical course of FTS usually occurs years after its formation as a slow-growing, dense, painless or mildly tender mass that is firmly attached to the tendon sheath.

In this case, the patient who had Turner syndrome had been treated with GH for years. Numerous case reports about FTS have been published. However, to our knowledge, there have been no reports of the co-occurrence of Turner syndrome and FTS in a young child during rGH treatment. We herein present a particular case of FTS in Turner syndrome, to emphasize its unusual clinical course through this case report and review of the literature.

Case Report

The patient was followed up for Turner syndrome in the Department of Pediatrics. The karyotype was 45,X[22]/47,XXX[8] mosaicism. After the condition was diagnosed at the age of six years and four months, she was treated with daily subcutaneous rGH to improve growth failure with a mean dose of 0.28 mg/kg/week (Table 1). The height standard deviation score increased from -2.17 to -0.09 and her growth velocity increased from 4 to 5 cm/year before treatment to 7 to 8 cm/year during treatment (Figure 1). During rGH treatment, she did not have an elevated glucose level or abnormal thyroid function and the level of IGF-1 was within the normal range. During the follow-up period, she complained about a mass in her hand and was referred to the Department of Plastic and Reconstructive Surgery for the treatment of a nontender, relatively rapid-growing mass of two-months duration.

The mass was located over the radial volar aspect of the right middle finger (Figure 2A). She had no neurological or vascular symptoms, and the range of movement of the right middle finger was not limited. She felt no discomfort but complained of the noticeable mass when flexing her finger. There was no history of previous penetrating or blunt trauma over the mass lesion. Sagittal fast spin-echo T2-weighted magnetic resonance image (MRI) revealed a mass with areas of iso-to-high signal intensity and dark signal foci at the periphery (Figure 3). Erosion of the adjacent bone was

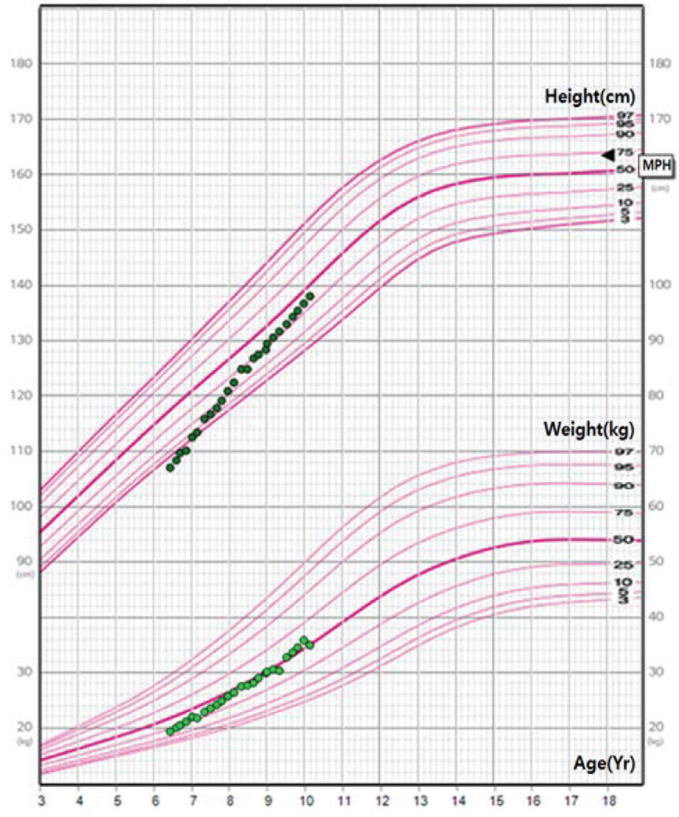

Figure 1. The patient's growth curve during the follow-up period. The patient was diagnosed with Turner syndrome at the age of 6.3 years and underwent recombinant human growth hormone treatment.

Table 1. Growth data, recombinant human growth hormone dose and serum insulin-like growth factor-1 levels in our patient

Chronological age	Height (cm)	Height percentile/SDS	rGH dose (mg/kg/week)	IGF-1 (ng/mL) (reference range)
6 years 4 months	107.2	1/-2.17	Started at 0.20	173.15 (100-446)
7 years	112.3	3-5/-1.85	0.20	320.30 (100-446)
8 years	120.6	10-15/-1.21	0.28	383.0 (100-446)
9 years	128.2	15-25/-0.82	0.30	483.0 (198-754)
9 years 8 months	136.4	25-50/-0.09	0.32	533.0 (198-754)

SDS: standard deviation score, rGH: recombinant human growth hormone, IGF-1: insulin-like growth factor-1

J Clin Res Pediatr Endocrinol
2021;13(1):104-108

not seen, but attachment to the 3rd flexor profundus tendon was marked. The neurovascular bundle was swept away laterally from the lesion. Originally, the first impression based on MRI was a giant cell tumor of tendon sheath.

The surgical procedure was performed under general anesthesia. Upon gross examination, the tumor appeared to be well-demarcated, lobulated, solid and oval-shaped. It was measured to be 2x1.8x1 cm, and its cut surface was white-tan and rubbery. Histopathologic examination confirmed the mass to be FTS (Figure 4).

As in our case, FTS can easily be confused with a giant cell tumor of tendon sheath, and a final accurate diagnosis is normally made by its histopathologic findings. Microscopically, a collagenous stroma and benign fibroblasts

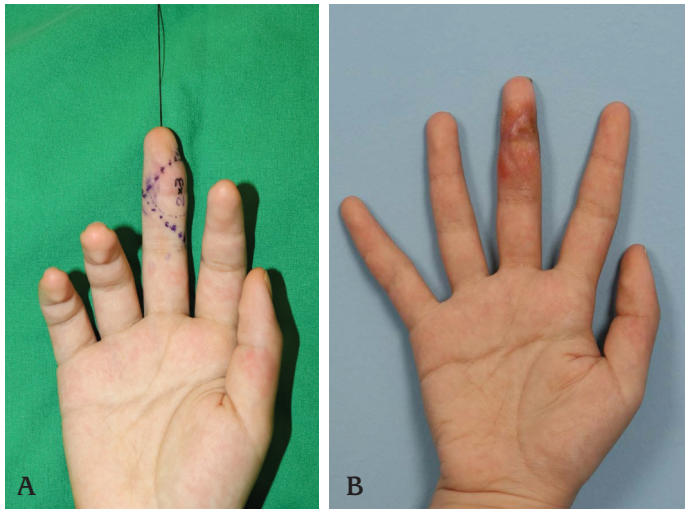

Figure 2. Appearance of the patient's hand before and after surgery. A) Note the mass on the right middle finger distal interphalangeal joint. B) Postoperative appearance of the patient's hand a month after the surgery.

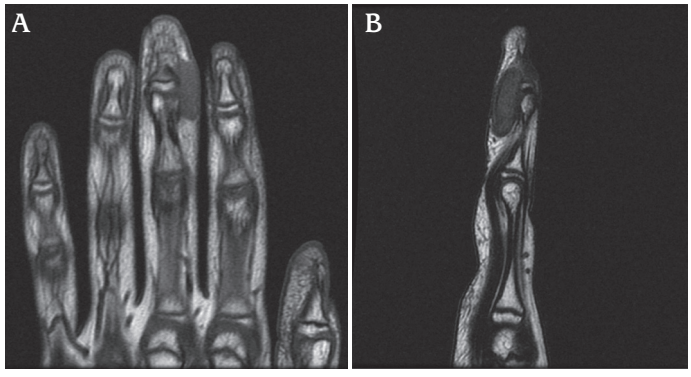

Figure 3. Preoperative magnetic resonance finding. A) Coronal fast spin-echo T1 image shows a mass of low signal intensity on the right middle finger distal interphalangeal joint. B) Sagittal fast spin echo T2 image shows a mass of equal-to-high signal intensity centrally with decreased signal peripherally.

with low cellularity were noted. Histopathologic findings and results of immunohistochemistry were consistent with that of FTS. No early postoperative complications such as infection, bleeding, or dehiscence were noted. The patient achieved full range of motion of the affected finger with no pain or tenderness by one month after the surgery (Figure 2B).

Discussion

The FTS in this present case developed in a patient with Turner syndrome. FTS is known to typically affect adults between 20 and 50 years with a predominance in males. In contrast, the patient in this case was female and only nine years old. Long-term studies have shown that early GH treatment can correct growth failure and normalize height in infants and children with Turner syndrome (7). Our patient had been treated with rGH for more than three years. Considering this unusual presentation and her special medial history, we supposed that the GH might affect the course of this patient. With this assumption, we investigated the clinical impact of Turner syndrome and GH treatment on the occurrence of this tumor through literature reviews.

Generally, it is accepted that the hormonal abnormalities and treatments for this syndrome might affect the risk of hormone-related cancers, and the chromosomal abnormality itself might affect cancer risk (8). Some large retrospective observational studies (8,9,10) have undertaken a comparison of cytogenetic and cancer registries data. They reported that the overall risk of cancer is possibly slightly raised (in one study only) (9) with standardized incidence ratios (SIR) between 0.9 and 1.34, but according to others (8,10), the overall risk of cancer was similar to that seen in the normal population. All reported that the incidence of breast cancer is reduced, the risk of melanoma increased between twofold and threefold, and the risk of nervous system malignancy increased between 4.3- and 6.6-fold with the SIR for meningioma increased between 12 and 14. Until prospective studies clarify the cost-effectiveness of routine screening, the International Turner Syndrome Consensus Group does not recommend a specific cancer screening protocol (11).

The final mediator of the growth promoting action of GH is IGF-1, which exerts potent anti-apoptotic and mitogenic activity in all cells and is expressed and secreted from many different types of cancer cells (12). There is considerable concern that GH treatment may be associated with tumor development. The potential relationship between GH treatment and increased risk of tumor development has been the subject of many studies. Although it is a reasonable

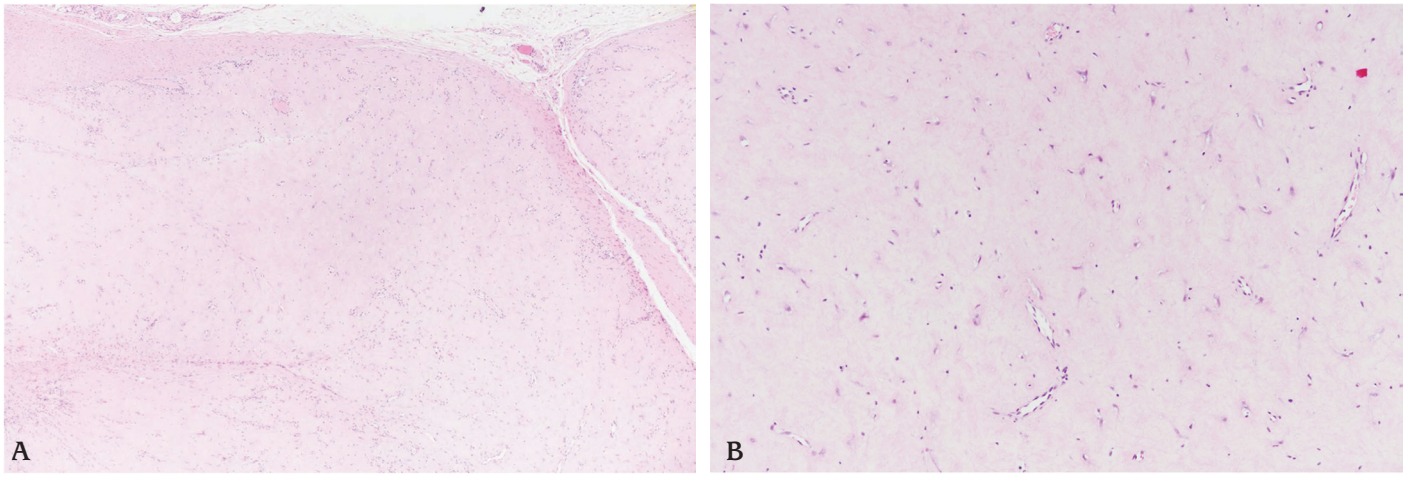

Figure 4. Histologic finding (hematoxylin and eosin stain). The tumor was a well-circumscribed nodule with clefts, attached to the flexor tendon (A, x40). It contained bland fibroblastic spindle cells in the dense collagenous stroma (B, x100).

assumption that there might be carcinogenic effects, still there is no evidence that GH treatment in young patients with growth disorders actually results in an increased risk of developing cancer relative to that expected in the normal population (13). Additionally, a recent, large, cross-European cohort study (14) also showed no clear, raised cancer risk in patients with growth failure without other major disease. The two aforementioned studies do not generally support a carcinogenic effect of rGH, but currently available experimental data does support the hypothesis that the GH/IGF-1 status may facilitate carcinogenesis (4).

The etiology of FTS is not certain, as it may represent a reactive fibrosing disease or a true neoplasm. However, after a (2;11) translocation was found by Dal Cin et al (15), it is now generally accepted that FTS is neoplastic. The presence of clonal chromosomal changes suggests a true neoplastic nature. Given the neoplastic nature of this lesion, a causal relationship between FTS and rGH treatment seems possible. This younger age of onset and relatively rapid growth also suggests a possibility that tumor growth might be affected by rGH treatment.

FTS is a benign process but may impose problematic anatomical and neurological complications if not treated promptly. About one-third of cases have been affected by neurologic symptoms due to compression (6). FTS has also been reported to cause a “trigger wrist” or limited flexion of fingers by adherence to the tendon (16). Therefore, in addition to timely management, early suspicion is also of great importance to avoid potential complications.

In conclusion, it was not clear that the rGH treatment facilitated the occurrence of FTS in a girl with Turner syndrome, but it seems possible because of the unusually young age of onset. The rGH treatment seems to affect the growth of the tumor in this case because of rapid growth compared with well-known characteristics of FTS in adults. To our knowledge, this is the first case of new-onset malignancy in a rGH-treated patient with Turner syndrome and without prior risk factors. If a hand mass occurs in Turner syndrome patients undergoing GH treatment, it may be worth considering FTS as a possible diagnosis in order to not miss appropriate management. For the present, decisive evidence should be explored to determine whether the relationship between rGH therapy and FTS occurrence is causal. Long-term clinical follow-up and further studies are required to highlight the link between FTS and GH treatment.

Ethics

Informed Consent: Written informed consents were obtained from all participants and their legal guardians. All procedures were performed in accordance with the Declaration of Helsinki.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Chang Yong Choi, Dong Gyu Kim, Concept: Yong Hee Hong, Design: Chang Yong Choi, Dong Gyu Kim, Data Collection or Processing: Min Jung Jung, Analysis or Interpretation: Jong Hyun Lee, Literature Search: Jong Hyun Lee, Writing: Yong Hee Hong.

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CASE REPORT

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6q25.1-q25.3 Microdeletion in a Chinese Girl

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What is already known on this topic?

6q25 microdeletion, a rare chromosome disorder, has been associated with growth restrictions, abnormal head shape, craniofacial anomalies, hypotonia, seizures, and mild to moderate intellectual disability.

What this study adds?

We reported a Chinese patient with an 8.1-Mb deletion involving 6q25.1-q25.3. Our patient shared the phenotypic features of the 6q25 microdeletion, including dysmorphic features with dysgenesis of the corpus callosum, growth retardation, intellectual disability, and language delay. In patients with dysmorphic features, microcephaly, growth retardation, intellectual disability, language delay and corpus callosum dysgenesis, 6q25 microdeletion should be considered in the differential diagnosis and chromosomal microarray analysis should be performed to confirm the diagnosis.

Abstract

Deletions of the long arm of chromosome 6 are rare and are characterized by great clinical variability according to the deletion breakpoint. Herein, we reported a 3-year-old girl evaluated for facial dysmorphism (long and connected eyebrows, big mouth, wide nasal bridge, high palatine arch, low set ears, and thin hair), growth retardation, intellectual disability, and language delay. Chromosomal microarray analysis revealed an 8.1-Mb deletion within 6q25.1-q25.3 ([hg19] chr6: 152,307,705-160,422,834) comprising 31 genes. Dysmorphic features, microcephaly, intellectual disability, language delay, growth retardation, and corpus callosum dysgenesis were commonly reported. Hence, 6q25 microdeletion is a rare condition. In patients with dysmorphic features, microcephaly, growth retardation, intellectual disability, language delay and corpus callosum dysgenesis, 6q25 microdeletion should be considered in the differential diagnosis and chromosomal microarray analysis should be performed to confirm the diagnosis.

Keywords: 6q25 microdeletion, facial dysmorphism, growth retardation, intellectual disability, language delay

Introduction

6q25 microdeletion, a rare chromosome disorder, has been associated with growth restrictions, abnormal head shape, craniofacial anomalies, hypotonia, seizures, and mild to moderate intellectual disability (1,2). Although a genotype–phenotype correlation has initially been proposed based on the proximal, medial, and distal location of the deletions, the reported cases with molecular karyotyping showed significant clinical heterogeneity, even with overlapping deletions (3,4). Most cases also share delayed verbal communication abilities, although detailed descriptions of speech have not generally been reported (5). 6q25 microdeletion has been extremely rare since the first report in 1975 (6). Herein, we reported a 3-year-old girl with 6q25 microdeletion to highlight this rare condition.

Case Report

The female patient was the first child of healthy non-consanguineous parents. Family history was negative for neurological disorders, behavioral problems, or congenital anomalies. She was born at 39^{+4} weeks gestation via

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vaginal delivery with uneventful pregnancy. Her birth weight and length were 2,600 g and 48 cm without documented occipital frontal circumference and APGAR. After birth, feeding difficulty was noted without history of nasal feeding. Her global development was delayed, with no improvement in her developmental skills with age. She could sit at the age of 15 months, and crawl at the age of two years old. She could not walk without support at the age of 37 months. She could only call "Dad" and "Grandma" at the age of 39 months.

She was first presented to our outpatient clinic at the age of 37 months because of growth retardation, intellectual disability, and language delay. Physical examination showed a height of 85.8 cm [< -2 standard deviation (SD)] and a weight of 11.8 kg (-1 ~ -2 SD). Characteristic facial dysmorphism, including long and connected eyebrows, big mouth, wide nasal bridge, high palatine arch, low set ears, and thin hair was noted. The heart, lung, abdomen, limb, and muscle tension were unremarked. Brain magnetic resonance imaging (MRI) revealed dysmorphism of the corpus callosum and stronger T2 signal at basal ganglia.

Standard chromosome banding analysis performed in a local hospital reported "balanced translocation" in chromosome 4 and 8 for this patient and no abnormality for her parents. Chromosomal microarray analysis (CMA) (CMA, CytoScan® HD, Affymetrix) performed in our Medical Genetics Laboratory did not find any microdeletion or microduplication in chromosome 4 or 8, but identified an 8.1-Mb deletion in 6q25.1-q25.3 ([hg19]chr6:152,307,705-160,422,834), which covered 31 genes *(ESR1, SYNE1, MYCT1, VIP, FBXO5, MTRF1L, RGS17, OPRM1, CNKSR3, SCAF8, TIAM2, TFB1M, NOX3, ARID1B, SNX9, SYNJ2, SERAC1, GTF2H5, TMEM181, DYNLT1, EZR, RSPH3, TAGAP, FNDC1, SOD2, WTAP, ACAT2, TCP1, MRPL18, MAS1* and *IGF2R*), as shown in Figure 1.

Figure 1. Chromosomal microarray analysis showed an 8.1-Mb deletion in 6q25.1-q25.3 (152,307,705-160,422,834), which covered 31 genes (100 x 65.9 mm)

Table 1. Clinical features observed in our patient and other reported patients with 6q25 microdeletion

No/Ref	Sex	Cytogenetic band	Deletion size	Intellectual disability	Language delay	Growth retardation	Dysmorphic features	Microcephaly	Corpus callosum dysgenesis	Limb anomalies	Genital hypoplasia	Hearing loss
Current case	F	6q25.1-q25.3	8.1 Mb	+	+	+	+	-	+	-	-	-
Case 1 (13)	F	6q25.1-q25.3	7.8Mb	+	+	+	+	-	+	-	-	NA
Case 2 (14)	M	6q25.1-q26	15.5 Mb	+	+	+	+	+	+	-	+	+
Case 3 (1)	M	6q25.2-q25.3	3.77 Mb	+	+	-	+	+	NA	-	-	+
Case 4 (1)	F	6q25.2-q26	6.3 Mb	+	+	-	+	+	-	-	NA	+
Case 5 (1)	F	6q24.3-q25.3	10.3 Mb	+	+	+	+	+	+	+	NA	+
Case 6 (1)	M	6q25.2-q27	13.81 Mb	+	+	+	+	+	+	-	+	+
Case 7 (15)	M	6q25.3	1.19 Mb	+	+	-	+	+	+	+	+	+
Case 8 (16)	F	6q25.3	1.1 Mb	+	+	+	+	-	+	+	NA	+
Case 9 (2)	M	6q25.3-qter	10.79 Mb	+	+	NA	+	+	+	+	NA	-
Case10 (5)	F	6q25.3-qter	11.1 Mb	+	+	-	+	-	-	-	NA	-
Case 11 (5)	M	6q25.3	403 Kb	+	+	-	-	-	NA	-	-	-
Case 12 (9)	M	6q25.1-q25.3	NA	+	+	+	+	+	NA	-	-	NA
Case 13 (14)	M	6q25.1-q25.3	NA	+	+	+	+	+	-	-	-	-
Case 14 (14)	M	6q25.1-q26	NA	NA	NA	+	+	+	+	-	+	+
Case 15 (12)	M	6q25-qter	NA	+	+	-	+	-	-	-	+	+
Case 16 (8)	F	6q25-qter	NA	+	+	-	+	+	-	NA	+	NA
Case 17 (17)	F	6q25-qter	NA	+	+	+	+	+	-	NA	NA	NA
Case 18 (10)	M	6q24.3-qter	NA	NA	NA	+	+	+	+	+	-	NA
Case 19 (10)	F	6q25.3-qter	NA	+	+	+	+	+	-	-	-	NA
Case 20 (7)	M	6q25-qter	NA	+	+	+	+	+	NA	+	+	NA
Case 21 (11)	F	6q25-q27	NA	+	+	+	+	+	NA	+	NA	NA
Case 22 (11)	M	6q25-qter	NA	+	+	-	+	-	NA	NA	NA	NA
Total	13 M			21/23	21/23	14/23	22/23	16/23	10/23	7/23	7/23	9/23

M: male, F, female; +: present, -: absent, NA: data no available

Discussion

Interstitial deletions of the long arm of chromosome 6 are rare. Since the first report in 1975 (6), the number of patients that have been described in the medical literature remains few (1,2,5,7,8,9,10,11,12,13,14,15,16,17). The phenotype of this syndrome is variable and depends on the breakpoints, location and size of the deletion. Facial dysmorphism, hand malformations, heart defects, microcephaly, intellectual disability, epilepsy, and other neurodevelopmental and neuropsychiatric conditions have been reported. A comparison of the clinical characteristics of our patients with those reported in the literature is shown in Table 1. The clinical features observed in our patient and the other 22 previously reported patients showed that 22 (95.7%) had dysmorphic features, 21 (91.3%) had intellectual disability and language delay, 16 (69.6%) had microcephaly, 14 (60.9%) had growth retardation, and 10 (43.5%) had corpus callosum dysgenesis. The clinical characteristics of our patient overlap with several of these patients. The most common ones include growth retardation, intellectual disability, language delay, and dysmorphic features as well as dysgenesis of the corpus callosum, while microcephaly, hearing loss, limb anomalies and genital hypoplasia are not noted in our patient. These differences may be partly attributed to the varying size and breakpoints of the deletion and more importantly, the gene content of the deleted segment (18). In our patient, CMA showed an 8.1-Mb deletion of 6q25.1-q25.3, which covers 31 genes and only four of these genes (*ARID1B, IGF2R, TIAM2* and *SYNJ2*) have been associated with pathogenicity. Short stature was observed in our patient, while it was not noted in some previous cases (5,8,11,12,15). This may be due to the deletion of the *IGF2R* gene in our patient. Most other case reports do not specify the deleted genes so further comparison is not possible. However, studies of mice have supported a major role for the *IGF* receptor pathway in growth: knockout of *IGF1, IGF2*, or *IGF1R* results in growth retardation, whereas overexpression of *IGF2* results in overgrowth (19,20). The identification of an *IGF2* mutation in patients with growth restriction suggests that *IGF2* is not only a mediator of intrauterine development but also contributes to postnatal growth (21). The importance of other deleted genes and their contribution to the 6q25 microdeletion are uncertain at this time. Additionally, links between brain anomalies and language delay has been noted in the literature. For instance, de Vasconcelos Hage et al (22) reported that 13 cases of perisylvian polymicrogyria and three cases of corpus callosum hypoplasia were found in 17 patients with language impairment. In typically developing young children, the developmental rate of the splenium of the corpus callosum was associated with vocabulary size (23). In individuals with disfluent speech, the anterior corpus callosum showed significantly lower fractional anisotropy than that of typical controls (24). Hence, in patients with dysmorphic features, microcephaly, intellectual disability, language delay, and corpus callosum dysgenesis, 6q25 microdeletion should be considered in the differential diagnosis and CMA should be performed to confirm the diagnosis.

The mechanism of 6q25 microdeletion is still unclear. The smallest critical region described so far for 6q25 microdeletion have restricted to a 6q25.3 region including two protein-coding genes, *ARID1B* and *ZDHHC14* which was considered to be responsible for the cognitive impairment and brain anomalies observed in their patients (15). The core phenotypic characteristics associated with the 6q25 microdeletion have been observed in a child with a deletion involving only *ARID1B* which suggested that ARID1B may be one key gene associated with these features (16). Additionally, *ARID1B* has been associated with multiple syndromes characterized by developmental delay and intellectual disability, such as Coffin-Siris syndrome, and with non-syndromic intellectual disability. It is reported that *ARID1B* is of great importance for normal human brain development and function. In one study, the phenotype-genotype correlation in seven patients who had various-sized deletions including *ARID1B,* has shown that haploinsufficiency of *ARID1B* is related with intellectual disability, speech impairment, and autism as well as corpus callosum abnormalities (25). Therefore, haploinsufficiency of *ARID1B* appears to be responsible for the clinical findings in our patient.

Conclusion

In summary, 6q25 microdeletion is a rare condition. In patients with dysmorphic features, microcephaly, growth retardation, intellectual disability, language delay, and corpus callosum dysgenesis, 6q25 microdeletion should be considered in the differential diagnosis and CMA should be performed to confirm the diagnosis. MRI of the brain should be considered in all patients with deletions involving 6q25.

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Ethics

Informed Consent: Written informed consent was obtained from the parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Mian-Ling Zhong, Chao-Chun Zou, Design: Mian-Ling Zhong, Ye-Mei Song, Chao-Chun Zou, Data Collection or Processing: Mian-Ling Zhong, Ye-Mei Song, Literature Search: Mian-Ling Zhong, Ye-Mei Song, Chao-Chun Zou, Writing: Mian-Ling Zhong.

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CASE REPORT

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Treatment Difficulties in Hypomagnesemia Secondary to the Transient Receptor Potential Melastatin 6 Gene: A Case Report with Novel Mutation

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What is already known on this topic?

Hypomagnesemia is one of the causes of hypocalcemia. Enteral replacement is the key treatment but the treatment should be individualized for each patient. Normalization of hypomagnesemia is not always easy and should not be the aim of the treatment.

What this study adds?

Genetic analysis revealed a novel frame shift variant in the transient receptor potential melastatin 6 gene. Magnesium levels varied during treatment with different preparations so in these patients treatment should be individualized for optimal replacement.

Abstract

Hypomagnesemia is a rare cause of seizures in childhood but should be kept in mind in recurrent and intractable seizures and hypocalcemia in communities where consanguineous marriages are common. Familial hypomagnesemia with secondary hypocalcemia is a rare genetic cause of hypomagnesemia, due to variants in the *transient receptor potential melastatin 6 (TRPM6)* genes. Here, a three year-old boy with a novel variant in this gene and had difficulties with enteral hypomagnesemia treatment is presented. He had recurrent seizures since two years of age and was diagnosed with epilepsy and treated with multiple antiepileptic drugs. Subsequently, he was diagnosed with rickets due to severe hypocalcemia at another center. The patient was hypotonic and neurodevelopmentally poor. The most prominent laboratory finding was of hypomagnesemia with secondary hypocalcemia. The genetic analysis revealed a novel variant in the *TRPM6* gene. After parental treatment of intravenous magnesium (Mg^{2+}) sulfate and calcium, the treatment was switched to enteral Mg^{2+} medications, due to persistent hypomagnesemia and the gastrointestinal side-effects, different oral preparations were used. The patient was stable on an oral maintenance dose of Mg^{2+} oxide with borderline blood Mg^{2+} levels and resolution of hypocalcemia. Hypomagnesemia is one of the causes of hypocalcemia. Enteral replacement is the key treatment but the treatment should be individualized for each patient. Normalization of hypomagnesemia is not always easy and should not be the aim of the treatment.
Keywords: Hypocalcemia, hypomagnesemia, TRPM6, transient receptor potential melastatin 6

Introduction

Familial hypomagnesemia with secondary hypocalcemia (HSH) is a rare autosomal recessive disorder that presents in infancy with neurological symptoms of magnesium (Mg^{2+}) dependent hypocalcemia (1,2). Variants in the gene for the distal convoluting tubules and colon specific apical Mg^{2+} channel, the transient receptor potential melastatin

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6 (*TRPM6*) gene, cause the most profound genetic hypomagnesemia (3). To date, there are a few reports of several variants of *TRPM6* in children (4). In addition, some reports have referred to the difficulties of maintaining serum Mg^{2+} levels in these patients (3,4). The treatment complexities, including the target serum Mg^{2+} levels and the different options for preparations to be given were rarely reported. Therefore, we present a patient with resistant seizures who was diagnosed with HSH due to a novel variant in *TRPM6* gene, discuss the importance of checking Mg^{2+} in seizures and to consider and discuss treatment strategies.

Case Report

A three-year-old Afghan boy was admitted to our hospital with a history of recurrent seizures since the age of two years. The first seizure was reported to be fever-induced at the age of four months. Cognitive and motor development was normal until the age of one, but thereafter neurodevelopmental decline was reported. He had been having generalized tonic-clonic seizures since the age of two years. The diagnosis of epilepsy was made in another center because of recurrent seizures and multiple antiepileptic drugs were started. He has been treated with levetiracetam, clonazepam, valproic acid, and pyridoxine treatments. The patient was admitted to our hospital from Afghanistan for further evaluation for recurrent seizures and hypocalcemia. He was born at full-term gestation after an uncomplicated pregnancy including an absence of polyhydramnios with a birth weight of 3500 gr from a consanguineous family (first degree cousins). His prenatal and natal history was uneventful. His family history was unremarkable. His family history was negative for epilepsy and neurological abnormalities, as well as any known renal, thyroid, or parathyroid disease. Physical examination revealed his growth parameters were within normal limits [height: 95 cm (25-50 p), weight: 15 kg (50-75 p)]. He did not show any dysmorphic or neurocutaneous features. He was conscious and had a speech delay. Bilateral horizontal nystagmus was prominent. He was hypotonic with normal reflexes. The rest of the physical examination was normal. Laboratory data included a serum Mg^{2+} level of 0.12 mmol/L (normal range: 0.7-0.86 mmol/L), calcium 4.7 mg/dL, phosphorus 6.2 mg/dL, alkaline phosphatase 222 U/L, parathyroid hormone 6 pg/mL (normal range: 11-67 pg/mL), 25-OH vitamin D3 60.1 ng/mL, sodium 139 mEq/L, potassium 4.24 mEq/L, albumin 3.2 g/dL, and uric acid 5.3 mg/dL. The urine fractional excretion of Mg^{2+} was 12% (normal range: <4%) with normal urine calcium/creatinine ratio. Laboratory examinations including acute phase reactants, serum glucose, and albumin levels, and liver and renal function tests were normal. The renal ultrasound did not show any medullary nephrocalcinosis. Electroencephalogram showed slow background activity without any epileptiform discharges and magnetic resonance imaging of brain showed mild diffuse cerebral and cerebellar atrophy (Figure 1).

Laboratory examination revealed the characteristic combination of severe hypomagnesemia, hypoparathyroidism, and profound hypocalcemia. Clinical and laboratory findings together suggested the diagnosis of HSH as responsible for disruption in Mg^{2+} homeostasis. The genetic analysis of the patient revealed a novel, homozygous variant in the *TRPM6* gene (NM017662.4: c.5473_5474insGCTTC (p.H18225Rfs*18) (p.His1825Argfs*18). This is a frameshift variant and based on American College of Medical Genetics and Genomics criteria this variant was classified as pathogenic. This is a null variant and is predicted to cause severe loss of gene function. *TRPM6* gene sequence analysis was performed using MiSeq next generation sequencing platform, an Food and Drug Administration approved diagnostic system (Illumina Inc., San Diego, CA, USA). Sequences were aligned to the hg 19 genome within MiSeq Reporter software (Illumina Inc.). Visualization of the data was performed with IGV 2.3 (Broad Institute-www.software.broadinstitute.org) software (Figure 2). Informed consent was obtained from the parents for genetic analysis. The parents were heterozygotes for the same variant.

Intravenous Mg^{2+} sulfate was administered at 50 mg/kg along with intravenous calcium for three days after which the serum Mg^{2+} increased and calcium levels normalized. The treatment was switched to oral Mg^{2+} sulfate 4x2000 mg (~533 mg/kg/d) but abdominal pain and diarrhea was

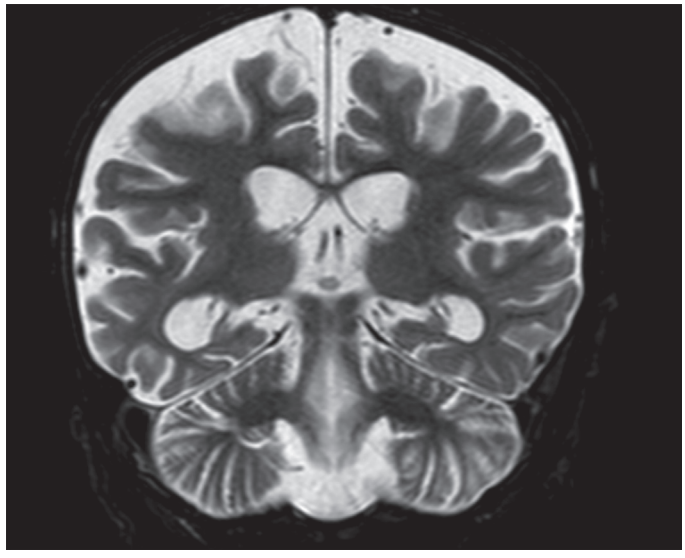

Figure 1. Cerebral and cerebellar atrophy in T2-weighted cranial magnetic resonance images

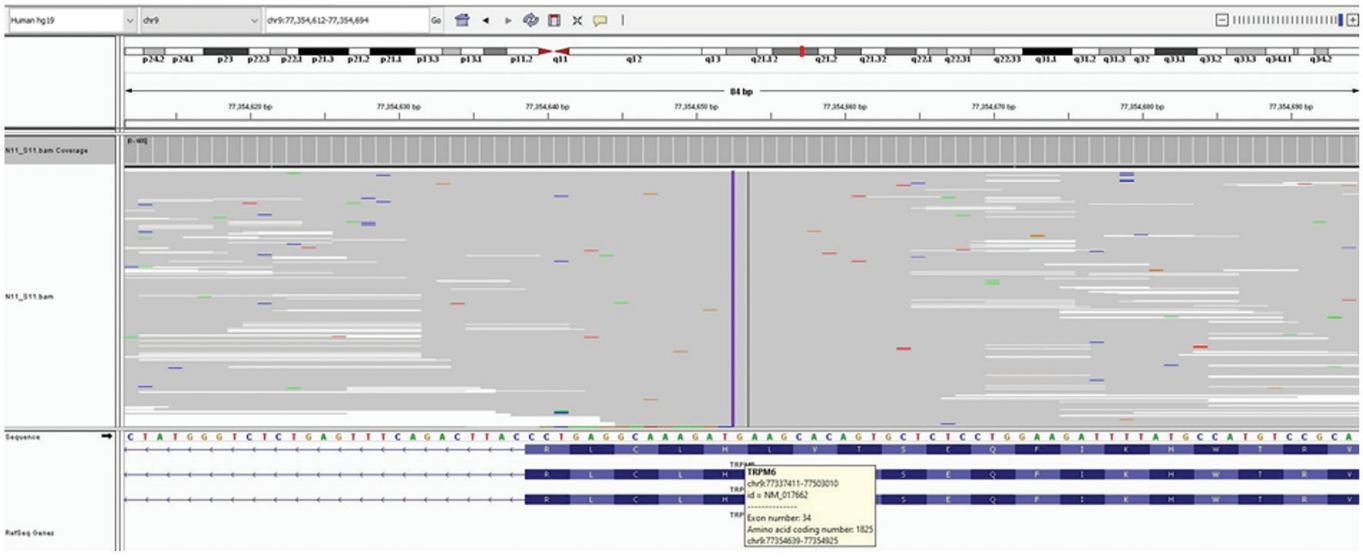

Figure 2. The figure of the pathogenic variant in the transient receptor potential melastatin 6 genes

significant. In addition, on this oral therapy, serum Mg^{2+} decreased to 0.2 mmol/L and convulsions re-occurred without hypocalcemia. The treatment was replaced with oral Mg^{2+} citrate and soon after switched again to Mg^{2+} carbonate because of persistent hypomagnesemia and gastrointestinal side-effects. Finally, Mg^{2+} oxide sachets were started and blood Mg^{2+} reached 0.78 mmol/L with high dose Mg^{2+} oxide (3x2 sachets ~ 150 mg/kg/d) (Figure 3). Antiepileptic treatment was reduced. His muscle tone, cognitive development, and motor development improved. He has been stable on an oral maintenance dose of 2000 mg of Mg^{2+} oxide daily with borderline blood Mg^{2+} levels without hypocalcemia.

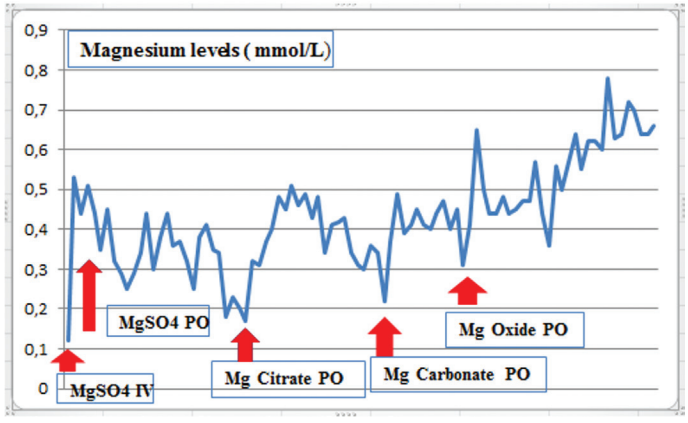

Figure 3. The blood magnesium levels by different magnesium preparations

Mg: magnesium, $MgSO_4$: magnesium sulphate, IV: intravenous; PO: per oral

Discussion

We presented a case of HSH due to a novel variant in the *TRPM6* gene. The patient's treatment was individually tailored according to blood Mg^{2+} levels and to minimize the side effects of a range of Mg^{2+}-containing medications. The patient benefitted from Mg^{2+} replacement for neurodevelopmental improvement and showed satisfactory progress.

HSH is a rare autosomal recessive disorder that affects the Mg^{2+} permeable ion channel encoded for by *TRPM6* gene on chromosome 9q22 (3). This gene is expressed in the distal segment of the intestine and the distal convoluted renal tubule. So the primary defect is impaired intestinal absorption of Mg^{2+} with a secondary defect of impaired renal conservation. The clinical presentation is usually in the early childhood period with hypocalcemia refractory to calcium supplementation. This secondary hypocalcemia is probably caused by inhibition of the parathyroid gland by the hypomagnesemia, resulting in low levels of parathyroid hormone which eventually results in hypocalcemia (1,4,5). The condition is treatable, but failure to diagnose early can lead to intractable seizures with irreversible cerebral damage and mental retardation (1). Some reports have revealed initial evaluations for neonates and infants presenting with seizures do not always include assessment for serum Mg^{2+} abnormalities (3,6). As far as the treatment is dependent on lifelong, high-dose supplementation of Mg^{2+} and the genetic diagnosis is relevant, this disorder should be included in the differential diagnosis of any infant presenting with seizures and hypomagnesemia. Our patient was being followed

for intractable epilepsy as well as rickets for two years. Neurodevelopmental delay and recurrent seizures increased the suspicion of neurometabolic disorders with the family history of consanguinity. The need to check Mg^{2+} levels in a severely hypocalcemic patient was overlooked.

Previous reports of HSH have demonstrated how well-timed diagnosis and rapid Mg^{2+} replacement accelerate normal development (3). One case series described considerably impaired neurodevelopment in two affected members of the same family who failed to receive supplementation (7). In another report, a patient who had HSH due to a *TRPM6* variant was followed-up over 29 years and demonstrated normal physical and mental development with treatment (3). The reported patient showed normal developmental milestones, she completed her education including getting a university science degree and went on to follow an academic career as an adult. The diagnosis age of the patients in the literature ranges from the neonatal period to four years old. The neurological outcome is reported to be related to the age at diagnosis and also the compliance to the treatment (5). Hypomagnesemia itself leads to lethargy, nystagmus and convulsions. In addition, without suitable treatment, it can lead to cerebral atrophy as was found in the present case (8,9). Even short-term follow-up of our patient demonstrated neurodevelopmental progress in our patient with appropriate treatment.

Oral or intravenous Mg^{2+} supplementation is the only existing treatment for hypomagnesemia of genetic origin. In the acute symptomatic situation of severely hypomagnesemic patient, intravenous Mg^{2+} supplementation is critical (1). The optimal rise in serum Mg^{2+} concentration often improves symptoms, such as seizures and secondary hypocalcemia, despite the fact that normal blood Mg^{2+} values are rarely reached (2,10). Extended correction of hypomagnesemia is generally delayed because of the gastrointestinal side effects frequently associated with oral Mg^{2+} supplementation. Paradoxically, higher doses of oral Mg^{2+} is damaging to intestines and results in diarrhea and also worsening hypomagnesemia (1,11). Thus, the type of oral Mg^{2+} preparation is important, since some preparations have been reported to have a better bioavailability than others (1,10). In a recent report, Mg^{2+} chloride or Mg^{2+} glycerophosphate was suggested rather than Mg^{2+} oxide or Mg^{2+} sulfate for oral Mg^{2+} supplementation (1). In a study conducted in mice, different Mg^{2+} preparations (Mg^{2+} acetyl taurate; Mg^{2+} malate; Mg^{2+} glycinate; Mg^{2+} citrate) were shown to be effective in increasing Mg^{2+} levels in different tissues like brain and muscle (12). In that study, blood Mg^{2+} levels were increased in all doses of Mg^{2+} acetyl taurate, malate, and glycinate, whereas Mg^{2+} citrate increased blood Mg^{2+} levels at high doses. Mg^{2+} citrate was reported to lead to a dose dependent increase in blood, brain, and muscle tissues (12). However, in our patient the highest serum Mg^{2+} levels without apparent side effects was achieved with Mg^{2+} oxide supplementation. This highlights the importance of tailoring the treatment to the individual patient as acceptability of Mg^{2+} supplement treatments vary from patient to patient. Treatment options may be optimized, based on future studies that investigate tissue-dependent Mg^{2+} concentrations in humans.

Milder clinical phenotypes may be due to different variants. However, due to the rarity of the condition, no definitive genotype–phenotype correlation has been established at present. The most commonly reported symptoms on admission were recurrent and intractable myoclonic or generalized tonic-clonic seizures (4). Mg^{2+} transport in the intestine occurs by both an active transcellular system, which is defective in HSH, and a passive paracellular pathway, which increases with rising intraluminal Mg^{2+} concentrations (1,2,3,4). Therefore, lifelong, enteral, high-dose Mg^{2+} is required in HSH to prevent symptoms and achieve at least subnormal serum Mg^{2+} levels. Optimal doses have been identified by trial and error and serial serum electrolyte monitoring. Previously reported cases have shown serum Mg^{2+} levels remain in the subnormal range (0.5-0.6 mmol/L) even with significant increases in supplemented dose (3). The published data suggests that the clinical aim should be normocalcaemia and the absence of features of neuroexcitability (3,8). There is no preparation of choice for oral Mg^{2+} replacement and preparation selection should be guided by follow-up in each individual patient and may also be affected by differences in the specific variant inherited.

TRPM6 variant is a cause of profound hypomagnesemia with secondary hypocalcemia. With appropriate treatment, the seizures can be controlled and neurocognitive development of the patients can be improved. Rapid diagnosis and treatment of this rare disorder can significantly improve the quality of life of affected individuals.

Hypomagnesemia is one of the causes of hypocalcemia. A diagnosis of primary HSH should be considered in all pediatric patients presenting with generalized seizures or tetany. Measurement of serum Mg^{2+} should be included in the work-up, especially during a seizure episode. This is especially true in those communities where consanguineous marriages are common. Enteral or parenteral Mg^{2+} replacement is key in managing this condition and the aim should be to normalize serum calcium and control the symptoms. The treatment of hypomagnesemia is not always easy and may depend on the dose and the content

of the medication. Individualized therapy and management should be tailored to each patient.

Ethics

Informed Consent: Informed consent was obtained from the parents for genetic analysis and for the writing of this.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Meltem Akçaboy, Saliha Şenel, Design: Çiğdem Seher Kasapkara, Çiğdem Genç Sel, Data Collection and Processing: Çiğdem Genç Sel, Gülin Karacan Küçükali, Senay Savas-Erdeve, Ülkühan Öztoprak, Analysis and Interpretations: Serdar Ceylaner, Literature Search: Meltem Akçaboy, Saliha Şenel, Hüsniye Yücel, Çiğdem Seher Kasapkara, Çiğdem Genç Sel, Gülin Karacan Küçükali, Senay Savas-Erdeve, Ülkühan Öztoprak, Writing: Meltem Akçaboy, Hüsniye Yücel.

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CASE REPORT

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Sirolimus Therapy and Follow-up in a Patient with Severe Congenital Hyperinsulinism Following Subtotal Pancreatectomy

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What is already known on this topic?

Congenital hyperinsulinism (CHI) is the most common cause of persistent hypoglycemia in neonates and infants. Sirolimus may be an effective treatment option in patients with CHI resistant to traditional medical therapy or failure of subtotal pancreatectomy, but experience is limited.

What this study adds?

This article further revealed the safety and efficacy of sirolimus in the very young patient with CHI, which can make us better understand the new treatment. The patient has a heterozygous ABCC8 mutation. It's also the first Chinese CHI patient with heterozygous ABCC8 mutation who used sirolimus.

Abstract

Congenital hyperinsulinism (CHI) is the most common cause of severe, persistent hypoglycemia in neonates and infants. If the patient does not respond to medical treatment the currently available treatment is subtotal pancreatectomy, but some patients still experience severe hypoglycemia after surgery. Sirolimus, a mammalian target of rapamycin inhibitor has recently been reported to be effective in the treatment of insulinoma and CHI patients. Here we report a patient with CHI who had prolonged hypoglycemia after subtotal pancreatectomy. The patient had a heterozygous mutation in *ABCC8* but was unresponsive to an optimal dose of diazoxide (15 mg/kg/day) and octreotide (30 µg/kg/day). The patient subsequently had subtotal pancreatectomy but severe and persistent hypoglycemia continued post-operatively. Sirolimus was commenced. There was a remarkable improvement in glycemic control without major adverse events, although he required a small dose of octreotide to maintain euglycemia. Sirolimus therapy was discontinued when the patient was 15 months old. At the time of this report, at an age of three years and eight months, the patient continues to maintain good glycemic control. This report suggests that sirolimus may be an effective treatment option in patients with CHI resistant to established medical therapy or failure of subtotal pancreatectomy. However, the long-term safety requires study in larger groups of very young patients.

Keywords: Congenital hyperinsulinism, hypoglycemia, mTOR, sirolimus, ABCC8

Introduction

Congenital hyperinsulinism (CHI), the major cause of persistent hypoglycemia in neonates and infants, is characterized by inappropriate insulin secretion from pancreatic beta cells in the presence of low blood glucose levels (1). Prompt and early management of these patients is very important for neurological prognosis (1,2). The incidence of CHI in the general population is estimated at 1/30,000-1/50,000 live births (3,4). Two major histologic subtypes have been described: diffuse (60-70 % of patients) and focal (30-40 % of patients) (5). Mutations in *ABCC8* and *KCNJ11* cause severe CHI that is unresponsive to medical treatment with diazoxide and octreotide (1). The current

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treatment for patients is a subtotal pancreatectomy (5,6). However, despite surgery, 40-59% of operated patients continue to experience severe and persistent hypoglycemia for months, or even years (7), and nearly 100% will develop diabetes mellitus within 11 years of surgery (8). Therefore, medical therapeutic alternatives should be considered with the aim of reducing insulin secretion and thereby preventing neurologic consequences. Constitutive activation of the mammalian target of rapamycin (mTOR) pathway has been postulated as a mechanism for hyperinsulinism and β-cell hyperplasia in diffuse CHI (9). Recent advances have shown the effectiveness of sirolimus, an mTOR inhibitor, in infants with severe diffuse CHI that had been unresponsive to medical therapy (10,11,12,13), one of whom had undergone subtotal pancreatectomy (10). During follow-up, no major adverse events was observed in the patients. We report a patient with CHI who failed to become euglycemic after pancreatectomy. The patient was successfully treated with sirolimus without further surgical intervention.

Case Report

The patient, a male infant, was born by cesarean at the 39th week of gestation to nonconsanguineous Chinese parents after an uneventful pregnancy. Birth weight was 3600 g. On the first day of his life, he was found to have severe hypoglycemia when he developed lethargy and seizures. He required high intravenous glucose infusion rate (GIR) (13 mg/kg/minute) to maintain normal blood glucose level. As he had persistent and severe hypoglycemia, he was transferred to our hospital for further management on postnatal day (PD) 13. The following results were obtained during an episode of hypoglycemia: glucose, 2.3 mmol/L; concomitant serum insulin, 13.52 (normal: 4.03-23.46) μIU/mL; C-peptide 4.25 (normal: 0.3-3.73) ng/mL; beta-hydroxybutyrate < 0.1 mmol/L. He had normal thyroid-stimulating hormone and free T4 levels. Metabolic screening profiles in plasma and urine were non-specific. Genetic analysis subsequently confirmed a novel mutation, c.1585_1587del, in exon 10 of the *ABCC8* gene (Figure 1), which resulted in the deletion of glutamic acid at position 529 (p.del529E) of *ABCC8* protein and produced *ABCC8* protein with a shorter topological domain (www.ncbi.nih.gov/orffinder and www.uniprot.org). The mutations in the topological domain affected the function of the *ABCC8* gene (14). According to American College of Medical Genetics criteria, the mutation is of uncertain significance and should be further studied (15). His father has the same mutation, but the phenotype is normal. Magnetic resonance imaging of the patient's brain showed bilateral abnormalities of the parietal white matter. Subsequently, maximal GIR was 16 mg/kg/min, administered parenterally via a central venous catheter. Diazoxide therapy was commenced on PD 14 and was gradually increased to an optimal dose of 15 mg/kg/day but with no response. On PD 20 Nifedipine was added to the therapeutic regimen, but it was discontinued after a week due to lack of response. Subcutaneous octreotide was initiated on PD 29. The octreotide dose was increased to a maximum dose (30 μg/kg/day), but resulted in only a 20% reduction in total glucose requirement. On PD 55, a subtotal pancreatectomy was performed at the Children's Hospital of Fudan University, Shanghai, China. Histopathological results confirmed diffuse hyperplasia of the islet cells (Figure 2). Subcutaneous octreotide was discontinued after the surgery,

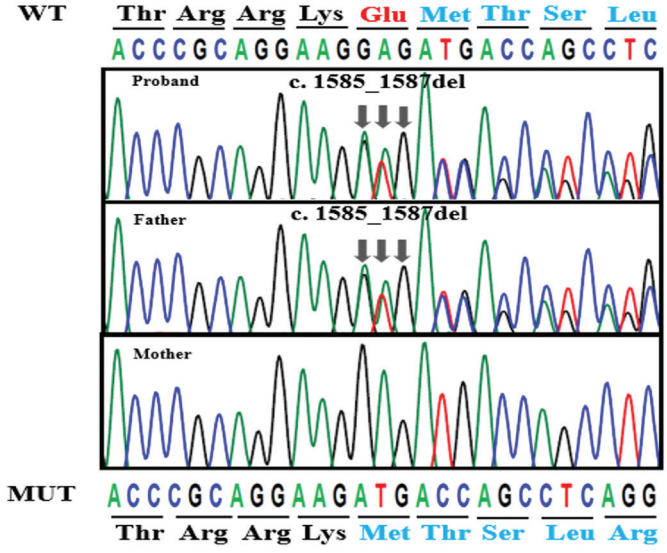

Figure 1. Sanger sequencing of *ABCC8* gene in the proband and his parents: the arrows showed the mutation site of the *ABCC8* gene

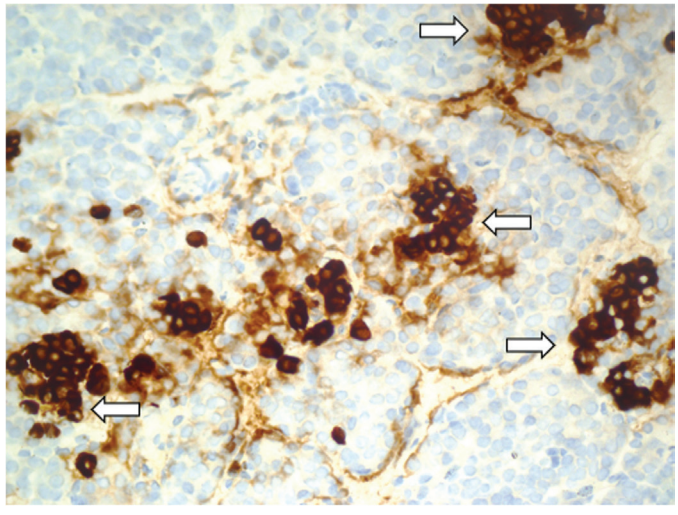

Figure 2. Histopathological result confirmed diffuse hyperplasia of the islet cells. The arrows showed hypersecretion of islet cells in islets

but the minimum GIR remained 10 mg/kg/min. Octreotide subcutaneous injection was resumed with the dose of 30 µg/kg/day. Over the next few weeks, there was no reduction in his glucose requirement. The total volume of nasogastric and parenteral fluids reached 190 mL/kg/d and it was also very difficult to establish a central venous line.

In view of the multiple medical problems, further surgery was being contemplated. After reviewing the risks and benefits, Sirolimus was considered as an alternative treatment option. Sirolimus treatment was begun at 4.5 months of age at a dose of 0.5 mg/m^2/day. The dose was gradually increased, with the goal of reaching a serum trough level of 5-15 ng/dL. The serum trough level of sirolimus was measured every 5-7 days. After 10 days of treatment with sirolimus, intravenous glucose infusion and subcutaneous octreotide were gradually tapered. Four weeks following initiation of sirolimus, stable blood glucose homeostasis was achieved without intravenous glucose infusion, and the octreotide dose was reduced from 30 µg/kg/day to 15 µg/kg/day. The patient was able to tolerate fasting for four hours while maintaining a blood glucose level >60 mg/dL prior to discharge as recorded by continuous glucose monitoring. The patient was followed regularly for assessment of glycemic control and measurement of serum sirolimus levels. Sirolimus was discontinued at 15 months of age. The maximum dose of sirolimus used was 3.2 mg/m^2/day.

The patient had good glycemic control after cessation of sirolimus. However, at times of poor appetite, there was still a requirement for low dose octreotide (2 µg/kg/day) to control blood glucose. Complete blood count, serum lipid profile, and renal and liver function have been monitored regularly and no significant side effects were observed, except for mildly elevated triglycerides at two years and three months old. At the time of writing the patient was three years and six months old. At the last visit, the patient was able to tolerate fasting for six hours according to continuous glucose monitoring. The blood glucose was 95 mg/dL and the insulin was 4.9 µIU/mL at the end of the six hours fasting.

Discussion

The management of diffuse CHI that is unresponsive to diazoxide poses a major therapeutic challenge. While subtotal pancreatectomy remains the procedure of choice following failure of medical therapy, the surgery is not completely curative and may still be associated with unsatisfactory glycemic control. Fluorine-18-dihydrophenylalanine positron emission tomography was not performed in our patient before surgery, since it was not available in children in China at the time, but based on the increased number of islets and enlarged volume of partial regional islets reported histopathologically, as well as the recurrent severe hypoglycemia after subtotal pancreatectomy, the patient likely had a diffuse CHI. The patient has a heterozygous mutation in ABCC8. Definitely, most dominant acting monoallelic potassium channel ATP gene mutations cause mild diazoxide responsive CHI. However, Saint-Martin et al (16) reported that some dominant *ABCC8* mutations are responsible for a subset of diffuse, diazoxide-unresponsive forms of CHI. The mechanism in these cases is unclear and needs further study. After pancreatectomy, the total amount of nasogastric and parenteral fluids had reached maximum, and it was also very difficult to establish a central venous line. Therefore, there was a need for an alternative treatment, minimizing the requirement for repeat pancreatectomy, and the burden of demanding medical and nutritional intervention in our patient.

Sirolimus has been reported as a treatment option for unresponsive CHI (10,11,12,13). No major adverse reactions were observed during follow-up period in these case reports, though a recent study in two large centers showed that mTOR inhibition achieved euglycemia, fasting tolerance and reduced medical therapy in only 30% of patients and more adverse events were observed (17). mTOR is a serine and threonine protein kinase that integrates signals from mitogens and nutrients, glucose and amino acids, to regulate cellular growth and proliferation (18). The mechanism of mTOR inhibitors in CHI has not been fully delineated. Hyperplasia of β-cells has been proposed to be involved in the trans-differentiation of mature acinar and ductal elements of exocrine pancreas into insulin-secreting cells, which is possibly mediated by the constitutive activation of the mTOR pathway (19). mTOR inhibition may also affect the number of insulin receptors that are present in pancreatic β-cells, which would reduce insulin production (20). Sirolimus has been used *in vitro* to induce fulminant diabetes by promoting insulin resistance and reducing β-cell mass through apoptosis induction (21,22). Furthermore, long-term management with sirolimus was found to cause glucose intolerance by up-regulating hepatic gluconeogenesis (23). It is postulated that the mechanism of mTOR inhibition is also reduced during islet cell proliferation (11,12). This was recently confirmed by genomic datasets implicating the insulin-like growth factor 1/mTOR/Akt pathway in the pathophysiology of CHI (9). Another study has shown that mTOR pathways are not downregulated in keeping with non-responsiveness to sirolimus and the observation that proliferation remains high after treatment with sirolimus (17).

The reported adverse effects of sirolimus treatment include stomatitis, fatigue, immunosuppression, increased risk of infections, renal function abnormalities, hyperlipidemia, and pneumonitis (22,24), which are reversible with dose reduction. Mild elevation of triglycerides was observed in our patient. Sirolimus appears to be well tolerated in children post renal transplant, even when initiated at higher doses (6 mg/m^2/day) and thereafter adjusted to achieve target trough levels in the range of 10-20 ng/mL (25). These studies suggest a reasonable safety profile for sirolimus, but the long-term safety remains unknown in younger children, particularly in neonates.

Conclusion

In conclusion, sirolimus was a well-tolerated treatment in our patient with CHI who otherwise would have required second surgery, and no major adverse events were observed during the period of 10 months of treatment. Sirolimus may be a feasible option for selected CHI patients with no contraindication, either before surgery or as an adjunctive therapy, although the mechanism and long-term adverse effects of such treatment require further study.

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Ethics

Informed Consent: Informed consent was obtained from the parents of this patient.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Yun Yan, Haiyan Wei, Design: Haiyan Wei, Yongxing Chen, Data Collection or Processing: Qiong Chen, Xiaohong Wang, Haihua Yang, Yingxian Zhang, Xiaojing Liu, Analysis or Interpretation: Yongxing Chen, Yun Yan, Literature Search: Yongxing Chen, Yun Yan, Writing: Qiong Chen, Yongxing Chen, Yun Yan, Haiyan Wei.

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