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Papers Published in Periodical Journals: Gungor N, Saad R, Janosky J, Arslanian S. Validation of surrogate estimates of insulin sensitivity and insulin secretion in children and adolescents. J Pediatr 2004;144:47-55.

Papers Only Published with DOI Numbers: Knops NB, Sneeuw KC, Brand R, Hile ET, de Ouden AL, Wit JM, Verloove-Vanhorick SP. Catch-up growth up to ten years of age in children born very preterm or with very low birth weight. BMC Pediatrics 2005 doi: 10.1186/1471-2431-5-26.

Book Chapters: Darendeliler F. Growth Hormone Treatment in Rare Disorders: The KIGS Experience. In: Ranke MB, Price DA, Reiter EO (eds). Growth Hormone Therapy in Pediatrics: 20 Years of KIGS. Basel, Karger, 2007;213-239.

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ORIGINAL ARTICLE

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Time Trends, Regional Variability and Seasonality Regarding the Incidence of Type 1 Diabetes Mellitus in Romanian Children Aged 0-14 Years, Between 1996 and 2015

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What is already known on this topic?

The incidence of type 1 diabetes mellitus is highly variable in the world. Epidemiological data for the EURODIAB Study and the DIAMOND Project were collected in Bucharest, Romania's capital city. Romania had a low incidence of the disease, but it increased continuously over a 10-year period (2002-2011) in children aged 0-17 years.

What this study adds?

Our study found a steeper rise in the incidence of childhood type 1 diabetes mellitus than the one reported globally. Romania now belongs with the countries having a high incidence of the disease. There are significant differences between geographic regions and there is a seasonality regarding the diagnosis.

Abstract

Objective: The incidence of type 1 diabetes mellitus in children is highly variable in the world. The aim of our study was to: 1) analyze the evolution of the incidence of childhood type 1 diabetes in Romania between 1996 and 2015, and: 2) to search for differences amongst age groups, gender, geographic regions and month of diagnosis.
Methods: Data on all new cases of type 1 diabetes, aged < 15 years, obtained from two independent sources, were included in the study. The statistical methods included modeling of the incidence rates, adjusting for age, sex, calendar year, geographic region and seasonality.
Results: The study group was composed of 5422 children, with overall completeness of ascertainment estimated at 93.7%. The incidence rate (per 100.000 person-years) rose continuously, from 4.7 [95% confidence interval (CI) 3.9-5.7] in 1996 to 11.0 (95% CI 9.9-12.2) in 2015, by a yearly rate of 5.1%, highest in the youngest and lowest in the oldest children. The mean incidence was significantly higher ($p < 0.0001$) in Transylvania (7.9, 95% CI 7.6-8.3) than in Moldavia (6.5, 95% CI 6.2-6.9) and Muntenia (7.0, 95% CI 6.7-7.3), probably due to differences regarding ethnicity and lifestyle. The monthly incidence showed a sinusoidal pattern, peaking in January and being minimum in June.
Conclusion: The incidence of type 1 diabetes mellitus in Romanian children increased continuously during the study period by a rate that, if maintained, would lead to its doubling every 14 years. Important differences were established between geographic regions and seasonality at diagnosis.
Keywords: Type 1 diabetes mellitus, Romania, children, incidence, seasonality

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Introduction

Knowledge about the epidemiology of type 1 diabetes mellitus (T1DM) has always been of interest among diabetologists. Epidemiological information is important not only for health care systems, but also for researchers, providing valuable information about the underlying mechanisms of this chronic disease. The currently accepted theory regarding the pathogenesis of T1DM states that, in genetically predisposed individuals, the intervention of some environmental factors triggers the activation of the immune system and leads to beta cell destruction and, consequently, to absolute insulin deficiency (1). However, the mechanisms are probably more complex, as there are cases of T1DM where insulin resistance probably plays an important role (2).

Most of the data regarding the incidence of T1DM concerns children, and they derive from two important multinational studies (3,4), as well as from national or regional reports. All these studies state that the incidence increases over time and is highly variable, depending on age, geographic region, and season. More specifically, T1DM is most common in children aged 10 to 14 years, has a probability of occurrence that increases with the distance from the Equator (suggesting the role of vitamin D deficiency), has its onset mainly in the cold season (underlining the role of viral infections), and increases by a mean annual rate of about 3%.

However, the knowledge regarding the secular trend of the incidence is far from being complete, since some data show continued increase (5,6), whereas others found that the increase has leveled off (7) or has a sinusoidal pattern (8).

Romania was part of the aforementioned multinational studies, but it provided data only from the capital, Bucharest, and its surroundings. In the last two decades, the Romanian Childhood Diabetes Registry was developed, and has provided information from all over the country. According to this source, Romania had a low incidence of the disease, with some regional differences (9), but the incidence has increased continuously over a 10-year period in children aged 0 to 17 years (10). The most recent data suggests that Romania may now be included in the group of countries that have a high incidence of pediatric T1DM (11).

The aim of this paper is to characterize the evolution of the incidence of T1DM in children aged 0 to 14 years during the 20 years that the Romanian Childhood Diabetes Registry has existed, and to establish the possible differences between various age groups and the two genders, as well as to reveal some regional and seasonal particularities.

Methods

This retrospective study was conducted on Romanian boys and girls under the age of 15, diagnosed with T1DM between 1996 and 2015. The study was approved by the Ethical Committee of "Victor Babes" University of Medicine and Pharmacy Timisoara (approval number: 43/11.05.2016). Due to the fact that it was a retrospective epidemiological study, informed consent was not requested.

The cases were collected from two independent sources. The primary source was the Romanian Childhood Diabetes Registry. This registry was started in 1996 by ONROCAD (Romanian acronym for the "Romanian National Organization for the Protection of Children and Adolescents with Diabetes"), and revised on a yearly basis, relying on the reports of the physicians who were managing these cases. Romania is divided into 41 administrative districts. Medical care for diabetic children is provided in a centralized manner. According to the rules imposed by the National Health Insurance Company, insulin and glucose strips are reimbursed only if prescribed by a limited number of physicians, up to four in each district (this person could be a pediatrician, a diabetologist or an endocrinologist). Consequently, the primary source of this study was based on the yearly reports of about 70 health care professionals, which included children with T1DM who constituted the majority of the cases compared to other types of diabetes. The records of the "Cristian Serban" Medical Center in Buzias, a public health care center specialized in the diagnosis, evaluation, education and treatment of children and young people with diabetes mellitus from all over the country, considered as a European Reference Center for the management of pediatric diabetes, constituted our second source (12).

The completeness of ascertainment of the cases was calculated by the capture-recapture method (13), using the number of patients diagnosed by each of these two sources to estimate the number of missing patients and the total number of children.

The diagnosis of T1DM was established by the lead physician for each case, based on internationally accepted guidelines (14). The date of onset of the disease was considered to be the date of the first insulin injection. Cases that were younger than six months old at diagnosis were excluded, since the probability of developing T1DM before this age is very low.

Statistical Analysis

The demographic data was retrieved from the National Institute of Statistics (15). These data were derived from the censuses performed in 1992, 2002, and 2011, also using estimates for the other years. The data were collected for each of the

41 administrative districts, for both genders, and for three standard age groups: 0-4 years, 5-9 years, and 10-14 years.

The incidence rates were expressed as new cases per 100.000 person-years at risk, with approximate 95% Poisson confidence intervals (CIs). Poisson regression was used to model the incidence rates, adjusting for age groups, sex, calendar year and geographical region, taking the respective background population into account as exposure.

The analysis of seasonality at diagnosis was based on all cases aggregated over sexes and all years, with stratification by age at diagnosis (0-4 years, 5-9 years, 10-14 years), as well as all ages combined. Using methodology described by Edwards (16), the analysis included a general test for seasonal variation [Total, 11 degrees of freedom (df)], a test for sinusoidal variation (Cyc. trend), as well as a test for seasonal variation unexplained by lack of fit of the sinusoidal model (Residual, 9 df).

Results

Completeness of Ascertainment

After the exclusion of other types of diabetes mellitus, the primary source (the Romanian Childhood Diabetes Registry) identified 5248 cases, whereas the secondary source (the medical records from the "Cristian Serban" Medical Center in Buzias) provided 1878 cases. Among the total 5422 cases ascertained, 1704 were captured independently by both sources. Based on these data, the total number of patients captured by the two sources was 5422, the number of missed cases was estimated to be 362, and the probable total number of patients was 5784.

The overall completeness of ascertainment was 93.7% (95% CI 93.1-94.4) and constant during the study period (data not shown). The value of the completeness of ascertainment for the primary source was 90.7% (95% CI 89.4-92.1), and for the secondary source 32.5% (95% CI 31.2-33.7).

Trends in the Incidence of Type 1 Diabetes Mellitus Between 1996 and 2015

The yearly incidence rate (per 100.000 person-years) showed a continuous increase during the study period, from 4.7 (95% CI 3.9-5.7) in 1996 to 11.0 (95% CI 9.9-12.2) in 2015 (Table 1), corresponding to an annual mean increase of 5.1% (95% CI 4.6-5.6). This phenomenon was due to the absolute increase in the number of new cases of T1DM, as well as to a decrease in the target population. The rise of

Table 1. Crude incidence rates for type 1 diabetes mellitus in Romanian children aged 0-14 years, between 1996 and 2015

Year	Boys		Girls		Total	
	Rate (/100.000)	95% CI	Rate (/100.000)	95% CI	Rate (/100.000)	95% CI
1996	4.6	3.7-5.5	4.9	4.0-5.9	4.7	4.1-5.4
1997	3.4	2.7-4.3	4.9	4.0-5.9	4.2	3.6-4.8
1998	3.8	3.0-4.6	5.1	4.2-6.2	4.4	3.8-5.1
1999	5.2	4.3-6.3	5.9	5.0-7.1	5.6	4.9-6.3
2000	5.8	4.9-7.0	5.2	4.3-6.3	5.6	4.9-6.3
2001	6.9	5.8-8.1	5.6	4.6-6.7	6.2	5.5-7.0
2002	6.9	5.8-8.1	5.2	4.3-6.4	6.1	5.3-6.9
2003	6.5	5.4-7.7	6.2	5.1-7.4	6.3	5.6-7.2
2004	7.5	6.3-8.8	6.0	5.0-7.3	6.8	6.0-7.7
2005	6.6	5.5-7.9	6.9	5.8-8.3	6.8	5.9-7.7
2006	7.8	6.6-9.2	7.1	5.9-8.5	7.5	6.6-8.4
2007	8.0	6.8-9.5	9.1	7.7-10.7	8.6	7.6-9.6
2008	8.1	6.9-9.6	9.2	7.8-10.7	8.7	7.7-9.7
2009	8.5	7.2-10.0	7.6	6.3-9.0	8.1	7.1-9.1
2010	8.6	7.2-10.0	8.2	6.9-9.7	8.4	7.4-9.4
2011	9.8	8.4-11.4	9.9	8.4-11.5	9.8	8.8-10.9
2012	9.1	7.8-10.6	9.0	7.6-10.5	9.0	8.1-10.1
2013	10.1	8.7-11.7	9.1	7.7-10.7	9.6	8.6-10.7
2014	11.3	9.8-13.0	10.7	9.2-12.5	11.0	9.9-12.2
2015	11.3	9.7-13.0	10.8	9.3-12.5	11.0	9.9-12.2
1996-2015	**7.3**	**7.0-7.5**	**7.1**	**6.9-7.4**	**7.2**	**7.0-7.4**

CI: confidence interval

encountered in all age groups, reaching peak values in the youngest children [annual rate 7.6% (95% CI 6.3-8.9)], and the lowest values in children aged 10-14 years [annual rate 3.9% (95% CI 3.1-5.8)]. The increasing trend affected both boys and girls, without significant differences between them, and it was present in all the geographic regions of the country.

The mean incidence of the disease in the study period was 7.2 (95% CI 7.0-7.4). It was not significantly ($p = 0.53$) higher in boys (7.3, 95% CI 7.0-7.5) than in girls (7.1, 95% CI 6.9-7.4). The differences between the age groups were statistically significant ($p < 0.0001$). The highest incidence was found in the age group 10-14 years (9.1, 95% CI 8.7-9.4), followed by the age groups 5-9 years (7.6, 95% CI 7.3-8.0), and 0-4 years (4.5, 95% CI 4.2-4.7). The mean age at diagnosis was similar in girls and in boys ($p = 0.81$).

Regional Differences in the Incidence of Type 1 Diabetes Mellitus

There were striking differences between geographic regions regarding the incidence of T1DM (Figure 1). The mean incidence during the study was significantly higher ($p < 0.0001$) in Transylvania (7.9, 95% CI 7.6-8.3), in comparison to Moldavia (6.5, 95% CI 6.2-6.9) and Muntenia (7.0, 95% CI 6.7-7.3).

Seasonal Variation in the Incidence of Type 1 Diabetes Mellitus

The incidence of T1DM differed by months in a pattern compatible with a sinusoidal variation (Figure 2). The incidence was higher during winter, with the maximum value registered in January, and lower values during summer, with the minimum value encountered in June. The pattern of seasonal variation was significant ($p < 0.001$) for all age groups except for the youngest (0-4 years), however with statistically significant evidence of residual variability.

Figure 1. Regional differences in the incidence of type 1 diabetes mellitus in Romanian children aged 0-14 years, in the period 1996-2015

CI: confidence interval

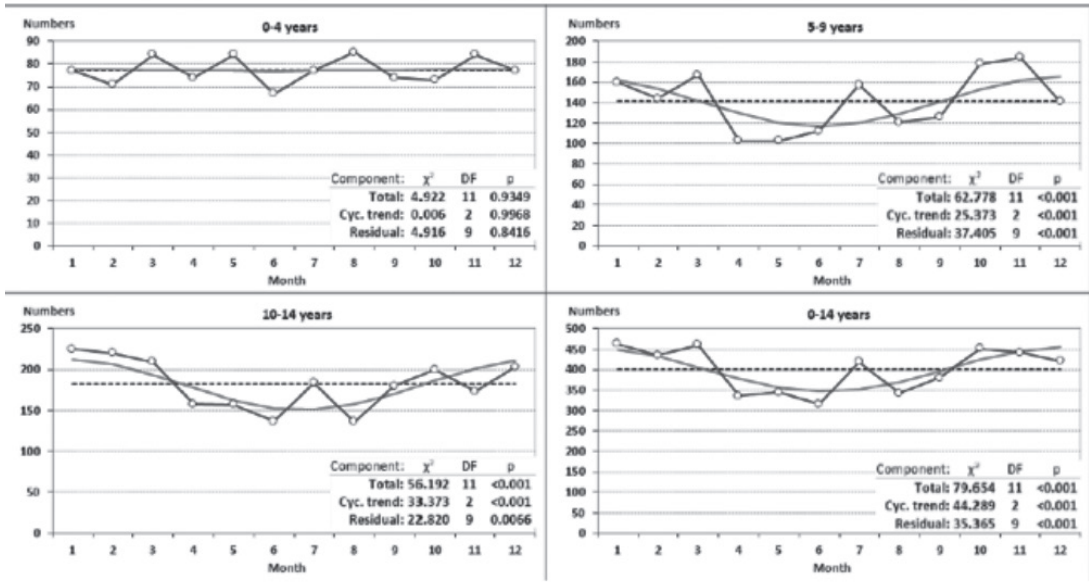

Figure 2. Seasonality of the incidence of type 1 diabetes mellitus in Romanian children aged 0-14 years (1996-2015). Blue lines with marks are observations, read lines are best fitted sinusoidal curves, dotted black lines are monthly average (ignoring differing days in months)

For each age group χ^2-values with corresponding degrees of freedom and p values are presented as total (test of heterogeneity by month), Cyc. trend (significance of accepting a sinusoidal model), and residual (test of variation unexplained by the sinusoidal model)

Discussion

The epidemiology of T1DM in Romanian children has been described previously. The first papers included only the capital, Bucharest (17), this region being part of the two important international epidemiological studies, EURODIAB (4) and DIAMOND (3). Later, data covering the whole country also became available (9,10). The present update is justified by the enlarged study material, now covering a full 20-years period from 1996, by the need to confirm the previously published age, gender and calendar time trends, and by the necessity to address the geographical distribution within Romania and the seasonality at diagnosis.

Completeness of Ascertainment

The incident cases were retrieved from two sources that diagnose and report new cases of childhood T1DM independently from each other. The primary source was the Romanian Childhood Diabetes Registry, in which records of children with diabetes are included, based on the evidence kept and forwarded by local diabetologists or endocrinologists. The secondary source was represented by the data files of patients admitted to the "Cristian Serban" Medical Center in Buzias, a public hospital where children with diabetes mellitus are admitted if referred by a physician or by direct registration via the diabetes association or website. The overall completeness of ascertainment was high (93.7%), and it was constant over the study period. The primary source had, by itself, a high completeness of ascertainment (90.7%). This could be explained by the fact that the medical care of children with diabetes mellitus is provided by a health system that can be considered centralized, as each of the 41 districts includes only few physicians (up to four) who are in charge of these cases. The relatively low number of physicians facilitates the achievement of almost complete data for the Romanian Childhood Diabetes Registry. The low completeness of ascertainment of the "Cristian Serban" Medical Center in Buzias may be explained by the fact that only a small percentage (about 30%) of all the children with diabetes from Romania benefited from evaluation, education and treatment within this hospital.

Trends in the Incidence of Type 1 Diabetes Mellitus Between 1996 and 2015

During the 20 years of follow-up, the incidence of T1DM in children increased constantly, with an annual rate of 5.1%, which is higher than the one reported by the EURODIAB study and the DIAMOND project (3,4). Due to the high completeness of ascertainment one can consider this information accurate and exclude bias due to under-reporting. In the past, Romania was included in the group of countries with a low incidence of T1DM in children (9), but, as shown by the present data, the country presently has a high incidence of this disease.

Such a rapid increase in incidence rates usually characterizes countries with lower overall incidence (18), while in those where the disease is more prevalent, the incidence rate increases more slowly or even levels off (6,7). However, in Romania, the increase continued to be steep even when the incidence became high, and, if this rate is maintained, one can expect a doubling of the incidence rate every 14 years. Even without valid information, one can speculate that, in the past, the incidence rate of T1DM was approximately constant, probably at a low value, and at a certain moment it started to increase (19). That specific moment was probably shortly after 1990, when the political changes in Romania led to important changes in lifestyle, represented by a higher mobility of the population and by the increased use of industrially processed food. These might have led to a greater exposure to infectious agents and to chemical substances found in food, putative triggers for the autoimmune destruction of the beta cells. It is very probable that new and different environmental factors contributed to the marked increase in the incidence of T1DM, as the study period is too short for the occurrence of genetic changes that could explain this rise in incidence. Enhanced economic development has been postulated as the reason for rapid increases in the incidence of T1DM by other authors (20).

It is known that Hungary (21) and Germany (22) have higher incidences of childhood T1DM compared to Romania. One can assume that the Hungarian and German ethnicities in Romania (two of the most important ethnic minorities), who have a similar genetic background to the population from the aforementioned countries, might have higher incidences of T1DM and that an increase in the percentage of those ethnicities might explain the rise in the incidence of T1DM. However, this demographic phenomenon was not shown by national censuses (15), which demonstrated, in fact, a decrease in the percentage of these populations, as a consequence of their emigration to Hungary and Germany, respectively.

Another possible explanation for this steep increase is the shift to a younger age at diagnosis. It is hypothesized that T1DM occurs at a younger age, due to an earlier effect of the environmental triggers, and that the overall incidence of the disease is not changed. This idea is supported by several trials that have analyzed the evolution of the

incidence of T1DM in children, as well as in adults (23), and was suggested by previous work carried out by our group, in which the analysis performed over a 10-year period reported an increase in the incidence of T1DM in the age groups 0-4, 5-9 and 10-14, and a decrease in the age group 15-17 (10). In the present study, the more rapid increase in the incidence of T1DM in the youngest children may be evidence to support the theory of the shift towards a younger age at diagnosis.

The highest incidence of T1DM was registered in the age group 10-14. This is the age when most of the children reach puberty. The insulin resistance induced by the release of sex hormones may play an important role in the onset of T1DM (2). Boys had a higher incidence of T1DM in most years and in all age subgroups. This is usually the case for populations with high incidence of the disease (24), without a clear explanation for this phenomenon. The mean age at diagnosis was significantly lower in girls as compared to boys. It is known that girls usually reach puberty at a younger age in comparison to boys, and this might be an explanation for the earlier onset. Our data are in concordance with reports of other authors regarding this (25).

Regional Differences in the Incidence of Type 1 Diabetes Mellitus

Romania is composed of three geographic regions (Transylvania, Moldavia and Muntenia), each of them including several administrative districts. Transylvania was, in the past, part of the Austro-Hungarian Empire. Nowadays, its population is more heterogeneous, as compared to Moldavia and Muntenia, the Hungarian and German ethnic minorities being more numerous here than in the other regions.

Our study has revealed significant differences between these three regions. The highest mean incidence was encountered in Transylvania, and this was significantly higher compared to Moldavia and Muntenia, respectively. In a previous paper published by our group (9), an analysis performed on these three geographic regions, between 1992-1995, showed similar differences, though the mean rates of incidence were much lower. The main explanation for this finding might be the ethnic heterogeneity of Transylvania, knowing that children from Hungary and Germany (who have a genetic background similar to the aforementioned minorities from our country) have higher rates of T1DM in comparison to Romania (21,22). However, we cannot test this hypothesis further, since neither the primary nor the secondary source contains information about the ethnicity of the patients. Another possible explanation could be the fact that Transylvania is a region with a higher standard of living, compared to the other two regions and, consequently, one may speculate that its population has a higher mobility (more frequent trips in different regions of the world), and adopted more modern eating habits, based on industrial processed food. These differences could facilitate the interaction of some environmental factors (infections, food antigens) in a genetically predisposed population and trigger the autoimmune destruction of pancreatic beta cells.

Seasonal Variation in the Incidence of Type 1 Diabetes Mellitus

The seasonal variability of T1DM underlines the role of infectious triggers and of the deficit of sun exposure in the pathogenesis of the disease (26,27). The putative infectious agents, notably viruses, are more common and long-lived in winter; consequently, given their involvement in the pathogenesis, the number of new cases should be higher in winter. In addition, the reduced number of sunny days in the winter induces a deficit in the subcutaneous synthesis of vitamin D, known to have an important role in immune modulation (28). However, due to the fact that the time elapsed between the intervention of the trigger and the onset of hyperglycemia is highly variable, the seasonal variability is not always obvious.

Our study has revealed significant seasonal variation of the incidence of T1DM, the highest values being recorded during winter (maximum incidence in January), and the lowest during summer (minimum incidence in June). This pattern was seen in children belonging to the age groups 5-9 and 10-14, but not for the youngest ones. The quite constant occurrence of the disease between ages 0-4 could be explained by the decreased exposure to infections, due to country's specifics: children are raised by their parents in the first two years of life (this is the length of the parental leave), by their grandparents in the following year. Admission to communities, where infections are more prevalent, usually takes place after the age of three years.

Similar data were reported by the EURODIAB investigators (27) for the majority of the 23 member centers, except for two amongst which Romania's capital, Bucharest. Since our study periods overlap substantially (1989-2008 in EURODIAB and 1996-2015 in our study) and the meteorological conditions do not differ significantly in Bucharest from the rest of the country, the different results could be explained by a lower prevalence of vitamin D deficiency in Bucharest, possibly due to better preventive measures.

Study Limitations

Our study has several limitations and strengths. The main limitations are represented by the lack of information regarding the ethnicity of the newly diagnosed patients, a finding that could have facilitated the interpretation of the regional differences in the incidence. The absence of data

about the dietary habits in early life and of the population from different geographic regions, as well as the lack of evidence about the evolution of the incidence of diabetes mellitus in adolescents and young adults, a finding that could have supported the theory of the shift towards a younger age at diagnosis, can be listed as other limitations. The strengths of our research consist in the existence of accurate data, provided by two high quality sources that cover the entire territory of the country over a long period.

Conclusion

We have analyzed the trends in the evolution of the incidence of T1DM in Romanian children aged 0-14 years, over a 20-year span (1996-2015), taking into consideration some particularities regarding age group, gender, geographic region, and seasonality. We found a steeper rise in the incidence than the one reported globally, for both genders and all age groups and, if this trend were to be maintained, it would lead to a doubling of the incidence every 14 years. Romania now belongs with the group of countries with a high incidence of childhood T1DM. There are significant differences between the geographic regions and there is a seasonality regarding the diagnosis of T1DM that concerns all age groups, except for the very youngest. The shortcomings of the research could be overcome by continuation of follow-up and the addition of new information about adult patients, ethnicity of the subjects and lifestyle with particular emphasis on changes in eating habits.

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Ethics

Ethics Committee Approval: Ethical Committee of "Victor Babes" University of Medicine and Pharmacy Timisoara (approval number: 43/11.05.2016).

Informed Consent: It was a retrospective epidemiological study, informed consent was not requested.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: Adrian Vlad, Viorel Serban, Design: Mihaela Vlad, Bogdan Timar, Data Collection or Processing: Adrian Vlad, Mihaela Vlad, Bogdan Timar, Alexandra Sima, Analysis or Interpretation: Adrian Vlad, Anders Green, Sören Möller, Literature Search: Viorel Serban, Mihaela Vlad, Bogdan Timar, Alexandra Sima, Writing: Adrian Vlad, Anders Green, Sören Möller.

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Klinefelter Syndrome in Childhood: Variability in Clinical and Molecular Findings

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What is already known on this topic?

Klinefelter syndrome (KS) is the most common chromosomal disorder in humans but patients with KS are often diagnosed late in life. Less than 10% of patients are diagnosed before puberty.

What this study adds?

Neurodevelopmental, psychological and verbal disorders with undescended testes are the findings of Klinefelter syndrome (KS) in childhood that should alert the physician. Further molecular analyses should also be considered in KS patients with ambiguous genitalia to exclude additional gene mutations.

Abstract

Objective: Klinefelter syndrome (KS) is the most common (1/500–1/1000) chromosomal disorder in males, but only 10% of cases are identified in childhood. This study aimed to review the data of children with KS to assess the age and presenting symptoms for diagnosis, clinical and laboratory findings, together with the presence of comorbidities.

Methods: Twenty-three KS patients were analyzed retrospectively. Age at admission, presenting symptoms, comorbid problems, height, weight, pubertal status, biochemical findings, hormone profiles, bone mineral density and karyotype were evaluated. Molecular analysis was also conducted in patients with ambiguous genitalia.

Results: The median age of patients at presentation was 3.0 (0.04-16.3) years. Most of the cases were diagnosed prenatally (n=15, 65.2%). Other reasons for admission were scrotal hypospadias (n=3, 14.3%), undescended testis (n=2, 9.5%), short stature (n=1, 4.8%), isolated micropenis (n=1, 4.8%) and a speech disorder (n=1, 4.8%). The most frequent clinical findings were neurocognitive disorders, speech impairment, social and behavioral problems and undescended testes. All except two patients were prepubertal at admission. Most of the patients (n=20, 86.9%) showed the classic 47,XXY karyotype. Steroid 5 alpha-reductase 2 gene and androgen receptor gene mutations were detected in two of the three cases with genital ambiguity.

Conclusion: Given the large number of underdiagnosed KS patients before adolescence, pediatricians need to be aware of the phenotypic variability of KS in childhood. Genetic analysis in KS patients may reveal mutations associated with other forms of disorders of sex development besides KS.

Keywords: Ambigious genitalia, cryptorchidism, disorders of sex development, speech impairment

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Introduction

Klinefelter syndrome (KS) is the most common (1/500–1/1000) chromosomal disorder in humans (1). Men with KS are often diagnosed late in life, usually during investigation for infertility and the mean age of diagnosis is commonly in the mid-30s (1,2). The diagnosis rate of KS is estimated to be only 25% (2,3). Furthermore, less than 10% of KS patients are diagnosed before puberty (4).

Although no firm diagnosis guidelines for KS exist and extreme heterogeneity in clinical and genetic presentation is found (5), the usual key findings in KS are primary testicular failure with small testes, hypergonadotrophic hypogonadism, tall stature with eunuchoid body proportions, neurocognitive impairment mainly related to language processing disability and varying degrees of social, behavioral, and learning difficulties (3,5). Gynecomastia, metabolic syndrome, osteoporosis, cryptorchidism, decreased penile size, psychiatric disturbances, inguinal hernia, mitral valve prolapse, growth hormone deficiency, hypothyroidism, hypoparathyroidism and increased risk of autoimmune diseases are some of the other reported abnormalities associated with KS (2,6). Newborns with KS generally present with a normal male phenotype, although ambiguous genitalia has been reported to be associated with KS (3,4). Phenotypic variation may depend on the severity of the expression of genetic defects, androgen deficiencies, androgen receptor (AR) sensitivities (i.e., CAG repeats polymorphism), or randomly skewed inactivation of the additional X chromosome material (4).

Patients who are unaware that they have KS are assumed to be relatively healthy and do not require treatment. Because of this, clinical description of the more mildly affected patients is not generally available leading to a limited account of the full spectrum of KS phenotypes (1). Given the insufficient awareness of this syndrome and the typical delay in diagnosis until after puberty, our aim was to review the data of our pediatric patients with KS to evaluate the age and reason for diagnosis, as well as the clinical and laboratory findings and presence of comorbid conditions.

Methods

A retrospective medical chart review was performed to collect data from the pediatric endocrinology outpatient clinics of İstanbul University, İstanbul, Turkey and Near East University Nicosia, Northern Cyprus, between January 1992 and February 2017. The definition of KS, as an inclusion criterion for this study, required the availability of a karyotype consisting of an X chromosome polysomy and at least one Y chromosome, either as a single lineage or as a mosaicism (7). Twenty-three patients with a confirmed diagnosis of KS and sufficient documented data were included in the study. Age at the time of diagnosis, chief complaint at admission and presenting symptoms, parents' ages during pregnancy and existing comorbidities were evaluated. Physical examination findings, pubertal status and secondary sex characteristics, presence of gonadal failure and gynecomastia were investigated. Clinical and laboratory parameters, including height, weight, body mass index (BMI), testicular volumes, fasting blood glucose levels, fasting insulin levels, homeostatic model assessment–insulin resistance (HOMA-IR), total testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid hormones, 25-hydroxyvitamin D [25(OH)D_3], bone mineral density (BMD) and different karyotype disorders of KS were recorded.

The standard deviation score (SDS) for height, weight and BMI were calculated according to reported data for healthy Turkish boys (8). To compare the data, the study group was divided into three age groups (<3, 3-9, >9 years) to determine the age-specific velocity in growth (3). A hologic anthropomorphic lumbar spine scan analysis was used and sex-specific z-scores for BMD were derived from the analysis. When BMD z-scores are between -1.0 and -1.9, "at risk for low BMD or bone mineral content for chronologic age" is used for terminology, and when BMD z-scores are less than or equal to -2.0, the terms "low BMD" or "low bone mineral content for chronologic age" were preferred instead of "osteoporosis" (9). 25(OH)D_3 levels were compared in cases with low and normal BMD values. The diagnosis of a neurocognitive disorder and the need for special education were retrieved from the medical records. Molecular analysis of the steroid 5 alpha-reductase 2 (*SRD5A2*) and *AR* genes was performed in three cases which presented with ambiguous genitalia. Patient records and information were anonymized and de-identified before analysis.

Statistical Analysis

Statistical Package for Social Sciences Software (SPSS 21, Chicago, IL, USA) was used for the analysis. All continuous variables were expressed as the median, minimum and maximum values. Age, height, weight, BMI and hormone levels were shown as median values. The Kruskal-Wallis test was used for the comparisons of height, weight and BMI SDS among the three age groups. Categorical variables were expressed as numbers and percentages. The Mann-Whitney U test was used to compare 25(OH)D_3 levels. A p value <0.05 was considered significant.

Results

Demographics and Clinical Characteristics

The median age of the patients at presentation was 3.0 (0.04-16.30) years. Most of the cases were diagnosed prenatally by amniocentesis because of advanced parental age. These prenatally diagnosed patients had no complaints at admission, except for two patients whose age at presentation was 3 years and whose additional complaints were short stature and undescended testis, respectively. The median maternal age and paternal age during pregnancy among all cases were 40.0 (22.0-46.0) and 38.5 (24.0-52.0) years, respectively. The details of the chief complaints on admission and the findings during the initial physical examination are shown in Table 1. Patients with speech disorder and amylogenesis imperfecta were diagnosed during genetic investigation for their main complaints in the department of physiotherapy and rehabilitation and pediatric dentistry. In the patient with short stature, all tests including growth hormone stimulation tests were found to be normal.

Most of the cases were prepubertal at admission. The pubertal patients (n = 2) presented with complaints of undescended testes or micropenis. Regarding the follow-up data of all patients, the early signs of pubertal development were observed in three of the prepubertal patients and in total we observed pubertal progress in five patients during follow-up. In three of the five pubertal patients, testicular

Table 1. Complaints and physical findings of Klinefelter syndrome patients at presentation and follow-up observation

Main complaints or reason for referral		**Physical findings at presentation**	
Prenatally diagnosed	n = 15, 65.2 % (thirteen without any complaints, one with undescended testis, one with short stature)	Pubertal status	• Minipuberty n = 4, 17.3 % • Prepubertal n = 17, 73.9 % • Puberty n = 2, 8.6 %
Scrotal hypospadias	n = 3, 13.0 %	Scrotal hypospadias	n = 3, 13.0 %
Undescended testis	n = 2, 8.7 % (one also had a prenatal diagnosis)	Undescended testis	n = 8, 34.7 %
Isolated micropenis	n = 1, 4.3 %	Isolated micropenis	n = 2, 8.7 %
Short stature	n = 1, 4.3 % (had a prenatal diagnosis)	Short stature	n = 1, 4.3 %
Tall stature	No	Tall stature	n = 3 13 %
*Speech impairment or disability of language processing	n = 1, 4.3 %	Speech impairment or disability of language processing	n = 11, 48.7 %
*Neurocognitive disorders and need for special education	n = 1, 4.3 %	Neurocognitive disorders and the need for special education	n = 11, 48.7 %
Amelogenesis imperfecta	n = 1, 4.3 %	Amelogenesis imperfecta	n = 1, 4.3 %
Macroglossia	n = 1, 4.3 %	Macroglossia	n = 1, 4.3 %
Social and behavioral problems	No	Social and behavioral problems	n = 9, 39.1 %
Gynecomastia	No	Gynecomastia	n = 2 (pubertal), 8.7 %
Follow-up observation			
Pubertal status		• Minipuberty n = 4, 17.3 % • Prepubertal n = 21, 91.3 % • Puberty n = 5, 21.7 %	
Gender identity		• Female n = 1, 4.3 % (one of the patient with scrotal hypospadias) • Male n = 22, 95.6 %	
Arrest in testicular volume during puberty		• n = 3 (testicular volumes were 5 mL, 12 mL and 15 mL) (the remaining 2 pubertal patients were at the beginning of puberty)	

*: same case

volumes had ceased to increase, pubertal testes remained small without additional enlargement, testicular failure had occurred and testosterone replacement therapy was started. The other two pubertal patients were in very early puberty with testicular volumes of 4 mL and they need more time to observe their pubertal progress (Table 1). Only two of the prepubertal patients had atrophic/hypoplasic testes (testis volume < 0.5 mL) at admission.

Undescended testes were found in almost one-third of all patients during the initial physical examination, but only two of them presented with this complaint. The cases with scrotal hypospadias or gynecomastia are also described in Table 1. One of the patients with scrotal hypospadias was raised with a female identity. This patient underwent bilateral laparoscopic gonadectomy and sex steroid therapy was initiated to stimulate pubertal changes in accordance with a female identity. Neurocognitive disorders and the need for special education were determined in almost half of the patients. Severe hypotonia and neuromotor retardation were observed in only one case.

Median height, weight and BMI SDS in KS patients younger than three years, between three and nine years, and after nine years are shown in Table 2. Despite the increase in growth status, which was observed clinically, the difference was not statistically significant.

Laboratory and Radiological Findings

At the time of minipuberty hormone levels showed variability. In the prepubertal period, LH, FSH and total testosterone levels were all in prepubertal ranges. During the pubertal period, the median testosterone level was found to be within normal ranges when the median LH and FSH levels began to increase, which indicated gonadal failure (Table 3).

Thyroid hormone levels and thyroid antibodies were normal in all patients. Hyperinsulinemia was observed in two patients (one prepubertal and one pubertal) who had BMI values > 2 SDS, although they had normal glucose tolerance. HOMA-IR results were 4.9 and 5.8 in these patients, respectively.

The L1-L4 vertebral BMD z-score was evaluated in 13 patients. The median (range) level of the BMD z-score was −1.0 (−3.6 to 0.7). Two pubertal patients had low BMD (z-score −3.2 and −3.6) and five patients (one pubertal and four prepubertal) were at risk for low BMD (z-score: −1.2, −1.3, −1.4, −1.4, and −1.8). The median 25(OH) D_3 of the whole cohort was 19.1 ng/mL (6.6-41.3). Median 25(OH)D_3 levels were 13 ng/mL (8.2-41.3) and 18.3 ng/mL (6.6-19.3) in the cases who had a BMD z score ≤ −1 and a BMD z score > −1. No statistically significant difference was found between these groups in terms of BMD z score.

Karyotype and Molecular Analyses

Most of the patients (n = 20, 86.9%) showed the classic 47,XXY karyotype (Table 4). All three of the cases presenting with ambiguous genitalia had the 47,XXY karyotype. One of these three cases was homozygous for the p.G196S mutation in exon 4 of the *SRD5A2* gene, and the other was heterozygous for the p.P892L mutation in exon 8 of the *AR* gene. However, no mutation was detected in either *AR* or *SRD5A2* in the third KS patient with scrotal hypospadias (Table 4). Both of the mutations detected have been previously described in the Human Genome Mutation Database.

Table 2. Height, weight and body mass index standard deviation score in Klinefelter syndrome patients by age at admission

Age (years)	Number of cases (n)	Height SDS median (range)	Weight SDS median (range)	BMI SDS median (range)
< 3	12	-0.29 (-4.09:1.33)	-0.73 (-3.24:0.97)	-0.51 (-1.82:1.28)
3-9	7	1.7 (-1.46:2.25)	0.85 (-1.39:1.91)	-0.17 (-1.40:2.42)
> 9	4	2.24 (-0.90:3.53)	0.74 (-1.04:2.97)	0.24 (-1.84:1.56)
*p value	-	0.08	0.14	0.47

*: Kruskal-Wallis test, SDS: standard deviation score, BMI: body mass index

Table 3. Hypothalamic-pituitary-gonadal axis hormone levels by pubertal status

Pubertal status	Minipuberty (n = 4) median (range)	Prepubertal (n = 21) median (range)	Pubertal (n = 5) median (range)
LH (mIU/mL)	3.09 (0.30:5.87)	0.10 (0.03:0.33)	15.0 (6.20:27.20)
FSH (mIU/mL)	1.01 (0.40:1.62)	0.82 (0.12:3.29)	23.2 (7.10:50.40)
T (ng/mL)	2.96 (0.12:5.80)	0.06 (0.01:0.49)	2.87 (2.43:3.30)

FSH: follicle-stimulating hormone, LH: luteinizing hormone, T: total testosterone

Table 4. Karyotypes of Klinefelter syndrome patients and molecular analysis of three cases with genital ambiguity

Karyotype			Molecular analysis of 3 cases with genital ambiguity			
Pure lineage	**n**	**%**	**Mutation**		**n**	**%**
47,XXY	20	86.9	*AR* gene	Heterozygous p.P892L	1	4.3
			SRD5A2 gene	Homozygous p.G196S	1	4.3
			No mutation		1	4.3
48,XXXY	1	4.3	*AR:* androgen receptor gene			
48,XXYY	1	4.3	*SRD5A2*: steroid 5 alpha-reductase 2			
Mosaicisms						
47,XXY/48,XXYY	1	4.3				

Discussion

KS is the most common sex chromosome aneuploidy in live male births, but less than 10% of cases are identified before puberty (10). This finding is worrying because these cases will present with complex comorbidities, such as hypogonadism, osteopenia/osteoporosis, metabolic syndrome, neurodevelopmental and psychosocial dysfunction all of which will adversely affect quality of life (3). With an early diagnosis, the complications of these comorbidities during follow-up can be minimized (11). According to our data, most of our patients were referred because of their prenatal diagnosis. Prenatal or early diagnosis can provide early and close monitoring of potential comorbidities (3). Early treatment of cryptorchidism, speech therapy with social training, close monitoring for learning disabilities and psychological support to both patients and parents are benefits which may be expected from early diagnosis. A special focus on nutrition and exercise for both bone mineralization and metabolic syndrome after the age of three years may be beneficial as has been suggested in the literature (3). In addition to the prenatal diagnosed patients, our series also includes a limited number of cases diagnosed in childhood and adolescence. The low ratio of cases diagnosed at pediatric ages leads to a concern about pediatric cases being missed. For this reason, increasing the rate of early diagnosis in childhood would be of immense benefit.

Speech disability may be the only sign during infancy (5). Disability in language processing, which requires additional educational help, was detected in almost half of our cases, but speech disorder as a primary complaint was present in only one single case. Language difficulties have been identified in 70%-80% of children with KS, starting at an early age (6,12). As language and learning disabilities become manifest during infancy, clinicians should bear in mind a possible diagnosis of KS in infants showing these disabilities. Health providers who deal with speech disorders should be informed about KS to prevent delay in diagnosis. It has been suggested that genital anomalies, such as micropenis, undescended testis and hypospadias, are rarely present at birth (5). Undescended testis was observed in almost one-third of the patients (n = 8) in our case series, and half of them (n = 4) also had a speech disorder. The almost 10-fold higher prevalence of KS in cryptorchid boys supports the indication for a karyotype analysis in these children (13). Cryptorchidism and mild developmental disorders may be warning signs for KS. Therefore, giving karyotype priority to children with undescended testes, particularly those who have accompanying speech impairment, is recommended.

Growth velocity is known to be accelerated by the age of three years with a modest increase in adolescence (3). Even though the statistical significance was not meaningful, the median height SDS increased after the age of three and seemed to increase again after puberty. Therefore, KS should also be considered in the differential diagnosis of boys whose height SDS increases after three years of age and increases further in puberty although this would be a delayed diagnosis. In our cohort, only one case, who had also a prenatal diagnosis, presented with short stature when he was aged three years. There are a few reported cases of KS with short stature, secondary to growth hormone deficiency (14,15). However, we did not detect growth hormone deficiency in this patient.

As traditionally described, patients with KS have small testes. The progressive increase in testes volume does not occur during puberty, both testes remaining small and firm (5). The course of puberty was observed in five patients in our case series, and three of them also had available adulthood records in the follow-up data. Their records showed that testicular volumes showed no increase and remained small without additional enlargement. Therefore, although pubertal findings may be initially observable there is a risk of pubertal arrest, and a close follow-up during

puberty is needed to begin hormonal replacement therapy at the right time.

Some of the earliest studies on minipuberty in infants with KS indicated that these boys could already have presented with biochemical signs of hypergonadotrophic hypogonadism. Conversely, recent large studies reported normal concentrations of testosterone and normal LH levels in minipuberty despite the finding that total testosterone concentrations were below the median of the total testosterone levels of the control group (3,16,17). In the current study, hormone levels showed variability, which was difficult to interpret, at the time of minipuberty.

Some clinical conditions associated with KS, such as diabetes and metabolic syndrome, worsen progressively with advancing age (5). IR and metabolic syndrome were reported in 24% and 7% of KS children of ages 4-12 years (3,18). We detected hyperinsulinemia in 8.7% (n = 2) of the patients. However, the rate of hyperinsulinemia would probably increase over time during the follow-up of remaining patients.

Low BMD is prevalent in patients with KS (5,19,20). This rate is 30.4% (n = 7) in our case series. The presence of osteopenia or osteoporosis in KS children may not manifest until puberty (19). However, KS patients have been reported to have an impaired bone mineral status that begins early in life (20). Four of the seven KS patients who had BMD SDS < -1 were also in the prepubertal period in the current study.

The $25(OH)D_3$ levels were previously reported to be significantly lower in KS patients than in controls (20). In our case series, nearly half of the patients had $25(OH)D_3$ levels lower than 20 ng/mL. However, no significant difference was found in median $25(OH)D_3$ levels between cases with normal BMD and those with BMD < -1. Except for two cases, all patients with BMD < -1 had $25(OH)D_3$ levels lower than 20 ng/mL. The etiopathogenesis of impaired bone mineral status in KS patients may be multifactorial, including KS-specific bone characteristics and/or low testosterone levels. Poor vitamin D levels may also contribute to impaired BMD in children with KS.

About 80%-90% of KS cases have 47,XXY karyotype, and the remaining cases may have a mosaic karyotype (46,XY/47,XXY), additional X or Y chromosomes (48,XXXY or 48,XXYY), or structurally abnormal X chromosomes (e.g., 47,X,iXq,Y) (1). Mosaicism (mainly 46,XY/47,XXY) is present in 10%-20% of KS patients (5). Interestingly, we did not find any 46,XY/47,XXY mosaicism, although we had 48,XXYY (n = 1), 48,XXXY (n = 1), and 47,XXY/48,XXYY (n = 1) karyotypes. Men with mosaic KS may be more androgenized, with larger testicular volumes and better hormonal profiles, than their non-mosaic counterparts (21). In the present case series, we did not have patients with 46,XY/47,XXY mosaicism. Some 46,XY/47,XXY cases could have been overlooked because of the silent phenotype. Therefore, the silent phenotype or minor findings of mosaic KS may result in the underdetection of KS children, a problem which pediatricians should be more aware of.

New disorders of sexual development (DSDs) nomenclature includes common entities such as Turner syndrome and KS under the title of sex chromosome DSDs (22,23). However, KS classically has complete male sex differentiation and ambiguous genitalia are generally not recognized as associated features of KS (23,24,25). The current study included three cases of ambiguous genitalia with KS. There are three cases of 47,XXY karyotype and *AR* gene mutation published in the literature (26,27,28). Two of them were reported to have complete androgen insensitivity syndrome whereas the other one had partial androgen insensitivity syndrome. Our case with *AR* gene mutation had partial androgen insensitivity syndrome, with heterozygous p.P892L mutation in exon 8. Partial androgen insensitivity syndrome developed in this case, probably because of the presence of the heterozygous AR mutation concurrent with random X inactivation of the healthy allele. It has been reported that the variation in phenotype could be explained by hormonal and genetic background differences, including androgen receptor polymorphism in the CAG_n repeat and skewed inactivation of additional genetic material on the X chromosome in KS patients (1,2,4,29). The one patient with homozygous *SRD5A2* gene mutation was considered a coincidence because of the consanguinity of his parents. The last patient with genital ambiguity had no detectable mutation in either *AR* or *SRD5A2* genes. This case could have been genetically investigated further for other genes associated with DSD [e.g., sex-determining region Y (*SRY*), dosage-sensitive sex reversal (*DSS*) or *DAX-1* gene locus on the X chromosome] that could cause genital anomalies. From our perspective, we suggest that analyzing some gene mutations, especially *AR* and *SRD5A2* genes, in KS cases with ambiguous genitalia would be useful. The evaluation of some gene mutations in KS cases with ambiguous genitalia is essential to provide accurate genetic counseling for other members of the family. Moreover, explaining the linkage between KS and ambiguous genitalia, by excluding the other gene mutations that cause genital ambiguity, may be possible through this evaluation.

Study Limitations

Our study has several limitations. First, due to the nature of the study, we had to rely on data from medical records. Secondly, the lipid profile and serum levels of other

reproductive hormones (e.g., serum estradiol, inhibin B, anti-Mullerian hormone and INSL3) were not examined. Thirdly, the small sample size limited us from obtaining statistically significant results. These shortcomings can be overcome in future prospective studies with samples of larger size.

Conclusion

Our data indicate that KS remains largely underdiagnosed in childhood. Pediatricians need to be aware of the phenotypic variability of KS. Specifically, neurodevelopmental, psychological and verbal disorders with undescended testes in childhood should prompt clinicians to evaluate the child in terms of KS. Further molecular analyses should be considered in KS patients with ambiguous genitalia to provide comprehensive genetic counseling to the family. Indeed, early diagnosis is essential to address age-specific challenges with timely treatment and rehabilitation to minimize the problems that patients with XXY face with and to mitigate some of the complications seen in late diagnosed cases.

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Ethics

Ethics Committee Approval: Retrospective study.

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Neşe Akcan, Şükran Poyrazoğlu, Firdevs Baş, Rüveyde Bundak, Feyza Darendeliler, Design: Neşe Akcan, Şükran Poyrazoğlu, Firdevs Baş, Rüveyde Bundak, Feyza Darendeliler, Data Collection and Processing: Neşe Akcan, Şükran Poyrazoğlu, Firdevs Baş, Rüveyde Bundak, Feyza Darendeliler, Literature Research: Neşe Akcan, Şükran Poyrazoğlu, Firdevs Baş, Rüveyde Bundak, Feyza Darendeliler, Writing: Neşe Akcan, Şükran Poyrazoğlu, Firdevs Baş, Rüveyde Bundak, Feyza Darendeliler.

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ORIGINAL ARTICLE

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Prevalence of ZnT8 Antibody in Turkish Children and Adolescents with New Onset Type 1 Diabetes

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What is already known on this topic?

The presence of zinc transporter 8 protein antibodies in diabetic children with type 1 diabetes changes according to countries. The prevalence is reported to range between 24 % and 80 %.

What this study adds?

Zinc transporter 8 protein (ZnT8A) antibody positivity was 58.2 % in Turkish children with type 1 diabetes. ZnT8A antibody was present in 46.6 % of cases with which were negative for classic type 1 diabetes associated autoantibodies.

Abstract

Objective: Zinc transporter 8 protein (ZnT8A) is a transmembrane protein which functions to transfer zinc to insulin vesicles. Antibodies formed against ZnT8A (ZnT8A) are regarded as an independent autoimmunity demonstrator in type 1 diabetes (T1D). The aim of this study was to investigate the prevalence of ZnT8A in Turkish children with new onset T1D.
Method: Eighty four patients between 1-18 years of age diagnosed with T1D between February 2015-March 2016 and the control group consisting of 50 healthy children without any autoimmune diseases were included in the study. Serum samples for ZnT8A testing were taken from the patient group within a week of diagnosis. A ZnT8A enzyme-linked immunosorbent assay was used in the analyses.
Results: ZnT8A positivity was detected in 58 % of the patients with new onset T1D and 8 % of the control group. ZnT8A were demonstrated in 5 of 11 patients with negative results for classical diabetes antibodies [insulinoma antigen-2 antibody (IA-2A), glutamic acid decarboxylase (GAD) or insulin autoantibodies]. No association was found between ZnT8A positivity and age, gender, presence or degree of ketoacidosis at presentation, hemoglobin A1c, insulin or C-peptide concentration, or the presence of either thyroid or celiac antibodies.
Conclusion: ZnT8A prevalence in children with T1D in Turkey was compatible with the literature. The ratio of patients who are clinically considered to have T1D but have negative routine diabetes auto-antibodies were observed to decrease nearly by 50 % when ZnT8 antibodies were added to the panel. ZnT8 measurement should be more widespread for clarifying the etiology in T1D.
Keywords: ZnT8 antibody, children, adolescent, type 1 diabetes

Introduction

Type 1 diabetes (T1D) is a chronic disease characterized by immune-mediated, selective destruction of pancreatic beta cells (1). Decrease in the induction effect of infections on negative immuno-regulatory genes, due to genetic predisposition and also due to a more hygienic way of life, which has become more significant in the last few decades, probably plays a role in the initiation of immune mediated degradation (2). The presence of DRB1*04-DQB1*0302 and DRB1*03 among human leukocyte antigen (HLA) class II haplotypes in at least 90 % of the individuals with the disease, detection of autoreactive anti-islet antigen specific T cells in the circulation of new onset or prediabetic individuals, demonstration of lymphocyte infiltration in the islet cells during the development of insulitis and increased

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predisposition to Addison's disease and celiac disease support the role of autoimmunity in the progression to T1D (2,3,4,5,6).

Although the initial steps stimulating the autoreactive cascade are unknown, it is suggested that autoreactive and cytotoxic T cells, activated by presentation of pancreas antigens to T cells by antigen presenting immune cells, cause beta cell destruction (7,8). Islet cell antigen (ICA), glutamic acid decarboxylase (GAD) 65, insulin and insulinoma antigen-2 (IA-2) are the main well-defined pancreatic antigens. Antibodies formed against some or all of these antigens are positive in more than 80% of new onset T1D patients (9). However, the increasing number of studies on new pancreas antigens, such as zinc transporter 8 (ZnT8), pancreatic duodenal homeobox factor-1, chromogranin A and islet amyloid polypeptide, may possibly lead to development of new treatment options and clarify etiology in idiopathic T1D patients (9,10,11,12).

Zinc is essential for the structural stabilization of insulin. Pancreas is one of the tissues with the highest zinc concentration. Zinc transportation to insulin vesicles is mediated by ZnT8, a transmembranic protein (13). It is encoded by the *SLC30A8* gene located in 8q24.11 (14). Antibodies formed against ZnT8 (ZnT8A) are regarded as an independent autoimmunity demonstrator in T1D diagnosis (9). When used in combination with IA-2 antibody (IA-2A), their predictivity for T1D and cost-effectivity compared to other antibody combinations is higher in "at risk" individuals, regardless of their age (15). Although their prevalence in children with new onset T1D changes by country and study, it is reported to be 24-80% and it is suggested that the presence of ZnT8A be investigated in all diabetes patients regardless of their ethnicity (16,17,18). ZnT8A presence has been shown in nearly 25% of patients accepted as idiopathic T1D who were negative for the classic autoantibodies (9,15). This study aimed to investigate ZnT8A prevalence in Turkish children with new onset T1D and the relation of ZnT8A to other antibodies.

Methods

A total of 84 patients, between 1-18 years of age, diagnosed with T1D in Ankara Pediatric Hematology Oncology Training and Research Hospital (n = 76) and in Gazi University Faculty of Medicine (n = 8) between February 2015 and March 2016 composed the subject group. Fifty healthy children with no autoimmune diseases were included in the study as controls (19).

Presence and degree of ketosis or ketoacidosis were recorded at the time of referral (pH 7.3-7.2 mild; 7.2-7.1 moderate < 7.1 severe ketoacidosis). C-peptide concentration was determined in serum samples taken during diagnosis, using the chemiluminescence immunoassay method. Patients with a C-peptide level above 1 ng/mL were excluded from the study. Hemoglobin A1c (HbA1c) was determined by immune turbidimetry using a modular P800 analyser (Roche Diagnostics, Basel, Switzerland). Cut-off for positivity for the following antibodies were: GAD antibody (GADA) concentration above 1 IU/mL; IA-2A concentration above 1 U/mL (both tested using radioimmunoassay method); IA concentration above 0.4 U/mL and anti-tissue transglutaminase IgA (tTG IgA) above 18 U/mL [both tested using the micro Enzyme-Linked ImmunoSorbent Assay (ELISA) method]. Thyroid function tests were performed following a ketotic or ketoacidotic period, after establishing euglycemia in the patients. Thyroid stimulating hormone (TSH) and free T4 (fT4) levels were determined using the two-region, two-stage enzymatic immunoassay method. According to the reference values of the TSH and fT4 kits, TSH lower and upper limit values were accepted as 0.34-5.6 mIU/mL and fT4 lower and upper limits as 0.6-1.2 ng/dL. Anti-thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-TG) antibodies were measured using the Beckman Coulter DX1800 chemiluminescence immunoassay method.

The ELISA method was used to determine ZnT8A concentration in serum samples which were taken within a week after diagnosis and stored at -80 °C. Medizym anti ZnT8 ELISA kit, which can detect antibodies to arginine (R-325), tryptophan (W-325) and other non-specific variants, was used to test for the presence of these antibodies. Concentrations above 15 U/mL were accepted as positive.

Ethic board consent for the study was granted by the ethic board of Ankara Pediatrics Hematology Oncology Training and Research Hospital (consent number: 2015-002). All parents were informed about the purpose of the study, and a signed consent for study participation was obtained.

Statistical Analysis

Statistical analysis of the data was carried out using "The Statistical Package for the Social Sciences 17.0" (SPSS, Inc. Chicago IL, USA, Microsoft) programme. Results were expressed as mean ± standard deviation for parametric data and median + range for nonparametric data. The Student t-test was used for the comparison of the medians for numeric variables and the chi-square test for comparing the medians for non-numeric variables. Mann-Whitney U test was preferred for the evaluation of numeric parameters without a normal distribution. Significance level was accepted as $p < 0.05$.

Results

The mean age of the 84 (49 female, 35 male) cases with T1D was 9.8 ± 4.0 years and 52.4% were prepubertal children. In the control group (25 females, 25 males) the mean age was 9.1 ± 4.0 years. Twenty-two cases (26.2%) had been referred with hyperglycemia, 23 (27.4%) with ketosis, 39 (46.4%) with ketoacidosis (12 mild, 8 moderate and 19 severe degrees of ketoacidosis). Mean HbA1c was 11.7 ± 2.3% and C-peptide level 0.41 ± 0.29 ng/mL. Accompanying hypothyroidism was not observed in any of the patients (TSH: 2.53 ± 1.2 mIU/L; fT4: 0.97 ± 0.22 ng/dL).

ZnT8A positivity was detected in 49 (58.2%) cases in the T1D patient group and in four (8%) individuals in the control group. ZnT8A was present in five out of 11 (13%) of cases all of which were negative for GADA, IA-2A and IA. Prevalence figures for tTG IgA, anti-TPO and/or anti-TG and diabetes autoantibodies in T1D patients are depicted in Figure 1. When ZnT8A positive (ZnT8A+) and ZnT8A negative (ZnT8A-) T1D cases were compared, no difference was detected in age, gender, presence and degree of ketoacidosis during referral, HbA1c concentration, insulin or C-peptide concentrations. When they were compared for prevalence of celiac, thyroid and other diabetes autoantibodies, it was observed that only the IA-2A positivity rate was significantly higher in ZnT8A+ cases with T1D (p = 0.024) (Table 1). It was also observed that ZnT8A titers in ZnT8A+ cases in T1D group were significantly higher (median 271.37 U/mL, range 23.28-501.00 U/mL) compared to the titres of the ZnT8A+ cases in the control group (median 26.96 U/mL, range 15.1-93.9 U/mL). While ZnT8A titer was not found to be related to age and body mass index (BMI), a week positive correlation was detected with C-peptide level (p = 0.034, r = 0.31). None of the four ZnT8A+ patients among control group were diagnosed T1D within two-year follow. Further follow-up is planned in these patients.

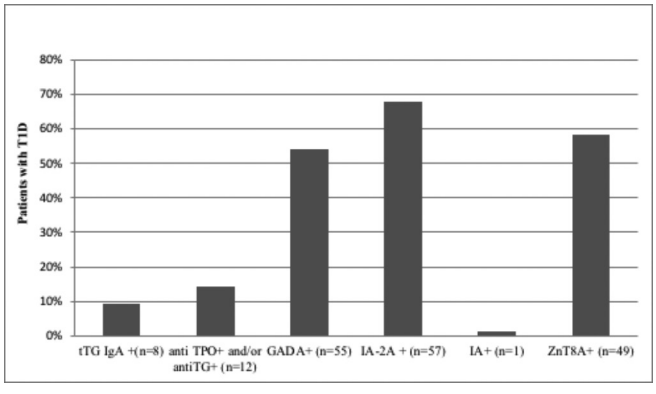

Figure 1. Frequency of celiac, thyroid and diabetic autoantibodies in 84 patients with new onset type 1 diabetes

T1D: type 1 diabetes, ZnT8A+: zinc transporter 8 protein positive, tTG: anti-tissue transglutaminase, IgA: immunoglobulin A, TPO: thyroid peroxidase, TG: thyroglobulin, GADA: glutamic acid decarboxylase antibodies, IA: insulinoma antigen, IA-2A: insulinoma antigen-2 antibody

Discussion

This study showed that the prevalence of ZnT8A is 58.6% in Turkish children with new onset T1D. This result is in accordance with most of the studies done in other countries. ZnT8A positivity was reported to be between 60-80% in Caucasians (1-18 years old) (9), 72 % in Czechs (1-19 years old) (20) and 65% in Argentinians (10-32 years old) (21), with new onset T1D. ZnT8A positivity was reported in 24% of Chinese new onset T1D patients (1-70 years old) and differences in HLA genotypes or other inter-ethnic genetic markers were thought to be a possible cause for this low rate (17). However, in another Asian population, Japanese acute onset T1D patients (19.1 ± 14.5 years old) had 58% ZnT8A positivity, which is very similar to our findings in a Turkish population (22). A study from Brazil, which encompassed both a Caucasian and a non-Caucasian new onset T1D population (30.3 ± 11.4 years old), found an overall ZnT8A positivity of 24% and it was stated that neither ZnT8A positivity nor concentration was associated with ethnicity (23).

The ZnT8A positivity prevalence of healthy controls from different countries was reported as 1-2.7%, which is a markedly lower rate than in this study (8%) (9,17,20,24). This difference may be attributed to the larger cohorts of the other studies which better reflects the population. However, the possible effect of ethnicity cannot be excluded. In line with studies using the same analysis method and cut-off

Table 1. Prevalence of specific autoantibodies in zinc transporter 8 protein positive and zinc transporter 8 protein negative patients with new onset type 1 diabetes. Prevalences are given as n (%)

	ZnT8A+	ZnT8A-	p value
Total	49 (58.2)	35 (41.6)	-
tTG IgA+	4 (8.2)	4 (11.4)	0.07
Anti-TPO and/or anti-TG+	5 (10.2)	7 (20.0)	0.21
GADA+	27 (60.0)	15 (42.9)	0.27
IA-2A+	38 (77.6)	19 (54.3)	0.02
IA+	0 (00.0)	1 (2.8)	-
Other autoantibodies	7 (15.6)	8 (22.9)	0.007

ZnT8A+: zinc transporter 8 protein positive, znT8A-: Zinc transporter 8 protein negative, tTG: anti-tissue transglutaminase, IgA: immunoglobulin A, TPO: thyroid peroxidase, TG: thyroglobulin, GADA: glutamic acid decarboxylase antibodies, IA: insulinoma antigen, IA-2A: insulinoma antigen-2 antibody

value, ZnT8A levels found in the control groups were lower than that found in the T1D patients (20,24). ZnT8A was shown to predict risk of progression to T1D in first degree relatives of T1D patients (15). Although these healthy controls had a negative T1D history in their families, they may have a higher risk for diabetes.

ZnT8A is directed to an epitope at the C terminal of the ZnT8 protein (residues 268-369). Gene polymorphism at the codon for the 325th aminoacid lead to different variants of the ZnT8 protein and antibodies are found which are specific to each of these. These are R325-ZnT8RA, W325-ZnT8WA and rarely Glutamine (Q325)-ZnT8QA variants (9). It has been demonstrated that the distribution of antibody variants differs between populations (25). The methodology used in this study is capable of detecting autoantibodies against all three of these ZnT8 variants ZnT8A variant distribution in Turkish T1D children could not be determined.

Prospective studies following up first-degree relatives of T1D patients or individuals with high risk HLA tissue types, starting from the first months of their lives until the development of T1D, demonstrated that ZnT8A developed many years prior to the development of T1D, in the 9th month of life at the earliest and mostly close to three years of age (9,26). The youngest ZnT8A + T1D case in our study was two years old. When present studies are considered, it may be predicted that the seroconversion starts around the age of one year. It was observed that ZnT8A + prevalence and concentration during diagnosis did not change with age in our study. Andersson et al (27) reported that ZnT8A prevalence during diagnosis was age-independent in 686 children with T1D. In a study examining 227 children and adolescents with T1D, it was shown that ZnT8A prevalence was not related to age at diagnosis, while ZnT8A titers increased with age (20). In studies in which the subjects are older, it is observed that both ZnT8A prevalence and titer decreases with a higher age at diagnosis (16).

In the present study, neither presence nor levels of ZnT8A were found to be related to BMI, a finding similar to some previous reports (16,17,18,19,20,21,22,23,24,25,26,27, 28). Conversely, there are reports indicating that ZnT8A positivity is more frequent in the leaner T1D patients than in the more obese, but it was mentioned that larger cohorts were necessary to verify this negative association (17).

Whether ZnT8A presence or levels predict residual beta cell function or not is still unclear, but there are reports indicating that the presence or levels of ZnT8 are unrelated to C-peptide levels (16,17). Andersson et al (27) found that both presence and levels of ZnT8RA and, to a lesser degree, ZnT8QA were associated with higher levels of stimulated C-peptide after diagnosis and during the follow-up of T1D. After excluding Znt8RA negative subjects and re-analyzing the relation between ZnT8A levels and stimulated C-peptide levels, that association failed to reach significance, a finding which may indicate that positivity rather than the level of Znt8RA has a protective role on beta-cell function (29). In contrast, the results of this present study showed that C-peptide levels were positively correlated with the concentration, but not with the presence of ZnT8A. Different cohort sizes, target ZnT8A epitope and type of C-peptide measurement, fasting or stimulated, may have caused these conflicting results.

Study Limitations

The study is limited by a relatively small number of subjects and lack of ICA antibody measurement.

Conclusion

ZnT8A is an independent marker of β-cell autoimmunity and its prevalence was found 58.6 % in Turkish children with new onset T1D. Nearly half of the T1D patients negative for IA-2A, GADA and IAA were detected to be pozitive for ZnT8A which supports that ZnT8 measurement should be more widespread for clarifying the etiology in T1D.

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Ethics

Ethics Committee Approval: Ethic board consent for the study was granted by the ethic board of Ankara Pediatrics Hematology Oncology Training and Research Hospital (consent number: 2015-002).

Informed Consent: All parents were informed about the purpose of the study, and a signed consent for study participation was obtained.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Medical Practices: Selin Elmaoğulları, Seyit Ahmet Uçaktürk, Şehri Elbeg, Concept: Selin Elmaoğulları, Design: Selin Elmaoğulları, Data Collection or Processing: Selin Elmaoğulları, Seyit Ahmet Uçaktürk, Şehri Elbeg, Fatih Gürbüz, Meltem Tayfun, Esra Döğer, Analysis or Interpretation: Selin Elmaoğulları, Aysun Bideci, Literature Search: Selin Elmaoğulları, Writing: Selin Elmaoğulları.

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ORIGINAL ARTICLE

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Retinal Neural and Vascular Structure in Isolated Growth Hormone Deficiency Children and Evaluation of Growth Hormone Treatment Effect

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What is already known on this topic?

Reduced retinal vasculature has been shown in patients with growth hormone (GH) deficiency and insensitivity. However, retinal neural morphology in these patients evaluated in different studies, reported different results. Unlike retinal vascularization, data regarding retinal neural structure are discrepant between different studies. Some studies reported increased, while others reported decreased retinal nerve fibre layer thickness or macular thickness. Additionally, different parameters have been used in the limited number of studies evaluating the effect of GH treatment on ocular tissues.

What this study adds?

Our findings suggest that GH deficiency may lead to decreased retinal vascularization. However, retinal neural growth and differentiation were not affected by GH deficiency. We also evaluated the effect of GH treatment on the retina and observed that GH treatment did not cause any retinal changes.

Abstract

Objective: To evaluate neural and vascular retinal morphology of children with isolated growth hormone deficiency (GHD) and to determine any retinal changes due to GH treatment.

Methods: Twenty-eight children with isolated GHD and 53 age-, gender- and body mass index-matched healthy volunteers were enrolled in this prospective study. The retinal nerve fibre layer (RNFL) and macular thickness (MT) were measured, as well as intraocular pressure (IOP). The number of retinal vascular branching points were calculated. Effect of GH treatment on the retina and IOP was evaluated after one year of treatment. Measurements were also made in the control group at baseline and following the initial examination. Pre- and post-treatment changes were compared. The findings were also compared with those of the controls. The correlation between ocular dimensions and insulin-like growth factor-I (IGF-1) levels were also analysed.

Results: The number of branching points was significantly lower in GHD patients as compared with control subjects (15.11 ± 2.67 and 19.70 ± 3.37, respectively, $p = 0.05$ for all comparisons). No statistically significant differences were found in mean RNFL, MT and IOP values between GHD patients and control subjects. GH treatment did not create any significant changes in the retinal vascularization or other retinal neural parameters and IOP either within the patient group or when compared with the control group. No correlations were observed between ocular dimensions and IGF-1 levels.

Conclusion: Our findings suggest that isolated GHD may lead to decreased retinal vascularization. However, retinal neural growth and differentiation were not affected by GHD. These findings may be related to the fetal development process of pituitary somatotropic cells and the retina. Additionally, GH treatment did not cause any changes in retinal neural and vascular tissues.

Keywords: Growth hormone deficiency, retinal neural development, retinal vascularization, growth hormone treatment

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Introduction

Growth hormone (GH) is released from pituitary somatotrophs into the circulation and is essential for postnatal growth and development. Experimental models have demonstrated the presence and effect of pituitary GH in many extrapituitary sites, including the nervous, reproductive, immune and vascular systems (1,2). The ocular neural and vascular system is one of these sites.

The possible role and effect of pituitary GH and subsequently generated insulin-like growth factor-1 (IGF-1) on retinal development is controversial. However, a few recent human studies have emphasized its functional role. Although GH and various growth factors (IGFs, vascular endothelial growth factor, fibroblast growth factor and transforming growth factor-beta) are often thought to be produced locally and act in autocrine/paracrine ways to promote the maintenance, survival and differentiation of retinal tissues, this is only partially true for the vascularization and neurogenesis of the retina before the functional differentiation of pituitary somatotrophs (3).

Abnormal ocular findings such as optic nerve hypoplasia, disc dysfunction, increased corneal thickness, reduced retinal vascularization and short axial length in GHD patients demonstrate the effect of reduced GH production on the developing ocular tissues (4,5,6,7,8). Presence of pituitary GH in the human retina and vitreous fluid also provides further evidence for a possible role of GH in ocular development (9,10). Consequently, ocular tissues seem to represent a target site for pituitary GH action, as suggested by several human and animal studies. Based on these studies, we aimed to evaluate retinal neural and vascular structure in isolated GHD patients. Another objective of this study was to assess any retinal changes developing as a result of GH treatment.

Methods

This prospective study consisted of 28 patients with severe short stature (height standard deviation score less than -3) at diagnosis and whose growth velocity was lower than 4 cm/year or below expectations for the pubertal stage. Patients were excluded if they had a history of being preterm or small for gestational age at birth; a history of cardiovascular, thyroid, hepatic or renal disease or obesity; current hypertension, chromosomal abnormalities or, in addition to their already known ocular disease, had severe refractive errors or a family history of ocular hypertension/glaucoma.

Pubertal staging was assessed by Tanner stage according to breast development in girls and genital development in boys (11). Routine biochemical tests, complete blood counts, thyroid function tests and serum tissue transglutaminase antibodies were obtained in all patients. Bone age was evaluated by using the Greulich and Pyle atlas. Pituitary magnetic resonance imaging (MRI) was performed in all to exclude presence of a structural anomaly. All patients underwent a GH stimulation test performed with L-Dopa and Clonidine. IGF-1 and IGF-binding protein-3 (IGFBP-3) levels were also measured. A peak GH responses below 10 μg/L after two stimulation tests was accepted as GH deficiency. The diagnosis of GHD was confirmed according to the clinical, auxological and biochemical criteria of the Growth Hormone Research Society (12). In diagnosed patients, recombinant human GH treatment was started at an initial daily dose of 0.025 mg/kg. During the study, IGF-1 and IGFBP-3 levels were measured at intervals of 3-6 months and the GH dose was adjusted to maintain serum IGF-1 levels above + 2 standard deviation (SD), but not exceeding + 3 SD levels.

Fifty-three healthy children who were carefully matched for age, gender, body mass index (BMI) and pubertal staging were recruited as the control group from the siblings of patients and children who had presented to the health care unit for a routine examination. Healthy volunteers were recalled at the end of the first year following the initial selection. The study protocol was approved by the ethics committee of Gazi University, Faculty of Medicine (approval number: 357-14/07/2014). Parents of the patients and controls were informed about the study and informed consent was obtained.

Assessment of Retinal Vascularization and Retinal Thickness

All patients and controls underwent a complete ophthalmologic examination, including an auto-refractometer (RM8900; Topcon), best-corrected visual acuity measurements with a 6 meter Snellen eye chart, slit-lamp biomicroscopy, fundus examination and intraocular pressure (IOP) measurement. The subjects also underwent an examination with the Heidelberg Spectralis-OCT (Spectralis; Heidelberg Engineering, Heidelberg, Germany). Central subfield macular thickness (MT) and peripapillary retinal nerve fibre layer (RNFL) thickness were assessed to evaluate retinal neurogenesis. The RNFL measurements were determined globally and for six regions including temporal, supertemporal, supernasal, nasal, inferotemporal and inferonasal regions. The number of retinal vascular branching points was obtained from infrared images. Optic nerve head (ONH) centered near-infrared reflectance pictures were obtained with a 30° field of view. A frame to

calculate retinal vascular branching points was set for each eye to minimize individual disparity. Temporal border of the fovea was defined as the temporal border of the frame. The other borders were calculated as 4000 µm away from the center of the ONH.

RNFL thickness, MT and retinal vascularity were evaluated again after 12 months of treatment and any retinal IOP changes, were recorded. Additionally, the correlation of changes with IGF-1 levels were calculated. The controls were also called back one year following their initial examination to record any retinal changes associated with age and puberty.

Statistical Analysis

All statistical calculations were performed using SPSS version 20 (SPSS, IBM Inc., Chicago). The subjects' right eyes were selected for statistical analysis. Descriptive statistics were computed as means ± SD. Parameters with normal distribution were analysed with the t-test and parameters with non-normal distribution with the Mann-Whitney U test. Differences between values before and after GH treatment were evaluated using paired samples t-tests. The linear relationships between variables were evaluated using Pearson's correlation tests. A p value < 0.05 was considered statistically significant.

Results

A total of 28 (female/male ratio: 14:14) isolated GHD patients and 53 (female/male ratio: 33:20) age- and gender-matched healthy children aged 12.46 ± 2.41 and 11.32 ± 3.1 years, respectively, were included in the study. No statistical difference was observed among the groups in terms of age, gender or BMI ($p > 0.05$). Puberty was compatible with Tanner stage 1 in nine patients and in 19 control subjects ($p > 0.05$). Tanner stage 2 and 3 occurred in the remaining subjects (8 patients and 15 control subjects in stage 2; 11 patients and 19 control subjects in stage 3). Routine biochemical tests, complete blood counts, thyroid function tests and serum tissue transglutaminase antibodies were within normal limits. Pituitary MRI was normal in all patients.

The best corrected visual acuities of all subjects were 6/6. None of the differences in spherical equivalent, MT and four quadrant RNFL thickness, IOP among patients and control subjects were significant ($p > 0.05$). The mean number of vascular branching points was 15.11 ± 2.67 in the study group and 19.70 ± 3.37 in the control group ($p < 0.01$, Figure 1). All ocular parameters of both groups are compared in Table 1.

At the end of the first year, eye parameters of all 28 patients were checked. No significant changes were observed in

Table 1. Ocular parameters of patient and control groups at baseline

Variables	Groups		
	Patients (n = 28)	Controls (n = 53)	p value
IOP	16.18 ± 2.79	15.47 ± 2.55	0.27
CSFMT	268.07 ± 18.70	266.38 ± 20.94	0.711
N	70.21 ± 13.44	70.58 ± 10.39	0.899
I	243.18 ± 42.16	255.19 ± 26.15	0.177
T	73.32 ± 8.87	74.53 ± 10.79	0.592
S	240.75 ± 41.64	250.09 ± 28.35	0.293
G	96.39 ± 12.28	99.72 ± 8.30	0.206
Number of branching points	15.11 ± 2.67	19.70 ± 3.37	< 0.001

n: number, IOP: intraocular pressure, CSFMT: central subfield macular thickness, N: nasal, I: inferior, T: temporal, S: superior, G: global

Table 2. Ocular parameters of patients before and after 1-year of growth hormone treatment

Variables	Patients (n = 28)		
	Before treatment	After treatment	p value
IOP	17.28 ± 1.90	16.46 ± 1.68	0.093
CSFMT	260.14 ± 20.15	260.79 ± 21.86	0.661
N	69.71 ± 12.04	72.93 ± 14	0.08
I	253.64 ± 35.50	241.36 ± 44.50	0.157
T	71.36 ± 7.38	71.21 ± 90	0.889
S	238.71 ± 48.53	238.57 ± 47	0.976
G	94.57 ± 10.17	95.93 ± 13.22	0.254
Number of branching points	14.43 ± 2.10	13.14 ± 1.90	0.079

n: number, IOP: intraocular pressure, CSFMT: central subfield macular thickness, N: nasal, I: inferior, T: temporal, S: superior, G: global

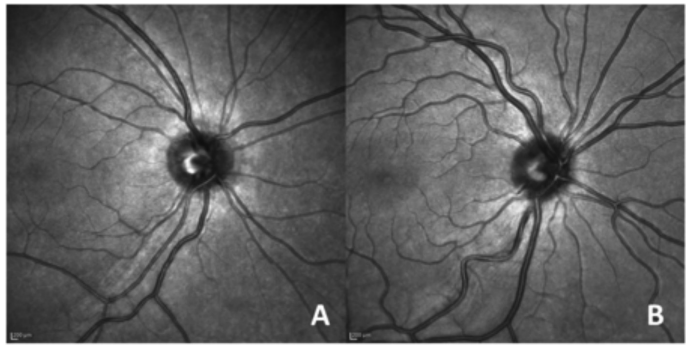

Figure 1. Infrared images; A) reduced retinal vascularization in a 10-year-old growth hormone deficiency patient, B) normal retinal vascularization in a 10-year-old healthy subject

the MT, RNFL thickness, IOP and the number of vascular branching points after treatment ($p > 0.05$; Table 2). In one GHD patient, optic disc drusen were detected and the patient was followed.

Additionally, retinal vascularization, IOP, MT and RNFL thickness did not show any significant correlation with an increase in IGF-1 levels ($r = 0.003$; $r = 0.12$; $r = -0.06$; $r = 0.16$, respectively, $p > 0.05$ for all).

The first-year evaluation of the control group could be performed in only 17 subjects. No significant differences in ocular parameters related to pubertal development and age were observed in these subjects. The stastistical results of the initial comparison were not different from the statistical results of the second comparison in which we compared the retinal measurements of the patients that were under treatment with GH and the controls.

Discussion

In this prospective study, we have essentially examined the retinal neural and vascular structures in patients with isolated GHD to explore whether GH treatment will cause any retinal change. We observed that the MT and RNFL thickness of patients was not different from healthy controls, while retinal vascularization decreased. On the other hand, GH treatment did not cause any retinal neuro-vascular changes.

Hellström et al (7) first reported reduced retinal vasculature in isolated GHD patients. These authors also drew attention to the importance of GH and IGF-1 for normal retinal vascularization. More recently, Pereira-Gurgel et al (13) reported moderate reduction of retinal vascular branching points in isolated GHD patients. Another recent study also evaluated the effects of the GH/IGF-1 axis on retinal vascular branching and other characteristics in patients with GH insensitivity and reported reduced retinal vasculature and tortuosity of the retinal vessels (14). Similarly, our patients also showed reduced retinal vasculature. Based on these data, we suggest that the GH/IGF-1 axis has an effect on retinal vasculature. However, we cannot show that pituitary GH has an effect on retinal neural development. In fact, GH and GH mRNA proteins have also been identified in retinal ganglion cells and can be traced within their axons in the retina within the optic fibre layer and outside the retina within the optic nerve, optic chiasm and optic tract (10,15,16). In view of this knowledge, we expected that the GHD would have led to greater thinning of the MT and RNFL thickness, but our findings did not support this expectation. Decreased peripapillary nerve fiber thickness and decreased optic disc size were previously described in some children with congenital GHD (17,18). The differences between these reports and our findings are probably due to the variety of causes of GHD as well as to differences in methodology.

The reason why retinal neural structure shows normal development while retinal vascularization is decreased in GH deficiency can be partially explained by the embryonic development process of these structures and somatotrop cells. GH producing cells can be identified at nine weeks of gestation (19), while embryonic neural development occurs in an earlier period. Therefore, it can be assumed that the development of the nervous system in its early stages is independent of GH and shows normal development, despite the lack of pituitary GH, or that its development is affected by GH produced in an extra pituitary site. Our findings also suggest that locally produced GH and factors are more effective in neural retinal development. This possibility is supported by the presence of GH immunoreactivity in the brain prior to the ontogeny of the pituitary gland and somatotroph differentiation, as has been demonstrated in the human and chicken brain (20,21). Vascularization of the retina normally starts at approximately 12 weeks of gestation, while pituitary somatotroph GH production has already begun and continues during fetal development, with little or no vascularization after birth (22,23). Normally, it is accepted that IGF-2 has a greater effect when fetal somatic and ocular development is considered. However, the effect of GH and IGF-1 on retinal vessels cannot be ignored. This hypothesis is supported by several studies showing a decrease in retinal vascularization in patients with GHD and GH insensitivity (24).

Another finding of this study was that GH treatment did not create any significant retinal changes, nor changes in IOP. The effect of a lack of effect of GH treatment on the retina can be explained in various ways. One proposal suggests that GH and IGF-1 cannot pass from the inner retinal barrier (25). Other proposals suggest that normalized GH and IGF-1 levels might not be sufficient to lead to significant retinal changes or that the follow-up time may be inadequate to evaluate retinal changes (26).

Previous data suggesting induction of neovascularization by IGF-1 following investigation of clinical conditions where an excess of IGF-1 is present in the serum (27,28,29,30). Treatment with GH, on the other hand, aims to normalize IGF-1 levels as much as possible and is not expected to induce a sustained excess of IGF-1, as in acromegaly or in patients with diabetic retinopathy. Another explanation may also be related to the impairment of the retinal blood barrier permeability and/or impairment of integrity in diabetic retinopathy.

Study Limitations

Our study has some limitations. As was the case with other similar studies, the lack of a genetic diagnosis was

the most significant limitation. Another limitation was the use of a semi-quantitative system to assess the retinal vascularization. Long-term treatment outcomes should be evaluated to determine correct and more extensive information about the effect of HGH treatment on the retina and this was also a potential limitation of our study.

Conclusion

The best of our knowledge, the current study is the first comprehensive prospective study to evaluate retinal structure (neural and vascular) in isolated GHD children. A selective reduction of retinal vascularization and normal retinal neural architecture may suggest that the GH/IGF-1 axis regulates retinal vascular development, but not the neural retina. Besides, our findings suggested that GH treatment is not associated with retinal changes. However, monitoring time, treatment dose and the etiology of GHD should be taken into consideration when stating that HGH treatment has no effect on ocular parameters. We recommend, therefore, that ophthalmologic evaluations should be performed in all GHD patients before institution of GH treatment and that these be repeated annually. Also, further studies with larger groups are required to clarify the functional role of GH on ocular growth and differentiation by using advanced measurement techniques.

Ethics

Ethics Committee Approval: The ethics committee Gazi University, Faculty of Medicine, Ankara (approval number: 357-14/07/2014).

Informed Consent: Informed consent was taken.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Aysun Bideci, Özge Yüce, Design: Aysun Bideci, Özge Yüce, Murat Hasanreisoğlu, Data Collection or Processing: Özge Yüce, Esra Döğer, Hamdi Cihan Emeksiz, Nuriye Gökçen Yalçın, Analysis or Interpretation: Özge Yüce, Nuriye Gökçen Yalçın, Zeynep Aktaş, Peyami Cinaz, Literature Search: Özge Yüce, Orhun Çamurdan, Writing: Özge Yüce, Nuriye Gökçen Yalçın, Zeynep Aktaş.

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ORIGINAL ARTICLE

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A Study of the Relationship Between Cystatin C and Metabolic Bone Disease in Preterm Infants

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What is already known on this topic?

Cystatin C is a valuable marker in the diagnosis of acute kidney injury in preterm infants. It was demonstrated that various parameters and conditions, such as respiratory distress, bilateral kidney anomalies, peripartum asphyxia, hemoglobin levels and sepsis, affect cystatin C values.

What this study adds?

To our knowledge, this is the first investigation of the measurement of cystatin C in infants with osteopenia of prematurity compared to infants without osteopenia. The presence or absence of osteopenia of prematurity had no effect on measured cystatin C levels in our cohort.

Abstract

Objective: Cystatin C (CysC) is commonly used as a marker of renal failure in premature infants. The aim of this study was to investigate serum CysC levels in osteopenia of prematurity (OP) and determine whether CysC could be safely used as a marker of renal insufficiency in infants with OP.

Methods: Subjects were 50 preterm infants (≤32 gestational weeks). Calcium (Ca), phosphorus (P) and alkaline phosphatase (ALP) serum levels were measured in postnatal week nine, and bone density was measured concurrently by quantitative ultrasonography. Patients with a Z score of <-2 were considered to have OP.

Results: The mean serum CysC levels in preterm infants in postnatal week nine were 1.50 ± 0.19 mg/L. Serum CysC levels were not correlated with speed of sound values, Z scores, serum Ca, P or ALP levels. Serum CysC levels were not significantly different between infants with OP [1.50 (1.35-1.61) mg/L] and in infants without OP [1.58 (1.28-1.70) mg/L].

Conclusion: The presence of OP does not affect the safety of CysC as a marker of renal insufficiency in preterm infants.

Keywords: Cystatin C, metabolic bone disease, osteopenia, premature, speed of sound, renal failure

Introduction

Premature infants are at risk of developing bone disease due to a low bone mineral content. Neonatal rickets or osteopenia of prematurity (OP), also known as metabolic bone disease in premature infants, is one of the most frequent problems encountered in neonatal units. OP adversely affects linear growth and height in the long term, while causing fractures, growth retardation and respiratory problems in the short term (1).

Dual photon X-ray absorptiometry (DEXA) is the gold standard method for the radiological measurement of bone mineral density. Nevertheless, time spent screening, artifacts caused by movement, exposure to radiation and cost limit the use of DEXA in newborns. A quantitative bedside ultrasonography (USG) assessment is a simple, inexpensive and noninvasive method, which can be used to obtain measurements related to bone mineral density and structure. Bedside assessment devices have been designed that quantify broad-band ultrasound attenuation or the

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speed of sound (SOS). Studies showed that quantitative ultrasound measurements correlated significantly with DEXA findings in both adults and children (2,3) and that they may be useful in evaluating structural and mechanical properties of bone (4). In preterm infants, a number of studies have demonstrated the potential clinical value of bedside ultrasound assessments of bone status (5,6,7).

Cystatin C (CysC) belongs to the cystatin family of cysteine proteinase inhibitors. It has a low molecular weight and is produced in virtually all nucleated cells in the body. The production rate of CysC does not change in inflammatory conditions (8,9) and it is commonly measured to determine the glomerular filtration rate (10,11,12).

Osteoclastic bone resorption depends on the activity of various proteolytic enzymes, particularly proteinases. CysC inhibits cysteine proteinase, a proteolytic lysosomal enzyme that prevents bone resorption. The association of CysC with bone metabolism has been demonstrated in a variety of *in vitro* studies (11,12). Clinical studies on bone metabolism and CysC are limited to adults, with no studies conducted in infants. Elevated serum CysC levels in postmenopausal women have been linked to increased bone fractures and reported to be a potentially promising biomarker for the risk of hip fractures (13,14).

Clinical studies of CysC in preterm infants have focused mainly on the relationship between CysC and renal function and aimed to determine the reference range. It was demonstrated that CysC is a valuable marker in the diagnosis of acute kidney injury in preterm infants (15,16,17,18). Other studies have demonstrated that various conditions, such as respiratory distress (19), bilateral kidney anomalies (20), peripartum asphyxia (21,22), abnormal hemoglobin levels (21,22), and sepsis (23), affect CysC values.

Premature infants have an increased risk of both kidney failure and osteopenia, and the CysC level can be used as a diagnostic marker of renal failure in preterms. However, no studies have examined whether CysC levels are altered, and therefore reliable, for renal function assessment in premature infants with OP. Based on the literature, we hypothesized that the CysC level would be altered in OP. To shed light on this issue, this study investigated the relationship between CysC concentrations, bone density and levels of biochemical markers of bone metabolism [serum calcium (Ca), phosphorus (P), and alkaline phosphatase (ALP)] to determine whether serum CysC levels were altered in OP.

Methods

Infants born between 24 and 32 gestational weeks who were admitted to the Newborn Unit of Erciyes University Faculty of Medicine were enrolled in the study. Infants with a severe congenital anomaly, congenital metabolic disease, perinatal asphyxia, who were diagnosed with acute renal injury or hypothyroidism up to postnatal age 9 weeks were not included in the study. Gestational week was determined by the last menstrual period of the mother. Infants with a birth weight below the 10th percentile according to the Fenton 2013 chart were accepted to be small for gestational age (SGA).

Clinical findings of OP become manifest between postnatal six and 12 weeks (1). In our subjects, bone density was assessed in the ninth week using quantitative USG. Serum Ca, P, ALP, creatinine and CysC levels were concomitantly measured.

Serum CysC level was analysed on an Abbott Architect C 16000 (Abbott, US) analyzer and an enhanced nephelometric immunoassay. An automatic biochemical analyzer (Cobas 8000 c701, Roche, Mannheim, Germany) was used to determine the values of P (phophomolybdate), Ca (o-cresolphthaleine), and ALP (kinetic p-nitrophenilphosphate).

SOS was measured using a Sunlight Omnisense 2000 (Sunlight Medical, Tel Aviv) quantitative ultrasound sonometer. Measurements were done on the right tibia. The SOS measurement is based on the fact that ultrasound waves propagate faster in bone than in soft tissue. SOS is influenced not only by bone mineralization but also by quantitative factors, such as micro-architecture, elasticity and cortical thickness. The results are reported as meter/second. The SOS measurements are compared with mean SOS measurements of the same age group using a reference database, the Z score is automatically calculated based on the difference between the patients' SOS scores and mean SOS scores of an age- and sex-matched group and expressed as a standard deviation by this sonometer. In this study, infants with Z scores <-2 were considered to have OP (7).

This study was approved by the ethical committee of Erciyes University Medical Faculty (02.10.2015/437). Informed consent from the parents of each newborn was provided.

Statistical Analysis

Visual (histograms and probability plots) and analytical methods (Shapiro-Wilk's test) were used to determine whether the data were normally distributed. Parametric data were presented as mean $\pm$ standard deviation. For intergroup comparisons, independent two-sample tests and the Mann-Whitney U test were conducted. Nonparametric data are presented as median values (25th percentile to 75th percentile). The correlation of serum Ca, P and ALP

levels, in addition to SOS and Z scores, with CysC levels was determined by Pearson's correlation analysis. The correlation analysis of the nonparametric data was tested by Spearman's correlation analysis. A chi-square test was conducted to determine the relationship between categorical variables. In all the tests, the level of statistical significance was accepted as $p < 0.05$.

Results

In total 50 premature infants were included in the study. The demographic features of the infants and their mothers are summarized in Table 1. Only 11 of the 50 patients were still in hospital at post-natal age nine weeks for follow-up. Thirty-nine patients were evaluated during follow-up visits at postnatal age nine weeks.

The mean serum CysC level of the whole cohort of preterm infants was 1.50 ± 0.19 mg/L. The median (25th percentile-75th percentile) and minimum-maximum serum creatinine levels of the whole cohort of preterm infants were 0.22 (0.19-0.29) mg/dL and (0.06-0.51) mg/dL, respectively.

Serum Ca, P, ALP, and CysC levels, in addition to SOS measurements, were grouped and compared according to gestational week and birth weight. In the group of infants with gestational ages of 26-29 weeks, the serum Ca levels ($p = 0.02$), p levels ($p = 0.01$) and SOS measurements ($p = 0.01$) were significantly higher. There was no difference in the serum CysC levels of the infant group with gestational ages 26-29 weeks versus those of the infant group with gestational ages 30-32 weeks. In the comparison of infants according to birth weight, serum Ca levels ($p = 0.04$) and P levels ($p = 0.02$) were significantly higher, whereas serum ALP levels ($p = 0.04$) were lower in those with birth weights ≥1500 g as compared with infants whose birth weight was < 1500 g. There was no between-group difference in serum CysC levels according to birth weight (Table 2). The mean serum CysC level of boys and girls was 1.48 ± 0.17 mg/L and 1.50 ± 0.24 mg/L, respectively, with no significant between-group difference.

Serum CysC levels were not correlated with serum Ca, P and ALP levels or with SOS measurements (Figure 1) and SOS Z scores. Serum CysC levels were also not correlated with birth weight or gestational age.

The SOS Z score values of 24 of 50 (48%) infants were < -2, and these infants were diagnosed with OP. Serum CysC levels were 1.50 (1.35-1.61) mg/L and 1.58 (1.28-1.70) mg/L in infants with OP and without OP, respectively, with no statistically significant different ($p = 0.34$). The demographic and clinical features of infants with OP and without OP are summarized in Table 3.

Discussion

Table 1. The demographic features of the infants and their mothers

Infants		
Birth weight (g)		
< 1500 g (n = 29)	1104 ± 227	(600-1450)$^{+}$
≥1500 g (n = 21)	1731 ± 239	(1500-2400)$^{+}$
Gestational age (weeks)		
30-32 weeks (n = 27)	31.2 ± 0.8	(30-32)$^{+}$
26-29 weeks (n = 23)	28.2 ± 1.4	(25-29)$^{+}$
Female (n, %)	28 (56)	
SGA (n, %)	6 (12)	
Mothers		
Age (years)	29.2 ± 6.1	
Gestational diabetes (n, %)	4 (8)	
Hypertension (n, %)	3 (6)	
PPROM (n, %)	10 (20)	
Prenatal steroid (n, %)	36 (72)	
Hypothyroidism (n, %)	5 (10)	

$^{+}$: Data are given as (minimum-maximum)

SGA: small for gestational age, PPROM: preterm premature rupture of membranes

Table 2. Measurements according to grouping by gestational week and birth weight

	30-32 weeks (n = 27)	26-29 weeks (n = 23)	p	≥1500 g (n = 21)	< 1500 g (n = 29)	p
Ca (mg/dL)	9.95 ± 0.39	9.23 ± 0.93	0.02	9.85 ± 0.32	9.45 ± 0.95	0.04
P (mg/dL)	6.02 ± 0.87	5.08 ± 1.46	0.01	6.19 ± 0.78	5.16 ± 1.37	0.02
ALP (IU/L)	366 ± 114	467 ± 258	0.07	347 ± 116	460 ± 232	0.04
CysC (mg/L)	1.51 ± 0.18	1.48 ± 0.21	0.51	1.51 ± 0.19	1.49 ± 0.20	0.74
SOS (m/s)	2875 ± 146	2780 ± 97	0.01	2874 ± 157	2800 ± 105	0.05
Z score	-1.7 ± 1.2	-2.2 ± 0.8	0.08	-1.7 ± 1.3	-2.1 ± 0.9	0.18

Ca: calcium, P: phosphorus, ALP: alkaline phosphatase, CysC: cystatin C, SOS: speed of sound

Table 3. Demographic and clinical features of infants with osteopenia of prematurity and without osteopenia of prematurity

	Without OP n = 26	With OP n = 24	p
Infants			
GA (weeks)	30.5 (29.4-32.0)	29.0 (28.2-31.0)	0.06
Birth weight (g)	1432 ± 399	1297 ± 371	0.22
Female (n, %)	15 (57.7)	13 (54.2)	1.00
Weight⁺ (g)	2666 ± 951	2556 ± 938	0.69
Head circumference⁺ (cm)	33.8 ± 3.0	32.7 ± 3.1	0.36
Height⁺ (cm)	48.0 (44.2-51.0)	47.5 (43.7-53.0)	0.73
SGA (n, %)	4 (15.4)	2 (8.3)	0.66
BPD (n, %)	5 (19.2)	13 (54.2)	0.01
TPN duration (day)	18.0 (10.0-34.0)	23.0 (11.0-30.0)	0.81
Mothers			
Prenatal steroid (n, %)	19 (73.1)	17 (70.8)	0.79
Hypothyroidism (n, %)	2 (7.7)	3 (12.5)	0.66
Gestational diabetes (n, %)	1 (3.8)	3 (12.5)	0.27
PPROM (n, %)	7 (26.9)	3 (12.5)	0.39

OP: osteopenia of prematurity, GA: gestational age, ⁺: at the moment of SOS measurement, SGA: small for gestational age, BPD: bronchopulmonary dysplasia, TPN: total parenteral nutrition, PPROM: preterm premature rupture of membranes

The serum CysC level was not correlated with serum Ca, P and ALP levels or SOS measurements and Z score values. There was no difference in the serum CysC levels of infants with and without OP. Mean serum CysC levels in preterm infants in postnatal week nine were 1.50 ± 0.19 mg/L.

Most previous studies in the English literature reported reference values for CysC levels in preterms in the postnatal first month (24). To our knowledge, there are no reports of CysC levels in preterm infants at nine weeks post-partum. Thus, this study aimed to provide reference data for CysC levels of preterm infants (born at ≤32 gestational weeks) in their ninth postnatal week.

Although some previous studies reported that serum CysC levels showed no gender difference in different age groups (17,18,25,26), others reported that they were higher in older males as compared with age-matched females (27). In the present study, although the mean CysC level was slightly lower in boys, we found no significant differences in serum CysC levels by gender.

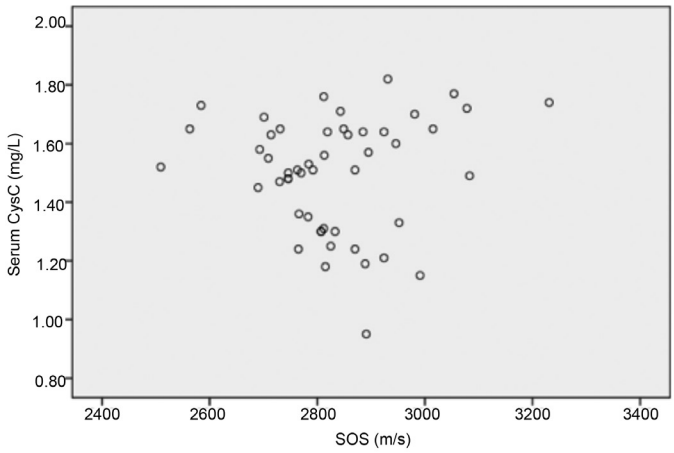

Figure 1. The serum CysC levels and SOS measurements of all infants

CysC: cystatin C, SOS: speed of sound. There was no correlation between serum cystatin C levels and speed of sound measurements (Rho = 0.04, p = 0.75)

Previous research showed that body weight did not affect the serum CysC level (26,28). The findings of the present study were consistent with those in the literature, with no difference found in serum CysC levels between infants with birth weights above and below 1500 g. Serum CysC levels show a gradual decrease with term in preterm neonates. The levels are higher in preterm than term neonates, with the highest values found in the most immature cases (24). In the present study, at the postnatal ninth week, mean CysC values of infants of gestational ages 26-29 weeks tended to be higher than those of infants of gestational ages 30-32 weeks. However, this difference was not statistically significant.

In the current study, SOS and SOS Z scores determined by USG were lower in osteopenic infants. Previous studies reported that bone SOS measurements were lower in preterm infants than term infants during early postnatal life, with SOS values of preterm infants decreasing until age 2 months and not reaching the levels of term newborns until age 12 months when measured longitudinally (29,30,31). Previous research also reported that SOS values showed a significant association with birth weight and gestational age (29,30,32). In the present study, bone SOS values were lower in the infant group with gestational ages 26-29 weeks (p = 0.01) and birth weights < 1500 g (p = 0.01).

An SOS Z score of less than -2 suggests low bone density (7,33). In the present study, infants with a tibia Z score of less than -2 were considered as having OP and the demographic and clinical features of infants with and without OP were compared. According to previous estimates, OP occurs in 30-50% of infants with birth weights < 1000 g and in 23-32% of infants with birth weights < 1500 g (34,35). In the present study, 24 of the 50 (48%) patients were diagnosed with OP.

The high rate of OP in our study may be explained by the timing of the SOS measurements, which were performed when the infants were two months old, a time when SOS values are lowest in premature infants. Previous studies reported that the incidence of OP was inversely correlated with gestational age and birth weight (34,35). In the present study, SOS values of infants were reduced in those with lower gestational weeks and lower birth weights. When the patient population was evaluated according to the presence or absence of OP, the gestational age and birth weight of infants with OP were lower than those without OP, but there was no statistical significance. This may be due to the Z score values, which were similar in both groups, as well as the small sample size.

Previous *in vitro* studies confirmed that CysC prevented bone resorption (36,37,38). In one study, Lerner and Grubb (36) showed that CysC in bone culture stimulated with parathyroid hormone and parathyroid hormone-related peptide was a potent inhibitor of mineral mobilization and matrix degradation. In an osteoblast cell culture system, Danjo et al (37) demonstrated that CysC affected bone morphogenetic protein signal pathways in osteoblasts, causing variation in osteoblasts and enabling mineralization and bone formation. Stralberg et al (38) showed that CysC inhibited osteoclast differentiation and formation. Clinical studies of bone resorption of CysC are limited to adults. Elevated serum CysC levels reported in postmenopausal women have been linked to an increased risk of bone fractures (39,40). Based on the results of these previous studies, we speculate that changes in the CysC level may act as a protective mechanism in OP and play a role in the pathogenesis of bone resorption.

In the present study, we investigated the relationship of serum Cys C levels with bone density and levels of biochemical markers of bone metabolism (Ca, P, and ALP). The results showed that serum CysC levels were not correlated with serum Ca, P and ALP levels or with SOS measurements and SOS Z score values.

Study Limitations

Assessment of osteopenia with QUS alone is a limitation of the current study. In subsequent studies, CysC levels should be investigated in patients with bone status assessed with DEXA.

Conclusion

CysC levels are not altered in OP. The presence of OP does not affect the safety of CysC as a marker of renal insufficiency in preterm infants.

Ethics

Ethics Committee Approval: This study approved by the Ethics Committee of Erciyes University Medical Faculty (02.10.2015/437).

Informed Consent: Informed consent was provided to parents of all patients.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Sabriye Korkut, Ahmet Özdemir, Osman Baştuğ, Şeyma Memur, Hülya Halis, Concept: Sabriye Korkut, Şeyma Memur, Selim Kurtoğlu, Tamer Güneş, Mehmet Adnan Öztürk, Design: Sabriye Korkut, Şeyma Memur, Selim Kurtoğlu, Tamer Güneş, Mehmet Adnan Öztürk, Data Collection or Processing: Sabriye Korkut, Ahmet Özdemir, Osman Baştuğ, Şeyma Memur, Hülya Halis, Analysis or Interpretation: Sabriye Korkut, Şeyma Memur, Hülya Halis, Osman Baştuğ, Levent Korkmaz, Ahmet Özdemir, Tamer Güneş, Mehmet Adnan Öztürk, Selim Kurtoğlu, Literature Search: Sabriye Korkut, Şeyma Memur, Hülya Halis, Osman Baştuğ, Levent Korkmaz, Ahmet Özdemir, Tamer Güneş, Mehmet Adnan Öztürk, Selim Kurtoğlu, Writing: Sabriye Korkut, Şeyma Memur, Hülya Halis, Osman Baştuğ, Levent Korkmaz, Ahmet Özdemir, Tamer Güneş, Mehmet Adnan Öztürk, Selim Kurtoğlu.

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The Distribution of Different Types of Diabetes in Childhood: A Single Center Experience

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What is already known on this topic?

Although type 1 diabetes is the most common type of diabetes in childhood, a variable increase in the prevalence of type 2 diabetes and maturity onset diabetes of the young has been reported by different multicenter studies depending on the ethnic background, the country of residence and the availability of genetic tests.

What this study adds?

Information on the distribution of type of diabetes in the Turkish pediatric population is scarce. Comparative data on the clinical characteristics of different types of diabetes, based on the experience of a tertiary pediatric diabetes center over the last 17 years, are presented in this paper. Also, this paper identified a trend towards increase in the frequency of type 2 diabetes in Turkish pediatric population.

Abstract

Objective: Type 1 diabetes (T1D) is the most common cause of diabetes in childhood but type 2 diabetes (T2D) and maturity onset diabetes of the young (MODY) are emerging as noteworthy causes of diabetes at young ages. The aim is to determine the distribution, trends and clinical features of the different types of diabetes in childhood in one tertiary center.

Methods: The records of children and adolescents aged 0-18 years who were diagnosed as "diabetes/persistent hyperglycemia" between January 1999 and December 2016, were reviewed. Clinical and laboratory characteristics of the patients at diagnosis and type of diabetes were recorded.

Results: The mean ± standard deviation age of 835 patients (48.7% females) at diagnosis was 8.8 ± 4.4 years. Eighty-four percent of the patients were diagnosed as T1D, 5.7% as T2D, 5.3% as clinical MODY and 5% as being cases of other types of diabetes. The frequency of diabetic ketoacidosis (DKA) and severe DKA in T1D were 48.4% and 11.6%, respectively. Fourteen patients (29.2%) with T2D presented with ketosis and two of them (4.2%) had DKA at diagnosis. Antibody positivity was 83.1% in T1D and 14.8% in T2D. A statistically significant increase in the frequency of T2D has clearly been demonstrated in recent years with a frequency of 1.9%, 2.4% and 7.9% in 1999-2004, 2005-2010 and 2011-2016, respectively ($p < 0.001$). In MODY, genetic analysis was performed in 26 (59%) patients and *HNF1A* and *GCK* gene mutations were detected in 3 (11.5%) and 14 (53.8%) patients, respectively.

Conclusion: Although the most frequent cause of DM is T1D in childhood, a trend towards increase in the frequency of T2D in recent years is notable in our population.

Keywords: Type 1 diabetes, type 2 diabetes, MODY, childhood

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Introduction

Type 1 diabetes (T1D) is the most common type of diabetes in childhood and its incidence is still rising in various parts of the world (1). However, the increasing worldwide rates of child obesity have also been associated with a variable increase in the prevalence of type 2 diabetes (T2D), depending on the ethnic background and the country of residence (2). While the prevalence of T2D in children was reported as 11% in the USA (3), this ratio was reported to be lower in Europe (1.3% in SWEET) (4).

Childhood T2D can be confused with maturity onset diabetes of the young (MODY) due to the presence of a family history, presenting features and a possible confounding factor of obesity/overweight (5,6). Furthermore, MODY, especially due to HNF1A mutations can be misclassified as T1D (7). Determining the type of diabetes is important for therapeutic considerations as well as genetic counseling (8).

The aims of the present study were: 1) to review the etiologic distribution and temporal changes in the etiology of childhood diabetes and; 2) to compare the clinical characteristics of the different types of diabetes encountered in a tertiary pediatric diabetes center over the last 17 years.

Methods

Data on 927 children and adolescents aged <18 years who were diagnosed as "diabetes" or "persistent hyperglycemia" and who were followed-up at the Pediatric Endocrinology and Diabetes Unit of Marmara University Faculty of Medicine in İstanbul, Turkey, between January 1999 and December 2016, were examined. Ninety-two patients with a follow up duration of less than one year were excluded, since the type of diabetes could not be specified because of insufficient data. Finally, 835 patients were included in this single-centered, observational, retrospective study.

The patients' gender, age of diagnosis, height (cm), weight (kg), body mass index (BMI, kg/m^2), c-peptide level (ng/mL), presence of pancreatic autoantibodies (islet cell antibodies, glutamic acid decarboxylase antibodies and insulin autoantibodies), presence of ketone bodies, pH and HCO_3 levels at the time of diagnosis, type of diabetes, treatment modalities (diet, oral antidiabetic drug, insulin) were recorded. The patients were classified according to the ISPAD Consensus 2014 (Table 1).

T1D was diagnosed in the presence of severe insulin deficiency, autoantibody positivity and the absence of any suggestive signs of other causes of diabetes. The diagnostic criteria for T2D were based on overweight/obesity, clinical findings of insulin resistance (acanthosis nigricans, hypertension, dyslipidemia), family history of T2D and good metabolic control with metformin or metformin combined with low dose, long-acting insulin (<0.5 U/kg/d). Patients who had a family history of diabetes of at least two generations in one side of the family, negative autoantibodies, no evidence of insulin resistance and good metabolic control with diet, sulphonylurea or low dose insulin were classified as clinically MODY. *HNF1A, HNF4A* and *GCK* genes were analysed for clinically suspected MODY cases. Children with an onset of diabetes before six months of age were diagnosed as neonatal diabetes mellitus (NDM) and relevant genetic tests were performed.

The study was approved by the local Ethical Committee of Marmara University (approval no: 09.2013.0408).

Statistical Analysis

All statistical data were analyzed using SPSS statistical software for Windows, version 17.0 (SPSS, Chicago, IL). Variables were summarized with descriptive statistics. Data were presented as mean ± standard deviation (SD). Normality was assessed using the Kolmogorov-Smirnov test. Parametric and nonparametric tests were used for inter-group comparisons. Chi-square test was used for categorical variables. Student's t-test was applied for continuous variables in independent groups. The Mann-Whitney U test was used for continuous variables that did not show normal distribution. The level of statistical significance was set as $p = 0.05$.

Results

After 92/927 patients (9.9%) were excluded, the mean age of 835 patients (48.7% females) at diagnosis was 8.8±4.4 (median 9.0, range 0.0-18.0) years. Seven hundred and one patients were diagnosed with T1D (84%), 48 with T2D (5.7%), 44 with clinical MODY (5.3%) and 42 with other types of diabetes (5%) (Table 1).

The clinical characteristics at diagnosis of T1D, T2D and MODY are shown in Table 2. In T1D, 23.7% (n=166) were younger than age 5 years and 1.6% (n=8) had a BMI standard deviation score (SDS) >2. The frequency of severe diabetic ketoacidosis (DKA) at diagnosis in T1D was 11.6%. T2D was more common in girls and older children. Fourteen patients with T2D (29.2%) presented with ketosis and two of these (4.2%) had DKA at diagnosis. Diabetes autoantibody positivity was 83.1% in T1D and 14.8% in T2D. The patients with antibody-positive T2D were compared with those with antibody-negative T2D in terms of age, BMI SDS, presence of DKA and use of insulin. The only statistically significant

Table 1. The distribution of the patients with diabetes

	n	%
T1D	701	84
T2D	48	5.7
Genetic defects of β-cell function		
MODY	44	5.3
NDM	7	0.8
Mitochondrial	2	0.2
Genetic defects in insulin action	2	0.2
Diseases of exocrine pancreas		
CFRD	11	1.3
Pancreatectomy	1	0.1
Endocrinopathies	1	0.1
Drug-induced	5	0.6
Infections	1	0.1
Genetic syndromes		
Wolfram	8	1
Others	4	0.5
Total	835	100

T1D: type 1 diabetes mellitus, T2D: type 2 diabetes mellitus, MODY: maturity onset diabetes of the young, NDM: neonatal diabetes mellitus, CFRD: cystic fibrosis related diabetes

difference was age at diagnosis. Antibody-positive T2D patients were younger than the antibody negative (11.8 ± 3.3 vs 13.7 ± 1.94, p = 0.045) patients.

A statistically significant increase in the frequency of T2D has clearly been demonstrated in recent years in our cohort with a frequency of 1.9%, 2.4% and 7.9% in the time periods 1999-2004, 2005-2010 and 2011-2016, respectively (p < 0.001) (Figure 1).

The frequency of DKA was 58.4% and there was also a statistically significant decrease in the proportion of ketoacidosis at diagnosis in T1D after year 2011 (55% vs 44.6%, p = 0.022). However, the decrement in the proportion of severe ketoacidosis was not statistically significant (15.1% vs 9.7%, p = 0.066).

Mean c-peptide levels at diagnosis were 0.7 ± 0.6 ng/mL in T1D, 3.2 ± 1.5 ng/mL in T2D and 1.3 ± 0.6 ng/mL in MODY patients (p < 0.001) (Table 2).

In MODY, genetic analysis was available in 26 (59%) patients and *HNF1A* and *GCK* gene mutations were detected in 3 (11.5%) and 14 (53.8%) patients, respectively.

Seven patients had NDM and it was molecularly confirmed in 6 of 7 patients in whom *KCNJ11* (n = 2), *6q24* (n = 1), *EIF2AK3* (n = 1), *SLC19A2* (n = 1) and *PTF1A* enhancer (n = 1) gene mutations were identified.

Table 2. The clinical features of the patients with type 1 diabetes mellitus, type 2 diabetes mellitus and maturity onset diabetes of the young at diagnosis

					p		
	n*	**T1D**	**T2D**	**MODY**	**T1D vs T2D**	**T1D vs MODY**	**T2D vs MODY**
Gender (F/M) (%)	793	49/51	71/29	32/68	0.003	0.028	< 0.001
Age at diagnosis (y)	793	8.4 ± 4.2	13.2 ± 2.5	10.2 ± 3.9	< 0.001	0.007	< 0.001
Age < 5 years, n (%)	793	166 (23.7%)	0	5 (11.4%)	< 0.001	0.06	0.016
BMI SDS	560	-0.5 ± 1.3	2.3 ± 1.0	-0.4 ± 1.1	< 0.001	0.91	< 0.001
BMI SDS ≥2, n (%)	560	8 (1.6%)	30 (69.8%)	0	< 0.001	0.46	< 0.001
C-peptide (ng/mL)	442	0.7 ± 0.6	3.2 ± 1.5	1.3 ± 0.6	< 0.001	< 0.001	< 0.001
Antibody positivity, n (%)	526	397 (83.1%)	4 (14.8%)	0	< 0.001	< 0.001	0.065
Anti-GAD, n (%)	519	301 (64.2%)	2 (7.1%)	0	< 0.001	< 0.001	0.20
ICA, n (%)	511	297 (64.8%)	1 (3.7%)	0	< 0.001	< 0.001	0.36
IAA, n (%)	494	147 (33%)	1 (3.7%)	0	< 0.001	< 0.001	0.37
pH	558	7.26 ± 0.15	7.38 ± 0.05	7.36 ± 0.03	< 0.001	0.008	0.13
HCO_3 (mMol/L)	547	15.1 ± 7.8	24.6 ± 5.2	22.7 ± 3.6	< 0.001	< 0.001	0.057
DKA, n (%)	566	251 (48.4%)	2 (6.5%)	0	< 0.001	< 0.001	0.29
Severe DKA, n (%)	566	60 (11.6%)	0	0	0.04	0.14	-

*: The n values are the number of patients who had available data, T1D: type 1 diabetes mellitus, T2D: type 2 diabetes mellitus, MODY: maturity onset diabetes of the young, F: female, M: male, BMI: body mass index, SDS: standard deviation score, GAD: glutamic acid decarboxylase, ICA: islet cell antibody, IAA: insulin autoantibody, pH: potential of hydrogen, HCO_3: bicarbonate, DKA: diabetic ketoacidosis

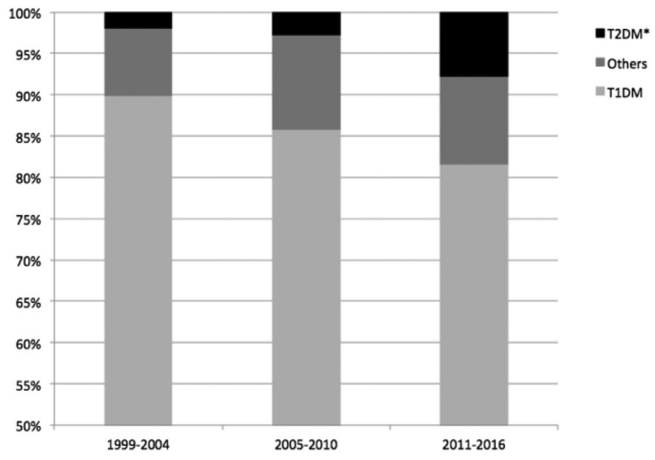

Figure 1. Frequency of type of diabetes in a single-center by 6 year periods

T1DM: type 1 diabetes mellitus, *: $p < 0.05$, T2DM: type 2 diabetes mellitus

In eight (2F/6M) patients with Wolfram syndrome from five families, three known homozygote/compound heterozygote mutations in *WFS* gene were detected and four of them had optic atrophy, one had cataract and one had diabetes insipidus.

Cystic fibrosis-related diabetes (CFRD) was detected in 11 patients (6F/5M, 1.3%) and the mean age and mean BMI SDS at diagnosis were 12.7 ± 4.1 (5.0-17.4) and -1.4 ± 1.5 (-3.7-1.1), respectively.

The frequency of drug-induced diabetes was 0.6% (n = 5), four of which were due to L-asparaginase and one due to tacrolimus.

Discussion

The present study illuminated some issues concerning the frequency of the different types of diabetes in our population and allowed us to make comparisons with other societies. The overall frequency of T1D, T2D, MODY and other specific types of diabetes were 84%, 5.7%, 5.3% and 5%, respectively.

T1D is still the most common cause of childhood diabetes and its frequency varies between 85-95% in different regions of the world (3,4,7). This variability originates from the number of children with T2D and MODY. The frequencies of T1D, T2D and MODY were 85.6%, 10.8% and 1.2% respectively in the SEARCH study (USA), while these ratios were 95.5%, 1.3% and 1.5% respectively in the SWEET study (Europe) (3,4,9). Also, the frequency of MODY was higher (5.5%) in a recent study from Italy (7). The variation in the frequencies could be explained by the availability of genetic testing and also by prevalence of obesity in that region. Misclassification of diabetes due to the lack of evidence-based clinical criteria for differential diagnosis is widespread and reported to be 7-15% (10). The diagnosis of MODY (5.3%) and T2D (5.7%) was found to be more common in this study as compared to the SWEET study. The present study is not a national multicenter study, so this difference may be explained by referral of the rare types of diabetes to our tertiary center.

The most confusing factor for classification of diabetes is obesity. BMI at the time of diagnosis is a less discriminatory feature for classification (10), since the increase in obesity has led to the appearance of children with obese T1D/MODY. In different studies, the frequency of obesity among patients with T1D at the time of diagnosis was 3.1-9% (11,12), but it was 1.6% in the present study. This could be due to lower obesity rates in our pediatric population (13) compared to North America and Western Europe. Although lower than these regions, obesity rates are also increasing in Turkey which may be the reason for the increase in the frequency of T2D observed over the time span of this study, from 1.9% to 7.9%. In accordance with previous reports (14), T2D was more common in girls and at pubertal ages.

The antibody positivity in T2D is reported up to 15% and these antibody-positive patients are usually younger, less overweight/obese and have higher hemoglobin A1c values (15). So, several terminologies have been recommended such as double diabetes, type 1.5 diabetes and latent autoimmune diabetes of youth. In the present study, the antibody positivity was 14.8% and there was a significant difference between antibody positive and negative subjects only at the age of diagnosis, with a younger age of diagnosis being seen in antibody positive patients, in line with other reports. Although, a few case reports described antibody positive MODY patients, the prevalence of antibody positivity in MODY is < 1% (16). Therefore, the antibody positivity was used as an exclusion criterion for MODY in the present study.

The frequency of DKA in T1D varied from 48% to 66% in the different studies in Turkey (17,18,19). Our study shows a decrease of 10% in the rate of DKA at the time of diagnosis, albeit, the current ratio is still high. DKA at the time of diagnosis of pediatric T2D is not infrequent and is reported to be as high as 40% of patients (15). However, it was not frequent in our study (4.2%) but nearly one-third of patients with T2D presented with ketosis without acidosis.

The frequency of MODY varies between 0.83-5.5% in different studies (4,6,7,20,21,22,23). GCK mutation (up to 95%) was the most common cause in the studies that reported higher MODY frequency (6,7,22). Similarly, we

detected GCK mutation in 53.8% of the clinically MODY patients who were genetically tested. This can be explained by the widespread use of random glucose measurement in general pediatric clinics in Turkey. On the other hand, the rate of genetic analysis in the clinical MODY patients was low (59%) in the present study, as it was not possible to perform this analysis prior to 2010. In 65.3% of these patients a mutation in one of the known MODY genes could be detected. This ratio varies between 27-89% in different studies (24). This variation and failure to detect mutations may result from inclusion criteria for genetic testing, may be due to a mutation in a gene not yet identified or to diagnostic overlap of different types of diabetes.

C peptide levels, although useful in long-standing diabetes cases, might not be discriminative in patients with new onset diabetes because of substantial overlap among different types of diabetes mellitus (25). Nevertheless, in addition to autoantibody positivity, c-peptide levels remain a relatively good diagnostic parameter. In the present study, c-peptide levels at the time of diagnosis were helpful, especially in differentiating between T1D and T2D.

Study Limitations

The limitation of this study is that it included a tertiary center data. Therefore, the frequency of some specific types of diabetes as CFRD may not reflect real frequency.

Conclusion

The present study provides trends over the last 17 years in pediatric diabetes in a large number of patients, from a single tertiary center and tries to identify the distinguishing features of each of different types of diabetes. The frequency of T2D is increasing but is still lower than that in North America. MODY is becoming more easily recognized in recent years owing to availability of autoantibody testing and genetic tests. Despite overlapping features such as obesity, ketosis and antibody positivity, there are demographic (age, puberty, gender, family history) as well as laboratory (autoantibody positivity, c-peptide) tools to correctly identify the type of diabetes in the pediatric population.

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Ethics

Ethics Committee Approval: This study approved by Ethical Committee of Marmara University (approval no: 09.2013.0408).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Zeynep Atay, Tülay Güran, Serap Turan, Serpil Baş, Concept: Abdullah Bereket, Design: Belma Haliloğlu, Data Collection or Processing: Saygın Abalı, Fuat Buğrul, Enes Çelik, Analysis or Interpretation: Saygın Abalı, Literature Search: Belma Haliloğlu, Writing: Belma Haliloğlu, Abdullah Bereket.

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ORIGINAL ARTICLE

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Restless Legs Syndrome and Poor Sleep Quality in Obese Children and Adolescents

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What is already known on this topic?

Restless Legs syndrome (RLS) is a sensory-motor disorder characterized by feelings of discomfort, causing the desire to move the legs. RLS is also common in the pediatric population affecting 2-4% of school-aged children and adolescents. Sleep disturbance has been shown to be a commonly associated feature of RLS in the pediatric population.

What this study adds?

This study demonstrated that the rate of Restless legs syndrome is higher in obese adolescents than in the general population. The rate is higher in patients with higher body mass index. Obese patients with RLS were found to have significantly more sleep-related symptoms.

Abstract

Objective: Adult epidemiological studies suggest that the rate of Restless Legs syndrome (RLS) in the general population may range from 5% to 15%. The aim of this study was to investigate the frequency of RLS in a community sample of obese adolescents aged 10-16 years and to assess the association with sleep quality and health-related glucose metabolism markers.

Methods: The study group comprised 144 obese and overweight children aged 10-16 yearsand the control group consisted of 66 age-matched healthy children. The RLS Questionnaire devised by the International RLS Study and the Pittsburgh Sleep Quality Index (PSQI), where a score >5 indicates poor sleep quality, was used to assess sleep quality.

Results: Mean body mass index (BMI) of the overweight/obese and control groups were 30.5 ± 0.5 and 18.7 ± 0.2, respectively. The frequency of RLS was higher in the obese group (21.7%) than the overweight (3.4%) and control (1.5%) ($p < 0.001$) groups. The frequency of a poor PSQI score was significantly higher ($p < 0.001$) in the obese group (37.3%) than the control group (24.2%). The obese with RLS group also had poorer sleep quality scores than the non-RLS obese group. Many symptoms of sleep disruption were more common in obese patients with RLS and RLS was independently correlated with a high PSQI score [odds ratio (OR): 2.25, confidence interval (CI): 0.96-5.28, $p < 0.001$)] and an increased BMI z-score (OR: 8.87, CI: 2.04-38.61, $p < 0.001$).

Conclusion: RLS is common in obese children and may be associated with altered sleep quality. Obese children with RLS need to be assessed since they may need support to improve their sleep quality.

Keywords: Obesity, restless legs syndrome, sleep quality, adolescent

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Introduction

Childhood obesity is associated with various adverse outcomes, such as poor academic performance, reduced psychological well-being, life-long obesity and cardiovascular diseases, which can all impair overall quality of life (1). Obese individuals are significantly more likely to report sleep disturbances (2). Today, there is increasing evidence indicating that sleep duration may be associated with obesity since sleeping plays a vital role in hormonal release, metabolic changes and lifestyle, which may result in obesity (3).

Restless Legs syndrome (RLS) is a sensory-motor disorder characterized by feelings of discomfort, causing the desire to move the legs (4). It manifests as an urge to move or the presence of unpleasant sensations in the extremities, symptoms that are worse with inactivity (while resting, sitting or lying down), which partially or wholly ease while moving the legs or walking, and are most severe at night (5,6). Currently, the pathophysiology of RLS is thought to be related with genetic predisposition, brain dopamine dysfunction and deficiencies in iron metabolism, although these factors have to date offered only a partial explanation (7).

RLS is usually associated with delayed sleep onset, difficulty in maintaining sleep, decreased total sleep time and reduced or absent slow-wave sleep (8). Sleep disturbance has been shown to be a commonly associated feature of pediatric RLS in population and clinic-based studies. Sleep disturbance is often the primary clinical complaint and more common in children with more severe RLS. Sleep disturbance is reported to be present in over 85% of pediatric patients with RLS (9,10,11). Six studies in adolescents reported that low sleep quality was negatively associated with body mass index (BMI) gain during the follow-up period (12).

The estimated prevalence of RLS has been reported to range between 4% and 29% in adults (13). RLS is less common in the pediatric population affecting 2-4% of school-aged children and adolescents (14,15). Some adult epidemiological studies have reported that BMI is associated with a higher likelihood of having RLS (16,17). However, unlike in adults, to date there have been no studies that have evaluated the prevalence of RLS and poor sleep quality in obese children.

The main focus of this present analysis was to determine the frequencies of RLS and poor sleep quality in obese pubertal children using the new International RLS Study Group (IRLSSG) criteria. The secondary objective was to assess the impact of RLS on sleep quality and the relationship between glucose metabolism markers and lipids. It was hypothesized that the frequency of RLS would increase progressively as adiposity and insulin resistance (IR) increased, and that RLS would have a negative impact on sleep quality scores in children with obesity.

Methods

Subjects

A total of 115 obese and 29 overweight adolescents with a mean age of 13.1 ± 1.7 years (range, 10-16 years), mean BMI of 30.5 ± 0.5 were randomly recruited from among obese children who were admitted to the Pediatric Endocrinology Unit of Antalya Research Hospital between January and October 2017. The adolescents were grouped according to their BMI percentile values. Adolescents were excluded if they had a history of major illness, including type 1 or type 2 diabetes, were taking any medications, or had a condition known to influence body composition, insulin action, or insulin secretion (e.g. glucocorticoid therapy, hypothyroidism, Cushing's disease). All subjects were in good health and had normal thyroid function. The control group consisted of 40 girls and 26 boys (mean age: 12.9 ± 2.7 years, mean BMI of 18.7 ± 0.2) who attended the hospital for minor illnesses such as common cold, conjunctivitis, or other similar condition.

BMI was calculated as weight (in kilograms) divided by height (in meters squared). Patients with a BMI of ≥95th percentile [BMI-standard deviation score (SDS) ≥1.64] according to reference curves for Turkish children were accepted as obese and BMI of 85-95th percentile (BMI-SDS = 1.04-1.64) as overweight (18). The pubertal development stages were assessed by a single pediatric endocrinologist using the criteria of Tanner stages. Staging for sexual maturation was >2 in all girls and boys (Tanner stages II–V) and considered as pubertal. The girls with menarche were excluded from the study.

The study was approved by the Local Ethics Committee of the Antalya Research Hospital Institutional Review Board (approval number: 19.01.2017-2/17). Signed informed consent was obtained from each subject over age 12 years. Informed parental consent was obtained for all children regardless of age.

Plasma glucose, insulin levels and other parameters were determined in blood samples collected between 08.00 and 10.00 am, after fasting for 12 hours overnight. Glucose was determined by the glucose oxidase method. Serum insulin levels were measured with an immulite immunoassay system (Diagnostic Products, Los Angeles,

CA). The homeostasis model assessment (HOMA) of IR was calculated as fasting insulin concentration (μU/mL) x fasting glucose concentration (mg/dL)/405. Iron and total iron binding capacity (TIBC) were studied using an Architect C8000 device (Abbott Laboratories, Abbott Park, IL, USA), ferritin on a DxI 600 device (Beckman-Coulter Inc., Pasadena, CA, USA) and hemoglobin on a Cell-Dyn Ruby device (Abbott Laboratories), all in accordance with the manufacturers' instructions. Serum concentrations of total cholesterol, high-density lipoprotein cholesterol, and triglycerides were measured using routine enzymatic methods with an Olympus 2700 analyzer (Olympus Diagnostica GmbH, Hamburg, Germany). Low-density lipoprotein (LDL) cholesterol levels were calculated using the Friedewald equation.

International Restless Legs Syndrome Study Group Rating (Symptom Severity) Scale

Pediatric or physical medicine residents asked face-to-face questions about the RLS diagnosis and severity based on the IRLSSG 2012 criteria. Pediatric diagnostic criteria are used for 10-12-year-old children while adult diagnosis criteria are used for 13-16-year-old children. Children were given a positive diagnosis of RLS if they met the following four criteria: 1) an urge to move due to uncomfortable sensations in the legs, 2) uncomfortable sensations are relieved by movement, 3) symptoms worsen during rest or inactivity, and 4) symptoms worsen in the evening (11).

Pittsburgh Sleep Quality Index

This is a questionnaire assessing sleep quality as well as the presence and severity of sleep disorder. It includes seven components and 19 self-rated questions, assessing subjective sleep quality (e.g. ''How would you rate your sleep quality overall?''), sleep latency (e.g. ''How long does it usually take you to fall asleep at night?''), sleep duration (e.g., ''How many hours of actual sleep do you get at night?''), habitual sleep efficiency (time asleep vs total time in bed), sleep disorder (e.g. ''How often do you have trouble sleeping because you wake up in the middle of the night or in the early morning?''), use of sleeping medications and daytime dysfunction (e.g. ''How often do you have trouble staying awake while driving, eating meals, or engaging in social activity?''). All questions were rated between 0 and 3 points; 0: not during the past month, 1: less than once a week, 2: once or twice a week, 3: three or more times a week. In addition, sleep quality is rated as follows; 0: very good, 1: fairly good, 2: fairly bad, 3: very bad. Component scores are totalled to obtain a global score ranging from 0-21 points. Higher global scores indicate worse sleep quality, where a score >5 indicated poor sleep quality. The diagnostic sensitivity and specificity of the scale are 89.6% and 86.5%, respectively (19). The Turkish validation and reliability study was performed by Agargun et al (20) in 1996.

Statistical Analysis

Mean and standard errors were used as descriptive statistics. Differences in the means of variables were tested using both parametric and non-parametric tests depending on the distribution of the variables. The Shapiro-Wilk W test was used to test for normality; $p < 0.05$ was considered evidence for non-normality. Categorical variables across groups were compared using the chi-square test or Fisher's exact test (if a cell number was five or less). Multivariable-adjusted logistic regression models were used to evaluate the association between the various risk factors and RLS and prevalent RLS. Odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) were calculated. In the model evaluating the association between risk factors and RLS, RLS status was the dependent variable and independent variables were the various risk factors such as obesity, Pittsburgh Sleep Quality Index (PSQI) score, hemoglobin, ferritin, plasma glucose, plasma insulin and HOMA. All tests were two-sided; the level of statistical significance was at $p < 0.05$. All analyses were performed with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).

Results

The characteristics of the 210 adolescents in the study are shown in Table 1. No differences were found among the three groups with respect to mean age and gender. Obese and overweight subjects had slightly higher hemoglobin levels than control subjects and the obese group had elevated ferritin levels compared to the other two groups, although the ferritin and hemoglobin levels were within normal limits in all groups. There was no significant difference between the groups with respect to TIBC levels. Fasting glucose, fasting insulin, LDL cholesterol, triglycerides levels and HOMA values were increased in the obese group compared to the other two groups. The overweight group had higher fasting insulin and triglyceride levels than the control group but the other glucose metabolism markers such as HOMA values were similar.

Frequency of Restless Legs Syndrome in Obese Children

Overall, 12.8% of the cohort met the diagnostic criteria for RLS. Within the three study groups, the frequency of RLS was higher in the obese group (21.7%) than in the overweight (3.4%) and control groups (1.5%) ($p < 0.001$). When compared to obese children diagnosed as RLS and

non-RLS, BMI-SDS was higher in the obese children with RLS than the non-RLS obese children (3.04 ± 0.46 vs 2.86 ± 0.43, p < 0.05) (Figure 1A).

Sleep Characteristics in Obese Subjects with Restless Legs Syndrome

Poor sleep quality was found in 32.8% of the adolescents of the study group. The PSQI score was found to be higher in the obese group (5.45 ± 0.2) than in the other two groups and the overweight group (4.21 ± 0.5) had a significantly higher score than the control group (3.91 ± 0.2) (Figure 1B).

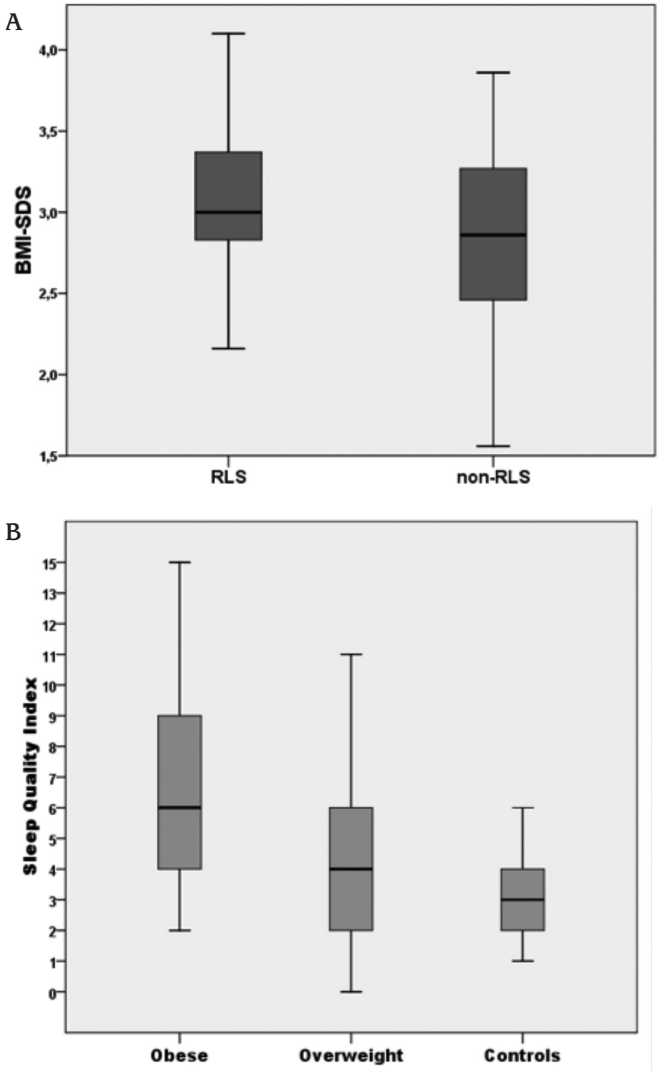

Figure 1. A) Boxplot for the distribution of body mass index - standard deviation score in obese children with restless legs syndrome and non- restless legs syndrome. B) Boxplot for the distribution of scores obtained through the Pittsburgh Sleep Quality Index used in children and adolescents according to their body mass index - standard deviation score

BMI: body mass index, SDS: standard deviation score, RLS: restless legs syndrome

These differences were statistically significant and the obese and overweight groups had higher scores than the control group. Therefore, the frequency of poor sleep quality (> 5 PSQI score) was higher in the obese group (37.3%) than in the control group (24.2%, p < 0.001). Gender difference was not statistically significant among the groups.

When the obese patients with RLS and non-RLS were compared, the scores of subjective sleep quality (p = 0.004), sleep latency (p < 0.001) and sleep disorders (p < 0.001) were significantly higher in the RLS obese subjects than in the non-RLS obese subjects, as reflected by the PSQI. The total PSQI score was significantly higher in obese subjects with RLS than in the non-RLS obese subjects (8.1 ± 0.7 vs 4.5 ± 0.2, p < 0.001) (Table 2). When the poor and good scores for total PSQI scores were compared in all obese subjects, poor sleep quality subjects were found to have higher BMI and BMI-SDS values than those with good sleep

Table 1. Characteristics of the study groups according to body mass index

	Obese	Overweight	Control
n	**115**	**29**	**66**
BMI	32.1 ± 0.3	26.7 ± 0.5*	18.7 ± 0.2#§
BMI-SDS	2.9 ± 0.45	1.39 ± 0.27*	-0.29 ± 0.85#§
Age (years)	13.5 ± 2.7	12.8 ± 2.2	12.9 ± 2.7
Gender (F/M)	70/45	20/9	40/26
Sleep disorders			
PSQI score	5.45 ± 0.2	4.21 ± 0.5*	3.91 ± 0.2#§
Poor PSQI (%)	43 (37.3)	10 (34.4)	16 (24.2)#
RLS (%)	25 (21.7)	1 (3.4)*	1 (1.5)#
Laboratory values			
Hemoglobin (g/dL)	13.3 ± 1.2	13.2 ± 0.8	12.9 ± 0.1§
Ferritin (ng/mL)	31.04 ± 3.8	21.4 ± 2.1	24.9 ± 3.8#
TIBC % (mcg/dL)	433 ± 40.1	376 ± 14.9	387 ± 6.2
Fasting glucose (mg/dL)	87.6 ± 1.03	85.3 ± 2.3	82.1 ± 3.5#
Fasting insulin (mIu/mL)	18.9 ± 1.5	10.8 ± 5.3	4.3 ± 2.5#§
HOMA	3.1 ± 0.3	2.1 ± 0.1	1.7 ± 1.2#
LDL-cholesterol (mg/dL)	96.3 ± 3.4	89 ± 14.2	88 ± 12.4#
HDL-cholesterol (mg/dL)	45.7 ± 1.2	42.6 ± 2.6	42.3 ± 4.5
Triglycerides (mg/dL)	120 ± 5.4	129 ± 20.2	94 ± 12.3#§

p < 0.01 for *: obese and overweight, #: obese and control, §: overweight and control, BMI: body mass index, SDS: standard deviation score, F: female, M: male, PSQI: Pittsburgh Sleep Quality Index, RLS: restless legs syndrome, TIBC: total iron binding capacity (%), HOMA: homeostatic model assessment of insulin resistance, LDL: low-density lipoprotein, HDL: high-density lipoprotein

quality (p = 0.04). No significant differences with respect of concentrations of hemoglobin, plasma glucose, plasma insulin and HOMA valueswere found between obese subjects with poor (> 5) and good PSQI (< 5) (Table 3).

Risk Factors for Restless Legs Syndrome

Multivariable logistic regression analysis revealed that increasing BMI was significantly associated with RLS when controlled for confounding factors. In this analysis, BMI-SDS (BMI-SDS > 1.64; OR: 8.87, 95% CI: 2.04-38.61, p < 0.001), and total PSQI scores (> 5 score; OR: 2.25, CI: 0.96-5.28, p < 0.001) were also independent significant risk factors for the incidence of RLS in adolescents. As was true for the full cohort, RLS in the obese group was independently and positively associated with age (OR: 0.83, CI: 0.35-1.98, p = 0.02) and plasma glucose (OR: 3.68, CI: 0.86-15.72, p < 0.001) but not with hemoglobin (OR: 1.98, CI: 0.25-15.8, p = 0.87), ferritin (OR: 1.42, CI: 0.57-3.56, p = 0.615), plasma insulin (OR: 1.29, CI: 0.51-3.27, p = 0.343) and the HOMA value (OR: 3.02, CI: 1.32-6.90, p = 0.086) (Table 4).

Table 2. Comparison of sleep quality scores (Pittsburgh Sleep Quality Index) in obese children with restless legs syndrome and non- Restless Legs syndrome

	Obese patients		
	RLS n = 25	Non-RLS n = 90	p value
Subjective sleep quality scale	1.76 ± 0.2	1.09 ± 0.1	0.004
Sleep latency	1.92 ± 0.3	0.66 ± 0.08	< 0.001
Sleep duration	0.44 ± 0.1	0.37 ± 0.06	0.616
Habitual sleep efficiency	0.44 ± 0.1	0.36 ± 0.05	0.525
Sleep disorders	1.32 ± 0.1	0.89 ± 0.04	< 0.001
Use of sleeping medication	0.14 ± 0.02	0.12 ± 0.05	0.219
Daytime dysfunction	1 ± 0.1	1.45 ± 0.1	0.234
Total PSQI score	8.1 ± 0.7	4.5 ± 0.2	< 0.001

PSQI: Pittsburgh Sleep Quality Index, RLS: Restless Legs syndrome

Table 3. Sleep quality Index in obese children with Restless Legs syndrome (cut-off score for poor sleep quality was over 5 according to Pittsburgh Sleep Quality Index)

	Sleep Quality Index (PSQI) scores		
Factors	Poor > 5	Good < 5	p value
BMI	32.9 ± 4.1	30.3 ± 3.8	0.04
BMI-SDS	2.9 ± 0.46	2.3 ± 0.42	0.04
Hemoglobin	13.5 ± 1.3	13.2 ± 1.1	0.20
Fasting insulin	19.4 ± 2.1	18.1 ± 2	0.67
Fasting glucose	87 ± 9.1	88.4 ± 10	0.50
HOMA	3.4 ± 0.4	2.7 ± 0.4	0.27

PSQI: Pittsburgh Sleep Quality Index, BMI: body mass index, SDS: standard deviation score, HOMA: homeostasis model assessment

Table 4. Relationships between risk factors and Restless Legs syndrome

		RLS	Non-RLS	Adjusted OR 95% CI	p value
Age	> 12	18	129	0.83 (0.35-1.98)	0.02
	< 12	9	54		
BMI-SDS	> 1.64	25	107	8.87 (2.04-38.61)	< 0.001
	< 1.64	2	76		
PSQI score	> 5	18	86	2.25 (0.96-5.28)	< 0.001
	< 5	9	97		
Hemoglobin	> 11	26	170	1.98 (0.25-15.8)	0.87
	< 11	1	13		
Ferritin	> 15	20	122	1.42 (0.57-3.56)	0.615
	< 15	7	61		
Fasting glucose	> 100	3	6	3.68 (0.86-15.72)	< 0.001
	< 100	24	177		
Fasting insulin	> 20	7	39	1.29 (0.51-3.27)	0.343
	< 20	20	144		
HOMA	> 2.5	14	48	3.02 (1.32-6.90)	0.086
	< 2.5	13	135		

RLS: restless legs syndrome, OR: odds ratio, CI: confidence interval, BMI: body mass index, SDS: standard deviation score, PSQI: Pittsburgh Sleep Quality Index, HOMA: homeostasis model assessment

Discussion

Firstly, the present study demonstrated that there is a significantly higher frequency of RLS in obese adolescents than in age-matched healthy control subjects (21.7% vs 1.5%). Secondly, obese patients with RLS were found to have many more sleep-related symptoms than those without RLS and RLS was found to be an independent predictor of poor sleep quality as reflected by the PSQI scores (OR: 2.25). RLS can be considered to be a common and clinically relevant sleep disorder in adolescents with obesity.

Although the pathophysiology of RLS is not yet fully understood, evidence exists for both iron/transferrin and dopaminergic abnormalities being factors in its etiology (10). Serum ferritin below 50 mcg/L was associated with increased severity of RLS in three adult studies (21,22,23). Recent pediatric case reports have also shown an improvement in RLS symptoms with oral iron therapy. However, iron deficiency is not common in all RLS sufferers and iron supplementation has shown variable success in RLS treatment (13,24,25). In this study, no relationships were found between RLS and serum levels of ferritin or hemoglobin, both of which have been reported to be related to the occurrence of RLS. However, it is possible that as the ferritin and TIBC levels were found within normal limits in all subjects, these were not detected as risk factors for RLS in the logistic analysis applied. In short, our findings suggest that low ferritin or iron deficiency has minimum or no impact on the development of RLS and that some undefined anemic condition might be required to increase the risk of the disorder. In most previous studies, anemia has been reported to be associated with increased risk for RLS, although approximately 70% of anemic adults do not develop RLS, and most patients with RLS do not have evidence of iron deficiency (7,26).

The diagnosis of idiopathic RLS is based on patient history as there are no physical characteristics or markers for the disorder. The disorder can be confirmed or ruled out on the basis of essential criteria defined by the IRLSSG. Two retrospective studies in adults have found the onset of RLS symptoms before the age of 20 years in approximately 40% of affected individuals (27,28). A large population-based prevalence study found RLS in 1.9% of children and 2% of adolescents in the United States and in the United Kingdom, respectively (29). More recently, a cross-sectional study carried out in Turkey estimated that the prevalence of RLS in non-obese children and adolescents was 2.9% (30). In the present study, the rate of RLS in the control group (1.5%) was found to be similar to the rate reported in previous studies on adolescents, while the frequency of RLS in obese patients was found to be significantly higher than that of the normal population (21.7%). Per et al (30) also reported that mean BMI value in adolescents was significantly higher in a group with RLS compared to those without RLS. These findings emphasize the importance of raising awareness of RLS among obese adolescents.

An association between obesity and a higher RLS prevalence has been observed in several adult studies (16,31,32). In a cross-sectional study including 1.803 men and women aged 18 years or older, each increase of 5 kg/m^2 BMI was associated with a 31% increased likelihood of having RLS (16). Several studies also suggest that RLS may be linked to key components of the metabolic syndrome, including diabetes, obesity and dyslipidemia. In an adult study, participants suffering from RLS were 4.7 times more likely to have impaired glucose tolerance and 8.5 times more likely to have elevated glycemia (fasting blood glucose $>$ 100 mg/dL) than the control group. Sleep disorders may have an association with decreased insulin sensitivity, independent of the association with adiposity (33,34). In the present study, obese patients had slightly elevated blood glucose levels but risk for RLS among the obese patients with elevated glucose levels or hyperinsulinemia was low. No correlation has been found among RLS and non-RLS adolescents for metabolic impairments such as glucose and insulin levels and HOMA, an IR marker.

A European primary-care study found that adult individuals whose RLS had a “high” negative impact on health had a significantly greater frequency of sleep disturbances (35). In another study by Picchietti et al (29), the sleep disorder rate was reported as 69.4% in adult patients with RLS. The excessive movements during sleep reported by obese patients with RLS may be secondary to the presence of periodic limb movements. In adults, leg movements are associated with 10-20% increases in heart rate and large elevations in blood pressure which begin at the time of leg movement onset and continue for 10-15 seconds afterwards (36). In the present study, RLS had a negative impact on sleep quality (OR: 2.25) in adolescents with obesity.

PSQI is a questionnaire which is useful in the evaluation of the quality and amount of sleep and the presence and severity of sleep disorders. In the present study, obese RLS patients were found to have elevated PSQI scores indicating poor sleep quality, especially in sleep latency, compared to non-RLS obese adolescents. There is also increasing evidence of an association between shortened sleep duration and/or poor sleep quality and obesity. In the current study, obesity was found to be significantly associated with an increased risk of developing RLS and poor sleep quality.

Study Limitations

There are limitations to our study. Firstly, the number of cases with RLS was relatively small. Secondly, while the questionnaire was based on criteria established by the IRLSSG for children, these questionnaires are not fully validated in the pediatric population and can lead to misclassification. Despite these limitations, the present study has established that RLS is common in obese children and adolescents and is a significant cause of sleep-related symptoms.

Conclusion

The results of this study demonstrated that the rate of RLS was higher in obese adolescents than in the general population and the rate increased as BMI values increased. It was also found that presence of RLS and a high BMI z-score, but not IR, have a significant impact on subjective sleep disturbances in obese patients. There is a clear need for further, randomized controlled RLS studies to better understand the metabolic response characteristics of the obese adolescent population.

Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of Antalya Training and Research Hospital (approval number: 19.01.2017-2/17).

Informed Consent: Consent form was obtained from patients and their families.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Rıza Taner Baran, Meral Filiz, Serkan Filiz, Concept: Özgür Pirgon, Rıza Taner Baran, Design: Rıza Taner Baran, Müge Atar, Meral Filiz, Serkan Filiz, Data Collection or Processing: Rıza Taner Baran, Meral Filiz, Serkan Filiz, Analysis or Interpretation: Meral Filiz, Serkan Filiz, Müge Atar, Literature Search: Müge Atar, Rıza Taner Baran, Writing: Rıza Taner Baran, Müge Atar, Rıza Taner Baran.

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ORIGINAL ARTICLE

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Optimal Cut-off Points of Fasting and Post-Glucose Stimulus Surrogates of Insulin Resistance as Predictors of Metabolic Syndrome in Adolescents According to Several Definitions

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What is already known on this topic?

There is no consensus for defining metabolic syndrome in the pediatric population. More than 40 different definitions for this population have been published to date. It is difficult to determine which definition is the most appropriate for clinical settings because of the variability of the prevalence reported.

What this study adds?

The use of an insulin resistance surrogate could be an adequate strategy to unify the diversity of diagnostic criteria. We are proposing, as surrogates of insulin resistance, cut-off points for post-stimulus insulin and glucose concentrations in a pediatric population.

Abstract

Objective: The aim of this study was to determine optimal cut-off points for fasting and post-glucose stimulus surrogates of insulin resistance to predict metabolic syndrome in adolescents according to several definitions.

Methods: One hundred fifty-five adolescents living in Mexico City were enrolled during 2011 and 2012. Waist circumference and blood pressure were recorded. Subjects received an oral glucose load of 1.75 g per kg up to a maximum dose of 75 g. Blood samples were drawn at baseline and 120 minutes. Concentrations of plasma glucose, triglycerides, high-density lipoprotein cholesterol and insulin were determined.

Results: The frequency of metabolic syndrome showed a large variability when using a variety of published definitions; in contrast, the optimal cut-off points for fasting insulin, homeostatic model assessment of insulin resistance and two-hour oral glucose tolerance test insulin were very similar in almost all the definitions considered and had adequate diagnostic performance: area under the curve >0.869, sensitivity >0.835 and specificity >0.755. Insulin resistance surrogates had substantial agreements with Ford, Cook and Salas definitions (Kappa ~ 0.62; agreement ~ 82%); moderate agreement was observed for International Diabetes Federation, Cruz and Ferranti definitions (Kappa ~ 0.41–0.59; agreement ~ 77%).

Conclusions: Insulin resistance surrogates may be a better approach for metabolic syndrome assessment in an adolescent population because of reduced variability and a higher predictive value.

Keywords: Insulin resistance surrogates, oral glucose tolerance test, HOMA-IR, cut-off points, metabolic syndrome, adolescents

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Introduction

In 1988, Reaven (1) coined the term "Syndrome X" for the cluster of clinical features: insulin resistance (IR), hypertension, raised triglycerides (TG) and decreased high-density lipoprotein-cholesterol (HDL-C). These individual criteria often co-occur and increase the risk of coronary artery disease. One decade later, in 1998, the World Health Organization (WHO) proposed a global definition for metabolic syndrome (MS) that consisted of impaired glucose tolerance or diabetes mellitus and/or IR determined by hyperinsulinemic-euglycemic clamp and two or more of the following components: elevated arterial pressure (≥140/90 mmHg), raised plasma TG, low HDL-C, microalbuminuria and, for the first time, central obesity (2). However, one year later, B. Balkau and M.A. Charles of the European Group for the Study of Insulin Resistance reviewed the WHO definition of MS. They advised to designate it as "IR syndrome" recognizing the importance of IR in its etiology and proposed to dispense with the hyperinsulinemic-euglycemic clamp and replace it by a surrogate of IR, that might be less invasive and more appropriate for epidemiological and clinical situations (3). Some years later, the National Cholesterol Education Program, developed one of the most widely accepted definitions (4) and finally, the International Diabetes Federation (IDF) proposed another in 2005, which has been quite controversial for use in pediatric population (5). Currently, MS in a pediatric population is defined as the combined and simultaneous presence of three or more of the following criteria: abdominal obesity, dyslipidemia (increased TG and/or decreased HDL-C), metabolic glucose impairment, and/or elevated blood pressure (BP) (5).

Nowadays there is no a standard, globally-accepted definition for MS in pediatric patients; although more than 40 sets of suggested criteria (definitions) have been published for this population to date. Golley et al (6) reported a prevalence variation of MS from 0 to 59% using six different definitions in a single population of pre-pubertal overweight children. Consequently, it is extremely difficult to determine which of them might be the most appropriate for the clinical setting (7).

Although initially IR was closely linked to MS, recent definitions do not consider a surrogate for IR as a formal component of it. Instead, fasting glucose concentrations are used as a marker of alterations in glucose metabolism, notwithstanding this usually manifests later in the natural history of the disease. Some authors have discussed the lack of sensitivity of fasting glucose for detecting impaired glucose tolerance (8,9,10).

The use of an IR surrogate could be a strategy to unify the diversity of diagnostic criteria published currently, reduce prevalence variability among populations and allow the identification of subjects at risk with a high predictive level. Therefore, the aim of this study was: 1) to determine optimal cut-off points of fasting and post-glucose stimulus IR surrogates to predict MS in adolescents, according to several definitions published in the pediatric population and; 2) to estimate the level of agreement between the analyzed definitions and the IR surrogates' cut-off points suggested.

Methods

Study Population

The data from this cross-sectional study correspond to two previous studies published by our group (11,12). One hundred fifty-five apparently healthy adolescents aged between 10 and 18 years from an open population living in Mexico City were enrolled during 2011 and 2012. The study protocol complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research in human subjects. The study protocol was approved by the Ethics Committee of the Instituto Mexicano del Seguro Social (registered as R-2010-3603-35). All subjects assented to participate in the study and informed consent was provided by their parents. Subjects with current chronic disease, such as type 2 diabetes mellitus, or using medications that affect glucose metabolism or a history of fever in the last 48 hours, were excluded. To avoid possible complications during the oral glucose tolerance test (OGTT), pre-study screening was carried out and patients with capillary blood glucose ≥126 mg/dL were not included.

Study Protocol

Voluntary participants arrived at the Unit of Medical Research in Nutrition with their parents or legal guardians at 8:00 am after an eight hour fast. Weight and height were recorded with light clothing and without shoes. Weight was assessed to the nearest 0.1 kg with a standard scale by using a fixed balance (Tanita, Arlington Heights, IL, TBF-300A) and height was measured to the nearest 0.1 cm using a wall stadiometer. Body mass index was calculated by dividing body weight (kg) by height squared (m^2). Waist circumference was determined with a non-elastic tape to the nearest millimeter at the midpoint between the lowest rib margin and the iliac crest, at the end of a gentle expiration. All measurements were obtained with the subject in a standing position. BP was measured in the right arm after a resting period of five minutes, while subjects were seated properly, as described by the National Heart, Lung, and Blood Institute (NHLBI) (13). Subjects received an oral glucose load of 1.75 g per kg of body weight up to a maximum dose of 75 g (ACS reagent; Sigma-Aldrich, St.

Louis, MO), dissolved in 150 mL of water for the OGTT. Blood samples were drawn at baseline and 120 minutes through an antecubital venipuncture into Vacutainer test tubes with ethylenediaminetetraacetic acid. Samples were centrifuged at 3000 rpm for 10 minutes. Plasma was preserved at -70 °C until analysis.

Blood Analysis

Concentrations of plasma glucose, TG and HDL-C were determined with commercial kits in a Spin 120 automated spectrophotometer (Spinreact, Girona, Spain; coefficients of variation ~3.9%). Insulin was determined by commercial radioimmunoassay kits (Millipore, Billerica, MA) with coefficient of variation of 7.5%. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated with the following formula (14):

$$\text{HOMA-IR} = \frac{[\text{fasting insulin } (\mu U/mL) * \text{fasting glucose } (mg/dL)]}{405}$$

Metabolic Syndrome Definitions

MS was assessed according to six different definitions: Cook et al (2003) (15), Cruz et al (2004) (16), de Ferranti et al (2004) (17), Ford et al (2005) (18), IDF (2007) (5) and Salas-Fernández et al (2015) (12). Definitions are summarized in Table 1.

The fourth report for the diagnosis, evaluation, and treatment of high BP in children and adolescents of the NHLBI (13) was used to establish elevated arterial pressure in the participants of our study. Waist circumference was assessed using the reference percentiles published by Fernández et al (19). Elevated plasma TG and low HDL-C for Cruz et al (16) definition were assessed against the reference tables from Hickman et al (20).

Statistical Analysis

Data analyses were performed with IBM SPSS Statistics for Windows software (SPSS version 22.0; IBM Corp., Armonk, NY). Kolmogorov-Smirnov test was used to assess data normality. Data are presented as median (range) as they were shown to be nonparametric. Mann-Whitney U test was used for inter-groups comparison according to gender. Several receiver operator characteristic (ROC) curves were constructed to determine optimal cut-off points for different IR surrogates to predict MS according to several definitions. ROC curves were computed by comparison to data from healthy subjects (exhibiting no components of MS) versus subjects with a high risk of MS (two components) and those with MS (three or more components), according to each definition. Area under the curve with 95% confidence interval was obtained; positive and negative predictive values were determined (positive predictive value and negative predictive value respectively). IR surrogates' cut-off points were selected according to performance for MS assessment on the ROC curve analysis. Those with higher levels of sensitivity and specificity were adopted for the purpose of this study. To assess agreement between the estimated cut-off points for IR surrogates and definitions of MS, a kappa coefficient was computed and percentage of agreement is also displayed.

Results

A total of 155 adolescents living in Mexico City were enrolled during 2011 and 2012 (83 males and 72 females). Clinical and metabolic characteristics of subjects are summarized in Table 2. Median age was 12.9 years for males and 13.6 for females. There was no statistical difference between most

Table 1. Metabolic syndrome definitions

Attribute	Cook et al (15)	Cruz et al (21)	de Ferranti et al (17)	Ford et al (18)	Salas-Fernández (12)	IDF
	3 or more of the criteria					
Abdominal obesity (waist circumference, cm)	≥90th percentile	≥90th percentile	>75th percentile	≥90th percentile	≥90th percentile	≥90th percentile
						And 2 more
High fasting glucose level (mg/dL)	≥110	≥110	≥110	≥100	≥100	≥100
High triglyceride level (mg/dL)	≥110	≥90th percentile	≥100	≥110	≥110	≥150
Low HDL level (mg/dL)	≤40	≤10th percentile	<50	≤40	<50	≤40
High blood pressure levels (mmHg)	≥90th percentile	≥90th percentile	>90th percentile	≥90th percentile	≥90th percentile	≥130/85 mmHg

IDF: International Diabetes Federation, HDL: high-density lipoprotein

of the biochemical and clinical variables when genders were compared. However, females had significantly higher levels of TG and lower concentrations of fasting glucose. Simple regression analyses were used to determine the influence of gender and Tanner stage on fasting insulin ($\beta = 1.4$; $p = 0.27$ and $\beta = -1.2$; $p = 0.14$, respectively), HOMA-IR ($\beta = 0.11$; $p = 0.71$ and $\beta = -0.4$; $p = 0.03$, respectively) and 2-hour insulin after an OGTT ($\beta = 11$; $p = 0.10$ and $\beta = -5.8$; $p = 0.17$). Since no substantial effects were observed in IR surrogates, subsequent analyses were not stratified by gender or Tanner stage.

The frequency of MS in the studied population showed large variability across different definitions; Cook and Ford had a similar frequency of 11 % and 11.6 % respectively, Ferranti 29.7 %, Salas 19.4 %, Cruz 4.5 % and IDF 3.2 %. In contrast, the optimal cut-off points for IR surrogates were very similar in almost all the studied definitions (Table 3). Only Ferranti's data partially disagreed. Furthermore, IR surrogates presented a high predictive level for MS, regardless of the definition used, as shown in Figure 1. Average fasting insulin cut-off point had a sensitivity and specificity of 0.835 and 0.808, respectively. Very similar values were found for HOMA-IR and 2 h OGTT insulin. Lower levels of sensitivity and specificity were observed for 2 h OGTT glucose (0.735 and 0.694, respectively).

Finally, Kappa coefficients (K) and percentage of agreement between the average cut-off points for IR surrogates and different definitions of MS are shown in table 4. IR surrogates had substantial agreements with the Ford, Salas and Cook definitions ($K \sim 0.62$; agreement ~ 82 %); moderate agreement was observed for IDF, Cruz and Ferranti definitions ($K \sim 0.41$-0.59; agreement ~ 77 %). In terms of agreement between IR surrogates, 2 h OGTT glucose showed the weakest match value to the other surrogates ($K \sim 0.18$-0.43; agreement ~ 69 %).

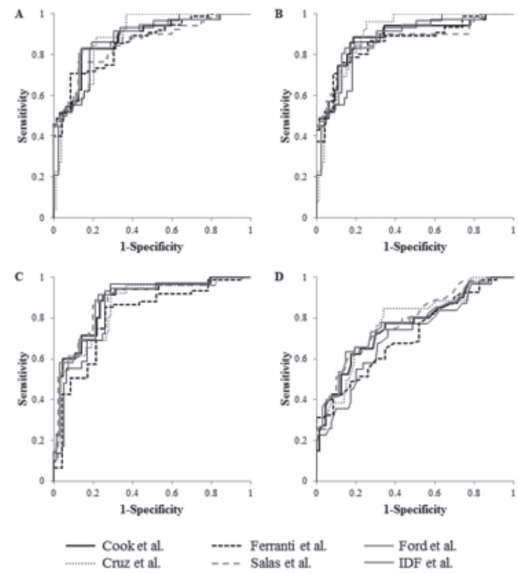

Figure 1. Performance of insulin resistance surrogates for metabolic syndrome assessment. Receiver operating characteristic curves evaluating the sensitivity and specificity of A) Fasting insulin, B) homeostatic model assessment of insulin resistance, C) 2 hours oral glucose tolerance test insulin and D) 2 hours oral glucose tolerance test glucose for assessment of metabolic syndrome according to different definitions

Table 2. Clinical and metabolic characteristics of study subjects

Characteristic	All (n = 155)	Female (n = 72)	Male (n = 83)	p value
Age (years)	13.3 (10.2-17.5)	13.6 (10.5-16.5)	12.9 (10.2-17.5)	0.022
BMI (kg/m^2)	22.8 (15.6-37.8)	23.0 (15.8-36.5)	22.7 (15.6-37.8)	0.577
WC (cm)	85.0 (63.0-129.0)	87.0 (64.5-129.0)	85.0 (63.0-126.0)	0.379
Systolic pressure (mmHg)	107.0 (68.0-135.0)	107.0 (89.0-129.0)	107.0 (68.0-135.0)	0.691
Diastolic pressure (mmHg)	62.0 (48.0-98.0)	62.0 (55.0-83.0)	63.0 (48.0-98.0)	0.725
TG (mg/dL)	90.5 (31.5-312.0)	99.5 (36.0-312.0)	81.5 (31.5-279.0)	**0.024**
HDL (mg/dL)	50.0 (25.5-461.0)	50.5 (32.5-76.0)	48.5 (25.5-461.0)	0.345
FPG (mg/dL)	85.0 (65.0-120.5)	83.0 (65.0-110.5)	88.0 (67.5-120.5)	**0.012**
FPI (µU/mL)	14.1 (4.5-43.6)	14.7 (4.5-43.6)	12.2 (5.5-37.8)	0.138
HOMA-IR	2.89 (0.6-9.8)	3.2 (0.6-9.8)	2.8 (1.1-8.5)	0.507
2 h OGTT glucose (mg/dL)[a]	96.5 (54.0-170.0)	95.3 (61.0-170.0)	98.0 (54.0-131.0)	0.679
2 h OGTT insulin (µU/mL)[b]	45.8 (9.9-266.9)	50.3 (18.5-163.2)	37.8 (9.9.3-266.9)	0.097

Data are presented as median (range). BMI: body mass index, WC: waist circumference, TG: triglycerides, HDL: high-density lipoprotein, FPG: fasting plasma glucose, FPI: fasting plasma insulin, HOMA-IR: homeostatic model assessment of insulin resistance, OGTT: oral glucose tolerance test, [a]: 2 hours oral glucose tolerance test glucose, [b]: 2 hours oral glucose tolerance test insulin

Table 3. Cut-off points for metabolic syndrome assessment in adolescents according to different definitions

Insulin resistance surrogates		Definitions used for metabolic syndrome assessment*						
		Cook et al (15) (2003)	Cruz et al (21) (2004)	de Ferranti et al (17) (2004)	Ford et al (18) (2005)	Salas-Fernández et al (12) (2015)	IDF (2007)	Average§
Fasting insulin (µU/mL)	**Cut-off points**	**14.87**	**14.87**	**10.91**	**14.63**	**12.89**	**14.63**	**14.38**
	AUC	0.877	0.880	0.857	0.882	0.855	0.871	0.873
	Sensitivity	0.829	0.846	0.853	0.833	0.804	0.862	0.835
	Specificity	0.859	0.797	0.696	0.855	0.725	0.805	0.808
	PPV	0.763	0.579	0.901	0.750	0.788	0.625	0.701
	NPV	0.901	0.941	0.592	0.898	0.743	0.939	0.884
HOMA-IR	**Cut-off points**	**2.99**	**3.12**	**2.39**	**2.86**	**2.79**	**3.07**	**2.97**
	AUC	0.885	0.900	0.860	0.890	0.862	0.888	0.885
	Sensitivity	0.886	0.885	0.867	0.889	0.824	0.862	0.869
	Specificity	0.812	0.772	0.739	0.823	0.825	0.792	0.805
	PPV	0.721	0.561	0.915	0.727	0.840	0.670	0.704
	NPV	0.928	0.954	0.629	0.927	0.780	0.938	0.905
2 h OGTT[a] insulin (µU/mL)	**Cut-off points**	**45.11**	**46.09**	**36.35**	**45.11**	**46.02**	**46.09**	**45.68**
	AUC	0.878	0.856	0.806	0.882	0.875	0.856	0.869
	Sensitivity	0.914	0.923	0.853	0.917	0.863	0.931	0.910
	Specificity	0.750	0.709	0.739	0.774	0.800	0.740	0.755
	PPV	0.653	0.510	0.914	0.688	0.846	0.574	0.654
	NPV	0.940	0.966	0.607	0.941	0.820	0.966	0.927
2 h OGTT[b] glucose (mg/dL)	**Cut-off points**	**98.75**	**98.75**	**96.25**	**98.75**	**98.75**	**96.85**	**98.37**
	AUC	0.760	0.775	0.715	0.777	0.780	0.713	0.761
	Sensitivity	0.725	0.769	0.650	0.732	0.709	0.742	0.735
	Specificity	0.698	0.696	0.652	0.721	0.718	0.637	0.694
	PPV	0.591	0.469	0.866	0.625	0.764	0.433	0.576
	NPV	0.796	0.900	0.349	0.796	0.619	0.862	0.795

*: all significant values <0.001. §: average values excepting Ferranti et al, IDF: International Diabetes Federation, AUC: area under the curve, PPV: positive predictive value, NPV: negative predictive value, HOMA-IR: homeostatic model assessment of insulin resistance, OGTT: oral glucose tolerance test, [a]: 2 hours oral glucose tolerance test glucose, [b]: 2 hours oral glucose tolerance test insulin

Table 4. Agreement between insulin resistance surrogates and metabolic syndrome definitions

Insulin resistance surrogates§		Metabolic syndrome definition*					
		Cook et al (15) (2003)	Cruz et al (21) (2004)	de Ferranti et al (17) (2004)	Ford et al (18) (2005)	Salas- Fernández (12) (2015)	IDF (2007)
Fasting insulin	Kappa	0.598	0.536	0.419	0.637	0.582	0.563
	% agreement	80.80	79.25	73.46	82.65	79.12	80.19
HOMA-IR	Kappa	0.664	0.546	0.464	0.661	0.603	0.587
	% agreement	83.84	79.25	75.51	83.67	80.22	81.13
2 h OGTT insulin[a]	Kappa	0.590	0.470	0.406	0.627	0.641	0.531
	% agreement	79.80	74.53	74.49	81.63	82.42	77.36
2 h OGTT glucose[b]	Kappa	0.391	0.384	0.184	0.425	0.395	0.304
	% agreement	69.90	70.64	61.17	71.57	70.21	67.57

*: all significant kappa values <0.001, §: average cut-off point values excepting Ferranti et al (Table 1), IDF: International Diabetes Federation, HOMA-IR: homeostatic model assessment of insulin resistance, OGTT: oral glucose tolerance test, [a]: 2 hours oral glucose tolerance test glucose, [b]: 2 hours oral glucose tolerance test insulin

Discussion

As expected, considerable variation of MS frequencies was found in the studied population using the different definitions we considered. The highest frequency was observed with Ferranti´s definition (29.7%) and the lowest with the IDF criteria (3.2%). This variability in MS frequencies is due to the diversity of cut-off points used by each author for the different components of MS. Ferranti's definition, for example, uses a lower threshold for waist circumference (75th percentile) in comparison with the other definitions (90th percentile). As a result, the number of subjects with central obesity is increased in Ferranti's definition, which in turn has a direct effect on higher prevalence. Conversely, IDF definition designated higher thresholds for elevated TG (>150 mg/dL) and raised BP (≥130/85 mmHg). According to the NHLBI reference tables (13), the 90th percentile for a girl or a boy of 12-years-old and height percentile >95th, corresponds to a value of 122-123 mmHg for systolic BP and 78-79 for diastolic BP, respectively. Therefore, from a physiological point of view, the BP threshold proposed by the IDF seems to be rarely found under normal conditions and could underestimate the presence of alterations on it. Finally, as in the IDF definition, Cruz et al (16) propose a higher cut-off points for serum TG (135 and 170 mg/dL for boys and girls between 12 and 15 years-old, respectively). The lower frequencies observed with these definitions are partially due to the low likelihood of exceeding these thresholds at an early age.

Independently of the variation of the frequencies observed among the MS definitions used in this study, IR surrogates seem to show more consistency. Proposed optimal cut-off points for fasting plasma insulin, HOMA-IR and 2 h OGTT insulin are almost the same when different definitions are compared (Figure 1). Only Ferranti's definition showed discrepant values and for this reason it was excluded from an average cut-off point that was computed for each IR surrogate: 14.38 µU/mL for fasting insulin, 2.97 for HOMA-IR and 45.68 µU/mL for 2h-OGTT insulin. Additionally, the suggested cut-off points showed an adequate diagnostic performance, as denoted by the diagnostic attributes summarized in Table 3 and displayed a substantial agreement across three of the definitions: Ford et al (18), Cook et al (15) and Salas-Fernández et al (12) (Table 4). As a whole, these data support that the use of an IR surrogate could be an adequate strategy to unify the diversity of diagnostic criterion for MS assessment published at present. In contrast, the proposed cut-off point for 2h-OGTT glucose (98.37 mg/dL) is not valid for MS assessment because it showed a poorer diagnostic performance and lower kappa coefficient and percentage of agreement (Table 3 and 4). It is well known that fasting and post-stimulus plasma glucose is not an adequate surrogate of IR, as it remains in the normal range due to the hyperinsulinemia that characterizes this alteration. Raised fasting and post-stimulus glucose levels (≥100 and ≥140 mg/dL, respectively) (21) are indicators of an advanced metabolic impairment (glucose intolerance) in which insulin can no longer maintain blood glucose levels in the normal range. Instead IR surrogates unmask an early alteration in glucose metabolism, which makes them more suitable for MS assessment.

The MS definitions used in this manuscript derive from a variety of studies that were performed in different population types and sample sizes. Data for Cook and Ferranti's definitions were extracted from the Third National Health and Nutrition Examination Survey (NHANES III; 1988-1994) using 2430 and 1960 children aged 12 to 19 years, respectively. Ford used data from 1366 participants aged 12-17 years from the NHANES (1999-2000). Cruz and Salas's definitions were in smaller samples of Hispanic children (n=126; 8-13 years living in Los angeles, California and n=155; 10-18 years from Mexico City, respectively). Regardless of these important differences, the estimated cut-off points for fasting insulin, HOMA-IR and 2h-OGTT insulin are remarkably homogeneous among the different populations used to create the definitions analyzed, so they could be extrapolated for similar populations.

Study Limitations

It is important to note that a disadvantage in the use of fasting and post-stimulus IR surrogates is the high within-subject variability reported previously by other authors. Reinehr et al (22) reported a coefficient of variation (CV) of 22% for HOMA-IR in children and adolescents. In Mexican adults, our group found similar CV for fasting insulin and HOMA-IR (20.7% and 19.3%, respectively) and for 2h-OGTT insulin a CV of 29.9% was observed (23). Additionally, Schousboe et al (24) found a CV of 54% for 2h-OGTT insulin in a population of Danish origin. Since fasting IR surrogates seem to have lower intrasubject variability, these could be a better alternative for MS assessment. Furthermore HOMA-IR has proved to be an adequate tool in clinical and epidemiological studies. According to a revision carried out by Wallace et al (25) this surrogate had shown a good correlation when compared with the euglycemic clamp ($r = 0.58\text{-}0.88$, $p<0.0001$).

A strength of this study is that we are proposing cut-off points for post-stimulus insulin and glucose concentrations. Due to

the scarcity of information related to this, we consider that this is a valuable contribution of this work.

Conclusion

Currently there is no standard definition for MS in pediatrics. This leads to underestimation or overestimation of its prevalence, severely limits the comparability of different studies and compromises its usefulness in the clinical setting. The use of an IR surrogate may be a better approach to assess subjects at risk of MS in pediatric populations as the IR surrogates used in this study exhibit less variability and a high predictive level.

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Ethics

Ethics Committee Approval: Yes. The Study Protocol was approved by the Ethics Committee of the Instituto Mexicano del Seguro Social (registered as R-2010-3603-35).

Informed Consent: Informed consent was taken.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Mónica Ivette Piña-Aguero, Jorge Maldonado-Hernández, Design: Mónica Ivette Piña-Aguero, Jorge Maldonado-Hernández, Azucena Martínez-Basila, Data Collection or Processing: Alejandra Salas-Fernández, Azucena Martínez-Basila, Analysis or Interpretation: Aranza Zaldívar-Delgado and Mariela Bernabe-García, Literature Search: Alejandra Salas-Fernández, Writing: Mónica Ivette Piña-Aguero, Jorge Maldonado-Hernández.

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Effectiveness of Continuous Subcutaneous Insulin Infusion Pump Therapy During Five Years of Treatment on Metabolic Control in Children and Adolescents with Type 1 Diabetes Mellitus

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What is already known on this topic?

With intensive insulin therapy, type 1 diabetic children and adolescents achieve good metabolic control.

What this study adds?

This data shows that with continuous subcutaneous insulin infusion treatment type 1 diabetic children and adolescents can achieve better metabolic control than multiple daily insulin treatment in the long term.

Abstract

Objective: To compare continuous subcutaneous insulin infusion (CSII) therapy with multiple daily insulin (MDI) therapy on metabolic control in children and adolescents with type 1 diabetes mellitus (T1DM) over the long term.

Methods: Fifty-two T1DM patients treated with CSII and monitored for at least one year prior to and at least five years following CSII were included. Thirty-eight age and sex-matched MDI controls with a 5-year follow up were recruited.

Results: Mean age of the subjects, duration of diabetes and CSII therapy were 17.0 ± 4.8 years, 10.7 ± 2.8 years and 7.7 ± 1.5 years respectively. Mean hemoglobin A1c (HbA1c) in the year prior to CSII, during the first year of treatment and after 5 years of CSII were 7.3 ± 1% (56 mmol/mol), 7.0 ± 0.7% (53 mmol/mol) and 7.8 ± 1.3% (62 mmol/mol) respectively. Initial and 5-year mean HbA1C levels of controls were 7.9 ± 1.08% and 8.6 ± 1.8%. Mean HbA1c values were significantly lower in those receiving CSII therapy throughout follow-up. Basal and total insulin doses were significantly lower in the CSII group at all times. HbA1c was compared between subjects by age (0-5, 6-11 and 12-18 years) with no significant difference between them.

Conclusion: Although CSII mean HbA1c values exceeded accepted good metabolic control limits after 5 years, CSII produces better HbA1c control at all times and in all age groups compared to MDI.

Keywords: Type 1 diabetes mellitus, continuous subcutaneous insulin infusion pump therapy, multiple daily insulin therapy, HbA1c

Introduction

Continuous subcutaneous insulin infusion (CSII) therapy has been used in the treatment of type 1 diabetes mellitus (T1DM) since the 1970's and is increasingly used as an alternative to multiple daily insulin (MDI) therapy as pumps have become more widely available. Its effectiveness has been confirmed by meta-analyses of various observational and randomized controlled studies and in childhood and adolescence studies (1,2). The therapeutic goal in T1DM is to establish good and close-to-normal glycemic control, without hypoglycemic attacks, in order to protect against microvascular and macrovascular complications (3). The International Society for Pediatric and Adolescent Diabetes (ISPAD), International Diabetes Federation (IDF) and the American Diabetes Association (ADA) cite a recommended hemoglobin A1c (HbA1c) value of < 7.5% in the pediatric age group.

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CSII therapy is the most physiological insulin therapy currently available, more closely mimicking daily insulin release and is also reported to improve patients' quality of life (4,5). Only a small percentage of patients achieve desired glycemic targets with MDI therapy (4,6). Although several studies have shown a decrease in HbA1c levels with CSII compared to MDI, the HbA1c levels recommended by the ISPAD/IDF/ADA have not been achieved in most studies. In some studies, however, no improvement was observed in HbA1c levels, or levels returned to pre-CSII values at the end of 3-4 years. The limitation of the majority of these studies is the short follow-up time (0.6-8.8 years). Long-term observation studies are therefore needed to determine the efficacy of CSII therapy (7,8,9).

The aim of this study was to assess the effect of CSII treatment on long-term metabolic control in children and adolescents diagnosed with T1DM and compare it to those treated with MDI therapy.

Methods

Type 1 diabetic patients, aged between 2-18 years, started on CSII therapy between January 2004 and December 2011 at a single centre and subsequently monitored for at least five years were included in the study. Demographic data of the patients, insulin doses, insulin/carbohydrate ratios, daily basal and bolus insulin (food and correction bolus) levels, frequency of capillary blood glucose monitoring, incidence of hypoglycemic attacks, episodes of diabetic ketoacidosis and HbA1c values were obtained retrospectively from file data recorded at every 3-monthly clinic visit. All patients were monitored by a team, consisting of a pediatric endocrinology specialist, a diabetes nurse and a dietician. Anthropometric data were converted to standard deviation scores using Turkish standard data (10). Body mass index (BMI) was calculated using the standard formula weight/height^2 (kg/m^2). HbA1c was measured by turbidimetric inhibition immunoassay (Tina-quant HbA1c Gen. 3) (Normal range: 4.8-5.9%). Severe hypoglycemia was recorded as an event, with symptoms consistent with hypoglycemia in which the patient required assistance from another person or resulted in seizure/coma (11). Incidence rate of severe hypoglycaemic episodes was calculated as number of episodes per 100 patient-years. All children were on Minimed Paradigm Insulin Pump (Minimed Medtronic; Northridge, USA). CSII data for all cases were evaluated with pump data transferred to computer (CareLink® Pro Therapy Management Software, Minimed Medtronic; Northridge, USA) at each visit. Patients aged between two and 18 years and treated with basal bolus regimen with MDI, with both the same duration of diabetes and a monitoring period of at least five years were enrolled as the control group. CSII patients were classified into three different groups: preschoolers (≤6 years old, n = 16), prepubertal (six years to Tanner stage 2, n = 18) and pubertal (n = 18). Patients who had at least Tanner 2 breast development or testicular volume ≥4 mL were included in the pubertal group (12). CSII patients were also stratified according to good (n = 37), moderate (n = 9) and poor metabolic control (n = 6) which was defined as: HbA1c: < 7.5%, 7.5-9% and > 9% or < 58, 58-75 and > 75 mmol/mol, respectively.

Before initiating CSII therapy, all patients and their families completed a training program. Patients declining informed voluntary consent, with diagnosed psychiatric disorders or a monitoring period of less than five years were excluded from the study. Ege University Medical Faculty Clinical Investigations Ethical committee approval was obtained for the study (no:16-6.1/13).

Statistical Analysis

Normal distribution of data was assessed using the Shapiro-Wilks test with $p > 0.05$ indicating normal distribution. Chi square analysis was performed for categoric variables. Two-group HbA1c comparisons were performed using the Mann-Whitney test. The Wilcoxon matched two samples test was used to determine variation over time in the groups. T test was used for comparisons between independent variables when comparing CSII and MDI groups. Analysis of variance was performed for recurring measurements in the analysis of groups determined on the basis of age groups. The Bonferroni test was used for multiple comparisons between times. Linear correlation between variables was evaluated using Pearson's correlation analysis.

Results

Demographic Data

Ninety cases diagnosed with type 1 DM were included in the study. Fifty-two patients (57%) were on CSII therapy and 38 (43%) were on MDI. 48.1% were male and 51.9% were female in the CSII group whilst 44.7% were male and 55.3% were female in the MDI group. Mean age and duration of diabetes in the CSII group at the time of enrolment was 17.0 ± 4.8 and 10.7 ± 2.8 years respectively. Mean duration of CSII therapy was 7.7 ± 1.5 years. Mean age of the subjects on MDI therapy was 17.6 ± 3.5 years and duration of diabetes was 10.1 ± 3.9 years There was no statistical difference in terms of age of the subjects and duration of diabetes between the CSII and MDI groups.

Metabolic Control

Mean HbA1c in the year prior to initiation of CSII was 7.3 ± 1.0% (56 mmol/mol) while mean HbA1c at the end of the first year of CSII was 7.0 ± 0.7% (53 mmol/mol). At the latter time point HbA1c levels were < 7.5% (< 58 mmol/mol) in 78.8% of cases. Mean HbA1c at the end of five years was 7.8 ± 1.3% (62 mmol/mol). In the CSII group 19 patients (39%) still had a mean HbA1c < 7.5% (< 58 mmol/mol) at the end of the fifth year. Mean initial and 5-year HbA1c levels of cases on MDI therapy were 7.7 ± 1.04% (61 mmol/mol) and 8.6 ± 1.8% (70 mmol/mol) respectively and nine (23%) of the patients' HbA1c were < 7.5% (< 58 mmol/mol) at the end of fifth year. Mean change in HbA1c at the end of five years in the CSII group was 0.5 ± 1.5% compared with 0.6 ± 1.9% in the MDI group and there was no significant difference between the groups with respect to change in HbA1c (Table 1). Mean HbA1c was significantly lower in the CSII group throughout the five years follow up ($p < 0.05$; Figure 1). No correlation was found between HbA1c levels and age, sex, duration of diabetes, duration of CSII or insulin doses used.

There was no significant difference in HbA1c in the CSII group when sub-grouped according to age (Figure 2). HbA1c levels increased during follow-up in all age groups.

At the end of the fifth year of CSII therapy, HbA1c of the patients in the well-controlled group (n = 37) increased to 7.6 ± 0.8% (60 mmol/mol) from 6.8 ± 0.6% (51 mmol/mol). The group with moderate control (n = 9) decreased HbA1c levels during the first year but HbA1c increased by the end of the fifth year. In the poor metabolic control group (n = 6), although HbA1c decreased in the first year, at the end of the fifth year it had again increased but in no patient did it exceed pre-treatment HbA1c levels (Table 2).

Insulin Dosage

Children using MDI therapy used lower total daily insulin doses compared to those treated with CSII at the beginning of therapy (MDI: 0.96 ± 0.21 U/kg/d; CSII: 1 ± 0.35 U/kg/d respectively). Daily insulin dose decreased to 0.83 ± 0.21 U/kg/d at the end of one year of CSII. No time-dependent changes in daily insulin dose were observed between the two groups during the subsequent years (Figure 3).

Basal insulin dose in the first year of treatment was 0.36 ± 0.14 U/kg/d in the CSII group vs 0.48 ± 0.19 U/kg/d in the MDI group. No time-dependent change was found in basal insulin throughout follow up in the two groups. Basal insulin dose was significantly lower in the CSII group compared to the MDI group in all of the time periods ($p < 0.05$; Figure 3).

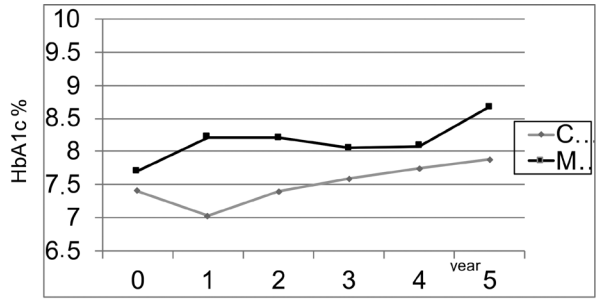

Figure 1. Comparison of the mean hemoglobin A1c values between the two treatment groups during the five year follow-up period

HbA1c: hemoglobin A1c

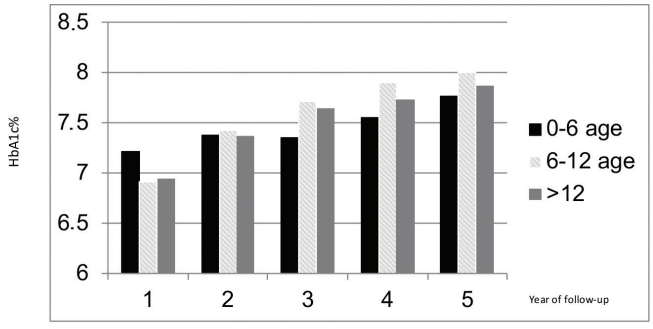

Figure 2. Mean hemoglobin A1c levels in the continuous subcutaneous insulin infusion patients by age group during the five year follow-up period

HbA1c: hemoglobin A1c

Table 1. Comparison of metabolic control during the five year follow up in continuous subcutaneous insulin infusion and multiple daily insulin groups

	At the beginning	End of the 1st year	End of the 5th year	ΔHbA1c (1st and 5th years)
Mean HbA1c (%)	7.4 ± 1.5	7.0 ± 0.7	7.8 ± 1.3	0.5 ± 1.5
CSII group				
(n/% of patients < 7.5%)	18/34%	41/78%	20/39%	
Mean HbA1c	7.7 ± 1.0	8.2 ± 1.3	8.6 ± 1.8	0.6 ± 1.9
MDI group				
(n/% of patients < 7.5%)	12/32%	10/25%	9/23%	
p	< 0.05	< 0.05	< 0.05	> 0.05

CSII: continuous subcutaneous insulin infusion, MDI: multiple daily insulin, HbA1c: hemoglobin A1c

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When cases were stratified by age within the CSII group, the basal insulin doses used by subjects aged over 12 years was higher than that in the other age groups.

Bolus insulin used in the first year of treatment was 0.46 ± 0.25 U/kg/d in the CSII group and 0.47 ± 0.17 U/kg/d in the MDI group. No statistically significant difference was found between the groups at the 5-year follow period (Figure 3). Neither was there a difference in terms of bolus insulin doses found between age groups.

Table 2. Comparison of metabolic control during the five year follow-up in the continuous subcutaneous insulin infusion group in terms of good, moderate and poor metabolic control prior to pump therapy initiation

Metabolic control at the beginning of the study	At the beginning	1st year	5th year
Good < 7.5 (n = 37)	6.8 ± 0.6*	6.9 ± 0.7**	7.6 ± 0.8
Moderate 7.5-9 (n = 9)	7.7 ± 0.2	6.9 ± 0.8	8.7 ± 2.0
Poor > 9 (n = 6)	9.6 ± 0.5*	7.6 ± 0.8***	8.2 ± 1.4

*: p < 0.05 between initiation of pump and 5th years

**: p < 0.05 between 1st and 5th years

***: p < 0.05 between initiation of pump and 1st years

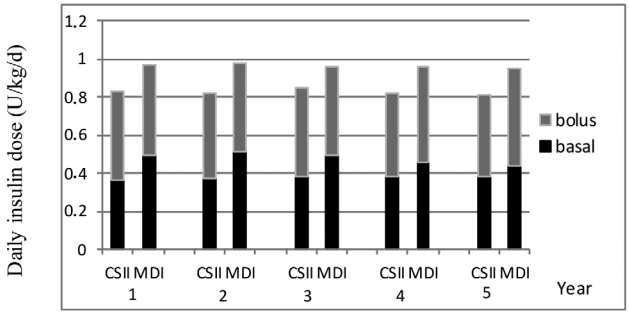

Figure 3. Comparison of the daily insulin dose between the two treatment groups during the five year follow-up period

CSII: continuous subcutaneous insulin infusion, MDI: multiple daily insulin

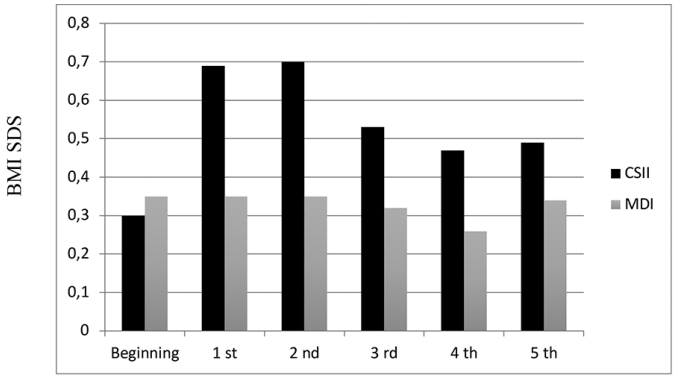

Figure 4. Body mass index standard deviation score values during the five-year follow-up period

CSII: continuous subcutaneous insulin infusion, MDI: multiple daily insulin, BMI: body mass score, SDS: standard deviation score

Anthropometric Data

BMI SDS at start of therapy was 0.39 ± 0.95 SD in the CSII group and 0.39 ± 0.85 SD in the MDI group. At the end of five years it was 0.49 ± 1.01 SD in the CSII group and 0.34 ± 0.87 SD in the MDI group. At the beginning of the study, there were three obese cases, but this increased to five at the end of the study period (two CSII, three MDI). Although BMI SDS in the CSII group increased in the first and second years, there was no statistically significant difference between the groups at 5-year follow up (Figure 4).

Adverse Events

Diabetic ketoacidosis was observed in one of the CSII cases (0.31/100-patient-years) and four of the patients on MDI treatment (2.1/100-patient-years) during monitoring, while severe hypoglycemia was seen in two patients in the CSII group (0.62/100-patient-years) and in one case in the MDI group (1.9/100-patient-years).

Discussion

CSII is a safe and effective therapeutic technique in children and adolescents diagnosed with type 1 DM. There has been a significant increase in its use in the last 10 years, although there are still differences in rates of use between countries (13). Various studies have shown that CSII improves glycemic control and increases patients' quality of life, without increasing the incidence of hypo or hyperglycemic episodes (14,15,16). However, target HbA1c levels of < 7.5%, based on ADA/IDF/ISPAD recommendations, have not been achieved in the majority of these studies. Although there have been several studies investigating the effectiveness of CSII, a short monitoring period has been a limitation in most of these studies (4). In one study over a two year follow up period, HbA1c levels, despite improving in the first six months, tended to increase over the subsequent 18-month period in pump patients (17). At longer term follow-up of up to five years, the initial decrease in HbA1c was described as a "temporary improvement", while an increase in HbA1c levels was observed in later periods (17). A meta-analysis of results of various randomized, controlled studies has shown that a decrease in HbA1c levels of 0-0.9% has been achieved with CSII when the duration of intervention ranged from six to 12 months (18). At the end of the first year in this analysis there was a significant decrease in HbA1c with 78.8% of the patients < 7.5% (58 mmol/mol). Although mean HbA1c levels were lower in the patients receiving CSII

during follow up, mean HbA1c increased to 7.8 ± 1.3% (62 mmol/mol) at the end of the fifth year from 7.3 ± 1.0% (56 mmol/mol) before CSII initiation. The increase in HbA1c in our patients after the first year could be due to increasing age, duration of diabetes or due to decreased compliance of the patients. One multi-center study reported lower HbA1c levels in all age groups in a group receiving CSII compared to MDI patients (13). Another study from Denmark showed lower HbA1c levels at all years in the CSII group, followed up for more than five years, in keeping with our findings (19). In the Danish study, although a marked improvement was observed in HbA1c levels in the first year of CSII, HbA1c levels tended to increase in subsequent years with the best metabolic control established one year after CSII initiation. We found exactly the same pattern of metabolic control in our study group. The lower mean HbA1c in the CSII group throughout the five years may be due to short duration of CSII treatment, which is a relatively recent treatment modality, compared to MDI treatment.

At the end of fifth year of therapy, HbA1c of the patients in the good control and moderate control groups increased. In the poorly controlled group, HbA1c decreased in the first year similarly to the other groups but the rate of increase after the first year was slower than the other groups. A future therapeutic aim should be to develop new approaches to prevent the impairment of metabolic control over the long-term, so that the short-term improvements seen in metabolic control in first year of therapy might be maintained. Repetition of periodic diabetes education and planning of practices that increase motivation, such as motivational interviewing, may be helpful and should be investigated.

When metabolic control was analyzed according to age groups no significant difference was observed throughout the five-year follow-up period between the age groups in our study. In a study by Johnson et al (20), with respect to different age groups, the older age groups (two groups; 6-12 years and >12 years) had the most dramatic initial improvement of glycemic control, compared with the youngest subject (<6-years) group upon commencement of insulin pump therapy, with HbA1c decreasing by 0.6 to 0.8% within three months. Over the following five years, each age group on CSII showed an improvement compared with non-CSII counterparts. However, the initial HbA1c was lowest in the <6-year-old group, followed by the six to 12 year olds and then the >12 year olds. The mean HbA1c of the <6-year-old pump cohort remained below 7.5% (58 mmol/mol) from six months through the first five years of follow-up.

An increase was found in BMI SDS in the first and second years of CSII in our study. This might be attributed to patients initially adopting a more flexible dietary model with CSII. However, at the end of 5-year follow-up, no significant difference was seen between the CSII and MDI groups in terms of mean BMI SDS. There was no relation between BMI SDS and poor metabolic control. The SWEET study group reported similar BMI SDS in CSII and MDI patients (13). Significant increase in BMI SDS was found in the 6-12 age group when compared with the MDI treatment group, but when linear regression analysis was performed on the basis of duration of diabetes, no significant variation was observed between the two treatment groups. Johnson et al (20) also reported a similar change in BMI SDS in CSII and MDI groups. No difference was found in change in BMI SDS during follow-up in terms of the age groups in our study ($p = 0.885$). There was also no correlation between HbA1c and BMI SDS. The impact of CSII treatment on BMI varies in the literature without any clear pattern emerging. Therefore longer follow-up periods may be helpful in drawing a conclusion on BMI in children and adolescents on pump therapy.

Total daily insulin doses recommended by IDF/ISPAD in prepubertal and pubertal children are 0.7-1U/kg/d and 1-2U/kg/d, respectively. A potentially greater insulin requirement has been reported due to insulin resistance in puberty (21). In our study, total insulin dose used before initiation of CSII was significantly higher than the total dose used after CSII therapy commencement and the MDI group's total daily insulin dose was higher than the CSII group throughout follow up. In the SWEET study CSII patients used lower-dose insulin compared to subjects on MDI (13). Similarly, Pickup et al (1) also observed a lower daily insulin dose in the CSII group. When patients in our study were analyzed according to age groups, daily and basal insulin doses were higher in subjects over 12 years, possibly reflecting early puberty insulin requirement increases or to a more flexible life style change with CSII, or a combination of the two factors. It has been reported that lower HbA1c levels are associated with higher basal insulin levels (22). However, no relation between HbA1c and basal insulin doses was observed in our study.

Study Limitation

The main limitation of this study is the retrospective design of data from a single center data. In addition, the frequency of patients' hypo- or hyperglycemic episodes, other than the most severe ones, are not included in the study. Conversely as a single center study, all the patients were monitored with the same treatment protocol which is a strength in terms of standardization of laboratory results, measurement techniques, patient counseling and team approach to management.

Conclusion

Although HbA1c values were not within recommended metabolic control limits with either treatment modality at the end of the five years follow up, CSII produces better metabolic control compared to MDI over the long-term.

Ethics

Ethics Committee Approval: Ege University Medical Faculty Clinical Investigations Ethical committee approval was obtained for the study (no:16-6.1/13).

Informed Consent: Retrospective design data collection.

Peer-review: External and internal peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Damla Gökşen, Şükran Darcan, Concept: Damla Gökşen, Design: Damla Gökşen, Şükran Darcan, Data Collection or Processing: Özlem Korkmaz, İlkin Mecidov, Günay Demir, Hafize Çetin, Analysis or Interpretation: Samim Özen, Damla Gökşen, Literature Search: Yasemin Atik Altınok,Damla Gökşen, Writing: Özlem Korkmaz, Damla Gökşen.

Financial Disclosure: The authors declare that there are no conflicts of interest.

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ORIGINAL ARTICLE

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The Association Between Maternal Subclinical Hypothyroidism and Growth, Development, and Childhood Intelligence: A Meta-analysis

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What is already known on this topic?

Thyroid hormone plays an important role in the differentiation, development and maturation of tissues. Insufficiency or lack of thyroid hormones during pregnancy have a negative effect on fetal neurological and intellectual development. Many studies suggest that hypothyroidism in pregnancy can increase the risk of adverse outcomes of pregnancy. To this date, there is no established consensus on routine screening for subclinical hypothyroidism in pregnant women due to lack of credible evidence.

What this study adds?

Our meta-analysis showed that maternal subclinical hypothyroidism in pregnancy is associated with an increased risk of several adverse neonatal outcomes. Thus, it is necessary to assess thyroid function in pregnant women and intervene when necessary. Because of the high methodological quality of the included trials, our data provide supportive evidence for initiating further investigations.

Abstract

Objective: To explore the association between maternal subclinical hypothyroidism (SCH) in pregnancy and the somatic and intellectual development of their offspring.

Methods: Using RevMan 5.3 software, a meta-analysis of cohort studies published from inception to May 2017, focusing on the association between maternal SCH in pregnancy and childhood growth, development and intelligence, was performed. Sources included the Cochrane Library, Pub-Med, Web of Science, China National Knowledge Infrastructure and Wan Fang Data.

Results: Analysis of a total of 15 cohort studies involving 1.896 pregnant women with SCH revealed that SCH in pregnancy was significantly associated with the intelligence ($p = 0.0007$) and motor development ($p < 0.00001$) of the offspring. SCH was also significantly associated with the child's weight in four studies involving 222 women ($p = 0.02$). Maternal SCH in pregnancy was identified as a risk factor for fetal growth restriction with a combined relative risk (RR) value of 2.4 [95% confidence interval (CI): 1.56, 3.7]. Meta-analysis of 10 studies that provided numbers of preterm infants revealed a significant association between maternal SCH in pregnancy and premature delivery, with a combined RR of 1.96 (95% CI: 1.34, 2.88). There was a significant effect of maternal SCH in pregnancy on fetal distress *in utero* ($p = 0.003$).

Conclusion: Maternal SCH in pregnancy is associated with increased risk of adverse neonatal outcomes, including delayed intellectual and motor development, low birth weight, premature delivery, fetal distress and fetal growth restriction.

Keywords: Gestation, subclinical hypothyroidism, child development, meta-analysis

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Introduction

Thyroid hormone (TH) promotes growth via its effect on protein synthesis. It also plays an important role in differentiation, development and tissue maturation (1). TH is essential for brain cell proliferation. Prior to gestational week 20, TH-dependent brain development fully or partly depends on maternal TH (2,3). Many studies provide evidence that hypothyroidism in pregnancy may increase the risk of adverse outcomes, including premature delivery, low birth weight (LBW), fetal demise and disrupted neurological/intellectual development of the fetus (4,5,6). Subclinical hypothyroidism (SCH) is defined as an elevated serum thyroid stimulating hormone (TSH) level in the context of normal triiodothyronine (T_3) and tetraiodothyronine (T_4) levels (7,8,9). Presence of SCH in pregnancy can be expected to have adverse effects on the growth and development of the fetus. In one survey, there was a higher incidence of maternal SCH in pregnancy (11.3%) compared with clinical hypothyroidism in pregnancy (2.4%) (10). Intelligence quotient scores in the offspring of women with untreated SCH were lower than those of the children of normal pregnant women (11). Currently, there is no consensus regarding routine SCH screening in pregnant women, owing to lack of credible evidence. Although many studies have investigated the effects of maternal SCH on childhood growth, development and intelligence level, the results have not been uniform. We therefore thought it would be worthwhile to conduct this meta-analysis of published studies to provide evidence-based research on the effects of maternal SCH on the growth, development and intelligence of the offspring.

Methods

This meta-analysis was based on the reports of domestic and international cohort studies that evaluated the effects of maternal SCH in pregnancy on childhood growth, development and intelligence. Only peer-reviewed articles published in English and Chinese were included in the analysis. The analysis only included studies on women with SCH in pregnancy with singleton fetuses. All subjects, apart from having SCH, were otherwise healthy. Maternal SCH was defined as having a serum TSH level higher than the upper limit of pregnancy-specified reference value (the 97.5th percentile of normal) and a serum free thyroxine (FT_4) level within normal range (between 2.5th to 97.5th percentiles of normal value) (12) or a TSH level between 2.5 and 10 mIU/L and a normal FT_4 level according to the definition established by the American Thyroid Association (13). All analyses were based on previous published studies, thus no ethical approval and patient consent are required.

We evaluated the following outcomes: growth; development and intelligence levels of the child (including growth, cognition and intelligence) measured by intelligence tests such as the Bayley Scales or the Gesell Scales. The Bayley Scales of Infant Development is one of the most psychometrically valid measurements for examining mental and psychomotor development in 1 to 30 months-old infants. This test consists of a mental scale (163 items) and a psychomotor scale (81 items) used to assess cognitive development, including visual performance function, memory, first verbal outcomes and fine and gross motor development. Age at assessment-adjusted raw scores were centered to a mean of 100 with a standard deviation of 15 to calculate index scores (mental and psychomotor scores). Gesell Scales can reflect the development level of children from five aspects: gross motor quotient; fine motor quotient; adaptive behavior quotient; individual social behavior quotient; and language quotient. Birth weight (BW); LBW (≤2500 g); premature birth (<37 weeks gestation); intrauterine distress (variously defined); and fetal growth restriction (IUGR, variously defined) were also considered in the analysis.

The following studies were excluded: studies which included pregnant women treated with thyroxine; studies that did not include pregnant women with normal thyroid function as controls; non-cohort studies; publications containing duplicate data from the same study; studies for which the full text was not available; studies published only as abstracts; studies that did not provide initial data and did not respond to our requests for more information; and studies with inconsistent data.

Cochrane Library, Pub-Med, Web of Science, China National Knowledge Infrastructure and Wan Fang Data were searched from the inception of each database to May 2017 for relevant studies. Search words in Chinese included: pregnancy, gestation, SCH, growth and development, intelligence; search words in English included pregnancy, pregnancies, gestation, SCH, cognition, growth, intelligence, premature birth, premature delivery, premature labor, preterm delivery, preterm birth, preterm labor, fetal distress, intrauterine distress, fetal growth restriction. As an example, the specific search strategy for Pub-Med is shown in Table 1.

Two investigators worked independently to screen articles according to the inclusion and exclusion criteria. Disagreements were resolved by discussion with a third reviewer, also an expert in the field. Extracted data included authors, year of publication, sample size, gestational age

at screening, intelligence score, BW, preterm delivery, intrauterine distress and fetal growth restriction.

The quality of selected studies was assessed independently by two investigators, using the Newcastle-Ottawa Scale (NOS) for cohort studies. The highest NOS score was 9 points, and consisted of the following three aspects: the definition and selection of subjects in the case and control groups (0-4 points); the comparability between study groups (0-2 points); and determination of exposure factors (0-3 points). A paper that scored ≥7 points or more was considered "high-quality".

Statistical Analysis

Meta-analysis was performed with Rev Man software (edition 5.3). Qualitative and quantitative analyses were performed, and 95% confidence intervals (CI) (95% CI) were calculated. Heterogeneity analysis was performed with the I^2 test. Meta-analysis was performed with a fixed effect model for studies without heterogeneity ($I^2 < 50\%$), and a random effect model for studies with heterogeneity ($I^2 \geq 50\%$) after data combination (14). Descriptive analysis only was conducted for clinical trials with data not suitable for meta-analysis. Effect size was represented as relative risk (RR) for categorical variables and as standard mean difference for continuous variables, with 95% CI.

Results

A total of 1.176 articles were identified with the prespecified search strategy. Ultimately, 15 articles were included in the analysis after step-by-step screening (5,10,15,16,17,18, 19,20,21,22,23,24,25,26,27). The screening flow chart is shown in Figure 1.

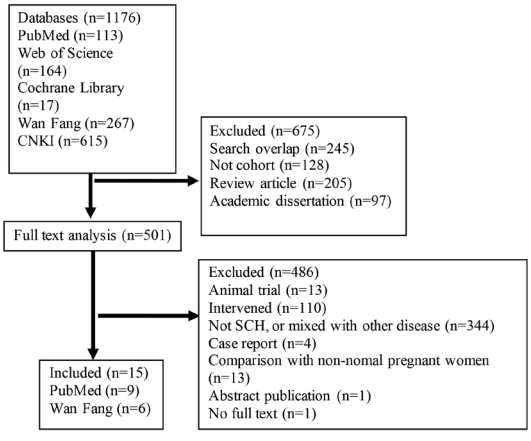

Figure 1. Flow chart of the article selection process

CNKI: China National Knowledge Infrastructure, SCH: subclinical hypothyroidism

Basic characteristics and quality evaluation of included studies: Of the 15 studies included, nine (5,10,15,16,17,18,19,20,21) were published in English and the remaining six were published in Chinese. A total of 1.896 patients met the inclusion criteria and were included in the study, with 37.968 controls. Fetal intrauterine distress was reported in five studies (17,19,20,22,27). The number of premature infants in various groups was reported in 10 studies (5,10,15,16,19-22,26,27). Neonatal BW was reported in four studies (10,19,24,26). The number of LBW neonates was reported in seven studies (5,15,17,20,21,22,27). Childhood intelligence was measured at age 12 to 30 months in three studies (18,24,25). In this meta-analysis, we chose cohort studies with high methodological quality. The principal characteristics and NOS scores are displayed in Table 2 and 3.

Maternal SCH in pregnancy and child intellectual and motor development: Because child intelligence and motor ability develop as children mature, studies with children of similar age were included in this meta-analysis (Table 4). In these three studies, the age range was 12 to 30 months. Intellectual and motor development were measured by Bayley Scales. The intellectual and motor development level of the children were compared between the SCH group and the control group. A fixed effect model was used because of the heterogeneity among studies ($p = 0.17$, $I^2 = 47\%$). There was a significant association between maternal SCH in pregnancy and child intellectual development (MD = -6.08, 95% CI: -9.57 ~ -2.58, $p = 0.0007$) and child motor development (MD = -7.29, 95% CI: -10.30 ~ -4.28, $p < 0.00001$).

The association between maternal SCH and BW: Data on BW were provided in four studies. The meta-analysis of these four studies revealed moderate heterogeneity ($p = 0.11$, $I^2 = 50\%$). Thus, a fixed effect model was used. We found a significant effect of maternal SCH in pregnancy on BW (MD = -0.27, 95% CI: -0.44 ~ -0.11, $p = 0.001$) (Figure 2).

The association between maternal SCH and LBW: Data on LBW were provided in seven studies. We found moderate heterogeneity among them ($I^2 = 58\%$). Thus, a random-effect model was used. Maternal SCH was a risk factor for LBW

Table 1. PubMed search strategy

#1 Pregnancy OR pregnancies OR gestation
#2 Subclinical hypothyroidism
#3 #1 AND #2
#4 Cognition OR growth OR intelligence
#5 Premature birth OR premature delivery OR premature labor
#6 Preterm delivery OR preterm birth OR preterm labor

(combined RR: 1.78, 95% CI: 1.04-3.07, p = 0.04). Figure 3A shows the association between maternal SCH and LBW. We also made a funnel plot as shown in Figure 3B. The funnel plot of this study is asymmetrical, indicating that there may be a publication bias.

The association between maternal SCH and fetal growth restriction: Data on fetal growth restriction was provided in three studies. The meta-analysis of these studies demonstrated a significant correlation between maternal SCH and fetal growth restriction, with no heterogeneity among results ($I^2 = 0\%$). Thus, a fixed-effect model was used and determined a combined RR of 2.4 (95% CI (1.56, 3.7), p < 0.0001), as shown in Figure 4.

The association between maternal SCH and fetal intrauterine distress: Data on fetal distress were provided

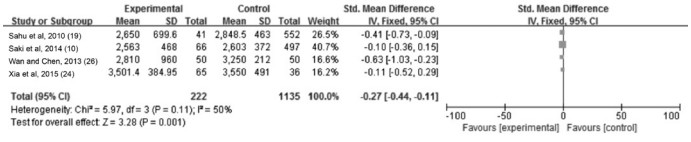

Figure 2. The forest plot of relative risk and 95% confidence interval of pooled studies comparing pregnant women with subclinical hypothyroidism to euthyroid pregnant women for risk of birth weight

SD: standard deviation, CI: confidence interval

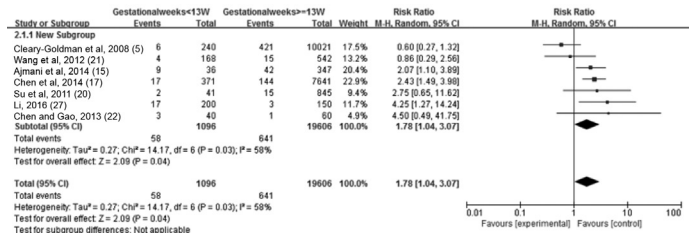

Figure 3A. The forest plot of relative risk and 95% confidence interval of pooled studies comparing pregnant women with subclinical hypothyroidism to euthyroid pregnant women for risk of low birth weight

CI: confidence interval

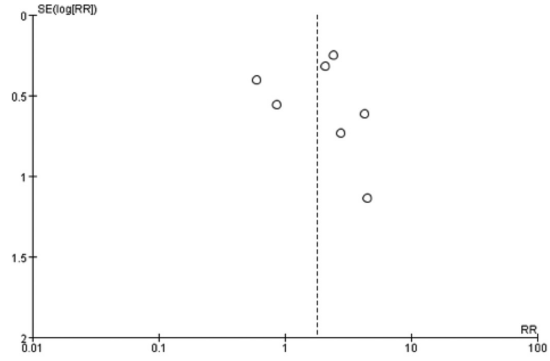

Figure 3B. The funnel plot of pooled studies comparing pregnant women with subclinical hypothyroidism to euthyroid pregnant women for risk of low birth weight

RR: relative risk

Table 2. Study characteristics

Author	Year	Country	SCH definition	SCH/normal (N)	GW	Outcomes					
Ajmani et al (15)	2014	India	TSH > 2.5 mIU/mL, FT_4 normal	36/347	13-26			C	D		F
Casey et al (16)	2005	USA	TSH > 97.5th percentile, FT_4 < 0.680 ng/dL	404/16894	< 20				D		
Chen et al (18)	2015	China	TSH > 97.5th percentile, FT_4 normal	106/106	12-24	A					
Chen et al (17)	2014	China	TSH > 97.5th percentile, FT_4 normal	371/7641	10-17			C		E	F
Cleary-Goldman et al (5)	2008	USA	TSH > 97.5th percentile, FT_4 2.5th–97.5th	240/10021	10-13			C	D		
Sahu et al (19)	2010	India	TSH > 5.5 mIU/L, FT_4 normal	41/552	13-26		B		D	E	
Saki et al (10)	2014	Iran	TSH > 97.5th percentile, FT_4 normal	66/497	15-28		B		D		F
Su et al (20)	2011	China	TSH > 95th percentile, FT_4 = 5-95th	41/845	≤20			C	D	E	
Wang et al (21)	2012	China	TSH > 2.5 mIU/L, FT_4 normal	168/542	≤12			C	D		
Xia et al (24)	2015	China	TSH > 3.5 mIU/L, FT_4 normal	65/36	≤12	A	B				
Li et al (23)	2015	China	TSH > 5.12 mIU/L, FT_4 normal	50/51	< 12	A					
Li (27)	2016	China	TSH > 2.5 mIU/L, FT_4 normal	200/150	38-41			C	D	E	
Chen and Gao (22)	2013	China	TSH > 2.5 mIU/L, FT_4 normal	40/60	unknow			C	D	E	
Li et al (25)	2008	China	TSH > 4.2 mIU/L, FT_4 normal	18/176	16-20	A					
Wan and Chen (26)	2013	China	TSH > 2.5-10 mIU/L, FT_4 normal	50/50	5-20		B		D		

SCH: subclinical hypothyroidism, GW: gestational weeks, TSH: thyroid stimulating hormone, FT_4: tetraiodothyronine

Outcomes; A: intelligence and growth; B: birth weight; C: low birth weight; D: preterm labor; E: intrauterine distress; F: fetal growth restrictriction

Table 3. Risk of bias

Author	Representativeness of exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Demonstration that outcome of interest was not present at start of study	Comparability of cohorts on the basis of the design or analysis	Assessment of outcome	Was follow-up long enough for outcomes to occur	Adequacy of follow up of cohorts
Ajmani et al, 2014 (15)	Somewhat representative	Same community	Secure record	Yes	Study controls for maternal age and BMI	Record linkage	Yes	Complete follow-up
Casey et al, 2005 (16)	Somewhat representative	Same community	Secure record	Yes	study does not control for additional factors	Record linkage	Yes	Complete follow-up
Chen et al, 2015 (18)	Somewhat representative	Same community	Secure record	Yes	Study controls for maternal age	Record linkage	Yes	Complete follow-up
Chen et al, 2014 (17)	Somewhat representative	Same community	Secure record	Yes	Study controls for maternal age	Record linkage	Yes	Complete follow-up
Cleary-Goldman et al, 2008 (5)	Somewhat representative	Same community	Secure record	Yes	Study controls for maternal age	Record linkage	Yes	Lost to follow-up unlikely to introduce bias
Sahu et al, 2010 (19)	Somewhat representative	Same community	Secure record	Yes	Study control for weight and age	Record linkage	Yes	Lost to follow-up rate >20%, and no description of those lost
Saki et al, 2014 (10)	Somewhat representative	Same community	Secure record	Yes	Study does not control for additional factors	Record linkage	Yes	Complete follow-up
Su et al, 2011 (20)	Somewhat representative	Same community	Secure record	Yes	Study control for age	Record linkage	Yes	Complete follow-up
Wang et al, 2012 (21)	Truly representative	Same community	Secure record	Yes	Study does not control for additional factors	Record linkage	Yes	Lost to follow-up unlikely to introduce bias
Xia et al, 2015 (24)	Somewhat representative	Same community	Secure record	Yes	Study control for age	Record linkage	Yes	Complete follow-up
Li et al, 2015 (23)	Somewhat representative	Same community	Secure record	Yes	Study control for age	Record linkage	Yes	Complete follow-up
Li, 2016 (27)	Somewhat representative	Same community	Secure record	Yes	Study control for age	Record linkage	Yes	Complete follow-up
Chen and Gao, 2013 (22)	Somewhat representative	Same community	Secure record	Yes	Study control for age	Record linkage	Yes	Complete follow-up
Li et al, 2008 (25)	Somewhat representative	Same community	Secure record	Yes	Study control for age	Record linkage	Yes	Complete follow-up
Wan and Chen, 2013 (26)	Somewhat representative	Same community	Secure record	Yes	Study control for age	Record linkage	Yes	Complete follow-up

BMI: body mass index

Table 4. Pooled relative risk with 95% confidence interval comparing pregnant women with subclinical hypothyroidism to pregnant euthyroid women

Outcomes	I^2	p	Pooled RR [95%CI]	Studies
MDI	47%	0.0007	-6.08 [-9.57 ~ -2.58]	(18,24,25)
PDI	47%	< 0.00001	-7.29 [-10.30 ~ -4.28]	(18,24,25)
BW	50%	0.001	-0.27 [-0.44 ~ -0.11]	(10,19,24,26)
LBW	58%	0.04	1.78 [1.04-3.07]	(5,15,17,20,21,22,27)
Preterm labor	61%	0.0006	1.96 [1.34-2.88]	(5,10,15,16,19,20,21,22,26,27)
IUGR	0%	< 0.0001	2.4 [1.56-3.7]	(10,15,17)
Fetal distress	20%	0.003	1.66 [1.19-2.31]	(17,19,20,22,27)

RR: relative risk, MDI: Mental Developmental Index, PDI: Psychomotor Developmental Index, BW: birth weight, LBW: low birth weight, IUGR: intrauterine growth restriction, CI: confidence interval

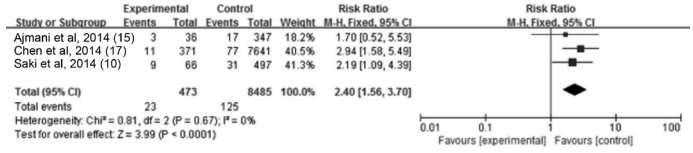

Figure 4. The forest plot of relative risk and 95% confidence of pooled studies comparing pregnant women with subclinical hypothyroidism to euthyroid pregnant women for risk of fetal growth restriction

CI: confidence interval

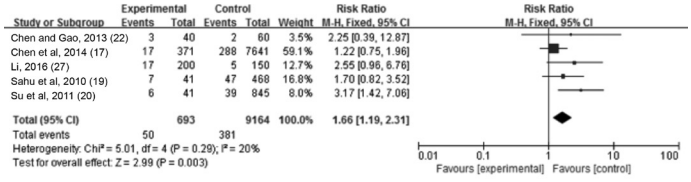

Figure 5. The forest plot of relative risk and 95% confidence interval of pooled studies comparing pregnant women with subclinical hypothyroidism to euthyroid pregnant women for risk of fetal intrauterine distress

CI: confidence interval

in five studies. Meta-analysis of these studies showed minor heterogeneity among results ($I^2 = 20\%$). Thus, a fixed-effect model was used and demonstrated a combined RR of 1.66 [95% CI (1.19, 2.31), p = 0.003], as shown in Figure 5.

The association between maternal SCH and premature delivery: Data on the number of preterm and term infants were provided in ten studies. Meta-analysis of these studies revealed moderate heterogeneity (p = 0.006, $I^2 = 61\%$). Thus, a random-effect model was used and demonstrated a combined RR of 1.96 [95% CI (1.34, 2.88), p = 0.0006]. The forest plot and funnel forest plot are displayed in Figure 6A and 6B. The funnel plot of this study is symmetrical, indicating that there is no publication bias.

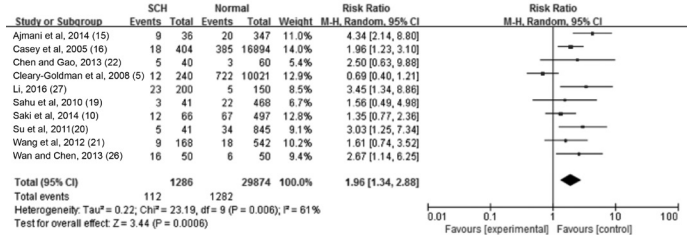

Figure 6A. The forest plot of relative risk and 95% confidence interval of pooled studies comparing pregnant women with subclinical hypothyroidism to euthyroid pregnant women for risk of premature delivery

CI: confidence interval

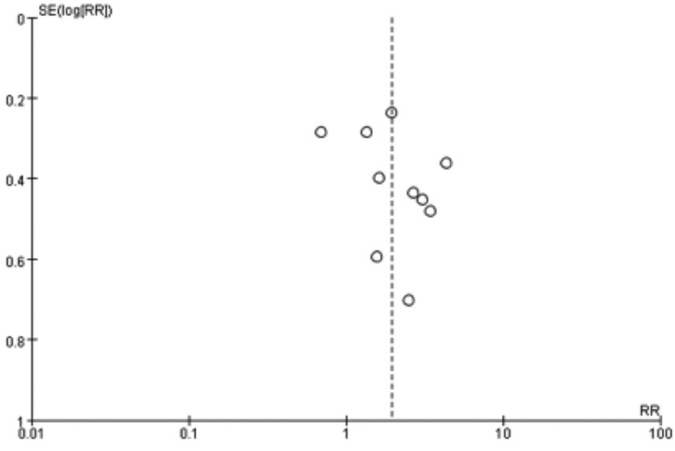

Figure 6B. The funnel plot of pooled studies comparing pregnant women with subclinical hypothyroidism to euthyroid pregnant women for risk of premature delivery

RR: relative risk

Discussion

TH is an important hormone in human metabolism throughout the whole lifespan. It is a particularly important

factor for brain development and corporal growth in the fetus. The first stage of the fetal brain growth-spurt occurs during the first and second trimesters of pregnancy. At this time TH is provided to the fetus mainly from transplacental delivery of THs (mainly T_4), because fetal thyroid follicular epithelial cells are immature and cannot yet synthesize TH during the first 12 weeks of pregnancy (3). An insufficient supply of maternal THs during this period can cause significant and irreversible neurodevelopmental defects. Vulsma et al (28) showed that the umbilical cord level of T_4 in fetuses with congenital hypothyroidism due to a total organification defect or thyroid agenesis was 30% to 60% that of normal fetuses, suggesting that maternal input of T_4 continues until birth. Therefore, brain development during late pregnancy is driven by both fetal and maternal THs. Deficient maternal THs in late pregnancy can cause neurodevelopmental defects, although the effect may not be as serious as the impact of maternal thyroid deficiency during the first trimester of pregnancy. Children of pregnant women with overt hypothyroidism were found to have a lower level of physical and intellectual development, as well as a lower level of responsiveness to external stimuli compared with children of pregnant women with normal thyroid function (4,5,6). It is unclear whether the same effect is true for children of women with SCH. Some investigators speculate that although women with SCH may have normal FT_4 levels, the increased TSH suggests these women need higher TH levels to ensure fetal development (29). Currently, overt hypothyroidism in pregnancy is an indication for TH replacement therapy. For women with SCH, however, it remains unclear whether TH supplementation would improve the developmental status of the child. Some investigators believe that thyroxine treatment would ameliorate adverse pregnancy outcomes, and other investigators believe the contrary (30,31). Our meta-analysis suggests that SCH is associated with delayed child intellectual and motor development, but the follow-up time of studies included in this meta-analysis was only up to two years of age, thus the impact of maternal SCH in the long-term was not investigated.

T_3 and T_4 promote the growth of long bones and teeth by stimulating the development of ossification centers. In addition, THs enhance glycogenolysis and inhibit glycogen synthesis by enhancing intestinal glucose absorption as well as by increasing glucose use by peripheral tissues. Thus, TH deficiency is a risk factor for fetal growth restriction and for LBW, both of which are risk factors for unsatisfactory neurological, motor, and intellectual development (2,32,33). Ohashi et al (34) reported a probability of 25% for fetal growth restriction in pregnant women with abnormal thyroid function and a probability of 16.25% for fetal growth restriction in pregnant women with SCH. Our study showed a 2.4-fold risk increase for fetal growth restriction in pregnant women with SCH as compared to pregnant women with normal thyroid function and a 1.78-fold risk for LBW. These results are consistent with those reported by Leung et al (35).

Zhang (36) reported a 6.62 - 7.86% incidence of abnormal thyroid function, SCH being the most common abnormality, prior to gestational week 20 in women without a previous history or family history of thyroid disease. SCH in pregnancy may be asymptomatic but, nevertheless, can negatively affect fetal neurodevelopment. Therefore, screening for thyroid abnormalities should be performed in the first trimester of pregnancy and if the TSH shows an abnormal level, more attention should be paid to the growth and development of the fetus. Some authors suggest that screening for SCH in pregnancy may be a cost-effective strategy in a wide range of circumstances (37). The effect of the intervention depends on the timing during gestation. Therefore, we suggest that there is an urgent need for large-scale, randomized trials to measure the intelligence of children whose mothers with SCH who were treated with thyroxine during pregnancy versus normal children,in theory,it should also be compared with non-intervention group, but this does not sound ethical to us, we may only be able to verify it in animal experiments.

Some studies suggest that SCH or elevated TSH in pregnancy result in premature delivery (15,38,39) while other studies reached contrary conclusions (10,19). Our meta-analysis showed that maternal SCH is associated with premature delivery. Maraka et al (14) also published a meta-analysis, showing that SCH during pregnancy is associated with multiple adverse maternal and neonatal outcomes. The effect of levothyroxine therapy in preventing these adverse outcomes remains uncertain. It is therefore necessary to do a large-scale trial to assess the value of levothyroxine therapy. In this study different TSH strata should be explored to identify the optimal treatment threshold, where the benefits of levothyroxine use outweigh the risks (40).

Study Limitations

The selection bias was small in this study due to a review of a vast literature and strict adherence to pre-specified inclusion and exclusion criteria. However, every meta-analysis has limitations. In our study, we did not take into account the effects of anti-thyroid antibodies, including anti-thyroglobulin antibodies and anti-thyroid peroxidase antibodies. As a result, we might have underestimated the effect of maternal SCH on growth and development or

intelligence level. In addition, the studies included in our analysis did not provide data on family economic status, parental education level, or the environment. All of these may be confounding factors affecting child development. Also, measurement methods and measuring instruments varied across the studies included in our analysis. This, too, might have affected the results.

Our meta-analysis suggests that maternal SCH is associated with fetal growth restriction, impaired intellectual and motor development, LBW, premature delivery and fetal distress. Therefore, more extensive screening for thyroid function during or even prior to pregnancy may be especially important for improved outcomes. We speculate that TH supplementation may promote normal fetal development and may prevent adverse pregnancy outcomes. As this is only speculation, large-scale randomized trials are needed.

Conclusion

Our meta-analysis showed that maternal SCH in pregnancy is associated with an increased risk of several adverse neonatal outcomes. Thus, it is necessary to assess thyroid function in pregnant women and intervene when necessary. Because of the high methodological quality of the included trials, our data provide supportive evidence for initiating further investigation. Additional cohort studies including large numbers of participants are needed to guide future investigation.

Acknowledgments

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Ethics

Ethics Committee Approval: All analyses were based on previous published studies, thus no ethical approval is required.

Informed Consent: All analyses were based on previous published studies, thus no patient consent are required.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Hui Chen, Design: Hui Chen, Data Collection or Processing: Yahong Liu, Fupin Li, Analysis or Interpretation: Yahong Liu, Chen Jing, Literature Search: Yahong Liu, Chen Jing, Writing: Yahong Liu.

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Psychometric Properties of the Turkish Version of the University of Virginia Parent Low Blood Sugar Survey

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What is already known on this topic?

It is well known that fear of hypoglycemia causes various problems in achieving metabolic control.

What this study adds?

This study showed that the Turkish Parent Low Blood Sugar Survey would aid pediatric diabetes nurses in Turkey in the evaluation of the fear of hypoglycemia experienced by parents.

Abstract

Objective: The aim of this study was to produce and validate a Turkish version of the University of Virginia Parent Low Blood Sugar Survey (P-LBSS). The P-LBSS is used to assess parental fear of their diabetic children's hypoglycemia.
Methods: Linguistic, content and face validity of the translated P-LBSS was tested. Afterwards, explanatory and confirmatory factor analyses were conducted in order to evaluate construct validity.
Results: The sample included 390 parents of type 1 diabetic adolescents aged 12-17 years. Results of the factor analysis showed that the Turkish P-LBSS had 2 subscales (behavior and worry) as in the original. The Cronbach's alpha coefficient of the Turkish version of the total P-LBSS was found to be 0.803, and the value was 0.865 for the behavior and 0.790 for the worry subscales. Psychometric investigation of the Turkish version of P-LBSS indicated high reliability and good retestability, content and construct validity.
Conclusion: The Turkish P-LBSS is a valid and reliable instrument to measure the fear of hypoglycemia experienced by parents of diabetic adolescents aged between 12-17 years in the Turkish population.
Keywords: Type 1 diabetes mellitus, adolescent, validity, reliability, Turkey

Introduction

Type 1 diabetes mellitus (T1DM) mostly presents during childhood and adolescence (1,2). Although seen in every age group, T1DM usually presents between 7 and 15 years of age (3). Factors such as having a chronic disease, poor adaptation to disease, lifestyle changes, the burden of disease on social life, lowered self-esteem, and fear of complications may negatively affect diabetes management (4,5).

Despite the recent progress in the treatment of T1DM, children and adolescents diagnosed with it are at risk of psychological problems due to the difficulties of diabetes management, such as diet adherence, blood glucose monitoring, insulin administration, physical exercise and self-care (5). Having diabetes brings about changes for the child and his/her family in their daily activities and lifestyle (5,6,7,8,9,10,11,12).

Hypoglycemia is the most commonly seen acute complication in children with T1DM and partially results from intense treatment regimes (13,14,15,16,17). Although transient, hypoglycemia, if untreated, may result in serious morbidity (16,18,19,20). Fear of hypoglycemia negatively

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affects quality of life (15,21,22,23). Patients and families have been reported to deliberately keep blood glucose levels high, despite having been informed of the risks, using strategies such as decreasing insulin doses, overeating, limiting activities and measuring blood glucose very often (16,19,22,24,25,26). It has been reported that parents may measure blood glucose repeatedly at night, increasing both patient and parental stress (15,17,27,28,29).

Diabetes training nurses have a major role to play in educating families and alleviating the fear of hypoglycemia and provide support to affected families (1,30,31). The Virginia Parent Low Blood Sugar Survey (P-LBSS) was developed by Gonder-Frederick et al (16) to assess parental attitudes to childrens' hypoglycemia.

The aim of this study was to assess a Turkish adaptation of the P-LBSS and to evaluate the fear of hypoglycemia experienced by parents of a large cohort of T1DM adolescents.

Methods

Methodological design was used to determine the validity and reliability of instruments in order to measure constructs used as variables in research (32).

Virginia P-LBSS: The P-LBSS consists of 25 questions in two subscales: the behavior subscale (questions 1-10) and the worry subscale (questions 11-25). Responses are scored on a Likert type scale (never: 0, rarely: 1, sometimes: 2, often: 3, almost always: 4). Total scores range between 0-100 with higher total scores indicating increased fear of hypoglycemia (16). The Cronbach's alpha reliability coefficients of the original total scale, the behavior subscale, and the worry subscale were found to be 0.89, 0.76 and 0.91, respectively, indicating good reliability.

The questionnaire was translated into Turkish by two competent English teachers. In order to test content validity, the scale was assessed for comprehensibility by 18 experts. The experts scored each item on a scale of 1 to 4 in accordance with the Davis technique (1992) (33). Finally, the scale was completed by 15 adolescents to evaluate scale legibility. Small corrections were made to achieve face validity. In addition, a Parent Identification Form was prepared in line with the literature (1,14,30) which consisted of 15 items with seven open ended and eight closed questions about sociodemographic characteristics.

Parents of adolescents aged between 12-17 years with T1DM were invited to participate in this study. Subjects were recruited from five hospitals, three in İstanbul and two in İzmir. Since adaptation to a chronic disease takes up to one year, parents of adolescents with a diabetes duration of one year or more, who regularly received routine three monthly follow-up care, were invited to participate. Sample size for questionnaire adaption studies should be between twice and ten times the number of questions included (34,35,36). The P-LBSS used in this study has 25 items and the aim was to recruit at least 250 families.

The data were collected between March and June 2016. Completion of the test took 15 to 20 minutes. For test retest reliability, the scale was repeated by the same parents three weeks later.

Ethical Considerations

Ethical board permission was taken from the Marmara University Health Sciences Institute Ethical Board (IRB no: 26.10.2015-14). Written permission from the institutions where the study would be conducted were obtained. Prior to data collection, the participants were informed about the study. Parents who agreed to participate in the study gave written informed consent.

Statistical Analysis

Number Cruncher Statistical System 2007 (Kaysville, Utah, USA) was used for statistical analyses. The expert views on the content validity of the scale were evaluated using the content validity index (CVI) (36). In order to evaluate structure validity, confirmatory and explanatory factor analyses were performed. In the reliability analysis, the Cronbach's alpha values and item-total score correlation coefficients were calculated. In test-retest reliability, the Intraclass Correlation Coefficient (ICC) was calculated. Descriptive statistical analyses [mean, standard deviation (SD), percentages] were also used. Data was evaluated at a 95% confidence interval. Significance level was set at $p < 0.05$.

Results

Mean age of the mothers and fathers was 42.44 ± 6.44 (range 29-56) and 46.37 ± 6.51 (28-56) years respectively. Mean $\pm$ SD time since diagnosis was 5.96 ± 3.47 (range 1-17) years. Mean hemoglobin A1c value was found to be $8.96 \pm 1.82\%$. Expert assessment of the questionnaire by CVI scoring gave a maximum value of 1.00.

Construct validity: Explanatory and confirmatory factor analyses were performed to test construct validity.

Explanatory factor analysis: The Kaiser-Meyer-Olkin (KMO) coefficient was found to be 0.881. Thus, it was shown that the sample size was sufficient for factor analysis. The Bartlett's sphericity test was found to be significant

($p < 0.05$), showing that the data set had multivariate normality (37,38). The result of the Bartlett's sphericity test in this study was found to be $\chi^2 = 3630$, df = 325, $p = 0.001$. A Varimax rotation was used and the scale was found to have two factors as in the original scale, factor 1 being the behavior subscale and factor 2 the worry subscale. Factor loadings in factor 1 were found to range between 0.315 and 0.879 and in factor 2 between 0.304 and 0.638 (Table 1). The two-factor structure explained 39.1 % of the total variance. Factor loadings were acceptable (Table 1).

Confirmatory factor analysis: Confirmatory factor analysis confirms the factors determined in explanatory factor analysis (39). For the structural validity of a scale to be confirmed, the "Goodness of fit statistics", which can be obtained via confirmatory factor analysis, should be at acceptable levels. In the current study, the factor model fit the data ($p < 0.001$) and the Root Mean Square Error of Approximation (RMSEA) was 0.086 (Figure 1). The χ^2/df index was found to be 3.88.

Reliability: Item-total correlations were found to vary between 0.019 and 0.595 (Table 1). The Cronbach's alpha coefficients of the scale are given in Table 2. The test-retest reliability results are given in Table 3. According to the results of the ICC analysis, the level of consistency between

Table 1. Factor Loads and Item to total correlation of University of Virginia Parent Low Blood Sugar Survey

Item no	Items	Factor loads		Item to total correlation
		Factor 1 (behavior)	Factor 2 (worry)	
1	Have my child eat large snacks at bedtime	0.674	-	0.293
2	Avoid having my child being alone when his/her sugar is likely to be low	0.315	-	0.312
3	Allow my child's blood sugar to be a little high to be on the safe side	0.768	-	0.477
4	Keep my child's sugar higher when he/she will be alone for a while	0.841	-	0.522
5	Have my child eat something as soon as he/she feels the first sign of low blood sugar	0.348	-	0.204
6	Reduce my child's insulin when I think his/her sugar is too low	0.745	-	0.466
7	Keep my child's blood sugar higher when he/she plans to be away from me for a while	0.879	-	0.537
8	Have my child carry fast-acting sugar	0.550	-	0.362
9	Have my child avoid a lot of exercise when I think his/her sugar is low	0.617	-	0.396
10	Check my child's sugar often when he/she plans to go on an outing	0.699	-	0.352
11	Child will not be able to recognize that he/she is having symptoms of hypoglycemia	-	0.358	0.289
12	Child is not carrying food, fruit, or juice with him/her	-	0.304	0.019
13	Child feeling dizzy or passing out in public	-	0.543	0.391
14	Child having a low blood sugar while asleep	-	0.565	0.362
15	Child embarrassing self or friends/family in a social situation	-	0.434	0.396
16	Child having a low while alone	-	0.595	0.417
17	Child appearing to be "stupid" or clumsy	-	0.454	0.357
18	Child losing control of behaviour due to low blood sugar	-	0.482	0.501
19	No one being around to help my child during a low		0.587	0.322
20	Child making a mistake or having an accident at school	-	0.638	0.370
21	Child getting a bad evaluation at school because of something that happens when his/her sugar is low	-	0.464	0.303
22	Child having seizures or convulsions	-	0.617	0.052
23	Child developing long term complications from frequent low blood sugar	-	0.599	0.220
24	Child feeling light-headed or faint	-	0.597	0.595
25	Child having a low	-	0.636	0.335

Table 2. Internal consistency of University of Virginia Parent Low Blood Sugar Survey

	Number of items	Internal consistency (Cronbach's alpha)
Behaviour	10	0.865
Worry	15	0.790
Total scale	25	0.803

Table 3. Test-retest reliability of University of Virginia Parent Low Blood Sugar Survey

n = 79	First measurement	Second measurement	ICC (%95 CI)	p
	Mean ± SD	Mean ± SD		
Behaviour	2.72 ± 0.85	2.75 ± 0.84	0.924	**0.000**
Worry	2.18 ± 0.74	2.24 ± 0.79	0.749	**0.000**
Total scale	2.39 ± 0.59	2.44 ± 0.63	0.825	**0.000**

p < 0.001

CI: confidence interval, ICC: intraclass correlation coefficient, SD: standard deviation

the first and last measurements was 92.4% for the behavior subscale, 74.9% for the worry subscale and 82.5% for the total scale.

Discussion

The main characteristics sought in a good instrument are reliability and validity (32). In the current study, the psychometric properties of the Turkish version of the P-LBSS were examined in detail.

Content validity represents the universe of content or the domain of given constructs (40,41). A CVI value above 0.80 indicates good content validity (36). In the present study, the CVI was found to be 1.00 indicating excellent content validity.

In the current study, the KMO and the Bartlett's sphericity test were used to evaluate sample size adequacy. KMO may range from 0 to 1, with higher values indicating appropriate sample size (34,38). In the literature, KMO values between 0.80 and 0.89 reflect a "very good" sample size (35).

In this study, the KMO was found to be 0.881, indicating sample size adequacy. The Bartlett's sphericity test was used to test the hypothesis that correlation matrices were similar, and this hypothesis was rejected at a value of p = 0.001. The results of the Bartlett's test being p < 0.01 showed that measurement was not affected by the sample size and that the sample size was adequate for factor analysis.

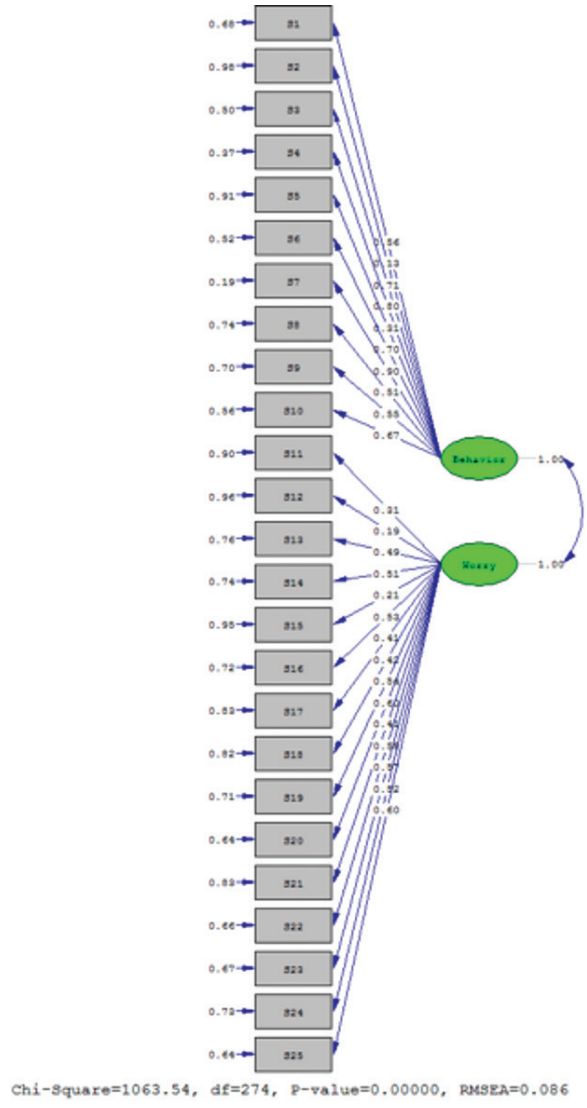

Figure 1. Path diagram of confirmatory factor analysis for University of Virginia Parent Low Blood Sugar Survey

Construct validity was tested using explanatory and confirmatory factor analysis. The factor analytical approach is a procedure that provides information about the extent to which a set of items measure the same underlying construct (40,41).

The Varimax rotation method showed that the two-factor structure explained 39.1% of the total variation. Higher variance rates indicate a stronger factor structure.

Factor loadings of items in factor 1 ranged between 0.315 and 0.879, and in factor 2 between 0.304 and 0.638. Factor loadings should be a minimum of 0.30 in scale development and adaptation to discriminate between the characteristics to be measured (38), thus adequate discrimination was present. In the interpretation of goodness of fit indices in confirmatory factor analysis, the RMSEA fit index is used

to assess goodness of fit indices in confirmatory factor testing with $0 < RMSEA < 0.05$ showing good fit, while $0.05 \leq RMSEA \leq 0.10$ shows acceptable fit (38,42). The RMSEA value of our instrument was 0.086 indicating acceptable fit. Similarly, a χ^2/df fit index of ≤3 shows perfect fit and ≤5 shows good fit (43). The χ^2/df value for our questionnaire was 3.88 indicating good fit.

Reliability of an instrument refers to the extent to which the instrument yields consistent results on repeated measures (40). There are five major techniques for reliability testing. These techniques include test-retest reliability, parallel or alternate forms, item-total correlation, split-half reliability, Kuder-Richardson-20, Cronbach's alpha, and inter-rater reliability (40). In the current study, the Cronbach's alpha coefficient, item-total correlations and test-retest reliability were used. In scale development and adaptation, scales with Cronbach's alpha values at and above 0.70 are accepted as reliable (38). Accordingly, the Cronbach's alpha reliability coefficients of the total scale and the behavior and worry subscales were found to be acceptable (Table 2). These values showed that the scale is a reliable instrument and parallel results were obtained with the original scale.

Item-total correlations show the reliability of each item in a scale (36). An item total score correlation of 0.30 and above shows that the items are adequate for measuring the desired characteristic and that the items are consistent with the total scale (38,39). In the present study, the item-total correlations ranged from 0.019 to 0.595 with four questions giving values below 0.30. However, since the factor structure was tested using confirmatory factor analysis, and since the Cronbach's alpha coefficient of the scale was above 0.70, the original structure of the scale was maintained. Thus, the four items were not removed from the scale.

Test-retest reliability is used for evaluating the consistency of the scale over time, with values above 0.70 indicating good retest reliability (38). The correlation values obtained in this study indicated perfect correlation and demonstrated the consistency of scale scores over time (Table 3).

The usability of the scale should also be tested in parents of adolescents with a diabetes duration of less than one year and adolescents with co-morbid disorders such as celiac disease or hypothyroidism.

Conclusion

The Turkish version of the P-LBSS had high reliability and good content and construct validity. The Turkish P-LBSS is a valid and reliable instrument to measure the fear of hypoglycemia experienced by parents of T1DM adolescents in the Turkish population. Additionally, the P-LBSS, which is easy for pediatric diabetes nurses to use, can help in evaluating parental fear of childrens' hypoglycemia. Thus, appropriate psychological help could be provided. Use of this questionnaire may have the effect of improving the quality of adolescent diabetic nursing care in Turkey.

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Ethics

Ethics Committee Approval: Ethical board permission was taken from the Marmara University Health Sciences Institute Ethical Board (IRB no: 26.10.2015-14).

Informed Consent: Parents who agreed to participate in the study gave written informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Design: Nesrin Şen Celasin, Çağrı Çövener Özçelik, Data Collection or Processing: Nesrin Şen Celasin, Şükriye Şahin, Analysis or Interpretation: Çağrı Çövener Özçelik, Literature Search: Nesrin Şen Celasin, Çağrı Çövener Özçelik, Writing: Nesrin Şen Celasin, Çağrı Çövener Özçelik.

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CASE REPORT

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Neonatal Diabetes: Two Cases with Isolated Pancreas Agenesis due to Homozygous PTF1A Enhancer Mutations and One with Developmental Delay, Epilepsy, and Neonatal Diabetes Syndrome due to KCNJ11 Mutation

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What is already known on this topic?

Permanent neonatal diabetes can be due to either disruption of pancreas development or of insulin secretion. Pancreas-specific transcription factor 1A is a transcription factor which is required for normal development of the pancreas. Mutations at this site cause pancreas agenesis. Closure of ATP sensitive potassium (K) channels depolarize the cell membrane and subsequently open voltage-dependent Ca channels that trigger release of stored insulin granules. KCNJ11 activating mutations result in the ATP-sensitive K channel remaining open and disrupting insulin secretion.

What this study adds?

In two patients with neonatal diabetes and exocrine pancreas insufficiency homozygous g. 23508363 > G and g.23508437A > G mutations in the distal pancreas-specific transcription factor 1A enhancer were identified. A previously reported heterozygous KCNJ11 missense mutation, p.C166Y, was identified in the third patient who had developmental delay, epilepsy, and neonatal diabetes syndrome. In patients with neonatal diabetes genetic causes should be investigated not just for finding the underlying cause but also for planning treatment.

Abstract

Neonatal diabetes mellitus is a rare form of monogenic diabetes which is diagnosed in the first six months of life. Here we report three patients with neonatal diabetes; two with isolated pancreas agenesis due to mutations in the pancreas-specific transcription factor 1A (PTF1A) enhancer and one with developmental delay, epilepsy, and neonatal diabetes (DEND) syndrome, due to a KCNJ11 mutation. The two cases with mutations in the distal enhancer of PTF1A had a homozygous g.23508363A > G and a homozygous g.23508437A > G mutation respectively. Previous functional analyses showed that these mutations can decrease expression of PTF1A which is involved in pancreas development. Both patients were born small for gestational age to consanguineous parents. Both were treated with insulin and pancreatic enzymes. One of these patients' fathers was also homozygous for the PTF1A mutation, whilst his partner and the parents of the other patient were heterozygous carriers. In the case with DEND sydrome, a previosly reported heterozygous KCNJ11 mutation, p.Cys166Tyr (c.497G > A), was identified. This patient was born to nonconsanguineous parents with normal birth weight. The majority of neonatal diabetes patients with KCNJ11 mutations will respond to sulphonylurea treatment. Therefore Glibenclamide, an oral antidiabetic of the sulphonylurea group, was started. This treatment regimen relatively improved blood glucose levels and neurological symptoms in the short term. Because we could not follow the patient in the long term, we are not able to draw conclusions about the efficacy of the treatment. Although neonatal diabetes mellitus can be diagnosed clinically, genetic analysis is important since it is a guide for the treatment and for prognosis.

Keywords: Neonatal diabetes, PTF1A, pancreas agenesis, KCNJ11

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Introduction

Neonatal diabetes mellitus (NDM) is a rare form of monogenic diabetes that can be caused by mutations in different genes and presents in the first six months of life (1). There are two main clinical groups; transient NDM (TNDM) and permanent NDM (PNDM). TNDM is a developmental insulin production disorder that resolves spontaneously postnatally. PNDM does not go into remission. The underlying genetic defect can be found in most of the patients with TNDM. The majority of cases with TNDM are due to methylation defects in the imprinted region on chromosome 6q24; these can be either paternal uniparental disomy, paternal duplication, or defective methylation of the maternal allele (2).

PNDM is a genetically heterogeneous disorder due to mutations in 23 different genes described to date: *KCNJ11*, *ABCC8*, *FOXP3*, *GCK*, *PDX1*, pancreas-specific transcription factor 1A *(PTF1A)*, *EIF2AK3*, *SLC2A2*, *GATA6*, *GATA4*, *SLC19A2*, *WFS1*, *NEUROD1*, *NEUROG3*, *RFX6*, *LRBA*, *NKX2-2*, *MNX1*, *IER3IP1*, *INS*, *STAT3*, *GLIS3* and *HNF1B* (3,4,5,6,7,8,9). These mutations can either compromise insulin secretion, disturb pancreas or islet cell development or result in autoimmune destruction of the beta cells. Genes associated with pancreatic agenesis are *PDX1, PTF1A, RFX6, HNF1B* and *GATA6* (5,6). Disruption of pancreas development leads to exocrine as well as endocine pancreatic insufficiency. Mutations in the genes encoding the ATP-sensivitive potassium channel (K_{ATP}) subunits, *KCNJ11 (Kir6.2), ABCC8* sulphonylurea receptor 1 *(SUR1)* and *INS* (insulin) compromise insulin secretion by affecting the mechanisms involved in insulin secretion (10,11,12,13,14).

In this report we describe three patients with neonatal diabetes. Two of these cases had isolated pancreatic agenesis due to mutations in a distal enhancer of the *PTF1A* gene. The third patient had normal pancreatic development and additional neurological symptoms due to a mutation in the *KCNJ11* gene.

Case Reports

Case 1

This patient was a female infant born to consanguineous parents (first cousins). She was born at 37 weeks gestation by normal vaginal delivery with a birth weight of 1900 g. After birth she was followed in the neonatal intensive care unit for respiratory distress and hyperglycemia. She was treated with subcutaneous (SC) regular insulin. She has two healthy siblings. There was no family history of diabetes.

At the age of one month she was referred to our clinic because of uncontrolled high blood glucose levels. On admission her body weight was 2330 g [-3.05 standard deviation score (SDS)], height was 47 cm (-2.67 SDS), head circumference was 35 cm (-1.72 SDS). Her physical examination was normal, except for hip dysplasia.

Laboratory tests revealed a venous glucose level of 354 mg/dL with glycosuria. She did not have ketonuria or acidosis. Serum C-peptide level was 0.01ng/mL (normal range: 0.9-4.3 ng/mL), hemoglobulin A1c (HbA1c) was 7.3% (normal range: 4.8-6%) and diabetes autoantibody tests [anti glutamic acid decarboxylase (GAD), islet cell antibodies (ICA), insulinoma antigen-2 (IA2)] were negative. Hb level was 9.5 mg/dL, and mean corpuscular volume (MCV) was 85.1 fL (normal range: 81-99 fL). The peripheral blood smear showed no signs of megaloblastic anemia. Serum folic acid, thiamine and vitamin B12 levels were normal. Serum thyroid hormones were within normal limits [thyrotrophin-stimulating hormone (TSH): 1.75 mIU/L, fT4: 1.06 ng/dL]. Renal and hepatic function tests were all within normal ranges. The patient was diagnosed as a case of neonatal diabetes and insulin regimen was changed from SC NPH insulin, with which blood glucose levels could not be stabilized, to detemir insulin, with rapid acting insulin adjustment when needed. But this regimen was also not successful in controlling the blood glucose levels. Finally detemir insulin was replaced with glargine insulin (1.0 U/day) that achieved more stable blood glucose levels. Humalog insulin (0.5 U/dose) was added when needed. Insulin doses were adjusted according to blood glucose levels. The patient also had episodes of significant diarrhea and stool tests revealed malabsorption. Abdominal ultrasonography revealed normal liver and kidneys, but the pancreas could not be visualized. Her echocardiography was normal. For exocrine pancreas insufficiency, enzyme replacement therapy was added to her treatment to which she responded well (Table 1). At her last visit she was 3.5 years old, her body weight was 13.9 kg (-0.64 SDS), height was 92.3 cm (-1.57 SDS), head circumference was 48 cm (-1.08 SDS) with normal mental and motor development. Her glucose regulation was in acceptable ranges with a HbA1c level of 7.3 %.

DNA Sequencing and Genetic Analysis

A homozygous g.23508363A>G mutation affecting a highly conserved nucleotide within a previously identified distal enhancer of PTF1A was identified (Figure 1). Previous functional analyses showed that this mutation disrupts enhancer activity and is likely to result in decreased PTF1A expression during pancreatic development (15). This result confirms a diagnosis of neonatal diabetes and exocrine pancreatic insufficiency due to a recessive PTF1A mutation.

Table 1. Clinical features, genetic defects and treatment modules of the patients

Cases	GA (weeks)/ BW (g)	Clinical features	Mutation/gene	Treatment
1	37/1900	Hyperglycemia, exocrine pancreas insufficiency/isolated pancreas agenesis	Homozygous g.23508363A > G/*PTF1A*	Insulin and pancreatic enymes
2	37/1520	Hyperglycemia, exocrine pancreas insufficiency/isolated pancreas agenesis	Homozygous g.23508437A > G/*PTF1A*	Insulin and pancreatic enzymes
3	35/3400	Hyperglycemia, convulsions, developmental delay/DEND syndrome	Heterozygous missense p.C166Y/*KCNJ11*	Glibenclamide and insulin

GA: gestational age, DEND: developmental delay, epilepsy, and neonatal diabetes, BW: body weight

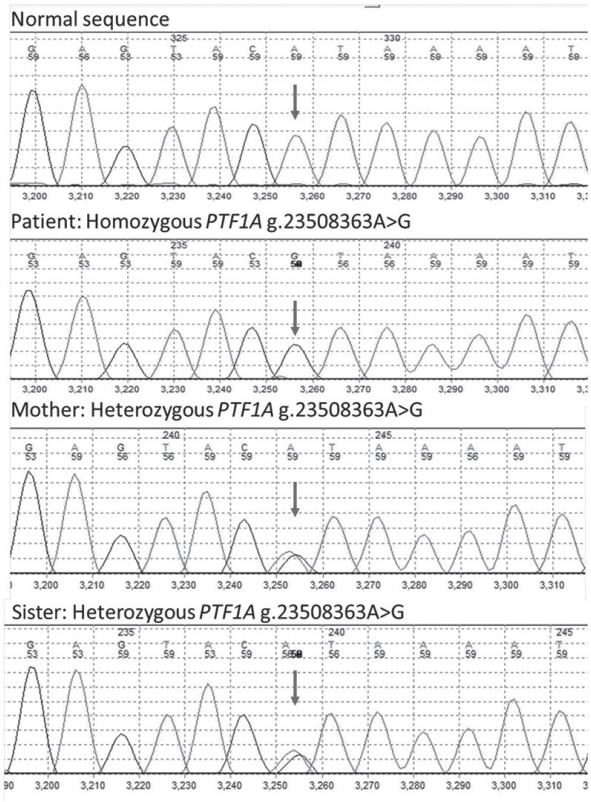

Figure 1. A homozygous g.23508363A > G mutation within distal enhancer of *PTF1* was identified in the first case. Mother and sister were heterozygous for the same mutation

Her mother and sister were heterozygous for the same mutation. Her brother did not carry the mutation. Father's sample was not available for testing.

Case 2

The second case was a female infant born to consanguineous parents (first cousins). She was born at 37 weeks of gestation with a birth weight of 1520 g by C-section delivery due to oligohydramnios and intrauterine growth restriction. She was followed in the neonatal intensive care unit for hyperglycemia and was treated with SC regular insulin. She was a first child with no siblings. There was no family history of diabetes.

She was referred to our clinic for glucose regulation when she was 44 days old. Her body weight was 1980 g (-3.93 SDS), height was 43 cm (-5.06), head circumference was 32 cm (-4.75). Her physical examination was normal.

Laboratory tests showed venous glucose was 300 mg/dL accompanied by glycosuria. She did not have ketonuria or acidosis. Her serum C-peptide level was 0.01 ng/mL (normal range: 0.9-4.3 ng/mL), HbA1c was 7.4% (normal range: 4.8-6%) and diabetes autoantibody tests (antiGAD, ICA, IA2) were negative. Hb level was 8 mg/dL, and MCV was 85 fL (normal range: 81-99 fL). The pheripheral blood smear showed no signs of megalobastic anemia. Serum folic acid, thiamine and vitamin B12 levels were normal. Thyroid function was normal (TSH: 5.8 mIU/L, fT4: 0.9 ng/dL). Renal and hepatic function tests were all within normal ranges. The patient was diagnosed with neonatal diabetes. We started glargine insulin (1 U/day). Humalog insulin (0.5 U /dose) was added when needed. Her mother was eager to use insulin pump therapy. Thus she was trained for it and a better glycemic control was achieved by continous SC insulin pump treatment. She was fed breast milk, thus her baseline insulin dose was 0.125 U/hour which was modified according to her blood glucose levels. Her bolus insulin dose was 0.5 U which was again modified according to her blood glucose levels.

Abdominal ultrasonography and MRI images failed to visualize the pancreas whilst liver and kidneys appeared normal. Her stool tests were significant for malabsorption. Her echocardiography revealed patent foramen ovale, thin patent ductus arteriosus and peripheral pulmonary stenosis. Enzyme replacement treatment for pancreatic insufficiency was added to her treatment regimen and she responded well.

At her last visit she was 4 months old, her body weight was 4500 g (-2.64 SDS), height was 53 cm (-3.92), head circumference was 38 cm (-2.68 SDS) with normal mental and motor development. Both her family and she were adapted to insulin pump therapy (Table 1). Her blood glucose levels were within appropriate levels with an HbA1c level of 7.1%.

DNA Sequencing and Genetic Analysis

A homozygous g.23508437A > G mutation was identified within the distal enhancer of the *PTF1A* gene which is known to affect a highly conserved nucleotide (Figure 2). Previous functional analysis had shown that this mutation disrupts enhancer activity and is likely to result in decreased PTF1A expression during pancreatic development (15). This result confirms a diagnosis of neonatal diabetes and exocrine pancreatic insufficiency due to a recessive PTF1A mutation. The patient's mother was heterozygous for the mutation whilst the unaffected father was also homozygous. One patient with a homozygous g.23508437A > G mutation who developed diabetes in adulthood has been previously reported (15). Her father is therefore at increased risk of developing diabetes and annual monitoring of his HbA1c was recommended. The risk for this couple's next pregnancy to be affected with neonatal diabetes is 1 in 2.

Case 3

The third case was a male infant born to non-consanguineous parents. He was born at 35 weeks gestation by spontaneous vaginal delivery with a birth weight of 3400 g. His seizures, manifesting as arm movements, started when he was one month old which then progressed as tonic clonic convulsions. He was the first and only child of his family and there was no family history of diabetes.

High blood glucose levels and failure to thrive were the reasons for referral to our clinic at the age of three months. On admission body weight was 4460 g (-2.24 SDS), height was 62.5 cm (0.44), head circumference was 40 cm (-0.81 SDS). His physical examination revealed hypotonia and decreased muscle strength of all four extremities. He was not following with his eyes.

Laboratory measurements of venous blood glucose level was 600 mg/dL with glycosuria, which was not accompanied by ketonuria or acidosis. Serum C-peptide level was 0.72 ng/mL (normal range: 0.9-4.3 ng/mL), HbA1c was 11.4% (normal range: 4.8-6%) and diabetes autoantibody tests (antiGAD, ICA, IA2) were negative. Hb level was 11.4 mg/dL, and MCV was 84 fL (normal range: 81-99 fL). The peripheral blood smear showed no signs of megalobastic anemia. Serum folic acid, thiamine and vitamin B12 levels were normal. Serum thyroid hormone measurements were within normal limits (TSH: 1.75 mIU/L, fT4: 1.06 ng/dL). Renal and hepatic function tests were all within normal ranges. In accordance with our previous experience, we started glargine insulin. Humalog insulin was added when needed according to the patient's blood glucose levels. His tonic clonic convulsions continued and were unrelated to blood sugar levels. An electro-encephalogram was performed and phenobarbital was started. Developmental delay, epilepsy and neonatal diabetes suggested developmental delay, epilepsy, and neonatal diabetes (DEND) syndrome. Genetic testing detected a heterozygous missense mutation, c.497G > A p.C166Y, in KCNJ11 which had been previously reported. Glibenclamide, an oral antidiabetic belonging to the sulfonylurea group, was started according to the protocol provided by the Exeter team (available at http://www.diabetesgenes.org/content/genetic-testing-neonatal-diabetes). Glibenclamide dose was gradually increased while insulin dose was decreased (Table 1). With this treatment regimen his blood sugar levels were well controlled and a relative improvement (normal muscle tone, eye contact) in his neurological status was observed at the seven month follow-up visit. At his last visit he was 10 months old, his body weight was 6190 g (-3.39 SDS), height was 74 cm (-0.29 SDS), head circumferance was 44 cm (-1.65 SDS). He was on glibenclamide and insulin treament at doses of 10mg/day and 4 U/day (2 U glargine and 2 U Humalog insulins) respectively.

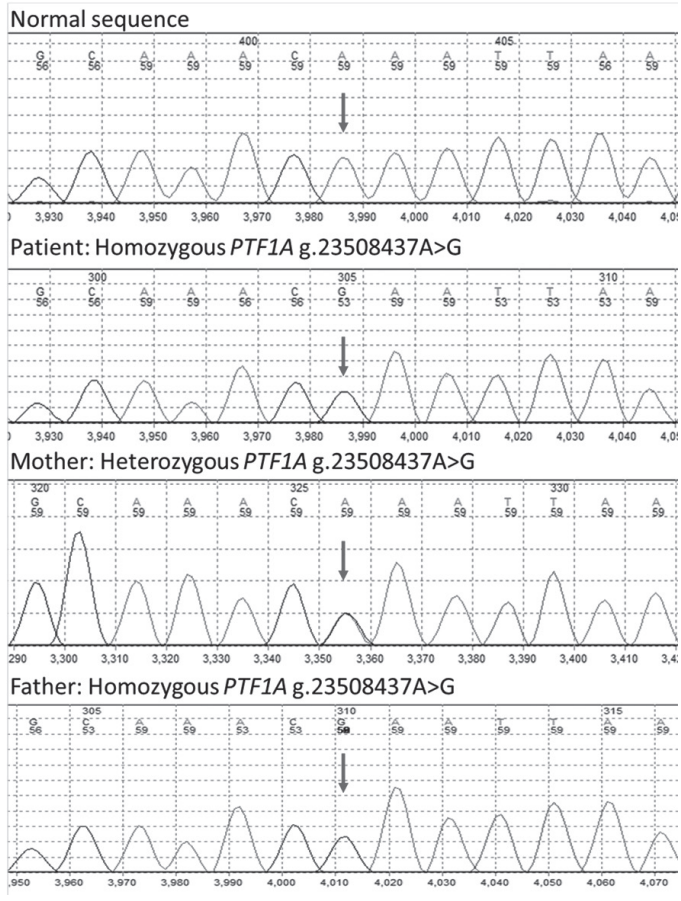

Figure 2. A homozygous g.23508437A > G mutation was identified within the distal enhancer of the *PTF1A* gene in the second case. For the same mutation mother was heterozygous, father was also homozygous

DNA Sequencing and Genetic Analysis

The patient was heterozygous for a previosly reported KCNJ11 missense mutation, p.C166Y (Figure 3). The p.C166Y mutation has been reported previously in patients with DEND syndrome. This mutation is predicted to be pathogenic and the result confirmed a diagnosis of neonatal diabetes, epilepsy and developmental delay due to a mutation in the Kir6.2 subunit of the K_{ATP} channel (16). The inform consent was taken from all the patients' parents for publication.

Discussion

Here we report three cases with neonatal diabetes caused by three different mutations, two homozygous mutations in the PTF1A enhancer and one heterozygous mutation in the *KCNJ11* gene. Their common finding was hyperglycemia before six months of age. Cases 1 and 2 were born small for gestational age and had exocrine pancreatic insufficiency. Case 3 was born with an appropriate for gestational age birth weight and had neurological symptoms. Although all were diagnosed with neonatal diabetes, their clinical findings suggested different modes of disease development.

Heterozygous activating mutations in KCNJ11, encoding the Kir6.2 subunit of the K_{ATP}, are common cause of neonatal diabetes and have been reported as being the reason in 30-58 % of neonatal diabetes cases (12,16,17,18,19). However, in populations with a high incidence of consanguineous marriages, homozygous mutations causing neonatal diabetes appear to be more common (20,21).

Exocrine pancreas insufficieny in the first two cases suggested pancreas agenesis. Thus, homozygous g. 23508363 > G and g.23508437A > G mutations in the distal PTF1A enhancer

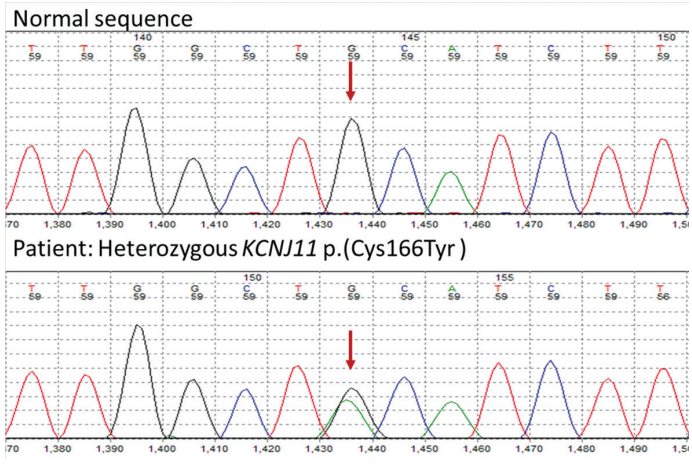

Figure 3. A heterozygous missense mutation, p.C166Y within *KCNJ11* gene was identified in the third casev

were identified. Spatiotemporally regulated expression of transcription factors is important for cell fate specification and organogenesis (22). PTF1A is a transcription factor that is required for the formation of the exocrine pancreas and the correct spatial organization of the endocrine pancreas in mice (23). In mice models, PTF1A inactivation reverted pancreatic cells to intestinal cells, suggesting its function as a switch between pancreatic and intestinal cell fates (24) and PTF1A dose reduction resulted in pancreatic hypoplasia and insufficient insulin secretion in a dosage dependent manner (22). Furthermore, in humans coding mutations in PTF1A were shown to cause neonatal diabetes due to pancreas agenesis (25). Weedon et al (15) have identified six different recessive mutations in a downstream enhancer of PTF1A in 10 families with isolated pancreas agenesis (15). This region acts as a developmental enhancer of PTF1A and the mutations abolish enhancer activity. It was interesting to find the same homozygous mutation in the distal PTF1A enhancer in the healthy father of the second case. However, a patient who developed diabetes in adulthood with a homozygous g.23508437A > G mutation has been previously reported (15). The reason for this situation is not known clearly. But it is well known that there is no genotype phenotype correalation in many genetic diseases. There should be more factors that effect gene functions other than the gene mutation it self.

Neurological symptoms in Case 3 suggested DEND syndrome and a previously reported heterozygous KCNJ11 missense mutation, p.C166Y, was identified. ATP sensitive K_{ATP} channels couple cell metabolism to membrane excitability in various cell types, including neurons, pancreatic beta cells, endocrine and muscle cells. The archetypal K_{ATP} channel is an octameric complex of Kir6.2 and either SUR1 or SUR2 subunits. Pancreatic beta cells and many neurons involve SUR1, muscle cells involve SUR2. Four Kir6.2 subunits form the channel pore, and each is associated with a SUR subunit that regulates channel gating (26). In pancreatic beta cells, ATP-potassium channels regulate glucose-induced insulin secretion. In the unstimulated state, the beta cell K_{ATP} channels are open. Following the uptake and metabolism of glucose, intracellular ATP/ADP ratio increases which results in closure of K_{ATP} channels, depolarization of the cell membrane, and subsequent opening of voltage-dependent calcium (Ca) channels. Increase in cytosolic Ca concentration triggers the release of stored insulin granules. KCNJ11 activating mutations result in the K_{ATP} channel remaining open and insulin secretion is therefore disrupted. Pancreas development is normal and diabetes is due to impaired insulin secretion. This channel is important in numerous sites such as neurological cells. Thus, mutations can result not only in diabetes but can also lead to neurological disorders.

KCNJ11 gene mutation can be treated with an oral antidiabetic agent, sulfonylurea, which can close the K_{ATP} channel and induce insulin secretion (27). This treatment has been shown to also improve neurological symptoms (28,29,30,31,32,33). However, in patients with mutations resulting in severe DEND syndrome, sulfonylurea treatment is often ineffective (10,16,27,34). Although in a Brazilian patient having the same mutation as our third patient (p.C166Y mutation in the *KCNJ11* gene) sulfonylurea treatment was unsuccesful in controlling blood glucose levels and neurological symptoms (35), we observed relative improvement in both blood glucose levels and neurological symptoms in the short term. Nevertheless, we cannot comment on treatment success because we were not able to follow this patient in the long term.

Although neonatal diabetes is a rare disorder, it should be promptly evaluated for additional clinical findings. Clinical findings can be a clue for choosing the genetic tests. Genetic testing is important because it not only reveals the underlying mechanism for the disorder but will also guide treatment and follow up of the patients.

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Ethics

Informed Consent: The inform consent was taken from the patients' parents for publication.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Olcay Evliyaoğlu, Oya Ercan, Emel Ataoğlu, Ümit Zübarioğlu, Bahar Özcabı, Aydilek Dağdeviren, Hande Erdoğan, Concept: Olcay Evliyaoğlu, Design: Olcay Evliyaoğlu, Oya Ercan, Data Collection or Processing: Olcay Evliyaoğlu, Emel Ataoğlu, Ümit Zübarioğlu, Bahar Özcabı, Analysis or Interpretation: Elisa De Franco, Sian Ellard, Literature Search: Olcay Evliyaoğlu, Writing: Olcay Evliyaoğlu

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CASE REPORT

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A Novel KCNJ11 Mutation Associated with Transient Neonatal Diabetes

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What is already known on this topic?

Neonatal diabetes is a monogenic disorder presenting as a transient or permanent type. Transient cases are usually due to abnormalities in the 6q24 region, while some patients may have mutations in the *KCNJ11* and *ABCC8* genes.

What this study adds?

We describe a novel *KCNJ11* gene mutation (p.P254Q) in a patient with neonatal diabetes that subsided at the age of 10 months. The p.P254Q mutation seems to cause mild impairment of the ATP-sensitive potassium channel function leading to transient neonatal diabetes.

Abstract

Neonatal diabetes mellitus (NDM) is a rare type of monogenic diabetes that presents in the first 6 months of life. Activating mutations in the *KCNJ11* gene encoding for the Kir6.2 subunit of the ATP-sensitive potassium (K_{ATP}) channel can lead to transient NDM (TNDM) or to permanent NDM (PNDM). A female infant presented on the 22nd day of life with severe hyperglycemia and ketoacidosis (glucose: 907mg/dL, blood gas pH: 6.84, HCO_3: 6 mmol/L). She was initially managed with intravenous (IV) fluids and IV insulin. Ketoacidosis resolved within 48 hours and she was started on subcutaneous insulin injections with intermediate acting insulin NPH twice daily requiring initially 0.75-1.35 IU/kg/d. Pre-prandial C-peptide levels were 0.51 ng/mL (normal: 1.77-4.68). Insulin requirements were gradually reduced and insulin administration was discontinued at the age of 10 months with subsequent normal glucose and HbA1c levels. C-peptide levels normalized (pre-prandial: 1.6 ng/mL, postprandial: 2 ng/mL). Genetic analysis identified a novel missense mutation (p.Pro254Gln) in the *KCNJ11* gene. We report a novel KCNJ11 mutation in a patient who presented in the first month of life with a phenotype of NDM that subsided at the age of 10 months. It is likely that the novel p.P254Q mutation results in mild impairment of the K_{ATP} channel function leading to TNDM.

Keywords: Neonatal diabetes, KCNJ11, hyperglycemia, transient

Introduction

Neonatal diabetes mellitus (NDM) is a rare form of monogenic diabetes, which usually presents before the age of six months (1). To date, abnormalities in 23 genetic loci have been associated with NDM (2,3,4). Clinically, NDM can be classified into two major categories, transient NDM (TNDM) and permanent NDM (PNDM). The reported incidence of NDM is quite variable and is lower in Western countries [Italy: 1 in 90.000 live births (5), UK: 1 in 400.000 (6)] and higher in Eastern countries [1 in 21.000 in Saudi Arabia (7)], which may be due to high rates of consanguinity. Turkey (particularly its South-Eastern Anatolian regions) has a high rate of consanguineous marriages (40%) and PNDM incidence is reported to be 1 in 48.000 live births (8).

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TNDM accounts for approximately 50% of the cases of neonatal diabetes. Children with TNDM are usually born with intrauterine growth retardation (IUGR) and tend to develop diabetes in the first weeks of life (9). Diabetes subsides in the following months, with a possible relapse to a permanent state during puberty or adult life. About 70% of TNDM cases are due to abnormalities in the 6q24 region, with the remainder of patients mainly having mutations in the *KCNJ11* and *ABCC8* genes encoding the Kir6.2 and SUR1 subunits of the pancreatic ATP-sensitive potassium (K_{ATP}) channel (10). This channel regulates insulin secretion by linking glucose metabolism and consequent ATP production to calcium-dependent release of insulin. Activating KCNJ11 or ABCC8 mutations lead to inappropriate activation of the K_{ATP} channel, thereby compromising membrane depolarization and insulin secretion (11). Some of these mutations have been reported to have less severe effects on channel function, causing TNDM (12).

In children with PNDM diabetes does not remit, and about half of them have K_{ATP} channel mutations (1). There is significant clinical overlap between the two types of neonatal diabetes and it is therefore not possible to predict the clinical course at the time of diagnosis. Sulfonylureas have inactivating effects on the K_{ATP} channel, hence most of the patients with confirmed KCNJ11 and ABCC8 mutations may discontinue insulin and be successfully managed with oral sulfonylureas (13). In this article, we describe a case of TNDM due to a novel mutation in the *KCNJ11* gene.

Case Report

A female infant was born to a single mother of Pakistani origin at 38 weeks of gestation. The mother was a refugee and had under her care three healthy children (age: 8, 5 and 2.5 years old), while the presumed father had presented with type 2 diabetes mellitus (T2DM) at the age of 30 years, which also affected many members of his family. The mother had inadequate antenatal care during this pregnancy. Delivery was uneventful and the infant had no dysmorphic features. Birth weight was 2500 g (-2 standard deviations according to World Health Organization growth charts). The patient was admitted to hospital at the age of 22 days, with respiratory distress and signs of severe dehydration. Diagnostic work-up revealed hyperglycemia with severe ketoacidosis (glucose: 907mg/dL, blood gas pH: 6.84, HCO_3: 6 mmol/L), that was managed with intravenous (IV) fluids and IV insulin administration. In addition, due to the patient's critical condition, the possibility of infection was also considered and IV antibiotic administration was started, which was discontinued as the results of blood cultures proved to be negative. Hemoglobin A1c (HbA1c) levels on admission were 7.5% (RR:4.0-6.0%). Due to the patient's young age (less than six months) and the laboratory findings of severe hyperglycemia and ketoacidosis, the diagnosis of NDM was considered. The infant recovered from ketoacidosis within 48 hours and was started on subcutaneous insulin with intermediate acting insulin NPH twice daily, requiring initially 0.75-1.35 IU/kg/d. Blood glucose levels were found to increase significantly after breastfeeding, therefore it was decided to start feeding with specific amounts of expressed breast milk at 150 mL/kg/day. With this regimen, hyperglycemia was well controlled with no episodes of hypoglycemia (blood glucose levels ranging between 91-109 mg/dL). Further investigations did not reveal any signs of autoimmunity with negative anti-glutamic acid decarboxylase (GAD) antibodies (GAD; 0.2 U/mL, RR: < 0.9) and antibodies against tyrosine phosphatase-related islet cell antigen 2 (IA-2; < 0.1 U/mL, RR < 0.75), while cardiologic, ophthalmologic and neurologic examinations revealed normal findings. At the time of diagnosis C-peptide was low (0.51 ng/mL, RR: 1.77-4.68).

During the patient's regular follow-up, insulin requirements were gradually reduced and at the age of eight months the patient was requiring 0.32 mg/kg/day of insulin NPH to achieve normoglycemia (HbA1c: 5.4%, RR: 4.0-6.0%). Growth and psychomotor development were normal (weight: 50th percentile, height: 75th-90th percentile, head circumference: 25-50th percentile). Informed consent was obtained from the patient's mother for genetic analysis and publication of the results. Sanger sequencing analysis of the *ABCC8*, *KCNJ11*, *INS* and *EIF2AK3* genes identified a novel missense variant in the *KCNJ11* gene p.Pro254Gln (p.P254Q) (Figure 1). This variant has not been reported before and is not listed in HGMDpro. In addition, the variant has not been identified in 138.487 individuals in the GnomAD database (http://gnomad.broadinstitute.org/). Testing for all the other known neonatal diabetes genes by targeted next generation sequencing (3)

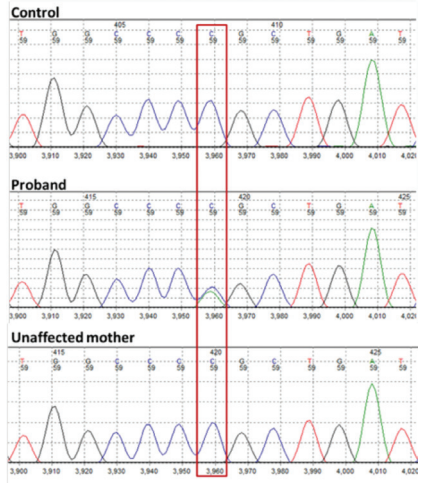

Figure 1. Sanger sequencing analysis of the *KCNJ11* gene. Detection of a novel mutation, c.761C > A (p.P254Q), in the proband

gave negative results, confirming that this was the only likely pathogenic variant identified in our patient. *In silico* analysis by SIFT and PolyPhen2 was performed. This analysis predicted that this mutation affects the protein's function.

The p.P254Q mutation was not detected in the mother's sample. The presumed father refused genetic testing. Trial of treatment with oral sulfonylureas was planned. However, at the age of 10 months, diabetes remitted and insulin injections were discontinued by the mother, before initiating the scheduled sulfonylurea treatment protocol. Blood glucose levels remained at the normal range without any treatment, HbA1c (4.9%, RR=4.0-6.0%) and C-peptide levels had normalized (pre-prandial: 1.6 ng/mL, postprandial: 2 ng/mL; RR: 1.1-4.4). During the following three months after insulin discontinuation, the patient's HbA1c (5.2%, RR=4.0-6.0%) and blood glucose levels remained normal.

Discussion

We report a patient with NDM caused by a novel heterozygous KCNJ11 mutation. Although the p.P254Q mutation has not been reported before, the phenotype of our patient, along with the fact that no mutations were found in the other known NDM genes, supports the pathogenicity of the mutation. This novel mutation (c.761C>A, p.P254Q) leads to the substitution of the non-polar proline at codon 254 for a polar glutamine in the cytoplasmic domain of the K_{ATP} channel. The proline residue at position 254 is highly conserved across species (up to C. Elegans, 23 species considered) and is predicted to be pathogenic by SIFT and PolyPhen2 as described above.

More than 30 activating KCNJ11 mutations have been associated with NDM so far (1). The majority of these mutations seem to affect the K_{ATP} channel's sensitivity to ATP and impair its function. Mutated K_{ATP} channels show reduced sensitivity to ATP inhibition, resulting in membrane hyperpolarization and impaired insulin secretion (14). Mutations within the ATP-binding site are known to be associated with milder phenotypes, whilst mutations located in areas responsible for channel opening and closure, affect ATP sensitivity indirectly and cause a more severe phenotype (15). The extent of membrane hyperpolarization caused by each mutation can explain the spectrum of variation of the clinical phenotype of the disease, ranging from TNDM (16) to PNDM with neurological complications (developmental delay, epilepsy and neonatal diabetes syndrome) (15). Psychomotor development was normal in our patient.

On the other hand, less severe KCNJ11 mutations result in remitting/relapsing neonatal diabetes, maturity onset diabetes of the young or T2DM at older ages (10). These mutations usually result in mild impairment of K_{ATP} channel function, as has been shown for the p.V252A mutation (17), that is located just two amino acids apart from the mutation identified in our patient. The mechanism proposed to explain these phenotypes suggests that there is a mild beta cell defect caused by some mutations that may be compensated transiently, and that the hyperglycemia may present again in periods of increased insulin requirements (16,18). Although we were not able to perform a functional study, considering the phenotype of our patient, we can hypothesize that the mutation identified in our patient causes a mild reduction in channel sensitivity to ATP. Likewise, our patient's presumed father developed T2DM at the age of 30 years, although we do not know if he has the same mutation with our patient since he refused genetic analysis.

Managing infants with NDM presents many problems, arising from the very small insulin doses required, the high risk of hypoglycemia, the lack of subcutaneous fat and the coordination of insulin therapy with the frequent and uncontrolled feeding schedule of the newborn period. Continuous subcutaneous insulin infusion (CSII) has been recommended as the treatment of choice in the initial management of infants with NDM (19,20,21). Rapid acting insulin preparations (Lispro, Aspart and Regular) may cause severe hypoglycemia and should be avoided, with the exception of CSII (1), while long acting or intermediate acting insulin has been successfully used in these patients (22,23,24). Due to the low socioeconomic and educational level of the family, our patient was managed with subcutaneous injections of insulin NPH, a treatment which proved to be successful. The patient achieved very good metabolic control with no significant glycemic variability and optimal growth and development.

Sulfonylurea is the treatment of choice in patients with KCNJ11 or ABCC8 mutations (13,25). Thus after genetic identification of a mutation in one of these genes, more than 400 patients have been successfully transferred from insulin to sulfonylurea and most of them responded well with improved glycemic control and less hypoglycemic events (25). In our patient, the diabetic state remitted before the planned sulfonylurea treatment initiation.

In conclusion, we report the case of an infant with transient neonatal diabetes associated with a novel mutation of the *KCNJII* gene. Diabetes remitted after 10 months with an uneventful course and good psychomotor development under subcutaneous insulin regimen. Studies describing the genotype-phenotype correlation of novel mutations can help clinicians to predict the severity of the disease and appropriately manage these patients.

Ethics

Informed Consent: Informed consent was obtained from the patient's mother.

Peer-Review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Evangelia Gole, Stavroula Oikonomou, Elisa De Franco, Sian Ellard, Kyriaki Karavanaki, Concept: Kyriaki Karavanaki, Elisa De Franco, Design: Kyriaki Karavanaki, Data Collection or Processing: Evangelia Gole, Stavroula Oikonomou, Sian Ellard, Analysis or Interpretation: Kyriaki Karavanaki, Elisa De Franco, Sian Ellard, Literature Search: Evangelia Gole, Stavroula Oikonomou, Writing: Evangelia Gole, Stavroula Oikonomou, Sian Ellard, Kyriaki Karavanaki.

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CASE REPORT

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Two Childhood Pheochromocytoma Cases due to von Hippel-Lindau Disease, One Associated with Pancreatic Neuroendocrine Tumor: A Very Rare Manifestation

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What is already known on this topic?

In childhood, pheochromocytomas (PCC) are mostly due to genetic causes, of which von Hippel-Lindau (VHL) disease is the most frequent disorder. VHL may be the only and/or initial manifestation of the disease, with delayed manifestations of the syndrome in other organs.

What this study adds?

We report two cases of von Hippel-Linda (VHL) disease who presented with pheochromocytomas (PCC). In the second case, pancreatic neuroendocrine tumor (PNET), a very rare manifestation of VHL disease, developed during follow-up. To the best of our knowledge, this is only the second case in the literature presenting with a combination of PNET and PCC in childhood.

Abstract

von Hippel-Lindau (VHL) disease is an autosomal dominantly inherited disorder, characterized by hemangioblastomas of the retina and central nervous system (CNS); renal cysts; clear cell carcinoma; pheochromocytoma (PCC); endolymphatic sac tumors; cystadenomas of the epididymis in males; broad ligament of uterus in females; pancreatic cysts; cystadenomas; and neuroendocrine tumors. We report two cases of VHL disease that presented with PCC as the first manifestation. Further clinical developments during follow-up, hemangioblastoma of CNS in one case and a pancreatic neuroendocrine tumor (PNET) in the second case led to the diagnosis of VHL disease. Genetic analyses of the two cases revealed p.Arg161Gln (c.482G > A) and p.Leu129Pro (c.386T > G) heterozygous missense mutations in the VHL gene, respectively. In children, PCC may be the only and/or initial manifestation of VHL with delayed manifestations of the syndrome in other organs. PNET is a very rare manifestation of VHL disease. To the best of our knowledge, this is only the second reported case presenting with a combination of a PNET and bilateral PCC as components of childhood VHL disease. Pediatric patients diagnosed with PCC should be investigated for genetic causes and especially for VHL.
Keywords: von Hippel-Lindau syndrome, pheochromocytoma, pancreatic neuroendocrine tumor, hemangioblastoma

Introduction

von Hippel-Lindau (VHL) disease is an autosomal dominantly inherited disorder caused by a germline mutation in the VHL tumor supressor gene. VHL is characterized by hemangioblastomas of the retina and central nervous system (CNS); renal cysts; clear cell carcinoma; pheochromocytomas (PCC); endolymphatic sac tumors; cystadenomas of the epididymis in males and broad ligament of uterus in females; pancreatic cysts, cystadenomas and neuroendocrine tumors (1,2). Incidence of VHL disease is estimated at 2-3 cases per 100 000 population (3). If a family history of VHL disease is

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present, a diagnosis of VHL disease can be made by finding only a single VHL-associated tumor. On the other hand, approximately 20% of VHL cases are sporadic and in these cases the presence of two VHL tumors is necessary to diagnose the disease, in the absence of a positive family history (4).

PCCs are uncommon neuroendocrine tumors that arise from chromaffin cells of the adrenal medulla and produce excessive amounts of catecholamines, which are responsible for hypertensive surges, palpitations, headache, and diaphoresis (5). PCCs are rare in childhood but represent a curable cause of hypertension and must be considered in the differential diagnosis of hypertension. Compared with adults, children with PCCs have a higher incidence of bilateral adrenal tumors, extra-adrenal tumors and multiple tumors (6). Although most PCC cases are sporadic, more than 25% are associated with an inherited mutation and this ratio can be as high as 55%, if diagnosed before 18 years of age (7). In childhood, PCCs are mostly due to genetic causes, in which VHL disease is the most frequent disorder (8). PCCs in VHL disease tend to be seen at younger ages, are often multiple and may be extra-adrenal (9,10).

Here we report two cases of VHL disease, who presented with PCC as the first manifestation. Further clinical developments during follow-up, hemangioblastoma of CNS in the first case and pancreatic neuroendocrine tumor (PNET) in the second case, led to the diagnosis of VHL disease.

Case Reports

Case 1

This patient was a twelve year old boy, admitted with complaints of weight loss, hot flushes, palpitation and diaphoresis, for the past one month. He was the first child of nonconsanguineus parents. His birth history was unremarkable. His family history was not significant for tumor occurrence. On physical examination, he weighed 43kg [-0,28 standard deviation (SD)]. Height was 150 cm (-0,48 SD). His blood pressure was 140/100 mmHg (95p 123/81 mmHg), heart rate was 115 beats per minute. General examination was otherwise normal.

Laboratory tests showed an elevated 24 hour (h) urinary vanillylmandelic acid (VMA) concentration of 115 mg/day (normal value < 15 mg/day). An abdominal ultasound revealed solid lesions, 27x35 mm at the right adrenal gland and 37x75 mm at the left adrenal gland. Abdominal magnetic resonance imaging (MRI) showed bilateral adrenal masses compatible with PCC. Bilateral subtotal adrenalectomy, including removal of the masses, was performed and the diagnosis of bilateral PCC was confirmed histologically. The patient remained asymptomatic with no laboratory or radiologic abnormalities for five years of follow-up. At the age of 17, he presented complaining of headache. Cranial MRI demonstrated a lesion of one centimetre diameter, located in the left frontal lobe. Positron emission tomography (PET) revealed a lesion of increased 18 fluorodeoxyglucose uptake in the right adrenal gland, compatible with recurrence, and a hypometabolic, hypodense focus in the left frontal lobe (Figure 1). The cranial mass was excised and hemangioblastoma was diagnosed histologically. Adrenalectomy was performed for the lesion in the right adrenal gland and recurrence of PCC was confirmed. Coexistence of PCC and cranial hemangioblastoma suggested the diagnosis of VHL disease. The previously reported heterozygous missense mutation c.482G > A (p. Arg161Gln) in the *VHL* gene was detected on genetic analysis.

Case 2

This 10 year old girl presented with intermittent fever for the past one month. Her birth history was unremarkable. She was the fourth child of nonconsanguineous parents. Her family history was not significant for tumor occurrence. Physical examination revealed a blood pressure level of 160/100 mmHg (95p: 120/79 mmHg) and a heart rate of 110 beats per minute. Laboratory tests showed an elevated 24 h urinary VMA level of 83 mg/day. Abdominal MRI revealed a 44x33 mm, well circumscribed mass with a necrotic core in the left adrenal gland. Subtotal adrenalectomy was performed and histologic examination showed that the tumor was PCC. During two years of follow-up, a 20x19x12 mm mass was detected in the right adrenal gland on abdominal MRI. PET-

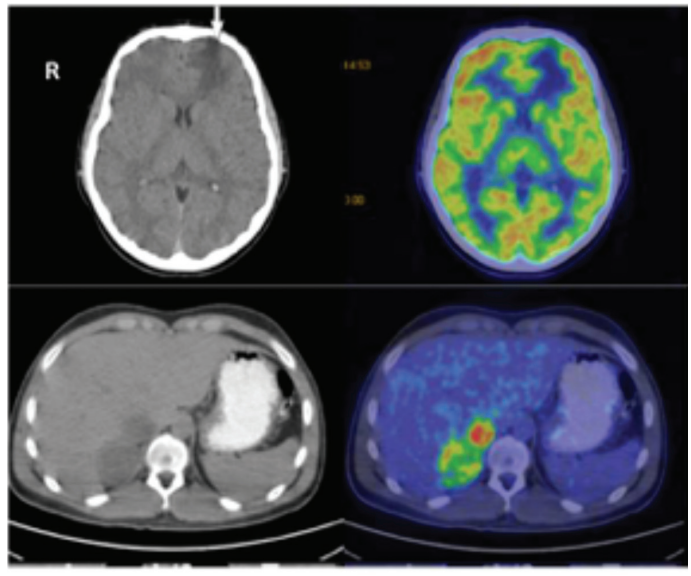

Figure 1. Positron emission tomography imaging showing increased fluorodeoxyglucose uptake in the right adrenal gland, compatible with recurrence, and hypometabolic, hypodense focus in the left frontal lobe

computed tomography (CT) with ^{68}Ga-DOTA-DPhe^{1}, Tyr^{3}-octreotate (^{68}Ga- DOTATATE) showed increased uptake in the right adrenal gland and a 11x10 mm nodular lesion in the corpus of the pancreas (Figure 2). The tumoral masses in the adrenal gland and pancreas were removed. Histologic investigation of adrenal and pancreas specimens confirmed the diagnosis of PCC and PNET (World Health Organisation grade 3) respectively. One year later, an 8x7 mm lesion in the pancreas, compatible with recurrence, was observed on abdominal MRI and confirmed with 68 Ga-DOTATATE PET-CT. Splenectomy and subtotal pancreatectomy were performed for removal of the lesion. Histologic examination of the pancreatic lesion reported a neuroendocrine tumor. Bilateral PCC with PNET suggested the diagnosis of VHL disease. Molecular genetic analysis of the *VHL* gene revealed a heterozygous missense mutation c. 386 T > G (p.Leu129Pro) which has been previously described. No additional VHL tumor developed during three years follow up.

Both patients are being followed up according to the recommended pediatric screening protocol for children carrying a VHL mutation (11).

Genetic Analysis

Molecular DNA was isolated from a 200 µL blood sample using the QIAamp DNA Blood Mini QIAcube Kit with a QIAcube instrument (QIAGEN, Hilden, Germany) according to the manufacturer's specifications. The full coding sequences, including the 5′ untranslated region (UTR) and the 3′ UTR of the *VHL* gene (OMIM*608537), were amplified and sequenced. PCR products were purified using ExoSAP-IT (GE Healthcare, Little Chalfont, UK). The PCR fragments were sequenced by using the BigDye terminator V3.1 Cycle Sequencing ready reaction system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequence analysis was performed on an ABI Prism 3100-Avant DNA sequencer (Applied Biosystems).

Discussion

PCC is an exceptionally rare neoplasm in children, accounting for 1% of pediatric hypertensive patients (12). Of all PCC cases, approximately 10-20% are reported to occur in the pediatric population (13). Childhood PCC is associated with sustained hypertension, whereas PCC in adults is characterized by hypertensive attacks with the classical triad of palpitation, headache and sweating (14). Episodic tachycardia, sweating and hot flush, the classic symptoms of PCC, accompanied by sustained hypertension, were present in our first patient. However, in the second case, the only symptom was intermittent fever. However, sustained hypertension was detected on physical examination.

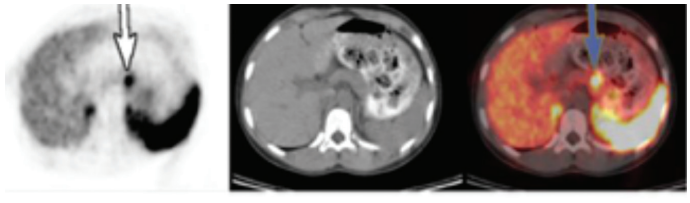

Figure 2. Positron emission tomography - computed tomography imaging showing increased uptake in the right adrenal gland and 11x10 mm nodular lesion in corpus of the pancreas

PCCs are seen both sporadically and in association with a number of familial cancer syndromes such as VHL disease, multiple endocrine neoplasia type 2, paraganglioma syndromes type 1, 3 and 4, and, rarely, in neurofibromatosis (13). Family history was negative for familial cancer syndromes in both cases. Even in patients with apparently sporadic PCCs, up to 25% will have unsuspected germline mutations. Younger age and multifocal tumors, as in our patients, are significantly associated with the presence of a mutation. Genetic testing may detect patients at risk for other associated tumors (15). The delayed diagnosis of VHL disease was made after the occurrence of cranial hemangioblastoma in the first case and PNET in the second case.

In childhood and adolescence, PCC may be the only initial manifestation of VHL disease with delayed manifestations of the syndrome in the eye, CNS or other organs (16).

VHL disease is classified into four subtypes. type 1 occurs without PCC while type 2A, 2B and 2C all carry the risk of development of PCC. Patients with type 2A have a low risk of renal cell carcinoma (RCC) while Type 2B patients have a high risk of RCC. VHL type 2C confers an increased risk of PCCs without other manifestations of the disease. In type 1 families, deletions in the *VHL* gene are often detected, whereas in type 2 disease, missense mutations are most often encountered. In our cases, the presence of PCC and the missense mutations in *VHL* gene suggested VHL type 2. Mutation found in the first patient, c.482G > A (p. Arg161Gln), is also known to be associated with RCC (17,18). However, to date, our patients have not shown any signs of RCC.

Involvement of the pancreas in VHL disease has been reported in 25% to 70% of cases (19). In most of the cases, pancreatic changes are characterized by benign cysts (20). In VHL disease, neuroendocrine tumors of the pancreas and PCCs are observed in 8-17%, and 10-20% of patients respectively (21). The association of neuroendocrine tumors of the pancreas with PCCs has been reported in 12% of patients with VHL disease (22). Pancreatic tumors rarely occur during childhood (23). The mean age at presentation for neuroendocrine tumours is 35 years (21). In our second case, PNET as a component of VHL was detected at the age of

twelve years, two years after diagnosis of PCC. Langrehr et al (24) reported a 12-year-old girl with c.695 G > A mutation in exon 3 of the *VHL* gene resulting in a neuroendocrine tumor of the pancreas and bilateral adrenal PCC. To the best of our knowledge, our patient is the second youngest reported case in the literature, presenting with a combination of PNET and bilateral PCC as components of childhood VHL disease.

To conclude, we have presented two childhood cases of VHL disease with bilateral PCC and an additional tumor, namely PNET and cranial hemangioblastoma diagnosed after two and five years after the initial diagnosis of PCC, respectively. The combination of PCC and PNET in childhood VHL disease is here reported for only the second time in the literature. Meticulous follow-up and early genetic testing in PCC may facilitate diagnosis and serve to prevent morbidity and mortality, as well as improving long term prognosis in VHL disease.

Ethics

Informed Consent: The inform consent was taken from the patients' parents for publication.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Aydilek Dağdevren Çakır, Hande Turan, Ayça Aykut, Asude Durmaz, Oya Ercan, Olcay Evliyaoğlu, Concept: Aydilek Dağdeviren Çakır, Olcay Evliyaoğlu, Oya Ercan, Design: Aydilek Dağdeviren Çakır, Olcay Evliyaoğlu, Oya Ercan, Data Collection or Processing: Aydilek Dağdeviren, Hande Turan, Ayça Aykut, Analysis or Interpretation: Ayça Aykut, Asude Durmaz, Literature Search: Aydilek Dağdeviren Çakır, Hande Turan, Writing: Aydilek Dağdeviren Çakır, Olcay Evliyaoğlu, Oya Ercan.

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CASE REPORT

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One Novel 2.43Kb Deletion and One Single Nucleotide Mutation of the *INSR* Gene in a Chinese Neonate with Rabson-Mendenhall Syndrome

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What is already known on this topic?

Mutation of the insulin receptor gene is responsible for Rabson-Mendenhall syndrome (RMS) which is an autosomal recessive disorder. Typical symptoms of RMS include growth retardation, elfin face, gingival hyperplasia, acanthosis nigricans, hypertrichosis and insulin resistance.

What this study adds?

We report an atypical and mild RMS patient due to a compound heterozygosity consisting of a novel 2.43Kb deletion and a known, pathogenic point mutation in the *INSR* gene.

Abstract

Mutations in the insulin receptor (*INSR*) gene are responsible for Donohue syndrome (DS) and Rabson-Mendenhall syndrome (RMS). Insulin resistance is a feature of both diseases.

Our patient was a Chinese neonate suffering from abnormal glucose homeostasis, hyperinsulinemia, dry skin, heavy hair, growth retardation and an elevated testosterone level. To search for candidate point mutations, small insertions or deletions and copy number variants, 2742 inherited disease-gene panel sequencing was performed. One pathogenic mutation (c.3355C>T, p.Arg1119Trp) and a novel 2.43Kb deletion (chr19:7150507-7152938) in INSR were found. The patient was diagnosed as RMS. Sanger sequencing and real-time quantitative polymerase chain reaction (PCR) confirmed the missense variant and microdeletion, respectively. We therefore supposed that these variants were candidate mutations in this case. We report a novel 2.43Kb deletion in *INSR* gene and provide further proof of the power of next generation sequencing in rare disease diagnosis.

Keywords: Insulin receptor gene, Rabson-Mendenhall syndrome, neonate, mutation, next generation sequencing

Introduction

Insulin receptor *(INSR)* is the gene responsible for a series of insulin resistance diseases, including hyperinsulinemic hypoglycemia, familial 5 [Online Mendelian Inheritance in Man (OMIM)#609968], Donohue syndrome [(DS), also called leprechaunism; OMIM#246200] and Rabson-Mendenhall syndrome [(RMS); OMIM#262190]. The inheritance pattern of DS and RMS is autosomal recessive. Typical symptoms of DS include growth retardation, elfin face, gingival hyperplasia, acanthosis nigricans, hypertrichosis and insulin resistance (1). RMS and DS share similar symptoms. The symptoms of DS are more severe, have an infantile onset and may lead to early death. RMS is often encountered as

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being of childhood-onset and with survival up to adulthood with milder symptoms. The differential diagnosis is based on the onset age and severity of the disease (2).

In this study, we describe a Chinese, male, newborn with hyperinsulinemia and hyperglycemia. Next generation sequencing (NGS) detected a compound heterozygous mutations of INSR, including one known mutation and one novel 2.43Kb deletion.

Case Report

The proband is a male infant and the first child of non-consanguineous parents. During the fetal period, he was diagnosed with intrauterine growth retardation and oligohydramnios and was delivered by natural labor at 36 weeks of gestation with a low birth weight at 1.7 kg. At age 13 days, he presented with dry skin and heavy hair over his whole body. The plantar grasp, Moro and sucking reflexes were weak. Facial malformation, abnormality of mouth size, acanthosis nigricans and abdominal distention were not observed. Clinical tests showed hyperglycemia (14.7 mmol/L), hyperinsulinemia (>300 IU/mL) and fasting hypoglycemia. C-peptide was 4.05 ng/mL (1.10-4.40 ng/mL). HbAlc (glycosylated hemoglobin) was normal (5.4%). Other abnormal laboratory test results are shown in Table 1. Insulin auto-antibodies were negative. Routine blood tests, liver function tests and thyroid-stimulating hormone levels were normal. Echocardiography suggested a ventricular septal defect, an atrial septal defect and ultrasonography indicated swelling of both kidneys. Magnetic resonance imaging of the brain indicated an impaired myelination of

Table 1. Clinical features of patient

	System or organ	Features	Proband
Syndromic features of RMS according to OMIM	Growth	Intrauterine growth retardation	+
		Small for gestational age	1.7 kg (GA: 36w)
	Face	Coarse face, prognathism	-
	Mouth	Large, fissured tongue; gingival hypoplasia; high-arched palate	-
	Teeth	Dental dysplasia, premature eruption of teeth	-
	Genitourinary	Large penis, clitoromegaly	-
	Skin	Acanthosis nigricans, lichenified skin	-
		Dry skin	+
	Nails	Onychauxis	-
	Hair	Hypertrichosis c	+
	Central nervous system	Pineal hypertrophy	-
		Developmental delay	+
	Endocrine features	Insulin resistant diabetes mellitus	-
		Diabetic ketoacidosis	-
		Altered melatonin secretion	/
		Precocious puberty	-
	Laboratory abnormalities	Postprandial hyperglycemia	+
		Fasting hypoglycemia	+
		Hyperinsulinemia	>300 IU/mL
Additional positive test results	Laboratory abnormalities	Testosterone in plasma (0.7-3.6 ng/mL)	18.2 ng/mL
		Urea in plasma (2.5-6.5 mmol/L)	0.6 mmol/L
		Creatinine in plasma (20-110 umol/L)	12 umol/L
		Hypokalemia (3.5-5.5 mmol/L)	3.2 mmol/L
	Ultrasonography	Ventricular septal defect, atrial septal defect	
		Swelling of both kidneys	
	MRI	Impaired myelination of white matter	

+: Patient owns this phenotype, -: This feature is absent in this patient, /: Whether this feature present in this patient is unknown,
GA: gestational age, OMIM: Online Mendelian Inheritance in Man, RMS: Rabson-Mendenhall syndrome, MRI: magnetic resonance imaging

white matter. The mother had transient hypothyroidism during pregnancy. Otherwise the family history is negative. At the last outpatient follow-up at age four months, the patient's neurodevelopment was found to be delayed and that the high postprandial blod glucose (> 11 mmol/L) and hyperinsulinemia (> 300 IU/mL) persisted.

Pre-test counseling was performed by physicians and appropriate informed consent was signed by the patient's parents in the clinic. The criteria of genetic testing received approval from the ethics committees of the Children's Hospital, Fudan University (2016-235). Genomic DNA samples were extracted from whole blood using the QIAamp DNA Blood Mini kit (QIAGEN, Germany) following the manufacturer's protocol. The quality and quantity of the DNA samples were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Nucleic acid preparation and high-throughput sequencing were performed using standard protocols in a Clinical Laboratory Improvement Amendments (CLIA) compliant sequencing laboratory in Wuxi NEXTCODE (288 Fute Zhong Road, Waigaoqiao Free Trade Zone Shanghai 200131, China CLIA ID 99D2064856). Inherited-Disease panel sequencing was generated using the Agilent ClearSeq Inherited Disease kit, Illumina Cluster and SBS kit. The sequencing was performed using NGS on the Illumina Hiseq 2000/2500 platform. This covers a minimum of 98 % of the genome with 20X coverage and was compared to a human reference sequence.

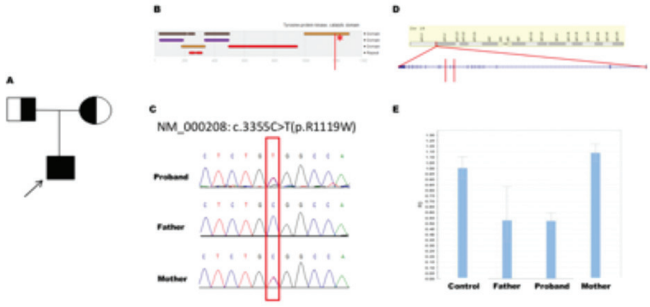

Figure 1. Insulin receptor gene compound heterozygous mutation: a known missense mutation and a novel microdeletion. A) Pedigree of the family. B) The SNV is located at the tyrosine-protein kinase catalytic domain and marked by red asterisk. C) Sanger sequencing shows the mutation is from mother. Insulin receptor gene locates at 19p13.3-19p13.2. D) The deletion fragment is marked within two red lines. This fragment contains Exon11 and part of Exon 10. E) Real-time quantitative polymerase chain reaction shows that the deletion is from father

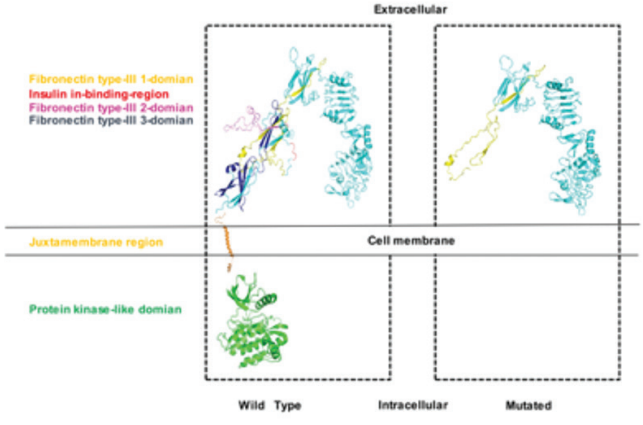

Figure 2. 3D structural modeling of insulin receptor protein. Comparing to the wild type, 3D structural modeling estimates a large portion of deficiency in the monomer form of the insulin receptor caused by the deletion. Different domains are marked by different colors with the color of the domain matched by the color of the domain name in the key

We identified a known-pathogenic mutation (c.3355C > T, p.Arg1119Trp) at exon 18 of the *INSR* gene (NM_000208). This mutation has been reported in a patient with DS (3). This mutation was recorded in HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) as CM1119. The Exac database (http://exac.broadinstitute.org/) and the 1000 gene database (http://www.internationalgenome.org/) have no record of this variant. This mutation was also the only record in our internal database, which contains sequencing data of ClearSeq of 4071 patients. Paired primers were designed by using Primer3 website and primer-BLAST (5'-GGGAGGAGAACCCTGGTGAG-3' and '-ATCCGAGGAGGCCAGGAG-3'). Sanger sequencing indicated that this mutation was inherited from the mother (Figure 1A, 1B, 1C).

We used CANOES (CNVs with an Arbitrary Number of Exome Samples) for basic detection of CNVs from NGS data at gene-level and region-level (4). Gene-level annotation was based on OMIM, Human Gene Mutation Database (HGMD), Swiss-Prot and RefSeq. Region-level information was annotated by Database of Genomic Variants (DGV), and Database of Genomic Variation and Phenotype in Humans using Ensembl Resources (DECIPHER). We detected a novel deletion of approximately 2.43 Kb (chr19:7150507-7152938) within the *INSR* gene (Figure 1D). This deletion is not found in HGMD, DECIPHER or DGV. Additionally, it is absent from our internal database. The detected mutation was confirmed using real-time quantitative PCR. PCR-amplified DNA products were subjected to direct automated sequencing (ABI step one plus v.2.0). Both strands of each amplicon were sequenced using the primers 5'-CCTGACCTGGGGACGAAAA-3' and 5'-GTCTCCACCATTCGAGTCTGA-3'. Real-time quantitative PCR indicated the deletion was from the father (Figure 1E). This region covers part of exon 10 and all of exon 11. The deletion is estimated to cause a truncated protein. We performed a three-dimensional (3D) structural modeling of

the monomer form of the INSR and mapped the deletion on to it. The PBD number of the INSR extracellular region is 4ZXB.E, that of the juxtamembrane region is 2MFR.A and that of the tyrosine kinase domain is 3BU3.A (5). The 3D structural modeling shows the monomer form of the INSR. The Fibronectin type-III 2-domain, Fibronectin type-III 3-domain, Insulin in-binding-region, protein kinase-like domain, juxtamembrane region and partial Fibronectin type-III 1-domain are absent (Figure 2) (5).

Discussion

In this Chinese newborn baby, we identified a novel microdeletion and a known missense mutation within the *INSR* gene, which caused a compound heterozygous mutation. The *INSR* gene is located at chromosome 19 and encodes the INSR. HGMD contains 178 mutations of *INSR*. For RMS, 26 mutations of INSR are reported. Of these, two compound heterozygous mutations, each containing one deletion and one single nucleotide mutation, have been previously reported. A gross deletion containing exons 9 and 10 was reported in a 15-year-old RMS patient (6). This patient carried a mutation (p.Ser635Leu) in INSR with a compound heterozygous genotype. The main phenotypes are hyperglycemia and hyperinsulinemia. Another gross deletion contains exon 18. This RMS patient, as a compound heterozygote, also carried a mutation (p.Val66Ala). Nephrocalcinosis was found to be one of the patient's dominant features (7). For the patient we report, the missense mutation (c.3355C > T, p.R1119W) has been reported from a DS patient who had symptoms at birth and died at three months of age (3). Our patient had symptoms 13 days after birth. Some typical RMS features of this patient include abnormal glucose homeostasis, hyperinsulinemia, dry skin, thick hair, elevated testosterone and growth retardation. We diagnosed this patient as RMS. With a microdeletion, our patient presented a mild and atypical phenotype, which may be explained by the unclear genotype-phenotype correlation of mutations in INSR. Different missense mutations in the same codon relate to different phenotypes (8). One DS patient, bearing a homozygous deletion in INSR resulting in inactivation of the INSR lived for 3.5 years (9). The coexistence of modifier genes and compensatory pathways may explain the phenotypic variability (10).

The insulin receptor is a tetramer of two α monomers and two β monomers. It is widely expressed and plays a vital role as a mediator between the extracellular and intracellular insulin signaling pathway. The whole region of the α-subunit is extracellular. The α-subunit contains a Leu-rich-compositionally biased region, Cys-rich-compositionally biased region, a Fibronectin type-III 1-domain and an insulin in-binding-region. The β-subunit extends through the cell membrane into the cytoplasm. The extracellular region of the β-subunit contains a Fibronectin type-III 2-domain and a Fibronectin type-III 3-domain. The cytoplasmic region of the β-subunit consists of several functional domains including a juxtamembrane region, a tyrosine kinase domain and the carboxy-terminal-region (5,11). The α-subunit is responsible for binding affinity to insulin. The Cys-rich-compositionally biased region is the main binding site of insulin. Fibronectin type-III domains 1 and 2 form the secondary insulin-binding site (5). Deficiency of the juxtamembrane region makes the folding of IR unstable and affects its downstream processing (12). The function of the Fibronectin type-III 3-domain remains unknown. A helical transmembrane region follows the Fibronectin type-III 3-domain. Through binding with insulin, IR initiates the phosphorylation of different phosphotyrosine residues in the tyrosine kinase domain (13). Downstream IR substrates bind to phosphotyrosine residues of IR and regulate two main signaling pathways: the phosphatidylinositol 3-kinase-AKT/protein kinase B (PI3K-AKT/PKB) pathway and the Ras-MAPK pathway. The PI3K-AKT/PKB pathway is responsible for controlling cell growth and differentiation. The metabolic action of insulin is mainly regulated by the Ras-MAPK pathway (14). 3D structural modeling indicates that with deficiencies in both α-subunit and β-subunit, IR may be unable to combine with insulin receptor substrates and recruit the downstream signaling molecules (15).

Our patient did not show some of the typical features of RMS including coarse face, gingival hyperplasia and acanthosis nigricans. These three symptoms can be absent in neonates and may develop in adolescence (16). The insulin receptor is expressed in the heart and regulates cardiac cell activity though the PI3K-AKT pathway. Insulin receptor deficiency possibly leads to cardiac dysfunction, as observed in some patients (10,17) and proven using animal models (18). There is no evidence indicating any relationship between heart structural malformation and insulin receptors, so we consider that the ventricle septal defect is not a consequence of insulin receptor deficiency in this case. The long-term prognosis of RMS patients is poor (19). Recombinant human insulin-like growth factor 1 and recombinant leptin are recommended for treatment of severe insulin resistance syndrome (19,20). However, the complications and safety of these drugs remain unknown (21)

In summary, we show an RMS patient carrying one known pathogenic mutation and one novel deletion in INSR. Since the presenting clinical features of patients with insulin resistance syndrome can be atypical, when the diagnosis

is in doubt genetic testing may help to identify the final diagnosis.

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Ethics

Informed Consent: The samples used in this study were collected with the appropriate informed consent and approval of the ethics committee of Children's Hospital, Fudan University. The methods used in this study were performed in accordance with the approved guidelines.

Peer-review: External and internal peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Lin Yang, Concept: Lin Yang, Wenhao Zhou, Design: Lin Yang, Wenhao Zhou, Xiang Chen, Data Collection or Processing: Bo Liu, Yulan Lu, Hongbo Chen, Analysis or Interpretation: Xinran Dong, Huijun Wang, Bingbing Wu, Literature Search: Xiang Chen, Writing: Xiang Chen.

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Can Pediatric Surgeons Become Truly Experienced for Thyroid Surgery on a Universal Scale?

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To the Editor,

We have read with great interest the paper titled "Management of Childhood Thyroid Nodules: Surgical and Endocrinological Findings in a Large Group of Cases" (1). This paper retrospectively analysed 103 children with thyroid nodules admitted to the authors' centre over a period of 9 years and indicated that there would be fewer resulting complications in more experienced hands. We have evaluated, from our perspective, the type of "experience" described in the paper as important for the success of pediatric thyroid surgeries.

Careful examination of the literature clearly shows the most significant criterion influencing experience in thyroid surgery is the number of patients operated on annually. Further, it is indicated that the fundamental criteria of surgical success are low rates of recurrent laryngeal nerve injury, post-operative hypocalcaemia and hospitalization time (2); the rate at which complications occur is inversely proportional to experience.

Surgeons interested in thyroid surgery have been categorized by researchers as belonging to different groups, such as high-volume surgeons, pediatric surgeons and others (3). For example, endocrine surgeons are a group found to perform an average of more than 100 thyroid surgeries per year (3,4). Tuggle et al (3) indicated that high-volume surgeons perform at least 30 thyroid surgeries and an average of 2 pediatric endocrine surgeries per year. Research has found that pediatric surgeons perform, on average, 2 pediatric thyroid surgeries per year. The same study emphasized that there were no pediatric surgeons in the high-volume group (3).

In conclusion, we think that it is not possible to categorize pediatric surgeons in the "experienced surgeon" or "endocrine surgeon" groups for thyroid surgery because pediatric surgeons do not have enough patient potential to complete a high volume of thyroid surgeries annually, as defined by the international literature. If thyroid surgeries are performed by pediatric surgeons without assistance, we believe that pediatric patients will frequently encounter recurrent laryngeal nerve injury, post-operative hypocalcaemia and long-term hospitalization issues. While experience is the most significant criteria in reducing the rate of complications in thyroid surgery, it is evident that a multidisciplinary approach is necessary for pediatric patients with different physiologies than adults. For optimal results, therefore, surgery on such patients, particularly for total thyroidectomy and lymph node and neck dissection operations, should be conducted by a team comprising pediatric surgeons and high-volume thyroid or endocrine surgeons.

Keywords: Thyroid nodule, thyroidectomy, surgical experience, pediatric surgeon

Ethics

Peer-review: Internally peer-reviewed.

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ERRATUM

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Karti Ö. Letter to the Editor Regarding "Assessment of Retinal Nerve Fiber Layer Thickness in Non-Diabetic Obese Children and Adolescents". J Clin Res Pediatr Endocrinol 2018;10:91-91.

The second reference of the article has been corrected as following:

Karti O, Nalbantoglu O, Abali S, Tunc S, Ozkan B. The assessment of peripapillary retinal nerve fiber layer and macular ganglion cell layer changes in obese children: a cross-sectional study using optical coherence tomography. Int Ophthalmol 2017;37:1031-1038. Epub 2016 Oct 7

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CONGRESS CALENDAR

ESPE 2017 (10th International Meeting of Pediatric Endocrinology)
14-17 September 2017, Washington, DC, USA

ISPAD 2017 (43rd Annual Conference, International Society for Pediatric and Adolescent Diabetes) October 18-21, 2017, Innsbruck, Austria