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Increased Carotid Intima-media Thickness and Its Association with Carbohydrate Metabolism and Adipocytokines in Children Treated with Recombinant Growth Hormone

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What is already known on this topic?

There is an ongoing discussion as to whether treatment with recombinant human growth hormone (rhGH) may increase the risk of cardiovascular events, including ischemic heart disease, stroke, cardiomyopathy, and aneurysm in adulthood. In addition, knowledge about possible early diagnostic markers and risk factors for cardiovascular events associated with rhGH therapy in childhood is limited.

What this study adds?

The present study showed that carotid intima-media thickness (cIMT) is increased in growth hormone deficiency children treated with rhGH when compared to healthy matched controls. Notably, the level of serum ghrelin was higher in those with high cIMT-standard deviation scores, which suggests that ghrelin may play a role in changes of vascular endothelium.

Abstract

Objective: Reports on the association between growth hormone (GH) therapy and cardiovascular risk factors in children are limited. This study aimed to evaluate carotid intima-media thickness (cIMT) in children treated with recombinant human GH (rhGH) and assess the effects of rhGH therapy and changes in serum carbohydrate metabolism, lipid profile and adipocytokines on cIMT.

Methods: Seventy-one isolated idiopathic GH deficiency (GHD) children and 44 age- and sex-matched healthy controls were enrolled in this study. The study group was divided into two subgroups according to insulin resistance (IR) on oral glucose tolerance tests. Insulin secretion [homeostatic model assessment (HOMA) B, total insulin] and sensitivity (HOMA-IR, QUICKI, Matsuda) indices were calculated. cIMT was measured and the standard deviation scores (SDS) were calculated. Associations between cIMT-SDS and insulin secretion and sensitivity indices, serum lipid levels, adipocytokines (leptin, resistin, ghrelin), and other rhGH treatment-related factors were evaluated.

Results: cIMT-SDS was increased in GHD children treated with rhGH compared to the controls [0.02 (2.27) vs. -1.01 (1.63), $p = 0.003$]. cIMT-SDS did not differ between those children on rhGH treatment with or without IR. High cIMT-SDS was significantly associated with higher serum ghrelin levels and lower serum high density lipoprotein (HDL) levels ($\beta = 0.491$, $p = 0.001$ and $\beta = -0.027$, $p = 0.017$), but not with BMI-SDS, blood pressure, insulin secretion and sensitivity indices, or the dose and duration of rhGH therapy.

Conclusion: Our findings showed that GHD children treated with rhGH have increased cIMT. Alterations in carbohydrate metabolism were not associated with cIMT in children treated with rhGH. GH therapy *per se* appears to be associated with this increased cIMT but causality should be elucidated in further studies. cIMT also appears to be associated with higher ghrelin and lower HDL levels.

Keywords: Recombinant growth hormone, carotis intima-media thickness, adipocytokines, carbohydrate metabolism

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Introduction

There are ongoing concerns about the long-term effects of recombinant growth hormone (rhGH) treatment on cardiovascular morbidity and mortality (1,2). Both growth hormone (GH) deficiency and GH overproduction in acromegaly are known to be associated with higher mortality from cardiovascular disease (CVD) (3,4,5). However, discussion of the cardiovascular safety of rhGH treatment was sparked in 2012 by a study in a French cohort of children, which reported an increased risk of cardiovascular mortality (6). Further studies also uncovered an increased risk of cerebrovascular morbidity (7). Although the overall cardiovascular safety profile of rhGH treatment has been considered favorable in several reviews and consensus statements (8,9,10), a nationwide, population-based cohort study from Sweden showed that treatment with rhGH in childhood was associated with an increased risk of cardiovascular events (2). However, the absolute risk of cardiovascular events was low and evidence of causality is still limited.

Carotid intima-media thickness (cIMT) is a noninvasive biomarker of atherosclerosis (11). The prodromal stage of atheromatous plaque begins very early in life (12,13) and it predicts CVD (14,15). Excessive GH secretion leading to acromegaly is a known cause of increased cIMT (4), but the results of the various studies which have examined cIMT in GH-deficient children treated with rhGH are controversial (16,17,18,19). Furthermore, none of these studies used sex- and height-specific normative cIMT values to calculate standard deviation (SD) scores (SDS) to compare with healthy children.

It has been suggested that the direct action of GH contributes to the inflammatory process in atherogenesis (20). In addition, GH and its polypeptide mediator, insulin-like growth factor 1 (IGF-1), alter lipid and carbohydrate metabolism (21), which may increase the risk of CVD. Regarding carbohydrate metabolism, studies have postulated a six-fold increase in the incidence of type 2 diabetes in children treated with rhGH (22). Before overt glucose abnormalities occur, rhGH increases insulin secretion and decreases insulin sensitivity (23,24). In recent years, a variety of different parameters and indices derived from the oral glucose tolerance test (OGTT) have been used to diagnose glucose abnormalities, but few reports have shown their potential application in studying the effects of rhGH treatment on glucose and insulin homeostasis (25,26,27). To the best of our knowledge, no study has evaluated the relationship between cIMT, glucose sensitivity and insulin secretion indices to date.

The aim of this study was to measure cIMT in a group of children with isolated GH deficiency (GHD) treated with rhGH and to compare their cIMT-SDS with healthy children. We also hypothesized that the direct effects of GH and its indirect effects via IGF-1 on carbohydrate metabolism, lipid profile, and adipocytokines might be associated with cIMT in children treated with rhGH. Therefore, we measured cIMT and analyzed its association with the carbohydrate metabolism indices derived from OGTT, adipocytokines (leptin, resistin, ghrelin), and other cardiovascular risk factors, such as hypertension, obesity, and dyslipidemia.

Methods

Study Design

This cross-sectional observational study included 71 children and adolescents with isolated idiopathic GHD [female, n = 17 (24%), mean age 13.7 ± 2.6 years] who were consulted and treated at İstanbul University, İstanbul Faculty of Medicine, Department of Pediatric Endocrinology (28). GHD was identified using clinical, auxological and biochemical criteria from the GH Research Society. The inclusion criteria were: (i) GHD, defined as an absence of GH (peak GH levels below 10 µg/L) in response to two stimuli (clonidine and L-Dopa test); and (ii) treatment with rhGH for at least one year. The exclusion criteria were: (i) multiple pituitary hormone deficiency, except for hypothyroidism; (ii) any cardiovascular, respiratory, renal, or liver diseases; (iii) personal or family history of lipid disorders; and (iv) bioinactive GH syndrome. All children with GHD were treated with biosynthetic rhGH once daily before bedtime, for a total of seven injections per week. The initial subcutaneous dose was 30.2 ± 4.1 mcg/kg/day which was gradually adjusted during follow-up based on growth velocity and IGF-1 concentration. The demographic, clinical, and radiologic information, including magnetic resonance imaging (MRI) findings, were obtained from the patient records.

The control group included 44 healthy, age- and sex-matched children [female, n = 15 (34.1%), mean age 13.4 ± 2.9 years]. Organic diseases were excluded, based on physical examination in our hospital. The control group was selected from children referred to our hospital for well-child care visits.

The procedure was performed with the written and informed consent of the parents or guardians of the minors and in accordance with all applicable ethical and legal rules for medical research involving human subjects, according to the Declaration of Helsinki ethical statement. The study protocol and this consent procedure were approved by the

İstanbul University, İstanbul Faculty of Medicine Local Ethics Committee (date: 20.06.2014, no: 2014/990).

Assessment of Anthropometric Measures and Blood Pressure

Height was measured to the nearest millimeter using a wall-mounted calibrated Harpenden rigid stadiometer (Holtain Ltd., Crymych, UK). Weight was measured in underwear to the nearest 0.1 kg using a calibrated balance scale (Seca, Hamburg, GER). Waist circumference (WC) was measured using a non-elastic tape with the subject in a standing position. The abdominal circumference midway between the lowest rib and the top of the iliac crest at the end of expiration was measured to obtain WC. Height, weight, body mass index (BMI) (kg/m^2) and WC were expressed as SDS, based on Turkish reference growth charts (29,30). Growth velocity SDS was determined (31). Pubertal status was assessed using Tanner staging (32).

Blood pressure (BP) was measured on the right arm after a 15-minute rest with an oscillatory device. Three repeated measurements were taken with five minute intervals between each, and the lowest values of systolic and diastolic BP were documented. Office BP values for patients younger than 16 years were assessed according to the age-, sex-, and height-specific normative values of the Fifth Report (33).

Assessment of Glucose Metabolism

All assessments were made after an overnight fast. Fasting glucose, fasting insulin, and hemoglobin A1c (HbA1c) were determined and an OGTT was performed (glucose load of 1.75 g/kg, maximum of 75 g). Blood samples were collected every 30 minutes for two hours for glucose and insulin measurements (34).

Altered glucose metabolism was defined according to the American Diabetes Association criteria for prediabetes and included impaired fasting glucose (IFG), impaired glucose tolerance (IGT) or impaired HbA1c (5.7-6.4%) (35). Diabetes was diagnosed if fasting glucose was ≥126 mg/dL or glucose at the 120-minute measurement was ≥200 mg/dL or HbA1c ≥6.5%. If there was no clear hyperglycemia, the results were confirmed by repeated testing (35).

Fasting insulin levels, homeostatic model assessment (HOMA)-insulin resistance (IR), QUICKI, and Matsuda index values were used to assess insulin sensitivity (36,37,38). Upper limits for HOMA-IR were 2.67 for prepubertal boys, 2.22 for prepubertal girls, 5.22 for pubertal boys, and 3.82 for pubertal girls (36). Fasting insulin, total insulin levels, and HOMA B were used to assess insulin secretory capacity (37). All formulae used for the calculation of indices are given in Supplementary Table 1.

The GHD children treated with rhGH were divided into two subgroups according to hyperinsulinemia: a) IR and b) without IR. Hyperinsulinemia was diagnosed when the total insulin level measured throughout OGTT (0, 30, 60, 90, and 120 minutes) was above 300 µU/mL or the 120-minute insulin level was above 75 µU/mL or the peak insulin level was above 150 µU/mL (39).

Assessment of Serum Lipid Profile and Adipocytokines

After an overnight fast, blood samples were collected from both the GHD and control groups to analyze serum lipid levels, including triglycerides (TG), total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and adipocytokines including leptin, resistin and ghrelin. The lipid profile was analyzed using routine laboratory methods. Serum samples were stored at -80 °C. Leptin was measured with a kit from Labsystems Diagnostics Oy (Vantaa, Finland), and resistin and ghrelin with kits from USCN Life Science Inc (Wuhan, PRC) using the enzyme-linked immunosorbent assay (ELISA) method. The sensitivities of the ELISA kits (detection limits) were 0.04 ng/mL, 0.2 ng/mL, and 21 pg/mL for leptin (Cat. No. 140101/A), resistin (Cat. No. L140718502), and ghrelin (Cat. No. L140718468), respectively. Intra-assay variability was 3.5-13.3%, <5.8% and 3.3-10.0% for leptin, resistin, and ghrelin, respectively. Inter-assay variability was 10.2%, 14.8% and 14.7% for these markers, respectively.

Assessment of cIMT

Images of the carotid artery were obtained by a single and experienced cardiologist (M.K.) from the children with GHD and the control group using a colored Doppler ultrasonographic device (Aplio SSA 770, Toshiba, Tokyo, Japan), with the linear probe set at 7.5 mHz according to the Mannheim cIMT consensus (40). Measurements were performed in the supine position with the neck slightly hyperextended. Three manual measurements were obtained in both carotid arteries, 1 to 2 cm proximal to the bifurcation at the far wall longitudinally, after a 10 minute rest, and then these measurements were averaged for each individual (11). The SDSs for cIMT were calculated using the LMS method and height-specific normative values (41).

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS), version 21 (SPSS Inc., Chicago, IL., USA). Normality of distribution for the variables was confirmed by a Shapiro-Wilk test. Continuous variables are presented as mean (± SD) or median and interquartile range according to the distribution of data. Student's t-test or the Mann-Whitney U test were used to compare

differences between two independent groups according to the distribution of data. The paired t-test and the Wilcoxon signed rank test were used to compare differences between two paired groups. Categorical variables are expressed as number (percentage). Fisher's exact test was used to compare categorical variables. Correlations between biomarkers and other clinical and laboratory variables were analyzed using Spearman's test. To identify independent factor(s) influencing cIMT, all variables which had a p value ≤0.1 on univariate analysis and were known clinical risk factors were tested in a stepwise multivariable linear regression analysis. Statistical significance was defined as a two-tailed p value < 0.05.

Results

Patient-related Characteristics

The mean age (minimum-maximum) of the children at the start of rhGH treatment was 11.2 (2.3-16.2) years, and the male to female ratio was 3.2:1.0. The frequency of consanguinity was 8% in the study group. The proportions of the study group with a family history of obesity, hypertension, diabetes, hyperlipidemia, and coronary heart disease were 7%, 4%, 14%, 1%, and 1%, respectively.

All the cranial MRI findings were normal, except in seven children (10%) who had Arnold Chiari malformation, small hypophysis (n = 3), microadenoma (n = 1), ectopic neurohypophysis (n = 1) and hypophyseal cyst (n = 1). Except for two term-born children with intrauterine growth retardation, all the rest of the GHD group was born with a gestational age-appropriate weight. Two of the children with GHD were treated with testosterone propionate before starting GH treatment because of micropenis. Hypothyroidism was diagnosed in twelve children (primary hypothyroidism in four children and secondary hypothyroidism in eight children) before starting rhGH treatment. All those children were under treatment and euthyroid at the time of this study.

The mean height-SDS (H-SDS) of the children with GHD (-2.42 ± 0.87) was 1.04 SD away from target H-SDS (-1.38 ± 0.78). GHD was supported by the absence of GH response in two stimulation tests (clonidine and L-Dopa test) with peak GH levels of 3.3 ± 2.3 µg/L and 4.2 ± 2.2 µg/L, respectively. The anthropometric findings and characteristics of the study group at the start of rhGH treatment are shown in Table 1.

At the time of investigation, the duration of rhGH therapy for the children with GHD was 2.5 ± 1.4 years. The percentage of children treated for 1-3 years and for more than 3 years were 73% (n = 52) and 27% (n = 19), respectively. The mean H-SDS increased significantly during the study period with rhGH treatment (-2.42 ± 0.87 vs. -1.65 ± 0.88, $p < 0.001$). The mean rhGH dose was 31.1 ± 4.0 mcg/kg/day. A consistent increase in IGF-1 SDS and IGF-binding protein 3 (IGFBP-3) SDS was noticed over the study period ($p < 0.001$) (Table 1). Eleven (15%) of the children with GHD were underweight

Table 1. Anthropometric and clinical features of growth hormone deficient children group (evaluation at diagnosis and at the time of study) and the control group

	GHD children at diagnosis (n = 71)	GHD children at time of study (n = 71)	Control group (n = 44)	p^1	p^2
Age (years)	12.0 ± 1.9	13.7 ± 2.6	13.4 ± 2.9	-	0.55
Pubertal children, n (%)	36 (51%)	60 (84%)	37 (84%)	-	0.20
H-SDS	-2.42 ± 0.87	-1.65 ± 0.88	-0.09 ± 0.98	**< 0.001**	**< 0.001**
Weight SDS	-1.34 (1.08)	-1.25 (0.96)	-0.39 (1.41)	0.059	**< 0.001**
BMI-SDS	-0.33 (1.23)	-0.68 (1.08)	-0.47 (1.22)	0.184	0.619
WC-SDS	-	0.36 (1.40)	0.49 (0.90)	-	0.90
Systolic BP-SDS	-0.12 ± 0.88	-0.11 ± 0.85	-0.29 ± 0.86	0.97	0.26
Diastolic BP-SDS	0.69 ± 0.69	0.70 ± 0.68	0.60 ± 0.77	0.97	0.44
Bone age/age	0.81 ± 0.13	0.87 ± 0.12	-	**< 0.001**	
Growth velocity-SDS	-0.96 ± 1.60	4.77 ± 3.89	-	**< 0.001**	
Target H-SDS	-1.32 ± 0.78		-		
Predicted H-SDS	2.28 (3.68)	0.96 (2.93)	-	**< 0.001**	
IGF-1 SDS	-2.13 (1.97)	1.15 (3.23)	0.37 (3.10)	**< 0.001**	0.056
IGFBP-3 SDS	-0.97 (2.22)	0.66 (1.65)	-0.45 (1.52)	**< 0.001**	**< 0.001**

p^1: comparison between GHD children at the start of the treatment and the time of study, p^2: comparison between GHD children at the time of study and control group. Results are given as mean ± SD or median (interquartile range) according to the distribution. Categorical results are given as n (%).
BMI: body mass index, BP: blood pressure, SDS: standard deviation (SD) score, IGF-1: insulin-like growth factor-1, IGFBP-3: IGF-binding protein 3, GHD: growth hormone deficiency, WC: waist circumference, H-SDS: height SDS

and four (5%) had obesity at the start of the treatment. At the time of this investigation, these frequencies were 10% and 7%, respectively, but weight SDS and BMI-SDS did not change significantly over the study period (Table 1). None of the controls were obese or underweight. At the time of this study, the BMI-SDS and WC-SDS of the children with GHD and the control group showed no statistical difference. Table 1 shows the anthropometric measurements, demographics, pubertal status, IGF-1 SDS, IGFBP-3 SDS and office BP of the children with GHD at the start of rhGH treatment and at the time of investigation, and their comparisons with the control group.

Carbohydrate Metabolism

Sixty-six children from the GHD group completed OGTT, while five children could not complete the test because of nausea and vomiting. None of the children had evidence of type 2 diabetes or IGT. Alterations in glucose metabolism were detected in three patients, one presenting with IFG and impaired HbA1c, and two presenting only with impaired HbA1c. In total, 30 (45.45%) had hyperinsulinemia and were grouped as IR.

Comparison of those children with GHD and those without IR did not show any difference in the frequency of family history of obesity, hypertension, diabetes, dyslipidemia or coronary heart disease. Furthermore, the proportion of patients with pathological MRI findings was not different between the GHD children with or without IR [1/30 (3.3%) vs. 6/36 (16.7%), respectively].

Age at start of rhGH treatment, peak GH levels of the two stimulation tests, SDSs of anthropometric measurements before treatment and growth velocities did not show any difference between the GHD children with or without IR (Table 2). Although the treatment times were similar between the groups, those children with IR were significantly older

Table 2. Anthropometric and clinical features of growth hormone deficient children with or those without insulin resistance and the control group

	GHD + IR (n = 30)	GHD without IR (n = 36)	Control (n = 44)	p
Female n (%)	5 (16.6)	12 (33.3)	15 (34.1)	-
At diagnosis				
Age at diagnosis (years)	11.8 ± 2.0	10.5 ± 2.8		**0.030**
Puberty at diagnosis, n (%)	20 (66.6)	13 (36.1)	-	**0.013**
Bone age/age	0.82 ± 0.14	0.80 ± 0.12	-	0.55
Weight SDS	-1.24 (1.09)	-1.49 (0.95)	-	0.17
H-SDS	-2.38 ± 0.84	-2.78 ± 1.00	-	0.10
BMI-SDS	-0.55 (1.55)	-0.17 (0.90)	-	0.50
Growth velocity SDS	-0.77 ± 1.59	-1.16 ± 1.63	-	0.33
Predicted H-SDS	2.21 (3.50)	2.07 (3.85)	-	0.63
At study time				
Age at study (years)	14.5 ± 1.7	13.0 ± 2.8	13.4 ± 2.9	0.06
Duration of rhGH treatment (years)	2.6 ± 1.4	2.5 ± 1.3		0.72
Pubertal children, n (%)	27 (90.0)	28 (77.7)	-	0.24
Bone age/age	0.86 ± 0.10**	0.87 ± 0.14**	0.97 ± 0.10	0.96
Weight SDS	-1.22 (0.77)**	-1.37 (1.31)**	-0.40 (1.41)	0.84
H-SDS	-1.50 ± 0.74**	-1.89 ± 0.96**	-0.09 ± 0.96	0.18
BMI-SDS	-0.68 (0.94)	-0.72 (1.14)	-0.47 (1.22)	0.88
Growth velocity SDS	5.55 ± 4.75	4.44 ± 3.13		0.26
Target H-SDS	-1.24 ± 0.72*	-1.42 ± 0.84**		0.64
Predicted H-SDS	1.29 (2.53)	0.26 (3.68)		0.12
WC-SDS	0.40 (0.93)	0.26 (1.49)	0.49 (0.90)	0.91
ΔWeight-SDS	0.02 (0.82)	0.09 (0.92)	-	0.38
ΔH-SDS	0.83 (1.07)	0.83 (0.69)	-	0.88
ΔBMI-SDS	0.72 (0.95)	0.79 (0.88)	-	0.70

p: comparison between GHD-IR and GHD without IR subgroups, *p < 0.05 for comparison with control group, **p < 0.01 for comparison with control group. Δ = change between the time of study and the time of diagnosis. Results are given as mean ± SD or median (interquartile range) according to the distribution. Categorical results are given as n (%).
BMI: body mass index, SDS: standard deviation (SD) score, rhGH: recombinant growth hormone, WC: waist circumference, H-SDS: height SDS, GHD: growth hormone deficiency, IR: insulin resistance

than the children without IR (p = 0.003). The proportion of pubertal children at onset of therapy was higher in the IR subgroup (n = 27; 90%) compared to the non-IR-subgroup (28; 78%; p = 0.013). Anthropometric measurements during the study of those GHD children with and those without IR are summarized in Table 2. Neither the BMI-SDS at study time nor the ΔBMI-SDS showed any difference between these subgroups.

The insulin sensitivity and secretion indices are summarized in Table 3. The children with GHD treated with rhGH showed significantly lower levels of fasting glucose and higher fasting insulin levels compared to the control group [81.0 ± 8.7 vs. 88.8 ± 9.1 mg/dL, p < 0.0001 and 11.5 (8.8) vs. 7.9 (5.6), p = 0.014]. Both HOMA-IR and HOMA-B indices were significantly higher in the GHD group compared to the controls [2.3 (1.9) vs. 1.7 (1.4), p = 0.039 and 160.6 (79.0) vs. 100.6 (49.0), p < 0.001] (Table 3).

Fasting insulin, HOMA-IR, total insulin level at OGTT, 120-minute glucose levels, HOMA-B, and Matsuda indices were significantly higher in the GHD children with IR than in those without IR (p = 0.001, p = 0.001, p < 0.001, p = 0.004, p = 0.044, and p < 0.001, respectively). Among the insulin

Table 3. Comparison of cardiovascular risk factors and carotid intima-media thickness measurement between growth hormone deficient children groups with or those without insulin resistance and the control group

	All children with GHD (n = 71)	Control (n = 44)	p[1]	GHD + IR (n = 30)	GHD without IR (n = 36)	p[2]
IGF-1 SDS (study time)	1.15 (3.23)	0.37 (3.10)	**0.019**	1.68 (3.32)	0.92 (3.09)	0.89
IGFBP-3 SDS (study time)	0.66 (1.65)	-0.45 (1.52)	**< 0.001**	0.65 (1.61)**	0.66 (1.74)**	0.68
Blood pressure						
Systolic BP-SDS	-0.11 ± 0.85	-0.29 ± 0.86	0.263	-0.05 ± 0.88	-0.17 ± 0.88	0.57
Diastolic BP-SDS	0.70 ± 0.68	0.59 ± 0.77	0.441	0.81 ± 0.69	0.57 ± 0.68	0.16
Glucose metabolism indices						
Fasting glucose (mg/dL)	80.1 ± 8.7	88.8 ± 9.1	**< 0.001**	82.8 ± 7.6*	78.8 ± 8.4**	0.050
2-h glucose (mg/dL)	105.4 ± 19.8	-	-	112.7 ± 19.5	98.8 ± 18.1	**0.004**
HbA1c (%)	5.1 ± 0.3	-	-	5.2 ± 0.3	5.1 ± 0.3	0.37
Insulin secretion indices						
Fasting insulin (μU/mL)	11.5 (8.8)	7.9 (5.6)	**0.014**	15.1 (11.9)**	10.0 (6.9)	**0.001**
HOMA-B	160.6 (79.0)	100.6 (49.0)	**< 0.001**	178.6 (90.1)**	148.9 (69.3)**	**0.044**
Total insulin (μU/mL)	253.1 (255.7)	-	-	432.5 (210.3)	180.8 (81.8)	**< 0.001**
Insulin sensitivity indices						
HOMA-IR	2.3 (1.9)	1.7 (1.4)	**0.039**	2.9 (2.3)**	1.9 (1.4)	**0.001**
QUICKI	2.8 (0.3)	3.1 (0.4)	**< 0.001**	2.8 (0.2)**	2.9 (0.3) *	**< 0.001**
MATSUDA	3.9 (3.1)	-	-	2.8 (1.4)	5.6 (3.3)	**< 0.001**
Adipocytokines						
Leptin (ng/mL)	0.26 (2.09)	0.78 (2.84)	0.728	0.38 (2.47)	0.24 (1.75)	0.80
Resistin (ng/mL)	1.36 (0.41)	1.51 (0.66)	**0.023**	1.28 (0.51) **	1.37 (0.37)	0.39
Ghrelin (pg/mL)	62.38 (52.49)	45.60 (31.26)	**0.001**	69.84 (67.46) *	60.82 (47.33)	0.65
Lipid profile at the time of the study						
Cholesterol (mg/dL)	166.3 ± 27.5	152.9 ± 34.2	**0.044**	168.9 ± 29.2	163.1 ± 26.2	0.77
Triglyceride (mg/dL)	86.4 ± 53.7	74.8 ± 30.1	0.256	83.0 ± 32.0	88.9 ± 67.2	0.90
LDL (mg/dL)	88.0 ± 24.0	83.6 ± 26.8	0.437	85.7 ± 22.9	88.6 ± 23.8	0.90
HDL (mg/dL)	60.0 ± 18.9	56.6 ± 17.0	0.395	64.0 ± 22.2	57.0 ± 16.3	0.35
VLDL (mg/dL)	15.5 ± 6.1	16.1 ± 6.0	0.781	15.3 ± 6.3	15.3 ± 5.4	0.97
Carotid intima-media thickness						
cIMT (mm)	0.50 (0.16)	0.42 (0.07)	**0.003**	0.50 (0.17) *	0.50 (0.14) *	0.52
cIMT-SDS	0.02 (2.27)	-1.01 (1.63)	**0.003**	0.02 (2.45)	0.01 (2.01)	0.86

p[1]: comparison between all GHD children and control group, p[2]: comparison between GHD-IR and without IR groups, *p < 0.05 for comparison with control group, **p < 0.01 for comparison with control group. Results are given as mean ± SD or median (interquartile range) according to the distribution. Categorical results are given as n (%).

BMI: body mass index, BP: blood pressure, IGF-1: insulin-like growth factor-1, IGFBP-3: insulin-like growth factor-binding protein-3, cIMT: carotid intima-media thickness, HbA1c: hemoglobin A1c, HOMA: homeostatic model assessment, LDL: low-density lipoprotein, VLDL: very LDL, HDL: high-density lipoprotein, SDS: standard deviation (SD) score, IR: insulin resistance, GHD: growth hormone deficiency

sensitivity indices, QUICKI was significantly lower in the GHD children with IR than in those without IR ($p < 0.001$) (Table 3).

Lipid Profile and Adipocytokines

Fourteen (20%) of the children in the GHD group had elevated serum lipid levels (Table 3). Seven children had hypercholesterolemia, four children had hypertriglyceridemia, and three had high LDL levels. The proportion of children with elevated serum lipids did not differ between the GHD group and the control group. Except for the total cholesterol level, which was significantly higher in GHD children treated with rhGH than in the controls ($p = 0.044$), none of the other serum lipid profile parameters differed between the groups. The serum lipid profile parameters and the number of children with elevated serum lipids did not differ between the GHD subgroups.

Whereas serum leptin levels in the GHD group did not differ from the control group, serum resistin levels were lower and ghrelin levels were higher in the GHD group than in the control group [1.36 (0.41) vs. 1.51 (0.66), $p = 0.023$ and 62.38 (52.49) vs. 45.60 (31.26), $p = 0.001$, respectively]. Although serum resistin levels tended to be lower and ghrelin levels tended to be higher in the GHD IR-subgroup compared to the GHD without IR subgroup, this was not significant. Resistin levels were lower in GHD children with IR than in the controls ($p = 0.008$), and ghrelin levels were higher than in the controls ($p = 0.014$). A comparison of adipocytokine levels between the groups is summarized in Table 3.

cIMT and Associated Factors

Both cIMT and cIMT-SDS were increased in the GHD children treated with rhGH compared to the controls [0.50 (0.16) vs. 0.42 (0.07) mm, $p = 0.003$ and 0.02 (2.27) vs. -1.01 (1.63), $p = 0.003$] (Figure 1). Both subgroups of GHD children with and those without IR showed increased cIMT and cIMT-SDS levels compared to the control group (Table 3) but there were no differences between the IR subgroups.

Higher CIMT-SDS only correlated significantly with higher ghrelin ($r = 0.338$, $p = 0.010$) but not with other adipocytokines (leptin, resistin), anthropometric measures (BMI-SDS, H-SDS, W-SDS, WC-SDS), BP-SDS, rhGH treatment related factors (dose of rhGH, IGF-1, growth velocity SDS, ΔH-SDS), or glucose metabolism indices (HOMA-IR, HOMA-B, Matsuda, QUICKI) (Table 4).

In multivariate linear regression analysis, a high cIMT-SDS was significantly associated with higher serum ghrelin levels [b-coefficient 0.491, 95% confidence interval (CI) = 0.007-0.028, $p = 0.001$] and lower serum HDL levels (b-coefficient -0.027, 95% CI = -0.049 – -0.005, $p = 0.017$). The cIMT and cIMT-SDS were not significantly associated with any other parameters (anthropometric measures, BP SDS, rhGH treatment related factors, or glucose metabolism indices) in multiple linear regression analyses.

Discussion

The present study showed that cIMT is increased in GHD children treated with rhGH when compared to healthy

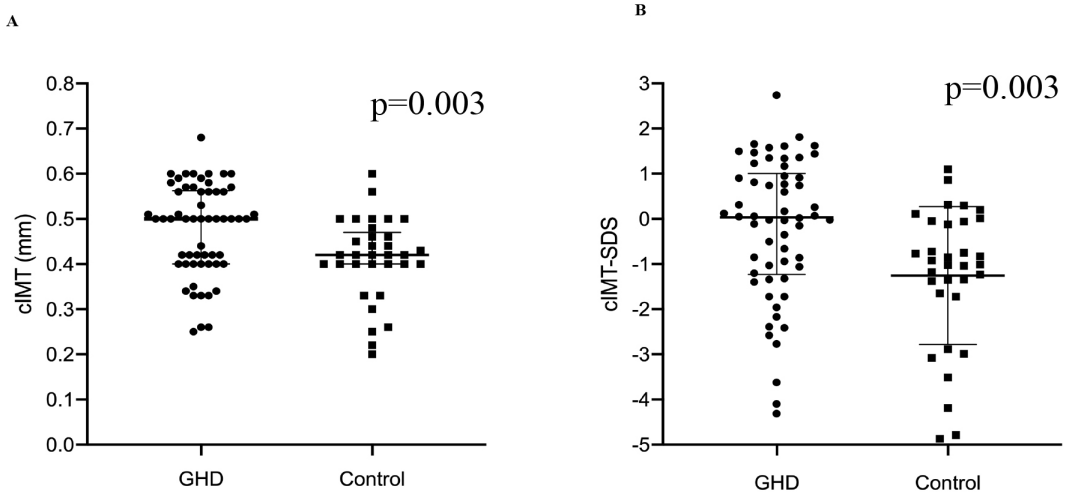

Figure 1. This figure shows the comparisons of cIMT and cIMT-SDS between healthy controls (n = 44) and GHD children treated with rhGH (n = 71). Original levels [median (interquartile range)] of cIMT and cIMT-SDS in healthy controls and GHD children treated with rhGH, A) cIMT levels: 0.50 (0.16) vs. 0.42 (0.07) mm, B) cIMT-SDS: 0.02 (2.27) vs. -1.01 (1.63)

cIMT: carotid intima-media thickness, SDS: standard deviation score, GHD: growth hormone deficiency

Table 4. Spearman's rank correlation between cIMT or cIMT-SDS and cardiovascular risk factors, age, IGF-1, IGFBP-3

	cIMT		cIMT-SDS	
	r	p	r	p
Age at diagnosis (years)	0.147	0.27	0.001	0.99
Age at study (years)	0.060	0.65	-0.107	0.42
Duration of rhGH treatment (years)	-0.101	0.45	-0.136	0.30
rhGH dose (mcg/kg/day)	-0.073	0.58	-0.077	0.56
H-SDS	-0.128	0.23	-0.171	0.11
BMI-SDS	-0.09	0.39	-0.069	0.52
WC-SDS	0.135	0.31	0.140	0.29
ΔH-SDS	-0.003	0.98	-0.013	0.92
ΔBMI-SDS	-0.233	0.08	-0.241	0.07
Mean growth velocity SDS	0.109	0.41	0.065	0.63
IGF-1 SDS	0.041	0.76	0.073	0.59
IGFBP-3 SDS	-0.105	0.44	-0.113	0.40
Systolic BP-SDS	0.002	0.99	-0.024	0.86
Diastolic BP-SDS	0.131	0.32	0.015	0.91
Fasting glucose (mg/dL)	0.138	0.30	0.069	0.60
120-minute glucose (mg/dL)	0.113	0.40	0.110	0.41
HbA1c (%)	-0.040	0.81	-0.077	0.64
Fasting insulin (μU/mL)	0.119	0.38	0.071	0.60
HOMA-B	0.111	0.41	0.137	0.31
Total insulin (μU/mL)	-0.003	0.98	-0.056	0.68
HOMA-IR	0.137	0.30	0.086	0.52
QUICKI	-0.097	0.47	-0.063	0.64
MATSUDA	-0.078	0.56	-0.019	0.89
Leptin (ng/mL)	-0.037	0.79	-0.013	0.92
Resistin (ng/mL)	-0.072	0.59	-0.086	0.52
Ghrelin (pg/mL)	0.306	**0.019**	0.338	**0.010**
Cholesterol (mg/dL)	-0.162	0.27	-0.069	0.64
Triglyceride (mg/dL)	0.120	0.41	0.103	0.48
LDL (mg/dL)	-0.063	0.67	0.023	0.87
HDL (mg/dL)	-0.259	0.07	-0.248	0.09

BMI: body mass index, BP: blood pressure, WC: waist circumference, rhGH: recombinant growth hormone, IGF-1: insulin-like growth factor-1, IGFBP-3: insulin-like growth factor-binding protein-3, SDS: standard deviation score, H-SDS: height SDS, cIMT: carotid intima-media thickness, HbA1c: hemoglobin A1c, HOMA: homeostatic model assessment, LDL: low-density lipoprotein, HDL: high-density lipoprotein

matched controls. This study also shows that the prevalence of alterations in glucose metabolism is low (5%) despite an increased risk of IR in children and adolescents treated with rhGH. Moreover, rhGH-treated children without IR exhibited increased insulin secretion capacity compared to the control group. However, there was no association between cIMT and loss of carbohydrate metabolism homeostasis, insulin secretion or sensitivity indices in these children. Furthermore, none of the factors related to the direct effects of rhGH treatment showed an association with changes in vascular endothelium. Adipocytokine levels and elements of the lipid profile also showed changes in GHD children treated with rhGH compared to the control group. Notably, serum HDL levels, which were lower, and ghrelin levels, which were higher in those with high cIMT-SDS scores, suggests that these adipocytokines and lipid profiles may play a role in changes of vascular endothelium.

We evaluated cIMT as an early marker of vascular changes in our cohort. Our results revealed that cIMT and cIMT-SDS were increased in GHD children treated with rhGH compared to the control group. In our study, all of the children were diagnosed as isolated GHD and treated with physiological rhGH dose and almost one third of the cohort were treated for more than three years. The results of the various studies which have examined cIMT in GH-deficient adults and children treated with rhGH are controversial. In a cohort of children and adults consisting of 105 subjects

over eight generations with GHD, there was no association between increased cIMT and GHD, but treatment with rhGH led to the appearance of carotid plaques (42). Rothermel et al. (16) demonstrated cIMT increase in children treated for three years with a supraphysiological rhGH dose but not in those treated with a physiological dose. Another study did not demonstrate any change after four years of rhGH treatment in cIMT levels in a cohort consisting of children and adolescents receiving rhGH for various indications (17). Two studies showed improvement in cIMT parameters in children and adolescents after one year of treatment with rhGH (18,19).

Atherosclerosis is a complex and multifactorial disease commonly associated with traditional cardiovascular risk factors; however, both GH and IGF-1 may influence vascular wall changes (42,43,44). The final effect of both of them in this process result from an interplay between their atherosclerotic and anti-atherosclerotic functions. On one hand, they promote atherogenesis, by stimulating vascular smooth muscle proliferation and inflammation, but on the other hand, they induce vasodilatation by stimulating nitric oxide production (42,43,44). It has been postulated that the direct action of GH contributes to the inflammatory process in atherogenesis (20). The longer the duration of rhGH treatment and the higher the cumulative dose in childhood, the stronger the association with CVD risk (2). A persistent very low IGF-1 level might have a protective role, whereas a milder decrease, such as in adult-onset GH-deficiency, might be predisposing to CVD risk (45). Rothermel et al. (16) showed a correlation between cIMT and changes in IGF-1 SDS after rhGH treatment in GHD children, but not with the dose of the rhGH treatment. In our study, there was no association between IGF-1 SDS and cIMT-SDS. Also, none of the parameters due to the direct effect of rhGH treatment (growth velocities, dose of rhGH and the duration of the treatment) were associated with vascular changes.

Alterations in body composition, carbohydrate metabolism and lipid profile have long been suspected of being risk factors for CVD in children with GHD (46), and in children treated with rhGH (16,17,25). In GHD children, rhGH therapy reduces insulin sensitivity and causes a compensatory hyperinsulinemia with normal glucose levels (26). Our current results are in line with these data. We found an increase in insulin values in half of the cohort, with a concomitant decrease in the insulin sensitivity indices QUICKI and Matsuda, after almost three years of treatment. In addition, GHD children without IR also showed an increased HOMA-B index compared to the control group, which indicates an increased insulin secretion capacity in these children. Although Cutfield et al. (22) reported an increased incidence of diabetes mellitus in children and adolescents receiving rhGH treatment, there was no individual with type 2 diabetes in our cohort. To the best of our knowledge, this is the first study evaluating the relationship between cIMT and parameters of carbohydrate metabolism in children with GHD treated with rhGH, but we could not find any association between the alterations in carbohydrate metabolism and changes in cardiovascular endothelium.

Studies have shown that rhGH treatment has a beneficial effect on body composition in GHD children (46,47). However, studies assessing the effect of rhGH treatment on lipid profile in children with GHD have reported conflicting results with some studies demonstrating a favorable effect on lipid profile with rhGH treatment (47,48), while others do not (49). The differences in severity and duration of GHD, and the variable presence of other hormone deficits might be the reason for these conflicting results. Due to the cross-sectional design of our study, we were able to compare the body composition and lipid profile of GHD children treated with rhGH and healthy controls, and there was no difference between the groups. Furthermore, there was no difference between the subgroups stratified by IR. Limited studies evaluating the effect of body composition and serum lipid profile on cIMT in children have shown a positive association between cIMT and BMI-SDS (17) and baseline LDL-cholesterol (16). In our study, there were no associations between cIMT-SDS and body composition parameters (BMI-SDS at diagnosis and study time, ΔBMI-SDS, and waist-SDS), but lower HDL-cholesterol levels showed an independent association with higher cIMT-SDS. However, longitudinal research is needed to evaluate the conflicting relationship between blood lipid profile and cIMT-SDS in GHD children treated with rhGH.

Adipocytokines, including leptin, resistin and ghrelin, are involved in the processes of utilizing and storing energy in a tight interaction with GH. GHD children treated with rhGH showed decreased levels of leptin and des-acyl ghrelin and increased acyl-ghrelin and serum resistin levels (50,51). The changes in leptin and ghrelin are thought to cause the metabolic effects of rhGH on lipid mobilization and promote fat loss (52). Ghrelin is especially known for its cardiovascular effects. Low circulating des-acyl ghrelin levels increase cardiovascular risk (53). However, Oliveira-Santos et al. (54) argued that there are favorable vascular and metabolic outcomes of reduced postprandial ghrelin secretion in GHD individuals. In our cohort, GHD children treated with rhGH showed higher total ghrelin and lower resistin levels than the control group.

Although some studies showed an increase in resistin levels after rhGH treatment in GHD children (50,55), others showed a decline in resistin after treatment (56,57). It has been shown that ghrelin decreases after rhGH treatment compared to the GHD period (56). Because of the lack of baseline results, we were unable to make any conclusions about the changes of these adipocytokines after rhGH treatment. Previously, no study had evaluated the relationship between ghrelin and cIMT in GHD children treated with rhGH. In our study, cIMT and cIMT-SDS showed a positive association with total ghrelin levels. High endogenous ghrelin levels are associated with increased carotid artery stenosis in the adult population, and they related with high IL-1β and vascular smooth muscle cell dysfunction (58,59). Our study showed that ghrelin should be considered as a potential early marker for vascular changes in GHD children treated with rhGH, but further studies are needed.

Study Limitations

The changes in carbohydrate metabolism in rhGH treated children and cardiovascular evaluation have been investigated in different studies. Our study attempted to elucidate the association between vascular and metabolic changes with a broad perspective. A major limitation of our study was that we did not use a glucose clamp test, which is the gold standard for the evaluation of IR. Secondly, our study protocol did not include pre-rhGH treatment evaluations of the lipid, carbohydrate or cardiovascular metabolism. Thirdly, our cohort was followed for a relatively short period of time and, as adulthood risks for CVD in this group of children are important, long-term follow-up studies are needed to evaluate the exact role of IR in CVD in GHD children treated with rhGH. Fourthly, we did not demonstrate any change between basal and post-treatment cIMT results in our cohort, due to the lack of evaluation at the start of the rhGH treatment. Lastly, our control group consisted of healthy children, but not of GHD children without rhGH treatment. There are several studies in children and adolescents showing improvements in cIMT after rhGH treatment. As we compared GHD children treated with rhGH with healthy controls, the difference of cIMT levels between the groups may also be effected by GHD itself.

Conclusion

In conclusion, this study found increased cIMT-SDS in GHD children treated with rhGH compared to a healthy control group. Alterations in carbohydrate metabolism were not directly associated with cIMT-SDS. Changes in ghrelin may be associated with early vascular changes. rhGH therapy *per se* appears to be associated with this increased cIMT but causality should be elucidated in future studies.

Ethics

Ethics Committee Approval: The study protocol and this consent procedure were approved by the İstanbul University, İstanbul Faculty of Medicine Local Ethics Committee (date: 20.06.2014, no: 2014/990).

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

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Seha Saygılı, Mehmet Kocaağa, Mine Şükür, Concept: Seha Saygılı, Feyza Darendeliler, Design: Seha Saygılı, Feyza Darendeliler, Data Collection or Processing: Seha Saygılı, Mehmet Kocaağa, Gamze Kaya, Mine Şükür, Firdevs Baş, Şükran Poyrazoğlu, Analysis or Interpretation: Seha Saygılı, Firdevs Baş, Şükran Poyrazoğlu, Rüveyde Bundak, Feyza Darendeliler, Literature Search: Seha Saygılı, Feyza Darendeliler, Writing: Seha Saygılı, Feyza Darendeliler.

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Click for Supplementary Table 1. Formulas for the assessment of insulin sensitivity and secretory capacity

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