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Platelet Hyperactivation in Maintained Growth Hormone-Deficient Childhood Patients after Therapy Withdrawal as a Putative Earlier Marker of Increased Cardiovascular Risk

Flávio Reis, Maria Vitor Campos, Margarida Bastos, Luis Almeida, Margarida Lourenço, Carlos A. Ferrer-Antunes, Aida Palmeiro, José D. Santos-Dias, José F. Mesquita, Manuela Carvalheiro, and Frederico Teixeira

Unit of Therapeutics (F.R., M.V.C., L.A., F.T.), Institute of Pharmacology and Experimental Therapeutics, and Electron Microscopy Laboratory (J.D.S.-D., J.F.M.), Botanic Department, Science and Technology Faculty, Coimbra University, 3004-504 Coimbra, Portugal; and Endocrinology, Diabetes, and Metabolism Unit (M.V.C., M.B., M.C.) and Hematology Laboratory (M.L., C.A.F.-A., A.P.), Coimbra University Hospital, 3049 Coimbra Codex, Portugal

GH deficiency (GHD) is associated with a higher risk of vascular disease, whose pathophysiological mechanisms remains not yet fully elucidated. This study aimed to assess the main cardiovascular risk indexes, plasma catecholamines content, and the platelet function in childhood-onset GHD patients.

Some of the main clinical examinations related with cardiovascular risk, plasma catecholamines content, as well as platelet intracellular free calcium concentration ($[Ca^{2+}]_i$), whole-blood aggregation, and morphology were evaluated in childhood-onset GHD patients treated with GH for a variable period and off GH therapy for at least 2 yr before entry into study and in sex-, age-, and body mass index-matched control groups. Among the patients, group 1 (GHD-1) has recovered GH levels after withdrawal, whereas group 2 (GHD-2) has remained GH deficient.

Minor differences on the cardiovascular risk indexes were observed between the groups. Plasma catecholamine concentrations in the GHD groups did not statistically differ from the control group, but higher adrenaline content was observed in the GHD-2 group when compared with the GHD-1 one. Basal and thrombin-evoked $[Ca^{2+}]_i$ and platelet aggregation were identical between the GHD-1 group and the matched control. However, the GHD-2 group has increased thrombin-evoked $[Ca^{2+}]_i$ (297.0 ± 15.7 Δnmol/liter; $P < 0.01$), collagen, and ADP-induced platelet aggregation (33.3 ± 4.3 and 12.5 ± 2.1 Ω, respectively; $P < 0.05$) *vs.* the control-2 group (Δ$[Ca^{2+}]_i$: 102.1 ± 13.6 Δnmol/liter; aggregation: 19.6 ± 2.9 and 6.2 ± 0.8 Ω). The platelet hyperreactivity state in the GHD-2 was reinforced by morphologic studies of electron microscopy.

In conclusion, there were minor differences between the GHD-1 group and the controls, which might be due to the recovery of GH levels after therapy withdrawal. However, the maintained GHD group, despite minor cardiovascular risk index differences, has increased $[Ca^{2+}]_i$ and aggregation, which could indicate a hyperactivation state that might be viewed as an earlier marker of cardiovascular disturbances. (*J Clin Endocrinol Metab* 90: 98–105, 2005)

GH DEFICIENCY (GHD) SINCE the developmental period may predict a higher risk for cardiovascular disease, which includes abdominal adiposity, dyslipoproteinemia and dyslipidemia, decreased fibrinolytic activity, insulin resistance, glucose intolerance, and increased prevalence of hypertension (1–5). Adults with hypopituitarism have twice the cardiocerebrovascular mortality rate of the normal population as well as reduced longevity, which seems to be due to untreated GHD (1, 3, 5, 6). The mechanisms underlying this condition remain poorly understood. However, several biochemical vascular abnormalities have been described in GH-deficient young adults who do not have the classic cardiovascular risk factors (diabetes, obesity, dyslipidemia, and hypertension) including increased peripheral resistance, reduction of aortic distensibility, and increased sympathetic nerve activity (7–9). Moreover, a significant increase in intima-media thickness, higher number of atheromatous plaques, and endothelial dysfunction have been reported in hypopituitary adults, despite adequate hormonal therapy except for GH, which support the causal role for GHD in determining premature atherosclerosis (10–13). Replacement therapy with GH has demonstrated favorable effects on some of the above-mentioned cardiovascular risk factors (2, 3, 14). However, this issue is not consensual in view of the fact that some studies did not confirm cardiovascular dysfunction in GH-deficient hypopituitary adults, whereas others reported no benefits for some of the disturbances associated with GHD (15–18). Most of the studies in the literature were based on clinical examination data, and few works are available concerning the cellular and molecular mechanisms underlying the pathophysiology of GHD-induced increased risk of vascular disease. So GH replacement

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Abbreviations: apo, Apoprotein; BMI, body mass index; $[Ca^{2+}]_i$, intracellular free calcium concentration; COGHD, childhood-onset GH-deficient; DBP, diastolic blood pressure; GHD, GH deficiency; HDL-Chol, high-density lipoprotein cholesterol; HRT, hormone replacement therapy; LDL-Chol, low-density lipoprotein cholesterol; PTT, partial thromboplastin time; SBP, systolic blood pressure; T-Chol, total cholesterol; TG, triglyceride; VSMC, vascular smooth muscle cell.

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therapy after completion of growth to prevent increased cardiovascular risk in the adult age remains controversial, and the clinical decision to do so is not wholly supported by scientific studies; thus, it is not licensed in several countries.

The diagnosis of GHD always was difficult to establish and remains controversial for several reasons: the onset of the disorder (childhood or adulthood-onset deficiency), the definition of the transition period or the need of therapy continuation after growth completion (19–21), the tests used for the GHD assessment (22), the causes of their hypopituitarism/etiology of the GHD (idiopathic or congenital/mutagenic) (23, 24), the nature of the hypopituitarism (isolated, combined, or multiple hormone deficiency) as well as the GHD persistence and severity in the adulthood (19–21, 25). Almost all the data available are derived from adult GHD, but it cannot be excluded that some metabolic and/or vascular abnormalities were already present when hypopituitarism set in. So if well defined and characterized, the model of childhood-onset GH-deficient (COGHD) patients might be particularly useful (19, 25, 26) and has been increasingly used to investigate GHD.

Platelets are mediators of thromboembolic complications and promoters of arteriosclerosis (27, 28). In addition, platelets produce, store, and/or release some vasoconstriction agents (such as serotonin and thromboxane A_2), which may affect both endothelium and vascular smooth muscle cell (VSMC) activity and influence vasoconstriction and peripheral vascular resistance. These corollaries, as well as their easy clinical accessibility and the features they have in common with VSMCs (29), make them useful tools in the study of pathophysiological abnormalities related with the vasculature, namely the widespread alterations in membrane and intracellular mechanisms underlying vascular disease development. Intracellular calcium is an essential second messenger in platelet responses and is the major determinant of vascular smooth muscle contractile activity (30, 31). Consequently, an increase in cytosolic Ca^{2+} in arterial smooth muscle leads to an increase in peripheral resistance that might contribute to vascular dysfunction. Therefore, an understanding of the general principles of cellular Ca^{2+} regulation in GHD patients might be crucial to find the main mechanisms underlying GHD-increased cardiovascular risks.

The purpose of this study was to assess some of the main cardiovascular risk indexes and plasma catecholamines levels and compare some platelet activation parameters (namely intracellular free calcium content, whole-blood platelet aggregation, and morphology) in two groups of COGHD patients (one with maintained GHD and the other with recovered GHD after withdrawal) treated with GH therapy for a variable period and in sex-, age-, and body mass index (BMI)-matched control groups.

Subjects and Methods

Subject selection and characterization

Four groups were tested: two groups of COGHD patients treated with GH therapy for a variable period until growth completion, and a sex-, age (± 2), and BMI (± 2)-matched control group of healthy volunteers for each GHD group of patients to avoid body weight interferences (Table 1). GHD had been diagnosed in their childhood by means of auxological data (stature, growth rate, and bone age) and biochemical tests (19). At least two stimulation tests were performed: clonidine and insulin-induced hypoglycemia (22). GH response after stimulation tests less than 7 ng/ml and IGF-I below the normal range for age and sex were determinant for their diagnosis. GH therapy had been administered at the dose of 0.5–0.7 UI/kg·wk in all patients for a period ranging from 3 to 8 yr and was withdrawn at least 2 yr before entry into the study. The criteria for therapy withdrawal were well-defined auxologic measures (growth rate and bone age) (19, 32). Patients from both GHD groups were stabilized on conventional hormone replacement therapy (HRT), when appropriate, with T_4, testosterone, estrogen/progestin, cortisone, and desmopressin. Each treatment corresponds to the dose needed for each particular subject and at each time point to repose the normal values for age and sex. All the HRTs were stopped for at least 2 yr before entry into the study and were at the normal levels.

The children were reevaluated 6 months to 6 yr after therapy withdrawal. The same stimulation tests, clonidine and insulin-induced hypoglycemia, were performed in these patients, and we did consider that the GHD was present when the peak level of GH was less than 3 ng/ml (33). Among the GHD subjects, some patients recovered GH levels after therapy withdrawal (group GHD-1: n = 10), whereas others remained GH deficient when retested for GH (group GHD-2: n = 8) after therapy was already withdrawn. There were several causes of hypopituitarism/etiology of the GHD in the two groups of COGHD patients. From the eight patients in the GHD-2 group (which maintained severe GHD when retested; peak GH level < 3 ng/ml), two had craniopharyngioma, one had Rathke's cleft cyst, one had optic nerve gioma, two had empty sella syndrome, one had pituitary hypoplasia, and one had idiopathic GHD. From the 10 patients in the GHD-1 group (with normal GH levels when retested for GH; > 7 ng/ml), two had operated craniopharyngioma, two had pituitary hypoplasia, and six had idiopathic GHD. One patient had panhypopituitarism with all anterior pituitary hormones and antidiuretic hormone deficiency (five-axis deficiency); two patients had a combination of GH, gonadal, adrenal, and thyroid axis deficiency (four-axis deficiency); two patients had a combination of GH, thyroid, and gonadal axis deficiency (three-axis deficiency); seven patients had a combination of GH and thyroid or GH and gonadal axis deficiency (two-axis deficiency); and six had isolated GHD. Two patients were submitted to radiotherapy. Control subjects (n = 7 for each control group) were recruited from students at the Institute of Pharmacology and Experimental Therapeutics, Medicine Faculty, Coimbra University. The study was performed according to the principles established in the Helsinki Declaration and was ethically approved by the Portuguese

TABLE 1. General characterization of the GHD groups and the sex-, age-, and BMI-matched control groups: anthropometric and blood pressure data

Parameters	Control-1 group (n = 7)	GHD-1 group (n = 10)	Control-2 group (n = 7)	GHD-2 group (n = 8)
Sex (male/female)	4/3	6/4	4/3	5/3
Age (yr)	22.3 ± 0.8	22.0 ± 1.0	24.9 ± 0.3	26.6 ± 1.7
BMI (kg/m^2)	26.3 ± 0.5	24.4 ± 1.7	24.6 ± 1.1	22.9 ± 1.7
WHR ratio	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
SBP (mm Hg)	111.3 ± 5.1	110.6 ± 2.7	113.7 ± 4.1	114.0 ± 5.1
DBP (mm Hg)	69.0 ± 7.0	67.8 ± 4.6	67.3 ± 5.9	70.7 ± 5.8

Data are expressed as means ± SEM of n individuals. GHD-1; GHD group 1; GHD-2; GHD group 2; WHR ratio, waist to hip ratio.

Ethics authorities. All the subjects, patients, and control healthy volunteers were informed and gave their written consent.

There were no hypertension, diabetes mellitus, or smoking habits in the four groups. No differences were observed in anthropometric evaluation or fat distribution. Body weight was measured in the morning, after the subjects had voided, to the nearest 0.1 kg. Body height was measured barefoot and to the nearest 0.5 cm. No differences were observed in BMI and waist to hip ratio in the four groups (Table 1). However, BMI values in the control groups were higher than the matched GHD patients groups, which might be recognized as a nontypical result that, in further studies, should be taken into consideration. The systolic and diastolic blood pressure (SBP and DBP) values were the mean of two measures on the right arm in a random zero sphygmomanometer with a cuff size corresponding to the size of the right arm. No differences were observed in the SBP and DBP values among the four groups (Table 1).

Blood collection and platelet preparation

Blood were collected from the control and GHD patients using appropriate tubes containing anticoagulant solution (0.1 ml/ml blood) containing (in millimoles per liter) citric acid (71), sodium citrate (85), and D-glucose (111). The blood was centrifuged (900 rpm for 10 min at 20 C) to obtain platelet-rich plasma, and the platelets were then recovered by a new centrifugation at 2500 rpm for 10 min at 20 C to be used in the other experimental protocols.

Clinical hematological and biochemical examinations

After one night fasting, blood samples were drawn to appropriated tubes provided with anticoagulant solution for the determination of hematological and biochemical parameters. Several laboratory examinations related with hemostase, coagulation, lipids, and apoproteins were made in each of the four groups, using standard laboratory techniques. It included platelet count, prothrombin time, partial thromboplastin time (PTT), fibrinogen, hemoglobin, total cholesterol (T-Chol), low-density lipoprotein cholesterol (LDL-Chol), high-density lipoprotein cholesterol (HDL-Chol), triglyceride (TG), apoprotein (apo) A1 and B, apo B to apo A1 ratio, pre-β- and β-electrophoretic bands of lipoproteins.

Plasma catecholamine concentration

Plasma catecholamines were measured as previously described (34). In brief, to 2 ml plasma, obtained as mentioned above, 100 ng/ml of the internal standard 3,4-dihydroxybenzylamine, 50 mg alumina, and 1 ml Tris buffer [1 mol/liter (pH 8.6)] were added. The catecholamines in the samples, extracted by using the method of alumina adsorption and appropriate microfilter systems, were finally resuspended in perchloric acid (100 mmol/liter) to be further quantified by HPLC with electrochemical detection, using the chromatographic conditions already described (34). Noradrenaline, adrenaline, and dopamine concentrations (in picograms per milliliter) were calculated by using the authentic standards of each one and the internal standard of 3,4-dihydroxybenzylamine (Sigma Chemicals, St. Louis, MO), intended to correct the possible catecholamine loss during the alumina extraction procedure. HPLC catecholamine recovery was greater than 95%, and within-assay and between-assay variability coefficients for the noradrenaline, adrenaline, and dopamine quantification were less than 2 and less than 4%, respectively.

Platelet intracellular free calcium concentration ($[Ca^{2+}]_i$)

The preparation of fura-2-loaded platelets was made as previously described (35). In brief, the platelet pellet, obtained as above described, containing 100 μmol/liter of acetyl-salicylic acid was resuspended and incubated for 10 min in a physiological saline solution containing 20 μg/ml apyrase and loaded with 5 μmol/liter fura-2/AM (Molecular Probes Inc., Eugene, OR) for 45 min at 37 C. The fluorescence of the platelet suspension finally obtained (2500 rpm, 10 min, 20 C) was measured at 37 C with continual agitation at the emission wavelength of 510 nm, with the excitation wavelength continuously switched between 340 and 380 nm (FluoroMax spectrofluorometer, SPEX Industries Inc., Edison, NJ), using the ratio of the fluorescence intensities to determine $[Ca^{2+}]_i$, according to the equation: $[Ca^{2+}]_i = Kd\ [(R - Rmin)/(Rmax - R)] \times \beta$ (36). $[Ca^{2+}]_i$ calibration was achieved by lysing the cells with 50 μmol/liter digitonin in the presence of 1 mmol/liter $CaCl_2$ or 10 mmol/liter EGTA (pH 9.0). $[Ca^{2+}]_i$ was tested at basal conditions and after addition of 0.1 U/ml thrombin (bovine, lyophilized, DiaMed AG, Cressier, Switzerland).

Whole-blood platelet aggregation

Whole-blood platelet aggregation was measured by assessing the electric impedance (37), using a Chrono-log aggregometer (Chrono Log Corp., Havertown, PA). This technique is based on the detection of changes in electrical resistance between two electrodes submerged in the sample. Then 0.5 ml of fresh heparinized whole blood and 0.5 ml of 0.9% NaCl were mixed using a magnetic stirrer and allowed to balance at 37 C for 5 min before adding the agonists (collagen-equine type I: 10 μg/ml; ADP: 1 μmol/liter; Chrono Log Corp.).

Electron microscopy study of platelet activation

The platelet samples from the blood of each of the four groups were prepared according to the protocol previously described (35). In brief, the platelet pellets, collected as described above, were fixed with glutaraldehyde, washed, and postfixed with osmium tetroxide. After a further washing, the platelets were then preembedded in agar, which was cut into 1-mm pieces, dehydrated with alcohol and propylene oxide, and finally embedded in a low-viscosity resin Epon 812 (TAAB, Aldermaston, UK). After polymerization, the blocks were cut on an ultramicrotome (LKB) using a diamond knife. Ultrathin sections were picked up on uncoated copper grids (300 mesh) and observed under a JEM-100SX (JEOL) transmission electron microscope, after uranyl/lead citrate staining. Ten samples of the 14 control individuals as well as all the samples of both GHD groups (10 of the GHD-1 and eight of the GHD-2) were analyzed. From each individual sample, a grid was prepared corresponding to 10 different cuts of the biological material to confirm that the pattern obtained in the picture was not due to the local of the cut. The quantification was made by calculating the percentage of each particular pattern (sign) observed in the 300-mesh grid.

Statistical analysis

Results are expressed as means ± SEM of n individuals for each group (as indicated). Groups were tested for differences by performing the ANOVA and Fisher's least protected significant difference test, using the Statview 4.53 software (Abacus Concepts Inc., Berkeley, CA). Differences were considered statistically significant at $P < 0.05$ (*, $P < 0.05$ and **, $P < 0.01$ *vs.* the matched control group and #, $P < 0.05$ and ##, $P < 0.01$ between GHD-2 and GHD-1 groups).

Results

Clinical hematological and biochemical examinations

Concerning the hemostasis and coagulation parameters, the GHD-1 group significantly differed only in hemoglobin: a lower value was obtained when compared with the matched control-1 group. In opposition, in the group that remained GH deficient after therapy withdrawal (GHD-2 group), there was a significant higher value of PTT and lower values of fibrinogen and hemoglobin than the matched control-2 group (Table 2). In relation to lipid profile and apoproteins, both GHD groups showed higher TG content and lower apo A value than the corresponding control group. The HDL-Chol was also numerically lower in both groups than the matched controls but without reaching statistical significance, whereas the LDL-Chol values were unchanged. In the group that remained GH deficient (GHD-2), there was also a higher T-Chol content when compared with the group that recovered the GH levels (GHD-1) (Table 2).

TABLE 2. Clinical hematological and biochemical examinations in the GHD patients (GHD-1 and GHD-2) and in the matched control volunteers

Parameters	Control-1 group (n = 7)	GHD-1 group (n = 10)	Control-2 group (n = 7)	GHD-2 group (n = 8)
Hemostasis and coagulation				
PTC (10^{12}/liter)	233 ± 22	234 ± 14	237 ± 30	242 ± 17
PT (sec)	12.5 ± 0.3	13.5 ± 0.3	12.5 ± 0.4	13.2 ± 0.5
PTT (sec)	27.0 ± 1.4	30.7 ± 1.1	25.4 ± 1.9	30.7 ± 2.1[a]
Fibrinogen (g/liter)	2.2 ± 0.2	2.6 ± 0.1	2.0 ± 0.2	2.9 ± 0.3[b]
Hemoglobin (mmol/liter)	10.2 ± 0.7	8.4 ± 0.3[a]	11.0 ± 0.7	8.4 ± 0.3[b]
Lipid profile				
T-Chol (mmol/liter)	5.1 ± 0.2	4.6 ± 0.2	5.1 ± 0.1	5.3 ± 0.3[c]
LDL-Chol (mmol/liter)	2.4 ± 0.2	2.6 ± 0.2	2.3 ± 0.2	2.4 ± 0.3
HDL-Chol (mmol/liter)	1.3 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
TGs (mmol/liter)	0.61 ± 0.05	1.34 ± 0.24[c]	0.55 ± 0.3	1.14 ± 0.15[b]
Apoproteins				
Apo A (mg/dl)	159 ± 8	131 ± 4[a]	174 ± 11	139 ± 6[a]
Apo B (mg/dl)	103 ± 3	90 ± 8	95 ± 5	98 ± 9
Apo B/apo A ratio	0.66 ± 0.03	0.70 ± 0.08	0.56 ± 0.05	0.70 ± 0.06
Alpha (%)	32.2 ± 2.8	35.2 ± 4.4	28.1 ± 2.2	29.0 ± 1.9
Pre-Beta (%)	24.3 ± 3.2	18.5 ± 3.2	26.8 ± 2.9	20.3 ± 3.1
Beta (%)	44.2 ± 1.6	44.1 ± 2.9	44.1 ± 2.1	49.9 ± 2.4

Data are expressed as means ± SEM of n individuals ([a] $P < 0.05$ and [b] $P < 0.01$ *vs.* the matched control group, and [c] $P < 0.05$ between GHD-2 and GHD-1 groups). PTC, Platelet count; PT, prothrombin time; GHD-1; GHD group 1; GHD-2; GHD group 2.

Plasma catecholamine concentration

Plasma noradrenaline concentration differences between the GHD groups and the matched control group did not reach statistical significance (Table 3). Plasma adrenaline content was especially higher in the GHD-2 group, being significant when compared with the GHD-1 group value. Plasma dopamine concentration also did not statistically differ between the GHD and the control groups (Table 3).

Platelet $[Ca^{2+}]_i$

Basal $[Ca^{2+}]_i$ was identical in the four groups (control-1: 86.4 ± 2.2 nmol/liter; GHD-1: 91.0 ± 1.8 nmol/liter; control-2: 81.7 ± 1.6 nmol/liter; GHD-2 group: 80.2 ± 1.7 nmol/liter) (Fig. 1). Thrombin-evoked $\Delta[Ca^{2+}]_i$ value was 95.7 ± 11.5 Δnmol/liter in the control-1 group and 80.0 ± 4.5 Δnmol/liter in the GHD-1 group. However, in the GHD-2 group, there was a statistically significant increased thrombin-evoked $\Delta[Ca^{2+}]_i$ (297.0 ± 15.7 Δnmol/liter; $P < 0.01$), compared with both the matched control-2 (102.1 ± 13.6 Δnmol/liter) and GHD-1 group (Fig. 1).

Whole-blood platelet aggregation

Collagen-induced whole-blood platelet aggregation values were similar between the control-1 and GHD-1 groups (control-1: 26.4 ± 1.5 Ω; GHD-1: 28.0 ± 2.0 Ω) (Fig. 2A). However, the GHD-2 group had a higher collagen-induced platelet aggregation (33.3 ± 4.3 Ω; $P < 0.05$) when compared with the matched control-2 group (19.6 ± 2.9 Ω; $P < 0.01$). ADP-induced whole-blood platelet aggregation values were similar between the control-1 (9.2 ± 1.9 Ω) and GHD-1 groups (6.4 ± 2.0 Ω). However, the GHD-2 group (12.5 ± 2.1 Ω) showed a statistically significantly ($P < 0.05$) higher value when compared with the matched control-2 group (6.2 ± 0.8 Ω) and GHD-1 group (Fig. 2B).

Electron microscopy study of platelet activation

The platelets of the GHD-1 patients showed identical morphological features to those of the control groups (Fig. 3, A1 *vs.* B1). In Fig. 3, C1 and C2, we observed signs of platelet activation for the platelets of the GHD-2 group, namely the pseudopodia formation, contrasting with the regular and well-defined shape of the platelets from the control volunteers (Fig. 3A1) and the GHD-1 patients (Fig. 3B1). In addition, extensive platelet aggregation was observed for the platelets of the GHD-2 group, which is evident when both their spatial distribution (how close to one another) and complementary shapes (Fig. 3C3) were compared with the platelets of the control (Fig. 3A1) and GHD-1 groups (Fig. 3B2). These results confirmed the significant increases in platelet aggregation obtained for the GHD-2 group when compared with the matched control-2 and GHD-1 groups, confirming the whole-blood platelet aggregation results described above.

TABLE 3. Plasma noradrenaline, adrenaline, and dopamine content in the GHD patients (GHD-1 and GHD-2) and in the matched control volunteers

Catecholamine (pg/ml)	Control-1 group (n = 7)	GHD-1 group (n = 10)	Control-2 group (n = 7)	GHD-2 group (n = 8)
Noradrenaline	93 ± 8	123 ± 34	69 ± 16	91 ± 6
Adrenaline	516 ± 138	549 ± 64	526 ± 108	731 ± 98[a]
Dopamine	79 ± 45	118 ± 42	96 ± 51	118 ± 16

Data are expressed as means ± SEM of n individuals ([a] $P < 0.05$ between GHD-2 and GHD-1 groups). GHD-1, GHD group 1; GHD-2; GHD group 2.

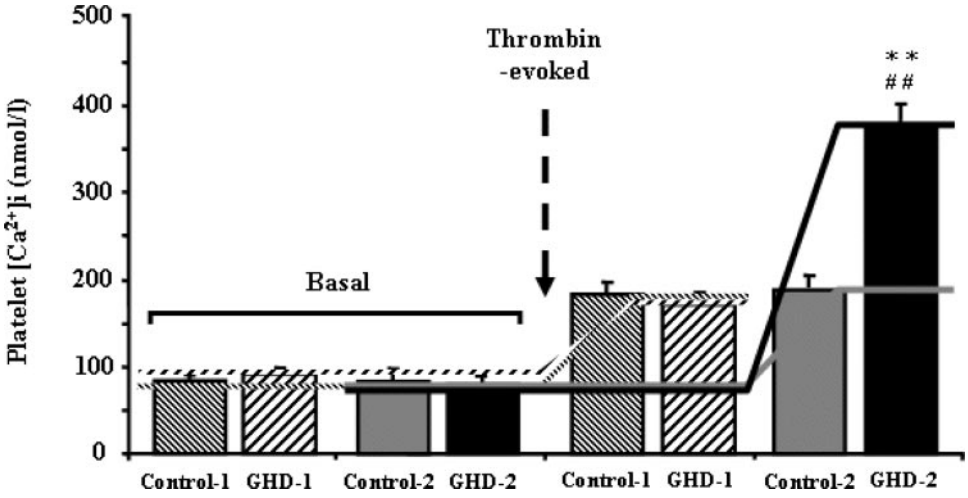

FIG. 1. Basal and thrombin-evoked platelet $[Ca^{2+}]_i$ in the GHD patients (GHD-1 and GHD-2) and the matched control volunteers. Platelets were incubated with fura-2/AM (5 μmol/liter) for 45 min and further stimulated with thrombin (0.1 U/ml) in the presence of 1 mmol/liter $CaCl_2$ to restore the external $[Ca^{2+}]$ (values obtained are the peak $[Ca^{2+}]_i$, achieved 1 min after the addition of the agonist). Each *bar* indicates the mean ± SEM of seven controls in each group, 10 GHD-1 patients, and eight GHD-2 individuals, each with two parallel determinations (**, $P < 0.01$ *vs.* the matched control group and ##, $P < 0.01$ between GHD-2 and GHD-1 groups).

Discussion

GH replacement therapy after growth completion to prevent increased cardiovascular risk in the adult age remains controversial, not only because of the high costs of these therapy but also, and especially, because of the lack of scientific studies undoubtedly proving the benefits of therapy prolongation after completion of growth. Benefits of GH replacement therapy on the cardiovascular indexes, such as those previously reported by other authors (38), who demonstrated intima-media thickness normalization and early atherosclerotic disturbances reversion, were not obtained by all the authors, as could be demonstrated by the nonconsensual results (15–18). So whether GH replacement therapy will restore cardiovascular mortality rates to normal remains to be undoubtedly shown.

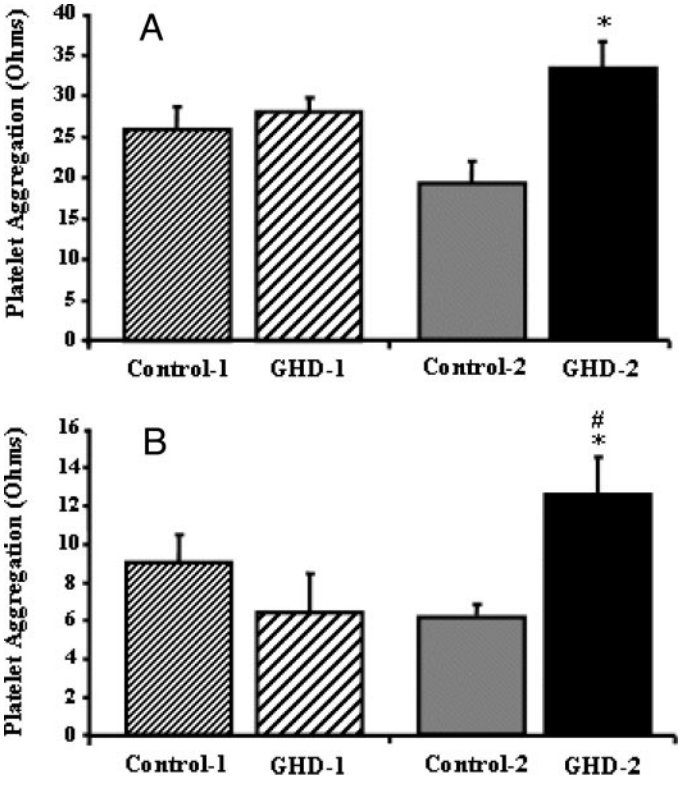

FIG. 2. Whole-blood platelet aggregation in control and GHD patients (GHD-1 and GHD-2) and the matched control volunteers: induced by 10 μg/ml collagen (A) and 1 μmol/liter ADP (B). Each *bar* indicates the mean ± SEM of seven controls in each group, 10 GHD-1 and eight GHD-2 individuals (*, $P < 0.05$ *vs.* the matched control group and #, $P < 0.05$ between GHD-2 and GHD-1 groups).

In our study, the lipid profile evaluation demonstrated that the GHD groups already had significant higher TG contents and lower apo A values when compared with the matched control groups, even though the age of patients was still young. All the other parameters analyzed did not significantly differ among the groups. However, even not reaching significance, the values for HDL-Chol in both GHD groups, compared with the controls, as well as the significant higher T-Chol in the GHD-2 group, compared with the groups that recovered GH levels after therapy (GHD-1), should be emphasized. GHD was previously associated with abnormalities of lipid and carbohydrate metabolism, thus contributing to the increased risk of vascular disease (3, 5). Elevations of T-Chol and LDL-Chol have been described in adult GHD patients (39, 40). Our study, in contrast, demonstrates no differences in T-Chol, LDL-Chol, and apo B apoprotein in both GHD groups when compared with the control volunteers. Once the subjects entering the study were retested COGHD patients off GH replacement therapy, the nonstatistically significant results could be due to their still young age at study. Therefore, most, but not all, of the studies clearly describing changes in those indexes were obtained from GHD patients already in the adulthood (3, 26, 39, 40).

A study performed by Gleeson *et al.* (41), assessing the lipid profile of GHD patients through the life span, reported an increased T-Chol and LDL-Chol, particularly patents in adulthood but less marked in child and adolescent GHD patients, which is not in opposition with our data. However, the absence of differences on both the TG and HDL-Chol values in children and also adult GHD patients seems to withdraw an age or GHD duration influence on these parameters. Another study also denied a pubertal or GH status influence in T-Chol, LDL-Chol, and TG abnormalities in healthy adults (42). In our study, even other lipid profile parameters, such as the decreased HDL-Chol contents, did not reach statistical significance, it was demonstrated a

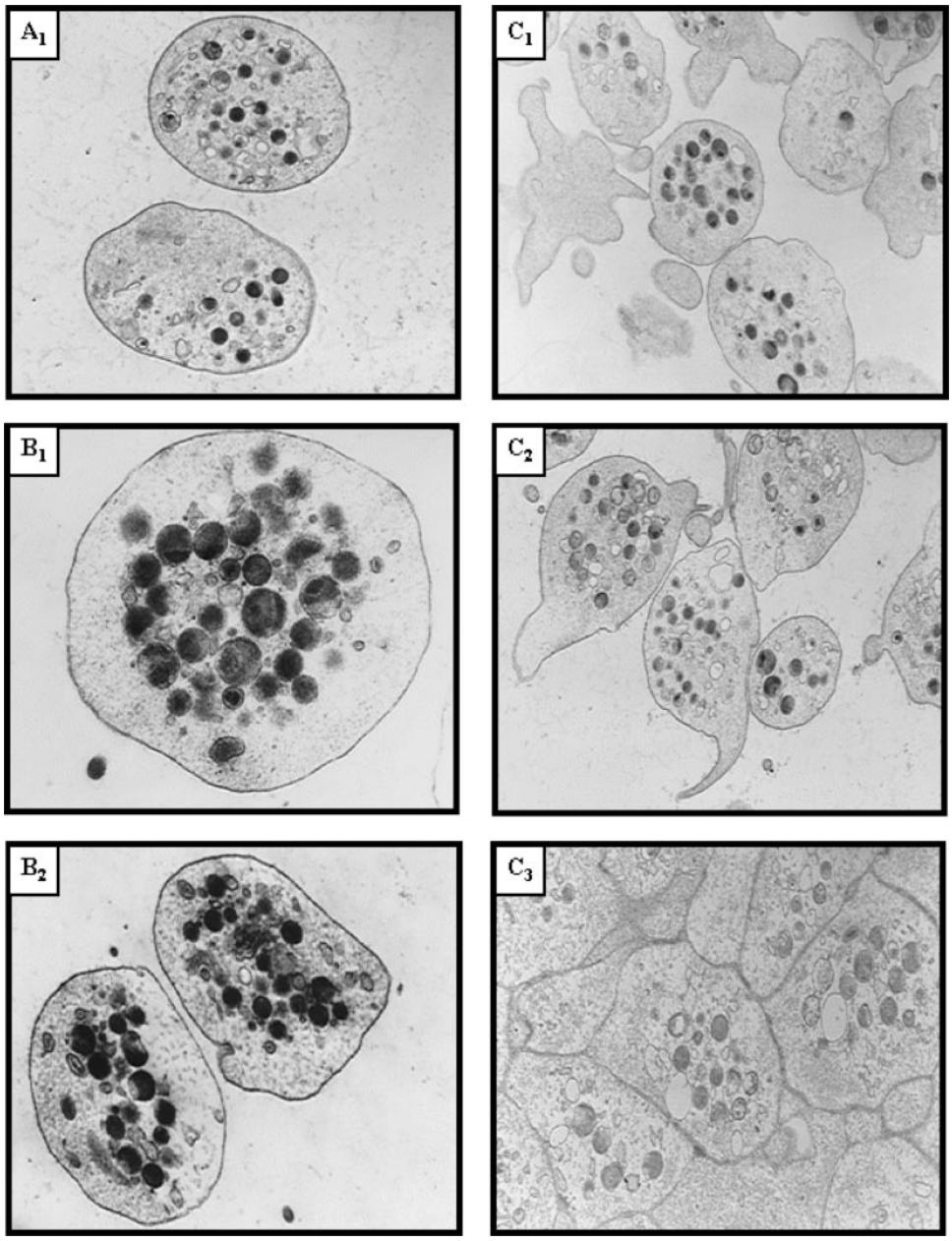

FIG. 3. Electron microscopy photographs of platelet morphology in the GHD patients (GHD-1 and GHD-2) and matched control volunteers (original magnification, ×16,800, except B1, ×33,600). A1, Platelets from the control groups (1 and 2); B1 and B2, Platelets from the GHD-1 group; C1–C3, Platelets from the GHD-2 group. In the control groups, 10 samples (individuals) of the 14 controls were analyzed: in nine of them, the pattern in A1 was seen in more than 95% of the grid holes, demonstrating the other one only little evidence of activation in less than 40% of the grid holes. In the GHD-1 group, the normal morphology (B1) was observed in all the samples in more than 85% of the holes, except two of them that have shown signs of membrane discontinuation (B2) and little evidence of pseudopodia formation but in only less than 25% of the holes. In the GHD-2 group, all eight samples demonstrated platelet activation. In seven of them, the grid demonstrated the signs seen in C1 and C2 in 70% of the holes. Furthermore, in five of the eight samples, signs of marked platelet aggregation were demonstrated by the photographs (C3), which corresponded to more than 85% of the holes.

higher TG concentration and a lower apo A apoprotein level in the GHD patients, compared with the controls, which would favor the increased cardiovascular risk and predisposition for premature atherosclerosis, previously suggested by others in childhood and adolescent GHD patients (11, 43). Another possible explanation for the differences, compared with other studies, is that our patients were under therapy until growth completion and were off GH treatment when assessed for the study. Therefore, some of the studies in the literature were related to untreated GHD patients; furthermore, improvement of lipid metabolism abnormalities in GHD patients after GH replacement therapy was seen in several studies (3, 5, 41, 44). So considering the abovementioned information, further studies comparing childhood GH-deficient patients before GH replacement therapy and after therapy withdrawal and adulthood GHD individuals might evaluate these influences and confirm or deny the tendencies of our results and the influence of the above-described factors.

Concerning the hemostasis and coagulation evaluation, our study demonstrated a significant difference on only one parameter analyzed in the GHD-1 group (a lower hemoglobin value), whereas in the maintained GH-deficient group (GHD-2), there was clear differences on the PTT and fibrinogen and hemoglobin values, compared with the matched control group. Other studies have already described GH-associated abnormalities in some of the main hemostatic, fibrinolytic, and inflammatory markers, such as hyperfibrinogenemia and increased PCR in GHD patients as well as increased hemoglobin levels in long-term replacement therapy with recombinant human GH (18, 45–47). The hemostasis and coagulation values obtained in our study also obligates a further evaluation in

adulthood to determine whether they represent potential increased cardiovascular and thromboembolic risk in advances stages of the deficiency.

Increased sympathetic nervous system activity has also been associated with GHD patients (8, 9). GHD in adulthood is commonly associated with several abnormal conditions that could influence and suggest a sympathetic nerve activity augmentation, such as excessive adiposity, insulin resistance, dyslipoproteinemia, increased prevalence of hypertension, arterial function and structure worsening (increase in intima-media thickness, atherosclerotic plaque development, and endothelial dysfunction), and cardiovascular morbidity/mortality usually seen in GHD patients (1, 3, 12). Two studies previously performed by other groups (8, 9) demonstrated an increased muscle sympathetic nerve activity in GHD adults. Whereas the first group has suggested a high correlation between muscle sympathetic nerve activity and increased DBP, the second demonstrated an increased peripheral vascular resistance that might be affected by the central sympathetic vasoconstrictor discharge to the peripheral vasculature, which results in a pronounced increased vasoconstriction. In our study, using plasma catecholamine concentration as indirect peripheral index for the sympathetic nervous system activity, the main finding was an increased adrenaline content in the GHD-2 group, compared with the (GHD-1) group, which is in agreement with a central sympathetic vasoconstrictor discharge to the peripheral vasculature, as suggested by the other two studies (8, 9), mainly in the group with maintained GHD. Even though the nonexistence of differences between the GHD groups and the matched controls, which could be due to the age of the subjects of our GHD group (all COGHD patients after the growth completion age), deserves further attention, our results do not deny that sympathetic hyperactivation might also have an important role on the vascular disease development in GHD patients, namely in advanced stages of the disturbance, as seems to be the case in the two other above-mentioned studies (8, 9) with adult GHD patients.

The platelet role in the physiology and pathophysiology of the cardiovascular system, their easier collection from humans, and the features they share with VSMCs make them useful tools to evaluate hypothetic vascular disturbances. Accordingly, platelets might be hypothetically implicated or might be a useful marker of cardiovascular risk in GHD patients. Because few studies have addressed the effects of GH, and particularly of GHD, on platelet activation, no elucidating comparisons could be made from our results. A previous study (48) testing the effect of GH-releasing mechanism on platelet function demonstrated an impairment of platelet aggregation in healthy male volunteers infused with GH release-inhibiting hormone. The authors suggested that the intracellular mechanisms associated with hormonal release of the pituitary and gut endocrine cells might resemble some metabolic mechanisms within platelets. In our study, directly testing GHD effect on platelet function showed no significant differences in the platelet activity parameters evaluated between the GHD-1 and control groups. This fact might be due to the recovery of GH levels after therapy withdrawal. However, patients with maintained GHD (GHD-2 group) had increased thrombin-evoked $[Ca^{2+}]_i$, collagen, and ADP-induced platelet aggregation *vs.* the matched control group, reinforced by the platelet morphology features revealed by the electron microscopy evaluation, which seemed to indicate a platelet hyperreactivity state and a putative increased cardiovascular risk, which might be related to GHD maintenance after therapy withdrawal.

GHD patients entering our study were previously submitted in most cases to other HRTs. The effect of HRT on the cardiovascular risk, particularly in women, is a widely investigated issue, its benefits or deleterious actions remaining controversial (49, 50). Less studied, but also unresolved, is the hypothetic influence of HRT on hemostatic and thrombosis parameters (51, 52). Even considering that in our study all the patients were already normal for all the other hormones except for GH and that HRT stopped for at least 2 yr before entry into the study, the potential influence on platelet aggregability could not be excluded at this point and deserves further investigation.

Conclusions

Our study demonstrates a platelet hyperactivation state, even in the absence of clear-cut abnormalities of the classic vascular risk factors, including blood pressures, adiposity, or sympathetic activity, in the GHD group of patients who do not recover GH levels after GH therapy but not in the patients who recovered GH levels. Minor changes in the cardiovascular risk indexes, increased aggregation, thrombin-evoked $[Ca^{2+}]_i$, and morphological characteristics of platelet activation, which might be further confirmed in adulthood, were observed in that group, which might be related to GHD maintenance after therapy withdrawal. So platelets might represent an important tool as earlier marker/predictor of future cardiovascular risk in the COGHD patients and might be viewed as a predisposing condition to premature vascular disease. The results suggest further studies to better evaluate whether earlier preventive antiaggregation therapy with low-dosage acetyl-salicylic acid or clopidogrel is recommended for those patients. This prospective study also recommends supplementary confirmation and investigation of the cellular and molecular mechanisms underlying these earlier cardiovascular biochemical disturbances and assess not only whether earlier preventive antiaggregation therapy is recommended for those patients but also whether GH replacement therapy after growth completion, although expensive, is scientifically recommended to prevent cardiovascular risk.

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Address all correspondence and requests for reprints to: Prof. Frederico Teixeira, M.D., Ph.D., Institute of Pharmacology and Experimental Therapeutics, Medicine Faculty, Coimbra University, 3004-504 Coimbra, Portugal. E-mail: fredjt@ci.uc.pt.

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