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## Chairman: Prof. Francesco Beguinot

## PH.D. THESIS

**NEW INSIGHTS IN CHILDHOOD-ONSET GROWTH HORMONE DEFICIENCY: FROM DIAGNOSIS TO LONG-TERM OUTCOMES**


TUTOR

**Prof. Mariacarolina Salerno**

PH.D. STUDENT

**Dr. Nicola Improda**

*Dedicated to my children Gabriele and Jacopo:*
*be proud of your uniqueness.*

**INDEX**

## CHAPTER 1

### 1a. Definition and etiology of Growth Hormone Deficiency

Growth hormone deficiency (GHD) is a rare but important cause of short stature in childhood with a prevalence estimated at approximately 1:4,000-1:10,000 patients (1).

GHD may result from a failure of hypothalamic GHRH production or release, from genetic or congenital disorders of pituitary development. GHD may be also secondary to central nervous system (CNS) insults including tumors, surgery, trauma, radiation or infiltration from inflammatory diseases (1).

Moreover, itmay be isolated or it may occur in the context of multiple pituitary hormone deficiency (MPHD)**(Table 1)**. This latter condition may present early in the neonatal period or later in childhood and may be associated with a number of midline defects or extra-pituitary abnormalities such as optic nerve hypoplasia, anophtalmia, microphtalmia, the corpus callosum dysgenesis, absence of the septum pellucidum, midbrain abnormalities, and olfactory bulbs and tract hypoplasia or agenesis. The majority of MPHD cases are idiopathic, while familial inheritance accounts for between 5 and 30% of all cases (1). The association of at least two features among (i) optic nerve hypoplasia, (ii) pituitary hormone abnormalities and (iii) midline brain defects identifies a condition called Septo-Optic Dysplasia (SOD). It is a poorly understood disorder with phenotypic heterogeneity even within families (2).

The question regarding GHD inevitably arises in those cases in which other more frequent causes of short stature (including genetic short stature, constitutional delay of growth and puberty, hypothyroidism, Turner syndrome, and chronic disease like celiac disease) have been ruled out (3). Tools for the diagnosis of GHD include auxology, radiographic assessment of bone age, measurement of insulin-like growth factor 1 (IGF-I) and IGF binding protein 3 (IGFBP-3), provocative GH testing, cranial magnetic resonance imaging (MRI), and, in selected cases, genetic testing (3) **(Table 2)**.

**Table 1. Etiology of GHD (Di Iorgi et al 2016).**

| | |
|---|---|
| Isolated GHD | Genetic<br>• GH1 Mutations (GHD type 1A)<br>• GHRH Mutations (GHD type 1B)<br>• GH1 Mutations (GHD type II with evolving pituitary deficiencies)<br>• GHD type III (XL Agammaglobulinemia)<br>• GH1 Kowarski Syndrome (Bioinactive GH)<br>• GHS Mutation/Variant<br>• Alstrom Syndrome |
| CPHD/MPHD | Genetic (Transcription factor defect, gene mutation, deletion or duplication)<br>• Genes implicated in early development of hypothalamic–pituitary region<br>• Genes implicated in early development of brain and hypothalamic–pituitary region<br>- Holoprosencephaly<br>- Septo-optic dysplasia and its spectrum involving eyes<br>- Midline defects (cleft-palate, persistence of craniopharyngeal canal, dental agenesis, …)<br>- Extra brain malformations (ARNT2, CHD7, IGSF1, …)<br>- Overlapping Kallmann syndrome (FGF8, FGFR1, PROKR2, PROK2 CDH7,WDR11, …)<br>- Other conditions<br>• Genes implicated in cellular differentiation<br>• Inducing tumor genes (SOX2, BRAF, …) |
| CPHD/MPHD | Congenital<br>• Midline brain and pituitary developmental defects<br>• Pituitary aplasia; ectopic posterior pituitary, anterior pituitary hypoplasia and pituitary stalk abnormalities (agenesis, hypoplasia); empty sella<br>• Congenital CNS mass (hamartoblastoma, hamartoma), cyst. encephalocele |
| IGHD/CPHD/MPHD | • Idiopathic permanent<br>• Idiopathic transitory |
| IGHD/CPHD/MPHD | Acquired<br>• CNS tumors (craniopharyngioma, germinoma, ependymoma, pituitary adenoma, meningioma, medulloblastoma, glioma, metastatic tumors (rare), Rathke's cleft cyst, arachnoid cyst)<br>• Radiotherapy (cranial irradiation for CNS tumors, other malignancies,BMT)<br>• TBI (accidental, after neurosurgery, subarachnoid hemorrhage)<br>• Infections (meningitis, encephalitis, tuberculosis, hypophysitis)<br>• Autoimmune (hypophysitis, APS, anti Pit1antibodies)<br>• Infiltration (LCH, hemochromatosis, chronic blood transfusions)<br>• Chemotherapy (cancer survivors) |

GHD, growth hormone deficiency; CPHD, combined pituitary hormone deficiency; MPHD, multiple pituitary hormone deficiency; CNS, central nervous system; BMT, bone marrow transplantation; TBI, traumatic brain injury; APS, autoimmune polyglandular syndrome; LCH, Langherans cell histiocytosis.

**Table 2. Consensus guidelines on the diagnosis of GHD in childen (GH Research Society).**

**When to consider investigation for GH deficiency**

1. Severe short stature, defined as a height more than 3 SD below the mean.
2. Height more than 1.5 SD below the mid-parental height.
3. Height more than 2 SD below the mean and a height velocity over 1 year more than 1 SD below the mean for chronological age, or a decrease in height SD of more than 0.5 over 1 year in children over 2 years of age.
4. In the absence of short stature, a height velocity more than 2 SD below the mean over 1 year or more than 1.5 SD sustained over 2 years; this may occur in growth hormone deficiency (GHD), presenting in infancy, or in organic acquired GHD.
5. Signs indicative of an intracranial lesion.
6. Signs of MPHD.
7. Neonatal symptoms and signs of GHD.

## 1b. Controversies in the diagnosis and outcomes of Growth Hormone Deficiency in childhood

Although rare, GHD is an important diagnosis to make correctly. In fact, GH replacement therapy (GHRT) in GHD is highly efficacious so amissed diagnosis will result in a poor outcome. Equally, a false positive diagnosis will lead to many years of unnecessary daily subcutaneous injections, with significant wasted expenses and exposure to potential adverse effects. Unfortunately, many aspects regarding diagnosis and treatment of GHD during childhood and adolescence are still subject of much controversy (4).

While the diagnosis of GHD in the context of MPHD and/or organic pathologies of the CNS generally appears straightforward, differentiating idiopathic GHD from non-GH deficient short children can be more challenging, especially in patients in the peripubertal phase or with delayed puberty, which may exhibit transient growth deceleration or short stature.

Although an integrated assessment of history and physical examination, stature, growth velocity and bone age represents is the most reliable diagnostic element, provocative GH testing continues to play a primary role in the diagnosis of GHD. Nevertheless, there are significant issues concerning the validity and reproducibility of GH testing, regarding optimal provocative stimuli, appropriate cut-off levels specific to GH assay and other factors such as BMI and pubertal status, utility of sex steroid priming, and standardization of testing protocols (4). Indeed, the vast majority of patients diagnosed with GHD in childhood and with no structural pituitary abnormality on MRI will have adequate GH secretion when retested in late adolescence or adulthood (66%–85% depending on the test and cutoff used) (5,6).

The presence of structural abnormalities of the hypothalamus-pituitary region, such as an ectopic posterior pituitary, has been previously reported to predict the development of severe GHD, but its role in predicting the evolution of endocrine deficits in MPHD or the persistence of GHD is still debated (7, 1).

Although recombinant human (rh) GH has been available since the 1980s, many clinical aspects related to GHD or to GH treatment itself still need to be defined.

It has been consistently shown that adults with untreated GHD have impaired cardiac performance, adverse metabolic profile and increased atherogenic risk, which can be restored by GH replacement therapy (GHRT) (8, 9). Moreover, in adults with GHD body composition is altered, with increased fat mass (FM) and decreased lean body mass (LBM), causing diminished muscle strength and physical fitness (10). There is usually an increase in muscle

mass in response to GH but whether this change results in increased strength is still debated (11-12). Nonetheless, overall data in adults suggest that GH treatment significantly improves aerobic exercise capacity and physical performance (13).

This topic is relevant even in childhood since, in addition to promoting linear growth, GH also exerts beneficial effects on early risk factors involved in the development of CV morbidity (14). Nonetheless, in childhood and adolescence the effects of GHD and GHRT on functional outcome measures characterizing the patient's physical fitness, such as cardiopulmonary functional capacity, muscle strength, flexibility, and endurance are still poorly delineated.

### 1c. Aims of the Study Project

Given the controversies regarding diagnosis and long-term outcomes of GHD, improved specificity of diagnostic testing, as well as a better understanding of the role of GHD and GHRT on metabolic and functional parameters are required in order to develop tailored therapeutic approaches. Indeed, functional parameters represent important therapeutic outcomes, as they are the most relevant for the patient's physical, motor and psychosocial development, thus significantly influencing the patient's quality of life.

Therefore, the present Ph.D. thesis aimed to investigate:

- Usefulness of priming with sex steroids in improving the diagnostic accuracy of GHST in the diagnosis of GHD.
- Endocrine morbidity and final height of children with MPHD and SOD.
- Metabolic profile, body composition, vascular morphology and function in children with GHD treated with rhGH.
- The effects of GHD and GHRT on functional parameters contributing to the health related fitness (exercise capacity, and muscle strength and flexibility).

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## CHAPTER 2. Contribution in improving diagnosis of GHD

**This work has been published as:**

Galazzi E*, **Improda N***, Cerbone M, Soranna D, Moro M, Fatti LM, Zambon A, Bonomi M, Salerno M, Dattani M, Persani L. Clinical benefits of sex steroids given as a priming prior to GH provocative test or as a growth-promoting therapy in peripubertal growth delays: Results of a retrospective study among ENDO-ERN centres.

**Clin Endocrinol (Oxf). 2021 Feb;94(2):219-228.**

### Background

Differentiating idiopathic GHD (GHD) from constitutionally delayed non-GH deficient short children (CDGP) in the peri-pubertal phase remains a challenge[1].

GH secretion is regulated by sex steroids, as demonstrated by the threefold increase in GH secretion occurring along with an increase in gonadal steroid concentrations during puberty[2].

Blunted GH peaks after physiological or pharmacological stimuli may be found in normal pre-pubertal children[3], and thus, although GH stimulation tests (GHST) are required to diagnose GHD, their results need to be evaluated carefully and integrated with other clinical, biochemical and neuro-imaging data.

Retesting 1-6 months after diagnosis of children labelled as idiopathic GHD upon unprimed GHST showed normal GH responses in up to 85% of subjects[4]. On the other hand, priming with sex steroids before GHST, aimed at sensitizing the pituitary gland of pre- / peri-pubertal children to sex steroids and increasing the secretion of GH under stimulation performed soon afterwards, has been shown to reduce false positive GHD results from 39% to 5%[3].

However, evidence supporting this approach is limited, as the studies available[3,5-9] report conflicting results, possibly due to the small cohorts reported, and the lack of long-term follow up. Widespread adoption of priming in clinical practice is also hampered by the fact that some clinicians consider it as an artificial stimulus leading to a transient increase in GH peaks, thus masking peri-pubertal GHD[10].

Other than being used to improve specificity of GHST, low dose sex steroids (LDSS) can also be given as a quasi-physiological growth-promoting therapy in those patients diagnosed as CDGP after GH deficiency has been excluded, in order to improve growth and sexual maturation and mitigate their psycho-social discomfort[11,12]. Nevertheless, this treatment is not universally accepted and in CDGP patients a wait-and-see approach is frequently adopted[11,].

**Study aims**

Primary aim of our multicenter retrospective study was to evaluate if priming with sex steroids improves the diagnostic accuracy of GHST in the diagnosis of GHD, by comparing the auxological outcomes of GHD subjects undergoing a primed GHST with GHD patients diagnosed without priming or untreated CDGP patients. Besides, we compared the reversal rates of idiopathic GHD at retesting between patients undergoing primed and unprimed GHST.

Secondary aim of our study was to assess whether LDSS administered as a growth-promoting therapy in CDGP subjects significantly influence their auxological outcomes.

**Material and methods**

*Population*

We retrospectively collected auxological data of 184 children (74 females) who underwent primed or unprimed (depending on local or individual preferences) GHST in the peripubertal phaseat 3 European tertiary centers from 2002 to 2015 (Naples and Milan) and from 2008 to 2015 (London). Data were retrieved from an internal audit conducted within the three hospitals. All patients sought medical attention for short stature (IH, initial height) per se and/orin relation to their target height (TH), as defined by IH-TH <- 1.5 SDS and/or for growth deceleration(definedas aheight velocity - 1.0 SDS below the mean for age and sex).All patients had attained FH(height measured at growth velocity < 2 cm/year) at the time of data collection. Testicular volume was measured using a Prader orchidometer[14] and pubertal stage was determined according to the criteria of Marshall and Tanner[15]. All boys diagnosed as having G1 stage of puberty had mean testes volume between <4 mL, whereas those classified as G2 stage had mean testes volume between 4 and 8 mL.

Inclusion criteria were: age 11-14 years for males or 10-13 years for females; Tanner stage < -1 SDS according to puberty nomograms 13; bone age no greater than chronological age. Exclusion criteria were:born small for gestational age; familial short stature; obesity or severe malnutrition; chronic diseases or steroid use; combined pituitary hormone deficiencies; syndromic patients; history of intracranial or systemic tumors.

The differential diagnosis between CDGP and GHD was based on the response entity to the provocative test for evaluation of GH reserve and supported by other clinical and/or laboratory criteria.

Patients were diagnosed as isolated GHD if peak GH upon GHST was< 8 µg/L, possibly together with IGF-1 concentrations below two Standard Deviation Score (SDS). Among

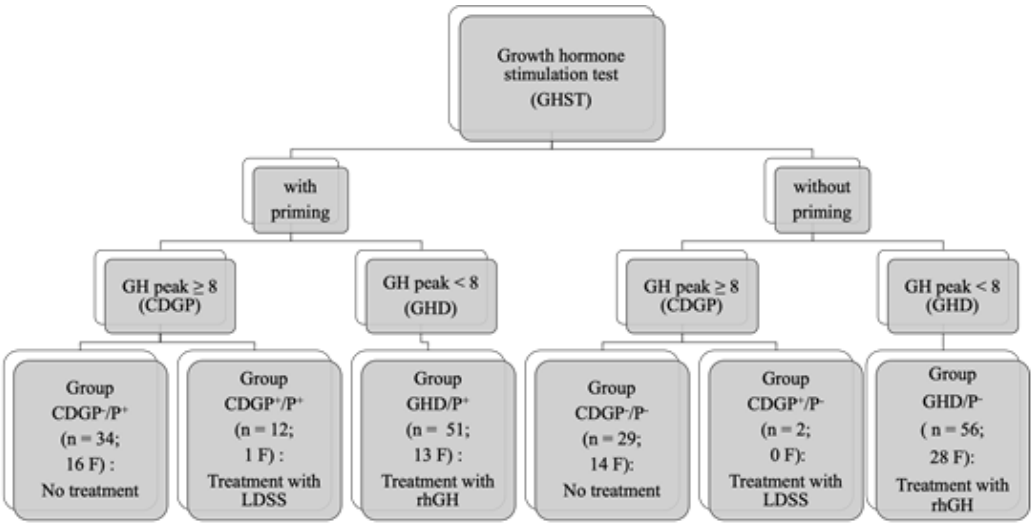
subjects with peak GH ≥ 8 µg/L, diagnosis of CDGP was established if puberty had not started yet or had a slow or stuttering progression (defined as Tanner stage < -1 SDS according to puberty nomograms) associated with at least two of these criteria; i) bone age delay > 1 year compared to chronological age; ii) being short for target height (TH); and iii) family history of pubertal delay[13].

CDGP patients were either untreated or received low-dose sex steroids (LDSS) on an individual basisuntil a Tanner stage 3 or 4 was achieved, in order to mitigate their psychosocial distress and improve their growth.

All patients diagnosed as GHD underwent a pituitary MRI. GHD children were treated with recombinant growth hormone (rhGH) at replacement doses (25-35 µg/kg/day).

Patients were divided into 6 groups: untreated CDGP diagnosed with or without priming (CDGP-/P+, n = 34; CDGP-/P-, n = 29), CDGP receiving LDSS diagnosed with or without priming (CDGP+/P+ n =12; CDGP+/P- n =2),GHD diagnosed with or without priming receiving rhGH therapy (GHD/P+ n = 51; GHD/P- n = 56) [**Figure 1**].

**Figure 1. Study groups.**




*Evaluation of GH reserve and priming*

Growth hormone reserve was investigated locally by using either primed or unprimedhypothalamic stimuli (arginine, clonidine, insulin tolerance or glucagontests, depending on local preferences). In males, primed GH stimulation was carried out following either low-dose(50 mg) or high-dose (100 mg) intramuscular Testosterone (Propionate or

Enanthate) injections for 4 to 7 days, depending on local preferences. In females, the two priming regimens used before GHST were Ethinylestradiol 100 ug/day for 3 days and Stilbestrol 1 mg twice daily for 2 days.
Retesting of GH reserve was performed by using either ITT or GHRH plus arginine testsat least one month after rhGH therapy discontinuation.

*Outcome assessments*

Anthropometric measurements [initial height (IH), weight and body mass index (BMI)] were standardized for age and gender and expressed as SDS, according to reference standards[16].TH was defined by Tanner's formula: (mother's height + father's heigh)/2 + 6.5 for males and – 6.5 for females. Bone age was evaluated by using either Greulich and Pyle or Tanner-Whitehouse methods[17].Height gain from presentation was defined as the difference between standard deviation score of FH and IH (ΔSDS FH-IH).The difference between FH and TH SDS was calculated as the difference between standard deviation of final height and target height (ΔSDS FH-TH). Degree of success, defined as the proportion of patients reaching a final height in the range TH ± 1 SDS, was considered as a dichotomic variable.

*Statistical analysis*

Continuous covariates were reported as mean andstandard deviation (or, if data were not normally distributed, as median and interquartile range), whereas categorical covariates were reported as number of patients and percentage. Normal distribution of the data was verified by means of Shapiro-Wilk test.Comparisons among groups were performed by means of Analysis of variance (or Wilcoxon test) for continuous covariates and Chi-square test (or Fisher test) for categorical ones.
For each continuous outcome ANOVA model was applied to verify that the outcomes, as well as the covariates, resulted statistically significantly different among groups. Analogously, for dichotomous outcomes, a log-binomial model was considered in order to prevent overestimation of odds ratio due to an uncommon outcome. All test were two tailed and a p-value $< 0.05$ was considered as statistically significant. All the analyses were performed by means of SAS software.

**Results**

*Description of the population*

Overall, 184 children were included: 104 from Milan, 54 from Naples and 26 from London. The main features of all groupsat diagnosis and at final height are presented in **Table 1**; data comparisons shown were confirmed in multivariate analyses and group CDGP+/P- was not considered due to its small size (n = 2).

**Table 1. General characteristics of the study population.**

| | CDGP$^-$/P$^+$ (N=34) | CDGP$^+$/P$^+$ (N=12) | GHD/P$^+$ (N=51) | CDGP$^-$/P$^-$ (N=29) | CDGP$^+$/P$^-$ (N=2) | GHD/P$^-$ (N=56) | P-value* |
|---|---|---|---|---|---|---|---|
| ***Characteristics at cohort entry*** | | | | | | | |
| Chronological age, years *Median [IQ]* | 12.60 [11.50 to 14.00] | 13.50 [13.10 to 14.10] | 13.00 [11.90 to 14.50] | 12.60 [11.10 to 14.00] | 13.75 (0.64) | 11.10 [10.00 to 13.00] | <0.0001ǂ |
| Male *N (%)* | 18 (53.0%) | 11 (92.0%) | 38 (75.0%) | 15 (52.0%) | 2 (100.0%) | 28 (50.0%) | 0.010‡ |
| BMI SDS *Median [IQ]* | -0.85 [-1.50 to -0.20] | -1.25 [-1.66 to -0.55] | -1.15 [-1.86 to -0.29] | -0.81 [-1.65 to 0.30] | -0.55 [-2.64 to 1.55] | -0.39 [-1.45 to 0.49] | 0.122ǂ |
| Puberty *N (%)* | | | | | | | |
| - G1 | 9 (26.5%) | 6 (50.0%) | 17 (33.3%) | 7 (24.1%) | 2 (100%) | 17 (30.4%) | |
| - G2 | 9 (26.5%) | 5 (41.7%) | 21 (41.2%) | 8 (27.6%) | 0 (0%) | 12 (21.4%) | 0.202‡ |
| - B1 | 11 (32.3%) | 0 (0.0%) | 8 (15.7%) | 11 (37.9%) | 0 (0%) | 17 (30.4%) | |
| - B2 | 5 (14.7%) | 1 (8.3%) | 5 (9.8%) | 3 (10.3%) | 0 (%) | 11 (19.6%) | |
| IGF-1 SDS *Mean (SD)* | -0.99 (0.97) | -1.50 (1.14) | -1.51 (1.34) | -1.40 (1.07) | -1.27 (.) | -1.39 (1.23) | 0.368† |
| IH SDS *Median [IQ]* | -2.10[-2.60 to -1.90] | -2.43 [-3.00 to -2.00] | -2.43 [-3.00 to -2.00] | -2.00 [-2.80 to -1.62] | -1.52[-2.06 to -0.97] | -2.15 [-2.47 to -1.80] | 0.017ǂ |
| TH SDS, cm *Mean (SD)* | -0.76 (0.66) | -0.37 (0.71) | -0.68 (0.83) | -0.53 (0.58) | -0.55 (0.96) | -0.62 (0.80) | 0.539† |
| Δ IH-TH SDS, cm *Median [IQ]* | -1.53 [-1.75 to -1.10] | -1.98[-2.40 to -1.00] | -1.98[-2.40 to -1.00] | -1.50[-2.10 to -1.20] | -0.97 [-1.10 to -0.83] | -1.60[-2.09 to -0.84] | 0.045ǂ |
| ***Characteristics at final visit*** | | | | | | | |
| BMI SDS *Median [IQ]* | -0.77 (1.30) | -0.86 (1.17) | -0.56 (1.16) | -0.70 (1.05) | -1.58 (1.42) | -0.35 (1.06) | 0.363† |
| FH SDS, cm *Mean (SD)* | -1.5 (0.88) | -0.86 (1.07) | -0.81 (0.93) | -0.9 (1.13) | -0.44 (1.41) | -0.93 (0.71) | 0.011† |
| FH-IH Δ SDS *Mean (SD)* | 0.79 (0.98) | 1.57 (0.68) | 1.68 (0.89) | 1.15 (0.87) | 1.08 (0.64) | 1.18 (0.82) | 0.0001† |
| FH-TH Δ SDS, *Mean (SD)* | -0.74 (0.99) | -0.48 (0.89) | -0.12 (0.72) | -0.37 (0.95) | 0.11 (0.45) | -0.31 (0.77) | 0.025† |
| Degree of success *N (%)* | 22 (65%) | 9 (75%) | 46 (90%) | 18 (62%) | 2 (100%) | 48 (86%) | 0.006‡ |

Within the whole cohort, GHD/P- patients were younger ($p < 0.0001$) and had bone ages more delayed compared to chronological age. Mean bone age at presentation was 11.90 years (10.10 to 12.50) for CGDP-/P+, 12.20 years (10.50 to 13.00) for CDGP+/P+, 12.00 years (10.70 to 13.00) for GHD/P+, 11.00 years (9.90 to 12.50) for CDGP-/P-, 11.20years (9.5 to12.90) for CDGP+/P- and 9.00 years (8 to 11.30) for the GHD/P- cohorts. For 3 of the groups, male predominance was greater than 70%. BMI and Tanner stage at presentation were similar between groups ($p = 0.122$ and 0.202 respectively). Among all groups, CDGP+/P+, GHD/P+ and GHD/P- had the most severely compromised median SDS IH ($p= 0.017$) and ΔSDS IH-TH ($p = 0.045$)at presentation.

SDS IGF-1 at diagnosis was not different between groups (p= 0.368).

Priming with sex steroids pre-GHST was overall performed in 97/184 children (30 females), of which 46 (17 females) were diagnosed with CDGP and 51 (13 females) with GHD.LDSS was given as a growth promoting therapyon an individual basis in 14/77CDGP patients, by using increasing dosages (up to 100 mg monthly) of either Propionate or Enanthate Testosterone for a median duration of 13 months (interquartile range 5-18 months). No concerns or side effects were reported in patients during or after LDSS treatment.

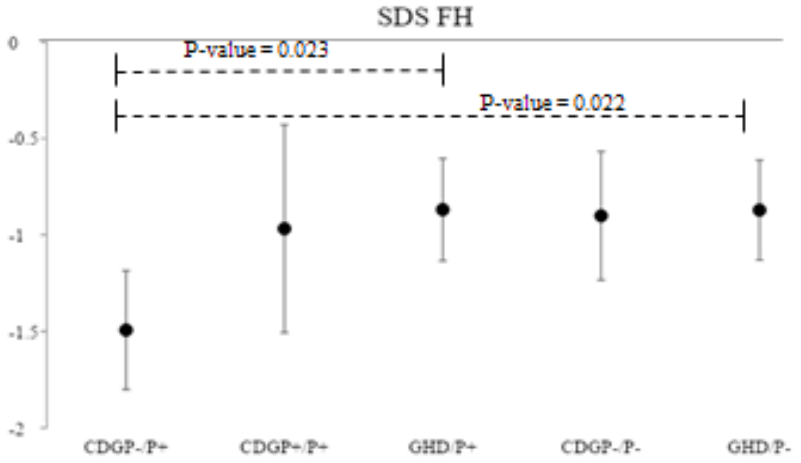
Mean GH peaks upon GHST were similar between CDGP/P+ and CDGP/P- and between GHD/P+ and GHD/P-cohorts (data not shown).

Pituitary hypoplasia was found in 12 out of 51 GHD/P+ children, and in 8 out of 56 GHD/P- patients, while the rest had a normal MRI. The prevalence of small anterior pituitary gland was not statistically different between the two groups.

*Multivariate analysis*

Mean SDS FH, ΔSDSFH-TH and ΔSDSFH-IH were statistically different between CDGP-/P+, CDGP+/P+,GHD/P+, CDGP-/P-, GHD/P- groups (p-value =0.017, p-value= 0.007 and p-value=0.002, respectively)**[Figure 2, 3 and 4].**

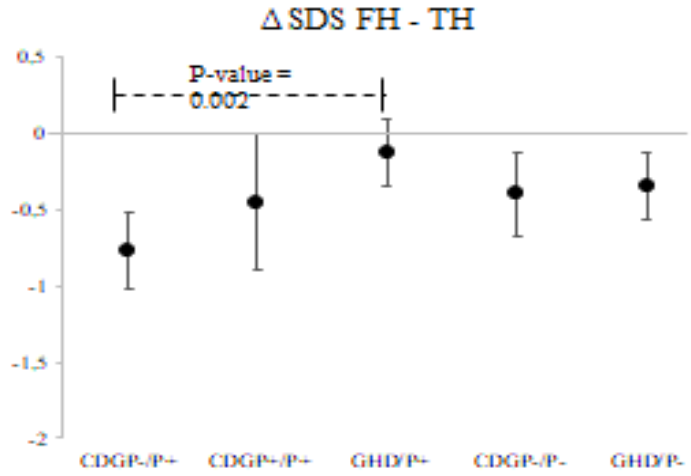
**Figure 2. Difference in SDS FH between the groups.**




| Comparisons overall | Mean difference (SE) | P-value* |
|---|---|---|
| CDGP- / P+ and CDGP- / P- vs GHD/P+ | -0.328 (0.177) | 0.065 |
| CDGP- / P+ and CDGP- / P- vs GHD/P- | -0.326 (0.174) | 0.064 |

* P-value adjusted with Bonferroni test

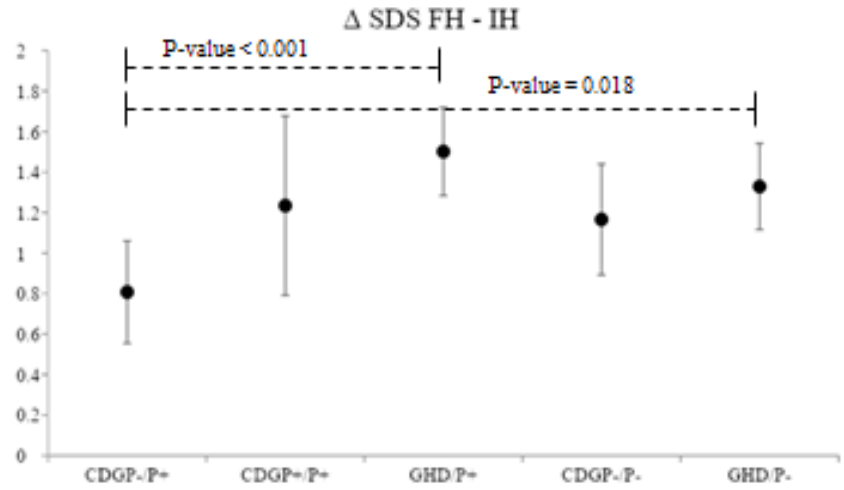
**Figure 3. Difference in ΔSDS FH-TH between the groups.**




| Comparison overall | Mean difference (SE) | P-value* |
|---|---|---|
| CDGP- / P+ and CDGP- / P- vs GHD/P+ | -0.454 (0.145) | 0.004 |
| CDGP- / P+ and CDGP- / P- vs GHD/P- | -0.238 (0.143) | 0.196 |

* P-value adjusted with Bonferroni test

**Figure 4. Difference in ΔSDS FH-IH between the groups.**




| Comparisons overall | Mean difference (SE) | P-value* |
|---|---|---|
| CDGP- / P+ and CDGP- / P- vs GHD/P+ | -0.515 (0.145) | 0.001 |
| CDGP- / P+ and CDGP- / P- vs GHD/P- | -0.342 (0.143) | 0.018 |

* P-value adjusted with Bonferroni test

More specifically, the head to head comparison for SDS FH proved to be lower in CDGP-/P+group compared to both GHDgroups : CDGP-/P+vs GHD/ P+LSmeans (SE): -1.49 (0.16) vs -0.87 (0.14), p-value =0.023; CDGP-/P+vs GHD/ P-: -1.49 (0.16) vs -0.87 (0.14), p-value=0.022 [**Figure 2**].

Concordantly, ΔSDSFH-TH proved to be significantly higher only between CDGP-/P+and GHD/P+[-0.77 (0.13) vs -0.35 (0.11), p-value =0.002] [**Figure 3**].

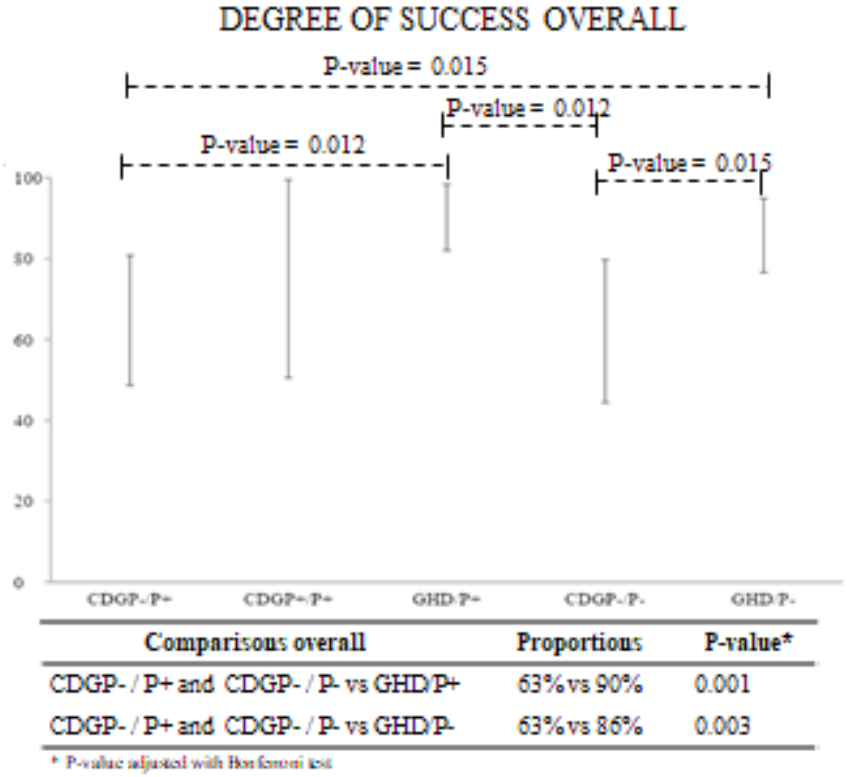
Hence, the differences in SDS FH and ΔSDS FH-TH between CDGP-/P-and both GHDgroups were similar [**Figure 2 and 3**].

Better long term auxological outcomes in GHD patients diagnosed upon a primed GHST (GHD/P+) were also confirmed in the comparison with overall untreated CDGP patients (groups CDGP-/P+and CDGP-/P-taken together). In fact, SDS FH nearly achieved statistical significance with a p-value approaching the significant threshold (p-value =0.065),whilst ΔSDSFH-TH achieved a significant statistical difference (p-value = 0.004) for GHD/P+ compared to untreated CDGP patients overall. In contrast, these outcomes were not both concordantly statistically significant among GHD/P- patients and untreated CDGP patients overall (SDS FH p-value= 0.064 and ΔSDSFH-TH p-value= 0.196) [**Figure 2 and 3**].

Analogously, catch-up growth from presentation (ΔSDS FH-IH) was lower in CDGP-/P+ compared to both GHD groups [CDGP-/P+vsGHD/ P+: 0.81 (0.13) vs 1.50 (0.11), p-value<0.001; and CDGP-/P+vs GHD/P-: 0.81 (0.13) vs 1.33 (0.11),p-value=0.018], whereasit was found to be similar between CDGP-/P- and both GHD groups [**Figure 4**].

Among all groups, the highest degree of success was gained by GHD/P+ (p = 0.006) [**Table 1**]. However, regardless of priming, treatment with rhGH was associated with better long term auxological outcomes compared to untreated CDGP. In fact, the head to head comparison between groups showed that GHD/P+hada higher degree of success compared to untreated CDGP groups (GHD/P+ vs CDGP-/P+: 90% vs 65%, p-value = 0.012; GHD/P+ vs CDGP-/P-: 90% vs 62% p-value = 0.012) and analogously that GHD/P- group had a higher degree of success compared to untreated CDGP (GHD/P- vs CDGP-/P+: 86% vs 65%, p-value = 0.015; GHD/P- vs CDGP-/P-: 86% vs 62%, p-value = 0.015) [**Figure 5**].

**Figure 5. Difference in degree of success between the groups.**

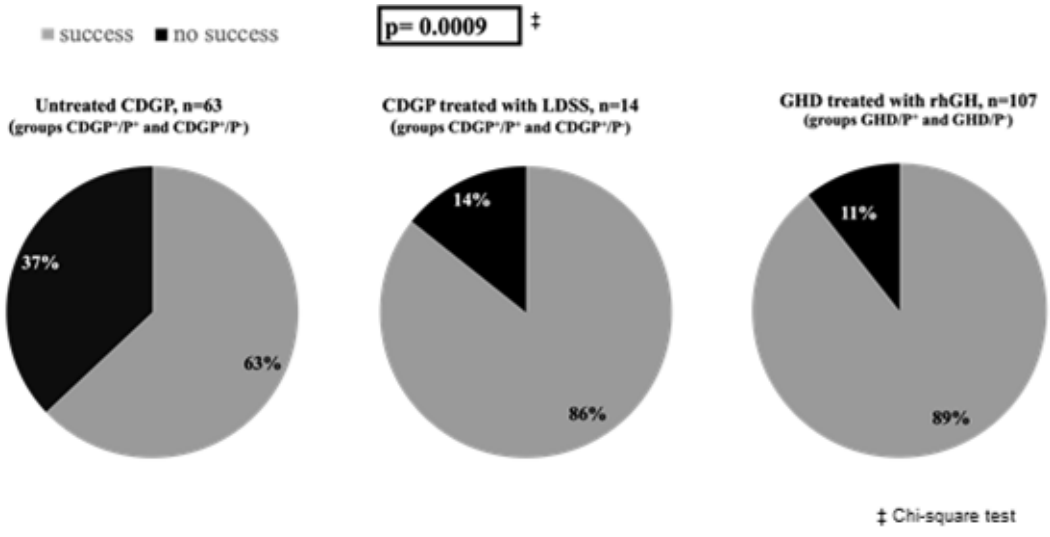



| Comparisons overall | Proportions | P-value* |
|---|---|---|
| CDGP- / P+ and CDGP- / P- vs GHD/P+ | 63% vs 90% | 0.001 |
| CDGP- / P+ and CDGP- / P- vs GHD/P- | 63% vs 86% | 0.003 |

* P-value adjusted with Bonferroni test

Finally, considering the entire cohort and grouping patients for diagnosis and treatment, regardless of the use of priming before GHST, both treatments with either rhGH or LDSS were associated with higher degrees of success compared to the untreated CDGP cohorts (GHD 89% vs CDGP+ 86% vs CDGP- 63% p= 0.0009)[**Figure 6**].

**Figure 6. Degree of success between the groups paired for treatment.**



*Retesting*

After attainment of final height, 46 out of 51 (90.19%) patients in group GHD/P+ (mean age 17.76±1.43 years) and 53 out of 56 (92.52%) patients in group GHD/P- (mean age 16.69±1.05 years) were retested for GH reserve and a trend towards a higher proportion of permanent GHD was documented in patients diagnosed upon a primed GHST (GHD/P+ vs GHD/P-: 30.43% vs 15.09%; p= 0.067).

No statistically significant differences were found in the reversal rates of GHD at retesting between subjects with normal (n=87) and abnormal (n=20) MRI (81% in those with normal MRI vs 70% in those with small anterior pituitary, N.S.).

No significant post therapy IGF-1 differences were observed between normalizedvspersistent GHD at retesting both in GHD/P+ (normalized: 0.23±1.21 vs persistent GHD: 0.97±1.21,N.S.) and in GHD/P-groups (normalized: -0.46±0.97 vs persistent GHD: -1.04±1.64, N.S.).

**Discussion**

To our knowledge this is the first study investigating long-term auxological outcomes in a large cohort of pre-/peri-pubertal children diagnosed with CDGP or GHD, undergoing a primed GHST, in comparison to a group of children who did not receive priming.

The results of our study indicate that priming with sex steroids prior to GHST may improve the diagnostic accuracy of the test in the diagnosis of GHD.

In fact, we documented that GHD patients diagnosed upon primed GHST reach a FH that is greater and closer to the TH, in comparison with untreated CDGP. Conversely, in patients in whom the diagnosis was made upon an unprimed GHST, we found no differences in auxological outcomes between treated GHD and untreated CDGP patients, suggesting that priming plays a key role in selecting those children who may benefit the most from rhGH treatment.

Important strengths of our study are both the involvement of a homogeneous cohort, as well as the inclusion of a control group of unprimedCDGP/GHD.In addition, we ruled out secondary causes of GHD or short stature. Therefore, our cohort is representative of Caucasian children presenting with idiopathic short stature and delayed puberty. However it has some limitations, inherent to the retrospective design of the study: low sample size of some groups, variabletests performed, different laboratory assays, use of different regimensof sex steroids either as priming before GHST either as a growth promoting therapy.

Along with the fact that during puberty, the increase of sex steroids circulating levels is associated with an increased GH pulse amplitude, higher IGF-I concentrations, and increased anterior pituitary size[18,19], most[3,6,7],but not all[9,20], studies reported that priming with sex steroids prior to GHST increases GH peaks in response to provocation in both normal and short children, improving diagnostic accuracy of GHST from 90% to 95% [6]. Although, as already mentioned, since 2016 priming before GHST has been recommended by Pediatric Endocrine Society Guidelines, its use in clinical practice is still limited, considered by some clinicians an unphysiological method leading to only a transient increase in GH peak and masking peri-pubertal GHD[10], possibly exposing patients to the risk of side effects, and due to the lack of robust evidence on its diagnostic advantages and long-term outcomes. In fact, so far, only one study explored final height in 50 untreated peripubertal boys with subnormal unprimed but normal primed stimulated GH peaks, reporting a normal FH that layed within the TH range, regardless of the priming protocol used [21].

Auxological outcomes of our GHD cohort (either/P+ or/P-) are in line with previous data for Caucasian patients reporting FH SDS ranging between -0.7 and -1.11 SDS and FH-TH ΔSDS between -0.17 and -0.6 [22,23].Growth response to rhGH has been previously found to correlate with anthropometric variables at the start of treatment (IH SDS, chronological age, bone age, pubertal status), severity of GHD and genetic potential[24]; however, the results of our study indicate the need for including also the use of priming in models predicting the response to rhGH.

Interestingly, although the overall proportion of re-confirmed idiopathic GHD at final height was similar to that reported in other papers [4,25,26] we found a trend towards a higher proportion of permanent GHD in primed compared to unprimed GHD patients. We believe that, although this comparison only approaches the significance threshold, it is of much interest and could become statistically significant by increasing the sample size. Taken together, these results indicate the poor diagnostic accuracy of unprimed GHST in the peri-pubertal phase, likely due to the physiological transient blunting of GH response to stimulation in this period of life[27].

IGF-1 values were not useful to differentiate between pre/peripubertal CDGP and GHD in the initial diagnostic process, nor to differentiate true and permanent GHD after retesting from transient forms of IGHD. This could be possibly related to the poor sensitivity of IGF-1 and its fluctuations in relation to pubertal and nutritional status.

Interestingly, no correlation was found between anterior pituitary size and reconfirmed GHD at retesting, possibly due to the conceptthat a borderline small anterior pituitary gland is

difficult to differentiate from low-normal sized pituitary glandgiven the very few studies available calculatingthe anterior pituitary volume/size for chronological age and gender[28,29] and its physiological variations with pubertal status [30].

An additional aim of our study was to evaluate the effects of LDSS given as a growth-promoting treatment in CDGP patients after GH deficiency was excluded.

In keeping with results of previous studies[31-33]our data indicate that, if left untreated, patients with CDGP fail to achieve their genetic potential, and this is even more evident when priming before GHST is performed.This result may be possibly due to the fact that theCDGP cohort diagnosed with the use of priming mayhave included some partial/mild forms of GHD, displaying normal transient GH peaks under primed stimuli. Even if there is no consensus on the treatment protocol to adopt for CDGP children, we have demonstrated that regardless of priming before GHST, administering variable schemes of LDSS is a safe and well tolerated approach and may favourthe achievement of height potential, avoiding the risk of premature closure of bone cartilages secondary to an excessive bone age maturation, as previously suggested in smaller cohorts[34,35].

Data evaluating long-term auxological outcomes for females are scanty, especially in CDGP, which typically shows a male preponderance [31]. In our study we failed to find any significant gender differences between CDGP and GHD, possibly due to the low female representation in our cohort.

Except from one female initially included into the CDGP+/P+ group, no patients had permanent hypogonadotrophic hypogonadism. This ideally confirms the prevailing clinical impression that the vast majority of children affected by hypogonadotropic hypogonadism escape retardation of linear growth.

**Conclusions**

In pre- or peri-pubertal short subjects, priming with sex steroids seems to improve the diagnostic accuracy of GHST, potentially avoiding cumbersome and less effective rhGH treatment in children with a false positive GHD diagnosis following an unprimed GHST. Indeed, we documented the highest degree of success, as well as the highest proportion of reconfirmed GHD at retesting after attainment of final height in GHD subjects diagnosed upon a primed GHST. Moreover, administration of LDSS in CDGP patients after exclusion of GHD proved to be effective in improving their auxological outcomes.

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## CHAPTER 3. Endocrine morbidity and final height of hypopituitarism

### 3.1 Endocrine morbidity in midline brain defects: Differences between septo-optic dysplasia and related disorders.

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#### Introduction

"Septo-optic dysplasia" (SOD), classically defined by the presence of two or more features of the "triad" optic nerve hypoplasia (ONH), pituitary deficits, and midline brain defects, is a rare condition with an estimated incidence of 5.5/100,000 live births [1]. It is a poorly understood disorder [2] and its diagnostic criteria have been much debated [2]. Significant phenotypic heterogeneity is observed, even within families [3]. Causative mutations in genes implicated in pituitary development are identified in only a small proportion (<10%) of cases [4], thus suggesting that other factors may play a pathogenic role [5]. Given the rarity of the condition, the extent of endocrine morbidity is largely unknown [1, 5-14].

#### Study aims

The aim of this study was to describe the endocrine morbidity and mortality of a large single-centrecohort of children and adolescents with SOD and to compare them with children with Multiple Pituitary Hormone Deficiencies (MPHD), and isolated ON Hypoplasia (ONH).

#### Patients and methods

*Patients and design*

Retrospective longitudinal data from 259 patients diagnosed with SOD (n=171), MPHD (n=53) or ONH (n=35) between 1994 and 2015 at our quaternary endocrine unit. The median duration of follow-up was 8.00 years for SOD, 6.62 years for MPHD and 6.90 years for ONH. We used SOD+ to indicate SOD patients with hypopituitarism and SOD- for those with no pituitary deficits. General and pubertal features as well as mortality data were compared between SOD, MPHD and ONH. The endocrine morbidity was studied in patients with SOD+

vs MPHD. The pituitary imaging findings were analysed in all groups and subgroups (SOD, MPHD, ONH, SOD+, SOD-).

*Diagnosis of Hypopituitarism*

Standard diagnostic criteria for GH, TSH, ACTH, and gonadotropins (Gn) Deficiencies and Central Diabetes Insipidus (DI) were adopted.

Additionally, in children with low growth factors, poor Growth Velocity (GV), structural hypothalamo-pituitary (H-P) abnormalities, the overnight GH secretion was considered disrupted when there were fewer than 3 GH peaks > 6.7 ng/L (20 minute GH sampling for 12 hours) [15]. The assessment of the pituitary-gonadal axis was performed either during mini-puberty (known to be variable, for the purpose of this study: <18 months of age) or at the expected time for puberty. Testicular function was assessed using the 3-day and 3-week testosterone response to human Chorionic Gonadotropin (hCG) stimulation. A testosterone peak < 3.6 nmol/L 3 days or <9.5 nmol/L 3 weeks after hCG stimulation was considered abnormal [16]. The degree of hypopituitarism was evaluated through the Endocrine Morbidity Score (EMS) from DeVile et al [17] ranging from 1 (one deficit) to 5 (panhypopituitarism).

*Statistics*

Body mass index (BMI) [18], GV [18] and IGF-1 [19] were expressed as SDS.

Anthropometric measures, age at diagnosis and last appointment, follow-up duration, pubertal and body weight disorders rates were compared between the SOD, MPHD and ONH subgroups using Kruskal-Wallis, ANOVA or chi-square, as appropriate.

Differences in the prevalence and age at onset of individual pituitary deficiencies were analyzed in children with SOD+ vs MPHD. Time to acquisition of each of the 5 endocrine deficits and to the first of these deficiencies were compared between diagnostic subgroups using Cox Proportional Hazards models. Differences according to demography and MRI findings were investigated. Interaction terms were added to the models to investigate differences in relationships with other factors between SOD+ and MPHD.

Differences in prevalence of deficits between groups were studied and Hazard ratios (HR) are presented with 95% confidence intervals (ci).

**Results**

1) General features, mortality and pubertal data in SOD vs MPHD vs ONH

*General features*

Most SOD patients presented with three diagnostic criteria of SOD (64.9%) and with ONH (89.9%) (87.4% bilateral, 12.6% unilateral).

MPHD were diagnosed earlier in life (median 0.44 vs 1.03 vs 1.68 years, in MPHD, SOD and ONH respectively, p=0.004), and more frequently in the neonatal period (30.2% vs 5.8% vs 0.0% respectively, p<0.0001) **(Table 1)**.

The prevalence of obesity was greater in MPHD than SOD and ONH (41.2% vs 31.1% vs 12.1%, respectively, p=0.015), whilst leanness was documented in SOD (5.59%) and ONH (6.06%) only, and "extreme obesity" in 3 SOD patients only **(Table 1)**.

In both SOD and MPHD, obesity was more frequent in subjects with DI, compared to those without DI [18/32 (56.3%) vs 53/180 (29.4%), diff (ci) 26.8% (8.5, 43.6%), p=0.003].

**Table 1. Characteristics of children with Septo-Optic Dysplasia (SOD), Multiple Pituitary Hormone Deficiencies (MPHD) and Optic Nerve Hypoplasia (ONH).**

| | SOD (n:171) | MPHD (n:53) | ONH (n:35) | p value |
|---|---|---|---|---|
| M/F (%) | 96/75 (56.1) | 30/23 (56.6) | 21/14 (60.0) | 0.915 |
| Age at SOD/MPHD/ONH diagnosis (years) median (IQR) (range) | 1.03 (1.92) (0.01-14.92) | 0.44 (3.37) (0.01-11.02) | 1.68 (1.86) (0.19-8.50) | **0.004** |
| Neonatal SOD, MPHD or ONH diagnosis n (%) | 10/171 (5.8) | 16/53 (30.2) | 0 (0.0) | **<0.0001** |
| Follow-up duration (years) median (IQR) (range) | 8.00 (6.19) (0.40-17.50) | 6.62 (5.59) (0.45-16.70) | 6.90 (7.19) (0.69-14.82) | 0.494 |
| Age at last appointment (years) mean ± SD (range) | 9.24 ± 4.64 (0.52-21.00) | 9.00 ± 4.66 (0.46-21.48) | 9.54 ± 3.82 (1.03-15.01) | 0.858 |
| Obesity at last appointment n (%) | 51/161 (31.1) | 21/51 (41.2) | 4/33 (12.1) | **0.015** |
| Leanness at last appointment n (%) | 9/161 (5.6) | 0/53 (0.0) | 2/33 (6.1) | 0.175 |

n: number; M: males, F: females; IQR: Interquartile Range; SD: Standard deviation; SDS: SD Score

*Mortality*

Mortality data were available from 144 SOD, 31 ONH and 50 MPHD. The mortality rate was 4.2% (6/144) in SOD (all of them had ONH) and 3.2% (1/31) in isolated ONH. No mortality was recorded in MPHD. In none of the deceased patients the endocrine morbidity was recorded as responsible for death, although one SOD had autonomic dysregulation suggestive of possible hypothalamic dysfunction. All deceased patients had complex phenotypes with cardiac, neurological, bone or respiratory involvement including one patient with cardiofaciocutaneous syndrome.

*Minipuberty*

In comparison to SOD, more males with MPHD were born with undervirilized external genitalia [70 vs 42.7%, diff (ci) 27.3% (6.8, 43.6%), p=0.027] **(Table 2a)**. Compared to SOD, MPHD with undervirilized genitalia had lower testosterone responses after 3 days [2.67 (1.45) vs 9.74 (12.77) nmol/L, p=0.011)] and 3 weeks [8.22 (4.33) vs 19.90 (20.10) nmol/L, p=0.052] of HCG stimulation, and lower LH [0.10 (0.15) vs 5.35 (9.45) IU/L, p<0.0001] and FSH [0.10 (0.00) vs 3.00 (6.65) IU/L, p<0.0001] responses after GnRH stimulation **(Table 2a)**. Three SOD patients presented with isolated hypospadias, without biochemical features of Gn Deficiency (GnD).

**Table 2a. Clinical and biochemical findings of likely GnRH Deficiency (GnD) and testicular dysfunction at minipuberty in males with Septo-Optic Dysplasia (SOD) compared to those with Multiple Pituitary Hormone Deficiencies (MPHD).**

| **Males** | **SOD (n:96)** | **MPHD (n:30)** | **p value** |
|---|---|---|---|
| Undervirilized genitalia n (%) | 41 (42.7) | 21 (70.0) | **0.027** |
| *Isolated micropenis* | *7 (7.3)* | *6 (20.0)* | |
| *Isolated hypospadias* | *3 (3.1)* | *0 (0.0)* | |
| *Isolated undescended testis/es* | *13 (13.5)* | *4 (13.3)* | |
| *2 of the previous features* | *16 (16.7)* | *8 (30.0)* | |
| *3 of the previous features* | *2 (2.1)* | *3 (26.7)* | |
| **Males with undervirilised genitalia** | **SOD (n:41)** | **MPHD (n:21)** | **p value** |
| Age at GnRH test (years) median (IQR) (range) | 0.62 (0.91) (0.18-1.50) *(n:16)* | 0.17 (0.09) (0.02-0.21) *(n:8)* | **<0.0001** |
| Peak LH response to GnRH test (IU/L) median (IQR) (range) | 5.35 (9.45) (0.20-27.90) *(n:16)* | 0.10 (0.15) (0.05-0.30) *(n:8)* | **<0.0001** |
| Peak FSH response to GnRH test (IU/L) median (IQR) (range) | 3.00 (6.65) (0.20-16.50) *(n:16)* | 0.10 (0.00) (0.05-0.20) *(n:8)* | **<0.0001** |
| LH peak < 5 IU/L to GnRH test n (%) | 8/16 (50.0) | 8/8 (100.0) | **0.0500** |
| Undetectable LH to GnRH test n (%) | 1/15 (6.6) | 5/7 (71.4) | **0.0015** |
| Age HCG test (years) median (IQR) (range) | 0.88 (0.90) (0.18-1.68) *(n:16)* | 0.18 (0.38) (0.01-1.63) *(n:12)* | **0.007** |
| Peak Testosterone to 3 day HCG test (nmol/L) median (IQR) (range) | 9.74 (12.77) (0.35-21.50) *(n:16)* | 2.67 (1.45) (0.35-6.03) *(n:12)* | **0.011** |
| Insufficient (< 3.6 nmol/L) 3 day Testosterone response to HCG stimulation n (%) | 3/14 (21.4) | 8/10 (80.0) | **0.011** |
| Peak Testosterone to 3 week HCG test (nmol/L) median (IQR) (range) | 19.90 (20.10) (2.91-32.50) *(n:9)* | 8.22 (4.33) (3.44-13.00) *(n:11)* | 0.052 |
| Insufficient (<9.5 nmol/L) 3 day Testosterone response to HCG stimulation n (%) | 3/9 (33.3) | 8/11 (72.7) | 0.078 |

IQR: Interquartile range, HCG: human chorionic gonadotropin

*Puberty*

More MPHD than SOD were diagnosed with GnD [37.5% vs 15.8%, diff (ci) -21.7% (2.7, 42.5%), p=0.023] and received treatment for delayed or "slowly–progressing" puberty [32.1% vs 9.5%, diff (ci) -22.7% (6.6, 41.7%), p=0.003] **(Table 2b)**.

Central Precocious Puberty (CPP) or "rapidly-progressing" puberty were diagnosed in 7.0% SOD, 8.6% ONH and none of MPHD patients **(Table 2b)**. 1 of 12 SOD and sexual precocity had an otherwise preserved pituitary function (SOD-). Among SOD with sexual precocity, 11/12 SOD (91.7%) had Small Anterior Pituitary (SAP), 8/12 (66.7%) had PP abnormalities (4 absence and 4 ectopia), 4/12 (27.3%) had Pituitary Stalk (PS) abnormalities (1 absence and 2 thinness), 1/12 (9.1%) had the Pituitary Stalk Interruption Syndrome (PSIS). 1/3 ONH with sexual precocity had SAP (33.3%).

Five out of 171 (2.9%) SOD and 2/35 (5.7%) ONH had isolated premature telarche; 3/171 (1.7%) SOD had isolated premature menarche; 4/171 (2.3%) SOD and 3/35 (8.6%) ONH had isolated premature adrenarche.

**Table 2b. Puberty in children with Septo-Optic Dysplasia (SOD), Multiple Pituitary Hormone Deficiencies (MPHD) and Optic Nerve Hypoplasia (ONH).**

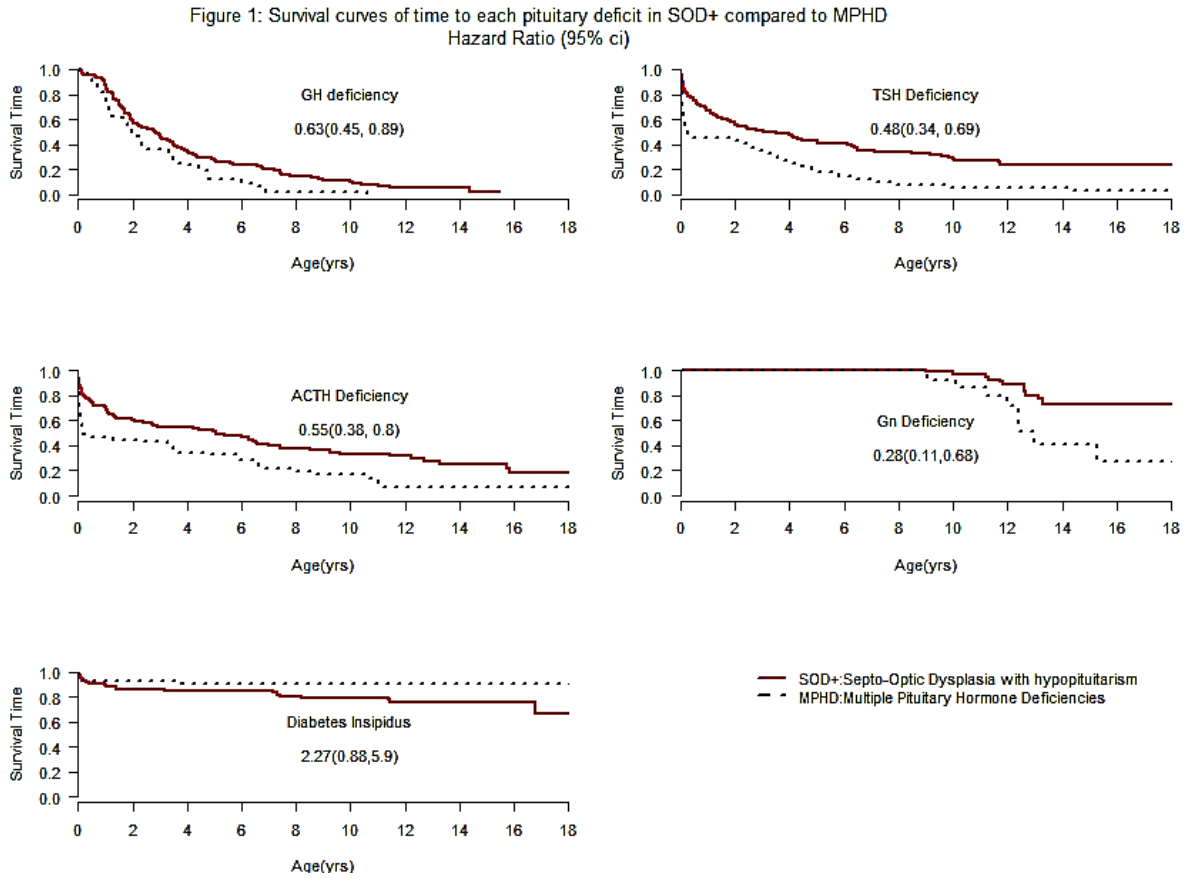
| | SOD (n:171) | MPHD (n:53) | ONH (n:35) | p value | Percentage Difference | Confidence Interval |
|---|---|---|---|---|---|---|
| Spontaneous puberty achieved at the expected ages (8-12y F, 9-13y M)<br>n (%) (M, F) | 54/66 (81.8)<br>(27M, 27F) | 7/15 (46.7)<br>(5M, 2F) | 13/14 (92.8)<br>(7M, 6F) | **0.004** | 36.7*<br>-9.5°<br>46.2# | 11.1, 59.6*<br>-21.8, 15.8°<br>12.6, 26.6# |
| Likely GnD (based on clinical and biochemical findings)<br>n (%) (M, F) | 12/76 (15.8)<br>(4M, 8F) | 9/24 (37.5)<br>(6M, 3F) | NA | **0.023** | 21.7 | 2.7, 42.5 |
| Treatment for delayed/slowly-progressing puberty<br>n (%) (M, F) | 9/95 (9.5)<br>6 DP (2M, 4F)<br>3 SPP (2M, 1F) | 9/28 (32.1)<br>7 DP (4M, 3F)<br>2 SPP (2M) | NA | **0.003** | 22.7 | 6.6, 41.7 |
| Treatment for precocious or early/rapidly-progressing puberty<br>n (%) (M, F) | 12/171 (7.0)<br>4 CPP (2M, 2F)<br>8 EP/RPP (5M, 3F) | 0/53 | 3/35 (8.6)<br>0 CPP<br>3 EP/RPP (2 M, 1F) | 0.078 | 7.0*<br>-1.6°<br>-8-6# | -0.4, 11.9*<br>-15.7, 5.9°<br>-22.4, 0.2# |

y: years; M: male, F: female, NA: Not Applicable; GnD: GnRH Deficiency; CPP: Central Precocious Puberty; EP: Early puberty; RPP: Rapidly-progressing puberty; DP: Delayed puberty, SPP: Slowly-progressing puberty
**SODvsMPHD °SODvsONH #MPHDvsONH*

2) Endocrine morbidity in SOD+ vs MPHD

Thirty-nine out of 171 (22.8%) SOD had preserved pituitary function (SOD-) over up to 14.12 years of follow-up [median (IQR) 6.25 (3.41, 8.06) years], whilst the remaining 77.2% had some degree of hypopituitarism.

Survival curves of times to each of the five pituitary deficits in SOD+ (n:132) and MPHD (n=53) are shown in **Figure 1**. All pituitary deficits were more frequent and occurred significantly earlier in MPHD [HR: 0.63(0.45,0.89) for GH, 0.48(0.34,0.69) for TSH, 0.55(0.38,0.80) for ACTH, 0.28(0.11,0.68) for Gn], except for DI [HR: 2.27(0.88,5.9)]. DI occurred before 4 years of age in all (5) MPHD, whilst 8/29 (27.6%) SOD+ were diagnosed with DI after 4 years (7.13 to 16.8 years) **(Figure 1)**.



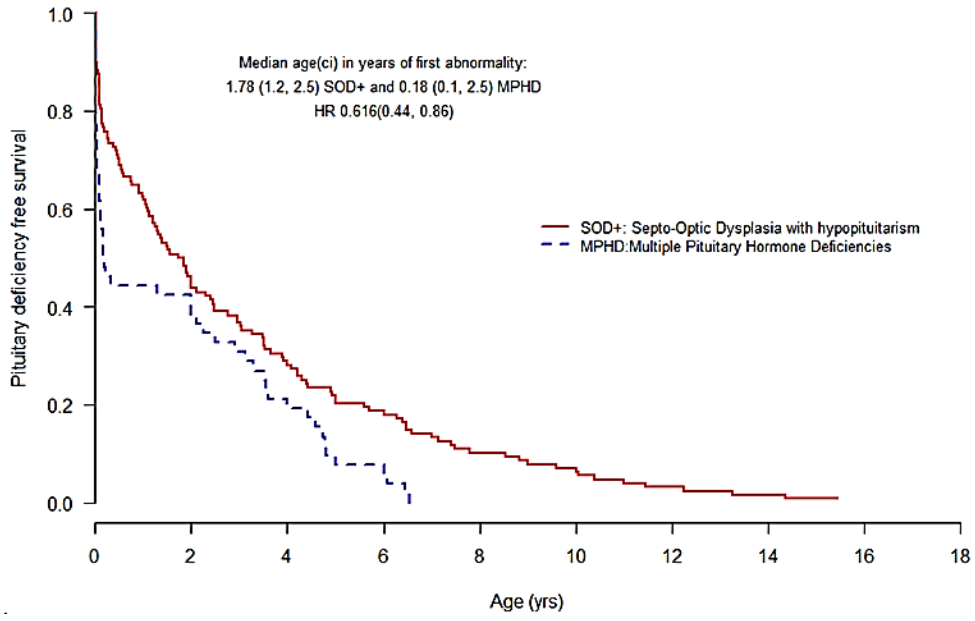

In our cohort, we identified up to 70 patterns of evolution of 16 types of associations of pituitary deficits (data not shown). The most frequent combination was GH+TSH+ACTH, in both the SOD+ (30.7%) and MPHD (49.0%). TSH (3.6%), ACTH (1.8%) and Gn (0.9%) deficiencies were rarely reported isolated in SOD+, whereas DI never presented in isolation.

The majority of patients with hypopituitarism had GHD with the exception of 11/132 (8.3%) SOD+ and 2/53 (3.8%) MPHD. The two MPHD patients without GHD were < 1 year old at last appointment (0.57 and 0.62 years), whereas 10/11 SOD+ with normal GH secretion were > 1 year of age (range 1.49-15.46 years). Four SOD+ without GHD had more than one pituitary deficit (two had TSH+ACTH+DI, one TSH+ACTH, one TSH+DI) whilst the remaining had isolated deficiencies (4 TSH, 2 ACTH, 1 Gn). The two MPHD without GHD had deficiencies in TSH+ACTH and TSH+ACTH+DI.

As Gn deficiency can only be diagnosed at the time of puberty and many of our patients had not attained the age at which puberty could be expected, the next section considers only the remaining 4 pituitary deficits.

As shown in **Figure 2**, 90% of the SOD+ patients had the first deficiency by 8.54 years, compared to 4.80 years for the MPHD. The time to first pituitary deficiency was significantly associated with the EMS [HR 1.59 (1.36, 1.85) higher for each additional abnormality subsequently seen]. The pattern was similar for SOD+ and MPHD (interaction p=0.896).

Figure 2: Survival curves of time to first pituitary deficit in SOD+ compared to MPHD




There were no differences in the biochemical diagnostic features of GH, TSH and ACTH deficiencies in SOD+ vs MPHD.

Five SOD+ (but none of the MPHD) had neurosecretory GH dysfunction (age at diagnosis 2.55-14.64 years). All had SAP, 2 had Posterior Pituitary Absence (PPA) and 1 had PSIS [SAP + Ectopic Posterior Pituitary (EPP) + Pituitary Stalk Absence (PSA)].
Raised (>6 mU/L) TSH concentrations (up to 9.8 mU/L in SOD and 16.1 mU/L in MPHD) where found in 10/78 SOD+ (13.8%) and 4/45 MPHD (8.9%) with TSH deficiency.

3) Pituitary imaging

A higher prevalence of PP abnormalities, which was largely due to EPP [80.0 vs 41.6%, diff (ci) 38.4%, (22.7, 50.4%), p<0.0001], and of PSIS [46.9 vs 29.5%, diff (ci) 17.4% (1.6, 33.0%), p=0.03] was documented in MPHD compared to SOD+, whilst rarer abnormalities such as AP and PP enlargement or PS thickening were documented in the SOD+ group only **(Table 3)**.

**Table 3. Comparison between the structural hypothalamo-pituitary abnormalities of the following 5 groups: Septo-optic dysplasia (SOD), Multiple Pituitary Hormone Deficiencies (MPHD), Optic Nerve Hypoplasia (ONH), SOD with pituitary deficits (SOD+), SOD without pituitary deficits (SOD-).**

| | SOD (n:171) | MPHD¶ (n:53) | ONH (35) | SOD+¶ (n:132) | SOD- (n:39) | p value¶ | Percentage¶ difference | Confidence¶ interval |
|---|---|---|---|---|---|---|---|---|
| AP abnormalities n (%) | 135/162 (83.3) | 49/50 (98.0) | 17/34 (50.0) | 108/125 (86.4) | 27/37 (73.0) | 0.127 | 11.6 | -1.8, 18.9 |
| AP Absence | 2/162 (1.2) | 1/50 (2.0) | 0/34 (0) | 2/125 (1.6) | 0/37 (0) | | | |
| Small AP (SAP) | 132/162 (81.5) | 48/50 (96.0) | 17/34 (50.0) | 105/125 (84.0) | 27/37 (73.0) | | | |
| AP Enlargement | 1/162 (0.6) | 0/50 (0) | 0/34 (0) | 1/125 (0.8) | 0/37 (0) | | | |
| PP abnormalities n (%) | 96/161 (59.3) | 43/50 (86.0) | 0/34 (0) | 84/125 (67.2) | 12/37 (29.6) | **<0.0001** | 18.8 | 4.4, 29.9 |
| PP Absence (PPA) | 34/162 (21.0) | 3/50 (6.0) | 0/34 (0) | 27/125 (21.6) | 7/37 (18.9) | | | |
| PP Hypoplasia | 8/162 (4.9) | 0/50 (0) | 0/34 (0) | 4/125 (3.2) | 4/37 (10.8) | | | |
| PP Enlargement | 1/162 (0.6) | 0/50 (0) | 0/34 (0) | 1/125 (0.8) | 0/37 (0) | | | |
| Ectopic PP (EPP) | 53/162 (32.7) | 40/50 (80.0) | 0/34 (0) | 52/125 (41.6) | 1/37 (2.7) | | | |
| PS abnormalities n (%) | 78/157 (49.7) | 23/49 (46.9) | 0/34 (0) | 67/122 (54.9) | 11/35 (31.4) | 0.629 | *8.0* | -8.3, 23.7 |
| PS Absence (PSA) | 32/157 (20.4) | 11/49 (25.4) | 0/34 (0) | 30/122 (24.6) | 2/35 (5.7) | | | |
| Thin PS | 43/157 (27.4) | 10/49 (10.4) | 0/34 (0) | 34/122 (27.9) | 9/35 (25.7) | | | |
| Interrupted PS | 2/157 (1.3) | 2/49 (4.1) | 0/34 (0) | 2/122 (1.6) | 0 (0) | | | |
| Thick PS | 1/157 (0.6) | 0/49 (0) | 0/34 (0) | 1/122 (0.8) | 0 (0) | | | |
| Pituitary Stalk Interruption Syndrome (PSIS) n (%) | 37/159 (23.3) | 23/49 (46.9) | 0/34 (0) | 36/122 (29.5) | 1/37 (2.7) | **0.030** | 17.4 | 1.6, 33.0 |

¶p values, percentage differences and confidence intervals for SOD+ vs MPHD. AP: Anterior pituitary; PP: Posterior pituitary; PS: Pituitary Stalk; PSIS: SAP + EPP + Absent/Thin/Interrupted stalk.

Half of patients with isolated ONH and 73% of the SOD- had SAP but preserved pituitary function at 10.22 (5.96, 12.98) years (median (IQR) (range 1.65-14.82) and 6.20 (3.38, 8.00) years (median (IQR) range 0.50-12.09) of follow-up, respectively.

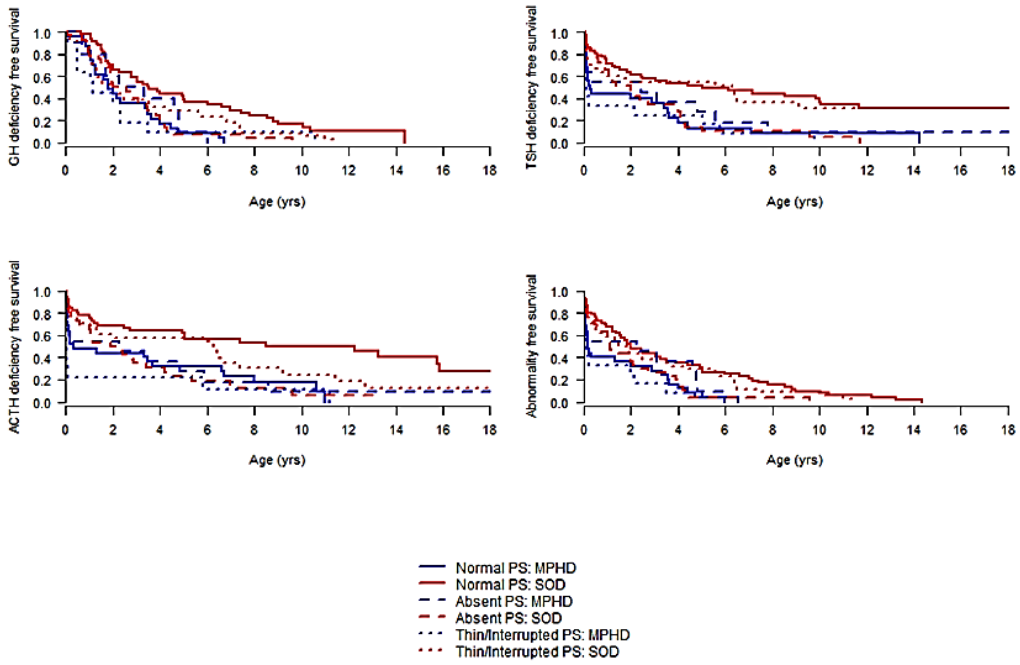
Among DI patients, 17/26 (65.4%) SOD and 1/5 (20.0%) MPHD had Posterior Pituitary Absence (PPA), 6/26 (23.1%) SOD and 3/5 (60.0%) MPHD had a normal PP, and 3/26 (11.5%) SOD and 1/5 (20.0%) MPHD had EPP. Among patients without DI, 18/128 (14.1%) SOD and 2/45 (4.4%) MPHD had PPA.

Patients with PPA [HR 2.70 (1.66, 4.38)] and EPP [HR 2.26 (1.53, 3.34)] were more likely to develop their first pituitary deficiency at a younger age.

The following MRI findings were significantly associated with an earlier onset of specific pituitary deficiencies: i) PPA with all pituitary deficits except GnD; ii) EPP and PSIS with all anterior pituitary deficits, whilst they were "protective" for DI; iii) PSA with GHD, TSHD and ACTHD.

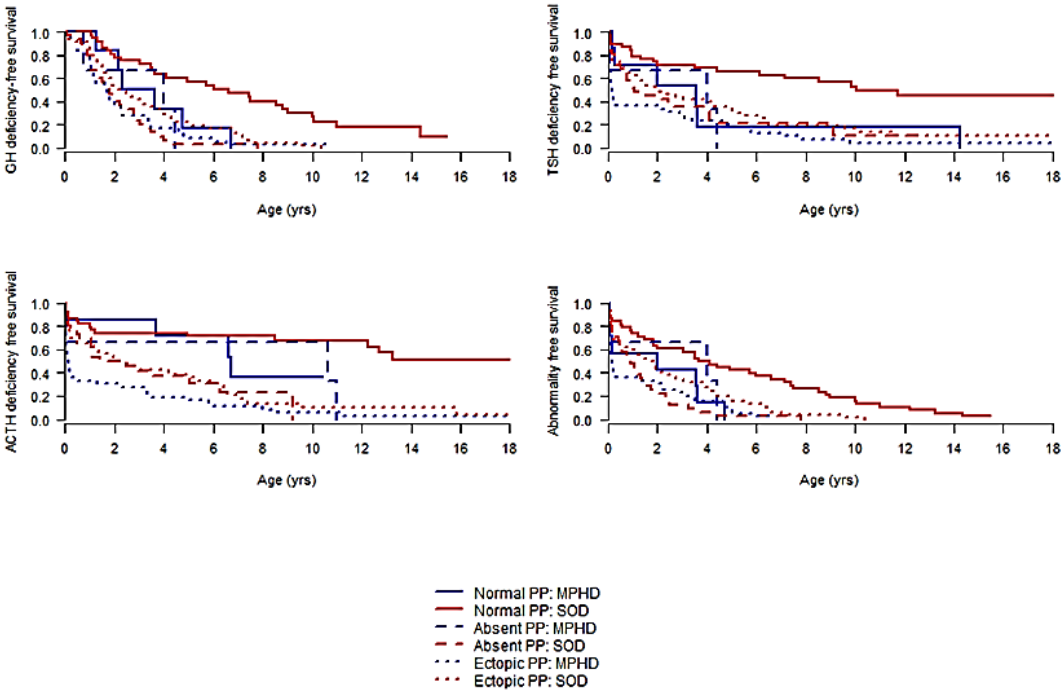
Further investigations revealed patterns of SOD+ times to first deficit and to GH/TSH/ACTH deficiencies being more similar to the MPHD amongst SOD+ with PP, PSA and PSIS abnormalities. In particular, there were significant interactions between PSA and diagnosis, resulting in SOD+ patients with PSA having times of GH/TSH/ACTH deficiencies, and to the first deficiency, in a similar pattern to MPHD (**Figure 3**, all p-values < 0.03).

Figure 3: Interactions between Pituitary Stalk (PS) abnormalities and SOD+ or MPHD diagnosis in relation to times to GH, TSH, ACTH deficiencies and first pituitary deficit



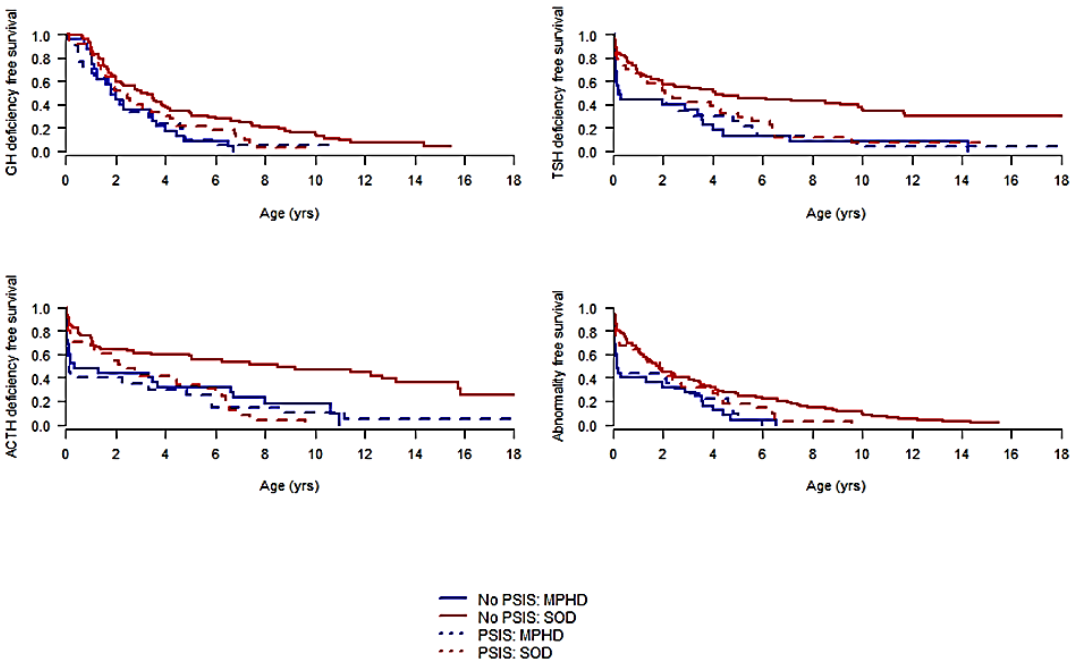
For Posterior Pituitary abnormalities, the patterns were similar (**Figure 4**), despite only attaining statistical significance for time to first abnormality (p=0.045) and being borderline for time to GHD (p=0.0585).

Figure 4: Interactions between Posterior Pituitary (PP) abnormalities and SOD+ or MPHD diagnosis in relation to times to GH, TSH, ACTH deficiencies and first pituitary deficit




Similarly, for PSIS, **Figure 5** clearly shows that SOD+ with PSIS had deficiencies at times comparable to MPHD, despite the interactions not being statistically significant, probably due to small numbers within subgroups (p-values ranged from 0.0686 for ACTHD to 0.25 for time to first abnormality).

Figure 5: Interactions between Pituitary Stalk Interruption Syndrome (PSIS) and SOD+ or MPHD diagnosis in relation to times to GH, TSH, ACTH deficiencies and first pituitary deficit




There was insufficient data to investigate the interactions for GnD, as this was not diagnosed until puberty, and for DI, which was uncommon in MPHD.

There was no clear evidence of association between ONH (bilateral vs unilateral) and the time to any pituitary deficiencies, although estimates are imprecise and some confidence intervals very wide.

**Discussion**

To our knowledge, this is the largest study on the endocrine morbidity and mortality of children with SOD and related disorders followed-up in a single centre over a relatively long period of time.

The comparison between our subgroups revealed three main endocrine phenotypes: 1) MPHD: higher occurrence and earlier onset of anterior pituitary deficits and higher prevalence of severe structural H-P abnormalities; 2) SOD+: wider range of age at presentation/onset of deficits and of body weight disorders (from leanness to extreme obesity), heterogeneous H-P structural abnormalities, higher prevalence of DI, possibly preserved GnRH secretion (albeit risk of developing CPP); 3) ONH/SOD-: preserved pituitary function but at risk of hypopituitarism (presence of SAP), possible CPP, obesity/leanness.

MPHD and SOD+ display distinct endocrine phenotypes, with the former condition being a more homogeneous disorder of early onset (and mainly anterior) pituitary dysfunction, and the latter being highly heterogeneous in the clinical and neuroimaging features. However, we have also identified associations between specific MRI abnormalities and the age at onset of pituitary deficits which place a subgroup of SOD+ patients with PS/PP abnormalities and PSIS at higher risk of developing pituitary deficiencies earlier making the endocrine phenotype of these children more similar to those with MPHD.

We were unable to identify specific combinations or "patterns of evolution" of deficits characteristic of SOD+ or MPHD, indicating unpredictable evolution. Indeed, in our sample, SOD+ continued to develop pituitary deficits throughout adolescence (up to 14 years for the first deficit, 17 years for DI and 16 years for ACTH deficiency). To our knowledge, this is the first study documenting "late onset" DI in children with SOD. Interestingly, in our cohort DI presented only in combination with AP deficits, suggesting that isolated DI should raise the suspicion of alternative diagnoses like acquired hypothalamic/PP dysfunction or genetic causes.

Both SOD+ and MPHD shared the association between the time to the first pituitary deficit and the number of pituitary hormone deficiencies, leading to the inference that an early onset may predict a more severe phenotype. An association between more severe endocrine phenotypes and early (neonatal) onset of hypopituitarism has previously been documented in patients with PSIS [20].

Asynchronous evolution of endocrinopathies has been extensively described in children with and without midline abnormalities [21]. However, most of the studies reported the development of additional deficits in children presenting with isolated GHD [7, 22], whilst in our cohort, a small number of SOD and MPHD patients had preserved GH reserve. This finding challenges previous assumptions that the first deficit to occur in children with hypopituitarism is always GHD, or that GH is invariably deficient in these patients [7, 22].

When interpreting our data about prevalence and evolution of deficits, it must be pointed out that i) in our cohort the majority of patients were pre-pubertal at the last appointment and thus, given the evolving nature of pituitary dysfunction, the prevalence of pituitary deficits (in particular GnD) might be underestimated, ii) our SOD population had a higher prevalence of presentation with the "classic triad" (65%) and hypopituitarism (77%) compared to previous studies (24-30% [5, 23] and 50-66% [5, 6, 11, 23], respectively), suggesting a possible selection bias due to the referral to our centre of the most severe phenotypes.

Although central hypothyroidism is classically diagnosed by low FT4 concentrations and inappropriately low/normal TSH, in our cohort a significant number of patients had mild-moderately raised TSH concentrations. The mechanism underlying the TSH elevation remains unexplained, but hypothalamic dysfunction or the secretion of biologically inactive TSH, have been previously suggested [24].

Striking differences in the range of pubertal disorders were identified between MPHD and SOD/ONH. Our data showed that SOD can present with the whole spectrum ranging from delayed to precocious puberty, whilst MPHD can only develop GnD and more frequently have a history of undervirilized genitalia and testicular dysfunction.

In our cohort, GnD was not associated with isolated hypospadias. This is in agreement with the recent European Consensus Statement on congenital GnD stating that, in contrast to cryptorchidism and micropenis, more severe genital anomalies such as hypospadias result from an early fetal developmental defect, before initiation of endogenous GnRH activity [25].

Interestingly, in our sample, a number of SOD and ONH patients, but none of those with MPHD, had CPP or pubertal/adrenarche "variants". These data are consistent with earlier reports of sexual precocity associated with SOD in smaller case series [8, 9, 26, 27], whilst a similar tendency has never been described in isolated ONH. The midline brain developmental insult in SOD likely starts between the 5th and 8th weeks of gestation [28]. The arrival of GnRH neurons in the hypothalamus after the development of a midline defect (by the 13th week) may explain how GnRH secretion can be retained even if secretion of other hypothalamic releasing factors is deficient. Moreover the abnormal H-P anatomy may alter the normal suppression of GnRH neurons from higher brain centers, leading to earlier onset of gonadotrophin secretion [26]. The presence of the two extreme forms of abnormal pubertal development in the same condition might be explained by the presence of lesions in different hypothalamic regions, with autopsy studies showing that lesions in the posterior hypothalamus are associated with sexual precocity, whereas lesions of the anterior hypothalamus are associated with hypogonadism [29]. In our SOD cohort, no patients evolved from precocious to delayed/absent puberty, in contrast to what has been reported in children with diencephalic and hypothalamic dysfunction due to optic gliomas [30]. However not all patients with CPP were post-pubertal at the last appointment, hence some may still develop GnD later in life.

Different degrees of hypothalamic involvement could explain the various body weight disorders between the three groups, with SOD/ONH again showing the most heterogeneous phenotypes ranging from leanness to extreme obesity. Leanness has been previously

documented in patients with optic gliomas and likely associated hypothalamic pathology [30]. The association found in our cohort between overweight/obesity and DI could also underline a common hypothalamic origin of the two clinical manifestations.

Although the majority of patients with SOD had ONH, it is interesting to point out that all SOD presenting with sexual precocity and leanness/extreme obesity had ONH, whist these pubertal and body weight disorders were not reported in the few patients with SOD without ON abnormalities.

Based on the above described shared phenotypes between SOD and ONH, it is not inconceivable to hypothesize that children with isolated ONH may have some degree of hypothalamic dysfunction, and that they form just one end of the SOD spectrum [2]. Previous data in patients with ONH also support this hypothesis [2]. It must be noted that, in our cohort, half of the patients with isolated ONH had a small anterior pituitary on MRI, and they may still develop pituitary deficits later in life to fulfill the criteria for SOD. Hence the boundaries between these two categories are rather blurred.

Data on neuroimaging abnormalities matched the distinctive endocrine phenotypes of SOD+ and MPHD and documented some common neuroimaging risk factors between the two groups. Specific abnormalities (PP/PS abnormalities and PSIS) were more strongly associated with earlier onset of anterior pituitary deficits, compared to others (e.g. SAP, PPA), in both groups. Importantly, they were more prevalent in MPHD than SOD+ and virtually absent in SOD- and isolated ONH.

EPP and PSIS have been previously associated with hypopituitarism [7, 10]. In a recent study, PP abnormalities were associated with pituitary dysfunction in patients with ONH [31]. In our cohort, most, but not all, patients with hypopituitarism had SAP. SAP has been reported in 74-100% of patients with hypopituitarism in previous studies [32]. This variability could be related to the relative lack of convincing age-related objective size criteria for the AP. Among patients with DI, 23% had a normal PP, whilst 14% of patients without DI had PPA. The relatively low predictive value of PP abnormalities for the risk of developing DI has been previously reported [10]. Interestingly, in our cohort, PPA was associated equally with the development of posterior and anterior pituitary deficits. Although an EPP usually points to an evolving anterior pituitary dysfunction [33], we documented DI in three SOD and one MPHD with EPP, all of whom developed DI at a relatively young age (0.05 to 1.38 years for SOD and 3.8 years for MPHD). This data are in agreement with a previous study demonstrating that patients with EPP may have a defect in the osmoreceptors regulating AVP secretion [12].

A specific correlation between H-P abnormalities and the type of endocrine deficits has been reported in a previous study, with EPP and PSA being correlated with hypocortisolism and hypothyroidism and a normal PP and PS with hypogonadism [34]. We documented, in a larger cohort, an association between EPP and PSIS and the occurrence of all AP deficits whilst they were protective for DI. We have also shown that the subgroup of patients with SOD+ and PS/PP abnormalities or PSIS have a risk of developing GH/ACTH/TSH Deficiencies earlier similar to those with MPHD. However, given the possible appearance of deficits up to late adolescence (particularly for GnD) and the relative low incidence of some deficits (e.g. DI), disentangling the association between MRI abnormalities and specific endocrinopathies is challenging.

We could not find any association between the presence of septum pellucidum and corpus callosum abnormalities and the age at onset of pituitary deficits. This observation reinforces recent views that abnormalities in midline brain structures are not linked with hypopituitarism [2]. Despite abnormalities in the corpus callosum being common in patients with ONH, they correlate more with the neurobehavioural (vs endocrine) features in these patients [35], whilst septum pellucidum abnormalities might even well be incidental as they do not correlate with vision status, nor with the endocrinopathies or developmental outcomes [2]. In contrast, the onset of pituitary deficits has been extensively associated with ONH, regardless of its laterality [2, 31]. A severe visual phenotype with blindness has been recently reported as a risk factor for hypopituitarism in a large cohort of patients with ONH [31], more than the presence of bilateral (vs unilateral) ONH, as also confirmed in our study. It could be hypothesized that the presence of abnormal connectivity between the optic nerves and hypothalamus/other brain structures might in part explain the complexity and heterogeneity in the presenting features of SOD/ONH children. However, given the poor resolution of conventional MRI techniques in the anatomical characterization of the hypothalamus, this hypothesis remains speculative.

Excess mortality has been previously documented in patients with hypopituitarism secondary to brain tumours [36]. This is the first study reporting mortality data in a large population of children with SOD and related complex disorders. In our cohort, premature death in childhood occurred exclusively in patients with ONH associated with complex phenotypes and never in patients with isolated MPHD, suggesting that hypopituitarism may be just a contributory factor for death in these patients.

**Conclusions**

SOD seems to represent a spectrum of malformative conditions involving different brain structures, characterized by heterogeneous, complex and unpredictable endocrine phenotypes. Conversely, children with MPHD tend to display a more homogeneous phenotype of (mainly) anterior pituitary failure. It can be speculated that insults at different stages of embryonic development affecting hypothalamo-pituitary and optic nerve development are responsible for the wide spectrum of endocrine morbidities observed in SOD.

MRI findings can predict the evolution of endocrine deficits only to some extent, hence lifelong regular surveillance is essential in all groups to enable prompt diagnosis of evolving endocrinopathies. Specific MRI abnormalities predispose to a higher risk of early onset pituitary deficiencies, placing some SOD+ at a similar risk compared to MPHD.

Our large scale data confirm recent views that ONH may represent just one component of the "SOD spectrum". ONH and hypopituitarism might be the core features of the erroneously called "Septo-Optic Dysplasia" syndrome, whilst additional midline (corpus callosum) or hemispheric brain abnormalities may or not be present in these patients.

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### 3.2 Final height in childhood-onset hypopituitarism

**This work has been presented as a poster at the 21st European Congress of Endocrinology (ECE) 2019:** Esposito A, **Improda N**, Moracas C, Barbieri F, Alfano S, Capalbo D, Salerno M. Final height in childhood-onset hypopituitarism.

**Background**

Growth hormone deficiency (GHD) in childhood is associated with impaired linear growth. GH therapy enables the achievement of normal adult height in most cases. However, the response is variable and the factors influencing final height (FH) are still not clearly defined.

**Objectives**

- To evaluate FH in a cohort of childhood-onset GHD patients treated with GH in a single centre.
- To investigate main predictors of FH in GHD patients receiving GH treatment.

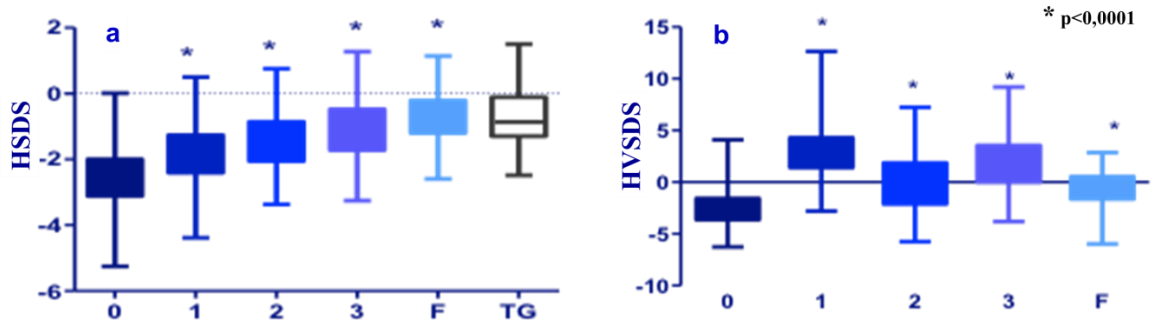
**Patients And Methods**

We collected auxological, biochemical (IGF-1) and neuroradiological data of 80 GHD patients (41 M/39 F) followed-up until the achievement of FH (defined as height velocity (HV) <2 cm/year). Structural abnormalities of the pituitary gland were detected in 40 patients. Overall, 79% of patients (63/80) had isolated GHD (IGHD), 21% (17/80) had multiple pituitary hormone deficiency (MPHD) and received hormone replacement as necessary.
Height, HV and IGF-1 were evaluated at diagnosis, at the end of the first year of treatment, at onset of puberty and at the attainment of FH and were expressed as standard deviation score (SDS) according to reference standards. Change in HSDS (Δ) from baseline to the different end points was calculated. Multiple regression analysis was used to evaluate predictors of FH.

**Results**

At diagnosis HSDS (-2.53±0.94), HVSDS (-2.47±1.69) and IGF1SDS (-1.23±1.24) were below normal ranges. Mean age at diagnosis was 9.9±4.0 years **(Figure 1)**.
After 1 year of GH, HSDS (-1.86±0.84, $p<0.0001$), HVSDS (3.03±2.79, $p<0.0001$) and IGFISDS (0.34±1.24, $p<0.0001$) significantly improved **(Figure 1)**.

**Figure 1: HSDS (a) and HVSDS (b) at different endpoints**




**0=baseline; 1=after 1y; 2=onset puberty; 3=end puberty; F=time at final height;TG=Target heigt**

At puberty onset, mean age of patients was 12.52±1.67 years; ΔHSDS from baseline to pubertal onset was significantly higher in MPHD vs IGHD (3.03±1.66vs0.6±0.68, p<0.0001) **(Figure2)**, while it was comparable between males and females **(Figure3)**.

**Figure 2: HSDS at puberty onset vs baseline and ΔHSDS in MPHD vs IGHD**

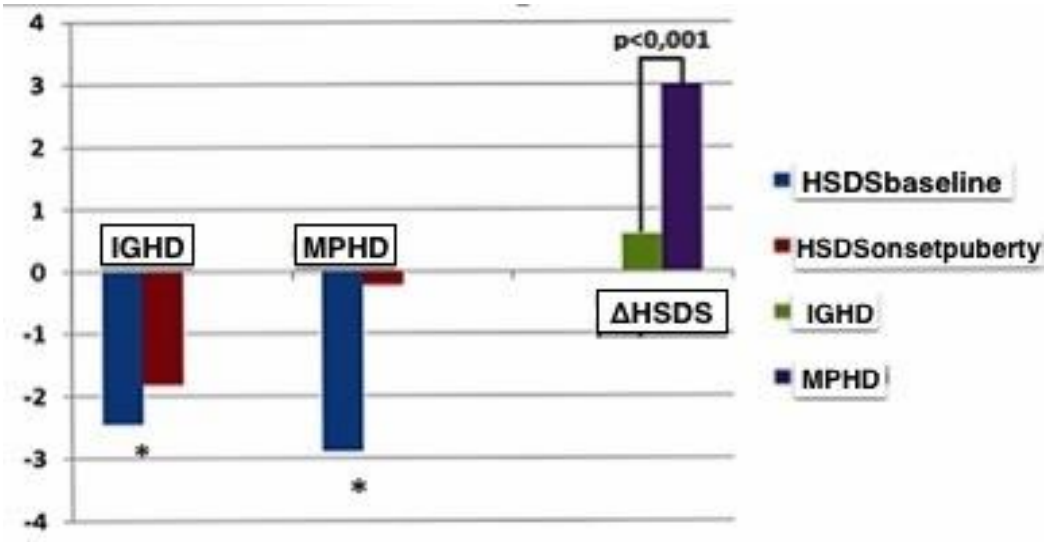
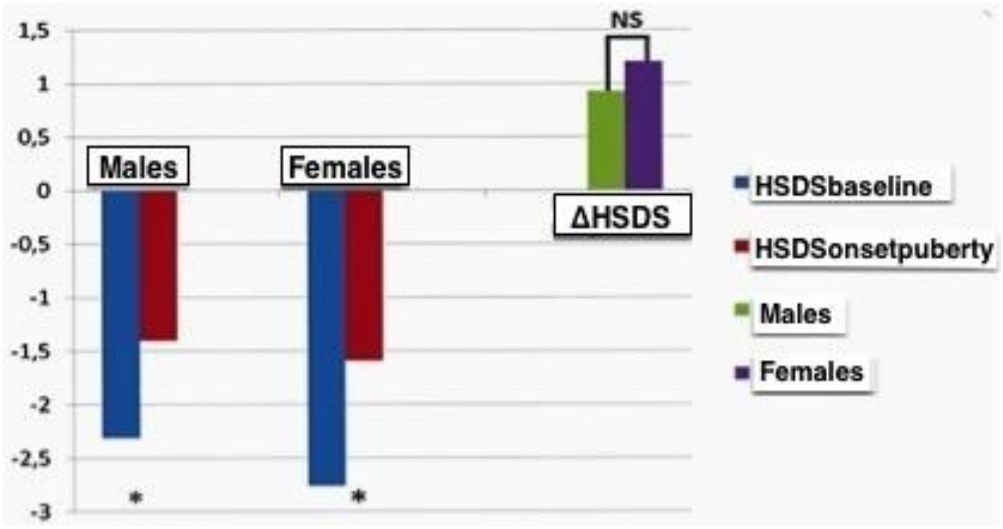


**Figure 3: HSDS at puberty onset vs baseline and ΔHSDS in males vs females**




FHSDS (-0.87±0.98), achieved at a mean age of 16.94±1.51 years, was significantly higher compared to baseline (p<0.0001) **(Figure 1)**, and was higher in patients with MPHD vs patients with IGHD (-0.47±1.15vs-0.98±0.91, p<0.05). Although FHSDS was higher in males vs females (-0.65±0.9vs-1.11±1.02, p<0.05), the total gain in HSDS was comparable between the two sexes (1.66±0.91vs1.64±1.09, p=ns).

Multiple regression analysis showed that FH correlated with:

- HV in the first year of treatment (p<0.0001)
- Type of diagnosis (MPHD>IGHD) (p<0.0001)
- Age at diagnosis (p<0.0001)
- H at onset of puberty (p<0.0001)
- Pre-pubertal ΔHSDS (p<0.0001)
- Duration of treatment (p<0.0001)
- Sex (M>F) (p<0.05**)**

**Conclusions**

GH treatment allows the achievement of normal FH. Early treatment and optimization of pre-pubertal growth are important to obtain a better growth response.

Patients with severe GH deficiency and MPHD have greater gain in height and FH than patients with mild GHD and IGHD.

## CHAPTER 4: Cardiovascular outcomes of GHD

### 4.1 Glucose homeostasis

**This work has been published as:** Capalbo D, Esposito A, **Improda N**, Wasniewska MG, Di Mase R, De Luca F, Bruzzese D, Salerno M.Glucose homeostasis in GHD children during long-term replacement therapy: a case-control study. **Endocrine. 2018 Mar;59(3):643-650.**

**Background**

Beyond the promotion of linear growth, Growth hormone (GH) significantly influences several processes of intermediate metabolism [1]. Untreated GH deficiency (GHD) in children has been associated to the development of early markers of cardiovascular risk, while GH replacement exerts beneficial effects on lipid profile, body composition and cardiac performance [2-5]. Despite these favourable changes, GH replacement therapy leads to reduction in insulin sensitivity (IS) and concern has been raised on the risk of type 2 diabetes mellitus after GH treatment [6].

GH influences glucose metabolism by increasing hepatic gluconeogenesis and glycogenolysis and by decreasing peripheral glucose utilization [1]. Furthermore, insulin-antagonistic effects of GH are attributed to its lipolytic activity, as suggested by the reversal of the GH-induced insulin-resistance by the coinfusion of GH with nicotine acid (an antilipolytic agent) [7]. Finally, GH also has direct effects on insulin sensitivity through shared signaling of GH and insulin receptors [8].

The relationship between GH and IS is intricate; paradoxically, insulin resistance has been associated with either GH deficiency and GH excess [5,9]. The effects of GH replacement treatment on glucose homeostasis are also controversial; in adults, short-term GH treatment has been related to derangement in IS while long-term treatment improved glucose homeostasis in some but not all studies [9]. Nevertheless, data from a large database reported an increased risk of type 2 diabetes after long-term GH replacement therapy in GHD adults with pre-existing risk factors as higher BMI, waist circumference, triglycerides concentration and blood pressure [6].

In children, after the neonatal period, untreated GHD has not been associated to overt abnormalities in glucose homeostasis [2,10-14], while insulin resistance compensated by hyperinsulinaemia has been documented during treatment in many studies [2,3,10,12,15]. Data from two large observational studies [16,17] documented a slight increase in the

incidence of type 2 diabetes during therapy in children with growth disorders receiving GH treatment up to 4 years; however, as in adults, glucose tolerance impairment was more common in children with pre-existing risk factors or underlying conditions which might per se alter IS (eg Turner syndrome or small for gestational age). In contrast, recent data from a French database of patients treated with GH during childhood for a mean period of 4.0±2.7 years did not document an increased risk of diabetes associated to GH therapy [18].

Most of the studies exploring glucose homeostasis in GHD children are limited by the short duration of treatment, the small sample size, the lack of an appropriate control group or the wide heterogeneity in the etiologies of growth disorder.

We designed this prospective, case-control study to evaluate glucose homeostasis in a large sample of children with GHD in comparison to healthy controls over a period of 5 years of treatment and to identify factors influencing glucose homeostasis during GH therapy.

**Subjects and Methods**

One hundred GHD children (60 males and 40 females) aged9.4 ± 3.7 years (range 3–4.3) were enrolled in the study;among them 82 patients were prepubertal, while 18 hadalready entered puberty at baseline. Diagnosis of GHD wasbased on clinical and biochemical criteria [19]. In order totest GH secretion all patients underwent two differentstimulation tests, namely, arginine and clonidine (meanpeak GH after arginine = 4.47 ± 2.42 ng/ml, range0.07–7.5; mean peak GH after clonidine = 4.77 ± 2.74 ng/ml, range 0.07–7.2). Ten patients had multiple pituitaryhormone deficiency (MPHD) and received adequate hormonereplacement with levo-thyroxine and hydrocortisoneas necessary. No patient had received GH before enteringthe study. Magnetic resonance imaging of thehypothalamus-pituitary region showed a normal gland in 55patients, while 44 patients had structural abnormalities ofpituitary gland and 1myelomeningocele and aplasia ofpituitary gland.

One hundred healthy children comparable to patients forage (8.96 ± 2.82 years, range 2.9–14.2), sex (60 males and40 females), and pubertal status (80 prepubertal, 20 at initialstage of puberty) were enrolled in the study as controls.

They were selected among children referred to our outpatientclinic for short stature or thyroid assessment becauseof family history of thyroid dysfunction. After diagnosticevaluation, they were all found to have familial short stature,constitutional delay of growth and puberty or to behealthy euthyroid children.

**Study protocol**

At study entry, all subjects underwent measurement forweight, height, waist, and hipcircumferences. BMI, waistto-height (WHtR) and waist-to-hip (WHR) ratios were thencalculated. Serum levels of IGF-1, triglycerides, totalcholesterol and HDL-cholesterol, glucose, insulin, andglycated hemoglobin (HbA1c) were measured. Insulinsensitivity was evaluated in the basal state, by the use of theHOmeostasis Model Assessment (HOMA-IR) [20] calculatedaccording to the following formula: HOMA-IR =insulin (μU/ml) × glucosio (mmol/l)/22.5 [20].

All GHD children were started on GH replacementtreatment at a dose of 25–30 mcg/kg/day; dose therapy wasthen titrated on the basis of growth pattern and IGF-1 levels.All the evaluations were repeated annually for 5 years inGHD children; healthy controls were evaluated after 1 and 5years of follow-up.

Informed parental consent for participation in the studywas obtained for patients and controls and the study wasauthorized by the Hospital Ethical Research Committee.

**Anthropometric measures**

Height was measured in the upright position using a Herpenden stadiometer (Holtain, Ltd., Crymmyth, UK); BMI was calculated by dividing weight in kilograms by the square of height in meters. Height and BMI were expressed as standard deviation score (SDS) according to Italian reference standards [22]. Waist circumference was measured by the same operator in the standing position with a non-elastic tape placed at the midpoint between the lower rib margin and the iliac crest. Hip circumference was measured at the level of widest portion of trochanters. All measurements were expressed in centimeters (cm) to the nearest 0.1 cm.

**Assays**

Serum GH and IGF-I concentrations were determined by a solid-phase, enzyme-labeled chemiluminescent immunometric assay (Immulite 2000 Siemens Healthcare Diagnostics Inc., New York, USA). IGF-1 concentrations were expressed as SDS according to the normative data provided by the manufacturer.

Glucose levels were measured through the colorimetric method of glucose oxidase. Insulin concentrations were determined by a solid-phase, enzyme-labeled chemiluminescent immunometric assay (Immulite 2000 Siemens Healthcare Diagnostics Inc., New York, USA). HbA1c was measured by high-performance liquid chromatography (HPLC).

Serum triglycerides, total- and HDL-cholesterol were determined by an enzymatic in vitro test using Roche automated clinical chemistry analyzers (Roche Diagnostics, Mannheim,

Germany). LDL cholesterol was calculated using the Friedewald formula [23]. AI was calculated by the ratio of total-/HDL-cholesterol [24].

**Statistical analysis**

Statistical analyses were carried out using R version 3.0.1. Data are expressed as mean ± standard deviation (SD) or occurrences and percentages. At baseline, differences between patients and controls were assessed using t-test for unpaired samples, Mann-Whiney U and chi square test.

Linear mixed models (LMM) were applied to assess changes over time in patients and healthy subjects with respect to the following variables: height, IGF-1, BMI, waist circumference, hip circumference, WHR, WHtR, total-, HDL- and LDL-cholesterol, triglycerides, AI, glucose, insulin, HOMA-IR, and HbA1c. Time was used as ordered factor in order to account for not linear relationship. Subject-specific random effects for the intercept were included. All the LMM's were adjusted for the following variables: age at baseline, gender and pubertal status (considered as time varying variable). For sake of readability, the mean (±SD) values reported in the results are based on the raw values but the p-values for between and within group comparisons were obtained from the LMM's and are thus adjusted for all the covariates in the models. Longitudinal association between outcome variables of glucose metabolism and anthropometric factors (weight, BMI, waist circumference, hip circumference, WHtR and WHR) and lipids (triglycerides, total-, LDL and HDL- cholesterol, AI) was assessed in GHD children. All these predictors were treated as time-varying covariates to account for changes in anthropometric and lipidic profile over time and entered individually in the model to avoid collinearity. The interaction between each predictor and time was also tested to assess whether the association pattern changed over time. In all these models, age at baseline, gender, severity of GHD, GH dose and pubertal status (considered as time varying variable) were entered as covariates. Significance was set at 0.05.

**Results**

*Clinical and anthropometric features*

Clinical and anthropometric details of subjects at study entry are shown in **Table 1**.

**Table 1. Details of GHD patients and controls at study entry**

| | GHD patients n=100 | Controls n=100 | P |
|---|---|---|---|
| Chronological age (years) | 9.42±3.65 | 8.96±2.82 | NS |
| Sex (M/F) | 60/40 | 60/40 | NS |
| Pre-pubertal/pubertal | 82/18 | 80/20 | NS |
| Height (SDS) | -2.42±0.80 | -1.13±1.30 | <0.001 |
| BMI ($kg/m^2$) | 17.53±2.99 | 17.07±2.57 | NS |
| BMI (SDS) | -0.41±1.10 | -0.35±1.20 | NS |
| IGF-1 (SDS) | -1.93±0.84 | -0.09±1.04 | <0.001 |
| Waist circumference (cm) | 64.07±9.79 | 60.84±9.27 | NS |
| Hip circumference (cm) | 67.69±10.86 | 66.13±9.28 | NS |
| WHtR | 0.52±0.06 | 0.48±0.05 | <0.001 |

Data are expressed as mean ± SD. NS, Not Significant; BMI, Body Mass Index; WHtR, Waist to Height Ratio.

As expected, at study entry GHD patients showed height and IGF-1 levels lower than controls ($p<0.001$). No difference was observed in BMI, waist circumference and hip circumference while WHtR resulted higher in GHD patients in comparison to controls ($p<0.001$) (**Table 1**). Height SDS increased significantly in GHD patients during treatment in comparison to baseline after 1 and 5 years of therapy ($p<0.001$) while remained stable in controls. Similarly, IGF-1 levels normalized after the first year of GH treatment ($p<0.001$) and remained stable during the following years (**Table 2**). Changes in BMI were comparable between the two groups over the study (**Table 2**) and waist circumference and hip circumference also showed a comparable longitudinal pattern between patients and controls during the entire follow up (data not shown). However, during GH treatment a significant reduction was observed in WHtR after 1 and 5 years ($p <0.001$) (**Table 2**).

**Table 2. Anthropometric parameters, lipid profile and glucose homeostasis in GHD patients and controls at baseline and at the 1st and 5th year of the study**

| | Baseline | 1 year | 5 years |
|---|---|---|---|
| Height (SDS) | | | |
| Patients | -2.42±0.80[b] | -1.91±0.80[a,b] | -1.23±0.94[a] |
| Controls | -1.13±1.30 | -1.31±1.50 | -0.97±1.60 |
| IGF-1(SDS) | | | |
| Patients | -1.93±0.84[b] | 0.28±1.20[a] | 0.38±1.19[a] |
| Controls | -0.09±1.04 | -0.15±0.78 | 0.14±0.88 |
| BMI (SDS) | | | |
| Patients | -0.41±1.10 | -0.50±1.0 | -0.40±1.0 |
| Controls | -0.35±1.20 | -0.40±1.20 | -0.36±1.20 |
| WHtR | | | |
| Patients | 0.52±0.06[b] | 0.49±0.04[a] | 0.46±0.05[a] |
| Controls | 0.48±0.05 | 0.48±0.06 | 0.46±0.06 |
| Total cholesterol (mg/dl) | | | |
| Patients | 165.23±26.20[b] | 155.63±26.22[a] | 154.04±23.29[a] |
| Controls | 150.75±20.60 | 151.78±23.27 | 149.09±22.64 |
| LDL-cholesterol (mg/dl) | | | |
| Patients | 98.02±25.63[b] | 85.02±24.45[a] | 85.81±18.57[a] |
| Controls | 88.53±27.80 | 90.05±15.43 | 81.49±16.00 |
| HDL-cholesterol (mg/dl) | | | |
| Patients | 56.38±13.24 | 59.39±14.24 | 57.54±13.50 |
| Controls | 55.69±9.68 | 54.54±13.88 | 57.63±12.34 |
| Triglycerides (mg/dl) | | | |
| Patients | 64.66±26.46[b] | 62.95±26.93 | 57.59±18.13 [a] |
| Controls | 55.96±20.35 | 55.82±24.32 | 53.48±15.67 |
| AI | | | |
| Patients | 3.05±0.69[b] | 2.72±0.62[a] | 2.67±0.55[a] |
| Controls | 2.79±0.59 | 2.79±0.69 | 2.68±0.66 |
| Glucose (mg/dl) | | | |
| Patients | 79.58±9.96 | 78.70±9.30 | 77.87±8.48 |
| Controls | 77.18±8.20 | 75.98±8.97 | 78.90±8.18 |
| HbA1C (%) | | | |
| Patients | 5.20±0.31 | 5.29±0.46 | 5.30±0.45 |
| Controls | 5.25±0.33 | 5.29±0.26 | 5.31±0.29 |
| Insulin (μU/ml) | | | |
| Patients | 4.50±3.24 | 7.21±4.84[a,b] | 7.50±4.00[a] |
| Controls | 4.30±2.60 | 4.40±2.60 | 6.50±3.50[a] |
| HOMA-IR | | | |
| Patients | 0.93±0.72 | 1.32±0.98[a,b] | 1.34±0.79[a] |
| Controls | 0.86±0.61 | 0.82±0.60 | 1.29±0.54[a] |

Data are expressed as means ± SD. [a] $p < 0.05$ vs baseline; [b] $p < 0.05$ vs controls. BMI, Body Mass Index; WHtR, Waist to Height Ratio, AI, Atherogenic Index; HOMA-IR, HOmeostasis Model Assessment; Mean ± SD reported in the table are based on raw values but p-values for between and within group comparison were obtained through LMM's.

After the first year of the study 36% of patients and 44% of controls had entered puberty, at the end of the study 55% of GHD patients and 66% of controls were still pubertal while 23% of patients and 22% of controls were post pubertal.

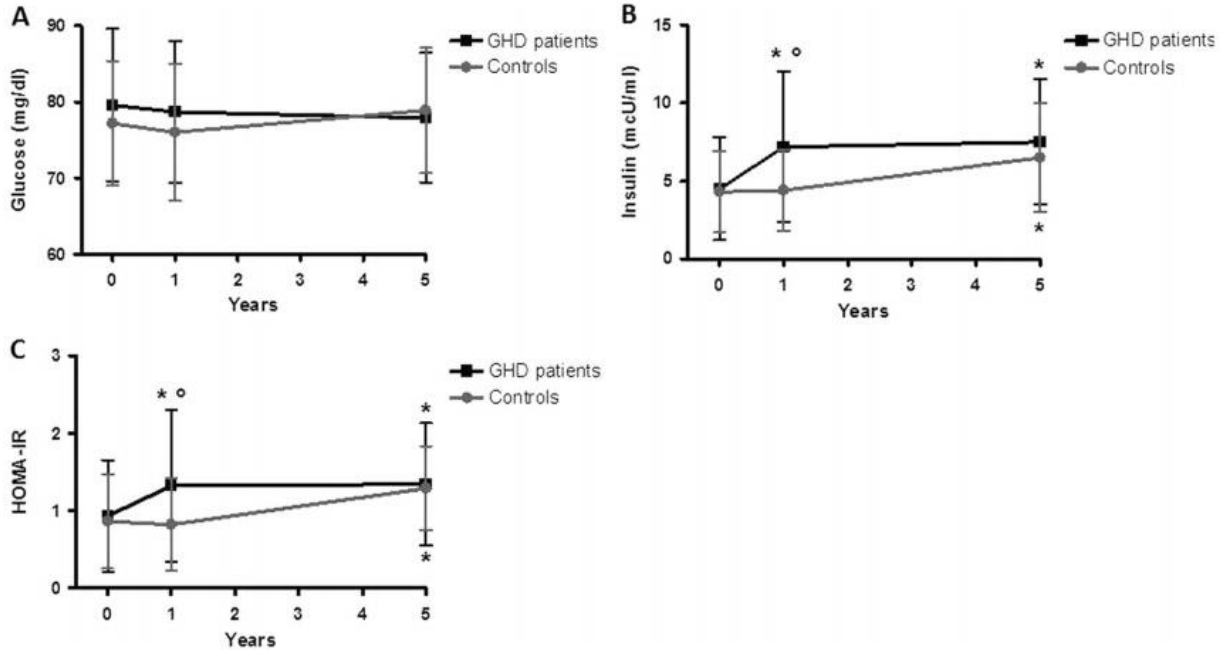
*Lipid profile*

GHD in comparison to healthy controls showed higherlevels of total-cholesterol ($p<0.001$) and LDL-cholesterol($p=0.022$), triglycerides ($p=0.028$), and AI ($p=0.047$);after 5 years of GH treatment significant changes wereobserved in total ($p=0.009$), LDL-cholesterol ($p = 0.026$),triglycerides ($p=0.002$) and AI ($p<0.001$). HDLcholesterolwas not significantly different between patientsand controls at baseline nor throughout the study (**Table 2**).

*Glucose metabolism*

No significant difference was detected between GHD patients and controls in glucose, insulin, HbA1c, and HOMA-IR at baseline (**Table 2, Fig. 1**).

**Figure 1: Longitudinal changes in glucose (a), insulin (b), HOMA-IR (c) in GHD patients in comparison to healthy controls**.




*p < 0.05 vs.baseline, °p < 0.05 vs. controls.

Mean ± SD shown in the figure arebased on raw values, but p-values for between and within group comparison were obtained through LMMs.

During the first year of treatment, insulin (4.5 ± 3.3 vs.7.2 ± 4.8 μU/ml; $p < 0.001$) and HOMA-IR (0.93 ± 0.72 vs.1.32 ± 0.98, $p < 0.001$) values significantly raised in GHD

children, while all these parameters remained stable incontrols (**Table 2, Fig. 1**); therefore, at the end of the first year of the study, there was a mean difference between patients and controls of 2.71 in insulin (95% CI 1.27–4.16,p < 0.001) and 0.47 in HOMA (95% CI 0.15–0.78, p =0.004). Fasting glucose and HbA1c levels did not change overthe first year of the study in GHD children nor controls (**Table 2, Fig. 1**).

Insulin and HOMA-IR levels did not show any further change in GHD patients over the following years of therapy (**Table 2, Table 3 and Fig. 1**). In contrast, healthy controls at the 5th year of follow-up showed a significant increase in insulin (p = 0.004) and HOMA-IR (p < 0.001) levels, all of which became comparable to those of GHD patients at the end of the study (Table 2, Fig. 1).With regard to fasting glucose and HbA1c no significant changes were observed in patients nor controls until the end of the study (**Table 2, Table 3**).

**Table 3. Glucose homeostasis in GHD patients at baseline and during 5 years of GH treatment**

| | **Baseline** | **1 year** | **2 year** | **3 year** | **4 year** | **5 years** |
|---|---|---|---|---|---|---|
| Glucose (mg/dl) | 79.58±9.96 | 78.70±9.30 | 79.90±8.10 | 78.95±9.90 | 77.87±8.48 | 77.87±8.48 |
| HbA1C (%) | 5.20±0.31 | 5.29±0.46 | 5.26±0.44 | 5.36±0.48 | 5.31±0.47 | 5.30±0.45 |
| Insulin (μU/ml) | 4.50±3.24 | 7.21±4.84[a] | 7.20±4.40[a] | 7.64±5.20[a] | 7.50±4.00[a] | 7.50±4.00[a] |
| HOMA-IR | 0.93±0.72 | 1.32±0.98[a] | 1.35±0.93[a] | 1.43±1.17[a] | 1.34±0.79[a] | 1.34±0.79[a] |

Data are expressed as means ± SD. [a] p < 0.05 vs baseline. HOMA-IR, HOmeostasis Model Assessment

Changes in insulin during treatment were positivelyassociated with changes in weight, BMI, waist circumference,hip circumference, IGF-1, and triglycerides(**Table 4**).

**Table 4. Longitudinal association between glucose metabolism, lipids and anthropometric factors**

| | Insulin | HOMA-IR |
|---|---|---|
| Weight | 0.101 [0.032 to 0.171]** | 0.013 [-0.002 to 0.029] |
| BMI | 0.401 [0.246 to 0.557]** | 0.065 [0.03 to 0.101]** |
| Waist Circumference | 0.106 [0.017 to 0.194]* | 0.017 [-0.003 to 0.037] |
| Hip Circumference | 0.11 [0.019 to 0.2]* | 0.014 [-0.006 to 0.034] |
| IGF1 SDS | 0.75 [0.44 to 1.06]** | 0.114 [0.051 to 0.177]** |
| Triglycerides | 0.022 [0.004 to 0.041]* | 0.003 [-0.001 to 0.007] |

* p <0.05; ** p < 0.01

The reported coefficients, with the corresponding 95% CIC, were estimated by using linear mixed models. Each coefficient measures the average change in the dependent variables (i.e. Insulin, HOMA_IR) for a unit change in each of the predictors, during the five years' follow-up period.

In particular, every unit increase in BMI andweight was associated with 0.40 (95% CI: 0.25–0.56, p <0.001) and 0.1 (95% CI: 0.03–0.17, p = 0.004) increase ininsulin levels, respectively, after controlling for measuredcovariates at any year of follow-up. Changes in HOMA-IRwere associated with changes in BMI and IGF-1.

GH dose was not correlated to variations in glucosemetabolism during treatment.

All these associations were stable over time as interactionterms between time and predictors were never significant.

## Discussion

Results of our longitudinal, case-control study indicate that nor GH deficiency or GH replacement in children are associated to significant impairment of glucose homeostasis. GH therapy was associated to a mild deterioration in insulin sensitivity during the first year of treatment without alterations in glucose levels and not followed by further derangement of glucose metabolism over the entire period of the study. Furthermore, after 5 years of therapy the mild insulin resistance observed in GHD subjects was comparable to that physiologically occurring during puberty in healthy controls [25].

The relationship between GH-IGF-1 axis and insulin sensitivity is puzzeling. Both GH deficiency and GH replacement have been associated with insulin resistance with the increased flux of free fatty acids (FFA) probably being the underlying mechanism in both conditions [5]. Moreover, during treatment the anti-insulin effect of GH seems to be

prevalent during the initial phase of therapy whereas in the long-term it can be counterbalanced by the positive effects of GH on metabolism and body composition [9].

The impact of GH therapy on glucose homeostasis in childhood still needs to be clarified. Analysis of data from KIGS database documented an increased incidence of type 2 diabetes in children treated with growth hormone in comparison to general population [16]; these data have been confirmed by a multinational observational study including 11.686 patients with growth disorders treated with GH which reported an increased incidence of type 2 diabetes in subjects with pre-existing risk factors [17]. On the contrary, a more recent study on a large French database failed to document increased incidence of diabetes in adults who had received GH during childhood [18]. However, it is worth to point out that these epidemiological studies are limited by the presence of potential confounding factors, the lack of a control group and the heterogeneity in study population which included conditions which are per se at increased risk for type 2 diabetes or usually receive GH at supraphysiological doses.

A few prospective studies exploring glucose homeostasis in selected populations of GHD children documented a deterioration of insulin sensitivity compensated by hyperinsulinaemia during treatment [2,3,10,12]. However most of these studies were limited by the short duration of treatment and follow-up or the lack of a comparison with an appropriate group of untreated subjects. Radetti [15] et al. evaluated glucose tolerance during standard oral glucose tolerance test (OGTT) in 128 GHD children over a long period of treatment; according to our results, authors found a slight impairment in IS during the first 12 months of treatment with no further worsening during the following 6 years of therapy. A limitation of this study was the lack of a control group. Ciresi et al [10] evaluated glucose metabolism in 34 GHD children during short-term GH replacement in comparison to a group of healthy controls and confirmed a slight decrease in insulin sensitivity in the short term without glucose intolerance; noteworthy, controls were only evaluated at baseline and all subjects included in the study were prepubertal.

Strenghts of our study are represented by the homogeneous study population, the large sample size and the presence of an appropriate control group. In particular, to the best of our knowledge, our study is the first comparing IS between GHD subjects and healthy children going through puberty. Several studies investigated the effects of puberty on glucose metabolism demonstrating that puberty is associated with a significant reduction in insulin sensitivity which is compensated by an increase in insulin secretion maintaining glucose homeostasis [25,26]. Longitudinal follow-up of GHD subjects in parallel to healthy controls

allowed us to document a mild but significant increase in insulin levels and a worsening of IS indexes in GHD subjects during the first year of treatment whereas further changes in IS were comparable to those observed in healthy adolescents going through puberty.

We can speculate that in our GHD cohort a complex interplay between beneficial metabolic effects of GH and its anti-insulin action regulates glucose homeostasis during long-term treatment. Indeed, in the current study we confirmed our previous data of a significant positive effect of GH on body composition and lipid profile [4] as GH therapy was associated with improvement in visceral adiposity, evaluated through WHtR, and levels of total- and LDL-cholesterol, AI and triglycerides. Although none of our GHD patients was obese or dyslipidemic, longitudinal association analysis revealed that measures of adiposity and trigliycerides levels were all significant predictors of changes in insulin sensitivity confirming the relevance of GH effects on these parameters in maintaining glucose homeostasis as well as the importance of controlling independent risk factors of insulin resistance as adiposity or lipids levels in children and adolescents receiving GH.

No associations were found between glucose homeostasis and GH posology, probably because we never used supraphysiological doses of the drug.

We acknowledge that a limitation of our study is represented by the evaluation of glucose homeostasis only in its basal state; however, fasting insulin levels and indexes derived from fasting sample, as HOMA-IR, have been proposed as surrogate markers of IS since the hyperinsulinemic euglycaemic clamp, which is the gold standard method, is invasive and labor intensive [27-29]. Even if we did not evaluate indexes of beta-cell function, the normal glucose levels during treatment suggested that insulin secretion was not impaired and compensated for the reduction in insulin sensitivity. Therefore, we suggest that in the absence of overt glucose homeostasis alterations insulin levels, HOMA-IR are simple indexes to monitor children and adolescents receiving GH and identify those subjects who deserve further evaluations of glucose metabolism.

In conclusion, the results of our study suggest that the chance of detecting abnormal glucose metabolism is not increased in children and adolescents receiving GH in comparison to healthy controls. In our large sample of GHD subjects, insulin sensitivity slightly reduced during the first year of therapy but remained stable in the long-term; after 5 years of GH, GHD adolescents had a mild insulin resistance comparable to that physiologically occurring in puberty.

Further longitudinal studies with longer follow-up are needed to better evaluate the impact of GH treatment on glucose homeostasis in subjects with childhood-onset GHD.

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### 4.2 Lipid profile, vascular morphology and function

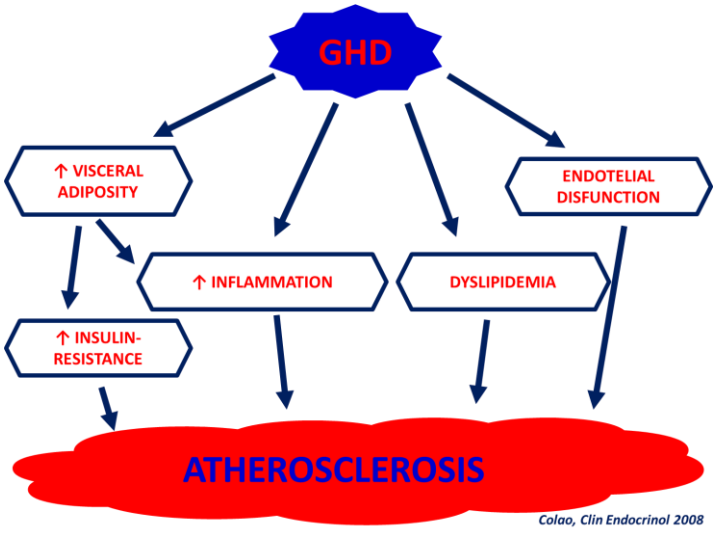
**This work has been presented as Poster presentation at ECE (European Congress of Endocrinology) meeting 2019**: F. Anselmi, **N Improda**, F.Barbieri, L.Bufalo, P.Lorello, D.Capalbo, M. Salerno. Effects of growth hormone deficiency (GHD) and GH treatment on early markers of atherosclerosis in children.

**A final manuscript is about to be submitted to an international peer-reviewed journal**

**Background**

Untreated GHD in adults can be associated with a cluster of cardiovascular (CV) risk factors, such as visceral obesity, dyslipidemia, altered glucose metabolism, reduced left ventricular mass and function (1-7), which can be reverted by GH replacement therapy (2-7).

**Figure 1: Pro-atherogenic alterations associated with GHD in adults**




Increasing evidences suggest that children and adolescents with GHD may already exhibit an unfavorable metabolic profile, with subtle alterations in body composition, lipid profile, and cardiac morphology and function (8-13), which may ameliorate upon initiating GH treatment (8-13).

In recent years, great attention has been paid at novel (emerging) CV risk factors, including ultrasound evidence of altered vascular morphology and/or function. Endothelial dysfunction and increased intima-media thickness (IMT) represent the earliest steps of clinically

detectable atherosclerosis and are independent predictors of the occurrence of major cardiovascular events later in life (14).

In contrast to adults affected with GHD, who have been consistently found to have altered flow-mediated dilatation (FMD) (13), and increased IMT (15-18), data in children and adolescents evaluating the effects of GHD and GHRT on vascular morphology and function are still scanty and inconclusive.

## Patients and methods

### *Patients and controls*

Twenty-four children affected with GHD (10.8±2.7 years) and 24 age-, sex- and BMI-matched controls were enrolled into the study. Diagnosis of GHD was made according to auxological criteria associated with insufficient GH response (peak GH <8 mg/L) after two stimulation tests. GH treatment was commenced at a mean dose of 30 µg/kg/day.

Controls were selected among children referred to our Unit for short stature or thyroid assessment, who were eventually found to have familial short stature, constitutional delay of growth and puberty or to be healthy euthyroid children. Exclusion criteria for entering the study in both GHD patients and controls were previous or current cardiovascular, respiratory, endocrine, or other systemic diseases.

### *Study design*

This was a 1-year non-pharmacological interventional prospective study. At study entry, we evaluated in all subjects: anthropometric measures, vascular morphology and function (brachial FMD and IMT of common (cIMT) and internal (iIMT) carotid artery), assessment of fasting lipid profile (triglycerides, total-, LDL, HDL cholesterol, non-HDL cholesterol, atherogenic index, non-HDL cholesterol) and serum IGF-1 concentrations. All the parameters were re-assessed after 1 year of GH treatment in GHD.

### *Serum assay*

Serum GH and IGF-I concentrations were determined by a solid-phase, enzyme-labeled chemiluminescent immunometric assay (Immulite 2000 Siemens Healthcare Diagnostics), and expressed as SDS according to the normative data provided by the manufacturer.

Serum triglycerides, total and HDL cholesterol were determined by an enzymatic in vitro test using Roche automated clinical chemistry analyzers (Roche Diagnostics, Mannheim,

Germany). LDL cholesterol was calculated using the Friedewald formula. Triglycerides, total, LDL and HDL cholesterol levels were normalized for age and sex and expressed as SDS.Non-HDL-C was calculated as total cholesterol minus HDL. The atherogenic index (AI) was defined as the ratio between total and HDL cholesterol.

*Flow-mediated dilation*

FMD was obtained by a single investigator for each center. All measurements were done after 8-h fasting with subjects in supine position in a quiet, temperaturecontrolled room. Brachial artery reactivity was evaluated in each subject using validated protocol (Corretti Journal of the American College of Cardiology 2002), with a 7.5MHz multifrequency linear array probe (Aplio XG Imaging System, Toshiba). Electrocardiographic leads were connected and a sphygmomanometer cuff was placed on the right arm. The brachial artery was imaged 2–5 cm proximal to the antecubital crease in a longitudinal axis, and the brachial artery diameter, from the intima–lumen interface on the near wall to the media–adventitia interface on the far wall, was measured at end-diastole cycle, on the electrocardiographic R-wave. Endothelium-dependent vasodilatation was assessed by measuring the maximum increase in brachial artery diameter during reactive hyperemia created by the inflation of the cuff (250mmHg for 5min) on the right arm. After cuff deflation, flow velocity indexes were measured in the first 15s; then brachial artery diameter was measured at least four times for the next 90s. FMD resulted from the formula: ((post-hyperemia diameter – baseline diameter)/ baseline diameter)×100.

*Intima–Media thickness*

Carotid ultrasound examination was performed in each subject, by experienced vascular sonographers, with a 7.5MHz multifrequency linear array probe. Ultrasound examination was made with the subject in a supine position, with a slight rotation of the neck. The probe was placed along the vessel axis, and carotid arteries were explored with longitudinal (anterior, lateral, and posterior) and transverse scans. Multiple long and short axis sections were employed, with special attention paid to defining the endothelial border from the origin of common artery beyond the carotid artery bifurcation. On each side, IMT was measured at the bifurcation and at its proximal and distal segments, 1cm before the bifurcation, on the echographic posterior wall of the common carotid artery, and 1cm after the bifurcation, on the echographic posterior wall of the internal carotid artery always in the longitudinal scan.

*Statistical analysis*

Statistical analysis was performed using SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY). Data are presented as mean±standard deviation (SD). Differences between patients and controls at baseline and at the end of the study were assessed using t-test for unpaired samples; differences in GHD patients before and after 1 year of GH therapy were evaluated through T test for paired samples. Statistical significance was set at 5%.

## Results

*General characteristics*

At the beginning of the study, as expected, height expressed in SDS was reduced in children with GHD ($p<0.0001$) and increased significantly after 1 year of GH replacement therapy ($p=0.0009$) (**Table 1**).

BMI expressed in SDS was comparable among GHD children and controls (-0.88±1.07 vs -0.31±1.00) (**Table 1**), and did not change after 1 year of GH therapy (-1.00±1.14).

**Table 1. Clinical characteristics of patients and controls at study entry.**

| | GHD | Controls | P |
|---|---|---|---|
| Patients | 24 | 24 | NS |
| Gender (M/F) | 15/9 | 15/9 | NS |
| Age (years) | 10.85±2.71 | 10.73±3.11 | NS |
| Height (SDS) | -2.36±0.46 | -0.34±1.62 | <0.0001 |
| BMI SDS | -0.88±1.07 | -0.31±1.02 | NS |
| IGF-1 (SDS) | -1.29±1.31 | -0.20±1.29 | 0.0005 |

Data are expressed as mean ±SD. NS = Not Significant; ND = Not available; M = male; F = female

*Lipid profile*

Compared with controls, GHD children at study entry had higher total cholesterol (162.83±18.33vs 149.83±20.63 mg/dl, $p=0.04$), LDL cholesterol (91.48 ±21.73 vs 77.08±19.73 mg/dl, $p=0.02$), non HDL cholesterol (102.4±20.23 vs 89.33±18.03 mg/dl, $p=0.04$) and AI (2.84±0.5 vs 2.56±0.4, $p=0.03$) (**Table 2**). No differences were found in triglycerides and HDL cholesterol between patients and controls (**Table 2**).

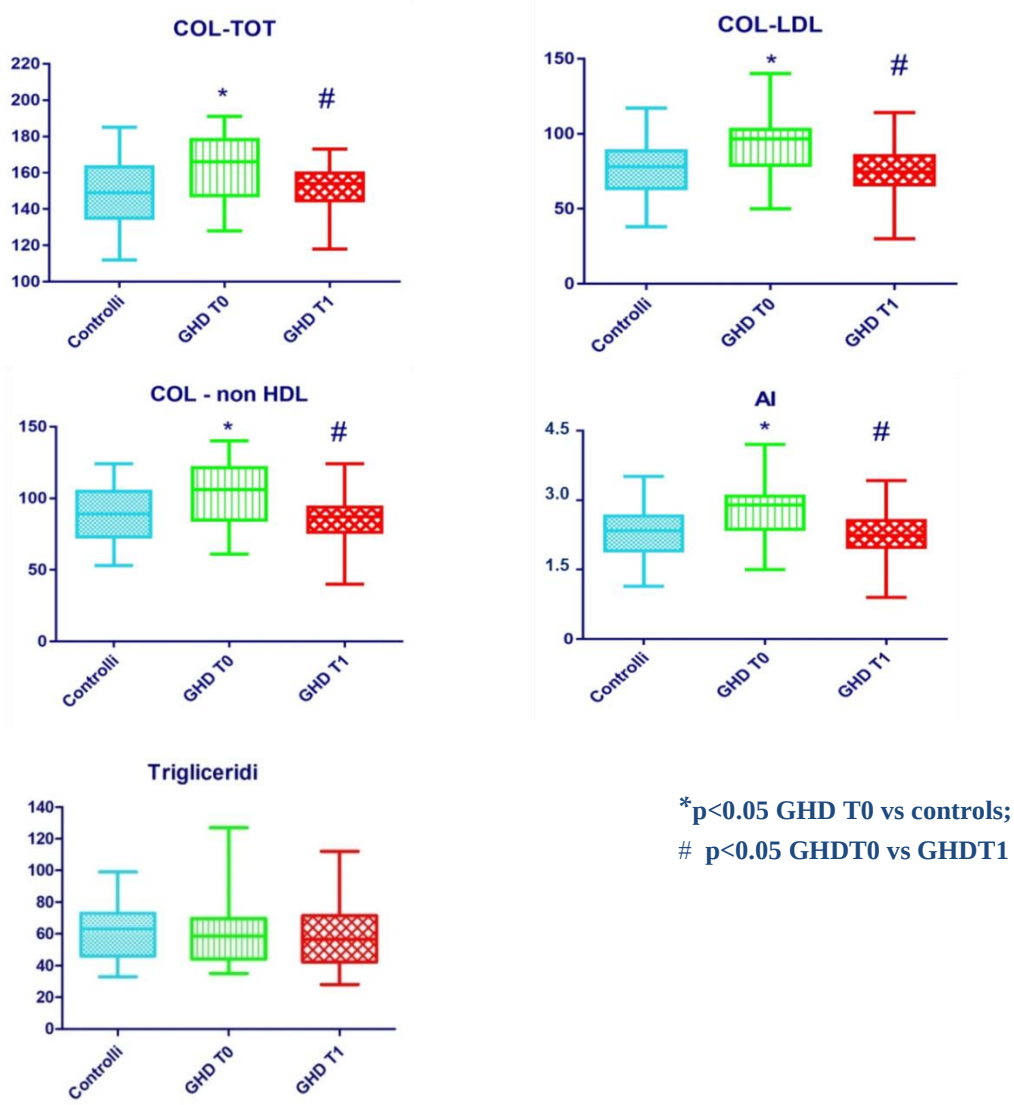
**Table2. Lipid profile before and after 1 year of GH in GHD patients, compared with controls.**

| | T0 | T1 | $p^a$ | $p^b$ |
|---|---|---|---|---|
| Triglycerides (mg/dl) | | | | |
| GHD | 61.08± 22.00 | 59.88± 26.50 | NS | NS |
| Controls | 60.14± 17.24 | NA | | |
| Total cholesterol (mg/dl) | | | | |
| GHD | 162.83±18.33 | 151.42±14.90 | 0.04 | 0.03 |
| Controls | 149.83±20.63 | NA | | |
| LDL-C (mg/dl) | | | | |
| GHD | 91.48±21.73 | 74.44±15.56 | 0.02 | 0.005 |
| Controls | 77.08±19.73 | NA | | |
| HDL-C (mg/dl) | | | | |
| GHD | 61.08±16.82 | 69.5±12.80 | NS | NS |
| Controls | 59.13±7.10 | NA | | |
| not HDL-C (mg/dl) | | | | |
| GHD | 102.04±23.00 | 86.42±17.81 | 0.04 | 0.01 |
| Controls | 89.33±18.03 | NA | | |
| AI | | | | |
| GHD | 2.84±0.50 | 2.29±0.35 | 0.03 | 0.0001 |
| Controls | 2.56±0.40 | NA | | |

$p^a$ GHD T0 vs controls; $p^b$ GHD T0 vs T1; Data expressed as mean ±SD. NS = Not Significant ; NA = Not available

GH therapy was associated with a significant reduction in total cholesterol (151.42±14.90 mg/dl, p=0.03), LDL cholesterol (74.44±15.56 mg/dl, p=0.005), non HDL cholesterol (86.42±17.81 mg/dl, p=0.01) and AI (2.29±0.35, p=0.0001), while triglycerides and HDL cholesterol did not change (**Figure 3**).

**Figure 3. Lipid profile in controls and GHD at study entry (GHD T0) and after GH (GHD T1)**



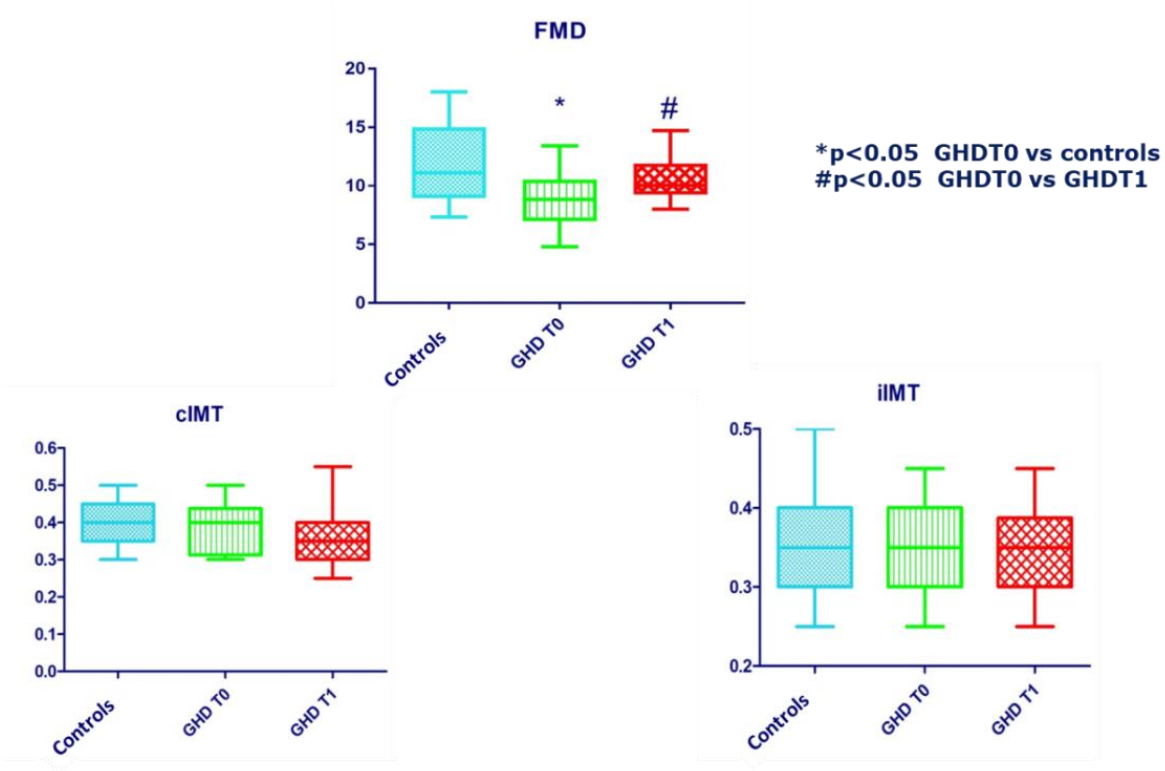

*Flow-Mediated Dilation and Intima–Media Thickness*

At study entry children with GHD showed lower values of FMD (8.75±2.44 vs 11.85±5.98%; p=0.02) compared to controls while no difference was found in cIMT (0.37±0.08 vs 0.40±0.06 mm) and iIMT (0.33±0.06 vs 0.36±0.07 mm) between the groups. GH treatment was associated to a significant improvement in FMD (10.60±1.69%, p= 0.04)**(Figure 3)**.

As shown in **Figure 2**, cIMT and iIMT slightly reduced in patients after 12 months of GH treatment although these differences did not reach statistical significance.

No correlations were found between changes in FMD and total cholesterol, LDL cholesterol, non HDL cholesterol and AI.

**Figure 4. Endothelial function in controls and GHD at study entry and after 1-yr GH**




## Discussion

The results of our study expand previous knowledge regarding metabolic and cardiovascular profile of GHD. Indeed, we documented that untreated GHD in children may be associated to a cluster of CV factors, characterized by unfavorable lipid profile and endothelial dysfunction, which improve upon starting GHRT.

Abnormalities in lipid profile have been reported in some (19, 20, 21), but not all (22, 23) previous studies in both adults and adolescents with untreated GHD. In particular, untreated GHD seem to display higher total and LDL cholesterol, triglycerides and atherogenic index, along with lower HDL cholesterol, compared with healthy controls. Such abnormalities improved with GHRT (8, 10, 13, 19, 24).

In agreement with these previous studies, we found higher total and LDL cholesterol, and atherogenic index, and lower HDL cholesterol than healthy controls, which improve after 1-year GHRT. Possible mechanisms underlying the positive effects of GH on lipid profile could be sought in its marked lipolytic effect, through the activation of hormone-sensitive lipase (1). GH also seems to stimulate the expression of hepatic receptors for LDL, thus favoring their

clearance, and to decrease LDL formation by accelerating the clearance of their precursor VLDL (1, 25).

We also evaluated the presence of novel (emerging) CV risk factors, by performing ultrasound assessment of vascular morphology and function. It is well known that endothelial dysfunction represents the first step which leads to permanent atherosclerotic changes, such as thickening of the arterial wall. An increased IMT is an independent predictor of the occurrence of major cardiovascular events later in life. An alteration of these surrogate markers of atherosclerosis has been demonstrated in children with several chronic conditions, characterized by metabolic and endocrine derangement.

In adults, GHD has been consistently associated with lower FMD values (13, 15-17), which increase after starting GHRT (13, 15-18). Moreover, GHD adults may exhibit increased carotid IMT (13, 19, 26), even though it still remains to be defined whether GH exerts beneficial effects on this surrogate marker of atherosclerosis (7, 19, 26). Data in children regarding vascular morphology and endothelial function are scanty an controversial.

Most studies (13, 27), but not all (28), reported IMT values comparable to controls in children and adolescents with GHD. Moreover, in this patients no clear association has been found between GH treatment and arterial vascular changes (27).

In keeping with this, we failed to find any significant differences in IMT between GHD children and healthy matched controls either at baseline or after 1-year GHRT.

However, consistent with one previous study involving adolescents with GHD (13), our patients showed reduced values of FMD in comparison to healthy controls, which normalized after 1 year of GHRT.

Detrimental effects of GHD on endothelial function might be due to the reduced availability of endothelial nitric oxide (NO), a vasodilatory compound. In fact, IGF-I has a direct NO-releasing effect on NO in cultured human endothelial cells and low basal IGF-I levels in serum are associated with low basal urinary nitrate and cAMP excretion (29). Moreover, there is evidence that GH increases volemia and diminishes peripheral resistance and arterialstiffness in treated patients (29). Finally, reduced FMD in untreated GHD patients may result from increased concentrations of asymmetric dimethylarginine (ADMA), an endogenous plasmatic inhibitor of endothelial NO synthase, which has been found to be increased in prepubertal patients with GHD in some reports (30), but not in others (31). Elevated ADMA levels are supposed to be associated with increased inhibition of the endothelial NO synthase and vasoconstriction, which represents the first phenomenon leading

to endothelial dysfunction. Interestingly, r-hGH therapy has been found to decrease ADMA levels,reaching values comparable to those found in control children (30).

Despite normal IMT values, we could speculate that decreased FMD may represent a precocious step, leading to morphological alterations of the blood vessels in the adult age. Further studies on larger cohorts are required to clarify this intricate topic and to establish usefulness of regular monitoring for CV abnormalities during GH treatment in childhood/adolescence.

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## CHAPTER 5. Health-related fitness in GHD children and adolescents, treated with rhGH.

### 5.1 Cardiopulmonary performance and body composition

**This work has been published as:**

Capalbo D, Barbieri F, **Improda N**, Giallauria F, Di Pietro E, Rapacciuolo A, Di Mase R, Vigorito C, Salerno M. Growth hormone improves cardiopulmonary capacity and body composition in children with growth hormone deficiency.

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**Background**

It has been well established that growth hormone (GH) regulates intermediate metabolism, body composition and cardiovascular (CV) health, thus influencing physical performance,general well-being and quality of life (1).

In adult onset GH deficiency (GHD) fat mass (FM) is increased and lean body mass(LBM) is reduced causing diminished muscle strength and physical fitness (2). There isusually an increase in muscle mass in response to GH but whether this change results inincreased strength is still debated. However, overall data suggest that GH treatmentsignificantly improves aerobic exercise capacity and physical performance (3, 4).

This is relevant even in childhood since, in addition to promoting linear growth, GH alsoexerts beneficial effects on early risk factors involved in the development of CV morbidityand mortality (5). Several studies have documented that untreated GHD during childhoodmay be associated to subtle alterations in body composition (6, 7), lipid profile (8-10), andcardiac performance (11, 12). In particular, untreated GHD children may display increasedvisceral adiposity expressed as both waist-hip (WHR) and Waist-to-Height (WHtR) ratio (7,8), increased FM and decreased LBM at dual-energy x-ray absorptiometry (DXA) (6), whichare improved by GH replacement therapy. Moreover, untreated GHD children exhibitreduced cardiac size and subclinical alterations in left ventricular (LV) systolic contractility,documented by higher wall stress, impaired mean velocity of circumferential fiber shorteningand stress-shortening index, with a normal global systolic function expressed by fractionalshortening (FS) and LV ejection fraction (LVEF) (13). All these abnormalities are reversibleafter GH replacement therapy (13). However, the relationship between such

metabolic andcardiac abnormalities and cardiopulmonary functional capacity in children with GHD isunknown.

Right Ventricle (RV) function is of essential clinical and prognostic importance in avariety of lung diseases, pulmonary hypertension (14), acquired and congenital heart disease(15). There are no data available about RV function and remodeling in GHD children withand without GH replacement therapy.

We designed this observational, case-control, prospective study to evaluate the effects ofGH deficiency and one-year GH replacement therapy on cardiopulmonary function, cardiacperformance and body composition in children.

## Materials and Methods

*Patients and controls*

Twenty-one children with untreated isolated GHD (17 boys and 4 girls) aged 11.3±0.8 yr (range 10.0 –12.5 yr) have been enrolled in the study. Diagnosis of GHD was made according to clinical and auxological criteria associated with insufficient GH response (peak GH <8 μg/liter) after two stimulation tests (mean GH peak 5.9±0.82 μg/liter after arginine and 5.4±1.37 μg/liter after glucagon) (16). Magnetic resonance imaging documented pituitary hypoplasia in four patients, empty sella in four and pars intermedia cyst in one.

None of the patients had been previously treated with GH. All the patients were commenced on GH at a mean dose of 30 μg/kg/day.

To assess the physical activity (PA) level, a modified version of the International Physical Activity Questionnaire (IPAQ) was administered to the all subjects' parents (17). The questionnaire comprises a set of four domains: (i) school-related PA, (ii) transportation, (iii) housework and (iv) leisure time. For each domain, the number of days per week and of PA periods per day (>10 min of walking, moderate or vigorous activity) were recorded. Based on these variables, three categories of physical activity level have been established namely high, moderate and low intensity (17). High level of PA was defined as vigorous activity in more than three days per week, accounting for at least 1500 MET-minutes/week; moderate level of PA was defined as 3 days or more of vigorous physical activity lasting at least 20 minutes or 5 or more days of moderate activity and/or walking at least 30 minutes per day; low level of PA was defined as no activity or some activity reported, but not enough to meet criteria for moderate level of PA.

Twenty-one sex-, pubertal status-, body mass index (BMI) and PA level-matched healthy subjects participated into the study as controls. These subjects were referred to our Unit for short stature and were diagnosed, following appropriate assessment, as having familial short stature or constitutional delay of growth and puberty.

All subjects were pre-pubertal at study entry. During the study a comparable proportion of GHD subjects (2 females and 2 males) and controls (3 females and 2 males) entered puberty.

Previous or current CV, respiratory, or endocrine diseases were exclusion criteria for entering the study in both GHD patients and controls.

The study was approved by our Institutional Ethical Committee. Informed parental consent for participation into the study was obtained for patients and controls.

*Study design*

This was a 1-year prospective case-control study. At study entry, we evaluated in all subjects: anthropometric measures, heart rate (HR), systolic (SBP) and diastolic (DBP) blood pressure,echocardiography, cardiopulmonary exercise testing, DXA scan,and serum IGF-1 concentrations. All the parameters were re-assessed after 1 year of GH treatment in GHD, whereas in controls was repeated after a 1-yr follow-up, with the exception ofDXA, because of ethical reasons.The study was approved by our Institutional Ethical Committee. Informed consent for participation into the study was obtained from the patients and/or their families.

*Anthropometric measurements*

Height was measured in the upright position using a Herpenden stadiometer and was expressed in standard deviation score (SDS) according to Italian reference standards (18). BMI was calculated by dividing weight in kilograms by the square of height in meters and then expressed as SDS according to Italian reference standards (18). Waist circumference was measured by the same operator in the standing position with a non-elastic tape placed at the midpoint between the lower rib margin and the iliac crest. Hip circumference was measured at the level of widest portion of trochanters. All measurements were expressed in centimeters (cm) to the nearest 0.1 cm. WHR and WHtR were used to evaluate visceral adiposity. Blood pressure was registered as the mean value of three measurements after 10 min resting.

*Serum assay*

Serum GH and IGF-I concentrations were determined by a solid-phase, enzyme-labeled chemiluminescent immunometric assay (Immulite 2000 Siemens Healthcare Diagnostics Inc., New York, USA). IGF-1 concentrations were expressed as SDS according to the normative data provided by the manufacturer.

*DXA*

In all subjects body composition was evaluated by DXA, using a Hologic QDR 1000 densitometer (Hologic, Waltham, MA, USA). Daily calibrations of the densitometer performed with a phantom during a 1-year period had provided a coefficient of variation of 0.56%. LBM and FM were measured in all patients and expressed both in absolute values and percentage of body composition.

*Echocardiography*

Transthoracic Echocardiography (TTE) was performed in conscious patients, in supine position to evaluate subcostal, parasternal and apical view. If required the apical view was obtained in left lateral decubitus position. It was recorded by Sequoia C256 system equipped with 7V3c (3-7 MHZ) and 3V2c (2-3 MHZ) probes.

In order to evaluate the left ventricle (LV) function and structure we measured LV End Diastolic (LVEDD) and End Systolic Diameter (LVESD) by M mode parasternal short axis view; LV mass by M mode parasternal short axis view (measuring septal, IVST, and posterior wall thickness, LVPWT); LV Fraction Shortening (FS) percentage calculated using the following formula=(LVEDD-LVESD)/LVEDD*100; LV Ejection Fraction (LVEF) obtained by the following formula=(LV end diastolic area-LV end systolic area)/LV end diastolic area*100; maximal early diastolic flow velocity (E wave), maximal late diastolic flow velocity (A wave); the ratio between E and A curves (E/A, normal value>1).

The LV mass (LVM) was calculated by using Devereux's formula according to Penn's convention with the regression-corrected cube formula LVM=1.04[(IVST + LVEDD + LVPWT)$^3$ - (LVEDD)3] - 13.8 g, and expressed by LVM index (LVMi) after correction for BSA.Similarly, in order to assess right ventricle function and structure we evaluated the tricuspid annular plane systolic excursion (TAPSE) detected by apical four chamber view placing the M-mode marker perpendicular to the tricuspid annular plane and measuring the distance from the base to apex of the M-mode curve obtained; right ventricular end diastolic diameter (RVEDD) by apical four chamber view; RV fractional area change (RVFAC) obtained by the following formula=(RV end diastolic area-RV end systolic area)/ RV end

diastolic area*100. Measurements were evaluated in all patients and controls at baseline and after 1 year of follow-up.

*Cardiopulmonary exercise testing*

Both patients and controls underwent an incremental cardiopulmonary exercise test on a bicycle ergometer (Ergoline Ergometrics 800; Bitz, Germany). Before each test, oxygen and carbon dioxide analyzers and a flow mass sensor were calibrated by the use of available precision gas mixtures and a 3-L syringe, respectively. All equipment were calibrated according to the instructions of the manufacturer before testing. To stabilize gas measurements, patients were asked to remain still on the ergometer for at least 3 min before starting exercise. After a 1-min warm-up period at 0 Workload, a ramp protocol of 15 W⁄min was started and continued until exhaustion. The pedalling was kept constant at 55–65 revolutions per minute. All patients were verbally encouraged to exercise to exhaustion, as assessed using a cutoff >1.1 for the respiratory exchange ratio at peak exercise. After maximal exercise has been reached, a cooling-down phase consisted of 5 min of pedaling at a slow rate (< 40 revolutions⁄ min) at a work rate of 0 W. A 12-lead electrocardiogram was monitored continuously during the test, and cuff blood pressure was manually recorded every 2 minutes. Respiratory gas exchange measurements were obtained breath by breath with the use of a computerized metabolic cart (Vmax 29C; Sensormedics, Yorba Linda, CA, USA). Peak oxygen consumption ($VO_2$peak) was recorded as the mean value of $VO_2$ during the last 20 s of the test and was expressed in milliliters per kilogram per minute. At the end of the cardiopulmonary exercise test, patients were asked to identify the primary reason for stopping. Medical treatment administered the day of exercise testing was recorded. $VO_2$peak was measured and compared with maximal predicted $VO_2$ by use of a sex-, age-,height- and weight-adjusted and protocol-specific formula outlined by Wassermann et al. (19). $O_2$ pulse (ml/beat) was automatically computed by dividing $VO_2$ by heart rate obtained every 10-s during cardiopulmonary exercise stress testing. The ventilatory anaerobic threshold was detected by two experienced reviewers (C.V. and F.G.) by use of the V-slope method (20).

*Statistical analysis*

Statistical analysis was performed using SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY). Data are presented as mean±standard deviation (SD). Differences between patients and controls at baseline and at the end of the study were assessed using t-test for unpaired samples; differences in GHD patients before

and after 1 year of GH therapy and differences in controls before and after one year of follow-up were evaluated through T test for paired samples. An additional evaluation between groups was performed for cardiopulmonary variables, using a linear regression model after adjustment for LBM. Covariates found significantly different between the two groups were then analysed by a stepwise multiple regression analysis in order to investigate the effects of several independent factors on cardiopulmonary functional capacity and cardiac performance endpoints in GHD patients. Statistical significance was set at 5%.

**Results**

*Anthropometric measurements and IGF-1*

As expected, at study entry patients were significantly shorter than controls (p=0.0001); height improved significantly after one year of treatment with GH (p=0.006), but was still significantly lower than controls after one year of treatment (p=0.001) (**Table 1**). BMI (18.84±3.20 *vs* 19.17±3.68 kg/m$^2$) , waist circumference (68.20±7.90 *vs* 67.80±8.10 cm), hip circumference (72.70±8.50 *vs* 72.60±9.00 cm) and WHR (0.94±0.05 *vs* 0.94±0.03) were similar in patients and controls at study entry and after one year of replacement therapy (**Table 1**). WHtR (0.48±0.05 *vs* 0.44±0.05, p=0.013) was higher at baseline in GHD patients compared to controls, but significantly reduced (0.45±0.04, p=0.04) after one year of GH therapy, becoming similar to the control group (**Table 1**).

SBP, DBP and HR were comparable between patients and controls and did not change in GHD subjects during therapy (**Table 1**).

At baseline, as expected, IGF-1 SDS was significantly reduced in patients as compared to controls (p=0.005) and significantly increased after 1 year of GH treatment (p=0.0001) (**Table 1**) being similar to controls at the end of the study.

**TABLE 1. Clinical characteristics of GHD patients and controls at study entry and after 1 year.**

| | Baseline | 1 year | $p^a$ | $p^b$ | $p^c$ |
|---|---|---|---|---|---|
| Height (SDS) | | | | | |
| GHD | -2.27±0.67 | -1.60±0.82 | 0.006 | <0.0001 | 0.001 |
| Controls | -0.60±1.20 | -0.40±1.30 | ns | | |
| BMI (kg/m2) | | | | | |
| GHD | 18.84±3.21 | 19.38±3.81 | ns | ns | ns |
| Controls | 19.17±3.68 | 19.72±3.20 | ns | | |
| BMI SDS | | | | | |
| GHD | -0.58±1.17 | -0.70±1.35 | ns | ns | ns |
| Controls | -0.42±1.15 | -0.36±1.10 | ns | | |
| WHR | | | | | |
| GHD | 0.94±0.05 | 0.92±0.04 | ns | ns | ns |
| Controls | 0.94±0.03 | 0.94±0.04 | ns | | |
| WHtR | | | | | |
| GHD | 0.48±0.05 | 0.45±0.04 | 0.04 | 0.013 | ns |
| Controls | 0.44±0.05 | 0.45±0.05 | ns | | |
| LBM% | | | | | |
| GHD | 65.36±7.84 | 75.36±7.59 | 0.0001 | <0.0001 | na |
| Controls | 76.13±8.23 | na | na | | |
| FM% | | | | | |
| GHD | 30.84±7.92 | 22.62±7.73 | 0.001 | 0.001 | na |
| Controls | 22.19±8.18 | na | na | | |
| SBP (mmHg) | | | | | |
| GHD | 102.24±9.71 | 99.05±9.44 | ns | ns | ns |
| Controls | 103.57±11.95 | 101.90±12.60 | ns | | |
| DBP (mmHg) | | | | | |
| GHD | 66.24±7.29 | 65.05±8.43 | ns | ns | ns |
| Controls | 68.33±8.85 | 67.62±8.89 | ns | | |
| HR (bpm) | | | | | |
| GHD | 81.76±10.87 | 78.10±9.62 | ns | ns | ns |
| Controls | 84.48±13.33 | 83.05±13.98 | ns | | |
| IGF-I (SDS) | | | | | |
| GHD | -1.22±1.23 | 0.40±1.22 | 0.0001 | 0.005 | ns |
| Controls | -0.25±0.88 | -0.15±0.76 | ns | | |

Data are expressed as mean ±SD. $p^a$ Baseline GHD *vs* 1 year GH replacement; $p^b$ Baseline GHD *vs* baseline controls; $p^c$ 1 year GHD *vs* 1 year controls; ns, not significant; na, not available

*Body composition*

At study entry LBM (22011±4998 *vs* 27275±5078 gr, p=0.006) and LBM% (65.36±7.84 *vs* 76.13±8.23%, p<0.0001) were significantly lower, while FM (11407±5933 *vs* 8066±2979 gr, p=0.014) and FM% (30.84±7.92 *vs* 22.19±8.18%, p=0.001) were significantly higher in GHD patients compared to controls (**Table 1**).

After one year of GH treatment an improvement in body composition was observed in GHD patients with a significant increase in LBM (33629±10863 gr, p<0.0001) and LBM% (75.36±7.59%, p=0.0001), a significant reduction in FM% (22.62±7.73%, p=0.001) and a trend toward reduction in FM (9589±4151 gr) (**Table 1**).

*Physical activity assessment*

According to the IPAQ categorical scoring system, 17/21 (81%) patients and 17/21 (81%) controls had a low intensity level of PA, and 4/21 (19%) in both patients and controls had a moderate intensity level of PA.

*Echocardiography*

Results of echocardiography are summarized in **Table 2.**

**TABLE 2. Echocardiography of GHD patients and controls at study entry and after 1 year.**

| | **Baseline** | **1 year** | **p[a]** | **p[b]** | **p[c]** |
|---|---|---|---|---|---|
| **Left ventricle** | | | | | |
| LVEDD (mm) | | | | | |
| GHD | 40.50±2.90 | 42.50±2.10 | <0.02 | 0.001 | 0.01 |
| Controls | 45.30±5.40 | 45.40±4.60 | ns | | |
| LVESD (mm) | | | | | |
| GHD | 25.00±2.30 | 26.60±2.70 | <0.05 | <0.05 | ns |
| Controls | 26.80±3.30 | 27.70±4.90 | ns | | |
| IVST (mm) | | | | | |
| GHD | 6.50±0.70 | 7.50±1.40 | 0.006 | 0.02 | ns |
| Controls | 7.80±2.40 | 8.30±2.20 | ns | | |
| LVPWT (mm) | | | | | |
| GHD | 6.80±0.80 | 7.60±1.20 | 0.01 | 0.01 | ns |
| Controls | 8.10±2.10 | 7.90±1.50 | ns | | |

| | | | | | |
|---|---|---|---|---|---|
| LV mass (gr/m$^2$) | | | | | |
| GHD | 63.32±7.80 | 72.01±15.88 | 0.03 | 0.006 | ns |
| Controls | 80.44±26.29 | 80.84±22.02 | ns | | |
| LVFS (%) | | | | | |
| GHD | 38.39±3.95 | 39.38±5.54 | ns | ns | ns |
| Controls | 40.36±8.97 | 41.35±10.04 | ns | | |
| LVEF (%) | | | | | |
| GHD | 61.19±3.61 | 64.29±6.62 | ns | ns | ns |
| Controls | 64.67±6.26 | 63.90±5.08 | ns | | |
| E/A | | | | | |
| GHD | 1.83±0.47 | 1.94±0.37 | ns | ns | ns |
| Controls | 1.90±0.46 | 2.03±0.39 | ns | | |
| **Right ventricle** | | | | | |
| RVEDD (mm) | | | | | |
| GHD | 23.20±3.80 | 22.20±4.20 | ns | ns | ns |
| Controls | 21.50±4.50 | 22.10±4.30 | ns | | |
| TAPSE (mm) | | | | | |
| GHD | 22.20±2.70 | 22.60±3.10 | ns | ns | ns |
| Controls | 21.10±2.70 | 21.50±2.80 | ns | | |
| RVFAC (%) | | | | | |
| GHD | 49.44±7.24 | 48.00±7.06 | ns | ns | ns |
| Controls | 49.20±9.46 | 50.30±6.37 | ns | | |

Data are expressed as mean ±SD. p[a] Baseline GHD *vs* 1 year GH replacement; p[b] Baseline GHD *vs* baseline controls; p[c] 1 year GHD *vs* 1 year controls; ns, not significant; na, not available

At study entry GHD patients presented significantly lower LV size than controls (LVEDD p=0.001; LVESD p<0.05; IVST p=0.02; LVPWT p=0.01), resulting in a reduced LV mass (p=0.006).

FS and LVEF were only slightly, but not significantly, reduced in GHD patients than in controls. E/A ratio was comparable between the two groups.

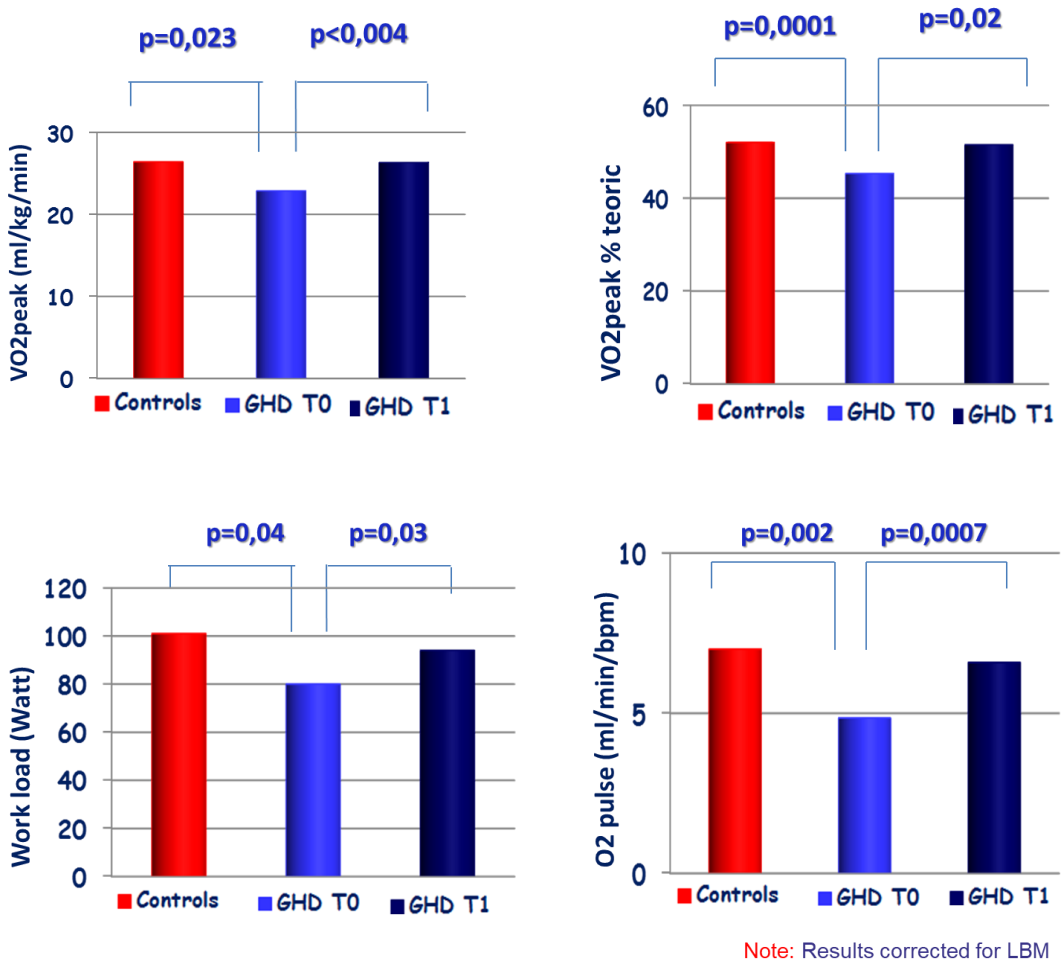
In GHD patients, 1-year GH replacement therapy significantly improved LVESD (p<0.05), IVST (p=0.006), LVPWT (p=0.01) and LV mass (p=0.03) which all became comparable to healthy subjects (**Table 2**); LVEDD also significantly improved after treatment (p<0.02) although at the end of the study was still slightly lower in GHD patients (p=0.01) (**Table 2**). No significant changes in FS, LVEF and E/A ratio were observed.

At study entry, no differences were found in RV diameter, RVFAC and TAPSE between GHD patients and controls. Moreover, these parameters did not significantly change during GH treatment.

*Cardiopulmonary exercise testing*

At baseline GHD children, compared to controls, showed significantly lower values of $VO_2$peak (22.92±4.80 *vs* 27.48±6.71 ml/Kg/min, p=0.01), theoretic $VO_2$peak% (45.19±10.02 *vs* 54.48±12.18 %, p=0.02), peak workload (80.62±29.32 *vs* 103.76±36.20 Watts, p=0.02) and $O_2$ pulse (4.93±1.30 *vs* 7.67±2.93 ml/beat, p=0.0003) (**Figure 2)**.

**Figure 2. Change from baseline in $VO_2$ peak, $O_2$ pulse and peak workload in healthy controls and in GHD patients following 1 year of GH treatment.**



After correction for lean body mass, differences in $VO_2$peak, theoretic $VO_2$peak% and $O_2$ pulse still remained significant (p=0.04, p=0.01 and p=0.001 respectively), whereas peak workload became comparable between the two groups.

GH therapy resulted in a significant improvement in all cardiopulmonary functional capacity parameters, which became comparable to those obtained in the control group (**Figure 2**). Anaerobic threshold was identified in 12 out of 21 patients at baseline and in only 2/21 after 1 year of GH treatment. HR (174.14±16.87 *vs* 169.58±14.45 bpm), SBP (127.48±18.62 *vs* 130.24±18.27 mmHg) and DPB (75.71±10.74 *vs* 75.62±9.88 mmHg) recorded at maximal exercise were similar between patients and controls at study entry, and did not change after one-year GH treatment.

Multiple regression analysis showed that changes in peak Workload were significantly correlated to changes in IGF-1 (β=0.59, p=0.004) and LBM (β=0.45, p=0.04).

**Discussion**

The results of this case-control, prospective study indicate that i) children with untreated GHD have reduced LV mass, impaired body composition and cardiopulmonary functional capacity compared to healthy controls; ii) short-term GH replacement therapy exerts beneficial effects on cardiac structure, body composition and cardiopulmonary functional capacity.

To the best of our knowledge, this is the first study investigating the effects of GHD and GH replacement therapy on cardiopulmonary functional capacity in a pediatric cohort. Although not having apparent limitations in their daily PA, our data indicate that GHD children may have limitation of exercise capacity. In particular, compared to healthy controls, patients with GHD exhibited significantly lower baseline $VO_2$peak, which is considered the gold standard measure of aerobic functional capacity, and reduced peak workload and oxygen pulse, which represents a non-invasive estimate of stroke volume and of cardiac output (21). A one-year course of GH treatment significantly improved all these measures of aerobic exercise performance which became comparable to healthy controls. These results are particularly interesting since a better cardiopulmonary performance in childhood is associated to long-term beneficial effects such as reduced CV risk and improved bone mineral density and psychological well-being (22, 23).

Our results are consistent with those reported in GHD adults. In fact, despite a few contrasting results (24), there is a general agreement that GHD adults have poor exercise tolerance and sub-optimal maximum oxygen uptake (25, 26). Several placebo-controlled studies have

documented a relevant increase in exercise capacity and maximum oxygen uptake after 6-12 months of GH replacement therapy (27-30). Furthermore, in a double-blind placebo-controlled study, GH restart in 20 childhood-onset GHD patients, off treatment for at least 2 years, was associated with a significant improvement of exercise capacity (31).

Favorable short-term effects of GH treatment on maximum power output and $VO_2$peak have been also confirmed by two recent meta-analysis including 268 patients from 11 studies (3) and 306 patients from 15 studies (4), respectively. In both meta-analysis, removal of studies with a predominance of childhood-onset GHD patients did not alter the significance of the results, thus suggesting that the effects of GHD and GH replacement therapy on physical performance are independent of the duration of GHD and/or GH replacement therapy (3, 4). In addition, no correlation was found between the improvement in exercise capacity variables, GH dose, initial IGF-1 concentrations or age at diagnosis (3).

Several lines of evidencein adult GHD suggest that decreased $VO_2$peak may be proportional to the reduction in skeletal muscle mass (32) and that improvement in physical performance in response to GH may reflect the parallel increase in LBM and muscle volume (27, 32). Indeed, the amount of skeletal muscle mass plays a key role in the regulation of metabolic changes and oxygen uptake during exercise (33-35).

Other abnormalities in body composition, such as increased amount of FM, which represents a mechanical limitation, disproportionate body fat distribution, and reduced extracellular water may further impair the ability to exercise (3).

Data on body composition in children and adolescents with GHD are scanty (6, 36, 37) but point toward a significant effect of GH deficiency and GH replacement. Accordingly, our data confirm that GHD is associated with lower LBM and higher FM in comparison to healthy controls while GH therapy induces beneficial effects on these parameters. Although it could be hypothesized a relationship between changes in body composition and aerobic exercise performance, regression analysis documented that in our children GH-induced changes in LBM correlated to changes in peak workload, but not to changes in $VO_2$ peak or $O_2$ pulse. In keeping with this, after adjustment for differences in LBM, $VO_2$ peak and $O_2$ pulse remained significantly lower in untreated GHD children suggesting that, in addition to alterations in body composition, other GH-related factors also influence cardiopulmonary capacity. However, the small sample size of our study and the lack of data on muscle volume and strength and on body fat distribution limit the ability to find significant correlations between changes in body composition and improvement in aerobic capacity measures. Furthermore, a major limitation of our study is that DXA was not repeated in controls after one year of

follow-up thus not allowing the evaluation of physiological effects of growth, age or puberty on changes in body composition.

Noteworthy, it is well known that GH has pleiotropic effects and an intricate combination of other factors may also account for the improvement of aerobic capacity observed after starting GH treatment, such as increased availability of energy substrate (i.e.free fatty acids and glycerol levels, reflecting its lipolytic effects), more efficient hematopoiesis, cardiac contractility, and thermogenesis (5, 38, 39). In this respect, we found, in agreement with our previous studies (12, 13), that GHD children have reduced LV mass, which significantly improves upon starting GH treatment. Therefore, we could hypothesize that favorable changes in ventricular size may play an important role in improving aerobic capacity in GHD patients.

Echocardiographic study in our patients also included the assessment of RV structure and function, that did not appear to be affected by GHD. There is no information available about RV modification by GHD; our results suggest that RV function and development are not influenced by GH. However, these data cannot be considered conclusive and, since echocardiography is not the gold standard for RV structure and function evaluation, further studies with other techniques such as cardiac magnetic resonance may clarify the role of GH in RV remodeling and function.

In conclusion, children with GHD have reduced LV mass, impaired body composition and cardiopulmonary functional capacity compared to healthy controls. Short-term GH replacement exerts beneficial effects on cardiac structure, body composition and cardiopulmonary functional capacity. Cardiopulmonary exercise stress testing could be considered in the baseline evaluation of untreated GHD patients in order to unmask mild alterations of aerobic capacity.

These results further support the evidence that GHD in children may be associated to a cluster of CV risk factors. Studies in larger populations are needed to confirm our findings and to further clarify the underlying mechanisms.

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## 5.2 Muscle health in GHD

This chapter provides a preliminary report of data obtained from a study protocol which is currently running at Federico II University.

**A final manuscript is about to be submitted to an international peer-reviewed journal.**

### Background

GH Deficiency (GHD) may be associated with unfavorable body composition, and increased cardiovascular risk, whereas knowledge regarding the effects of GHD and GH replacement therapy (GHRT) on functional outcome measures of physical fitness, such as muscular strength and flexibility,and exercise tolerance is limited (1-3).

GHRT may affect muscle strength by increasing muscle mass and/or inducing structural changes within myocytes (1-3). Studies in GHD adults have yielded controversial results on this topic (4,5). GHD adults exhibit decreased muscle mass and increased fat mass compared with age- and gender-matched controls, with a normalization of body composition after starting GHRT (6,7). GHD adults also exhibit poor exercise tolerance, with reduced maximum oxygen uptake (7,8). Results of a recent meta-analysis demonstrated a significant increase in maximum power output and oxygen uptake after 6-month GHRT (5). Despite positive effects of GHRT on body composition and exercise capacity, a recent meta-analysis failed to demonstrate an increase in muscle strength over 12 months (9). Interestingly, a long-term study demonstrated that adult-onset GHD have reduced isometric and isokinetic muscle strength with a progressive increase in muscle strength observed during GHRT up to 7 years (10).

GHD children/adolescents and young adults in the transition period exhibit abnormal body composition, with reduced lean mass and increased fat mass, which normalize upon starting GHRT (11). Moreover, we recently demonstrated that GHD adolescents can exhibit reduced VO2max compared to matched healthy controls, in addition to altered body composition and reduced cardiac mass, which can be reverted by 1-year GHRT (12). Nevertheless, the effects of GHD and GHRT on muscle strength and flexibility in children are largely unknown.

### Projects aims

Our study aims to characterize the components of health-related fitness (body composition, exercise tolerance, muscle strength and flexibility) in children and adolescents with GHD.

Moreover, we aim to establish whether GHRT exerts beneficial short-term effects on such functional outcomes.

**Methods:**

*Patients*

-Cohort A: 19 children and adolescents with untreated GHD, aged 9-13 years.

-Cohort B: 19 healthy children with normal response to GH stimulation matched for age,stature and sex.

Inclusion criteria: Diagnosis of GHD according to clinical criteria associated with insufficient GH response (peak GH <8 μg/liter) after two different stimulation tests (Cohort A). Short stature and normal response to GH stimulation test (Cohort B).

Exclusion criteria: Chronic diseases, genetic syndromes.

*Study design*

This was a 1-year prospective case-control study. At study entry, we evaluated in all subjects: anthropometric measures, systolic (SBP) and diastolic (DBP) blood pressure, a battery of tests evaluating musculoskeletal fitness,multifrequency bioimpedance (BIA)IPAQ questionnaire andserum IGF-1 concentrations. All the parameters were re-assessed after 1 year of GH treatment in GHD subjects.The study was approved by our Institutional Ethical Committee. Informed consent for participation into the study was obtained from the patients and/or their families.

*Anthropometric measurements*

Height was measured in the upright position using a Herpenden stadiometer and was expressed in standard deviation score (SDS) according to Italian reference standards (13). BMI was calculated by dividing weight in kilograms by the square of height in meters and then expressed as SDS according to Italian reference standards (13). Waist circumference was measured by the same operator in the standing position with a non-elastic tape placed at the midpoint between the lower rib margin and the iliac crest. Hip circumference was measured at the level of widest portion of trochanters. All measurements were expressed in centimeters (cm) to the nearest 0.1 cm. WHR and WHtR were used to evaluate visceral adiposity. Blood pressure was registered as the mean value of three measurements after 10 min resting.

*Serum assay*

Serum GH and IGF-I concentrations were determined by a solid-phase, enzyme-labeled chemiluminescent immunometric assay (Immulite 2000 Siemens Healthcare Diagnostics Inc., New York, USA). IGF-1 concentrations were expressed as SDS according to the normative data provided by the manufacturer.

*Musculoskeletal fitness*

This will be assessed throughout an extensive test battery including measurements that have shown to be strongly related with the current and future health status of children (14).

- *Jump test:* this test evaluates lower body explosive strength. The subjects will perform a series of five consecutive jumps with maximal effort and the values of the flight (Tf) and contact time (Tc) of feet with ground relative to each jump will be measured with a digital timer connected with an optical acquisition system (Optojump). Average results will be calculated on the series of the five jumps for each subject.
- *Hand-grip strength:* Dominant hand-grip strength will be measured isometrically using a dynamometer. Each subject will be asked to carry out three maximal efforts, lasting 4-5 s, with a 2- min interval in-between. The subject will be encouraged to improve his previous score; only the highest value will be retained for analysis.
- *Chair sit and reach:* this test evaluates flexibility of the lower part of the body. The subject sits on the edge a chair with one foot flat on the floor and the other leg extended forward with the knee straight, heel on the floor. The subject is asked to reach forward toward the toes by bending at the hip. The distance between the tip of the fingertips and the toes is measured. If they do not touch, the distance between the finger tips is measured and considered as a negative score, while an eventual overlap is considered as a positive score. Sit and reach test: this measures the flexibility of the lower back and hamstring muscles. The soles of the feet are placed flat against a box, with both knees locked at the floor. With the palms facing downwards, the subject reaches forward along the measuring line as far as possible.
- *Sit to stand test (STS):* This test assesses leg strength and endurance. The subject sits with the feet shoulder width apart, flat on the floor. The arms are crossed at the wrists and held close to the chest. From the sitting position, the subject stands completely up, then completely back down, repeatedly for 30 seconds. The total number of complete chair stands is recorded.
- *Sit to stand test - 5 repeats (STS-5R):*The participant is asked to repeat this sit-to-stand action five times as quickly as possible, and the time taken to complete the five repetitions is recorded.

- *Six minute walking test (6MWT):*About one hour after completing the battery of muscle functional teststhe patient is encouraged to walk on a 30 m, flat, straight corridor and the distance (6MWD)is measured. Continuous measurements of SpO2 and HR are performed using a finger pulse oximeter from 1 minute before the 6MWT to the fourth minute after.
- *KIDMED score:*The KIDMED questionnairewas used to evaluate the adherence to a Mediterranean diet. It consists of 16 items, where there are 4 questions denoting a negative connotation to the Mediterranean diet (consumption of fast food, baked goods, sweets, and skipping breakfast) and 12 questions denoting a positive connonation (consumption of oil, fish, fruits, vegetables, cereals, nuts, pulses, pasta or rice, dairy products, and yoghurt). Questions denoting negative connotation are scored with−1, while positive connotation questions are scored with +1. A scoreof0–3reflectspooradherencetotheMediterraneandiet,ascoreof4–7describesaverage adherence, and a score of 8–12 good adherence.

*Body composition*

Multifrequency BIA has been performed with a tetra-polar technique in standardized conditions (ambient temperature between 23 and 25°C, fast >3 h, empty bladder, supine position for at least 10 minutes). Patients have been asked to lie down with their legs and arms slightly abducted at 30°. Z and phase angle have been determined at different frequencies (5-10-50-100-250 kHz) for both dominant and non-dominantsides of the body injecting an electrical alternating current of 800 mA. Raw BIA variables providing information on hydration status, cellular mass and quality ,such as the impedance ratio between Z at high (50-250 kHz) to Z at low (5 kHz) frequencies and phase angle at 50 kHz have been collected. FFM has been predicted from BIA data using the available predictive equations for children.

*Physical activity level*

In order to determine the level of physical activity among adolescents at this age, the International Physical Activity Questionnaire (IPAQ) was used. IPAQ describes physical activity in energy expenditure units – minutes per week (MET). MET is used to estimate the metabolic cost (energy expenditure as reflected by oxygen consumption) of physical activity – resting metabolic rate.

Selected items from the survey were used in the study concerning adolescents' physical activity, which is reflected the short version of the IPAQ. The survey contains 7 questions covering all types of physical activity:

– physical activity associated with the occupation performed, or at school;
– physical activity at home and around the house;
– moving to various places and mobility during free time devoted to recreation, playing games, sports, tourism, or other muscular work.
Only the physical activity lasting longer than 10 minutes was estimated, without rest breaks, and within the last 7 days. An average number of hours of therespondent's remaining in a sitting position daily (sitting time) was noted.

*Statistical analysis*

Quantitative variables have been reported as mean and standard deviation (SD) or median and Interquartile Range (Q1-Q3). Categorical variables have been reported as number (n) and percentage (%).Shapiro Wilk test and graphics methods have been used to asses normality assumptions.T Student test or Mann Whitney test have been used to compare Muscle strength index, endurance and Muscle flexibility of two groups (GHD patients vs no GHD patients) at baseline.In primary endpoint analysis paired t test or Wilcoxon paired test has been used to evaluate change in Musclestrength in GHD group between T1 and T0. A p value <0.05 has been considered statistically significant.

**Preliminary results**

*General characteristics*

As expected, at study entry patients were significantly shorter than controls (p=0.02); height improved significantly after one year of treatment with GH (p=0.0001) (Table 1). Baseline BMI SDS (-0.33±1.13 vs -0.5±1.29) and WHR (0.94±0.04 vs 0.92±0.06) were comparable between patients and controls (**Table 1**). However, WHR significantly decreased (p=0.02) after one year of replacement therapy (**Table 1**). WHtR (0.50±0.08 vs 0.45±0.03, p=0.02) was higher at baseline in GHD patients compared to controls, but significantly reduced (0.45±0.06, p=0.02) after one year of GH therapy, becoming similar to the control group (**Table 1**). SBP and DBP were comparable between patients and controls and did not change in GHD subjects during therapy (**Table 1**).

As expected, baseline IGF-1 SDS was significantly lower in patients as compared to controls (p=0.03) and significantly increased after 1 year of GH treatment (p=0.0008) (**Table 1**).

Physical activity level, as measured by total METs (min/week), was significantly lower in GHD patients (1969.77±1884.59 vs 3017.07±1125.70, p=0.01) than controls, and

significantly improved at the end of the study (p=0.02). Moreover, untreated GHD patients exhibited a more sedentary behavior compared to healthy controls (sitting time 2897.00±1067,21 vs 1989.38±1001.16 min/week, p=0.02), which significantly improved after one year of treatment with GH (p=0.02) (**Table 1**).

**TABLE 1. General characteristics of GHD patients and controls at study entry and after 1 year.**

| | **Baseline** n=19 | **1 year** n=19 | **p[a]** | **p[b]** |
|---|---|---|---|---|
| Age (yrs) | | | | |
| GHD | 10.54±1.66 | 11.57±1.7 | Ns | Ns |
| Controls | 11.08±2.61 | Na | | |
| Height (SDS) | | | | |
| GHD | -2.06±0.74 | -1.4±0.78 | 0.02 | <0.0001 |
| Controls | -1.42±0.84 | Na | | |
| BMI SDS | | | | |
| GHD | -0.33±1.13 | -0.26±1.0 | Ns | Ns |
| Controls | -0.5±1.29 | Na | | |
| WHtR | | | | |
| GHD | 0.50±0.08 | 0.45±0.06 | 0.02 | 0.02 |
| Controls | 0.45±0.03 | Na | | |
| SBP (mmHg) | | | | |
| GHD | 103.93±11.1 | 99.4±12.51 | Ns | Ns |
| Controls | 98.24±8.97 | Na | | |
| DBP (mmHg) | | | | |
| GHD | 66.24±7.29 | 69.4±7.62 | Ns | Ns |
| Controls | 66.7±7.5 | Na | | |
| IGF-I (SDS) | | | | |
| GHD | -0.75±1.04 | 0.94±0.73 | 0.03 | 0.0008 |
| Controls | -0.03±0.71 | Na | | |
| Sitting time (min/week) | | | | |
| GHD | 2897.00±1067.21 | 1348.43±912.17 | 0.02 | 0.02 |
| Controls | 1989.38±1001.16 | Na | | |
| Mets (min/week) | | | | |
| GHD | 1969.77±1884.59 | 4136.33±4113.88 | 0.01 | 0.02 |
| Controls | 3017.07±1125.70 | Na | | |
| Kidmed score | | | | |

| | | | | |
|---|---|---|---|---|
| GHD | 4.42±2.09 | 3.5±2.5 | Ns | Ns |
| Controls | 3.21±2.36 | Na | | |

Data are expressed as mean ±SD. p[a] Baseline GHD *vs* baseline controls; p[b] Baseline GHD *vs* 1 year GH replacement
ns, not significant; na, not available

*Body composition*

At study entry FFM (22.45±5.11vs 28.68±7.39 kg, p=0.03) was significantly lower, while FM% (26.99±6.03vs 22.78±5.52 %, p=0.03) was significantly higher in GHD patients compared to controls (**Table 2**).

After 1year of GHRT an improvement in body composition was observed in GHD patients with a significant increase in FFM (28.68±7.39 kg, p=0.004), and reduction in FM% (22.61±6.84%, p=0.03) (**Table 2**). Baseline values of bicipital, tricipital and subscapular folds were all comparable between patients and controls at baseline; however, a significant reduction in both bicipital (p=0.01) and subscapular (p=0.02) foldswas observed (**Table 2**).

**TABLE2. Body composition of GHD patients and controls at study entry and after 1 year.**

| | **Baseline** n=19 | **1 year** n=19 | **P[a]** | **P[b]** |
|---|---|---|---|---|
| FFM (kg) | | | | |
| GHD | 22.45±5.11 | 28.68±7.39 | 0.03 | 0.004 |
| Controls | 26.63±6.01 | Na | | |
| FM% | | | | |
| GHD | 26.99±6.03 | 22.67±6.84 | 0.03 | 0.04 |
| Controls | 22.78±5.52 | Na | | |
| Phase angle | | | | |
| GHD | 5.21±0.64 | 5.35±0.99 | Ns | Ns |
| Controls | 5.32±0.8 | Na | | |
| Bicipital folds (mm) | | | | |
| GHD | 9.91±3.97 | 7.4±3.25 | Ns | 0.01 |
| Controls | 10.07±5.90 | Na | | |
| Tricipital folds (mm) | | | | |
| GHD | 13.62±5.64 | 11.51±4.55 | Ns | Ns |
| Controls | 14.28 ±5.89 | Na | | |
| Subscapular folds (mm) | | | | |
| GHD | 28.9±4.07 | 27.86±3.43 | Ns | 0.02 |
| Controls | 28.12±4.30 | Na | | |

Data are expressed as mean ±SD. p[a] Baseline GHD *vs* baseline controls; p[b] Baseline GHD *vs* 1 year GH replacement
ns, not significant; na, not available

*6MWT, muscle strength and flexibility*

At baseline, GHD subjects exhibited values of hand-grip strength (11.29±3.49 vs 14.03±4.7 kg; p=0.04) and jumping capacity (vertical jump 12.78±4.17 vs 15.42±2.99, p=0.03; long jump 104.91±28 vs114.03±34.3, p=0.03) lower than healthy controls (**Table 3**). Moreover, baseline 6MWT distance was lower in patients than in controls (505.31±67.25 vs 547.12±52.09 m, p=0.04), indicanting reduced exercise tolerance. One year of GH replacement was ossociated to an improvement of both muscle strength (handgrip 14.56±4.91, p=0.001; vertical jump 16.02±4.81, p=0.03; long jump 120.81±34.73, p=0.03) and 6MWT distance (603.31±79.45, p=0.005) (**Table 3**). No differences were found in baseline and 1-year values for the STS test (19.13±4.67 vs 18.61±4.98, ns), while, despite baseline values of sit-to-stand test-5 repeats comparable to controls, a significant improvement was observed in such parameter after 1 year of GH (7.32±2.15, p=0.04) (**Table 3**).Finally, untreated GHD patients exhibited lower results at the sit and reach test (8.50±9.21 vs 14.50±7.75, p= 0.03) compared to controls, which improved with GHRT (15.60±8.95, p=0.02) (**Table 3**).

**TABLE 3. Results of tests evaluating exercise tolerance and muscle strength and flexibility of GHD patients and controls at study entry and after 1 year.**

| | **Baseline** n=19 | **1 year** n=19 | **P[a]** | **P[b]** |
|---|---|---|---|---|
| Handgrip | | | | |
| GHD | 11.29±3.49 | 14.56±4.91 | 0.04 | 0.001 |
| Controls | 14.03±4.7 | Na | | |
| Vertical jump (cm) | | | | |
| GHD | 12.78±4.17 | 16.02±4.81 | 0.03 | 0.03 |
| Controls | 15.42±2.99 | Na | | |
| Long jump (cm) | | | | |
| GHD | 104.91±28.0 | 120.81±34.73 | 0.03 | 0.03 |
| Controls | 114.03±34.3 | Na | | |
| STS test (n) | | | | |
| GHD | 19.13±4.67 | 21.81±5.39Na | NS | Ns |
| Controls | 18.61±4.98 | | | |
| Sit-to-stand- 5R (sec) | | | | |
| GHD | 8.23±2.23 | 7.32±2.15 | NS | 0.04 |
| Controls | 8.99±2.18 | Na | | |
| Sit and reach test | | | | |
| GHD | 8.50±9.21 | 15.60±8.95 | 0.03 | 0.02 |
| Controls | 14.50±7.75 | Na | | |
| 6MWT (m) | | | | |

| | | | | |
|---|---|---|---|---|
| GHD | 505.31±67.25 | 603.31±79.45 | 0.04 | 0.005 |
| Controls | 547.12±52.09 | Na | | |

Data are expressed as mean ±SD. $p^a$ Baseline GHD *vs* baseline controls; $p^b$ Baseline GHD *vs* 1 year GH replacement
ns, not significant; na, not available*

**Discussion**

The preliminary results of our study demonstrate for the first time that untreated GHD may be associated with reduced muscle strength, and body flexibility, possibily contributing to reduced exercise tolerance. Important strength of our study is the use of an easily reproducible, non-invasive, standardized battery of tests and/or techniques evaluating muscle functional outcomes and body composition.

An important role for endogenous GH in physical fitness was first proposed a few decades ago when it was observed that increased GH concentrations during exercise resulted in increased free fatty acids, thus improving the availability of oxidisable fat to exercising muscle, and prolonging the ability to exercise (1-3). GH could also influence physical fitness through anabolic modifications of various organs/systems such as improved cardiac and skeletal muscle performance, body composition, and more efficient thermoregulation (1-3). The effects of GH on muscle strength may be mediated by an increase in muscle mass and/or induction of structural changes within myocytes (3).

Adults with GHD, although able to carry out normal daily activities, have increased fat mass (FM) and reduced fat-free mass (FFM), which normalize after starting GHRT (6,7) and may suffer from general fatigue and weakness, leading to diminished productivity and social isolation (1,8). Moreover, GHD adults exhibit poor exercise tolerance, with reduced peak oxygen uptake and maximum power, which improve after 6-month GHRT (8).

We recently reported for the first time that pediatric GHD patients may also havesignificantly lower $VO_2$peak, Wmax and oxygen pulse (a non-invasive estimate of stroke volume and of cardiac output), together with abnormal body composition and reduced left ventricular mass, compared to healthy controls. A one-year course of GH treatment significantly improved all these outcome measures (12). In this respect, the results of the present study provide further evidence that untreated GHD may be associated with altered body composition, with increased fat mass and reduced fat-free mass, and visceral adiposity, as assessed with WHtR.

Moreover, we found that 1-year GHRT is able to ameliorate such abnormalities.It is worth highlighting that body composition has been assessed through multifrequency BIA, which is an easy, non-invasive, relatively inexpensive and portable technique allowing reliable evaluation of body composition without radiation exposure.

Both patients and controls had a similar level of adherence to the Mediterranean diet, indicating that the detrimental effects of GHD on body composition and fat distribution are independent from dietary habits.

The effects of GHD and GHRT on muscle strength in children are unknown, but some information could be extrapolated from other patients receiving GH. In fact, an increase in muscle strength has been found in children born small for gestational age treated with GH over 12 months (15) as well as in children with Prader-Willi syndrome, where GH also exerts beneficial effects on muscle mass and aerobic capacity (16). Additionally, although some studies in GHD adults reported reduced isometric and isokinetic muscle strength (4), others revealed measures of jumping capacity, postural (quadriceps) and non-postural (handgrip) strength comparable to controls after correction for muscle area (7). In adults with childhood-onset GHD who had stopped GHRT at final height, vertical jump, quadriceps and handgrip strength normalized for muscle area were also comparable to controls (17). Indeed, a recent meta-analysis failed to demonstrate an increase in muscle strength over 12 months (9). Interestingly, a long-term study found that adult-onset GHD have reduced isometric and isokinetic muscle strength with a progressive increase in muscle strength observed during GHRT up to 7 years (10). Furthermore, there is evidence that patients who discontinue GH in the transition period may experience a reduction of isometric muscle contraction and muscle mass or do not gain muscle strength when compared with GH-sufficient and healthy subjects (18).

In this context, preliminary results of our study provide the first evidence that untreated GHD may be associated with reduced muscle strength. Indeed GHD patients had significantly lower handgrip andleg power output, which improved upon starting GHRT. We also found that GHD children and adolescents have a more sedentary behaviour and lower exercise tolerance (as evaluated by 6MWT) in comparison to their healthy counterpart, which were significantly improved after 1 year of GH. Thus, we could speculate that the postive action exerted by GH on muscle mass and strength results in improved physical activity level and exercise tolerance. Whether this has a relevant influence on their quality of life or other psycho-social aspects needs to be evaluated in further studies.

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## CHAPTER 6: Conclusive remarks

Our study project has provided novel insights regarding diagnosis, phenotypic characterization and management of hypopituitarism and GHD.

The results of the firstpart of the present study project (chapters 2 and 3), obtained from large databases of children diagnosed with hypopituitarism and/or GHD, contributed to expand knowledge on the differential diagnosis between GHD and CDGP, as well as on endocrine morbidity, height outcomes and mortality of children affected with hypopituitarism and/or midline brain defects. Indeed, we documented in a large multicentre cohort of peripubertal children that priming with sex steroids before GHST improves diagnostic accuracy of GHST for idiopathic GHD. Moreover, the use of low-dose sex steroids as a growth-promoting treatment may improve auxological outcomes of CDGP.

Comparison between different conditions characterized by midline brain defects, revealed striking differences between Septo-Optic Dysplasia (SOD) and multiple pituitary hormone deficiency (MPHD). In fact, the former is heterogeneous in terms of brain structures involved and dynamic and sequential nature of endocrinopathies, while the latter displaysamore homogeneous phenotype of (mainly) early-onset anterior pituitary failure. Finally, we have showed that pituitary stalk and posterior pituitary abnormalities are predictive of an earlier onset of endocrine deficits within the SOD spectrum.

The second part of the study project (chapters 4-5) was focused on health outcomes of GHDand GHRT in children and adolescents.We provided further evidence that childhood-onset GHD may be associated with a cluster of cardiovascular (CV) risk factors, such as visceral adiposity, dyslipidemia, reduced left ventricular mass and function and endothelial dysfunction, which can be reverted by GH replacement therapy.On the other hand, our studies contributed to establish that long-term treatment with GH does not impair glucose metabolism.

Given that GHD may exert detrimental effects on many organs/systems, it configures the prototype of a disease at risk to develop clinical or subclinical muscle impairment and reduced exercise capacity. However, so far little attention has been paid at functional outcome measures and health-related fitness.

In this scenario, the results or our study provided the first evidence that children and adolescents with GHD have impaired cardiorespiratory fitness compared to healthy controls, with significantly lower VO2peak, Wmax and oxygen pulse (a non-invasive estimate of stroke volume and cardiac output). In addition, GHD children and adolescents exhibited lower

measures of exercise tolerance (6MWTD), along with lower muscle strength (as evaluated by handgrip strength and jumping capacity) and flexibility (as evaluated by the sit and reach test), compared to matched healthy controls. Such abnormalities are likely to contribute significantly to reduced physical activity level found in GHD patients. Of note, we documented that all these measures significantly improve after 1 year of GH replacement therapy, supporting the concept that replacement therapy with GH exerts beneficial effects also on functional parameters, thus warranting an improvement of physical self-perception and social life of GHD patients, especially during the challenging period of childhood/adolescence.

Therefore, future directions of the study project will consist in confirming our results on larger popuations, also including the evaluation of the effects of changes in functional outcome measures on quality of life and psychosocial health of GHD children and adolescents. This will potentially help to identify strategies to promote physical and mental health of GHD patients, aiming to optimize social life, productivity and school performances of these subjects. We also aim to perform combined evaluation with non-invasive techniques of muscle function and oxidative metabolism.

## CHAPTER 7: Other research or academic items

This chapter contains a collection of all papers resulting from other research projects, as well as other academic activitiesconducted in the course of the Ph.D. Program (2018-2021).

**Full Papers:**

**1. Research article:** Donatella Capalbo, Sara Alfano, Miriam Polizzi, Raffaella Di Mase, **Nicola Improda**, Andrea Esposito, Carmela Bravaccio, Mariacarolina Salerno.
Cognitive function in children with idiopathic subclinical hypothyroidism: effects of two years of levothyroxine therapy.
**J Clin Endocrinol Metab (IF 5.6) 2020 1;105(3):dgaa046.**

**2. Review article:** M Salerno**, N Improda**, D Capalbo. Subclinical hypothyroidism in children.
**Eur J Endocrinol (IF 5.107).2020 Aug;183(2):R13-R28.**

**3. Review article: Improda N,** Barbieri F, Ciccarelli GP, Capalbo D, Salerno M.
Cardiovascular Health in Children and Adolescents With Congenital Adrenal Hyperplasia Due to 21-Hydroxilase Deficiency.
**Front Endocrinol (Lausanne) (IF 3.67). 2019 Apr 11;10:212.**

**4. Research article:** Nettore IC, Desiderio S, De Nisco E, Cacace V, Albano L, **Improda N**, Ungaro P, Salerno M, Colao A, Macchia PE.
High-resolution melting analysis (HRM) for mutational screening of Dnajc17 gene in patients affected by thyroid dysgenesis.
**J Endocrinol Invest (IF 3.397). 2018 Jun;41(6):711-717.**

**5. Research article: Nicola Improda**, Angela Mauro, Letizia Zenzeri, Francesco Valitutti, Erica Vecchione, Sara Esposito, Vincenzo Tipo.
Infection control strategy and primary care assistance in Campania region during the national lockdown due to COVID-19 outbreak: the experience of two tertiary emergency centers.
**Italian Journal of Pediatrics (IF2.185) 2021 47:19.**

**Book chapters:**

**1. Improda N**, Salerno M, Capalbo D

**Genetics of Autoimmune Regulator (AIRE) and clinical implications in childhood.**

Polyendocrine Disorders and Endocrine Neoplastic Syndromes

Springer Nature (accepted 19 october 2018)

**2. Improda N**, Salerno M, Capalbo D

**Patologie andrologiche pediatriche e obesità.**

Manuale di Andrologia Pediatrica e dell'Adolescenza

A cura di Matteo Sulpasso. Patrocinato SIP, SIA, SIEDP

Il Pensiero Scientifico Editore 2020.

**3. Improda N**

Ipoglicemia e disordini elettrolitici (ipocalcemia, ipopotassiemia, iperpotassiemia).

**Manuale di Emergenze pediatriche SIMEUP 2021**

**Abstracts and Oral Communications**

1) **ESPE 2018:**Manuela Cerbone, Maria Güemes, **Nicola Improda**, Mehul T Dattani Growth Pattern and Final Height Outcome in Children With Septo-Optic Dysplasia and Isolated Hypopituitarism treated with rhGH in a SingleCentre.
2) **ESPE 2018:**Manuela Cerbone, Maria Güemes, AngieWade, **Nicola Improda**, Mehul T Dattani.Can Neuroimaging Predict Endocrine Morbidity in Congenital Hypothalamo-Pituitary (H-P) Disorders?
3) **SIE 2019: Oral communication** for the abstract entitled: "Long term outcomes of precocious puberty"
4) **SIE 2019:** F. Anselmi, **N Improda**, F.Barbieri, L.Bufalo, P.Lorello, D.Capalbo, M. Salerno. Effects of growth hormone deficiency (ghd) and gh treatment on early markers of atherosclerosis in children

5) Scientific update Department of Medical Translational Sciences 3rd ofDicembre 2019: "Health-related fitness in children and adolescents with GHD"

6) **ESPE 2019:** Elena Galazzi, **Nicola Improda**, Manuela Cerbone, Davide Soranna, Mirella Moro, Letizia, Maria Fatti, Antonella Zambon, Mariacarolina Salerno, MehulDattani, Luca Persani. Role of priming in peri-pubertal growth delays: preliminary results of a large multicenter study.

7) **ESPE 2019:Nicola Improda**, Sara Alfano, Federica Anselmi, Valeria Gaeta, Lorenzo Bufalo, Fabiana Santamaria, Raffaella Di Mase, Mariacarolina Salerno. Long-term outcome in young women treated for central precocious puberty.

8) **ESPE 2019:Nicola Improda**, Cristina Moracas, Gian Paolo Ciccarelli, Donatella Capalbo, Mariacarolina Salerno. Metabolic Outcome in Adolescents with Growth Hormone Deficiency During Transition Phase.

9) **ESPE 2019:**Flavia Barbieri, Andrea Esposito, Ida D'Acunzo, Paola Lorello, Raffaella Di Mase, **Nicola Improda**, Donatella Capalbo. Bone homeostasis in children with subclinical hypothyroidism: Effects of two-years treatment with levothyroxine.

10) **SIE 2019:**G. P. Ciccarelli, F. Anselmi, **N. Improda**, A. Esposito, D. Capalbo, M. Salerno. Final height in childhood-onset hypopituitarism.

11) **SIE 2019:** V. Gaeta, G. P. Ciccarelli, **N. Improda**, R. Di Mase, S. A. Wudy, G. Parenti, L. Baldazzi, S. Menabò, D. Capalbo, M. Salerno. An unusual association of p450 oxidoreductase deficiency and argininosuccinatelyase deficiency.

**Invited as a Speaker**

1. TALENT (Transition and AdoLescenceENdocrine diseases management) meeting 15th December 2020: Adrenal insufficiency and congenital adrenal hyperplasia: old and new treatments.
2. SIEDP (Italian society of Pediatric endocrinology) 2019 Milan: "Yearbook on Adrenal Diseases 2018-2019".
3. Molecular Diagnostic in Pediatric Endocrinology 1st of December 2018 Potenza (Italy): "Short Stature".

4. Update in Pediatric endocrinology SIEDP Naples 2019: “Nutritional an non-nutritional rickets”.
5. Update in Endocrinology, Naples palazzo Alabardieri 16/12/2019: “Precocious pseudopuberty”.
6. Participation in the ESPE Summer School, Delphy (Grece) Settembre 2018, with a case report on differential diagnosis between GHD and CDGP.

**Other academic activities**

1. **Tutor and Correlator** for:

- Residency thesis (Candidate Dr Sara Esposito) entitled: Infection control strategy and primary care assistance in Campania region during the national lockdown due to COVID-19 outbreak: the experience of two tertiary emergency centers.

- First degree thesis in Pediatric Nursing (Candidate Dr Federica Coppola) entitled: Effects of the national lockdown due to COVID-19 outbreak on the activity of the pediatric Emergency Department.

2. **Teacher for the ONSP national meeting 2019**: “Management of Hypocalcemia”.
3. **Reviewer** for “Endocrine” and “Medical Principles and Practice” Journals.
4. **Co-investigator of the project entitled** “Physical fitness and muscle health in children and adolescents with Growth Hormone Deficiency”, funded by Serono-Merk.
5. **Member of the “Youth Committee” of the Italian Society of Pediatric Endocrinology (SIEDP) 2017-2018**.
6. **Participation in the “Journal Club” of the Italian Society of Pediatric Endocrinology (SIEDP)** from 2017.