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ORIGINAL ARTICLE

Growth hormone of dried blood spot for the diagnosis of growth hormone deficiency

Hormona de crecimiento en sangre de papel filtro para el diagnóstico de deficiencia de hormona de crecimiento

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Abstract

Introduction: The diagnosis of growth hormone deficiency (GHD) is difficult to determine, and could be associated with severe complications, especially in the neonatal period. The stimulation test of growth hormone (GH) secretion is considered the gold standard for diagnosis, but it has methodological complications and is associated with adverse effects. Neonates present physiological increased secretion of GH, representing a diagnostic window. **Objective:** To evaluate if the dried blood spot on filter paper obtained in the neonatal period, as part of a neonatal screening for congenital hypothyroidism and phenylketonuria, allows differentiating patients with GHD from those who do not have it. **Patients and Method:** Study of cases and controls by measuring the GH concentration in dried blood spot on filter paper obtained in the neonatal period, comparing controls with GHD with cases with discarded deficiency. The sample was extracted from the filter paper, obtaining two 0.125 inch discs per each patient from the center of the blood spot on the paper, for a highly sensitive ELISA assay for human GH based on the use of polyclonal antibodies against 22 kDa recombinant human GH. **Results:** Seven cases of GHD and ten controls were obtained. The median GH concentration of the dried blood spot in the cases is 2.0 ng/ml (Interquartile range 3.6 ng/ml) and 2.05 ng/ml (Interquartile range 2.0 ng/ml) in the controls, Mann-Whitney U test 30.5 ($p = 0.68$). The two cases with multiple pituitary-hormone deficiency (MPHD) present concentrations lower than 1 ng/ml. **Conclusion:** The dried blood spot sample did not differentiate GHD patients from control cases, although MPHD cases present much lower concentrations compared to isolated growth hormone deficiency (IGHD).

Keywords:
Growth hormone deficiency;
pituitary gland;
dried blood spot testing;
dwarfism

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Introduction

Growth hormone deficiency (GHD) is a rare cause of short stature but can be associated with severe metabolic disorders, including hypoglycemia, especially in the neonatal period. A 1 in 4,000 to 1 in 10,000 prevalence is estimated[1]. The GHD diagnosis in childhood and adolescence is controversial. Diagnostic methods use clinical and auxological elements, biochemical tests of the growth hormone (GH) axis and type 1 insulin-like growth factor (IGF-1), radiological elements, and in some cases include genetic studies[2]. To confirm the diagnosis, different pharmacological stimulus tests (i.e., using clonidine, arginine, levodopa, glucagon, among others), and physiological tests (e.g., exercise test and determination of GH secretion during sleep) are used for the GH secretion. There are different protocols for the study of the GH production involving drugs that stimulate its secretion. Stimulus tests are not standardized, and many of them are associated with adverse effects; thus, in some centers, they are contraindicated in children under two years of age. It is therefore necessary to find new, more standardized and reliable diagnostic methods.

Currently, there are no specific and reproducible methods to definitively diagnose of GHD, especially in breastfeeding and preschool-age patients. Alternative methods of diagnosis in these patients have been sought, including the use of filter paper samples for neonatal screening for congenital hypothyroidism and phenylketonuria. This is because in the first few days of life, there is an increased spontaneous GH secretion[3], where this is a window period in the diagnosis of GH production sufficiency, a concentration that remains increased for a longer time in preterm newborns. Cases with GHD should have deseased GH secretion in this period without the physiological increase of GH production that normally occurs in newborns. It has been proposed to determine neonatal GH concentration in filter paper sample as another element in the study of the somatotropic axis function. Binder et al.[4] demonstrated the usefulness of this method in patients with multiple pituitary hormone deficiencies (MPHDs), but not in patients with isolated GH deficiency (IGHD).

The objective of this study is to determine whether filter paper blood sample taken in the neonatal period allows patients who have congenital GHD, both isolated and associated with deficiency of other pituitary hormones, to be distinguished from patients whom this pathology can be disregarded.

Patients and Method

Subjects and controls

A case-control study was conducted. Cases included patients with GHD diagnosis who were born in the UC-Christus Health Network between 2010 and 2017 and had at least one altered stimulus test of GH secretion, with a plasma concentration less than or equal to 7.0 ng/ml (used cut-off point as a reference in the Chilean Ministry of Health unpublished guideline for the treatment of GH deficiency) (1), associated with auxological criteria (length or height < -2 SD) and/or growth rate loss (< -1 SD), and biochemical and radiological studies highly suggestive of GHD (hypoglycemia, neonatal jaundice, micropenis, altered pituitary anatomy, decreased IGF-1 and IGF binding protein-3 (IGFBP-3), decreased GH in critical sample obtained in hypoglycemia (<15.0 ng/ml in the newborn period or < 7.0 ng/ml after the newborn period).

As controls, patients with low height and a GH stimulus test result of > 7.0 ng/ml were selected.

Candidates were identified by reviewing the clinical records database and by direct contact with pediatric endocrinologists in the UC-Christus Health Network. Informed consent was requested from the parents or legal guardians of each patient. After obtaining this signed document, the clinical file was accessed to acquire the corresponding clinical and laboratory data for each candidate. All children in control in our health center were included, and all those without blood sample on filter paper or the clinical and laboratory information needed to classify them as GHD were excluded. Collected data including date of birth, current age (time of filter paper preservation), diagnosis, sex, gestational age, birth weight, clinical elements suggestive of GHD, the results of somatotropic axis and GH secretion stimulation test, age at diagnosis, and results indicating involvement of other pituitary axes.

Conditions for sample taking on filter paper and storage

Individuals in both groups had a blood sample on filter paper taken in the neonatal period and stored in the clinical laboratory of our center. Dried blood samples on paper filters kept at room temperature (5,6) were analyzed. The filter paper age, which was defined as the conservation period from sampling to analysis in our study, was considered a likely confusing variable.

Tests and laboratory techniques

The sample for each patient was taken from the filter paper by obtaining two 0.125 inch disks from the

center of the blood spot on the paper using the method indicated by the filter paper manufacturer (Whatman, United Kingdom). The obtained sample was diluted with commercially available buffer (human GH ELISA buffer), provided by the manufacturer. The GH content in the eluate was measured by a highly sensitive ELISA method (Mediagnost, Reutlingen, Germany). The test is based on the use of polyclonal antibodies directed against recombinant human GH of 22 kDa molecular weight. The test is calibrated according to International Standard 98/574. The obtained results were expressed in ng/ml. The determinations were made in duplicate. An analysis was performed on all available samples.

Statistics

The collected data were tabulated in Microsoft Excel® spreadsheets. SPSS® and Prism® GraphPad software were used for statistical analysis. The Mann-Whitney test was used for reporting medians and interquartile range. Spearman's test was used for correlation analysis. $P < 0.05$ was considered significant.

Ethics

The study was approved by the ethics committee of the Faculty of Medicine of the Pontifical Catholic University of Chile, project number 16-121. All parents or legal guardians were asked to sign an informed consent form.

Results

Sample characteristics

Twenty participants were recruited during the analysis period. Ten were patients with GHD, of whom only seven had neonatal blood sample on filter paper stored in the UC-Christus Health Network laboratory (Table 1), and ten individuals were controls.

Fig. 1 compares the newborn period characteristics of GHD patients and the controls. Both groups were similar in gestational age (fig. 1A), birth weight Z score (fig. 1B), and birth length Z score (fig. 1C).

GH results in filter paper samples

In patients diagnosed with GHD, GH concentrations ranged from 0.2 to 7.1 ng/mL, while individuals without GH deficiency had GH concentrations ranged from 1.0 to 6.6 ng/mL. Although no differences were observed between the medians of GH concentration between both groups, it is important to note that the two participants with MPHDs presented concentrations below the lower limit observed in those without GH deficiency (less than 1.0 ng/mL) (fig. 2B).

The median filter paper age was 18 months (IQR=3-52 months) in cases and 67 months for controls (IQR=56.5-83.75 months); two-sided Mann-Whitney U = 3.0; P = 0.02. There was no association between the filter paper age and the result of the GH

Table 1. Patients description

	Sex	Diagnosis age (months)	Diagnosis	Age at blood sample (days)	Diagnostic GH (pg/mL)
Cases					
Case 1	F	0	MPHDs	11	0.05
Case 2	M	0	IGHD	15	5.65
Case 3	F	0	IGHD	7	4.06
Case 4	M	18	IGHD	2	3.20
Case 5	M	0	IGHD	2	2.07
Case 6	M	8	IGHD	2	7.00
Case 7	M	44	MPHDs	2	6.00
Controls					
Control 1	F	16	Short stature	8	11.20
Control 2	F	39	Short stature	7	8.05
Control 3	F	65	Short stature	2	12.10
Control 4	M	8	Short stature	9	20.80
Control 5	F	79	Short stature	3	12.30
Control 6	M	26	Short stature	8	18.30
Control 7	F	65	Short stature	2	10.70
Control 8	F	67	Short stature	1	11.30
Control 9	F	20	Short stature	2	9.54
Control 10	M	61	Short stature	3	19.10

GH: Growth hormone, F: female, M: male, MPHDs: Multiple Pituitary Hormone Deficiencies; IGHD: Isolated Growth Hormone Deficiency.

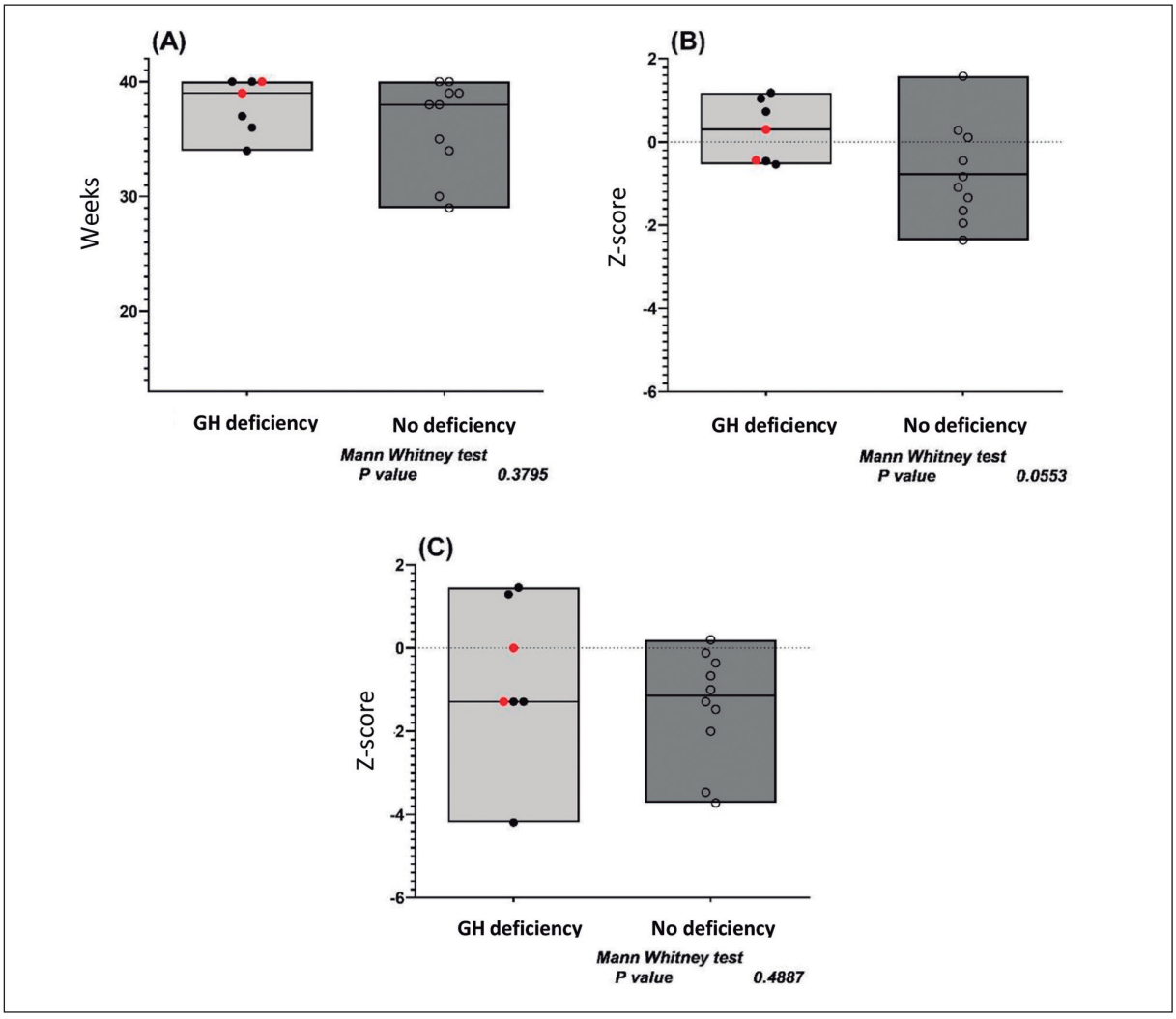

Figure 1. (A) Gestational age, **(B)** Wight at birth, **(C)** Length at birth. Red circles: Multiple pituitary hormone deficiencies. Black circles: Isolated growth hormone deficiency. GH: Growth Hormone.

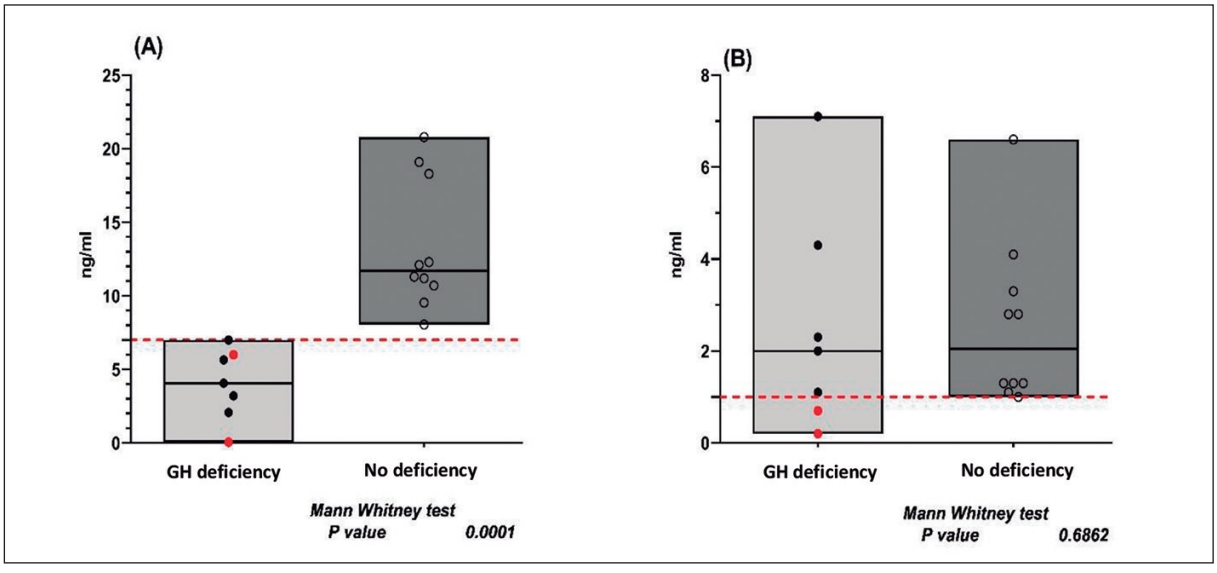

Figure 2. (A) Growth hormone concentration at diagnosis. **(B)** Growth Hormone on blood spot sample.

concentration in these samples (Spearman's Rho = -0.1; p = 0.704; fig. 3).

Discussion

Participants with MPHDs had significantly lower concentrations of GH in neonatal filter paper samples than did individuals with IGHD or without GH deficiency. The GH determination on filter paper stored at room temperature[5,7] was not useful for distinguishing between individuals with IGHD or without GH deficiency.

Binder et al.[7,8] demonstrated the feasibility of determining GH in blood sample on filter paper, validating laboratory tests for analysis, but in these cases, the sample were stored at at 6° C. In our center, filter paper samples were stored at room temperature protected from humidity and sunlight. As shown in the results, there is no direct correlation between storage times of the samples under these conditions and the obtained GH concentration results.

From our results, we can observe that there is no significant difference between the GH concentration obtained from filter paper samples of patients with an IGHD diagnosis and of control participants.

The two MPHDs individuals had GH concentrations lower than the minimum range observed in both IGHD and control participants, with filter paper GH concentrations of < 1 ng/ml. These results are consistent with what was observed in a previous study[7]. It should be noted that one of the patients with MPHDs was diagnosed at the age of 44 months and could lead to the increased risk of hypoglycemia and neurodevelopmental involvement.

Case 1 was diagnosed in the neonatal period in the context of a critical sample due to severe hypoglycemia (17 mg/dl) and the presence of craniofacial malformations. MR shows pituitary aplasia. Case 7 was diagnosed at 44 months of age, and a study was performed due to the presentation of ketotic hypoglycemia (18 mg/dl blood sugar) at three years of age associated with a seizure. The study was completed with GH secretion stimulus test with clonidine that showed GH deficiency.

Patients with MPHDs constitute a broad clinical spectrum in which simple phenotypes without cerebral anatomical alterations and that correspond to pathogenic variants of the late genes involved in pituitary gland formation have been described. On the other hand, there are subjects with MPHDs with a complex phenotype secondary to pathogenic variants of genes involved in early brain development, such as septo-optic dysplasia and holoprosencephaly, among others. Patients with MPHDs often have severe hypoglycemia due to the absence of cortisol and GH, two essential hormones for maintaining glucose homeostasis. Failure to make a timely diagnosis in the neonatal period could be associated with irreversible neurological sequelae due to repeated hypoglycemia.

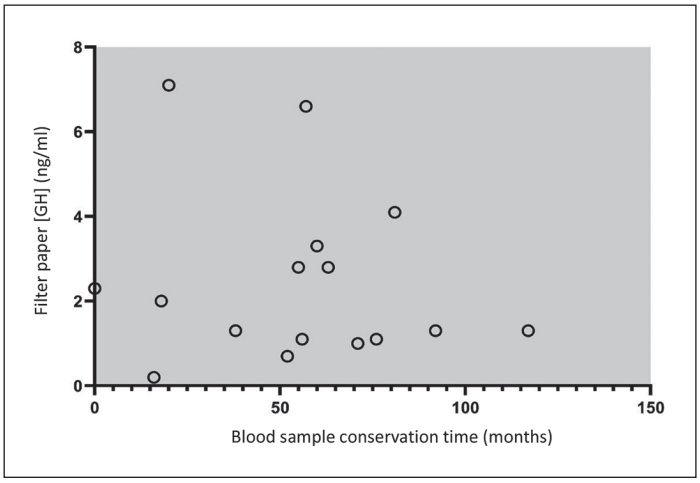

Figure 3. Correlation between time of conservation of filter paper sample and growth hormone concentration ([GH]) in filter paper sample.

The Ministry of Health suggests that stored filter paper samples for neonatal screening should preferably be refrigerated but can be stored at room temperature, nevertheless, the maximum time they can be stored in this way is not clear[9]. However, samples storage at room temperature appears to directly affect the result obtained in the laboratory test, such as decreased GH concentration in all samples. This could explain why the results previously reported in the literature, in which filter papers are stored at 6°C, are completely different from ours.

As already mentioned in other reports, GH concentrations detected in filter paper samples stored at 6ºC decreased by a relatively small magnitude[8]. It should be noted that the control patients, in whom a higher GH concentration would be expected, had filter papers stored for a longer period of time, which could have a direct impact on the results obtained in our research.

Further evaluation of the viability of blood samples on filter paper stored for long periods to that will be used as a diagnostic method for GHD or other pathologies is required, especially considering the effect storage conditions have on the sample stability.

The blood sample on filter paper is easy to obtain, is minimally invasive and easily accessible via puncture and requires a limited amount of blood, allowing it to be performed on even the smallest newborns[5]. The GH determination in blood stored in filter paper could be useful in identifying severe cases of GHD or MPHDs, especially in infants and preschoolers without a critical

sample during hypoglycemia and in whom performing a pharmacological stimulus test to release GH might be concerning. A new study with filter paper stored refrigerated and not at room temperature is required to validate this hypothesis.

The results of our study may be limited given the insufficient sample size, as we have a limited number of patients at our health center who had their filter paper available. In addition, samples at other centers were not accessible.

We suggest changing the current storage of filter paper with blood obtained in the newborn period; therefore, we propose that these samples be refrigerated and stored in individual bags to prevent moisture formation at least at 6 º C and for a period of five years.

Determination of GH and other hormones, such as cortisol, from the filter paper sample obtained during neonatal screening may be useful for patients in whom no critical hypoglycemic sample is available and thus may have additionallt support the diagnosis of MPHDs, which may carry life-threatening risk.

Ethical Responsibilities

Human Beings and animals protection: Disclosure the authors state that the procedures were followed according to the Declaration of Helsinki and the World Medical Association regarding human experimentation developed for the medical community.

Data confidentiality: The authors state that they have followed the protocols of their Center and Local regulations on the publication of patient data.

Rights to privacy and informed consent: The authors have obtained the informed consent of the patients and/or subjects referred to in the article. This document is in the possession of the correspondence author.

Financial Disclosure

Authors state that no economic support has been associated with the present study.

Conflicts of Interest

Authors declare no conflict of interest regarding the present study.

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