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Original Research

GH-IGF1 Axis in Children with Cystic Fibrosis

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ABSTRACT

Objective: To verify whether growth hormone receptor gene expression plays a role in Cystic fibrosis (CF)children'sgrowth, as a consequence of the chronic inflammatory condition and malnutrition.

Design: We enrolled forty-nine prepubertal patients (24 males and 25 females) affected by CF in a stable clinical condition, 19 of whom had been diagnosed through newborn screening and 30 following presentation of symptoms. Patients had no significant comorbidity affecting growth or CFTR (Cystic Fibrosis Transmembrane conductance Regulator)-related diabetes requiring insulin therapy. Blood was collected during two follow-up visitsto measure IGF-I, GHBP and GH-R gene expression. Fifty-two healthy children, sex- and age-matched, were recruited as a control group.

Methods: In this study we compared BMI, height, weight, IGF-I, GHBP and GHR gene expression values (evaluated by Chemiluminescent Immunometric assay; ELISA and Real time PCR respectively) inCF patients diagnosed throughNBS (New born screening), or by symptoms (Late Diagnosis (LD)) and in healthy controls.

Results BMI increased significantly in patients between the time of diagnosis and check-ups ($p<0.001$) and particularly in the LD group, the median value was lower at diagnosis and significantly higher ($p<0.001$) at follow up visitscompared to controls. At the initial evaluation, higher levels of IGF-I (not statistically significant) were found in both theNBS groupand in the LDgroupcompared to the control group. At the second evaluation, significantly higher levels of IGF-I ($p=0.003$) were found in both the NBS and LD groups compared to controls; GHR mRNA expression had significantlyincreased ($p=0.013$) in LD patientsin comparison with the first evaluation and was significantly higher in the NBS and LD groups than in controls. GHBP values had significantly increased ($p=0.047$) in the NBS group after one year of therapy compared to first visit levelsand were significantly higher ($p<0,0001$) in the NBS and LDgroupscompared to controls.

Conclusion In our LD patients during childhood, we observed good auxological values and a GH/IGF-I axis function within normal range for the factor evaluated. However, earlier diagnosis through NBS might further minimize and prevent growth retardation, by reducing the duration of symptoms before treatment.

INTRODUCTION

Cystic fibrosis (CF) is a genetic disorder transmitted as an autosomal recessive mutation in the transmembrane conductance regulator (CFTR) gene. This gene codifies a protein, which is a chloride channel present in the apical membrane of epithelial cells,although it also has many other important regulatory roles.[1,2] In fact, as CFTR is widely expressed, CF affects most organs including the lung, pancreas, liver and kidney.[3,4]Moreover, individuals with CF show a high degree of variability in disease severity, complications and survival[3], due to the fact thatdifferentCFTR mutations cause a number of defects in the CFTR protein synthesis, trafficking or function.[5]

Like most chronic inflammatory diseases in childhood, CF is associated withimpaired growth.[2, 6, 7]The exact mechanisms of this effect are not known, although it is generally thought to be caused by concomitant severe disease complications due totheinflammation itself, as well as prolonged use of glucocorticoids and suboptimal nutrition.[8,9,10]Moreover, an important correlation has been found between poor growth and reduced long-term lung function in CF children.[11]

The GH-IGF axis is the most important endocrine axis involved in linear growth in children and adolescents. In children with chronic inflammation, growth failure may be affected by several mechanisms including GH\IGF-I defect, GH\IGF-I resistance, down-regulation of GH\IGF receptors, disruptions in downstream GH\IGF signalling pathways, or deregulation of IGF binding protein (IGFBPs)[7]. In CF children, growth impairment may be associated with abnormally low IGF-I concentrations. In fact, IGF-I signalling is mediated by CFTR and consequently CFTR dysfunction may impact linear growth, adding to indirect effects due to malnutrition.[10,11]Furthermore, IGF-I plays an important role in inflammation and in turn, is related to lung function and nutritional status in CF.

Management of CF has improved significantly in recent years. While infants born in the past would have been unlikely to live beyond their first year, infants today are likely to live well into adulthood, thanks to the introduction of newborn screening (NBS), more effective treatment, greater availability and improved genetic testing, using more specific and sensitive diagnostic criteria. The cornerstones of CF management are the treatment of respiratory infections, promotionof good nutrition and an active lifestyle.[12]The increased life expectancyof these patients has meant that attentionis now focusedon new complications of the disease, such

as endocrine dysfunctions;[13,14,15] in particular, dysfunctions involving the growth hormone (GH), the insulin-like factor (IGF) system, thyroid hormones, insulin and sex steroids.[2]

Therefore, even though the current strategy to improve growth and weightin CF children tends to focus on correction of exocrine pancreas insufficiency and nutritional supplements,careful evaluation of other endocrine complications such as growth hormone deficiency (GHD)should not be overlooked.In fact, the prevalence of GHD in pediatric CF patients ishigher than in the general population, although it should be noted that the relevant studies centered on children with growth delay.[13,16] Furthermore, it is thought that the anabolic action of GH and IGF-I could theoretically have beneficial effects in CF patients with growth failure, regardless of GH deficiency.[17]

The involvement of the GH\IGF-I axis in poor linear growth in CF children prompted us to verify whether growth hormone receptor (GHR) gene expression plays a role in CFchildren'sgrowth, as a consequence of the chronic inflammatory condition and malnutrition. We measured in serum IGF-I andGH binding protein levels; GHR gene expression was evaluated in lymphocytes, obtained from peripheral blood of CFpatients and from age-sex-matched controls. In CF patients, we analysedthese parameters at various points: when patients were in a clinically stable condition, a fewyearsafter diagnosis, and at one year after the first evaluation;we thenanalysed the valuesobtained together with anthropometric data and clinical activity, in order to discover whether they may be influenced by therapy in childhood.

METHODS

Patients

We enrolled forty-nine consecutive CF patients (23 males and 26 females) attending regular follow up at Bambino Gesù Children's Hospital in Rome and Federico II University Hospitalin Naples. Patients were enrolled if they were diagnosed with CF according to current diagnostic criteria,[18] prepubertal and in stable clinical condition. Exclusion criteria were diagnosis of another significant comorbidity affecting growth and CFTR-related diabetes requiring insulin therapy. Of these CF patients, 19 had been diagnosed by newborn screening (NBS) and 30 had been diagnosed following presentation of symptoms (Late diagnoses LD). Spirometry was performed in patients aged 6 years or more. Mild lung disease (FEV1 >70%) were found in 18 patients whereas 6 patients had moderate lung disease (FEV1 >40%<70%). None of the patients had severe lung disease (FEV1 <40%). Standard therapy for CF was

prescribed (pancreatic enzymes in pancreatic insufficient patients, liposoluble vitamin supplementation in all patients, ursodeoxycholic acid in patients with CF-liver disease, and dornase alfainhalation, inhaled antibiotics, or oral azithromycin according to the patient's clinical condition) Table 1 shows the characteristics of the CF patients and healthy subjects.

Patients were prospectively followed for 1 year. Blood was collected during follow-up visits in clinically stable conditions (a few years after diagnosis) and again one year later the first evaluation, to measure IGF-I, GHBP and GH-R gene expression.

Fifty-two healthy children, sex- and age-matched (mean age: 8.95 ± 0.38 years), were recruited as a control group.

Written informed consent was obtained from parents or legal guardians of all children, and patients over 13 years of age signed a statement of assent. The study was approved by the Ethics Committee of the centers involved in the study.

GHBP Evaluation

Serum levels of GHBP were measured usinga commercially available ELISA (DSL-10-48100 ACTIVE hGHBP Elisa-Webster, Texas, USA). The minimum detectable concentration was 1.69 pmol/l. The intra- and inter-assay coefficients of variation were 5.59%-4.78% and 8.36%-5.11%, for a quality control range of 20.25–198.24 pmol/l and 19.99–195.78 pmol/l, respectively.

IGF-I Determination

The serum IGF-I concentration was measured usingan automatic assay that utilizes a solid-phase, enzyme-labeled chemiluminescent immunometric assay (Immulite 2 000 IGF-I-DPC, Los Angeles, CA and Immulite Analyzer). The intra-assay coefficients of variation were 3.9%-2.4%, for a quality control range of 77-1 358 ng/ml. IGF-I values are expressed as mean standard deviation score (SDS) according to Elmlinger MW et. Al.[19]

GHR Gene Expression

Peripheral blood mononuclear cells (PBMC) of patients and age-matched controls were separated by Ficoll density gradient centrifugation using a standard procedure (centrifugation

at 1800 rpm for 30 minutes at room temperature, followed by the recovery of the PBMC ring at the interface).

For Real-Time GHR gene expression analysis, total RNA was isolated from PBMC using RNAeasy mini-columns (Qiagen, Hilden, Germany). RT-PCR was carried out usingthe SuperScript First-Strand Synthesis System. An RNA/primer mixture containing total RNA, oligo dT (50 ng/µl), 10mM dNTP mix and DEPC water was prepared. The samples were incubated at 65°C for 5 minutes and then on ice for at least 1 minute. A master reaction mixture, containing 10X RT buffer, 25 mM MgCl2, 0.1 M DTT and RNAase OUT was prepared for each sample. The reaction mixture was then added to the RNA/primer mixture, samples were mixed briefly and kept at room temperature for 2 mins. Fifty units of SuperScript II RT wereadded to each tube, the samples were mixed and incubated at 25°C for 10 mins, and the tubes were then incubated at 42°C for 50 mins, heat inactivated at 70°C for 15 mins, and chilled on ice. First strand cDNA was stored at –20°C until use for real-time PCR.

Quantitation of GHR mRNA expression was determined by quantitative real-time RT-PCR (Real-Time PCR 3500-Applied Biosystems) and assays on demand were used (Hs00174872_m1 Applied Biosystems). Normalization and validation of the data were carried out using GADPH as a housekeeping control gene. Each GHR or GAPDH probe was labeled with a fluorescent reporter (FAM. Specifically, a twenty-five microliter volume reaction mixture containing 1.25 µl Assay, 12.5 µl Master Mix, 10.25 µl H2O and 1 µl cDNA was treated under the following conditions: 95°C for 10 mins, 95°C for 15 s, 60°C for 1 min, for 40 cycles).

Quantitative Real-time PCR data were calculated using a standard curve and expressed as agGHR/5X105 agGAPDH.

The amplification efficiency of GHR in relation to GAPDH mRNA expression was evaluated by analyzing the ΔCt variation with template dilutions in the 1000-fold range.

Statistical Analysis

Data are expressed as the median±interquartile range. Statistical differences between patients and controls were determined using the Mann-Whitney U test, whereas the non-parametric Wilcoxon test for paired samples was used to compare values in patients at baselineand after

one year. Statistical differences between groups of different subjects were determined using the one-way ANOVA test, when a normal distributionof data was observed, and the non-parametric Kruskall-Wallis test when the data were not normally distributed. If a statistical significance was found, an adequate post-test identified which group differed from which. A value of $p< 0.05$ was considered statistically significant.

RESULTS

In this study we compared BMI, height, weight, IGF-I, GHBP and GHR gene expression values inCF patients diagnosed by NBS, or by symptoms (LD) and in healthy controls. We evaluated length, weight and BMI (Table 1) at diagnosis, at a follow-up visit (5 years from diagnosis), in stable clinical conditions, and one year after the first measurement.IGF-I, GHBP and GHR gene expression were measuredat two follow up visits only (5 years from diagnosis and one year after the first measurement.

Median height at time of diagnosis was higher in patients than in controls but the difference was statistically significant ($p=0.005$) only in NBS patients. In the LD group, height showed a significant increase ($p<0,05$) at the first follow up visit compared to diagnosis. For all patients, height was significantly higher compared to the control group ($p<0.0001$) at the second follow up visit (Table 1).

BMI increased significantly in patients between the time of diagnosis and check-ups ($p<0.001$) and in particular in the LD group, the median value was lower than the controls' value at diagnosis and significantly higher ($p<0.001$) at follow up visits. The NBS group also displayed significantly higher BMI at follow up visits compared to controls (Table 1).

Overall, patients' weight significantly increased ($p<0.002$) between diagnosis and follow up visits but no significant differences were found between the two groups of subjects, although the median level of LD patients was consistently lower than that of NBS patients. No significant difference was found in weight between patients and controls (Table 1).

In summary: LD children were found to have reduced weight, height and BMI values compared to NBS children. In particular, 5 patients were under -2 SD for weight in the LD group compared to just one in the NBS group; four children had pathological height in the LD group but no children were found to be under 2 SD in the NBS group

At the initial evaluation, higher levels of IGF-I were found in the NBS group (median level 0.24SDS within aninterquartile range of 0.04 to 0.59)and in the LD group (median level 0.37SDSwithin an interquartile range of0.09 to 1.24) compared to the control group (median value -0.5SDS within aninterquartile range of -0.75 to 0.09), although the differenceswere not statistically significant(Fig. 1). On the contrary, GHBP was significantly lower ($p<0.0001$) in the NBS and LD groups (median 25.ng/ml within an interquartile range of 21.35 to 27.9; median 36 ng/ml within aninterquartile range of 23.6 to 47respectively) compared to controls (median: 145ng/ml within an interquartile range of 95 to 201.25) (Fig. 2).

GH-R mRNA expression was significantly ($p=0.036$) higher in the NBS group (median value 681.3.agGHR/5X10^5agGAPDH within an interquartile range of 287.36 to 5090.86) but not in the LC-FC group (median value 768.89agGHR/5X10^5agGAPDH within an interquartile range of 50.45 to 7851.79)compared to controls (median value 71.83agGHR/5X10^5agGAPDH within an interquartile range of 28.56 to 981.14) (Fig. 3).

At the second evaluation, during the 1-year follow-up visit, significantly higher levels of IGF-I ($p=0.003$) were found in the NBSgroup (median level 0.91SDS within aninterquartile range of0.38 to 1.14) and in the LD group (median level 1.02SDS within aninterquartile range of0.87 to 1.52) compared to controls.

Furthermore, GHR mRNA expression had significantlyincreased ($p=0.013$) in LC-FC patients (median value 4338.67agGHR/5X10^5agGAPDHwithin an interquartile range of240.86 to 37690.16**)** in comparison with the first evaluation. Moreover, GHR mRNA expression levels were significantly ($p=0.022$) higher in the NBS group (median value 646.86agGHR/5X10^5agGAPDHwithin an interquartile range of268.39 to 1000**)** and the LD group ($p=0.0003$) than in controls.

In addition, GHBP values had significantly increased ($p=0.047$) in the NBS group after one more year of therapy (median value 48 ng/ml within an interquartile range of 30.5 to 50.75) compared to first visit levels. Furthermore, GHBP levels were significantly higher ($p<0,0001$) in the NBS and LD groups (median value 35within an interquartile range of 25.8 to 54.8**)** compared to controls.

DISCUSSION

Growth failure is a common feature in many children with chronic diseasessuch as CF. Both altered GH metabolism and organ resistance to GH have been implicated as major contributors of growth retardation in CF subjects.[20]Chronic undernutrition and inflammatory cytokines are the principal and interrelated determinants of GH resistance, whose negative effect may be compounded by long-term steroid therapy. On the contrary, there is evidence that higher serum IGF-I levels are associated with better health in CF because they reflect a link between nutritional sufficiency and lung function in CF.[11]

Before NBS, many patients showed persistent growth failure due to a delay in the diagnosis of CF. The advent of NBS improved clinical outcomes of CF patients, in particular regardingweight and stature.[21]Therefore, in our study we consideredboth CF children who underwent NBS and thosewho were diagnosedlate. We analyzed the differences in weight, height and BMI at diagnosis and again some years later, when they were in a clinically stable condition, in order to confirm data on benefits of NBS. Furthermore, we evaluated some parameters relating to the GH/IGF-I axis (GHR gene expression, IGF-I and GHBP) to verify if significant differences existamong children diagnosed by NBS, subjects diagnosed by symptoms, and controls.

Our results showeddifferences inheight, weight and BMI between NBS children and LDchildrenat diagnosis. Even though these differences were not statistically significant at median level, LD children were found to havereduced weight, height and BMI values compared to NBS children. In particular, 5 patients wereunder -2 SD for weight in the LD group compared to just one in the NBS group; four children had pathological height in the LD group but no children were found to be under 2 SD in the NBS group. These data show that the LD children wereat a disadvantage compared to the NBS group at diagnosis, probably because of thelonger interval between the onset of symptoms and diagnosis in this group. Some years later, the children in both these groups showed significantly higher weight-for-age values compared with the values recorded at diagnosis, as well as significantly higher BMI and height comparedto healthy controls, thanks to appropriate and prompt nutritional intervention, although the LD group's values continue to be lower than those of the NBS children. Darrah et al.[22]identified a relationship between birth weight of newborns affected by CF and later childhood pulmonary function. In contrast to these findings, we did not find any significant correlation between weight at diagnosis and pulmonary function expressed as FEV_1 (%). The different sampling of

subjects and the longer time between the diagnosis and the evaluation of lung function may explain the differences in our results.

Previous studies[21]showed that despite NBS and prompt nutritional intervention, a sub-group of patients with CF was unable to overcome growth deficits, suggesting that other factors contributed to poor weight gain and linear growth.Hence, we evaluated IGF-I, GHR gene expression and GHBP levels during childhood, when patients were in clinical stable condition.We found no significant differences in these parameters between NBS and LD children,but there were significantly higher values of IGF-I and GHR expression and lower levels of GHBP in CF patients compared to healthy controls. In particular, in the NBS patients, GHR gene expression and serum GHBP values werealready significantly different at the first visit compared to controls. Therefore, in our LD patients during childhood, we observed a good auxological condition and a GH/IGF-I axis function in normal range regarding factor evaluated. However, earlier diagnosis with NBS might further minimize and prevent growth retardation, i.e., in height and weight, by reducing the duration of symptoms before treatment, as opposed to late diagnosis based on symptom presentation and treatment aimed at rectifying the growth inhibiting effects of malnutrition and inflammatory cytokines.

Furthermore, our data showthat GHBP level and GHR function are not closely correlated, as demonstrated also in other physiological and pathological conditions.[23] GHR regulation and its cleavage to GHBP istissue-specific. Therefore, it is possible that, in our patients, these mechanisms lead to an increase in GHR availability on the cell's surface, thus improving GH action. [24]

Further studies are mandatory in order to confirm our results and improve the quality of life of CF sufferers.

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FIGURES

Figure 1: Serum values of IGF-I in NBS, LD patients and controls, at first evaluation(1) and at 1-year follow-up (2), expressed as SDS (Standard Deviation Score).

***:** p= p=0.003Patients NBS (2) vs Controls

****:** p= p=0.003 Patients LD (2) vs Controls

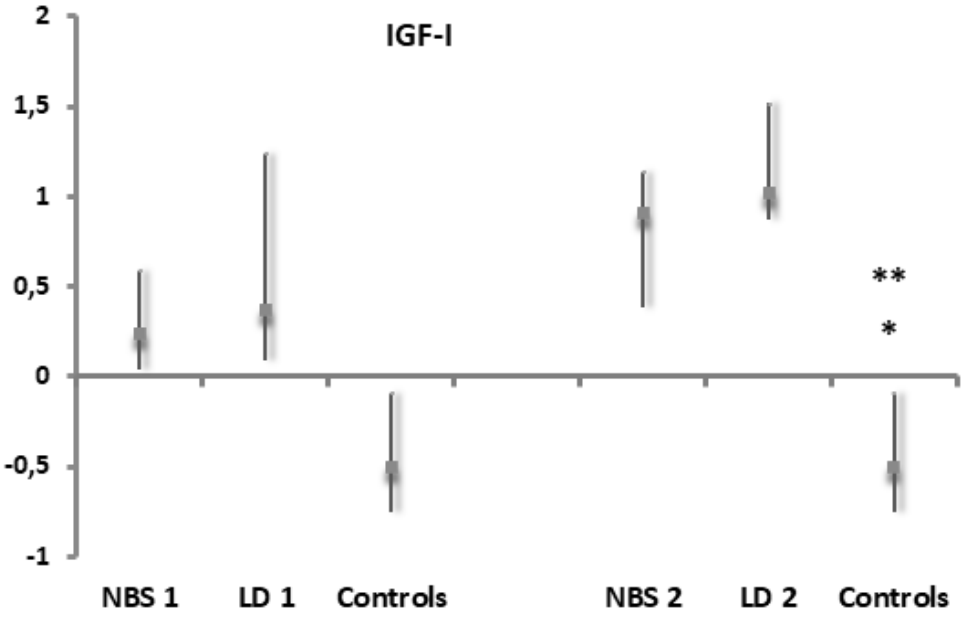

Figure 2: Serum value of GHBP in NBS, LD patients and controls, at first evaluation (1) and at 1-year follow-up (2), expressed as ng/ml.

***:** p<0.0001 Patients NBS (1) vs Controls and Patients NBS (2) vs Controls

**: p<0.0001 Patients LD-FC (1) vs Controls and Patients LD (2) vs Controls

0.047 Patients NBS (2) vs Patients NBS (1) vs

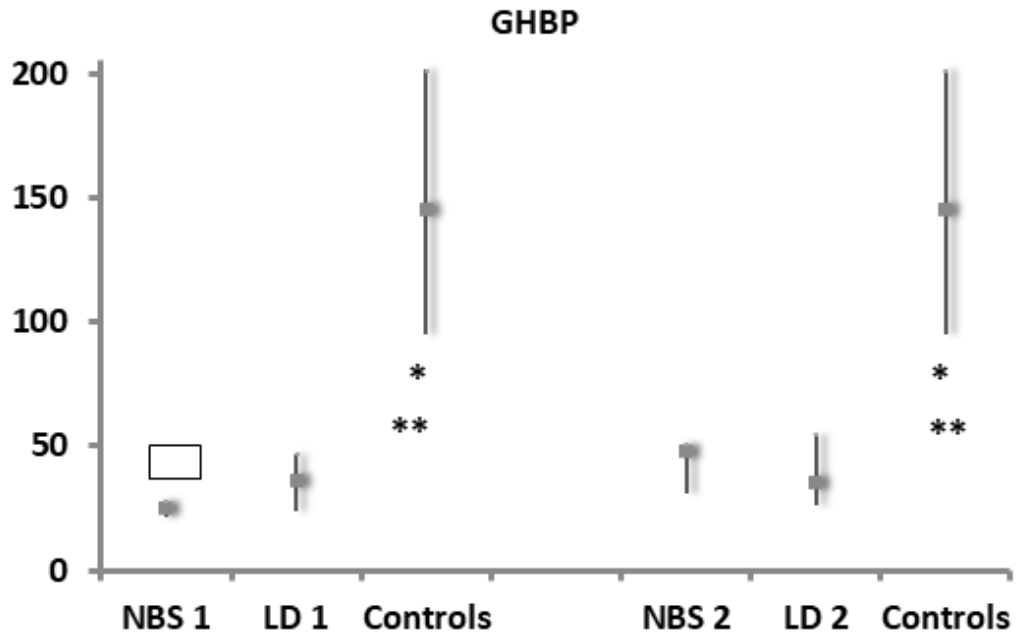

Figure 3: GHR gene expression values in NBS, LD patients and controls, at first evaluation (1) and at 1-year follow-up (2), expressed as agGHR/5X10^{5}agGAPDH.

***:** p= p=0.036 Patients NBS (1) vs Controls and p=0.022 Patients NBS (2) vs Controls

**:p=0.0003 Patients LD (2) vs Controls

▭**:** p=0.013 Patients LD (2) vs Patients LD (1) vs

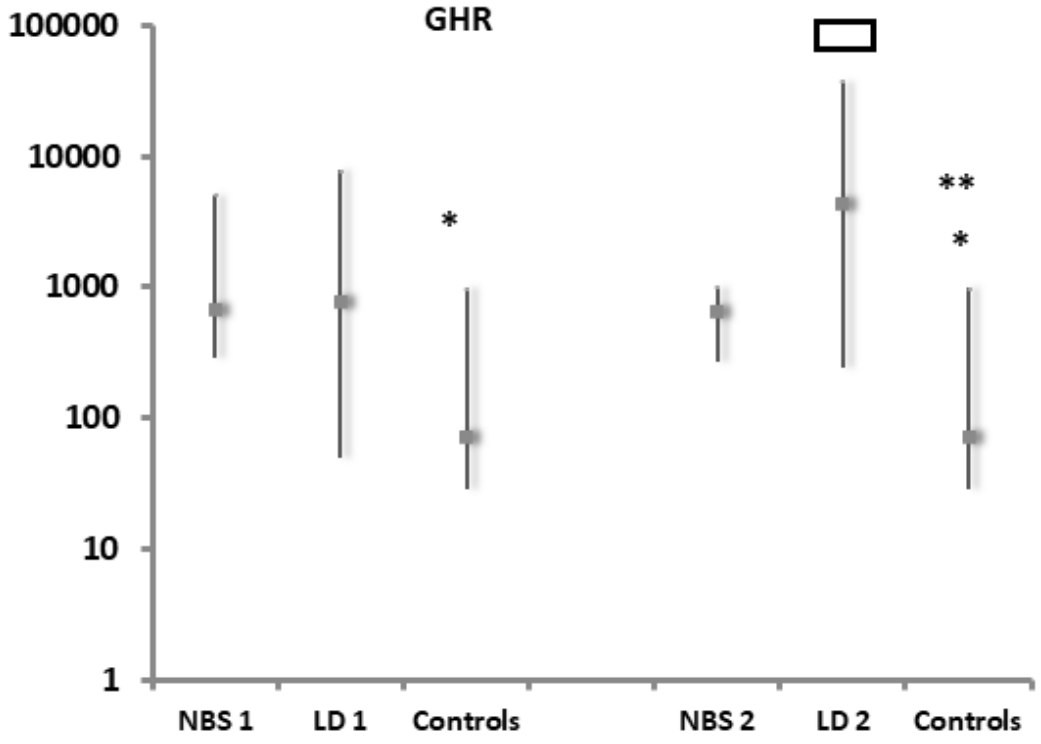

Table 1. Demographic and anthropometric characteristics of patients and controls.

	NBS	LD-FC	CTRL
N	19	29	52
Age I (Years)	5.09±0.46	7.16±0.43	8.9±0.38
Height-0 (SDS)	-0.21*(-0.72—0.88)	-0.59°(-1.85-0.34)	
Height-1 (SDS)	0.99*(0.19-1.49)	0.35**(-0.36-1.2)	-0.84(-0.99—0.49)
Height-2 (SDS)	0.76*(0.02-0.94)	0.29**(0.55-1.49)	
Weight 0 (SDS)	-0.7(-1.38-0.48)	-1.3(-1.9—0.2)	
Weight 1 (SDS)	0.63*°(0.015-1.56)	0.44**° (0.07-1.01)	-0.97(-1.33—0.02)
Weight 2 (SDS)	0.74°°(0.17-1.28)	0.59°°(-0.2-1.2)	
BMI 0 (SDS)	-0.325(-2.445-0.32)	-1.435(-4.14-2.18)	
BMI 1 (SDS)	0.4°*(-0.51-1.09)	0.09°*(-47-0.74)	-1.1(-1.59—0.2)
BMI 2 (SDS)	0.1°°** (-0.4-0.86)	-0.14**(-0.6-1.18)	
$FEV_1$1 (%)	88.5(80-103)	94(83.75-108.25)	
$FEV_1$2 (%)	96(84.25-105.75)	101(91-110)	

Except age (expressed as mean ± SE), all data are expressed as median value and interquartile range.
Key: *NB patients vs controls; ** LD patients vs controls °LD or NB patients at diagnosis vs LD or NB patients at first follow up visit respectively; °° LD or NB patients at diagnosis vs LD or NB patients at second follow up visit respectively.
0: patients at diagnosis; 1: patients at the first follow-up visit; 2: patients at the second follow-up visit.
SDS= Standard Deviation Score