Changes in Growth Hormone Receptor Gene Expression during Therapy in Children with Juvenile Idiopathic Arthritis

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Abstract

Background: High levels of cytokines in juvenile idiopathic arthritis (JIA) can alter target cell sensitivity to growth hormone (GH) leading to short stature in adulthood. We hypothesized that the down-regulation of GH receptor (GHR) gene expression could be involved in growth failure of children with JIA. **Methods:** In 18 (12 F and 6 M) prepubertal JIA patients and 13 age- and sex-matched healthy children, we evaluated serum growth-promoting factors and inflammatory indexes. We also measured GHR gene expression, by real-time PCR, in lymphocytes of patients and controls. All parameters were evaluated in patients before and after treatment of JIA. **Results:** The most interesting (p = 0.007) result was the increase in GHR mRNA expression in all JIA patients. Moreover, we observed a significant (p = 0.0156) decrease in IL-6 levels in JIA patients after 2 years of therapy (19.37 ± 41.01) with respect to basal values (90.84 ± 124.71). On the contrary, IGF-I significantly (p = 0.0005) increased to a mean SDS value of 0 (range –1.69 to +1.70 SDS) with respect to values at disease onset (–0.64 SDS). **Conclusions:** Our preliminary data suggest that the restoration of both GHR gene expression and IGF-I secretion correlate with inactive disease in JIA children.

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Introduction

Growth is often negatively affected in pediatric diseases characterized by chronic inflammation, such as juvenile idiopathic arthritis (JIA) [1, 2]. The goals of JIA treatment at onset are to suppress chronic synovitis, control systemic inflammation, relieve pain, avoid deformities and improve linear growth [1]. Chronic inflammation is considered the basis of the pathology in JIA [1]. In detail, evidence has shown that pro-inflammatory cytokines such as interleukin-1 (IL-1), 6 (IL-6) and tumor necrosis factor α (TNF-α) are important mediators of chronic inflammation in JIA [2–4]. Markedly elevated levels of circulating IL-6 have been observed in children suffering from JIA in association with laboratory and clinical variables of disease activity [2, 3]. Moreover, in children affected by systemic arthritis, an inverse association between increased IL-6 and low serum levels of insulin-like growth factor-I (IGF-I) and IGF-I binding

Table 1. Patient demographic and clinical characteristics at recruitment

Characteristics at enrollment	JIA	Control group
Female, n (%)	12 (66.6)	7 (53.8)
Age at enrollment, years, mean	7.6	8.6
Disease subtypes		
Oligoarticular, n (%)	16 cases (88.8)	
Polyarticular, n (%)	1 case (5.6)	
Psoriatic, n (%)	1 case (5.6)	
Antinuclear antibodies positive, n (%)	16 cases (88.8)	
Therapy		
Non-steroidal anti-inflammatory drugs	13 cases (72.2%)	
No therapy	5 cases (38.5%)	
Height, SD, mean and range	0.03 (−2.12/2.38)	−0.86 (−1.6/0.98)
ESR, mm/h, mean and normal value	49 (<20)	
CRP, mg/dl, mean and normal value	3 (<0.05)	

protein-3 (IGFBP-3) raises the possibility of a direct link between inflammation and the growth hormone (GH)-IGF-I axis [4]. Although chronic inflammation is described in the pathogenesis of JIA, few data are available on the cytokine-growth factor relationship. Data on this relationship would be considered of interest in the study of the mechanisms involved in growth failure in JIA patients.

In the literature, there are very few reports evaluating growth velocity in JIA subjects not exposed to GH therapy [1, 2–7]. Moreover, in these few studies, children affected by systemic arthritis receiving steroid therapy have also been included. The results indicated that reduced growth velocity was associated not only with inflammatory activity and serum levels of IGF-I, but also with glucocorticoid therapy [2, 5, 6].

To the best of our knowledge, no reports have been conducted on GH receptor (GHR) gene expression levels in JIA patients. GH is the main regulator of longitudinal growth and its responsiveness in target cells is mainly dependent upon the expression of GHR. In fact, several studies have already shown that GH activity can be modulated through interference with GHR regulation [8].

The aim of this study was to verify whether GHR gene expression may play a role in the growth failure of children with JIA as a consequence of the chronic inflammatory condition. For this reason, we measured GHR gene expression by real-time PCR (RT-PCR) in lymphocytes from peripheral blood of prepubertal patients with JIA and from age- and sex-matched controls. We also compared circulating IGF-I in children with JIA and in controls in order to investigate whether the disease interferes with GH activity in cartilage and bone. Moreover, we correlated growth hormone binding protein (GHBP) levels

to cellular GHR gene expression. Finally, in JIA patients we analyzed the same parameters after 2 years and correlated these with anthropometric data and clinical activity during active disease, in order to discover whether they may be considered surrogate measures of disease activity in JIA. Inflammatory parameters were also evaluated to assess the activity of disease and the effects of therapy.

Patients and Methods

Patients

Eighteen (12 females and 6 males) prepubertal patients who fulfilled the diagnostic criteria for JIA at onset, according to the International League of Associations for Rheumatology, were enrolled at the Rheumatology Unit of Bambino Gesù Children's Hospital [9]. For each patient, joint involvement was clinically assessed by the same rheumatologist. All JIA patients had evidence of synovitis in at least one joint. Patients were consecutively recruited, over 8 months and were evaluated prospectively for 2 years. Exclusion criteria included systemic arthritis, which is usually treated with oral prednisone and may represent a confounding factor in the analysis of hormonal values and growth velocity [6, 10] since corticosteroids are known to be inhibitors of IGF-I biosynthesis [11]. Moreover, we excluded systemic JIA which may necessitate anti-IL-6 receptor antibody therapy, a confounding factor in the interpretation of IL-6 results [12].

Patients' demographic and clinical characteristics at recruitment are reported in table 1. Anthropometric measurements were performed by a professional nurse and reported in the clinical diary, according to World Health Organization standards. Stature was measured in patients when barefoot with a stadiometer. Height was expressed according to chronological age as the standard deviation (Z-score).

Puberty was assessed according to the Tanner classification at baseline and during the follow-up period. Children in the growth-spurt phase were stratified, characterized as Tanner 2 for girls and Tanner 3 for boys. The clinical assessment was repeated during

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the follow-up and 2 years after recruitment and during this period only 2 patients (1 male and 1 female) entered puberty.

The initial treatment of chronic arthritis was non-steroidal anti-inflammatory medication combined with local intra-articular injections of triamcinolone hexacetonide or methylprednisolone. When needed, second-line antirheumatics (sulfasalazine, methotrexate, etanercept) were added to the regimen, solely or in combination. Oral prednisone was never prescribed during the follow-up period (table 2). Blood was collected on the same day as the baseline clinical evaluation to measure erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), white blood cells, IL-6 levels, IGF-I, GHBP and GH-R gene expression.

At the Auxology Unit, 13 healthy children, sex- and age-matched, were recruited as a control group. Two years later, enrolled patients affected by JIA were reevaluated. Five patients (all female) were lost at follow-up, refusing the second year blood analysis and consequently dropped from the study.

Informed consent was obtained from each patient, a parent or legal guardian. The study was approved by the Ethics Committee at both the Bambino Gesù Hospital and Fondazione IRCCS Policlinico San Matteo Hospital.

Methods
IL-6 Determination

Serum levels of IL-6 were measured with a commercially available enzyme-linked immunosorbent assay (ELISA DuoSet; R&D Systems, Minneapolis, Minn., USA). The minimum detectable concentration by this assay was 4.68 pg/ml. The samples were diluted 1:5 in assay buffer. The intra- and interassay coefficients of variation were <7%.

GHBP Evaluation

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

IGF-I Determination

The serum IGF-I concentration was measured with an automatic assay that utilizes a solid-phase, enzyme-labeled chemiluminescent immunometric analyzer (Immulite 2000 IGF-I-DPC, Los Angeles, Calif. 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.

GHR Gene Expression

PBMC of patients and age-matched controls were separated by Ficoll density gradient centrifugation using a standard procedure (centrifugation at 1,800 rpm for 30 min 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 with the SuperScript First-Strand Synthesis System. An RNA/primer mixture containing total RNA, oligo dT (50 ng/µl), 10 mM dNTP mix and DEPC water was prepared. The samples were incubated at 65°C for 5 min and then on ice for at least 1 min. A master reaction mixture for each sample, containing 10× RT buffer, 25 mM MgCl$_2$, 0.1 M DTT

and RNAase OUT was prepared. The reaction mixture was then added to the RNA/primer mixture, samples were mixed briefly and kept at room temperature for 2 min. 50 units of SuperScript II RT was added to each tube, then the samples were mixed and incubated at 25°C for 10 min. The tubes were incubated at 42°C for 50 min, heat inactivated at 70°C for 15 min, and chilled on ice. First-strand cDNA was stored at –20°C until use for RT-PCR.

Quantization of GHR mRNA expression was determined by quantitative 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. The GHR probe was labeled with a fluorescent reporter (FAM) and the GAPDH probe was labeled with FAM. In detail, a 25-µl volume reaction mixture containing 1.25 µl assay, 12.5 µl Master Mix, 10.25 µl H$_2$O and 1 µl cDNA was treated under the following conditions: 95°C for 10 min, 95°C for 15 s, 60°C for 1 min, for 40 cycles.

Relative quantification (RQ) was obtained using the $2^{-\Delta\Delta Ct}$ method, with respect to the control condition (RQ = 1). Variations in input cDNA mass were corrected by normalizing all data with the corresponding GAPDH levels. The amplification efficiency of GHR with respect to GAPDH mRNA expression was evaluated by analyzing the ΔCt variation with template dilutions having a 1,000-fold range.

Statistical Analysis

Data were expressed as the mean ± SEM. Statistical differences between patients before therapy and controls were determined using the Mann-Whitney U test; the non-parametric Wil-
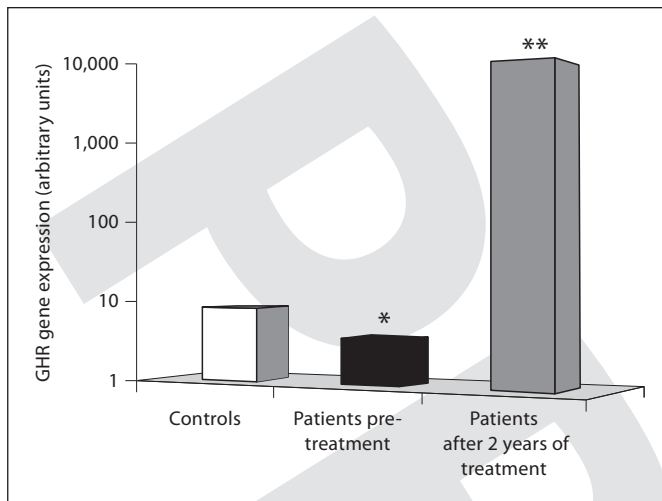
Table 2. Patient therapy

Pa-tients	Arthrocentesis n	FANS months	Metho-trexate months	Etaner-cept months	Sulfasala-zine months
1	1 (at onset)	3	0	0	0
2	0	4	0	0	0
3	1	2	0	0	0
4	1 (at onset)	1	0	0	0
5	0	4	0	0	0
6	2	1	6	0	0
7	0	7	0	0	0
8	0	1	16	15	0
9	1 (at onset)	5	0	0	0
10	0	5	0	0	0
11	1 (at onset)	2	0	0	0
12	1 (at onset) +1	5	0	0	0
13	1	3	20	17	0
14	0	2	8	6	0
15	0	2	7	6	0
16	0	2	2	2	0
17	0	1	20	20	0
18	1 (at onset) +1	3	0	0	10

FANS = Non-steroidal anti-inflammatory drugs.

Fig. 1. GHR gene expression values in control, JIA patients pre-treatment and JIA patients after 2 years of treatment expressed in arbitrary units (RQ). * p < 0.005 patients vs. controls; ** p = 0.007 patient after treatment vs. patients before treatment.

coxon test for paired samples was used to compare values in patients before and after 2 years of treatment. Correlations were analyzed using Spearman's rank correlation test. A value of p < 0.05 was considered statistically significant.

Results

At recruitment, JIA children presented with a mean height of 0.03 SD (range –2.12 to +2.38). The mean target height was 0.3 ± 0.9 SD. Two patients presented with short stature, Z-score ≤2, lower than their target height (respectively of –0.8 and –1.3 SD). As for inflammatory indexes, CRP was moderately elevated in 41% of patients, while ESR was increased in 67% of the patients. White blood cells and hemoglobin levels were normal in all patients. All JIA patients had evidence of synovitis in at least one joint.

At disease onset, lower levels of IGF-I were found in the JIA group (mean value –0.64 SDS; range –3.65 to +1.41 SDS with 3 patients below –2.0 SDS) compared to the control group (mean level –0.1 SDS), even if the difference was not statistically significant (p = 0.08). On the contrary, GHBP was higher in patients compared to controls (1,455 ± 301.1 and 889.17 ± 182.73 pmol/l respectively), but this datum was not statistically significant (p = 0.085). GH-R mRNA expression was significantly lower in patients (RQ 3.7 ± 1.2) compared to the control group (RQ 8.2 ± 1.7) (fig. 1).

Two years later, 9 children had a persistent oligoarticular form, 2 an extended-oligoarticular form, 1 a polyarticular subtype and 1 a psoriatic subtype. Regardless of disease activity, at clinical observation, 9 patients had a negative rheumatologic examination. Two patients had fair disease activity, while just 1 patient had an extra-articular manifestation (anterior uveitis).

At follow-up, the mean height increased even if not significantly, to 0.41 ± 1.46 SDS. In 2 cases, stature was persistently lower than –2 SDS, but with a mild improvement (–2.12 and –2.38 at the onset compared to –2.07 and –2.06 at the follow-up). Two patients experienced a growth spurt during the follow-up period, but they presented with stable disease activity.

A reduction in inflammatory indexes was observed in the majority of patients. In fact, CRP remained moderately elevated in 3 patients and ESR decreased in all but 1 patient. In detail, ESR was significantly reduced at follow-up compared to onset with a mean value of 12.9 mm/h (p = 0.0039), in accordance with inactive arthritis. White blood cells and hemoglobin levels were persistently normal in all patients.

The most interesting result was the significant (p = 0.007) increase in GHR mRNA expression in all patients at follow-up with respect to the basal value (fig. 1, 2). Moreover, IL-6 levels significantly (p = 0.0156) decreased in JIA patients (90.84 ±124.71 pg/ml at onset and 19.37 ± 41.01 pg/ml at follow-up). On the contrary, mean IGF-I significantly (p = 0.0005) improved compared to levels at disease onset (0 SDS; range –1.69 to +1.70). In detail, in all but 1 patient we noted an improvement in IGF-I. In this patient, IGF-I was lower at the follow-up than at onset (–0.82 SDS in the first blood sample and –1.06 at the second). The decrease in IGF-I value may be correlated to disease activity, as this patient experienced an ocular complication (uveitis) a few months before. GHBP values did not significantly change after 2 years of therapy (1,205.83 ± 924.4 pmol/l) compared to basal levels (1,327.5 ± 1,152 pmol/l). Finally, we observed significant correlations between height, GHBP and IGF-I at the follow-up (height vs. GHBP: p = 0.037; height vs. IGF-I: p = 0.037; GHBP vs. IGF-I: p = 0.007).

Since local intra-articular injections of triamcinolone hexacetonide or methylprednisolone can produce a systemic effect in JIA patients, we separately analyzed patients with or without intra-articular injections. We found no significant difference between patients treated with arthrocentesis (IGF-I: –0.61667 ± 0.7, GHBP 1,691.68 ± 778.7, GHR mRNA expression 0.8955 ± 0.21)

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Fig. 2. Fold increase in GHR gene expression after 2 years therapy compared to basal values in each patient. * = Patients who entered puberty; ° = patients with psoriatic JIA.
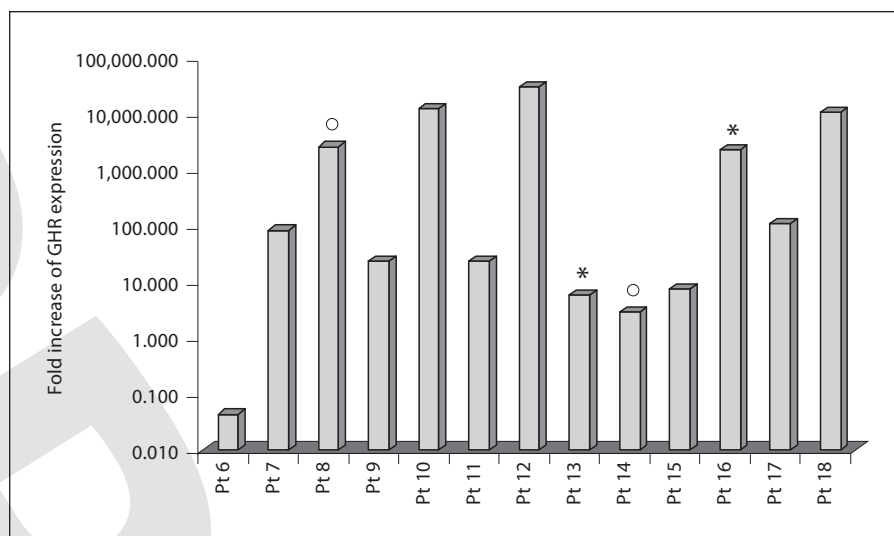
and patients not treated (IGF-I: −0.5825 ± 0.39, GHBP 1,336.667 ± 314.76, GHR mRNA expression 5.1215 ± 1.79).

Discussion

JIA is known to be associated with elevated levels of pro-inflammatory cytokines [1, 10]. In affected children, these cytokines, mainly IL-6, IL-1 and TNF-α, may act individually or in concert to influence children's growth through systemic and local effects on long bones [2, 3]. The chronic inflammatory process itself has been associated with disease activity [1, 10]. In studies where GH secretion and response to GH therapy in JIA were measured, disease activity was assumed to have a suppressive effect on growth [13, 14]. The cellular mechanism through which cytokines act is of interest and their impact on JIA needs to be clarified. In fact, knowledge of the pathogenic events in inflammation is essential for the identification of new therapeutic strategies to prevent inflammation-induced diseases. In particular, IL-6 has been studied as a mediator of chronic inflammation in animal models [15–18].

In the NSE/hIL-6 transgenic murine model IL-6, circulating IGF-I was overexpressed, GHBP levels were reduced, GH was unchanged, while the inflammatory process was enhanced [3]. On the contrary, mice deficient in IL-6 were protected from rat collagen-induced arthritis (CIA) [17]. In addition, at CIA disease induction, the administration of IL-6-neutralizing antibodies abolished

the inflammatory response, suggesting that IL-6 plays an important role in disease induction [17]. High disease activity and therefore high levels of inflammatory cytokines, mainly IL-6, have been reported in JIA [2, 3]. In our experience, as well, IL-6 levels were initially elevated, which is compatible with an inflammation status. At follow-up, in accordance with the reduction in joint involvement, a significant decrease in IL-6 values was observed. In order to better understand the role of IL-6 in JIA, patients affected by a systemic form, which may necessitate anti-IL-6 receptor antibody therapy, were excluded [12]. In addition to IL-6, ESR was significantly reduced at follow-up compared to onset, as in inactive arthritis. This confirms that ESR grossly reflects disease activity.

While the inflammatory process is enhanced by pro-inflammatory cytokines, the reparation response is influenced by peptide growth factors, which improve both the number of cell divisions and the biosynthesis of extracellular matrix components [11].

Nevertheless, precise information on IGF-I, IGFBP and GHR in association with clinical variables of disease activity is not available. IGF-I is an important metabolic and mitogenic factor involved in cell growth and differentiation. In plasma and tissues it is complexed to binding proteins (BP) which are important regulators of its biological activity [11]. Under normal conditions, the majority of IGF-I circulates in a form that is bound to a high-molecular-weight complex, containing IGFBP-3 as an active constituent. It has been suggested that this complex prolongs the half-life of IGF-I and increases cell responsiveness to IGF-I stimulation. Another factor that may be

important in regulating the availability of IGF-I to its receptor is the level of IGFBP1, which inhibits IGF-I correlated functions, and the IGFBP3 cleavage product, as proteolysis of IGFBP-3 produces a fragment that can still bind IGF-I but with reduced affinity. It is also known that the peptide growth factors, especially IGF-I and IGFBPs, play an important role in the regulation of cell division and maintain equilibrium between synthesis and degradation of extracellular matrix components [11].

IGF-I receptor binding initiates an IGF-I signaling cascade which may be altered by pro-inflammatory cytokines at one or more points [3]. Increased concentrations of pro-inflammatory cytokines may alter the response of IGF-I to GH, thereby affecting the growth plate or interfering with GHR signaling. Cytokines may induce the expression of SOCS (suppressor of cytokine signaling protein) which down-regulates cytokine signaling and alters GH signaling, which may explain GH resistance. In fact, genetically modified mice that are SOCS-2-deficient show gigantism accompanied by deregulation of GH signaling [3].

Previous studies on IGF-I concentrations in JIA have reported conflicting results, either reduced or normal values [7, 13, 19]. In these studies most patients had received steroid therapy. Glucocorticoids themselves may alter IGF-I values, interfering with the GH-IGF-I axis, by reducing the GH level and affecting growth rates [6, 11, 20]. For this reason, we decided to study patients never treated with oral steroids, from onset over the entire follow-up period. In our series, similar to previous studies, the IGF-I level was lower in JIA patients than in the controls, consistent with GH resistance [3, 11]. However, this result is not statistically significant probably because our patients were at the onset of an oligoarticular form of JIA with a milder clinical history when compared with a systemic form. Nevertheless, the inverse association between serum concentrations of IGF-I and IL-6 in JIA patients suggests the possibility of a direct link between inflammation and the GH-IGF-I axis. Further larger studies are required in order to confirm our hypothesis.

At follow-up, the IGF-I value of all but 1 patient increased, reaching normal values. In detail, the IGF-I increase was significant and concomitant with an improved rheumatologic examination. This probably reflects the diminished activity of inflammation during disease control. The only child who did not show improvement in IGF-I values was affected a few months prior by an arthritic ocular complication (anterior uveitis). This finding supports the concept of GH resistance in patients with active JIA. We believe that the activity of the inflammatory process was the most important growth-impairing factor and that the extra-articular complication aggravated this effect.

Moreover, in our report, we observed an improvement in patient height at follow-up, in accordance with optimal disease control and improvement in IGF-I and GHR gene expression values. This is in line with a previous study in which height SDS significantly correlated with serum IGF-I levels [7]. These findings underline that catch-up growth in disease remission may have an endocrinological component.

Cell surface levels of GHR are the primary determinant of GH responsiveness [8]. Pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6, have been demonstrated to down-regulate hepatic GHR expression during active processes, for example in sepsis [21]. In our series, GHR mRNA levels were significantly lower at onset, in association with an inflammatory state. Two years later, when disease was under control, GHR value significantly increased, in association with the IGF-I level. We suppose that the return to healthy state after a period of illness contributes to the increase of GHR values. Further studies should investigate whether there is an association between the increase in GHR gene expression and lymphocyte subsets, although there are large variations among age ranges and through different laboratories analyzing the same sample [22]. These findings were in apparent contrast with equal values of GHBP in JIA patients and in controls but, even if several clinical conditions are accompanied with reduced GHBP levels, this is not universally true. In fact, in other physiological and pathological conditions, GHBP level and GHR function are not tightly correlated [23]. For example, GHBP levels in patients with GH insensitivity due to GHR gene mutations vary according to the compromised domain of the GHR: they are low when the extracellular domain is affected, and high when the intracellular domain is mutated. GHBP possesses tissue-specific properties, in terms of GHR regulation and its cleavage to GHBP. Furthermore, even if GHBP is generated mainly from the liver GHR, many other tissues express GHR and probably also contribute to the total GHBP level [24].

To the best of our knowledge, no previous studies have been performed on GHR gene expression values at onset and during follow-up in JIA children. Our study raises the possibility that there is another mechanism through which cytokines interact with the GH/IGF-I axis. This mechanism involves GHR before the start of signal transduction.

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Conclusion

Serum IGF and GHR gene expression levels monitored on a longitudinal basis during the course of therapy correlates with the clinical picture, suggesting that disease activity is accompanied by changes in hypothalamic/pituitary-related hormones. During disease control, GHR mRNA expression returns to normal values. This finding supports the idea of a strong correlation between growth factors and the inflammatory process. We believe that the increase in GHR gene expression is the outcome of an improved of GH/IGF-I axis. In fact, also IGF-I levels increase when the acute inflammatory condition of JIA is reduced. These observations stress the importance of immediate action in controlling inflammatory activity to prevent stunted growth and support the idea of a good overall outcome in mild to moderate JIA. However, because of the small sample size in our study, a larger series should be studied to confirm the conclusions of this study.

Acknowledgement

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