Insulin-like growth factor 1 gene (CA)n repeats and a variable number of tandem repeats of the insulin gene in Brazilian children born small for gestational age

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OBJECTIVE: To investigate the influence of (CA)n repeats in the insulin-like growth factor 1 gene and a variable number of tandem repeats of the insulin gene on birth size in children who are small or adequate-sized for gestational age and to correlate these polymorphisms with serum insulin-like growth factor 1 levels and insulin sensitivity in children who are small for gestational age, with and without catch-up growth.

PATIENTS AND METHODS: We evaluated 439 infants: 297 that were adequate-sized for gestational age and 142 that were small for gestational age (66 with and 76 without catch-up). The number of (CA)n repeat in the insulin-like growth factor 1 gene and a variable number of tandem repeats in the insulin gene were analyzed using GENESCAN software and polymerase chain reaction followed by enzymatic digestion, respectively. Clinical and laboratory data were obtained from all patients.

RESULTS: The height, body mass index, paternal height, target height and insulin-like growth factor 1 serum levels were higher in children who were small for gestational age with catch-up. There was no difference in the allelic and genotypic distributions of both polymorphisms between the adequate-sized and small infants or among small infants with and without catch-up. Similarly, the polymorphisms were not associated with clinical or laboratory variables.

CONCLUSION: Polymorphisms of the (CA)n repeats of the insulin-like growth factor 1 gene and a variable number of tandem repeats of the insulin gene, separately or in combination, did not influence pre- or postnatal growth, insulin-like growth factor 1 serum levels or insulin resistance.

KEYWORDS: IGF1 Gene; Small For Gestational Age; Serum IGF1 Levels; IGF1 Polymorphism; VNTR of the Insulin Gene; Growth.

Coletta RR, Jorge AA, D’ Alva CB, Pinto EM, Billerbeck AE, Pachi PR, et al. Insulin-like growth factor 1 gene (CA)n repeats and a variable number of tandem repeats of the insulin gene in Brazilian children born small for gestational age. Clinics. 2013;68(6):785-791.

Received for publication on February 13, 2013; First review completed on February 13, 2013; Accepted for publication on February 13, 2013

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INTRODUCTION

Several endocrine and environmental factors have been implicated in fetal growth. Among the endocrine factors, the insulin and the insulin-like growth factor systems have a critical role in mediating pre- and postnatal growth. Their strong stimulatory effect on growth is supported by the observation of marked pre- and postnatal growth failure in Igf1 or insulin knock-out animal models (1) and in humans with IGF1 gene defects (2–4).

The substantial contribution of genetic factors to the inter-individual variation in circulating IGF-1 levels is well recognized (5). Lower circulating IGF-1 levels have been
observed in children that present with intrauterine growth retardation (6–8). Because there are few reports of mutations in the IGF1 gene in children born small for gestational age (SGA) (2–4), associations between growth and polymorphisms in the non-coding regions of IGF1, which may affect transcription or messenger RNA processing, have been analyzed (9,10).

5′-(CA)n repeats in the IGF1 gene are common and are the most-investigated polymorphism in association studies. (CA)n repeats in the IGF1 gene comprise a microsatellite characterized by a variable number of CA repeats (10 to 24) in the promoter region of the IGF1 gene (Genbank accession number: M12659, data base: UnSTS); this polymorphism is characterized by alleles ranging in length from 174–202 bp that start 947 bp upstream from the initiation site (11–17). As summarized in Table 1, the involvement of the wild-type 192-bp allele (19 CA repeats) in clinical disorders, birth size and IGF1 serum levels is still controversial in the literature (9,10,14,16–19).

IGF1 secretion and prenatal growth are regulated by the glucose-insulin-IGF1 axis. The placenta transfers glucose to the fetus, thereby stimulating the secretion of fetal insulin, which determines the amount of IGF1 secretion (20). In postnatal life, growth hormone is the main regulator of IGF1 expression; however, in the prenatal period, growth hormone exerts little influence on fetal growth.

The VNTR (variable number of tandem repeats) of the insulin gene (INS VNTR) consists of repetitions of the variable oligonucleotide ACAGGGGT(G/C)(T/C)GGGG and is located 596 bp upstream of the transcript starting codon. According to the number of repetitions, the alleles can be divided into: class I alleles with 26 to 63 repetitions (approximately 570 bp), class II alleles with 64 to 140 repetitions (approximately 1,200 bp) and class III alleles with 141 to 209 repetitions (approximately 2,200 bp) (21).

The variation in the INS VNTR length regulates the transcription of the insulin gene and the contiguous IGF2 gene (22). Similarly to the (CA)n repeats, the association of the INS VNTR with low birth weight and insulin resistance syndrome is controversial; the research on this topic is summarized in Table 2.

It is worth noting that 90% of children who are born SGA achieve a normal weight and length during the first two years of life (9,23). However, SGA without postnatal catch-up is a clinically complex entity and most likely has a polygenic etiology. For this reason, the aim of the present study was to analyze two polymorphisms, i.e., (CA)n repeats in the IGF1 gene and the INS VNTR of the insulin gene, separately or in combination, in relation to birth size and catch-up growth in Brazilian children born adequately sized for gestational age (AGA) and SGA. Additionally, we compared these polymorphisms with IGF1 serum levels and insulin sensitivity in SGA children with and without catch-up growth.

### Patients and Methods

The study was approved by the local research ethical committee, and informed written consent was obtained from all patients’ parents or guardians. Information concerning health status, birth weight, birth length, birth head circumference, gestational age and maternal conditions during the pregnancy (e.g., previous or gestational diabetes mellitus and smoking) were ascertained by reviewing the patients’ medical records. The anthropometric data were expressed as standard deviation scores (SDS) adjusted for sex and gestational age (24). We evaluated 439 infants over 2 years of age from three different Brazilian centers; these infants were divided into two groups, i.e., born small for gestational age (SGA group) or adequate for gestational age (AGA group), according to their birth length and birth weight SDS (25,26). The AGA group consisted of 297 children whose birth length and birth weight SDS were $<-2.0$, and the SGA group consisted of 142 children whose birth length and/or birth weight SDS were $\geq -2.0$. None of these children showed signs of severe hypoxia in the neonatal period (defined as a 5-minute Apgar score <6).

Additionally, the SGA group was divided into two subgroups according to height after two years of age: one group contained 66 children with catch-up growth (height SDS <–2), whereas the other group contained 76 children without catch-up growth (height SDS $\geq -2$) (27).

Children with endocrine or metabolic disorders, chromosomal defects, genetic syndromes (except Silver Russell syndrome) or growth failure caused by other conditions (e.g., malnutrition, emotional deprivation, severe chronic illness and chondrodysplasia) were excluded.

### Biochemical Measurements

Serum IGF1 levels were measured in the SGA children using a specific immunoradiometric assay (IRMA) or

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**Table 1 - Relevant studies associating promoter IGF1 5′-(CA)n repeats with clinical disorders, birth size and IGF1 serum levels.**

| Population                  | N   | (CA)n repeats | Results                                                                 | References              |
|-----------------------------|-----|---------------|------------------------------------------------------------------------|-------------------------|
| Adults                      | 900 | 192 bp non-carriers | ↓ IGF1 serum levels, lower final height, higher risk of type 2 diabetes and myocardial infarction | Vaessen et al., 2001 (17) |
| AGA Adults                  | 463 | 192 bp non-carriers | Lower birth weight                                                     | Vaessen et al., 2002 (18) |
| Adults                      | 171 | 192 bp carriers | ↓ IGF1 serum levels and osteoporosis in men                             | Rosen et al., 1998 (16) |
| AGA Adults                  | 640 | 192 bp carriers | ↓ IGF1 serum levels and no association with type 2 diabetes and birth weight | Frayling et al., 2002 (14) |
| AGA Children                | 738 | 192 bp non-carriers | Association with smaller fetal size, followed by ↓ growth from mid-pregnancy to early infancy | Geelhoed et al., 2008 (19) |
| Short Stature SGA Children  | 124 | 198 bp carriers | Minimal transmission from parents to SGA children                       | Arends et al., 2002 (9) |
| SGA                         | 174 | 198 bp carriers | S-SGA only in Swedish population: No association with IGF1 serum levels | Johnston et al., 2003 (10) |
| Short Stature SGA Children  | 201 | 192 bp carriers | No association with birth size and post-natal growth                    | Ester et al., 2009 (46) |
| SGA and AGA Infants         | 142/297 | 192 bp non-carriers | No association with birth size and IGF1 serum levels                   | Present study           |

SGA: small for gestational age; AGA: adequate size for gestational age; ↓: decreased; S-SGA: short-small for gestational age.
enzyme-labeled chemiluminescent immunometric assay (ICMA) (28,29), and the values were transformed into SDS adjusted for sex and age. Blood glucose levels were determined by the enzymatic colorimetric method (glucose-oxidase) (30). Insulin levels were determined by an immunofluorometric assay (IFMA) (31,32). Insulin resistance was determined using the HOMA-IR index (fasting insulin (µU/ml) × fasting plasma glucose (mmol/l)/22.5) (33).

Molecular analysis
Genomic DNA from peripheral blood lymphocytes was amplified by polymerase chain reaction (PCR) with specific primers for the region containing the polymorphic promoter cytosine-adenine [(CA)n] repeats, which are located approximately 1 kb upstream of the human IGF1 gene (9). The PCR reaction was performed in a final volume of 25 µl containing 50 ng of genomic DNA, 0.5 nmol of each primer, 1.5 mM MgCl₂, 250 µM dNTP and 2.5U of Taq DNA polymerase (Invitrogen®). After the initial denaturation for 10 min at 94°C, the samples were subjected to 25 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C; these cycles were followed by a final extension step of 10 minutes at 72°C. The forward primers were labeled with FAM, which is a fluorescent size marker, to determine the size of PCR products using an autosequencer (ABI Prism 3100 Genetic analyzer); this size determination was followed by analysis with GENESCAN Fragment Analysis software (Applied Biosystems, Foster City, CA, USA).

All samples were amplified in a GeneAmp PCR Instrument System 9600 automatic thermocycler (Perkin-Elmer/Cetus, Norwalk, CT, USA), and all amplifications were accompanied by a negative control.

Statistical analysis
The Hardy-Weinberg equilibrium of the IGF1 and insulin promoter polymorphism genotypes was tested, and the allele and genotype frequencies were all in Hardy-Weinberg equilibrium. The data were expressed as the mean ± SD. Birth length, birth weight, head circumference, height and IGF1 levels were expressed as SDS. In addition, glucose, insulin and HOMA-IR were analyzed in combination with the clinical variables. Cross tabulation paired with the Chi-Squared Test or Fisher Exact Test was used to analyze categorical data; a t-Test or ANOVA was used for comparisons of the mean between normally distributed variables; and the Mann-Whitney or Kruskal-Wallis test was used for comparisons among skewed variables. A p<0.05 was considered statistically significant.

To verify the correlation between dependent and independent variables, a logistic regression analysis (not linear) was used for binary dependent variables, and a linear regression analysis was used for numerical variables. Spearman’s correlation was used for the selection of independent variables with a significance of p<0.20.

All analyses were performed using the SPSS program (Statistical Package Social Sciences graph), Version 13.0, and the threshold for statistical significance was p<0.05.

RESULTS

Clinical results
All children from the AGA group presented with normal birth weight (0.5±1.2 SDS) and length (−0.5±1.1 SDS).

The clinical data for the SGA groups with and without catch-up growth are displayed in Table 3. The birth weight and length SDS were similar between the SGA groups. Children with catch-up, however, presented with significantly higher height, target height (TH) and BMI (index body mass) SDS than those without catch-up growth (p<0.05). Unexpectedly, the head circumference was smaller in children with catch-up.

Table 2 - Relevant studies associating promoter INS VNTR of the insulin gene with anthropometric data and clinical disorders.

| Studied Population | n   | INS VNTR | Associations                                      | References                     |
|--------------------|-----|----------|--------------------------------------------------|-------------------------------|
| AGA Children       | 758 | II       | ↑ Birth weight, without catch-up growth           | Dunger et al., 1998 (47)      |
| AGA Boys          | 141 | Allele I | ↓ Birth weight, higher HOMA-IR                    | Ibáñez et al., 2001 (50)      |
| AGA Men           | 105 | Allele II| ↓ Birth weight, without catch-up growth, DM2      | Ong et al., 1999 (48)         |
| AGA Pima Indians  | 660 | Allele III|                                                   | Lindsay et al., 2003 (49)     |
| AGA               | 5646| Allele I and III | No association with birth size and insulin resistance | Mitchell et al., 2004 (34) |
| SGA and AGA Infants| 758| Allele I and III | No association with birth size | Vu-Hong et al., 2006 (52) |
| SGA and AGA Children| 735/886| Allele I and III | Higher insulin resistance in SGA children | Present study |

SGA: small for gestational age; AGA: adequate size for gestational age; ↑ : increased; S-AGA: short-small for gestational age; DM2: type 2 diabetes mellitus.
In the laboratory analysis, serum IGF1 levels were significantly higher in the SGA catch-up group than in the SGA group without catch-up. Although the mean insulin serum concentration and HOMA-IR index were higher in SGA children with catch-up growth, the difference was not statistically significant.

The frequency of maternal smoking during pregnancy was significantly higher in SGA children who had catch-up growth (p<0.05). The frequency of gestational or previous diabetes mellitus was similar in both SGA groups.

Logistic regression analysis revealed that the highest probability of catch-up growth (99.93%) was related to the maximal values of independent variables, such as the height SDS of the father and the IGF1 SDS. Linear regression analysis demonstrated a lack of influence of any of the clinical variables analyzed on the height SDS and the IGF1 SDS.

### Molecular results

The molecular results are displayed in Table 4. The length of the PCR products that contained the IGF1 5′-(CA)n repeats ranged from 184 to 204 bp in our cohort, and the PCR products represented nine distinct alleles. The 192-bp repeats were the most common in our population and was found in three different genotypic groups: children who were homozygous for the 192-bp allele (192/192), children who were heterozygous for the 192-bp allele (192/*), and children who did not carry the 192-bp allele (*/*). There was no difference in the allelic and genotypic frequency of IGF1 5′-(CA)n repeats between the AGA and SGA groups.

With regard to the distribution and frequency of the INS VNTR class I and III alleles, no difference was found in the allelic and genotypic distribution between the AGA and SGA groups or between the SGA groups with and without catch-up. Similarly, this polymorphism was not associated with the clinical and laboratory variables analyzed in this study. In addition, the IGF1 (CA)n repeats and the INS VNTR were not related to pre- and postnatal growth; furthermore, there was no association between these polymorphisms and serum IGF1 levels or insulin resistance in our cohort of individuals born SGA.

#### DISCUSSION

Intrauterine and postnatal growth, as well as height, are complex clinical traits that are influenced by many genes. This study showed that the parents, particularly fathers, of children SGA without catch-up growth were significantly shorter than those of children SGA with catch-up. Our findings suggest that the genetic factors that determine permanent short stature in children born SGA are mainly of paternal origin; this phenomenon has been previously described in the literature (46).

Ong et al. (35) demonstrated that catch-up growth in the first two years of life is a risk factor for central obesity in childhood. Similarly, we observed that the BMI SDS was higher in children with catch-up growth, which indicates that these children must be followed until adult age to prevent the onset of obesity and future co-morbidities.

Gluckman et al. (20) demonstrated that birth size is directly correlated with serum concentrations of IGF1 in the umbilical cord, which in turn are directly influenced by the nutritional status of the fetus. In addition, Gluckman et al. observed that serum concentrations of IGF1 during the first year of life in children born SGA are positively associated with catch-up growth and may reflect insulin secretory capacity (36). Therefore, higher serum concentrations of IGF1 in SGA children with spontaneous catch-up may be an indicator of relative resistance to insulin and IGF1 and may constitute an increased risk for the onset of Type 2 diabetes mellitus in adulthood. We also found higher IGF1 serum concentrations in children born SGA with catch-up growth.

### Table 3 - Clinical data of children born small (SGA) for gestational age with and without catch-up growth.

| Data                      | Catch-up growth n = 142 | No catch-up (n = 76) | p-value |
|---------------------------|-------------------------|----------------------|---------|
| Birth weight SDS          | -2.47 ± 0.85            | -2.33 ± 1.07         | NS      |
| Birth length SDS          | -3.45 ± 1.16            | -3.42 ± 1.28         | NS      |
| Head circumference SDS    | -2.25 ± 1.28            | -1.22 ± 1.47         | <0.05   |
| Height SDS (2–4 years)    | -0.58 ± 0.83            | -3.14 ± 1            | <0.05   |
| Target height SDS         | -0.72 ± 0.98            | -1.21 ± 0.94         | <0.05   |
| BMI SDS                   | -0.29 ± 1.71            | -1.16 ± 1.56         | <0.05   |
| IGF1 SDS (2–4 years)      | 0.032 ± 1.44            | -0.86 ± 1.26         | <0.05   |
| Glucose                   | 81.25 ± 10.22           | 81.61 ± 7.11         | NS      |
| Insulin                   | 6.32 ± 5.35             | 4.45 ± 0.70          | NS      |
| HOMA-IR                   | 1.32 ± 1.15             | 0.91 ± 0.68          | NS      |

SGA: small for gestational age; AGA: adequate size for gestational age; NS: non-significant.

### Table 4 - Genotype frequency of IGF1 5′-(CA)n repeats and INS VNTR of the insulin gene in the SGA and AGA groups.

| Polymorphism | Genotype | SGA = 142 Total n (%) | AGA = 297 Total n (%) | p-value |
|--------------|----------|-----------------------|-----------------------|---------|
| IGF1         | 192/192  | 41 (29)               | 83 (28)               | NS      |
|              | 192/*    | 76 (54)               | 163 (55)              | NS      |
|              | */*      | 25 (17)               | 51 (17)               | NS      |
| INS VNTR     | I/I      | 45 (32)               | 101 (34)              | NS      |
|              | I/III    | 40 (28)               | 48 (16)               | NS      |
|              | III/III  | 57 (40)               | 148 (50)              | NS      |

SGA: small for gestational age; AGA: adequate size for gestational age; NS: non-significant (p>0.05).
but the insulin levels were similar in SGA individuals with and without catch-up growth.

In the last few decades, several studies have demonstrated a higher frequency of insulin resistance in children and adults born SGA (37-42). Soto et al. (43) showed higher insulin resistance in children born SGA who had catch-up growth. Although their results are controversial, other studies have also indicated that low birth weight is associated with an increased risk of insulin resistance syndrome when accompanied by stature recovery, which strengthens the evidence that nongenetic mechanisms determine insulin resistance and low birth weight. If insulin resistance syndrome is multifactorial in origin, the INS VNTR may represent a genetic risk factor. In this study, insulin sensitivity, as measured by the HOMA-IR index, was slightly higher in subjects born SGA with catch-up growth, but this correlation did not reach significance, most likely because of the small number of children involved in the present study and their chronological age (they are very young). It is possible that if these children are analyzed in the future, an association may be observed between insulin resistance and these polymorphisms.

Among maternal causes of SGA, smoking is one of the most common preventable causes of intrauterine growth retardation (23). Newborns born to smoking mothers generally have lower weight, length and head circumference at birth (44,45). We observed that the incidence of maternal smoking was higher in SGA children with catch-up growth, which suggests that in postnatal life, outside of the hostile intrauterine environment, these children will express their genetic potential for growth.

Because of the crucial role of IGF1 in pre- and postnatal growth, some studies have addressed the influence of IGF1 \(5\)-(CA)n repeats on birth length and weight as well as child growth. The 192-bp allele of IGF1 (CA)n repeats has been found in the homozygous and heterozygous states in 37 – 47% and 42 – 49% of UK and Dutch populations, respectively (14,17). A similar frequency was observed in our cohort, despite the high miscegenation that is found in the Brazilian population.

\(5\)-(CA)n repeats in IGF1 were first reported in a study that investigated the influence of IGF1 \(5\)-(CA)n repeats on serum IGF1 levels and bone mineral density in a small group of adult patients. In this first report, adults homozygous for the 192-bp allele presented with lower IGF1 levels and BMD (body mass density) relative to those with other (CA)n genotypes (16). However, in a subsequent study that evaluated this polymorphism in a large group of diabetic patients and controls, the presence of the 192-bp allele was associated with higher IGF1 levels as well as an increase in height. Additionally, non-carriers of the 192-bp allele presented with an increased relative risk for type 2 diabetes and myocardial infarction (17). In contrast, some studies of adults born AGA showed an association of the wild-type allele (192) with low IGF1 circulating levels and a lack of correlation between the 192-bp allele and weight, height and head circumference SDS at birth (14,16).

AGA children who are non-carriers of the 192-bp allele tend to have a smaller fetal size during gestation, followed by increased growth from mid-pregnancy to early infancy (19); the birth weight of these children was also shown to be 215 g lower than that of individuals who were homozygous for this allele (Table 1). In another study, four IGF1 gene polymorphisms were analyzed in 201 short-SGA children (46), and no association of CA repeats with birth size or postnatal growth was observed (Table 1).

Regarding children born SGA, only three studies (9,10,46), all from the same research group, have assessed the influence of IGF1 \(5\)-(CA)n repeats in pre- and postnatal growth parameters in children from two distinct Caucasian populations (Sweden and the Netherlands). In the first study, transmission disequilibrium of the 198-bp allele was observed; this allele was transmitted less frequently from parent to child. Because of the low frequency of this allele (0.4%), this result should be cautiously considered (9). In a subsequent study, Johnston et al. (11) demonstrated the association of a haplotype that combined the 192-bp allele of IGF1 \(5\)-(CA)n repeats and another IGF1 promoter SNP (T-1148C) with the short SGA phenotype. Finally, another haplotype, which combined the 192-bp allele of the IGF1 \(5\)-(CA)n repeats and the G1245A SNP, was associated with a smaller head circumference SDS during spontaneous postnatal growth, but not during growth hormone (GH) treatment. In addition, no associations were found with birth size and postnatal growth (46).

In the present study, we screened AGA and SGA patients for the IGF1 \(5\)-(CA)n repeat polymorphism, and no association with birth size or birth length was found. In our SGA children, we also failed to find an association of the IGF1 \(5\)-(CA)n repeats with birth size, the presence or absence of catch-up growth and IGF1 serum levels.

The absence of functional studies* regarding IGF1 \(5\)-(CA)n repeats raises the question of whether this polymorphism itself participates in the regulation of IGF-I expression or merely flags another polymorphism in the promoter region that is functionally involved in IGF1 expression.

It is known that insulin is a relevant endocrine factor that determines fetal growth. However, most studies correlating the INS VNTR (genotypes or allele class I or III) with low birth weight, insulin resistance and absence of catch-up growth were conducted in subjects born with a size adequate for their gestational age (47,34,48–50) (Table 2). Some of these studies analyzed the relationship of birth size with insulin resistance and found no association of alleles I and III of the INS VNTR with birth size (34,51–53). However, Mitchell et al. (34) described an association of the class III allele with increased insulin resistance (Table 2). Maas et al. (52) studied the INS VNTR and CA repeats of the IGF1 gene in AGA children but did not find interactions between these genotypes and birth weight or measures of body composition. Only one study (52) comparing subjects born SGA and AGA showed higher insulin resistance in individuals who carried allele III, but there was no association of the INS VNTR with birth size (Table 2).

In the present study, we confirmed a lack of association of the INS VNTR with birth size, postnatal growth or the presence of insulin resistance.

When analyzing the two polymorphisms, i.e., the INS VNTR and IGF1 (CA)n repeats, in combination, we failed to find any association with birth size in the SGA group, as previously demonstrated in AGA children (54).

Studies correlating IGF1 \(5\)-(CA)n repeats and INS VNTR polymorphisms with growth, IGF1 serum levels and insulin sensitivity are controversial independently of the population characteristics (children or adults born SGA or AGA). Various reasons might explain the discrepancies observed among these studies (55–57): 1) false-positive results caused
by multiple testing (type 1 error); 2) false-negative results caused by insufficient power (type 2 error); 3) population stratification; 4) or finally, a real difference between studied populations. Similarly, in this study, it was not possible to eliminate these biases.

Therefore, further studies are needed to establish an association of IGF1 5′-(CA)n repeats and INS VNTR with fetal growth. Furthermore, a meta-analysis and/or functional studies will be able to confirm the relevant role of these polymorphisms in pre- and postnatal growth, as well as their association with the serum IGF1 levels.

In conclusion, by comparing SGA children with and without catch-up growth and AGA children, this study demonstrates for the first time that IGF1 (CA)n repeats and INS VNTR separately or in combination do not influence birth size, postnatal growth, IGF1 serum levels and insulin resistance. The young age of the patients in this study may explain why we failed to find any association with insulin levels.

*An “in vitro” or functional study is a molecular tool used to demonstrate the effect of a mutation or polymorphism on gene function.

**ACKNOWLEDGMENTS**

This work was supported by grants from Fundação de Amparo a Pesquisa do Estado de São Paulo - FAPESP (03/30999-5 and 03/04726-0), Conselho Nacional de Desenvolvimento Científico e Tecnológico (301246/95-5, to B.B.M.; 300982/09-7, to I.J.P.A.; 306000/04-0, to M.C.S.B., and 301477/2009-4, to A.A.L.J.) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); PRODOC 00031/03-8 to E.M.F.C.

**AUTHOR CONTRIBUTIONS**

Coletta RR conducted the clinical and molecular analysis of all children involved in the study and wrote this article. Coletta RR, D’Ava CB and Garcia RM conducted the selection and clinical evaluation of the children involved in the study. Jorge AA contributed to the statistical methodology used in the study and to the revision of the article. Finto EM and Billerbeck AE assisted in performing the molecular techniques used in the study. Pacheco PR and Longui CA were the physicians responsible for the pediatric clinic where most of the patients were selected, and they contributed to the patient selection. Boguszewski M contributed seven patients in our series. Arnhold Jr and Mendonca BB contributed to the development and production of this article. Costa E formulated and supervised the doctoral thesis of Rocio RD and the production of this article.

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This work was supported by grants from Fundação de Amparo a Pesquisa do Estado de São Paulo - FAPESP (03/30999-5 and 03/04726-0), Conselho Nacional de Desenvolvimento Científico e Tecnológico (301246/95-5, to B.B.M.; 300982/09-7, to I.J.P.A.; 306000/04-0, to M.C.S.B., and 301477/2009-4, to A.A.L.J.) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); PRODOC 00031/03-8 to E.M.F.C.

**AUTHOR CONTRIBUTIONS**

Coletta RR conducted the clinical and molecular analysis of all children involved in the study and wrote this article. Coletta RR, D’Ava CB and Garcia RM conducted the selection and clinical evaluation of the children involved in the study. Jorge AA contributed to the statistical methodology used in the study and to the revision of the article. Finto EM and Billerbeck AE assisted in performing the molecular techniques used in the study. Pacheco PR and Longui CA were the physicians responsible for the pediatric clinic where most of the patients were selected, and they contributed to the patient selection. Boguszewski M contributed seven patients in our series. Arnhold Jr and Mendonca BB contributed to the development and production of this article. Costa E formulated and supervised the doctoral thesis of Rocio RD and the production of this article.

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