Abstract

Context: McCune–Albright syndrome (MAS) is a disorder caused by a post-zygotic gain-of-function mutation in the gene encoding the Gs-α protein. Sexual precocity, common in girls, has been reported in only 15% of boys, and little is known on the long-term evolution of MAS in males.

Objective: In a boy with MAS, we studied spermatogenesis, testis histology, and immunohistochemistry with the aim to shed light on seminiferous tubule activity.

Design: A boy who presented at the age of 2.9 years with sexual precocity, monolateral macroorchidism, increased testosterone levels, and suppressed gonadotropins was followed up until the age of 18.

Results: Throughout follow-up testicular asymmetry persisted and gonadotropin and testosterone pattern did not change. At the age of 18, inhibin B was undetectable while α-immunoreactive inhibin was within normal range. Anti-Müllerian hormone level was slightly subnormal. Sperm cells were 3 900 000 per ejaculate. Histology of both testes showed spermatogonia, spermatocytes, and, in some tubes, matured spermatozoa. Sertoli cells were markedly stained with anti-inhibin α-subunit antibody in both the testes. There was no immunostaining of Sertoli, Leydig, or germ cells with anti-βA or anti-βB antibody. MAS R201H mutation was identified in both the testes.

Conclusion: The 15-year follow-up in this boy with MAS demonstrated that autonomous testicular activation and gonadotropin suppression persisted over time. This provides an interesting model of active spermatogenesis despite long-term FSH suppression. It also suggests that FSH is needed for the full expression of the inhibin βB-subunit gene, an expression previously reported in the germ and Leydig cells of normal adult subjects.
of Messina University Hospital owing to sexual precocity, monolateral right testicular enlargement, accelerated growth, and bone maturation (Table 1). At that time, he exhibited marked muscle development and adult timbre of the voice. His testosterone level was in the adult male range. Baseline follicle-stimulating hormone (FSH) and luteinizing hormone (LH) serum levels were in the prepubertal range and did not increase after gonadotrophin-releasing hormone stimulation (Table 1).

Ketoconazole was given: 800 mg/day from 3 to 6 years of age and 1200 mg/day till the age of 7.6. Testosterone levels decreased sharply during ketoconazole treatment, but secretion was never totally suppressed. The lowest level was achieved at the age of 7.6 when ketoconazole dosage was 1200 mg/day. At this time, treatment was withdrawn because of a marked increase in aminotransferase serum. After ketoconazole withdrawal, an important increase in testosterone levels was again observed. Normal liver enzyme levels were recovered within 3 months.

The patient remained relatively tall until the age of 9 (Fig. 1) and bone age was advanced until the age of 12, when pubertal development was already complete; right testis volume achieved its zenith and height was already near final (Table 1). No significant changes of the echogenic architecture of both the testes as shown by ultrasound imaging were demonstrated during the period from 2.9 to 12 years of age.

Other MAS phenotypical manifestations occurred during follow-up: polyostotic fibrous dysplasia at 9.0 years of age and hypophosphatemic rickets at 12.3 years. No other clinical or biological endocrine abnormalities were observed during the entire follow-up, despite repeated screening.

At the age of 12.3 the patient dropped out of follow-up and we lost his traces until the age of 18 years, when he suddenly presented again due to his embarrassment for the persistent testicular asymmetry. On physical examination the patient appeared well developed, virilized, and stocky. He exhibited a complete pubertal development with persisting testicular asymmetry and satisfactory libido and potency. Final height was distinctly lower than target height (cm 155.0 vs 162.0). At ultrasound examination, bilateral hyperchogenic multiple spots compatible with testicular microlithiasis grade 3 (7) were observed.

Both testosterone and estradiol were in the adult male range, and FSH and LH remained markedly suppressed (Table 1).

### Table 1 Auxological, clinical, and endocrinological data during the entire 15-year follow-up, both on and off therapy.

<table>
<thead>
<tr>
<th>Chronological age (year)</th>
<th>3</th>
<th>4.6</th>
<th>6</th>
<th>7.6</th>
<th>9</th>
<th>12.3</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone age (year)</td>
<td>6</td>
<td>11.3</td>
<td>12.5</td>
<td>13.3</td>
<td>14.9</td>
<td>17.0</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>108</td>
<td>125.4</td>
<td>132.4</td>
<td>144.2</td>
<td>150.7</td>
<td>154.8</td>
<td>155</td>
</tr>
<tr>
<td>Height SDS</td>
<td>5</td>
<td>4.2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>0.5</td>
<td>-1.95</td>
</tr>
<tr>
<td>Left testis (ml)</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Right testis (ml)</td>
<td>20</td>
<td>12</td>
<td>9</td>
<td>15</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>LH basal (IU/I)</td>
<td>&lt;0.05 (0.1–1.3)</td>
<td>0.2 (0.1–1.3)</td>
<td>0.2 (0.2–1.6)</td>
<td>0.2 (0.3–2)</td>
<td>&lt;0.05 (0.9–2.5)</td>
<td>0.33 (5.5–15)</td>
<td></td>
</tr>
<tr>
<td>LH peak post-GnRH (IU/I)</td>
<td>&lt;0.05 (0.4–6.0)</td>
<td>0.43 (0.9–4.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH basal (IU/I)</td>
<td>0.05 (0.1–1.6)</td>
<td>0.06 (0.1–0.8)</td>
<td>0.1 (0.2–2.2)</td>
<td>0.06 (0.2–2.5)</td>
<td>0.05 (0.6–4.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH peak post-GnRH (IU/I)</td>
<td>&lt;0.05 (2.3–6.9)</td>
<td>0.08 (1.5–6.1)</td>
<td></td>
<td></td>
<td>0.18 (1.2–6.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>18.5 (0.1–0.5)</td>
<td>6.3 (0.1–1.0)</td>
<td>5.5 (0.1–1.0)</td>
<td>2.4 (0.1–1.0)</td>
<td>7.3 (0.1–1.0)</td>
<td>5.7 (0.7–10)</td>
<td>13.16 (4.2–24)</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>14 (7–44)</td>
<td>9 (7–51)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28 (22–117)</td>
</tr>
<tr>
<td>Inhibin B (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;6 (125–330)</td>
</tr>
<tr>
<td>Inhibin A (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;2</td>
</tr>
<tr>
<td>Anti-Mullerian hormone (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;2</td>
</tr>
<tr>
<td>Total a-inhibin (U/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>397 (280–700)</td>
</tr>
</tbody>
</table>

Italics indicate ages at which the individual was receiving therapy.
For the hormone levels, the reference values for age are given within brackets.
*According to Greulich & Pyle (5).
*According to Tanner & Whitehouse (6).
Hormone assays

Serum testosterone was measured by RIA using CIS Biointernational reagents (Gif sur Yvette, France) as previously described (8).

Estradiol was measured by RIA using DiaSorin reagents (Antony, France) as previously described (9).

FSH and LH were measured by time-resolved fluoroimmunometric assay using Wallac Delfia reagents (Perkin–Elmer, Courtaboeuf, France) as previously described (9).

Serum inhibin A and inhibin B were measured by ELISA using Oxford Biolinnovation reagents (DSL-France, Cergy-Pontoise, France), as previously described (8).

Immunoreactive α-inhibin (or total α inhibin) was measured by RIA, as previously described (10) using antiserum 1989 raised to purified 31 kDa bovine

Figure 1 Growth chart from 3 to 18 years of age.
inhibin, kindly donated by D M de Kretser (Monash University, Melbourne, Australia). Purified bovine 31-kDa inhibin iodinated by the lactoperoxidase method was used as a tracer. A pool of human follicular fluid was used as a standard. Its bioactivity assessed by in vitro bioassay with dispersed rat pituitary cells in culture was 280 U/ml. The sensitivity was 28 U/ml, and the intra- and inter-assay coefficients of variation were 2.1 and 6.5% respectively at the level 420 U/l.

Serum anti-Mullerian hormone (AMH) was measured by ELISA using Diagnostic System Laboratory reagents (DSL-France), as previously described (8).

**Testicular histological and immunohistochemical studies**

Testicular biopsy was performed at the age of 18. The biopsies of both the testes were fixed in 10% buffered formalin, paraffin embedded, and routinely processed. Multiple 2 μm thick sections were histologically examined using hematoxylin–eosin staining. Testicular paraffin sections were processed for immunohistochemistry by using the immunoperoxidase method as previously described (8).

Briefly, dewaxed sections were microwed in 0.01 citrate buffer, then preincubated in 0.3% triton–5% normal sheep serum in potassium PBS. They were incubated with monoclonal primary antibodies directed against inhibin α, βA, and βB-subunits (Oxford BiolInnovation, Oxford, UK) for 72 h at 4 °C at a concentration of 1:100, 1:5, and 1:5 respectively. The sections were sequentially incubated for 90 min in 1:150 biotinylated sheep anti-mouse immunoglobulin G (IgG) diluted in potassium PBS, and for 90 min in avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, AbCys, Paris, France) and were then exposed to biotinyl tyramide solution (Perkin–Elmer). The sections were exposed for 90 min to 1:200 streptavidin-peroxidase (Amersham Biosciences). Peroxidase activity was revealed with diaminobenzidine (DAB) (Sigma, St Louis, MO, USA) for 5 min and then counterstained with hematoxylin. The specificity of the primary antibodies was previously established (11, 12). Control incubations included omission of the primary antibodies to test non-specific binding of the secondary antibodies.

**Molecular analyses**

Genomic DNA was isolated from leukocytes and a frozen histological section of both the testes with the ‘Puregene TM’ DNA purification system kit (Gentra, Minneapolis, MN, 55441, USA).

A PCR-based technique for the selective enrichment of the Arg201 mutant allele in exon 8 of the GNAS1 gene was used (13). Briefly, it consists of PCRs with a modified 5′-primer that introduces a unique Eag I restriction site (CGGCGG) into the amplification product of the normal allele only and several 3′-primers to obtain a series of nested PCR amplifications. Five rounds of amplification each followed by restriction endonuclease digestion with Eag I (New England Biolabs, Beverly, MA, USA) were used to enrich the mutated allele and destroy the normal alleles. The amplified fragments were purified (QiAquick Gel Extraction Kit; Qiagen) and directly sequenced with a semi-automated detection system (ABI 373A sequencer, Applied Biosystem, Foster City, CA, USA). A control sample was added to the molecular DNA analysis.

**Informed consent**

The local Institutional Review Board was informed of the investigations being carried out. Because only one fully informed patient was involved, a research protocol approval was not required.

**Results**

**Cross-sectional evaluation of inhibins and AMH at the age of 18**

Inhibin A and inhibin B were undetectable, while α-immunoreactive inhibin was within the normal adult male range: 397 U/ml. AMH level was slightly below the lower limit of normal range: 0.87 ng/ml (Table 1).

**Semen analysis**

At the age of 18, the volume of the ejaculate was 6.5 ml (normal > 2 ml) and pH was 7.4 (normal 7.2). Bacterial culture of the semen showed no growth of any microorganism. Sperm concentration was low (600,000/ml; normal 20,000,000/ml), whereas motility was 60% within 60 min after ejaculation (normal > 50%) and morphology was normal in 80% of sperm; normal > 50% according to World Health Organization criteria (14).

**Histological findings**

The size of each biopsy sample was 3 × 3 × 3 mm. Each biopsy sample consisted of more than 100 sections of seminiferous tubules with a decreased mean diameter of 160 μm (normal 250–300 μm). The lamina propria was slightly thickened (7 μm thick, normal 3–5 μm). The histological phenotype of the left testis was homogeneous Sertoli cell only (SCO) with Sertoli cells normal in size. Conversely, the right testis had a mixed phenotype with predominantly SCO tubules and three adjacent tubules displaying complete spermatogenesis progressing through elongated spermatids/spermatocytes. These tubules had a normal diameter of 240 μm.
In both the testes, Leydig cells were scarce and non-hyperplastic.

**Immunohistochemistry**

In both the testes, Sertoli cells were immunolabeled with inhibin \(\alpha\)-subunit. In the testis with spermatogenesis, germ cells were not labeled. In both the testes there was no immunostaining by Sertoli, Leydig, or germ cells with the anti-\(\beta\)A or the anti-\(\beta\)B antibody.

**Molecular analyses**

A mutation at codon 201 resulting in Arg > His substitution (R201H), classically responsible for MAS, was identified in all the analyzed DNA samples from the biopsy of both the testes and from leucocytes.

**Discussion**

We report here, for the first time, that spermatogenesis is effective, although quantitatively impaired, in an adolescent boy with MAS, which gives evidence for germ cell maturation despite sustained and marked FSH suppression.

Clinical presentation was characterized by unilateral testicular enlargement in the context of a sexual precocity, which remained for many years the only manifestation of MAS in this boy. Sexual precocity is an infrequent presenting symptom of MAS in boys (1) and testicular enlargement is generally bilateral (15, 16).

There is only another report that demonstrates some similarities with our case (17). Also in that patient testicular enlargement was unilateral and occurred very early. However, macroorchidism was not associated with manifestations of hyperandrogenism, probably due to an autonomous hyperfunction of Sertoli cells, with no activation of Leydig cells. In fact, it has been recently demonstrated that GNAS1 allele mutation may be present only in Sertoli cells (18), resulting in isolated Sertoli cell hyperfunction (15). In the present case, on the contrary, clinical, biochemical, and histological findings altogether suggest an isolated Leydig cell hyperfunction, with no premature activation of Sertoli cells. Unfortunately, we could not separately analyze GNAS1 gene in the different testicular cells.

In this patient, the distinct asymmetry between the testes persisted unchanged from childhood to adulthood. This suggests that only the right testis was affected, whereas the left testis was rendered quiescent even during adolescence, due to the lack of FSH stimulation, as a result of the sex steroid-rich endocrine environment. It has been known for a long time that testosterone can exert an inhibitory effect upon FSH secretion through its conversion to estradiol and/or to dihydrotestosterone in the hypothalamus and/or the pituitary (19, 20). The same hypothesis has been proposed to explain the persistence of unilateral autonomous ovarian function in an adult woman with MAS (21). In that case, however, the mutation was only found in the affected ovary, while in our patient the mutation was present in both the testes, but presumably in a smaller number of cells in the left one.

It could also be hypothesized that the mutation affects some Sertoli cells in the right testis, thus resulting in the focal stimulation of Sertoli cell proliferation and focal germ cell maturation. This assumption is supported by the increase in the size of the right testis relative to the left one. However, in such a situation an overproduction of total \(\alpha\)-inhibin should be expected. In our patient, the inhibin \(\alpha\)-subunit gene is indeed expressed in Sertoli cells from both the testes, but the secretion of \(\alpha\)-inhibin into blood at 18 years of age is quite normal. That does not support the assumption that the mutation involves Sertoli cells, although it cannot be precluded.

On the other hand, the normal immunoreactive \(\alpha\)-inhibin level gives evidence that the Sertoli cells have been exposed to the physiological postnatal FSH rise. It could also be assumed that FSH suppression in the early pubertal age was not as marked as it was found at the age of 18 years, and that it could have initiated spermatogenesis. Unfortunately the gonadotropin levels at the age of 12 had not been evaluated.

The very long follow-up of our patient enabled us to reconstruct for the first time the long-term evolution of pituitary–testicular function in MAS and to shed some light on the pathophysiology of testicular autonomous function in this syndrome. The persistently elevated sex steroid levels, with low or undetectable gonadotropin levels during the entire follow-up, reflect that the autonomous testis hyperfunction lasted until adulthood, in contrast with the usual picture seen in the familial male-limited precocious puberty (FMPP) (8).

Finally, at the time of the last observation, testicular US in our patient revealed a picture of bilateral hyperecho-genic multiple spots compatible with testicular micro-lithiasis. In agreement with previous reports, testicular micro-lithiasis may be considered as a manifestation of this syndrome representing an additional diagnostic marker in boys (22).

Thanks to the long-term follow-up, we have evidenced another surprising finding in this case, i.e. a significant decrease in testis volume during ketoconazole administration. Since FSH levels remained unchanged, which is barely detectable during treatment, the decrease in testosterone level should be responsible for such a change in testis volume. As discussed below, experiments in rodents have shown that testosterone alone is able to stimulate seminiferous tube activity.

This is the first demonstration of an actual, although impaired, spermatogenesis in a subject with documented Leydig activation due to GNAS1 gene mutation. This gives evidence that, despite the longstanding gonadotropin suppression. the physiological Sertoli cell proliferation occurs normally in the postnatal and
pubertal periods (23). At the age of 18, the gene encoding for the inhibin βB-subunit was not expressed in any type of testis cells, in contrast to previous reports showing that the gene was expressed either in Sertoli cells and germ cells in normal boys (24), or in Leydig cells in boys with testotoxicosis or FMPP (8). In both of these disorders, the constitutional activation of the testosterone biosynthesis pathway results in premature testosterone secretion leading to sexual precocity. At the same time, the Sertoli cells lose their ability to express the inhibin βB-subunit gene, which could be interpreted as a consequence of the activation of the Sertoli cell androgen receptors. In FMPP, the genetic activation of the LH receptor induces a premature production of inhibin B (8), in contrast with the present situation, where only the receptor-associated Gs protein subunit is activated by the somatic mutation. That suggests that different post-receptor pathways are involved in FMPP and MAS regarding the relationship between LH receptor and inhibin βB gene expression in Leydig cells.

The contrast between germ cell maturation and low prepubertal FSH levels raises the question of the relative roles of FSH and testosterone in promoting spermatogenesis. FSH and testosterone, according to experiments carried out in rodents, synergically act in germ cell maturation (25). However, testosterone alone can induce significant germ cell maturation, independently of FSH activity (26). In the Rhesus monkey the proliferation of type A spermatogonia is relatively gonadotropin independent (27) and differentiation of spermatogonia from type A to type B can be driven by either testosterone or FSH (28). Moreover, our observation demonstrates that prolonged exposure to testosterone can result in sperm cell production, even though FSH levels are undetectable. In the present case, Sertoli cell production of α-immunoreactive inhibin was within normal adult range despite undetectable FSH levels, in agreement with the immunohistochemical staining of all Sertoli cells from both the testes, whilst the inhibin βB gene was not expressed in any type of testis cells, in contrast with inhibin β gene expression in normal pubertal boys. These findings may suggest that the expression of the α-subunit gene is less FSH-dependent than the expression of the βB-subunit gene. In addition they raise the question of the role of autonomous testosterone secretion on the inhibin β gene expression. However, as discussed above, a focal post-receptor activation of the FSH stimulation pathway due to mutation mosaicism cannot be precluded and that might account for focal stimulation of spermatogenesis.

Conclusion

In this boy with MAS, the 15-year follow-up of pituitary–testicular axis has demonstrated that both autonomous testicular function and gonadotropin suppression in MAS may persist over time. This long-term observation provides an interesting human model of spermatogenesis despite FSH suppression, which suggests that testosterone alone can maintain a certain level of germ cell maturation. On the other hand, there is no secretion of dimeric inhibin B, due to the lack of inhibin βB expression. Indeed it has been reported that in normal adult males, expression of the inhibin ββ-subunit is localized in germ cells and Leydig cells. Our findings are consistent with the assumption that, in human males, FSH is needed for the full expression of the inhibin ββ-subunit, although no FSH receptors have been evidenced so far in germ cells and Leydig cells.

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