ORIGINAL ARTICLE

Elevated anti-Müllerian hormone (AMH) and inhibin B levels in prepubertal girls with type 1 diabetes mellitus

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Summary

Objective Elevated anti-Müllerian hormone (AMH) and adrenal androgen levels have been observed during childhood in girls at risk of developing polycystic ovarian syndrome (PCOS). The aim of this study was to evaluate ovarian function and adrenal steroid levels in prepubertal girls with type 1 diabetes mellitus (T1D).

Design Cross-sectional study.

Patients/Measurements We evaluated hormonal and ultrasonographic characteristics in girls with T1D ($N = 73$) and compared them to characteristics found in a control group of healthy girls ($N = 86$). Data are reported as geometric means (95% CI).

Results Prepubertal girls with T1D had higher levels of AMH (29·1 pmol/l (23·2–36·3) *vs* 20·9 pmol/l (16·6–26·1), $P = 0·038$), inhibin B (arithmetic mean: 16·7 pg/ml (11·6–21·7) *vs* 11·7 pg/ml (10·0–13·5), $P = 0·044$) and dehydroepiandrosterone sulphate (DHEAS) (0·3 nmol/l (0·2–0·6) *vs* 0·2 nmol/l (0·1–0·3)) than controls ($P = 0·045$). During puberty, decreasing AMH levels were observed in girls with T1D only ($P < 0·0001$). Girls with T1D in Tanner stages 4–5 had lower AMH levels than their paired healthy controls (10·1 pmol/l (7·4–13·9) *vs* 15·7 pmol/l (11·6–21·3), respectively, $P = 0·047$).

Conclusions Our observations indicate that prepubertal girls with T1D may exhibit similar endocrine findings to those of other girls at risk of developing PCOS. The elevated levels of AMH and inhibin B suggest that higher numbers of follicles are present in the ovary during childhood in these patients and that insulin treatment may act as a local growth factor. In addition, AMH levels differed in prepubertal and pubertal girls, suggesting that the effect of T1D on ovarian folliculogenesis changes once gonadotrophin levels rise during puberty.

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Introduction

Several risk factors have been identified in girls at risk of developing polycystic ovarian syndrome (PCOS) later in life.[1] Exaggerated adrenarche, defined as elevated adrenal steroids during childhood, is a recognized phenomenon that is associated with ovarian hyperandrogenism during adolescence or adulthood.[2] Girls with type 1 diabetes mellitus (T1D) represent another group at high risk of developing hyperandrogenism;[3] this is likely related to intensive insulin therapy.[4] Recently, higher adrenal androgen metabolites have been observed in the urine of children younger than 10 years of age with T1D.[5] However, no systematic study of circulating adrenal steroid levels during childhood or adolescence while considering specific Tanner stages has been performed in girls with T1D undergoing intensive insulin treatment.

Anti-Müllerian hormone (AMH), a glycoprotein produced by granulosa cells of small ovarian follicles, is useful to evaluate ovarian function in prepubertal girls.[6,7] Two groups of girls at risk of developing PCOS, daughters of PCOS women[6,7] and infants small for their gestational age,[6,7] have elevated AMH levels at a young age. Girls with T1D are also at risk of developing PCOS,[3,4,8] but whether these girls also exhibit elevated AMH levels is not known.

We postulate that ovarian and adrenal function in prepubertal girls may be affected by T1D, which may lead to higher AMH and adrenal steroid levels. To investigate this hypothesis, we performed a cross-sectional study and compared ovarian function in prepubertal and pubertal girls with and without T1D.

Materials and methods

Subjects

We studied ovarian function in a group of prepubertal (N: 20) and pubertal (N: 53) girls with T1D. As a control group, 86 healthy girls

(without any chronic diseases) with normal fasting glucose levels were recruited from nearby schools or day care centres. The T1D and control girls were matched according to their Tanner stage, chronological age, socioeconomic status and BMI. Pubertal stage was classified as prepubertal (Tanner stage 1), early puberty (Tanner stage 2–3) or late puberty (Tanner stage 4–5). The ages of the girls ranged from 2·8 to 16·2; pubertal girls were included up to 2 years post-menarche. The steroidogenic ovarian profile of the pubertal group has been reported previously.[3]

All the girls with T1D who attended the diabetes clinic in Hospital San Borja Arriarán, Santiago, and had experienced menarche at least 2·5 years earlier were invited to participate. This diabetes unit takes care of all patients with T1D living in central Santiago and in the public health system. In addition, prepubertal girls older than 2·5 years of age with T1D who attended Hospital Sótero del Río, which is located in the southern area of Santiago, were recruited. All the girls with T1D needed insulin treatment from the time of diagnosis. Exclusion criteria included the following: other specific types of diabetes mellitus (DM) according to the American Diabetes Association classification[9] (including genetic defects in β-cell function, genetic defects in insulin action, diseases of the exocrine pancreas, endocrinopathies, drug- or chemical-induced DM, infections, uncommon forms of immune-mediated diabetes and other genetic syndromes sometimes associated with DM), type 2 DM, honeymoon period, abnormal thyroid function, use of sex steroids and presence of other concomitant chronic conditions such as genetic syndromes, coeliac disease, renal, liver or cardiac disease or undernourishment. Daily insulin doses that were administered during the 15-day period prior to the initiation of the study were recorded.

Study protocol

We performed a complete physical examination and pubertal development assessment according to Marshall and Tanner. Weight was measured using a conventional Seca scale with a precision of 100 g, and height was measured with a Harpenden stadiometer. Standard deviation scores were calculated for height, weight and BMI using current NCHS standard curves.[10] These growth curves have been shown to be applicable to contemporary Chilean populations.[11]

An early morning fasting blood sample was obtained for the measurement of AMH, inhibin B, oestradiol, LH and FSH levels. In post-menarcheal girls, the test was performed during the follicular phase (days 3–8), as previously described.[3] Ovarian volume was calculated by transabdominal ultrasonography using the simplified formula for a prolate ellipsoid;[12] the mean volume of both ovaries was calculated.

The protocol was approved by the Institutional Review Board of San Borja Arriarán Hospital. All parents signed informed consents, and all girls gave their assent before entering the study.

Hormone assays

Serum testosterone [sensitivity (S) = 0·0035 nmol/l], androstenedione (S = 0·07 nmol/l), 17-OH progesterone (S = 0·03 nmol/l), dehydroepiandrosterone sulphate (DHEAS) (S = 0·07 nmol/l) and oestradiol (S = 18·4 pmol/l) were measured by competitive specific binding Radioimmunoassay (RIA, Diagnostic System Laboratories,Webster, TX, USA); interassay coefficients of variation (CVs) were 8·1, 8·9, 7·3, 7·7 and 6·1%, respectively; intra-assay CVs were 5·3, 4·2, 7·7, 5·3 and 4·1%, respectively, as previously described.[3,13] Serum LH (S = 0·10 mUI/ml), FSH (S = 0·10 mUI/ml) and sex-hormone-binding globulin (SHBG) (S = 0·5 nmol/l) levels were measured by immunoradiometric assays from Siemens Healthcare Diagnostics (USA). Intra-assay CVs were 6·5% for LH, 3·6% for FSH and 3·9% for SHBG. Interassay CVs were 7·6% for LH, 6·2% for FSH and 6·9% for SHBG.[3,13]

The free androgen index (FAI) was calculated using the formula $[100 \times \text{testosterone (nmol/l)}]/[\text{SHBG (nmol/l)}]$.[14] Serum AMH and inhibin B levels were measured as previously described.[15] HbA1c levels were measured using a commercially available automatic system (DCA 2000, Bayer Diagnostics, Tarrytown, NY, USA).

Statistical analysis

Normal distribution was evaluated using the Kolmogorov–Smirnov and skewness/kurtosis tests for normality. Inhibin b and SHBG showed normal distributions and are reported as arithmetic means (and 95% CI). The remaining hormones did not pass the normality test and were transformed to their logarithms for analyses. These hormones, after being logarithmically transformed, showed normal distributions and are reported as geometric means (and 95% CI).

Comparisons of the means between the two groups (T1D and C) for each Tanner stage were made using the unpaired Student's *t*-test. Differences within each group among the various Tanner stages were assessed by one-way ANOVA, followed by the Bonferroni correction for multiple comparisons. Correlation analysis was performed using the Pearson's test.

All statistical calculations were run on SPSS for Windows, version 18.0, and GraphPad Prism, version 5.00. A *P*-value of <0·05 was considered to be statistically significant.

Results

The clinical characteristics of the patients are shown in Table 1. All the prepubertal girls with T1D were treated with three or four daily insulin injections of short-acting insulin analogues (N = 18) or soluble insulin (N = 2). Basal insulin replacement was performed with glargine (N = 8), detemir (N = 1) or NPH (N = 11) insulin. The HbA1c range in this group was 6·6–8·8%. The age of menarche in the post-menarcheal girls was similar in both groups: 12·5 years (95% CI: 11·9–13·0 years) and 12·0 years (95% CI 11·7–12·5 years) in T1D and C girls, respectively. A positive family history of PCOS was observed in a similar proportion for the T1D and C girls (5·5 and 7·0%, respectively).

Steroid and gonadotrophin levels and ultrasonographic findings of the prepubertal girls are shown in Table 2. Girls with T1D had higher levels of SHBG and DHEAS than control girls (*P* = 0·037 and *P* = 0·045, respectively). Androstenedione levels had a tendency to be higher in those with T1D, although the difference did

Table 1. Clinical and anthropometric characteristics of girls with type 1 diabetes mellitus (T1D) and controls (C), as well as metabolic controls in girls with T1D according to the Tanner stage (T). Data are shown as arithmetic means (95% CI)

	Tanner 1		Tanner 2 to 3		Tanner 4 to 5	
	T1D	C	T1D	C	T1D	C
n	20	24	28	28	25	34
Age (years)	6·1 (5·2 to 7·0)	6·2 (5·6 to 6·9)	11·2 (10·8 to 11·7)	10·8 (10·3 to 11·3)	13·2 (12·6 to 13·7)	12·7 (12·4 to 13·1)
BMI SDS	1·0 (0·6 to 1·5)	1·0 (0·6 to 1·4)	−0·1 (−0·4 to 0·3)	0·4 (−0·1 to 0·8)	0·6 (0·3 to 0·8)	0·7 (0·4 to 1·0)
Height SDS	0·2 (−0·3 to 0·7)	0·4 (−0·1 to 0·9)	−0·4 (−1·0 to 0·2)	−0·4 (−0·8 to 0·1)	−0·3 (−0·7 to 0·2)	0·1 (−0·2 to 0·3)
DM duration (years)	1·9 (1·3 to 2·5)		3·6 (2·5 to 4·7)		5·0 (3·7 to 6·3)	
HbA1c (%)	7·7 (7·4 to 8·0)		8·7 (7·9 to 9·4)		9·3 (8·4 to 10·1)	
Insulin dose (U/kg per day)	0·8 (0·7 to 0·9)		0·8 (0·7 to 1·0)		1·2 (1·0 to 1·3)	

SDS, standard deviation score.

not reach statistical significance ($P = 0.07$). The results from the transabdominal ultrasonography revealed that both groups of girls had similar ovarian volumes.

Girls with T1D exhibited higher AMH levels than control girls during childhood (29·1 pmol/l (95% CI: 23·2–36·3 pmol/l) *vs* 20·9 pmol/l (95% CI: 16·6–26·1 pmol/l), $P = 0.038$, Fig. 1a). During the final stages of puberty, girls with T1D had lower levels of AMH than C girls in the same pubertal stage ($P = 0.047$). During puberty, AMH levels decreased in girls with T1D (ANOVA $P < 0.0001$), but not in controls ($P = 0.4$).

Table 2. Steroid and gonadotrophin levels, as well as ultrasonographic findings, in prepubertal girls with type 1 diabetes mellitus (T1D) and controls (C). Data are shown as geometric means (95% CI), except those that have a normal distribution (sign), which are shown as arithmetic means (95% CI). Statistical analysis was performed using the unpaired Student's *t*-test. To convert to metric: Oestradiol (pmol/l) × 0·272 = Oestradiol (pg/ml); 17-OH progesterone (nmol/l) × 0·33 = 17-OH progesterone (ng/ml); testosterone (nmol/l) × 0·288 = testosterone (ng/ml); dehydroepiandrosterone sulphate (DHEAS) (nmol/l) × 370·4 = DHEAS (ng/ml); androstenedione (nmol/l) × 0·287 = androstenedione (ng/ml)

	Tanner 1	
	T1D	C
n	20	24
LH (IU/l)	0·6 (0·4–0·7)	0·5 (0·4–0·5)
FSH (IU/l)	2·0 (1·7–2·4)	2·1 (1·8–2·4)
LH/FSH ratio	0·3 (0·2–0·3)	0·2 (0·2–0·3)
Oestradiol (pmol/l)	29·8 (23·5–37·7)	29·8 (25·0–35·6)
Testosterone (nmol/l)	0·30 (0·22–0·41)	0·33 (0·26–0·41)
Free androgen index	0·3 (0·2–0·5)	0·4 (0·3–0·5)
SHBG (nmol/l)†	95·5 (81·6–109·4)	80·9 (74·5–87·2)*
17-OH progesterone (nmol/l)	1·5 (1·0–2·1)	1·7 (1·3–2·2)
DHEAS (nmol/l)	0·3 (0·2–0·6)	0·2 (0·1–0·3)**
Androstenedione (nmol/l)	3·1 (2·5–3·9)	2·6 (2·0–3·3)***
Mean ovarian volume (ml)	0·8 (0·6–1·2)	0·7 (0·5–0·9)

SHBG, sex-hormone-binding globulin.
*$P = 0.037$; **$P = 0.045$; ***$P = 0.07$.
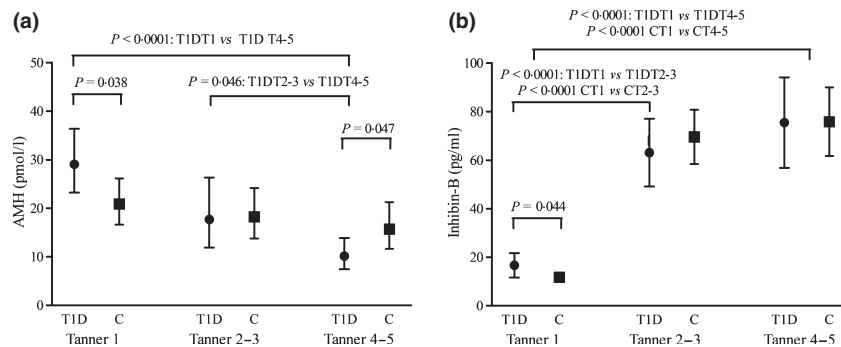†Data are shown as arithmetic means (95% CI).

Inhibin B levels were slightly, but significantly, higher in girls with T1D during childhood ($P = 0.044$, Fig. 1b). During puberty, inhibin B increased in both the T1D and control girls, and differences between groups were no longer observed.

Discussion

Our results suggest that girls with T1D exhibit higher adrenal androgen, AMH and inhibin B levels in childhood. The magnitude of the observed increase in AMH and androgen levels in this group of girls was greater than what has been previously reported for daughters of women with PCOS.[6] During puberty, AMH levels progressively decreased; by the end of puberty, the levels were lower in girls with T1D compared to the controls, whereas inhibin B levels normalized during pubertal development.

In T1D, nonphysiological insulin replacement therapy is performed by administering insulin into the subcutaneous tissue, with subsequent absorption into the systemic circulation. Usually, the liver eliminates 50–70% of the insulin delivered into the portal vein,[16–18] a step that is bypassed by subcutaneous insulin administration in patients with T1D. This may lead to exposure of the ovary and adrenal gland to elevated insulin levels and may explain the hyperandrogenism and polycystic ovarian morphology observed in these patients.[3,4,8] In fact, insulin binds to insulin, IGF-1 and hybrid insulin-IGF-1 receptors in the ovary and the zona reticularis, thus stimulating androgen production.[19,20] Conversely, the direct effect of insulin on aromatase activity in granulosa cells seems to be minor.[21] On the other hand, insulin has co-gonadotropic actions,[22] which increases LH-induced androgen synthesis and secretion[19,23] and FSH-dependent recruitment and growth of preovulatory follicles.[24,25]

AMH is secreted mostly by early-growing, preantral and early antral follicles.[26] Ovarian folliculogenesis has two distinct stages.[27] During the first stage, known as the noncyclic recruitment stage, the primordial follicle grows through small primary, secondary and small antral stages to approximately 2 mm in diameter. The growth of these small follicles, which are usually observed in the prepubertal ovary, is dependent on the presence of local growth factors that act through autocrine and paracrine mechanisms.[28,29] The elevated levels of AMH and inhibin B observed in prepubertal girls with

Fig. 1 Anti-Müllerian hormone (AMH) and inhibin B levels in girls with type 1 diabetes mellitus (T1D) and control (C) girls. (a) AMH levels are shown as geometric means (and 95% CI). (b) Inhibin B levels are shown as arithmetic means (and 95% CIs). Differences between T1D and C girls in the same pubertal stage were evaluated using the unpaired Student's *t*-test. Differences between the different Tanner stages (T) were assessed by one-way ANOVA, followed by Bonferroni correction for multiple comparisons. T: Tanner.

T1D suggest that higher numbers of follicles are present in their ovaries. It is possible that during childhood, insulin treatment acts as a local growth factor, which stimulates the growth of small follicles (Fig. 2).

After the onset of puberty, the second stage of folliculogenesis, or the cyclic recruitment stage, occurs under the control of gonadotrophins and other metabolic signals.[28,29] Above a size of 2 mm, follicles become more FSH-dependent. The proliferation rate of granulosa cells increases, and the follicle, reaching a diameter of 5 mm in approximately 5 days, becomes recruitable for the next ovulatory cycle.[27] Recruited follicles progressively lose AMH expression but retain inhibin B production.[30] In the pubertal T1D girls, AMH and inhibin B levels were no longer higher than in the controls. A possible explanation may be that once gonadotrophin secretion increases during puberty, insulin acts as a co-gonado-

trophin, stimulating the recruitment and growth of larger follicles,[24,25] which do not secrete AMH but rather maintain inhibin B secretion (Fig. 2).

A relationship might exist between increased AMH levels in prepubertal girls and the earlier age of menopause in women with T1D.[31] We have recently shown an earlier decline of ovarian reserve in adult women with T1D.[32] Studies that were conducted in animals have suggested that a chronic state of hyperglycaemia leads to follicular apoptosis.[33] Future studies are needed to assess whether the earlier decline in ovarian function in adult women with T1D is related to an exaggerated noncyclic recruitment and subsequent apoptosis of ovarian follicles at an early age.

The discrepancy in the results of the AMH and inhibin B levels that were observed in prepubertal and pubertal girls with T1D can also be explained by the pathophysiology of abnormalities in
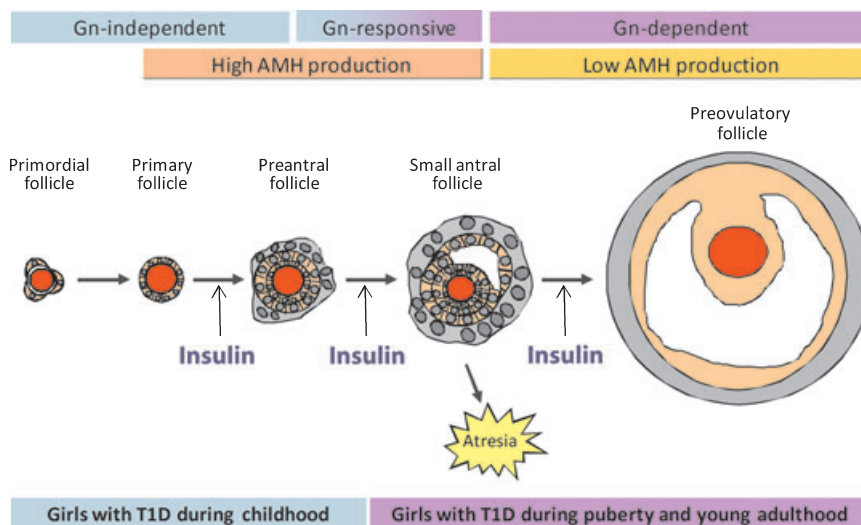
Fig. 2 Proposed model to explain anti-Müllerian hormone (AMH) levels in prepubertal and pubertal patients with T1D. AMH is highly expressed by preantral and small antral follicles, whereas large follicles produce less AMH. The first phase of ovarian folliculogenesis, involving the noncyclic recruitment stage of primordial follicles that grow until the small antral stage (approximately 2–5 mm), occurs from foetal life through adulthood and is gonadotrophin (Gn)-independent. The growth of these small follicles, usually observed in the prepubertal ovary, is dependent on the presence of local factors that act through autocrine and paracrine mechanisms. The elevated levels of AMH observed in prepubertal girls with T1D suggest that higher numbers of small follicles are present in their ovaries, probably in response to insulin treatment. After the onset of puberty, the second phase of folliculogenesis, or the cyclic recruitment stage, occurs under the control of gonadotrophins and other metabolic signals. Insulin acts as a co-gonadotrophin, stimulating the recruitment and growth of larger follicles,[24,25] which only secrete a small amount of AMH.

ovarian function observed in these patients. Recently, we have shown that women with PCOS and T1D do not show increased AMH levels despite having an elevated number of ovarian follicles.[15] This finding is in direct contrast to women with PCOS without T1D who have elevated levels of AMH. We postulate that normal or lower AMH levels observed in early puberty and late puberty, respectively, can be explained by follicular growth stimulated by insulin to the point of decreasing AMH levels (Fig. 2). Another possible mechanism explaining the lower AMH serum levels observed at the end of puberty in girls with T1D may be related to exacerbated insulin resistance during puberty observed in these patients[34] because a negative correlation between AMH and insulin resistance has been described.[35]

Increased adrenal androgen levels in association with premature pubarche, known as exaggerated adrenarche, are associated with a higher risk of developing PCOS. Here, we show that prepubertal girls with T1D without pubarche exhibit high DHEAS levels compared to controls. Similar results have been recently shown by Remer *et al.*[5] who studied urinary steroid levels in a group of prepubertal children and pubertal girls with T1D; higher adrenal androgens, especially DHEAS metabolites, were observed in these patients compared to the controls. The zona reticularis of the adrenal gland, similar to the ovary, has insulin and insulin growth factor receptors. Insulin binds to this receptor and is able to enhance adrenal androgen secretion.[36]

A limitation of this study is that the measurement of oestradiol and testosterone was taken with RIAs, with an assay sensitivity close to the physiological levels observed during childhood. This problem may limit the conclusions regarding how the levels of these steroids are affected by T1D in prepubertal girls with T1D and could be addressed only with ultrasensitive assays.[37]

In summary, we have shown that AMH, inhibin B and adrenal androgen levels are increased in girls with T1D during childhood. In addition, the levels of AMH decrease during puberty in girls with T1D; this suggests that T1D modulates ovarian follicle growth differently once gonadotrophin levels rise. These endocrine findings suggest that girls with T1D exhibit similar endocrine findings to other groups at increased risk of developing PCOS. Prospective follow-up of this cohort may help elucidate their prognosis in adulthood.

Disclosure

Acknowledgements

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