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The pituitary–testicular axis in Klinefelter's syndrome and in oligo-azoospermic patients with and without deletions of the Y chromosome long arm

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Summary

OBJECTIVE The most frequent known genetic causes of severe oligospermia (< 5 million sperm/ml) or azoospermia in men are Klinefelter's syndrome (KS), and deletions in the Y chromosome long arm (Yq). We aimed to compare the function of the pituitary–testicular axis in patients with severe oligospermia or azoospermia, idiopathic or associated with Y chromosome deletions or Klinefelter's syndrome (KS) and in control subjects.

PATIENTS We studied 47 men with idiopathic oligo-azoospermia, 42 with Yq deletions (27 *AZFc*, 13 *AZFb* and two *AZFa*) and oligo-azoospermia, 14 with KS and 39 control subjects (total 143).

MEASUREMENTS We analysed levels of FSH, inhibin-B, LH, free testosterone and oestradiol in all subjects, and we calculated indexes based on those hormones.

RESULTS Inhibin-B levels were indistinguishable between patients with idiopathic and Y deletion-associated oligo-azoospermia, lowest in the Klinefelter's patients and highest in controls. FSH levels followed the reverse pattern: indistinguishable between patients with idiopathic and deletion-associated oligo-azoospermia, highest in Klinefelter's patients and lowest in controls. Oestradiol, free testosterone and the derived indeces were not different in subjects with Yq deletions compared to those with idiopathic oligo-azoospermia. Among the Yq-deleted patients, no measured or derived parameter differed between the subjects with *AZFc* deletion and those with *AZFb* deletion. When non-KS oligo-azoospermic patients were classified according to histology [Sertoli cell-only (SCO), *n* = 18 or non-Sertoli cell only (non-SCO), *n* = 18] and compared to KS patients, the hormonal pattern did not differ between SCO and non-SCO subjects, but levels in KS patients were significantly different for FSH, inhibin-B and the FSH/inhibin-B ratio. KS patients not only had lower inhibin-B than SCO and non-SCO oligo-azoospermic men, but also higher FSH levels for any given inhibin-B concentration.

CONCLUSION Our data show that Y-deleted patients do not have a lesser impairment of Sertoli cell function than patients with idiopathic oligo-azoospermia, and support the concept that the main determinant of inhibin-B production is the germ cell mass. Also, our results suggest that one or more other factors, apart from inhibin-B, may contribute to increased pituitary secretion of FSH in KS patients.

Deletions of portions of the long arm of the Y chromosome that can be identified by karyotyping or molecular methods (Yq deletions) are present in approximately 7–11% of male subjects with azoospermia or severe oligospermia (less than 5 million sperm/ml) and are considered the most common cause of genetic infertility in the male (Simoni *et al*., 1997; Liow *et al*., 1998; Krausz *et al*., 1999, 2001; Krausz & McElreavey, 2001). Although fertility has been described in patients with Yq deletions (Chang *et al*., 1999), it is now acknowledged that all patients with Yq deletions have a substantial impairment of spermatogenesis, and sperm count is never within the normal range according to the WHO definition (Krausz *et al*., 2001). Submicroscopic characterization of Yq identified three broad subregions where most interstitial deletions occur, termed *AZFa*, *AZFb* and *AZFc* (Vogt *et al*., 1996).

The genes that are situated in Yq broadly fall into two categories: those that are testis-specific (expressed only in testicular tissue) and genes that are widely expressed (Lahn & Page, 1997). Notwithstanding this observation, there are no confirmed reports of abnormal phenotypes in men with Yq deletions, with the

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exception of altered spermatogenesis and subsequent infertility or subfertility (Fagerli *et al*., 1999; Oates *et al*., 2002). Furthermore, testis expression of Y genes may be limited to the germ cell lineage and absent in somatic cells (Sertoli cells, myoid cells, Leydig cells, etc.; Schnieders *et al*., 1996; Menke *et al*., 1997), although expression of at least one gene (RBMY) has also been detected in Sertoli cells (Osterlund *et al*., 2001).

For this reason, it may be hypothesized that Yq deletions only affect germ cell function, and that other testicular somatic cells may be unaffected. Indeed, a recent study (Foresta *et al*., 2001) has found that patients with oligo-azoospermia due to Yq deletions have inhibin-B and FSH levels that are significantly different from those of patients with idiopathic oligo-azoospermia, whereas the LH/testosterone axis was not affected.

However, inhibin-B production by Sertoli cells is considered to reflect the global efficiency of spermatogenesis, as men with various degrees of spermatogenic failure have lower levels of inhibin-B (and higher FSH) regardless of the cause of the dysfunction (Anawalt *et al*., 1996).

We thus set out to evaluate the basal activity of the hypothalamic–pituitary–testicular axis in patients with azoospermia or severe oligospermia, with or without deletions of Yq, and used two comparison groups, a control group consisting of a general population of males and a group of Klinefelter's syndrome patients presenting for infertility.

Furthermore, recent data suggest that most, if not all, complete *AZFb* deletions by the criteria of Vogt *et al*. (1996) encompass a part of the *AZFc* region, and are the largest of all human interstitial deletions for which deletion junctions and complete intervening sequence are available (Repping *et al*., 2002); because even partial *AZFc* deletions are associated with spermatogenetic failure (de Vries *et al*., 2002), this may contribute to explain the more severe spermatogenetic phenotype observed in *AZFb* compared to *AZFc* deletions.

For this reason, we also compared the hormonal values between the patients with *AZFb* deletions (including terminal deletions) and those with interstitial *AZFc* deletions.

Patients and methods

The study was approved by the MIT Institutional Review Board, and all patients gave written informed consent.

Patients and controls

We studied four groups of subjects:

1 Patients being evaluated for azoospermia or severe oligospermia, and who had a deletion in the Y chromosome long arm ('Y-deleted', $n = 42$);

2 Patients as above, but without Y chromosome deletions, and with normal 46,XY karyotype ('idiopathic', $n = 47$);

3 Control subjects taken from a general population of males undergoing thyroid function tests, which were later reported as normal ('controls', $n = 39$);

4 Patients with azoospermia, who were clinically and cytogenetically diagnosed as having Klinefelter's syndrome ('KS patients', $n = 14$).

'Non-KS' refers to the two groups (with and without Yq deletion) of non-Klinefelter's azoospermic or severely oligospermic patients. To allow us to compare histological data, we combined non-KS patients; biopsy data were available for 36 of these 89 patients, with 18 patients displaying a Sertoli cell only (SCO) histological pattern, and 18 patients with non-SCO oligo-azoospermia.

None of the non-KS patients reported previous scrotal trauma or infection; endocrinopathies including hypogonadotropic hypogonadism were also excluded. Obstructive azoospermia was ruled out based on a combination of clinical exam, semen analysis and hormonal levels and testicular biopsy in selected cases.

Laboratory evaluation

Semen analysis was performed at the Boston University Medical Center, according to WHO guidelines; severe oligospermia was defined as fewer than 5 million sperm/ml.

Yq deletion testing was done by ± PCR, using described methods (Vollrath *et al*., 1992).

FSH and LH levels were assayed using commercial enzyme-linked, two-site, solid-phase immunoassays (DSL Laboratories, Webster, TX, USA); the intra- and interassay coefficients of variation (CV) at medium levels are 3·4% and 3·5% for FSH, and 5·6% and 7·6% for LH. Oestradiol (E2) and free testosterone were determined using competitive, enzyme-linked, solid-phase immunoassays (DSL Laboratories); intra- and interassay CVs at low levels are 4·8% and 6·5% for E2, and 6·8% and 9·2% for free testosterone.

Inhibin-B was measured using a specific enzyme-linked, two-site, solid-phase immunoassay (Oxford Bio-Innovation Ltd, Oxford, UK), whose intra-assay CV was 6·5% and sensitivity 7 pg/ml. The mean of the internal control samples (supplied separately by the same manufacturer), was within the acceptable range. However, this assay has been shown to have a poor interassay precision (Meachem *et al*., 2001), and there are no internationally recognized reference preparations of inhibin-B. Consequently, in order to allow an easier comparison of our data to those published in the literature, inhibin-B-values were normalized by multiplying them to a factor obtained by dividing the expected *vs.* observed mean concentration of the internal control samples.

For completeness, inhibin-B levels were also assayed in a second control group, comprised of 26 subjects with normal semen exam (putative fathers of at least one child, before were undergoing

vasectomy), to confirm the suitability of the control group for the present analysis.

Each of the five hormones examined (LH, FSH, inhibin-B, free testosterone and E2) was assayed in all subjects at the same time; inhibin-B was determined in all subjects with a single standard curve, using kits from the same batch.

We calculated derivative indices of Sertoli and Leydig cell function using the original (nonlog-transformed) values. These were the LH/free testosterone ratio (a measure of Leydig cell 'resistance'), the FSH/inhibin-B ratio (a measure of Sertoli cell 'resistance'; Mahmoud *et al*., 2000) and the LH/FSH ratio.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance, with *post hoc* mean separation using the Tukey's honestly significant differences (HSD) test at the 0·05 significance level. As a preliminary analysis of the variables by the Levene's test showed that none of the hormones tested had homogeneity of variance among groups, all measured parameters were log-transformed before further analysis. However, for clarity, means (± standard error of the mean, SEM) and other values are reported in their original values, in graphs and text.

When indicated, the χ^2 test was performed, using Yates' correction if appropriate.

Correlation between inhibin-B and FSH values was determined using the Spearman coefficient. Regression lines were calculated using inhibin-B as the independent and FSH as the dependent variable. Analysis of covariance, with the Sidak adjustment, was also calculated.

Finally, multivariate analysis was performed on the subset composed of the two groups of non-KS patients (idiopathic and Y-deleted), using discriminant analysis.

Graphs of individual variables are reproduced as boxplots, which show the median and interquartile range (boxes) and outliers (as dots outside the graph).

All statistical calculations were done using SPSS for Windows, version 8·0.

Results

Y deletion detection

Of the 42 Y-deleted patients, 27 had an *AZFc* deletion; 13 had an *AZFb* deletion, which in all but one case included part or all of the *AZFc* region; and two patients had an *AZFa* deletion. All *AZFc* deletions were interstitial and spanned the same deletion interval; the *AZFb* group was more varied and included terminal deletions as well as different interstitial deletions. Details of the different deletions are shown in Table 1 and Fig. 1 (also according to the recently published nomenclature; Repping *et al*., 2002).

Semen analysis

Semen parameters were not statistically different between Y-deleted and idiopathic oligo-azoospermic patients. All KS patients were azoospermic.

Semen analysis was not performed on controls; however, inhibin-B levels in controls were closely matched by those in proven normospermic subjects (165·3 ± 13·3 *vs.* 175·6 ± 23·6 pg/ml, $P = 0{\cdot}986$ by Tukey's HSD). This confirms that unselected control subjects are suitable in studies of inhibin-B levels.

Hormonal univariate analysis

Inhibin-B levels (Fig. 2) were significantly lower in KS patients (16·8 ± 3·5 pg/ml, $P = 0{\cdot}001$) than in the two non-KS groups (Y-deleted patients, 63·1 ± 8·6 or idiopathic subjects, 59·4 ± 10·5). These two groups in turn had significantly lower inhibin-B values than control subjects (165·3 ± 13·3, $P = 0{\cdot}001$). Analysis of the Y-deleted *vs.* idiopathic groups confirmed practically identical means ($P = 0{\cdot}71$). Normal subjects (and the additional control group of proven normospermic subjects) however, display ample variability of inhibin-B levels, so there is significant overlap with the values of non-KS patients, whether Y-deleted or not.

Table 1 Classification of Y chromosome deletions

General group (*n*)	Type of deletion	Approximate length	Previous classification	Patients (*n*)
AZFc (27)	AZFc (interstitial)	3·5 MB	Classic *AZFc*	27
AZFb (13)	Terminal deletions, with breakpoints proximal to P4	9·5–14·3 MB	Terminal *AZFb* + *AZFc*	5
	P5/distal P1 (interstitial)	7·7 MB	*AZFb* + *AZFc*	6
	P3/distal P1 (interstitial)	6·2 MB	Partial *AZFb* + *AZFc*	1
	Unique interstitial	2 MB	Partial *AZFb*	1
AZFa (2)	*AZFa* (interstitial)	0·8 MB	*AZFa*	2

P1-P5: Y palindromes 1–5 (Kuroda-Kawaguchi *et al*., 2001). P5/P1 indicates a deletion that completely eliminates palindromes P4 to P1 (Repping *et al*., 2002).

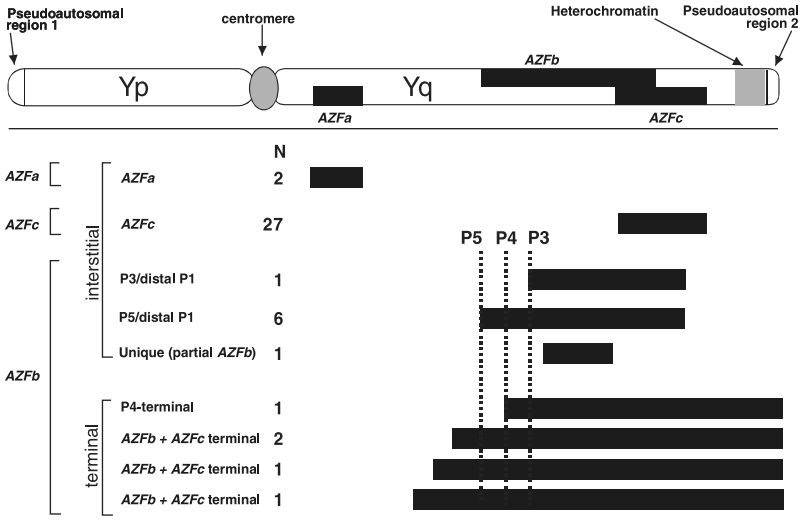

Fig. 1 (Y) chromosome deletion types (see also Repping *et al*., 2002 for a detailed description of *AZFb* deletions). Heterochromatin and pseudoautosomal regions are not to scale.

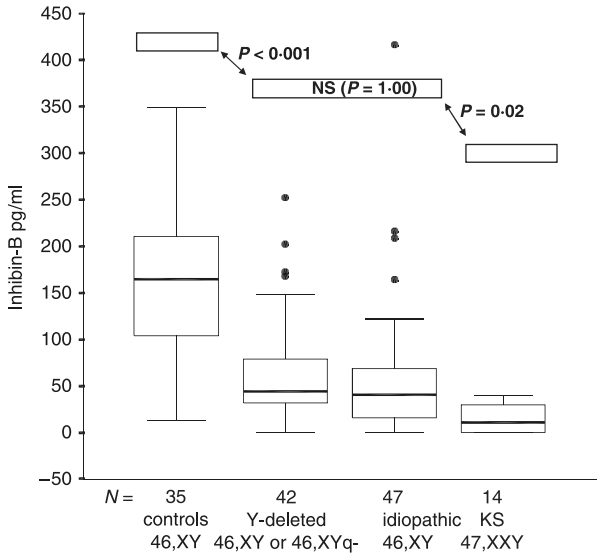

Fig. 2 Boxplot of inhibin-B levels in the four groups. Horizontal rectangular boxes show homogeneous groups by Tukey's test. Boxes include the median and interquartile range, outliers are shown as dots outside the graphs.

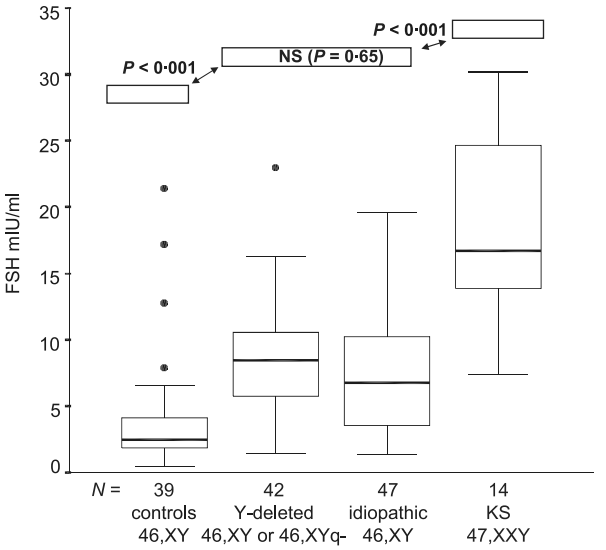

Fig. 3 Boxplot of FSH levels in the four groups. Horizontal rectangular boxes show homogeneous groups by Tukey's test.

The ranges of inhibin-B were: 12·8–348·5 in normal subjects, 0·01–252·6 in Y-deleted, 0·01–416·5 in idiopathic infertile and 0·01–40·2 in KS patients.

FSH levels (Fig. 3) were lowest in the control group ($3{\cdot}97 \pm 0{\cdot}7$ mIU/ml, $P < 0{\cdot}001$), and highest in the KS patients ($18{\cdot}12 \pm 1{\cdot}8$, $P < 0{\cdot}001$), whereas the two groups of non-KS patients had intermediate values ($8{\cdot}6 \pm 0{\cdot}7$ in Y-deleted patients, and $7{\cdot}7 \pm 0{\cdot}7$ in idiopathic infertile patients) and did not differ significantly ($P = 0{\cdot}65$ by Tukey's HSD).

The FSH/inhibin-B ratio was also unable to differentiate between Y-deleted and idiopathic infertile patients. It was much higher in KS patients ($2{\cdot}10 \pm 0{\cdot}6$) than in the other three groups ($0{\cdot}001 \pm 0{\cdot}0$ in controls, $0{\cdot}35 \pm 0{\cdot}1$ in Y-deleted patients and $0{\cdot}55 \pm 0{\cdot}1$ in idiopathic infertile patients, $P < 0{\cdot}001$ *vs.* KS subjects in each case). However, these three groups did not differ among themselves ($P > 0{\cdot}09$ in all cases).

LH values were significantly higher in KS patients ($12{\cdot}2 \pm 1{\cdot}7$ mIU/ml) than in the three other groups, which were homogeneous among them (control subjects, $4{\cdot}59 \pm 0{\cdot}6$; Y-deleted infertile patients, $5{\cdot}98 \pm 1{\cdot}0$; idiopathic infertile subjects, $4{\cdot}22 \pm 0{\cdot}6$). The

Table 2 Oestradiol and free testosterone values in the four groups (means ± SEM)

	Controls (n = 39)	Idiopathic (n = 47)	Y-deleted (n = 42)	KS (n = 14)	P-value
Oestradiol (pg/ml)	80·8 ± 10·3	86·8 ± 12·3	84·6 ± 10·6	103 ± 24·0	0·95
Free testosterone (pg/ml)	6·9 ± 0·7	5·7 ± 0·4	6·2 ± 0·7	4·9 ± 0·5	0·83

Table 3 Hormonal values in *AZFc* and *AZFb* deleted subjects (means ± SEM)

	AZFc (n = 27)	*AZFb* (n = 13)	P-value
Inhibin-B (pg/ml)	62·7 ± 10·1	70·6 ± 18·3	0·76
FSH (mIU/ml)	8·4 ± 0·8	8·8 ± 1·2	0·94
LH (mIU/ml)	6·8 ± 1·5	4·3 ± 1·1	0·26
Oestradiol (pg/ml)	78·3 ± 12·8	100·9 ± 21·9	0·63
Free testosterone (pg/ml)	6·7 ± 1·0	5·6 ± 0·6	0·98
LH/FSH	0·34 ± 0·05	0·29 ± 0·06	0·86
FSH/Inhibin-B	0·37 ± 0·1	0·21 ± 0·05	0·56
LH/free testosterone	1·07 ± 0·2	0·78 ± 0·3	0·67

same was true for the LH/free testosterone ratio, which was significantly higher in KS patients (2·95 ± 0·5) than in the three groups just listed (0·91 ± 0·1, 1·0 ± 0·1 and 0·87 ± 0·1, respectively).

Control subjects had significantly higher LH/FSH ratios (0·93 ± 0·1, $P < 0·001$) than all three groups of patients (KS, Y-deleted and idiopathic infertile patients). We observed no significant differences among these three groups (Y-deleted, 0·34 ± 0·1; idiopathic, 0·40 ± 0·1; KS, 0·13 ± 0·1; $P > 0·23$ in all cases).

E2 and free testosterone levels did not differ significantly among the four groups (Table 2). Hormonal levels and derived indexes did not differ significantly between subjects with *AZFb* deletions and those with *AZFc* deletions (P always greater than 0·26; Table 3).

Hormonal multivariate analysis

Linear discriminant analysis was performed on the idiopathic and Y-deleted infertile patients using LH, FSH, inhibin-B, E2 and free testosterone as variables. The calculated equation failed to correctly assign patients to either group; the best equation was able to predict only 51·6% of the cases, i.e. not different from a random guess. Also, the same statistical technique was unable to discriminate *AZFc* from *AZFb* deleted patients (percentage correct: 52·4).

In the total set (non-KS patients, KS patients and controls), inhibin-B-values were inversely correlated with FSH, with high significance, as expected ($R = -0·65$, $P < 0·01$). However, when single subgroups were analysed (Fig. 4), inhibin-B and FSH were correlated in controls ($R = -0·37$, $P = 0·028$) and in non-KS

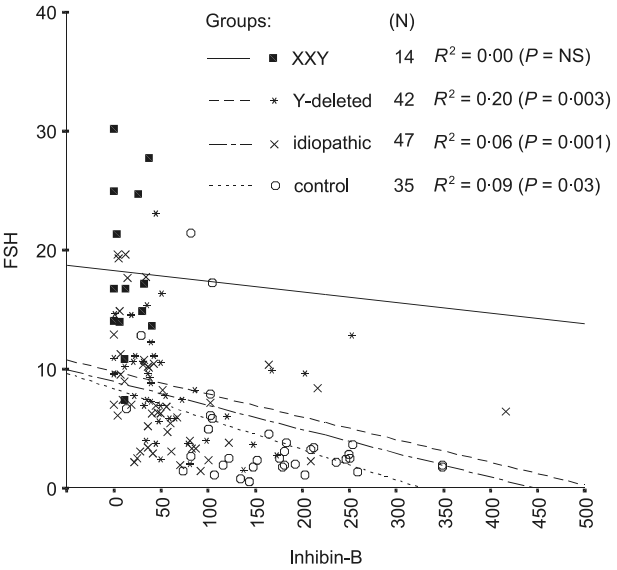

Fig. 4 Regression of FSH on inhibin-B in the four subgroups based on genetic diagnosis. Spearman correlation coefficients and significance are shown.

patients ($R = -0·52$, $P = 0·001$ for idiopathic and $R = -0·44$, $P = 0·03$ for Y-deleted patients), but not in Klinefelter's subjects ($R = -0·10$, $P = 0·74$).

Analysis on histological classification subgroups

Inhibin-B levels did not differ between non-SCO and SCO subjects (58·6 ± 8·6 *vs.* 75·5 ± 19·6 pg/ml, $P = 0·71$; Fig. 5), but were significantly lower in Klinefelter's patients (17·5 ± 3·6, $P = 0·022$). Of the subjects with KS, 5/14 had undetectable inhibin-B, compared to 5/18 in SCO patients ($\chi^2 = 0·01$, P = ns) and 1/18 in non-SCO patients ($\chi^2 = 3·34$, P = ns). Ranges of inhibin-B were 6·4–138 in non-SCO and 0·01–252·6 in SCO patients.

FSH levels were significantly different in the three groups (Fig. 6), with lowest values in non-SCO patients (6·4 ± 0·9 mIU/ml), intermediate in SCO (11·0 ± 1·1, $P = 0·001$) and highest in Klinefelter's patients (17·6 ± 1·8, $P = 0·032$). The FSH/inhibin-B ratio (Fig. 7) was not different between non-SCO and SCO subjects (0·26 ± 0·1 *vs.* 0·65 ± 1·7, $P = 0·65$), but was significantly higher in Klinefelter's patients (2·10 ± 0·6, $P = 0·01$). E2 and free testosterone did not differ significantly among the three groups ($P = 0·65$ and 0·76, respectively).

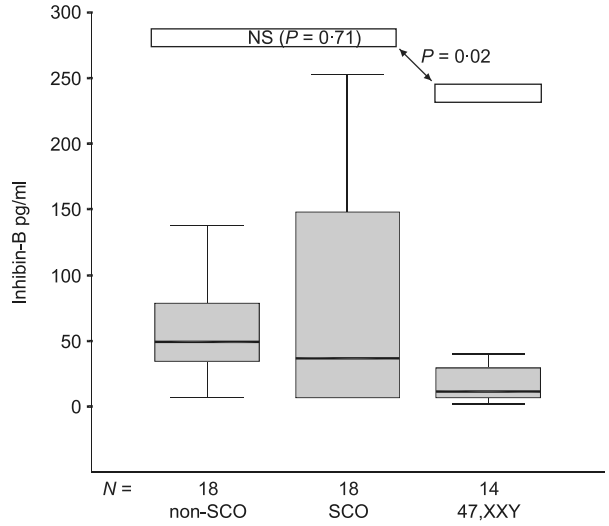

Fig. 5 Boxplot of inhibin-B levels in subjects with non-SCO and SCO histology, and in Klinefelter's syndrome patients. Horizontal rectangular boxes show homogeneous groups by Tukey's test.

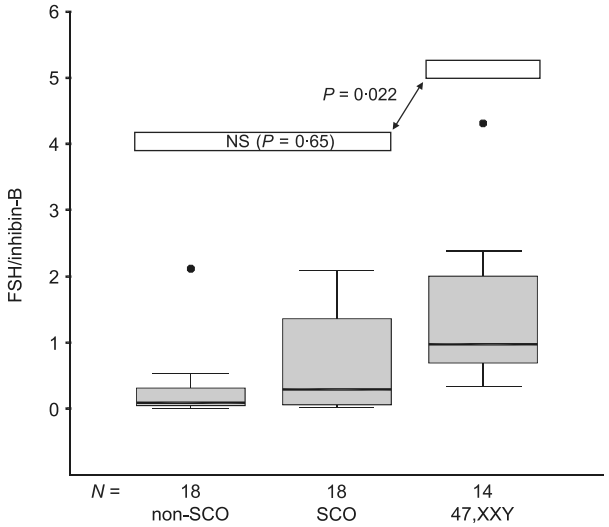

Fig. 7 Boxplot of the FSH/inhibin-B ratio in subjects with non-SCO and SCO histology, and in Klinefelter's syndrome patients. Horizontal rectangular boxes show homogeneous groups by Tukey's test.

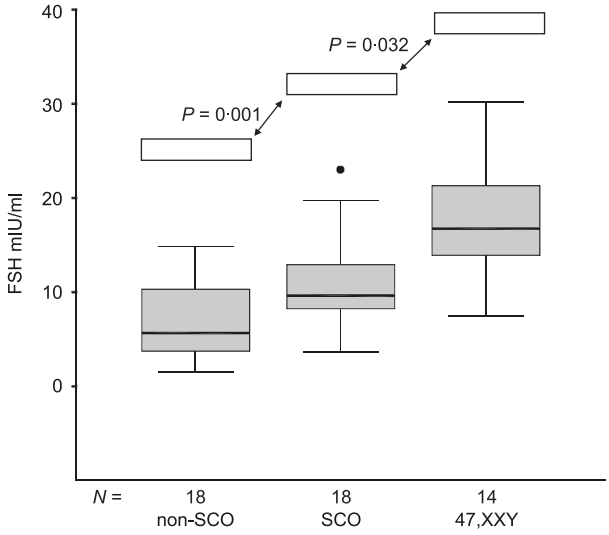

Fig. 6 Boxplot of FSH levels in subjects with non-SCO and SCO histology, and in Klinefelter's syndrome patients. Horizontal rectangular boxes show homogeneous groups by Tukey's test.

Analysis of covariance (using inhibin-B as covariate and FSH as independent variable) confirmed that lower inhibin-B concentrations alone, although a significant component (F = 11·828, P = 0·001), do not completely explain the higher FSH levels observed in SCO and KS subjects, as there is a residual independent and significant effect of the histological pattern (F = 8·53, P < 0·001).

Discussion

FSH levels clearly identified three different subgroups, with 47,XXY (KS) patients having the highest levels, 46,XY (non-KS) patients having intermediate levels and control subjects having the lowest levels as expected. Among non-KS patients, FSH levels were similar in Y-deleted and Y-intact patients.

Inhibin-B displayed an inverse pattern: KS patients had lower levels than oligo-azoospermic non-KS patients, and these in turn had lower levels than controls. However, although inhibin-B was much higher on average in controls, there was a wide overlap of values with those in oligo-azoospermic non-KS patients, confirming that inhibin-B levels are of limited value in the differential diagnosis of oligo-azoospermia (Mahmoud *et al*., 1998).

In an attempt to differentiate between idiopathic and Yq deletion-associated oligo-azoospermia, we calculated a putative index of Sertoli cell resistance, using the FSH/inhibin-B ratio, which had been suggested as a means to improve the significance of inhibin-B determination (Mahmoud *et al*., 1998); this index failed to identify a difference between the two sets of non-KS patients.

In summary, both FSH and inhibin-B levels, and their ratios, were nearly identical in patients with idiopathic oligo-azoospermia and in those with Y deletions.

LH values and LH/free testosterone ratio were highest in Klinefelter's patients, as expected given the primary Leydig cell failure which is a component of the syndrome. On the contrary, the LH/free testosterone ratio was similar across the three other groups, including controls. The LH/FSH ratio again did not

discriminate between Y-deleted and idiopathic oligo-azoospermic patients.

Our results are not consistent with published reports (Foresta *et al*., 2001) of different levels of inhibin-B in patients with Y deletions *vs.* idiopathic oligo-azoospermic patients. In addition, these authors reported a correlation of inhibin-B levels with the class of Yq deletion, a finding that we have also been unable to replicate. It has recently been demonstrated that most interstitial *AZFb* deletions are larger that *AZFc* deletions, and include at least part of the *AZFc* region (Repping *et al*., 2002). Our group of patients with *AZFb* deletions included five subjects with terminal deletions and seven with interstitial deletions, all of whom are at least twice the size of the *AZFc* deletions, and only one subject with a unique, smaller deletion. Notwithstanding this, and the fact that many more genes are lacking in *AZFb* deletions, hormonal levels in our patients with the grouped *AZFb* deletions were indistinguishable from those of patients with *AZFc* deletions. Although *AZFb* deletions tend to cause a greater impairment of spermatogenesis than *AZFc* deletions, we hypothesize that the relationship between inhibin-B production by the tubules, and the histology may not be sensitive enough to discriminate between these two groups of patients. In conclusion, patients with Y chromosome deletions have a pituitary–testicular basal endocrine pattern that is indistinguishable from that of idiopathic infertile subjects.

Globally, our data do not support the hypothesis of a lesser impairment of Sertoli cell function in patients with Y chromosome deletions, as compared to patients with idiopathic oligo-azoospermia. This hypothesis derived from the observation that the only clinical phenotype seen in patients with Y chromosome deletions is an impairment of the germ cell lineage, with spermatogenetic arrest; somatic cells do not appear affected, in the testis and elsewhere. Thus, it was reasonable to suppose that Sertoli function might be more conserved in patients with Yq deletions than in patients with idiopathic causes of infertility, even after excluding patients with secondary causes that might be expected to affect the testis as a whole (infection, trauma, cryptorchidism and, perhaps, varicocele). Indeed, a previous study reported higher inhibin-B levels in Y-deleted patients, compared to those in patients with idiopathic infertility, with an inverted FSH pattern (Foresta *et al*., 2001).

However, there are reasons to suggest that Y-deleted and idiopathic oligo-azoospermic subjects should have a similar impairment of Sertoli cell function. First, inhibin-B production is considered to be a mirror of the global efficiency of spermatogenesis, and is lower when spermatogenesis is altered, regardless of the cause. This is true even in patients who have no anatomic or genetic defect of the germ cell lineage, for example patients with hypogonadotropic hypogonadism (Anawalt *et al*., 1996).

Furthermore, it has been reported that although inhibin-B is secreted by the Sertoli cell, in the human adult testis its β-B subunit may be synthesized also in germ cells (and to a lesser extent in Leydig cells), whereas the α subunit is manufactured only in Sertoli cells (Andersson *et al*., 1998). This leads to the possibility that inhibin-B is a joint product of both Sertoli and germ cells (Andersson *et al*., 1998), which would support the results of Anawalt *et al*. (1996), and is consistent with the observation that, at least in the human fetus, inhibin α-subunit mRNA expression is specific for steroidogenic cells, whereas β-B mRNA is expressed in several extragonadal tissues (Tuuri *et al*., 1994).

However, at least in experimental animals isolated Sertoli cells are capable of producing complete inhibin-B (Morris *et al*., 1988; Klaij *et al*., 1992). Also, FSH is able to increase the secretion of inhibin-B in isolated rat Sertoli cells (Pineau *et al*., 1990); on the other hand, in the same study only the expression of α-subunit mRNA (but not of β-B-subunit mRNA) was induced by FSH, and addition of germ cells further enhanced inhibin-B secretion (Pineau *et al*., 1990).

In summary, in our opinion the available evidence suggests that Sertoli cells can produce and secrete complete inhibin-B, but β-B subunit production in Sertoli cells is probably a rate-limiting step; thus a significant germ cell contribution to β-B subunit production and total inhibin-B secretion cannot be excluded.

Even if inhibin-B synthesis and production occurred exclusively in the Sertoli cell, with no β-B subunit contribution from spermatocytes, a reduced inhibin-B secretion may result from an altered germ cell–Sertoli cell interaction.

Finally, although by definition the aetiology of idiopathic oligo-azoospermia is unknown, it is possible, if not likely, that in many cases genetic causes are responsible for the observed phenotype. If genetic causes do underly many cases of 'idiopathic' oligo-azoospermia, this condition would not be very different from deletion-associated oligo-azoospermia.

Our data are in agreement with these considerations, and do not show any difference in the pituitary–testicular axis parameters of Y-deleted and idiopathic oligo-azoospermic patients. After submission of this manuscript, another group has shown no difference in inhibin-B levels between patients with AZFc deletions and idiopathic oligo-azoospermia (Frydelund-Larsen *et al*., 2002).

Interestingly, the inhibin-B–FSH axis appears to behave differently in Klinefelter's patients, compared to non-KS patients, whether classified on an aetiological or on a phenotypic basis. If inhibin-B secretion by Sertoli cells is only related to the amount of residual spermatogenesis (Anawalt *et al*., 1996), then comparable levels might be expected in SCO and KS patients.

On the contrary, our data indicate that KS patients have significantly lower inhibin-B levels, thus suggesting that inhibin-B production is indeed lower in KS patients. We speculate that this may be due to a decreased total Sertoli cell mass in KS testes. Although formal comparative morphometric studies have not been reported, it is well recognized that many tubules in KS

patients are fibrous and devoid of any cells, either germinal or Sertoli.

However, the decreased inhibin-B production in KS patients is not sufficient to explain the higher FSH levels observed in these patients, compared to patients with SCO histology. In fact, FSH levels in KS patients are higher at any given inhibin-B level, and this is reflected in the significantly higher FSH/inhibin-B ratios displayed by KS patients. The same observation holds true also if KS patients are compared to Y-deleted or nondeleted patients.

Thus, the higher levels of FSH in KS patients cannot be explained on the basis of inhibin-B feedback alone, and other regulators should be involved. Although it is possible that KS patients may have a more severe spermatogenetic damage than patients with SCO, we do not consider it likely: literature data suggest that the chances of finding sperm at TESE, if at all different, are probably lower and not higher in patients with SCO (Meng *et al*., 2000; Seo & Ko, 2001) than with KS (Friedler *et al*., 2001; Madgar *et al*., 2002).

Free testosterone levels were not significantly different in our Klinefelter's patients. This finding did not surprise us, as there is clearly a selection bias in our Klinefelter's patients, who were under evaluation for infertility and had not been diagnosed before. This subset of patients may be different from the classic 'textbook' description of KS (Yoshida *et al*., 1997). Although testosterone is not a principal regulator of FSH or inhibin-B in males (Anderson & Sharpe, 2000; Hayes *et al*., 2001a, 2001b), the normal levels of free testosterone are important to exclude this as a potential factor in causing lower levels of inhibin-B and/or higher FSH in Klinefelter's patients compared to subjects with SCO histology.

E2 does seem to play a role in FSH feedback in males (Hayes *et al*., 2001a), but again levels in our Klinefelter's patients were not significantly different.

We speculate that in males FSH secretion could be regulated by inhibin-B only down to a certain level of inhibin-B concentration, after which FSH levels become independent of inhibin-B and other regulators, possibly paracrine, may play a principal role.

Finally, our data confirm the limited clinical utility of the inhibin-B assay, given the substantial overlap of values in normal and pathologic subjects. The FSH/inhibin-B ratio was also not significantly different between controls and oligo-azoospermic patients, whether Y-deleted or idiopathic; thus inhibin-B and the ratio do not appear to be useful diagnostic tools in Y-deleted patients, as in oligo-azoospermic patients in general (Meachem *et al*., 2001).

Note added in proof

After submission of our manuscript, Frydelund-Larsen *et al*. (2002) have reported similar findings in patients with deletions of AZFc.

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