Could androgen receptor gene CAG tract polymorphism affect spermatogenesis in men with idiopathic infertility?

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Abstract

Purpose This study examined whether the AR-CAG repeat length might affect clinical characteristics (testis volume) seminal parameters (sperm count and its mobility) along with hormonal serum profile [FSH, LH, Testosterone (T) and Inhibin B (InhB)] both in idiopathic male infertility (IM) and in infertility due to a previous condition of cryptorchidism (CryM) or to Y chromosome long arm microdeletions (YM).
Design Observational study without intervention(s).

Capsule No concrete evidence about the pathogenetic role of androgen receptor (AR) CAG repeat length polymorphism in infertile men has been obtained so far. This study aims to search it, measuring AR-CAG polymorphism both in fertile and in infertile men with idiopathic infertility or with well-known causes of infertility (i.e. cryptorchidism and Y-chromosomal microdeletions). The shortest tract as well as the longest one of AR-CAG repeat polymorphism may negatively affect the spermatogenesis in fertile and infertile men.

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Patients One hundred and ten IM patients [90 idiopathic oligozoospermic males (IOM) and 20 idiopathic azoospermic males (IAM)], 19 CryM male and 10 YM patients were included. Sixty-one age-matched healthy men who had fathered within 3 years were involved representing the control group (FM).

Results AR-CAG repeats stretch was significantly longer in IOM ($p<0.05$), CryM ($p<0.05$) and YM ($p<0.001$) than FM. When the AR-CAG repeat tracts were subdivided in three subgroups according to the length of CAG repeats tract assessed in fertile subjects (the one with the middle (n 19–21) belonging to the 25 and 75 % inter-quartile, the ends belonging to the <25 % inter-quartile and >75 % inter-quartile, respectively), there was a statistically significant difference of distribution of AR-CAG tract length among fertile and different groups of infertile men ($p<0.0005$; chi-square test). Moreover, the subgroup of AR-CAG repeat stretch with 22–28 triplets was associated with lower levels of InhB both in idiopathic oligozoospermic (Scheffe, Bonferroni and Dunett tests $p<0.01$) and azoospermic men (Scheffe, Bonferroni and Dunett test $p<0.05$), while, when FM and men with idiopathic infertility were gathered in a single group, both the subgroup of AR-CAG tract with 15–18 repeats and the one with 22–28 repeats are associated with lower testis volume, reduced sperm count and serum InhB levels.

Conclusions Our study showed that the outliers of AR-CAG repeat length seem to influence the function of AR, affecting testis volume and Sertoli cell function and consequently sperm production in both fertile and idiopathic infertile men.

Keywords CAG repeat length polymorphism · Idiopathic male infertility · Y chromosome long arm microdeletions · Sertoli cell · Leydig cell · Inhibin B and testosterone

Introduction

Nowadays, in almost 40 % of infertile couples the fertility is due to a male factor, even though the cause often remains unknown [1, 2]. Idiopathic male infertility is the most frequently encountered condition; it comprises an heterogeneous group of patients showing different level of spermatogenesis impairment and variable plasma levels of Testosterone (T), Inhibin B (InhB), Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), and their ratio (T/LH and InhB/FSH, respectively), suggesting that a variety of genetic and environmental factors by means of different pathogenic mechanisms can play roles to bring about the male infertility coupled with various stages of both Sertoli (CS) and Leydig (LC) cells damage. Indeed, approximately 50 % of idiopathic infertile men show low serum InhB compared to fertile men [3], whereas almost 20 % of them have lower plasma T levels and/or calculated T/LH ratio [4, 5].

Beside serum FSH, plasma T levels seem to play a key role in regulating InhB secretion by Sertoli cell [6, 7], whereas LH together with other different hormones (e.g. Growth hormone, Thyroid hormone, Insulin, etc.) [8] and non-hormonal factors (smoking, lifestyle, obesity, etc.) [8–10] can affect plasma levels of T in men [11]. However, the serum T sensitivity of target tissues, especially at the hypothalamus and pituitary gland levels, is a major factor regulating the circulating T levels in men [11, 12]. Finally, environmental substances may be accountable for serum T levels variations in infertile men [13].

Recently, CAG repeats length in exon 1 of the androgen receptor (AR) gene (AR-CAG) has been shown to play a role in AR function [14], modulating the T activity at target tissues and being instrumental in part of the variability of serum T in adult and older men [15, 16]. Therefore, AR-CAG polymorphism has been intensely studied in several androgen-related clinical conditions, such as prostate cancer, male infertility [17] and cryptorchidism [18]. However, in male infertility evidence about the role of AR-CAG repeats length has remained remote, open and even conflicting so far [19].

In order to gain further insight about its role in the contest of male infertility, the present study examined the AR-CAG length polymorphism in relation to the clinical characteristics, the seminal parameters and the serum levels of FSH, LH, T, and InhB as well as to the estimated T/LH ratio and the InhB/FSH ratio in non-obese male subjects belonging to three different groups: 1) healthy men with proved fertility (FM), 2) idiopathic infertile men (IM) and 3) infertile men with either a history of cryptorchidism (ex-cryptorchid subjects) (CryM) or with Y chromosome long arm (Yq) microdeletions (YM). Thus, it was aimed to assess a possible relationship among the AR-CAG length polymorphisms genotype and the clinical and the hormonal milieu phenotype in men with different forms of spermatogenesis impairment.

Materials and methods

Subjects selection and study design

Subjects involved in our study satisfied the WHO criteria for the infertile couples (failure to achieve pregnancy within a 12-month period of unprotected intercourse at normal frequency) [20]. To be considered male affected by idiopathic infertility, we selected those men who did not suffer from evident causes of male infertility such as hypogonadotropic or hypergonadotropic hypogonadism, hyperprolactinaemia, Klinefelter's syndrome, testicular atrophy, Yq microdeletions, ductal obstruction or agenesis, epididymitis and cryptorchidism [1, 2, 21] and whose female partner presented normal ovulatory cycles as suggested by basal body temperature and luteal phase progesterone levels (>10 ng / mL), and had patent tubae as shown by hysterosalpingography [22]. Since the expected distribution of AR-CAG repeat length polymorphism might depend on different ethnic groups, all of our fertile and infertile men included in this study were Caucasian people [23]. Therefore, 110 non-obese men with idiopathic infertility (IM) who fulfilled the above reported criteria, were involved in the study. According to the sperm count, they were divided into idiopathic azoospermic males (IAM) (age = 33.8 ± 5.8 ; $n=20$) and idiopathic oligozoospermic males (IOM) (age = 32.5 ± 6.0 $n=90$); IOM were considered those showing a sperm count below the 10th percentile of the control group, consisting of 61 non-obese healthy age-matched men (FM) (age = 34.5 ± 5.7) whose fertility was proved by having fathered a child within the previous 3 years. Moreover, both a group of 10 non-obese and non-cryptorchid infertile patients suffering from Yq microdeletions (YM) and a group of 19 non-obese infertile males who had suffered from idiopathic cryptorchidism (ex-cryptorchid men) (CryM) whilst not affected by primary (i.e. Klinefelter syndrome) or secondary hypogonadism (i.e. Kallmann syndrome), were enrolled in our study. From the medical history and the clinical setting, 3 out of 19 of the ex-cryptorchid males were affected by bilateral cryptorchidism and 5 out of 19 suffered from azoospermia [24]. Furthermore, none of our formerly cryptorchid men presented Y chromosome microdeletions [25].

Given that the length of trinucleotide CAG repeats might affect AR function [14, 17] and, moreover, may be in a non-linear genotype-phenotype association model in relation to AR CAG repeats length [23], we subdivided the AR-CAG repeats polymorphism in three subgroups according to the length of AR-CAG repeats triplets interquartile (<25 %, 25 – 75 % and >75 %) measured in FM: the middle subgroup had the AR CAG repeat triplets ranging from 19 to 21 and the ends had made up of AR CAG repeat triplets with 15–18 and 22–28, respectively

All infertile and fertile subjects were examined by a single physician and their clinical history and their physical

examination were addressed to evaluate specific signs and symptoms associated with infertility [1, 2]. Testis size (TS) was assessed using the Prader orchidometer and reported as mean volume of both testes (sum of volume of both right testis plus the left testes divided by 2). All the participants were not obese (body mass index (BMI) $<30 \text{ Kg/m}^2$) and presented fasting serum glucose levels lower than 100 mg/dl as well as normal kidney, heart and liver functions. In order to evaluate sperm characteristics, both groups of fertile and infertile men were asked to collect their sperm by masturbation twice at interval of 14 days. Seventy per cent of our infertile men were not under treatment with any drugs at the time of the enrolment, while the remaining 30 % were enrolled in the study after withdrawing any therapy for at least 3 months. In all subjects, blood samples for hormones assays were drawn before each sperm analysis, while blood samples for genetic tests (Yq microdeletions and AR- CAG repeat triplets determinations) were obtained before the first sperm analysis.

All men involved in the study signed an informed consent. The Local Ethical Committee of Biomedical Research Association “Guglielmo Telesforo” (Foggia, Italy) approved the study protocol.

Hormonal assessments

Blood samples were drawn between 08:00 and 09:00 h after an overnight fast, and the plasma was kept at -20°C until processing. Plasma specimens of both infertile and proved fertile men were proportionally distributed in each hormone assay run.

Serum T, FSH and LH levels were assessed by commercial immunometric assays (Immulite, EURO/DPC, UK), while circulating InhB levels were determined by a kit form Serotec (Oxford UK). As previously described [7], all these methods showed a intra- and inter-assay coefficient of variation (CV) $<10 \%$ and $<15 \%$, respectively.

Genetic determinations

Yq microdeletions determination method

The Y microdeletions evaluation was performed using a commercial kit (AB Analitica srl, Advance Biomedicine, AZF-MX cod.04-23, Padova, I) which showed the markers and PCR characteristics, according to the recent evidence and guidelines [26–28]. The method consists on DNA extraction, Polymerase Chain Reaction (PCR) amplification and separation on agarose gel. The extracted DNA, after a short incubation at 56°C for 10 min, was washed in filter column by ethyl alcohol and was eluted by sterile distilled water. PCR was carried out using three multiples with 9 primers (mix 1:

sY254, sY 86, sY 127, sY 255; mix 2: sY 95, sY 117, sY 125; mix 3: DBY, DFFRY). In order to obtain PCR amplification, all samples were put in a Thermal Cycler (PTC-100, Genenco, MJ Research Inc., Watertown, MA, USA) employing the following standard program: one cycle at 95°C for 5 min; 35 cycles: denaturation 94°C for 60 s; annealing at 56°C for 60 s and extension at 65°C for 90 s; one cycle at 65°C for 7 min and storage at 4°C . All amplified samples were identified comparing to known marker molecular wait after an electrophoresis run on agarose gel. All samples were analysed for the detection of the SRY gene as internal control for reaction and female, fertile men and DNA negative controls (water) were included in each electrophoresis run. In order to confirm the deletion of that sequence-tagged site (STS) marker deletion, the specimen amplifications found to be negative were repeated three times [27, 28].

AR- CAG repeats polymorphisms determination

Androgen receptor polymorphism was assessed as previously described [29]. In brief, CAG triplets repeats number was determined by counting genomic DNA extracted [Microgeno DNA kit (AB Analitica srl, Advance Biomedicine, REF. 05-42, Padova, Italy)]. Fifty to two hundred milligram of genomic DNA were amplified in a PCR assay that included both $4 \mu\text{l}$ PCR $2 \mu\text{l}$ primer mix (forward primer: $5'\text{-GTGCGCGAAGTGATCCAGA-3'}$, and reverse primer: $5'\text{-GTTTCCTCATCCAGGACCAGGTA-3'}$) (Primer Express, Perkin-Elmer Corp., Norwalk, CT, USA) and 2.5 U Taq DNA polymerase (Perkin-Elmer Corp.) in a final volume of $25 \mu\text{l}$. Thermal cycling (PTC-100, Genenco, MJ Research Inc., Watertown, MA, USA) included: 94°C for 4 min, 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, then 72°C for 10 min. Following ethanol precipitation of each PCR sample, amplified fragments were directly sequenced by an automated capillary electrophoresis unit; the size of the CAG repeats detected was analysed by ABI PRISM 3100 Genetic Analyzer (Perkin-Elmer Corp.), and were found to range from 260 to 320 bp. Each determination of CAG repeat extensions was repeated twice in two distinct tests.

Sperm analysis

Semen specimens were obtained by masturbation after 3–5 days of abstinence and delivered to the laboratory within 30 min. Sperm analysis was performed according to the World Health Organization criteria [30].

Statistics

Since some variables in the study turned out to be skewed, these data were given as median and interquartile

range (25th–75th percentile), while others were reported as mean $\pm$ standard deviation (SD). The *t* Student test was employed to assess the differences between groups studied showing normal distribution; whereas the difference among fertile group and the groups of infertile patients, was evaluated by the Mann–Whitney *U* test for non-normally distributed variables.

Analysis of variance (ANOVA test) was employed in order to evaluate the difference among the intrer-groups of trinucleotide CAG repeats length and, when there was a statistical significance, post-hoc tests (Scheffe, Bonferroni and Dunnett tests) were employed to evaluate the differences among the clinical characteristics, the seminal parameters, the serum hormonal levels and AR-CAG repeat stretch in our fertile and infertile subjects.

Chi-squared test (χ^2 test) was employed to assess the differences of frequency distribution among the trinucleotide CAG repeat strength in fertile and infertile participants.

An “a priori” 5 % was deemed statistically significant. Statistical analysis was performed by SPSS version 10.0 (Chicago, USA).

Results

The group of YM consisted of 7 oligozoospermic men (sperm count $<9 \times 10^6$ /ml) affected by AZF B-C and AZF C forms, and 3 azoospermic males affected by 3 different forms: AZF A, B and C. In this group, the AR-CAG repeats length was between 22 and 28 triplets.

Table 1 provides clinical characteristics, seminal parameters, AR-CAG repeat length, serum levels of FSH, LH, T, InhB and the T/LH and InhB/FSH ratios of the different groups: oligozoospermic men (IOM) and azoospermic males (AM), both with idiopathic infertility, and patients with established causes of infertility (CryM and YM), as well as healthy fertile men (FM). Besides the sperm parameters characterizing the clinical diagnosis of our infertile men, the CYM and YM presented significantly lower testis size (TS) ($p < 0.01$) compared to FM. In comparison with FM showing a median number of AR-CAG triplets = 20 (inter-quartile 25–75 % percentile = 19–21), AR-CAG repeat length were significantly longer in IOM [median = 22; 20–23 inter-quartile 25–75 % percentile, $n = 90$ ($p < 0.05$)], cryptorchidism (CryM) [median = 22, 20–24 inter-quartile 25–75 % percentile, $n = 19$ ($p < 0.05$)] and Yq microdeletions (YM) [median = 26, 24–26 inter-quartile 25–75 % percentile, $n = 10$ ($p < 0.001$)], whereas this was not the case of IAM. As expected, serum FSH and IB levels were significantly higher and lower respectively in infertile men than in controls. Consequently the estimated InhB/FSH ratio resulted to be significantly lower in all groups of infertile men. Serum LH and T levels were significantly higher and lower respectively in IAM, CryM and YM than in

Table 1 Clinical and sperm characteristics, AR-CAG number repeats (CAG n), FSH, LH, T, InhB levels and ratio of T/LH and InhB/FSH in fertile (FM), Idiopathic Oligozoospermic (IOM), Idiopathic Azoospermic (IAM), ex-cryptorchid (CryM) and Yq microdeletion males (YM) involved in the study

	Age (yrs)	TS (ml)	SpC/ejac (10^6 /ejac)	SpC (10^6 /ml)	PM %	NM %	CAG n	FSH (mU/l)	LH (mU/l)	T (nMol/l)	T/LH	InhB (pg/ml)	InhB/FSH
FM (n 61)	34.5 $\pm$ 5.7*	20.5 $\pm$ 3.5	145 (98–226)	42.5 ⁺ (22.5–55.5)	36.5 (28–59)	10 (8–30)	20 ⁺ (19–21)	5.4 $\pm$ 2.1	5.3 $\pm$ 1.6	18.5 $\pm$ 2.2	4.2 $\pm$ 1.9	209.1 $\pm$ 26.2	52.7 $\pm$ 35.2
IOM (n 90)	33.8 $\pm$ 5.8	18.5 $\pm$ 8.5	13.5 (7.5–22.4)	4.5 (2.5–7.5)	5.8 (3.6–7.7)	3 (0.0–5.5)	22 (20–23) ^a	7.9 $\pm$ 5.1 ^a	6.2 $\pm$ 2.8	17.7 $\pm$ 3.2	3.7 $\pm$ 2.7	114.8 $\pm$ 40.6 ^b	21.4 $\pm$ 17.9 ^b
IAM (n 20)	33.5 $\pm$ 6.0	18.0 $\pm$ 4.5 ^a	0.0	0.0	0.0	0.0	21 (20–22)	17.2 $\pm$ 8.9 ^c	9.8 $\pm$ 2.6 ^c	16.1 $\pm$ 1.9 ^a	1.8 $\pm$ 0.6 ^b	94.5 $\pm$ 24.8 ^c	7.9 $\pm$ 6.5 ^c
CryM (n 19)	32.5 $\pm$ 8.5	17.0 $\pm$ 6.5 ^b	2.5 (0.0–11.5)	4.0 (0.0–12.0)	3.0 (0.0–6.5)	3.5 (0.0–5.0)	22 (20–24) ^a	15.8 $\pm$ 8.9 ^b	8.6 $\pm$ 2.9 ^b	15.1 $\pm$ 2.1 ^b	1.9 $\pm$ 0.4 ^b	68.5 $\pm$ 10.1 ^c	4.3 $\pm$ 2.3 ^c
YM (n 10)	32.8 $\pm$ 5.5	16.5 $\pm$ 2.5 ^b	4.7 (0.8–34.9)	1.0 (0.1–3.3)	3.6 (0.0–7.3)	4.0 (0.7–5.5)	26 (24–26) ^c	14.3 $\pm$ 8.9 ^b	7.4 $\pm$ 3.9 ^a	13.3 $\pm$ 3.8 ^c	2.6 $\pm$ 1.5 ^a	77.8 $\pm$ 9.3 ^c	7.2 $\pm$ 3.1 ^c

Entries are * means $\pm$ SD (range) or ⁺ medians (inter-quartile 25th–75th percentile) for normally or non-normally distributed variables, respectively

a= $p < 0.05$; b= $p < 0.01$; c= $p < 0.001$ compared with FM

TS testis size; SpC/ejac sperm count/ejaculate; SpC sperm count; PM % of progressive motility; NM % of normal morphology; FSH follicle stimulating hormone; LH luteinizing hormone; T testosterone; InhB inhibin B

FM, while the T/LH ratio was significantly reduced in IAM ($p<0.01$), CryM ($p<0.01$) and YM ($p<0.05$).

Figure 1 provides the frequency distribution of trinucleotide CAG repeats number in FM and in those with infertility. It turned out to be significantly different ($p<0.0005$ from χ^2 test) at variance with the other groups and, especially, we did not find a number of CAG repeats of 15–18 and 19–21 in YM. On the other hand, a number of triples 22–28 was very rare in FM (7.5 %) and IAM (9 %), whereas it was well represented in IOM (57 %).

Table 2 reports the means $\pm$ SD of clinical characteristics, sperm parameters, hormonal plasma levels and their relationship with the three categories of AR-CAG repeats polymorphism in FM, IOM and IAM. There were no statistically significant differences among those clinical and sperm characteristics as well as the endocrine plasma parameters and length of trinucleotide CAG repeats subgroups in FM, while the three categories of AR-CAG repeats stretch showed a statistically significant difference for InhB serum levels in both IAM ($p<0.05$) and IOM ($p<0.001$). Employing post-hoc tests for InhB and trinucleotide CAG repeats stretch, in IAM there was a statistically significant difference between the sub-group 16–18 of trinucleotide CAG and the 22–24 subgroup (Scheffe, Bonferroni and Dunnett tests $p<0.05$), while in IOM there was a statistically significant difference among the subgroup of CAG number 16–18, 19–21, 22–28 and 19–21 (Scheffe, Bonferroni and Dunnett tests $p<0.05$). Finally, there was no difference for serum InhB levels between the sub-groups 16–18 and 22–28.

Fig. 1 The distribution frequency of fertile participants (FM), idiopathic oligozoospermic men (IOM), idiopathic azoospermic male (IAM), ex-cryptorchid men (CryM) and subjects with Yq microdeletions (YM). $P<0.0005$ from chi-square test

Table 2 Different subgroups of CAG repeat length versus clinical, seminal and endocrine parameters in different groups of fertile, idiopathic oligozoospermic and azoospermic men

CAG length	Fertile men				Idiopathic oligozoospermic men				Idiopathic azoospermic men			
	n 16–18	n 19–21	n 22–24	p value	n 15–18	n 19–21	n 22–28	p value	n 16–18	n 19–21	n 22–24	p value
	(n 16)	(n 36)	(n 9)		(n 18)	(n 25)	(n 47)		(n 3)	(n 10)	(n 7)	
Et _a (yrs)	39.1 $\pm$ 5.1	36.9 $\pm$ 5.9	35.0 $\pm$ 7.8	0.45	32.2 $\pm$ 6.3	32.7 $\pm$ 5.6	34.2 $\pm$ 6.2	0.42	35.3 $\pm$ 3.6	29.6 $\pm$ 4.9	35.47 $\pm$ 0.4	0.12
TS (ml)	21.1 $\pm$ 3.1	20.1 $\pm$ 2.6	21.07 $\pm$ 2.6	0.33	15.3 $\pm$ 1.4	16.5 $\pm$ 2.8	15.6 $\pm$ 1.7	0.11	13.0 $\pm$ 1.0	15.3 $\pm$ 1.0	14.1 $\pm$ 2.7	0.14
SV (ml)	3.3 $\pm$ 0.4	3.6 $\pm$ 0.6	4.0 $\pm$ 0.8	0.06	2.8 $\pm$ 0.5	3.2 $\pm$ 0.5	3.1 $\pm$ 0.5	0.09	2.9 $\pm$ 1.0	3.0 $\pm$ 0.7	2.8 $\pm$ 0.7	0.82
SpC (10 ⁶ /ml)	40.2 $\pm$ 23.4	44.4 $\pm$ 10.1	43.4 $\pm$ 13.4	0.77	4.9 $\pm$ 2.9	7.4 $\pm$ 5.4	6.4 $\pm$ 4.5	0.26	0.00	0.00	0.00	
PM %	19.8 $\pm$ 3.4	19.2 $\pm$ 2.0	21.8 $\pm$ 2.7	0.07	6.6 $\pm$ 4.1	6.5 $\pm$ 2.8	9.6 $\pm$ 1.1	0.30	0.00	0.00	0.00	
IM %	60.2 $\pm$ 10.1	61.3 $\pm$ 6.7	61.2 $\pm$ 5.9	0.95	78.5 $\pm$ 14.4	75.1 $\pm$ 6.4	77.6 $\pm$ 8.8	0.46	0.00	0.00	0.00	
FSH (mU/l)	4.5 $\pm$ 2.8	4.7 $\pm$ 1.9	3.3 $\pm$ 1.4	0.35	9.4 $\pm$ 7.4	7.7 $\pm$ 3.9	8.5 $\pm$ 5.6	0.65	25.3 $\pm$ 15.6	15.6 $\pm$ 8.2	15.9 $\pm$ 6.9	0.42
LH (mU/l)	5.7 $\pm$ 1.7	5.51 $\pm$ 0.5	4.1 $\pm$ 1.7	0.07	5.8 $\pm$ 2.1	5.9 $\pm$ 3.0	6.7 $\pm$ 2.8	0.45	9.7 $\pm$ 4.8	10.3 $\pm$ 2.9	9.21 $\pm$ 0.3	0.76
T (nMol/l)	19.4 $\pm$ 1.1	19.7 $\pm$ 1.8	20.7 $\pm$ 2.0	0.02	15.7 $\pm$ 3.7	17.5 $\pm$ 3.9	17.2 $\pm$ 3.5	0.25	15.7 $\pm$ 1.0	15.7 $\pm$ 1.6	16.9 $\pm$ 2.6	0.42
InhB (pg/ml)	280.0 $\pm$ 33.1	214.7 $\pm$ 27.4	207.2 $\pm$ 22.3	0.35	96.6 $\pm$ 16.1	130.3 $\pm$ 40.3	108.0 $\pm$ 35.0	<0.01	126.7 $\pm$ 23.8	94.9 $\pm$ 15.2	79.7 $\pm$ 23.8	<0.05
T/LH	3.9 $\pm$ 2.2	3.9 $\pm$ 1.7	5.5 $\pm$ 1.8	0.14	3.1 $\pm$ 1.5	4.3 $\pm$ 3.3	3.3 $\pm$ 2.4	0.26	1.9 $\pm$ 0.9	1.7 $\pm$ 0.8	1.9 $\pm$ 0.5	0.87
InhB/FSH	70.4 $\pm$ 41.5	53.6 $\pm$ 28.5	80.7 $\pm$ 40.5	0.18	18.5 $\pm$ 15.6	25.0 $\pm$ 22.6	18.5 $\pm$ 15.2	0.16	8.2 $\pm$ 8.3	8.9 $\pm$ 7.8	6.5 $\pm$ 4.6	0.77

TS testis size; SV sperm volume; SpC sperm count; PM progressive motility; IM immobile sperm; FSH follicle stimulating hormone; LH luteinizing hormone; T testosterone; InhB inhibin B. p value from ANOVA test on different subgroups of CAG repeats length. Statistically significant p values are in bold

In the CryM patients no statistic difference was found correlating CAG repeats length and clinical characteristics, sperm parameters and endocrine parameters levels (data not shown).

Table 3 provides (means $\pm$ SD) clinical characteristics, sperm parameters, plasma hormonal levels and their relationship with the three categories of AR-CAG polymorphism in all of fertile and infertile participants with idiopathic infertility taken together. There were statistically significant difference (ANOVA test) for testis size ($p<0.05$), sperm count ($p<0.005$), mobile sperm ($p<0.01$), InhB levels ($p<0.005$) and InhB/FSH ratio ($p<0.05$) in the three subgroups of AR-CAG repeats. Applying the post hoc analysis in the group of FM and in those with idiopathic infertility (IM and IAM) there were a significant difference for testis size (Scheffe, Bonferroni and Dunnett tests $p<0.05$), sperm count (Scheffe, Bonferroni and Dunnett tests $p<0.05$), mobile sperm (Scheffe, Bonferroni and Dunnett tests $p<0.05$), InhB/FSH ratio in the two subgroups of trinucleotide CAG repeat 19–21 and 22–28, while there were a significant difference for serum inhB levels (Scheffe, Bonferroni and Dunnett tests $p<0.05$) in the two subgroups of AR-CAG repeats stretch 15–18 and 19–21 as well as for plasma InhB levels in the two categories of CAG repeats 19–21 and 22–28. There was, however, no significant difference for serum InhB levels in the subgroups of AR-CAG polymorphism 16–18 and 22–28.

Table 3 Different subgroups of CAG repeats length versus clinical characteristics, seminal parameters and endocrine plasma levels in all of fertile and infertile men with idiopathic infertility taken together

CAG length	Fertile subjects and men with idiopathic infertility			<i>p</i> value
	n 15–18 (n 37)	n 19–21 (n 72)	n 22–28 (n 62)	
Età (yrs)	33.3 $\pm$ 7.2	33.5 $\pm$ 6.6	33.2 $\pm$ 6.7	0.97
TS (ml)	16.4 $\pm$ 3.5	17.2 $\pm$ 3.6	15.5 $\pm$ 2.8	<0.05
SV (ml)	2.9 $\pm$ 0.5	3.2 $\pm$ 0.6	3.1 $\pm$ 0.7	0.23
SpC (10 ⁶ $\times$ ml)	13.5 $\pm$ 20.2	19.9 $\pm$ 20.2	8.2 $\pm$ 11.3	<0.005
PM %	8.8 $\pm$ 7.9	9.9 $\pm$ 7.9	5.8 $\pm$ 5.7	<0.01
IM %	60.8 $\pm$ 31.1	55.2 $\pm$ 29.3	65.3 $\pm$ 30.6	0.15
FSH (mU/l)	10.1 $\pm$ 9.0	8.5 $\pm$ 6.3	10.5 $\pm$ 7.8	0.30
LH (mU/l)	6.3 $\pm$ 2.6	6.6 $\pm$ 3.0	7.1 $\pm$ 3.0	0.39
T (nMol/l)	16.6 $\pm$ 3.3	17.8 $\pm$ 3.3	16.7 $\pm$ 3.7	0.12
InhB (pg/ml)	129.6 $\pm$ 60.1	149.7 $\pm$ 57.3	104.6 $\pm$ 45.8	<0.005
T/LH	3.1 $\pm$ 1.7	3.6 $\pm$ 2.5	3.0 $\pm$ 2.2	0.33
InhB/FSH	28.6 $\pm$ 34.1	31.9 $\pm$ 9.1	18.9 $\pm$ 24.7	<0.05

p value from ANOVA test on different subgroups of CAG repeats length. Statistically significant *p* values are in bold. Legend: see Tables 1 and 2

Discussion

The so-called idiopathic form is the most common cause of male infertility, designated as a “diagnosis by exclusion” of other forms. Conversely, cryptorchidism and Yq microdeletions are two important and clinically specific conditions of male infertility [1, 2]. However, although the genetic cause of spermatogenesis damage is well-identified in men with Yq microdeletions [26], several and complex pathogenic mechanisms may be responsible of infertility in the idiopathic form [31]. Indeed, in a hormonal setting these infertile subjects may have common issues that are characterized by a continuous spermatogenesis damage and a parallel impairment of both Sertoli cell and Leydig cell function as documented by a progressive rise in serum FSH and LH levels coupled with a significant reduction of both plasma InhB and T levels, respectively, as well as of InhB/FSH and T/LH ratios (Table 1) [3–5]. However, as different non-hormonal factors such as lifestyle, smoking, adiposity, etc. [8, 9], as well as environmental substances [13] can be accountable for serum T levels in men, it could be regarded as an imperfect parameter of both Leydig cell function and spermatogenesis damage in infertile men. In addition, the plasma levels of T might influence the secretion of InhB by Sertoli cell [6, 7], which could belittle the significance of serum IB levels as the reliable marker of spermatogenesis [32]. Consequently, the study of genetic factors that might have affect the spermatogenesis, has drawn the attention of researchers who struggle to identify the main factors causing infertility, in order to remove those comorbidity [8, 9] and/or environmental agents that could play a confounding role [33–35].

It is generally accepted that T and its receptor (AR) have a key role in differentiation and development of male genitalia as well as to initiate and to maintain a normal spermatogenesis [36]. Therefore, AR mutations generating androgen resistance can bring about male sexual disturbs ranging from complete genitalia feminization (Morrison Syndrome) to simple spermatogenesis failure without genitalia differentiation defects [37]. As a result, that evidence bears out the role of AR not only in developing male genitalia but also in determining a completely effective spermatogenesis.

Recently, trinucleotide CAG repeat polymorphism, situated in exon 1 of AR gene, has been shown to be the most important tract in transactivating amino-terminal domain of AR, modulating its function [14]. As a matter of fact, it is commonly accepted that the number of AR-CAG repeats co-regulate the AR transactivation capacity in an inverse manner: the higher the number of AR-CAG repeats the lower the activity [17]. The identification of Kennedy syndrome (>40 CAG repeats with hypoandrogenism due to mild androgen insensitivity coupled with spinobulbar muscular atrophy), the results of two clinical observational studies [higher serum

(free) T levels in those men with higher number of AR-CAG repeats] [15, 16] and a recent meta-analysis (infertile men had <1 number of AR-CAG repeats than infertile men) [38] have supported that hypothesis. Conversely, both two recent “in vitro” papers [14, 39] and a meta-analysis [23] have provided data that are in disagreement with the previous evidence showing a lack of a stepwise reduction in activity with a rise in AR-CAG length across the polymorphic range.

With specific reference to the latest results, we subdivided all participants in three categories showing a short, a middle and a long sequence of CAG repeats in AR gene, corresponding respectively below 25 % inter-quartile, between 25 and 75 % inter-quartile and above the 75 % inter-quartile of those assessed in fertile men, with the aim to verify if there was a different distribution of CAG repeat length among fertile men and men with idiopathic oligozoospermia and azoospermia as well as in patients affected by former cryptorchidism or microdeletions of Y long arm. As Table 1 shows, the number of trinucleotide CAG repeat stretch assessed in IOM, CryM and YM was significantly higher than in FM. In addition, the distribution of AR-CAG repeat length among fertile participants and those with idiopathic oligozoospermia and azoospermia as well as cryptorchidism and Yq microdeletions was statistically different (Fig. 1), indicating that trinucleotide CAG repeat length might co-regulate the AR function, being different the distribution both between fertile and infertile male and even between the well-known forms of infertility and the idiopathic ones. These data can gain more weight if one considers that the range of our fertile participants was not different from those reported by other Italian Authors in previously published works [40–42]. Indeed, in a paper by Canale et al. [42] the Authors showed a range of 18–26 CAG repeats with only one case with CAG repeat number >25 in the control group, while in another paper by Ferlin et al. [41], the control group had a wide range of 9–30, even though the patients with CAG repeat number below 15 were only 3 % and those above 25 were less than 10 %. Finally, delli Muti et al. [40] showed a range of 15–27 with only 3 % of subjects with trinucleotide CAG repeat number above 25 in their control group.

In order to further study the three genotype of trinucleotide CAG repeat length (n 15–18; n 19–21; n 22–28), we analysed the statistical difference among clinical and seminal parameters along with the hormonal serum levels in both fertile group and infertile participants with idiopathic and non-idiopathic infertility. We observed that in idiopathic oligozoospermic and azoospermic men, the subgroup of 22–28 CAG repeat number might affect the serum levels of InhB, resulting lower than in the subjects belonging to the other two phenotypes (Table 2). In addition, when all fertile and infertile participants with idiopathic infertility were considered together, the phenotype 15–18 seems to affect serum levels of InhB and sperm count,

while the subgroup 22–28 seems to co-regulate the testis volume, the sperm count, the sperm motility, serum InhB levels as well as the InhB/FSH ratio. This evidence sheds light on the fact that the AR with both shorter sequences of AR-CAG repeat (n 15–18) and longer sequences of AR CAG repeat (n 22–28) might have a more reduced activity compared with the subgroup of participants with a number of 19–21 trinucleotide CAG repeats, negatively influencing the testis volume, the sperm count and the Sertoli cell function as well as reducing serum InhB levels and the InhB/FSH ratio. These results are in agreement with those that have been published so far. Previous studies, in fact, carried out by means of immunohistomorphometric techniques on testicular bioptic specimens from men with complete androgen insensitivity syndrome, non-obstructive azoospermia, “Sertoli Cell only” syndrome, oligozoospermic men with germ-cell arrest and cryptorchid subjects, showed both immature Sertoli cells [43, 44] and the absence of AR expression in the nucleus of these cells [45, 46].

Our idiopathic infertile subjects belonging to the former-cryptorchid group (CryG) showed a statistically significant difference of frequency distribution of three subgroups AR-CAG repeats in comparison with FM and the other infertile participants (Fig. 1). Moreover, the number of AR-CAG repeat stretch was statistically higher in comparison with those assessed in FM (Table 1). Our data are in agreement with most results published until now [47–51] except for those of a more recent study [18]. These conflicting evidence, though, can be explained by the following factors: 1) the choice of selection criteria for the patients enrolment, taken the complexity of the syndrome characterized by several clinical aspects such as idiopathic (mono or bilateral) cryptorchidism with or without comorbidity (inguinal hernia, micropenis, hypospadias etc.) and secondary cryptorchidism (hypogonadotropic hypogonadism, Klinefelter S.) [25]; 2) the close partnership with other genes and/or hormones in testicular descent process [25]; 3) the complexity of androgen action (genomic and/or nongenomic action) in different target tissues [19, 51]. Conversely, the ethnic difference of number of AR-CAG repeats should not be retained as a significant cause for these contrasting data since all groups that have been studied so far, with the exception of study by Davis-Dao C et al [18], have reported a longer AR-CAG repeat length in comparison with the fertile men independently of their ethnic origin [41, 47–50].

We found a higher number of AR-CAG repeats in YM in comparison with controls (FM) (Table 1 and Fig. 1), which could be quite surprising in the light of consistent evidence showing that Yq microdeletions, involving three Azoospermia Factors (AZF) loci (AZFa, AZFb and AZFc), remove many genes likely involved in male germ cell development and maintenance [26–28]. However, recent evidence provided by

Gatta et al. [52] suggested that in Yq microdeletion men, beside the three AZF loci, other genes (e.g. AR genes) might be implicated in spermatogenesis failure. Therefore, more additional data should be provided before drawing definitive conclusions about the potential role of CAG triples in the spermatogenesis damage in males with Yq microdeletions.

To sum up, our study indicates that the AR-CAG repeat polymorphism may affect the function of AR according to non-linear association as previously thought. Furthermore, the outliers in trinucleotide CAG repeat stretch might bring about different activity of AR compared with the phenotype with CAG repeat length close to the median number. As a result, in infertile subjects the AR-CAG polymorphism might affect testis volume and consequently sperm count along with Sertoli cell function, as both low serum InhB levels and reduced InhB/FSH ratio pointed out. However, it is arguably advisable to further evaluate the pathogenic role of the AR-CAG repeat length in infertile men, testing larger numbers of patients to confirm our results.

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