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Original Article

Androgen receptor gene polymorphism and sex hormones in elderly men: the Tromsø study

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Abstract

The aim of this study was to examine whether CAG/GGN repeats are significant modulators of serum concentrations of total and free testosterone (T) as well as of luteinizing hormone (LH) in elderly men. Sixty-nine 60- to 80-year-old men with subnormal T levels ($\leq$ 11.0 nmol L^{-1}) and 104 men with normal T levels taking part in a nested case-control study were used for these analyses. Sex hormones were measured and free T was calculated. The CAG and GGN polymorphisms in the androgen receptor gene were determined by polymerase chain reaction and subsequent direct sequencing. There were no differences in the CAG and GGN repeat lengths between the groups. In cross-sectional analyses of the whole cohort, total and free T were positively associated with CAG length (all $P < 0.05$) before, but not after, waist circumference or body mass index was added to the model. CAG repeat lengths were weakly, but not independently, associated with total and free T. These findings indicate that when clinically evaluating T and LH levels in elderly men, the CAG and GGN repeat lengths do not need to be taken into consideration.

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1 Introduction

The decrease in testosterone (T) levels during aging is characterized by high inter-individual variability [1]. The mechanisms responsible for the age-related decrease in T are still being debated and probably involve the hypothalamic and testicular portions of the hypothalamo–pituitary–testicular axis to some degree [2]. In addition to aging, lifestyle factors and health status might influence individual T levels [1]. However, discrepancies in the sensitivity to biological effects of the androgens, exerted through the binding of the hormone to the androgen receptor (AR), may also be involved in the inter-individual variation of T as well as in age-related decline.

The human AR, located on the X-chromosome at Xq11.2–q12 [3, 4], belongs to the nuclear receptor family of ligand-activated transcription factors. Its regulation of transcription is crucial for androgenic effects in individuals of both genders. The AR encodes a protein with three major functional domains: the NH_2-terminal

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transactivation domain [5, 6], the DNA-binding domain and the C-terminal ligand-binding domain [7].

The NH_2-terminal domain, which is an important determinant of the transactivating capacity, contains a glutamine repeat, encoded by (CAG)nCAA, and a glycine repeat, encoded by (GGT)3(GGG)(GGT)2(GGC)n [8]. These sequences are commonly referred to as the CAG and the GGN repeats, respectively. The length of the CAG repeat sequence spans from approximately 10 to 30 repeats, with an average length of 22 repeats in Caucasians [9]. Pathological expansion of the CAG repeat length (> 40 CAG) causes the neuromuscular disease known as Kennedy's disease (spinal bulbar muscular atrophy), which is often associated with androgen insensitivity in men [10]. Regarding the GGN segment, little is known so far about its normal physiological function or associated pathophysiological conditions. There are no strong associations with any diseases reported, although short GGN repeats have been reported to correlate with decreased semen volume [9], which is indicative of lower androgen activity.

In vitro experiments have indicated an inverse relationship between the CAG stretch and the transactivating capacity of the AR [11, 12]. Similar studies on the impact of GGN length indicate that the most common length (23) is associated with superior AR function, with shorter and longer repeats implying less active receptors [13].

No studies have yet shown an association between GGN number and reproductive hormone levels [9], and studies considering the relationship between the CAG repeat length and concentrations of luteinizing hormone (LH) and T in men are conflicting. A positive, significant association between CAG length and free T has been reported by Krithivas *et al*. [14], Mifsud *et al*. [15], Giwercman *et al.* [16] and Crabbe *et al*. [17], but not by Van Pottelbergh *et al*. [18], Zitzmann *et al*. [19], Harkonen *et al*. [20], Walsh *et al*. [21], Canale *et al*. [22] and Campbell *et al*. [23]. The studies by Krithivas *et al.* [14] and Crabbe *et al.* [17] also report a positive and significant association between CAG length and total T. A positive, significant association between CAG length and LH level was found by Giwercman *et al*. [16] Crabbe *et al.* [17] and Zitzmann *et al*. [19].

Owing to the earlier contradictory results, our aim in this study was to examine whether CAG/GGN repeat lengths had an impact on endogenous T and LH levels in elderly men participating in the Tromsø study. If polymorphisms in the AR gene have a significant and independent impact on serum levels of reproductive hormones, this could imply that CAG and/or GGN numbers need to be taken into consideration when clinically evaluating T and LH levels in relation to the diagnosis of hypogonadism.

2 Materials and methods

2.1 Subjects

The study population and the methods have been presented earlier [24]. The Tromsø study is an ongoing general health survey that was performed for the fifth time in 2001 in a manner similar to the earlier surveys [25]. All men and women 30 years and older who were living in the municipality of Tromsø, and who participated in the second phase of the fourth Tromsø study in 1994–1995 [1] or turned 30, 40, 45, 60 or 75 years old during 2001, were invited to participate. Non-fasting blood samples were drawn between 08:00 and 18:30 hours and analyzed for serum total T.

In 2005, all men aged 60–80 years, with a serum total T level ≤ 11.0 nmol L^{-1} in the survey in 2001, were invited to a follow-up examination at the Clinical Research Unit at the University Hospital of North Norway. For each subject, a randomly selected age-matched control subject from the same survey with serum total T levels > 11.0 nmol L^{-1} was also invited to participate. The invitation letter informed the subjects about the purpose of the study but did not disclose the subject's T level. Of the 3 447 men with serum total T measurements in the fifth Tromsø study in 2001, 335 men aged 60–80 years in 2005 had a serum total T level ≤ 11.0 nmol L^{-1}, and were invited to the follow-up study. Of these, 157 participated, 69 of whom still had total T levels ≤ 11.0 nmol L^{-1}. The majority of the men had T levels between 6.0 and 11.0 nmol L^{-1}, and they are therefore referred to as having subnormal T levels. As age-matched controls for this group, 335 subjects with serum total T levels > 11.0 nmol/L^{-1} in the fifth Tromsø Study were invited to participate. Of the 124 who attended, 104 still had a total T concentration in the normal range (> 11.0 nmol L^{-1}).

Those who still had total T levels in the same range were informed that their T levels were similar to those in the fifth Tromsø study and were invited to attend additional examinations. Those consenting returned on a separate day to the Clinical Research Unit for more extensive investigations. Fasting blood samples, including whole blood for the AR analyses, were drawn

at 08:00 hours. Height, weight and waist circumference (WC) were measured with subjects wearing light clothing and no shoes. Body mass index (BMI) was calculated (kg/m^2). The Aging Males Symptoms (AMS) scale was used for evaluating well-being [26].

The study was approved by the Tromsø Regional Research Ethics Committee, and all participants gave written informed consent to participate.

2.2 Sex hormone analyses and biochemistry

Serum total T, follicle stimulating hormone (FSH), LH and prostate specific antigen (PSA) were analyzed through electrochemical luminescence immunoassay using an automated clinical chemistry analyzer (Modular E; Roche Diagnostics GmbH, Mannheim, Germany). The total analytical values, expressed as the sum of intra-assay and inter-assay coefficients of variation (CV_a), were 5.6%, 4.0%, 2.2% and 3.2%, and the reference ranges were 10–28 nmol L^{-1}, < 12 IU L^{-1}, < 9 IU L^{-1} and < 4.0 μg L^{-1}, respectively. Serum sex hormone-binding globulin (SHBG) was analyzed by immunometry based on chemiluminescence, using an automated clinical chemistry analyzer (Immulite 2000; Diagnostic Product Corp., Los Angeles, CA, USA). The coefficients of variation CV_a was 5.2%.

Free T was calculated from total T and SHBG using a fixed albumin value, according to the Vermeulen formula [27].

2.3 Analysis of AR gene polymorphisms

Genomic DNA was extracted from peripheral leukocytes, and the GGN repeat was amplified in a 50-μL polymerase chain reaction (PCR) containing approximately 10 ng DNA; 0.5 μmol L^{-1} of each of the primers, F-CGGTTCTGGGTCACCCTCA and R-TCACCATGCCGCCAGGGTA (Invitrogen Corp., San Diego, CA, USA); 1.5 mmol L^{-1} $MgCl_2$; 200 μmol L^{-1} each of dATP, dCTP and dTTP; 100 μmol L^{-1} dGTP and 7-deaza-dGTP (Roche Diagnostics GmbH, Mannheim, Germany), respectively; 45 mmol L^{-1} KCl; 10 mmol L^{-1} Tris-HCl (pH 8.4 at 70ºC); 0.1% Tween 20 and 0.5 unit of Dynazyme DNA polymerase (Finnzymes Oy, Espoo, Finland). Amplification proceeded for 35 cycles in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). Each cycle included denaturation at 96ºC for 45 s, primer annealing at 61ºC for 45 s and primer extension at 72ºC for 1 min, with an initial denaturation step at 96ºC for 3 min and a final extension step at 72ºC for 5 min.

The CAG repeat was amplified in a 50-μL PCR containing approximately 10 ng DNA; 0.3 μmol L^{-1} of each of the primers, F-TTAGGGCTGGGAAGGGTCTA and R-TGGGGCCTCTACGATGGGCT; 1.5 mmol L^{-1} $MgCl_2$; 200 μmol L^{-1} dNTPs; 45 mmol L^{-1} KCl; 10 mmol L^{-1} Tris-HCl (pH 8.4 at 70ºC) and 0.5 units of Dynazyme DNA polymerase. Amplification proceeded for 35 cycles. Each cycle included denaturation at 96ºC for 1 min, primer annealing at 61ºC for 45 s and primer extension at 72ºC for 2 min, with an initial denaturation step at 96ºC for 3 min and a final extension step at 72ºC for 5 min.

PCR products were purified, directly sequenced with the reverse primers from the PCR, precipitated, resuspended, and run externally on an eight-capillary Beckman Coulter CEQ 2000XL (Beckman Coulter, Bromma, Sweden) sequencing machine.

2.4 Statistical analysis

Normal distribution was evaluated with determination of skew and histograms. Total and free T and SHBG were considered to be normally distributed. LH was slightly skewed, but because it was not used as a dependent variable it was not log-transformed. Men with subnormal and normal T levels were compared using *t*-test. Age-adjusted and BMI-adjusted partial correlations were used when analyzing for univariate associations. Analysis of variance was used to calculate means of total and free T and SHBG by quartiles of CAG and three groups (< 23; = 23; > 23) of GGN repeat lengths. All statistical tests were two-tailed, with statistical significance defined as $P < 0.05$. The data were analyzed using the SPSS statistical package for Windows version 15.0 (SPSS, Chicago, IL, USA).

3 Results

CAG and GGN results were missing in one of the men with subnormal T levels; thus, the statistical analyses were performed on 68 men with subnormal T and 104 men with normal T levels. The characteristics of the study population are presented in Table 1; in addition to the known difference in total T, free T levels were significantly lower in the subnormal T group. Serum sex hormone-binding globulin levels were significantly lower in the subnormal T group, but there was no difference in LH levels. As also seen in Table 1, there was no difference in the CAG and GGN repeat lengths between the groups, and the distribution of the number of CAG trinucleotide repeats for the whole study population is shown in Figure 1A. There were

Table 1. Characteristics including CAG and GGN repeat lengths and anthropometry in men 60–80 years old, with subnormal testosterone and normal testosterone levels (mean ± SD).

	Subnormal testosterone (n = 68)	Normal testosterone (n = 104)	P-value
Age	69.4 ± 4.7	68.7 ± 4.9	0.345
Total testosterone (nmol L^{-1})	8.3 ± 1.9	18.4 ± 4.4	< 0.001
Free testosterone (pmol L^{-1})	180 ± 47	308 ± 65	< 0.001
Luteinizing hormone (IU L^{-1})	7.8 ± 7.6	6.5 ± 3.0	0.144
SHBG (nmol L^{-1})	29.2 ± 12.1	50.8 ± 16.1	< 0.001
CAG (n)	21.2 ± 3.6	21.9 ± 2.5	0.149
GGN (n)	23.1 ± 1.8	23.2 ± 1.4	0.657
Body mass index (kg/m^2)	30.1 ± 3.8	26.5 ± 3.1	< 0.001
Waist circumference (cm)	107 ± 10	97 ± 9	< 0.001
Aging Males Symptoms Scale score			
Psychological	6.6 ± 2.0	6.9 ± 2.2	0.394
Somatic	14.3 ± 4.0	12.4 ± 3.7	0.005
Sexual	12.0 ± 3.3	10.9 ± 3.1	0.054
Total	32.9 ± 7.0	30.0 ± 6.6	0.016

Abbreviation: SHBG, serum sex hormone-binding globulin.

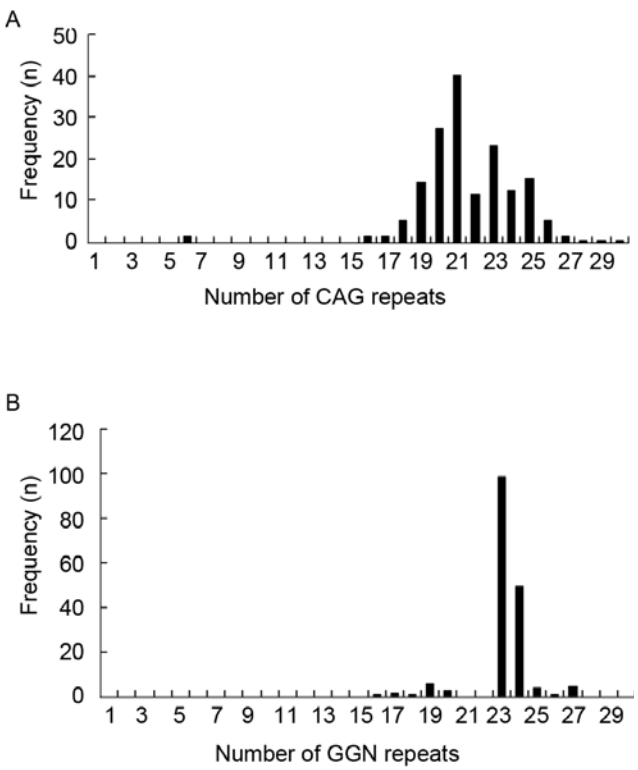

Figure 1. The CAG (A) and GGN (B) distribution in 172 elderly Norwegian men. There were 16 CAG alleles, ranging from 6 to 30 repeats, with the median allele length being 21, and 10 GGN alleles, ranging from 16 to 27 repeats, with 23 and 24 as the most common alleles.

16 CAG alleles, ranging from 6 to 30 repeats, with the median allele length being 21. In contrast to CAG, the GGN locus was less variable: 10 different alleles, ranging from 16 to 27 repeats, with 23 and 24 as the most common alleles (Figure 1B). Table 1 also shows that the quality of life according to the AMS scale was better in men with normal T levels than in men with subnormal T levels, with significant differences in the total score (P = 0.016) and the somatic domain (P = 0.005) and a borderline difference in the sexual domain (P = 0.054).

The entire study population was used to study the cross-sectional associations between the CAG and GGN repeat lengths and sex hormones. In age-adjusted partial correlations, total and free T were positively correlated with CAG repeat lengths (R = 0.16, P = 0.038; R = 0.15, P = 0.047, respectively), as shown in Table 2. However, when adjusting for BMI or WC, the significance was lost. There was no association between GGN repeat lengths and sex hormones. There were also no associations with CAG or GGN repeats and the AMS scale (data not shown).

In Table 3, the distribution of sex hormones is presented by quartiles of CAG repeat lengths; there were no significant linear trends. Likewise, we found no significant differences between the three GGN repeat groups with regard to the same variables. We found no important interaction that could have affected the results.

Table 2. Adjusted partial correlations (R) with CAG and GGN repeat lengths and hormone levels.

	CAG	*P*-value	GGN	*P*-value
Total testosterone (nmol L^{-1})				
Age-adjusted	0.16	0.038	0.06	0.475
Age-adjusted and BMI adjusted[a]	0.12	0.135	0.05	0.499
Free testosterone (pmol L^{-1})				
Age-adjusted	0.15	0.047	0.085	0.272
Age-adjusted and BMI adjusted	0.12	0.114	0.08	0.284

Abbreviation: BMI, body mass index.
[a]Adjusting for waist circumference instead of BMI yielded the same results.

Table 3. The distribution of age, anthropometrics and sex hormones by quartiles of CAG repeat length (≤ 20, 21, 22–23 and > 23) (mean ± SD).

	CAG quartiles			
	1 (n = 55)	2 (n = 41)	3 (n = 36)	4 (n = 40)
Age	69.3 ± 5.2	69.8 ± 5.4	67.6 ± 4.1	69.0 ± 4.5
BMI (kg m^{-2})	27.6 ± 4.2	28.6 ± 3.8	28.3 ± 3.3	27.3 ± 3.8
Waist circumference (cm)	100 ± 11	103 ± 10	101 ± 11	99 ± 3.8
Total testosterone (nmol L^{-1})	14.0 ± 6.2	14.6 ± 5.8	15.0 ± 6.4	15.4 ± 7.1
Free testosterone (pmol L^{-1})	244 ± 79	255 ± 92	273 ± 75	262 ± 87
SHBG (nmol L^{-1})	42 ± 20	43 ± 14	40 ± 18	44 ± 19
LH (IU L^{-1})	7.9 ± 7.3	6.6 ± 4.0	5.6 ± 2.8	7.7 ± 5.0

Abbreviations: LH, luteinizing hormone; SHBG, serum sex hormone-binding globulin.

4 Discussion

In this nested case-control study in elderly men, we found no difference in CAG or GGN repeat length between men with subnormal T levels and men with normal T levels. In cross-sectional analyses of the whole cohort, we found an age-adjusted positive correlation between the length of AR CAG repeats and total and free T. When adjusting for BMI or WC, however, these associations were no longer significant.

Our observations are in line with several cross-sectional studies. Van Pottelbergh *et al.* [18] reported a non-significant association between either total or free T and CAG in 273 healthy men aged 71–86 years. Lack of significant associations was also reported by Harkonen *et al*. [20], Walsh *et al*. [21], Canale *et al.* [22] and Campbell *et al*. [23]. In contrast, Krithivas *et al*. [14] reported a significant association between AR CAG and total and free T upon follow-up examination in a large cross-sectional study of 882 healthy men (mean age 61 ± 8 years). However, no association was found between the AR CAG repeat and T levels at baseline examination 8 years earlier (when aged 40–70 years). Mifsud *et al.* [15] reported a significant positive correlation between the AR CAG repeat length and free T in a cohort of 91 healthy men; however, no adjustments for possible confounders were performed. Giwercman *et al.* [16] reported a positive correlation in unadjusted analyses between the AR CAG length and serum concentrations of LH and free T among 274 young men aged 18–21 years. The study by Crabbe *et al*. [17] which consisted of two cohorts, Beltress (857 men aged 35–59 years) and Siblos (358 men aged 25–45 years), reported a positive association between AR CAG repeat length and total and free T in both of the cohorts. A positive association was also found between the AR CAG repeat length and LH in the Beltress population. It is difficult to compare the results from these studies because most of the studies—including ours—are small, the age in the different cohorts varies greatly and adjustments for important covariates, such as age and BMI, are missing in four of the 11 studies, including two of the positive studies.

Although this study does not support any significant relationship between the AR CAG repeat and the

reproductive hormones T and LH, it does not exclude the possibility that the contribution of genetic markers, as predictors of T levels, is padded by other factors. Elderly men may possess traits, such as obesity, that conceal the relationship of CAG repeat lengths to T and LH. It has been suggested that obesity plays a causal role in the decline of total and free T [28]; in contrast, low-dose androgen treatment decreases abdominal fat mass in older men regardless of the cause of the decreased T level [29]. Therefore, whether obesity causes a reduction in T levels or vice versa, it is a strong predictor of endogenous T levels in men. This was also the case in this study; the men with subnormal T levels had significantly higher BMI and WC. In addition, the correlations found in age-adjusted analyses were lost after adjusting for BMI and WC. Therefore, in younger men the impact of AR sensitivity might be significant, but in elderly men lifestyle factors are probably more important predictors of T levels. This lack of association among elderly men, especially concerning LH, might also be explained by age-related alterations in androgen feedback regulation. Several changes in neuroendocrine control of Leydig cell function have been described in aging men. The circadian rhythm of both LH and T secretion is blunted [30], and even if the LH pulse frequency remains relatively unchanged the mean LH pulse amplitude is decreased [31]. Indirect evidence suggests that the main neuroendocrine changes occur at the hypothalamic level, as the responsiveness of the pituitary is preserved when stimulated with physiological doses of gonadotropin-releasing hormone [2]. In addition, an age-related reduction of Leydig cell function is indicated by a reduced secretory response to stimulation with human choriogonadotropin [32], as well as to gonadotropin-releasing hormone infusion [33]. A reduced number of Leydig cells appear to account for this decrease in testicular secretory reserve [34], and, although LH levels do increase in older men, the increase is not sufficient to normalize T levels. In this study, adjusting for age did not change the associations between CAG repeat length and reproductive hormones. However, the age range examined was narrow, and these factors might be more important in another population.

Men with normal T levels had better results on the AMS scale than expected, but there was no association with CAG or GGN repeats. Therefore, even though we found a weak association between total and free T levels and CAG repeats in the cross-sectional analyses, the lack of association between the result from the AMS scale and the CAG repeats suggests that these findings are probably of little clinical relevance.

Limitations of this study should be noted. We did not measure free T but used calculated values. However, these calculations have been evaluated by different investigators and found to be a reliable index of free T [27, 35].

In summary, there were no differences in AR CAG or GGN repeat length between men with subnormal total T levels and men with normal T levels. In cross-sectional analyses, the lengths of the CAG tract in exon 1 of the AR gene were weakly, but not independently, associated with total and free T. These findings indicate that CAG and GGN repeat numbers do not need to be taken into consideration when evaluating reproductive hormone levels in elderly men.

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