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X-Chromosome Inactivation Patterns and Androgen Receptor Functionality Influence Phenotype and Social Characteristics as Well as Pharmacogenetics of Testosterone Therapy in Klinefelter Patients

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Klinefelter syndrome is characterized by a vast range of phenotypes related to androgen effects. Testosterone (T) acts via the X-linked androgen receptor gene carrying the CAG repeat (CAGn) polymorphism, the length of which is inversely associated with androgen action and might account for the marked variation in phenotypes. In 77 newly diagnosed and untreated Klinefelter patients with a 47,XXY karyotype we assessed phenotype and social traits in relation to X-weighted biallelic CAGn length using X-chromosome inactivation analysis after digestion of leukocyte DNA with methylation-sensitive *Hpa*II. Forty-eight men were hypogonadal and received T substitution therapy; in these, pharmacogenetic effects were investigated. The shorter CAGn allele was preferentially inactive. CAGn length was positively associated with body height. Bone density and the relation of arm span to body height were inversely related to CAGn length. The presence of long CAGn was predictive for gynecomastia and smaller testes, whereas short CAGn were associated with a stable partnership and professions requiring higher standards of education also when corrected for family background. There was a trend for men with longer CAGn to be diagnosed earlier in life. Under T substitution, men with shorter CAGn exhibited a more profound suppression of LH levels, augmented prostate growth, and higher hemoglobin concentrations. A significant genotype-phenotype association exists in Klinefelter patients: androgen effects on appearance and social characteristics are modulated by the androgen receptor CAGn polymorphism. The effects of T substitution are pharmacogenetically modified. This finding is magnified by preferential inactivation of the more functional short CAGn allele. (*J Clin Endocrinol Metab* 89: 6208–6217, 2004)

KLINEFELTER SYNDROME, THE most frequent form of male hypogonadism, is an endocrine disorder based on sex chromosome aneuploidy. Descriptions of the clinical picture include small testes, hypogonadism, gynecomastia, and elevated concentrations of gonadotropins, whereas testosterone (T) levels tend to be low (1–5). A marked variation in neurological and cognitive perturbations (language/behavioral problems) exists as well as shifts in body proportions and androgen deficiency-related problems (loss or initial absence of libido, decreased muscle strength, osteoporosis, and anemia) (2–4, 6).

Klinefelter syndrome has a prevalence of 0.1–0.2% within the male population (4, 6). Eighty percent of cases are due to a 47,XXY karyotype; the others relate to higher grade aneuploidies or mosaicism (2, 5, 6). Although Klinefelter syndrome is not rare, many patients escape diagnosis. Only 10% are detected before or during puberty, and about two thirds of all men with X-chromosome polyploidies fail to be identified during their lifetime (6, 7).

It remains unresolved why so many Klinefelter patients are not diagnosed, and it must be speculated that the clinical picture we observe is a biased one, showing only the extreme cases, whereas those men with more unobtrusive phenotypes lead normal lives. Nevertheless, because spermatogenesis is affected by meiotic problems in all Klinefelter patients, they may be detected at fertility centers (8).

Recently, the NIH sponsored a meeting on the topic of variations in Klinefelter phenotypes; new research directions were identified, especially the roles of androgens and the X-linked androgen receptor (AR) (4).

Differences in the AR sequence are characterized mostly by a highly polymorphic trinucleotide repeat (CAGn) in exon 1 (9), the normal length of which is 9–37 (10); expanded numbers are observed in the neurological disorder of X-linked spinobulbar muscular atrophy (X-SBMA) (11). *In vitro*, the T-induced transactivation activity of the AR is inversely associated with the length of CAGn due to reduced binding of AR coactivators (12), and accordingly, marked features of hypogonadism are noticed in X-SBMA (13, 14).

Also, in healthy men with repeat numbers within the normal range, there are numerous reports on how the CAGn polymorphism modulates physiological androgen effects (reviewed in Ref. 10). Various targets are affected in eugonadal men: prostate size (15); concentrations of lipids, insulin, and leptin (16); endothelial functions (17); bone density (18, 19); mood/cognition (20, 21); and sperm concentrations (22). The risk for prostate cancer is increased in men with shorter repeats (23), and a pharmacogenetic implication for prostate growth during androgen substitution of hypogonadal men has been described (24).

Abbreviations: AR, Androgen receptor; DF, degree of freedom; SS, sum of squares; T, testosterone; X-SMBA, X-linked spinobulbar muscular atrophy.

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In Klinefelter patients, the case is complicated by the presence of at least two AR alleles undergoing the phenomenon of X inactivation; one gene in every cell becomes inactive (25). In general, this process take places in cells containing more than one X-chromosome. It is facilitated by methylation of specific genes on the X-chromosome; a methylated gene cannot be transcribed and, as a consequence, cannot be translated into a protein. Hence, one of the AR alleles is methylated and inactive; the other AR allele is not methylated and active. Inactivation is believed to be random on a cell to cell basis (25). Nevertheless, in certain pathological environments in women, nonrandom inactivation of AR alleles can occur; it has been described in conditions related to increased androgen activity (hirsutism and polycystic ovaries) (26–28). In contrast to normal men, Klinefelter patients have two AR alleles, as do women. It can be speculated, that nonrandom inactivation of AR alleles may take place in this condition. Correspondingly, a study of 17 men with 47,XXY demonstrated skewed inactivation, but provided no information on CAGn length or phenotypes (29).

The present study analyses phenotype and clinical traits in newly diagnosed and untreated Klinefelter patients with a 47,XXY karyotype in regard to the putative influence of X-chromosome inactivation and AR CAGn length. It also investigates pharmacogenetic effects occurring under T substitution. The study concerns Klinefelter patients with endocrine disturbances or those with infertility who were previously undetected. The range of Klinefelter phenotypes was unusually broad, because patients presented at an andrology unit treating both endocrine and infertility patients; they were examined identically by the same physicians within the same setting.

Subjects and Methods

Patients

Among 244 Klinefelter patients of a total of 15,600 patients, 77 men (age, 18–65 yr at diagnosis) fulfilled the inclusion criteria for entry into a retrospective data analysis: Caucasian origin, nonmosaic 47,XXY karyotype, and no previous exposure to exogenous androgens. They were referred because of endocrine disorders suspected by external physicians (n = 45) or unwanted childlessness (n = 32). The patients' histories were obtained during a standardized interview. Body hair pattern was categorized according to a four-degree scale (feminine, scantly male, normally male, or extraordinarily male) by experienced clinicians. The social status of patients and family members was assessed in terms of profession and partnership. All men gave written informed consent for diagnostics, treatment, and the use of genomic material for scientific evaluation (approved by the ethics committee of the medical faculty and the State Medical Board). Of the 50 patients who were hypogonadal (total T, <12 nmol/liter; see *Results*), 48 men agreed to receive T substitution by either every 2-wk im injections of T enanthate (250 mg; n = 40) or daily nonscrotal patches (two patches, 2.5 mg T each; n = 8). In these 48 men, a pharmacogenetic evaluation was performed of prostate volume during treatment (assessments after 2.1 ± 0.4 yr). Also determined was the suppression of LH levels during T treatment, and data refer to suppression maximally achieved during that time period.

Ultrasound examinations, semen analysis, and hormone measurements

Determination of testicular and prostate volumes, assessment of bone density by phalangeal ultrasound as amplitude-dependent speed of sound (meters per second), and semen analysis were performed using established procedures (5, 18, 24, 30, 31). Determination of hormones followed methods previously described (17). Whole EDTA-blood for subsequent DNA analysis was stored continuously at −20 C.

All blood sampling was performed between 0800–1200 h. To test for effective T substitution, samples from patients being treated with im injected T enanthate were obtained at time points indicating individual average levels, preferentially in the second week after injection. Patients treated with the transdermal scrotal T system were sampled 2–5 h after administration of the patch.

Chromosome analysis

Karyotyping of metaphase peripheral blood lymphocytes according to standard methods at the Institute of Human Genetics, University of Munster.

Determination of CAGn length

DNA was isolated from EDTA blood samples using the Nucleon Kit (Amersham Biosciences Freiburg, Germany), and analysis of the AR gene microsatellite residues was performed as previously published (17). Patients with two detected bands of CAGn length were considered heterozygous and subjected to X-chromosome inactivation analysis.

X-Chromosome inactivation analysis

Analysis using leukocyte DNA from heterozygous patients was performed as previously described (27, 29). The methylation status of AR alleles, hence inactivation, was assessed using the methylation-sensitive restriction enzyme *Hpa*II (Roche Diagnostic Systems, Mannheim, Germany). Nonmethylated, hence active, DNA segments are digested by the enzyme and are unavailable for PCR amplification, whereas the methylated sites are not digested, remain intact, and thus provide substrate for amplification.

Equivalent 100-ng DNA aliquots were either digested with 10 U *Hpa*II or mock-digested in respective buffer containing no enzyme. Samples were digested overnight at 37 C in a 30-μl reaction volume, followed by a final enzyme denaturation step at 95 C for 5 min. Aliquots of 5 μl were amplified by PCR and run on an LI-COR sequencer (Biosciences, Bad Homburg, Germany). From every fifth blood sample, DNA was again isolated, and the methylation status was reanalyzed to assure the reproducibility of the methods being used (interassay coefficient of variation, 7.3%). Total fluorescent peak areas for both alleles in digested and undigested samples were calculated by ChemiImages software (Biozym, Oldesdorf, Germany). The total fluorescent peak areas for both alleles in digested and undigested samples were recorded and used for the following calculations of allele inactivation. The variables were signal allele 1 digested (a), signal allele 2 digested (b), signal allele 1 undigested (c), and signal allele 2 undigested (d). In an ideal case, c should equal d, which, in practice, is rarely the case. To compensate for unequal amplification of alleles due to confounding factors not caused by methylation, signals c and d (undigested samples) are necessary to create a correction factor: inactivation of allele 1 = $(a/c)/(a/c + b/d)$ (equation I) and inactivation of allele 2 = $(b/d)/(a/c + b/d)$ (equation II). Equations 1 and 2 were used throughout this study. An inactivation value of 0 equals no inactivation, 1 would be complete inactivation, and 0.5 is random inactivation. Inactivation values of alleles 1 and 2 always sum up to 1 (equation I + equation II = 1, normalization). Equations I and II have been used in a previously published study (29). Hence, in the case of $c = d$, no presence of a confounder of amplification, the equations equal to: equation I = equation III [inactivation of allele 1 = $a/(a + b)$] and equation II = equation IV [inactivation of allele 2 = $b/(a + b)$].

To calculate the physiologically active means of CAGn, the method previously described (27) was used. Each CAGn allele length in a genotypic pair was multiplied by its total expression (1 minus inactivity) and the two adjusted CAGn values were then added to obtain the X-weighted biallelic mean, which can differ markedly from the arithmetic mean in the case of skewed inactivation. For homozygous patients, simple CAGn length was used for additional analysis.

Statistical evaluation

Associations between continuous parameters and CAGn length were calculated (Spearman's rank correlation). Other associations were in-

TABLE 1. General data of 77 untreated Klinefelter patients (nonmosaic 47,XXY)

Parameter	Unit	Value (range, mean ± SD)	No data
Anthropometrical data			
Age at diagnosis	yr	18–65, 29.4 ± 8.5	
Age of mother at birth	yr	17–45, 28.1 ± 6.1	n = 3
Age of father at birth	yr	17–50, 31.4 ± 7.4	n = 4
BMI	kg/m^2	15.9–36.4, 24.6 ± 4.4	
Body height	cm	169–201, 184.9 ± 7.5	
Body weight	kg	50.0–130.0, 83.9 ± 16.3	
Arm span	cm	165–200, 184.5 ± 7.7	n = 5
Ratio arm span/height		0.95–1.08, 0.99 ± 0.02	n = 5
Sociometrical data			
Partnership	n	Yes: 43 No: 34	
Profession			
Unskilled worker	n	26	
Craftsman	n	41	
University education	n	10	
Reason for referral			
Fertility problems	n	34	
Endocrine diagnostics	n	43	
Hormone levels			
Total T	nmol/liter	1.3–25.9, 10.5 ± 5.3	
SHBG	nmol/liter	13.0–82.0, 38.5 ± 17.5	n = 4
Free T	pmol/liter	29–592, 212 ± 118	n = 4
Estradiol	pmol/liter	20.3–139, 51.3 ± 22.5	n = 1
LH	U/liter	8.5–40.0, 19.2 ± 6.1	n = 2
FSH	U/liter	11.1–81.0, 34.3 ± 13.4	n = 2
Prostate-specific antigen		0.2–3.9, 0.6 ± 0.5	n = 6
AR alleles			
Homozygous	n	31	
CAGn length	n	15–28, 21.4 ± 2.9	
Heterozygous	n	46	
CAGn length short allele	n	12–24, 19.4 ± 2.1	
CAGn length long allele	n	18–30, 23.2 ± 2.6	
Arithmetic mean		17.5–25.0, 21.2 ± 1.9	
Inactivation short allele		0.20–0.88, 0.59 ± 0.15	
Inactivation long allele		0.11–0.79, 0.41 ± 0.15	
X-weighted biallelic mean		16.8–26.5, 21.8 ± 2.1	
All patients		15.0–28.0, 21.6 ± 2.5	
X-weighted biallelic mean			
Clinical data			
Gynecomastia	n	Yes: 39 No: 38	
Body hair pattern			n = 1
Feminine	n	10	
Scantly male	n	50	
Normally male	n	13	
Extraordinarily male	n	3	
Bitesticular volume	ml	0.9–14.7, 5.5 ± 2.6	n = 2
Prostate volume	ml	5.0–30.0, 14.5 ± 4.7	n = 5
Azoospermia	n	Yes: 54 No: 1	n = 22
Hematocrit	%	0.36–0.51, 0.44 ± 0.32	n = 3
Hemoglobin	g/liter	120–169, 146 ± 10.3	n = 3
Bone density	m/sec	1790–2172, 2071 ± 72.4	n = 9
Data from the 48 T-treated hypogonadal Klinefelter patients			
CAGn length	n	15.0–28.0, 21.2 ± 3.1	
X-weighted biallelic mean			
Total T baseline	nmol/liter	1.3–11.0, 6.6 ± 2.2	
Total T under treatment	nmol/liter	12.1–33.1, 18.9 ± 5.5	
LH baseline	U/liter	8.5–31.8, 19.0 ± 5.0	
LH maximally suppressed	U/liter	0.2–7.7, 2.1 ± 1.9	
Prostate volume baseline	ml	5–28, 14.3 ± 5.1	
Prostate volume under treatment	ml	10–45, 21.6 ± 6.4	
Hemoglobin baseline	g/liter	120–169, 142 ± 12	
Hemoglobin under treatment	g/liter	130–181, 154 ± 12	

BMI, Body mass index.

vestigated and adjusted for potentially confounding variables using linear regression models (see Table 3) for the association of CAGn allele length with the respective continuous parameter and stepwise binomial logistic regression models for association with dichotomous variables. To this end, all variables were checked for normal distribution by the Kolmogorov-Smirnov one-sample test for goodness of fit and were log-transformed if necessary.

In the regression models (see Table 3) various putative predictors

were included in backward stepwise multiple linear regression analyses according to the clinical plausibility of possible influence on a clinical feature (called the variable in this report). Those putative predictors that did not contribute to the regression model were excluded (exclusion threshold, $P > 0.10$), but those predictors that significantly influenced the clinical feature are given with the standard coefficient β and the respective P value. In this approach, the estradiol/T ratio was not used, but, rather, total T and estradiol concentrations as raw values standing on their own were used to discriminate the activity of each substance.

Contingency tables of categorical variables (gynecomastia, partnership, reason for referral, and profession) were created according to tertiles of X-weighted biallelic CAGn length (see Fig. 3). The professions of fathers and brothers were categorized accordingly, and differences from the patient category were expressed as the score (see Fig. 3 for details).

For a comparison between a linear and a nonlinear association of T levels with hemoglobin concentrations, the goodness of fit of both regression models was quantified by the sum of squares. The F ratio quantifies the relationship between the relative increase in sum of squares (SS) and the relative increase in degrees of freedom (DF) between two models, here a linear (1) and a nonlinear (2) association: $F = [(SS1 - SS2)/SS2]/[(DF1 - DF2)/DF2]$. If the linear model is correct, one would expect an F ratio near 1.0. If the ratio is much greater than 1.0, there are two possibilities: the nonlinear model is correct, or the linear model is correct, but random scatter lets the more complicated model fit better. The P value yielded by the F test will show how rare the latter coincidence would be. Thus, $P < 0.05$ can be considered a significant confirmation of the nonlinear model.

Computations were performed using the statistical software package SPSS (Chicago, IL; release 11.0) and PRISM (GraphPad, Inc., San Diego, CA; release 3.2).

Results

Anthropomorphic and clinical data are shown in Table 1; basic correlations to allele length are given in Table 2. There was a trend for men with shorter repeats to be diagnosed later in life (Table 2). Fifty of 77 patients (65%) were hypogonadal (T, <12 nmol/liter).

Forty-six of 77 investigated Klinefelter patients exhibited a heterozygous genotype of AR alleles; 31 men were homozygous. In the heterozygous patients, nonrandom inactivation of AR alleles was found; the average inactivations of the short and long alleles were 0.59 ± 0.15 and 0.41 ± 0.15, respectively (0 = no inactivation; 1 = complete inactivation). Testing inactivation against random was significant (Fig. 1A). The X-weighted biallelic mean and the arithmetic mean of both CAGn residues were significantly different (Fig. 1B).

TABLE 2. Basic correlations

Parameter	Basic correlations with the X-weighted biallelic mean of CAG repeats in the AR gene		Basic correlations with the E_2/T ratio	
	Spearman's r_s	P	Spearman's r_s	P
Anthropometrical data				
Age at diagnosis	**−0.19**	**0.07**	**−0.18**	**0.08**
Age of mother at birth	−0.05	0.67	−0.12	0.35
Age of father at birth	−0.13	0.32	−0.14	0.29
BMI	0.02	0.90	**0.29**	**0.007**
Body height	**0.46**	**<0.001**	0.12	0.28
Body weight	0.12	0.37	**0.33**	**0.002**
Arm span	**0.26**	**0.02**	0.13	0.29
Ratio arm span/height	**−0.37**	**0.001**	−0.03	0.81
Hormone levels				
Total T	0.10	0.37		
SHBG	−0.09	0.47	−0.10	0.39
Free T	0.12	0.33		
Estradiol	0.11	0.32		
LH	−0.07	0.56	−0.04	0.71
FSH	−0.05	0.68	**−0.26**	**0.02**
Prostate-specific antigen	0.02	0.90	0.02	0.89
Clinical data				
Body hair pattern	0.04	0.63	**−0.31**	**0.006**
Bitesticular volume	**−0.24**	**0.04**	−0.14	0.22
Prostate volume	0.03	0.89	−0.16	0.19
Hematocrit	−0.08	0.49	**−0.26**	**0.02**
Hemoglobin	−0.08	0.49	**−0.27**	**0.02**
Bone density	**−0.36**	**0.003**	**−0.33**	**0.007**
Pharmacogenetic data from the 48 T-treated hypogonadal patients				
Total T baseline	−0.03	0.85		
Total T under treatment	0.02	0.91		
LH baseline	−0.16	0.28	−0.03	0.74
LH maximally suppressed	**0.39**	**0.005**	0.12	0.35
Prostate volume baseline	−0.17	0.46	−0.17	0.25
Prostate volume under treatment	**−0.34**	0.02	*−0.25*	*0.09*
Hemoglobin baseline	0.17	0.24	**−0.33**	**0.03**
Hemoglobin under treatment	**−0.36**	**0.009**	*−0.25*	*0.08*

Basic correlations according to Spearman's rank correlation test of continuous parameters (clinical features) to the X-weighted biallelic CAGn length or the estradiol (E_2)/testosterone (T) ratio: two-sided $P < 0.05$ was considered significant, and results are given in *bold* numbers; a trend ($0.05 < P < 0.10$) is given in *italics*. For the significant associations of CAGn length or E_2/T ratio to partnership, profession, gynecomastia, and reason for referral see *Results* and Fig. 3. BMI, Body mass index.

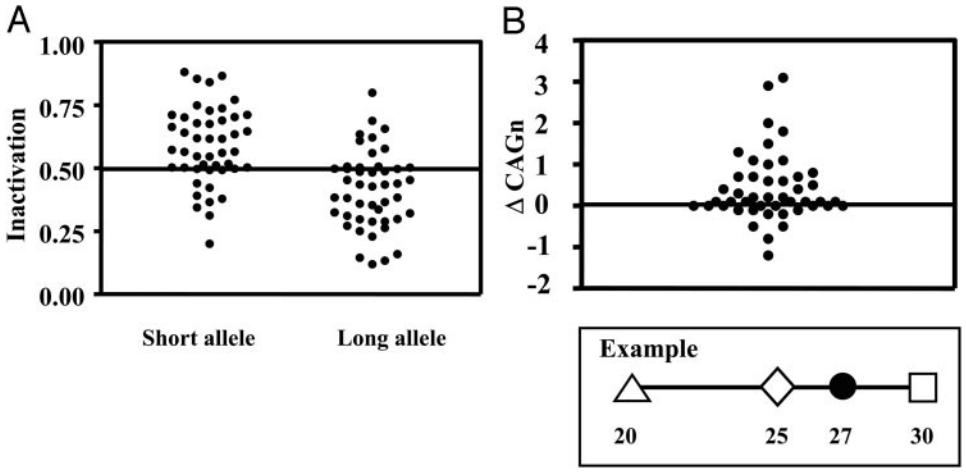

FIG. 1. A, Inactivation patterns of short and long AR alleles in heterozygous Klinefelter patients (n = 46). There was a significant difference from random (Wilcoxon's signed rank test, $P < 0.001$). End to end line, Random inactivation. B, The difference of the X-weighted biallelic mean and the arithmetic mean of CAGn length of the AR gene in heterozygous Klinefelter patients (n = 46). Example: △, short allele; □, long allele; ◇, arithmetic mean; ●, X-weighted biallelic mean; numbers indicate CAGn length. In this case, the difference would be 2. Overall, this difference was significantly different from zero, the X-weighted biallelic mean was longer than the arithmetic mean in most cases (by Wilcoxon signed rank test, $P < 0.001$).

For additional analyses in all Klinefelter patients, CAGn length was used as combined data from X-weighted biallelic mean in heterozygous men and simple CAGn length in homozygous men.

Multiple regression models (Table 3) confirmed the basic associations (Table 2) of body height and the ratio of arm span/body height to CAGn length. For example, the average height of men with short CAGn (180.7 ± 6.4 cm) differed markedly from the height of men with long CAGn (189.4 ± 7.3 cm; Fig. 2A, *inset*). Bone density was also lower in men with longer CAGn (Tables 2 and 3). The estradiol/T ratio was mostly related to different clinical parameters than was CAGn length, such as body mass index and hair pattern (Table 2).

Gynecomastia was found significantly more often in patients with longer CAGn (Fig. 3A). This was confirmed by binomial logistic regression; long CAGn was a predictor ($P = 0.009$) as were low T levels ($P = 0.005$), high estradiol concentrations ($P = 0.02$), and high body mass index ($P = 0.06$). The results were corroborated when the estradiol/T ratio was incorporated in the regression model (CAGn, $P = 0.01$; estradiol/T ratio, $P < 0.001$). Men with shorter CAGn were significantly more likely to live within a stable relationship and seek advice for reasons of childlessness than other patients (Fig. 3, B and C), whereas the estradiol/T ratio was not related to these parameters.

Klinefelter patients with shorter CAGn were more often employed in professions requiring a higher level of education. This was corroborated when correcting for professions of family members, hence background education and environment; patients with short CAGn tended to maintain the family status compared with fathers or brothers, whereas a deterioration of educational levels was mostly and significantly observed in those with longer CAGn (Fig. 3D). The estradiol/T ratio was not related to professional status.

The other parameters were, with 65% of the patients being hypogonadal and, hence, without current sufficiency of AR activation, not dependent on genomically determined androgen activity. Respective associations in multiple regression models are shown in Table 3. Within this mixed cohort of hypogonadal and eugonadal Klinefelter patients, hemoglobin concentrations were dependent on T levels in a log-linear manner, a regression model superior to a linear association (by F test, $P < 0.01$; Fig. 4A).

Among the 55 patients who donated a semen sample, all except one were azoospermic. In the respective patient, less than 0.1 million sperm/ml were found. This 35-yr-old carpenter had a bitesticular volume of 7.2 ml, eugonadal total T concentrations of 13.5 nmol/liter, and was heterozygous for AR alleles (20 and 24 CAGn). He was one of the few patients in whom the shorter allele was preferably active (X-weighted biallelic mean, 21.2; arithmetic mean, 22; see Fig. 1B).

The age of parents at birth was not related to any parameter, neither homozygocity nor heterozygosity of patients.

Pharmacogenetic data

In cross-sectional analyses of data before and under treatment in the 48 treated men, baseline concentrations of LH and hemoglobin as well as prostate size were not related to CAGn length. When T levels were elevated by substitution therapy, suppression of LH concentrations, elevation of hemoglobin concentrations, and prostate growth were strongly related to the AR polymorphism (Tables 2 and 3 and Fig. 4, B and C).

These approaches are confirmed by analysis of covariance for repeated measurements using age, changes in T levels, and CAGn length as covariables. In this model, suppression of LH concentrations was positively influenced by the degree of T increment during therapy ($P = 0.04$) and was inversely associated with CAGn length ($P = 0.009$). Also, prostate growth during therapy was inversely influenced by CAGn length ($P = 0.004$), whereas higher age ($P = 0.06$) and more pronounced increment in T levels during substitution ($P = 0.05$) were positively related to prostate volume. Initial prostate size was included as a confounder in this longitudinal model as well; it influenced the outcome (prostate size under therapy) significantly ($P < 0.001$). The effect of T treatment on hemoglobin concentrations was more pronounced in men with

TABLE 3. Backward stepwise multiple linear regression models

Variable	Unit	Predictor	Standard coefficient (β)	P	Excluded
Body height	cm	CAGn	**0.47**	**<0.001**	TT, log age, estradiol
Relation of arm span to body height		CAGn	**−0.39**	**0.001**	TT, log age, estradiol
Log bitesticular volume	ml	CAGn	**−0.26**	**0.02**	Log age, height
BMI	kg/m^2	TT	−0.43	0.01	
		Estradiol	0.45	0.01	CAGn
		Log age	0.20	0.05	
Body hair pattern	TT		0.26	0.03	CAGn, log age, estradiol
		Body height	−0.32	0.02	
LH	U/liter	Bitesticular volume	−0.47	<0.001	CAGn, log age, TT, estradiol
		BMI	−0.33	0.002	
FSH	U/liter	Bitesticular volume	−0.29	0.006	CAGn, log age, TT, estradiol
		BMI	−0.45	<0.001	
TT	nmol/liter	Log age	−0.23	0.04	CAGn, BMI, LH
		Bitesticular volume	0.24	0.03	
SHBG	nmol/liter	BMI	−0.37	0.002	CAGn, estradiol
		Log age	0.23	0.04	
Log free T	pmol/liter	Log age	−0.36	0.003	CAGn, BMI
Prostate volume	ml	TT	0.27	0.02	CAGn, estradiol
		Log age	0.25	0.03	
Hemoglobin	mg/dl	TT	0.36	0.002	CAGn, log age, estradiol
Arcsin hematocrit	%	TT	0.33	0.004	CAGn, log age, estradiol
Bone density	m/sec	CAGn	**−0.34**	**0.003**	Log age, estradiol, BMI
		TT	**0.32**	**0.005**	
Pharmacogenetic data from the 48 T-treated hypogonadal patients					
Prostate volume under treatment	ml	CAGn	**−0.34**	0.002	Estradiol
		Log age	**0.18**	**0.08**	
		TT under treatment	**0.21**	**0.07**	
		Initial prostate volume	**0.49**	**<0.001**	
Log LH max. suppressed	U/liter	CAGn	**0.39**	**0.005**	Log age, estradiol
		TT under treatment	**−0.25**	**0.07**	
Hemoglobin under treatment	mg/dl	CAGn	**−0.41**	**0.001**	Log age, estradiol
		TT under treatment	**0.34**	**0.01**	

Bold numbers indicate significant involvement of the AR polymorphisms. BMI, Body mass index.

shorter CAGn ($P = 0.003$) and higher increases in T levels ($P = 0.02$).

Discussion

This study presents novel information concerning the unresolved variety of phenotypes in Klinefelter patients. In a cohort comprising a broad range of Klinefelter phenotypes, analysis of the CAGn polymorphism of the AR gene in combination with X-chromosome inactivation demonstrated that a modulation of morphological traits as well as social aspects is exerted via this genomically determined entity.

In contrast to women, of whom 15% are homozygous in terms of CAGn length (27), 31 of 77 Klinefelter patients (40%) had the same allele length. This can be explained by the cytogenetic origin of 47,XXY. Paternal meiosis I errors account for 50% of the cases; the rest derive from maternal meiosis I and II failure as well as postzygotic errors (4). Although a relation of paternal age to the origin of 47,XXY is probably nonexistent, maternal age has been associated with meiosis I errors (4); the prevalence of Klinefelter cases increases with maternal age (6). Because a maternal meiosis II error accounts for about 15% of all Klinefelter patients (4),

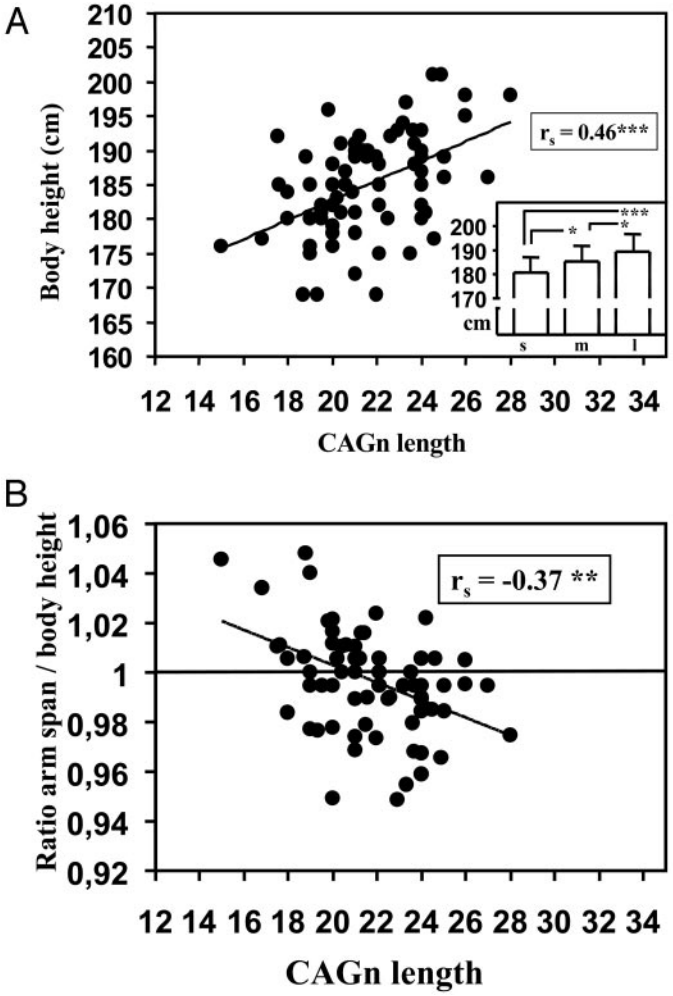

FIG. 2. A, Body height in relation to CAGn length of the AR genes of Klinefelter patients (X-weighted biallelic mean in heterozygous men, simple triplet length in homozygous men). Spearman's rank correlation is given as well as the regression line. *Inset*, Height distribution according to tertiles of X-weighted biallelic CAGn length [short: CAGn, ≤20.0 (n = 27); medium: 20.0 < CAGn, ≤ 23.0 (n = 27); long: CAGn, >23.0 (n = 23)]. Significant differences according to Kruskal-Wallis and *post hoc* tests. Levels of statistical significance are given as asterisks (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.001$). B, The arm span/body height relation and CAGn length of the AR genes of Klinefelter patients (X-weighted biallelic mean in heterozygous men, simple triplet length in homozygous men). Spearman's rank correlation is given as well as the regression line. *Data points above the horizontal line* show patients with arm span longer than body height.

the higher number of homozygous men than women is explained by cloning of one maternal X-chromosome. In female mice, inactivation of the paternal X-chromosome during early embryonal stages is present, whereas a globalized inactivation pattern on a gene to gene basis occurs in later life (32). Preferential inactivation of paternal X-chromosomes could speculatively account for some of the variation in phenotypes; this aspect was not assessed here.

Forty-six of 77 men were heterozygous for the CAGn polymorphism of the AR gene. In these men, a nonrandom inactivation of AR alleles can be described with a preference of the longer allele to be more active. Preferential expression of longer AR alleles augments the effects discussed below. How such a nonrandom inactivation, which has also been observed in women, is facilitated remains speculative (26–28). Some researchers suggested that other genes located on the X-chromosome affect inactivation, *e.g.* in the vicinity of Xq27 (33). It can also be speculated that the enzyme methyltransferase, which provides the methyl adducts to guanosine, will statistically inactivate higher proportions of a short CAGn sequence, compared with a long sequence, once it binds to the androgen receptor region. There are no data yet to confirm such a hypothesis.

Body height is increased in Klinefelter patients, with their mean height falling within the 75th percentile (4, 5). In general, tall stature is considered a typical feature of hypogonadism due to a retardation in the androgen-induced closure of epiphyses (2, 3). In agreement, we describe a strong association of height and bone density with the length of CAGn. Possibly, the clinically typical growth excess is augmented in those Klinefelter patients with longer repeats. Long-leggedness especially accounts for the increased body height of Klinefelter patients (34), a feature that cannot be reported here.

In contrast to body height, eunuchoid proportions with arm span exceeding body height are not generally seen in Klinefelter patients. Publications mention either no difference in arm span to body height or relatively long or relatively short arms (2, 3, 34, 35). On the average, arm span equaled body height in our patients. Nevertheless, those men with short CAGn had a longer arm span in relation to body height, whereas the opposite effect was seen in men with longer repeats. Our results point toward a positive association of androgens or androgen activity with the relation arm span/body height. Corresponding observations were made in adolescent boys compared with girls or Africans compared with Asians (36, 37); Africans have, on the average, three or four fewer CAGn than Asians (10). Hence, both androgen concentrations as well as genetically determined androgen activity seem to have an impact on body proportions.

Gynecomastia was present in half the patients, in agreement with other observations (2–4, 6). Especially those men with longer CAGn exhibited gynecomastia. Correspondingly, intrinsic androgen activity exerted by the AR polymorphism as well as T and estradiol concentrations influenced the presence of gynecomastia in multiple binomial regression models. Accordingly, in patients with X-SBMA, gynecomastia is a common clinical feature (13, 14).

We describe a marked influence of CAGn length on the social status of Klinefelter patients; men with higher androgenic activity (shorter CAGn) were more likely to live with a partner and present because of fertility problems than because of endocrine disorders. It has to be remarked that profound disturbances of spermatogenesis occur in all Klinefelter patients due to meiotic problems. Only those men sufficiently virilized to find a partner will then present with the desire for paternity (8). Those subjects with short CAGn were also more likely to work in highly skilled professions. The latter result has to be regarded with some caution, because the number of men with university degrees was low. Nevertheless, the finding is corroborated by an analysis involving relative shifts to professions of fathers and brothers;

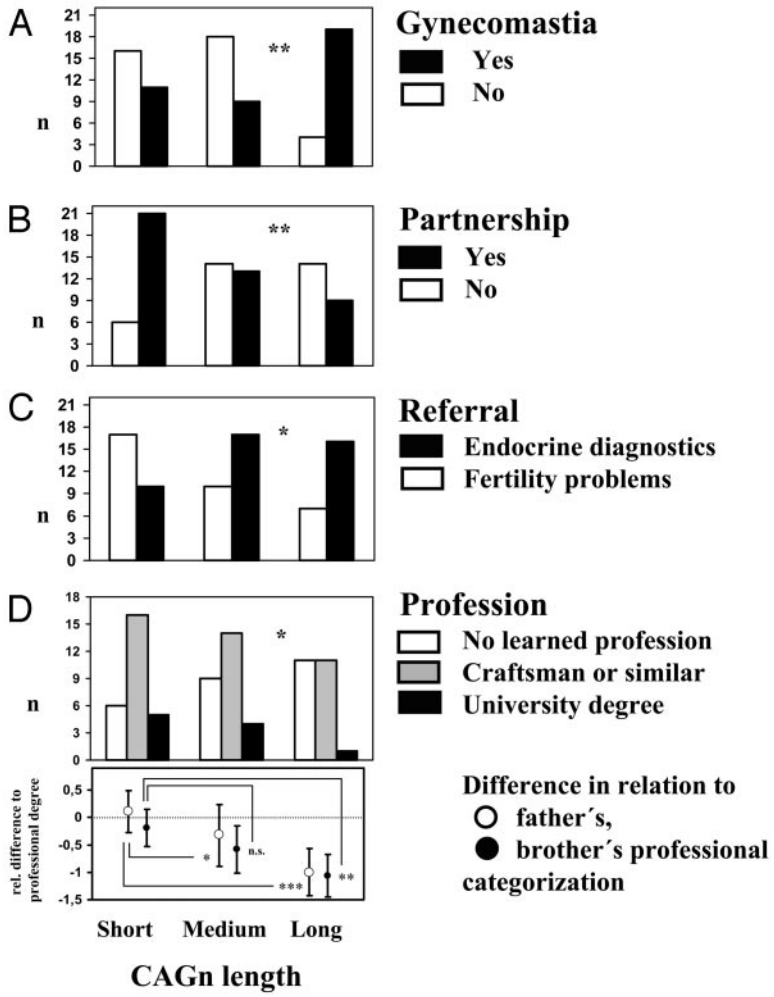

FIG. 3. Distribution of categorical parameters according to tertiles of X-weighted biallelic CAGn length [short: CAGn, ≤20.0 (n = 27); medium: 20.0 < CAGn ≤ 23.0 (n = 27); long: CAGn, >23.0 (n = 23)]. A, Gynecomastia; B, partnership; C, reason for referral; D, profession. *Upper part*, Raw data on professional categorization; *lower part*, relative difference in patient professional categorization score to the score of the father (○) or brother(s) (●; n = 18, 16, or 13 respective to tertile of CAGn length; in case of more than one brother, the mean of brother scores was considered). Scoring system (raw scores: no learned profession = 0, craftsman = 1, university degree = 2); the score of the Klinefelter patient (KSc) minus the score of the father (FSc) or brother (BSc) results in the relative difference. Example: Klinefelter patient is craftsman (KSc = 1), father is professor of economics (FSc = 2), relative score (RSc) = KSc − FSc = −1. Maintenance of family educational level results in RSc = 0, an improvement of the Klinefelter patient over the family level in RSc > 0, a deterioration in RSc < 0, the scale ranges from −2 to 2 and is ordinal. Given are mean and 95% confidence intervals; the *dotted line* indicates maintenance of social status in relation to fathers or brothers. Data for mothers or sisters are not reported, because gender-specific differences could create bias and confound results. Significance levels demonstrate a dependence of the respective parameter on CAGn length according to Somer's D test with CAGn as independent variable or, in the case of score comparison with father/brother, nonparametric Kruskal-Wallis and *post hoc* tests and are given as asterisks (*, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$).

especially those Klinefelter patients with long X-weighted biallelic means of CAGn length tended to achieve educational qualifications below their family level. Particularly those Klinefelter patients who are markedly taller than their brothers might be affected by educational underachievement. Because data on the body height of brothers are not available in this study, this remains speculative. The results confirm previous findings and explain the marked variety found in Klinefelter patients, who, as individuals, generally fall within the normal range of mental abilities, but lower than that of euploid siblings (38). An increased risk for difficulties in social interaction is reported; the phenomenon seems to relate to limited expressive vocabulary skills and restrictions in language processing (38–40).

The AR polymorphism is associated with characteristics that are subject to slow or no change once they are determined: height, arm span, bone density, profession, gynecomastia, and partnership. Consideration of the CAGn polymorphism obviously allows a view into the androgenic past of Klinefelter patients, a time when these parameters were determined in an environment of sufficient androgen concentrations and are conserved in a *status quo ante*. One may speculate that in an environment of slowly decreasing androgen levels, those men with short CAGn maintain androgenicity for a longer time. This may merely be the crucial years during which the course is set for later life. At clinical diagnosis, however, 65% of the men were hypogonadal and without current sufficiency of AR activation, and the effects of the AR polymorphism were not visible; the more volatile parameters (*e.g.* hemoglobin, prostate size, body weight, body mass index, and hair pattern) were dependent on T levels or the estradiol/T ratio. Bone density may be seen as an intermediate parameter; it was dependent on both CAGn length and T levels. Nevertheless, when patients received T substitution, the effect of the AR polymorphism to modulate androgen action emerged as various degrees of LH suppression, elevated hemoglobin levels, or increased prostate growth (24).

The discrepancy of normal T levels and elevated LH concentrations, which is often found in Klinefelter patients, has not been resolved. One explanation is a Leydig cell dysfunction resulting in compensated hypergonadotropic hypogonadism; another hypothesis is that Klinefelter patients are moderately androgen insensitive. This study supports the latter concept, especially with the pharmacogenetic data on LH suppression. Nevertheless, both physiological explanations could be simultaneously present.

One can assume that the androgenic difference due to T

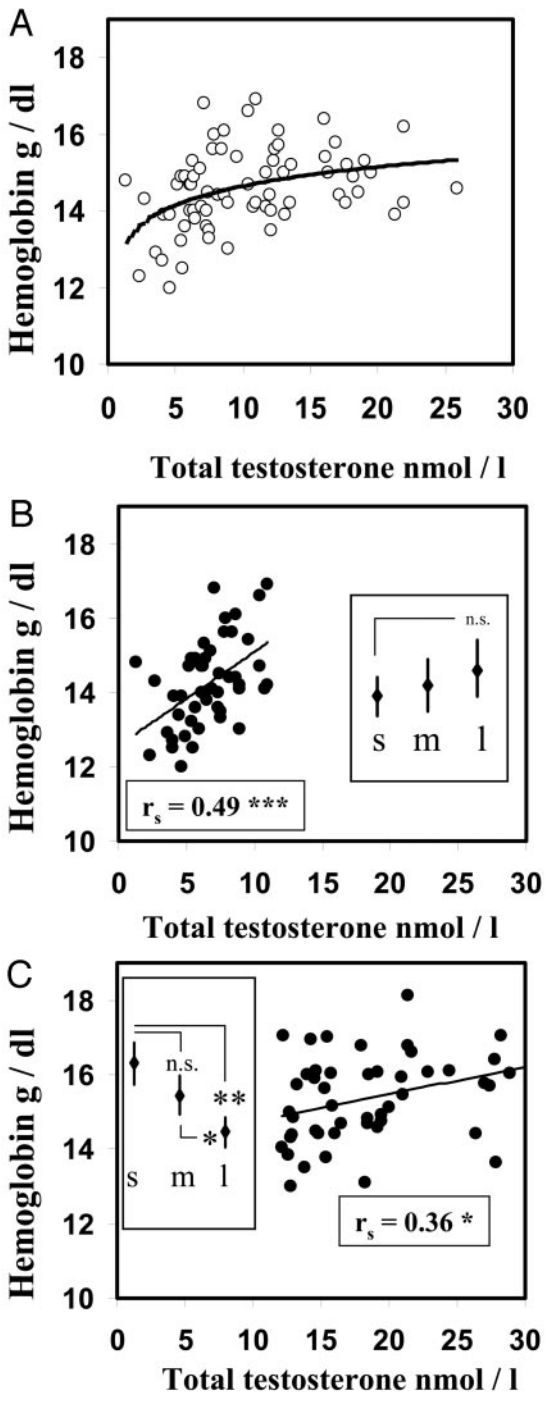

FIG. 4. A, Relation of total T concentrations to hemoglobin concentrations in all 77 Klinefelter patients. The association is log-linear [$y = 0.7 \times \mathrm{Ln}(x) + 12.9$], and its fit is superior to a linear regression (by F test, $P = 0.01$). B, Relation of total T to hemoglobin levels in the 48 hypogonadal Klinefelter patients, who were, at this time point, not yet receiving T substitution therapy. Spearman's rank correlation is given as well as the regression line. C, The similar association, but during T substitution therapy. *Insets*, Distribution of hemoglobin levels according to tertiles of X-weighted biallelic CAGn length in the hypogonadal men who were treated [short: CAGn, ≤20.0 (n = 17); medium: 20.0 < CAGn ≤ 23.0 (n = 16); long: CAGn, >23.0 (n = 15)]. The mean and 95% confidence intervals are given; the y-axis of the diagram applies. Significant differences according to Kruskal-Wallis and *post hoc* tests are indicated. Levels of statistical significance are given as asterisks (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.001$). Note that the association of hemoglobin levels to T concentrations is less pronounced in the eugonadal than in the hypogonadal range, but that CAGn length is only related to hemoglobin concentrations when T levels are within the normal range.

levels is mainly observed when hypogonadal men are compared with eugonadal men. Once sufficient androgen levels are reached, the CAGn polymorphism gains influence on androgen effects, whereas the actual T levels only account for smaller differences (see Table 3, prostate volume and LH levels under treatment).

From our results, it becomes clear that especially those Klinefelter patients with a long X-weighted biallelic mean of CAG repeats tend to encounter problems in life, such as professional underachievement as well as difficulties in finding a partner. Health issues are also affected, *e.g.* bone density is lower in these men. In general, life expectancy seems to be decreased in Klinefelter patients, but whether this is due to hypogonadism *per se* or socioeconomic factors has not been resolved (41). Although especially those Klinefelter patients with longer CAG repeats tend to be diagnosed earlier in life due to their more noticeable phenotype (see above), it might still be too late for these individuals to take countermeasures (*i.e.* T treatment or special education programs; the efficacy of which to improve social factors has yet to be demonstrated). This means that especially tall young boys with gynecomastia and learning disorders should be presented for endocrinological/andrological diagnosis. Although the Klinefelter syndrome is well known and will hardly be overlooked by specialists, there is a discrepancy between the high incidence of this most common form of male hypogonadism and the low number of specialized physicians. It is important to deliver information concerning Klinefelter syndrome to physicians working in other fields and in general practice so that the general awareness of this health problem will be increased. Reviews in journals with a wide, general spectrum of readers will be most helpful in this effort (42).

The CAG repeat polymorphism of the AR gene in conjunction with X-chromosome inactivation patterns can explain variations in phenotype and social traits of Klinefelter patients. Although this is scientifically fascinating, the practical clinical value of our data lies with the pharmacogenetic modulation of T treatment.

In conclusion, significant modulation of androgen effects on the phenotype of Klinefelter patients is exerted via the CAGn polymorphism of the AR gene, especially during those phases of life in which sufficient androgen levels and, hence, AR activation can be assumed. This modulation is magnified by preferential inactivation of the more functional short CAGn allele. The study supports efforts of early identification and T treatment of Klinefelter adolescents (4, 6) to override symptoms of hypogonadism (42). This approach may be especially indicated in those boys with an X-weighted biallelic mean above 23 CAGn to help them to achieve sufficient androgenicity during a pivotal time period to set the course for a normal male life within the intrinsic settings of their family. This study also yields important information with regard to pharmacogenetic surveillance of patients, *e.g.* concerning prostate growth during T substitution therapy; those patients with shorter CAGn may need closer monitoring and possibly less T.

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