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CAG-repeats in the *Androgen Receptor* Gene Relate with Plasma Androgen Levels in the Bouvier Des Flandres

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Abstract. *Background: The Bouvier des Flandres (BdF) dog is predisposed to develop prostate carcinoma (PCA). In humans, ethnic groups with higher prevalence of PCA have higher serum androgens concentrations and shorter polyglutamine (CAG) repeat lengths in the androgen receptor (AR) gene. In dogs, shorter CAG-I lengths are associated with increased PCA risk. Objective: To compare serum androgens concentrations in the BdF with other breeds and to determine whether CAG repeats length and plasma androgens concentrations are correlated. Materials and Methods: Androgens were measured in 46 BdF and in 53 other dog breed. Length of CAG-I and CAG-III repeats were measured in 41 BdF and correlated with androgen levels. Results: In the BdF group shorter CAG-I and longer CAG-III repeat lengths were associated with lower androgen concentrations. Conclusion: As the shorter CAG-I repeat is associated with an increased PCA risk, higher androgen concentrations may protect against the development and progression of PCA.*

An epidemiological study of prostate disease in dogs has shown that the Bouvier des Flandres (BdF) has a more than 8-times higher risk of developing prostate carcinoma (PCA) compared to a large reference population of dogs of a wide range of breeds (1). In other reports, middle to large breed dogs are reported to be over-represented among dogs with PCA (2, 3).

In humans there is a well-documented difference in incidence of PCA between different ethnic groups (4, 5). African-American men have the highest incidence of PCA in the world, while Asian-American have an increased high-risk disease (4, 6, 7). However, it is uncertain whether this difference is truly due to ethnicity, as a number of other factors may also explain the predisposition of a certain group to PCA. These include differences in exposure to carcinogenic substances (particularly caused by dietary differences), differences in detection and genetic differences (4). Only the latter can be of importance in dogs, since there are no breed-related differences in nutrition and veterinary treatment in the dog population. In humans, differences in serum concentrations of androgens are a possible cause of the racial differences in PCA risk. In African-American men higher concentrations of testosterone and sex hormone-binding globulin (SHBG) have been found, compared to the Caucasian population (5, 8). It has been hypothesized that SHBG can activate androgen-responsive genes (5). Other race-related differences reported include the activity of the enzyme 5α-reductase (9, 10), concentrations of insulin-like growth factor-I (IGF-I) and IGF-binding protein-3 (11, 12) as well as differences in the microsatellite alleles at the androgen receptor (*AR*) locus. In particular the length of polyglutamine (CAG) repeats in these microsatellite alleles may be a risk factor for the development of PCA (13, 14). African-American men were found to have shorter CAG repeats with higher risk for PCA (15) and Japanese men longer CAG repeats with lower risk for PCA (16) compared to white men.

The objectives of the present study were to investigate whether the higher incidence of PCA in the BdF compared to dogs of other breeds may be associated with a higher plasma concentration of androgens in this breed. In addition, the relationship between the length of the CAG repeats in the *Ar* gene and the plasma concentration of androgens was investigated.

Materials and Methods

Animals. Blood samples were collected from 99 intact male dogs. Approximately 4 ml of blood was obtained from the cephalic or jugular vein of each dog and transferred to EDTA-coated tubes. All dogs were either healthy dogs (BdFs, recruited through breed club internet websites and discussion forums or by direct contact with

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breeders) or dogs presented to the clinic (Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University) for other conditions than prostatic disease. One group consisted of 46 BdF while the other group consisted of 53 dogs of other different breeds. After centrifugation the plasma samples were stored at –20˚C until hormone measurement.

Hormone assays. Total testosterone was measured using a Coat-A-Count solid phase radioimmunoassay kit obtained from the Diagnostic Products Corporation (Los Angeles, CA). Before the assay, 0.25 ml of plasma was extracted with 1 ml ether. After centrifugation the aqueous phase was frozen and the ether extract was decanted, evaporated to dryness and dissolved in 0.25 ml of buffer phosphate-buffered saline (PBS) with 0.5% (w/v) bovine serum albumin (BSA).

The intra-assay coefficient of variation was 16.6% and the sensitivity was 0.14 nmol/l. The accuracy of the assay, measured by recovery, was 91.2%. Cross-reactivities were as follows: 4-estren-17-ol-3-one (20%), 11-ketotestosterone (16%), 5α-dihydrotestosterone (3.3%), 19-hydroxyandrostenedione (2.0%), methyltestosterone (1.7%) and 4-estren-7x-methyl-17B-ol-3-one (1.1%).

Androstenedione was measured directly using a Coat-A-Count radioimmunoassay obtained from the Diagnostic Products Corporation. The intra-assay coefficient of variation was 4.2% and the sensitivity was 0.14 nmol/l. The cross-reactivities were as follows: testosterone (1.49%), 5α-dihydrotestosterone (0.212%), dehydroepiandrosteron (DHEA) (0.164%), progesterone (0.160%) and adrenosterone (0.135%).

Dihydrotestosterone (DHT) was measured after extraction using a radioimmunoassay kit obtained from Intertech (Strassen, Luxembourg). From each plasma sample, 300 μl was extracted by 3 ml of diethyl ether. The tubes were vortexed for 2 min and then centrifuged at 2,000 rpm for 5 min. The aqueous phase was frozen and the ether extract was decanted and evaporated to dryness. After adding PBS buffer, vortexing and adding 50 μl oxidation solution, the tubes were incubated at 37˚C for 20 min. The solution was extracted again by 3 ml mixture of hexane/ethanol 98/2. The tubes were vortexed, centrifuged and 2 ml of the organic layer was transferred into glass tubes and evaporated to dryness. The sensitivity of the assay was 0.07 nmol/l, the intra-assay coefficient of variation was 6%. The cross-reactivities were as follows: estradiol (1.20%), estriol (0.04%), diethylstilbestrol (DES) (<0.01%), pregnenolone (<0.01%), progesterone (<0.01%), testosterone (<0.01%) and aldosterone (<0.01%).

Androgen receptor gene polyglutamine repeats. The length of the polyglutamine repeats of each polymorphic region (CAG-I and CAG-III) were measured in 41 of the 46 BdF dogs as described elsewhere (17). Briefly, DNA was extracted from blood, both CAG repeats were amplified by polymerase chain reaction (PCR). The length of the PCR products was determined on an ABI 3100 Genetic Analyzer (Life Technologies Europe BV, Bleiswijk The Netherlands). Haplotype is defined as the combination of alleles at the two CAG regions of the *Ar* gene.

Statistical analysis. Analysis of data was performed by the statistical software SPSS 15.0 for Windows (Statistical Package for Social Sciences, SPSS Inc, Chicago, IL, USA). Mean hormone concentrations in the BdF group and the control group were compared for significant differences using an independent sample *t*-test, after testing for normality with the one-sample Kolmogorov-Smirnov test. The correlation between age and androgen concentrations and between the different androgens was analyzed by linear regression analysis. The relationship between CAG repeat length and serum androgen concentration was analyzed by bivariate correlation analysis (Spearman's rho, 2-tailed). A *p*-value below 0.05 was considered significant.

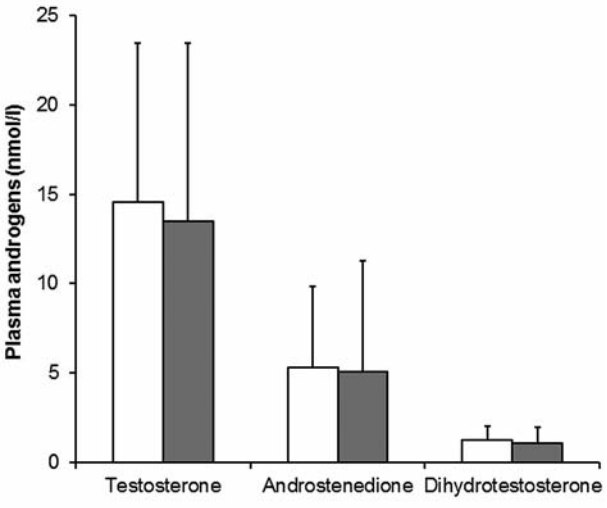

Figure 1. *Plasma androgen concentrations in the Bouvier des Flandres (□) and control (■) groups (Mean±SD).*

Results

Animals. The median age was 5.9 years in the BdF group (range, 8 months to 11 years) and 3.2 years in the control group (range, 11 months to 12 years). The control group consisted of 26 different breeds. The most represented breeds were the Labrador retriever (6 dogs), the German Shepherd dog (5 dogs) and cross breed dogs (5 dogs).

Hormones. In the BdF group, the mean plasma testosterone concentration was 14.53±8.90 nmol/l (mean±SD). In the control group the testosterone concentration was 13.49±9.98 nmol/l (Figure 1). There was no statistically significant difference between the groups (p=0.623). The mean plasma DHT concentration was 1.27±0.74 nmol/l in the BdF group and 1.08±0.87 nmol/l in the control group. The difference between groups was not significant (p=0.234). The mean plasma androstenedione concentration was 5.30±4.57 nmol/l and 5.07±6.19 nmol/l in the BdF and control groups, respectively. There was no statistically significant difference between groups regarding the androstenedione concentration either (p=0.867).

No significant correlation between age and the concentrations of testosterone and DHT was found in the controls or the BdF. However, a significant positive correlation was found between age and androstenedione concentrations in the control group (p=0.036), which was not

Table I. *Plasma androgen concentration related to the length of polyglutamine (CAG)-I repeat (mean±SD).*

Length of CAG-I repeat	Testosterone (nmol/l)	Dihydrotestosterone (nmol/l)	Androstenedione (nmol/l
10	11.29±7.09	0.99±0.33	5.17±5.05
11	13.69±10.05	1.19±0.59	4.12±3.00
12	21.12±5.14	2.63±1.56	12.58±10.65

Table II. *Plasma androgen concentration related to the length of polyglutamine (CAG)-III repeat (mean±SD).*

Length of CAG-III repeat	Testosterone (nmol/l)	Dihydrotestosterone (nmol/l)	Androstenedione (nmol/l)
11	15.62±9.61	1.42±0.77	5.22±4.63
12	9.85±7.84	0.87±0.33	2.11±1.83
13	6.96±3.80	0.88±0.34	6.25±6.43

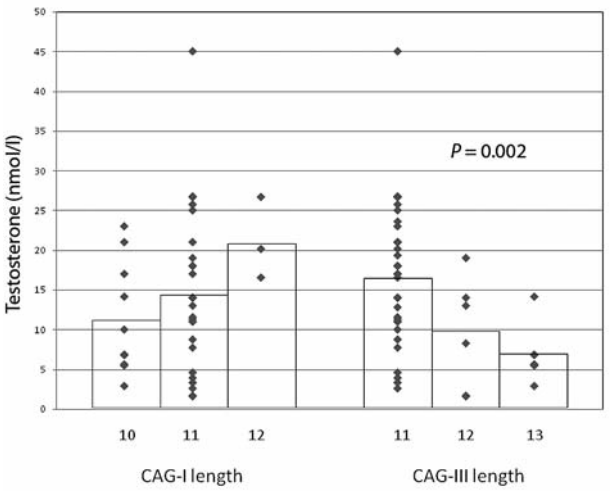

Figure 2. *Correlation between polyglutamine (CAG) repeat length and plasma testosterone concentration. Boxes show the mean for each group. Diamonds (♦) indicate individual values. Where a significant (p<0.05) correlation was found using Spearman's rho correlation analysis, the P value is indicated.*

found in the BdF group (p=0.172). In addition, a significant positive correlation between testosterone and androstenedione concentrations was found in the BdF group (p<0.01), which was not found in the control group (p=0.85). Similarly, a significantly positive correlation was found between androstenedione and DHT in the BdF group (p<0.01), but not in the control group (p=0.31).

Androgen receptor gene polyglutamine repeats. In the first polymorphic region (CAG-I), 10 BdFs had 10 repeats, 28 had 11 repeats and 3 had 12 repeats. The mean (±SD) concentration of testosterone, DHT and androstenedione for each of these groups is shown in Table I. In the other polymorphic region (CAG-III), 30 BdFs had 11 repeats, 5 had 12 repeats and 6 had 13 repeats. The mean (±SD) concentration of testosterone, DHT and androstenedione for each of these groups is shown in Table II. Individual data and means for testosterone and DHT are plotted as a function of CAG repeat lengths (Figures 2 and 3). There was a significant positive correlation between the length of CAG-I repeats and the plasma concentration of DHT, whereas the length of CAG-III repeats was significantly negatively correlated to both the plasma concentration of testosterone and DHT. The highest plasma testosterone and DHT concentrations were found for haplotypes CAG-I/CAG-III of 12/11 and the lowest plasma testosterone concentration for haplotypes CAG-I/CAG-III of 10/13. No significant correlation was found between CAG-I repeat length or CAG-III repeat length and the concentration of androstenedione.

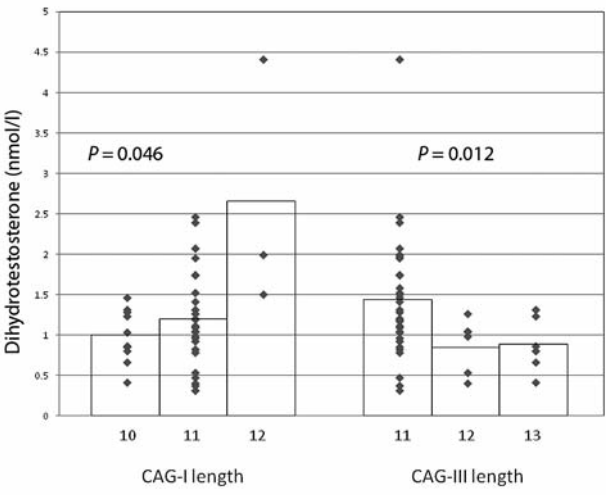

Figure 3. *Correlation between polyglutamine (CAG) repeat length and plasma dihydrostestosterone (DHT) concentration. Boxes show the mean for each group. Diamonds (♦) indicate individual values.*

Discussion

As most of the studies on androgen concentrations in the dog present their data in non-SI units for comparative reason, our plasma testosterone concentrations found in this study equal 4.03±2.59 ng/ml (BdF) and 3.14±3.23 ng/ml (control). These results are higher than the mean testosterone concentrations in normal dogs reported in other studies where results ranged

from 2.1 to 3.1 ng/ml (18-22), but lower than in two other studies with mean testosterone concentrations of 4.5 ng/ml (23) and 4.6 ng/ml (24). The present study included many more dogs than the previous reports mentioned, which all included less than 10 dogs. The variation in the testosterone concentration found in this study is larger in comparison to previous reports (18-22), although the two studies with higher reference values for testosterone also had higher standard deviations (23). Our study using a larger group of dogs emphasizes the larger variation of plasma testosterone concentrations in the dog. Whether this is due to variation in sex hormone binding globulin (SHBG) concentrations in the dog is unknown.

In humans, it has been hypothesized that the serum SHBG concentrations might be an important factor initiating the development of prostate cancer (5) and further studies to examine the serum concentration of SHBG in BdF may yield additional information on possible causes for the apparent predisposition of this breed for prostate cancer. Although canine SHBG can be measured (25), human SHBG radioimmunoassays cannot be used as the molecular structure of canine SHBG differs too much from human SHBG.

In a recent study, CAG repeats in the *Ar* gene were examined in dogs with and without PCA (17). Similarly to the results found in humans, it was found that longer CAG-I repeat length may have a protective effect and shorter repeat length may assist in the development of PCA. No difference was seen in the length of CAG-III repeats between dogs with PCA and those without.

Although no overall significant differences in androgen plasma levels existed between BdF and control dogs, significant differences within the BdF in androgen plasma levels were found for the different CAG-I and CAG-III repeat numbers. Haplotype 10/13 had the lowest testosterone and DHT levels while haplotype 12/11 the highest.

The correlation found in this study between shorter CAG-I repeat length and longer CAG-III repeat length with lower plasma testosterone and DHT concentrations may reflect differences in the effect of these repeats on recruiting of coactivators that play a role in transactivation of DHT-responsive genes. Moreover they may indicate a possible protective effect of androgens on PCA development, which is in agreement with previous findings that castrated dogs have a higher risk of developing PCA and that PCA in castrated dogs is less well-differentiated than in intact males (1, 26). The mechanism of this protective effect is unknown. In humans, androgens are required for the development and progression of PCA and treatment to eliminate androgens is effective in inhibiting progression and preventing the development of PCA (27, 28). However, there is also evidence that lower concentrations of androgens are related to higher histological grades of PCA (28, 29).

In conclusion, differences in plasma concentrations of androgens cannot explain breed differences concerning the risk of developing PCA in dogs in the same way as they are related to ethnic differences in men. However, shorter CAG-I repeat length in the Bouvier des Flandres, a breed predisposed to the development of PCA, is associated with a higher risk of PCA and lower concentrations of androgens, suggesting a possible protective role of androgens in the development of PCA.

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