Male Endocrinology

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ORIGINAL ARTICLE

Bulbocavernosus muscle area measurement: a novel method to assess androgenic activity

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Serum testosterone does not correlate with androgen tissue activity, and it is critical to optimize tools to evaluate such activity in males. Ultrasound measurement of bulbocavernosus muscle (BCM) was used to assess the relationship between the number of CAG repeats (CAGn) in the androgen receptor (AR) and the BCM size; the changes in the number of CAGn over age were also evaluated. Transperineal ultrasound measurement of the BCM was also performed. AR CAGn were determined by high performance liquid chromatography, and morning hormone levels were determined using immunoassays. Forty-eight men had CAG repeat analysis. Twenty-five were <30 years of age, mean 23.7 years (s.d. = 3.24) and 23 were >45 years of age, mean 53 years (s.d. = 5.58). The median CAGn was 21 (13–29). BCM area was greater when the number of CAGn were <18 as compared to the number of CAGn >24 ($P = 0.04$). There was a linear correlation between the number of CAGn and the BCM area $R^2 = 16\%$ ($P = 0.01$). In the 45 to 65-years-old group, a much stronger negative correlation ($R^2 = 29\%$, $P = 0.01$) was noticed. In the 19 to 29-years-old group, no such correlation was found ($R^2 = 4\%$, $P = 0.36$). In older men, the number of CAGn increased with age ($R^2 = 32\%$, $P = 0.01$). The number of CAGn in the AR correlates with the area of the BCM. Ultrasound assessment of the BCM is an effective surrogate to evaluate end-organ activity of androgens. The number of CAGn may increase with age.

INTRODUCTION

Androgens and their mediated effect through the androgen receptor (AR) are critical to the development of the male phenotype. An AR is a member of the steroid nuclear receptor superfamily of ligand-activated transactivation factors, and is encoded by eight exons located on chromosome Xq11-12.[1,2] The AR is expressed in the developing human penis, urethra and multiple other organs.[3] The gene exhibits two polymorphic sites in exon 1, characterized by varying numbers of CAG and GGC repeats, resulting in different lengths of polyglutamine and polyglycine stretches in the N-terminal region of the AR protein. This region normally ranges from 11 to 31 amino acids,[4] and varies with race.[2,4]

The suggested inverse association between AR sensitivity and length of CAG repeats (CAGn) is based on the androgen resistance in men with spinobulbar dystrophy (Kennedy syndrome),[5] and is further supported by *in vitro* studies.[6,7] There is mounting evidence that patients with increased CAGn, as exhibited by patients with spinal bulbar muscular atrophy, have reduced AR function and suffer from under virilization, testicular atrophy and possibly reduced fertility presenting as oligospermia or azoospermia.[8–12] This inverse correlation, where shorter length of CAGn displays an increase in androgen sensitivity[13] and longer repeats lead to more androgen resistance with under-masculinized genitalia in XY males,[14] has been demonstrated in multiple studies. Men with CAGn lengths above 26 exhibit substantially shorter anogenital distance when compared to men with shorter CAGn.[15] Short AR CAG alleles are associated with prostate cancer that is androgen-dependent. Moreover, shorter number of CAGn results in abnormally high stimulation of prostatic tissue and earlier age of

onset of prostate cancer,[16] increased tumor grade and increased risk of extra-prostatic extension.[11]

AR contains an exonic polymorphic trinucleotide CAGn in which 95% of individuals have a germ line allele length between 16 and 29 CAGn.[17] However, it is unknown whether the number of repeats are static or change during the life span. AR CAGn are inherently genomically unstable; in humans the CAG microsatellite region expands leading to the increased number of CAGn.[18-20] Models have confirmed that this instability that leads to expansion and contraction is propagated by both DNA replication and DNA damage response.[21,22] The instability in the CAG region in humans and mice can occur in post-mitotic cells, leading to somatic mosaicism and tissue-specific trinucleotide repeat instability.[22] Therefore, the current study postulates that with age, the number of CAGn changes within an individual and the AR response might also be altered. Such genetic heterogeneity can introduce AR somatic functional mosaicism, which can have important physiological effects within specific organs.[23] For example, in the case of AR, different sexual organs might react differently to the same serum androgen levels. More importantly, the AR sensitivity might change with age. Hence, the clinical signs and symptoms of hypogonadism associated with age can be quite variable. For example, the loss of libido, depression, erectile dysfunction and diabetes mellitus occurs at different testosterone concentrations.[24] This observed difference is linked to specific risk factors,[25] and some of these risk factors operate independently of AR function.

The size of both the spinal nucleus of the bulbocavernosus (SNB) motor neurons and the target organ, bulbocavernosus muscle (BCM), require androgen for proper development and to be maintained in

adulthood. Castration will reduce the size of the SNB and BCM, while androgen treatment reverses this effect, indicating that SNB motor neurons and their target muscles are sensitive to androgens.[26,27] BCM is highly sensitive to androgen and commonly used to assess androgenic activity. Male rats carrying a testicular feminization mutation, a ubiquitous mutation that causes androgen insensitivity or treated with an AR antagonist have a feminine SNB system and a BCM that are completely absent.[27] Many groups have examined the relationship between the length of the polyglutamine repeat in the AR and male AR activity.[28] Most of the published literature on CAGn in AR is focused on predicting the phenotype from the number of CAGn. However, no studies evaluated the effect of instability and somatic mosaicism on the function of the AR. The objective of the current study is to determine the relationship between the BCM measurement and the AR CAGn in healthy volunteers, and to determine whether age has an effect on the length of the AR repeat.

MATERIALS AND METHODS
Healthy men (18–65 years) were prospectively evaluated at a single tertiary care center in the United States according to an Institutional Review Board-approved protocol. Volunteers responded to the American Urological Association/International Prostate Symptom Score, International Index of Erectile Function-15 and Male Sexual Health Questionnaires to rule out any confounding medical or urological history, including orgasmic, erectile and ejaculatory dysfunction. Baseline hormonal evaluation and transperineal ultrasonography recording of the BCM was performed. Inclusion criteria were normal erectile, ejaculatory, orgasmic and voiding function without use of prescribed or over-the-counter medications. All subjects had normal psychiatric history and were capable of consent. All subjects were compensated for their time.

Genital measurements
Genital measurements were performed by placing the patient in the supine position, with slight frog-legged position of the lower extremities. Transperineal ultrasound was performed to measure the BCM length and thickness with MyLab 25 Gold (Esaote North America, Inc; Indianapolis, IN) ultrasound machine as depicted in **Figure 1**. The BCM area was calculated using the initial measurements. From the same position, stretched penile length (PL) and erect PL were measured from the base of the dorsal surface of the penis to the tip of the glans. Previously published studies have shown no evidence for measurement error being proportional to the magnitude of the measurement.[29] Most measurements were reproducible when done by the same clinician.

Hormone values
Morning testosterone (Beckman Coulter, Webster, TX, USA), luteinizing hormone (Beckman Coulter, Webster, TX, USA), follicle-stimulating hormone (ALPCO, Salem, NH, USA), epinephrine (Rocky Mountain Diagnostics, Colorado Springs, CO, USA), dehydroepiandrosterone (Beckman Coulter, Webster, TX, USA), dehydroepiandrosterone sulfate (Alpco, Salem, NH, USA), dopamine (Rocky Mountain Diagnostics, Colorado Springs, CO, USA), estradiol (ALPCO, Salem, NH, USA), inhibin B (Beckman Coulter, Webster, TX, USA), norepinephrine (Rocky Mountain Diagnostics, Colorado Springs, CO, USA), prolactin (Beckman Coulter, Webster, TX, USA), serotonin (Rocky Mountain Diagnostics, Colorado Springs, CO, USA) and sex hormone binding globulin (ALPCO, Salem, NH, USA) were collected on all subjects. All baseline hormone assays were obtained, at minimum, 12 h prior to ultrasound recording. All specimens were processed by our laboratory with validated assays for
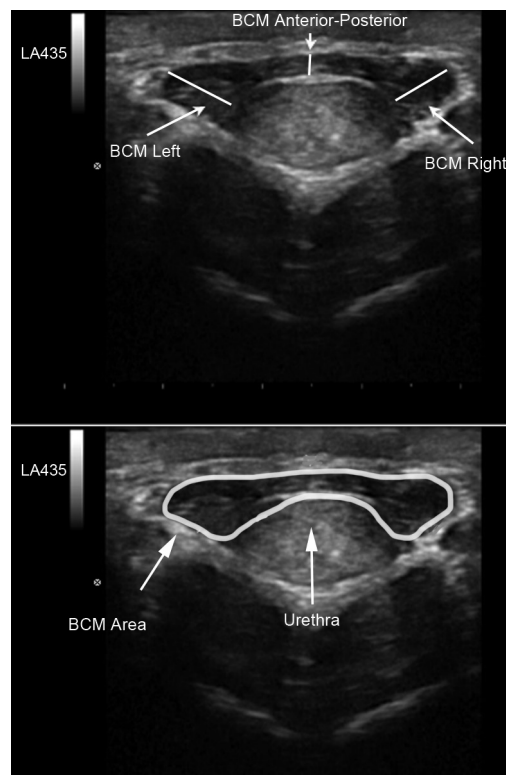

Figure 1: Method to measure BCM's length, thickness and area using ultrasound images. BCM: bulbocavernosus muscle.

these tests, and assays' details are listed in **Table 1**. Testosterone assay was validated with liquid chromatography-tandem mass spectrometry.

DNA isolation and analysis
Genomic DNA from all study subjects was extracted from peripheral blood leukocytes to examine the length of CAGn. Primers for 20S nucleotide amplicon encompassing polyglutamine-repeat track in exon 1 of the AR were designed (L-5'-CGCGAAGTGATCCAGAACC-3' and R-5'-CTTGGGGAGAACCATCCTC-3'). AR amplicons were generated through standard PCR technique using patient genomic DNA as a template. The amplified products were then analyzed for size determination using a non-denaturing method of high performance liquid chromatography with the Transgenomic Wave™ System platform (Transgenomic, Inc., Omaha, NE, USA). Ten microliters of each sample was injected and run through the high performance liquid chromatography column at 50°C using sizing method. Chromatogram of each run was then analyzed and retention times were recorded. The number of CAGn was directly calculated (interpolated) with Graph Pad Prism™ (GraphPad Software, Inc, La Jolla, CA, USA) using a standard curve generated from eight male control samples whose amplified products were sequenced for exact size determination and CAGn content. The results obtained by this method were confirmed using direct sequencing of exon 1 of the AR gene in 10 randomly selected subjects.

Statistical analysis
Descriptive analysis, scatter plot with linear regression and logistic regression was completed. Paired *t*-test and Student's *t*-test were performed to compare the mean of the CAGn between each of the categories. All statistical analysis was performed using JMP, Version 10.0 (SAS Institute Inc, Cary, NC, 1989–2011).

RESULTS

Forty-eight men had CAGn analysis performed. Twenty-five men were younger than 30 years (range 19–29 years) with a mean age of 23.7 years (standard deviation (s.d.) 3.24) and 23 men were older than 45 years (range 45–65 years) with a mean age of 53 years (s.d. 5.58). Eight percent of the cohort were Asian, 34% black, 14% Hispanic and 44% white non-Hispanic. The median number of repeats was 21 (range 13–29), with a mean of 21.35 and a mode of 22. The frequency and distribution of the CAGn in all 48 men is shown in **Figure 2**.

Subjects were divided into CAGn ranges: low (13–18), mid (19–24) and high (25 or greater) based on bimodal distribution of the results. The number of repeats is summarized with the mean measurement for rigid PL, flaccid PL, BCM area and BCM thickness in **Table 2**. Statistical significance was noted in the BCM area (cm^2) when extreme high and low ranges were compared. BCM was higher when CAGn was less than 18, as compared when CAGn was greater than 24 ($P = 0.04$). However, there was no statistically significant difference between the one-dimensional measurement (thickness and length) between the groups with CAGn less than 18 and greater than 25 repeats (anterior posterior thickness $P = 0.46$, left thickness $P = 0.09$ and right thickness $P = 0.18$). There was also no difference in the flaccid and the rigid PL between CAGn groups. Linear regression analysis to evaluate the relationship between the number of CAGn and BCM area showed a statistically significant linear correlation ($P = 0.01$, $R^2 = 16\%$) [**Figure 3**]. On further statistical analysis, the cohort was divided into two groups. In the younger group (18–30 years) no correlation was found between the BCM area and number of CAGn ($P = 0.36$, $R^2 = 4\%$). However, in
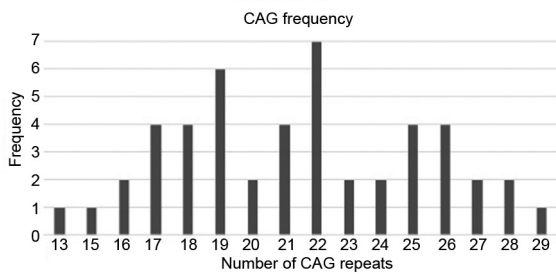
the older age group (45–65 years) a much stronger negative correlation was identified ($P = 0.01$, $R^2 = 29\%$). In this age group it was also found that the number of CAGn and age were positively correlated ($P = 0.01$, $R^2 = 32\%$). Hence, it is plausible that CAGn increases with age secondary to microsatellite instability in the CAG region.

Each subject's hormonal profile was evaluated, and all evaluated subjects had values within the normal range. The mean hormonal values for the group with a CAGn number less than 21 were compared to the mean hormonal values for the group with CAGn greater than 21. In the current cohort, no correlation was found between the pituitary, adrenal or testicular hormones and the number of the CAGn in the AR as shown in **Table 1**. Furthermore, all subjects had testosterone in the normal range and the testosterone levels were not correlated with the BCM area. Thus, this indicates that the BCM area is heavily dependent on the length of the CAGn.

DISCUSSION

The role of CAGn in androgen signaling is the source of unprecedented interest in recent years because CAG expansion beyond 40 leads to
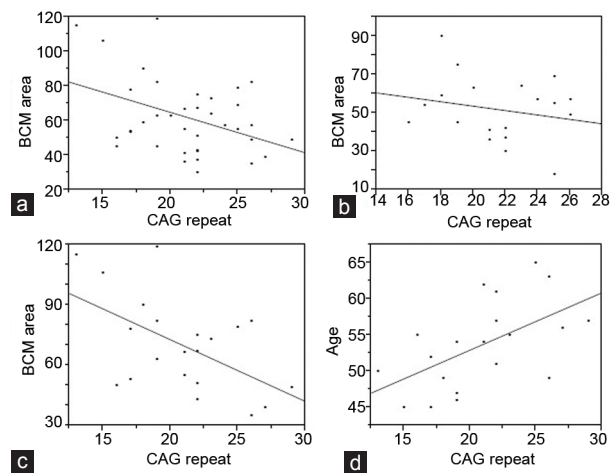

Figure 3: (**a**) Scatter plot of CAG repeats vs BCM area (cm^2) ($R^2 = 16\%$, $P = 0.01$), all subjects. (**b**) CAG repeats and BCM area (cm^2) ($R^2 = 4\%$, $P = 0.36$), 18–30 years age group. (**c**) CAG repeats and BCM area (cm^2) ($R^2 = 29\%$, $P = 0.01$), 45–65 years age group. (**d**) CAG repeats and age ($R^2 = 32\%$, $P = 0.01$), 45–65 age group. BCM: bulbocavernosus muscle.

Figure 2: Frequency of the number of CAG repeats.

Table 1: Number of CAG repeats (CAGn) and correlation to hormone levels

Repeats (n)	Manufacturer	Company location	Catalogue number	CAGn<21 (n=10) [a]	CAGn>21 (n=14) [a]	P value
Epinephrine (pg ml^{-1})	Rocky Mountain Diagnostics	Colorado Springs, CO	BA 10-1601	3.3	3.2	0.92
DHEA (ng ml^{-1})	Beckman Coulter	Webster, TX	DSL-10-9000	8.4	10.9	0.29
DHEA-S (µmol ml^{-1})	Alpco	Salem, NH	11-DHEHU-E01	2.9	2.4	0.50
Dopamine (pg ml^{-1})	Rocky Mountain Diagnostics	Colorado Springs, CO	BA 10-1603	4.8	6	0.36
Estradiol (pg ml^{-1})	ALPCO	Salem, NH	20-DR-4399	14.2	19.5	0.28
FSH (mIU ml^{-1})	ALPCO	Salem, NH	11-FSHHU-E01	3.6	2.3	0.24
Inhibin B (pg ml^{-1})	Beckman Coulter	Webster, TX	A81301	154.6	155.4	0.98
LH (mIU ml^{-1})	Beckman Coulter	Webster, TX	DSL-10-4600	5.6	4.6	0.54
Norepinephrine (pg ml^{-1})	Rocky Mountain Diagnostics	Colorado Springs, CO	BA 10-1602	43.7	36.9	0.48
Prolactin (µg l^{-1})	Beckman Coulter	Webster, TX	DSL-10-4500	4.9	7.9	0.12
Serotonin (ng ml^{-1})	Rocky Mountain Diagnostics	Colorado Springs, CO	BA 10-0900	174.4	181	0.87
SHBG (nmol l^{-1})	ALPCO	Salem, NH	11-SHBHU-E01	31.9	27.7	0.49
Testosterone free (pg ml^{-1})	Beckman Coulter	Webster, TX	DSL-10-49100	69.9	63.5	0.73
Testosterone total (ng dl^{-1})	Beckman Coulter	Webster, TX	DSL-10-4000	666.4	711	0.73

DHEA: dehydroepiandrosterone; DHEA-S: dehydroepiandrosterone sulfate; FSH: follicle-stimulating hormone; LH: luteinizing hormone; SHBG: sex hormone binding globulin.
[a]Mean hormone levels

Table 2: CAG repeats and BCM measurement

CAGn	Penis rigid (cm)	Penis flaccid (cm)	BCM area (mm²)	BCM thickness (mm)	Thickness AP (mm)	Thickness left (mm)	Thickness right (mm)
(L) 13–18	16.2	13.6	74.3	22.8	2.0	5.3	5.2
(M)19–24	16.9	13.2	96.5	22.6	1.8	4.1	4.1
(H) 25–29	16.9	12.9	53.2	22.2	1.8	4	4.1
P value (L *vs* H)	0.65	0.28	0.04	0.24	0.46	0.09	0.18

AP: anterior posterior; BCM: bulbocavernosus muscle; CAGn: CAG repeats ; L: low; M: mid; H: high

multiple fatal neuromuscular diseases.[5] Furthermore, increases in the AR CAGn have been associated with androgen resistance, decreased virilization, oligospermia or azoospermia, testicular atrophy[8-11] and abnormal sperm parameters in normal men.[13,30,31] One of the major challenges of clinical andrology is differentiating men with clinical hypogonadism and normal testosterone levels from classic hypogonadism and low testosterone. The authors believe that there is a group of men with normal testosterone with hypogonadal symptoms because of AR dysfunction secondary to an increase in the AR CAGn. Definitive diagnosis of the number of CAGn is limited to CAGn analysis, such as sequencing. And there are very few clinical tools available to screen patients who are suspected to have an increased number of CAGn. A paucity of studies in the literature has attempted to correlate image findings with clinical and genetic data in this context. A study that looked at patients with trinucleotide repeat expansion diseases found substantial correlation of both the brain stem and cerebellar atrophy with CAGn length, age, disease duration and degree of disability. This and similar studies concluded that volumetric analysis of the brain might be used as a prognostic tool in the management of these patients.[32]

In the current study, the aim was to identify a clinical or radiological finding that may further support the CAGn in AR role in BCM parameters and determine androgen activity at the tissue level. The results indicate that an increased number of CAGn is associated with decreased BCM area. The authors' technique of ultrasound measurement of BCM can be a reliable screening tool for patients suspected to have CAG expansion and AR resistance. BCM ultrasonography may prevent the indiscriminate use of CAGn analysis that tends to be expensive and not covered by most health insurances.[33] Even though androgenic activity can be inferred from a patient's symptoms, objective clinical measurement at the tissue level is proven to be difficult to measure. Proxy measures like frequency of shaving, morning erections, second to fourth digit ratio and so on, have been used with little success.[34] Multiple studies have indicated the inverse correlation between the number of CAGn and virilization.[13,14] However, most clinicians do not use this information in clinical decision-making and there is a critical need to measure androgenic activity in an objective way. Therefore, measurement of anogenital distance,[15] or BCM area, to determine the presence of CAG expansion (CAG > 25) and the level of androgen activity at the tissue level will be of clinical value. The use of area over one dimensional measurement when assessing BCM mass minimizes variation and intraobserver bias. Therefore, the use of BCM area, a three-dimensional measurement will minimize the potential for errors and variation. In this study, no correlation was found between testosterone levels and the number of the CAGn in the AR. Other studies that have evaluated the interaction of AR CAGn polymorphism and serum testosterone levels collaborate these findings, and do not find any association between testosterone levels and the number of CAGn.[35]

This study is the first, to the authors' knowledge, in the published literature to evaluate the relationship between BCM parameters and

CAGn in humans. The BCM measurement is an efficient, bed-side, point-of-care measurement that can guide physician decision-making. As have been shown in the current results, BCM area can be used as a proxy to help determine the CAGn range and tissue level androgen activity. Men with BCM area above 72 mm² indicate good androgen activity.

In the current cohort, the number of CAGn increases with age, similar to what is demonstrated in the published literature illustrating CAG region expanding.[36,37] This increase in the number of CAGn confirms previous models that indicate the presence of CAG microsatellite instability.[21,22] The instability in the number of CAGn occurs in both humans and mice leading to somatic mosaicism and tissue-specific trinucleotide repeat polymorphism.[22] Such genetic heterogeneity can introduce AR somatic functional mosaicism and polymorphic physiological effects of androgens on end organs.[23] In the case of AR, different sexual organs may react differently to the same serum androgen levels. As the number of CAGn change with age, the response of the AR to androgens might also be altered, and it is conceivable that older men with normal testosterone levels and longer AR CAGn lengths will have a higher risk of developing andropausal symptoms.[35] The number of CAGn and BCM area may vary by race.[2] The older age group of the current study was from a mixed group of ethnic backgrounds, therefore, minimizing its effect as a confounding factor.

Age-related loss of skeletal muscle mass and strength has been well-established. However, in elderly men, the decrease in muscle strength outweighs the decrease in muscle mass. Change in muscle mass is dependent on multiple factors, such as physical activity, tobacco smoking, individual wellbeing and age.[38] The age-related decline in muscle mass usually does not start until the 7th decade of life, and the detection of loss of muscle mass is highly influenced by the method and indices used to assess body composition.[39] Moreover, muscle mass is highly dependent on androgen levels, and testosterone administration is associated with dose dependent increase in muscle mass.[40] In the current cohort, all subjects were healthy, under the age of 65 years, with normal testosterone levels who BCM mass was mainly dependent on androgen activity.

This study is the first to look at the BCM muscle parameters and the number of CAGn to determine androgen activity at the tissue level. The authors have further presented that the number of CAGn is susceptible to changes with age and this might account for andropausal symptoms in men. However, this is a very small study and further longitudinal studies are necessary to fully elucidate the effect of age on the number of CAGn and androgen activity. Moreover, it is critical for future studies to examine the interactions among serum testosterone level as well as other androgens, AR CAGn length and age. A larger sample size than the current study would be required to provide sufficient data to examine these interactions and to further define our findings, including patients with CAGn disease and erectile/ejaculatory dysfunction. However, it can be concluded that the BCM area can be correlated with the number of CAGn in the AR and this relationship can be used to assess androgen status of the patient.

CONCLUSIONS
This study of normal male subjects indicates that an increased number of CAGn is associated with decreased BCM area and androgen activity. This supports prior studies demonstrating that longer CAGn in ARs have been shown to result in reduced AR activity and decreased virilization. Furthermore, the possible increase in the number of CAGn over age might account for andropausal symptoms which older men may experience. The association of AR CAGn with BCM is a novel finding that may help to select the most appropriate patients for AR CAGn testing in the future. Further, the findings of this study may be

used for future comparison in other populations with known CAGn disease and erectile and ejaculatory pathology.

AUTHOR CONTRIBUTIONS

AAD carried out analyses, designed the study, helped in data collection, drafted and finalized the manuscript. MSW carried out analyses, reviewed and revised the manuscript. AM performed the AR CAG size analysis and molecular genetic study, performed data collection and revised the manuscript. AB designed the data collection instruments and coordinated data collection. PNS reviewed and finalized the analyses and the manuscript. DAP conceptualized and designed the study; collected the clinical data; and drafted, reviewed and revised the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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