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Published in:
Thrombosis and Haemostasis

DOI:
10.1055/a-1346-3384

Publication date:
2021

Document version:
Accepted manuscript

Citation for published version (APA):

Go to publication entry in University of Southern Denmark's Research Portal

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Download date: 14. Sep. 2023
The effect of anabolic-androgenic steroid abuse on the contact activation system

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Abstract

The effect of anabolic-androgenic steroid (AAS) abuse on the contact activation system (CAS) is not known in detail. We hypothesized that current AAS abuse reduces the kallikrein generating capacity of CAS significantly and investigated the impact of AAS on the proteins and capacity of CAS in current and former AAS abusers and healthy age matched controls. Men 18 to 50 years of age were included as current AAS abusers, former AAS abusers or controls. Blood samples were collected after overnight fasting. Kallikrein generation (lag time, peak height, and endogenous kallikrein potential (EKP)), coagulation factor XII (FXII), prekallikrein, high molecular weight kininogen (HK), and C1 esterase inhibitor (C1inh) were assessed. Groups were compared by ANOVA or Kruskal–Wallis test and probabilities were corrected for multiple comparisons. Associations were evaluated by linear regression models.

The EKP was significantly reduced in current (n=37) AAS abusers (984 ± 328 nmol/L x min) compared to former (n=33) abusers (1543 ± 481 nmol/L x min) and controls (n=30) (1521 ± 339 nmol/L x min), p<0.001. Current abusers had higher levels of FXII and C1inh and lower levels of prekallikrein and HK than controls, p ≤ 0.025. Stepwise regression analysis showed that EKP was associated with C1inh and prekallikrein in current AAS abusers, R² = 0.70, p<0.001. We conclude that current AAS abuse reduces the kallikrein generating capacity of CAS by increasing the concentration of C1inh and reducing the concentration of prekallikrein. These changes may contribute to the anti-inflammatory effect of testosterone.

Keywords

Factor XII, prekallikrein, C1 esterase inhibitor, high molecular weight kininogen, anabolic-androgenic steroids
Summary table

<table>
<thead>
<tr>
<th>What is known about this topic?</th>
<th>What does this paper add?</th>
</tr>
</thead>
<tbody>
<tr>
<td>The effect of synthetic testosterone on the contact activation system (CAS) has been briefly addressed in controlled clinical settings</td>
<td>The effect of abuse of wild type anabolic-androgenic steroids (AAS) on CAS is studied in a real-life setting</td>
</tr>
<tr>
<td>The plasma concentration of the proteins of CAS is influenced by synthetic testosterone</td>
<td>Abuse of AAS reduces the kallikrein generating capacity of CAS significantly by increasing the plasma concentration of complement C1 esterase inhibitor and reducing the plasma concentration of prekallikrein</td>
</tr>
</tbody>
</table>

Introduction

Anabolic-androgenic steroids (AAS) are used for therapeutic as well as illegitimate purposes. The therapeutic applications include supplementation of natural testosterone to males deficient in testosterone (1) and lifelong gender-affirming hormonal treatment of transgender males (2). Synthetic AAS were previously considered as drugs with potential use in treatment of patients with coagulation factor deficiencies or deficiencies in the regulatory mechanisms of coagulation or fibrinolysis (3), as synthetic AAS increase the plasma concentration of many proteins including proteins of the haemostatic system (3-5).

Illicit use of synthetic AAS in combination with numerous other drugs has been reported in professional athletes and is in particular prevailing among recreational strength athletes (3) in order to enhance muscle strength and size. AAS abuse, however, may cause many adverse reactions and is associated with cardiovascular, neuroendocrine, psychiatric, and hepatic side effects (3-10).

Of notice is the effect of synthetic AAS on the contact activation system (CAS) consisting of coagulation factor XII (FXII), prekallikrein, and high molecular weight kininogen (HK). Activation and propagation of this system lead to cleavage of HK and formation of bradykinin (BK) which mediates vasodilation through binding to the BK B2 receptor on vascular endothelial cells (11) and
stimulates inflammatory reactions by this action. Moreover, CAS is associated with the complement system (12, 13), contributes to thrombus growth and stability, and is significantly involved in plasminogen activation, thus contributing to fibrinolysis (14, 15). C1-esterase inhibitor (C1inh) is the major inhibitor of CAS. Compromised regulation of the system due to impaired functional C1inh leads to abundant generation of BK causing the edema formation that characterizes patients with hereditary angioedema (HAE). AAS are used therapeutically for long-term prevention of HAE (16), because AAS induce a profound increase in the plasma concentration of C1inh (17). This prophylactic treatment reduces the bradykinin mediated edema attacks suggesting that AAS affect the regulation of CAS significantly.

Although specific attention has been paid to the prophylactic effect of AAS on HAE in a clinical setting, only few older studies have briefly addressed the effect of synthetic testosterone on CAS (18, 19). Thus, a thorough investigation of the effect of AAS abuse on the plasma proteins of CAS, including an evaluation of the overall performance of the system has not been performed. The purpose of the present cross-sectional study was to investigate in detail the impact of AAS on the proteins and the capacity of CAS in current and former AAS abusers as well as healthy age matched controls.

Materials and Methods

Participants and design

We performed this community-based cross-sectional case-control study in the greater Copenhagen area from November 2014 to December 2015. The study is part of a comprehensive study investigating health-related outcomes among current and former male AAS abusers. We have previously published the study design and data on the primary hypotheses (7-9). In brief: Men (18–50 years) involved in recreational strength training were enrolled in one of three groups: 1) current
AAS abusers (n=37), 2) former AAS abusers who had discontinued AAS abuse ≥ 3 months before enrolling in the study (n=33), and 3) age-matched healthy control participants who denied ever having used AAS (n=30). We recruited the participants primarily from fitness centers and by internet advertising. The groups were matched by age. A clinical interview using a structured questionnaire registered the duration of AAS abuse, as well as the compounds and doses used. Congenital hypogonadal conditions, medically prescribed testosterone therapy, known cardiovascular disease and diabetes mellitus were exclusion criteria. The participants were allocated to one of the three groups based on the self-reported history of AAS abuse supported by measurement of biochemical markers well known to be influenced by supra-physiological concentrations of androgens such as testosterone, gonadotropins, sexual hormone-binding globulin, and hematocrit (19). All current AAS abusers reported that they administered the last dose of AAS within one week before they entered the study. Testicular size (ml) assessed using Prader’s orchidometer was recorded in all participants as previously reported (9).

**Ethics**

The Capital Regional Committee on Health Research Ethics in Denmark (H-3–2014–127) and the Danish Data Protection Agency (HEH-2014–095, I-Suite: 03250) approved the study. The participants gave informed consent before inclusion and the study was conducted in accordance with the Helsinki Declaration.

**Blood Sampling**

All procedures were performed at a single visit to the Centre of Endocrinology and Metabolism, Department of Internal Medicine, Copenhagen University Hospital, Herlev, Denmark, as described previously (9). In brief: participants attended the research lab between 07:30 and 09:00 a.m. after a
minimum of eight hours of overnight fasting and placed in the supine position for a minimum of 30 minutes. Blood aimed for serum preparation was collected into Greiner Bio-One 4 mL Vacuette tubes with serum clot activator. Blood aimed for preparation of citrate stabilized plasma was collected in Greiner Bio-One 3 mL Vacuette® 9NC tubes containing 0.3 mL 3.2% citrate. Both tubes were from Greiner Bio-One, Kremsmünster, Austria. Within 90 minutes serum and platelet-poor plasma were prepared by centrifuging at 3,000 \( g \) for 10 minutes and frozen at -80°C.

*Laboratory assays*

The plasma protein concentration of FXII and prekallikrein was determined with enzyme-linked immunosorbent assays (ELISA) as described previously (20, 21). The protein concentration of HK was assessed by in-house prepared ELISA employing specific monoclonal antibodies. Briefly, 96-well polystyrene flat bottom MicroWell™ MaxiSorp™ plates (Thermo Fisher Scientific, Lilleroed, Denmark) were coated with 1.0 \( \mu \text{g} \) MAb HK-6 per mL. Plasma samples were analyzed at a 1:4000 dilution and a citrate plasma pool from 30 healthy volunteers was used as calibrator. Biotinylated MAb HK 19-31-18 (0.14 \( \mu \text{g/mL} \)) was used as detection antibody and plates were visualized using HRP-conjugated streptavidin and the TMB One-substrate (Kementek, Taastrup, Denmark). The plates were read at 450 nm with 650 nm as reference using a Tecan Sunrise ELISA reader (Tecan, Männedorf, Switzerland). The protein concentration of C1inh was determined using N antiserum against human C1inh, buffers, and reagents, employing the BN II analyzer (all from Siemens Healthcare Diagnostics, Marburg, Germany). Measures of kallikrein generation, i.e. the lag time, peak kallikrein concentration, and endogenous kallikrein potential (EKP) were recorded by the automated kallikrein generation assay as previously published (22).
Statistics

Statistical calculations were performed using IBM SPSS Statistics version 24.0 (Chicago, IL, USA). The Kolmogorov Smirnov test and Q-Q plots of residuals were used to test the distribution of results. The \( \chi^2 \)-test and Fishers Exact Test were employed for comparison of dichotomous variables, as appropriate. ANOVA was used for between-group comparisons of normally distributed data and the results are expressed as mean and standard deviation (SD). The Kruskal-Wallis test was used for between-group comparisons of non-normally distributed data and the results are expressed as median and 25-75 percentile range. Bonferroni and Dunn’s test, respectively, were used as post-hoc tests. Association between variables was investigated using Pearson product-moment correlation analysis. Stepwise regression analysis evaluated the significance of the variables associated with EKP. P < 0.05 was considered statistically significant.

Results

The participants in the three study groups were comparable with respect to age, \( p = 0.11 \). Current AAS abusers had significantly higher BMI and estradiol levels than controls, \( p<0.001 \). Body fat mass was significantly different among the three study groups, \( p<0.001 \), with the highest values in the former AAS abusers and the lowest values in the current abusers. Current AAS abusers had the lowest and controls the highest testicular size, \( p<0.001 \). The testosterone levels were significantly different among the three groups with the highest values in the current AAS abusers and the lowest in the former AAS abusers, \( p<0.001 \). The accumulated duration of AAS abuse and the number of AAS compounds used did not differ significantly among former and current AAS abusers, \( p=0.22 \) and \( p=0.32 \), respectively, Table 1.
Table 1. Characteristics and measures of the contact activation system and kallikrein generation in control individuals, former and current abusers of androgenic anabolic steroids.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (n=30)</th>
<th>Former abusers (n=33)</th>
<th>Current abusers (n=37)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>31.5 ± 1.2</td>
<td>34.8 ± 1.2</td>
<td>31.4 ± 1.4</td>
<td>0.11</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27.2 ± 0.6</td>
<td>28.5 ± 0.6</td>
<td>30.8 ± 0.4</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body fat (kg)</td>
<td>17.5 ± 0.7</td>
<td>19.4 ± 0.6</td>
<td>14.1 ± 0.4</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Accumulated duration of AAS abuse (wk)*</td>
<td>-</td>
<td>142 (100-203)</td>
<td>112 (81-154)</td>
<td>0.22</td>
</tr>
<tr>
<td>Number of AAS compounds used (n)</td>
<td>-</td>
<td>6 (4-9)</td>
<td>8 (4-9)</td>
<td>0.32</td>
</tr>
<tr>
<td>Testicular size (mL)**</td>
<td>22.3 (0.6)</td>
<td>17.4 (0.8)</td>
<td>12.2 (0.7)</td>
<td>&lt;0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total testosterone (nmol/L)</td>
<td>18.9 (17.3-20.6)</td>
<td>14.8 (13.1-16.8)</td>
<td>75.6 (54.2-105.3)</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Estradiol (pmol/L)</td>
<td>21 (2-38)</td>
<td>33 (5-51)</td>
<td>100 (13-270)</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Measures of the contact activation system</th>
<th>Controls (n=30)</th>
<th>Former abusers (n=33)</th>
<th>Current abusers (n=37)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor XII (mg/L)</td>
<td>40.6 ± 6.4</td>
<td>42.9 ± 5.8</td>
<td>45.4 ± 8.4</td>
<td>0.025&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Prekallikrein (%)</td>
<td>112 ± 18</td>
<td>102 ± 23.5</td>
<td>91 ± 24</td>
<td>0.001&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>High molecular weight kininogen (%)</td>
<td>115 ± 22</td>
<td>102 ± 21</td>
<td>103 ± 14</td>
<td>0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>C1 esterase inhibitor (g/L)</td>
<td>0.22 (0.21-0.24)</td>
<td>0.22 (0.20-0.24)</td>
<td>0.32 (0.27-0.48)</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Measures of kallikrein generation</th>
<th>Controls (n=30)</th>
<th>Former abusers (n=33)</th>
<th>Current abusers (n=37)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>1.0 (0.8 – 1.2)</td>
<td>0.8 (0.8 – 1.1)</td>
<td>0.8 (0.6 – 1.2)</td>
<td>0.38</td>
</tr>
<tr>
<td>Peak kallikrein concentration (nmol/L)</td>
<td>909 ± 283</td>
<td>929 ±</td>
<td>767 ± 221</td>
<td>0.025&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The plasma protein concentration of C1inh was higher in current AAS abusers than former AAS abusers or controls, p <0.001. Also the plasma concentration of FXII was higher in current AAS abusers than in controls, p=0.025, whereas the concentration of prekallikrein was significantly lower in the current AAS abusers than in controls, p = 0.001. The plasma concentration of HK was significantly lower in both current and former AAS abusers compared with the controls, p=0.01,
Table 1. The lag time of kallikrein generation was not significantly different among the groups, \( p = 0.38 \). The peak kallikrein concentration formed during activation of the contact system was significantly lower in current AAS abusers compared with former AAS abusers or controls, \( p=0.025 \). Table 1.

The EKP was significantly lower in current AAS abusers (984 ± 328 nmol/L x min) compared with former AAS abusers (1543 ± 481 nmol/L x min) or controls (1521 ± 339 nmol/L x min), \( p < 0.001 \), Fig. 1.

![Graph](image)

**Figure 1.** The endogenous kallikrein potential in controls, former, and current abusers of anabolic-androgenic steroids.

**Correlation and stepwise regression analysis**

Correlation analyses in the three groups of individuals revealed that the EKP was correlated with C1inh, \( p<0.04 \), and prekallikrein, \( p=0.001 \), in control subjects, with prekallikrein, \( p<0.001 \), in
former AAS abusers, and with C1inh, p<0.001, prekallikrein, p=0.003, and HK, p=0.023 in current AAS abusers.

Stepwise regression analyses with EKP as the dependent variable and FXII, C1inh, and HK as independent variables demonstrated that EKP was significantly associated with prekallikrein in the control group, \( R^2 = 0.28, p = 0.002 \). Similar results were obtained in the former AAS abusers with EKP significantly associated with prekallikrein, \( R^2 = 0.51, p < 0.001 \). In the current AAS abusers EKP was significantly associated with C1inh, \( R^2 = 0.55, p<0.001 \) and inclusion of prekallikrein also contributed significantly to the model, \( R^2 = 0.70, p<0.001 \), Table 2.

Table 2. Stepwise regression analysis between endogenous kallikrein potential (EKP) and factors contributing to the variance in EKP in current and former abusers of androgenic-anabolic steroids and control subjects.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>( \beta )</th>
<th>p-value</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls (n=30)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prekallikrein</td>
<td>0.53</td>
<td>0.002</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>Former AAS Abusers (n=33)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prekallikrein</td>
<td>0.73</td>
<td>&lt;0.001</td>
<td>0.51</td>
</tr>
<tr>
<td><strong>Current AAS abusers (n=37)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1 esterase inhibitor</td>
<td>-0.72</td>
<td>&lt;0.001</td>
<td>0.55</td>
</tr>
<tr>
<td>Prekallikrein</td>
<td>0.40</td>
<td>&lt;0.001</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Estimated standardized regression coefficients (\( \beta \)) and variance explained (\( R^2 \)) are presented.

**Discussion**

The present study demonstrates that AAS abuse affects CAS significantly. The key finding is that the kallikrein generating capacity induced by contact activation is significantly reduced by AAS. Current AAS abusers express significantly higher plasma concentration of FXII and significantly lower concentrations of prekallikrein than controls. The concentration of C1inh is significantly
higher in current AAS abusers than in former abusers and controls, whereas the concentration of HK is significantly lower in both former and current AAS abusers than controls. These findings are in agreement with previous publications dealing with the effect of synthetic testosterones on the proteins of CAS (18, 19) as recently reviewed (3). The results, however, indicate that the effect of AAS on the plasma levels of HK persists after refrain from AAS abuse as significantly reduced HK is observed in former AAS abusers compared to controls.

Notably, the former trials were conducted in a controlled setting studying the effect of a single dose of specific AAS. Presently we extended these studies and focused on the effect of wild type AAS on CAS in an uncontrolled real-life setting where the formulation of the AAS component is unknown and where the accumulated duration of AAS abuse spans several months. The integrated and dynamic effects of AAS on CAS were studied by means of the kallikrein generation assay (22). The outcome of this assay depends potentially on the concentration of FXII, prekallikrein, and C1inh as previously demonstrated in vitro (22). In the present setting, we demonstrated that the peak kallikrein concentration and the kallikrein generating capacity of CAS in terms of EKP were significantly reduced in current AAS abusers compared to former abusers and controls. Correlation analysis and stepwise regression analysis showed that the kallikrein generating capacity of CAS was dependent on the concentration of prekallikrein in former AAS abusers and controls, but that also C1inh contributed to the variance in EKP in current ASS abusers, with prekallikrein and C1inh explaining 70% of the variance in EKP.

Notably, reduced capacity of CAS induced by AAS may translate into reduced BK formation. This is the rationale behind the administration of AAS, in particular Danazol, to patients suffering from HAE in order to reduce the BK-mediated attacks characterizing the disease (16). Our results on the kallikrein generating potential of CAS indicate that not only the AAS-induced increase in C1inh,
but also the AAS-induced reduction in prekallikrein may contribute to the effect of AAS in the long-term prevention of HAE.

Presently we observe that AAS abuse increases both testosterone and estrogen significantly. Estrogen increases the plasma concentration of FXII and PK and decreases the concentration of C1inh (23-25), and may by these actions, in contrast to testosterone increase the kallikrein generation potential. The effect of synthetic AAS however apparently overwhelms the effect of genuine estradiol on CAS.

It is of interest that testosterone has anti-inflammatory effects and low testosterone levels are associated with increased expression of inflammatory markers as recently reviewed (26). Administration of testosterone to men low in testosterone reduces the plasma levels of these markers. The findings in the present sub-study suggest that also the effect of AAS on CAS, with impaired kallikrein generation and by that decreased BK formation capacity, may contribute to the anti-inflammatory effect of testosterone.

It might be speculated whether the observed effect of AAS abuse on CAS is acute or permanent. We have previously observed increased endogenous thrombin generation potential and elevated concentrations of plasmin inhibitor in both current and former AAS abusers when compared with controls (4, 5). In line with these findings, we presently observe elevated concentrations of HK in both current and former AAS abusers. Thus, our results suggest that the plasma concentration of some specific proteins remains elevated years after cessation of abuse, indicating a long-lasting effect of AAS on hemostasis.

There are several noted limitations in the present study as previously published (9). The cross-sectional design does not allow clinical conclusions and the modest number of participants available for inclusion in the study may induce statistical type II errors. The AAS abusers enrolled in the current study apply and combine a number of drugs. We have previously reported that the AAS
Abusers on average had experience with four to nine different AAS compounds and 50% of the AAS abusers regularly used human chorionic gonadotropin (HCG) preparations or aromatase inhibitors (9). Few studies have demonstrated that administration of HCG to women with ovarian hyper-stimulation syndrome reduces the plasma concentration of prekallikrein significantly (27, 28). Moreover, administration of HCG or aromatase inhibitors stimulates the testicular production of testosterone (29, 30). Thus, the reduced capacity of CAS in our patients abusing AAS may not solely be associated to the testosterone formulation applied, but may also be related to the effect of HCG or aromatase inhibitors.

The validity of the self-reported history of AAS administration given by the study participants may be questioned. Current AAS abusers, however, display increased testosterone and estradiol levels and, as previously published significantly reduced concentrations of FSH and LH (9). These outcomes, together with a significantly reduced testis volume observed in current AAS abusers strongly indicate that the current AAS abusers administer AAS on a regular basis, providing a persistent effect on hormone levels.

In conclusion: current abuse of AAS reduces the kallikrein generating capacity of CAS significantly by increasing the plasma concentration of C1inh and reducing the concentration of prekallikrein. These changes may contribute to anti-inflammatory effects of testosterone.

**Conflicts of interest**

None declared.
References