STUDY PROTOCOL

Cross-sectional study of 800 recreational athletes with current and former use of performance and image enhancing drugs (PIED), focusing on androgenic anabolic steroids (AAS)

Short title: **FI**tness **DO**ping in **D**enmar**K**

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Research Ethical Committee

The Regional Scientific Ethical Committee of the Region of Copenhagen

Content

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Background

Epidemiology of illicit use of anabolic androgenic steroids

Anabolic androgenic steroids (AAS) are synthetic derivates of the primary male reproductive hormone, testosterone, but have been chemically modified and thereby featuring a higher anabolic/androgenic ratio, longer plasma half-life and stronger affinity for the androgen receptor [1]. During the 1980's, illicit AAS use was limited to elite athletes and bodybuilder communities, thereby restricting the incidence of complications of AAS use to a small, highly selected group. But within recent years studies suggest illicit AAS use has moved to the broader population and is now widespread among young men and should therefore be considered as a public health concern [1]. A meta-analysis from 2014 based on all accessible studies on illicit AAS use worldwide, estimated that almost 20% of male recreational athletes and approximately 6% of all men worldwide have experience with illicit AAS use [2]. Data on the prevalence of AAS use among women is virtually non-existent.

Health-related complications of illicit AAS use

A recent Danish register-based study noted a factor three increased risk of all-cause mortality and hospital outpatient contacts among men who had tested positive for AAS as compared with a randomly selected age- and sex-matched cohort [3]. Within recent years, we and others have reported several adverse effects of AAS abuse among men that is supportive of long-term AAS complications years after AAS cessation in terms of long-lasting male hypogonadism, impaired fertility, decreased cardiac function as well as impaired lipid and glucose metabolism, and incipient atherosclerosis [4–9]. Furthermore, sustained AAS use has been associated with adverse behavioral consequences, including impulsivity, aggression, and violence. AAS users may also develop a condition of aberrant body image, often referred to as muscle dysmorphia or bigorexia. A condition defined by a warped perception of one's body size being smaller than what can objectively be measured. Loss of muscle triggers anxiety and contributes to AAS dependence and relapse.

In summary, current information on adverse effects related to illicit AAS use is based on cross-sectional studies demonstrating only momentary health status among AAS users and therefore not the true impact of illicit AAS use on health during following many years. Furthermore, studies based on registers only provide even less valuable information since absolutely no conclusions on causality can be drawn. Therefore, it would be of high interest to examine a large cohort of current and former AAS users for detailed information with regular follow-up the following years to assess their health status and potentially find screening biomarkers which could suggest development of severe health adverse effects.

AAS-induced male hypogonadism

Male hypogonadism is not frequently observed among younger men and has traditionally been attributed congenital conditions such as Klinefelter and Kallmann's syndrome or cryptorchidism [10,11]. Interestingly, a recent US study reported approximately 55% of younger men referred to urologic outpatient clinics with profound male hypogonadotropic hypogonadism (plasma total

testosterone < 1.7 nmol/L) were related to prior AAS use [11]. These findings are corroborated by a recent Danish register-based study reporting substantially increased risk of male hypogonadism among AAS users: 22-fold increased risk of testicular dysfunction, 2.5-fold increased risk of male infertility, 15-fold more likely of being treated with testosterone supplementation [3]. The mechanisms behind AAS-induced male hypogonadism relates to the hypothalamic-pituitary-gonadal (HPG)-axis suppression after supraphysiologic plasma androgen concentrations and leads to a rapid decline in plasma testosterone following AAS cessation [9,12] with various concomitant hypogonadal symptoms, such as erectile dysfunction, decreased sexual desire, fatigue, depression even after 2-3 years since AAS discontinuation [9]. Indeed, from our own and others experience, many of these patients will resume their AAS abuse due to these severe symptoms which lead to AAS-dependence [13]. The current knowledge on impaired fertility after AAS cessation is sparse being primarily from case reports, [14–19] although we reported higher prevalence of previous AAS abusers (median of 2.6 years since AAS cessation) with plasma inhibin B, a fertility marker, below the lower cut off level abusers compared with non-AAS users [9]. Thus, suggesting that oligo- or azoospermia may become permanent or long-lasting. New state-of-art methods of investigating fertility among men, such as DNA fragmenting and acrosome reaction, could be of high interest in men with previous abuse of AAS [20,21]. Although testosterone is presently the best biomarker for the symptoms following AAS cessation, regarding the pleiotropic adverse effect following AAS use, and psychological well-being especially, testosterone alone may be limited in its ability to capture important characteristics needed to guide clinical decision-making, why further studies using a broader approach are warranted to investigate the clinical, biomedical and physical well-being in these young individuals.

Illicit use of AAS among women

Information on AAS use among women is very sparse. Information on prevalence, patterns of AAS use, AAS compounds used have not been reported from systematic studies in the literature. We now from case-reports, and from Anti Doping Denmark that illicit AAS use seems to have a masculinizing effect in women and therefore give rise to several adverse effects such as: hirsutism, acne, irreversible deepening of voice, absent or irregular menstrual cycle and clitoris enlargement. Nevertheless, scientific literature on the subject is extremely limited. However, a recent minor qualitative study in female AAS users confirmed several of the proposed side-effects. **(***Havnes et el. International journal of drug policy, 2021)***.** Thus, it would be of high interest to perform a cohort study including both current and former female AAS users to gain information on illicit AAS use among women to develop prevention strategies and therapy options.

Cardiovascular disease among AAS users

An increased risk of cardiovascular disease (CVD) and impaired cardiac function has been suggested among former male AAS abusers[7,10]. The precise mechanisms linking AAS with myocardial dysfunction are unknown, however, a direct toxic effect of supraphysiologic levels of androgens could be implicated [9]. We have previously reported in a Danish community-based cross-sectional study that past illicit male AAS use is associated impaired left ventricular (LV) systolic function expressed as impaired global longitudinal strain (GLS) [9]. Impaired LV GLS independently predicted

adverse cardiovascular outcomes, including heart failure in a low risk general population [27]. By using coronary computed tomography angiography, a US study, demonstrated higher coronary artery plaque volume and reduced LV systolic function in AAS users than nonusers[28]. Interestingly, both current and former AAS abusers exhibited higher aortic stiffness [18].

Notably, AAS may have even worse adverse effects on the heart and metabolism among women, since they by biology only have very low testosterone levels, and increasing knowledge from the condition polycystic ovary syndrome, which is characterized by increased testosterone levels, we know that these patients have a markedly increased risk of type 2 diabetes and CVD. Therefore, it could be hypothesized that myocardial blood flow reserve will be impaired among former male and female AAS abusers.

Early signs of AAS-induced cardiomyopathy, such as microvascular dysfunction with reduced myocardial perfusion can be measured as myocardial blood flow reserve (MFR) quantitatively as ml per gram per minute, and noninvasively by cardiac positron emission tomography (PET) with Rubidium-82 (Rb-82). Several advantages using PET with Rb-82 should be addressed, such as higher diagnostic accuracy, improved image quality, less radiation dose to patient and staff, and especially rapid examinations time [29], and the Rb-82 PET CT method has not previously been used among AAS users. Therefore, it will be relevant to use this highly sensitive Rb-82 PET CT to further examine the mechanism behind cardiovascular disease in former AAS abusers.

Dysmetabolism among AAS users

We have previously noted a dysmetabolic state in both current and former male AAS users compared with age-matched healthy controls (9). Interestingly, both current and former AAS users showed increased volume of visceral adipose tissue (VAT) and impaired insulin sensitivity compared with healthy controls [6]. Increased VAT among AAS users was associated with decreased insulin sensitivity in a multivariate linear regression [6]. Further, a few case reports noted debut of diabetes among current AAS users. Thus, it would be of interest to perform a dual X-ray absorption (DXA) scan, which is a low dose x ray scan, to assess body composition, especially VAT, and to assess a potential link with later development of fulminant metabolic syndrome including diabetes in male and female AAS abusers.

Objective

The overarching aim of this study is to characterize a large cohort of former and current male and female AAS users and to identify whether certain characteristics such as duration or intensity of illicit AAS use could play a role for development of cardiovascular disease, diabetes, male hypogonadism, female infertility, and psychiatric disease during the following years. Furthermore, we will pay attention to the prevalence of subclinical disease including impaired gonadal function and fertility, psychological well-being and cardiovascular disease and the predictive value of markers subclinical disease in terms of developing overt disease requiring therapy during the following years.

The study will especially focus on the following among current and former male and fremale illicit AAS users:

- Long-term complications and outcomes related to: cardiovascular disease, diabetes, gonadal disease (women and men) and psychiatric disease using data from Danish registries including addressing central questions such as whether the following characteristics play a role for development of overt disease: duration of and intensity of AAS use, increased risk of certain AAS compounds, age when starting AAS use and other characteristics.
- Characterization of illicit AAS use among women: duration and intensity of AAS use, AAS compounds used, apparent side-effects including reproductive health and hirsutism.
- Current male reproductive health: reproductive serum hormone levels, semen status and erectile dysfunction and gynecomastia
- Current psychological well-being, aggressive tendencies, cognitive function and quality of life
- Current cardiovascular and metabolic status: blood pressure, serum levels of lipids, insulin resistance, cardiac function, body composition

Study Methodology

The study design is a combined cross-sectional and prospective cohort study including male and female participants with an ongoing or former illicit AAS use. The study will establish a cohort with participants of current and former users of AAS containing clinical data, and a biobank, consisting of blood and urine samples. Participants in the AAS cohort are to be followed-up every third year from inclusion of the last study participants for 15 years with regard to diagnoses related to gonadal function, mental health, cardiovascular disease and diabetes mellitus using national registry data. The study aims at including a total of 800 participants during an inclusion period of three to four years.
To ensure a contemporary cohort of comparison (control), a concurrent, smaller cohort, including 100 healthy athletes (80 men and 20 women) without current or former AAS use, will constitute a control cohort for in-depth analysis of clinical characteristics. Further, a larger age- and sex-matched cohort based on national registry-data (n = 8000) will be used to assess the clinical implication of clinical characteristics in prospective follow-up.

Participants

Participants will be recruited from the outpatient clinic at the Department of Endocrinology and from the local community through advertisement in fitness- and weightlifting gyms and the internet: (Social media such as :Facebook, Instagram, homepage of AntiDoping Denmark, forsoegspersonen.dk).

Inclusion criteria (AAS Cohort)

- Male and female recreational athletes (≥18 years of age) with current or former illicit use (> 3 months since AAS cessation) of PIEDs (e.g. AAS) for a minimum four weeks.

Inclusion criteria (Control cohort)

- Male or female recreational athletes (≥18 years of age) with NO current or former illicit use of AAS.

Exclusion criteria

- Severe psychiatric or somatic diseases which makes it impossible to give informed consent or comply with the investigatory program.
- Known pregnancy.

Procedures

Core study programme

Participants are to undergo a semi-structured medical interview, a clinical examination, fill out questionnaires, and be submitted to blood and urine sampling (Table 1). This core study program will be performed during a single clinical visit. Furthermore, the participants will be presented to participate in the extended research programs (Table 3).

Table 1: Core study program

Procedures	Outcome
Semi-structured interview	- Medical history, including concomitant chronic disease, medication and dietary supplements. - PIED history - Alcohol, tobacco and substance abuse - Fertility (characteristics of menstrual cycle) and sexual complaints - Diet and physical activity - Socioeconomic status
Clinical examination	- Anthropomorphic measures (Age, height, weight, waist and hip circumference, body mass index (BMI, kg/m^2)) - Dermatology Score (Acne) - Alopecia and face and body hair (Ferriman-Gallway Score); (Women)
	- Office blood pressure (OBP)
	- Orchidometer to determine testicular size (Men) - Gynecomastia (Tanner-staging; Men)
Patient reported outcome	- "Dit helbred og velbefindende" (SF-36®; *Quality of life*) - Inventory of Interpersonal problems ® *(Interpersonal relations)* - Body Q (*Bodily perception*) - Buss-Perry Aggression Questionnaire (BPA) *(Aggressive tendencies)* - General Anxiety Disorder – 7 (GAD 7) *(Anxiety and depression)* - Major Depression Index (MDI) *(Depression score)* - Cognitive complaints in bipolar disorder rating (COBRA) *(Cognition)*

	- Sexual function (IIEF5) *(Sexual function)* - Inventory of interpersonal problems (IIP32) - Testosterone deficiency (ADAM) - Physical activity and sports injury questionnaire
Blood and urine samples (please see Table 2 for details)	Routine biochemical panel Biomarkers of: - Organ function (TAME biomarkers) - Hormone function (Hormones and their binding proteins) - Adipose tissue function (derived hormones and inflammation markers) - Endothelial function - Extracellular matrix - Skeletal muscles growth - Hemostatic system - Circulating micro-RNA - AAS derivatives in urine *Extra biological material acquired will constitute a research biobank of future research. (See Consent regarding biobank of future research)*
Dual x-ray absorptiometry	- Body composition including distribution of VAT

Description of procedures

Clinical examination

The following anthropomorphic measures will be acquired for every participant: Age, height, weight, waist and hip circumference, body mass index (BMI, kg/m2). Acne is estimated by dermatology score. Testicular size is measured using an orchidometer and gynecomastia is estimated using the Tanner score (men). Alopecia and face and body hair are scored using the Ferriman-Gallway Score (women).

Office blood pressure (OBP)

An automatic, oscillometric apparatus is used to measure blood pressure on the non-dominant arm of the participant after a predefined period of rest (above 5 min). The measurement will be repeated two times divided by a minute of additional rest.

Blood and urine sampling

Venous blood will be drawn through a cannula in antecubital vein. Urine sampling will be acquired by participants providing a fresh urine-sample performed on-site supervised by one of the investigators. A research biobank will be established for blood and urine for analysis not performed immediately. A total of 300 ml blood and 200 mL urine will be sampled from the participants in the study.

Prespecified routine blood analyses

The routine blood panel analyses are performed at the local hospital laboratory immediately after acquisition (Table 2). Surplus biological material from samples sent for the routine analyses will be destroyed following analyses.

Whole-body dual x-ray absorptiometry

Full body dual x-ray absorptiometry (DXA - lumbar spine, hip, and whole body) is used to measure body composition including VAT and bone density. Radiation amounts to 0.004 mSv (See: *Ethical considerations*). The estimated examination time is 5 minutes.

Biobank

As part of the protocol a subset of samples are frozen as part of a biobank, where extra material, if any, acquired will constitute a biobank of future research. The biobank will be used to perform measurements according to the prespecified list of biomarkers from the protocol. The biobank of future research will consist of the extra material acquired at the scheduled visit (See *Consent regarding biobank of future research*).

Blood and urine samples will be frozen and stored in a secure freezer at Rigshospitalet, University Hospital. Samples will be stored in aliquots at -80°C as part of the abovementioned biobank, established at the Department of Endocrinology, Rigshospitalet according to specifications in this protocol. Samples will be stored as pseudo-anonymized form until use. The purpose of the biobank is to be able to perform analyses in one assay batch, whereby assay variation is minimized and a more exact analysis is achieved.

After the prespecified analyses in the protocol, any material remaining in the biobank will stored in a biobank for future research until the completion of register-based follow-up of specified in the study and then destroyed, however, the samples will at most be stored for 15 years. If biomarkers not pre-specified are to be analysed, the investigators will apply VEK for permission to perform these laboratories measurements without obtaining novel informed consent from the participants.

The biobank will be registered at the National Biobank Register and will comply with the legislation of the Danish Data Protection Agency. No analyses, besides the prespecified in this protocol, will be performed without a new assessment and subsequent approval from the regional ethics committee on health research.

Biomarker analyses by international associates

Regarding the urine samples, samples to be used in AAS analyses will be sent to Centre for Preventative Doping Research at the German Sports University Cologne, Germany (B), as specified in the protocol. The urine samples will subject to a specialized analysis for traces of AAS and PIEDs (B). The urine samples will be pseudo-anonymized prior to shipment. The legislation and regulations of the Danish Data Protection Agency will apply to these samples, including the Data Protection Act, chapter V (C). The transfer and handling of samples will follow the above stated legislation and regulations.

Registry-based follow-up

All citizens of Denmark are given a permanent unique civil registry number allowing us to perform individual-level linkage in nationwide registries. Data for these studies will be obtained from: 1) the Danish Civil Registration System registry (sex, date of birth, immigration, emigration and vital status), 2) the Danish National Patient Registry (discharge diagnoses coded according to the ICD-10 system since 1994), 3) the Danish National Prescription Registry (claimed prescriptions), 4) the Danish National Cause of Death Registry (primary and underlying causes of death from death

certificates), 5) the Population's Education Register (sociodemographic characteristics and education level), and 6) the Danish Psychiatric Center Research Register (depression) 7) participants hospital records. It is essential to gain access to the participants' hospital records since information such as examination results (CT scans, echocardiography, ultrasound, blood samples) van verify diagnoses given to a participant. Furthermore, examination results in the hospital record may reveal diagnoses the participant have not formally been given but fulfill criteria for. We will seek information on previous relevant conditions (from the 4 conditions of interest) from the hospital records as from a maximum of 10 years before the participant entered the study.

Registry-based prospective follow-up will be performed every third year until 15 years past initial establishment of the cohort. This follow-up will be centered on the 4 primary conditions of interest: reproductive health, type 2 diabetes, cardiovascular complications and mental health (depression, anxiety, psychotic episodes, schizophrenia) which leads to subsequent hospitalization or outpatient visits in the tertiary hospital sector, for the duration of the follow-up. Moreover, date of death and cause will be obtained. Data on prescriptions and ICD10-diagnoses will internally validate data registration and determine severity of underlying disease (e.g. the more severe disease, the more aggressive the treatment is). Data extracts will be obtained for both the AAS cohort, baseline controls and the excerpt of the general population.
Data will be kept until the end of the trial. The duration of the trial is 15 years.
The following ICD codes will be applied in the Danish National Patient registry every third year:
Gonadal diseases: Testicular hypofunction (men) (ICD-10: DE291-C); erectile dysfunction of any kind (men): ICD-10 codes: DN484, DF522-A; Female infertility (women) (DN970-9); Hirsutism (women) (ICD-10: DL680)
Cardiovascular diseases: cardiac diseases including arrythmias (ICD-10: DI44- 49), ischemic heart disease/nonfatal myocardial infarction (ICD-10: I20-I25), heart failure (ICD-10: DI110, DI500-501, DI509, DI420-422, DI426, DI429, DJ819) and valve diseases (ICD-10: DI05, DI06, DI34-36, DZ952); nonfatal hemorrhagic or ischemic stroke (ICD-10: DI60-64); venous thromboembolism (ICD-10: DI26, DI74, DI81, DI646, DI801-803, DI822- 823, DI828-829); Hypertension (ICD-10: DI10-109)
Diabetes mellitus: established diabetes of any kind including previous gestational diabetes (ICD-10: DE10, DE11, DE13, DE14, and DO244).
Mental health: depression (DF33-DF339); Psychotic episodes of any kind (DF22-DF28); Anxiety of any kind (DF40-DF419); Schizophrenia (ICD-10 codes: DF20-DF209)

1 **Table 2: Prespecified biomarker analyses**

	Blood			Urine
Local routine lab, immediate analysis	**Hormones** LH FSH Total T Free T SHBG Estradiol, Prolactin TSH ACTH Cortisol Aldosterone, FGF23, Ghrelin TT3 TT4 T3R 17OH Progesterone	**Metabolism and inflammation** Lipids p-glucose, HbA1c (TAME) 25-OHD NT-proBNP (TAME) TNT, TNI	**Other** Hemoglobin Hematocrit Creatinine Sodium, Potassium, ALAT, GGT, BASF Ionized-Ca	NA
Frozen for later analysis in batches		C-peptide Adiponectin, leptin sCD36 SuPAR, IGF-II, IGFBPs, bioactive IGF- I, IGF activating enzymes and regulators (PAPP-A and PAPP-A2) INSL-3 Stanniocalcins YKL40, IL6, TNFα Ferritin, PTH Inhibin B, AMH, TAME markers: IL-6, TNFα-receptor I or II, CRP, GDF15, fasting insulin, IGF1, cystatin C, NT-	Autoantibodies Myostatin, Follistatin Fibulin 1 CK Hemostatic tests ACE White cell count, Albumin Urea Endocrine disruptors (PFAS, phthalate and metabolites)	Metabolomics endocrine disruptors (PFAS, phthalate and metabolites), suPAR, markers of oxidative stress

		proBNP, and hemoglobin A1c.		
Amount of biological material	300 ml blood			200 ml urine

Extended study programmes (3 sub studies)

1) **Semen sample (in total 200 current and former male AAS users and all male controls (n = 80))**
2) **GnRH and hCG stimulation tests (30 former male AAS users and 30 male controls)**
3) **Rubidium PET CT scan (90 men: 30 current and 30 former AAS users and 30 controls and 60 women: 20 current, 20 former and 20 controls).**

All participants in the core study program are eligible for participation the extended study programmes. Recruitment for the extended study programmes will be in a consecutive manner, thus all who consent to participate in the core study programme will be offered participation in the extended study programmes until recruitment for these sub studies is complete. Participants who choose to participate in the extended study programs will be asked to sign an additional informed consents for each sub study Procedures related to the extended study programmes will, if possible, be performed during the same day as the core study programme or within two weeks.

Table 3: Extended study program 1 (semen analyses)		
Algorithm	Investigation	Outcome
N = 280 (200 men from the AAS cohort included + all controls)	Semen analyses	morphology, motility, volume, pH, acrosome reaction, DNA fragmentation
Table 4: Extended study program 2 (hormonal gonadal axis)		
Algorithm	Investigation	Outcome
N = 60 (30 former male AAS users and all + 30 male controls)	GnRH and hCG stimulation tests	Serum gonadotropins and serum total testosterone, respectively
Table 5: Extended study program 3 (cardiac microcirculation)		
Algorithm	Investigation	Outcome

N = 90 males (30 former and 30 current male AAS users + 30 male controls) **N = 60 women (20 former and 20 current female AAS users and 20 controls**	Rubidium PET CT	Myocardial flow reserve and myocardial microcirculation

Semen samples (Extended study program 1)

Participants will provide a semen sample by masturbation in a private room at the laboratory at Rigshospitalet following at least 48 hours of sexual abstinence. The samples will be analyzed immediately for: sperm count, morphology, vitality, motility, pH, acrosome reaction and DNA fragmenting.

GnRH stimulation test (Extended study program 2)

Intravenous injection with 100 mikrogram of GnRH will administered through a cannula in an antecubital vein. Blood for analyses of FSH, LH and total testosterone (freezed for later analyses in batches using LC-MS) will be drawn at time 0 min and +30 min.

hCG stimulation test (Extended Study program 2)

Following GnRH test, at the same visit, an intramuscular injection with 5000 IE of hCG will be administered intramuscularly. 72 hours after the injection, blood for analyses of serum total testosterone (freezed for later analyzes in batches using LC-MS) will be drawn in an overnight fasting state during morning hours.

Ultrasound of testes (Extended Study Program 2)

Ultrasound of testes will be performed to measure size and morphology. Examination time: 5 minutes

Rubidium-82 PET cardiac scan (Extended Study Program 3)

Rubidium-82 is a potassium analogue with a short half-life, which permits quantitative measurements of myocardial perfusion in absolute values (mL/g/min) during stress and rest, as the myocardial flow reserve (MFR) [37]. PET scans will be performed using a Siemens Biograph mCT/PET 64-slice scanner (Siemens Medical Solutions, Knoxville, USA). Serial rest followed by stress imaging with Rb-82 from a CardioGen-82 Sr-82/Rb-82 generator (Bracco Diagnostics, Inc., Princeton, New Jersey, USA). A standard clinical protocol will be used. Stress is induced with adenosine infusion of 140 μg/kg/min for 6 min. Myocardial blood flow quantification is performed ECG gating under pharmacological stress will be used for measurement of LVEF and wall motion in relation to peak hyperemia. Rubidium-PET procedures will be performed at the Department of Nuclear Physiology, Rigshospitalet.

Study visits

Participants eligible for inclusion in the study will participate either solely in the core study program (1 visit) or in both the core program as well as the extended study programs.

Core program

Each participant enters the study facility (Total examination time 2 h)

Table 5: 1 Visit	
Investigations	Time/hours
- **Written informed consent** - **Semi-structured medical interview** - **Physical examination** - **Acquisition of blood and urine sample** - **DXA scan**	2 hours in total

Extended program 1

Table 6: 1 Visit	
Investigations	Time/hours
- **Semen sample**	10 minutes

Extended program 2

Table 7: 2 Visits	
Investigations	Time/hours
Visit 1	
- **GnRH test**	40 minutes
- **hCG test (injection)**	10 min
Visit 2 (72 hours later)	
- **hCG test (blood sample)**	5 min

Extended program 3

Table 8: 1 Visit	
Investigations	Time/hours
- **Rubidium PET CT**	35 minutes

Statistical considerations

Sample size

In general, exact sample size calculation for observational studies is not unproblematic and is primarily used for randomized controlled trials, but to do the best to ensure an appropriate sample

size, we have calculated sample sizes for the prospective cohort study with registry follow-up and the three sub studies.

For the **prospective cohort study** with registry-based follow-up, a sample size of 800 AAS users is appropriate based on the following calculations:
A major interest in the present study is risk of CVD among AAS users and therefore chosen as primary endpoint for the prospective cohort study. A previous registry-based study noted a 3% incidence of "other forms of heart diseases" during a mean follow-up of seven years among 545 AAS users and that the risk was 3-fold lower among healthy age- and sex-matched controls than AAS users [3].
Using a reduced two-sided alpha (significance) level of 0.01 in our cohort study, due to expected multiple comparisons, and furthermore, using a factor 10 larger sample size of age- and sex matched registry-based control group (n = 8000) we find a power of 90% of detecting a 3-fold difference in heart diseases between the AAS group (n =800) and registry-based control group (n = 8000). With these assumptions and calculations, the risk of statistical type I and II errors both seem to be appropriately low in the prospective cohort study.

The sample sizes in the extended programs are based on a number of recent cross-sectional studies [8-9, 35-36].

For the **extended sub study 1** sample size is based on the following considerations:
Data from a recent study demonstrated that the total motile sperm count decreased among recreational athletes after one cycle of illicit AAS use and was persistently low one year following AAS cessation, decreased by 14 x 10^6 compared with prior to the AAS cycle [36]. We expect a major decrease in the total motile sperm count among current AAS users whereas a large variation could occur among former AAS users due to expected variation in elapsed duration since AAS cessation. Including a total of 200 AAS (approximately 100 current and 100 former AAS users) and 80 non-AAS users as control participants and using a 90% power and a reduced two-sided alpha level of 0.01 (due to three study groups and multiple comparisons in the study), we will be able to detect a difference in total motile sperm count of 5 x 10^6 (SD = 9) between the three study groups. We find this difference clinically relevant.

For the **extended sub study 2** sample size is based on the following considerations:
Data from a prior study demonstrated lower mean serum total testosterone of 10.2 nmol/L in persistently hypogonadal former AAS users following a hCG stimulation test compared with non-AAS users who were hypogonadal due to unknown reasons [35]. We expect a lesser group difference in mean serum total testosterone following the hCG stimulation test in our study as the participants are not all hypogonadal, although a large individual variation in serum total testosterone could occur as we expect a more heterogenic group of participants. Including 30 participants in each of the two study groups (former AAS users and controls) and using a 90% power and a reduced two-sided alpha level of 0.01 (due to multiple comparisons in the study), we will be able to detect a difference in

serum total testosterone of 5 nmol/L (SD = 5) between the two study groups following the hCG stimulation test. We find this difference in serum total testosterone clinically relevant during a hCG stimulation test.

For the **extended sub study 3** sample size is based on the following considerations:

A difference in Myocardial Blood Flow of 0.5 ml/g/min is considered clinically relevant. With a SD of measurements of global MBF by Rb-82 PET of 0.5, based on recent studies in the department; a sample size of 30 participants is required is each of the three male study groups with a power of 90 % and a reduced two-sided alpha level of 0.015 (three study groups and multiple comparisons in the study) to detect a difference of 0.5 ml/g/min.

Data analyses

Analyses of basic anthropometric data, clinical characteristics and the measured biomarkers will be performed to elucidate the underlying phenotypic characteristics on the AAS cohort, with the healthy athlete cohort acting as controls. Cross-sectional data will be analyzed using conventional parametric and non-parametric analyses as appropriate and with post-hoc adjustment for multiple comparisons. Prospective follow-up will rely on registry-based data from national registries as described above and is planned to consist of data on the three cohorts.
Planned prospective analyses include survival analyses of time-to-event data testing the impact of comorbidities and baseline characteristics distinguishing the cohort from controls. Competing risks analysis or extending analyses to adjust for recurrent events will be performed if deemed necessary.
Though no machine learning-based statistical methods are planned, future exploratory analyses investigating the predictive power of the found clinical characteristics will adhere to the following:

- In case the statistical analyses need a random seed, the seed chosen will be '343635' (FIDO-DK) to ensure integrity of approach and reproducibility of results.
- The statistical models are to be subject to cross-validation, using an 80/20-split to train the model and subsequently test the predictive power of the models, respectively. Further, a 5-fold cross-validation will be used unless suggestions from an independent statistical advisor provides evidence of a different approach providing a more robust estimate.
- The final model will use all data and be the product of the best performing test.

Time schedule

- September 2020– April 2022: Application for funding and approval from local ethical committee on health research
- June 2022: First participant first visit
- June 2025: Last participant last visit
- July 2025 – June 2026: Lab and statistical analyses
- July 2026 – November 2027: writing of manuscripts and publications
- Data extraction recurring every three years counting from date of last participant visit +/- 3 months until 15 years of follow-up (i.e. approximately July 2040)

Feasibility

Conducting the study is deemed highly feasible. All equipment and personnel with the necessary experience and expertise for the practical conduction of the study are employed at Rigshospitalet in Copenhagen. Principal investigator, Professor Caroline Kistorp and co-investigator post.doc Jon Rasmussen have previously conducted community-based clinical research among current and former AAS abusers and published several papers on the subject in well-recognized international peer-reviewed journals [4–7,9]. Our experience from previous studies in illicit AAS users is that recruitment of several hundreds of AAS as many of these are highly motivated to participate and, furthermore, in- and exclusion criteria are few which increases the probability of recruiting a study cohort which is representative of illicit AAS users in the community. The current study is an apparent continuation of our previous research in the field. Furthermore, the research group has previously been involved in the conduction of several clinical trials including other patient groups. Jon Rasmussen is employed as post-doctoral scientist, 20% of full-time for six years, and work on the study during this time and a PhD student (Yeliz Bulut) will be involved with the study full-time. In addition, a collaboration has been established with Professor and Head of Department, Anders Juul and Consultant, Niels Jørgensen, Department of Growth and Reproduction, Rigshospitalet, Associate Professor Philip Hasbak, Department of Nuclear Physiology, Professor, Finn Gustafsson, Department of Cardiology, Rigshospitalet and Professor Lars Kessing, Department of Psychiatry, Rigshospitalet and Associate Professor, Morten Schou, Department of Cardiology, Herlev-Gentofte Hospital, Professor Marianne Andersen and Professor Jan Frystyk, Department of Endocrinology, Odense University Hospital, Associate Professor, Ebbe Eldrup, Herlev and Gentofte University Hospital, and Professor, Michael Kjær, Bispebjerg and Frederiksberg University Hospital.

Data extraction from participant files and hospital records

Source data will be composed of the participants' hospital records, files and charts (including electronic charts), and laboratory/scan reports up to 15 years after participation in the study. Direct access to source data is allowed during audit and inspection from national or local ethics committee on biomedical research, the Danish Data Protection Agency and other relevant health authorities. Direct access to source data is allowed for the primary investigator, sponsor and the sponsor's representatives. This must be accepted by participants and is explicitly stated in the informed written consent. All legislations, regulations and laws of the Danish Data Protection Agency will be complied with.

The participants consent to investigators reading and the transfering information from the electronical hospital records to investigators and REDCap. The transfered data include medical history, blood and urine test results, previous DXA scan results, echocardiographic and CT scans, and prescribed as well as over-the-counter medication. Consent includes giving permission for the study group to see all blood test results as AAS use may be associated with damage or illness in every organ system. Furthermore, consent includes permission to retrieve information on prescribed and over-the-counter

medication, as it may influence the risk and progression of disease. hence all information on medication is relevant.
In the initial assessment of the eligibility of participants, who have contacted the investigators, will give oral consent of access to their hospital records to allow the investigators the ability to assess whether the individual characteristics is in line with the stated inclusion and exclusion criteria. Investigators will only tranfer source data after the acquisition of oral and written informed consent.

Registry follow-up

To avoid bias due to inevitable data-loss, we want to compare participants who drop out or who are lost-to-follow-up to those continuing in the study. Therefore, the present application from this study group applies for permission to collect registry data after anonymization regarding ICD10 diagnostic codes, medical treatment, socioeconomic status, mortality and morbidity in all participants, including those who drop out or are lost to follow-up.
The data will be kept in encrypted format for the duration of the trial. The duration of the trial is 15 years.

Data protection of Personal Information in the Study

All legislations, regulations and laws of the Danish Data Protection Agency will be complied with. Permission to handle personal data will be sought from the Danish Data Protection Agency. All data are stored pseudo-anonymized and analyzed electronically and no unauthorized access to data is allowed. Original data is filed according to a unique participant number. REDCap, hosted by OPEN (Open Patient data Explorative Network) will be used for registration of clinical data. REDCap meets the safety requirements set by the Danish Data Protection Agency for storage of person-sensitive data; prescribed medication, medical history, height, weight are examples of such data. Data will be encrypted and stored for 15 years in accordance with recommendations on data storage from the Danish Data Protection Agency and thereafter transferred to the Danish Data Archives.
The study is reported to the Danish Data Protection Agency Pactius in Region H and will be handled according to the regulations of the General Data Protection Regulation: GDPR: REGULATION (EU) 2016/679 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 27th of April 2016 on the protection of natural persons with regard to the processing of personal data and on the free movement of such data, and repealing Directive 95/46/EC and the Act on Processing of Personal Data. Data is stored for 15 years.

Economy, Funding and Participant Insurance

The initiator of the study Caroline Kistorp, a professor and researcher at the Department of Medical Endocrinology at Rigshospitalet, University Hospital, Denmark. The study is solely funded by external private and public foundations. Any funding is deposited in a research account administered at Rigshospitalet, University Hospital, Denmark. None of the investigators or departments will have any financial gain from conducting the study. The participants will not receive payment, but transportation cost will be covered according to the guidelines of the Capital Region of Denmark.
The investigators have received an unrestricted grant from the Novo Nordisk Foundation of approximately 10 million Dkr for the interdisciplinary project: “Fitness Doping in Denmark (FIDO)

novel treatment strategies and somatic and mental health complications with anabolic androgenic steroids', grant number: 0065138, which comprises several studies including the present trial. Furthermore, Jon Rasmussen, post.doc, MD, PhD has received 590.000 Dkr from "Region Hovedstadens Forskningsfond til Sundhedsforskning" to be used for conducting the present trial. Funding ensuring the completion of the trial is therefore in place.

Participants in the trial are covered by the existing patient insurance "Patienterstatningsordningen og ordningen om erstatning for lægemiddelskader". Healthy athletes participating as controls are covered by the existing insurance "Arbejdsskadeforsinkringen".

Recruitment of Participants and Informed Consent

Initial contact

Patients from the endocrine outpatient clinic at the Department of Medical Endocrinology, Center of Cancer and Organ Disease at Rigshospitalet, who are eligible to participate in the study, will be informed of the possibility to participate in study by a physician when they attend the outpatient clinic for a scheduled visit. If the patient is interested in further information on the study, contact information on the investigators will be given to the patient. If the patient favors contact being initiated by an investigator from the study instead and provides oral consent, the patients contact information will be forwarded to an investigator. Posters informing of the study will be displayed in the hospital departments and information about the study will be advertised on the facebook, and on varies homepages (sundhed.dk, antidoping.dk and forsoegspersonen.dk), where contact information on investigators can be found. Over the years we have gained much experience in recruiting AAS users for research studies in a discrete manner without incrimination using social media and we find that this way of communicating with potential participants works for both parties. Potential participants can contact the Departments of Endocrinology, Rigshospitalet by email or telephone. Regarding advertisements on Facebook, any advertisement/post will have its ability to be shared or write a comment to the advertisement/post disabled.

Following contact with one of the investigators if the individual is interested further written and oral information on the study will be given to the patient during an initial meeting or sent by mail. In addition to the written information pertaining to study, it will be accompanied by the brochure: "Forsøgspersoners rettigheder i et sundhedsvidenskabeligt forskningsprojekt" (Your rights as a participant in medical research). If the individual accepts further information on the study, a meeting in a quiet, undisturbed room will be scheduled with one of the investigators, and the person will be informed of the right to have a bystander present at that moment.

Scheduled meeting

At the scheduled meeting, the individual will receive additional oral information, including the pamphlet "Dit væv, din ret", from an investigator, and it will be clarified whether the potential participant fulfill the general participation criteria. The potential participant will be informed about the terms and restrictions of the form of 'informed written consent' and is informed of 24 hours deciding time. The investigator will ensure that the patient is adequately informed about the study

background and design both orally and in writing. It will be made clear that the patient can withdraw from the study at any time.

Acquisition of informed consent

If the person is still interested in participating in the study after the initial information meeting and after the allotted deciding time, a new meeting will be scheduled where oral and written informed consent will be obtained. After consent to participate in the study has been obtained, the visit (Core program: visit 1) will be scheduled.

No study-related examinations will be conducted prior the acquisition of informed consent. Investigators will only access and transfer electronic hospital records after acquisition of written informed consent.

Informed consent

The participant will be required to sign the individual forms of informed consent applicable.

Consent regarding core program, registry-based follow-up and research biobank

The participant's consent includes the right to read and transfer information from electronical hospital records to RED-Cap by investigators. The data transferred include information such as medical history, blood and urine test results, prescribed and over-the-counter medication. Further, the informed consent includes permission to establish a research biobank regarding the analyses of biomarkers specified in the present protocol. The informed written consent grants permission to use registry data prospectively after anonymization, including information on ICD10 diagnostic codes, medical treatment, socioeconomic status, morbidity and mortality.

The informed consent will be valid for the duration of the trial. The duration of the trial is 15 years.

Consent regarding extended program

Participants undergoing additional procedures will be required to sign additional forms of informed consent for each extended program. Data will be stored in REDcap as previously described and kept in encrypted form for the duration of the trial.

The informed consent will be valid for the duration of the trial. The duration of the trial is 15 years.

Consent regarding establishment of a biobank of future research

A separate form of informed consent gives permission of the establishment of a biobank of future research consisting of extra material, if any, acquired at the initial visit of the core program. The aim of the future research biobank is to ensure the ability of the cohort to answer contemporary research questions in a rapidly evolving field. Future analyses may therefore include, but not be limited to analyses of circulating levels of hormones, proteins, DNA-excerpts, not specified in the current protocol. Future analyses will be required to be approved by the regional ethics committee prior to analysis.

The informed consent will be valid for the duration of the trial. The duration of the trial is 15 years.

Risks and discomforts

Phlebotomy to acquire blood can be uncomfortable, but is considered safe. In comparison to blood donation, where 500 mL is donated, the current study will require a sample of 300 mL, which may induce slight lightheadedness, but is otherwise not associated with any known risk. No genome-

mapping is planned in the current protocol. In the event the routine blood panel gives indication of organ damage, which requires immediate action or a referral for further analysis, the attending physician is obliged to do so.
The radiation dose delivered by the DXA scans (0.001-0.004 mSV per scan) during the entire trial is comparable to less than or equal to half a day of radiation when compared to the natural background radiation (0.008 mSV/day) and hence does not form any known health risk [34]. Radiation has a stochastic risk of inducing spontaneous mutation, why pregnant women are ineligible to participate in the trial. The Rb-82 PET scan delivers radiation much less than a single photon emission tomography (SPECT) scan which is the current noninvasive clinical standard of diagnosing coronary artery disease. Radiation doses of Rb-82 PET scans (Participants in the extended Study program 3) are approximately 4 mSv per investigation including rest and stress.. In general, Danish citizens' lifetime risk of cancer is approximately 25.00%. Participating in this extended study program 3 increases the risk of cancer to approximately 25,02% (risk category: IIb) (International Commission on Radiation Protection (ICRP) and Europe Commission).

Ethical considerations

The study is in accordance with the Helsinki II declaration and the regulations of the General Data Protection Regulation. The study will be approved by the Danish Data Protection Agency and the Regional Ethics Committee of Copenhagen Denmark.
Our study involves the recruitment of AAS users, who have self-administered AAS for long periods and may continue to do so. All study participants will receive information material of the hazardous effects of AAS created in collaboration with AntiDoping Denmark. Further, to protect the participants from future stigma or potential prosecution, information specific to the use of PIEDs by each individual will only be registered in the database and will not appear in the medical journal. If we encounter persons, we find are in need of acute medical or psychiatric therapy (including suicidal risk) we will refer or admit the participant as appropriate to the relevant specialty.
In summary, illicit AAS misuse is associated numerous adverse effects on health related to almost every organ system. Furthermore, previous AAS users often suffer from prolonged male hypogonadism, increased cardiovascular risk, increased risk of infertility and a range of psychological symptoms related to this condition. We find that the potential therapeutic gain and benefits for the participants and future patients outweigh the risks and discomforts of participating in the study and therefore, we believe conducting the study is justified.

Dissemination of Results

The investigators oblige themselves to publish all clinically relevant findings in peer-reviewed journals irrespective of their ability to achieve statistical significance. Positive, inconclusive and negative results will be presented. Results will be published following the International Committee of Medical Journal Editors (ICMJE) recommendations. Further, the findings will be presented at national and international conferences.

Future perspectives

To the best of our knowledge, no trials have previously been conducted among men with AAS-induced hypogonadism and no consensus on therapy exists to mitigate the risks following the condition in Denmark or abroad. This study will provide incremental value to the evidence-based risk assessment of men and women current and former, which will be crucial in maintaining a normal health condition or mitigating the adverse effects of continued AAS use.

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