## PEER REVIEW HISTORY

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## ARTICLE DETAILS

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| **TITLE (PROVISIONAL)** | Impact of androgenic anabolic steroid use on cardiovascular and mental health in Danish recreational athletes: protocol for a nationwide cross-sectional cohort study as a part of the Fitness Doping in Denmark (FIDO-DK) study |
| **AUTHORS** | Buhl, Laust; Lehmann Christensen, Louise; Diederichsen, Axel; Lindholt, Jes; Kistorp, Caroline; Glintborg, Dorte; Andersen, Marianne; Frystyk, Jan |

## VERSION 1 – REVIEW

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| **REVIEWER** | Tsarouhas, Konstantinos<br>General University Hospital of Larisa |
| **REVIEW RETURNED** | 20-Nov-2023 |

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| **GENERAL COMMENTS** | The study protocol is novel and the new knowledge in the field would be significant. It would be interesting if the authors would include in their study apart from grip strength an evaluation of the intensity and volume of exercise on a weekly basis by their participants. Also, objective evidence of their cardiorespiratory fitness would be significant to be included.<br>A second very important point to be addressed is the study enrollment criteria. Objective evidence of anabolic use has to be included for the athletes of the study group at the time of evaluation. Along with self-reporting Hair analysis could prove long term use terminated at the time of the enrollment. Urine analysis could evaluate and quantify the current use of anabolics. The same applies for the control group, as contamination of supplements of athletes with anabolics has been widely reported. |

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| **REVIEWER** | Bond, Peter<br>PeterBond.org |
| **REVIEW RETURNED** | 25-Dec-2023 |

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| **GENERAL COMMENTS** | The authors describe a protocol to assess the cardiovascular and mental health in Danish recreational athletes with former or current use of androgens. The study will be cross-sectional in nature. The protocol is well-described, the measurements are comprehensive and adequately cover the research question, and the sample size is generous. The results of such a study would be of much interest to the field.<br><br>I have little to comment on this protocol, but I do have some minor questions and suggestions for the authors to consider.<br><br>The current inclusion criteria require current or former AAS use for at least 3 months and your primary endpoint is non-calcified |

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| | plaque volume (NCPV). I think it's unlikely that 3 months of AAS use will significantly impact NCPV. It might be interesting to increase the minimum time of exposure to, for example, a year, to increase the possibility of finding a difference in NCPV when there actually is one. Additionally I would recommend a minimum weekly androgen dose as a requirement too. You might now run the risk of AAS users on self-initiated TRTs that might be included (e.g. at a dosage of 150 mg testosterone weekly), which might skew results and also doesn't accurately fit the research question. Suggestion: a minimum weekly total androgen dose of 200 mg. I feel this would be more representative of actual AAS abuse.<br><br>By extension: you include both men and women. Patterns of AAS use among women differ markedly from that of men - as I'm sure you're well aware of. In many cases this involves steroid cycles aching to 5-10 mg of oxandrolone weekly for a couple of weeks. Also for this reason I think it's worth to consider a minimum weekly androgen dose to minimize null findings as a result of relatively low androgen exposure in women. (Inclusing criteria could perhaps differentiate between women and men in terms of androgen exposure, as it should also not make inclusion of women in practice unfeasible).<br><br>On line 161 you write: "The controls have to perform exercise at least two times per week.". I would suggest to explicitely require resistance exercise rather than exercise, as I believe it's more likely to lead to overlap in subject characteristics with (former) AAS users, thus strenghtening the results of your research when finding differences between groups.<br><br>You also measure hand grip strength: be sure to have a backup device as these dynamometers are prone to be broken by strength athletes.<br><br>On line 260 you describe measurement of cardiovascular risk markers in serum or plasma. I'm curious to which ones you'll be measuring. Will it be limited to total, HDL and LDL cholesterol, or will it also include ApoB, ApoA1 and lp(a)? I can imagine these more expensive markers are not feasible due to budget constraints. With regard to CRP: ensure it's the high sensitive CRP as this one in particular is used for prognostic values in terms of CVD risk.<br><br>In the statistics section you write: "We will test the individual data sets by performing appropriate statistical analyses dpeneding on the outcome and distribution of results". This is too brief.<br><br>Finally, as cumulative time (and dose) of exposure are likely to hold predictive value, I would urge to inquire about this with each subject as extensively as possible. It might help to have a structurized list of questions for this, as former AAS users sometimes "forget" about AAS cycles they've done depending on how the questions are asked. Additionally, in my experience they also often omit to note use of growth hormone, insulin, and experimental peptides when inquiring about other PIEDs unless explicitely mentioning the names of these drugs for example. |

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| **REVIEWER** | de Ronde , Pim<br>Spaarne Gasthuis |
| **REVIEW RETURNED** | 04-Jan-2024 |

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| **GENERAL COMMENTS** | This study protocol is well written in proper English. The introduction is elaborate and complete, properly highlighting the limits of current knowledge. Users of anabolic steroids are difficult to investigate. This has to do with ethical reasons; the substances used are usually obtained illegally and of dubious, often unknown composition. In addition, the method of use is heterogeneous, with major differences in type, dose and duration of use.<br><br>For this reason, I would recommend to introduce as much rigidity as possible into the study protocol in order to ultimately be able to draw meaningful conclusions.<br>The main problem with the present protocol is that, by design, it offers very little structure. Both men and women are included, exposure to steroids can vary from just 3 months to years of use, no threshold is mentioned for the minimum dose for inclusion and the studies can take place during or (years after) use.<br><br>These limitations add to the already high variability of studied population. This will lead to enormous heterogeneity of the data that is probably insufficiently compensated by the relatively low number of participants to be included. I therefore fear that the results of the current study will suffer from the same limitations as the results of already known studies; too limited to actually add substantially to current knowledge.<br><br>For instance:<br>-sex steroid levels are very different between men and women. In women, sex steroids fluctuate during the menstrual cycle or are highly influenced by hormonal contraceptives. In both men and women, sex steroid levels are highly influenced by steroid use and are therefore highly variable. As a result, random steroid hormone testing in men and women who are using of have recently used steroids is of little value.<br><br>-Other studied variables, such as myocardial structure and function, body composition, blood cardiovascular risk markers and psychological variables may vary dependent on whether the studied subject is on or off steroids (see for instance PMID: 36304014). In the current protocol participants may be either on or off steroids adding to the already high variability of studied population.<br><br>I have a fundamental remark concerning the inclusion of both men and women.<br>-as stated by the authors, the proportion of female AAS users is much lower compared to men. As a result, in the proposed protocol, potentially only a limited number of women may be included making it highly doubtful that these limited observations allow meaningful conclusions.<br><br>-steroid abuse and its medical consequences are fundamentally different between men and women. Women use much lower doses and obviously, have very different default steroid levels. As a result, cardiovascular effects are expected to be less compared to men. Similarly, sex steroid levels and mental effects are expected to be largely different compared to men.<br>Therefore, I would recommend to study men and women separately, securing a sizable number of female participants . |

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| | Also, the authors state that data on the effects of AAs in women are virtually absent. However, they neglect the substantial evidence that can be derived from studies in female-male transsexuals. |

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| **REVIEWER** | Gestsdottir, Sunna<br>Reykjavik University |
| **REVIEW RETURNED** | 05-Jan-2024 |

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| **GENERAL COMMENTS** | This is a comprehensive and very interesting study protocol that will add a great deal of new and vital information to the literature on the health of AAS users, and having input from long term AAS users on the study is of great importance.<br><br>The main concern is the balance and emphasis between mental and physical health parts. The physical health part is described in much more detail than the mental health part. This is especially concerning because in the Legend to Table 1 (p.21) you write: “Thus, at the time of writing we do not know whether we will perform the examinations marked as (X) in the cohort study [b]. This depends on findings in the pilot study [a]”. All the examinations marked as (X) in the cohort study [b] in table 1 are physical health measures. Therefore, the measures with the most detailed description in the protocol will be left out.<br><br>Many things are unclear about the questionnaire, e.g., what is measured by SF-36, what is your hypothesis on the association between AAS and ICAT (verbal memory, working memory, and psychomotor speed)? The rationale for including many of the measures in the questionnaire is missing, and there are no references regarding validation of the scales used.<br><br>The aim of the study only addresses mental health by saying “increased mental problems”.<br><br>In the last sentence of the aim: “Furthermore, we hypothesize that the changes are related to the magnitude and length of AAS use”. It is unclear what changes you are referring to.<br><br>As this is a comprehensive work with a lot of variables and measures it would be beneficial and make it easier for the reader if the same terms for the measures are used through the protocol e.g. in the abstract you use cardiovascular risk markers and body compositions parameters vs. in methods p.9 you use biochemical risk factors and body proportions. In table 1 you use clinical examination, but not in the methods, there is no mention of urine analysis in the table only in methods.<br><br>Even though your emphasis is on the outcome of AAS use it would be of interest to know the reason behind the start of AAS use in the first place, as studies have indicated that female AAS users started using AAS after sexual assault (Gruber, A. J., & Pope Jr, H. G. (1999). Compulsive weight lifting and anabolic drug abuse among women rape victims. Comprehensive psychiatry, 40(4), 273-277.). |

## VERSION 1 – AUTHOR RESPONSE

**Response to comments from reviewer 1:**

1. Inclusion of exercise metrics:

We appreciate the insightful suggestion regarding the evaluation of exercise intensity, volume, and cardiorespiratory fitness of our participants. To enhance the comprehensiveness of our study, we will incorporate an assessment of participants' weekly exercise intensity and volume using questionnaires. This will allow us to gather detailed information on both strength training and endurance training patterns for both AAS users and controls.

2. Objective evidence of anabolic use:

The peer reviewer rightly highlights the importance of objective evidence for anabolic use in both the study and control groups. We acknowledge the potential limitations associated with self-reporting and have revised our study enrollment criteria accordingly. To provide a more accurate assessment of AAS use, we will incorporate urine analysis to quantify the current use of anabolics. Additionally, blood tests measuring testosterone, FSH, and LH levels will be employed to distinguish and differentiate between AAS users and controls. This multifaceted approach aims to enhance the robustness of our study and address potential sources of bias.

We also recognize concerns about the control group and the possibility of supplement contamination. To address this, we will implement thorough screening measures, including urine analysis, to ensure that the control group remains free from anabolic substances. This will strengthen the validity of our findings and ensure that observed effects are attributable to anabolic steroid use rather than external factors.

We appreciate the constructive feedback and believe that these additions will significantly strengthen the study's methodology, providing a more comprehensive understanding of the relationship between anabolic steroid use, exercise metrics, and cardiovascular health.

**Response to comments from reviewer 2:**

We appreciate the thoughtful and detailed review of our study protocol. We have carefully considered each of the suggestions and questions raised and would like to address them as follows:

1. Duration and dose of AAS use:

We acknowledge the concern regarding the impact of a 3-month minimum duration of AAS use on our primary endpoint, non-calcified plaque volume (NCPV). Our study has aimed to investigate the continuously growing and heterogeneous population of AAS users, encompassing individuals with diverse fitness objectives, ages, genders, and socio-economic backgrounds. This inclusivity is the reason for setting a minimum requirement of 3 months without specifying a particular weekly androgen dose.

We also aim to ensure the recruitment of a diverse group of long-term users, allowing for subgroup analyses to investigate the relationship between long-term use and various pertinent endpoints.

In our preliminary analyses, we have observed a notable increase in the prevalence of both non-calcified plaque (NCP) and coronary artery calcium (CAC) among long-term AAS users. This early insight emphasizes the relevance of our study in uncovering potential cardiovascular implications associated with AAS use.

2. Gender-specific androgen exposure:

Recognizing the marked differences in AAS use patterns between men and women, we will conduct separate analyses for men and women. We believe it is highly relevant, especially given that women have never been systematically studied in this particular domain. This approach aims to contribute valuable insights into the gender-specific effects and risks associated with AAS use, filling a significant gap in the existing literature.

3. Exercise criteria for controls:
We appreciate the suggestion to explicitly specify resistance exercise as a requirement for controls, minimizing overlap in subject characteristics with AAS users. In light of our goal to examine the broad population of AAS users, encompassing individuals beyond strength trainers and weightlifters, we recognize the importance of ensuring a diverse and representative control group. Therefore, we will modify the inclusion criteria accordingly to include individuals engaging in both resistance exercise and regular exercise, for both AAS users and controls. This adjustment aims to provide a more comprehensive understanding of the associations between AAS use and cardiovascular health in a broader context, comparing with a healthy general population.
4. Measurement of cardiovascular risk markers:
Concerning the assessment of cardiovascular risk markers, our strategy extends beyond conventional evaluations of total, HDL, and LDL cholesterol. We aim to delve deeper by incorporating additional markers, including ProBNP, TNT, stanniocalcin, various IGF values, among others, all while adhering to budget constraints. The consideration of ApoB, ApoA1, and lp(a) as well as high-sensitive CRP may be introduced in subsequent stages of the research. This comprehensive analysis is designed to furnish a more intricate understanding of the cardiovascular profile associated with AAS use, contributing to a nuanced evaluation of potential health implications.
5. Statistics section clarification:
We acknowledge the brevity of the statistics section and will provide a more detailed description of the statistical analyses, specifying the methods chosen based on the outcome and distribution of results.
6. Cumulative exposure inquiry:
Recognizing the potential predictive value of cumulative exposure, we will enhance our data collection process by incorporating a structured list of questions. This will aid in capturing comprehensive information on AAS use, growth hormone use, insulin, and experimental peptides, reducing the likelihood of omissions or inaccuracies.
We sincerely thank for these valuable suggestions, which will undoubtedly strengthen the robustness and applicability of our study.

**Response to comments from reviewer 3:**
We appreciate the thorough review of our study protocol and the valuable insights provided. We acknowledge the complexity of investigating users of anabolic steroids and are grateful for the constructive feedback. We have carefully considered each point raised and provide the following responses:
1. Rigidity and study design:
We recognize the importance of introducing rigidity into the study protocol to enhance the interpretability of results. Our study is specifically crafted to investigate the continually expanding and heterogeneous group of AAS users, incorporating individuals with diverse fitness objectives, mental health purposes, ages, genders, and socio-economic backgrounds. These individuals may have diverse objectives in their AAS use, with some seeking aesthetic enhancements, while others aim to sustain their mental well-being. This rationale underlies the inclusion criterion of a minimum 3-month duration without specifying a required weekly androgen dose, aiming to capture the complexity within this evolving population.
In later stages of the analyses, we will conduct a detailed examination of various subgroups within the population. This will include an investigation into long-term users, exploring correlations between extended AAS use and various endpoints.
2. Inclusion of both genders:
We appreciate your concern about the potential limitations of including both men and women. In response, we will conduct separate analyses for men and women to address the differences in sex steroid levels, cardiovascular effects, and mental health consequences. By doing so, we aim to provide more meaningful and gender-specific conclusions. We will also make efforts to secure a sizable number of female participants to ensure the validity of our observations.
3. Consideration of female-male transsexual studies:

We appreciate the suggestion to consider evidence from studies involving female-male transsexuals. While we acknowledge the potential insights these studies may offer, we exercise caution in drawing direct comparisons with AAS users. Significantly differing testosterone doses between the two groups and the controlled administration of testosterone in transsexuals, in contrast to the less regulated conditions in AAS users, underline the need for careful interpretation. We will thoroughly review the literature and integrate relevant findings from these studies into our discussion, contributing to a more nuanced understanding of the effects of anabolic steroids in women. This addition aims to address the knowledge gap in the existing literature.

4. Timing of steroid use and variable effects:

We acknowledge the concern about the timing of steroid use and its potential impact on studied variables. To address this, we will gather detailed information on the current status of steroid use among participants and ensure that analyses consider the on/off status of steroid use, minimizing variability and providing a more nuanced interpretation of the results.

We are grateful for the insightful comments and will implement these recommendations to strengthen the study's design and improve the quality of our findings. Your feedback is instrumental in refining our approach to ensure the study's validity and contribution to the field.

**Response to comments from reviewer 4:**

We appreciate the thorough review of our study protocol and the thoughtful comments provided. We acknowledge the importance of balancing the emphasis between mental and physical health aspects and have carefully considered each point raised. Here are our responses and planned adjustments:

1. Balance between mental and physical health:

We acknowledge the concern about the perceived imbalance between the descriptions of mental and physical health measures. We will revise the protocol to provide more detailed descriptions of the mental health measures, addressing the specific concerns raised, such as the content of SF-36 and the hypothesis regarding the association between AAS and ICAT.

2. Unclear hypothesis and changes:

We recognize that the hypothesis related to mental health needs further clarification. We will explicitly outline the anticipated changes in mental health and specify how they are related to the magnitude and length of AAS use. This clarification will be reflected in both the aim and relevant sections of the protocol.

3. Consistency in terminology:

We appreciate the suggestion to maintain consistency in terminology throughout the protocol. We will ensure that the same terms are used consistently for measures and outcomes, addressing discrepancies noted in the abstract, methods, and table. This will enhance clarity and facilitate understanding for the readers.

4. Inclusion of reason for AAS use:

We acknowledge the significance of understanding the reasons behind the initiation of AAS use. We will incorporate this information into the study, capturing the motivations and circumstances leading to the commencement of AAS use, as suggested. This addition will contribute valuable context to the study findings.

5. Clarification of questionnaire rationale and references:

We will provide a more comprehensive rationale for the inclusion of each measure in the questionnaire, including specific details on SF-36 and the hypothesis regarding AAS and ICAT. Additionally, we will include references regarding the validation of the scales used, ensuring transparency and reliability of the questionnaire.

6. Addressing unclear terms in the aim:

We will revise the aim to explicitly outline the mental health aspects under investigation and clarify the specific changes hypothesized in relation to AAS use.

We appreciate the constructive feedback and will implement these adjustments to enhance the clarity, rigor, and overall quality of the study protocol. Your input is invaluable in refining our work and contributing to its significance in the literature on AAS users' health.

## VERSION 2 – REVIEW

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| **REVIEWER** | Bond, Peter<br>PeterBond.org |
| **REVIEW RETURNED** | 07-Mar-2024 |

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| **GENERAL COMMENTS** | The authors have adequately addressed or commented on the points that I raised. While I do not necessarily agree with all decisions, I do feel it is justified to accept the manuscript and study design in its current form. |

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| **REVIEWER** | de Ronde , Pim<br>Spaarne Gasthuis |
| **REVIEW RETURNED** | 26-Mar-2024 |

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| **GENERAL COMMENTS** | Although the authors substantiate their methodological choices in their answers to my questions, they do not remove my fundamental doubts about the chosen protocol. |