PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Masculinizing testosterone treatment and effects on preclinical cardiovascular disease, muscle strength and power, aggression, physical fitness and respiratory function in transgender men: Protocol for a ten-year, prospective, observational cohort study in Denmark at the Body Identity Clinic (BIC)
AUTHORS	Lehmann Christensen, Louise; Glintborg, Dorte; Taulbjerg Kristensen, Tine; Diederichsen, Axel; T'Sjoen, Guy; Frystyk, Jan; Skovsager Andersen, Marianne

VERSION 1 – REVIEW

REVIEWER	Mats Holmberg ANOVA, Karolinska University Hospital, Sweden
REVIEW RETURNED	21-Jul-2020

GENERAL COMMENTS	Review Manuscript ID bmjopen-2020-042607 Cardiovascular risk, respiratory function, muscle strength, and quality of life in transgender men Body Identity Clinic (BIC), Odense General comments: In general, this is a much awaited study and I strongly sympathize with the general aim of this rather large effort. As I sympathize with protocol publications. Prospective studies of adverse effects of cross-sex hormonal treatment in transgender individuals are scarce and even if most of these patients most likely will be too young to develop coronary disease during the 10 years this study will most likely generate new, important knowledge. A general comment is also that I think you are being too general in your description of the motifs behind and the design of the study. How about hypotheses and primary endpoints? How do you handle drop-outs? Power calculations? Can you be more specific regarding the included methodology and how you handle the multi-location setting? How do you plan to handle the combination of prospective data with registry data? How do you plan to differentiate changes from increased age from pathological changes? And so on. To put more work into these questions before starting the study not only strengthen your research but also makes your coming publications easier Below some specific comments Introduction R 60 why is this the most likely reason for the increse? Ref? R 61 masculinity? This word is not commonly used but interesting, masculinity is a much broader term than what we ever can achieve with hormones. I would use “Masculinizing treatment”.

	R 64 muscle growth is only one of the factor that changes the body shape, redistribution of fat is also important R 65 100-fold. This is true for very few extremes but at the same time misleading since the vast majority of transmen increase their testosterone levels more closer to 10-fold than 100-fold. Respiratory function R 85 It is possible that hormones are involved in differences in respiratory function between cismen and ciswomen but there may be other explanations such as genetic and lifestyle differences R 88 VO2max is dependant on Hb concentration. Which likely increases in most transmen. Don´t you expect that to increase VO2max? Questionnaires on aggression and quality of life (QoL) R102 I think using this reference for the first line is not correct. The article refers to WPATH SOC 7 but in itself contradicts that warning. Aim Fine but how about hypotheses? Methods and analysis Study population and recruitment All transgender men? But they have to be untreated at inclusion won´t they? Transgender is a wide definition and so is “treated” but I suppose this is meant to be individuals with a diagnose of gender dysphoria who are treated with hormones. Don´t mean to be picky but I think you can be more specific. Does it include those who have selfadministered testosterone prior to diagnose? It is also clear that the study is a multicentre study. Will you bring all the patients to Odense for investigation or will they be investigated at different locations? How do you handle this? Study design R 126 What will be done at each of these timepoints?, I think it´s a good idea to add this information here just to make reading esier. R 127 and those who already have started therapy? Will they be accepted? R 128 Do you intend to use these registries? You write previously (r55) that you lack hard endpoints but it´s hard to imagine a harder one than death! You need to be more specific so we understand more …. Outcomes Data analysis plan R 144 There is lot´s of data available for power calculations but in order to calculate you need to have a primary endpoint. What about dropouts? You have a 10 year follow-up which is one of the great qualities of this study, but the long follow-up also increases the risk of drop-outs. So how many do you need remaining after 10 years in order to find clinically relevant, significant differences in your primary endpoint? I would spend more time calculating and thinking about this. Discussion R168 “female and birth” should be “female at birth” R 169 “We have some experience with elevated testosterone levels in cisgender women”. Suggest: “In certain conditions, elevated endogenous testosterone levels are found in cisgender women”. R 173 100-fold, I would change this since it is not standard. It also sounds strange when you mention previously that PCOS can have 2 fold (3 nmol/L as an example) while transmen can have 100 fold (usually 10-20 nmol/L).

	R 178 CT at three different locations? Or are you bringing all to Odense? How do you secure that all investigations are done the same way? R 179 Echocardiography. This is very user dependant, how do you handle that? R 189 The article you are referring to included hypogonadal men over 65. You have to inform the reader about this fact since your study most likely will include much younger patients. It wouldn´t hurt to inform the reader a little bit more about the great debate about cardiovascular risk with testosterone treatment of hypogonadal men. There are tons of publications on this. R 191 “we have some information” sound a little bit like it´s information that only you have, but you are referring to previous publications? R 199 reference? Respiratory function R 214 reference? I think you can skip prepubertal information since you are not including these patients. R 231 This looks like a hypothesis. I would not say that it “is” expected. It is you that hypothesize that the VO2max will be higher in testosterone treated transgender men compared to ciswomen. I think it is a reasonable hypothesis but when? Already after 1 year? Or more? R 233 data is lacking? There is data on both increased muscle mass and increase haematocrit. R 239 “hyperandrogenism, PCOS”, two groups or one? Write hyperandrogenism (PCOS) or just PCOS instead. Muscle strength and body composition R 260 Here is another interesting study: Effects of moderately increased testosterone concentration on physical performance in young women: a double blind, randomised, placebo controlled study, Br J Sports Med 2019 Questionnaires on aggression and QoL 280 ref till WPATH warning Table 1 Why have you skipped two cardioinvestigations at 10 years? If there is an effect on the cardiovascular system it is most likely a slow one and thus this is the timepoint where you are most likely to find any difference. What is your primary endpoint? Table 2 There is no need to be so general about what testing you are planning. As an example: Instead of “hormones and binding proteins” write what you plan to test. Which hormones and by which methods a.s.o.

REVIEWER	Sohaib Haseeb James Cook University, Australia
REVIEW RETURNED	01-Aug-2020

GENERAL COMMENTS	The paper by Lehmann Christensen et al. presents a protocol of a prospective long-term follow-up study of transgender men during testosterone therapy. The authors aim to investigate the short- and long-term effects of testosterone on vascular risk, respiratory function, muscle strength, and QoL in transgender men living in Denmark. This mixed-methods analysis sheds light on an important topic that is relevant to medical care. The paper is written in a satisfactory

	way, and the need to conduct this study is clearly defined. However, this is an ambitious attempt to broadly assess many outcomes in one study, which, at points, may make it tedious to weigh the entire piece together. Specific comments: The “outcomes” section should be expanded upon, ideally mentioning primary and secondary outcomes. A short paragraph on statistical analysis will be beneficial. How will incomplete answers be dealt with? Will open-ended questions be organized thematically? Et cetera. To date, cardiovascular research in the transgender population has focused on the association of hormone therapy on myocardial infarction, stroke, venous thromboembolism, diabetes, and hypertension. The incidence of arrhythmia due to hormone therapy has not been adequately evaluated. Does your study assess electrocardiographic parameters or document arrhythmic episodes (AF, PVCs and VT)? This may be important to further delineate the role of hormone therapy on the cardiovascular system. The study has several limitations that will need to be considered when interpreting its results in the future. There should be a section addressing them. The mention of the recruitment site in the title may be confusing to international readers. Perhaps re-consider its inclusion in the title.

VERSION 1 – AUTHOR RESPONSE

Reviewer Name: Mats Holmberg, ANOVA, Karolinska University Hospital, Sweden
General comments
In general, this is a much awaited study and I strongly sympathize with the general aim of this rather large effort. As I sympathize with protocol publications. (A) Prospective studies of adverse effects of cross-sex hormonal treatment in transgender individuals are scarce and even if most of these patients most likely will be too young to develop coronary disease during the 10 years this study will most likely generate new, important knowledge. (B) A general comment is also that I think you are being too general in your description of the motifs behind and the design of the study. (C) How about hypotheses and primary endpoints? (D) How do you handle drop-outs? (E) Power calculations? (F) Can you be more specific regarding the included methodology and how you handle the multi-location setting? (G) How do you plan to handle the combination of prospective data with registry data? (H) How do you plan to differentiate changes from increased age from pathological changes? And so on. To put more work into these questions before starting the study not only strengthen your research but also makes your coming publications easier

Regarding general comments
Thank you so much for reviewing our paper and for acknowledging that this is an important study ‘that will most likely generate new, important knowledge’. Your thorough comments give us the opportunity to improve our protocol paper, substantially.

A: ‘Prospective studies of adverse effects of cross-sex hormonal treatment in transgender individuals are scarce and even if most of these patients most likely will be too young to develop coronary disease during the 10 years this study will most likely generate new, important knowledge’.
Re A: We fully agree, that coronary disease is not very prevalent in young men. Therefore, we assess non-calcified coronary artery plaque volume to detect preclinical coronary involvement. We use contrast cardiac CT as it is a very sensitive method in combination with a semi-automated computer program (Dey et al. Eur. Radiology 2018, Otaki et al. European Heart Journal- cardiovascular imaging 2015). A participant will be his own control. Furthermore, the availability of national registers will give us access to data from individuals, who are lost to follow-up in the clinical study.

B: ‘Too general in your description of the motifs behind and the design of the study’
Re B: We apologize for the lack of clarity and we fully agree that we can improve the manuscript. Already during the planning phase and before funding, we discussed the design with Guy T’Sjoen, (T’Sjoen et al. Endocrine Reviews 2019), who has initiated European Network for the Investigation of Gender Incongruence (ENIGI) (Dekker et al. J Sex Med 2016). According to professor Guy T’Sjoen, Ghent, Belgium and others within the research community on transgender related issues, there was a lack of knowledge regarding coronary pathology, muscle strength and power, physical fitness, respiratory function and, especially, aggression among other psychological issues.
Often muscle function is estimated by grip strength or lower body muscle strength, however, as androgen receptors are most abundant in upper body muscles (Kadi et al. Histochemistry and cell biology 2000), it would be of interest to thoroughly test the strength and power of these muscles. We collaborate closely with Per Aagaard https://orcid.org/0000-0002-9773-7361 who will be handling the practical part, as well as co-authoring papers on muscle function.

C: ‘How about hypotheses and primary endpoints?’
Re C:
Hypotheses

- Testosterone therapy is associated with non-calcified coronary plaque development in transgender men.
- Long-term beneficial changes in muscle strength and power, VO_2max, cardiac and respiratory function will be seen during testosterone therapy in transgender men.
- Aggression scores will be significantly higher during initiation of testosterone therapy, but the scores will decrease during long-term testosterone therapy.

Primary endpoint
Non-calcified coronary artery plaque volume
Secondary endpoints
Upper body muscle strength and power
Aggression and quality of life
VO_2max
Ejection fraction and left ventricular muscle mass
Respiratory function
Cardiovascular risk markers in blood and serum
Inflammation markers in blood and serum
Urinary and hair cortisol

D: ‘How do you handle drop-outs?’
Re D: We highly acknowledge the risk of participants being lost to follow-up during a 10-years-study. By combining the clinical outcomes (figure 1 and table 1) with registry data (figure 1) we will be able to compare study participants with participants lost to follow-up. The study group has ethical permission to use registry data regarding International Statistical Classification of Diseases and Related Health Problems (ICD-10) diagnostic codes, medical treatment, socioeconomics and causes of death in all

participants including those, who are lost to follow-up. The participants accepts by written informed consent. All individuals in Denmark are registered with a civil registration number (CPR) and when the CPR is changed during legal transitioning, the two CPR numbers are merged.

E: 'Power calculations?'
Re E:
Power calculation
Primary endpoint: Non-calcified coronary artery plaque volume

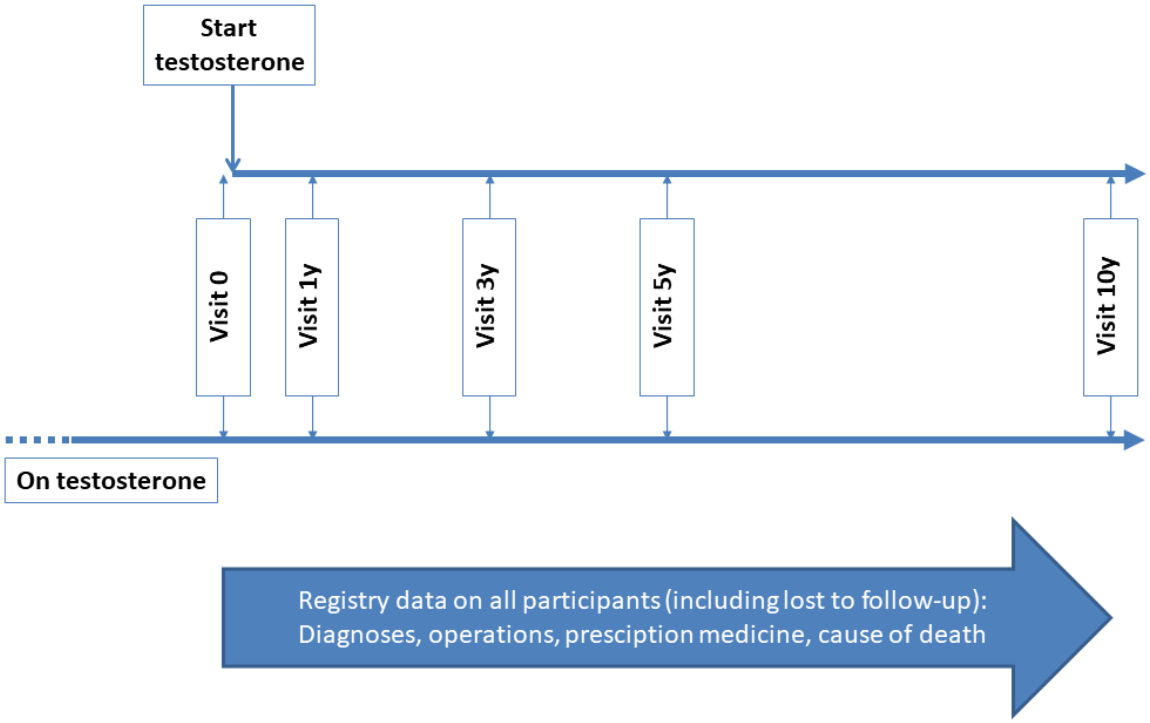
We believe, that even a small increase in non-calcified coronary artery plaque volume ($1mm^3$), in these young people should be regarded as subclinical disease and accordingly treated as such. We acknowledge the risk of a negative result, but a negative result would be very reassuring and clinically relevant. We do understand the quest for a proper power calculation. However, in line with a previous paper (Wiik et al. JCEM 2020), we must try to estimate as there is no valid data.
In a large cardiac CT registry study 545 participants (mean age 38 ±6 years, 70% men) were registered as having no cardiovascular symptoms (Otaki et al. European Heart Journal Cardiovascular Imaging 2015). Coronary plaques were observed in 22% of men and 15% of women, respectively. The coronaries were assessed by experienced readers. We intend to increase the sensitivity of the method by using a semi-automatic program, comparable to Autoplaque (Dey et al. Eur Radiol 2019), for plaque characterization making it is possible to report non-calcified coronary artery plaque volume (mm^3). We do not expect that any of our participants will have non-calcified coronary artery plaques. However, we expect during a 10-years follow-up period at least 15% of our participants will have detectable non-calcified coronary plaques.

Power calculation
Main secondary endpoint: Upper body muscle strength and power
Sample size estimation for upper extremity muscle strength and power was based on data (mean baseline/ standard deviation) in a similar cohort (Seo et al. J Sports Sci Med 2012). The estimation was based on a 10 % within-participant difference (deemed as functional relevant) with an alpha level of 0.05 and a statistical power of 0.80. On the basis of these data, a sample size of 39 was deemed sufficient to detect within-participants differences with two-tailed comparison. To account for potential dropouts (estimated to 20 %) 50 individuals approved to start testosterone treatment will be recruited for the trial.

F: 'Can you be more specific regarding the included methodology and how you handle the multi-location setting?'
Re F: Our study is a single-center observational cohort study at Odense University Hospital, Denmark. The participants in the study will spend one day in Odense, where all examinations are carried out using the same equipment and protocols, we pay for transportation.

G: 'How do you plan to handle the combination of prospective data with registry data?'
Re G: By combining the clinical outcomes (figure 1 and table 1) with registry data (figure 1) we will be able to compare study participants with participants lost to follow-up. The study group has ethical permission to use registry data regarding International Statistical Classification of Diseases and Related Health Problems (ICD-10) diagnostic codes, medical treatment, socioeconomics and causes of death in all participants including those, who are lost to follow-up. The participants accepts by written informed consent. All individuals in Denmark are registered with a civil registration number (CPR) and when the CPR is changed during legal transitioning, the two CPR numbers are merged.

Figure 1, study outline.



H: 'How do you plan to differentiate changes from increased age from pathological changes?'
Re H: It is very difficult to differentiate if sub-clinical changes are due to biological ageing per se or testosterone treatment. We address this issue in two ways. Firstly, our subjects will be their own control for within subject comparison, prospectively. Secondly, we compare data from the included individuals with data from other cohorts. We have performed a large cross sectional study in young healthy men (20-29 years old and somewhat comparable with the expected age of the included transgender men) representative of the background population in Fünen, Denmark (Nielsen et al. 2007) and we are about to reinvestigate these men in 2021 (follow-up time 14 years). We have similar data in women with PCOS with planned reinvestigation after 10 years.

Introduction
R 60: 'why is this the most likely reason for the increase? Ref?'
Re 60: We agree that this is speculative and will revise our paper.
The number of transgender individuals attending health care facilities in Denmark is increasing, and may be due to growing awareness and availability of care facilities.

R 61: 'masculinity? This word is not commonly used but interesting, masculinity is a much broader term than what we ever can achieve with hormones. I would use "Masculinizing treatment".'
Re R61: We agree to use 'Masculinizing treatment'.
Masculinizing treatment is testosterone therapy.

R 64: 'Muscle growth is only one of the factors that changes the body shape, redistribution of fat is also important'
Re R64: The redistribution of fat is certainly important and we have added this to the paper
The androgen effects of testosterone include increased terminal hair growth and deepening of the voice, whereas the anabolic effects include stimulation of muscle growth. In combination with the redistribution of subcutaneous fat associated with testosterone treatment (Magnussen et al. Andrology 2017, Frederiksen et al. Age 2012) body shape changes from feminine to masculine.

R 65: ‘100-fold. This is true for very few extremes but at the same time misleading since the vast majority of transmen increase their testosterone levels more closer to 10-fold than 100-fold.’
Re R65: In healthy, non-obese men, the 95% reference interval for total testosterone is 12.5-37.6 nmol/l (Nielsen et al. 2007). Conversely, the reference interval in premenopausal women is 0.35-1.97 nmol/l (Glintborg et al. 2018). Many transgender men aim at testosterone levels within the high range of the reference interval, and thus some transgender man may have up to 100 fold increase in testosterone levels when initiating testosterone therapy. However, we agree, that the average increase may be around 10-20- fold. We will revise this section in the paper.
Circulating testosterone levels in transgender men are within the normal range for cisgender men, which is up to 10020-fold higher compared to cisgender premenopausal women.

Respiratory function
R 85: ‘It is possible that hormones are involved in differences in respiratory function between cismen and ciswomen but there may be other explanations such as genetic and lifestyle differences.’
Re R85: We agree and will revise the paper.
Suggesting that sex hormones, in contribution with genetic factors and lifestyle, regulate respiratory function.

R 88: ‘VO_2max is dependent on Hb concentration. Which likely increases in most transmen. Don´t you expect that to increase VO_2max?’
Re R88: We hypothesize, that testosterone therapy in transgender men will induce beneficial changes in VO_2max during the first year, and, in theory, the VO_2max will stay above or equal to baseline. In men already on testosterone, we expect VO_2max to be above female reference range but below male reference range of VO_2max.

Questionnaires on aggression and quality of life (QoL)
R102 ‘I think using this reference for the first line is not correct. The article refers to WPATH SOC 7 but in itself contradicts that warning.’
Re R102: We apologize, we have inserted this reference.
Increased risk of aggression is listed as an adverse event of testosterone therapy in transgender men (Coleman et al. International Journal of Transgenderism 2012).

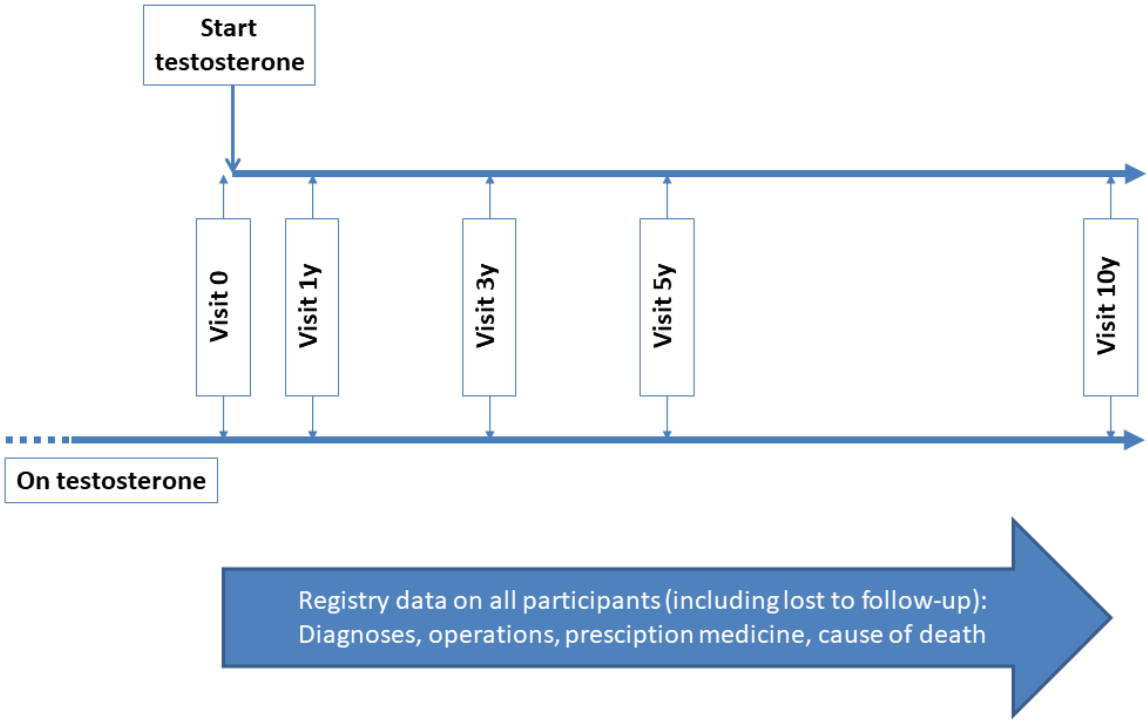
Aim
‘Fine but how about hypotheses?’
Re Aim: Hypotheses

- Testosterone therapy is associated with non-calcified coronary plaque development in transgender men.
- Long-term beneficial changes in muscle strength and power, VO_2max, cardiac and respiratory function will be seen during testosterone therapy in transgender men.
- Aggression scores will be significantly higher during initiation of testosterone therapy, but the scores will decrease during long-term testosterone therapy.

Methods and analyses
Study population and recruitment
‘All transgender men? But they have to be untreated at inclusion won´t they? Transgender is a wide definition and so is “treated” but I suppose this is meant to be individuals with a diagnosis of gender dysphoria who are treated with hormones. Don´t mean to be picky but I think you can be more specific. Does it include those who have self-administered testosterone prior to diagnose? It is also clear that the study is a multi-center study. Will you bring all the patients to Odense for investigation or will they be investigated at different locations? How do you handle this?’
Re study population and recruitment: Thank you very much for this question. Our study is a prospective single center observational cohort study at Odense University Hospital, Odense,

Denmark. The participants in the study will spend one day in Odense, where all examinations are carried out using the same equipment and protocols. Participants are individuals with a diagnosis of gender dysphoria who are assigned female at birth and treated with testosterone or approved to start treatment with testosterone. We include only individuals associated with one of the 3 centers of gender identity in Denmark: Odense, Aalborg and Copenhagen. No individual will be included, where we know they use 'self-prescribed' hormones. The study design is outlined in figure 1 and will be included in the paper. The study description has been revised.
The study is a prospective single-center observational cohort study at Odense University Hospital with duration of ten years.

Figure 1, study outline.



Study design
R 126: ‘What will be done at each of these time points? I think it´s a good idea to add this information here just to make reading easier.’
Re R126:
All investigations will be performed at each time point (inclusion, 1, 3, 5 and 10 years) with the exception of 3 and 5 years where CT is not performed. We have moved the CT from 5 to 10 years in accordance with your suggestion.

Modified table 1
Table 1

Investigations	Inclusion	1 yr.	3 yr.	5 yr.	10 yr.
Contrast cardiac CT	X	X			X
Muscle strength and power	X	X	X	X	X
Aggression, quality of life questionnaires	X	X	X	X	X
VO_2max	X	X	X	X	X
Echocardiography	X	X	X	X	X
Spirometry	X	X	X	X	X
Blood, urine and hair samples	X	X	X	X	X
Dual x-ray absorptiometry whole-body and bone density	X	X	X	X	X
Medical history	X	X	X	X	X
Physical examination	X	X	X	X	X

Our study is a single-center observational cohort study at Odense University Hospital, Denmark. The participants in the study will spend one day in Odense, where all examinations are carried out using the same equipment and protocols.

R 127: ‘and those who already have started therapy? Will they be accepted?’
Re R127: Participants are individuals with a diagnosis of gender dysphoria, who are assigned female at birth, and treated with testosterone or approved to start treatment with testosterone. We include only individuals associated with one of the three gender identity centers in Denmark: Odense, Aalborg and Copenhagen. No individuals will be included, where we know they use ‘self-prescribed’ hormones (figure 1).

R 128: ‘Do you intend to use these registries? You write previously (r55) that you lack hard endpoints but it´s hard to imagine a harder one than death! You need to be more specific so we understand more’

Re R128: By combining the clinical outcomes (figure 1 and table 1) with registry data (figure 1) we will be able to compare study participants with participants lost to follow-up. The study group has ethical permission to use registry data regarding International Statistical Classification of Diseases and Related Health Problems (ICD-10) diagnostic codes, medical treatment, socioeconomics and causes of death in all participants including those, who are lost to follow-up. The participants accepts by written informed consent. All individuals in Denmark are registered with a civil registration number (CPR) and when the CPR is changed during legal transitioning the two CPR numbers are merged.

Outcomes
Data analysis plan
R 144: 'There is lot´s of data available for power calculations but in order to calculate you need to have a primary endpoint. What about dropouts? You have a 10 year follow-up which is one of the great qualities of this study, but the long follow-up also increases the risk of drop-outs. So how many do you need remaining after 10 years in order to find clinically relevant, significant differences in your primary endpoint? I would spend more time calculating and thinking about this.'
Re R144:
Primary endpoint: Non-calcified coronary artery plaque volume.

We highly acknowledge the risk of participants being lost to follow-up during a 10-years-study. However, we have obtained ethical permission to access register data from all participants including those lost to follow-up. Register data will enable us to have knowledge on both active participants and participants lost to follow-up regarding diagnoses from all Danish hospitals (ICD-10), operations, prescription medicine and causes of death, these data will give us the possibility to describe drop-outs and to compare these individuals to those who stay in the clinical study. We will perform sensitivity analyses including and excluding drop-outs.
It is not possible to calculate the exact number of participants we 'need remaining after 10 years in order to find clinically relevant, significant differences in your primary endpoint'. We have based the number of included participants (N= 200) on an estimate of what is realistically obtainable (around 300 referrals for gender dysphoria and testosterone treatment in Denmark per year, we estimate that up to 1/3 will participate per year), we plan for a high percentage of individuals included before testosterone treatment is initiated (50 of the 200).
We hope that 50 % will remain in the study for 10 years or reach a hard endpoint based on hospital diagnoses. We acknowledge the risk of a negative result, but a negative result would be reassuring and clinically relevant.

Discussion
R168: '"female and birth" should be "female at birth"'
Re R168: We apologize and have corrected this in the paper.
Transgender men are assigned female at birth and are exposed to high levels of exogenous testosterone.

R 169: '"We have some experience with elevated testosterone levels in cisgender women". Suggest: "In certain conditions, elevated endogenous testosterone levels are found in cisgender women".'
R R169: Thank you for the suggestion and we have changed accordingly in the paper.
In certain conditions, elevated endogenous testosterone levels are found in cisgender women.

R 173: '100-fold, I would change this since it is not standard. It also sounds strange when you mention previously that PCOS can have 2 fold (3 nmol/L as an example) while transmen can have 100-fold (usually 10-20 nmol/L).'

Re R173: In healthy, non-obese men, the 95% reference interval for total testosterone is 12.5-37.6 nmol/l (Nielsen et al. 2007). Conversely, the reference interval in premenopausal women is 0.35-1.97 nmol/l (Glintborg et al. 2018). Many transgender men aim at testosterone levels within the high range of the reference interval, and thus some transgender man may have up to 100-fold increase in testosterone levels when initiating testosterone therapy. However, we agree, that the average increase may be around 10-20- fold. We will revise this section in the paper.
Testosterone levels are increased up to 20100-fold during testosterone therapy in transgender men and the testosterone levels need to stay high life-long.

R 178: 'CT at three different locations? Or are you bringing all to Odense? How do you secure that all investigations are done the same way?'
Re R178: All investigations are performed in one day in Odense with the same investigation protocol and equipment.

R 179: 'Echocardiography. This is very user dependent, how do you handle that?'
Re R179: Thank you for the very important comment, we are so happy that Axel Diederichsen ORCID ID: https://orcid.org/0000-0002-1285-4826 is responsible for echocardiography.

R 189: 'The article you are referring to included hypogonadal men over 65. You have to inform the reader about this fact since your study most likely will include much younger patients. It wouldn´t hurt to inform the reader a little bit more about the great debate about cardiovascular risk with testosterone treatment of hypogonadal men. There are tons of publications on this.'
Re R189: Thank you, we agree, that there is an ongoing debate regarding the risk and benefits of testosterone replacement therapy in cisgender men. (Basaria et al. N Engl J Med. 2010) and we have also contributed regarding this field (Magnussen et al. Diabetes Obes Metab 2016, Magnussen et al. Andrology 2017, Frederiksen et al. Age 2011, Frederiksen et al. EJE 2012).
We know that testosterone therapy in male hypogonadism increased coronary artery plaque formation already after 1 year in ageing cisgender men[26] however, there are no data in transgender men. Transgender men are, however, often young adults when testosterone is initiated and it is unknown if non-calcified coronary plaque formation is associated with testosterone therapy in these men. It is a limitation that we cannot perform a RCT, and a proper control group is not available. However, we still think, that we have the possibility to obtain reassuring data.

R 191: '"we have some information" sound a little bit like it´s information that only you have, but you are referring to previous publications?'
Re R191: We apologize for the misleading wording.
Furthermore, there is data we have some information on increased cardiovascular risk in women with PCOS or CAH who have endogenously elevated testosterone levels.

R 199: 'reference?'
Re R199: We apologize, we have inserted the reference.
Testosterone levels often tend to be above the female reference range, even after suppression of testosterone with glucocorticoids (Jones et al. J Clin Endocrinol Metab. 2017).

Respiratory function
R 214: 'reference? I think you can skip pre-pubertal information since you are not including these patients.'
Re R214: The reference is only included to underline the impact of sex hormones in relation to asthma. We have clarified in the manuscript.
Around puberty, the frequency of asthma changes from being higher in cisgender men to being higher in cisgender women, and continues to be higher in cisgender women through adulthood[12]. Hence, sex hormones are suggested to alter pathways in the pathogenesis of asthma[12].

R 231: ‘This looks like a hypothesis. I would not say that it “is” expected. It is you that hypothesize that the VO_2max will be higher in testosterone treated transgender men compared to ciswomen. I think it is a reasonable hypothesis but when? Already after 1 year? Or more?’
Re R231: We hypothesize that VO_2max increases after 1 year of testosterone therapy. We agree that hemoglobin increases within the first three months of testosterone treatment and muscle mass increase within the first year of testosterone treatment (T’Sjoen et al. endocrine reviews 2019).
Higher Cardiorespiratory fitness estimated by VO_2max is expected may be higher in general in transgender men compared to cisgender women, as testosterone has marked effects on key elements in the cardiorespiratory system, for example increased muscle mass and hematocrit, however data on VO_2max in transgender men is lacking.

R 233: ‘data is lacking? There is data on both increased muscle mass and increase hematocrit.’
Re R233: Thank you for the comment
We do agree that we can clarify, that it is data on VO_2max that is lacking.
Higher Cardiorespiratory fitness estimated by VO_2max is expected may be higher in general in transgender men compared to cisgender women, as testosterone has marked effects on key elements in the cardiorespiratory system, for example increased muscle mass and hematocrit, however data on VO_2max in transgender men is lacking.

R 239: “hyperandrogenism, PCOS”, two groups or one? Write hyperandrogenism (PCOS) or just PCOS instead.
Re R239: This is noted and changed accordingly in the paper.
Data in women with hyperandrogenism, PCOS, are limited but overall cardiorespiratory fitness is impaired compared to healthy cisgender women.

Muscle strength and body composition
R 260: ‘Here is another interesting study: Effects of moderately increased testosterone concentration on physical performance in young women: a double blind, randomized, placebo controlled study, Br J Sports Med 2019 Questionnaires on aggression and QoL’
Re R260: We thank you for this very important reference.
..suggesting a positive effect of elevated testosterone on muscle strength in cisgender women.
There are data on physical fitness and exogenous testosterone administration in cisgender women.
Aerobic running time increased in young physically active cisgender women during 10 weeks of testosterone treatment with 10 mg cream/day, which is a much lower dose compared to the dose of testosterone used for treatment in transgender men (Hirschberg AL Br J Sports Med 2020).

R 280: ‘ref till WPATH warning’
Re R280: We apologize, we have inserted the reference.
The warning is recommended by the World Professional Association for Transgender Health (WPATH)
(Coleman et al. International Journal of Transgenderism 2012).

Table 1
‘Why have you skipped two cardio-investigations at 10 years? If there is an effect on the cardiovascular system it is most likely a slow one and thus this is the time point where you are most likely to find any difference. What is your primary endpoint?’
Re table 1:
We apologize. Echocardiography is performed at all time points, inclusion, 1, 3, 5 and 10 years (table 1, figure 1).
Primary endpoint: Non-calcified coronary artery plaque volume.

We have ethical permission to perform three cardiac CTs during the study period of 10 years, due to the amount of radiation from contrast CT. According to the Danish national ethical committee radiation for < 10 mSv are acceptable for our kind of study and radiations amounts to 2.5 mSv per contrast CT and 0.1 mSv for 5 dual x-ray absorptiometry whole body scans.
Thank you for your comment regarding CT and time points. We had not included 10 years, as we were afraid of drop outs. However, we have reevaluated the design, and have decided to move the cardiac CT at 5 years to 10 years.

Table 2
'There is no need to be so general about what testing you are planning. As an example: Instead of "hormones and binding proteins" write what you plan to test. Which hormones and by which methods a.s.o.'
Re table 2: We have discarded table 2 and elaborated in the methods section.
Contrast Cardiac Computed Tomography (CT)
Cardiac CT scans will be performed using a high-end CT scanner at the first visit, after 1 year and at 10 years follow-up. To examine presence of coronary plaques, an ordinary contrast cardiac CT will be performed. The scanning protocol depends on the patient heart rate. In patients with a stable heart rate above 60 beats per minute, orally or intravenously β-blocker are administered until the heart rate is appropriate (if possible below 60), and a prospectively gated protocol is used. In patients with a heart rate > 70 bpm despite β-blocker pretreatment a retrospectively gated scan with dose modulation will be performed. Additionally, sublingual nitrates are administered prior to the scan. Administrating of β-blocker and nitrates are in accordance to daily clinical practice. Analyses of non–calcified coronary plaques/ stenosis and pericardial fat are performed off-line. Data analyses are performed by an experienced cardiologist. Radiation amounts to 2.5 mSv per CT.

Upper body muscle strength and power
Participants will be tested in a "Low Row" weight stack resisted exercise machine (Technogym, Italy), hence involving arm and upper back muscles during the pulling movement. The "Low Row" weight stack resisted exercise machine is adjusted to fit the individual participant relative to body height and arm length. Initial loading is estimated based on gender, body weight, training history and age. Warmup comprised 3 x 10 and 1 x 5 repetitions at the estimated test load, with 1 min of rest in between sets. Subsequently maximal effort (1 repetitions maximum, 1RM) was obtained using single repetitions with increasing load until task failure (cf. unable to complete the full range of motion) with 2-min of rest in between sets. Hereafter participants are instructed to perform at least 3 sets of one repetition at forceful and fast as possible to evaluate muscle power with a load corresponding to 80 % of 1RM and 2-min of rest in between sets. During each repetition, peak and mean power will be measured. To evaluate muscle power, a PUSH 2.0 inertial motion device (PUSH, Toronto, Canada) was attached to the weight stack. The PUSH device includes a 3-axis accelerometer and gyroscope, enabling measurements of human movement kinematics at a sampling rate of 1000 Hz. PUSH has previously been validated to evaluate power using resistance exercises (Hughes et al 2019). Peak and mean power (Watt), respectively, were calculated based on kinematic data derived by the PUSH software. The best results (1RM and muscle power) will be stored for further analysis.

Questionnaires
Buss-Perry Aggression Questionnaire
Quality of life: SF-36®
Inventory of Interpersonal problems ®
Gender Q (Development in progress)
GAD 7 (General Anxiety Disorder – 7)

PHQ9 (Patient Health Questionnaire – 8)

VO_2max
Measurement of VO_2max is performed on a bike ergometer and Vyntus®CPS system. The resistance will start low and increase gradually until maximum capacity or exhaustion.

Echocardiography
A comprehensive transthoracic echocardiography is performed by a training cardiologist. The recordings are stored digitally for blinded analysis. The following are included: size and mass of left ventricle, systolic and diastolic function of cardiac chambers and heart valve function. The rhythm during the investigation is recorded. Echocardiography uses ultrasound and no radiation is applied.

Respiratory function
Respiratory function is tested using a spirometer (Vyntus®CPS system) measuring forced expiratory volume in one second (FEV1), forced vital capacity (FVC) and peak flow.

Biological material
Blood samples are taken in the morning after an overnight fast. After sample collection, routine analyses will be performed at the local hospital laboratory at Odense University Hospital. For other analyses, serum and plasma as well as urine will be frozen and kept secured in a freezer house at Odense University Hospital. During the study, the analyses will be performed in batches, e.g. after 1 year, 3 year etc. (please refer to table below)
24 h urine is collected and frozen and hair samples (2 strands, 3 mm thick from the back of the head) are stored dry.

Cardiovascular risk markers in blood and serum
HbA1c, lipids, hemoglobin, hematocrit, hemostatic markers, adiponectin, suPAR.

Inflammation marker in blood and serum
White cell count, hsCRP, IL6, TNFα.

Urinary and hair cortisol
Cortisol metabolites.

Dual x-ray absorptiometry
Dual x-ray absorptiometry, Horizon A Discovery (whole body) is used to measure lean body mass, fat mass and bone density. Radiation amounts to 0.1 mSv.

Medical history
Chronic diseases, medication and supplements, alcohol, tobacco and abuse, gynecological history, previous treatment with testosterone, information on diet and physical activity.

Physical examination
Age, height, waist and hip circumference, body mass index (BMI, kg/m^2), blood pressure, alopecia and face and body hair (Ferriman-Gallwey Score).

Reviewer Name: Sohaib Haseeb, James Cook University, Australia
‘The paper by Lehmann Christensen et al. presents a protocol of a prospective long-term follow-up study of transgender men during testosterone therapy. The authors aim to investigate the short- and long-term effects of testosterone on vascular risk, respiratory function, muscle strength, and QoL in transgender men living in Denmark. This mixed-methods analysis sheds light on an important topic

that is relevant to medical care. The paper is written in a satisfactory way, and the need to conduct this study is clearly defined. However, this is an ambitious attempt to broadly assess many outcomes in one study, which, at points, may make it tedious to weigh the entire piece together. ‘

Regarding general comment
Thank you for kindly reviewing our paper and for the acknowledgment of the relevance of the study. We are grateful for your comments and the opportunity to improve our protocol paper, substantially.

Specific comments:
1: ‘The “outcomes” section should be expanded upon, ideally mentioning primary and secondary outcomes.’
Re 1: We apologize.
Primary endpoint
Non-calcified coronary artery plaque volume
Secondary endpoints
Upper body muscle strength and power
Aggression and quality of life
VO_2max
Ejection fraction and left ventricular muscle mass
Respiratory function
Cardiovascular risk markers in blood and serum
Inflammation markers in blood and serum
Urinary and hair cortisol

2: ‘A short paragraph on statistical analysis will be beneficial. How will incomplete answers be dealt with? Will open-ended questions be organized thematically? Et cetera. ‘
Re 2: We apologize, the statistic section has been elaborated, as follows:
Normally and non-normally distributed data will be analyzed using parametric and non-parametric statistics, respectively. Associations between testosterone and endpoint will be investigated with multiple linear regression modeling. Random mixed-effects linear regression models will be applied to investigate associations between testosterone and longitudinal repeated markers of assessed outcomes. We will collaborate with a statistician regarding random mixed-effects linear regression modeling.
A directed acyclic graph (DAG) will be performed depicted in order to transparently identify a priori assumptions of causal relations between exposure, outcome, potential confounders, intermediate factors, and selection bias.
Missing data will be handled according to type: MCAR (Missing Completely at Random), MAR (Missing At Random) or NMAR (Not Missing At Random) (Sterne et. al. BMJ 2009). Depending on data, analyses will be performed based on either complete case analyses or, if appropriate, by imputing missing data in collaboration with the statistical department.
Analyses will be conducted using STATA 14 (StataCorp 2015). Post regression diagnostics/Model validation will be performed. Assumptions of linearity between predictors and the outcome variable will be inspected by using scatter plots and augmented component-plus-residual plots. Normality of predicted residuals will be checked with quantile-normal and probability-normal plots. Homogeneity of variance (homoscedasticity) of the residuals will be investigated by plotting residuals against the fitted (predicted) values. Multicollinearity will be assessed using variance inflation factors. Open-ended questions will not be included.
Analyses will be conducted using STATA 14 (StataCorp 2015). Data are anonymized according to Danish law and regulations (Regional ethical committee in southern Denmark Project-ID: S-20190108, The Danish Data Protection Agency, journal no. 19/27572), and therefore analyses will be performed through a remote VPN access to Statistics Denmark.

3: ‘To date, cardiovascular research in the transgender population has focused on the association of hormone therapy on myocardial infarction, stroke, venous thromboembolism, diabetes, and hypertension. The incidence of arrhythmia due to hormone therapy has not been adequately evaluated. Does your study assess electrocardiographic parameters or document arrhythmic episodes (AF, PVCs and VT)? This may be important to further delineate the role of hormone therapy on the cardiovascular system.’
Re 3: Thank you for your comments regarding heart rhythm. We record the actual heart rhythm during the echocardiography and are trying to get access to Loop Recorders in collaboration with Axel Diederichsen ORCID ID: https://orcid.org/0000-0002-1285-4826. The cost is substantial, so we are uncertain, if we will succeed, and it will only be in a subgroup of men and only after an addendum to the protocol and ethical and authority permissions.

4: ‘The study has several limitations that will need to be considered when interpreting its results in the future. There should be a section addressing them.’
Re 4: We will elaborate and change the discussion, please see part of the strength and limitation section:
Strengths and limitations
The strength of our study is the thorough evaluation of a substantial number of transgender men regarding physical health as well as psychological issues, especially aggression (Defreyne et al. Hormones and Behavior 2019; Kristensen et al. in revision, Hormones and Behavior 2020). However, the study also has several limitations. Firstly as the study is a non-representative cohort study there is a risk of selection bias regarding age, BMI, smoking, ethnicity, fitness level and morbidity. However, we are performing a national registry study, including all transgender men in Denmark, so we have a possibility to compare the included individuals with the national register-based cohort of transgender men, and, in addition, we will have access to register-data on men who leave the clinical study regarding hospital diagnoses, prescription medicine and cause of death. Secondly, we cannot perform a proper power calculation regarding our primary endpoint, as we have no previous relevant data for the calculation. However, we think that, we can get important new information regarding vascular risk of testosterone therapy in transgender men. Thirdly, it is a strength that the study will be performed at one center using the same equipment and the same protocols, but, some individuals may not want to spend a day in Odense. Fourth, a long-term placebo-controlled RCT on testosterone therapy is not possible in transgender men. We address this issue in two ways: Participants will be their own control for within subject comparison, prospectively. We compare data from the included individuals with data from other cohorts. We have performed a large cross sectional study in young healthy men (20-29 years old), however, we do not know if it is feasible to use them as controls regarding the effect of ageing (Nielsen et al. 2007) and we are about to reinvestigate these men in 2021 (follow-up time 14 years) We have similar data in women with PCOS with planned reinvestigation after 10 years.

5: ‘The mention of the recruitment site in the title may be confusing to international readers. Perhaps re-consider its inclusion in the title.’
Re 5: We have changed the title accordingly and added the type of study to the title as requested by the editorial feedback.
Body Identity Clinic - Cardiovascular risk, physical fitness, respiratory function and psychological well-being in transgender men: a protocol for a prospective observational cohort study

Additional Editorial Feedback:
Thank you so much for the editorial feedback. We will revise in accordance with the comments.
1: ‘The title should include your study design. This is the preferred format for the journal. The title also needs to make it clear this is a protocol’

Re 1: We have revised the title according to the preferred format
Body Identity Clinic - Cardiovascular risk, physical fitness, respiratory function and psychological well-being in transgender men: a protocol for a prospective observational cohort study

2: 'Please avoid using bullet points in the abstract'
Re 2: bullets have been removed from the in the abstract
Study outcomes include: Non-calcified coronary artery plaque volume, upper body muscle strength and power, aggression and QoL, VO_2max, cardiac and respiratory function, cardiovascular risk markers, inflammation markers and urinary and hair cortisol

3: 'The strengths and limitations section after the abstract needs revising. It should contain up to five short bullet points, no longer than one sentence each, that relate specifically to the methods of the study reported (see: http://bmjopen.bmj.com/site/about/guidelines.xhtml#articletypes). It needs to be clearer why each point is a methodological strength or limitation. Each bullet point should also be a separate strength or limitation.'
Re 3: We will revise accordingly.

- Prospective longitudinal cohort study in transgender men assessing non-calcified coronary artery plaque volume as a marker of preclinical coronary pathology.
- Contrast cardiac CT is a very sensitive state-of-the-art method to assess non-calcified coronary artery plaque volume.
- Important new data on upper body muscle strength and power, aggression, VO_2max, cardiac and respiratory function in transgender men on testosterone therapy.
- Limitations of the study include selection bias, lack of appropriate control group and risk of being lost to follow-up during the 10 years study.

4: 'The discussion section needs revising. Most of the information presented in this section can be incorporated into the introduction and methods sections. Whilst we recommend including a discussion section in study protocol articles, it should be mostly used to reflect on the study's strengths and limitations (and in greater detail than what is presented at the beginning of the paper).'
Re 4: We apologize for not adhering to instructions. If we get the chance to resubmit we will thoroughly revise the manuscript according to instructions.

We hope that you will reconsider our manuscript for resubmission.

VERSION 2 – REVIEW

REVIEWER	Sohaib Haseeb James Cook University, Australia
REVIEW RETURNED	22-Oct-2020

GENERAL COMMENTS	This protocol presented by Lehmann Christensen and colleagues is a De Novo submission. This prospective single-center study will aim to investigate the short- and long-term effects of testosterone on clinical coronary disease, muscle strength and power, respiratory function, and QoL in transgender men living in Denmark. The authors shed light on an important topic that is relevant to medical care. After reviewing this resubmission, I still believe that this study is an ambitious attempt to broadly assess many outcomes in one study, which may make it tedious to weigh the entire piece together. However, I also believe that this is a much awaited study in an understudied population which has the

	potential to generate new data, and I am also sympathetic towards the need to publish protocols. My comments were properly addressed and the overall quality of this paper was substantially improved in this revised version. The paper now has primary and secondary endpoints and a specific methodology. It is clear that the authors put much work into addressing comments from both reviewers.

REVIEWER	Mats Holmberg ANOVA, Karolinska University Hospital, Stockholm, Sweden
REVIEW RETURNED	28-Oct-2020

GENERAL COMMENTS	Review Manuscript ID bmjopen-2020-045714 Masculinizing testosterone treatment and effects on preclinical cardiovascular disease, muscle strength and power, aggression, physical fitness and respiratory function in transgender men: Protocol on a ten-years, prospective, observational cohort study at the Body Identity Clinic (BIC). General comments: First of all I think you´ve done a good job in specifying the issues you aim at addressing. The data on long-term safety with cross-sex hormonal treatment is scarce and this study will definitely fill it´s purpose. I am not a native English speaker and my guess is that your manuscript wasn´t checked by anyone who is. I think it would be a good idea to do that to further increase the clarity of the manuscript. I have a few minor comments and have used the row numberings to guide you. Title: I there really such a thing as preclinical cardiovascular disease? This is a semantic question and I found one previous publication that used the term. Not a big issue perhaps but if you check plaque formation, are you investigating a disease or ageing? I would use “risk factors for cardiovascular disease” or “coronary atherosclerosis” or something similar. R67 “Masculinizing treatment is testosterone treatment and is initiated both in transgender men and if requested by non-binary individuals”. Testosterone is one of the masculinizing treatment regimens. I would write: Testosterone is the cornerstone of masculinizing treatment and is initiated… R111 “Low cardiorespiratory fitness has been associated with an increased risk of premature death from all causes, but primarily from cardiovascular disease, in cisgender individuals”. R139 For clarity I think it is better to name the direction of an association? Also what do you compare with? “Masculinizing testosterone treatment is associated with NCP development and progression”. You could write something like: Masculinizing treatment results in a higher prevalence of NCP compared to agematched cis-gender males. It is great that you have included hypotheses but I think you could specify all of them. R208 “Radiation from CCTA is lower than expected and we are applying for an additional CCTA at five years”. Why is this? Who expected the radiation to be higher? Technical development?

	R224 “repetition at forceful and”. Should be: “as forceful” R263 why not measure albumin? R296 “previous treatment with testosterone” Do you plan to include those who have used it and stopped? This is an important question since previous use leaves trace. To get a cleaner study I would exclude all who have used testosterone previously. R362 “experienced readers”? Experienced radiologists? R368 “Also, our study will elaborate on interesting data on the possible activating effect of testosterone therapy on aggression and the temporal relation between masculinizing testosterone treatment and levels of aggression”. A sentence difficult to understand. I suppose you will investigate if testosterone treatment increases the level of aggression? R381 just a thought. Are you not able to match subsamples of both groups at least? R382 “however, we do not know if it is feasible to use them as controls regarding the effects of ageing”. I really appreciate your openness with doubts which is part of every researchers life. But even better would be if you can specifiy what you actually can use the comparaison with your controls to an how to do that as meaningful as possible. R387. You can specify this. Can you really compare? What data from the registry? Cardiovascular diagnoses? That is something else than the other part of your study will capture. To me this sound like a separate (and interesting) study but not possible to use as a control group. A registry study will be easier to use to compare transgender men with matched persons from the general population.

VERSION 2 – AUTHOR RESPONSE

REVIEWER COMMENTS

Reviewer: 1

Comments to the Author

This protocol presented by Lehmann Christensen and colleagues is a De Novo submission. This prospective single-center study will aim to investigate the short- and long-term effects of testosterone on clinical coronary disease, muscle strength and power, respiratory function, and QoL in transgender men living in Denmark.

The authors shed light on an important topic that is relevant to medical care. After reviewing this resubmission, I still believe that this study is an ambitious attempt to broadly assess many outcomes

in one study, which may make it tedious to weigh the entire piece together. However, I also believe that this is a much awaited study in an understudied population which has the potential to generate new data, and I am also sympathetic towards the need to publish protocols.

My comments were properly addressed and the overall quality of this paper was substantially improved in this revised version. The paper now has primary and secondary endpoints and a specific methodology. It is clear that the authors put much work into addressing comments from both reviewers.

Re comments to the author: Thank you very much for your kind comments.

Reviewer: 2

Comments to the Author

Review Manuscript ID bmjopen-2020-045714 Masculinizing testosterone treatment and effects on preclinical cardiovascular disease, muscle strength and power, aggression, physical fitness and respiratory function in transgender men: Protocol on a ten-years, prospective, observational cohort study at the Body Identity Clinic (BIC).

General comments:

First of all I think you´ve done a good job in specifying the issues you aim at addressing. The data on long-term safety with cross-sex hormonal treatment is scarce and this study will definitely fill it´s purpose.

I am not a native English speaker and my guess is that your manuscript wasn´t checked by anyone who is. I think it would be a good idea to do that to further increase the clarity of the manuscript. I have a few minor comments and I have used the row numberings to guide you.

Re general comments: Thank you very much for your comments. We have thoroughly checked the manuscript as requested and addressed your comments by row-numbers below.

Title: I there really such a thing as preclinical cardiovascular disease? This is a semantic question and I found one previous publication that used the term. Not a big issue perhaps but if you check plaque formation, are you investigating a disease or ageing? I would use “risk factors for cardiovascular disease” or “coronary atherosclerosis” or something similar.

Re title: We fully understand the point raised. However, we have chosen the term preclinical cardiovascular disease, as risk factors are often smoking, high cholesterol levels, hypertension etc. We assess a pathological finding (NCP), however, with unknown clinical impact. Please, tell us, if you want another term.

R67 “Masculinizing treatment is testosterone treatment and is initiated both in transgender men and if requested by non-binary individuals”. Testosterone is one of the masculinizing treatment regimens. I would write: Testosterone is the cornerstone of masculinizing treatment and is initiated…

Re R67/current R70: Thank you so much for your suggestion. We have changed the text, accordingly.

'Testosterone treatment is the cornerstone of masculinizing treatment and is initiated both in transgender men and if requested by non-binary individuals.'

R111 "Low cardiorespiratory fitness has been associated with an increased risk of premature death from all causes, but primarily from cardiovascular disease, in cisgender individuals".

Re R111/current R117: We have revised the sentence.

'Low cardiorespiratory fitness is associated with increased risk of premature death from all causes, but cardiovascular disease is the most common cause of death in cisgender individuals.'

R139 For clarity I think it is better to name the direction of an association?(A) Also what do you compare with?(B) "Masculinizing testosterone treatment is associated with NCP development and progression". You could write something like: Masculinizing treatment results in a higher prevalence of NCP compared to agematched cis-gender males. It is great that you have included hypotheses but I think you could specify all of them.

Re R139/current R144: (A) Thank you for the comment and we agree we can name the direction. We have changed the text accordingly: 'Masculinizing testosterone treatment accelerates NCP development and progression' (B) We fully agree that it would be best to have a relevant control group, but it is not possible, and we will not have access to data from CCTA from matching controls.

R208 "Radiation from CCTA is lower than expected and we are applying for an additional CCTA at five years". Why is this? Who expected the radiation to be higher? Technical development?

Re R208: A very relevant question. We have written the protocol in collaboration with the department of cardiology. In the planning phase, the estimates for radiation from CCTA were based on other protocols. There has been development in CT technology and in the technical CT protocol. Furthermore, participants are leaner compared to previous CCTA protocols and therefore the radiation applied was lower.

R224 "repetition at forceful and". Should be: "as forceful"

Re R224/current R218: Thank you and we have revised accordingly: 'Hereafter participants are instructed to perform at least 3 sets of one repetition as forceful and fast as possible to evaluate muscle power with a load corresponding to 80 % of 1RM and 2 minutes of rest in between sets'.

R263 why not measure albumin?

Re R263: Thank you for the comment. According to Vermeulen et al. JCEM 1999, free testosterone could be calculated for routine purposes assuming an albumin concentration of 43 g/L if we are not dealing with sera from patients with marked abnormalities in plasma protein composition, such as in nephrotic syndrome or cirrhosis of the liver. Therefore, we do not consider assessment of albumin necessary.

R296 “previous treatment with testosterone” Do you plan to include those who have used it and stopped? This is an important question since previous use leaves trace. To get a cleaner study I would exclude all who have used testosterone previously.

Re R296: Thank you for the comment. We plan to include those who are on stable testosterone treatment and transgender men who are about to start testosterone therapy (figure 1 in the manuscript). We obtain information on previous testosterone treatment including prescriptions and interviews with participants. We believe that only very few participants will stop testosterone treatment because less than 1 % have regrets (The Amsterdam Cohort of Gender Dysphoria Study (1972-2015): Wiepjes et al. J Sex Med. 2018).

R362 “experienced readers”? Experienced radiologists?

Re R362/current R353:

Thank you for the comment. This is the text from the cited paper: All CCTA readers were Level III equivalent physicians with extensive experience interpreting CCTAs (Otaki et al European Heart Journal – Cardiovascular Imaging, 2005). We have revised the manuscript: ‘…by cardiologists’.

R368 “Also, our study will elaborate on interesting data on the possible activating effect of testosterone therapy on aggression and the temporal relation between masculinizing testosterone treatment and levels of aggression”. A sentence difficult to understand. I suppose you will investigate if testosterone treatment increases the level of aggression?

Re R368/current R360: Thank for your comment, we have revised the sentence for clarity.

‘Also, our study will elaborate on interesting data on possible increased levels of aggression during testosterone therapy and the temporal relation between masculinizing testosterone treatment and levels of aggression’

R381 just a thought. Are you not able to match subsamples of both groups at least?

Re R381: Thank you for your comment. It would have been very nice to be able to match sub-samples of both groups, however, we are not able to match sub-samples, as we do not have these data from the protocol on Odense Androgen Study, unfortunately.

R382 “however, we do not know if it is feasible to use them as controls regarding the effects of ageing”. I really appreciate your openness with doubts, which is part of every researcher’s life. But even better would be if you can specify what you actually can use the comparison with your controls to an how to do that as meaningful as possible.

Re R382: Thank you so much for your comment. We plan to use data from Odense Androgen Study (Nielsen et al. 2007) regarding BMI, waist circumference, muscle mass, fat mass, blood pressure, Hba1c, testosterone and estradiol levels, lipid status and VO2 max.

R387. You can specify this. Can you really compare? What data from the registry? Cardiovascular diagnoses? That is something else than the other part of your study will capture. To me this sound like a separate (and interesting) study but not possible to use as a control group. A registry study will be easier to use to compare transgender men with matched persons from the general population.

Re R387: Thank you for the comment. Sorry that we have not clarified this part.

We have revised the text in the abstract:

(R41): 'Our cohort (BIC), including dropouts, will be an embedded sub-cohort in a future national registry study in all individuals with gender dysphoria and controls. Data are available on International Statistical Classification of Diseases and Related Health Problems (ICD-10) diagnostic codes, prescriptions, socioeconomics and causes of death.'

We have revised the text in the discussion section

(R370): 'Future access to national registry data on all individuals with registered gender dysphoria and controls will allow us to study our cohort (BIC) as an embedded sub-cohort including individuals, who remain in the ten-years study as well as drop-outs; we have previously used this design in women with polycystic ovary syndrome.'

VERSION 3 – REVIEW

REVIEWER	Mats Holmberg ANOVA, Karolinska University Hospital, Stockholm, Sweden
REVIEW RETURNED	30-Nov-2020

GENERAL COMMENTS	Thank you again for improving the manuscript. I accept this for publication but still suggest you do a language check in order to further improve the script. Good luck with the study.