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Peer Review File

Humans with function-disrupting variants in the myostatin gene (MSTN) have increased skeletal muscle mass and strength, and less adiposity

Corresponding Author: Dr David Glass

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript focusses on an established suppressor of skeletal muscle growth, myostatin. By analysing the exome sequencing data of over 1 million subjects, the authors identified rare myostatin variants with possible loss-of-function effects. Subsequent association analyses indicated that carriers of those variants had higher grip strength and muscle mass, and lower adiposity compared with the carriers of wildtype myostatin. Overall, the study indicates that variants of myostatin exerts similar effects on human muscles and adipose tissue as observed in several mammalian species. Importantly, no adverse indications for the cardiometabolic indices were found. The latter observation appears new and valuable addition to the literature on myostatin.

Major
Although the authors state identifying 38 rare variants predicted to cause loss of function in myostatin (44 such variants were alluded to later in the text), only four, rs34094280, rs535776509, rs143242500, rs965957357, are presented in supplementary tables. The entire set of the captured rare myostatin variants would be useful for research community.

Size of the skeleton (reflected in the height measurement) is positively associated with body weight as well as the mass of various tissues and organs, including skeletal muscle because growing long bones stimulates elongation of muscle fibres. Not adjusting for height might have inflated the estimates of the effects of myostatin variants particularly because myostatin appears to be associated with height. It is not clear how introducing a concept of deviation from the derived “expected values” helps to address this issue of potential inflation.

Minor
Results, page 5, 1 para. “While there was no association with changes in overall body mass,…”. Word “changes” might be misleading as it implies longitudinal measures. Whereas it appears that comparison is made between groups.
Page 5, last para. “we sought to confirm these results using direct measures of muscle and fat volume from obtained from whole-body Dixon MRI data” Remove one “from”.
Page 5. What is the difference between pLoF and “function-disrupting variants”? Where are they presented?
Page 6, para 2. Not clear why 18 pLoF variants were selected from the total set of 38. In general, the text appears as a fragmented presentation of the results with little explanation for why it was done one way or the other.
Page 7, para 1. Indicate what the p-values belong to and separate them by comma.
Discussion, page 9, top para. The closing sentence of the paragraph cites reference #29 in support of the statement that blockade of myostatin in adult humans increased muscle by only few percents. Although the reference is still under review, its title suggests that the study was done in mice and primates, not humans.
Page 11, para 2. Not clear what is the basis for the fertility hypothesis in other species. Mice carrying mutant myostatin allele appear to be adequate breeders.
Figure 1. What is the meaning of the arrows merged with the error bars?
Figure 2. What does AAF stand for? Alternative allele frequency? It would be helpful spelling it out.
Methods, page 38, Association analyses. What does the GWAS refer to in this section? GWAS customary stands for genome-wide association study, but this report is on a single gene rather than genome-wide.
In the same section, please describe the gene burden association analysis in sufficient detail for replication.

Extended Data Figure 1. Percentage difference, as in the x-axis annotation, seems a more accurate reflection of what is presented, than percentage change.
Supplementary Tables, page 40. Please indicate Build of human genome used in Supplementary Table 2 and 3.

Reviewer #2

(Remarks to the Author)
This a compelling and important study examining associations between human genetic variants in MSTN and muscle mass/strength and adiposity. I recommend that this paper be published essentially as is. My only minor comment is that there are several citations in the Introduction that should be corrected:

1. For the sentence describing the latent complex, the role of the prodomain in blocking the mature domain was first described in the following two references: (i) Lee SJ, McPherron AC. 2001. Regulation of myostatin activity and muscle growth. PNAS 98(16):9306–11 (this is reference #1 in the paper); (ii) Thies RS, Chen T, Davies MV, Tomkinson KN, Pearson AA, et al. 2001. GDF-8 propeptide binds to GDF-8 and antagonizes biological activity by inhibiting GDF-8 receptor binding. Growth Factors 18(4):251–59.
2. For the next sentence, the activation of the latent complex by BMP1/Tolloid proteases was first described in: Wolfman NM, McPherron AC, Pappano WN, Davies MV, Song K, et al. 2003. Activation of latent myostatin by the BMP-1/tolloid family of metalloproteinases. PNAS 100(26):15842–46 (this is reference #16 in the paper).
3. For the next sentence describing the receptors, the role of ActRII was first described in Lee SJ, McPherron AC. 2001. Regulation of myostatin activity and muscle growth. PNAS 98(16):9306–11 (this is reference #1 in the paper).

Reviewer #3

(Remarks to the Author)
This study reports that the burden of deleterious rare variants in the myostatin gene, identified through whole exome sequencing, is positively related to muscle mass. Similar findings were observed in relation to four specific myostatin variants. Taken together, these findings suggest that many instances exist of heterozygous loss of function variants in myostatin, leading to an increase in lean mass. This significantly extends knowledge in this area, given that till now there has just been a single case report of homozygous loss of function myostatin mutation in humans, with no reports of heterozygous loss having any effect. This in turn has implications for drug discovery programs aiming to develop new therapeutics for sarcopenia based on blockade of myostatin.

The work supports the main conclusions, which are primarily based on findings from UKB, namely bioimpedance data obtained in the whole cohort, and MRI data from the subset included in the imaging data. That said, whereas measures of fat and lean mass were derived from MRI using a somewhat complex AI method, it was somewhat surprising that fat and leam mass obtained from whole body DXA scans were not utilised, particularly as these provide a measure of whole body body composition unlike the case with MRI.

Further comments:-
1. Though the study is referred to as being multi-cohort, it seems like the great majority of the main outcome variables have been obtained from UKB. It needs to be made clearer in those instances where other cohorts are contributing to the results, and if so which ones, eg by providing this information in the Figure/Table legends.

2. Myostatin variants were also found to influence height, if anything to a greater extent than height-independent effects on lean mass, which would be worthy of further discussion. At first sight it's hard to think height might be affected secondary to effects on lean mass, so presumably this reflects a separate mechanism?

3. Were any cases of heterozygous loss observed in relation to the previously reported homozygous mutation in reference 8

Minor points:-
Extended Figure 1 doesn't appear to be cited in the text

What is meant by "PCOS diagnosis or Rotterdam"

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
All my comments have been adressed.

Reviewer #3

(Remarks to the Author)

The points raised by the reviewers have largely been dealt with satisfactorily. That said, they have not addressed the request for discussion of the possible mechanisms by which myostatin variants might affect height (reviewer 3 point 2).

Thank you very much to the reviewers for their comments on our manuscript, which helped improve it quite a bit. Also, just so the reviewers are aware – the delay in re-submitting was due to our desire to incorporate some additional data from other populations, so that we could give as complete a report as possible. You'll notice that several groups are now co-authors on the manuscript, which gives you an idea of the wide-range of data inputted.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript focusses on an established suppressor of skeletal muscle growth, myostatin. By analysing the exome sequencing data of over 1 million subjects, the authors identified rare myostatin variants with possible loss-of-function effects. Subsequent association analyses indicated that carriers of those variants had higher grip strength and muscle mass, and lower adiposity compared with the carriers of wildtype myostatin. Overall, the study indicates that variants of myostatin exerts similar effects on human muscles and adipose tissue as observed in several mammalian species. Importantly, no adverse indications for the cardiometabolic indices were found. The latter observation appears new and valuable addition to the literature on myostatin.

Major

Although the authors state identifying 38 rare variants predicted to cause loss of function in myostatin (44 such variants were alluded to later in the text), only four, rs34094280, rs535776509, rs143242500, rs965957357, are presented in supplementary tables. The entire set of the captured rare myostatin variants would be useful for research community.

We thank the reviewer for this comment. We agree that a supplemental table listing all MSTN predicted loss of function (pLoF) variants identified in our study may be helpful for the research community. To address this, we have added a list of all 38 pLoF variants to Supplementary Table 2.

Size of the skeleton (reflected in the height measurement) is positively associated with body weight as well as the mass of various tissues and organs, including skeletal muscle because growing long bones stimulates elongation of muscle fibres. Not adjusting for height might have inflated the estimates of the effects of myostatin variants particularly because myostatin appears to be associated with height. It is not clear how

introducing a concept of deviation from the derived “expected values” helps to address this issue of potential inflation.

This is a question we address in the manuscript, namely the extent to which effects on muscle/fat mass are mediated by differences in body size. As noted, function-disrupting variants in MSTN are associated with increased height; since effects mediated by differences in height may not be reproducible via therapeutic intervention later in life, this question is relevant from a clinical perspective. To quantify effects on lean mass and muscle size in excess of what could be attributed to differences in body size we analyzed the associations with “percentage (%) above/below expected”, where the expected value was computed using a model that included height and height squared as predictors (see Extended Data Figure 8 for an example of the observed vs. expected values for BIA lean mass using this model). Since the % difference from expected takes the form 100(observed-expected)/expected, any additive effect mediated via height will be removed in the numerator; division by the expected value and multiplication by 100 converts the covariate-adjusted differences in the numerator into percentages, to aid with interpretability of these adjusted effect sizes. An effect size of +10% from the expected value can then be interpreted as a 10% increase relative to other individuals with matched age, sex, height and other covariates, and represents the effect in excess of what can be explained by these other factors.*

We have added a reference to Extended Data Figure 8 inside the caption for Figure 1 where this % difference from expected is first introduced, to make the methodological details clearer to the reader.

Minor
Results, page 5, 1 para. “While there was no association with changes in overall body mass,…”. Word “changes” might be misleading as it implies longitudinal measures. Whereas it appears that comparison is made between groups.

We agree with the reviewer and have edited the manuscript accordingly:

Previous text: “While there was no association with changes in overall body mass,….”

Edited text: While there was no association with overall body mass, ….”

Page 5, last para. “we sought to confirm these results using direct measures of muscle and fat volume from obtained from whole-body Dixon MRI data” Remove one “from”.

The text is edited accordingly.

Page 5. What is the difference between pLoF and “function-disrupting variants”? Where are they presented?

The term “pLoF” indicates variants that cause changes typically associated with inability to transcribe and/or translate the gene (e.g. premature stop codons, frameshifts, etc.). The term “function-disrupting variant” is a more inclusive term and includes (1) pLoFs or (2) missense variants that are predicted to partially or fully impair protein function. This information was presented in the Methods, but we agree that it could be clearer in text. As such, we edited the first paragraph of the results section to better explain this terminology, along with a direct reference to the methods section that includes citations for the algorithms used to annotate said variants:

> *Previous text: “Of these variants, 226 were predicted to disrupt protein function, including 38 predicted to induce loss-of-function (pLoF).”*
>
> *Edited text: “Of these variants, 226 were predicted to partially or fully disrupt protein function: 38 were predicted to result in inability to generate functional protein (referred to as predicted loss-of-function, abbreviated as pLoF; Supplementary Table 2), and 188 were predicted to impair protein function (see Methods).*

Page 6, para 2. Not clear why 18 pLoF variants were selected from the total set of 38. In general, the text appears as a fragmented presentation of the results with little explanation for why it was done one way or the other.

The set of 38 pLoFs refers to those observed among 1.1m individuals across 11 different cohorts. The subset of 18 variants mentioned later refers to those that were observed in UK Biobank; since we have bioimpedance and imaging data only for UK Biobank, we used only these 18 variants when analyzing the effects of pLoFs on these traits. We edited the text to make this clearer as follows:

> *(1) We specifically mention data noted in paragraph 1, sentence 1 of results are from a multi-cohort analysis:*
>
> *Previous text: “Analyzing whole exome sequencing data from a meta-analysis across 1.1 million participants, we observed 13,454 individuals carrying one or more of 506 unique nonsynonymous rare variants (allele frequency ≤0.5%) within the myostatin gene (MSTN).”*
>
> *Edited text: “Analyzing whole exome sequencing data from 1.1 million participants across 11 cohorts (Supplementary Table 1), we observed 13,454*

individuals carrying one or more of 506 unique nonsynonymous rare variants (allele frequency ≤0.5%) within the myostatin gene (MSTN).”

(2) We note that, as we transition to analysis of imaging data, we state that we are analyzing data from the “UK Biobank imaging subcohort”:

Original, unedited text: “….we sought to confirm these results using direct measures of muscle and fat volume obtained from whole-body Dixon MRI data in the UKB imaging subcohort (N= 62,308).” [please note, the sample size in this sentence has now been changed to 77,572 with an updated analysis]

(3) We now state that, in transitioning to the subset of 18 pLoFs on page 6, that these are carried in UK Biobank participants:

Original text: “For comparison, we also analyzed the effects of gene burden restricted to the set of 18 pLoF variants observed in MSTN, …..”

Edited text: “For comparison, we also analyzed the effects of the gene burden restricted to the set of 18 MSTN pLoF variants found in participants in the UKB cohort, ……”

Page 7, para 1. Indicate what the p-values belong to and separate them by comma.

We have edited the text as follows:

*Previous text: “We observed that the single homozygote carrier of Arg65His has a higher lean mass (+12.3%), higher lean percentage (+19.2%) and creatinine:cystatin C ratio (+37.8%) compared to equivalent heterozygote carriers of this mutation (p=0.11, p=0.045 p=0.10; **Table 1, Figure 4**).”*

Edited text: “We observed that the single homozygote carrier of Arg65His had a higher fat-free mass (+12.3%, p=0.11), higher fat-free percentage (+19.2%, p=0.045) and creatinine:cystatin C ratio (+37.8%, p=0.10) compared to heterozygote carriers of this mutation (Extended Data Table 1, Extended Data Figure 3)”

Discussion, page 9, top para. The closing sentence of the paragraph cites reference #29 in support of the statement that blockade of myostatin in adult humans increased muscle by only few percents. Although the reference is still under review, its title suggests that the study was done in mice and primates, not humans.

Thank you for this point. The reference has been updated.

Page 11, para 2. Not clear what is the basis for the fertility hypothesis in other species. Mice carrying mutant myostatin allele appear to be adequate breeders.

Thank you for this comment. We agree that there is not in fact a problem with breeding myostatin knockouts. We sought to make sure this was the case in humans, because previous work published by Ongaro et al (Science 2025, Ref 36 in the manuscript) reported an effect of myostatin on FSH expression in mice and rats which was the source of the fertility hypothesis. We sought to investigate the relevance of these findings in humans by examining the association between MSTN disrupting variants and relevant hormonal and reproductive outcomes. We did not observe evidence of relevant associations via human genetics.

Figure 1. What is the meaning of the arrows merged with the error bars?

The arrows on the error bars indicate that the error bar extends beyond the axis limit. We use this convention to allow us to best represent the majority of data visually when a subset of error bars are much larger than the others. Since the 95% confidence intervals are also listed in numerical form in the forest plot, the error bars are primarily to aid with visual comparison of the effect magnitudes and confidence intervals across traits. We have added a comment to figure legends to explicitly state what the arrow indicates.

Figure 2. What does AAF stand for? Alternative allele frequency? It would be helpful spelling it out.

AAF stands for alternative allele frequency, and pLoF, AAF<0.5% indicates that the pLoF variants included in the burden mask had a frequency of less than 0.5%. We have added this information to the respective figure legends.

Methods, page 38, Association analyses. What does the GWAS refer to in this section? GWAS customary stands for genome-wide association study, but this report is on a single gene rather than genome-wide.

We thank the reviewer for bringing this point to our attention. This project was a gene-centric focused analysis and not a genome-wide analysis. As such, we have removed the sentence referring to GWAS from the text to avoid confusion.

In the same section, please describe the gene burden association analysis in sufficient detail for replication.

We have added edits to text to ensure the different sections of analyses are explicitly split. In brief:

(1) We note that quantitative traits undergo a rank inverse normal transformation (RINT) prior to genetic analyses
(2) We outline and cite the software we use to run the association analysis (i.e. REGENIE), including what pieces of genetic information we use in STEP 1 and STEP 2 of REGENIE analyses.
(3) We outline specific covariates used to run the association analysis
(4) We indicate how variants were annotated in a previous section and, now, specifically cite that section within the association analysis methods section
(5) We state the allele frequency cutoff as ≤0.5%
(6) Finally, we state how the data is presented and how we estimate clinical units for the quantitative traits

We believe this provides sufficient detail needed to replicate the burden test results presented in the manuscript.

Extended Data Figure 1. Percentage difference, as in the x-axis annotation, seems a more accurate reflection of what is presented, than percentage change.

We thank the reviewer for the suggestion. We have now updated the analyses of the single MSTN variants and show these results across all variants in Supplementary Table 5; we have therefore removed this figure from the extended data section.

Supplementary Tables, page 40. Please indicate Build of human genome used in Supplementary Table 2 and 3.

We have indicated the genome build in the "Genetic data preparation" subsection of the Supplementary Methods as requested.

Reviewer #2 (Remarks to the Author):

This a compelling and important study examining associations between human genetic variants in MSTN and muscle mass/strength and adiposity. I recommend that this paper be published essentially as is. My only minor comment is that there are several citations in the Introduction that should be corrected:

1. For the sentence describing the latent complex, the role of the prodomain in blocking the mature domain was first described in the following two references: (i) Lee SJ, McPherron AC. 2001. Regulation of myostatin activity and muscle growth. PNAS 98(16):9306–11 (this is reference #1 in the paper); (ii) Thies RS, Chen T, Davies MV, Tomkinson KN, Pearson AA, et al. 2001. GDF-8 propeptide binds to GDF-8 and antagonizes biological activity by inhibiting GDF-8 receptor binding. Growth Factors 18(4):251–59.

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3. For the next sentence describing the receptors, the role of ActRII was first described in Lee SJ, McPherron AC. 2001. Regulation of myostatin activity and muscle growth. PNAS 98(16):9306–11 (this is reference #1 in the paper).

We appreciated the reviewer's comments, and have incorporated the recommended references in our manuscript.

Reviewer #3 (Remarks to the Author):

This study reports that the burden of deleterious rare variants in the myostatin gene, identified through whole exome sequencing, is positively related to muscle mass. Similar findings were observed in relation to four specific myostatin variants. Taken together, these findings suggest that many instances exist of heterozygous loss of function variants in myostatin, leading to an increase in lean mass. This significantly extends knowledge in this area, given that till now there has just been a single case report of homozygous loss of function myostatin mutation in humans, with no reports of heterozygous loss having any effect. This in turn has implications for drug discovery programs aiming to develop new therapeutics for sarcopenia based on blockade of myostatin.

The work supports the main conclusions, which are primarily based on findings from UKB, namely bioimpedance data obtained in the whole cohort, and MRI data from the subset included in the imaging data. That said, whereas measures of fat and lean mass were derived from MRI using a somewhat complex AI method, it was somewhat surprising that fat and leam mass obtained from whole body DXA scans were not utilised, particularly as these provide a measure of whole body body composition unlike the case with MRI.

We have included results for fat and lean mass derived from DXA as Supplementary Table 4 for comparison to address the reviewer's question. Initially these were omitted, since MRI provides higher resolution, and allows for precise quantification of muscle volume in specific muscles and muscle groups, as well as whole-body fat volume and total water across all muscles. In contrast DXA can only provide estimates of total and regional lean mass, and these totals includes contributions from tissues such as internal organs and cartilage as well as muscle (Basty N, et al. medRxiv 2024; doi: https://doi.org/10.1101/2024.12.12.24318943). Furthermore, DXA-derived body composition estimates are available only for a subset of up to ~40k UKB participants, resulting in lower power for detecting genetic associations, hence providing limited additional value beyond what can be derived from the MRI data in the context of this study (which includes close to 80,000 subjects).

In regard to the comment about the complexity of the AI algorithms used for processing the MRI data, it is worth noting that these were only used to automate the segmentation of regions of interest within each scan, and were benchmarked on training sets derived via manual annotation. In most cases these segmentations perform similarly to a manual annotator (see Extended Data Tables 2-3 and Reference [69]), and are able to accurately identify individual muscle groups (see Figure 4 for an example).

Further comments:
1. Though the study is referred to as being multi-cohort, it seems like the great majority of the main outcome variables have been obtained from UKB. It needs to be made clearer in those instances where other cohorts are contributing to the results, and if so which ones, eg by providing this information in the Figure/Table legends.

To address this comment, we created a supplementary table (see Supplementary Table 1 in revised manuscript) that indicates the cohorts used for each phenotype or outcome that was part of a meta-analysis. The table is now cited in each figure legend and in text wherever a meta-analysis was incorporated.

2. Myostatin variants were also found to influence height, if anything to a greater extent than height-independent effects on lean mass, which would be worthy of further discussion. At first sight it's hard to think height might be affected secondary to effects on lean mass, so presumably this reflects a separate mechanism?

This is a question we address in the manuscript, namely the extent to which effects on muscle/fat mass are mediated by differences in body size. As noted, function-disrupting variants in MSTN are associated with increased height; since effects mediated by differences in height may not be reproducible via therapeutic intervention later in life, this question is relevant from a clinical perspective. To quantify effects on lean mass and muscle size in excess of what could be attributed to differences in body size we analyzed the associations with "percentage (%) above/below expected", where the expected value was computed using a model that included height and height squared as predictors (see Extended Data Figure 8 for an example of the observed vs. expected values for BIA lean mass using this model). Since the % difference from expected takes the form 100(observed-expected)/expected, any additive effect mediated via height will be removed in the numerator; division by the expected value and multiplication by 100 converts the covariate-adjusted differences in the numerator into percentages, to aid with interpretability of these adjusted effect sizes. An effect size of +10% from the expected value can then be interpreted as a 10% increase relative to other individuals with matched age, sex, height and other covariates, and represents the effect in excess of what can be explained by these other factors.*

We have added a reference to Extended Data Figure 8 inside the caption for Figure 1 where this % difference from expected is first introduced, to make the methodological details clearer to the reader.

3. Were any cases of heterozygous loss observed in relation to the previously reported homozygous mutation in reference 8

We observe 11 heterozygous carriers (and no homozygotes) of the intronic MSTN variant highlighted in Schuelke et al 2004 NEJM (2:190062219:C:T, rs397515373) across our cohorts. Of these, we observe only 1 carrier in the subset of UKB with BIA data (this individual was not in the UKB imaging subcohort).

Minor points:-
Extended Figure 1 doesn't appear to be cited in the text

We thank the reviewer for catching this discrepancy; we have decided to remove the figure from the manuscript.

What is meant by "PCOS diagnosis or Rotterdam"

We have added the text which was present in the Methods section to the legend of Extended Data Figure 5.

> *Polycystic ovary syndrome (PCOS) cases were defined based on an electronic health record or self-reported diagnosis of PCOS. An additional, broader version was defined that also considered women as cases if they had clinical evidence of both hyperandrogenism and ovulatory dysfunction (i.e. according to Rotterdam criteria). Samples with a history of PCOS, hyperandrogenism or ovulatory dysfunction were removed from the control groups for these two phenotype versions.*