nature portfolio

Peer Review File

GDF8 and activin A are the key negative regulators of muscle mass in postmenopausal females: a randomized phase I trial

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

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors previously identified ActA as the key second negative regulator acting via ActRIIA/B, as blockade of both GDF8 and ActA in mice/monkeys matches the muscle growth of ActRIIA/B blockade. The authors now studied humans in a randomized placebo-controlled Phase 1 trial. Again , combining GDF8 and ActA blocking antibodies led to greater muscle growth than either antibody alone; in addition increases in muscle were accompanied by reductions in fat. Thus combination ligand blockade with the GDF8 (trevogrumab) and ActA (garetosmab) mAbs led to substantial dose dependent increases in MRI-quantitated thigh muscle volume over placebo that was greater with combination than treatment with either mAb alone. The combination was generally well tolerated. The authors conclude that combined blockade may be a promising therapeutic approach in muscle atrophy and obesity settings.
The study represents exciting biology and the finding that the combined blockade seems to equivalent to blockade of the relevant receptor, is of course interesting, (and supported by preclinical studies). So far so good.
Clearly the side effect profile is the most important result of this study and the previous experiences with trevogrumab are not comforting. To this reviewer, the dose dependent and high frequency of headache, muscle spasm and mouth ulcerations does not sound nice.
The findings presented here are based on Dexa scanning or MRI of muscle mass. I can read that body weight was not increased. So I guess there is a limit to the magnitude of the muscle response, although apparently loss of fat mass may have been obscured these changes. Many of the observed findings were not terribly significant. But wat about energy expenditure? If increased EE was to be responsible for the fat loss it would certainly be of interest to measure this?
To me the most important is whether this apparently increased muscle mass was functional. So what happened to exercise capacity, muscle strength, VO2max and what about patient reported outcomes in general and pertaining to muscle mass?

Reviewer #2

(Remarks to the Author)
This is an important study that should be published. My main criticism is that in order to make the conclusion that targeting both myostatin and activin A gives greater effects on lean mass and fat mass than targeting either alone, the statistical analysis should be between combination versus each alone. In the figures, all comparisons are made only against placebo. The authors show that there is a difference between targeting both versus placebo and that there is no statistical difference between targeting myostatin alone versus placebo or between targeting activin A alone versus placebo. They need to show the statistical analysis for comparing targeting both versus targeting myostatin alone and comparing targeting both versus targeting activin A alone.

Reviewer #3

(Remarks to the Author)
Trotter et al. report on two clinical trials investigating the impact of combined inhibition of GDF8 ActA on muscle mass in women. This is an innovative and potentially important therapeutic approach to treat muscle atrophy. I have only minor suggestions to improve the manuscript, detailed below:

1. It would be helpful if the authors included data from the animal studies investigating reproductive toxicity in rat and

nonhuman primate models, currently described in the supplement.
2. Given that the combination therapy was only tested in females, generalizability to males should be tempered and it should be made more clear in the title and abstract that the results on combination therapy reported are specific to women.
3. It would be helpful if the authors explained what the potential mechanisms are by which muscle mass returns to baseline after washout.
4. Its not clear to me why none of the data from the multi-dose trial are included in the main figures. Also, it would be helpful if the authors comment on why the effect of a single dose and multiple doses was similar, was this expected?

Reviewer #4

(Remarks to the Author)
The paper reports results from a randomised placebo-controlled phase 1 trial investigating changes in body composition following exposure to two monoclonal antibodies (mAbs): a GDF8 mAb (trevogrumab) and to an ActA mAb (garetosmab) given alone or in combination. The trial design, sample size and statistical methods are in line with best practice for this type of investigations. The conclusions drawn are supported by the objectives, trial design and reported results. While early in development the compounds investigated could address important unmet medical needs in the future.

Major comments:
- none

Minor comments:
- The trial completed 2019-04-30 and for transparency it would be prudent to provide a statement revealing the status for the two mAbs, e.g. reported and ongoing trials including pursued and abandoned indications.

- As described in the protocol, single dose administration of each mAb alone had already been safety cleared in human up to and exceeding the dose levels used in the present trial. This should be mentioned in the introduction and references to the relevant clinical trials should be provided if available.

- Please check the y-axis of all five panels in Figure 3, something is off with e.g. the yellow bar in panel A reaching far above 2%.

- In the discussion section (line 248 to 252) it is stated that weight loss following bariatric surgery or GLP1 therapy “can often also result in profound muscle loss” and “while weight regain following these treatments is predominately in fat” providing an unmet medical need for the mAb combination investigated in the trial. Readers could easily get the impression that there is evidence to claim that body composition after weight regain is worse than prior to the surgery or GLP1 induced weight loss. The two referenced papers for the weight regain claim provide no such evidence. Reference 22 is a retrospective observational study trying to predict weight regain based on changes in weight and body composition following GLP1 treatment. And reference 23 is a cross-sectional database study in sleeve gastrectomy providing no insight into why patients in the weight regain group weighed more or had more fat mass (and muscle mass) than patients who had lost weight – other than the obvious selection bias. Evidence for the claim “while weight regain following these treatments is predominately in fat” should be substantiated or alternatively the sentence should be revised.

Reviewer #5

(Remarks to the Author)
Myostatin (MSTN), also known as growth and differentiation factor 8 (GDF8), is an extensively studied negative regulator of skeletal muscle mass. This TGF-beta superfamily signaling protein is high conserved amongst mammalian species and, for example, it has been demonstrated using CRISPR/Cas9 that removing MSTN/GDF8 signaling can substantially augment muscle mass in goats (He Z et al. Bioscience Reports, 2018). GDF8 exerts its muscle degradation effects via binding to activin type II receptors (ActRIIA/B) in skeletal muscle, which activates SMAD 2/3 and prompts the transcription of atrogenes. Consequently various inhibitors of GDF8 and ActRIIA/B have been developed over recent years as potential therapies to prevent or reverse muscle degradation. However, the translation of GDF8 or ActRIIA/B inhibition into human scenarios of muscle wasting, including men with prostate cancer (Padhi et al, J Clin Endocrinol Metab, 2014), boys with muscular dystrophy (Campbell et al. Muscle Nerve, 2017), or older adults with sarcopenia (Rooks et al. JAMA Netw Open 2020), have each been clinically underwhelming to date. Of note, bimagrumab (ActRIIA/B inhibitor) is being studied with and without semaglutide in patients with obesity to determine efficacy and safety in the preservation of lean mass during fat mass loss (e.g. NCT05616013). Also of relevance is the recent publication of a phase 2 study of ponsegromab, a mAb inhibiting GDF15, which demonstrated a 2.8 kg mean bodyweight increase in the maximal dosing group over 12 weeks of administration, versus placebo, amongst adults with cancer cachexia (Groarke et al. NEJM, 2024).

This manuscript reports a phase I study assessing human safety, tolerability, pharmacokinetics, and pharmacodynamics of an GDF8 mAb (trevogrumab) and an ActA mAb (garetosmab). Each were administered alone, as well as GDF8 mAb given in combination with increasing doses of the ActA mAb, with a view towards the development of therapies to reverse skeletal muscle wasting. The study initially recruited only healthy post-menopausal females due to pre-clinical concerns about testicular toxicity; in a subsequent part of the study healthy males not intending to reproduce were also recruited. Body composition was assessed at weeks 4 and 8 of drug delivery. MRI was used for thigh muscle volume assessment and DXA for total lean mass, appendicular lean mass, android fat mass and total fat mass. The main finding of the study was that the combination of GDF8 inhibition wth trevogrumab and ActA inhibition with garetosmab achieved dose-dependent increases

in thigh muscle volume per MRI imaging, as compared to placebo, with the combination of agents having greater efficacy that either mAb alone. Fat mass decreased with combination GDF8 and ActA inhibition. The primary endpoint was the incidence and severity of adverse events to week 16 for the single-dose study and to week 40 for the multiple-dose study; there were no significant safety concerns and no antibodies were noted to either mAbs. The male participants were primarily included to assess the tolerability of ActA alone but did not demonstrate any changes in thigh muscle volume nor lean or fat mass. The authors conclude that combination blockade of both GDF8 and ActA may have more clinically significant effects on maintenance or recovery of skeletal mass in scenarios with muscle wasting, including muscular disorders, sarcopenia and during weight loss.

The manuscript is clearly written, of appropriate length, and strikes a good balance between supplying comprehensive data on the safety and efficacy parameters of this new therapeutic approach while still focusing on a few key messages. The figures are clear and augment the written results. The topic is one of considerable interest to the scientific and medical community, not only in the context of medical illnesses with wasting components such as heart failure, but also with growing recognition of the potential for clinically-meaningful lean muscle loss with therapeutic use of incretin agonists for weight loss. We have the following questions and comments please:

1. Magnitude of lean mass change with combination mAbs: The placebo-adjusted lean mean squares difference for DXA lean mass in the combination groups was substantial for a short 8-week study drug period, at 3.37 (SE 1.10) kg for the 6 mg/kg and 3 mg/kg combination therapy and 3.00 (SE 0.89) kg for the 6 mg/kg and 10 mg/kg combination therapy. Even more surprising was the magnitude of the appendicular lean mass increases for these two groups at 6.39 (SE 1.28) kg and 4.97 (SE 1.03) kg, respectively, suggesting a disproportionate expansion of skeletal muscle mass in the extremities. This is a particularly remarkable increase when considering these were healthy participants who were not anticipated to have baseline skeletal muscle wasting from which augmentation could easily be achieved. By comparison, a 48-week study of bimagrumab showed a lean mass increase of 1.7 kg in adults with type 2 diabetes and overweight/obesity (Heymsfield et al. JAMA Netw Open, 2021) and a DXA lean mass gain of 2-3 kg over an 8-12 week period would ordinarily be considered clinically significant.

2. Indicators of regional adiposity: Given that MRI thigh images were obtained for body composition assessment, is it possible to obtain any measures of intramuscular vs intermuscular fat volume changes? A decrease in intermuscular fat is often observed with favorable changes in body composition. Did the MRI images extend to include any assessment of visceral versus subcutaneous adiposity? Location of adipose tissue loss can help to indicate the metabolic implications of the change in body composition.

3. Strength or functional capacity measures: Changes in skeletal muscle volume or overall lean mass are most relevant when coupled with metrics of muscle strength or functional status. Were any such measured obtained within this study protocol?

4. Dietary changes or impact on appetite: GDF15 inhibition induces increases in appetite, which may mediate some of the gains in bodyweight observed in studies of ponsegromab. Were changes in appetite assessed within this protocol? Given the magnitude of lean mass increases in the combination mAb administration groups, it would be anticipate that dietary macronutrient intake must have increased over the 8-week interval to support the degree of muscle mass increase observed.

5. Relevance of the body composition assessments with ActA inhibition in males?: Do the authors assign any sex-specific relevance to the absence of muscle mass effects in the small group of males receiving ActA mAb administration alone? It would be useful to hear if this finding was interpreted as a consequence of the small sample size and monotherapy, or if there is any reason to suspect that females may have a greater lean mass response than males. This is particularly clinically relevant given that some of the prior neutral studies of GDF8 inhibition amongst patients with muscle wasting were limited only to males (Duchenne muscular dystrophy, prostate cancer). It is unfortunate in this regard that the cohort of females receiving monotherapy ActA mAb Q4W x 4 doses “did not have any MRI or DXA assessments and was therefore not included in those analyses.” Such data would have been helpful in clarifying whether there was sexual dimorphism in response. Why were these assessments not performed in this group?

6. Please clarify (perhaps in the Supplementary materials) the rationale for the dosing scheme in both phase 1 and phase 2 of the study. For phase 1, the investigators did follow the classical pattern for evaluation of a “combination product,” that is of testing each component separately and then in combination. But why was the dose of the anti-GDF8 fixed at 6 mg/kg while that of the anti-ActA ranged from 1 to 10 mg/kg? Was this based upon prior data with the anti-GDF8 mAB showing toxicity or a plateau of dose-response at or above 6 mg/kg? For phase 2, please explain the rationale for the asymmetry in dosing arms – why were different dosing intervals used for the two cohorts of post-menopausal females (Q4wks for the anti-ActA and Q2W for the anti-GDF8 and anti-ActA combination), and why was only the anti-ActA agent, rather than the combination which is the main topic of this study, tested in the male cohort?

7. Please clarify how and why two baseline MRIs and 2 baseline DXAs were obtained for each subject (Figure 1). What was the rationale for two baselines (while subsequent evaluations were single) and how were they handled – what was the time interval between the studies, were the results averaged?

8. How were the safety reviews performed between each of the dosing cohorts in Phase 1? Were the safety reviews performed after safety data on each cohort had been obtained through Week 16 (the timepoint of the primary safety endpoint)? What data or safety measures were assessed, and by whom – was there an independent unblinded Data Safety Monitoring Committee or did investigators or sponsor have access to the interim safety data?

9. Regarding trial oversight - were there any investigators independent of the trial sponsor (Regeneron), and if so, did any investigators have the right to make an independent decision regarding contents of the publication of results?

10. The high frequency of mouth ulcers and muscle spasms is interesting. Are there any data regarding the time course of these events relative to dosing?

11. The description of and results for the ligand assay are somewhat confusing. In the Methods section, it is stated that "To evaluate ligands, serum samples were analyzed for total GDF8 and total ActA using non-validated ELISAs. Total ligand is defined as the sum of free and bound ligand (i.e., ligand not bound to antibody plus ligand bound to antibody)." If the assay was able to detect free GDF8 and ActA ligand that is not bound to antibody, why were levels of these endogenous ligands undetectable before administration of the mAbs (Figure 4B and 4D)?

12. Much of the first paragraph of the Discussion is repetition of what is presented in the Introduction and could be shortened.

13. The text in lines 240-242 – "…demonstrate that GDF8 and ActA likely explain the effects previously demonstrated by broad blockade of downstream receptors, and are almost certainly the key negative regulators of muscle mass in humans" - is overstated, as it is based upon the limited strength of evidence from indirect comparisons between this trial and the bimagrumab studies. Even if we could accept a similar magnitude of treatment effect between studies, it is impossible to know whether similar findings would have been obtained by combination of anti-GDF8 with an inhibitor of other ligands of activin type II receptors. The authors should modify this speculative statement.

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
No further comments

Reviewer #2

(Remarks to the Author)
The authors have addressed my comments.

Reviewer #3

(Remarks to the Author)
The authors include descriptions of multiple preclinical studies that point to potential negative side effects without including any data. This issue was raised in the first review and appears to not have been addressed. Data must be included for all studies presented in the manuscript.

Reviewer #4

(Remarks to the Author)
Thanks for the thorough replies to my review comments. No further comments.

Reviewer #5

(Remarks to the Author)
The authors have effectively addressed our comments in this current revision.
I have no suggestions for further revision.

Reviewer #6

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 4:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
no further comments

Response to Reviewers
Reviewer #1 (Remarks to the Author):

The authors previously identified ActA as the key second negative regulator acting via ActRIIA/B, as blockade of both GDF8 and ActA in mice/monkeys matches the muscle growth of ActRIIA/B blockade. The authors now studied humans in a randomized placebo-controlled Phase 1 trial. Again , combining GDF8 and ActA blocking antibodies led to greater muscle growth than either antibody alone; in addition increases in muscle were accompanied by reductions in fat. Thus combination ligand blockade with the GDF8 (trevogrumab) and ActA (garetosmab) mAbs led to substantial dose dependent increases in MRI-quantitated thigh muscle volume over placebo that was greater with combination than treatment with either mAb alone. The combination was generally well tolerated. The authors conclude that combined blockade may be a promising therapeutic approach in muscle atrophy and obesity settings.

Response: Thank you for this detailed summary.

The study represents exciting biology and the finding that the combined blockade seems to equivalent to blockade of the relevant receptor, is of course interesting, (and supported by preclinical studies). So far so good.

Response: Thank you. We agree that the biology is exciting.

Clearly the side effect profile is the most important result of this study and the previous experiences with trevogrumab are not comforting. To this reviewer, the dose dependent and high frequency of headache, muscle spasm and mouth ulcerations does not sound nice.

Response: We understand the concerns expressed by the reviewer. The adverse events (AEs) from this study have been transparently portrayed in Table 3 and Supplementary Table 7 (previously Supplementary Table 5). All AEs in the single-dose part of the study were mild-to-moderate and non-serious, and, importantly, no AEs led to discontinuation. In the multiple-dose part of the study, only one serious AE of diverticulitis occurred, which was considered not related to treatment by the investigator. There was also one non-serious AE of herpes zoster that occurred in the anti-ActA alone group, which lead to withdrawal from the trial; this AE was considered related to treatment by the investigator. Importantly, the AEs that occurred in this study did not preclude further development, and do not undermine the central premise that ActA and GDF8 appear to be the dominant negative regulators of muscle mass in humans.

Furthermore, it is important to interpret these AEs in the context of a Phase 1 clinical trial. These are preliminary results in a small number of subjects, and it is typical to observe AEs in clinical studies of experimental drugs. To address the reviewer's underlying concern about benefit:risk of the drug, please note that there is an ongoing Phase 2 study that is cited in the Discussion (www.clinicaltrials.gov, NCT06299098) that will further characterize the safety and tolerability of these mAbs. This Phase 2 study is evaluating a range of doses of the anti-GDF8 mAb (trevogrumab) with and without the anti-ActA mAb (garetosmab) and semaglutide in a larger population, allowing us to put into context the tolerability profile of these drugs relative to an approved GLP-1 agonist, which itself is associated with profound gastrointestinal AEs[1] often resulting in discontinuation.

Finally, we would appreciate further clarity on reviewer's comment that "previous experiences with trevogrumab are not comforting". From our experience, this mAb has an acceptable safety

profile. To our knowledge, there are no trevogrumab publications that raise safety concerns precluding further clinical development.

The findings presented here are based on Dexa scanning or MRI of muscle mass. I can read that body weight was not increased. So I guess there is a limit to the magnitude of the muscle response, although apparently loss of fat mass may have been obscured these changes. Many of the observed findings were not terribly significant. But wat about energy expenditure? If increased EE was to be responsible for the fat loss it would certainly be of interest to measure this?

Response: We agree with the reviewer that effects on body weight are neutral. We hypothesize that the increase in muscle mass compensates for the loss of fat mass, consistent with preclinical findings.[2]

The reviewer raises interesting questions to explain the observed effects, which are worthwhile exploring. The suggestion from the reviewer that energy expenditure may be increased is, in fact, a subject of future investigation. However, in context of a completed Phase 1 trial with prespecified endpoints, post-hoc examination of energy expenditure in this study population is unfortunately not possible and beyond the scope of the paper.

To me the most important is whether this apparently increased muscle mass was functional. So what happened to exercise capacity, muscle strength, VO2max and what about patient reported outcomes in general and pertaining to muscle mass?

Response: We agree with the reviewer that understanding whether increased muscle mass will translate into functional activity is a central question. Preclinical studies support this hypothesis: combined inhibition of ActA and GDF8 in mice and monkeys led to pronounced muscle hypertrophy and increased force production.[3]

However, given this is a completed Phase 1 trial with prespecified endpoints, post-hoc examination of exercise capacity, muscle strength, VO_2 max, and patient-reported outcomes in this study population is unfortunately not possible and beyond the scope of the paper. Moreover, this does not limit the central premise that ActA and GDF8 appear to be the dominant negative regulators of muscle mass in humans.

Reviewer #2 (Remarks to the Author):

This is an important study that should be published. My main criticism is that in order to make the conclusion that targeting both myostatin and activin A gives greater effects on lean mass and fat mass than targeting either alone, the statistical analysis should be between combination versus each alone. In the figures, all comparisons are made only against placebo. The authors show that there is a difference between targeting both versus placebo and that there is no statistical difference between targeting myostatin alone versus placebo or between targeting activin A alone versus placebo. They need to show the statistical analysis for comparing targeting both versus targeting myostatin alone and comparing targeting both versus targeting activin A alone.

Response: Thank you for this feedback. We are pleased to hear that you agree the work should be published. It is fair to ask whether the conclusion – combination blockade of both ActA and GDF8 has greater effects on muscle size than either alone – is supported by statistical comparisons of the treatment arms, and not just against placebo. It should be noted that this study was not designed to formally assess the contribution of each ligand individually, given that the sample size was small in this early phase of development and that the arms were not concurrently randomized. Comparison of active treatment arms versus placebo (rather than to one another directly) is common in this context and in others when sample size becomes a consideration (e.g., dose ranging studies which are not powered for differences between arms, but for each arm versus placebo). For these reasons, comparisons between treatment arms were not pre-specified or planned for in our analyses. Given the question, however, we utilized the same statistical methodology as we did for the formal comparisons on thigh muscle volume, the most sensitive endpoint and the one most relevant for our conclusions, on the effects of these factors on regulating muscle size. In **Supplementary Table 3** below, combination blockade of ActA and GDF8 was compared to blockade of GDF8 alone: the LS mean difference and corresponding 95% CI was 3.12% (-0.50, 6.75) with a nominal p-value of 0.0896. In **Supplementary Table 4** below, combination blockade of ActA and GDF8 was compared to blockade of ActA alone: the LS mean difference and corresponding 95% CI was 4.88% (1.26, 8.50) with a nominal p-value of 0.0095. Despite the design limitations and the sample size constraints (n = 6 for the ActA and GDF8 groups and n = 12 for the combination group), the differences appear striking. We believe these post-hoc comparisons support the conclusions in the manuscript that each ligand contributes to the negative regulation of muscle size and that combination blockade is superior to blockade of either alone.

We have now added text on the post-hoc analysis to the main manuscript (please see lines 116-119 in the in the Results and lines 384-388 in the Methods) and have included both tables below in the Supplement (**Supplementary Table 3**; **Supplementary Table 4**).

Supplementary Table 3: Post-hoc analysis: Percent change from baseline to week 8 in thigh muscle volume to compare combination therapy vs GDF8 monotherapy (healthy postmenopausal females)

	Anti-GDF8 6 mg/kg (*n* = 6)	Anti-GDF8 6 mg/kg + anti-ActA 1 mg/kg (*n* = 6)	Anti-GDF8 6 mg/kg + anti-ActA 3 mg/kg (*n* = 6)	Anti-GDF8 6 mg/kg + anti-ActA 10 mg/kg (*n* = 12)
Thigh muscle volume (cm^3) by MRI				
Week 4				
LS Mean (95% CI)	3.27 (1.12, 5.41)	4.96 (2.81, 7.11)	4.61 (2.47, 6.76)	5.92 (4.41, 7.44)
LS Mean difference vs anti-GDF8 (95% CI)	-	1.69 (-1.34, 4.72)	1.35 (-1.68, 4.37)	2.65 (0.02, 5.28)
Nominal *P*-value	-	0.2658	0.3743	0.0481
Week 8				
LS Mean (95% CI)	4.61 (1.65, 7.57)	3.51 (0.54, 6.48)	6.19 (3.24, 9.14)	7.73 (5.64, 9.82)
LS Mean difference vs anti-GDF8 (95% CI)	-	-1.1 (-5.28, 3.08)	1.58 (-2.60, 5.76)	3.12 (-0.50, 6.75)
Nominal *P*-value	-	0.5978	0.449	0.0896

Full analysis set presented. LS means, 95% confidence intervals, and p-values are taken from ANCOVA. The model includes baseline measurement as covariate and the treatment as fixed factor. P-values were not adjusted for multiple testing.
ActA activin A, *CI* confidence interval, *GDF8* growth differentiation factor 8, *LS* least squares, *MRI* magnetic resonance imaging.

Supplementary Table 4: Post-hoc analysis: Percent change from baseline to week 8 in thigh muscle volume to compare combination therapy vs anti-ActA monotherapy (healthy postmenopausal females)

	Anti-ActA 10 mg/kg (n = 6)	Anti-GDF8 6 mg/kg + anti-ActA 1 mg/kg (*n* = 6)	Anti-GDF8 6 mg/kg + anti-ActA 3 mg/kg (*n* = 6)	Anti-GDF8 6 mg/kg + anti-ActA 10 mg/kg (*n* = 12)
Thigh muscle volume (cm^3) by MRI				
Week 4				
LS Mean (95% CI)	0.27 (-1.88, 2.42)	4.96 (2.81, 7.11)	4.61 (2.47, 6.76)	5.92 (4.41, 7.44)
LS Mean difference vs anti-ActA (95% CI)	-	4.69 (1.64, 7.75)	4.35 (1.31, 7.39)	5.66 (3.03, 8.28)
Nominal *P*-value	-	0.0035	0.0061	<.0001
Week 8				
LS Mean (95% CI)	2.85 (-0.11, 5.82)	3.51 (0.54, 6.48)	6.19 (3.24, 9.14)	7.73 (5.64, 9.82)
LS Mean difference vs anti-ActA (95% CI)	-	0.66 (-3.56, 4.87)	3.34 (-0.86, 7.53)	4.88 (1.26, 8.50)
Nominal *P*-value	-	0.7552	0.1157	0.0095

LS means, 95% confidence intervals, and p-values are taken from ANCOVA. The model includes baseline measurement as covariate and the treatment as fixed factor. P-values were not adjusted for multiple testing.
ActA activin A, *CI* confidence interval, *GDF8* growth differentiation factor 8, *LS* least squares, *MRI* magnetic resonance imaging.

Reviewer #3 (Remarks to the Author):

Trotter et al. report on two clinical trials investigating the impact of combined inhibition of GDF8 ActA on muscle mass in women. This is an innovative and potentially important therapeutic approach to treat muscle atrophy. I have only minor suggestions to improve the manuscript, detailed below:

1. It would be helpful if the authors included data from the animal studies investigating reproductive toxicity in rat and nonhuman primate models, currently described in the supplement.

Response: We agree it is helpful to provide data from animal studies, and therefore have provided the results from these studies in the Supplement as the reviewer notes. The results in the Supplement are the complete nature of the data one gets from these toxicity studies. Tables with number of animals and/or toxicokinetics are beyond the scope of *Nature Communications* and do not provide any additional needed information for the audience regarding the rationale for initially excluding males from the single-dose part of the study.

2. Given that the combination therapy was only tested in females, generalizability to males should be tempered and it should be made more clear in the title and abstract that the results on combination therapy reported are specific to women.

Response: We acknowledge and appreciate the reviewer's feedback. We have clarified the sex in the title of the manuscript, added additional detail into the abstract regarding sex for both parts of the study, and have tempered references to "humans" throughout the manuscript.

Of note, we also state the following in the limitations section of the Discussion: "The key parts of the study and conclusions of the impact of the combination of ActA mAb and GDF8 mAb were based on a study in postmenopausal women only (males did not receive the combination)."

3. It would be helpful if the authors explained what the potential mechanisms are by which muscle mass returns to baseline after washout.

Response: GDF8/myostatin is a muscle rheostat (see Lee 2010)[4] and GDF8 levels increase in proportion to muscle mass (see Orioli et al. 2024).[5] We speculate this is in an effort to keep muscle growth in check because growing muscle is such an energy intensive process. Having no GDF8 signal is a non-physiologic state akin to leptin deficiency for adipose; the absence of GDF8 signaling likely suggests to the muscle that it has wasted away to nothing and needs to promote growth. Thus, once the antibody levels decline to the point that the GDF8 signal returns, the muscle stops trying to grow and shrinks back to its pre-treatment size.

This above hypothesis suggests that these ligands need to be constitutively blocked in order to maintain the effects on lean mass. As observed in the single-dose part of the study, maintenance of inert complexes between the individual mAbs and the respective ligands were consistent with the coincident effects on muscle. In the multiple-dose part of the study, serum concentrations of the mAbs paralleled the effects on muscle volume (i.e., as concentrations declined muscle volume returned to baseline).

Importantly, the duration of this study was short and there was no exercise intervention because we wanted to understand, in the least-confounded way, the effects of inhibiting these factors on

muscle growth. Thus, we did not ask subjects to do anything to retain muscle mass (e.g., exercise).

4. Its not clear to me why none of the data from the multi-dose trial are included in the main figures. Also, it would be helpful if the authors comment on why the effect of a single dose and multiple doses was similar, was this expected?

Response: The reviewer raises a valid query regarding placement of the multiple-dose data in the Supplement. Our preference was to tell a focused story. The single-dose part of the trial (Part I) was designed to address the central question of how inhibition of the two ligands impacts body composition, while the multiple-dose part of the study (Part II) was an amendment put in place to corroborate the findings from the single-dose part of the study as well as provide safety data in men to enable continued development of the compound in a Phase 2 study. Thus, we thought placement of the multiple-dose data in the Supplement was appropriate.

Regarding the reviewer's question on expectations of the effect of single- and multiple-dose administration, we didn't know what to expect. We think single-dose effects are maximal or near-maximal, and this is corroborated by the multiple-dose information data. This is consistent with effects in animals where near-maximal effects are apparent acutely.[2] We now comment on this in the Discussion (see lines 204-207): "Consistent effects of the combination treatment on thigh muscle MRI were observed following either single or multiple doses**, suggesting maximal or near-maximal effects were observed after a single dose**."

Reviewer #4 (Remarks to the Author):

The paper reports results from a randomised placebo-controlled phase 1 trial investigating changes in body composition following exposure to two monoclonal antibodies (mAbs): a GDF8 mAb (trevogrumab) and to an ActA mAb (garetosmab) given alone or in combination. The trial design, sample size and statistical methods are in line with best practice for this type of investigations. The conclusions drawn are supported by the objectives, trial design and reported results. While early in development the compounds investigated could address important unmet medical needs in the future.

Response: Thank you for this detailed summary.

Major comments:
- none

Minor comments:
- The trial completed 2019-04-30 and for transparency it would be prudent to provide a statement revealing the status for the two mAbs, e.g. reported and ongoing trials including pursued and abandoned indications.

Response: We agree with the reviewer. In the Discussion section, we note that the Act mAb alone is being studied in fibrodysplasia ossificans progressiva (FOP) and cite results of the LUMINA-1 study recently published in *Nature Medicine.* Please see lines 212-223 of the Discussion for details.

Additionally, we discuss and reference the NCT number (www.clinicaltrials.gov, NCT06299098) for the ongoing Phase 2 trial of the GDF8 and ActA mAbs in combination with a GLP1-receptor agonist (semaglutide) in Discussion (see lines 244-262). In addition to what is discussed in the manuscript, the GDF8 + ActA combination is a subject of active investigation, with additional trials currently being planned in subjects with obesity.

- As described in the protocol, single dose administration of each mAb alone had already been safety cleared in human up to and exceeding the dose levels used in the present trial. This should be mentioned in the introduction and references to the relevant clinical trials should be provided if available.

Response: Another reviewer has requested the rationale for dose selection to be added in detail to the Supplement. We therefore added the information requested here to that same section and now reference these Supplementary Methods in the Methods section of the main manuscript. This information is supported with references if available; only the FIH data for the ActA mAb has been published. For the GDF8 mAb, we have cited the NCT numbers for the relevant studies, since these data are not published.

- Please check the y-axis of all five panels in Figure 3, something is off with e.g. the yellow bar in panel A reaching far above 2%.

Response: We double checked the figures. Regretfully, we do not understand the reviewer's query. Can you kindly provide clarity on this request? Perhaps there is a misunderstanding of the numbers above the bars. As indicated in the figure legend, "In panels A-E, column data labels indicate difference from placebo."

Perhaps it would be helpful to evaluate the yellow bar in panel A of Figure 3 in more detail (see first screenshot below). The data supporting the yellow bar can be found in Table 2 (see second screenshot below). The yellow bar represents the least-squares mean and standard error (as indicated in the figure legend), so a value of 4.61 with an error bar of 1.49. The "+3.7" above the yellow bar is the LS mean difference vs placebo in Table 2 (3.73), which was a nominally statistically significant difference vs placebo (nominal *P* = 0.047 in Table 2) and therefore given an asterisk in the figure.

We hope the description above helps to address the reviewer's query.

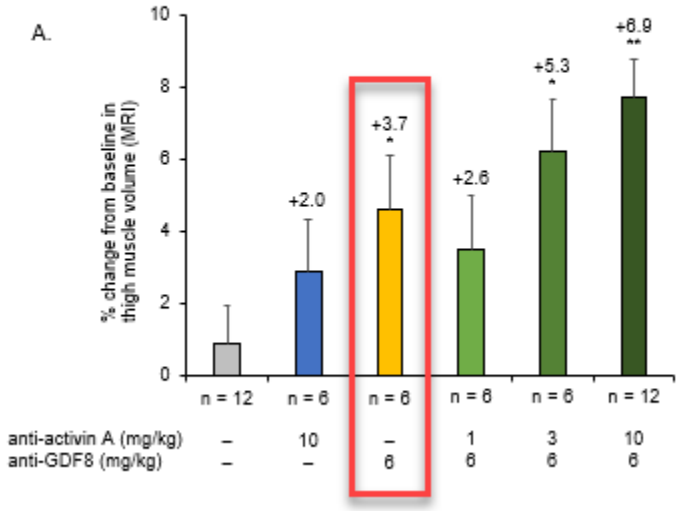

Table 2 | Percent change from baseline to week 8 in lean body mass and fat mass following single-dose blockade of GDF8 and ActA (healthy postmenopausal females)

	Pooled placebo (*n* = 12)	Anti-ActA 10 mg/kg (*n* = 6)	Anti-GDF8 6 mg/kg (*n* = 6)	Anti-GDF8 6 mg/kg + anti-ActA 1 mg/kg (*n* = 6)	Anti-GDF8 6 mg/kg + anti-ActA 3 mg/kg (*n* = 6)	Anti-GDF8 6 mg/kg + anti-ActA 10 mg/kg (*n* = 12)
Thigh muscle volume (cm^3) by MRI						
Baseline						
Mean (SD)	411.50 (45.17)	425.72 (55.28)	401.08 (65.05)	393.56 (78.84)	405.58 (62.99)	416.33 (66.50)
Percent change from baseline						
Week 4						
LS mean (SE)	0.37 (0.76)	0.27 (1.08)	3.27 (1.08)	4.96 (1.08)	4.61 (1.07)	5.92 (0.76)
LS mean difference vs placebo (SE)		–0.11 (1.32)	2.90 (1.32)	4.59 (1.32)	4.24 (1.32)	5.55 (1.07)
95% CI		–2.77, 2.55	0.24, 5.55	1.92, 7.25	1.59, 6.90	3.38, 7.72
Nominal *P*-value		0.94	0.03	0.0012	0.0025	< 0.0001
Week 8						
LS mean (SE)	0.88 (1.05)	2.85 (1.49)	4.61 (1.49)	3.51 (1.49)	6.19 (1.48)	7.73 (1.05)
LS mean difference vs placebo (SE)		1.97 (1.82)	3.73 (1.82)	2.63 (1.82)	5.31 (1.82)	6.85 (1.48)
95% CI		–1.70, 5.65	0.06, 7.40	–1.05, 6.31	1.64, 8.98	3.86, 9.85
Nominal *P*-value		0.28	0.047	0.16	0.0056	< 0.0001

- In the discussion section (line 248 to 252) it is stated that weight loss following bariatric surgery or GLP1 therapy "can often also result in profound muscle loss" and "while weight regain following these treatments is predominately in fat" providing an unmet medical need for the mAb combination investigated in the trial. Readers could easily get the impression that there is evidence to claim that body composition after weight regain is worse than prior to the surgery or GLP1 induced weight loss. The two referenced papers for the weight regain claim provide no such evidence. Reference 22 is a retrospective observational study trying to predict weight

regain based on changes in weight and body composition following GLP1 treatment. And reference 23 is a cross-sectional database study in sleeve gastrectomy providing no insight into why patients in the weight regain group weighed more or had more fat mass (and muscle mass) than patients who had lost weight – other than the obvious selection bias. Evidence for the claim "while weight regain following these treatments is predominately in fat" should be substantiated or alternatively the sentence should be revised.

Response: Thank you for this feedback. We have deleted the text in question. Please see the revised statement below (as now reflected in the manuscript).

Revision implemented in manuscript: Moreover, it has recently been appreciated that emerging approaches to combat obesity by causing profound weight loss – such as with bariatric surgery or glucagon-like peptide-1 (GLP1)-receptor agonists – can often also result in profound muscle loss, with up to 40% of the associated weight loss accounted for by lean mass loss.[6-8] ~~(while weight regain following these treatments is predominately in fat[9,10]).~~

Reviewer #5 (Remarks to the Author):

Myostatin (MSTN), also known as growth and differentiation factor 8 (GDF8), is an extensively studied negative regulator of skeletal muscle mass. This TGF-beta superfamily signaling protein is high conserved amongst mammalian species and, for example, it has been demonstrated using CRISPR/Cas9 that removing MSTN/GDF8 signaling can substantially augment muscle mass in goats (He Z et al. Bioscience Reports, 2018). GDF8 exerts its muscle degradation effects via binding to activin type II receptors (ActRIIA/B) in skeletal muscle, which activates SMAD 2/3 and prompts the transcription of atrogenes. Consequently various inhibitors of GDF8 and ActRIIA/B have been developed over recent years as potential therapies to prevent or reverse muscle degradation. However, the translation of GDF8 or ActRIIA/B inhibition into human scenarios of muscle wasting, including men with prostate cancer (Padhi et al, J Clin Endocrinol Metab, 2014), boys with muscular dystrophy (Campbell et al. Muscle Nerve, 2017), or older adults with sarcopenia (Rooks et al. JAMA Netw Open 2020), have each been clinically underwhelming to date. Of note, bimagrumab (ActRIIA/B inhibitor) is being studied with and without semaglutide in patients with obesity to determine efficacy and safety in the preservation of lean mass during fat mass loss (e.g. NCT05616013). Also of relevance is the recent publication of a phase 2 study of ponsegromab, a mAb inhibiting GDF15, which demonstrated a 2.8 kg mean bodyweight increase in the maximal dosing group over 12 weeks of administration, versus placebo, amongst adults with cancer cachexia (Groarke et al. NEJM, 2024).

This manuscript reports a phase I study assessing human safety, tolerability, pharmacokinetics, and pharmacodynamics of an GDF8 mAb (trevogrumab) and an ActA mAb (garetosmab). Each were administered alone, as well as GDF8 mAb given in combination with increasing doses of the ActA mAb, with a view towards the development of therapies to reverse skeletal muscle wasting. The study initially recruited only healthy post-menopausal females due to pre-clinical concerns about testicular toxicity; in a subsequent part of the study healthy males not intending to reproduce were also recruited. Body composition was assessed at weeks 4 and 8 of drug delivery. MRI was used for thigh muscle volume assessment and DXA for total lean mass, appendicular lean mass, android fat mass and total fat mass. The main finding of the study was that the combination of GDF8 inhibition wth trevogrumab and ActA inhibition with garetosmab achieved dose-dependent increases in thigh muscle volume per MRI imaging, as compared to placebo, with the combination of agents having greater efficacy that either mAb alone. Fat mass decreased with combination GDF8 and ActA inhibition. The primary endpoint was the incidence and severity of adverse events to week 16 for the single-dose study and to week 40 for the multiple-dose study; there were no significant safety concerns and no antibodies were noted to either mAbs. The male participants were primarily included to assess the tolerability of ActA alone but did not demonstrate any changes in thigh muscle volume nor lean or fat mass. The authors conclude that combination blockade of both GDF8 and ActA may have more clinically significant effects on maintenance or recovery of skeletal mass in scenarios with muscle wasting, including muscular disorders, sarcopenia and during weight loss.

Response: Thank you for this detailed summary.

The manuscript is clearly written, of appropriate length, and strikes a good balance between supplying comprehensive data on the safety and efficacy parameters of this new therapeutic approach while still focusing on a few key messages. The figures are clear and augment the written results. The topic is one of considerable interest to the scientific and medical community, not only in the context of medical illnesses with wasting components such as heart failure, but also with growing recognition of the potential for clinically-meaningful lean muscle loss with therapeutic use of incretin agonists for weight loss. We have the following questions and

comments please:

1. Magnitude of lean mass change with combination mAbs: The placebo-adjusted lean mean squares difference for DXA lean mass in the combination groups was substantial for a short 8-week study drug period, at 3.37 (SE 1.10) kg for the 6 mg/kg and 3 mg/kg combination therapy and 3.00 (SE 0.89) kg for the 6 mg/kg and 10 mg/kg combination therapy. Even more surprising was the magnitude of the appendicular lean mass increases for these two groups at 6.39 (SE 1.28) kg and 4.97 (SE 1.03) kg, respectively, suggesting a disproportionate expansion of skeletal muscle mass in the extremities. This is a particularly remarkable increase when considering these were healthy participants who were not anticipated to have baseline skeletal muscle wasting from which augmentation could easily be achieved. By comparison, a 48-week study of bimagrumab showed a lean mass increase of 1.7 kg in adults with type 2 diabetes and overweight/obesity (Heymsfield et al. JAMA Netw Open, 2021) and a DXA lean mass gain of 2-3 kg over an 8-12 week period would ordinarily be considered clinically significant.

Response: We agree with the reviewer that the comparison of our results to the 48-week, multiple-dose bimagrumab study in in adults with diabetes and obesity (Heymsfield et al.) is striking.[11] In the manuscript we instead discuss cross-study comparisons to the Rooks et al. bimagrumab study.[12] Rooks et al. was conducted in older healthy adults (mean age 60.4 years) treated for 4 weeks with a single-dose of bimagrumab. We feel this design more closely aligns with our study. Following single administration of 30 mg/kg bimagrumab, thigh muscle volume increased 6.1% at week 4 and total fat mass decreased by –1.6%. Similarly, following single administration of GDF8 and ActA mAbs in combination in our study, thigh muscle volume increased 5.92% and 7.73% at weeks 4 and 8 and total fat mass decreased by –2.58% and –4.57%, respectively. However since this is not a formal comparison, we were more conservative in our comparison to Rooks et al. in the Discussion section of the manuscript, simply stating, “Although no head-to-head trials are available, cross-study comparisons suggest that the efficacy of GDF8 and ActA mAbs in combination is similar to bimagrumab, a human mAb that binds and blocks activin type II receptors.[12]”

2. Indicators of regional adiposity: Given that MRI thigh images were obtained for body composition assessment, is it possible to obtain any measures of intramuscular vs intermuscular fat volume changes? A decrease in intermuscular fat is often observed with favorable changes in body composition. Did the MRI images extend to include any assessment of visceral versus subcutaneous adiposity? Location of adipose tissue loss can help to indicate the metabolic implications of the change in body composition.

Response: We did assess intramuscular adipose tissue in this study. In this subject population the volumes were quite modest (a few cubic cm), difficult to segment, and no differences were detected among groups. In this study, MRI assessments were limited to the thighs. Additional MRI assessments of body composition including visceral fat and liver fat fraction are included in an ongoing Phase 2 study in patients with obesity referenced in the Discussion of the manuscript (www.clinicaltrials.gov, NCT06299098).

3. Strength or functional capacity measures: Changes in skeletal muscle volume or overall lean mass are most relevant when coupled with metrics of muscle strength or functional status. Were any such measured obtained within this study protocol?

Response: We agree with the reviewer that understanding whether increased muscle volume or overall lean mass will translate into functional activity is a central question. Preclinical studies

support this hypothesis: combined inhibition of ActA and GDF8 in mice and monkeys led to pronounced muscle hypertrophy and increased force production.[3]

However, given this is a completed Phase 1 trial with prespecified endpoints, post-hoc examination of muscle strength or functional status in this study population is unfortunately not possible and beyond the scope of the paper. Moreover, this does not limit the central premise that ActA and GDF8 appear to be the dominant negative regulators of muscle mass in humans.

4. Dietary changes or impact on appetite: GDF15 inhibition induces increases in appetite, which may mediate some of the gains in bodyweight observed in studies of ponsegromab. Were changes in appetite assessed within this protocol? Given the magnitude of lean mass increases in the combination mAb administration groups, it would be anticipate that dietary macronutrient intake must have increased over the 8-week interval to support the degree of muscle mass increase observed.

Response: The reviewer raises a valid hypothesis. Unfortunately, we did not measure changes in appetite in this study, as it was a Phase 1 trial with the primary of objective of assessing safety and tolerability. Given the trial is complete, it is not possible to measure appetite changes in the study population. Nevertheless, the reviewer raises interesting questions to explain the observed effects, which are worthwhile exploring in future investigations.

5. Relevance of the body composition assessments with ActA inhibition in males?: Do the authors assign any sex-specific relevance to the absence of muscle mass effects in the small group of males receiving ActA mAb administration alone? It would be useful to hear if this finding was interpreted as a consequence of the small sample size and monotherapy, or if there is any reason to suspect that females may have a greater lean mass response than males. This is particularly clinically relevant given that some of the prior neutral studies of GDF8 inhibition amongst patients with muscle wasting were limited only to males (Duchenne muscular dystrophy, prostate cancer). It is unfortunate in this regard that the cohort of females receiving monotherapy ActA mAb Q4W x 4 doses “did not have any MRI or DXA assessments and was therefore not included in those analyses.” Such data would have been helpful in clarifying whether there was sexual dimorphism in response. Why were these assessments not performed in this group?

Response: This is the initial study of the ActA + GDF8 combination in humans. As noted in the Discussion section of the manuscript, one of the limitations of the central findings on muscle growth is that combination data are confined to females (see lines 265-268). As noted in the manuscript (lines 268-270), this was done for practical reasons at an early stage in development when there was uncertainty about potential adverse consequences of ActA blockade on reproductive potential in men based on preclinical rat toxicology findings. Thus, we initially took a conservative approach and didn’t expose men. However, subsequent toxicology results in non-human primates revealed an absence of testicular toxicity, so we added in an ActA monotherapy group in males to the multiple-dose part of the study based on an FDA request.

The FDA also requested additional safety data with greater exposure in females, though this was not a central question of the study. This is the reason we included the ActA monotherapy Q4W x 4 group in females for safety evaluation only.

We have not seen gender-specific differences in mixed-gender preclinical studies in non-human primates,[3] and we saw similar reductions in fat mass and gains in lean mass in male monkeys in the co-submitted preclinical manuscript.[2] Therefore we hypothesize that the observations in the

current study are unlikely a gender-specific phenomenon. We touch on this in the Discussion section in the context of the generalizability of the results (see lines 270-272). However, the ongoing Phase 2 trial (www.clinicaltrials.gov, NCT06299098), which is enrolling men and women, will confirm this hypothesis.

6. Please clarify (perhaps in the Supplementary materials) the rationale for the dosing scheme in both phase 1 and phase 2 of the study. For phase 1, the investigators did follow the classical pattern for evaluation of a "combination product," that is of testing each component separately and then in combination. But why was the dose of the anti-GDF8 fixed at 6 mg/kg while that of the anti-ActA ranged from 1 to 10 mg/kg? Was this based upon prior data with the anti-GDF8 mAB showing toxicity or a plateau of dose-response at or above 6 mg/kg? For phase 2, please explain the rationale for the asymmetry in dosing arms – why were different dosing intervals used for the two cohorts of post-menopausal females (Q4wks for the anti-ActA and Q2W for the anti-GDF8 and anti-ActA combination), and why was only the anti-ActA agent, rather than the combination which is the main topic of this study, tested in the male cohort?

Response: The rationale for the study design, as described in section 3.2 of the protocol included with the submission. This information has now been added to the Supplement.

Briefly, the single ascending-dose part of the study (Part I) was intended to provide initial characterization of the safety, tolerability, and pharmacodynamic (PD) effect of the ActA mAb in combination with the GDF8 mAb. The combination of anti-ActA with anti-GDF8 was hypothesized to have additive (or synergistic) effects on increasing muscle mass based upon preclinical and clinical data from related compounds. The 6 mg/kg fixed dose of the GDF8 mAb used in the study was lower than the highest tolerated dose, and was well-characterized based on previous studies of anti-GDF8 monotherapy. The doses of ActA mAb took into consideration both preclinical and clinical data.[13] The 1 mg/kg dose (low dose) was selected based on exposures from nonclinical mouse studies, which showed a threshold of effect with the combination; the 3 mg/kg dose was an intermediate dose between the high- and low-dose groups; and the 10 mg/kg dose (high dose) was the highest tolerated dose of the first-in-human study of anti-ActA monotherapy.

For the multiple-dose part of the study (Part II) the anti-ActA 10 mg/kg Q4W X 4 group was intended to provide preliminary safety and tolerability data, as well as an initial characterization of the pharmacokinetic (PK) profile. The anti-GDF8 6 mg/kg + anti-ActA 10 mg/kg Q2W × 3 dosing of the combination was studied to understand safety, tolerability, the PK profile, and PD effects with a more frequent dosing regimen, with drug exposures and target saturation over a longer period of time compared to the single-dose part of the study.

7. Please clarify how and why two baseline MRIs and 2 baseline DXAs were obtained for each subject (Figure 1). What was the rationale for two baselines (while subsequent evaluations were single) and how were they handled – what was the time interval between the studies, were the results averaged?

Response: Two scans were acquired before dosing in order to assess test-retest repeatability. The scan most proximal to the date of dosing was used as baseline.

8. How were the safety reviews performed between each of the dosing cohorts in Phase 1? Were the safety reviews performed after safety data on each cohort had been obtained through Week 16 (the timepoint of the primary safety endpoint)? What data or safety measures were

assessed, and by whom – was there an independent unblinded Data Safety Monitoring Committee or did investigators or sponsor have access to the interim safety data?

Response: As described in section 6.1 of the protocol included with the submission, dosing for the single ascending dose part of the study (Part I) was by group (panel), with each group divided into blocks.

The low-dose combination group (anti-GDF8 6 mg/kg + anti-ActA 1 mg/kg) included 3 blocks. Block 1 included 2 subjects: one placebo and one combination therapy. Dosing of the remaining 6 subjects in the group could only begin after the subjects in block 1 successfully completed the day 2 safety assessments, and the blinded study data had been reviewed. These 6 subjects were divided into 2 blocks (block 2 and block 3), with 2 subjects in block 2 and 4 subjects in block 3. Dosing of block 2 and block 3 was performed on different days.

Dose escalation from the low-dose combination group (anti-GDF8 6 mg/kg + anti-ActA 1 mg/kg) to the medium-dose combination group (anti-GDF8 6 mg/kg + anti-ActA 3 mg/kg) could only proceed once all subjects had completed day 15 safety assessments (adverse events, clinical laboratory tests, assessment of vital signs, and ECGs), the blinded safety data had been reviewed at a Dose Escalation Review meeting, and the decision for dose escalation had been approved. The Dose Escalation Review meeting was led by a designated member of the Regeneron clinical team (generally either the medical monitor or the clinical trial manager) and at a minimum was attended by the Regeneron Medical Monitor and the Risk Management Lead or designee. Other individuals, including the Investigator, may have been included. Dose escalation decisions were determined as outlined in protocol section 6.1.1.

The remaining group escalations were able to proceed once all subjects in the prior group had completed day 15 safety assessments and the blinded safety data had been reviewed at a Dose Escalation Review meeting. The remaining groups were also divided into blocks, which are described in further detail in protocol section 6.1.

9. Regarding trial oversight - were there any investigators independent of the trial sponsor (Regeneron), and if so, did any investigators have the right to make an independent decision regarding contents of the publication of results?

Response: No, there were no investigators independent of the trial sponsor. A Data Monitoring Committee (DMC) is not usually warranted for a Phase 1 clinical trial per the FDA guidance entitled “Guidance for clinical trials sponsors: On the establishment and operation of clinical trial data monitoring committees” available at www.fda.gov/media/75398/download.

10. The high frequency of mouth ulcers and muscle spasms is interesting. Are there any data regarding the time course of these events relative to dosing?

Response: For AEs related to mouth ulcers, these events started 8 to 35 days post first dose administration of either GDF8 + ActA (3 mg/kg) or GDF8 + ActA (10 mg/kg) and lasted for 10 to 68 days. These were mild to moderate in severity, non-serious, and did not lead to study discontinuation. No AEs related to mouth ulcers were reported in the multi-dose cohorts except for 1 event that occurred in the placebo arm at day 189.

In single-dose combination treatment groups, 18 AEs related to muscle spasms occurred in 14 participants. The median time to onset and range for the GDF8 + ActA (1 mg/kg), GDF8 + ActA (3 mg/kg), and GDF8 + ActA (10 mg/kg) arms were 19 days (4-80), 39 days (19- 64), and 32

days (8-57), respectively. All but one of these AEs were deemed by the investigator to be related to study drug; all were mild to moderate in severity. In the multi-dose cohorts, 12 AEs related to muscle spasms occurred in 8 participants including 1 placebo participant. In the GDF8 + ActA (10 mg/kg) Q2W X 3 arm, the median time to onset was 14 days (range: 4 to 50 days) across 6 events whereas the ActA (10 mg/kg) Q4W X 4 monotherapy arm was 15 days (range: 5 to 43 days) across 5 events. These events were mild to moderate in severity, non-serious, and did not lead to discontinuation of treatment. No muscle spasm events occurred in the multi-dose monotherapy GDF8 arm.

LUMINA-1 was a phase 2 clinical in FOP participants evaluating ActA (10 mg/kg) given Q4W.[14] In the double-blind period, mouth ulcers and muscle spasms occurred in 10% (2/20) and 10% (2/20) of participants after 7 doses, respectively. Additionally, please note that there is an ongoing Phase 2 study that is cited in the Discussion (www.clinicaltrials.gov, NCT06299098) that will further characterize the safety and tolerability of these mAbs. This Phase 2 study is evaluating a range of doses of the anti-GDF8 mAb (trevogrumab) with and without the anti-ActA mAb (garetosmab) and semaglutide in a larger population, allowing us to further evaluate the safety profile.

11. The description of and results for the ligand assay are somewhat confusing. In the Methods section, it is stated that “To evaluate ligands, serum samples were analyzed for total GDF8 and total ActA using non-validated ELISAs. Total ligand is defined as the sum of free and bound ligand (i.e., ligand not bound to antibody plus ligand bound to antibody).” If the The was able to detect free GDF8 and ActA ligand that is not bound to antibody, why were levels of these endogenous ligands undetectable before administration of the mAbs (Figure 4B and 4D)?

Response: At baseline (before administration of the mAbs), the concentrations of the total GDF8 and/or total ActA are below the lower limit of quantification (LLOQ) in the respective assays. For reference, the LLOQ for the total GDF8 and total ActA are 4 ng/mL and 0.313 ng/mL, respectively. This suggests that these ligands are either produced at low concentrations or they clear very fast from the blood. Following administration of the administration of the mAbs, the ligands bind to the antibody. The antibody-ligand complex clears slowly because the clearance or elimination of the complex is driven by the pathways that clear the antibody, and this is much slower than the free ligand. This results in a gradual increase of total ligand concentrations in serum, and the assay can detect and quantify the total concentrations.

12. Much of the first paragraph of the Discussion is repetition of what is presented in the Introduction and could be shortened.

Response: We have removed the parts of the Discussion that are repetition of what is presented in the Introduction.

13. The text in lines 240-242 – “…demonstrate that GDF8 and ActA likely explain the effects previously demonstrated by broad blockade of downstream receptors, and are almost certainly the key negative regulators of muscle mass in humans” - is overstated, as it is based upon the limited strength of evidence from indirect comparisons between this trial and the bimagrumab studies. Even if we could accept a similar magnitude of treatment effect between studies, it is impossible to know whether similar findings would have been obtained by combination of anti-GDF8 with an inhibitor of other ligands of activin type II receptors. The authors should modify this speculative statement.

Response: We have removed this speculative statement from the Discussion section. Please see the revised paragraph below:

Although no head-to-head trials are available, cross-study comparisons suggest that the efficacy of GDF8 and ActA mAbs in combination is similar to bimagrumab, a human mAb that binds and blocks activin type II receptors.[12] Thus, specific blockade using just our GDF8 and ActA mAbs produces similar benefits as does a receptor-blocking mAb that blocks multiple ligand pathways, while potentially avoiding adverse effects that might emerge as a result of complete ActRIIA and ActRIIB blockade. ~~Taken together, these findings corroborate the previous preclinical studies[3] and now demonstrate that GDF8 and ActA likely explain the effects previously demonstrated by broad blockade of downstream receptors, and are almost certainly the key negative regulators of muscle mass in humans.~~

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Response to Reviewers

Reviewer #1 (Remarks to the Author):

No further comments
Response: Thank you for your review.

Reviewer #2 (Remarks to the Author):

The authors have addressed my comments.
Response: Thank you for your review.

Reviewer #3 (Remarks to the Author):

The authors include descriptions of multiple preclinical studies that point to potential negative side effects without including any data. This issue was raised in the first review and appears to not have been addressed. Data must be included for all studies presented in the manuscript.
Response: We appreciate the reviewer's comment. Our desire is to be as transparent as possible. We now recognize that the previous summary of preclinical data regarding possible adverse consequences on reproductive potential was not optimal and understandably did not fully address the reviewer's query during the previous round of review. **We have now added detailed tables to provide granular information on the methods and findings for each preclinical study** previously discussed in the Supplementary Materials. Please see pages 4-9 of the Supplementary Materials – the section called Supplementary Results – for the added information. It is important to note that the results in the Supplementary Materials are the nature of the data one gets from toxicity studies conducted by a contracted organization, and that the data now presented in the Supplementary Materials is reflective of the confidential information shared with trial clinicians and regulatory agencies in the Investigator Brochure.

We have also added language to the Discussion section to make the preclinical toxicity issues more transparent and have added qualifying language to the 'generally well tolerated' safety profile by reminding readers of the muscle spasms and aphthous ulcers that were observed in the study.

Additionally, we recognize that the preclinical data previously added into the Supplementary Materials may not have been clear to the reviewer (and to potential readers), so we have revised the text in the manuscript to explicitly direct the reader to the data available to support the statements in the manuscripts regarding the:

1) Rationale for inclusion of only postmenopausal females based on preclinical toxicity studies in Sprague Dawley rats and cynomolgus monkeys – see lines 80-84 (Results), 282-285 (Discussion), and 311-315 (Methods).
2) Rationale for the initial exclusion of males from the study based on testicular toxicity findings in Sprague Dawley rats – see lines 84-88 (Results), 282-285 (Discussion), and 316-319 (Methods).
3) Rationale for including an additional panel of males in part II of the study once subsequent toxicology findings in non-human primates revealed an absence of testicular toxicity – see lines 91-95 (Results), 282-285 (Discussion), and 319-322 (Methods).

Regarding the inclusion of males in part II of the study, early on in development we intentionally avoided the inclusion of males due to initial toxicity findings in rats. However, we could not

recapitulate the rat findings in non-human primates. We therefore added a panel of male subjects in part II of the study (primarily for safety evaluations) and a monitoring plan for males was applied to clinical trials with these agents, which was approved by several regulatory agencies. In the current study, no TEAEs of special interest applicable to male subjects – epididymitis, orchitis (inflammation of the testicles), hydrocele (fluid buildup around 1 or both testicles), scrotum pain, and scrotum swelling – occurred in males (see Supplementary Table 7 for details). It is also important to note that we have forthcoming clinical data in both male and female human participants age ≥18 to ≤55 years treated with the GDF8 mAb alone (healthy volunteers), as well as the GDF8 mAb + semaglutide (GLP1-receptor agonist) and the GDF8 mAb + the ActA mAb + semaglutide (patients with obesity) that will ultimately further characterize the potential benefit risk profile of these agents (this has also been elaborated further in the Discussion). Participants must be willing to use highly-effective methods of contraceptives to be included in the study, and the study will include contraception monitoring as part of the safety assessment. We reference this study in the manuscript (www.clinicaltrials.gov, NCT06299098), and the report of this study, once available, will include full safety data in human participants.

Finally, we also recognize that we sometimes referred to preclinical “studies” (plural) in the manuscript text when we were only talking about a single study. We have therefore modified the manuscript text accordingly, which may help to limit confusion.

We hope these revisions address the reviewer’s concerns.

Reviewer #4 (Remarks to the Author):

Thanks for the thorough replies to my review comments. No further comments.
Response: Thank you for your review.

Reviewer #5 (Remarks to the Author):

The authors have effectively addressed our comments in this current revision.
I have no suggestions for further revision.
Response: Thank you for your review.

Reviewer #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.
Response: Thank you for your review.