nature portfolio

Peer Review File

Efficacy and safety of GLP-1 analog ecnoglutide in patients with type 2 diabetes: a randomized phase 2 trial

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This paper describes a phase 2 clinical trial with ecnoglutide, a new GLP-1 receptor agonist. Ecnoglutide is a recombinant GLP-1 analogue that has a modified primary sequence with valine as the second amino acid (GLP-1 [1-36] position 8), which prevents inactivation by DPP-4 and potentially ease of synthesis. The peptide has shown bias at the GLP-1R toward G-protein compared to arrestins, and has PK compatible with once weekly dosing. The study described in this paper was an RCT of 3 graded doses of ecnoglutide and placebo with a primary outcome measure of A1c after 20 weeks of therapy. Participants had an average A1c of 8.5 and BMI of 26 and were taking either oral agents or controlling glycemia with diet and exercise there was good balance of age, sex, weight and A1c across the four study groups. The key findings presented in this paper are: a) subjects receiving ecnoglutide had reduction of A1c from 1.8 to 2.4 % compared to 0.5% for the placebo treated subjects; b) there was small but significant weight loss in the ecnoglutide group; c) the side effect profile of the ecnoglutide treated subjects was typical of drugs in the GLP-1RA class with primarily nausea and diarrhea that was dose related and minimal hypoglycemia; d) PK was proportional to the dose of ecnoglutide with a linear increase to steady state. The authors conclude from this study that ecnoglutide is effective for reducing A1c and body weight in subjects with type 2 diabetes.

Overall this is a well designed and executed phase 2 study. The analytic approach is straightforward and meets current standards; it would be useful to understand how the 3 groups of placebo treated subjects were defined. The effects of treatment with ecnoglutide on A1c over the relatively short study period were strong and in keeping with other once weekly GLP-1RA such as semaglutide. The safety profile also followed a pattern expected for a drug of this class. Thus, while a proper demonstration of the efficacy of a new GLP-1 based drug, this paper does not extend understanding of the GLP-1R in diabetes or therapeutics. The results, while clear, are completely predictable. The interesting features of ecnoglutide, e.g. its bias at the GLP-1R and its putative advantage for synthesis, are not addressed directly here. This work supports advancing ecnoglutide to larger and longer trials, but otherwise adds very little new information except that the Ala-Val substitution in the primary sequence of GLP-1 does not impact its activity as an agonist.

Reviewer #2 (Remarks to the Author):

The manuscript evaluates the efficacy and safety of Ecnoglutide (XW003), a Glucagon-like peptide-1 (GLP-1) analog, in Type 2 diabetic patients through a placebo-controlled, double-blind, randomized controlled trial. This study explores a novel GLP-1 with potentially significant therapeutic implications.

Overall, the study exhibits robust design elements: well-defined inclusion and exclusion criteria, primary and secondary outcome measures, pharmacokinetic endpoints, and rigorous safety assessments, including the evaluation of adverse events (AE) and serious adverse events (SAE). It will be helpful to provide the primary objective of the study in the Abstract.

However, the manuscript lacks comprehensive information about the statistical methodologies employed for data analysis. This impacts the clarity and rigor of the models and their alignment with the results. I have summarized some of the statistical issues below.

Sample Size Calculation: The manuscript justifies the sample size calculation based on a one-sided significance level, but it fails to provide reasoning for not considering a two-sided significance level. In randomized controlled trials (RCTs), a two-sided approach is often preferred due to a lack of a priori knowledge about the direction of the treatment effect.

Clinical Significance: The assumption that a 1.0% difference in mean change from baseline in HbA1c between the Ecnoglutide group and the placebo is clinically significant requires substantiation with evidence.

Placebo Arm: The placebo arm had a similar three Ecnoglutide regimens (0.4, 0.8, and 1.2 mg), however, it looks arbitrary that patients in the placebo arm were pooled without any further reasonings. The fact that the sample size was calculated as a single placebo arm cannot be an adequate justification.

Imputation Strategy: The last observation carried forward (LOCF) imputation strategy for the missing values is not an ideal approach unless a strong justification is given. The protocol version indicates that LOCF might have been applied for a different reason. It states that for the subjects with a lack of change in the HbA1c at Week 20 from the baseline, the LOCF was applied. If the data were available, the lack of change or not, it is not advisable to employ LOCF.

Repeated Measures Model: The manuscript should provide a comprehensive explanation of the repeated measures model, including details such as the model specification to account for the correlation between multiple measurements within a subject, the covariance structure or random effects structure used, estimation methods for fixed and random effects, and methods for estimating standard errors and 95% confidence intervals. If fitted models are different for the primary and secondary outcomes, provide full descriptions along with the predictors used.

Estimated Difference: For example, it is not clear if Figures 1A and 1B present the same model or two different models. The manuscript should clarify how the estimated mean difference and the corresponding SE and 95% CI (presented in these and other Figures) were obtained. If the model included other covariates (age, sex), state how the adjusted mean differences accounting for these covariates were obtained (as presented in Figures 1, 2 and 3).

Baseline Adjustment: The manuscript should clarify if the model adjusted for the baseline HbA1c and provide details about the response variable for such a model. The protocol version (with a similar indication in the manuscript) of modelling the difference with the baseline HbA1c as a covariate is not an appropriate approach.

Multiple Comparisons: An appropriate and simple contrast could be comparing the average of all doses in the treatment arm versus the control arm. It is important to state if the stated p-values were adjusted to account for the inflation of type 1 error due to multiple comparisons.

Interaction Effects: The manuscript should indicate whether interaction effects, such as treatment by time interaction or treatment by baseline value interaction, were explored. If not, please justify since the two-way interaction effect of treatment by time may look reasonable as indicated in the Figures.

Multicenter Trial: The RCT was conducted as a multicenter trial. It is a standard practice to include the center as a random effect. It is not mentioned in the statistical analysis section.

Model Assumptions: The manuscript should demonstrate that all model assumptions have been thoroughly checked and confirmed.

Per-Protocol Analysis: The approved study protocol mentions conducting a Per-Protocol analysis. The manuscript should provide details on the outcomes of this analysis and present additional results as supplementary information.

Sub-Group Analysis: The manuscript should specify whether any sub-group analyses were conducted.

Secondary Efficacy Indicators: The comments provided above are equally applicable to secondary efficacy indicators such as changes in glucose and glucagon from baseline and mean change in body weight from baseline, as well as the pharmacokinetics of Ecnoglutide.

Code Availability: It would be helpful to include all SAS codes with relevant details and simulated data as supplementary information to facilitate results and plots reproducibility. A statement regarding code availability should be incorporated.

Data Presentation: The 'Data Presentation' statement does not conform to the manuscript. For example, the manuscript does not present individual data points on figures or provide sufficient clarity on the distribution of relevant data.

Reviewer #3 (Remarks to the Author):

Ecnoglutide is a once-weekly injectable GLP-1R agonist which was evaluated as a monotherapy in the drug naïve or mono-OAD treated T2D patients who were randomized 1:1:1:1 to placebo: 0.4 mg:0.8mg:1.2mg groups for a treatment of 20 weeks followed by a 5-week safety observation follow-up. Significant HbA1c reductions achieved in each dose group compared with placebo. Body weight reduction is moderate with 1.2mg group in which equal or greater than 5% weight loss is 33.3%.

At lower doses, the Ecnoglutide showed good glucose reduction effect comparable with Semaglutide at lower doses.

Overall it is well designed and executed study. Results are presented in line with the expectation.

There are a few questions need to be discussion:

1. Could the author explain the reasons for relatively high % of dyslipidemia and hypoglycemia with Ecnoglutice in this study?

2. The weight reduction is moderate at highest dose of 1.2mg. What is the reason for not testing Ecnoglutide at higher dose to enhance the weight reduction effect?

3. The plasma levels of the tested drug at 0.8 and 1.2 mg differ in day 50 (completed titration) and day 134. Will these possible drug cumulation continue when it is used for a longer period of treatment?

RESPONSE TO REVIEWER COMMENTS

Reviewer requests have been numbered and responses are in purple.

Reviewer #1 (Remarks to the Author):

This paper describes a phase 2 clinical trial with ecnoglutide, a new GLP-1 receptor agonist. Ecnoglutide is a recombinant GLP-1 analogue that has a modified primary sequence with valine as the second amino acid (GLP-1 [1-36] position 8), which prevents inactivation by DPP-4 and potentially ease of synthesis. The peptide has shown bias at the GLP-1R toward G-protein compared to arrestins, and has PK compatible with once weekly dosing. The study described in this paper was an RCT of 3 graded doses of ecnoglutide and placebo with a primary outcome measure of A1c after 20 weeks of therapy. Participants had an average A1c of 8.5 and BMI of 26 and were taking either oral agents or controlling glycemia with diet and exercise there was good balance of age, sex, weight and A1c across the four study groups. The key findings presented in this paper are: a) subjects receiving ecnoglutide had reduction of A1c from 1.8 to 2.4 % compared to 0.5% for the placebo treated subjects; b) there was small but significant weight loss in the ecnoglutide group; c) the side effect profile of the ecnoglutide treated subjects was typical of drugs in the GLP-1RA class with primarily nausea and diarrhea that was dose related and minimal hypoglycemia; d) PK was proportional to the dose of ecnoglutide with a linear increase to steady state. The authors conclude from this study that ecnoglutide is effective for reducing A1c and body weight in subjects with type 2 diabetes.

Overall this is a well designed and executed phase 2 study.

1. The analytic approach is straightforward and meets current standards; it would be useful to understand how the 3 groups of placebo treated subjects were defined.

   We thank the reviewer for this feedback. The placebo participants were randomized to receive an injection volume matching that of one of the three ecnoglutide arms. This was required as the injection volumes of the ecnoglutide doses were different, and the injector pen volume is pre-set.

   We have added text to explain the placebo dosing and the rationale for pooling the placebo participants to the Study Design (**Page 4**) and Statistical Analyses methods (**Page 6**) sections.

2. The effects of treatment with ecnoglutide on A1c over the relatively short study period were strong and in keeping with other once weekly GLP-1RA such as semaglutide. The safety profile also followed a pattern expected for a drug of this class. Thus, while a proper demonstration of the efficacy of a new GLP-1 based drug, this paper does not extend understanding of the GLP-1R in diabetes or therapeutics. The results, while clear,

are completely predictable. The interesting features of ecnoglutide, e.g. its bias at the GLP-1R and its putative advantage for synthesis, are not addressed directly here. This work supports advancing ecnoglutide to larger and longer trials, but otherwise adds very little new information except that the Ala-Val substitution in the primary sequence of GLP-1 does not impact its activity as an agonist.

Ecnoglutide is unique among clinically developed single GLP-1 analogs in that it was designed to have bias for cAMP signaling. Bias is also observed for dual GLP-1/GIP analogs, such as tirzepatide. The impact of signaling bias on efficacy has been debated. Ecnoglutide is just 4 amino acids different in peptide sequence to semaglutide, which is an unbiased GLP-1 analog. Here, the HbA1c lowering effect of the highest ecnoglutide dose tested (1.2 mg weekly at 20 weeks, -2.39%) surpassed that of 1.0 mg semaglutide (-1.86%) at 40 weeks, and was comparable to 15 mg weekly tirzepatide (−2.30%) at 40 weeks (Frias et al NEJM 2021). While the present study does not compare ecnoglutide to other GLP-1 single or dual agonists head-to-head, the strong efficacy results observed for HbA1c reduction support the hypothesis that cAMP signaling bias is a key contributing factor to the efficacy of GLP-1 receptor agonists.

The introduction and discussion have been updated to highlight that the outcomes of this clinical study contribute to our understanding GLP-1 receptor agonism. **(Page 3 and Page 17).**

Reviewer #2 (Remarks to the Author):

The manuscript evaluates the efficacy and safety of Ecnoglutide (XW003), a Glucagon-like peptide-1 (GLP-1) analog, in Type 2 diabetic patients through a placebo-controlled, double-blind, randomized controlled trial. This study explores a novel GLP-1 with potentially significant therapeutic implications.

Overall, the study exhibits robust design elements: well-defined inclusion and exclusion criteria, primary and secondary outcome measures, pharmacokinetic endpoints, and rigorous safety assessments, including the evaluation of adverse events (AE) and serious adverse events (SAE).

1. It will be helpful to provide the primary objective of the study in the Abstract.

   The primary objective and endpoint have been added to the abstract **(Page 2).**

2. However, the manuscript lacks comprehensive information about the statistical methodologies employed for data analysis. This impacts the clarity and rigor of the models and their alignment with the results. I have summarized some of the statistical issues below.

a. Sample Size Calculation: The manuscript justifies the sample size calculation based on a one-sided significance level, but it fails to provide reasoning for not considering a two-sided significance level. In randomized controlled trials (RCTs), a two-sided approach is often preferred due to a lack of a priori knowledge about the direction of the treatment effect.

One-sided significance testing was chosen as only a treatment effect superior to the placebo would be relevant. The set superiority margin of 0.3% with one-sided significance of 0.025 is equivalent to a 0.5% margin for two-sided significance testing.

The rationale for one-sided significant testing has been added to **Page 5.**

b. Clinical Significance: The assumption that a 1.0% difference in mean change from baseline in HbA1c between the Ecnoglutide group and the placebo is clinically significant requires substantiation with evidence.

We agree that the clinical relevance of HbA1c as a surrogate endpoint for diabetes control and mortality has been questioned (e.g., PMC5350060)

HbA1c remains, however, a validated endpoint for risk reduction of microvascular disease. Current updated draft FDA guidelines focus on HbA1c as "an acceptable endpoint to support a glycemic-control indication." (FDA, May 2023 https://www.fda.gov/regulatory-information/search-fda-guidance-documents/diabetes-mellitus-efficacy-endpoints-clinical-trials-investigating-antidiabetic-drugs-and-biological)

While a 1% change in HbA1c from placebo is the industry standard for clinical trial design, we acknowledge that change in HbA1c is not used as the standard of clinical care. Rather patients are evaluated using pre-defined ranges set by the American Diabetes Association (ADA, <7.0%) and American Association of Clinical Endocrinologists (AACE, ≤6.5%). Here, we also report the proportion of patients achieving these targets during the study.

We added text to highlight that attaining the target ranges is a key metric in clinical care **(Page 16)**.

c. Placebo Arm: The placebo arm had a similar three Ecnoglutide regimens (0.4, 0.8, and 1.2 mg), however, it looks arbitrary that patients in the placebo arm were pooled without any further reasonings. The fact that the sample size was calculated as a single placebo arm cannot be an adequate justification.

The design of the placebo arms is due to ethical considerations and clinical operational constraints. The placebo doses are volume matched to ecnoglutide using injector pens premade with a set volume. It is logistically not possible to have the same

injector volume for all three groups. In order for all groups to have identical injections, the number of injections per subject would have to be increased, which is not in line with ethical best practices. Similarly, increasing the number of placebo subjects is practically difficult and unethical, as clinical studies try to limit the number of placebo participants.

Pooling the placebos was warranted as during randomization all participants had the same opportunity to be assigned into any dose group on active or placebo. Subjects could know the dose volume but not if the agent was active or placebo.

We have added text to explain that placebo participants received different injection volumes that were matched with ecnoglutide and the rationale for pooling the placebo participants to the Study Design (**Page 4**) and Statistical Analyses methods (**Page 6**) sections.

d. Imputation Strategy: The last observation carried forward (LOCF) imputation strategy for the missing values is not an ideal approach unless a strong justification is given. The protocol version indicates that LOCF might have been applied for a different reason. It states that for the subjects with a lack of change in the HbA1c at Week 20 from the baseline, the LOCF was applied. If the data were available, the lack of change or not, it is not advisable to employ LOCF.

LOCF imputation was used only for calculation of the proportion of participants with HbA1c values of ≤6.5% and<7% at Day 134. The method was also used for sensitivity analyses that are now included in **Supplemental Table S1**.

We have removed the LOCF analysis from Figure 1 and replaced it with the proportion of participants determined without LOCF. The two results are very similar. (**See updated Figure 1 C and D**).

e. Repeated Measures Model: The manuscript should provide a comprehensive explanation of the repeated measures model, including details such as the model specification to account for the correlation between multiple measurements within a subject, the covariance structure or random effects structure used, estimation methods for fixed and random effects, and methods for estimating standard errors and 95% confidence intervals. If fitted models are different for the primary and secondary outcomes, provide full descriptions along with the predictors used.

Additional details of the MMRM have been added to the manuscript **(Page 6).**

f. Estimated Difference: For example, it is not clear if Figures 1A and 1B present the same model or two different models. The manuscript should clarify how the estimated mean difference and the corresponding SE and 95% CI (presented in these and other Figures) were obtained. If the model included other covariates (age, sex),

state how the adjusted mean differences accounting for these covariates were obtained (as presented in Figures 1, 2 and 3).

Figures 1A and 1B (HbA1c) and Figure 3A and 3B (weight) present the same MMRM analysis for change from baseline (in A) over time and change from baseline/change from placebo on Day 134 (in B). Figure 2 shows absolute values for SMBG (C) and change from baseline/difference from placebo as derived from ANOVA (D).

The **Figure legends of Figures 1-3** have been updated to state the analysis used for each part. The Methods section has also been updated to indicate the analyses used for primary and secondary endpoints (**Page 6**)

For each covariate used in the MMRM model, the values used are indicated in the **Tables below.** We are happy to include these tables as Supplemental Information at the Editor's request.

Table 1 Solution of MMRM

Effect	Estimate	SE	df	t-stat	p-value
Intercept	-0.3463	0.2230	135	-1.55	0.1228
Sex					
Male (Ref)	---	---	---	---	---
Female	0.0768	0.0864	135	0.89	0.3756
Age	0.0079	0.0042	135	1.90	0.0591
Baseline HbA1c					
≤8.5% (Ref)	---	---	---	---	---
＞8.5%	-0.1008	0.0814	135	-1.24	0.2175
Group					
Placebo (Ref)	---	---	---	---	---
XW003-0.4mg	-0.4886	0.1199	135	-4.07	<.0001
XW003-0.8mg	-0.5105	0.1220	135	-4.18	<.0001
XW003-1.2mg	-0.6112	0.1198	135	-5.10	<.0001
Visit					
D22 (Ref)	---	---	---	---	---
D50	-0.0528	0.0707	135	-0.75	0.4567
D92	-0.3000	0.1096	135	-2.74	0.0070
D134	-0.5821	0.1271	135	-4.58	<.0001
D169	-0.6947	0.1448	135	-4.80	<.0001
Visit*Group					
D22 * Placebo (Ref)	---	---	---	---	---
D22 * XW003-0.4mg (Ref)	---	---	---	---	---
D22 * XW003-0.8mg (Ref)	---	---	---	---	---
D22 * XW003-1.2mg (Ref)	---	---	---	---	---
D50 * Placebo (Ref)	---	---	---	---	---
D50 * XW003-0.4mg	-0.5582	0.1003	135	-5.56	<.0001
D50 * XW003-0.8mg	-0.5228	0.1011	135	-5.17	<.0001
D50 * XW003-1.2mg	-0.6729	0.1007	135	-6.68	<.0001
D92 * Placebo (Ref)	---	---	---	---	---
D92 * XW003-0.4mg	-0.9157	0.1547	135	-5.92	<.0001
D92 * XW003-0.8mg	-0.8369	0.1562	135	-5.36	<.0001
D92 * XW003-1.2mg	-1.2819	0.1564	135	-8.19	<.0001
D134 * Placebo (Ref)	---	---	---	---	---
D134 * XW003-0.4mg	-0.7724	0.1798	135	-4.30	<.0001
D134 * XW003-0.8mg	-0.8434	0.1825	135	-4.62	<.0001
D134 * XW003-1.2mg	-1.2287	0.1819	135	-6.76	<.0001
D169 * Placebo (Ref)	---	---	---	---	---
D169 * XW003-0.4mg	-0.2113	0.2032	135	-1.04	0.3001
D169 * XW003-0.8mg	-0.3366	0.2085	135	-1.61	0.1088
D169 * XW003-1.2mg	-0.6202	0.2054	135	-3.02	0.0030

Table 2 Summary of Baseline Characteristics

Baseline Characteristics	statistics	XW003-0.4mg (N=37)	XW003-0.8mg (N=36)	XW003-1.2mg (N=36)	Pooled Placebo (N=36)	Pooled XW003 (N=109)	Overall (N=145)
Age (Years)	N (nmiss)	37 (0)	36 (0)	36 (0)	36 (0)	109 (0)	145 (0)
	Mean (SD)	49.1 (8.87)	51.8 (10.34)	49.6 (9.65)	49.7 (10.54)	50.2 (9.62)	50.1 (9.82)
	Median	51.0	55.0	48.0	48.0	52.0	52.0
	Q1, Q3	41.0,57.0	46.0,60.5	43.0,58.0	42.5,59.5	43.0,58.0	43.0,58.0
	Min, Max	32,63	26,65	31,65	31,66	26,65	26,66
Sex	Male, n(%)	25 (67.6)	28 (77.8)	21 (58.3)	19 (52.8)	74 (67.9)	93 (64.1)
	Female, n(%)	12 (32.4)	8 (22.2)	15 (41.7)	17 (47.2)	35 (32.1)	52 (35.9)
	Sum (nmiss)	37 (0)	36 (0)	36 (0)	36 (0)	109 (0)	145 (0)
Baseline HbA1c classification	≤8.5%, n(%)	22 (59.5)	19 (52.8)	19 (52.8)	18 (50.0)	60 (55.0)	78 (53.8)
	＞8.5%, n(%)	15 (40.5)	17 (47.2)	17 (47.2)	18 (50.0)	49 (45.0)	67 (46.2)
	Sum (nmiss)	37 (0)	36 (0)	36 (0)	36 (0)	109 (0)	145 (0)

The results might be slightly different between the LSMs showed in the report and the results of X β using the parameters showed above due to the precision of the decimals.

The covariates values we used in the LSMs calculation is the mean of age of each group and the proportion of Female and ＞8.5% of each group. The proportion can also be interpreted as the mean of 0 (for reference level) and 1 (for non-reference level).

g. Baseline Adjustment: The manuscript should clarify if the model adjusted for the baseline HbA1c and provide details about the response variable for such a model. The protocol version (with a similar indication in the manuscript) of modelling the difference with the baseline HbA1c as a covariate is not an appropriate approach.

Baseline HbA1c only was used as a covariate, along with age and gender, to account for baseline heterogeneity. The primary endpoint (change from baseline in HbA1c) is the response variable for the model. Methods have been modified to clarify that baseline HbA1c is an explanatory variable in the MMRM analysis **(Page 6).**

h. Multiple Comparisons: An appropriate and simple contrast could be comparing the average of all doses in the treatment arm versus the control arm. It is important to state if the stated p-values were adjusted to account for the inflation of type 1 error due to multiple comparisons.

We thank the reviewer for this suggestion. The study was pre-defined to compare each treatment group vs the pooled placebo arm and not designed for a post hoc analysis comparing a combined treatment arm versus the control. We have therefore not included this analysis.

We did not adjust for type 1 error inflation due to multiple comparisons because the trial was designed as a Phase 2, exploratory dose finding study. This information has been added to the statistical methods (**Page 6**).

i. Interaction Effects: The manuscript should indicate whether interaction effects, such as treatment by time interaction or treatment by baseline value interaction, were explored. If not, please justify since the two-way interaction effect of treatment by time may look reasonable as indicated in the Figures.

Treatment by time/visit was included in the model. We did not include additional interaction terms, but rather followed only the prespecified MMRM model.

Details of the MMRM have been added on **Page 6**.

j. Multicenter Trial: The RCT was conducted as a multicenter trial. It is a standard practice to include the center as a random effect. It is not mentioned in the statistical analysis section.

A sensitivity analysis has been conducted to include center as a covariate. We grouped sites by number of participants (≤5 [12 sites], 6-9 [6 sites] and >10 [2 sites] subjects) and evaluated HbA1c change from baseline for the three groups. Results are consistent regardless of the numbers of subjects per site.

The results of the analysis are shown in the **Table below.** We are happy to include this table as Supplemental Information at the Editor's request.

Table 3. Summary of Day 134 HbA1c Change from baseline by Site Classification

Treatment	statistics	5 or less Subjects	6-9 Subjects	10 or more Subjects	p-value
Pooled Placebo	N (nmiss)	11 (1)	13 (0)	13 (1)	0.1230
	Mean (SD)	-0.90 (0.746)	-0.94 (1.086)	0.05 (0.649)	
	Median (Q1, Q3)	-1.00(-1.50, -0.20)	-0.60(-1.40, -0.20)	0.00(-0.40, 0.60)	
	Min, Max	-1.9, 0.4	-3.1, 0.3	-0.9, 1.1	
XW003-0.4mg	N (nmiss)	15 (0)	16 (1)	9 (2)	0.9740
	Mean (SD)	-1.84 (0.667)	-1.79 (0.786)	-1.91 (0.819)	
	Median (Q1, Q3)	-1.70(-2.30, -1.60)	-2.05(-2.35, -1.30)	-2.00(-2.30, -1.70)	
	Min, Max	-2.9, -0.3	-2.9, -0.3	-3.0, -0.6	
XW003-0.8mg	N (nmiss)	11 (1)	11 (1)	13 (3)	0.9212
	Mean (SD)	-1.78 (1.264)	-1.97 (1.316)	-1.77 (0.840)	
	Median (Q1, Q3)	-1.70(-2.90, -1.30)	-1.80(-3.00, -1.60)	-2.00(-2.20, -1.00)	
	Min, Max	-3.7, 1.0	-3.9, 1.0	-3.1, -0.6	
XW003-1.2mg	N (nmiss)	11 (0)	16 (2)	10 (2)	0.4535
	Mean (SD)	-2.66 (0.842)	-2.41 (0.936)	-2.24 (0.700)	
	Median (Q1, Q3)	-2.50(-3.50, -1.70)	-2.50(-3.05, -1.70)	-2.10(-2.70, -1.70)	
	Min, Max	-3.9, -1.5	-3.8, -0.7	-3.6, -1.3	

The p-values derived from ANOVA showed that the difference between different site classification are not significant.

k. Model Assumptions: The manuscript should demonstrate that all model assumptions have been thoroughly checked and confirmed.

For the analysis of primary efficacy endpoint (change in HbA1c from baseline), MMRM with imputation was used, with covariances of baseline HbA1c, age, gender, treatment and visit time, in both ITT and PP populations. Sensitivity was checked using ANCOVA with or without LOCF, which confirmed the trend was robust.

This sensitivity analysis has been added as **Supplemental Table S1** and indicated in the Methods (**Page 6**) and Results (**Page 8).**

l. Per-Protocol Analysis: The approved study protocol mentions conducting a Per-Protocol analysis. The manuscript should provide details on the outcomes of this analysis and present additional results as supplementary information.

Per-Protocol analysis of the primary efficacy endpoint (HbA1c) has been added as **Supplemental Table S1.** Results of HbA1c reduction from baseline and difference from placebo were very similar between the two populations.

This has been indicated in the Results section on **Page 8.**

m. Sub-Group Analysis: The manuscript should specify whether any sub-group analyses were conducted.

Two subgroup analyses were conducted:

1. Effects on change in HbA1c from baseline by factors: baseline HbA1c >8.5% or ≤8.5%, prior treatment (yes/no), sex (M/F)

2. Effects on change in body weight from baseline by factors: baseline body weight (>75 kg or ≤75 kg) and sex (M/F)

No significant difference was detected between any of the subgroups. We have noted these analyses in the Methods section **(Page 6).**

n. Secondary Efficacy Indicators: The comments provided above are equally applicable to secondary efficacy indicators such as changes in glucose and glucagon from baseline and mean change in body weight from baseline, as well as the pharmacokinetics of Ecnoglutide.

Modifications and clarifications have been made to address the above questions for both primary and secondary outcomes, as outlined in the responses above.

o. Code Availability: It would be helpful to include all SAS codes with relevant details and simulated data as supplementary information to facilitate results and plots reproducibility. A statement regarding code availability should be incorporated.

Code is not publicly available. A statement has been added to the manuscript **(Page 18).**

p. Data Presentation: The 'Data Presentation' statement does not conform to the manuscript. For example, the manuscript does not present individual data points on figures or provide sufficient clarity on the distribution of relevant data.

We have reviewed the Data Presentation statement. It is not practical to display individual data points on the Figures due to the number of subjects in this trial. The data presentation statement requires display for n<10, which does not apply to any of the Figures we present.

All Figures in the manuscript present the standard error, standard deviation, or confidence intervals, where applicable.

Reviewer #3 (Remarks to the Author):

Ecnoglutide is a once-weekly injectable GLP-1R agonist which was evaluated as a monotherapy in the drug naïve or mono-OAD treated T2D patients who were randomized 1:1:1:1 to placebo: 0.4 mg:0.8mg:1.2mg groups for a treatment of 20 weeks followed by a 5-week safety observation follow-up. Significant HbA1c reductions achieved in each dose group compared with placebo. Body weight reduction is moderate with 1.2mg group in which equal or greater than 5% weight loss is 33.3%.

At lower doses, the Ecnoglutide showed good glucose reduction effect comparable with Semaglutide at lower doses.

Overall it is well designed and executed study. Results are presented in line with the expectation.

There are a few questions need to be discussion:

1. Could the author explain the reasons for relatively high % of dyslipidemia and hypoglycemia with Ecnoglutice in this study?

Dyslipidemia events were reported by 1 (2.8%) participant in the placebo group and 4 (3.7%) treated with the two lower doses of ecnoglutide (2 with ecnoglutide 0.4 mg and 2 with ecnoglutide 0.8 mg), without an indication of dose relationship. This finding may be due to a relatively high proportion of participants in the ecnoglutide groups (19.3%) having a known history of requiring lipid lowering medications prior to the study. In comparison, 8.3% of participants in the placebo group had a history of these medications.

Hypoglycemic events were reported by 1 (2.8%) participant in the placebo group and 6 (5.5%) receiving ecnoglutide (1 with 0.4 mg, 4 with 0.8 mg and 1 with 1.2 mg). These hypoglycemic events were reported as mild in severity and mainly due to skipped meals and/or increased intensity of physical activity. Fisher's probability tests were performed comparing the incidence

of hypoglycemic events between ecnoglutide group and the placebo group, which showed no significant difference in any of the ecnoglutide doses.

A discussion of the incidence of hypoglycemia has been added to **Pages 14 and 17**.

2. The weight reduction is moderate at highest dose of 1.2mg. What is the reason for not testing Ecnoglutide at higher dose to enhance the weight reduction effect?

Since this study was the first Phase 2 trial of ecnoglutide, doses were chosen based on the Phase 1 studies in healthy participants as well as information from the similar peptide, semaglutide. Based on tolerated doses in these studies, a starting dose of 0.2 to 0.3 mg and top dose of 1.2 mg ecnoglutide were defined in the protocol.

Current Phase 3 studies of ecnoglutide are investigating a top dose of 2.4 mg for the obesity indication.

3. The plasma levels of the tested drug at 0.8 and 1.2 mg differ in day 50 (completed titration) and day 134. Will these possible drug cumulation continue when it is used for a longer period of treatment?

No, the increasing ecnoglutide plasma exposure between Day 50 and 134 is not accumulation but the slow path to steady state exposure generally observed with once-a-week compounds with a long half life.

Briefly, on Day 56 the subjects in C2 and C3 start their top dose of 0.8 mg and 1.2 mg, respectively. Since ecnoglutide has a long (~140 h) half-life, multiple doses (4 to 6 doses) are needed for the peptide to reach steady-state plasma exposure. The climb to steady state plasma exposure in C2 and C3 is visible between the plasma samples collected on Day 50 and Day 92. By Day 92 most subjects have achieved steady state plasma exposure, and the increase is slower to Day 134.

This has been clarified in the pharmacokinetic Results section on **Page 15.**

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

No new comments.

Reviewer #2 (Remarks to the Author):

I appreciate the authors' comprehensive responses to the reviewers' comments, which have notably improved the clarity of the revised manuscript.

I have a few minor suggestions:

Figures:

- Ensure that all figure legends contain minimal abbreviations (e.g., MMRM) unless they are self-evident (e.g., SE).

- All figures include upper cases next to the figure but lower cases (a, b, c, d etc.) in the legends.

Tables:

In response to Reviewer 2 comments, the authors have presented "Table 1 Solution of MMRM" (primary outcome?) with effect, SE, df, t-stat and p-value. I recommend including Tables with the solutions of all models (both primary and secondary outcomes) in the supplementary material. This additional information will be invaluable for readers and future researchers to understand the size and precision of all estimates.

Other tables (Table 2: Summary of Baseline Characteristics; Table 3: Summary of Day 134 HbA1c Change from baseline by Site Classification) presented in the 'Response' document are not required.

Model Assumptions:

The response to Reviewer 2's comments on model assumptions is not sufficiently addressed. It is essential that all fitted models adhere to standard assumptions of the properties of residuals and their distributions. I suggest including a statement in the Methods section something along the lines of: "All MMRM conform to the underlying model assumptions."

Reviewer #3 (Remarks to the Author):

I have no further comments to the manuscript. I support it to be published in Nature Communication.

Li

April 1, 2024

Response to Reviewers

Reviewer requests have been numbered and responses are in purple.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

No new comments.

Reviewer #2 (Remarks to the Author):

I appreciate the authors' comprehensive responses to the reviewers' comments, which have notably improved the clarity of the revised manuscript.

I have a few minor suggestions:

Figures:

- Ensure that all figure legends contain minimal abbreviations (e.g., MMRM) unless they are self-evident (e.g., SE).

The abbreviations have now been spelled out at first use in the Figure legends.

- All figures include upper cases next to the figure but lower cases (a, b, c, d etc.) in the legends.

The Figures have been revised to contain lower case letters for parts a, b, c, etc.

Tables:

In response to Reviewer 2 comments, the authors have presented "Table 1 Solution of MMRM" (primary outcome?) with effect, SE, df, t-stat and p-value. I recommend including Tables with the solutions of all models (both primary and secondary outcomes) in the supplementary material. This additional information will be invaluable for readers and future researchers to understand the size and precision of all estimates.

These tables have been included in the Supplemental Material as Supplemental Tables S2, S3, S4 and S5. These tables have also been referenced in the Statistical Analyses Methods section.

Other tables (Table 2: Summary of Baseline Characteristics; Table 3: Summary of Day 134 HbA1c Change from baseline by Site Classification) presented in the 'Response' document are not required.

These tables will not be included in the Supplemental Material, as agreed.

Model Assumptions:

The response to Reviewer 2's comments on model assumptions is not sufficiently addressed. It is essential that all fitted models adhere to standard assumptions of the properties of residuals and their distributions. I suggest including a statement in the Methods section something along the lines of: "All MMRM conform to the underlying model assumptions."

It is confirmed that all MMRM conform to the underlying model assumptions and the statement has been added to the Statistical Analyses Methods section.

Reviewer #3 (Remarks to the Author):

I have no further comments to the manuscript. I support it to be published in Nature Communication.

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

I thank the authors for their detailed responses. I agree with all the changes incorporated by the authors. After reviewing all MMRM solution tables (S2 to S5), I feel that the clarity of the MMRM modeling approach is still lacking. An overview of the solution table suggests that the full modelling description is not reflected in the Statistical Analysis section. Having said that, I feel this aspect is relevant to the technical details of statistical modelling, hence, no further changes in the main manuscript are required.

However, I believe it would benefit readers to include the SAS script for each supplementary table (S2 to S5) as a footnote. In my first review, I requested to provide the SAS code which would have explained the models better. The repeated measures data in SAS can be fitted using multiple approaches: PROC GLM (using either univariate or multivariate methods), PROC MIXED and PROC GENMOD. The SAS scripts would assist discerning readers and facilitate power calculations for future studies with similar designs. The SAS script can explain the full modeling perspective and how multiple observations within subjects were considered. Authors should also include estimates of between and within subject variances as footnotes to support prospective power calculation. It may be presented slightly differently in the SAS outputs depending on the PROC used. Authors do not need to present detailed scripts relevant to data preparation leading to model fitting or explain the model terms. A simple presentation of PROC GLM or PROC MIXED scripts will be adequate.

I assume that providing the SAS script on modeling as a footnote is a minor revision and should be readily available to the authors. This additional information will enhance the clarity, replicability and transparency of the reported study.

May 30, 2024

Response to Reviewers

Reviewer requests have been numbered and responses are in purple.

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

1. I believe it would benefit readers to include the SAS script for each supplementary table (S2 to S5) as a footnote. In my first review, I requested to provide the SAS code which would have explained the models better. The repeated measures data in SAS can be fitted using multiple approaches: PROC GLM (using either univariate or multivariate methods), PROC MIXED and PROC GENMOD. The SAS scripts would assist discerning readers and facilitate power calculations for future studies with similar designs. The SAS script can explain the full modeling perspective and how multiple observations within subjects were considered.

   The SAS code has been added under each table in the Supplemental Material.

2. Authors should also include estimates of between and within subject variances as footnotes to support prospective power calculation. It may be presented slightly differently in the SAS outputs depending on the PROC used. Authors do not need to present detailed scripts relevant to data preparation leading to model fitting or explain the model terms. A simple presentation of PROC GLM or PROC MIXED scripts will be adequate.

   The requested information has been added under each table in the Supplemental Material.

   As the added information is lengthy, we leave it up to the Editor to decide whether to include the expanded Supplemental Material or abbreviated Supplemental Material as part of the publication.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

I thank the authors for their detailed comments and for incorporating the SAS scripts and outputs for each model. The information would facilitate power calculations for future clinical trials and the reproducibility of the work. There are some duplications of SAS outputs and the tabular data. I am happy to retain the extended Supplementary Material (with some duplications of information) if the Editor agrees.