MOLECULAR
METABOLISM

Monomeric GLP-1/GIP/glucagon triagonism corrects obesity, hepatosteatosis, and dyslipidemia in female mice

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ABSTRACT

Objective: Obesity is a major health threat that affects men and women equally. Despite this fact, weight-loss potential of pharmacotherapies is typically first evaluated in male mouse models of diet-induced obesity (DIO). To address this disparity we herein determined whether a monomeric peptide with agonism at the receptors for glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), and glucagon is equally efficient in correcting DIO, dyslipidemia, and glucose metabolism in DIO female mice as it has been previously established for DIO male mice.

Methods: Female C57BL/6J mice and a cohort of fatmass-matched C57BL/6J male mice were treated for 27 days via subcutaneous injections with either the GLP-1/GIP/glucagon triagonist or PBS. A second cohort of C57BL/6J male mice was included to match the females in the duration of the high-fat, high-sugar diet (HFD) exposure.

Results: Our results show that GLP-1/GIP/glucagon triple agonism inhibits food intake and decreases body weight and body fat mass with comparable potency in male and female mice that have been matched for body fat mass. Treatment improved dyslipidemia in both sexes and reversed diet-induced steatohepatitis to a larger extent in female mice compared to male mice.

Conclusions: We herein show that a recently developed unimolecular peptide triagonist is equally efficient in both sexes, suggesting that this polypharmaceutical strategy might be a relevant alternative to bariatric surgery for the treatment of obesity and related metabolic disorders.

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1. INTRODUCTION

Obesity and its metabolic comorbidities like type 2 diabetes impose major threats to global public health and socioeconomic prosperity [1,2]. Lifestyle modification as a first intervention proves mostly ineffective to fight excess adiposity [3,4]. The acceptance for therapeutic or surgical intervention is considerably high, albeit constrained by substantial side effects [5]. Long-term studies clearly suggest bariatric surgery as the most effective, yet most cost-intensive therapy for sustained body weight normalization [6,7]. Approximately 80% of patients undergoing bariatric surgery are women, although no differences in eligibility criteria between sexes exist [8,9]. In sharp contrast to this, preclinical obesity studies largely neglect female rodents, because of a relative resistance to diet-induced obesity and glucose intolerance that is typically observed in most conventional strains [10,11]. A further concern is that sex hormones and fluctuations in the estrous cycle can have an impact on key metabolic endpoints and can increase the natural variance of drug effects, resulting in the necessity for larger group sizes to detect metabolic benefits [12,13]. Recent evidence shows that some pharmacological treatment strategies have differing effects in women and men, as well as higher rates of adverse drug reactions in women [14,15]. This signifies that detailed pre-clinical investigations of differences between the sexes are warranted to accurately assess the therapeutic utility of drug candidates. In line with this notion, an initiative of the U.S. National Institutes of Health (NIH) recently

suggested to expand preclinical studies to also include female rodents [16].

Whereas most previous weight-loss pharmacotherapies are hampered by limited efficacy or unacceptable adverse effects, there is hope resting on recent advances in the development of single molecules which promote their biological action through simultaneous agonism at multiple key metabolic receptors [17,18]. In this regard, a monomeric peptide with balanced agonism at the receptors for glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), and glucagon has previously been shown to correct diet-induced obesity (DIO), dyslipidemia, and insulin resistance in male mice [19]. The principle underlying this molecule is that the anorectic action of central GLP-1 receptor (GLP-1R) agonism synergizes with the action of glucagon to increase energy expenditure, resulting in a net loss of body weight. The combined glycemic action of GLP-1R and GIP receptor (GIPR) agonism restrains the hyperglycemic effect of glucagon, improves insulin sensitivity, and results in body weight improvements. While the efficacy of this GLP-1/GIP/glucagon triagonist to correct the metabolic syndrome has been shown in male mice [19], its metabolic effects in female mice remain unknown. Accordingly, the aim of this study was to comparatively evaluate the metabolic efficacy of this triple agonist in female and male DIO mice.

2. MATERIALS AND METHODS

2.1. Animals and diet
Due to the fact that progression of obesity differs between both sexes, with male mice gaining body fat more rapidly compared to female mice [20], we determined metabolic effects of the triagonist in age-matched male and female mice with similar body fat mass as well as in female and male mice cohorts exposed for equal periods of time to high fat diet feeding. Eight-week old female and male C57BL/6J mice (Charles River Laboratories, Wilmington, MA) were fed a high-fat, high-sugar diet (HFD) comprising 58% kcal from fat (D12331; Research Diets, New Brunswick, NJ, USA). To match females in body fat mass, another cohort of male C57BL/6J mice was switched from a regular diet to HFD at 30 weeks of age. The mice were maintained at $23 \pm 1\,°C$, constant humidity, and on a 12-h light—dark cycle with free access to food and water. At the age of 38 weeks, mice were randomized within the three cohorts and equally distributed according to body composition. All procedures were approved by the Animal Use and Care Committee of Bavaria, Germany in accordance with the Guide for the Care and Use of Laboratory Animals [21].

2.2. GLP-1/GIP/glucagon triple agonist
The synthesis, purification, and characterization of the GLP-1/GIP/glucagon triagonist was described previously and was used without any further chemical modification or change in formulation [19].

2.3. Evaluation of GLP-1/GIP/glucagon triagonist in females and males *in vivo*
All female and male mice were treated daily via subcutaneous injections (5 μl/g body weight) at the indicated doses. Vehicle mice received an equivalent volume of PBS. Whole-body composition was analyzed using nuclear magnetic resonance technology (EchoMRI, Houston, TX, USA). In accordance with previous reports, glucose tolerance was analyzed in 6-h fasted mice that received an intraperitoneal injection of 1.5 g glucose per kg body weight [22]. For the tolerance test, glucose was measured in blood samples from the tail veins at the indicated time points using a handheld glucometer (Abbott GmbH & Co. KG, Wiesbaden, Germany).

2.4. Biochemical analysis
For tissue analysis, mice were injected with the respective treatment dose of the triagonist or vehicle and immediately fasted for 4 h prior to sample collection. Plasma levels of insulin (ALPCO Diagnostics, Salem, NH, USA), cholesterol (Thermo Fisher Scientific, Waltham, MA, USA), free fatty acids, triglycerides (Wako Chemicals, Neuss, Germany), leptin (ALPCO Diagnostics), fibroblast growth factor 21 (FGF21) (Merck Millipore, Darmstadt, Germany), L-alanine:2-oxoglutarate aminotransferase (ALT), and aspartate aminotransferase (AST) (Thermo Fisher Scientific) were measured with the respective kits according to the manufacturers' instructions. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated using the formula: HOMA-IR = [fasting insulin (mU/l) * fasting glucose (mg/dl)/405] [23]. For lipoprotein separation, samples from the different treatment groups were pooled and analyzed in a fast-performance liquid chromatography gel filtration on two Superose 6 columns connected in series [24]. For evaluation of steatosis, formalin fixed liver samples were embedded in paraffin. Tissue was cut in 4 μm sections and stained with hematoxylin and eosin. Morphological features were recorded and summarized in an activity score that is recommended for diagnosis of steatohepatitis in non-alcoholic fatty liver disease (NAFLD) in humans [25]. The NAFLD activity score (NAS) is defined as the unweighted sum of the three individual scores for steatosis, lobular inflammation, and ballooning degeneration. Steatosis is graded by the presence of fat vacuoles in liver cells according to the percentage of affected tissue (0: <5%; 1: 5—33%; 2: 33—66%; 3: >66%). Lobular inflammation is scored by overall assessment of inflammatory foci per 200× field (0: no foci; 1: <2 foci; 2: 2—4 foci; 3: >4 foci). The individual score for ballooning degeneration ranges from 0 (none), 1 (few cells) to 2 (many cells). Thus, NAS scores range from 0 to 8, with scores ≤2 considered as non-steatohepatitis, scores from 3 to 4 considered as possible/borderline steatohepatitis, scores ≥5 are diagnostic for definite steatohepatitis.

2.5. Statistics
Differences between treatment groups were assessed by two-way ANOVA followed by Tukey's *post hoc* analysis as appropriate or an unpaired two-tailed Student's *t*-test. All results are presented as mean ± s.e.m. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Triagonist treatment normalizes body weight with equal efficiency in DIO female and male mice matched for fat mass
Because of the well-known delay in diet-induced body weight gain in female mice, and in order to achieve a single cohort of mice matched for age and body composition, we delayed the introduction of HFD to male mice relative to female mice in this single cohort study. To achieve a comparable body fat mass of 16.05 ± 0.79 g and 15.10 ± 0.87 g ($p > 0.05$) in females and males respectively, the duration of HFD exposure before treatment initiation was 30 weeks for females and 8 weeks for males. Female and male DIO mice (age 38 weeks) were randomized by body fat mass and injected daily for 27 days with either vehicle or the triagonist at doses of 5 or 10 nmol/kg. Treatment with the triagonist resulted in a dose-dependent decrease in body weight with identical relative weight loss in both sexes (Figure 1A). The triagonist-induced weight loss was accompanied by a dose-dependent decrease in food intake (Figure 1B) and body fat mass (Figure 1C) with negligible inter-sexual variation at both doses tested. The weight loss induced by the triagonist slightly decreased lean tissue mass in male mice with no effect in females (Figure 1D). In line with the marked reduction in body weight and body fat mass, plasma levels of
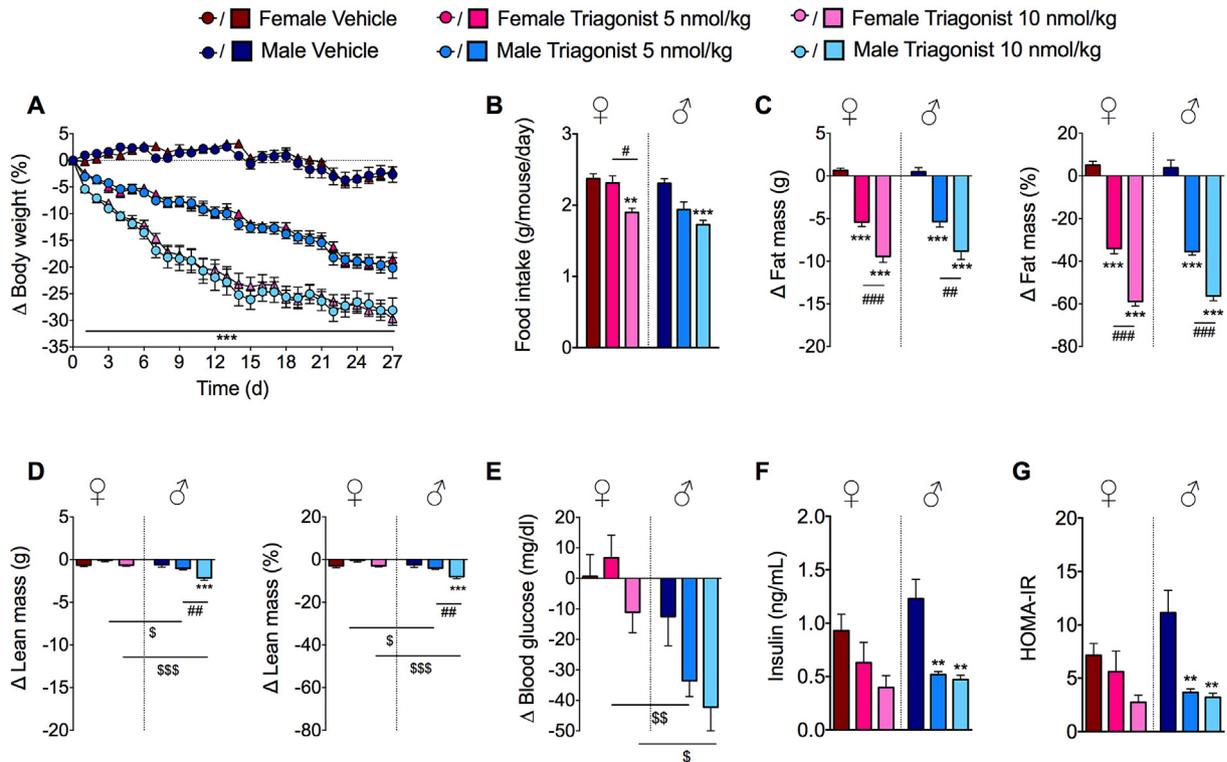
Figure 1: Equal efficiency of the monomeric triagonist to normalize body weight in female and fat mass-matched male mice (A-G). Effects on body weight change (**A**), daily food intake (**B**), fat mass change in females and in males in gram and percent (**C**), and lean mass change in females and males in gram and percent (d0 - d20) (**D**). Effects of triagonist treatment on fasted blood glucose change (d0 - d22) in female and male mice (**E**), plasma insulin levels (d22) (**F**), HOMA-IR (d22) (**G**) of fat mass-matched female and male mice (age 9 months; ♀ $n = 6-10$; ♂ $n = 6-8$ per group) treated daily with vehicle and the GLP-1/GIP/glucagon triagonist at 5 nmol/kg or 10 nmol/kg. Data represent mean ± s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, determined by two-way ANOVA comparing vehicle with compound injections in both sexes. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ determined by two-way ANOVA comparing 5 nmol/kg and 10 nmol/kg doses of the triagonist in both sexes. $$P < 0.05$, $$$P < 0.01$, $$$$P < 0.001$ determined by two-way ANOVA comparing both sexes. ANOVA was followed by Tukey *post hoc* multiple comparison analysis to determine statistical significance.

leptin were dose-dependently decreased in both sexes (Suppl. Table 1). In female mice, but not male mice, we observed an increase in plasma levels of free fatty acids ($p < 0.001$) and FGF21 ($p < 0.05$) following treatment with 10 nmol/kg and 5 nmol/kg of the triagonist, while levels of triglycerides were unchanged (Suppl. Table 1).

3.2. Triagonist treatment does not further enhance normal glucose tolerance in DIO female mice in contrast to male mice matched for body fat mass

In line with previous findings (19), we observed a dose-dependent improvement in glucose metabolism, in particular in male mice, reflected by lower fasting levels of blood glucose (Figure 1E), decreased levels of fasting insulin (Figure 1F), an improved glucose tolerance (Suppl. Fig. 1A,C), and insulin sensitivity, as indicated by the HOMA-IR (Figure 1G). Triagonist treatment did not further enhance an already normal glucose tolerance in female mice (Suppl. Fig. 1B,C). Although female mice are known to be largely protected from the development of diet-induced glucose intolerance and insulin resistance [10,26], we still observed a mild improvement of diet-induced hyperinsulinemia in DIO females (Figure 1F,G).

3.3. Triagonist treatment improves diet-induced hypercholesterolemia in both sexes with a pronounced effect on steatohepatitis in female mice

As previously reported in obese male mice [19], treatment with the triagonist potently improves diet-induced dyslipidemia and NAFLD.

Thus, it was of great interest to investigate potential sex differences in changes of NAFLD and hypercholesterolemia following triagonist treatment. In females, plasma cholesterol levels were only significantly decreased in high-dose triagonist treated mice ($p < 0.05$), whereas in males, both doses significantly reduced plasma cholesterol ($p < 0.001$) (Figure 2A). This decrease in plasma cholesterol was attributed to a substantial reduction in LDL in the high-dose treated mice and a slight reduction in HDL (Figure 2B,C) in both sexes.

Histological analysis of the liver showed that 88.9% of the vehicle treated females and 62.5% of the vehicle treated males were diagnosed with a definite steatohepatitis (Figure 2D). Treatment with the triagonist dose-dependently improved steatohepatitis. The majority of mice that have been treated with 10 nmol/kg of the triagonist showed either a complete resolution of steatohepatitis (females) or only a borderline steatohepatitis (males) at the end of the study (Figure 2D−F). We observed a pronounced effect on lowering hepatic lipid content and hepatocellular vacuolation in females that have been treated with the higher dose of the triagonist (Figure 2D,E). Although hepatic lipid content was diminished also in males treated with the same dose of the triagonist, they displayed a greater variability in reactive changes like hepatocyte ballooning, polyploidy (red dotted arrow, Figure 2F), and sustained inflammatory process (black arrow, Figure 2F), resulting in mild periportal fibrosis (black dotted arrow, Figure 2F). Moreover, treatment with the highest dose of the triagonist lowered plasma levels of ALT to a larger extent in female mice than in males (Suppl. Table 1). Levels of AST in the plasma of
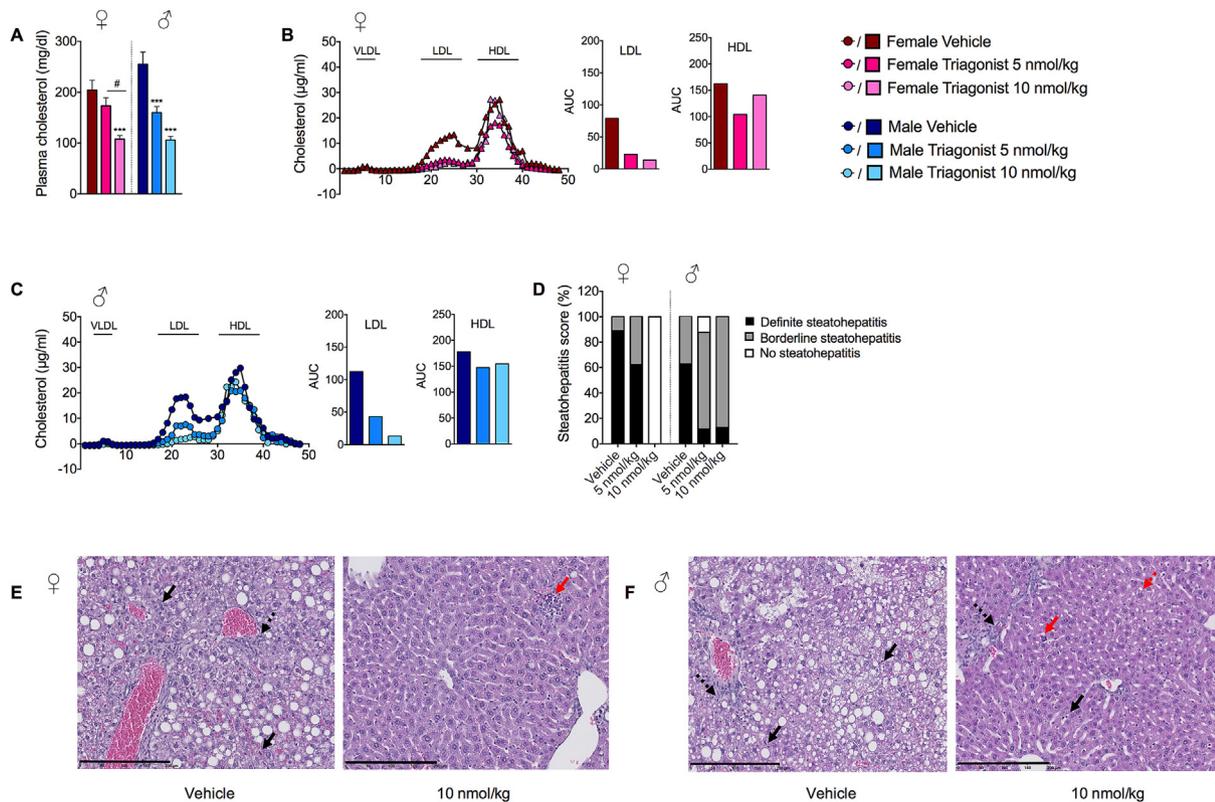
Figure 2: GLP-1/GIP/glucagon triple agonism reverses dyslipidemia and NAFLD in female and fat mass-matched male mice (A–F). Plasma cholesterol (d22) (**A**), plasma lipoprotein fractions in females (**B**) and males (d27) (**C**). Hepatic steatosis score of liver samples (**D**), effects on hepatocellular vacuolation after 27 days of treatment in female (**E**) and male mice (**F**) (age 9 months; ♀ $n = 7$−10; ♂ $n = 7$−8 per group) treated daily with vehicle and the GLP-1/GIP/glucagon triagonist at 5 nmol/kg or 10 nmol/kg. Scale bars in **E** and **F,** 200 μm. Beginning fibrosis periportal (black, dotted arrow), inflammatory intraparenchymal cells (black arrow), microgranuloma (red arrow), focal hepatocyte ballooning and polyploidy (red, dotted arrow). Data represent mean ± s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, determined by two-way ANOVA comparing vehicle with compound injections in both sexes. #$P < 0.05$ determined by two-way ANOVA comparing 5 nmol/kg and 10 nmol/kg doses of the triagonist in both sexes. ANOVA was followed by Tukey *post hoc* multiple comparison analysis to determine statistical significance.

female and male mice remained unaltered by the treatment with the triagonist (Suppl. Table 1).

3.4. Triagonist treatment normalizes body weight without inducing excessive fat mass loss in male and female mice matched for the duration of HFD exposure

To evaluate whether the duration of HFD exposure affects the efficacy of the triagonist to improve metabolic disease, we also compared female mice to a cohort of age-matched male mice, which were kept on a HFD for the same duration as the female mice (30 weeks). Body weight and body fat mass substantially differed between these cohorts with female mice weighing 39.63 ± 0.92 g and having 16.05 ± 0.79 g body fat mass and male mice weighing 54.20 ± 0.59 g and having 22.44 ± 0.53 g body fat. Relative weight-loss induced by 5 nmol/kg of the triagonist was remarkably similar between both sexes (♀ −18.55± 1.28% and ♂ −25.33± 1.67% relative to baseline, Figure 3A). When treated with 10 nmol/kg of the triagonist, male mice had significantly greater relative weight-loss compared to female mice with females losing 29.64± 1.29% compared to baseline and males 42.82± 1.56% (Figure 3A). The enhanced relative weight loss in males was accompanied by a greater reduction in food intake compared to vehicle treated controls (Figure 3B) and was reflected by a greater absolute and relative loss of fat and lean mass (Figure 3C,D). As shown in Figure 3A, the slopes of body weight loss curves flattened

in both, male and female mice after 15 days of treatment with 10 nmol/kg indicating that normal healthy body composition was achieved and no excessive fat mass loss was observed. As stated earlier, due to the fact that female mice are protected against diet-induced insulin resistance [10], improvement in glucose metabolism relative to vehicle controls was evident only in male mice (Figure 3E,F).

4. DISCUSSION

Our data show that chronic treatment of DIO mice with the GLP-1/GIP/glucagon triagonist potently improves metabolic disease in both sexes with comparable efficiency in female and male mice when pre-treatment body composition is considered. Side by side comparisons between sexes, to determine the efficacy of a pharmacological compound to improve DIO in mouse models, are hampered by the fact that progression of obesity differs between females and males [20]. Herein, we thus opted first for a comparison at a time point when fat mass was similar between male and female mice. In our case, the duration of HFD exposure to achieve similar fat mass in both sexes was 8 weeks in males and 30 weeks in females. With this experimental design we were not able to determine whether the period of HFD feeding itself may alter pharmacological effects of the triagonist to improve metabolic disease. Thus, we included a group of DIO male mice that were fed a HFD for the same period of time (30 weeks) as the DIO female mice. Treatment with

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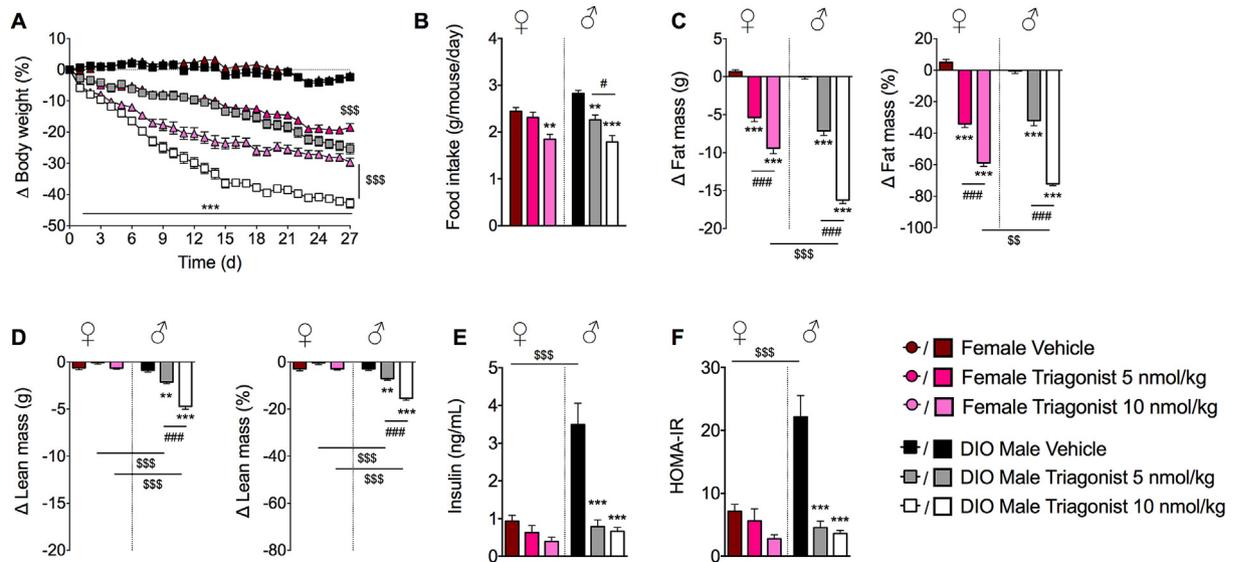
Figure 3: Efficiency of the monomeric triagonist to normalize body weight and reverse hyperinsulinemia in HFD-matched DIO female and male mice (A-F). Effects on body weight change (A), average daily food intake (B), fat mass change (C), and lean mass change in HFD-matched DIO male compared to female mice (d0 - d20) (D), plasma insulin levels (d22) (E), and HOMA-IR (d22) (F) of male DIO mice compared to female mice (age 9 months; ♀ $n = 6-10$; ♂ $n = 6-8$ per group) treated daily with vehicle and the GLP-1/GIP/glucagon triagonist at 5 nmol/kg or 10 nmol/kg. Data represent mean ± s.e.m. $**P < 0.01$, $***P < 0.001$, determined by two-way ANOVA comparing vehicle with compound injections in both sexes. $\#P < 0.05$, $\#\#\#P < 0.001$ determined by two-way ANOVA comparing 5 nmol/kg and 10 nmol/kg doses of the triagonist in both sexes. $\$\$P < 0.01$, $\$\$\$P < 0.001$ determined by two-way ANOVA comparing both sexes. ANOVA was followed by Tukey *post hoc* multiple comparison analysis to determine statistical significance.

10 nmol/kg of the triagonist normalized body weight in both sexes with a higher weight-lowering potency in the DIO male group due to the fact that this group had markedly more fat mass at study start than DIO female mice. DIO male mice lost significantly more body fat than male mice that matched females with respect to body fat, although food intake for both male cohorts did not differ. This strongly suggests a prominent role for the GLP-1/GIP/glucagon triagonist in directly targeting the fat mass in mice. Importantly, throughout our study, we did not observe excessive body weight or fat mass loss in the triagonist treated mice suggesting that triagonist treatment normalizes fat mass up to healthy conditions. These results imply that the triagonist may be a safe drug to use, because even at high dosages it does not lead to excessive body weight and fat mass loss.

Similarly to previous studies [19], triagonist treatment potently improved glycemic control in male DIO mice. Due to the evidence in literature and in this study showing that DIO female mice of the C57BL/6 strain do not become as hyperglycemic as male DIO mice and become only moderately hyperinsulinemic [10,26], triagonist treatment was not able to further improve glucose tolerance in female DIO mice. Female mice did develop a mild hyperinsulinemia under HFD feeding, which the triagonist treatment was able to reduce along with improving HOMA-IR. Based on the fact that one prominent and very early sign of metabolic disease in men and women is fasting hyperinsulinemia [27], we propose that the observed insulin lowering effect of the triagonist in both sexes presents a promising novel therapeutic option for men as well as for women even before onset of type 2 diabetes.

It has been previously reported that female C57BL/6J mice fed a diet rich in fructose are more susceptible to develop a NAFLD compared to male mice [28]. We here extend this finding to DIO female mice, as nearly 100% of vehicle treated female mice were diagnosed with a full-blown steatohepatitis after 30 weeks of HFD, while fat mass-matched male mice were not as severely affected. The male cohort

was on a HFD for 8 weeks to match females in fat mass content, which could also have an impact on the observed difference. Conversely, treatment with the GLP-1/GIP/glucagon triagonist for 27 days completely resolved NAFLD-associated complications in female mice. Interestingly, both sexes of the high-dose treated mice displayed a nearly total diminishment of hepatic lipid content, but, in males, sustained inflammatory processes resulted in mild periportal fibrosis. Our results are in line with previous findings by Ganz M. et al. reporting that obese female mice develop steatosis without inflammation in contrast to steatohepatitis found in obese male mice [29]. Based on a recent study showing that astaxanthin reversed advanced non-alcoholic steatohepatitis (NASH) in male mice after 12 weeks of treatment [30] we hypothesize that a comparable amelioration of NAFLD hallmarks may occur in males after an elongation of triagonist treatment duration. When we measured ALT and AST in plasma of triagonist treated male and female mice, we detected a significant reduction in ALT levels in female mice and a clear trend of reduced ALT levels in male mice. Plasma levels of AST were not significantly altered in neither sex. These findings are in line with the results published recently by Finan B. et al. [19] demonstrating a significant reduction in ALT but not AST in DIO obese male mice upon chronic triagonist treatment. Compared to AST, ALT is especially expressed in liver and thus the reduction of ALT plasma levels may reflect a reduction in diet-induced liver cell injury by the triagonist treatment [31]. Our histological findings of a significant improvement of NAFLD in triagonist treated female and male mice, together with the observed reduction of plasma ALT levels in both sexes, underscore the profound effect of the triagonist to resolve the HFD induced liver damage

5. CONCLUSION

For translational applicability, the inclusion of female mice in pharmacological research is indispensible. To this end, we here

demonstrate equal efficiency of the GLP-1/GIP/glucagon triagonist in reversing DIO and liver steatosis in female and male rodent models of adiposity. Reports on body weight loss 4 weeks after bariatric surgery in comparably obese male and female mice show reductions of approximately 30% of the starting body weight mirroring the loss in body weight we observed in the high-dose triagonist treated mice [32,33]. In conclusion, our findings indicate that triagonist treatment may reduce body weight as efficiently as bariatric surgery highlighting the potential of the monomeric triagonist as an effective treatment option for severe obesity also in women.

AUTHOR CONTRIBUTIONS

S.J. performed the experiments, evaluated the data, and drafted the manuscript. S.S. performed the experiments, evaluated the data, and helped composing the manuscript. C.C. made substantial contributions in the study design, helped with the in vivo experiments, evaluated the data, and helped draft the manuscript. B.F. and R.D.D. designed, synthesized, and characterized compounds, made substantial contributions in the study design and interpretation of data, and helped edit the manuscript. F.N. interpreted histopathological hepatic data. M.H.T. made substantial contributions in the study design and interpretation of data and helped edit the manuscript. T.D.M. oversaw the in vivo experiments, evaluated the data, helped draft the manuscript, made substantial contributions in the study design and interpretation of data, and helped edit the manuscript. S.M.H. headed the lipoprotein profile measurements and analysis, oversaw S.S. in this project and mentored S.J. for the generation of this manuscript. T.D.M. and S.M.H. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

DUALITY OF INTEREST

No potential conflicts of interest relevant to this article were reported.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.02.002.

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