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Original article

Liraglutide and sitagliptin have no effect on intestinal microbiota composition: A 12-week randomized placebo-controlled trial in adults with type 2 diabetes

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ABSTRACT

Aim. – Preclinical data suggest that treatment with either glucagon-like peptide (GLP)-1 receptor agonists or dipeptidyl peptidase (DPP)-4 inhibitors could change the intestinal microbiome and thereby contribute to their beneficial (cardio)metabolic effects. Therefore, our study aimed to investigate the effects of these agents on microbiota composition in adults with type 2 diabetes (T2D).

Methods. – A total of 51 adults with T2D (mean ± SD: age 62.8 ± 6.9 years, BMI 31.8 ± 4.1 kg/m^2, HbA_{1c} 7.3 ± 0.6%) treated with metformin and/or sulphonylureas were included in the 12-week randomized, double-blind trial. Patients were given the GLP-1 receptor agonist liraglutide (1.8 mg sc) or the DPP-4 inhibitor sitagliptin (100 mg), or matching placebos, once daily for 12 weeks. Faecal samples were collected at baseline and at 12 weeks after the start of the intervention. Microbiota analyses were performed by 16S rRNA gene-sequencing analysis. Bile acids were measured in faeces and plasma.

Results. – Liraglutide decreased HbA_{1c} by 1.3% (95% CI: -1.7 to -0.9) and tended to reduce body weight (-1.7 kg, 95% CI: -3.6 to 0.3), but increased faecal secondary bile acid deoxycholic acid. Sitagliptin lowered HbA_{1c} by 0.8% (95% CI: -1.4 to -0.4) while body weight remained stable (-0.8 kg, 95% CI: -2.7 to 1.0), but increased faecal levels of cholic acid, chenodeoxycholic acid and ursodeoxycholic acid. However, neither liraglutide nor sitagliptin affected either alpha or beta diversity of the intestinal microbiota, nor were changes in microbial composition related to clinical parameters.

Conclusion. – These data suggest that the beneficial effects of liraglutide and sitagliptin on glucose metabolism, body weight and bile acids, when used as add-on therapies to metformin or sulphonylureas, are not linked to changes in the intestinal microbiota (NCT01744236).

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Introduction

Intestinal microbiota are critical for many biological processes in the human body, including digestion, glucose metabolism, immunomodulation and gut barrier function. Intestinal dysbiosis, with its reduced bacterial load and diversity, has extensively been linked to the development of obesity and its related disorders, such as insulin resistance and type 2 diabetes (T2D) [1,2]. Modulation of intestinal microbiota could therefore be a tool to prevent or reduce symptoms of these highly prevalent metabolic conditions. In line with this hypothesis, faecal microbiota transplantation from lean donors to obese recipients with the metabolic syndrome (MetS) has been associated with improved insulin sensitivity [3,4], although further studies are still needed to clarify whether this improvement has any long-term clinical relevance.

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Microbial metabolites, including short-chain fatty acids and bile acids, as well as microbial-mediated metabolism of xenobiotics such as oral medications, have been postulated as mechanisms through which microbiota might alter metabolism and facilitate medication efficacy. Indeed, metformin has been shown to alter the intestinal microbiota that, in turn, mediated some of metformin's glucose-lowering effects [5,6], including those related to changes in bile acid metabolism [7].

Incretin-based therapies using glucagon-like peptide (GLP)-1 receptor agonists and dipeptidyl peptidase (DPP)-4 inhibitors rely on the insulinotropic actions of the gut hormone GLP-1. Their mechanisms of action either mimic GLP-1 (GLP-1 receptor agonists) or increase levels of endogenous GLP-1 (DPP-4 inhibitors). Both agents increase insulin, reduce glucagon secretion and improve hepatic glucose metabolism [8]. In addition, both reduce body weight, increase insulin sensitivity, reduce inflammation and alter bile acid composition, especially GLP-1 receptor agonists, but also DPP-4 inhibitors to a lesser extent [9–13].

In preclinical studies, these beneficial effects have been postulated to be mediated in part by medication-induced changes in the intestinal microbiome. Liraglutide was shown, in hyperglycaemic and obese mice, to augment microbial diversity and to increase the presence of beneficial bacteria such as *Lactobacillus* and *Turicibacter* [14]. However, whether this was a liraglutide-specific effect or simply an effect of (liraglutide-induced) weight loss and reduced caloric intake was not determined. Beneficial effects on the intestinal microbiome were also reported with the DPP-4 inhibitor sitagliptin in a rat model of diabetes [15]. Nevertheless, to date, the effects of GLP-1-based therapies on the intestinal microbiota have yet to be studied in an adequate placebo-controlled randomized clinical trial, nor have such actions been linked to their metabolic effects in an extensively phenotyped cohort of adults with T2D.

Materials and methods

The present randomized placebo-controlled, double-blind, parallel-group trial aimed to assess the effects of 12-week treatment with either liraglutide (Novo Nordisk A/S, Bagsvaerd, Denmark) or sitagliptin (Merck & Co., Inc., Kenilworth, NJ, USA), or matching placebos, on faecal microbiota. The full protocol has been previously published elsewhere [16]. The present study was performed at the Amsterdam University Medical Centers, location VU University Medical Center (VUmc), between July 2013 and August 2015; it was approved by the ethics review board of the VUmc, registered at ClinicalTrials.gov (NCT01744236), and conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization (ICH) Good Clinical Practice (GCP) Guideline. All participants provided their written informed consent before participation.

Participants, treatments and randomization

A total of 60 Caucasian men and postmenopausal women, aged 35–75 years, with T2D [HbA_{1c} 6.5–9.0% (48–75 mmol/mol) treated with stable doses of metformin and/or sulphonylurea (SU) derivatives for at least 3 months] and a body mass index (BMI) of 25–40 kg/m^2 participated in the study. Relevant exclusion criteria included: current treatment with either insulin or GLP-1-based therapy or use of an antimicrobial agent; cholecystectomy; history of hepatic, pancreatic, cardiovascular or renal disease; and alcohol intakes $>$ 3 units/day. Eligible patients were randomly assigned, using computer-generated numbers, to receive either liraglutide 1.8 mg, sitagliptin 100 mg or a matching placebo (1:1:1 allocation ratio; block size of 6).

Study procedures

Endpoint measurements were taken at baseline (after a run-in period of 4 weeks) and after 12 weeks of treatment. In the original protocol, faecal microbiota were included as the exploratory endpoint, while markers of intestinal inflammation [faecal calprotectin, serum intestinal fatty-acid-binding protein (I-FABP)] were considered secondary endpoints [16]. Blood was drawn during test visits after an overnight fast and processed immediately. A standardized mixed meal tolerance test (905.7 kcal: 50 g of fat, 75 g of carbohydrate, 36.8 g of protein) was performed for assessment of bile acid kinetics [13]. Participants were instructed to collect two faecal samples from the same defecation within 2 days of the next study visit. Collection was independent of the patient's defecation pattern or stool consistency (with the exception of watery diarrhoea). Stool samples were collected in a sterile stool collection tube (Sarstedt AG & Co., Newton, NC, USA) for faecal microbiota analysis and stored at -20 °C until transported to the study centre in a chilled Styrofoam container. Samples were subsequently stored on site at -80 °C. A second stool sample was stored in a cool and dark place at the patient's home, then transferred within 2 days to be stored at -20 °C at the study centre for eventual determination of calprotectin and I-FABP levels.

Faecal microbiota analyses

Faecal deoxyribonucleic acid (DNA) was extracted using a repeated bead-beating protocol [17]. DNA was purified using Maxwell® RSC Whole Blood DNA Kits, and 16S ribosomal ribonucleic acid (rRNA) gene amplicons were generated using a single-step polymerase chain reaction (PCR) protocol targetting the V3–V4 region [18]. PCR products were purified using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA) and the purified products equimolarly pooled. Libraries were sequenced with the MiSeq System platform using v3 chemistry at 2 $\times$ 251 cycles. Forward and reverse reads were truncated to 240 and 210 bases, respectively, and merged using the USEARCH sequence analysis tool [19]. Merged reads that did not pass the Illumina chastity filter had an expected error rate higher than two or were shorter than 380 bases. Amplicon sequence variants (ASVs) were inferred for each sample individually with a minimum abundance of four reads [20]. Unfiltered reads were thereafter mapped against the collective ASV set to determine abundances. Taxonomy was assigned using the RDP classifier [21] and SILVA 16S ribosomal database V132 [22].

Laboratory measurements

Faecal calprotectin levels were assessed using EliA™ assays with an ImmunoCAP® 250 system (ThermoFisher Scientific, Waltham, MA, USA), and an intra-run variance of 2.8–7.0% and inter-run variance of 1.9–7.3%. Serum levels of I-FABP were determined by sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer's specifications (R&D Systems, Inc., Minneapolis, MN, USA), with an intra-assay variance of 6.3% and an interassay variance of 9.4%. Finally, venous blood glucose, HbA_{1c}, triglycerides, cholesterol, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were assayed at the Department of Clinical Chemistry in our hospital using conventional methods.

Bile acid measurement

The methodology used and the results of faecal and (postprandial) serum bile acids in this study have been previously published elsewhere [13]. However, as explained below (see the Results

Table 1
Study participants' baseline characteristics and effects of treatment.

Parameter	Placebo (n = 15)			Sitagliptin (n = 18)				Liraglutide (n = 16)			
	Before	After	Time effect* *P*	Before	After	Time effect* *P*	Treatment effect** *P*	Before	After	Time effect* *P*	Treatment effect** *P*
Characteristics											
Age (years)	65.3 ± 1.5			61.7 ± 1.6				60.0 ± 1.7			
Male gender [n (%)]	12 (80)			16 (84.2)				12 (70.6)			
Duration of T2D (years)	7.7 ± 1.2			8.1 ± 5.8				7.2 ± 1.1			
Metformin use [n (%)]	13 (86.7)			18 (94.7)				17 (100)			
Sulphonylurea use [n (%)]	8 (53.3)			9 (47.4)				6 (35.3)			
General											
Weight (kg)	96.2 ± 2.5	96.4 ± 2.6	0.778	99.4 ± 3.8	98.4 ± 4.0	0.069	0.208	105.4 ± 4.4	103.9 ± 4.2	0.056	0.144
Body mass index (kg/m^2)	30.6 ± 0.8	30.6 ± 0.9	0.988	31.5 ± 1.0	31.1 ± 1.0	**0.042**	0.222	33.3 ± 1.2	32.8 ± 0.1	0.068	0.194
FPG (mmol/L)	9.0 ± 0.5	9.7 ± 0.6	0.232	8.0 ± 0.2	7.0 ± 0.2	< **0.001**	< **0.001**	8.4 ± 0.4	7.5 ± 0.4	**0.031**	**0.001**
HbA1c (%)	7.5 ± 0.2	8.2 ± 0.3	**0.006**	7.1 ± 0.1	6.8 ± 0.2	**0.027**	< **0.001**	7.4 ± 0.2	6.7 ± 0.2	< **0.001**	< **0.001**
HbA1c (mmol/mol)	58.8 ± 2.1	66.2 ± 3.7	**0.006**	53.8 ± 1.2	50.5 ± 1.9	**0.027**	< **0.001**	57.2 ± 1.9	49.7 ± 1.9	< **0.001**	< **0.001**
Systolic BP (mmHg)	138.7 ± 3.9	139.6 ± 3.7	0.807	132.5 ± 3.0	132.1 ± 3.1	0.903	0.457	137.8 ± 4.1	130.0 ± 5.1	0.057	0.078
Diastolic BP (mmHg)	77.2 ± 1.8	76.6 ± 1.6	0.618	75.2 ± 1.7	76.5 ± 1.6	0.464	0.574	77.2 ± 1.4	77.3 ± 2.5	0.975	0.772
Gut inflammation											
I-FABP	114.0 ± 24.9	117.8 ± 47.3	0.846	85.3 ± 12.2	105.3 ± 11.4	**0.047**	0.625	131.7 ± 25.2	130.6 ± 32.4	0.961	0.759
Calprotectin	19.9 ± 7.2	18.2 ± 5.7	0.677	14.6 ± 2.4	18.7 ± 5.0	0.363	0.483	12.9 ± 2.5	25.8 ± 10.3	0.444	0.579
Bile acids											
Serum CA (AUC)	12.9 ± 4.9	4.0 ± 0.5	0.102	13.8 ± 7.7	17.4 ± 10.4	0.765	0.176	18.7 ± 1.6	4.6 ± 0.7	0.227	0.997
Serum DCA (AUC)	79.1 ± 14.0	54.6 ± 6.6	0.060	88.9 ± 12.9	101.4 ± 26.6	0.571	0.107	100.4 ± 21.6	106.5 ± 1 5.3	0.925	0.184
Serum CDCA (AUC)	46.5 ± 17.1	20.1 ± 4.4	0.119	37.4 ± 11.7	40.0 ± 12.6	0.883	0.112	37.6 ± 15.4	20.8 ± 2.8	0.288	0.940
Serum UDCA (AUC)	5.2 ± 0.7	4.4 ± 0.7	0.375	6.9 ± 1.4	7.1 ± 1.4	0.819	0.309	5.8 ± 1.1	5.9 ± 1.3	0.781	0.651
Serum LCA (AUC)	4.5 ± 0.5	4.4 ± 0.6	0.944	5.6 ± 0.6	5.4 ± 0.1	0.851	0.806	5.4 ± 1.0	6.5 ± 0.9	0.236	0.275
Faecal CA (μmol/kg)#	0.01 [0.00–0.03]	0.01 [0.01–0.02]	0.925	0.02 [0.01–0.05]	0.03 [0.01–0.11]	0.177	**0.013**	0.01 [0.00–0.02]	0.03 [0.01–0.09]	0.069	0.142
Faecal DCA (μmol/kg)#	2.2 [1.4–4.0]	2.4 [0.9–3.0]	0.140	2.3 [1.2–3.3]	2.3 [1.6–3.0]	0.981	0.075	3.1 [1.8–4.9]	3.4 [2.2–4.2]	0.691	**0.017**
Faecal CDCA (μmol/kg)#	0.04 [0.00–0.08]	0.03 [0.00–0.06]	0.158	0.04 [0.02–0.07]	0.09 [0.02–0.14]	0.055	**0.009**	0.02 [0.01–0.05]	0.06 [0.01–0.08]	0.061	0.122
Faecal UDCA (μmol/kg)#	0.005 [0.00–0.05]	0.005 [0.00–0.03]	0.826	0.03 [0.00–0.06]	0.09 [0.01–0.16]	**0.031**	**0.005**	0.03 [0.01–0.07]	0.05 [0.01–0.09]	0.233	0.105
Faecal LCA (μmol/kg)#	1.4 [0.96–1.87]	1.02 [0.65–1.82]	**0.035**	1.5 [0.9–2.1]	1.4 [0.7–2.2]	0.356	0.194	1.7 [0.9–2.8]	1.5 [1.00–2.2]	0.427	0.173

Data are presented as means ± standard error of mean (SEM) for continuous data following a Gaussian distribution or as medians [interquartile range] for data which does not (faecal bile acid results) unless otherwise specified; * difference between after and before treatment intervention; ** placebo- and baseline-corrected effect of intervention with mean, 95 % CI and *P* value; # log-transformed analyses of treatment-induced effects.
T2D, type 2 diabetes; FPG, fasting plasma glucose; BP, blood pressure; I-FABP, intestinal fatty-acid-binding protein; CA, cholic acid; AUC, area under the curve; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; LCA, lithocholic acid.

section), the present analysis included a slightly smaller study population than did the original analysis. Because the effects of GLP-1-based therapy on bile acid physiology are considered important in terms of changes in the microbiota, as it points to an aspect of microbiome function, the present study also included this updated analysis.

Sample size, data management and statistical analysis

Microbiota data were analyzed and visualized using R 3.5.2 software (R Foundation for Statistical Computing, Vienna, Austria), using phyloseq [23], vegan [24], picante [25] and lme4 [26] packages. Data were rarefied without replacement to 35,000 counts. Mixed models were used to test treatment-induced shifts in several alpha diversity metrics and abundances of the top 250 ASVs with prevalence rates $\geq$ 30%. Permutational multivariate analysis of variance (PERMANOVA) was used to test clinical parameters and compositional differences after the study interventions using both Bray–Curtis (BC) dissimilarity and weighted UniFrac distances. In addition, multilevel principal component analysis (PCA) was used on centered log-ratio (CLR)-transformed counts to test compositional shifts [27]. The first 10 components of the multilevel PCA were tested using 1000-repetition PERMANOVA. Multiple testing correction was performed for clinical variables and taxa analysis. Also, as metformin can change microbiota composition, subgroup analyses were performed for the use and dose of metformin.

For non-microbiota data, multivariable regression models were used for the per-protocol population. Treatment with either liraglutide or sitagliptin was added as a dummy variable, while baseline variables of the tested endpoint were included as covariates to correct for baseline differences between treatment arms. The results of these tests are presented as treatment-induced effects with 95% confidence intervals (CIs) corrected for baseline values. Table 1 presents the results of within-group analyses as per paired *t* tests. All non-microbiota analyses were performed using SPSS version 22.0 software (IBM Corp., Armonk, NY, USA), and a two-sided *P* value $<$ 0.05 was considered statistically significant.

Results

Study population

Prior to randomization, two patients withdrew their informed consent and two patients were excluded because of incidental findings (malignancy). Therefore, 56 patients were randomized to receive either liraglutide (n = 19), sitagliptin (n = 20) or placebo (n = 17) (Fig. 1). None of these participants were using either pro- or antimicrobial agents. In the sitagliptin group, one further patient withdrew from the trial because of dizziness and pollakiuria. Thus, due to missing samples and technical failures, the per-protocol analyses were performed on 16 patients taking liraglutide,

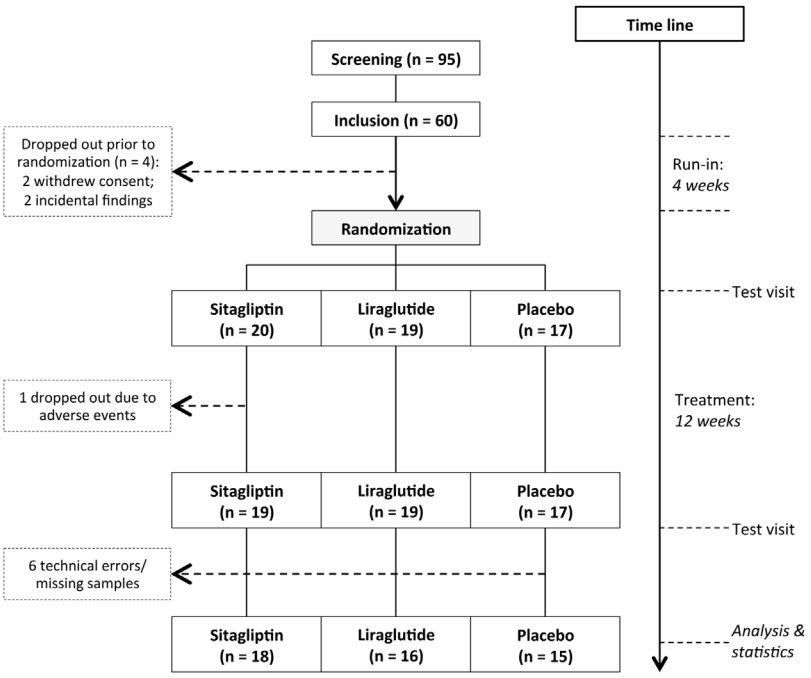


Fig. 1. Flow chart of study participant recruitment.

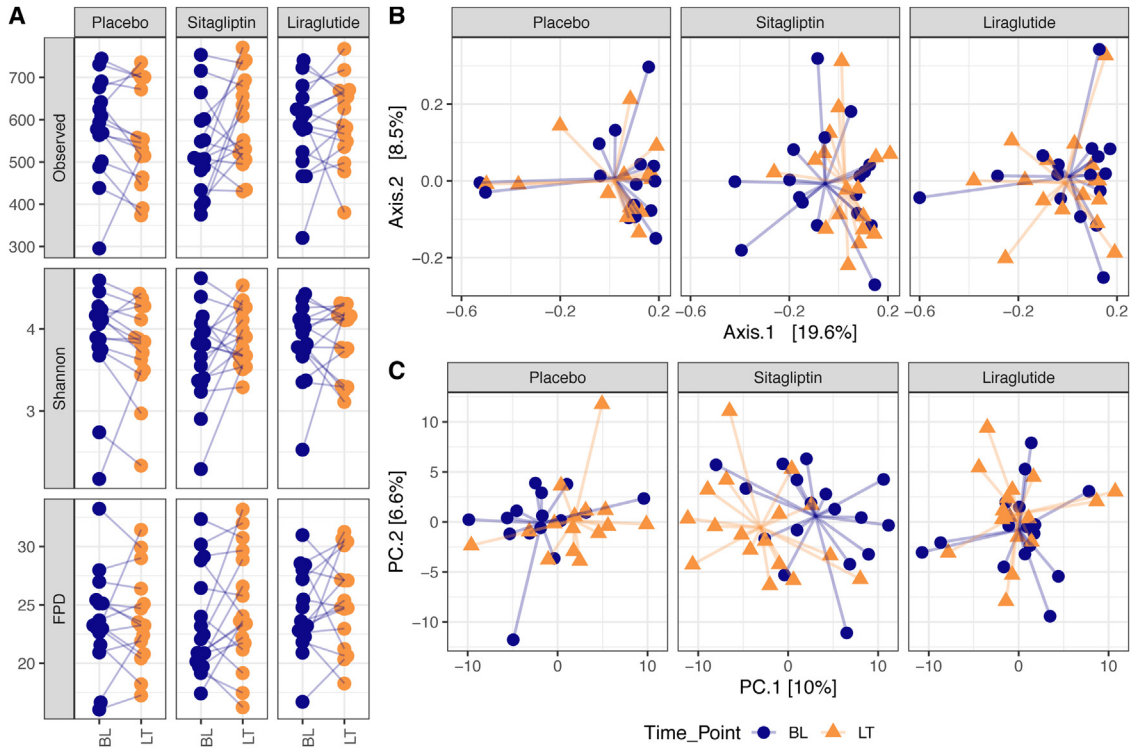


Fig. 2. Microbiota alpha and beta diversity were not affected by glucagon-like peptide (GLP)-1 based therapies in subjects with type 2 diabetes (T2D): (A) Alpha diversity (microbial community diversity within subjects) at baseline (BL) *vs* long-term (LT) after indicated interventions, as represented by Shannon diversity (sample richness corrected for relative abundances) and Faith's phylogenetic diversity [FPD; based on the phylogenetic distance between amplicon sequence variants (ASVs)], a marker of genetic diversity; (B) principal coordinate analysis (PCoA) plots of Bray–Curtis dissimilarity indicating no significant differences among the three treatment groups; interindividual variations in microbiota composition were the strongest determinant of variation in beta diversity at both BL and after treatment; and (C) multilevel principal component (PC) analysis corrected for interindividual variance revealing no significant treatment effects either.

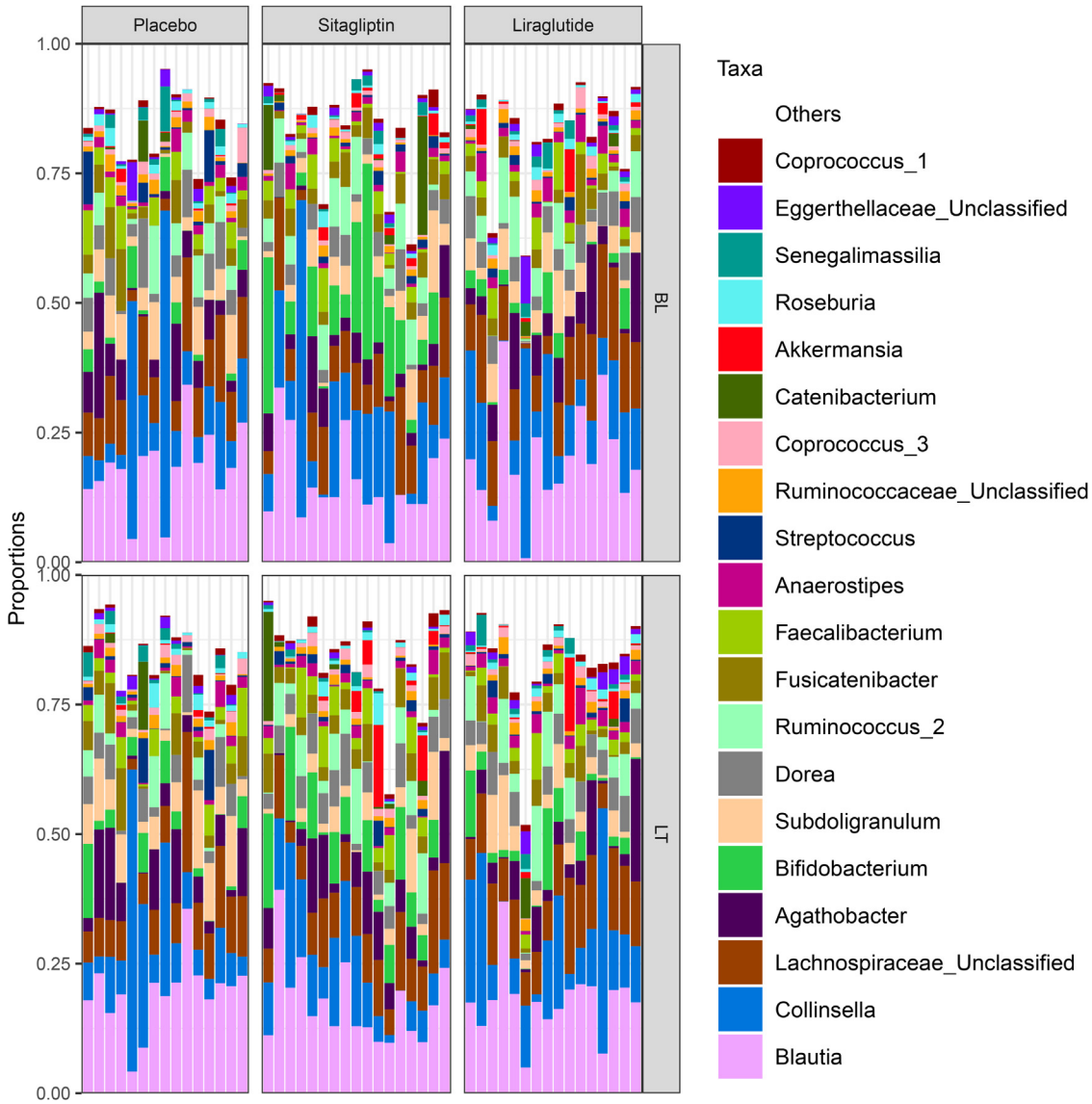


Fig. 3. Glucagon-like peptide (GLP)-1-based therapies such as liraglutide and sitagliptin have no effect on microbiota composition, including the top 20 bacterial genera, in patients with type 2 diabetes (T2D): (upper) baseline (BL) composition (prior to study interventions); and (lower) long-term (LT) composition (after 12 weeks of study interventions). 'Others' represent taxa found in low abundance.

18 taking sitagliptin and 15 taking a placebo. Baseline characteristics were similar among these three groups (Table 1).

Intestinal microbiota

Faecal microbiota composition was determined by sequencing the V3–V4 region of the 16S rRNA gene. Alpha diversity of the microbial community after study interventions remained unaltered [Fig. 2A; Faith's phylogenetic diversity (FPD): P = 0.72; Shannon index: P = 0.22]. Principal coordinates analysis (PCoA) of the microbiota indicated no significant differences in beta diversity across the three treatment groups (Fig. 2B; PERMANOVA BC: P = 0.23). However, PERMANOVA analysis did reveal that subject identification was the strongest variable associated with microbial composition (PERMANOVA BC: P = 0.001, R^2 = 0.81), indicating between-subject differences as the best explanatory variable. To correct for interindividual variances, multilevel PCA was employed to test treatment-induced compositional shifts (Fig. 2C). The results indicated that neither treatment had any significant effect on microbial composition compared with placebo (P = 0.091). In fact, neither placebo nor sitagliptin nor liraglutide treatment for 12 weeks altered the relative abundance of individual microbial species as measured by detected ASVs compared with baseline (Fig. 3). In addition, no associations were observed between post-interventional microbiota composition and clinical parameters (including anthropometrics, glycaemic values, inflammatory markers and bile acids).

Subgroup analyses were performed of patients using metformin monotherapy or metformin/SU combination therapy [SU monotherapy was not tested because the number of patients was too small (n = 3)], but no differences were observed within these subgroups. There was, however, a significant effect of total daily metformin dose on beta diversity (weighted UniFrac: P = 0.107 at baseline; P = 0.028 post-intervention) whereas no interactions were found with either treatment allocation or time.

Intestinal inflammation and bile acids

The present study found that 12-week treatment with either liraglutide or sitagliptin failed to change either faecal calprotectin

or I-FABP levels (Table 1). Neither liraglutide nor sitagliptin affected the postprandial area under the curve (AUC) for any bile acid compared with placebo. On the other hand, liraglutide significantly increased faecal levels of deoxycholic acid (DCA; Table 1) while sitagliptin increased faecal levels of cholic acid (CA), chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA).

Anthropometric and glycaemic effects

Liraglutide tended to reduce body weight by -1.7 kg (95% CI: -3.6 to 0.3; $P = 0.09$), whereas body weight remained stable with sitagliptin (-0.8 kg, 95% CI: -2.7 to 1.0; $P = 0.374$). In addition, liraglutide decreased HbA_{1c} by -1.3% [95% CI: -1.7 to -0.9; $P < 0.001$ (-14.1 mmol/mol, 95% CI: -18.7 to -9.4)] while sitagliptin lowered HbA_{1c} by -0.8% [95% CI: -1.4 to -0.4; $P = 0.001$ (-8.6 mmol/mol, 95% CI: -13.4 to -3.8)]. Moreover, both liraglutide and sitagliptin reduced fasting plasma glucose [-1.5 mmol/L, 95% CI: -2.3 to -0.8 ($P < 0.001$) and -0.9 mmol/L, 95% CI: 1.7 to -0.2 ($P = 0.015$), respectively] compared with placebo.

Discussion

Our present study has demonstrated that neither the GLP-1 receptor agonist liraglutide nor the DPP-4 inhibitor sitagliptin, when used as add-on therapy in metformin- and/or SU-treated adults with T2D, altered the faecal microbiota to any significant extent compared with placebo. Microbiota analyses were focused on two important aspects, alpha and beta diversity. Alpha diversity is an ecological measure that indicates the richness and diversity of the microbiota in one sample (for example, the abundance distribution of detected microbes in a particular sample). Beta diversity indicates the compositional differences between samples (for example, the microbiota composition in different subjects from different treatment groups). Our present data suggest that the cardiometabolic changes effected by these drugs are not mediated by changes in faecal microbiota.

These present findings differ from observations made in rodents. In a series of experiments wherein Sprague–Dawley rats were fed a high-fat diet, followed by a streptozotocin injection to induce diabetes and then treatment with sitagliptin for 12 weeks, the DPP-4 inhibitor normalized faecal microbiota composition back to baseline (lean, normoglycaemic) state [15]. In another experiment involving 60 C57BL/6 $ApoE^{-/-}$ mice (half of which received streptozotocin) randomized to 8 weeks of treatment with either liraglutide or saxagliptin, the former (liraglutide), but not the latter (saxagliptin), substantially changed gut microbiota composition as well as the relative abundance of weight-relevant phylotypes [14]. In a mouse model of non-alcoholic fatty liver disease (NAFLD), liraglutide changed the overall composition as well as relative abundance of weight-relevant phylotypes [28]. In 27 C57BL/6 J mice fed a typical Western diet, vildagliptin modulated the gut microbiota and their metabolic activities [29]. Finally, in a randomized trial involving rats, liraglutide decreased the abundance and diversity of gut microbiota *vs* placebo [30].

Thus, the animal data suggest that both GLP-1 receptor agonists and DPP-4 inhibitors may affect the gut microbiota. In the only available human study, 37 T2D patients taking metformin were randomized to either treatment with liraglutide (after halting metformin) or continuing metformin [31]. Microbiota composition in the liraglutide group differed significantly from the metformin group (mainly, a greater number of genus *Akkermansia* bacteria). However, it remains unclear whether this difference was due to the administration of liraglutide or the withdrawal of metformin (known to affect gut microbiota [6]).

Nevertheless, the present study data are novel and add to the accumulating evidence, given that this was the first double-blind, placebo-controlled trial in patients with T2D involving the use of a GLP-1 agonist or a DPP-4 inhibitor as add-on therapy to metformin or SU instead of monotherapy. In fact, such a setting is more in line with the usual clinical practice. Moreover, the withdrawal of metformin might explain the difference between the two clinical studies, while the differences compared with the animal studies may more likely be explained by the more homogeneous data found in the latter studies.

As regards standardization, there are two factors that should be mentioned in the present study. First, it included patients with and without metformin, and allowed different daily dosages. Also, although not among our research goals, it was observed that the metformin dosage affected beta diversity, which is in line with previous data [5,6]. This finding may have impacted our results. However, the lack of any statistically significant interaction between metformin dose and the study drugs argues against the possibility that metformin use affected our results. However, it may be hypothesized that patients taking high doses of metformin already have a 'beneficial' microbiota profile, thereby resulting in less of an impact with GLP-1-based therapies, although this idea could not be tested in our study.

The second important factor is diet, a well-known modulator of intestinal microbiota composition. In animal experiments, all are fed the same food throughout the intervention period, which minimizes the risk of potential confounders. As GLP-1 receptor agonists are associated with decreases in caloric intakes [32] (although it is not clear whether longer-term treatment would retain this effect [33]), microbiome results might be altered if the diet is not standardized. Unfortunately, given the already burdensome protocol [16] of our clinical trial, the participants' diets were not standardized, nor were their dietary intakes assessed. Thus, whether or not the liraglutide-treated patients changed their food intakes, which could have affected our outcomes, is unclear. While this may be seen as a limitation in our attempt to identify the 'pure' effects of liraglutide, it should be noted that: (*i*) in clinical practice, patients do actually change their diets, so the present analysis represents real-life results; and (*ii*) the direction and magnitude of changes in the intestinal microbiota *via* dietary change are as yet still unclear. Finally, it must be mentioned that, as DPP-4 inhibitors have not been linked to dietary changes, the above discussion is not applicable to our sitagliptin analysis.

As previously reported [13], both liraglutide and sitagliptin affect bile acid concentrations. However, given our slightly different and smaller study population, these measurements were repeated. The outcome of the present analyses was that plasma bile acid levels were not affected. On the other hand, liraglutide did increase faecal DCA, a secondary bile acid produced by microbial 7-dehydroxylation of the primary bile acid CA, which might indicate a liraglutide-induced effect on the intestinal microbiota [13]. Also, sitagliptin increased faecal levels of several bile acids, which might be an indication of increased production or microbial involvement. In any event, the discrepancy between faecal and plasma bile acid levels cannot be explained by our present data. One explanation could be a lack of statistical power. Alternatively, while the plasma bile acid pool is largely absorbed in the distal ileum (enterohepatic circulation), faecal samples represent colonic bile acids, and any changes in bile acids in the colon might explain the differences. The obvious option here would be a change in the intestinal microbiome.

Nevertheless, no associations were observed between the effects of liraglutide or sitagliptin on bile acids and microbiota composition. More important, this study measured microbiota composition. In theory, an effect of GLP-1-based therapies on

microbial function with no alteration of microbiota composition could be involved. However, whether the change in faecal bile acids was caused by changes in microbial function cannot be deduced from the present study, as other aspects of microbial function were not measured and would require other techniques, such as shotgun metagenomic sequencing and metabolomics. Moreover, other mechanisms, such as an increase in bile acid production/secretion or slower intestinal transit times, might also explain the altered bile acids in faeces.

In the present study, faecal markers of intestinal inflammation (calprotectin) and mucosal damage (I-FABP) were primarily measured to determine whether liraglutide and/or sitagliptin could affect the microbiota. However, our data show that neither drug affected either calprotectin or I-FABP, indicating that GLP-1-based therapies have no adverse effects on the intestine. Nonetheless, as our baseline values for calprotectin and I-FABP were low, it is not possible to ascertain whether these therapies could reduce inflammatory damage as previously suggested [34].

The strengths of the present study include its design (as a double-blind randomized study). Several limitations have already been mentioned, including the lack of dietary monitoring/standardization and control of co-medication use, and the fact that many aspects of microbiome function were not measured. Furthermore, because of technical errors and missing samples, the full number of participants could not be analyzed, thereby reducing statistical power. However, any sampling bias is unlikely, given that the errors were random. Finally, the effects of GLP-1-based therapies on diet were not assessed.

In conclusion, 12-week treatment with either the GLP-1 receptor agonist liraglutide or the DPP-4 inhibitor sitagliptin, when used as add-on therapy to metformin or SU, induced metabolic improvement with no effects on faecal microbiota composition. However, whether or not changes in microbial function arise should now be tested in dedicated trials with emphases on dietary standardization and co-medication use.

Authors' contributions

M.M. Smits developed the study protocol, performed the measurements and analyses, and wrote the manuscript (ms). C. Belzer, W.M. de Vos and M. Davids took the measurements, and contributed to the discussion and writing of the ms. H. Herrema, K.S. Fluitman, M.H.H. Kramer, A.K. Groen, D.L. Cahen, M. Nieuwdorp and D.H. van Raalte contributed to the discussion and edited the ms. All authors had full access to all the data, and take full responsibility for the integrity of the data and accuracy of the data analysis.

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Conflicts of interest

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References

[1] Hartstra AV, Bouter KEC, Bäckhed F, Nieuwdorp M. Insights into the role of the microbiome in obesity and type 2 diabetes. Diabetes Care 2015;38:159–65. http://dx.doi.org/10.2337/dc14-0769.
[2] Tremaroli V, Bäckhed F. Functional interactions between the gut microbiota and host metabolism. Nature 2012;489:242–9. http://dx.doi.org/10.1038/nature11552.
[3] Kootte RS, Levin E, Salojärvi J, Smits LP, Hartstra AV, Udayappan SD, et al. Improvement of insulin sensitivity after lean donor feces in metabolic syndrome is driven by baseline intestinal microbiota composition. Cell Metab 2017;26:611–9. http://dx.doi.org/10.1016/j.cmet.2017.09.008. e6.
[4] Vrieze A, Van Nood E, Holleman F, Salojärvi J, Kootte RS, Bartelsman JFWM, et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology 2012;143:913–6. http://dx.doi.org/10.1053/j.gastro.2012.06.031. e7.
[5] Forslund K, Hildebrand F, Nielsen T, Falony G, Le Chatelier E, Sunagawa S, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. Nature 2015;528:262–6. http://dx.doi.org/10.1038/nature15766.
[6] Wu H, Esteve E, Tremaroli V, Khan MT, Caesar R, Mannerås-Holm L, et al. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. Nat Med 2017;23:850–8. http://dx.doi.org/10.1038/nm.4345.
[7] Sun L, Xie C, Wang G, Wu Y, Wu Q, Wang X, et al. Gut microbiota and intestinal FXR mediate the clinical benefits of metformin. Nat Med 2018;24:1919–29. http://dx.doi.org/10.1038/s41591-018-0222-4.
[8] Smits MM, Tonneijck L, Muskiet MHA, Kramer MHH, Cahen DL, van Raalte DH. Gastrointestinal actions of GLP-1 based therapies: glycaemic control beyond the pancreas. Diabetes Obes Metab 2016;18:178–85. http://dx.doi.org/10.1111/dom.12593.
[9] Sjoberg KA, Rattigan S, Jeppesen JF, Lundsgaard AM, Holst JJ, Kiens B, et al. Differential effects of glucagon-like peptide-1 on microvascular recruitment and glucose metabolism in short- and long-term insulin resistance. J Physiol (Paris) 2015;593:2185–98. http://dx.doi.org/10.1113/JP270129.
[10] Jung YA, Choi YK, Jung GS, Seo HY, Kim HS, Jang BK, et al. Sitagliptin attenuates methionine/choline-deficient diet-induced steatohepatitis. Diabetes Res Clin Pract 2014;105:47–57. http://dx.doi.org/10.1016/j.diabres.2014.04.028.
[11] Klein T, Fujii M, Sandel J, Shibazaki Y, Wakamatsu K, Mark M, et al. Linagliptin alleviates hepatic steatosis and inflammation in a mouse model of non-alcoholic steatohepatitis. Med Mol Morphol 2014;47:137–49. http://dx.doi.org/10.1007/s00795-013-0053-9.
[12] Sun F, Chai S, Li L, Yu K, Yang Z, Wu S, et al. Effects of glucagon-like peptide-1 receptor agonists on weight loss in patients with type 2 diabetes: a systematic review and network meta-analysis. J Diabetes Res 2015;2015:1–9. http://dx.doi.org/10.1155/2015/157201.
[13] Smits MM, Tonneijck L, Muskiet MHA, Hoekstra T, Kramer MHH, Diamant M, et al. Biliary effects of liraglutide and sitagliptin, a 12-week randomized placebo-controlled trial in type 2 diabetes patients. Diabetes Obes Metab 2016;18:1217–25. http://dx.doi.org/10.1111/dom.12748.
[14] Wang L, Li P, Tang Z, Yan X, Feng B. Structural modulation of the gut microbiota and the relationship with body weight: compared evaluation of liraglutide and saxagliptin treatment. Sci Rep 2016;6:33251. http://dx.doi.org/10.1038/srep33251.
[15] Yan X, Feng B, Li P, Tang Z, Wang L. Microflora disturbance during progression of glucose intolerance and effect of sitagliptin: an animal study. J Diabetes Res 2016;2016:1–10. http://dx.doi.org/10.1155/2016/2093171.
[16] Smits MM, Tonneijck L, Muskiet MHA, Hoekstra T, Kramer MHH, Pieters IC, et al. Cardiovascular, renal and gastrointestinal effects of incretin-based therapies: an acute and 12-week randomised, double-blind, placebo-controlled, mechanistic intervention trial in type 2 diabetes. BMJ Open 2015;5e009579. http://dx.doi.org/10.1136/bmjopen-2015-009579.
[17] Costea PI, Zeller G, Sunagawa S, Pelletier E, Alberti A, Levenez F, et al. Towards standards for human fecal sample processing in metagenomic studies. Nat Biotechnol 2017;35:1069–76. http://dx.doi.org/10.1038/nbt.3960.
[18] Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol 2013;79:5112–20. http://dx.doi.org/10.1128/AEM.01043-13.

[19] Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;26:2460–1. http://dx.doi.org/10.1093/bioinformatics/btq461.
[20] Edgar RC. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. BioRxiv 2016081257. http://dx.doi.org/10.1101/081257.
[21] Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73:5261–7. http://dx.doi.org/10.1128/AEM.00062-07.
[22] Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 2013;41:D590–6. http://dx.doi.org/10.1093/nar/gks1219.
[23] McMurdie PJ, Holmes S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 2013;8e61217. http://dx.doi.org/10.1371/journal.pone.0061217.
[24] Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, Mcglinn D, et al. Package "vegan". Community Ecology Package; 2019.
[25] Kembel SW, Cowan PD, Helmus MR, Cornwell WK, Morlon H, Ackerly DD, et al. Picante: r tools for integrating phylogenies and ecology. Bioinformatics 2010;26:1463–4. http://dx.doi.org/10.1093/bioinformatics/btq166.
[26] Bates D, Mächler M, Bolker BM, Walker SC. Fitting linear mixed-effects models using lme4. J Stat Softw 2015;67. http://dx.doi.org/10.18637/jss.v067.i01.
[27] Rohart F, Gautier B, Singh A, Lê Cao K-A. mixOmics: an R package for 'omics feature selection and multiple data integration. PLoS Comput Biol 2017;13e1005752. http://dx.doi.org/10.1371/journal.pcbi.1005752.
[28] Moreira G, Azevedo F, Ribeiro L, Santos A, Guadagnini D, Gama P, et al. Liraglutide modulates gut microbiota and reduces NAFLD in obese mice. J Nutr Biochem 2018;62:143–54. http://dx.doi.org/10.1016/j.jnutbio.2018.07.009.
[29] Olivares M, Neyrinck AM, Pötgens SA, Beaumont M, Salazar N, Cani PD, et al. The DPP-4 inhibitor vildagliptin impacts the gut microbiota and prevents disruption of intestinal homeostasis induced by a Western diet in mice. Diabetologia 2018;61:1838–48. http://dx.doi.org/10.1007/s00125-018-4647-6.
[30] Zhao L, Chen Y, Xia F, Abudukerimu B, Zhang W, Guo Y, et al. A glucagon-like peptide-1 receptor agonist lowers weight by modulating the structure of gut microbiota. Front Endocrinol (Lausanne) 2018;9. http://dx.doi.org/10.3389/fendo.2018.00233.
[31] Wang Z, Saha S, Van Horn S, Thomas E, Traini C, Sathe G, et al. Gut microbiome differences between metformin- and liraglutide-treated T2DM subjects. Endocrinol Diabetes Metab Case Rep 2018;1e00009. http://dx.doi.org/10.1002/edm2.9.
[32] van Bloemendaal L, IJzerman RG, Ten Kulve JS, Barkhof F, Konrad RJ, Drent ML, et al. GLP-1 receptor activation modulates appetite- and reward-related brain areas in humans. Diabetes 2014;63:4186–96. http://dx.doi.org/10.2337/db14-0849.
[33] ten Kulve JS, Veltman DJ, van Bloemendaal L, Barkhof F, Drent ML, Diamant M, et al. Liraglutide reduces CNS activation in response to visual food cues only after short-term treatment in patients with type 2 diabetes. Diabetes Care 2016;39:214–21. http://dx.doi.org/10.2337/dc15-0772.
[34] Smits MM, van Raalte DH, Tonneijck L, Muskiet MHA, Kramer MHH, Cahen DL. GLP-1 based therapies: clinical implications for gastroenterologists. Gut 2016;65:702–11. http://dx.doi.org/10.1136/gutjnl-2015-310572.