The metabolomic effects of tripeptide gut hormone infusion compared to Roux-en-Y gastric bypass and caloric restriction

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Abstract:

**Context:** The gut-derived peptide hormones glucagon-like peptide-1 (GLP-1), oxyntomodulin (OXM), and peptide YY (PYY) are regulators of energy intake and glucose homeostasis, and are thought to contribute to the glucose-lowering effects of bariatric surgery.

**Objective:** To establish the metabolomic effects of a combined infusion of GLP-1, OXM and PYY (tripeptide “GOP”) in comparison to a placebo infusion, Roux-en-Y gastric bypass (RYGB) surgery, and a very low-calorie diet (VLCD).

**Design and setting:** Sub-analysis of a single-blind, randomised, placebo-controlled study of GOP infusion (ClinicalTrials.gov NCT01945840), including VLCD and RYGB comparator groups.

**Patients and interventions:** 25 obese patients with type 2 diabetes or prediabetes were randomly allocated to receive a 4-week subcutaneous infusion of GOP (n=14) or 0.9% saline control (SAL; n=11). An additional 22 patients followed a VLCD, and 21 underwent RYGB surgery.

**Main outcome measures:** Plasma and urine samples collected at baseline and 4 weeks into each intervention were subjected to cross-platform metabolomic analysis, followed by unsupervised and supervised modelling approaches to identify similarities and differences between the effects of each intervention.

**Results:** Aside from glucose, very few metabolites were affected by GOP, contrasting with major metabolomic changes seen with VLCD and RYGB.

**Conclusions:** Treatment with GOP provides a powerful glucose-lowering effect but does not replicate the broader metabolomic changes seen with VLCD and RYGB. The contribution of these metabolomic changes to the clinical benefits of RYGB remains to be elucidated.

**Keywords:** GLP-1, oxyntomodulin, PYY, bariatric surgery, caloric restriction, metabolomics
1 Introduction

Type 2 diabetes (T2D) affects 1 in 10 people and is responsible for 4.2 million deaths every year (1), as well as severe complications including cardiovascular disease, retinopathy, nephropathy and amputations (2). The increasing prevalence of obesity drives a parallel increase in T2D due to the shared pathophysiological processes of these two metabolic diseases (3). Bariatric surgery is the most effective treatment for both morbid obesity and T2D, significantly outperforming lifestyle interventions and pharmacotherapy (4). One proposed mechanism for the beneficial metabolic effects of bariatric surgery is the observed increase in post-prandial release of glucoregulatory and satiety gut hormones including glucagon-like peptide-1 (GLP-1), oxyntomodulin (OXM) and peptide YY (PYY) (5). To probe this, we have previously administered these three gut hormones in combination (triptide “GOP”) as a daily subcutaneous infusion for 4 weeks in overweight patients with T2D (6) to achieve circulating concentrations comparable to peak levels seen after Roux-en-Y gastric bypass (RYGB) surgery. In fact, triptide GOP infusion led to better improvements in glycaemia compared to parallel groups undergoing RYGB or caloric restriction (very low-calorie diet, “VLCD”), despite losing less weight. This implies that the triptide infusion exerts a potent effect on glucose homeostasis that is at least partly independent of weight loss (6), and also provides further evidence that other mechanisms beyond elevations in gut hormones may be operative after RYGB.

Metabolic profiling analyses based on proton nuclear magnetic resonance (¹H NMR) spectroscopy and mass spectrometry have made investigation of the biochemically diverse human metabolome and lipidome accessible, helping further the understanding of normal physiology and numerous disease states (7). A number of previous studies have demonstrated that both RYGB (8-16) and VLCD (17-21) result in profound changes to the plasma and urinary metabolome. Certain features suggest common underlying metabolic processes, such as increases in plasma ketone derivatives, fatty acids and acylcarnitine species as responses to caloric restriction (22). RYGB appears to exert additional effects such as an early reduction (within weeks) in circulating branched-chain amino acids (BCAAs), a change which is suggested by some to exert beneficial effects on glucose metabolism (23-25). However, a recent study showed that the glucose-lowering effects of bariatric surgery are not dependent on a reduction in BCAAs (26). Moreover, some metabolomic changes apparently specific to RYGB may in fact be due to a pre-operative period of caloric restriction which is commonly recommended prior to the procedure (27). The nature and importance of bariatric surgery-associated metabolomic changes are therefore unresolved. Furthermore, the effect of combined gut hormone infusion on the metabolome is not known, although effects of GLP-1 receptor agonist treatment on lipid and lipoprotein parameters have been reported in the literature (28,29).

With the aim of investigating potential shared or distinct mechanisms through which GOP, RYGB and VLCD achieve their glucoregulatory and weight lowering benefits, we provide in this study comprehensive analyses of the effects of a 4-week infusion of GOP or vehicle control (saline – SAL), RYGB and VLCD on the circulating and urinary metabolome. In our study patients that underwent RYGB did not follow a VLCD prior to the procedure, allowing us to discount this potential
confounder. Overall, we observed very limited impact on the metabolome of tripeptide GOP infusion, contrasting with the wide-ranging and generally well correlated effects of both VLCD and RYGB.

2 Materials and methods

2.1 Study design and participants

This cohort of patients took part in a mechanistic study at the National Institute for Health Research (NIHR) Imperial Clinical Research Unit Facility at Hammersmith Hospital, London, UK, from July 2016 to October 2018. See Figure 1A for patient flow through the study. Inclusion and exclusion criteria, demographic characteristics as well as data on weight, fructosamine, fasting glucose and insulin levels at baseline and four weeks post each intervention have been published previously (6). Briefly, the study was a single-blind, randomised, placebo-controlled study comparing two 28-day infusion groups: tripeptide GOP or 0.9% saline vehicle (SAL) in patients with obesity and prediabetes or T2D. The weight-adjusted doses of each GOP component were 4 pmol/kg/min (GLP-1), 4 pmol/kg/min (oxyntomodulin) and 0.4 pmol/kg/min (PYY), delivered for 12 hours per day, starting an hour prior to breakfast and finishing after the last meal of the day. All infusion participants also received dietetic advice on healthy eating and weight loss from a qualified dietician.

Two similar nonblinded groups of patients, either undergoing RYGB surgery or following a VLCD meal replacement diet (c. 800 kcal/day for four weeks), were also recruited as comparators. The participants undergoing RYGB attended the research unit for a baseline visit prior to surgery and were reviewed 2, 4 and 12 weeks post-surgery. VLCD participants attended the research unit for a baseline visit, before starting a complete meal replacement VLCD of 800 kcal/day for 4 weeks (Cambridge Weight Plan). They were reviewed by a dietician pre-diet and then on a weekly basis until completion.

Blood samples for metabolomic analysis were collected in the fasting state into lithium heparin tubes with no protease inhibitors, prior to and four weeks into each intervention. For the GOP and SAL groups, the second sample was collected on the last day of the study, at least two hours after the initiation of the infusion. Plasma was obtained after centrifuging the blood samples at 2500 × g for 10 min at 4°C. Urinary samples were collected at a fasting state on the same study days as the plasma samples. All the samples were kept at -80°C before metabolomic analysis.

2.2 Metabolomic analysis

A total of 136 plasma and 136 urine samples from 68 subjects (see Supplementary Figure 1A (30)) were analysed using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) and 1H NMR spectroscopy. Full analytical details, following previously described sample preparation, analytical, and quality control (QC) procedures (31-33), are provided in the Supplementary Material (30).
NMR and UPLC-MS assays were applied to maximise coverage of a broad range of metabolite classes including lipophilic, hydrophilic, small and macromolecular analytes (see Supplementary Figure 1B (30)). Specifically, UPLC-MS assays for plasma analysis were tailored for the separation of lipophilic analytes (e.g. complex and neutral lipids) by reversed-phase chromatography and the separation of hydrophilic analytes (e.g. polar and charged metabolites) by hydrophilic interaction liquid chromatography (lipid RPC and HILIC assays respectively). UPLC-MS assays for urine analysis were tailored for broad small molecule coverage using reversed-phase chromatography (SmMol RPC) and the separation of hydrophilic analytes by HILIC. When coupled to positive and or negative mode ionisation the following datasets were produced: plasma lipid positive (lipid RPC+), lipid negative (lipid RPC-) and HILIC positive (HILIC+); urine small molecule positive (SmMol RPC+) and negative (SmMol RPC-) and HILIC positive (HILIC+). For both plasma and urine samples, a standard one-dimensional (1D) \(^1\)H NMR profile experiment was acquired using the 1D-NOESY pre-sat pulse sequence with water pre-saturation. For plasma, an additional spin-echo experiment using the 1D Carr-Purcell-Meiboom-Gill (CPMG) pre-sat pulse sequence was carried out to better visualise signals of the small metabolites.

Data from each assay was processed to include both global profiling and targeted extraction datasets. These two approaches are highly complementary. Global profiling provides a comprehensive analysis of all measurable metabolites in a sample, but results in datasets with multiple variables per analyte, the identities of which are typically unknown. In contrast, by targeted extraction of a pre-defined set of metabolites, pre-annotated datasets are immediately more interpretable but are limited in coverage to those metabolites in the pre-defined set. For UPLC-MS, targeted extraction of metabolites from each assay was performed using PeakPantheR (https://github.com/phenomecentre/peakPantheR), and for NMR targeted extraction was performed using the in vitro diagnostics platform (IVDr) from Bruker Biospin (www.bruker.com) to generate plasma SmMol IVDr, plasma lipoprotein IVDr and urine SmMol IVDr datasets.

2.3 Statistical analysis

2.3.1 Unsupervised analysis by principal component analysis

To provide a broad overview of the data, including identification of trends, clusters and any biological outliers, principal component analysis (PCA) was performed on the global profiling metabolomic datasets. For brevity, datasets were combined before analysis to generate a biofluid/platform-specific overview, with four overall groupings: plasma UPLC-MS (containing lipid RPC+, lipid RPC- and HILIC+ datasets, 3641 total variables), plasma NMR (containing the plasma standard 1D and CPMG datasets, 944 total variables), urine UPLC-MS (containing SmMol RPC+, SmMol RPC-, and HILIC+ datasets, 24077 total variables) and urine NMR (containing the urine standard 1D dataset, 585 total variables). To better visualise grouping patterns among the treatment
groups and to take the time course-related changes into consideration, PCA was performed on fold-change data, where, for each variable and each individual, values were calculated as the log₂ fold-change between 4-week and baseline time-points. PCA was performed on these derived datasets using the Scikit-learn Python package (34). To provide additional insight, one-way ANOVAs were performed between treatment groups (SAL, GOP, RYGB and VLCD) for the scores of each PCA component using Prism 8.0 (GraphPad Software).

### 2.3.2 Supervised analysis by linear mixed effect modelling

To further investigate treatment group specific differences over the study time-course linear mixed effects (LME) modelling was performed on the targeted extraction datasets.Datasets were selectively combined to generate a metabolite class-specific overview, with four overall groupings: plasma small molecules (containing the SmMol IVDr dataset and 38 of the HILIC+ metabolites, 67 total metabolites), plasma lipoproteins (containing the lipoprotein IVDr dataset, 112 total metabolites), plasma lipids (containing the lipid RPC- and HILIC+ datasets, and 11 of the HILIC+ metabolites, 269 total metabolites) and urine small molecules (containing SmMol IVRr, SmMol RPC+, SmMol RPC- and HILIC+ datasets, 178 total metabolites). LME models were generated using the lme4 R software package (35) according to the formula:

\[
\text{model} \leftarrow \log(\text{variable}) \sim \text{time-point} \times \text{treatment group} + (1|\text{subject})
\]

A model was generated for each variable including fixed effects for interaction between time-point (baseline or 4 weeks post intervention) and treatment group (SAL, GOP, RYGB or VLCD), allowing for subject specific random effects. Metabolites with a false discovery rate (FDR) \(\alpha<0.05\) controlled using the Benjamini-Hochberg procedure (36) were considered to be statistically significantly different between treatment groups (specifically compared to the SAL group). To investigate the influence of potential confounding/covarying factors additional models were generated according to the adapted formula:

\[
\text{model} \leftarrow \log(\text{variable}) \sim \text{time-point} \times \text{treatment group} + \text{time-point} : \text{treatment group} : \text{factor} + (1|\text{subject})
\]

Factors investigated included difference (baseline to 4-week time-points) in weight, fasting glucose, fasting insulin, triglycerides and total cholesterol. As for the main model, significant metabolites were determined after adjustment of appropriate model estimates for multiple corrections by FDR control.

Subsequently, to investigate metabolic changes specifically associated with each of these clinical factors, the supervised multivariate method of partial least squares (PLS) was performed between metabolomic datasets (X) and each clinical factor (Y) in sample sets containing all groups (the full cohort) and each treatment group (SAL, GOP, VLCD and RYGB) individually. The validity of PLS models was assessed by cross-validation and generation of the Y prediction performance statistic \(Q^2_Y\) and permutation testing of the true model against 1000 null models with mis-matched Y. Where valid models were obtained (\(Q^2_Y>0.15\) and permutation p-value<0.01), metabolites associated with Y (clinical factor) were selected empirically by comparing the true model weights against the null model weight distribution and selecting those where the true weight exceeded the null model weight distribution with p<0.05.
3 Results

3.1 Tripeptide GOP infusion achieves blood glucose control without significantly altering the circulating or excretory metabolome

The study design and participant disposition are summarised in Figure 1A. Baseline demographic and clinical characteristics are shown in Supplementary Table 1 (30). We previously reported that tripeptide GOP infusion led to better glucose tolerance in response to a mixed meal test but less weight loss than VLCD and RYGB (6). Individual participant changes from baseline in weight, fasting glucose, insulin and lipid parameters from the current sub-analysis, which includes one additional RYGB participant that had not completed follow-up in the original study, are presented in Figure 1B.

We first performed untargeted metabolomic analysis on pre- and post-intervention plasma and urine samples to identify patterns of change in an unbiased manner, without focusing on specific metabolites. It is immediately apparent from visual inspection of the metabolomic changes between pre- and post-intervention timepoints (Figure 2A) that the impact of GOP was less marked than of RYGB and VLCD, and in many cases similar to that seen with SAL. This was further investigated using LME modelling to formally determine the proportion of changes that were significantly different between interventions (Figure 2B). This indicated that, when compared to SAL, up to one third of the observed plasma LC-MS features were distinctly altered by RYGB or VLCD, whereas no features were significantly altered by GOP after FDR correction. However, calculating the similarity between the effects of each intervention by correlating average fold changes for each variable showed that VLCD and RYGB effects were highly congruent (Figure 2C). In fact, even features that were statistically significantly altered only by VLCD or by RYGB still showed a high degree of correlation (Supplementary Figure 2A (30)), i.e. the directions of change, if not the magnitudes, were generally consistent. On the other hand, urine LC-MS features identified as “uniquely” affected by VLCD or RYGB were not consistently as well correlated (Supplementary Figure 2B (30)).

As an additional analysis aiming to identify patterns in the metabolomic effects of each treatment, PCA was also performed on all recorded log₂ fold-changes. No principal components in any biofluid were significantly different between GOP and SAL, whereas VLCD and RYGB showed a number of differences when compared to SAL, GOP, and each other (Supplementary Figure 2C, 2D (30)).

This main message from this global analysis is that GOP treatment has a far more minor effect on the metabolome than VLCD or RYGB, despite being more effective for lowering blood glucose. A second message is that the metabolomic impacts of VLCD and RYGB share many common elements.
3.2 Lipidomic responses to VLCD and RYGB reflect caloric restriction

Following this analysis of the untargeted profiling data, we next repeated the LME modelling to determine the proportion of changes that were significantly different between interventions in specific groups of metabolites from the targeted extraction datasets. Overall changes across major plasma metabolite classes relative to SAL are summarised in Figure 3A. See Supplementary Figure 1B (30) for information about the plasma and urine datasets, and Supplementary Figures 3-7 (30) for full characterisation of individual metabolites, statistical analysis of differences between groups, and associations with clinical responses. As for the untargeted datasets, no metabolites were affected by GOP compared to SAL with FDR correction (Figure 3A). In contrast, VLCD and RYGB led to significant changes in a substantial proportion of lipid species compared to SAL (Figure 3A). From the lipids quantified, VLCD led to a larger number of changes in acylglycerols, fatty acids, phospholipids and lysophospholipids, whereas RYGB affected ceramides and sphingomyelins to a greater extent. However, despite some variation in the statistical significance obtained for SAL-VLCD and SAL-RYGB comparisons, the lipidomic effects of these two interventions were very well correlated (Figure 3B), suggesting overlap between the mechanisms driving these changes.

The pattern of lipidomic changes seen with RYGB and VLCD, characterised by decreases in acylglycerols, increases in fatty acids and acylcarnitines, but decreases in phospholipids and their lysophospholipid derivatives (Figure 3A), is consistent with the known effects of caloric restriction (19,37). These changes reflect an energetic switch to beta oxidation, facilitated by mobilisation of fatty acids from diverse sources (acylglycerols, phospholipids and their derivatives) which are then converted to acylcarnitines for transport into mitochondria, where they undergo beta oxidation (Figure 3C). Generation of acetyl-CoA from beta oxidation provides a substrate for both ketogenesis and the tricarboxylic acid (TCA) cycles, and accordingly, elevations in ketones (e.g. 3-hydroxybutyric acid and acetoacetic acid) and circulating TCA cycle intermediates (e.g. citric and succinic acid) were observed, particularly after VLCD (Figure 3D, Supplementary Figure 3 (30)). Ketones in urine tended to also be increased after VLCD and RYGB (Supplementary Figure 4 (30)). The marked reductions in triacylglycerol levels with VLCD and RYGB tended to apply particularly to shorter chain, saturated forms (Figure 3E, Supplementary Figure 5 (30)), a pattern previously noted during caloric restriction (37).

Resolution of individual acyl chain carbon content in phospholipids and lysophospholipids enabled us to observe how these lipid species changed in parallel to fatty acids and acylcarnitines with the same chain length (Figure 3F, 3G). Note that phosphatidylcholines (a major group of phospholipids) contain two acyl chains (sn-1 and sn-2 positions); in this case we matched free fatty acids to the acyl chain at the sn-2 position, meaning there is more than one “match” for many fatty acids, with the mean change for each treatment represented by a single data point for each “match” (Figure 3G, left panel). Similarly, inclusion of several different lysophospholipid subclasses (Figure 3G, right panel) means that each fatty acid has a number of lysophospholipid matches. With these caveats in mind, it was still apparent that phospholipids and lysophospholipids tended to reduce in parallel with
increases in free fatty acids of the same chain length (Figure 3G), which is consistent with the notion that (lysophospholipids are a key source of fatty acids during caloric restriction, e.g. sourced from cell membranes. Lysophosphatidylethanolamines (LPE) and lysophosphatidylinositides (LPI) showed the clearest correlations ($r = 0.68$ and $0.62$, respectively) with their matched fatty acids.

Whilst a majority of fatty acids and acylcarnitines were increased with VLCD and RYGB, we observed that the very long chain C24:0 (lignoceric acid) or C26:0 (cerotic acid) species tended to be reduced (Figure 3F, Supplementary Figures 5 and 6 (30)). The divergent effects on these very long chain fatty acids versus shorter chain counterparts may be a feature of caloric restriction (19,37), and are compatible with the observation that reductions in triglycerides seen with VLCD and RYGB applied preferentially to species with a lesser carbon content (Figure 3E), as these are presumably unlikely to contain very long acyl chains.

Showing the opposite pattern to that observed with phospholipids, ceramide and sphingomyelin species across the entire cohort tended to increase or decrease in tandem with fatty acids or acylcarnitines sharing the same acyl chain length (Figure 3H). C24:0- and C26:0-containing sphingomyelins and ceramides were well represented in our pre-annotated lipidomic panel, but not in the phospholipid or lysophospholipid panels; due to the contrasting direction of change seen with these very long chain species compared to their shorter counterparts, this could explain why the effects of RYGB and VLCD on sphingomyelin/ceramide lipids appeared (somewhat artefactually) more heterogenous than for phospholipids/lysophospholipids (Figure 3A).

We also investigated the relationship between changes in the abovementioned lipids or energetic metabolites and changes in clinical parameters including weight, fasting glucose, insulin, triglycerides and cholesterol, across the whole cohort and within individual interventions. Approximately 60% of quantified lipids showed a significant correlation with weight loss across the entire cohort, with a smaller number (~23%, mainly fatty acids) significantly correlated with glucose, and 56%, 44% and 40%, respectively, correlated with changes in insulin, cholesterol and triglycerides (Supplementary Figures 5 and 6 (30)). However, using LME modelling, owing to the observed group-specific differences in these clinical parameters (Figure 1B) it is hard to mathematically separate the impact of treatment from the impact of differences in clinical values on the metabolome. This was therefore further investigated using PLS analysis, by building models between the metabolomic data and each clinical parameter across all samples (i.e. all treatment groups combined), and separately for individual interventions. Interestingly, even where valid models were obtained, for the majority of lipids the observed associations did not meet an FDR corrected $p$-value threshold of 0.05 (Supplementary Figures 5 and 6 (30)). Those lipid species that did show a statistically significant association with weight loss (but not glucose) across the whole cohort included CAR(24:0) and selected ceramide, sphingomyelin and phosphatidylcholine species. Of these however, only the two ceramide species (d16:1/22:0 and d16:1/24:0) showed any associations with weight loss when individual groups were modelled separately (Figure 3I); significant Pearson’s correlations with blood glucose were seen for these lipids in the SAL group, but these were not significant by PLS modelling.
Acetoacetic acid remained predictive of weight changes across the entire group (Figure 3I).

In summary, we observed that VLCD and RYGB led to marked and well correlated changes in several lipid parameters that can be explained by caloric restriction. A relationship between some of these changes and weight loss was observed. The effects of GOP on the lipidome were minor.

3.3 Effects on amino acid metabolism

Caloric restriction can affect amino acid metabolism, in part as several amino acids serve as substrates for gluconeogenesis, ketogenesis, or both (19,37,38). In our study, only one amino acid (tyrosine) was significantly altered by an active treatment (RYGB) compared to SAL treatment (Figure 3A, Supplementary Figure 3 (30)). However, there were a number of other statistically significant inter-group comparisons and non-significant but informative trends (Figure 4A, Supplementary Figure 3 (30)). For example, with VLCD and RYGB, trends towards reductions in alanine and glutamic acid, and increases in glycine were observed. This pattern is reported in the context of starvation (37,38), with the drop in alanine levels thought to represent increased alanine utilisation as a gluconeogenic substrate, thereby fitting with reduced levels of pyruvic acid (another gluconeogenic substrate) after VLCD (Figure 3D). Reductions in blood glucose for the RYGB group, but not weight loss, were associated with greater reductions in alanine in the PLS (Supplementary Figure 3 (30), Figure 4B); this did not apply to other interventions or across the cohort as a whole. Changes in glycine were inversely associated with weight loss across the whole cohort by Pearson’s correlation (Figure 4B), but this was not significant by PLS modelling for the entire group or for individual interventions (Supplementary Figure 3 (30)). The fact that alanine and glycine, both considered gluconeogenic amino acids, showed divergent changes with VLCD indicates that the effects of caloric restriction on amino acid metabolism cannot be considered only within the framework of fuel utilisation.

RYGB has previously been associated with a specific lowering effect on BCAAs, with this effect predictive of improvements in glucose homeostasis (23). Notably, in our study all three BCAAs (valine, leucine, isoleucine) were numerically decreased, albeit non-significantly, after RYGB compared to SAL (Figure 4A), whereas for VLCD there was no change. Interestingly, isoleucine was also reduced after GOP treatment compared to baseline, although this was driven primarily by three participants who showed quite marked reductions in this BCAA (Figure 4B). The same three GOP participants also showed the greatest reductions in blood glucose and, accordingly, changes in isoleucine were correlated with changes in blood glucose across the whole cohort (Figure 4B). This was the case for leucine (Supplementary Figure 3 (30)), and the glucose-isoleucine association within the GOP treatment group remained significant in the PLS model (Supplementary Figure 3 (30)). The effects of RYGB and GOP on BCAAs are unlikely to be explained by caloric restriction, which is known to cause an initial increase in circulating BCAAs and a subsequent decline if starvation is prolonged.
over many days (37,38). Compared to VLCD, for which the metabolomic effects of caloric restriction are most pronounced (Figure 3), consistently lower BCAAs in the RYGB group are suggestive of a distinct mechanism unrelated to caloric restriction.

The RYGB-mediated reduction in plasma tyrosine (Figure 4A) was associated with weight loss across the entire cohort, both by Pearson’s correlation (Figure 4B) and by PLS analysis (Supplementary Figure 3 (30)). Urinary tyrosine was also significantly reduced after RYGB versus SAL (Supplementary Figure 4 (30)), similar to a previous study with sleeve gastrectomy (39).

These data therefore reflect the effects of caloric restriction on amino acid metabolism, along with superimposed intervention-specific effects, mainly of RYGB, apparently achieved via a different mechanism.

3.4 Identification of additional VLCD- and RYGB-discordant metabolite changes

Analysis of both global profiling and pre-annotated datasets thus far highlight many similarities between the effects of VLCD and RYGB, in many cases (especially for lipid species) explicable by caloric restriction. On the other hand, differences in plasma and urine amino acid responses indicate these interventions are not entirely equivalent at the metabolomic level (Figure 4). We therefore sought to identify additional metabolites showing differential responses between RYGB and VLCD, focussing on the non-lipid small molecule pre-annotated datasets in plasma and urine (the corresponding analysis for plasma lipids is already shown in Figure 3B and shows a high degree of correlation).

We identified metabolites that were significantly affected by either VLCD or RYGB, compared to SAL, and categorised these changes as “uniquely significant” to one intervention or “shared” (Figure 5A). A number of metabolites, such as plasma pantothenate, plasma creatine, urine succinic acid, and urine kynurenic acid, showed concordant responses, i.e. both significantly altered compared to SAL, and both in the same direction. Other metabolites clearly showed similar direction of change but were only significant for one intervention compared to SAL, e.g. plasma 3-hydroxybutyric acid, plasma citric acid, and urine suberic acid. We interpret these as reflecting a lack of statistical power rather than true intervention-specific changes. However, a smaller number of metabolites were identified that did not show a consistent pattern between interventions. For example, plasma caffeine and its metabolites trigonelline and paraxanthine were all decreased with RYGB but unchanged with VLCD (Figure 5A), with trends in the same direction for the same metabolites (and also theophylline) in urine (Supplementary Figure 4 (30)). Plasma trimethylamine-N-oxide (TMAO) was increased with RYGB and slightly decreased with VLCD (Figure 5A); this effect of RYGB has been described on other occasions (40,41). Urine indoxyl glucuronide, a metabolite of tryptophan, was increased with RYGB...
but unchanged with VLCD (Figure 5A). This may reflect accelerated tryptophan metabolism as urinary tryptophan was particularly reduced with RYGB (Supplementary Figure 4 (30)). Of these highlighted metabolite changes, only urinary indoxyl glucuronide showed an association with changes in clinical parameters (weight, fasting glucose) across the entire cohort (Figure 5B), but this was not significant by PLS modelling (Supplementary Figure 4 (30)).

### 3.5 Intervention-specific effects on circulating lipoproteins and their lipid cargo

In the original description of this trial, minor changes only were observed in the standard clinical lipid profile after 4 weeks of each intervention, with the most notable effects being a reduction in triglycerides with VLCD and a paradoxical increase with RYGB, compared to SAL (6). This pattern was confirmed using NMR lipoprotein analysis, which is able to subclassify a large number of lipoprotein subfractions and their lipid content (Figure 6A, Supplementary Figure 7 (30)). VLCD also reduced total apolipoprotein B100 (apoB), the protein compound of very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL), whereas RYGB did not (Figure 6B). Examining the abundance and content of differently sized apoB-containing lipoprotein subfractions revealed a similar pattern for both GOP and VLCD, in which particle number and carriage of triglycerides, cholesterol, free cholesterol and phospholipids on the smaller, denser LDL subfractions (LDL4-6) tended to be reduced (albeit not significantly compared to SAL), whilst the larger, lighter subfractions (LDL1-3) were less affected (Figure 6C). Moreover, the effect of VLCD on VLDL-associated lipids particularly affected the very lowest density subfraction (VLDL1, Figure 6C), for which insulin is a key regulator of production (42) and thus improvements in insulin sensitivity may be expected to exert a prominent effect. Although no lipoprotein-related differences passed adjustment for weight loss (Supplementary Figure 7 (30)), no valid models were obtained for PLS between the lipoprotein data and weight (either across all groups or for individual groups), perhaps indicating that these changes are indeed treatment- rather than weight loss- associated.

### 4 Discussion

This study provides a comprehensive profiling and comparison of the plasma and urine metabolomic changes across three different active interventions (i.e. GOP, VLCD, RYGB) for weight loss and diabetes improvement, along with a placebo control group (SAL). The effects of the tripeptide gut hormone infusion on the metabolome have not previously been investigated but are of significant clinical interest given the current drive to develop pharmacological agonists that target multiple gut hormone receptors (43). VLCD and RYGB have been subjected to several metabolomic investigations in the past, although in many cases without a suitable control group. The potential role of gut hormones in mediating metabolic effects of RYGB has been often discussed, so our direct comparison of these two interventions within the same study provides an opportunity to evaluate this hypothesis. The two main messages from our study are that 1) GOP treatment results in very few changes to the global metabolome, yet exerts a powerful glucose-lowering effect, and 2) RYGB
and VLCD exert profound metabolomic changes which are in many cases well correlated, although with a number of exceptions that are discussed in more detail below.

Our original study showed that the glucoregulatory effects of the tripeptide GOP combination exceed those of VLCD and RYGB in spite of lesser weight loss (6). The individual components of the tripeptide treatment, i.e. GLP-1(7-36) amide, OXM and PYY(3-36), are known to exert distinct effects on glucose and energy homeostasis. GLP-1 stimulates insulin secretion, slows gastric emptying and suppresses appetite (44). OXM, through its joint targeting of GLP-1 and glucagon receptors, reinforces the effects of GLP-1 itself, but additionally increases energy expenditure, enhancing achievable weight loss (45). PYY(3-36) is primarily known for its anorectic action and has no significant acute effects on insulin secretion and sensitivity (46,47). To our knowledge, neither OXM nor PYY have been evaluated for their effects on the metabolome in the clinical or preclinical setting, although some clues into the anticipated effects of OXM are provided by an earlier study demonstrating glucagon-induced suppression of plasma amino acid levels to provide fuel for gluconeogenesis (48), and a more recent study in mice showing that the dual glucagon/GLP-1 receptor agonist cotadutide modifies the amino acid and lipid hepatic metabolome (49).

Some studies of the effects of pharmacological GLP-1R agonists on metabolomic parameters have been reported and provide context to our observations with GOP tripeptide. Specifically, treatment with the GLP-1R agonist liraglutide over a similar time period to our study led to a reduction in small dense LDL particles (29), a trend for which was also seen in our GOP cohort. This profile is typically associated with reduced atherogenic potential and is in keeping with the known cardiovascular benefits of GLP-1R agonist treatment (50). One year of liraglutide treatment resulted in reductions in total apoB (28), which was not the case with GOP in our study. Women with polycystic ovarian syndrome treated with exenatide for three months showed a reduction in BCAAs compared to baseline (51); we also observed a trend for a reduction in BCAAs with GOP, although this was not statistically significant compared to SAL.

However, whilst some metabolically plausible trends were seen with the tripeptide combination, it is important to highlight that that not a single plasma or urinary metabolite in either the profiling or pre-annotated datasets reached statistical significance with GOP compared to SAL after correction for false discovery. This may be partly due to the relatively small sample sizes in our study, and the observed subtle degree of weight loss in the SAL group “diluting” the potential for observing differences with the GOP group. We performed a power analysis using the SAL group as a control by simulating the effect of increasing the size of the GOP group to that of the RYGB group on power to detect metabolomic changes (Supplementary Methods and Supplementary Figure 8 (30)); this indicated that the slight differences in sample size were unlikely to explain the large changes in number of metabolomic changes observed, i.e. the metabolomic effects of GOP are almost certainly more minor than for VLCD and RYGB, despite outperforming these two interventions for anti-hyperglycaemic effect. Whether this “glucocentric” action of tripeptide treatment represents a therapeutic advantage or disadvantage is not clear, but it may hold some relevance to
pharmacological approaches targeting the same receptors. Notably, the on-infusion steady state plasma concentration of GLP-1 achieved in the GOP cohort were comparable to the calculated free concentration of semaglutide in humans (52), although there are no equivalent comparisons available for OXM or PYY. On the other hand, GOP was administered as a 12-hour infusion with an overnight break, contrasting with the extended pharmacokinetic profiles of long-acting GLP-1R agonists. The potential for GLP-1R tachyphylaxis to influence metabolic responses (53,54) should therefore be borne in mind when extrapolating our findings to the pharmacological setting.

The effects of bariatric surgery and dietary manipulation have been subject to intense investigation using metabolomic approaches (22). A key area of controversy is how much of the weight loss and metabolic perturbation seen with bariatric surgery is due to caloric restriction versus specific physiological changes (e.g. the gut microbiota) induced by the procedure. The effects of acute (48 hour) caloric restriction have been comprehensively documented (19) and include increased generation of ketones and ketone derivatives, increased flux through the TCA cycle leading to increases in circulating TCA cycle intermediates, increased lipolysis resulting in reduced acylglycerols but increases in fatty acids and acylcarnitines, as well as reductions in most phospholipids and lysophospholipids, also as sources of free fatty acids destined for beta oxidation. All of these patterns have been described for RYGB and diet-induced weight loss (12,23), and were observed in our RYGB and VLCD cohorts.

Notably, some phospholipid species that were not decreased by acute caloric restriction in the study by Collet et al. (19), were also conspicuously unaffected by RYGB and VLCD in our study, including PC(16:0/16:0), PC(16:0/22:6), PE(16:0/20:4). This pattern was not universally the case though; for example, PC(16:0/20:4) was decreased by RYGB and VLCD in our study, but increased with acute caloric restriction (19). In contrast, whilst Collet et al. found increases in several sphingomyelins (19), we observed a diversity of impacts on both sphingomyelins and ceramides, with some species increased [e.g. SM(d18:1/18:0)] and some decreased [e.g. SM(d16:0/22:0)]. This may be due to having measured different sphingomyelin species in the different studies, and indeed, our pre-annotation pipeline identifies several examples of ceramides or sphingomyelins containing very long chain (C22:0+) acyl groups which, as highlighted above, behaved somewhat differently to shorter chain fatty acids. Alternatively, this discrepancy could indicate adaptation to the different intervention durations.

Nevertheless, an important message from both the global profiling data and our pre-annotated datasets was that both VLCD and RYGB effects were often very well correlated, even if the effect magnitudes differed in some cases, implying at least some common underlying mechanisms driving these changes. This interpretation is consistent with the conclusions of Herzog et al., who demonstrated that several metabolomic changes commonly attributed to bariatric surgery are also seen with pre-surgical dietary restriction in a longitudinal study which examined the metabolomic changes in patients after undergoing a 4-week pre-operative VLCD prior to RYGB (27). We point out here again that our RYGB group did not undergo a pre-operative VLCD, i.e. the metabolomic

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similarities between RYGB and VLCD are not confounded by the imposition of a VLCD during pre-operative preparation.

On the other hand, in the context of somewhat congruent metabolomic impacts of RYGB and VLCD, the smaller number of discrepant analytes are of particular interest as they provide more insights into physiological differences of each intervention. For example, caffeine and its metabolites trigonelline and paraxanthine were markedly decreased in our RYGB cohort, which was not the case after VLCD. In our study, all participants were requested to refrain from caffeine consumption for 24 hours before sample collection. A reduction in paraxanthine after caffeine loading in post-RYGB patients was previously reported (55), which was attributed to the effects of the surgical procedure on hepatic cytochrome P450 enzyme induction (56). Of broader relevance, RYGB surgery profoundly alters the gut bacterial composition, e.g. increased Gammaproteobacteria, and metabolic functions, e.g. increased protein putrefaction (57,58), with the latter reflected by increased urinary host-bacterial metabolites following RYGB, such as 4-cresyl sulfate, 4-hydroxyphenylacetate phenylacetylyglutamine, and indoxyl sulfate, the precursors of which are tyrosine, phenylalanine and tryptophan (59). Thus, the significantly lower levels of plasma tyrosine, together with a trend towards lower phenylalanine, in the RYGB group compared to SAL or GOP could result from the incomplete digestion of protein and absorption of these amino acids in the foregut post-surgery. As a result, partly digested peptides could be more abundant in the colon as substrates for bacterial putrefaction. Consistent with these observations, we found an increased urinary concentration of indoxyl glucuronide after RYGB, suggesting a higher degree of bacterial conversion of tryptophan to indole and subsequent conjugation with glucuronide in the liver. Moreover, we observed relative elevations in TMAO after RYGB compared to VLCD, in line with other studies and thought to depend on the effect of the procedure on the gut microbiota (60).

There are several limitations of our study. Firstly, our post-intervention sample was collected at 4 weeks, meaning we were unable to examine the sustainability of changes and how these could potentially impact on the longer-term effects of each intervention. Studies with serial sampling have demonstrated that some of the changes seen after bariatric surgery are transient (61). Whilst in keeping with the sample sizes of analogous interventional metabolic studies, the relatively small number of participants in the SAL and GOP groups may have reduced our ability to robustly identify small but important metabolomic changes, especially when investigating the potential influence of confounding/covarying clinical factors such as weight loss. Our use of pre-annotation and semi-automated feature identification markedly streamlines the analytical pipeline compared to traditional approaches, facilitating biological insights by documenting changes in specific analyte groups. However, this strategy is dependent on the analytes included in the pre-annotated panel, which risk interpretative bias. Therefore, the global profiles provided in the current study are complementary to the pre-annotated datasets. An important point when interpreting metabolomic studies is that a large number of metabolites reflect recent dietary intake (62), meaning that care must be taken not to overinterpret changes within individuals. Whilst this frequently can be a random “nuisance” factor, in our study changes to dietary habits are, to varying extents, inherent to the intervention. Patients consumed set calorie-restricted meals as part of the VLCD, and strict post-
procedure dietary changes in the RYGB group are required as part of post-operative recovery. Changes to food preference with bariatric surgery, and indeed with GLP-1 treatment, are well recognised (63). As a further limitation, whilst our study benefits from analysis of both circulating (plasma) and excretory (urine) metabolome, a more comprehensive description of the intervention-specific changes could be provided by including additional analyses such as of the faecal metabolome and microbiome. Indeed, the use of GLP-1R agonists has been associated with significant changes to the intestinal microbiome (64) and, in reverse, the microbiome may modulate responses to GLP-1R agonist treatment (65). Finally, the descriptive nature of this study is inherently hypothesis-generating rather than mechanistic; further experimental work will be required to unveil the role of metabolomic changes described herein.

In conclusion, our study provides a comprehensive profiling of the metabolomic phenotypes of three different interventions for weight loss and T2D. We have shown that the effects of GOP on the metabolome are far more minor than for VLCD and RYGB. Overall, tripeptide GOP treatment possesses more powerful effects on glucose than VLCD and RYGB, but does not replicate the more profound effects of VLCD and RYGB on the metabolome, the duration and mechanistic importance of which are still to be fully elucidated.

5 Data availability

All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.
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7 Main figure legends

Figure 1. CONSORT diagram and summary of effects of each intervention on clinical parameters. (A) CONSORT flow chart. Note that one patient in the original GOP group was randomised after the metabolomic analysis for other participants was completed and is not included in the current manuscript. (B) Changes in clinical parameters from baseline for each individual.

Figure 2. Overview of changes to untargeted plasma and urinary metabolome after each intervention. (A) Heatmap summary of profiling data from plasma and urine, represented as mean log₂ fold change, with analytical mode indicated. NMR 1D refers to NMR NOESY 1D. (B) Summary of pairwise comparisons between interventions by LME model, indicating the percentage of features in each dataset that were statistically significantly different, with control of FDR at 5% applied. (C) Correlations (Pearson’s r) between each intervention pair of mean log₂ fold changes for each analytical dataset.

Figure 3. Intervention-specific changes to energetic metabolites and the plasma lipidome from pre-annotated datasets. (A) Percentage of species within major metabolite groups that were significantly increased or decreased by each active treatment versus SAL. (B) Correlations (Pearson’s r) between mean log₂ fold change for individual lipid species after VLCD or RYGB treatment; all correlations were p<0.05. (C) Metabolic links between lipid subclasses and energy metabolism. (D) Intervention-specific mean log₂ fold changes ± 95% confidence intervals for selected energy metabolism-associated metabolites, with statistical comparisons versus SAL by LME model (* q<0.05). (E) Intervention-specific mean log₂ fold changes ± 95% confidence intervals for selected triacylglycerols (the minimum number of saturations available for each chain length was chosen for display). (F) Association between mean log₂ fold changes for fatty acids and acylcarnitine species with matched acyl chains. Pearson’s correlation r across the full cohort is shown. (G) As for (F) but for fatty acids versus phosphatidylcholine or lysophospholipid species. PC = phosphatidylcholine; LPC = lysophosphatidylcholine; LPA = lysophosphatidic acid; LPE = lysophosphatidylethanolamine; LPI = lysophosphatidylinositol. For phosphatidylcholines, the sn-2 acyl chain is matched to the fatty acid, with species with different sn-1 acyl chains differentiated on the graph. Each “match” is represented by 4 datapoints (one per intervention). Pearson’s correlation r for each combined dataset is shown. (H) As for (G) but showing ceramides (Cer) and sphingomyelins (SM). The sn-2 acyl chain is matched to the fatty acid, with species with different sn-1 acyl chains differentiated on the graph. (I) Correlations between weight loss or change in fasting glucose and different metabolites. Pearson’s correlation r is shown only where p<0.05, either for the whole cohort (black) or individual groups (with corresponding colour). See Supplementary Figures 3-6 (30) for additional correlation analysis and PLS modelling.
Figure 4. Effects on amino acid metabolism. (A) Intervention-specific mean log₂ fold changes ± 95% confidence intervals for plasma amino acids, with statistical comparisons between groups by LME model (* q<0.05). (B) Correlations between weight loss or change in fasting glucose and selected amino acids. Pearson’s correlation r is shown only where p<0.05, either for the whole cohort (black) or individual groups (with corresponding colour). See Supplementary Figures 3-4 (30) for additional correlation analysis and PLS modelling.

Figure 5. Intervention-specific changes to non-lipid small molecule plasma metabolites from pre-annotated datasets. (A) Scatter plots indicating the relationship between VLCD and RYGB effects (mean log₂ fold change versus baseline) on plasma and urine metabolites; Pearson’s correlations between VLCD and RYGB effects are reported for non-significant plus jointly affected metabolites (“not unique”) versus for metabolites “uniquely” affected by one intervention only, as identified by the LME model. 95% confidence intervals are shown for significantly affected analytes. (B) Associations between weight loss or change in fasting glucose and selected plasma (P) or urine (U) metabolites identified as VLCD/RYGB discordant in (A). Pearson’s correlation r is shown only where p<0.05, either for the whole cohort (black) or individual groups (with corresponding colour).

Figure 6. Intervention-specific changes to plasma lipoproteins. (A) Intervention-specific mean log₂ fold changes ± 95% confidence intervals for total cholesterol (Chol), low-density lipoprotein-associated cholesterol (LDL-chol), high-density lipoprotein-associated cholesterol (HDL-chol), and triglycerides, as determined by NMR. Statistical significance determined by LME model, with control of FDR at 5% applied. (B) As for (A) but showing apolipoprotein A1 (ApoA1), apolipoprotein A2 (ApoA2), and apolipoprotein B100 (ApoB100). (C) Heatmap representation of mean log₂ fold changes for apoB-containing subfraction parameters, arranged in order of decreasing size / increasing density, including particle number (PN) and content; the latter includes triglycerides (TG), cholesterol (CH), free cholesterol (FC) and phospholipids (PL). Very low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL) fractions, as well as LDL, are shown. Also shown are same data for LDL particle number and VLDL triglycerides, but including 95% confidence intervals and statistical significance as determined by LME model, with control of FDR at 5% applied. *p<0.05.
Figure 1

A

Infusions
Screened 80

Randomised
35

Saline 14

Died after screening 7
Discontinued 1
Completed follow up 11

Completed follow up 12

Discontinued 6

Discontinued 5

Not randomised

Completed follow up 13

Diabetes screening 5
Coronary artery disease 5
Family history of diabetes 2

Diabetes screening 6

Completed follow up 14

Died after screening 15

Not randomised

Died after screening 16

Completed follow up 15

B

Weight

Glucose

Insulin

Total cholesterol

Triglycerides

Δ vs baseline (%)

Δ vs baseline (mmol/L)

Δ vs baseline (mmol/L)

Δ vs baseline (mmol/L)

Δ vs baseline (mmol/L)
Figure 2

A

Plasma

log2 fc

SAL
GOP
VLCD
RYGB

Urine

log2 fc

SAL
GOP
VLCD
RYGB

B

LME model: % significantly altered variables between interventions

% LME <0.05

0 25 50

0.0 0.1 0.5 1.0 2.5 5.0 10.0 20.0 50.0

SAL

0.0

0.1

0.5

1.0

2.5

5.0

10.0

20.0

50.0

GOP

0.0

0.1

0.5

1.0

2.5

5.0

10.0

20.0

50.0

VLCD

0.0

0.1

0.5

1.0

2.5

5.0

10.0

20.0

50.0

RYGB

0.0

0.1

0.5

1.0

2.5

5.0

10.0

20.0

50.0

C

Correlation of changes between interventions

SAL

0.0 0.00 0.01 0.10 0.17 0.12 0.11 0.20 0.29

0.0 0.01 0.10 0.17 0.12 0.11 0.20 0.29

0.0 0.01 0.10 0.17 0.12 0.11 0.20 0.29

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0.0 0.01 0.10 0.17 0.12 0.11 0.20 0.29
Figure 4

A

Log₂ fold change

Amino acids

Glucogenic

Glucogenic and ketogenic

Ketogenic

B

Alanine

Glutamine

Glycine

Isoleucine

Tyrosine

Log₂ fold change

Weight

Weight or glucose log₂ fold change

r = 0.48

r = -0.39

r = 0.57

r = 0.57

r = 0.71
Figure 5

A. VLCD vs. RYGB: Identification of shared/uniquely modified metabolites vs. SAL

- LME p>0.05 or ▲ not unique, r = 0.79 *
- VLCD or ▲ RYGB unique, r = 0.62 *
- LME p>0.05 or ▲ not unique, r = 0.67 *
- VLCD or ▲ RYGB unique, r = 0.57 (ns)

B. (P) TMAO, (P) Caffeine, (P) Paryxanthine, (U) Indoxyl glucuronide

- ▲ SAL
- ■ GOP
- □ VLCD
- ▲ RYGB

- Weight or glucose log2 fold change
- Metabolite log2 fold change

- r = -0.67
- r = -0.59
Figure 6

A

B

C

D

Log₂ fold change

SAL

GOP

VLCD

RYGB

Log₂ fold change

PN

TG

CH

FC

PL

Increasing density / decreasing size

Log₂ fold change

VLDL-triglycerides

LDL particle number