# PHARMACOLOGICAL AND NON-PHARMACOLOGICAL THERAPY FOR OBESITY AND DIABETES

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# PHARMACOLOGICAL AND NON-PHARMACOLOGICAL THERAPY FOR OBESITY AND DIABETES

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# Analysis of the Adherence and Safety of Second Oral Glucose-Lowering Therapy in Routine Practice From the Mediterranean Area: A Retrospective Cohort Study

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The aims of our study was compare adherence measured by the medical possession ratio (MPR), time until discontinuation and describe adverse events after adding a DPP-4i, SGLT-2i, or sulfonylureas (SU) to metformin in a primary care population with insufficient glycemic control. We used routinely-collected health data from the SIDIAP database. The included subjects were matched by propensity score. The follow-up period was up to 24 months or premature discontinuation. The primary outcomes were the percentage of subjects with good adherence, treatment discontinuation and adverse events among treatment groups. The proportion of patients with good adherence (MPR> 0.8) after the addition of DPP-4i, SGLT-2i or SU was 53.6%, 68.7%, and 43.0%, respectively. SGLT-2i users were 1.7 times more likely to achieve good adherence compared with DPP-4i users (odds ratio [OR]:1.72, 98% confidence interval [CI]:1.51, 1.96), and 2.8 times more likely compared with SU users (OR: 0.35, 98% CI: 0.07, 0.29). The discontinuation hazard ratios were 1.43 (98%CI: 1.26; 1.62) and 1.60 (98%CI: 1.42; 1.81) times higher among SGLT-2i and SU users than DPP-4i users during the follow-up period. No differences were observed for adverse events among the treatment groups. In conclusion, in our real-world setting, the combination of SGLT-2i with metformin was associated with better adherence. The mean time until discontinuation was longer in the SGLT-2i group in comparison with the DPP-4i or SU groups.

**Keywords: adherence - compliance – persistence, glycemia control, type 2 diabetes, primary care, observational study**

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## INTRODUCTION

Good quality management of type 2 diabetes mellitus (T2DM) involves a combination of changes in lifestyle and pharmacological interventions to achieve target glycated hemoglobin (HbA1c) and, thus, reduced risk of macrovascular and microvascular complications (1). However, over time, insulin secretory capacity declines, and most people with T2DM will require escalation of pharmacotherapy to achieve good metabolic control (2). This is common in real clinical practice where first-line treatment with metformin will, in time, require intensification with a second antidiabetic drug to achieve good glycemic control (3–5). According to the current therapeutic guidelines, the selection of a second antidiabetic drug should be based on patient-specific treatment goals, presence of comorbidities, and drug characteristics (6–10). Unfortunately, intensification with additional antidiabetic drugs is often delayed, leaving patients with prolonged periods of poor glycemic control with worse long term outcomes (11). We recently reported a lack of treatment intensification in 1 in 5 patients with HbA1c values >8% (12) and only 20% of the persons with T2DM were treated with dual antidiabetic therapy (13).

There are a number of complex barriers to the proper implementation of antidiabetic treatment both on the healthcare professionals' and patients' side. Adherence to pharmacological treatment plays an important role in achieving treatment goals (2). Moreover, data from a meta-analysis suggests that good adherence to antidiabetic treatment was associated with a lower hospitalization rate and all-cause mortality among the persons with T2DM (14). Treatment adherence and persistence are similar, yet distinct, measurements of the degree to which a patient continues treatment after initiation (15). Adherence is defined as "the extent to which a patient acts following the prescribed interval and dosing regimen" (16). Treatment persistence is defined as the length of time from initiation until discontinuation of therapy (16), measured by the drug's availability, expressed as the continuous filling of prescriptions (17).

Evidence suggests that adherence to medication in T2DM is less than optimal, and many patient factors could influence it, such as comprehension of the treatment regimen and its benefits, emotional well-being, regimen complexity, medication cost and adverse events (18). RWE (real-world evidence) studies have shown that non-adherence to oral antidiabetic drugs is frequent: over 50% in the first year and even higher at the two-year follow-up (19). Low adherence may explain, at least in part, the efficacy gap in the reduction of HbA1c between RWE studies and randomized clinical trials (RCT) (20).

In RWE studies, where prescription or pharmacy claims data are available, adherence is usually measured through the medication possession ratio (MPR), where a value of 0.80 (80%) is the cut-off point that stratifies adherent and non-adherent patients (21). Results from a recently published meta-analysis confirm the high variability in adherence (38.5 to 93.1%) among different observational studies (2). In another meta-analysis, the proportion of adherent patients was found to be suboptimal (67.9%), while the persistence to initial oral antihyperglycemic agents ranged from 41.0% to 81.1% (22). Adverse events can directly influence adherence and persistence to antidiabetic treatment. Hypoglycemias associated with sulphonylureas (SU) and genital tract infections associated with sodium-glucose Cotransporter 2 Inhibitors (SGLT-2i) combined with metformin were the most frequently reported adverse events in recently published meta-analyses (23–25).

We previously published efficacy results regarding the addition of dipeptidyl peptidase-4 inhibitor (DPP-4i), SGLT-2i, or SU as second-line therapies to metformin, showing that users initiating SGLT-2i in combination with metformin achieved greater reduction in weight and combined target HbA1c (≥0.5%) and weight (≥3%) reduction among the cohorts (26). In the present study, we assessed adherence using the MPR and time till discontinuation of DPP-4i, SGLT-2i, or SU added to metformin in subjects with T2DM with insufficient glycemic control in a primary care setting. Additionally, we described the adverse events associated with these drug combinations.

## MATERIAL AND METHODS

### Study Design and Data Source

This was a retrospective cohort study to compare subjects initiating add-on treatment with DPP-4i, SGLT-2i, or SU to metformin. Exposure to these drugs was defined if the user had more than one drug dispensation/prescription register for the first time between January 1st, 2010, and December 31st, 2017. Subjects were followed up for a period of 24 months or until premature discontinuation.

Data were obtained from the primary care SIDIAP database (The Information System for the development of Primary Care Research) (27). This database contains anonymized data from electronic medical records of the people attended in the 279 Primary Care Teams that belong to the Catalan Health Institute, Catalonia, Spain. The Institute's assigned population is about 5,835,000 individuals (75% of the total Catalan population). Furthermore, the SIDIAP database incorporates laboratory data, prescriptions, and data on drug dispensations extracted from pharmacy-billing records provided by the Catalan Health Service (CatSalut). The SIDIAP database has been extensively used for other epidemiologic and pharmacoepidemiologic national and international research studies, and it is established as a well-validated primary care Spanish database for the study of diabetes (28, 29).

### Inclusion and Exclusion Criteria

Patients were included if they were 18 years or older, diagnosed with T2DM (ICD-10: E11), and had poor glycemic control (HbA1c ≥7%). We defined the inclusion date when the second add-on treatment (DPP-4i, SGLT-2i, or SU) was introduced to metformin for the first time. For each treatment group, we identified drug exposure (index medication) using ATC codes (Anatomical Therapeutic Chemical classification system) from the World Health Organization (WHO) (30), the date of

prescription and dispensation. Patients registered with other types of diabetes such as diabetes mellitus type 1, gestational or secondary (ICD-10: E8, E9, E10, O24, E13), and those subjects with missing baseline values for HbA1c and weight were excluded. Subjects could enter the study groups only once.

## Study Variables

At inclusion, we collected routine information on the social-demographic characteristics of subjects (age, gender and toxic habits) and clinical characteristics such as laboratory and clinical parameters related to diabetes control and comorbidities. We collected information about drug prescriptions in each treatment group, dispensations, and both adverse events and discontinuation events during the follow-up period.

### Outcomes

Adherence was estimated using the medication possession ratio (MPR), calculated as the number of days covered by dispensation divided by the number of days covered by prescription, which is defined as days between the date of initiation of index medication and discontinuation event or up to 24 months. MPR is a validated and standard method to evaluate adherence in studies with routinely-collected health data; good adherence was defined as an MPR value >0.8 (>80%), whereas poor adherence was defined as an MPR value ≤0.8 (≤80%) (21).

Persistence was defined as the time between index treatment initiation and the first discontinuation event. For this study, we considered treatment discontinuation events if there was any gap of at least 90 days (15) without index medication dispensation, any changes in antidiabetic treatment, death, or moving to another healthcare provider. We calculated the proportion of subjects who discontinued treatment for each treatment group at 6, 12, and up to 24 months of follow-up period.

Adverse events were classified into eight categories based on the affected system organ class (SOC) (metabolic, gastrointestinal, hepatic, renal, musculoskeletal, dermatological, hematological, and genitourinary events); these SOCs were chosen as they are the most frequently reported adverse reactions in the summary of product characteristics for each drug group. We described the mortality events (any cause) for the three groups during the follow-up period.

## Statistical Methods

### Propensity Score Matching

The matching criteria were the same as for the previously published effectiveness analysis related to changes in glycated hemoglobin (HbA1c) and the effect on body weight following the addition of DPP-4i, SGLT-2i, or SU as second-line therapies to metformin (26). The three treatments groups were matched for the following baseline characteristics: weight, HbA1c, sex, age, diabetes duration, year of inclusion, and kidney function. Matching was done by the "Nearest Neighbor algorithm" (caliper=0.01), using the "MatchIt" library of the R (v3.6.1) statistical package (31).

### Main Analysis

The MPR and persistence were described by mean, standard deviation, median and interquartile range, while good and poor adherence and adverse events were reported by frequency and percentage. We used linear regression models to analyze the differences in MPR as an interval variable among the three treatment groups. The associations between good/poor adherence among the treatment groups were analyzed by logistic regression models, summarized as odds ratios (OR), with 98% confidence intervals (CI). All pairwise comparisons (2X2) was conducted between the three groups, where the family significance level (alpha=0.05) was corrected for multiple paired groups (Bonferroni correction), so the individual test was prefixed at 0.017, and the confidence level at 98%. To analyze the time to a discontinuation event, we used Cox proportional hazards analysis, and hazard ratio (HR), CI, and p-value were summarized. We used Kaplan-Meier curves to graphicly visualize treatment persistence up to 24 months of the observation period in each treatment group. As a sensitivity analysis, adjusted estimates were calculated with multivariable models. The variables used for adjustment were age, sex, number of comorbidities, weight, HbA1c, year of inclusion, duration of diabetes, and glomerular filtration rate. The statistical analyses were performed using R3.6.1 software (https://www.r-project.org/).

## Ethical Review

The study was approved by the Ethics Committee of the Primary Health Care University Research Institute (IDIAP) Jordi Gol, Barcelona (approval code: P17/205).

# RESULTS

## Patient Characteristics

A total of 75,808 poorly controlled T2DM subjects initiating a second antidiabetic drug in addition to metformin were included: 27,878 (36.7%) initiated a DPP-4i, 2,198 (2.89%) a SGLT-2i and 45,732 (60.3%) an SU. The study flow chart is shown in **Supplementary Figure 1**. After matching, 6,310 subjects were compared: 2,124 for DPP-4i, 2,124 for SGLT-2i and 2,062 for SU (**Supplementary Figure 2**). The baseline characteristics of subjects in each study group are shown in **Supplementary Table 1**. Overall, the mean age was 60.8 years ( ± 11.7), with a mean diabetes duration of 7.61 years ( ± 6.59), and an HbA1c of 8.8% ( ± 1.45) (72.3 mmol/mol ( ± 15.9). Subjects in the DPP-4i group were older with a mean age of 61.2 ( ± 12.1), while those in the SGLT-2i treatment group had a longer diabetes duration of 7.89 ( ± 6.67) and had a higher BMI 33.9 ( ± 5.80) compared to the other groups. SGLT-2i users also had slightly higher triglycerides and a worse comorbidity profile, especially for cardiovascular complications. The baseline characteristics and analysis of effectiveness among the three treatment groups have been recently published (26).

## Adherence to Treatment

**Table 1** summarises the data related to adherence and drug dispensations. Comparison between study groups showed that good adherence (MPR>0.8) was achieved for most of the SGLT-2i and DPP-4i treated subjects (68.7% and 53.6%, respectively), while the majority of SU users had poor adherence (43.0%).

**Supplementary Table 2** shows adherence for different drug within the drug groups. Alogliptin in combination with metformin had the highest mean MPR in the DPP-4i group (0.81 ± 0.28), canagliflozin in combination with metformin in the SGLT-2i group (0.82 ± 0.30), and glimepiride in combination with metformin in the SU group (0.91 ± 0.21). Multiple logistic regression analysis showed that SGLT-2i users were 1.7 and 2.8 times more likely to be associated with good adherence than DPP-4i users (adjusted OR: 1.72, 98% CI: 1.51, 1.96), or SU users (adjusted OR: 0.35, 98% CI: 0.07, 0.29), respectively.

The DPP-4i users were 1.6 times more likely to be associated with good adherence than SU users (OR: 0.59, 98% CI: 0.52, 0.67). A mean difference in MPR of 6% was observed between SGLT-2i users and DPP-4i users (adjusted DR: 0.06, 98% CI: 0.04, 0.08) and 14% compared with SU users (adjusted DR: -0.14, 98% CI: -0.16, -0.11); the difference was 8% between DPP-4i and SU users (adjusted DR: -0.08, 98% CI: -0.10, -0.06). Comparing the difference in number of packages dispensed between groups, we only observed statistical differences between SU and DPP-4i users (1.02 fewer packages in the former group; adjusted DR: -1.02, 98% CI: -1.59, -0.46). The odds ratios for good adherence, MPR differences and the number of dispensed packages among the treatment groups are shown in **Table 2**.

## Treatment Persistence

**Table 1** summarizes the results of discontinuation and persistence in the 3 study groups. The mean time until discontinuation was longer in the SGLT-2i group in comparison with the DPP-4i or SU groups: 385 ( ± 289), 372 ( ± 330) and 343 ( ± 306) days, respectively. During the initial six month period, 21.3% of SU users discontinued treatment, compared with 18.6% of SGLT-2i users and only 12.9% of DPP-4i users. At the end of the 24-month follow-up period, 43.0% of SU users, 39.7% of SGLT-2i users, and 28.8% of DPP-4i users had ceased treatment.

The Kaplan-Meier curves of persistence are shown in **Figure 1** and summarized in **Supplementary Table 3**. We performed a Cox proportional hazards analysis to compare the hazard risk ratios for discontinuation events. The risk of discontinuation was 1.4 times higher for SGLT-2i (HR: 1.43, 98% CI: 1.26, 1.62) and 1.6 times higher for SU (HR: 1.60, 98% CI: 1.42, 1.81) compared to DPP-4i. Furthermore, the risk of

**TABLE 1** | Medical possession ratio, adherence, persistence (time until discontinuation and discontinuations) among the three treatment groups.

| | MET+ DPP-4i (n = 2113) | MET+SGLT-2i (n = 2117) | MET+ SU (n = 2056) |
|---|---|---|---|
| **Medical possession ratio (MPR)** | | | |
| **Medication possession ratio, Mean (SD)** | 0.71 (0.34)** | 0.78 (0.34)** | 0.63 (0.35)** |
| **Medication possession ratio, Median [IQR: 25th;75th]** | 0.86 [0.43;1.00]** | 1.00 [0.62;1.00]** | 0.64[0.33;1.00]** |
| **Number medicine packages dispensed, Mean (SD)** | 10.6 (8.62)* | 10.7 (8.52)* | 9.88 (12.9)* |
| **Poor adherence (≤ 0.8)** | 981 (46.4%)** | 662 (31.3%)** | 1172 (57.0%)** |
| **Good adherence (>0.8)** | 1132 (53.6%)** | 1455 (68.7%)** | 884 (43.0%)** |
| **Persistence** | | | |
| **Persistence time on treatment, Mean (SD)** | 372 (330)** | 385 (289)** | 343 (306)** |
| **Persistence time on treatment, Median, [IQR: 25th;75th]** | 274 [121;548]** | 333 [150;600]** | 272 [91.2;486]** |
| **Discontinuation events** | | | |
| **Discontinuation of treatment 6 m: % (98% CI Linf, Lsup)** | 12.9 (11.4, 14.5) | 18.6 (16.8, 20.4) | 21.3 (19.4, 23.2) |
| **Discontinuation of treatment 12 m: % (98% CI Linf, Lsup)** | 20.1(18.0, 22.0) | 28.6 (26.4, 30.7) | 32.1 (29.7, 34.3) |
| **Discontinuation of treatment 24 m: % (98% CI Linf, Lsup)** | 28.8 (26.0, 31.4) | 39.7(36.8, 42.5) | 43.0 (39.8, 48.9) |

*CI, confidence interval; DPP-4i, dipeptidyl peptidase-4 inhibitors; IQR, inter-quartile range; Linf, inferior limit; Lsup, superior limit; m, months; MET, metformin; SD, standard deviation; SGLT-2i, sodium/glucose cotransporter 2 inhibitors; SU, sulphonylureas; *p-value =0.018; **p-value <0.001.*

**TABLE 2** | Odds ratios for good adherence, MPR differences and number of dispensed packages among the cohorts.

| | Good adherence (MPR>0.8) | | MPR differences | | Number of dispensed packages differences | |
|---|---|---|---|---|---|---|
| | Unadjusted OR (98% CI) | Adjusted OR (98% CI) | Unadjusted DR (98% CI) | Adjusted DR (98% CI) | Unadjusted DR (98% CI) | Adjusted DR (98% CI) |
| **SGLT-2i+MET, ref: DPP-4i+MET** | 1.90 (1.67, 2.16)* | 1.72 (1.51, 1.96)* | 0.08 (0.06, 0.1)* | 0.06 (0.04, 0.08)* | 0.13 (-0.49, 0.74) | -0.63 (-1.19,-0.06) |
| **SU+ MET, ref: DPP-4i+MET** | 0.65 (0.57,0.73)* | 0.59 (0.52, 0.67)* | -0.07 (-0.09,-0.05)* | -0.08 (-0.10, -0.06)* | -0.71 (-1.33,-0.08) | -1.02 (-1.59,-0.46)* |
| **SU +MET, ref: SGLT-2i+MET** | 0.34 (0.29, 0.40)* | 0.35 (0.07, 0.29)* | -0.15(-0.17, -0.13)* | -0.14 (-0.16, -0.11)* | -0.83 (-1.6,-0.08)* | -0.34 (-1.09,0.29) |

**Statistically significant p-value (p-value <0.017).*

*CI, confidence interval; IDPP-4i, dipeptidyl peptidase-4 inhibitors; OR, odds ratio; DR: differences; SGLT-2i, sodium/glucose cotransporter 2 inhibitors; SU, sulphonylureas; MET, metformin.*

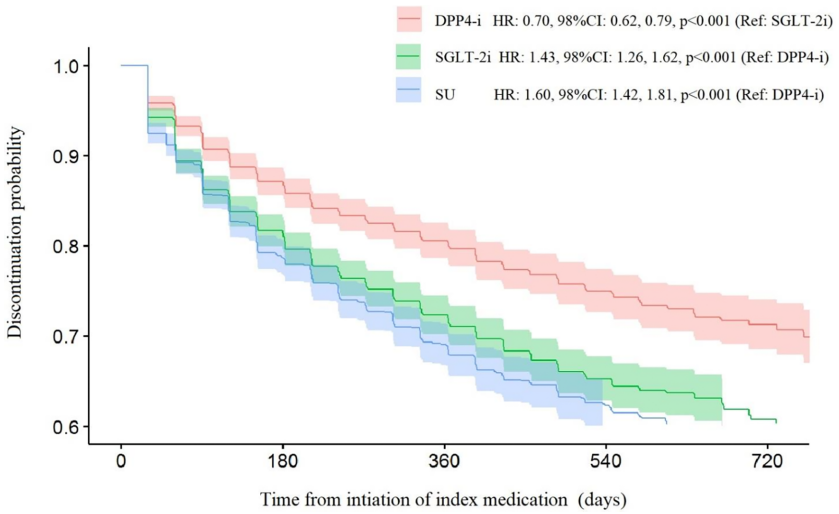


**FIGURE 1** | Kaplan-Meier discontinuation probability curves for the three treatment groups.

discontinuation among SU users was 1.1 times higher than that of SGLT-2i users (HR: 1.12, 98% CI: 1.00, 1.26).

## Adverse Events

The results for adverse events are reported in **Table 3**. We observed that gastrointestinal, musculoskeletal, dermatological, and urogenital were the most frequent adverse events during the follow-up period in all three groups. In the SGLT-2i group, urogenital, metabolic and dermatological adverse reactions were more frequent than in the other treatment groups (10.5%, 0.19% and 3.01%, respectively) but without statistically significant differences between groups. There were no significant differences in the frequency of gastrointestinal adverse events between the groups.

# DISCUSSION

In the current study, among 6,310 propensity score-matched users who initiated a second line add-on therapy to metformin with DPP-4i, SGLT-2i or SU in Catalonia, the highest adherence and persistence was observed in SGLT-2i users.

Comparing the adherence among the study groups, 68.7% of users in the SGLT-2i treatment group had good adherence (MPR>0.8), while this percentage was lower in both DPP-4i and SU users (53.6% and 43%, respectively). In an observational study with 11,961 subjects in the US, the percentage of good adherence (MPR≥0.8) for subjects initiating an SGLT-2i was 56.2-58.8% for canagliflozin, 36.4-36.7% for dapagliflozin and 45.7% for sitagliptin after 12 months (15). Our study showed a similar tendency, although with a higher level of good adherence: canagliflozin 62.5-73.7%, dapagliflozin 71.2-71.7%, and sitagliptin 49.9-51.8%. In an observational study with 171,220 T2DM subjects from Sweden during 2005 and 2006, the refill adherence for dual therapy with SUs (glibenclamide, glipizide, glimepiride) was high (91.3%, 91.0%, and 91.7%, respectively) (32), however the proportion of subjects with good adherence in our study was lower for the same drugs (50%, 85.3% and 44.4%, respectively).

Our results show that SGLT-2i users were more likely to have good adherence to treatment than DPP-4i and SU users (1.7 and 2.8 times higher, respectively). Results from an administrative-claims study in the US reported that patients who initiated an

**TABLE 3** | Adverse events among the treatment groups.

| Adverse event, n (%) | MET+ DPP-4i (n = 2113) | MET+SGLT-2i (n = 2117) | MET+ SU (n = 2056) |
|---|---|---|---|
| **Metabolic adverse event** | 0 (0.00%) | 4 (0.19%) | 0 (0.00%) |
| **Gastrointestinal adverse events** | 156 (7.34%) | 149 (7.02%) | 145 (7.03%) |
| **Hepatic adverse events** | 26 (1.22%) | 20 (0.94%) | 23 (1.12%) |
| **Kidney adverse events** | 25 (1.18%) | 28 (1.32%) | 12 (0.58%) |
| **Musculoskeletal system adverse events** | 57 (2.68%) | 57 (2.68%) | 48 (2.37%) |
| **Dermatological adverse events** | 54 (2.54%) | 64 (3.01%) | 49 (2.38%) |
| **Hematological adverse events** | 1 (0.05%) | 2 (0.09%) | 0 (0.00%) |
| **Urogenital adverse events** | 161 (7.58%) | 223 (10.5%) | 160 (7.76%) |
| **Death by any cause** | 33 (1.55%) | 29 (1.37%) | 39 (1.89%) |

**No statistically significant difference were observed among the groups; DPP-4i, dipeptidyl peptidase-4 inhibitors; SGLT-2i, sodium/glucose cotransporter 2 inhibitors; SU, sulphonylureas; MET, metformin.*

SGLT-2 inhibitor were 1.36 times more likely to be adherent to their medication and 1.35 times less likely to discontinue their medication than patients who initiated an SU (33). In another RWE study from the US, comparing the DPP-4i sitagliptin with SUs as an add-on therapy to metformin, subjects in the SU group had lower adherence and persistence (34). Similar findings were observed in a retrospective RWE study with 238,372 subjects, where DPP-4i users had a significantly greater OR of being adherent than users initiating an SU; the authors pointed to a better tolerability profile of DPP-4i as an explanation of their findings (19).

About 79.9% of DPP-4i users, 71.4% of SGLT-2i users, and 67.9% of SU users persisted with their initial therapy during the first year of treatment in our study. In an RWE study by Farr et al. (19), the authors reported that over 40% of SU initiators stopped refiling in the first year. In another RWE study from Hungary, the persistence rate after the first 12 months was 69.6% for DPP-4i users and 67.8% for SGLT-2i users (35). A meta-analysis of previous studies of treatment persistence to oral antidiabetic drugs reported persistence rates ranging from 33 to 61%, with an overall mean percentage of persistence of 49.2% (95% CI: 40.1%– 58.3%) among the studies that investigated only persistence to the index medication (22). We found that the risk of treatment discontinuation among SGLT-2i and SU users compared with DPP-4i users during the follow-up period was 43% and 60% higher, respectively. These results are in line with other RWE studies, where the risk of discontinuation was 40% higher among SU initiators (adjusted HR: 1.390, 95% CI: 1.363, 1.418) (19) and 6% higher among SGLT-2i users (HR: 1.066, 95% CI: 1.036–1.096) (35) compared with DPP-4i users. High discontinuation rates and poor adherence are important factors that may induce possible issues with the initially prescribed treatment. In the current study, among the users who initiated SU in combination with metformin, despite the relatively large gap period (90 days) without dispensation, two of ten subjects stopped the initial treatment during the initial six months. On the other hand, in the SGLT-2i group, the percentage of users with good adherence was higher, but discontinuation rates were higher than in the DPP-4i users. A possible explanation for this could be an improved tolerability in the DPP-4i group (36).

With regards to safety, the most frequently reported adverse events were gastrointestinal and urogenital disturbances. We found higher percentages of urogenital, metabolic and dermatological adverse events in the SGLT-2i group and more frequent gastrointestinal events in the DPP-4i group but without significant differences. Indeed, it is well reported that SGLT-2i drugs are often associated with a higher incidence of urogenital infections (mycotic genital infections such as vaginitis in women and balanitis in man) (37–39). However, despite the occurrence of these adverse effects, these episodes are often regarded as mild by patients (40); additionally, their incidence tends to decline over time without the need for halting SGLT-2i therapy (41).

Studies have previously shown that achieving better adherence is associated with improved glycemic control (17), but many factors could influence adherence and persistence to treatment. The patient is the primary driver of treatment adherence and may be influenced by both efficacy and tolerability; a subject who experiences undesired side effects of medication is less likely to take the prescribed drug (15). One UK study reported that gastrointestinal side effects, hypoglycemia, weight change, and efficacy were the most important factors determining patient preferences for oral antidiabetic drugs (42).

In our study, efficacy in combination with weight reduction could be the reason for good adherence and persistence among the SGLT-2i and DPP-4i users. Our previous study showed that the addition of SGLT-2i or DPP-4i to metformin was associated with a greater weight reduction (3.47 kg and 1.21 kg, respectively) (26). Additionally, the proportion of subjects who achieved a combined target of HbA1c (≥0.5%) and weight (≥3%) reduction was greater in these two treatment groups (26). Previous studies have shown that the presence of certain conditions, such as depression and mental disorders, before the first antidiabetic drug prescription is associated with non-persistence to antidiabetic treatment (43). In our study, the lowest persistence and adherence were observed among SU users; however, at baseline, these users had fewer mental disorder comorbidities than the users included in the other treatment groups.

There are some limitations to our study. As per the study design, we only included subjects with complete data for baseline HbA1c and weight; the study population is a highly selected sample which potentially diminishes the external validity. Moreover, we cannot rule out that patients having both variables at baseline were treated more proactively to favor a better T2DM control; however, due to the matching process, we would expect this limitation to be the same for the three study groups. Another limitation is the relatively small sample size to observe the number of adverse events, mainly due to the propensity score matching, which drastically reduces the population size and, thus, the total number of adverse events. However, our goal was to describe the number of events among the treatment groups as opposed to analyzing statistically significant differences. Finally, the retrospective nature of the study precludes explaining the reason for treatment discontinuation, so we are not able to discern whether the differences in adherence could be due to the drug itself, to the risk of adverse events, to the number of pills per day or to the use of available fixed-dose combinations. Strengths of our study include a population-based cohort, long follow-up of two years, propensity matching and outcomes for adherence, persistence and adverse events.

In conclusion, the results of the present study show better drug adherence and longer persistence among subjects on SGLT-2i as an add-on to metformin compared with DPP-4i or SU users. Subjects being treated with DPP-4i combined with metformin had the fewest discontinuation events during the follow-up period. These results may help clinicians better understand the treatment trajectory following the addition of DPP-4i, SGLT-2i, or SU to metformin. However, further studies in real-world conditions are needed to identify factors related to good adherence, persistence and safety amongst these three commonly prescribed drug combinations.

## DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The data controller for SIDIAP does not allow the sharing of raw data. The source code is available at https://github.com/jrealgatius/METPLUS. Requests to access these datasets should be directed to JF-N, dap.cat.info@gmail.com.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Primary Health Care University Research Institute (IDIAP) Jordi Gol, Barcelona (approval code: P17/205). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

## AUTHOR CONTRIBUTIONS

JF-N, MM-C, JR, DM, XM-T, JAV-C, and BV conceived the research and participated in its design. JR performed the statistical analysis. BV wrote the initial draft of the manuscript, which JF-N, MM-C, JR, DM, XM-T, JAV-C, XC MF and KK edited. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.708372/full#supplementary-material

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OPINION
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# A Combination of Glucagon-Like Peptide-1 Receptor Agonist and Dietary Intervention Could Be a Promising Approach for Obesity Treatment

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**Keywords: combination therapy, diet, glucagon-like peptide-1 agonist, gut-brain axis, obesity**

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## INTRODUCTION

Obesity continues to be a global health issue. According to the World Health Organization (WHO), its prevalence has increased almost 3-fold between 1975 and 2016; thirteen percent of the adult population worldwide was obese in 2016 (1). The real concern behind the increasing number of obese individuals is that obesity is a risk factor of many debilitating diseases (1). Obesity was also found to worsen the effects of COVID-19 (2), the pandemic that has cost millions of lives globally (3). Obesity increases the risk of hospitalization and death among COVID-19 patients (4). It is therefore never 'enough' to discuss about existing strategies used in the management of obesity, more so when there are newer findings that could enlighten us on the underlying pathophysiology of obesity, thereby offering us new perspectives of the role of these approaches. This article focuses on glucagon-like peptide-1 (GLP1) - revisiting the physiological effects of GLP1, and proposing ways of optimizing the effects of GLP1 in the management of obesity.

## WHAT DO WE ALREADY KNEW ABOUT GLP1?

Glucagon-like peptide-1 (GLP1) is a peptide hormone produced by the L cells of the small intestine when the cells detected food. By binding to its receptor on the pancreatic β-cells, GLP1 stimulates insulin secretion in a glucose-dependent manner. GLP1 also exerts the following effects: suppresses pancreatic glucagon release as well as hepatic glucose production, promotes the proliferation of pancreatic β-cells, prevents apoptosis of β-cells, and delays gastric emptying (5). These physiological effects of GLP1 have led to the extensive development of GLP1-based anti-diabetic drugs. Interestingly, not only did GLP1 lower the hemoglobin A1C, subcutaneous GLP1 infusion reduces the body weight and appetite of diabetic patients (6). Zander and team were probably the first to report the presence of an inverse relationship between GLP1 and body weight and appetite, which has then sparked the interest of using GLP1 as an anti-obesity agent. Their findings were supported by studies conducted by other researchers subsequently. A review published a decade later (7) confirmed that GLP1 secretion is reduced in obese subjects, while weight loss normalizes the levels, and GLP1 replacement restores satiety.

Currently, Liraglutide is the only FDA-approved GLP1 receptor agonist for weight loss treatment in non-diabetic patients. Semaglutide was added to the list by FDA recently for chronic weight

management in obese or overweight adults with at least one weight-related condition such as hypertension, diabetes, or hypercholesterolemia (8). However, the most effective clinical procedure that has resulted in significant long-term weight loss is bariatric surgery (9). Obese patients who underwent bariatric surgery have elevated levels of GLP1 and diminished appetite (10). The increased GLP1 may be one of the main factors that has contributed to the weight loss effect of bariatric surgery (11), although the metabolic improvements post-bariatric surgery are more likely to be contributed by several mechanisms (12). But as with other surgeries, complications may develop, and not all obese patients are suitable to undergo bariatric surgery. Taken into consideration the cost and safety, therapeutic agent such as GLP1 receptor agonist may be preferred over bariatric surgery as a treatment option, especially in non-morbidly obese patients. The effects of GLP1 receptor agonist can be optimized through our understanding of the pathophysiology of obesity as well as the effect of obesity on GLP1 production.

## WHAT IS NEW AND NOT SO NEW ABOUT OBESITY PATHOPHYSIOLOGY AND ITS IMPACT ON GLP1?

A cohort study reported that individuals who are obese and overweight had a 20% reduction in the GLP1 response to oral glucose compared with subjects who have normal weight (13). While these results were recently debated by some researchers based upon their findings from animal studies (14), the causal-effect relationship remains possible, as discussed below. Variation in the effect of glucose response observed by different group of researchers can be attributed to the differences in the experimental environment, the animal strain used, and the experimental design. The physiological effect of GLP1 is not limited to insulin secretion, but a range of other effects, as mentioned above. One of the most important beneficial effects of GLP1 in obesity treatment is promoting satiety. This is because increased access to tasty, low-cost and energy-dense food is partially responsible for the increase in obesity prevalence (15, 16).

The appetite-suppressant effect of GLP1 is well known. Briefly, two sets of neuronal circuitry are present within the arcuate nucleus (ARC) of the hypothalamus. These neuronal circuits signal to the paraventricular nucleus and modulate feeding behavior. The neuronal circuit that expresses neuropeptide Y (NPY) and agouti-related peptide (AgRP) stimulates food intake, while the ARC neuron that expresses pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) reduces food intake. GLP1 suppresses the expression of NPY and AgRP, while increases the activity of POMC and CART (17). Besides GLP1 receptor agonists, another class of drug that has gained increasing interest for having an effect on appetite is the cannabinoid receptor type 1 (CB1R) antagonists. Peripherally-acting CB1R antagonists effectively reduced the body weight and appetite of diet-induced obesity (DIO) mice while improving the metabolic dysfunction of the animal (18). The insulinotropic effect of GLP1 was enhanced in CB1R-deficient mice (19). The combination of peripherally-acting CB1R antagonist and GLP1 agonist, Semaglutide was significantly more effective than CB1R antagonist alone or Semaglutide alone in lowering the body weight of DIO mice (20). The weight reduction effect of the CB1R antagonist was negated when GLP1 receptor was absent, while CB1R antagonist potentiated the weight loss effect of Semaglutide and improved Semaglutide's response to glucose. The combination of CB1R antagonist, Rimonabant and Semaglutide also reduced food intake to a greater extent than monotherapies but this effect is not dose-dependent (20). The amplified anorectic effect of GLP1 by CB1R antagonist may be due to the fact that both receptors are expressed on the vagal afferent neurons, enabling a cross talk between the two receptors (21).

The concept that gut plays a critical role in obesity has been widely described. Most of these studies involved rodent treated with high fat diet (HFD), as the DIO model presents metabolic status that is closest to the human condition. Two months of HFD feeding increases the sensitivity of the gut to luminal nutrients, but the increased luminal sensitivity may not have stimulated GLP1 secretion (22). The possibility of HFD induces GLP1 secretion was not confirmed because the same group of researchers did not see a significant increase in GLP1 level in the distal small intestine where GLP1 is abundantly produced (23). In contrast to these findings, a few studies involving chronic HFD feeding (2-4 months) in rodent reported a decrease in GLP1 secretion in response to oral glucose (24–26). Several studies on the other hand showed that HFD terminates the gut lipid sensing mechanisms (27). For example, HFD diminishes the effect of cholecystokinin-8 (28) and long chain fatty acyl-coenzyme A (29) in reducing the hepatic glucose production. HFD also suppresses peroxisome proliferator-activated receptor-α (30) and N-acylphosphatidylethanolamine secretion (31) resulting in increased hunger. Collectively, it seems that HFD may increase luminal sensitivity but this effect is likely to adversely affect the metabolic status of obese subjects. It is more likely that the HFD-induced GLP1 secretion observed by some researchers was short-term or intermittent because HFD impaired the secretory function of the L cells (32), and GLP1 inversely correlates with appetite and body weight gain.

Human studies reported the presence of inflammatory proteins in the systemic circulation of obese patients, which correlates positively with body weight (33, 34). The inflammation observed in obese human subjects was supported by animal studies, which indicated that inflammation occurred during the course of obesity development (35). The interaction between HFD and gut microbiota triggers the inflammatory cascade causing intestinal inflammation. This effect was not found in germ-free mice (35), which were also resistant to DIO (36). By acting through G protein-coupled receptor 41 (Gpr41), microbiota increase adiposity and leptin production. Conversely, Gpr41-deficient mice have a decrease in peptide YY secretion as well as hepatic lipogenesis (37). It is possible that the effect of microbiota on the Gpr expressed on the enteroendocrine cells, which contributes to adiposity is exaggerated by HFD. This is

because Gpr are fat sensors that mediate gastrointestinal peptides secretion. In another study, mice fed with 14 weeks of HFD had a significant increase in body weight and gastrointestinal methanogens compared with chow-fed mice. In conjunction with the increased abundance of fecal methanogens, there was elevated circulating GLP1 level (38). However, the elevated GLP1 level was observed at 10 minutes after oral glucose and not thereafter suggesting that the increase is transient. An *in vitro* study conducted subsequently by the same group of researchers, shows that the increased GLP1 in the cell culture media as induced by methane, was not dose-dependent. It is therefore not clear if the enhanced GLP1 secretion in DIO mice (38), which was thought to be contributed by methane is sustainable, and if this effect would lead to a reduction in body weight as well as appetite eventually. Prolonging the *in vivo* study beyond 14 weeks and measuring relevant parameters could confirm the methane effect on GLP1 secretion. In short, what is clearer at the moment is that HFD induces changes in the gut microbiome, and that causes inflammation and adiposity.

## HOW SHALL WE PROGRESS FROM HERE?

Research over the past two decades has established that GLP1 acts along the gut-brain axis, producing anti-obesity effects. But obesity negatively influences the effect of GLP1 centrally and peripherally through the appetite-regulating centre and the gut, respectively. A treatment regime that targets on both axes instead of one axis may therefore be more beneficial. This could mean increasing the appetite-suppressant effect of GLP1 and increasing or keeping the intestinal production of GLP1, both of which are achieved by improving the systemic bioavailability of GLP1. The bioavailability of GLP1 can be improved by developing GLP1 receptor agonists, which are resistant to enzyme dipeptidyl peptidase IV degradation. This approach has been widely adopted and most of the agonists have been approved for use in diabetic treatment (39). Liraglutide and Semaglutide, as mentioned above have been approved for use as weight loss agents. What is next then? Maintaining gut health appeared essential if one were to benefit from the physiological effects of GLP1, and 'fend off' the masking effects of DIO on GLP1 or the wide-ranging adverse effects of HFD or the impact of obesity on the gut.

## EFFECTS OF DIETARY INTERVENTIONS ON THE GUT AND BODY WEIGHT

Many researchers have turned to dietary intervention, hoping that the approach would increase GLP1 secretion. Highly soluble and fermentable fibers, through the production of short chain

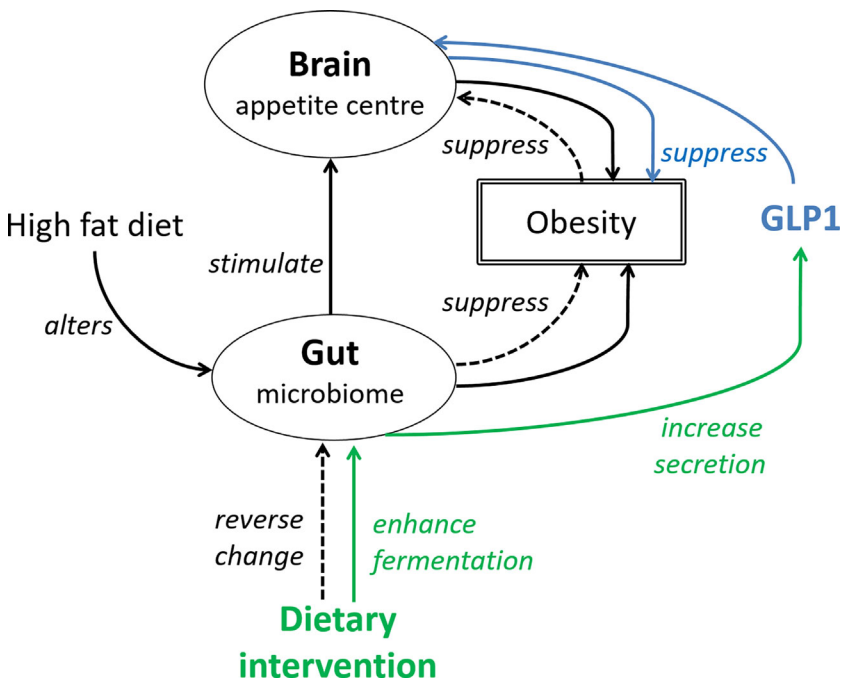


**FIGURE 1** | The gut-brain axis is central to the effects of GLP1. Solid black line: High fat diet alters the gut microbiome, which then stimulate the appetite leading to increased food intake and body weight gain. The interaction between HFD and gut microbiome also increases adiposity. Blue line (pharmacological approach): GLP1 or GLP1 receptor agonist suppresses appetite and reverses the increase in body weight. Green line (non-pharmacological approach): dietary fibers by enhancing fermentation in the large intestine, increase GLP1 secretion. Dotted line (non-pharmacological approach): dietary antioxidants may reverse changes of the gut microbiome induced by high fat diet, subsequently suppressing obesity and food intake.

fatty acids, promote GLP1 secretion (40). Fructooligosaccharides (41) and flavonoids (42) also acutely stimulated GLP1 secretion in rats. The use of oligosaccharides however may not be preferred as it may exacerbate irritable bowel syndrome, causing bloating, abdominal discomfort, and altering the movement of the intestine. Flavonoids consumption on the other hand should be encouraged. Gut antioxidants not only protect intestinal cells from inflammation, but also stress-induced body weight gain and food intake (43). Due to the hydrophilic nature of flavonoids, this plant-based antioxidant is poorly absorbed, and accumulated in the stomach and intestinal lumen. As proposed before (22), dietary antioxidant is an ideal alternative to conventional drugs for obesity treatment, more so if it could increase GLP1 secretion. Separately, if we are able to identify the specific microbial strains that influence the synthesis of GLP1, ingesting prebiotics that modify the activity of those strains may also be useful in increasing GLP1 secretion.

Dietary strategies are effective in reducing body weight. The effect is prolonged if the strategies are combined with behavioral counseling and ongoing support (44). High protein diet and low carbohydrate diet were suggested to be more beneficial than other types of diet (44). A meta-analysis of randomized controlled trials comparing low carbohydrate diet with low fat diet showed that the former improved weight loss more significantly than low fat diet in studies that ranged from 8 weeks to 24 months in duration (45). Mediterranean diet has been widely discussed lately (46, 47). There is no consensus on the definition of Mediterranean diet but the diet generally includes high levels of low carbohydrate and low protein food, and moderate levels of red wine and dairy products. Consumption of Mediterranean diet was found to produce greater weight reduction compared with low fat diets at 2 years (48, 49). The mechanism of the effects of Mediterranean has not been well investigated but an 8 weeks randomized controlled trial showed that the diet changed the gut microbiome of healthy obese and overweight subjects, decreasing microbial species that is proinflammatory, and had reduced systemic inflammation of the subjects (46). Regardless of which dietary strategy is implemented, adherence to the diet is the key to the long-term success of weight management or obesity treatment (46, 47, 50).

Various plant-based food, which have weight and fat mass reduction effects (51, 52), and food not targeting at weight control or increasing GLP1 secretion but reducing inflammation, have been suggested (53). The latter approach is sensible because inflammation has been reported to contribute either to the progression or the manifestation of obesity. Overall, there is adequate evidence to show that dietary intervention is promising. A concerted effort between endocrinologist and dietitian may be more effective in improving the treatment outcomes of obesity than a single modality treatment. **Figure 1** gives an overview of the effect of HFD and gut microbiome on obesity development, and how targeting the gut-brain axis is beneficial.

## CONCLUSION

Currently available therapy has not been effective in curbing the upward trend of obesity prevalence, which should prompt us to reconsider the traditional approach of obesity treatment. The outcomes of various clinical trials suggest that adopting a holistic approach in weight management may be more effective than focusing on one specific strategy involving either drug or non-drug treatment. The holistic approach may comprise of a pharmacological therapy, implemented alongside a dietary intervention. Liraglutide and Semaglutide are promising weight reduction pharmacological agents. The treatment should be accompanied by behavioral therapy and on-going support for dietary changes. Long-term adherence to dietary intervention such as Mediterranean diet and low carbohydrate diet may help to produce a sustainable weight loss, while the consumption of food or herbal products with antioxidant and anti-inflammatory properties synergizes the effect of GLP1 receptor agonist and improves the overall health.

## AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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# Restoration of mRNA Expression of Solute Carrier Proteins in Liver of Diet-Induced Obese Mice by Metformin

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Metformin (MET), the most common medicine for type 2 diabetes (T2DM), improves insulin sensitivity by targeting the liver, intestine and other organs. Its impact on expression of the solute carrier (*Slc*) transporter genes have not been reported in the mechanism of insulin sensitization. In this study, we examined *Slc* gene expression in the liver and colon of diet-induced obese (DIO) mice treated with MET by transcriptomic analysis. There were 939 differentially expressed genes (DEGs) in the liver of DIO mice *vs* lean mice, which included 34 *Slc* genes. MET altered 489 DEGs in the liver of DIO mice, in which 23 were *Slc* genes. Expression of 20 MET-responsive *Slc* DEGs was confirmed by qRT-PCR, in which 15 *Slc* genes were altered in DIO mice and their expressions were restored by MET, including *Slc2a10*, *Slc2a13*, *Slc5a9, Slc6a14*, *Slc7a9*, *Slc9a2*, *Slc9a3*, *Slc13a2*, *Slc15a2*, *Slc26a3*, *Slc34a2*, *Slc37a1*, *Slc44a4*, *Slc51b* and *Slc52a3*. While, there were only 97 DEGs in the colon of DIO mice with 5 *Slc* genes, whose expression was not restored by MET. The data suggest that more genes were altered in the liver over the colon by the high fat diet (HFD). There were 20 *Slc* genes with alteration confirmed in the liver of DIO mice and 15 of them were restored by MET, which was associated with improvement of insulin sensitivity and obesity. The restoration may improve the uptake of glucose, amino acids, mannose, fructose, 1,5-anhydro-D-glucitol and bumetanide in hepatocytes of the liver of DIO mice. The study provides new insight into the mechanism of metformin action in insulin sensitization and obesity.

**Keywords: metformin, transcriptome, solute carrier transporter, insulin sensitivity, obesity**

## INTRODUCTION

Metformin (MET), a guanidine derivative initially extracted from the plant *Galega officinalis* (French lilac), is the first-line medicine in the treatment of type 2 diabetes (T2DM) (1). In addition, MET activity has been reported in the treatment of many other diseases, such as cancer, obesity, nonalcoholic fatty liver disease (NAFLD) and inflammation (2). In the treatment of T2DM, MET is considered to act in the liver by inhibition of gluconeogenesis through a couple of mechanisms, such as inhibition of mitochondrial redox shuttle (3), suppression of the Complex I of mitochondrial respiratory chain (4), activation of the cellular energy sensor AMP-activated protein kinase (AMPK) (5). Additionally, MET is reported to act in the intestine to regulate bile acids (6), microbiota (7) and GLP-1 secretion (8) in the improvement of insulin sensitivity. However, the relative importance of liver and intestine remains to be established in the mechanism of insulin sensitization by MET.

Solute carrier (*Slc*) proteins are a group of transmembrane transporters that mediate solute influx and efflux across the plasma and intracellular membranes. The members of *Slc*5, *Slc*13, *Slc*16, *Slc*25, and *Slc*30 families, which are studied in the liver, intestine, pancreas, skeletal muscle, adrenal glands, adipose tissue, etc., have been linked to the metabolic disease, such as obesity, NAFLD and T2DM in human and mouse studies (9). It was reported that *Slc*22A1, *Slc*22A2, *Slc*22A3, *Slc*47A1 played an important role in the kidney, fat and liver for bioavailability, clearance, and pharmacological action of metformin in T2DM (10–12). However, systemic examination of *Slc* genes has not been reported in obesity.

MET inhibits hepatic gluconeogenesis through a direct action in hepatocytes. It is up taken by the organic cation transporters (*Slc*22A1 and *Slc*22A3) and secreted to the bile through an efflux transporter (*Slc*47A1). DNA methylation of these transporter genes (*Slc*22A1, *Slc*22A 3 and *Slc*47A1) are reduced by MET in the human liver, and increased by hyperglycemia and obesity (13). Metformin increases free fatty acid (FFA) uptake under hypoxic conditions, partially through up-regulation of fatty acid transporter *Slc*27A4 gene expression in the rat L6 skeletal muscle cells (14). However, an impact of MET in expression of the *Slc* transporters has not been examined extensively using transcriptome in the liver and colon. In this study, we systematically investigated the MET impact in the liver and colon of DIO mice using transcriptomic analysis, and provided new insight into the mechanism of metformin action in *Slc* mRNA expression.

## MATERIALS AND METHODS

### Animals and Materials

Male C57BL/6J mice at 8 weeks of age (SPF grade) were obtained from the Shanghai Slac Laboratory Animal Co. Ltd. (Shanghai, China). The mice were kept in the animal facility of Shanghai Jiao Tong University with a controlled temperature (20 ± 2°C), humidity (60 ± 5%) and a 12 h dark/light cycle. The control mice were fed a regular chow diet (NCD mice, n=10, A5002,13.5% Kcal from fat, Shanghai Slac Laboratory Animal Co. Ltd). A total of 20 mice were fed on HFD (# D12492, 60% Kcal from fat, Research diets) for 16 weeks to generate DIO mice as previously described (15). The DIO mice were divided randomly into 2 groups: the HFD control group (HFD mice, n=10) and the MET treated HFD group (HFD+MET mice, n=10). MET was administrated at 100 mg/kg/day through the drinking water. MET (metformin hydrochloride) was purchased from Sigma-Aldrich Co. Ltd. (Shanghai, China). The mice were treated with MET for 8 weeks.

At the end of the treatment, the mice were subjected to tissue collection under anesthesia with intraperitoneal injection of pentobarbital (35 mg/kg). Orbital bleeding was applied in the blood collection. Serum was isolated by centrifugation at 3000 g at 4°C for 10 min and stored at −80°C until the biochemical assays. The mice were sacrificed by cervical dislocation after blood collection. The visceral adipose tissues, livers and colons were collected from the animals and immediately weighed. The samples were flushed with phosphate-buffered saline (PBS, pH7.4) and instantly frozen in liquid nitrogen and then stored at −80°C until subsequent analysis. The animal experiments were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University. Other chemicals were purchased from Sigma-Aldrich Co. Ltd. (Shanghai, China) unless stated otherwise.

### Histological Analysis

The livers were fixed in 10% phosphate-buffered formalin acetate at 4°C overnight and embedded in paraffin wax. Paraffin sections (5 μm) were cut and mounted on glass slides for hematoxylin and eosin (H&E) staining. Cryosections of the livers were stained by oil red O and counterstained with hematoxylin to visualize the lipid droplets.

### Glucose Tolerance Test and Insulin Tolerance Test

Glucose tolerance test (GTT) and Insulin tolerance test (ITT) were respectively performed in the mice after 16 h fasting with peritoneal injection of glucose (2 g/kg) and 6 h fasting with peritoneal injection of insulin (1 U/kg). Blood glucose was tested in the tail vein blood at 0, 15, 30, 60, and 120 min using a One Touch glucometer (ACCU-CHEK® performa, Roche).

### Insulin Sensitivity and Blood Lipids

The insulin sensitivity index HOMA-IR [=fasting insulin (mU/l) × fasting glucose (mmol/l)/22.5] was calculated according to the fasting insulin and glucose concentration as previously described (15). Blood lipid profile was examined for serum triglyceride, total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol using an autoanalyzer (Hitachi 7600-020, automatic analyzer).

### RNA Extraction

Total RNA from liver and colon were extracted using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to manual instruction. About 60 mg of tissues were ground into powder by liquid nitrogen in a 2 mL tube, followed by being homogenized

for 2 minutes and rested horizontally for 5 minutes. The mix was centrifuged for 5 minutes at 12,000×g at 4°C, then the supernatant was transferred into a new EP tube with 0.3 mL chloroform/isoamyl alcohol (24:1). The mix was shacked vigorously for 15s, and then centrifuged at 12,000×g for 10 minutes at 4°C. After centrifugation, the upper aqueous phase where RNA remained was transferred into a new tube with equal volume of supernatant of isopropyl alcohol, then centrifuged at 13,600 rpm for 20 minutes at 4°C. After deserting the supernatant, the RNA pellet was washed twice with 1 mL 75% ethanol, then the mix was centrifuged at 13,600 rpm for 3 minutes at 4°C to collect residual ethanol, followed by the pellet air dry for 5-10 minutes in the biosafety cabinet. Finally, 25μL~100μL of DEPC-treated water was added to dissolve the RNA. Subsequently, total RNA was qualified and quantified using a Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA).

## mRNA Library Construction and Sequencing

Oligo(dT)-attached magnetic beads were used to purified mRNA. Purified mRNA was fragmented into small pieces with fragment buffer at appropriate temperature. Then First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by a second-strand cDNA synthesis. afterwards, A-Tailing Mix and RNA Index Adapters were added by incubating to end repair. The cDNA fragments obtained from previous step were amplified by PCR, and products were purified by Ampure XP Beads, then dissolved in EB solution. The product was validated on the Agilent Technologies 2100 bioanalyzer for quality control. The double stranded PCR products from previous step were heated denatured and circularized by the splint oligo sequence to get the final library. The single strand circle DNA (ssCir DNA) was formatted as the final library. The final library was amplified with phi29 to make DNA nanoball (DNB) which had more than 300 copies of one molecular, DNBs were loaded into the patterned nanoarray and pair end 100 bases reads were generated on BGIseq500 platform (BGI-Shenzhen, China).

## RNA-Seq Data Quality Analysis

In order to ensure data quality for the following analyses, the raw data of 9 samples were firstly filtered with SOAPnuke (v1.5.2) (https://github.com/BGI-flexlab/SOAPnuke) (16). Finally, clean data were obtained by (1) removing reads sequencing adapter, (2) removing reads whose low-quality base ratio (base quality less than or equal to 5) is more than 20%, (3) removing reads whose unknown base ('N' base) ratio is more than 5%. All the downstream analyses were based on the clean data with high quality.

## Differentially Expressed Genes Analysis

The differentially expressed genes (DEGs) analysis was performed using the DESeq2 (v1.4.5) (http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html) (17). We identified genes with a fold change (FC) ≥ 2 and Q value ≤ 0.05 in a comparison as significant DEGs.

## Gene Ontology and KEGG Pathway Enrichment Analysis

To take insight to the change of phenotype, Gene Ontology (GO) (http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp/) enrichment analysis of annotated different expressed gene was performed by Phyper (https://en.wikipedia.org/wiki/Hypergeometric_distribution) based on Hypergeometric test. The significant levels of terms and pathways were corrected by Q value with a rigorous threshold (Q value ≤ 0.05), as previously described (18).

## Real Time Quantitative RT-PCR

To confirm the transcriptomic data, MET-responsive *Slc* transporters DEGs were selected and validated through qRT-PCR. Total RNA was extracted using the EastepTM Total RNA Super Extraction Kit (promega, Shanghai, China) according to the manufacturer's instruction and quantified with a Denovix DS-11 Spectrophotometer (Denovix, Inc., Wilmington DE, USA). cDNA was synthesized from total RNA (1 μg: 20 μl final reaction volume) using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO Bio-Technology, CO., Shanghai, China) in a SimpliAmp Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA). A 20 μl PCR reaction system included 2 μl cDNA, 10 μl TB mixture, 0.4 μl forward primer, 0.4 μl reverse primer, 0.4 μl ROX Reference Dye II and 6.8 μl deionized water. After mixing, the PCR reaction was performed using ABI Prism TM 7500 Real-Time qPCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The GAPDH was used as a house gene to normalize the expression level of the test genes, and the relative gene expression level was analyzed using the $2^{-\Delta\Delta CT}$ method. All samples were analyzed in triplicate. Primers were synthesized by GENEWIZ (Suzhou, China) and were listed in the **Supplementary Table 1**.

## Statistical Analysis

The results are expressed as the mean ± SEM. The tissue weight, serum blood lipid levels, and mRNA expression were analysed using one-way ANOVA followed by Brown-Forsythe and Welch multiple comparisons tests. The body weight, fasting blood glucose, fasting serum insulin, HOMA-IR index, $AUC_{glucose}$ during GTT and ITT were analysed by two-way (repeated measures) ANOVA followed by Holm-Šídák multiple comparisons tests (note: each time was analysed separately). All statistical analyses were performed using GraphPad Prism 9.0 software (La Jolla, CA, USA) with a statistical significance set at $P < 0.05$.

# RESULTS

## Inhibitory Effect of MET on Hepatic Steatosis, Hyperlipidemia, and Obesity

The DIO mice were generated in C57BL/6 mice with HFD feeding for 16 wks. In the MET-treated DIO mice, the hepatic steatosis was decreased for a reduction in the hepatic intracellular vacuoles observed by H&E staining and oil red O staining of the

liver tissue (**Figures 1A, B**). The pathological changes in liver for pale fatty color, the number of hepatic fat vacuoles and hepatomegaly were reversed by MET (**Figures 1A–C**). A reduction in the fat mass (epididymal and perirenal fat) and liver weight were observed in the MET-treated mice (**Figure 1C**). An improvement in hyperlipidemia was observed with parameters including the low-density lipoprotein C, total cholesterol, and total triglyceride (**Figure 1D**). Moreover, the high-density lipoprotein C was increased (**Figure 1D**). A reduction in the body weight gain was observed in the MET-treated group as indicated by the weekly body weight data (**Figure 1E**). These data suggest that the model was successfully established for MET efficacy in the DIO mice.

## Improvement of Insulin Sensitivity by MET

Glucose metabolism was examined in the DIO mice at the 0, 4 and 8 weeks of the MET-treatment. An improvement in insulin sensitivity by MET was supported by the reduction in fasting glucose and fasting insulin (**Figures 2A, B**), which led to a favorite change in the index of HOMA-IR for insulin sensitization (**Figure 2C**). The improvement was extended in other tests including GTT and ITT at 4 and 8 weeks (**Figures 2D, E**). This group of data suggests that the insulin sensitivity was improved by MET in the DIO mice to support the MET efficacy in the DIO model.

## DEGs in liver of DIO mice

The liver gene expression was examined using the RNA-seq technology to investigate the mechanism of MET action. The differentially expressed genes (DEGs) were identified by the gene expression levels determined with fragments per kilobase of exon per million fragments mapped (FPKM) method. The differential expression in all samples was comprehensively analyzed with the criteria of FC≥2 and $P \leq 0.01$. In the liver, 939 DEGs were identified in the DIO mice (HFD *vs* NCD), and 489 DEGs were found in the MET-treated DIO mice (HFD+MET *vs* HFD) (**Figures 3A, B**). In DEGs of DIO mice, 137 were upregulated and 802 downregulated (**Figure 3A**). In the DEGs of MET-treated group, 451 were upregulated and 38 downregulated (**Figure 3B**). Among the HFD-responsive DEGs, there were 34 *Slc* genes with 32 downregulated and 2 upregulated in the DIO mice (HFD *vs* NCD) (**Table 1**), and in the MET-responsive DEGs, there were 23 *Slc* genes upregulated in the MET-treated DIO mice (HFD+MET *vs* HFD) (**Table 2**). Venn diagram analysis of the DEGs revealed that MET did not up-regulate any DEGs that were up in the liver of DIO mice (**Figure 3C**). MET did not down-regulate any DEGs that were down in the DIO mice (**Figure 3D**). However, MET upregulated 365 out of 802 DEGs that were downregulated in the DIO mice (HFD+MET *vs* HFD) (**Figure 3E**). MET downregulated 11 DEGs out of 137 that were upregulated in the liver of DIO mice (HFD+MET

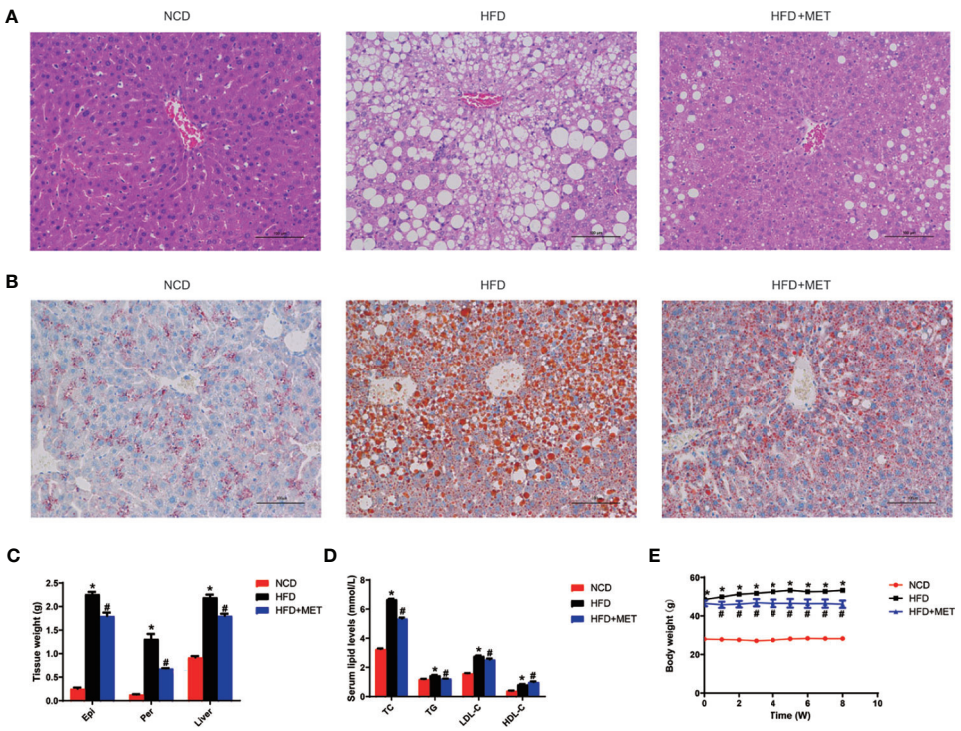


**FIGURE 1** | Inhibition of hepatic steatosis, hyperlipidemia, and obesity by MET. **(A)** Liver hematoxylin and eosin (H&E) staining. **(B)** Liver oil red O staining, ×200 (scale bars, 100 μm). **(C)** Tissue weight of perirenal fat, epididymal fat and liver after 8 weeks of treatment. **(D)** Serum lipid levels after 8 weeks of treatment. **(E)** Curve of body weight change from 0 to 8 weeks of treatment. The MET treatment was administrated for 8 weeks in HFD mice after 16 weeks on high-fat diet. Data are presented as the mean ± SEM (n = 10). $^{*}P < 0.05$ HFD *versus* NCD, $^{\#}P < 0.05$ HFD+MET *versus* HFD.

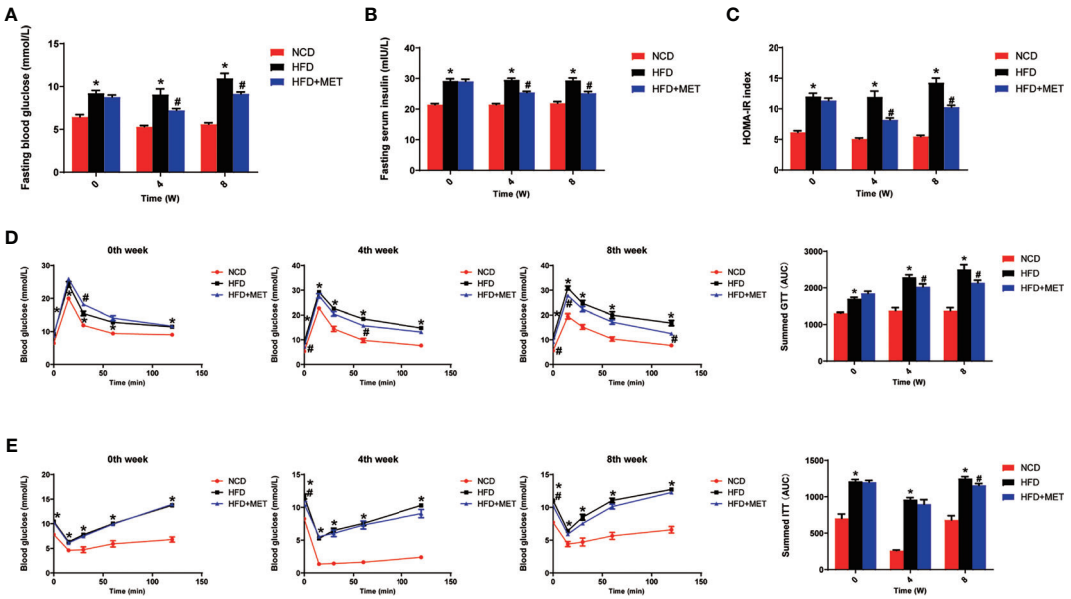


**FIGURE 2** | Improvement of insulin sensitivity by MET. **(A)** Fasting blood glucose. **(B)** Fasting serum insulin. **(C)** HOMA-IR. **(D)** GTT at 0, 4, 8 weeks of MET treatment. **(E)** ITT at 0, 4, 8 weeks of MET treatment. GTT and ITT was performed by intraperitoneal injection of glucose and insulin. Data are presented as the mean ± SEM (n = 10). *$P < 0.05$ HFD *versus* NCD, #$P < 0.05$ HFD+MET *versus* HFD.

*vs* HFD) (**Figure 3F**). mRNA of 20 out of 32 *Slc* genes that were downregulated in the DIO mice were upregulated in the MET-treated DIO mice (**Table 3**). The data suggest that the liver had a dramatic response to HFD with mRNA expression as suggested by DEGs, and MET were able to counter-regulate almost half of them.

## DEGs in Colon of DIO Mice

The gene expression was examined in the colon tissue in the same mice by RNA-seq to test the impact of MET in intestine. In response to HFD, 97 DEGs were identified in the colon of DIO mice (HFD *vs* NCD) and 92 DEGs were identified in the MET-treated DIO mice (HFD+MET *vs* HFD) with the criteria of FC≥2 and P ≤ 0.01 (**Figures 4A, B**). In the DIO mice, 23 DEGs were upregulated and 74 downregulated (**Figure 4A**). In the MET-treated DIO mice, 62 DEGs were upregulated and 30 downregulated (**Figure 4B**). There were 5 *Slc* genes in the DEGs of DIO mice, and 2 *Slc* genes in the MET-treaded DIO mice, respectively (**Supplementary Table 2**). Venn diagram analysis of the DEGs revealed that HFD-responsive and MET-responsive DEGs did not share the same direction of changes (**Figures 4C, D**). In contrast, MET counter-regulated 24 DEGs that were downregulated in the DIO mice (**Figure 4E**) and 3 DEGs that were upregulated in the DIO mice (**Figure 4F**). Expression of several *Slc* transporters was changed in the DIO, and none of them were restored by MET in the DIO mice (**Supplementary Table 2**). The data suggest that the colon responded to HFD with fewer DGEs, which was 1/8 of those in the liver. MET upregulated 24 downregulated DEGs in the colon of DIO mice and none of them was *Slc* gene.

## GO Enrichment Analysis of MET-Responsive Slc Genes in Liver

To investigate the function of the candidate DEGs of *Slc* transporters, we conducted GO enrichment analyses on the 20 common DEGs of *Slc* transporter genes (**Figure 5**). As shown in **Figure 5A**, the localization, cellular process and biological regulation were the subcategory of highest percentages in the biological processes (BP); the membrane and membrane part were the main significant terms in the cellular component (CC); the transporter activity was the most representative functions in the molecular function (MF). The 20 common DEGs of *Slc* genes in liver were mainly enriched in the BP associated with the transmembrane transport of sodium, ion, carbohydrate, anion transmembrane, glucose transmembrane, proton transmembrane, oxalate, glucose, sulfate, monocarboxylic acid, bicarbonate, propanoate (**Figure 5B**). As for the CC, the *Slc* gene products were mainly enriched in the brush border membrane, apical plasma membrane, integral component of membrane, plasma membrane, integral component of plasma membrane, microvillus membrane and vesicle (**Figure 5C**). The results for MF analysis demonstrated that the *Slc* gene products were mainly related to the functions of transmembrane transporter including symporter, antiporter, glucose: sodium symporter, potassium: porton antiporter, oxalate, secondary active sulfat, sulfate, sodium: proton antiporter, myo-insositol: proton symporter, high-affinity oligopeptide, propionate, short-chain fatty acid, thiamine pyrophosphate (**Figure 5D**). The data suggest that the cross-membrane transporter activities may be decreased by alteration in their mRNA expression in the liver of DIO mice. MET may restore the transporter activities by rescuing the gene expression.

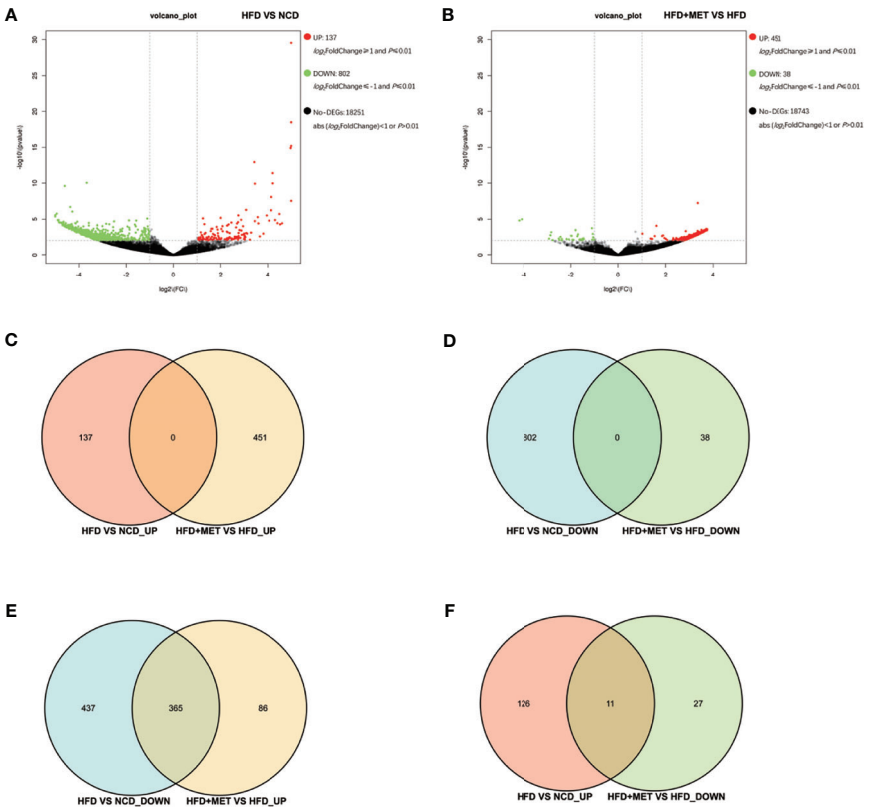


**FIGURE 3** | Differentially expressed genes (DEGs) analysis in liver. The volcano plot showed distribution pattern of DEGs in the comparison of HFD *vs* NCD **(A)** and HFD+MET *vs* HFD **(B)**. Venn diagram of upregulated common DEGs **(C)** and downregulated common DEGs **(D)** in two comparisons of HFD *vs* NCD and HFD+MET *vs* HFD. Venn diagram of common DEGs showing opposite expression changes in two comparisons of HFD *vs* NCD and HFD+MET *vs* HFD **(E, F)**. Red color or positive number indicates upregulation, and green color or negative number indicates down-regulation.

## KEGG Pathway Enrichment Analysis of MET-Responsive Slc Genes in Liver

To investigate the impact of MET-responsive *Slc* genes in the liver metabolism, we conducted an analysis of the metabolic pathway using the Kyoto Encyclopedia of Genes and Genomes (KEGG). The 20 *Slc* genes were clustered into 2 main categories, including organismal systems and human diseases, which were further divided into 4 subcategories (**Figure 6A**). They were mainly enriched in the mineral absorption, bile secretion, protein digestion and absorption, vitamin digestion and absorption, carbohydrate digestion and absorption, synthesis, secretion and action of parathyroid hormone, pancreatic secretion, which were associated with metabolic balance (**Figure 6B**). The data suggests that HFD may change digestion and absorption of proteins, carbohydrates, minerals, and vitamins in the liver. MET may target these pathways by restoring the expression of *Slc* genes in the improvement of insulin sensitivity.

## Validation of MET-Responsive *Slc* Genes in Liver

The 20 MET-responsive *Slc* genes were validated with qRT-PCR assays (**Figure 7**). Among the 20 genes, 15 genes with downregulation in the DIO mice were confirmed in qRT-PCR, which included *Slc2a10*, *Slc2a13*, *Slc5a9, Slc6a14*, *Slc7a9*, *Slc9a2*, *Slc9a3*, *Slc13a2*, *Slc15a2*, *Slc26a3*, *Slc34a2*, *Slc37a1*, *Slc44a4*, *Slc51b* and *Slc52a3* (**Figures 7A–G, J–O**). Their up-regulation by MET was also confirmed in qRT-PCR (**Figures 7A–G, J–O**). Three of them with upregulation in the DIO mice (including *Slc16a5*, *Slc25a24* and *Slc26a2*) were confirmed in qRT-PCR and their expression was further upregulated by MET as confirmed in qRT-PCR (**Figures 7H–J**). MET upregulated two *Slc* genes, *Slc5a1* and *Slc5a8*, which were not changed in the untreated DIO mice (**Figure 7B**).

# DISCUSSION

The transmembrane transporters are the gatekeeper proteins, which regulate the import and export of molecules, such as sugars, amino acids, fatty acids, nucleotides, metals, organic anions, inorganic ions, oligopeptides, and drugs (19). They are crucial for cellular homeostasis and their dysfunctions may contribute to the metabolic diseases. In fact, around 10% of all human genes encode the transporter proteins (20). The

**TABLE 1** | Slc transporters DEGs in liver compared between HFD group and NCD group (\log2FC\≥1 and P<0.01).

| Gene ID | Symbol | Description | $log_2FC$ | P value | Variation trend |
|---|---|---|---|---|---|
| 20510 | Slc1a1 | excitatory amino acid transporter 3 | -3.58192 | 0.002005 | Down |
| 20514 | Slc1a5 | neutral amino acid transporter B (0) | -2.55003 | 0.004563 | Down |
| 170441 | Slc2a10 | solute carrier family 2 (facilitated glucose transporter), member 10 | -2.95141 | 0.002313 | Down |
| 239606 | Slc2a13 | proton myo-inositol cotransporter | -3.63064 | 0.000488 | Down |
| 20537 | Slc5a1 | unnamed protein product | -3.77914 | 0.000583 | Down |
| 216225 | Slc5a8 | sodium-coupled monocarboxylate transporter 1 | -4.25732 | 0.000236 | Down |
| 230612 | Slc5a9 | solute carrier family 5 (sodium/glucose cotransporter), member 9, isoform CRA_b, partial | -3.88485 | 0.001178 | Down |
| 15567 | Slc6a4 | sodium-dependent serotonin transporter | -2.96903 | 0.003356 | Down |
| 102857 | Slc6a8 | sodium- and chloride-dependent creatine transporter 1 isoform 3; sodium- and chloride-dependent creatine transporter 1 isoform 1; solute carrier family 6 (neurotransmitter transporter, creatine), member 8, isoform CRA_a, partial | -3.50688 | 0.000971 | Down |
| 56774 | Slc6a14 | sodium- and chloride-dependent neutral and basic amino acid transporter B(0+) | -4.50093 | 9.50E-05 | Down |
| 224022 | Slc7a4 | cationic amino acid transporter 4 isoform X1 | -2.23075 | 0.000335 | Down |
| 30962 | Slc7a9 | solute carrier family 7 (cationic amino acid transporter, y+ system), member 9, isoform CRA_b, partial | -3.32269 | 0.006116 | Down |
| 226999 | Slc9a2 | solute carrier family 9 (sodium/hydrogen exchanger), member 2, isoform CRA_a, partial | -4.27668 | 0.000267 | Down |
| 105243 | Slc9a3 | sodium/hydrogen exchanger 3 precursor | -3.51883 | 0.001746 | Down |
| 20500 | Slc13a2 | solute carrier family 13 member 2 | -4.64711 | 2.27E-05 | Down |
| 790911 | Slc13a2os | mCG146185, partial | -3.47566 | 0.002674 | Down |
| 243755 | Slc13a4 | solute carrier family 13 member 4 | 2.680519 | 0.003954 | Up |
| 56643 | Slc15a1 | solute carrier family 15 member 1 | -3.90485 | 0.000646 | Down |
| 57738 | Slc15a2 | solute carrier family 15 member 2 isoform 1; solute carrier family 15 (H+/peptide transporter), member 2, isoform CRA_b | -4.59956 | 2.39E-10 | Down |
| 277898 | Slc15a5 | solute carrier family 15 member 5 | 2.394322 | 0.004192 | Up |
| 217316 | Slc16a5 | monocarboxylate transporter 6 | -1.35628 | 0.001467 | Down |
| 24059 | Slc21a2 | solute carrier organic anion transporter family member 2A1 | -1.78776 | 0.004075 | Down |
| 22626 | Slc23a3 | solute carrier family 23 (nucleobase transporters), member 3, partial | -2.68098 | 0.006767 | Down |
| 229731 | Slc25a24 | calcium-binding mitochondrial carrier protein SCaMC-1 | -3.52426 | 0.000856 | Down |
| 13521 | Slc26a2 | sulfate transporter | -3.65957 | 0.001049 | Down |
| 13487 | Slc26a3 | chloride anion exchanger | -4.68552 | 7.22E-05 | Down |
| 20531 | Slc34a2 | sodium-dependent phosphate transport protein 2B isoform X1 | -5.07452 | 2.78E-06 | Down |
| 224674 | Slc37a1 | glycerol-3-phosphate transporter | -2.94471 | 0.006447 | Down |
| 56857 | Slc37a2 | solute carrier family 37 (glycerol-3-phosphate transporter), member 2, isoform CRA_a, partial | -4.14538 | 0.00032 | Down |
| 72027 | Slc39a4 | zinc transporter ZIP4 precursor | -1.60956 | 0.006416 | Down |
| 72002 | Slc39a5 | solute carrier family 39 (metal ion transporter), member 5, isoform CRA_b, partial | -3.01113 | 0.001091 | Down |
| 70129 | Slc44a4 | choline transporter-like protein 4 | -4.6403 | 5.94E-05 | Down |
| 330962 | Slc51b | organic solute transporter subunit beta | -3.8114 | 0.000104 | Down |
| 69698 | Slc52a3 | solute carrier family 52, riboflavin transporter, member 3 isoform 1 precursor; solute carrier family 52, riboflavin transporter, member 3 isoform 2 | -3.69715 | 0.000307 | Down |

transporters include the ATP-binding cassette (ABC) transporters, ion channels, and solute carrier proteins (*Slc*s). The *Slc* transporters are the largest group of the transmembrane transporter proteins, which comprise over 60 subfamilies with more than 400 genes. *Slc*s are localized in the membrane surface to mediate passive and secondary active transportation of substrates (20). The *Slc* transporters are highly expressed in the metabolically active organs, such as the liver, kidney, brain, and intestine (9). Their roles in insulin resistance remains largely unknown.

Emerging evidence suggests that *Slc* transporters are closely associated with the metabolic diseases, and are potential candidates of drug targets for insulin resistance, T2DM, hypertension, chronic kidney disease (CKD), gout, asthma, inflammatory bowel disease (IBD), cancer, dementia, and anxiety disorders (9, 21–23). The activities are related to tissue specificity of the *Slc* genes. In our study, the *Slc* genes were examined in the liver and alteration in their expression was associated with hepatic steatosis, hyperlipidemia, obesity and insulin sensitivity in the DIO mice. We identified 20 MET-responsive *Slc* genes *via* transcriptome analysis, whose tissue distribution, physiological functions and pathological roles are shown in the **Supplementary Table 3**. Among them, expression of 15 *Slc* genes that were reduced in the liver of DIO mice was restored by MET. The 15 *Slc* members are *Slc2a10, Slc2a13, Slc5a9, Slc6a14, Slc7a9, Slc9a2, Slc9a3, Slc13a2, Slc15a2, Slc26a3, Slc34a2, Slc37a1, Slc44a4, Slc51b and Slc52a3*. Additionally, mRNA of 3 *Slc* genes (*Slc16a5*, *Slc25a24*, *Slc26a2)* were upregulated in DIO mice and further upregulated in MET-treated DIO mice. Expression of *Slc5a1* and *Slc5a8* were upregulated by MET although they were not altered in the liver of DIO mice. The data suggest that the 15 *Slc* genes may contribute to the mechanism of MET activity in insulin sensitization.

The exact functions of the MET-responsive *Slc* genes are largely unknown in obesity and type 2 diabetes. However, there are reports on six of *Slc* genes (*Slc2a10*, *Slc5A1*, *Slc5a9*, *Slc6A14*, *Slc16A5*, *Slc25A24*) in metabolic diseases including obesity, dyslipidemia, NAFLD, and T2DM. The *Slc2a* gene encodes the glucose transporter (GLUT) that has a family of 13 isoforms (24). *Slc2a10* is also known as *Glut10*, whose expression was reduced in the liver of DIO mice in this study. *Glut10* gene is highly

**TABLE 2 |** Slc transporters DEGs in liver compared between HFD+MET group and HFD group (\log2FC\≥1 and P<0.01).

| Gene ID | Symbol | Description | $log_2FC$ | P value | Variation trend |
|---|---|---|---|---|---|
| 170441 | Slc2a10 | solute carrier family 2 (facilitated glucose transporter), member 10 | 2.976387 | 0.001975 | Up |
| 239606 | Slc2a13 | proton myo-inositol cotransporter | 2.712812 | 0.006263 | Up |
| 20537 | Slc5a1 | unnamed protein product | 3.032958 | 0.002372 | Up |
| 216225 | Slc5a8 | sodium-coupled monocarboxylate transporter 1 | 3.207696 | 0.001732 | Up |
| 230612 | Slc5a9 | solute carrier family 5 (sodium/glucose cotransporter), member 9, isoform CRA_b, partial | 2.893464 | 0.006372 | Up |
| 56774 | Slc6a14 | sodium- and chloride-dependent neutral and basic amino acid transporter B(0+) | 3.645746 | 0.000411 | Up |
| 30962 | Slc7a9 | solute carrier family 7 (cationic amino acid transporter, y+ system), member 9, isoform CRA_b, partial | 2.678152 | 0.009553 | Up |
| 226999 | Slc9a2 | solute carrier family 9 (sodium/hydrogen exchanger), member 2, isoform CRA_a, partial | 3.39624 | 0.00124 | Up |
| 105243 | Slc9a3 | sodium/hydrogen exchanger 3 precursor | 2.985228 | 0.004804 | Up |
| 171286 | Slc12a8 | solute carrier family 12 member 8 isoform 1; solute carrier family 12 member 8 isoform X5 | 2.600641 | 0.009193 | Up |
| 20500 | Slc13a2 | solute carrier family 13 member 2 | 3.493265 | 0.000674 | Up |
| 57738 | Slc15a2 | solute carrier family 15 member 2 isoform 1; solute carrier family 15 (H+/peptide transporter), member 2, isoform CRA_b | 3.353095 | 5.65E-08 | Up |
| 217316 | Slc16a5 | monocarboxylate transporter 6 | 1.42968 | 0.005747 | Up |
| 56517 | Slc22a21 | solute carrier family 22 member 21 | 2.552414 | 0.007362 | Up |
| 229731 | Slc25a24 | calcium-binding mitochondrial carrier protein SCaMC-1 | 3.169503 | 0.001724 | Up |
| 384071 | Slc25a34 | solute carrier family 25 member 34 | 1.37105 | 0.005277 | Up |
| 13521 | Slc26a2 | sulfate transporter | 3.081388 | 0.002561 | Up |
| 13487 | Slc26a3 | chloride anion exchanger | 3.250563 | 0.001134 | Up |
| 20531 | Slc34a2 | sodium-dependent phosphate transport protein 2B isoform X1 | 2.628209 | 0.004297 | Up |
| 224674 | Slc37a1 | glycerol-3-phosphate transporter | 2.919821 | 0.002715 | Up |
| 70129 | Slc44a4 | choline transporter-like protein 4 | 3.65143 | 0.000433 | Up |
| 330962 | Slc51b | organic solute transporter subunit beta | 3.391635 | 0.001222 | Up |
| 69698 | Slc52a3 | solute carrier family 52, riboflavin transporter, member 3 isoform 1 precursor; solute carrier family 52, riboflavin transporter, member 3 isoform 2 | 3.071518 | 0.00165 | Up |

**TABLE 3 |** MET-responsive Slc transporters DEGs in liver (\log2FC\≥1 and P<0.01).

| Gene ID | Symbol | HFD *VS* NCD | | | HFD+MET *VS* HFD | | |
|---|---|---|---|---|---|---|---|
| | | $log_2FC$ | P value | Variation trend | $log_2FC$ | P value | Variation trend |
| 170441 | Slc2a10 | -2.95141 | 0.002313 | Down | 2.976387 | 0.001975 | Up |
| 239606 | Slc2a13 | -3.63064 | 0.000488 | Down | 2.712812 | 0.006263 | Up |
| 20537 | Slc5a1 | -3.77914 | 0.000583 | Down | 3.032958 | 0.002372 | Up |
| 216225 | Slc5a8 | -4.25732 | 0.000236 | Down | 3.207696 | 0.001732 | Up |
| 230612 | Slc5a9 | -3.88485 | 0.001178 | Down | 2.893464 | 0.006372 | Up |
| 56774 | Slc6a14 | -4.50093 | 9.50E-05 | Down | 3.645746 | 0.000411 | Up |
| 30962 | Slc7a9 | -3.32269 | 0.006116 | Down | 2.678152 | 0.009553 | Up |
| 226999 | Slc9a2 | -4.27668 | 0.000267 | Down | 3.39624 | 0.00124 | Up |
| 105243 | Slc9a3 | -3.51883 | 0.001746 | Down | 2.985228 | 0.004804 | Up |
| 20500 | Slc13a2 | -4.64711 | 2.27E-05 | Down | 3.493265 | 0.000674 | Up |
| 57738 | Slc15a2 | -4.59956 | 2.39E-10 | Down | 3.353095 | 5.65E-08 | Up |
| 217316 | Slc16a5 | -1.35628 | 0.001467 | Down | 1.42968 | 0.005747 | Up |
| 229731 | Slc25a24 | -3.52426 | 0.000856 | Down | 3.169503 | 0.001724 | Up |
| 13521 | Slc26a2 | -3.65957 | 0.001049 | Down | 3.081388 | 0.002561 | Up |
| 13487 | Slc26a3 | -4.68552 | 7.22E-05 | Down | 3.250563 | 0.001134 | Up |
| 20531 | Slc34a2 | -5.07452 | 2.78E-06 | Down | 2.628209 | 0.004297 | Up |
| 224674 | Slc37a1 | -2.94471 | 0.006447 | Down | 2.919821 | 0.002715 | Up |
| 70129 | Slc44a4 | -4.6403 | 5.94E-05 | Down | 3.65143 | 0.000433 | Up |
| 330962 | Slc51b | -3.8114 | 0.000104 | Down | 3.391635 | 0.001222 | Up |
| 69698 | Slc52a3 | -3.69715 | 0.000307 | Down | 3.071518 | 0.00165 | Up |

expressed in the pancreas and liver with a function in transportation of glucose (25). However, GLUT10 is found to mediate ascorbic uptake in cells in a recent study. *Glut10* was studied in adipocytes through genetic analysis, in which mutation-induced inactivation of *Glut10* gene impaired adipogenesis and reduced adipose tissue development *via* an ascorbic acid-mediated pathway leading to insulin resistance, suggesting that *Glut10* may mediate ascorbic acid uptake in cells (26). However, the role of *Glut10* (*Slc2a10*) remains unknown in the control of insulin sensitivity in the liver. In our study, the downregulation of *Glut10* gene in the liver of DIO mice was restored by MET-treatment, suggesting that MET may induce ascorbic acid uptake in the liver in the mechanism of insulin sensitization.

The *Slc5a* gene encodes the sodium-dependent glucose transporters (SLGTs) that has a family of 11 isoforms (27).

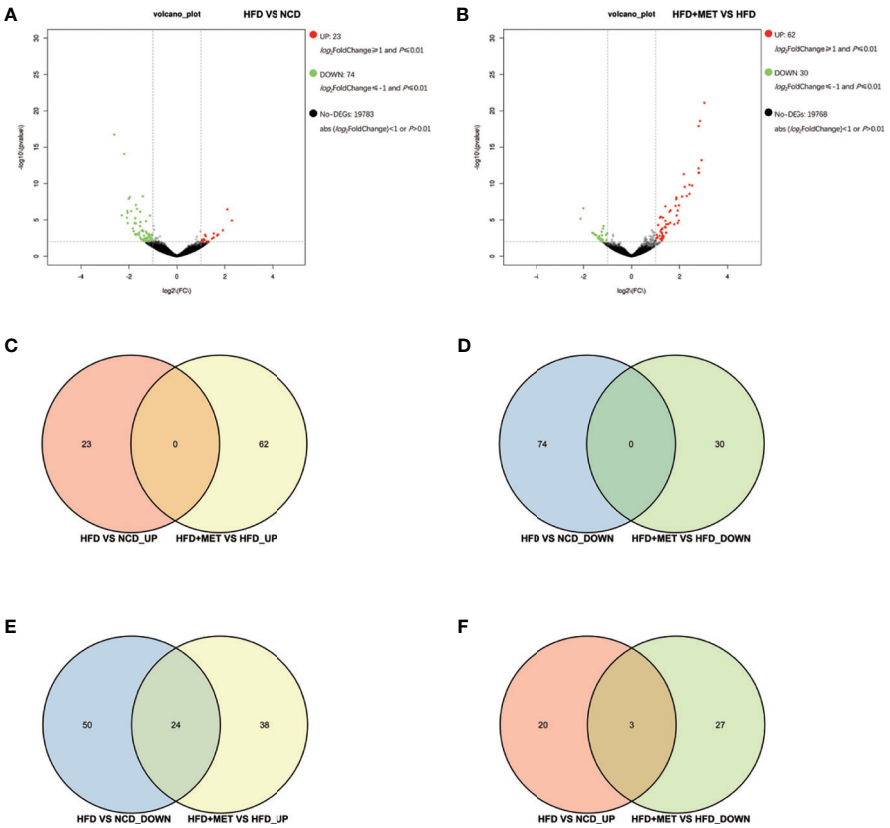


**FIGURE 4** | Differentially expressed genes (DEGs) analysis in colon. The volcano plot showed distribution pattern of DEGs in the comparison of HFD *vs* NCD **(A)** and HFD+MET *vs* HFD **(B)**. Venn diagram of upregulated common DEGs **(C)** and downregulated common DEGs **(D)** in two comparisons of HFD *vs* NCD and HFD+MET *vs* HFD. Venn diagram of common DEGs showing opposite expression changes in two comparisons of HFD *vs* NCD and HFD+MET *vs* HFD **(E, F)**. Red color or positive number indicates upregulation, and green color or negative number indicates down-regulation.

SGLT2 is a well-known member in the kidney for glucose reabsorption from urine, and SGLT2 inhibitors are effective medicines in the treatment of type 2 diabetes for promotion of glucose discharge in urine. *Slc5a1* gene encodes SGLT-1 in the intestine for absorption of glucose (Glc) and galactose (Gal) (28). *Slc5a1* expression is reported to be reduced by the inhibitors of mitochondrial electron transport chain complexes (such as metformin, rotenone and antimycin A) in mouse ileal cultures mouse duodenal organoid derived 2D monolayer cultures (29). In human, *Slc5a1* expression is increased in the duodenal of individuals with impaired glucose tolerance and T2DM (30). *Slc5a1* activity remains unknown in the liver. We found that *Slc5a1* expression was induced in the liver of DIO mice by MET, suggesting that glucose uptake by liver may be increased by MET in the improvement of insulin sensitivity. *Slc5a1* expression was not changed in the colon of DIO mice and MET-treated DIO mice.

In this study, mRNA expression of *Slc5a9* and *Slc25a24* was elevated in the liver of DIO mice and the expression was further increased by MET. *Slc5A9* is also known as SGLT4, a sodium-dependent glucose transporter, which acts as an essential transporter for mannose, fructose and 1,5-anhydro-D-glucitol in the intestine and kidney (31). A mutation within *Slc5a9* gene is reported in the development of proliferative diabetic retinopathy (32). However, *Slc5a9* activity remains unknown in the liver. We observed that *Slc5a9* expression was induced by MET, suggesting that MET may promote uptake of mannose, fructose and 1,5-anhydro-D-glucitol in the liver through induction of *Slc5a9*. In DIO mice, it was reported that *Slc25a24* expression was increased in the white adipose tissues (WAT) of DIO mice during tissue expansion, and *Slc25a24*-knockout mice exhibited an obesity-resistant phenotype (33). This study suggests that *Slc25a24* may be required for adipose tissue expansion and a novel candidate gene in the control of obesity.

We found that *Slc6a14* and *Slc16a5* were upregulated by MET in the DIO mice, which is consistent with the published studies. *Slc6a14* is a $Na^+$ and $Cl^-$ dependent transporter for glutamine and other amino acids except glutamate and aspartate (34). Sathish Sivaprakasam, et al. suggested that the deficiency of *Slc6a14* was related to obesity, and dietary/pharmacologic interventions-induced *Slc6a14* expression in the intestinal tract might play a role in the prevention of obesity (35). *Slc16a5* is known as monocarboxylate transporter 6 (MCT6), which transports bumetanide in a pH- and membrane potential-

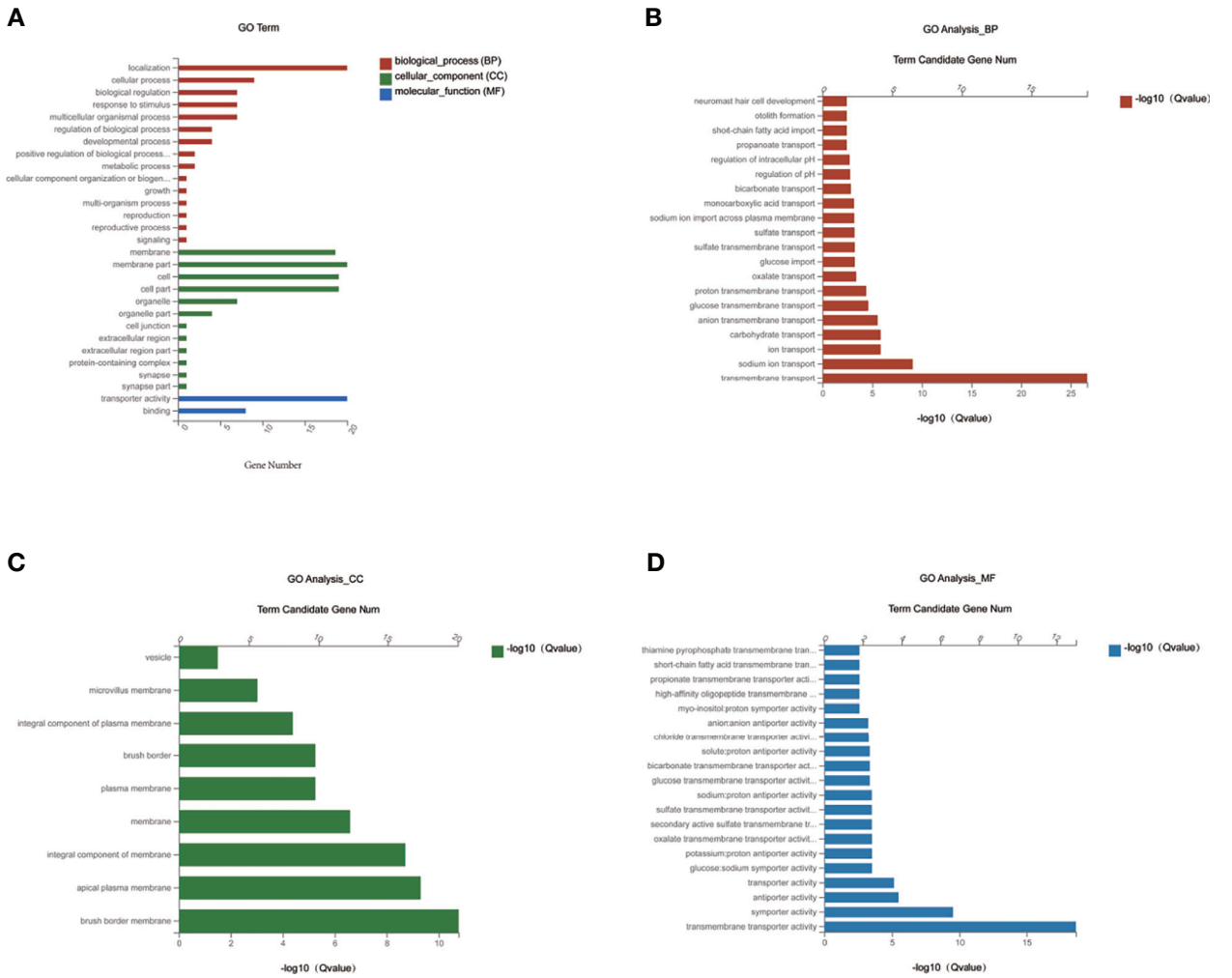


**FIGURE 5** | GO enrichment analysis of MET-responsive Slc genes in liver. **(A)** The annotation and classification of GO functional enrichment analysis with the common DEGs of Slc transporters in the liver. The functional enriched classes of the common DEGs of Slc transporters in the liver annotated by biological processes (BP) **(B)**, and cell components (CC) **(C)**, molecular function (MF) **(D)** sub-ontologies of GO enrichment analysis.

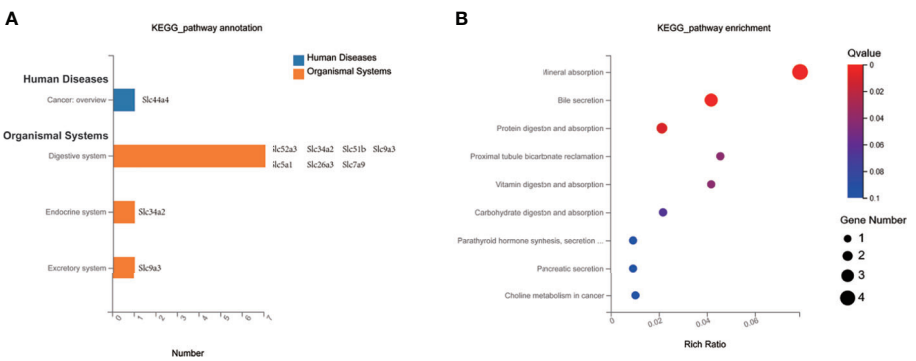


**FIGURE 6** | KEGG pathway enrichment analysis of MET-responsive Slc genes in liver. **(A)** KEGG pathway annotation and classification of common Slc transporters DEGs in liver. **(B)** Scatterplot of KEGG enrichment pathways associated with metabolic balance of common Slc transporters DEGs in liver.

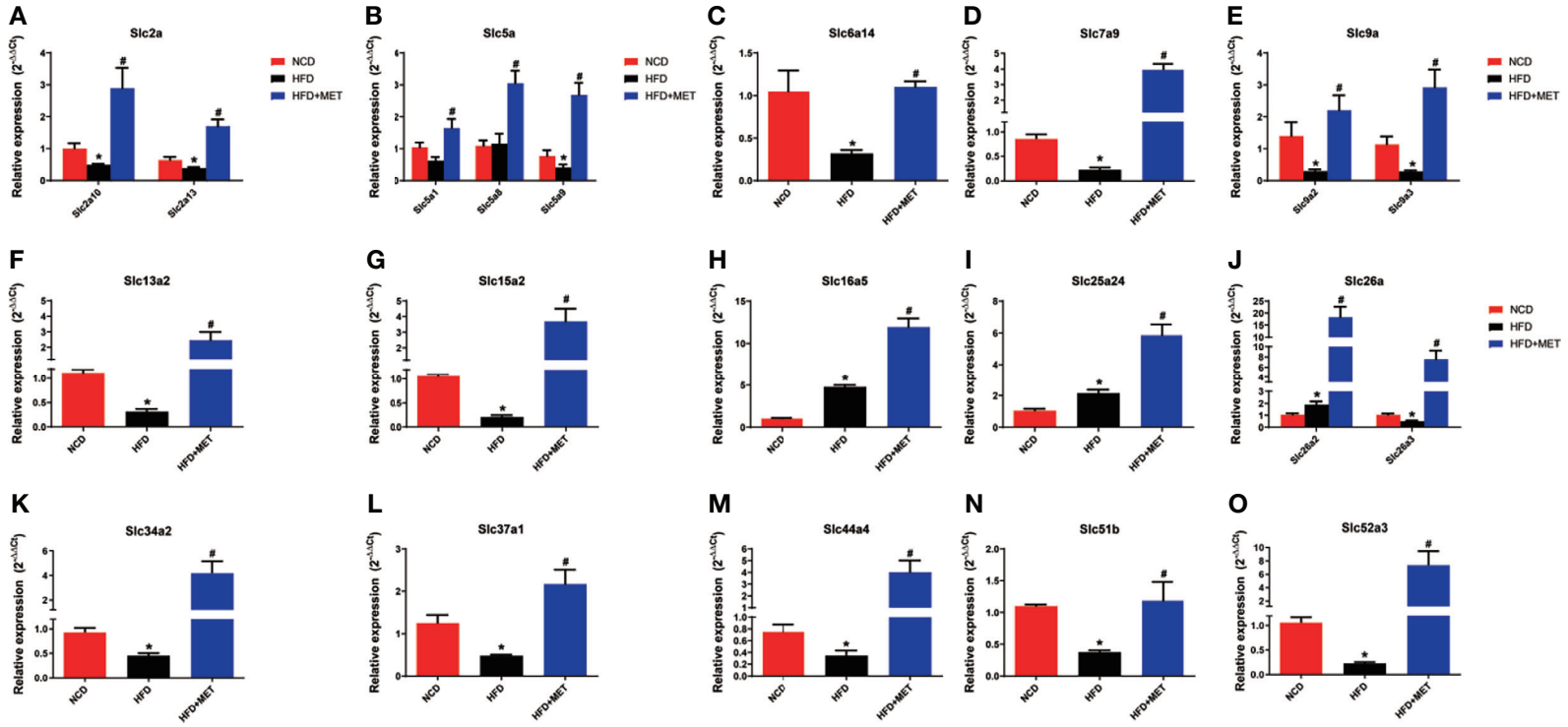


**FIGURE 7** | Expression validation of the 20 MET-responsive Slc genes in liver via RT-qPCR. The mRNA levels of the 20 common Slc DEGs were validated by RT-qPCR (2−ΔΔCT method) **(A–O)**. The housekeeping gene GAPDH was used to normalize the relative expression level. Data are presented as the mean ± SEM (n = 3). *$P < 0.05$ HFD *versus* NCD, #$P < 0.05$ HFD+MET *versus* HFD.

sensitive manner independently of proton gradient (36). Lu, et al. reported that the treatment of mice with fenofibrate resulted in 3- to 6-fold upregulation of *Slc16a5* in the liver of mice, which was associated with an improvement in insulin sensitivity (37). After 24-hour fasting, *Slc16a5* was also detected as the 6th most increased gene with about a 5-fold upregulation in gene expression, compared with gene expression after a normal diet (38). These studies confirm that *Slc6a14* and *Slc16a5* may play a role in lipid metabolism due to its differential regulation under various interventions. Our data suggest that MET may increase liver uptake of amino acids and bumetanide in the improvement of insulin sensitivity and obesity.

In the liver analysis by RNA-seq, there are three other studies in the literature. Those were designed to understand MET mechanism in the regulation of liver gene expression in the normal mice or human hepatocytes. These studies provide evidence for MET direct impact in gene transcription. Meng Y et al. revealed that metformin changed gene expression profile in the mouse liver, which was associated with the beneficial/deleterious effects in the healthy mice (39). Lien F et al. found that MET regulated the bile acid homeostasis through gene transcription mediated by the AMPK-FXR pathway in the liver of normal mice (40). Luizon MR et al. reported that the AMPK-ATF3 pathway was activated by MET to regulate gene transcription in the normal human hepatocytes in the cell culture, which was associated with inhibition of gluconeogenesis by MET (41). We analyzed the RNA-seq data in the study by Luizon, et al, to understand the MET direct and indirect effect in the expression of *Slc* genes. The result suggests that MET does not alter expression of the 15 *Slc* genes identified in the DIO mice. The negative result from the cellular model suggest that MET may use an indirect mechanism in the restoration of the 15 *Slc* genes in the liver of DIO mice in current study. The restoration may a secondary effect of improved insulin sensitivity or hepatocyte function in the liver of DIO mice.

Although the mechanism by which MET restored the 15 *Slc* gene expression remains unclear, significance of *Slc* genes in the pathogenesis of metabolic disease is supported by a couple of studies. A study suggests that *Slc*13a5 expression is required for development of diet- and aging-induced obesity. *Slc*13a5 gene inactivation were protected the knockout mice from high-fat diet (HFD)- and aging-induced obesity, hepatic steatosis, and insulin resistance (42). Consistently, knockdown of human *Slc*13a5 resulted in lower lipid levels in a human hepatocyte cell line (43). *Slc*25a24 seems to have the same activity in the knockout mice, which were protected from HFD-induced obesity and hepatic steatosis (33). Conversely, a reduction in *Slc*16a11 activity promoted T2DM and induction of the gene expression decreased the risk of T2DM (44, 45). Similarly, a reduction in *Slc*30a8 activity increased the risk of T2DM, and overexpression of *Slc*30a8 improved glucose tolerance in mice (46, 47). These studies were conducted in the global transgenic mice, and the results might reflect the *Slc* activities in organs other than the liver.

In summary, we observed that more *Slc* genes exhibited alteration in the liver over the colon under in the DIO mice. In the liver of DIO mice, there were 34 *Slc* genes with mRNA alteration, in which 15 *Slc* genes were downregulated in mRNA expression, and the reduced expressions were restored in the liver by MET. The restoration of the *Slc* gene expression may be a secondary effect of the improved insulin sensitivity in liver to promote uptake of glucose, amino acids, mannose, fructose, 1,5-anhydro-D-glucitol and bumetanide in the hepatocytes. The *Slc* genes may contribute to liver metabolism through regulation of substrate exchange in hepatocytes. However, the possibilities remain to be tested in experiments. The upstream events for the *Slc* gene restoration remain to be identified in the MET action.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA735274.

## ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiaotong University.

## AUTHOR CONTRIBUTIONS

JY contributed to the concept and design of the study. JLe, YF, QH, XW, HJ, and XZ performed the experiments. JLe, YC, QW, PP, JLi, XL, and YZ processed the experimental data and prepared table and picture. JLe wrote the manuscript. JLe and JY revised the manuscript. JLe and JY are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.720784/full#supplementary-material

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# Spatial Analysis of Incidence of Diagnosed Type 2 Diabetes Mellitus and Its Association With Obesity and Physical Inactivity

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**Objectives:** To investigate the spatial distribution of 10-year incidence of diagnosed type 2 diabetes mellitus (T2DM) and its association with obesity and physical inactivity at a reginal level breakdown.

**Methods:** Demographic, behavioral, medical and pharmaceutical and diagnosed T2DM incidence data were collected from a cohort of 232,064 participants who were free of diabetes at enrolment in the 45 and Up Study, conducted in the state of New South Wales (NSW), Australia. We examined the geographical trend and correlation between obesity prevalence, physical inactivity rate and age-and-gender-adjusted cumulative incidence of T2DM, aggregated based on geographical regions.

**Result:** The T2DM incidence, prevalence of obesity and physical inactivity rate at baseline were 6.32%, 20.24%, and 18.7%, respectively. The spatial variation of T2DM incidence was significant (Moran's I=0.52; p<0.01), with the lowest incidence of 2.76% in Richmond Valley-Coastal and the highest of 12.27% in Mount Druitt. T2DM incidence was significantly correlated with the prevalence of obesity (Spearman r=0.62, p<0.001), percentage of participants having five sessions of physical activities or less per week (r=0.79, p<0.001) and percentage of participants walked to work (r=-0.44, p<0.001). The geographical variations in obesity prevalence and physical inactivity rate resembled the geographical variation in the incidence of T2DM.

**Conclusion:** The spatial distribution of T2DM incidence is significantly associated with the geographical prevalence of obesity and physical inactivity rate. Regional campaigns advocating the importance of physical activities in response to the alarming T2DM epidemic should be promoted.

**Keywords: diabetes, incidence, GIS, Australian, risk factors, obesity, physical inactivity**

## INTRODUCTION

Diabetes is a key non-communicable disease (NCDs) targeted for global action (1, 2). The prevalence of diabetes has increased from 4.7% in 1980 to 8.5% in 2014 in the adult population globally (1). The World Health Organization (WHO) projected that the number of diabetes cases worldwide would reach 366 million by 2030 (3). Type 2 diabetes mellitus (T2DM), diagnosed when the pancreas could no longer produce sufficient insulin, accounts for 90-95% of all diabetic cases (4). The management of T2DM is complex and time-consuming, often involving regular health consultations, lifestyle modifications, frequent blood glucose and podiatry monitoring and complex medication regimes (5–8). The estimated annual spending on T2DM in Australia is around $6 billion. The average annual healthcare cost per person is as high as $4,025, even if there are no associated complications (9).

Previous studies (10–12) have revealed that the development of T2DM results from complex interactions between genetic, environmental, lifestyle and other risk factors. It is also revealed that obesity and physical inactivity are highly correlated with T2DM (13, 14). Most of these associated studies have been conducted on an individual level, providing essential information to identify key modifiable health behaviours for the general health of people living with T2DM. However, understanding the geographical trends of T2DM at a population level may provide important evidence to inform better health policies and population-based prevention programs.

Several existing studies have reported the geographical variance in T2DM disease burden (15–19). Angela et al. (16) evaluated the geographical variations of T2DM incidence among teenagers in the United States. Douglas et al. (17) identified regions with a high disease burden of T2DM in the city of London, UK. Similar studies were also conducted in Ukraine (19) and China (20). Geospatial correlation studies that focused on the association between the risk factors and population effects on T2DM were also conducted in Europe, the US and China as evidence to facilitate political commitment and implementation of community-based programs to curb the epidemic of T2DM (21–23). They reported the association of T2DM with obesity and physical activity, and some studies also highlighted that these geographical variances could result from social determinants, such as income and employment (24–26). In Australia, a typical developed country with the world's 12$^{th}$ largest economy, several government-issued reports unveiled the geographical variations of T2DM incidence nationwide but without in-depth spatial analysis of T2DM and its interaction with population risk factors (9, 27–29). In particular, more than 90% of T2DM in Australia were found in the middle-aged and elderly population of more than 45 years old. Therefore, understanding the geographical trends and the relevant population factors that contribute to T2DM in this group is key for the prevention of T2DM in the ageing Australian population.

We hypothesised that there are significant geographical variations in T2DM incidence across the Australian state of New South Wales, and these variations are potentially associated with the obesity prevalence and physical inactivity rate in the population. We used demographic, behavioural, medical, and pharmaceutical data from a ten-year follow-up study cohort with 266,896 participants to explore the geographical variation in the incidence of T2DM and associated population factors. Findings from this study will add new evidence to inform health policies to be modified based on geographical variations across Australia at a population level.

## METHODS

### Data Source

The data used in this study was mainly collected from the Sax Institute's 45 and Up Study, a population-based cohort study with participants aged 45 and over in NSW, Australia (30). A total of 266,896 participants, who were first randomly sampled from Services Australia's (formerly the Australian Government Department of Human Services) Medicare enrolment database, joined the Study between January 2006 and December 2009. Each participant completed a baseline questionnaire and provided signed consent for follow-up and linkage of one's information to a routine health database. The baseline questionnaire captured a broad range of information related to socioeconomic, health, and lifestyle factors. The 45 and Up Study data was also linked to the Medicare Benefits Schedule (MBS), and Pharmaceutical Benefits Scheme (PBS) claims from 2004 to 2016 with deterministic matching. Hence, detailed medical procedures (identified by MBS code) and medications prescribed by clinicians (identified by PBS code) could be tracked for each participant. Data of population walking to work was obtained from a census study by Zander et al. in 2011 (31), where they aggregated the raw data from the ABS.

### Geographical Measures

Statistical Area Level 3 (SA3) is a regional breakdown of Australia based on the Australian Statistical Geography Standard (ASGS), using a standardised set of numeric codes issued by the ABS to uniformly identify geographical entities (32). The delimitation of a total of 91 SA3s in New South Wales was based on the relative homogeneity in the demographic, functional, geographical, and socioeconomic characteristics (32). SA3 code for each participant was derived from the self-reported baseline questionnaire in the 45 and Up Study, and the SA3 maps of Digital Boundaries for the year 2011 were downloaded from ABS website (32).

### Ethics Considerations

Ethics approval for the 45 and Up Study was granted by the University of New South Wales Human Research Ethics Committee. Ethics approval for this specific study was granted by the Royal Victorian Eye and Ear Hospital Human Research Ethics Committee.

### Inclusion and Exclusion Criteria

We excluded participants with established diabetes at baseline, defined as those who:

- self-reported to have established diabetes;
- applied diabetes medications before the baseline implied by the PBS database (33);
- defined as a diagnosis of diabetes earlier than the last childbirth, but without diabetes medication use subsequently;
- had missing or invalid BMI or Statistical Area 3 data;
- reported age of diabetes diagnosis older than the age at baseline survey. We also excluded participants who are from the SA3 regions with less than 100 participants (10702, 10803 and 12402).

After exclusions, a total of 232,064 residents were selected in this study (**Figure 1**).

## Outcomes and Associated Factors

The SA3-level 10-year diabetes incidence rates were estimated in 232,064 residents for the period from 2006 to 2017. Age- and-gender adjusted diabetes incidence was calculated using the direct adjustment method, based on the age and gender distribution of 2011 census population data. We defined diabetes incidence as the first occurrence of any kind of medications prescribed for T2DM (including oral hypoglycaemic agents and insulin) with their corresponding PBS codes. As all participants were aged above 45 years, we assumed that all incident cases of new diabetes medication use were for T2DM rather than type 1 diabetes mellitus.

Each participants' body mass index (BMI) was derived from the self-reported baseline questionnaire in the 45 and Up Study. Definition of obesity is based on body mass index calculated from height and weight, which was previously validated in this study cohort (34, 35). According to the definition by WHO (34), a person with a BMI of more than 30kg/m$^2$ is considered obese in our study. Using this standard, we calculated the obesity prevalence for each SA3 region.

We applied two methods to evaluate physical inactivity in our study. Using the first method, we defined people who do physical activities of less than five sessions (metabolically adjusted) per week as participants "having insufficient physical activities", based on recommendations by the US Centers for Disease Control (CDC) (36, 37). Since information about the number of physical activity sessions for all participants were available in the self-reported baseline questionnaires in the 45 and Up Study, we aggregated the percentage of the population with insufficient physical activities for each SA3 region. To strengthen our physical inactivity measures, we applied a secondary indicator to measure physical inactivity. A previous study (31) conducted in Australia has shown that the percentage of the population walking to work, which is the ratio of the number of journeys to work by walking to the total number of journeys to work, is a good indicator of physical activity in Australia. A low percentage of the population walking to work presented the lack of physical activity in the population and a high rate of physical inactivity. Since the data from the previous study were aggregated based on a different region breakdown standard (38), we mapped each region in that study to the SA3 region in our study using the correspondence data from ABS and re-calculated the percentage of the population walking to work for each SA3 region.

## ANALYSES FOR T2DM INCIDENCE

SAS version 9.4 (SAS Institute Incorporation) and R Studio was used for non-spatial data analyses. To understand the distributions in diabetes incidence across different regions in Australia,

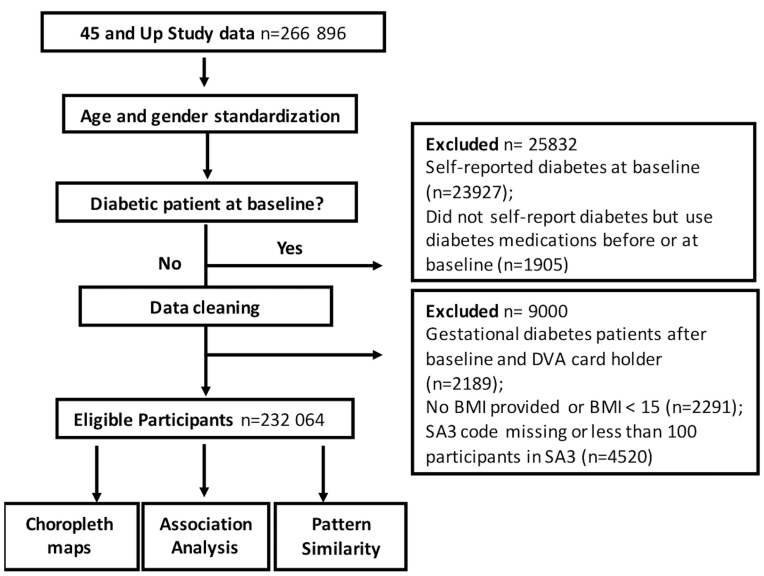


**FIGURE 1** | Spatial Analysis of Diabetes Study Flow Diagram.

we calculated the range, mean and standard deviation for each potential association factor, including obesity prevalence, percentage of the population with insufficient physical activities and percentage of the population walking to walk. We further analysed the non-spatial correlation between diabetes incidence and each factor using Spearman correlation.

For spatial analysis, Choropleth maps of the outcome and associated factors were plotted using ArcGIS 10.4 (ESRI, Redlands, CA) and GeoDa 1.12. We applied univariate Moran' I to identify if there are significant geographical variations in diabetes incidence, obesity prevalence, percentage of the population with insufficient physical activities and percentage of the population walking to walk, respectively. If a univariate Moran' I value is close to 1, it indicates a significant geographical variation, whereas a value close to 0 indicates no clear geographical variations. To assess colocation between T2DM incidence and each of the association factors, we calculated the bivariate Moran's I and intraclass correlation coefficient for the associated factors and diabetes incidence. Bivariate Moran's I was a global measure of spatial correlation to measure the influence one variable has on the occurrence of another variable in close proximity (39). A value close to 1 represents a clear spatial correlation, whereas a value close to 0 means no clear spatial correlation.

# RESULTS

## Characteristics of Participants Based on SA3

Amongst the 232,064 individuals in the 88 SA3s aged 45 and over, the average 10-year incidence of diabetes in the period of 2006–2017 was 6.32% (95% CI, 2.76-12.27%). The average prevalence of obesity across all SA3s was 20.24% (10.36-31.40%). The average percentage of the population with insufficient physical activities was 18.70% (10.39-25.66%), and only 4.43% (1.45 -23.11%) of the population walk to work in metropolitan areas.

## Correlation Between Diabetes and Associated Factors

As shown in **Figure 2**, the prevalence of obesity and the percentage of the population with insufficient physical activities showed a strong positive correlation with T2DM incidence (obesity prevalence: $r$=0.62, $p$<0.001, insufficient physical activities: $r$=0.79, $p$<0.001). The percentage of the population walking to work showed a negative correlation with T2DM incidence ($r$=-0.44, $p$<0.001) in metropolitan areas.

## Geographical Variation of Diabetes Incidence and Associated Factors

Geographical variations in T2DM incidence and associated factors were shown in **Figure 3**. T2DM incidence presented a significant uneven geographical distribution (Univariate Moran's I=0.52; $p$=0.001). Similar findings were also identified in the geographical distribution of prevalence of obesity (Univariate Moran's I=0.67; $p$=0.001), percentage of the population with insufficient physical activities (Univariate Moran's I=0.59; $p$=0.001) and percentage of the population walking to work in metropolitan areas (Univariate Moran's I=0.44; $p$=0.001).

The choropleth maps (**Figure 3**) shows that the north-western regions of NSW and the western regions of Sydney had relatively high T2DM incidence, high obesity prevalence, a high percentage of the population with insufficient physical activities and a low percentage of the population walking to work. In comparison, areas along the coastal lines had relatively low T2DM incidence, low prevalence of obesity, low percentage of the population with insufficient physical activities and a high percentage of the population walking to work.

The highest T2DM incidence was found in Mount Druitt (**Figures 3A, E**), which also showed the highest obesity prevalence (**Figures 3B, F**) and the second-highest percentage of the population with insufficient physical activities (following its neighbour Blacktown-North, **Figures 3C, G**). Consistently, both Blacktown-North and Mount Druitt showed the lowest percentage of the population walking to walk (**Figures 3D, H**). In contrast, the lowest T2DM incidence was found in Richmond Valley-Coastal and Manly (**Figures 3A, E**), which also showed

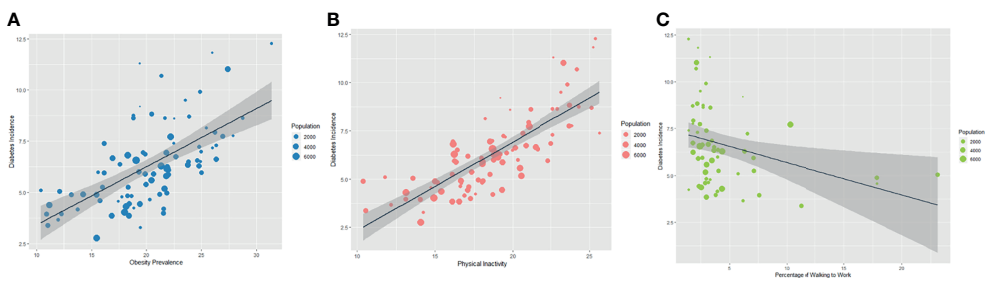


**FIGURE 2** | Association in Prevalence of Obesity **(A)** and Physical Inactivity Rate **(B, C)** with Incidence of Diabetes **(A)** shows the correlation between T2DM incidence and obesity prevalence with a spearman correlation coefficient of 0.62 ($p < 0.001$); **(B)** shows the correlation between T2DM incidence and percentage of the population with insufficient physical activities with a spearman correlation coefficient of 0.79 ($p < 0.001$); **(C)** shows the correlation between T2DM incidence and percentage of the population walking to work with a spearman correlation coefficient of -0.44 ($p < 0.001$).

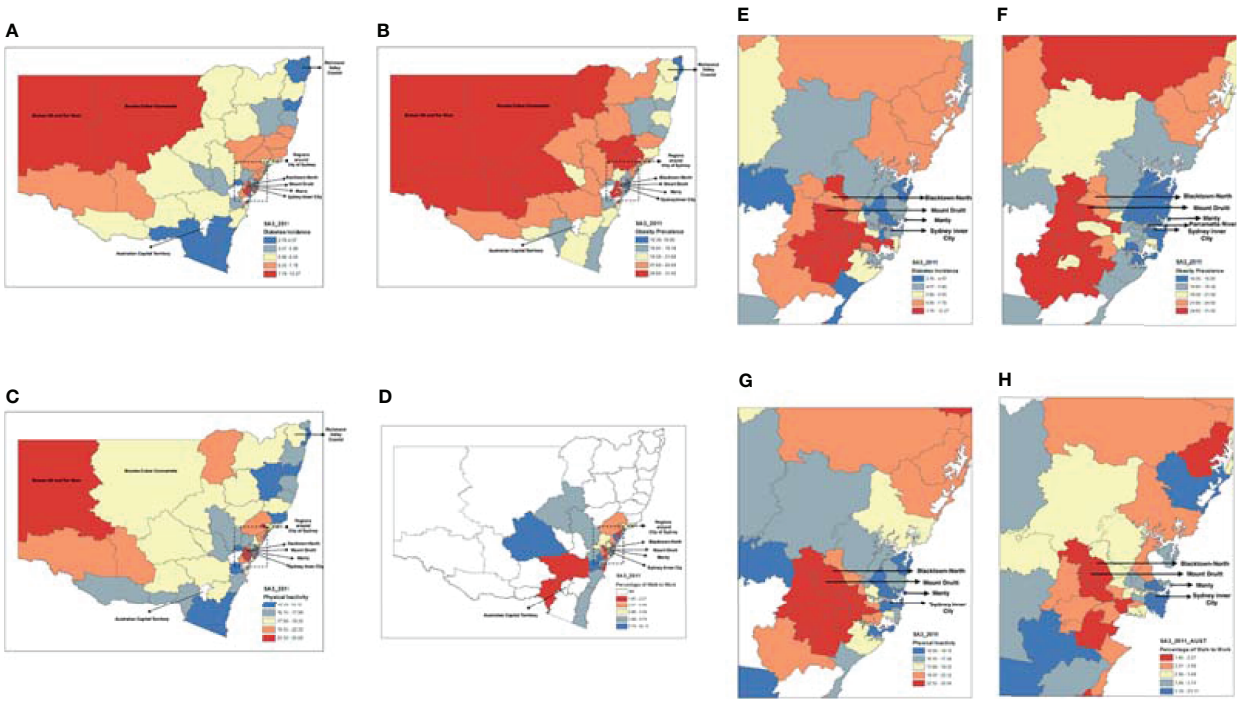


**FIGURE 3** | Geographic Variations in the Incidence of Diabetes, Obesity Prevalence, Percentage of the Population with Insufficient Physical Activities and Percentage of the Population Walking to Work. **(A)** shows the variations in T2DM incidence at SA3 level with a Univariate Moran's I of 0.52 (p=0.001) while **(E)** shows T2DM incidence in the magnified Sydney region; **(B)** shows the variance of obesity prevalence at SA3 level with a Univariate Moran's I of 0.67 (p=0.001) while **(F)** shows obesity prevalence in the magnified Sydney region, and by comparing between **(A, B)**, it can be identified a very similar pattern between them with a Bivariate Moran's I of 0.37 (p=0.001) and an intraclass correlation coefficient of 0.6 (p<0.001); **(C)** shows the variance of the percentage of the population with insufficient physical activities at SA3 level with a Univariate Moran's I of 0.59 (p=0.001) while **(G)** shows the percentage of the population with insufficient physical activities in the magnified Sydney region, by comparing between **(A, C)**, it can be identified a very similar pattern between them with a Bivariate Moran's I of 0.54 (p=0.001) and an intraclass correlation coefficient of 0.8 (p < 0.001); **(D)** shows the variance of the percentage of the population walking to work at SA3 level with a Univariate Moran's I of 0.44 (p=0.001) while **(H)** shows the percentage of the population walking to work in the magnified Sydney region, and by comparing between **(E, H)**, it can be identified a similar pattern between them with a Bivariate Moran's I of -0.23 (p=0.001) and an intraclass correlation coefficient between T2DM incidence and percentage of the population walking to work of -0.47 (p < 0.001); by comparing between, it can be identified a very similar pattern between them with and an intraclass correlation coefficient of 0.61 (p < 0.001).

the lowest obesity prevalence. In addition, Sydney Inner City (**Figure 3G**) showed the lowest percentage of the population with insufficient physical activities. In Southeast Sydney, SA3s with high T2DM incidence also presented high obesity prevalence and physical inactivity rate (**Figures 3E–G**). Two SA3s, Bourke-Cobar-Coonamble, and Broken Hill, together with far west regions, showed very high T2DM incidence (**Figure 3A**).

## Colocation Between the 10-Years Incidence of T2DM and Associated Factors

Results of the spatial correlation analyses indicate significant colocation between T2DM incidence and its associated factors, such as the prevalence of obesity (Bivariate Moran's I=0.37, p=0.001), the percentage of the population with insufficient physical activities (Bivariate Moran's I=0.54, p=0.001) and the percentage of the population walking to work in metropolitan areas (Bivariate Moran's I=-0.23, p=0.001, **Figure 3**). Similarly, intraclass correlation analysis based on the choropleth maps showed a significant spatial correlation between T2DM incidence and its associated factors (p<0.001 for all, **Figure 3**).

# DISCUSSION

In this large-cohort GIS study of Australians aged 45 and above, we identified significant geographical variations in T2DM incidence across NSW, Australia. We demonstrate that T2DM incidence is significantly correlated with obesity prevalence and physical inactivity rate at a population level.

The geographical variation and spatial clustering of T2DM incidence identified in this study are consistent with the results from previous studies that compare the diabetes prevalence among major cities and remote areas in Australia (40). Besides, the very high T2DM incidence in the outback region of NSW could result from the arid local climate and the lack of health resources in these regions (41).

Our finding that the onset of T2DM was closely associated with obesity and physical inactivity is consistent with the findings from existing studies (13, 42, 43). Although multiple epidemiological studies identified obesity as the most significant associated factor for T2DM due to its pro-inflammatory contribution to the development of insulin resistance and disease progression (44, 45), our study revealed that physical inactivity rate is more important contributing factor to T2DM than obesity in an Australian population. This is supported by Ansari (42) who found that physical activities, including stair climbing and cycling, may reduce the risk of T2DM at a population level.

Previous studies have identified that the spatial variance of T2DM, obesity and physical inactivity can result from a few socioeconomic factors, such as income, education and occupational status (46, 47). It is found that one's education level is most likely to affect one's understanding and knowledge of the health benefits of preventative behaviors (48). Low income and education level have also shown to be associated with a high risk of metabolic system, leading to over-weight and obesity (49, 50). In addition, employment status categorized by occupation, has shown an inverse relation with glucose intolerance (47, 51). Therefore, more educational resources and occupational guidance can be provided to population in the high-risk regions for T2DM control.

The major contribution of this study is that we identified a significant colocation of T2DM incidence with obesity prevalence and physical inactivity rate at a population level. A population with a high prevalence of obesity (BMI>30kg/$m^2$) and a high percentage of insufficient activity (physical activities of <5 sessions/week) was strongly associated with a high incidence of T2DM. To our knowledge, our study was the first to investigate the spatial correlation of T2DM with its associated lifestyle factors, contributing to the sociocultural perspective of T2DM prevention. The advances in technology and transportation, long hours of sedentary office work, and increasing access to processed food increase the risk of obesity and physical inactivity (52), leading to an increased risk of T2DM onset in adults. For instance, in our study, the Mount Druitt region has the highest obesity prevalence, the second-highest percentage of population physical inactivity; therefore, substantial changes in public policies to create an environment that promotes the wellbeing of the whole community is a priority in the region. Further, the government may consider allocating resources for health promotion campaigns and increasing accessible exercise facilities at the workplace to reduce T2DM incidence (53). Another strength of this study is that the use of a sizable study cohort with 232,064 participants in Australia with a long follow-up period, and the comprehensiveness of the captured information for each participant, can lead to more reliable study findings. T2DM incidence was captured based on the Medicare record system, which effectively minimized the recall bias from the participants, especially the elderly.

However, the study has a number of limitations. First, our definition of T2DM might overlook cases of gestational diabetes. Second, our study was conducted based on the assumption that participants did not move to a new location, and their body weights were relatively stable during the study period. Third, the data of working to work used in this study only relates to the regions around the City of Sydney not the whole of NSW and is limited to the people who are employed. Fourth, data for obesity prevalence and physical inactivity rate were collected from individual-level questionnaires with the response rate of only 18%, raising issues regarding the overall representativeness of the recruited participants from each SA3 region. But we found that the characteristics of participants in our study are, in fact, very similar to the characteristics presented in the New South Wales Population Health Survey conducted by the NSW government (54).

In conclusion, this study is a large-scale GIS study that addressed the geographical disparities in T2DM incidence and its associations with the prevalence of obesity and physical inactivity across an Australian state. This study highlights that a high prevalence of obesity and physical inactivity in a population may contribute to a high incidence of T2DM in the population. Community-based intervention on healthy lifestyles and behaviors should be prioritized to help control T2DM incidence in a population.

## DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: This research was completed using data collected through the 45 and Up Study (www.saxinstitute.org.au) supplied by Services Australia and the Australian Bureau of Statistics (ABS). The 45 and Up Study is managed by the Sax Institute. Requests to access these datasets should be directed to www.saxinstitute.org.au.

## AUTHOR CONTRIBUTIONS

JW was in charge of the project experiments and analysis, and writing the draft for the paper. YW was responsible for assuring the experiment the results are consistent and modifying the paper. XX was providing method advice and tool advice to make sure appropriate data analysis. XS is responsible for data preprocessing to extract proper data for our project. MH and LZ supervised the project, gave advice on the techniques and direction advice on the project, and helped in paper revision. All authors contributed to the article and approved the submitted version.

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# Aerobic Physical Exercise Improves Exercise Tolerance and Fasting Glycemia Independent of Body Weight Change in Obese Females

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Obesity is associated with increased risk of several chronic diseases and the loss of disease-free years, which has increased the focus of much research for the discovery of therapy to combat it. Under healthy conditions, women tend to store more fat in subcutaneous deposits. However, this sexual dimorphism tends to be lost in the presence of comorbidities, such as type 2 diabetes mellitus (T2DM). Aerobic physical exercise (APE) has been applied in the management of obesity, however, is still necessary to better understand the effects of APE in obese female. Thus, we investigated the effect of APE on body weight, adiposity, exercise tolerance and glucose metabolism in female ob/ob mice. Eight-weeks-old female wild-type C57BL/6J and leptin-deficient ob/ob mice ($Lep^{ob}$) were distributed into three groups: wild-type sedentary group (Wt; n = 6), leptin-deficient sedentary group ($Lep^{ob}$S; n = 5) and leptin-deficient trained group ($Lep^{ob}$T; n = 8). The $Lep^{ob}$T mice were subjected to 8 weeks of aerobic physical exercise (APE) at 60% of the maximum velocity achieved in the running capacity test. The APE had no effect in attenuating body weight gain, and did not reduce subcutaneous and retroperitoneal white adipose tissue (SC-WAT and RP-WAT, respectively) and interscapular brown adipose tissue (iBAT) weights. The APE neither improved glucose intolerance nor insulin resistance in the $Lep^{ob}$T group. Also, the APE did not reduce the diameter or the area of RP-WAT adipocytes, but the APE reduced the diameter and the area of SC-WAT adipocytes, which was associated with lower fasting glycemia and islet/pancreas area ratio in the $Lep^{ob}$T group. In addition, the APE increased exercise tolerance and this response was also associated with lower fasting glycemia in the $Lep^{ob}$T group. In conclusion, starting APE at a later age with a more severe degree of obesity did not attenuate the excessive body

weight gain, however the APE promoted benefits that can improve the female health, and for this reason it should be recommended as a non-pharmacological therapy for obesity.

**Keywords: obesity, insulin resistance, physical exercise, female, leptin deficiency**

## INTRODUCTION

Obesity is characterized by an exaggerated accumulation of body fat in white adipose tissue (WAT) accompanied by a different distribution in its body deposits, implying the increase in the visceral WAT/subcutaneous WAT ratio. Under healthy conditions, women tend to store more fat in subcutaneous deposits, while men are prone to greater visceral fat deposition (1). However, in the presence of comorbidities, such as type 2 diabetes mellitus (T2DM), this sexual dimorphism tends to be lost, so that 70% of women with T2DM have visceral obesity versus 40% of men (2). It is no coincidence that in both women and men, the new distribution of WAT in obesity is associated with increased risk of several chronic diseases and the loss of disease-free years (3).

The percentage of visceral WAT in obese subjects has a strong correlation with insulin resistance and deficient control of glucose metabolism, which in turn increases the risk of cardiovascular disease (4–6). The higher percentage of visceral WAT is followed by an alteration in the profile of adipokines produced by the individual. Also, the WAT of an obese person shows infiltration with lymphocytes and macrophages and characteristics of a subclinical inflamed tissue (7). Although the mechanism involved in increasing cardiovascular risk are the same in both sexes, it has been demonstrated that women showed a risk of death from cardiovascular disease 1.5 times higher than men. When diagnosed with obesity and T2DM, the risk of myocardial infarction followed by death was 3 to 6 times higher (8).

Different strategies have been applied in the management of obesity and the prescription of aerobic physical exercise (APE) has been very efficient for this purpose. In this sense, there is already much evidence to support the notion that in overweight or obese individuals, the later physical activity is incorporated into the routine, the higher the metabolic cost, as numerous health-related variables worsen in short periods, especially in those who already have metabolic damage (for review, please read reference 9). Among the many benefits of APE, are the reduction in adiposity and improvement in glucose metabolism (9–12), reduction in pro-inflammatory adipokines and higher secretion of anti-inflammatory ones (adiponectin and IL-10) (13), and the increase in exercise tolerance regardless of weight loss (14). Regarding the sex, both males and females respond positively to APE for the control of blood glucose (15, 16), body mass (17, 18), blood pressure (19) and lipid profile (20, 21), which are crucial to maintaining cardiovascular health. But the magnitude of response, the type of exercise and the time taken to obtain the benefits should differ between males and females, mainly due to the female hormones (22).

The studies performed with women or female models is increasing, however it is still necessary to better understand the effects of APE in obese female (23). Considering APE benefits to the characteristics of the female organism, the purpose of this study was to investigate the effect of APE on body weight, adiposity, exercise tolerance and glucose metabolism in female ob/ob mice, a rodent lineage lacking in leptin secretion that presents a phenotype of obesity at birth.

## MATERIALS AND METHODS

### Animals

Eight-week-old female wild-type C57BL/6J and leptin-deficient mice ($Lep^{ob}$) were obtained from the Laboratório de Gastroenterologia Clínica e Experimental (LIM-07) of University of São Paulo. The animals were separated into three groups: wild-type sedentary group (Wt; n = 6), leptin-deficient sedentary group ($Lep^{ob}S$; n = 5) and leptin-deficient trained group ($Lep^{ob}T$; n = 8). Animals were kept under the same housing conditions (12- h light/12- h dark cycle, temperature 22 ± 2°C) with water and food *ad libitum*. All procedures were approved by the Ethics Committee of School of Arts, Sciences and Humanities of University of São Paulo (# 001/2017). The *in vivo* evaluations were conducted during non-ovulatory phase of the estrous cycle.

### Aerobic Physical Exercise

Animals were submitted to aerobic physical exercise (APE) during the dark cycle (i.e., during their active period) on a motorized treadmill for 1 h/day at 60% of maximal speed achieved in the running capacity test (speed range between 0.6 km/h and 0.8 km/h), five times per week for eight weeks (24). The duration was progressively increased, starting with 30 minutes in the first week and reaching 60 minutes in the fourth week. This training duration was maintained until the end of the protocol. To minimize the influence of the treadmill stress, Wt and $Lep^{ob}S$ mice were placed on the treadmill for 5 min twice weekly at 0.2 km/h.

### Exercise Tolerance Test

Running capacity was assessed before, in the fourth and in the eighth week of APE using a progressive test without incline on a treadmill until exhaustion as described by Ferreira et al. (24). The initial speed was 0.4 km/h and the speed was increased by 0.2 km/h every three minutes until exhaustion of the animal, which was characterized by the impossibility of maintaining the standard rate. In the 4th week of the protocol, the test was assessed only in groups trained to readjust the intensity of APE.

### Indirect Calorimetry

In the eighth week, the animals were acclimatized in the Oxylet Calorimetry System (Panlab, Barcelona, Spain) and the

measurements were done during rest. Firstly, the animals were fasted (2-h) and then the volumes of oxygen consumption ($VO_2$) and carbon dioxide production ($VCO_2$) were measured during 30 min of resting. The non-protein respiratory exchange ratio (RER), a measurement of metabolic substrate preference, was calculated as the molar ratio of $VCO_2$ to $VO_2$. Energy expenditure (EE) was calculated by the formula [3.815 + (1.232 x R)] x $VO_2$ x 1.44 and the result was expressed as $kcal.Kg^{-1}.min^{-1}$. The rate of oxidation of carbohydrate (CHO) and lipids (LIP) were calculated using the formulas (25): CHO = (4.55 x $VO_2$) - (3.21 x $VCO_2$) and LIP= (1.67 x $VO_2$) - (1.67 x $VCO_2$). Both data were expressed as $mg.min^{-1}.kg^{-1}$.

After the resting measurement, the animal was subjected to the exercise tolerance test using the protocol described previously. The volumes of $VO_2$ and $VCO_2$ were continuously measured until the animal reaches the exhaustion. The maximum $VO_2$ ($VO_2max$) and $VCO_2$ ($VCO_2max$) were considered the average obtained in the last stage of the test. The running intensity at which $VO_2max$ was reached (iVO2max) was measured as described by Machado et al. (26). The results were expressed as $mg.min^{-1}.kg^{-1}$.

## Body Weight and Food Intake

The animals were weighed weekly on a digital scale (Gehaka/ model BG4001, São Paulo, Brazil), on the same day and time. In addition to the evolution of body weight, we calculated the body weight gain through the difference between final body weight (week 8) and initial body weight (week 0). The 24-h food intake was determined weekly throughout the study in mice's groups that were housed in the same cage.

## Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

Fasting glycemia, GTT and ITT were performed after the 8-week of APE protocol. The experiments were done in awake animals after an 8-h fast. The glucose load (1 g/kg body weight) was injected as a bolus intraperitoneally, and the blood glucose levels were determined in caudal blood sampled at 0, 15, 30, 60, 90 and 120 min after glucose infusion. The glucose concentration was determined using a glucometer (AccuChek Advantage Roche Diagnostics). After 72 h of GTT test, a similar procedure was performed for ITT. The insulin load (0.75 U/kg body weight) was injected as a bolus intraperitoneally, and the blood glucose levels were determined in caudal blood samples collected at 0, 5, 10, 15, 20, 25 and 30 min after injection. The values obtained between 5 and 30 min were used to calculate the rate constant for the disappearance of plasma glucose (kITT) according to the method proposed by Bonora et al. (27).

## Tissue and Blood Collection

Forty-eight hours after the end of the last APE session, the animals were sacrificed with an intraperitoneal injection of pentobarbital sodium (4 mg/100 g body weight) following exanguination. The animal was weighed and then the subcutaneous (inguinal) and visceral (retroperitoneal) WAT fat pads (SC-WAT and RP-WAT, respectively), interscapular brown adipose tissue (iBAT), skeletal muscles (gastrocnemius, soleus and plantaris) and pancreas were harvested and weighed. The splenic portion of the pancreas was used for the histological analysis.

## Histological Analysis

The morphology of adipocytes was measured in paraffin sections of SC-WAT and RP-WAT fat pads (5μm) stained with hematoxylin and eosin (Sigma). Digital images from 50 adipocytes per animal were obtained using a light microscope (Axio observator. A1, Zeiss, Jena, Germany), at 400x magnification. After digitalization, adipocyte diameter and area were traced and calculated using a computerized morphometric analysis system (Image Pro-Plus 4.1; Media Cybernetics, Silver Spring, MD, USA). In addition, the splenic portion of pancreas included in paraffin was cut (4 μm) and stained with hematoxylin and eosin (Sigma). The images of pancreatic islets and pancreas were acquired in a digital light microscopy (Axio observator. A1, Zeiss, Jena, Germany), at 400x and 100x magnification, respectively. The pancreatic islets area (AI) and the islet/pancreas area ratio (AI/AP) were analyzed in the Image Pro-Plus 4.1 program (Media Cibernetics Inc, Rockville, USA). All analysis was done by a single observer (Boschetti D) blinded to mice identities.

## Statistical Analyses

All values are expressed as mean ± SEM. Data were analyzed with one-way or two-way analyses of variance (ANOVA). The Tuckey *post hoc* test was used to determine differences between means when a significant change was observed using ANOVA. Pearson correlation was used to analyze the association between variables. *p* value equal to or less than 0.05 was statistically significant (GraphPad Prism, v.7.0).

# RESULTS

## Body Weight, Tissues Weight and Food Intake

From the beginning until the end of the experimental protocol, body weight of $Lep^{ob}S$ and $Lep^{ob}T$ were higher than Wt group (**Figure 1A**). Also, $Lep^{ob}S$ and $Lep^{ob}T$ groups showed higher body weight gain compared with Wt group (**Figure 1B**). Both results confirmed that APE did not reduce the body weight gain typically observed in $Lep^{ob}$ mice. In addition, both $Lep^{ob}S$ and $Lep^{ob}T$ groups increased daily food intake compared with Wt group, which revealed that PA did not counteract the hyperphagic response in the $Lep^{ob}T$ group (**Figure 1C**).

As shown in **Table 1**, the groups $Lep^{ob}S$ and $Lep^{ob}T$ increased the weight of both SC-WAT and RP-WAT compared with Wt group. Although the $Lep^{ob}T$ group had shown 18% less RP-WAT weight compared to the $Lep^{ob}S$ group, this difference was not statistically significant. In addition, the weight of iBAT was higher in the $Lep^{ob}T$ group compared with Wt group. Regarding the weight of skeletal muscles, it was observed that both gastrocnemius and plantar muscles of the animals of $Lep^{ob}S$ and $Lep^{ob}T$ were lighter compared with Wt group, while no

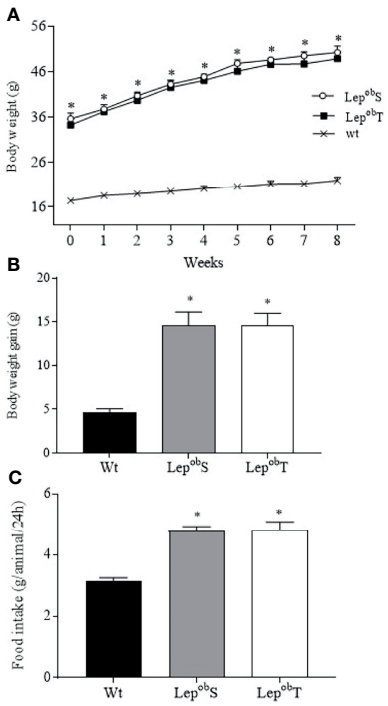


**FIGURE 1** | Body weight evolution **(A)**, body weight gain **(B)**, and food intake **(C)**. Wt (n = 6), Lep$^{ob}$S (n = 5) and Lep$^{ob}$T (n = 8). Error bars indicate the SEM. *p ≤ 0.05 vs. Wt. The results of body weight evolution were compared by two-way ANOVA and body weight gain and food intake by one-way ANOVA plus Tuckey *post hoc* test.

difference was found in the soleus muscle. However, the gastrocnemius and soleus muscles were heavier in the Lep$^{ob}$T group when compared with the Lep$^{ob}$S. Finally, there was no statistical difference in the pancreas weight among the groups (**Table 1**).

Morphometric analyses of the WAT are shown in **Figure 2**. Increased adipocyte diameter and area were observed in the SC-WAT fat pad in the Lep$^{ob}$S group compared with Wt group (**Figures 2A, C, E**). The APE was efficient in decreasing both the diameter and the area of adipocytes in Lep$^{ob}$T animals compared to Lep$^{ob}$S (**Figures 2A, C, E**). The hypertrophy of adipocyte was also found in the RP-WAT as showed by the higher diameter and area of adipocyte in the Lep$^{ob}$S group compared with Wt group. However, APE did not prevent these responses in the Lep$^{ob}$T animals (**Figures 2A, D, E**).

We also evaluated pancreatic islet morphometry and although the pancreas weight did not differ statistically among groups, the pancreatic islets of Lep$^{ob}$S (3.2 x $10^5$ ± 1 x $10^5$ μm$^2$) were almost three times larger than those in groups Wt (1.24 x1 $0^5$ ± 0.98 x $10^5$ μm$^2$) and Lep$^{ob}$T (1.21 x $10^5$ ± 0.43 x $10^5$ μm$^2$; **Figure 3A**). In addition, the animals in the Lep$^{ob}$S group had a higher islet/pancreas area ratio compared with Wt group, which was countered by APE in the Lep$^{ob}$T group (**Figure 3B**). Also, we observed a positive correlation between islet/pancreas area ratio and SC-WAT adipocytes area (r = 0.56, p = 0.0041) (**Figure 3C**).

## Metabolic Parameters

**Table 2** shows it was showed the results of indirect calorimetry during the rest and at maximal running test. There was no difference in the values of $VO_2$, $VCO_2$, RER and EE among groups during rest. Also, carbohydrate and lipid oxidation rates during rest were not different among groups. When the metabolic parameters were evaluated during the exercise tolerance test, reduction in $VO_2$ max, $VCO_2$ max, i$VO_2$ and EE in both Lep$^{ob}$S and Lep$^{ob}$T groups was found when compared with Wt group. However, the time until exhaustion (exercise performance) and the running intensity at which $VO_2$ max was reached (iVO2) were higher in the Lep$^{ob}$T compared with Lep$^{ob}$S group. These results revealing that the leptin deficiency-induced obesity caused significant damage in the aerobic capacity, but that the APE mitigated this loss in the Lep$^{ob}$T group (**Table 2**).

Glucose metabolism was evaluated at the end of the experimental protocol, and as shown in the **Figure 4A**, the Lep$^{ob}$S and Lep$^{ob}$T groups increased fasting glycemia (127.2 ± 3.88 mg/dL and 109.5 ± 63.56 mg/dL, respectively) compared with Wt group (84.5 ± 1.61 mg/dL). In addition, fasting glycemia was significantly lower in Lep$^{ob}$T group compared with Lep$^{ob}$S group. Reductions in the glucose tolerance were observed in the Lep$^{ob}$S and Lep$^{ob}$T groups compared with Wt group (**Figures 4B, C**). In the same way, damages in the insulin resistance were found in Lep$^{ob}$S and Lep$^{ob}$T groups compared with Wt group (**Figures 4D, E**).

A negative correlation was observed between fasting blood glucose and exercise performance (minutes) (r = -0.84, p = 0.0001) (**Figure 5A**). A positive correlation was observed between fasting blood glucose and SC-WAT adipocytes area (r = 0.82, p = 0.0007) (**Figure 5B**), fasting blood glucose and RP-WAT adipocytes area

**TABLE 1** | The weight of adipose tissue, skeletal muscles, and pancreas.

| | Wt (n = 6) | Lep$^{ob}$S (n = 5) | Lep$^{ob}$T (n = 8) |
|---|---|---|---|
| SC-WAT (mg/g) | 17.47 ± 2.43 | 104.07 ± 2.08* | 110.11 ± 6.35* |
| RP-WAT (mg/g) | 5.75 ± 1.08 | 43.52 ± 8.44* | 35.63 ± 2.41* |
| iBAT (mg/g) | 3.57 ± 0.49 | 17.06 ± 4.90 | 21.00 ± 5.63* |
| Gastrocnemius (mg/g) | 7.93 ± 0.55 | 2.37 ± 0.18* | 3.72 ± 0.2*# |
| Plantar (mg/g) | 1.12 ± 0.11 | 0.33 ± 0.05* | 0.59 ± 0.13* |
| Soleo (mg/g) | 0.78 ± 0.14 | 0.31 ± 0.04 | 1.08 ± 0.27# |
| Pancreas (mg/g) | 20.10 ± 3.89 | 21.64 ± 8.86 | 18.85 ± 6.10 |

*Data are presented as mean ± SEM. Wt (n = 6), Lep$^{ob}$S (n = 5) and Lep$^{ob}$T (n = 8). SC-WAT, subcutaneous white adipose tissue; RP-WAT, retroperitoneal white adipose tissue; iBAT, brown adipose tissue. *p ≤ 0.05 vs. Wt; #p ≤ 0.05 vs. Lep$^{ob}$S. The results were compared by the one-way ANOVA plus Tuckey post hoc test.*

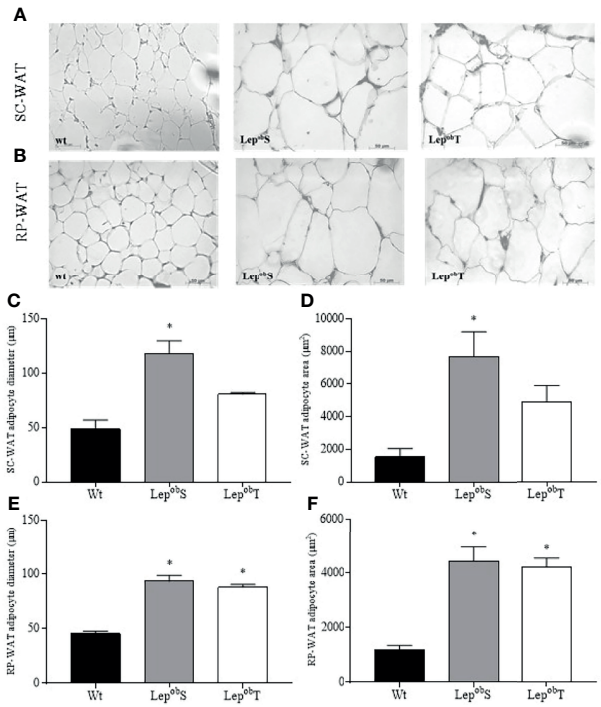


**FIGURE 2** | Representative histological sections of SC-WAT **(A)** and RP-WAT **(B)** to visualize the morphology of adipocytes at 400X magnification, SC-WAT adipocyte diameter **(C)**, SC-WAT adipocyte area **(D)**, RP-WAT adipocyte diameter **(E)**, RP-WAT adipocyte area **(F)**. Wt (n = 5), Lep$^{ob}$S (n = 4) and Lep$^{ob}$T (n = 4). Error bars indicate the SEM. *p ≤ 0.05 vs. Wt. The results were compared by the one-way ANOVA plus Tuckey *post hoc* test.

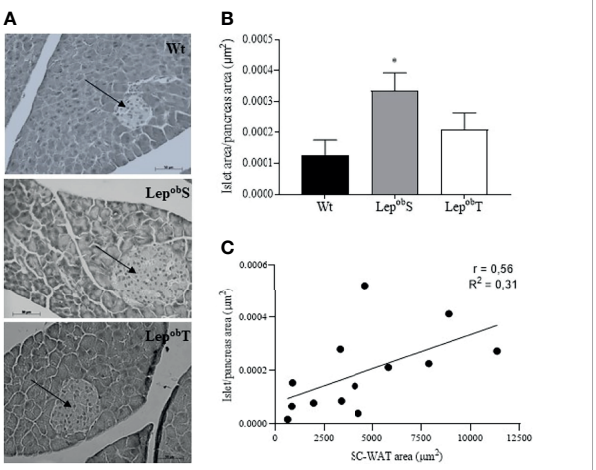


**FIGURE 3** | Representative histological sections of pancreas to visualize the morphology of pancreatic islet at 400X magnification **(A)**, pancreatic islet area to pancreas area ration **(B)**, and correlation between pancreatic islet area to pancreas area ratio and SC-WAT adipocytes area **(C)**. Wt (n = 6), Lep$^{ob}$S (n = 5) and Lep$^{ob}$T (n = 5). The arrows point to the pancreatic islets. Error bars indicate the SEM. *p ≤ 0.05 vs. Wt. The results were compared by the one-way ANOVA plus Tuckey *post hoc* test.

(r = 0.83, p = 0.001) and fasting blood glucose and islet area to pancreas area ratio (r = 0.68, p = 0.004) (**Figures 5C, D**).

## DISCUSSION

The growing number of obese women and the negative repercussions for health has motivated the search for understanding the prevention and treatment of obesity. Despite that our group previously reported a reduction in body weight gain in male ob/ob mice with the same age and exercise protocol as applied here (28, 29), we revealed that APE did not avoid the excessive body weight gain in female ob/ob mice. We also observed that females ob/ob of the present study seem to gain more weight than males from our previous study (28, 29). The sex-dependent obesity response in ob/ob mice has been characterized in studies with metabolomic and lipidomic analysis (30, 31), and our research adds novel knowledge by suggesting that the APE-mediated benefits on the body weight of ob/ob mice is also sex dependent, which reinforces the importance of considering the differences between the sexes for the management of body weight. However, further studies are still needed to unravel such mechanisms.

The reduction of body weight has been shown in female ob/ob mice when the APE started earlier at five or six weeks of age (32, 33). In our study the animals started the exercise protocol at eight weeks old (with severe degree of obesity), which could, in part, explain the discrepancy between our and other results. The adiposity negatively influences the skeletal muscle function, including damage to force generation, gait speed and locomotor pattern (34, 35), which can increase the difficulty of exercising and early exhaustion. In this sense, our APE protocol was enough to promote some beneficial adaptations, however the earlier start of the APE may be more efficient for the control of body weight.

The unchanged body weight gain in the Lep$^{ob}$T group also can be explained by the maintenance of hyperphagia. The body weight stems from caloric intake and energy expenditure. In turn, energy expenditure is determined by resting and exercise energy expenditures and thermogenic effect of food. As observed in the results, the resting energy expenditure was not different among the groups, and despite the resting measurement consider only a short period of 24h and we did not evaluate of thermogenic effect of food, it is possible that the energy expenditure promoted by APE was not enough to reduce body weight gain because it was compensated by the high caloric intake of Lep$^{ob}$T group. According to the literature, the effect of APE on food consumption is controversial since the authors showed increases (36) or no changes in food consumption (12, 37). In this sense, our results allow us to suggest that for severe obesity cases, weight control will be efficient if the practice of physical exercise is associated with caloric restriction.

The reduction of WAT can improve insulin sensitivity and reduce the risk of T2DM (9, 12). Here, the APE decreased the diameter and the area of SC-WAT adipocytes in Lep$^{ob}$T compared to Lep$^{ob}$S, which may have contributed to the better fasting glycemia after APE. In fact, we observed a positive

**TABLE 2** | Metabolic response at rest and at maximal running test.

| | Wt (n = 6) | Lep$^{ob}$S (n = 5) | Lep$^{ob}$T (n = 8) |
|---|---|---|---|
| *Resting* | | | |
| $VO_2$ (ml.min$^{-1}$.kg$^{-1}$) | 36.08 ± 4.77 | 28.38 ± 3.15 | 31.07 ± 1.81 |
| $VCO_2$ (ml.min$^{-1}$.kg$^{-1}$) | 28.96 ± 3.75 | 22.32 ± 2.37 | 23.93 ± 1.47 |
| RER | 0.81 ± 0.05 | 0.79 ± 0.04 | 0.71 ± 0.09 |
| EE (kcal. kg$^{-1}$.min$^{-1}$) | 249.58 ± 32.36 | 195.21 ± 21.22 | 216.40 ± 13.43 |
| CHO oxidation (mg.min$^{-1}$.kg$^{-1}$) | 71.20 ± 11.83 | 57.45 ± 7.76 | 64.58 ± 5.06 |
| Lipid oxidation (mg.min$^{-1}$.kg$^{-1}$) | 11.89 ± 3.43 | 10.11 ± 2.13 | 11.93 ± 1.69 |
| *Running test* | | | |
| Exercise performance (minutes) | 35.17 ± 1.08 | 15.20 ± 1.36* | 21.25 ± 1.54*# |
| $VO_2$ max (ml.min$^{-1}$.kg$^{-1}$) | 46.78 ± 2.11 | 30.10 ± 1.39* | 32.22 ± 2.36* |
| $VCO_2$ max (ml.min$^{-1}$.kg$^{-1}$) | 45.47 ± 1.81 | 25.28 ± 0.30* | 30.67 ± 2.24* |
| RER | 0.91 ± 0.07 | 0.91 ± 0.05 | 0.92 ± 0.05 |
| i$VO_2$ (km/h) | 2.06 ± 0.06 | 0.70 ± 0.06* | 1.13 ± 0.12*# |
| EE (kcal. kg$^{-1}$.min$^{-1}$) | 324.93 ± 20.54 | 213.67 ± 10.05* | 240.70 ± 15.10* |
| CHO oxidation (mg.min$^{-1}$.kg$^{-1}$) | 66.91 ± 11.75 | 49.53 ± 5.77 | 49.55 ± 12.59 |
| Lipid oxidation (mg.min$^{-1}$.kg$^{-1}$) | 2.19 ± 4.88 | 4.79 ± 2.70 | 3.09 ± 5.21 |

*Data are presented as mean ± SEM. $VO_2$, oxygen consumption; $VCO_2$, carbon dioxide production; RER, respiratory exchange ratio; EE, energy expenditure; CHO, carbohydrate; i$VO_2$, running intensity at which $VO_2$ max was reached. The results were compared by the one-way ANOVA plus Tuckey post hoc test. *p ≤ 0.05 vs. Wt; #p ≤ 0.05 vs. Lep$^{ob}$S.*

correlation between SC-WAT adipocytes area and fasting blood glucose. Stanford et al. (10) showed that transplanting SC-WAT from exercise-trained mice in sedentary ones improved glucose tolerance and insulin sensitivity. They also observed that the deleterious effects of high-fat on glucose homeostasis were completely reversed in high-fat fed mice transplanted with SC-WAT from exercise-trained mice. On the other hand, we did not find changes in the diameter and the area of RP-WAT adipocytes, but a positive correlation between RP-WAT adipocytes area and fasting blood glucose. These data reinforce the idea that APE induced depot-specific effects, which was previously observed in genes involved in mitochondrial activity, glucose metabolism, and fatty acid uptake and oxidation by Lehnig et al. (2019) (11).

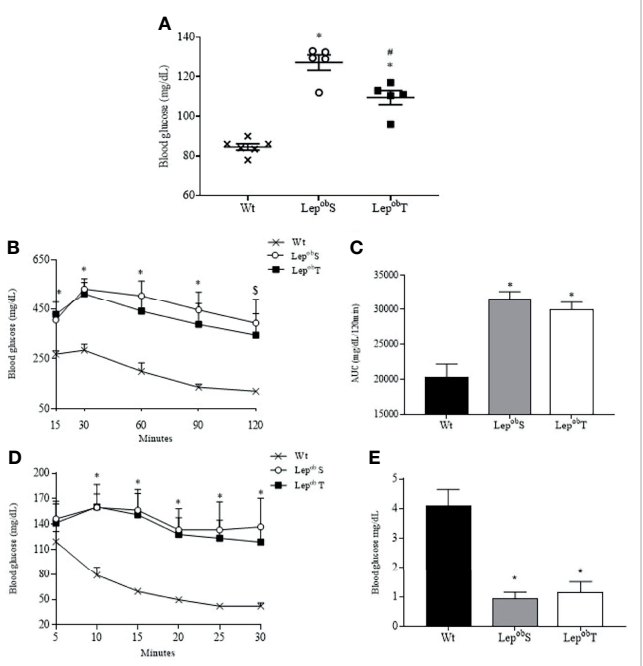


**FIGURE 4** | Fasting blood glucose **(A)**, glucose tolerance test **(B)**, area under the curve **(C)**, insulin tolerance test **(D)** and kITT **(E)**. Wt (n = 6), Lep$^{ob}$S (n = 5) and Lep$^{ob}$T (n = 5). AUC = area under the curve, kITT = glucose disappearance constant rate. Error bars indicate the SEM. *p ≤ 0.05 vs. Wt, #p ≤ 0.05 vs. Lep$^{ob}$S, $p ≤ 0.05 Wt vs. Lep$^{ob}$S. The results were compared by the one-way ANOVA (Figure A, C and E) and by the two-way ANOVA (Figure B and D) plus Tuckey *post hoc* test.

Leptin deficiency is associated with reduction in the sympathetic innervation of BAT, which results in damage of oxidative capacity and thermogenesis (38, 39). In a thermogenesis-reduced state, the conversion of brown adipocyte to white-like unilocular cells (BAT whitening) associated with lower lipid oxidation results in increased BAT (40). Although the APE is a stimulus that counteracts this typical BAT phenotype in obesity, our results showed that the APE was not efficient to reverse the high weight of the iBAT in the Lep$^{ob}$T animals.

While metabolic diseases are associated with damages in energy metabolism and reduced aerobic capacity, the greater the aerobic capacity, the lower is the risk of cardiovascular and metabolic diseases (41). The APE reduced the negative effect of leptin deficiency in the Lep$^{ob}$T group by increasing the exercise performance, the running intensity at which $VO_2$max was reached (i$VO_2$max), and the weight of soleus and gastrocnemius muscles. Furthermore, we found a negative correlation between fasting blood glucose and exercise performance (minutes), which reinforces that APE is efficient to improve glucose metabolism and that is a good therapeutic strategy to ameliorate metabolic diseases. We also expected that Lep$^{ob}$T group could improve $VO_2$max, however additional studies are necessary to understand if the $VO_2$max result is a specific pattern of female ob/ob mice and/or is due to the exercise protocol used in the treadmill test.

Since leptin contributes to the maintenance of glucose homeostasis, ob/ob mice showed damages in glycemic control. In addition, the islet size in ob/ob mice can be up to ten times higher than in control mice, depending on the age (42). Here, we found greater islet/pancreas area ratio,

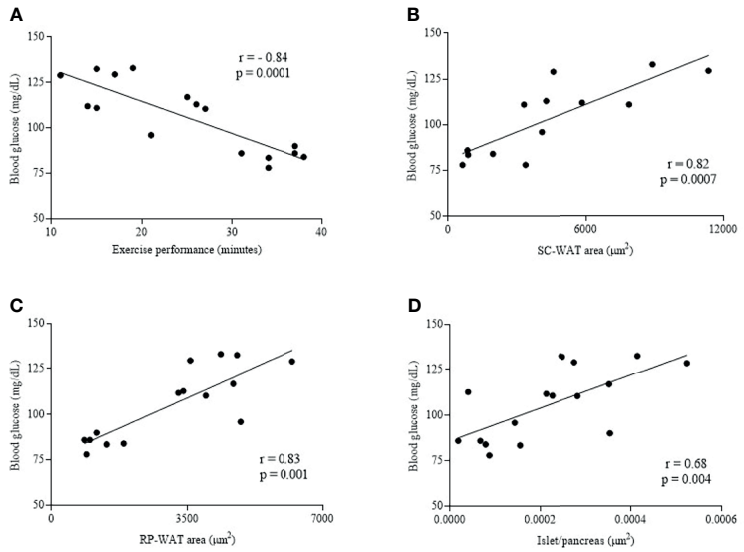


**FIGURE 5** | Correlation of fasting blood glucose and exercise performance (minutes) **(A)**, fasting blood glucose and SC-WAT adipocytes area **(B)**, fasting blood glucose and RP-WAT adipocytes area **(C)** and fasting blood glucose and islet area to pancreas area ratio **(D)**. Wt (n = 5), Lep$^{ob}$S (n = 4-5) and Lep$^{ob}$T (n = 4-5).

hyperglycemia, glucose intolerance and insulin resistance in the Lep$^{ob}$S compared with Wt group. The APE decreased the islet/pancreas area ratio and hyperglycemia in the Lep$^{ob}$T group without changes in glycemic tests. These results are different from the study of Jimenez-Maldonado et al. (43), in which it was observed that healthy male rats trained with high or moderate intensity APE showed β-cells hypertrophy, while the moderate intensity increased the number of β-cells per islet, without change on islet/pancreas area ratio.

Interesting that the positive correlation between islet/pancreas area ratio and fasting blood glucose and islet/pancreas area ratio and SC-WAT adipocytes area revealed that the smallest islet size was associated with the lower blood glucose levels and SC-WAT adipocytes size. Furthermore, considering that increasing in islet size is much more due to the elevated demand for insulin than the leptin deficiency (44) and the reduction of fasting glycemia, it is possible that APE has improved the glycemic control in an insulin-independent manner by the activation of AMP-activated protein kinase (AMPK) in the skeletal muscle (45), and therefore reduced the islets workload of the ob/ob animals. Further study is still needed to investigate the effect of AET on AMPK in ob/ob mice.

In conclusion, the results provide evidence that starting APE at a later age with a more severe degree of obesity did not avoid the excessive body weight gain in female ob/ob mice. However, the APE had positive effects in reducing SC-WAT adipocytes size which was associated with lower fasting glycemia and islet/pancreas area ratio. In addition, the APE increased exercise tolerance and this response was also associated with lower fasting glycemia. Thus, despite not changing the weight value on the scale, the training promoted benefits that can improve the female health, and for this reason it should be recommended as a non-pharmacological therapy for obesity.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of School of Arts, Sciences and Humanities of University of São Paulo (protocol number 001/2017).

## AUTHOR CONTRIBUTIONS

Conceptualization and design: AA-M, FE, and CO. Data collection and analysis: CM, AA, BV, LM, RP, and PF. Data curation: AA-M and FE. Manuscript drafting: DB, AA-M, and FE. Supervision: AA-M and FE. All authors contributed to the article and approved the submitted version.

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# Chlorogenic Acid-Induced Gut Microbiota Improves Metabolic Endotoxemia

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**Background:** Coffee can regulate glucose homeostasis but the underlying mechanism is unclear. This study investigated the preventive and therapeutic effects of chlorogenic acid (CGA), a polyphenol that is found in coffee, on obesity and obesity-related metabolic endotoxemia.

**Method:** Male 4-week-old C57BL/6 mice were fed either normal chow or a high-fat diet or 20 weeks and half the mice in each group were gavaged with CGA. Oral glucose tolerance tests (OGTTs) and insulin tolerance tests (ITTs) were performed. Markers of inflammation and intestinal barrier function were assayed. The composition of the gut microbiota was analyzed by 16S rRNA high-throughput pyrosequencing. The role of CGA-altered microbiota in metabolic endotoxemia was verified by fecal microbiota transplantation.

**Results:** CGA protected against HFD-induced weight gain, decreased the relative weight of subcutaneous and visceral adipose, improved intestinal barrier integrity, and prevented glucose metabolic disorders and endotoxemia ($P$ <0.05). CGA significantly changed the composition of the gut microbiota and increased the abundance of short chain fatty acid (SCFA)-producers (e.g., *Dubosiella*, *Romboutsia*, *Mucispirillum*, and *Faecalibaculum*) and *Akkermansia*, which can protect the intestinal barrier. In addition, mice with the CGA-altered microbiota had decreased body weight and fat content and inhibited metabolic endotoxemia.

**Conclusion:** CGA-induced changes in the gut microbiota played an important role in the inhibition of metabolic endotoxemia in HFD-fed mice.

**Keywords: obesity, gut microbiota, chlorogenic acid, lipopolysaccharide, metabolic endotoxemia, insulin resistance**

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## INTRODUCTION

The worldwide incidence of obesity has risen in recent decades together with the rapid economic development of societies and changes in lifestyle and dietary habits. About 2 billion adults worldwide are estimated to be overweight or obese, and the proportion of overweight and obese children is also on the rise (1). Obesity results from a long-term positive energy balance, an ongoing

increased food intake and decreased energy expenditure, and the influence of genetic and environmental factors. Long-term obesity leads to a series of metabolic diseases, such as type 2 diabetes, cardiovascular disease, nonalcoholic fatty liver disease, hypertension, malignant tumors, and others has become a global public health threat (2).

Obesity is characterized by chronic low-grade inflammation. In animal models, high-fat diets (HFDs) eventually lead to gut microbiota imbalance, an increase of intestinal permeability, and entry of lipopolysaccharide (LPS) into the blood, inducing the release of proinflammatory factors, such as TNF-α, IL-1β and monocyte chemotactic protein (MCP)-1 (3, 4). The increase of LPS and release of proinflammatory cytokines results in metabolic endotoxemia, which promotes the development of metabolic syndrome, including impaired insulin signaling and chronic low-grade inflammation. Inhibiting the occurrence of chronic low-grade inflammation would be significant in the management of obesity (5, 6).

Because obesity is largely influenced by lifestyle, dietary habits play an important role in its prevention and treatment. Chlorogenic acid (CGA) is a widely distributed polyphenol with a high content in coffee, fruits, and vegetables. It has been reported to have multiple health benefits including antioxidant and anti-inflammatory properties (7–9). Recent human and animal studies have shown that CGA can reduce body weight and decrease serum total cholesterol (TC), total triglyceride (TG) levels (10, 11). A twice-daily dietary supplement based on CGA improved blood glucose, insulin sensitivity, and other metabolic parameters (TC, TG, and visceral adipose tissue, etc.) in overweight patients (12).

However, as most of those studies have been observational, the underlying mechanisms of those effects have not been determined. To address that issue, we investigated the effects of CGA on obesity, gut microbiota, and metabolic endotoxemia. The purpose of this study was to verify the effectiveness of CGA in the treatment of metabolic syndrome.

# METHODS

## Animals

Procedures involving animals followed the guidelines of the Institutional Animal Care and Use Committee of China Medical University Affiliated Shengjing Hospital and were approved by same (approval no. 2020PS034K). Four-week-old male C57Bl/6 mice were purchased from Beijing HFK Bioscience Co. Mice were kept in a specific pathogen-free hospital facility with a 12-h dark/light cycle, a feeding temperature maintained at 18°C to 19°C, and a humidity maintained at 40% to 70%. Mice were fed normal chow and water for 1 week before being randomly divided into four groups. The groups included normal chow with 150 mg/kg CGA dissolved in water and administered daily (NCGA), normal chow with the water vehicle by garage (NFD), an HFD with 150 mg/kg CGA administered daily by gavage (HCGA), and HFD with water by gavage. The general condition, food, and water intake of the mice were observed every day. The mice were weighed every week. Food intake was measured as previously described (13). The mice were euthanized after 20 weeks of treatment.

## Fecal Microbiota Transplantation

Ten-week-old male C57Bl/6 mice were acclimated to a normal chow for 1 week and then randomly divided into four groups that were transplanted with HFD-group microbiota (HFD-R), or HCGA-group microbiota (HCGA-R). Mice in the microbiota transplantation group were given an antibiotic cocktail and then recolonized with donor microbiota as previously reported (13).

## Oral Glucose Tolerance Test (OGTT)

Mice were fasted for 12 h before taking a blood sample from the tail vein for measurement of blood glucose concentration at time zero, after which 1.5 g/kg of a 0.4 g/ml glucose solution was rapidly gavaged. The blood glucose concentration was measured and recorded with a glucometer at 30, 60, 90 and 120 min after injection, a blood sugar versus time curve was calculated and the mice returned to a normal diet.

## Insulin Tolerance Test (ITT)

The mice were fasted for 6 hours before taking a blood sample from the tail vein followed by intraperitoneal injection of 0.75 μ/kg of a 0.2 μ/mL saline solution of insulin. The blood glucose concentration was measured and recorded at 15, 30, 60 and 120 min, a blood sugar versus time curve was calculated, and the mice were returned to a normal diet.

## Biochemical Analysis

Serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, monocyte chemoattractant protein (MCP)- 1, and insulin were determined with commercial enzyme-linked immunosorbent assay kits (Boster Biological Technology, Wuhan, China) read at OD 450 nm. Concentrations were determined by comparison to standard curves. Plasma lipopolysaccharide (LPS) concentrations were determined using a chromogenic Limulus amebocyte lysate endotoxin assay kit (ToxinSensor, GenScript) as previously described (5).

## Intestinal Epithelial Barrier Permeability *In Vivo*

After 12 h fasting, the mice were given 600 mg/kg fluorescein Isothiocyanate-dextran (80 mg/mL) by gavage. Blood samples were collected before and 2 h after gavage. Plasma fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 520 nm as previously described (13).

## Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Trizol (Invitrogen) was used to extract total RNA and PrimeScript RT reagent kits (TaKaRa, Mountain View, CA) were used to reverse transcribe the RNA samples. The qPCR assays were performed with SYBR Premix Ex Kit (TaKaRa) and a Bio-Rad IQ5 system as previously published using the primers described in **Supplemental Table I** (14).

## Western Blot Assays

Total protein was extracted with radioimmunoprecipitation assay and phenylmethane sulfonyl fluoride lysate buffers. The protein concentrations were determined with a bicinchoninic acid protein assay kit. The western blotting procedures were performed as previously described (14). The blots were incubated with primary antibodies (**Supplemental Table II**) at 4°C overnight. The membranes were incubated with secondary antibodies for 2 h. The blots were visualized using enhanced chemiluminescence substrate kits (Thermo Fisher Scientific, Rockford, IL, USA), and the results were read and analyzed with Image J software (https://imagej.net/Welcome).

## Histological Analysis

Tissues were fixed with 4% paraformaldehyde solution, paraffin-embedded, and cut into 4 μm serial sections. Immunofluorescence was carried out as previously reported (15), intestinal tissue sections were incubated with Claudin-1 (Abcam), diluted at 1:200 at 4°C for 12 hours, and then incubated with a secondary antibody for 1 h. Images were captured by a laser scanning fluorescence microscope (TCS SP5, Leica, Germany) at 200× magnification.

## Gut Microbiota Analysis

Total genome DNA was extracted from six samples from each study group using sodium dodecyl sulfate and cetyltrimethylammonium bromide. The 16S ribosomal (r)RNA genes of distinct regions (16S V3-V4) were amplified using specific barcoded primers. Polymerase chain reaction (PCR) assays were carried out with 15 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, and about 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s and 72°C for 5 min. Sequencing libraries were generated using TruSeq DNA PCR-Free Sample Preparation Kits (Illumina, USA) following the manufacturer's recommendations; index codes were added. The library quality was assessed with a Qubit@2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated. Microbiome sequencing data were analyzed with QIIME, a plugin-based microbiome analysis platform. Briefly, raw sequencing reads with exact barcode matches were assigned to respective samples and identified as valid sequences. Paired-end reads were merged using FLASH, Quality filtering on the raw tags was performed under specific filtering conditions to obtain high-quality clean tags according to the QIIME quality-control process. After chimera detection, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity by UCLUST. Alpha (Chao1 and Shannon) and beta diversity metrics (weighted UniFrac) and principal component analysis (PCA) were calculated with QIIME software. LEfSe analysis was carried out for comparisons among intergroup samples. An alpha significance level of 0.05 and an effect size threshold of 4 were used for all biomarkers (16).

## Statistical Analysis

Data were analyzed with SPSS 21.0 (IBM Corp., Armonk, NY, USA) Continuous variables were reported as means ± standard deviation. Student's *t*-tests were used to compare between-group differences. Differences among three or more groups were compared by analysis of variance with Bonferroni's *post-hoc* test. Bar plots were generated with GraphPad Prism 8.0 (GraphPad Software, San Diego, USA). *P*-values < 0.05 indicated statistical significance.

# RESULTS

## CGA Inhibits HFD-Induced Body Weight Gain

After 20 weeks of treatment, the mean body weight of CGA-treated mice was significantly lower than that of the controls (**Figure 1A**). In HFD-fed mice, the reduced weight gain of those given CGA was mainly attributable to a significant reduction in overall fat mass that included both subcutaneous and visceral adipose tissue (**Figure 1B**). Differences in the food intake of the four groups were not significant, indicating that the weight loss induced by CGA was not caused by a reduction in energy intake (**Figure 1C**). These results indicated that CGA treatment inhibited body weight gain

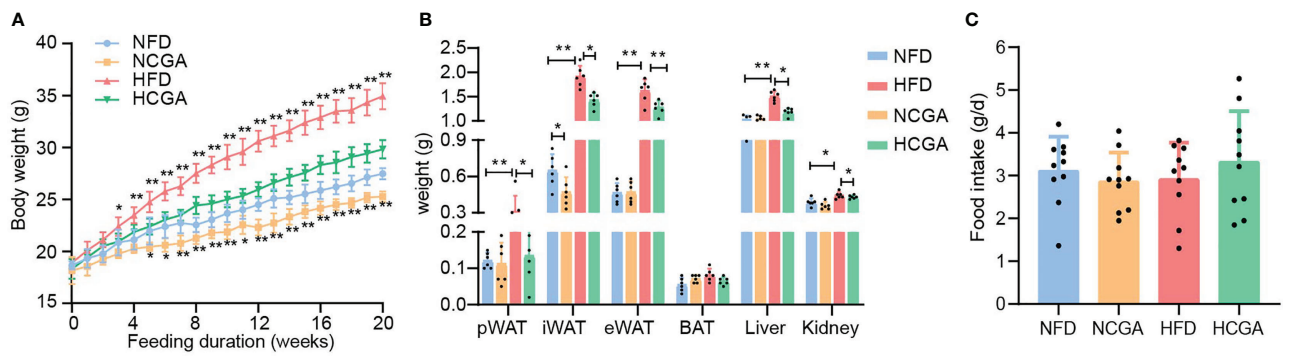


**FIGURE 1** | CGA inhibits HFD-induced body weight gain and adipose tissue accumulation. **(A)** Body weight, **(B)** weight of inguinal white adipose tissue (iWAT), perirenal (p)WAT and epididymal (e)WAT, brown adipose tissue, liver, and kidneys, **(C)** food intake, Data are means ± standard deviation. *$P < 0.05$, **$P < 0.01$.

and adipose tissue accumulation in HFD-fed mice without limiting food intake.

## CGA Improves Glucose Homeostasis and Insulin Sensitivity in HFD-Fed Mice

It is well known that insulin resistance is a key component of metabolic syndrome along with T2DM and obesity. To study the effect of CGA on insulin resistance induced in mice by different dietary patterns, fasting glucose, fasting insulin, homeostasis model assessment of insulin resistance (HOMA-IR) index, OGTT, and ITT were measured in mice fed normal chow or the HFD with or without CGA. The results showed that fasting blood glucose, fasting insulin concentrations and the HOMA-IR index in the HCGA group were significantly lower than those in the HFD group, and insulin sensitivity and glucose tolerance were higher than those in the HFD group. Differences between the NCGA and NFD groups were not significant (**Figures 2A–E**). These results showed that CGA treatment improved glucose homeostasis and reduced IR in mice fed with the HFD.

## CGA Inhibits Low-Grade Inflammation

Obesity is associated with low-grade chronic inflammation, and that may contribute to many associated complications, such as insulin resistance (IR) and subsequent type 2 diabetes (17, 18). LPS is a normal component of the outer cell wall of Gram-negative bacteria, and long-term exposure to LPS results in the development of low-grade inflammation (19, 20). In this study, we compared the plasma LPS levels in four groups of mice. plasma LPS was significantly increased in the HFD group compared with the NFD group, and CGA-treated mice had

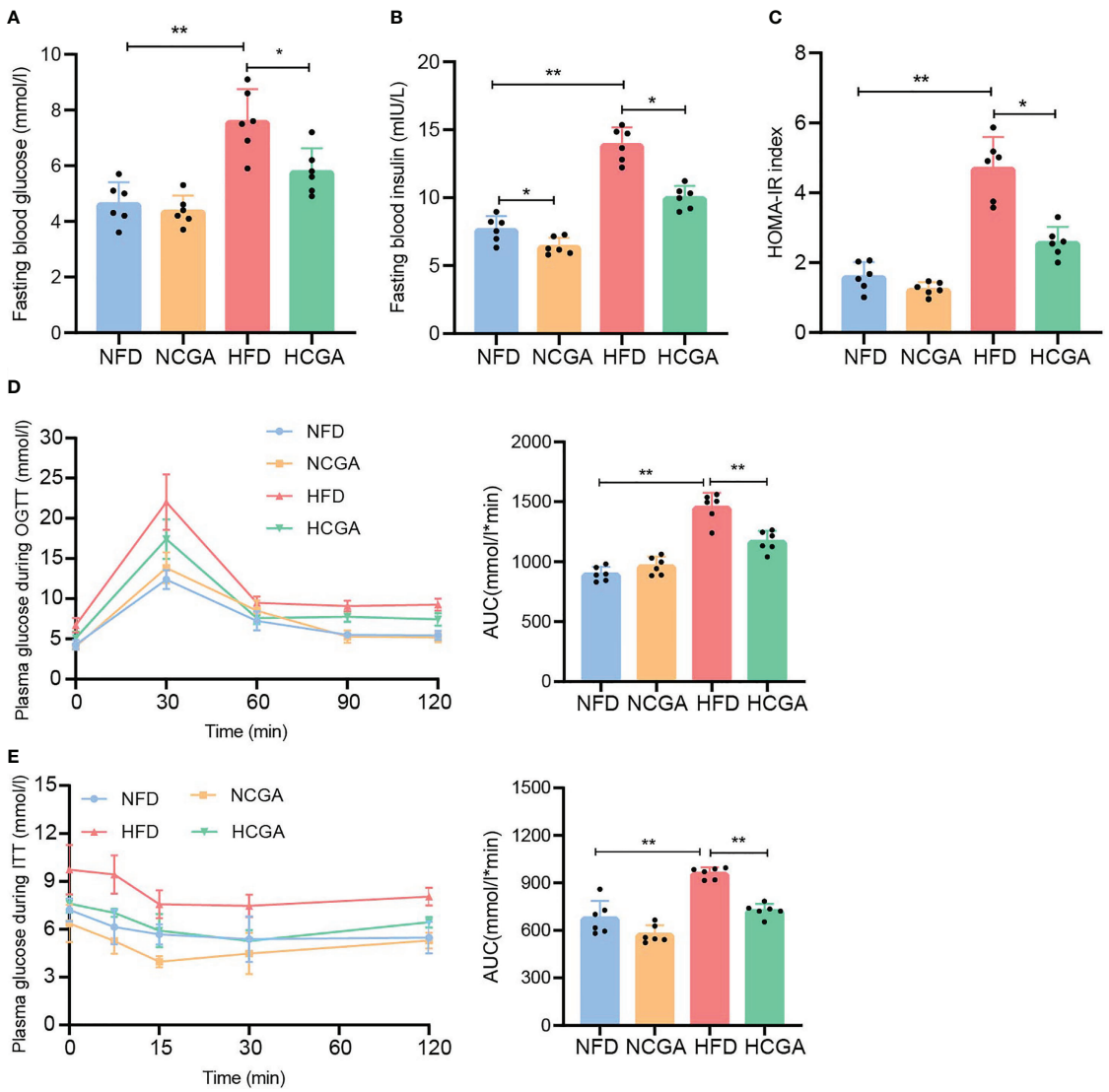


**FIGURE 2** | CGA improves glucose homeostasis in HFD-fed mice. **(A)** fasting glucose, **(B)** fasting insulin, **(C)** HOMA-IR index calculated using fasting glucose and insulin, **(D)** oral glucose tolerance test (OGTT), and **(E)** insulin tolerance test (ITT). Data are means ± standard deviation, $^{*}P < 0.05$, $^{**}P < 0.01$.

lower plasma LPS levels than their controls (**Figure 3A**). After LPS enters the bloodstream, it binds to Toll-like receptor 4 on the surface of immune cells to form a complex that promotes the occurrence and development of an inflammatory response (21). To investigate whether CGA improves IR and obesity by regulating the TLR-4 pathway, we assayed TLR-4 expression in liver and epididymal fat. The results indicated that the expression of TLR4 in liver and epididymal adipose tissue from CGA-treated mice was significantly lower than that in the corresponding control group (**Figures 3E, F**). To further investigate whether CGA inhibited LPS-induced low-grade inflammation, we assayed the expression of the proinflammatory mediators TNF-α, IL-1β, and MCP-1in serum, liver, and epididymal adipose tissue from obese mice with IR. The results showed that TNF-α, IL-1β, and MCP-1 levels in serum, liver, and epididymal adipose tissue in the HFD group were significantly higher than those in the NFD group, with lower expression levels in CGA-treated mice than in their controls (**Figures 3B–F**). These results suggested that CGA could inhibit low-grade chronic inflammation in mice.

## CGA Improves the Intestinal Barrier

Previous studies have confirmed that impaired intestinal mucosal barrier function and increased intestinal permeability in people with obesity-related related metabolic syndrome is responsible for entry of LPS into the blood circulation (22, 23). Intestinal epithelial tight junctions are a major component of the intestinal mechanical barrier, maintaining intestinal permeability and integrity. The main proteins involved in the formation of tight junctions are occludin, claudins, and junctional adhesion molecules. The three transmembrane proteins and peripheral cytoplasmic zonula occludens (ZO) proteins form tight junctions. Injury to the intestinal mechanical barrier leads to increased intestinal permeability that may allow translocation of intestinal bacterial, entry of LPS into the bloodstream, and the occurrence of enteric endotoxemia (24). The effects of CGA on intestinal permeability and expression of tight-junction proteins were investigated. We found that the colons of mice in the HFD group were significantly shorter than those in the NFD group, and that the colons of CGA-treated mice were longer than those in their control group (**Figure 4A**). Assay of intestinal mucosal permeability found that the plasma fluorescein isothiocyanate (FITC)-dextran level was significantly higher in HFD group than it was in the NFD group, The plasma FITC-Dextran level was significantly lower in the CGA treatment group than that in the corresponding control group. The results indicated that CGA improved intestinal mucosal permeability (**Figure 4B**). In addition, mRNA and protein expression of the tight-junction proteins (ZO-1, occludin, and claudin-1) in the ileum were increased in CGA-treated mice compared with the control mice (**Figures 4C, D**), Similar trends were observed in claudin-1 immunofluorescence observed by confocal microscopy (**Figure 4E**). These results suggested that CGA could inhibit damage to the intestinal mucosal barrier in mice fed an HFD and reduced the amount of LPS entering the blood circulation.

## Effects of CGA Treatment on the Diversity of Gut Microbiota

The effects of CGA treatment included an analysis of the changes in gut microbiota composition. Richness estimates obtained from

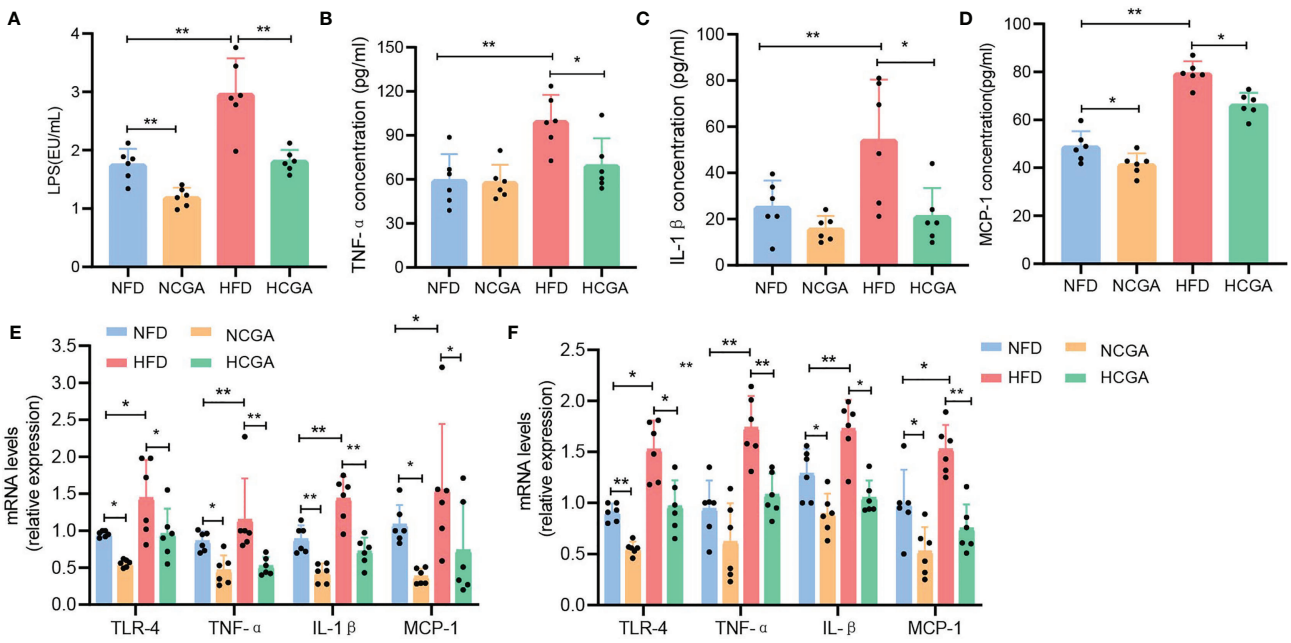


**FIGURE 3** | CGA inhibited low-grade inflammation as shown by **(A)** circulating LPS levels. **(B–D)** Serum TNF-α, Il-1β, and MCP-1 cytokine levels **(E, F)** proinflammatory cytokine expression in liver and epididymal fat. Data are means ± standard deviation. *$P < 0.05$, **$P < 0.01$.

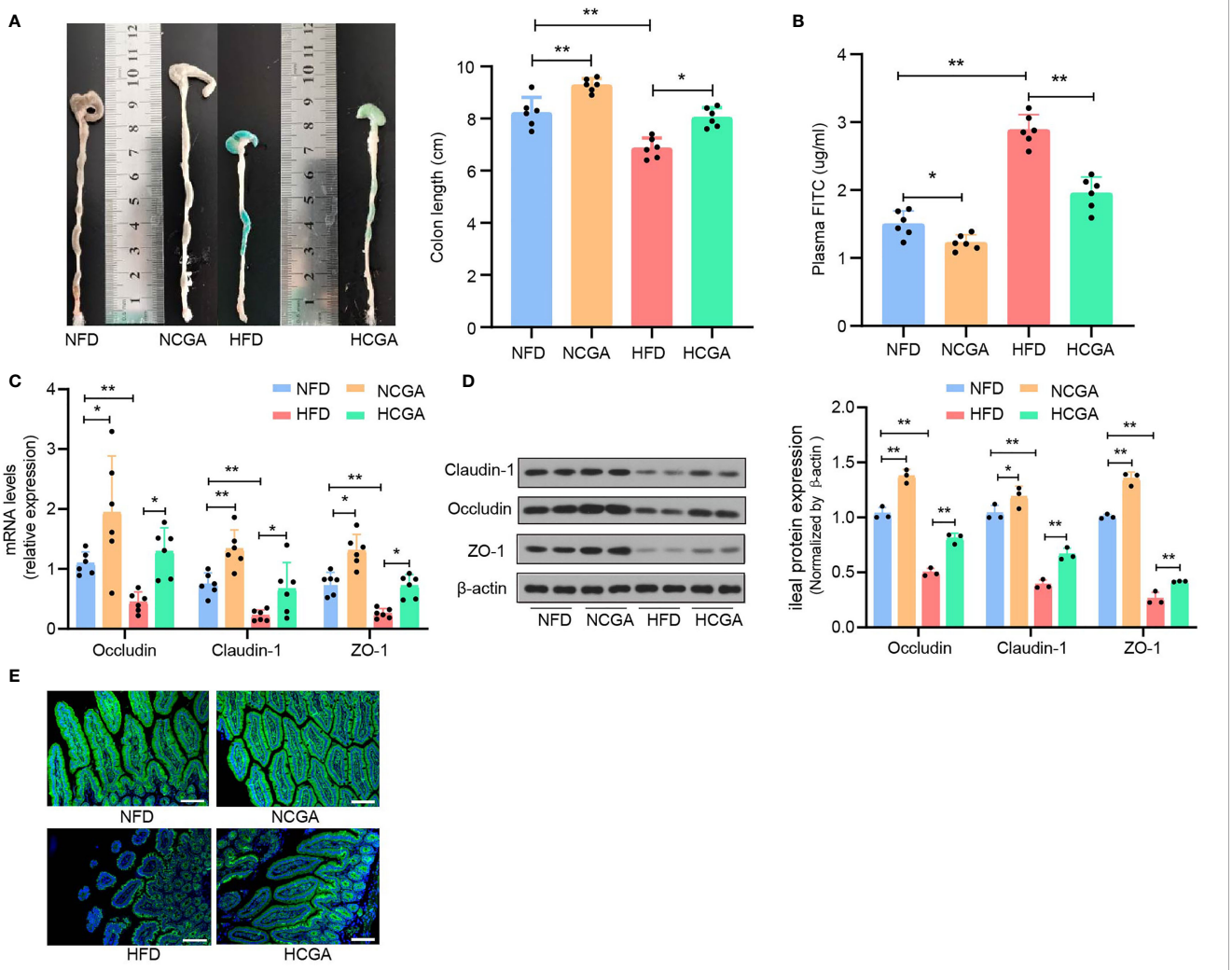


**FIGURE 4** | CGA treatment improves the intestinal barrier. **(A)** Representative photographs show the length of colon, **(B)** plasma FITC-Dextran levels, **(C)** claudin-1, occludin, and ZO-1 mRNA expression in the ileum, **(D)** claudin-1, occludin, and ZO-1 protein expression in the ileum, **(E)** representative images of claudin-1. $^{*}P < 0.05$, $^{**}P < 0.01$.

the observed number of species by extrapolation using Chao1 and Shannon indices showed that CGA had no significant effect on the richness of the gut microbiota (**Figures 5A, B**). β-Diversity calculated with weighted UniFrac algorithms indicated that the CGA treatment groups had significant structural differences in the first spatial dimension compared with their controls (**Figure 5C**). PCA revealed distinct clustering of intestinal microbiota communities within each group (**Figure 5D**).

## Effects of CGA Treatment on the Gut Microbiota Composition

At the phylum level, *Firmicutes* and *Actinobacteria* were significantly increased, and *Bacteroidetes, Verrucomicrobia*, and *Proteobacteria* were significantly decreased in the HFD group compared with the NFD group. CGA treatment decreased *Firmicutes* and increased *Bacteroidetes* and *Verrucomicrobia* compared with their controls. The results indicated that CGA intervention resulted in a phylum-level shift in the gut microbiota of mice fed the HFD, toward the population seen in the mice fed a normal diet. Effects of CGA on the gut microbiota at the family and genus level are given below (**Figure 6A**).

At the family level, Muribaculaceae, Erysipelotrichaceae, Prevotellaceae, Deferribacteraceae, and Desulfovibrionaceae were decreased, and Lachnospiraceae, Peptostreptococcaceae and Ruminococcaceae were increased in the HFD group compared with the NFD group. The NCGA group had a higher abundance of Muribaculaceae, Prevotellaceae, and Akkermansiaceae and a lower abundance of Lachnospiraceae, Erysipelotrichaceae, Peptostreptococcaceae, Desulfovibrionaceae, and Deferri-bacteraceae than the NFD group. The HCGA group had a higher abundance of Akkermansiaceae,

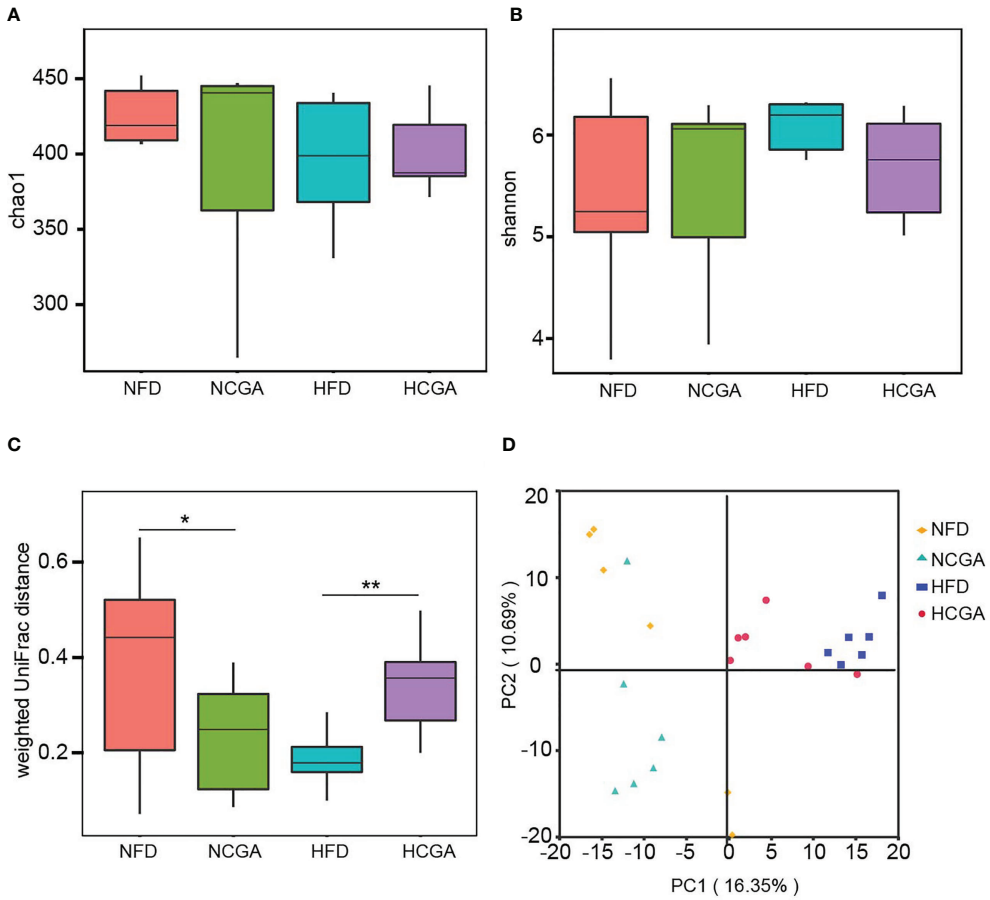


**FIGURE 5** | CGA treatment reshaped the gut microbiota of the mice as shown by **(A)** Chao index α-diversity, **(B)** Shannon index α-diversity, **(C)** weighted UniFrac β-diversity, and **(D)** principal component analysis (PCA) of the gut microbiota metagenomes. *$P < 0.05$, **$P < 0.01$.

Muribaculaceae, Erysipelotrichaceae, Peptostreptococcaceae, Desulfovibrionaceae, and Deferribacteraceae and a lower abundance of Lachnospiraceae, Ruminococcaceae, and Marinifilaceae than the HFD group (**Figure 6B**).

At the genus level *Dubosiella, Akkermansia, Alloprevotella*, and *Desulfovibrio* were decreased, and *Romboutsia, Mucispirillum, Odoribacter*, and *Faecalibaculum* were increased in the HFD group, compared with the NFD group. The NCGA group had a higher abundance of *Akkermansia* and *Alloprevotella* and a lower abundance of *Dubosiella, Romboutsia, Desulfovibrio*, and *Faecalibaculum* than the NFD group. The HCGA group had a higher abundance of *Dubosiella, Romboutsia, Akkermansia, Desulfovibrio, Mucispirillum*, and *Faecalibaculum*, and a lower abundance of *Odoribacter* than the HFD group (**Figure 6C**).

Analysis of the changes in OTUs showed that compared with the NFD group, Ileibacterium, Paenibacillus, Raoultella, Proteus, Parabacteroides, Bacteroides, and Alistipes were increased in NCGA group, and Turicibacter, Lactobacillus, Intestinimonas, Enterococcus, Acinetobacter, Rhodococcus, and Mucispirillum were decreased in NCGA group. Compared with the HFD group, Paenibacillus, Spingobium, Parasutterella, Alistipes, and Akkermansia were increased in HCGA group, and Blautia, Lachnoclostridium, Oscillibacter, Lachnospiraceae, and Ruminococcaceae were decreased in HCGA group (**Figures 6D, E**).

Linear discriminant analysis effect size (LEfSe) revealed the phylotypes responsible for the differences among the study groups. *Desulfovibrio* and *Turicibacter* were most abundant in the NFD group, *Alloprevotella* was most abundant in the NCGA group, *Oscillibacter*, *Ruminiclostridium*, and *Blautia* were most abundant in the HFD group, and *Romboutsia*, *Akkermansia*, and *Faecalibaculum* were most abundant in the HCGA group (**Figures 6F, G**).

## Effects of CGA-Derived Gut Microbiota on Obesity

The effects of CGA-derived microbiota on obesity and metabolic syndrome were evaluated by fecal microbiota transplantation. After microbiome depletion treatment, C57BL/6 mice were transplanted with cecal and colon contents obtained from HFD, and HCGA mice. PCA of the gut microbiota composition of the recolonized HFD (HFD-R), and HCGA (HCGA-R) mice was similar to that of the original donor

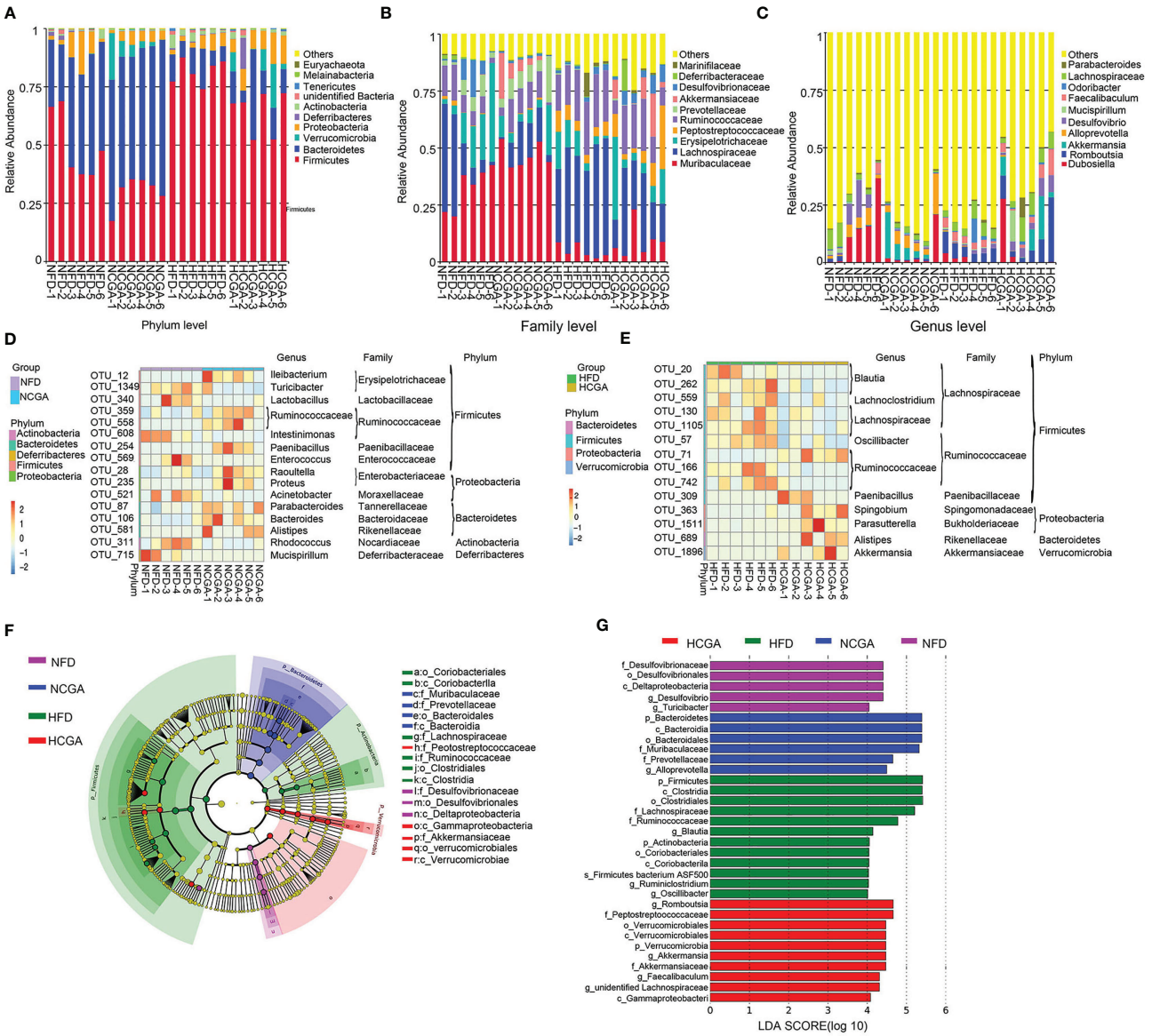


**FIGURE 6** | Taxonomic differences in the gut microbiota among the study groups. Six mice were evaluated in each group. **(A–C)** Relative abundance distribution at phylum, family and genus levels. **(D, E)** The heatmap indicates the abundance of 30 OTUs that were significantly changed by CGA. Under-represented units are blue; over-represented units are red. **(F, G)** LEfSe analysis showed phylogenetic differences induced by CGA treatment. Only taxa meeting a linear discriminant analysis significance threshold > 4 are shown.

(**Figure 7A**). The relative abundance distribution of gut microbiota in the study four groups at the phylum, family and genus levels are shown in **Supplementary Figure 1**. As shown in **Figures 7B–F**, the HCGA-R group had a lower body weight, liver weight, liver percentage of whole body weight, epididymal fat pad weight, and epididymal fat pad weight percentage of whole body weight compared with HFD-R group. The results indicate that the changes in the microbiota caused by CGA resulted in reduced body weight and body fat percentage in the HFD-fed mice. We further compared glucose metabolism in the two groups, finding that compared with the HFD-R group, mice in the HCGA-R group had better glucose tolerance and insulin sensitivity (**Figures 7G–H**) and that CGA-altered microbiota were associated with significantly improved insulin sensitivity and glucose tolerance in the C57Bl/6 mice. The study confirmed that CGA inhibited the increase of intestinal mucosal permeability, reduced plasma LPS levels, and ultimately inhibited chronic low-grade inflammation. Next, we further clarified the role of CGA-altered gut microbiota in intestinal mucosal permeability. As shown in **Figures 7I, J**, the plasma FITC-dextran and LPS levels were significantly lower in the HCGA-R group than in the HFD-R group. The results suggest

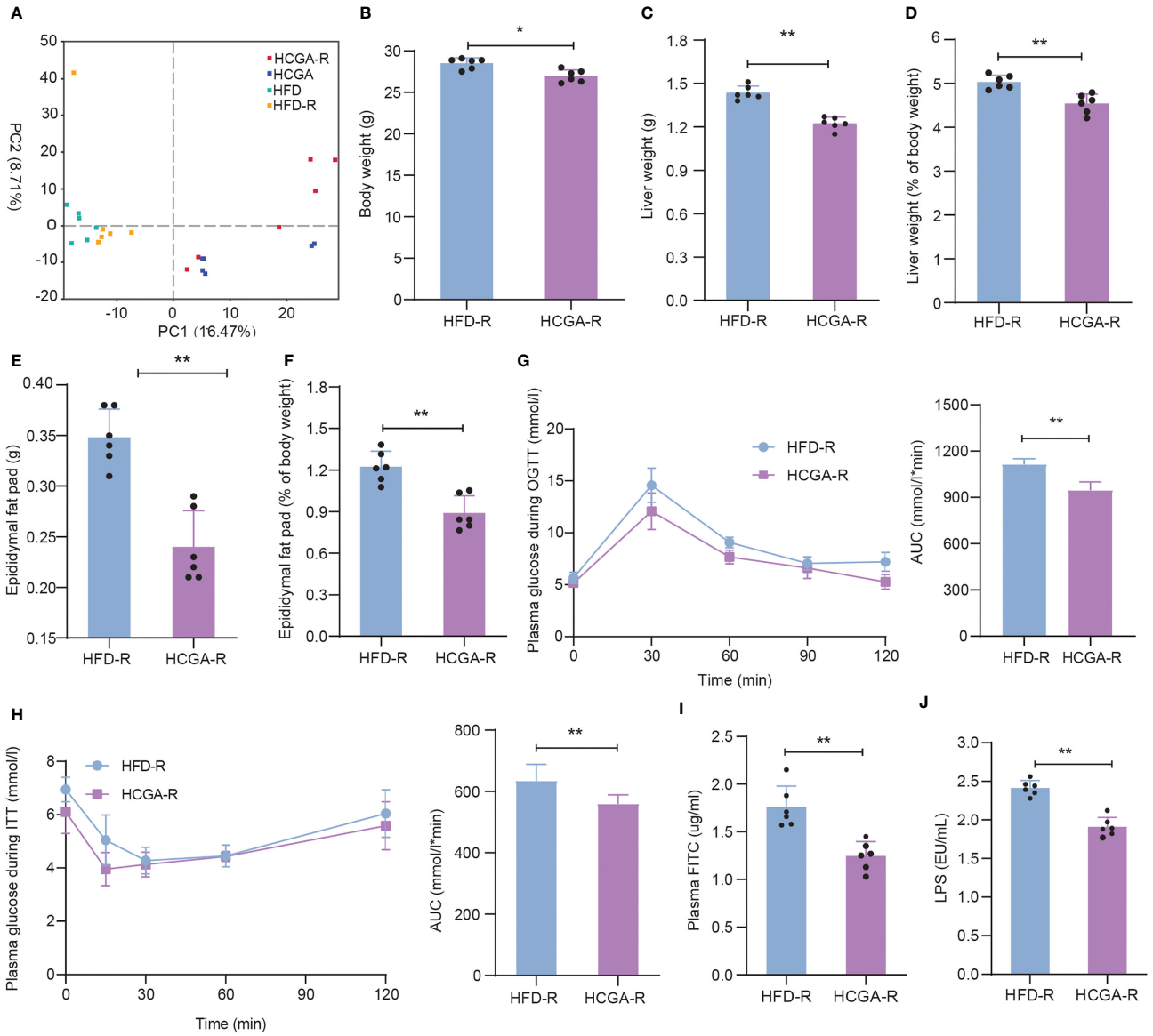


**FIGURE 7** | Effects of CGA-derived microbiota on obesity and metabolic endotoxemia. **(A)** PCA of the gut microbiota metagenomes, **(B)** Body weight, **(C)** liver weight, **(D)** liver percentage of whole body weight, **(E)** epididymal fat pad weight, **(F)** epididymal fat pad percentage of whole body weight, **(G)** OGTT, **(H)** ITT. **(I)** Plasma FITC level. **(J)** Plasma LPS level. Data are means ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$.

that the CGA-derived microbiota improved intestinal permeability. These data demonstrate that the gut microbiota is a primary mediator of improvements in obesity and metabolic endotoxemia.

## DISCUSSION

CGA is widely distributed in plants and is one of the main polyphenols in the human diet. It has been widely studied for its health-promoting effects (7). In this study, the role of gut microbiota in the protective effect of CGA on obesity and metabolic endotoxemia was identified in mice. We found that CGA may inhibit metabolic endotoxemia by regulating gut microbiota, thus improving obesity and IR.

In this study, we observed that CGA significantly inhibited increases in body weight and body fat content induced in HFD-fed mice without affecting the total food intake. Ma et al. reported that CGA significantly blocked the development of diet-induced obesity but did not affect body weight in obese mice (25). Mice were given 150 mg/kg CGA for up to 20 weeks, a larger dose and longer duration than used by Ma et al., and that might be the reason for the difference between our results and theirs. Furthermore, in this study, CGA prevented HFD-induced

subcutaneous and visceral adipose weight gain, which is consistent with the findings of previous studies (25, 26). We conclude that the effect of CGA on weight gain mainly resulted from the loss of adipose weight. As visceral fat is strongly related to obesity and its complications, the data suggest that the beneficial effects of CGA may not be limited to weight loss. Additional benefits of CGA are supported by the significant improvements in glucose metabolism, promotion of insulin sensitivity, which are consistent with the findings of Aidilla et al. (27). The study results indicate that CGA treatment inhibited weight gain, reduced fat accumulation, and significantly improved glucose metabolism disorders in obese mice induced by an HFD. The results are consistent with a protective effect of CGA on inhibiting obesity and improving metabolic syndrome, and that the protective effect was not the result of reduced food intake. We plan a further investigation into the mechanism of CGA-improved obesity.

Low-grade inflammation is known to be involved in the pathogenesis of obesity-related metabolic syndrome, insulin secretion defects, and impaired energy homeostasis (28–30). Inflammation of visceral adipose tissue is an important driver of IR (31–33). Mice fed an HFD have higher intestinal permeability and circulating LPS levels than mice fed an NFD, both of which leading to a release of a variety of cytokines and inflammatory mediators (34). On the contrary, lowering the LPS level can reduce the release of proinflammatory factors, downregulate the expression of LPS-related inflammatory proteins, and increase insulin sensitivity (35). In this study, serum LPS concentration, and inflammatory cytokines were higher in the HFD than in the NFD group, which is consistent with previous findings (36). CGA significantly reduced plasma LPS levels, inhibited TLR-4, TNF-α, IL-1β, and MCP-1 expression in liver and epididymal adipose tissue, which further confirmed that CGA reduced low-grade inflammation in the C57Bl/6 mice.

Intestinal mucosal barrier injury is an important cause of metabolic endotoxemia. An intact barrier inhibits the translocation of LPS and bacteria out of the enteric cavity. The structural integrity of tight-junction complexes is key to maintaining the integrity of the intestinal epithelial barrier (37). In this study, after 20 weeks of CGA treatment, intestinal permeability was decreased, ZO-1, claudin-1, and occludin expression in the ileal mucosa were significantly increased. Based on those results, we believe that CGA may inhibit adipose-related low-grade inflammation by improving the intestinal barrier.

The occurrence of chronic low-grade inflammation is closely related to the composition of the gut bacterial community. The imbalance of gut microbiota leads to the increased production and absorption of intestinal endotoxin, causes endotoxemia, promotes the occurrence and development of inflammation, and finally leads to the onset of obesity, IR, T2DM, and other metabolic diseases (6). Compelling evidence shows that there is a link between HFD, body weight regulation, and the gut microbiota (38, 39). Changes in the gut microbiota have been associated with a decrease in the serum LPS level (40). Imbalance in the gut microbiota can lead to an increased uptake of bacterial LPS from the gut into the bloodstream, leading to inflammation, obesity, and IR in obese mice (41). In our study, the gut microbiota of mice fed the HFD had fewer *Bacteroidetes* and more *Firmicutes* bacteria than mice fed the NFD, which are changes that are typical in obesity (42, 43). However, CGA significantly reduced the *Firmicutes/Bacteroidetes* ratio in the gut microbiota of HFD-fed mice, which is consistent with previous studies (26). At the family level, *Bacteroidetes* was reduced in the HFD group and *Lachnospiraceae* and *Ruminococcus* were increased, which is also consistent with previous findings (44). The family *Muribaculaceae* has been shown to inhibit, and *Lachnospiraceae* has been shown to aggravate, obesity. CGA reduced *Lachnospiraceae* and increased the levels of *Muribaculaceae* and *Akkermansiaceae*, which have been negatively correlated with obesity, IR, inflammation, and increased intestinal permeability. In addition, we found that the *Akkermansia*, *Romboutsia*, *Faecalibaculum*, *Dubosiella*, and *Mucispirillum* were significantly increased in mice in the HCGA group compared with those in the HFD group. *Romboutsia, Faecalibaculum, Dubosiella*, and *Mucispirillum* have been shown to produce SCFAs (45, 46). *Akkermansia* belongs to phylum *Verrucomicrobia*, which has been shown to reduce intestinal permeability and maintain intestinal barrier integrity (47). The return to a healthy metabolic state in obese patients after diet restriction may be related to an increase of *Akkermansia* in the gut (48). Gavage of *Akkermansia* in obese mice was reported to inhibit weight gain, IR, and reduce metabolic endotoxemia (49). In addition, *Akkermansia* has significant anti-inflammatory activity. Studies have shown that *Akkermansia* can improve the intestinal mucosal barrier and reduce intestinal inflammation by increasing the intestinal expression of endocannabinoids, proving its important role in maintaining intestinal health and the balance of glucose and lipid metabolism. These results suggest that CGA can improve metabolic endotoxemia by enrichment of SCFA-producing bacteria (e.g., *Dubosiella*, *Romboutsia, Mucispirillum*, and *Faecalibaculum*) and *Akkermansia*, which protects the intestinal barrier.

The effects of CGA-altered microbiota were investigated in C57Bl/6 mice that were transplanted with fecal microbiota from mice in the HFD, and HCGA groups. The transplanted CGA-altered microbiota inhibited body weight gain, decreased adipose tissue content, improved glucose metabolism, and reduced plasma FITC-dextran and LPS level. The results suggest that the CGA-induced changes in gut microbiota are the main reason for the decreased percentage of adipose weight and the inhibited metabolic endotoxemia that occurred. In the future, multi-omics (genomics, metabonomics and transcriptomics) should be used to explore the effects of CGA on gut microbiota structure, intestinal metabolite profile and intestinal mucosal gene expression profile to clarify the effects of CGA on intestinal metabolites and intestinal mucosal core gene expression.

In conclusion, the protective effect of CGA on obesity and related metabolic syndrome was based on its regulation of gut microbiota structure, diversity, and changes in relative abundance at the phylum to genus levels. CGA inhibited systemic low-grade inflammation by increasing the expression of tight-junction proteins in intestinal epithelial cells and

inhibiting the translocation of LPS from the enteric cavity into the blood circulation.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below:

NCBI SRA and BioSample: PRJNA715381 and PRJNA766296.

## ETHICS STATEMENT

Procedures involving animals followed the guidelines of the Institutional Animal Care and Use Committee of China Medical University Affiliated Shengjing Hospital and were approved by same (approval no. 2020PS034K).

## AUTHOR CONTRIBUTIONS

Conceptualization, DW. Methodology, XY and YG. Software, XY, JH, and YG. Validation, YL. Formal analysis, XY. Data curation, XY. Writing and preparation of the original draft, XY. Writing, review and editing, DW and YM. Visualization, XY. Supervision, YL. Project administration, DW. Funding acquisition, DW. All authors contributed to the article and approved the submitted version.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.762691/full#supplementary-material

**Supplementary Figure 1 |** Relative abundance distribution at phylum (a), family (b) and genus (c) levels between HFD-R and HCGA-R mice.

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# Metabolomics Analysis on Obesity-Related Obstructive Sleep Apnea After Weight Loss Management: A Preliminary Study

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**Objective:** Roux-en-Y gastric bypass (RYGB) surgery is an effective type of weight loss management and may improve obesity-related obstructive sleep apnea (OSA). Obese subjects who meet the criteria for surgery with OSA were enrolled. We investigated the metabolomic effects of RYGB on OSA.

**Methods:** Clinical data, serum measurements including indices of glycolipid metabolism, and polysomnography (PSG) measurements were collected at baseline and 6 months after RYGB surgery. Metabolomic analysis was performed using ultra-performance liquid chromatography-mass spectrometry.

**Results:** A group of 37 patients with obesity, type 2 diabetes (T2DM) and suspected OSA were enrolled of which 27 were OSA subjects. After RYGB surgery, metabolic outcomes and sleep parameters were all significantly improved. The OSA remission group had lower valine, isoleucine, and C24:1(cis-15) levels, and higher trimethylamine N-oxide, hippurate, and indole-3-propionic acid levels after RYGB surgery. A combination of preoperative indices (age, apnea-hypopnea index (AHI), fasting C-peptide level, and hippurate level) predicted the RYGB effect size in obese patients with T2DM and OSA, with an area under receiver operating characteristic curve of 0.947, specificity of 82.4%, and sensitivity of 100%.

**Conclusions:** RYGB surgery may significantly improve the metabolic status of patients with obesity, T2DM and OSA. A combination of preoperative indices (age, AHI, fasting C peptide level, and hippurate level) may be useful for predicting the effect size of RYGB in obese patients with T2DM and OSA. The mechanisms underlying OSA remission need to be explored.

**Keywords: obstructive sleep apnea, metabolomics, Roux-en-Y gastric bypass, obesity, type 2 diabetes**

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## INTRODUCTION

Obstructive sleep apnea (OSA) has a high prevalence and is characterized by recurrent episodes of upper airway obstruction during sleep. The resulting abnormal breathing leads to intermittent hypoxia and sleep fragmentation. OSA increases all-cause mortality (1, 2) by further progression of pathological changes involving sympathetic activation, oxidative stress, inflammation, exaggerated negative intrathoracic pressure, insulin resistance, endothelial dysfunction, or other factors related to OSA such as excessive daytime sleepiness, obesity and lung disease (2, 3). All of these in turn contribute to the pathogenesis of cardiovascular diseases, metabolic dysregulation, and other systemic disorders and consequently threaten patients' lives (4). In total, 120 million people are at high risk of OSA in China (5), which has a prevalence exceeding 5% (5). These subjects are withstanding the adverse effects causing by OSA. Thus, OSA is not only a serious health problem but also a socioeconomic issue (6, 7).

OSA patients are more likely to be obese and have type 2 diabetes (T2DM). As a bariatric surgery technique, Roux-en-Y gastric bypass (RYGB) surgery is an effective type of weight loss management which can improve or resolve obesity-related comorbidities such as T2DM (8, 9), and may also improve accompanied OSA. RYGB surgery can resolve abnormal sleep architecture and restore normal hypoxic oxygen status (10–13). Former study revealed serum metabolites changes of OSA patients after multilevel sleep surgery (14). However, the serum metabolites changes of OSA patients who have undergone RYGB remains unclear.

Recently, metabolomics, a novel tool for exploring the physiological and pathological mechanisms underlying disease, has been used to identify new biomarkers by exploring the correlation between the biochemical reactions of small molecules in cells and the internal state of the body (14–17). Previous studies revealed that free fatty acids, acylcarnitines, amino acids, bile acids, and lipid species could predict T2DM remission after bariatric surgery more effectively than existing prediction models (18, 19). Thus, understanding the characteristic serum metabolites associated with OSA after RYGB is important for finding potential pathways underlying and may be helpful in developing strategies for intervention or efficacy prediction.

In this study, we performed targeted metabolomics, which provided quantitative data on 52 metabolites (mainly fatty acids and amino acids), to assess metabolomic changes after RYGB, and to identify biomarkers and pathways that are helpful for predicting the progress and prognosis of OSA.

**Abbreviations:** OSA, obstructive sleep apnea; T2DM, type 2 diabetes; RYGB, Roux-en-Y gastric bypass surgery; BMI, body mass index; CPAP, continuous positive airway pressure; PSG, polysomnography; SBP, systolic blood pressure; DBP, diastolic blood pressure; GHb, glycated hemoglobin; GA, glycated albumin; TC, cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Apo, apolipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; Lp (a), lipoprotein (a); AHI, apnea-hypopnea index; LSpO2, lowest pulse oxygen saturation; SpO2, pulse oxygen saturation; ODI, oxygen desaturation index; MAI, microarousal index; ESS, Epworth Sleepiness Scale; AASM, American Academic Sleep Medicine; ROC, receiver operating characteristic; AUC, area under the curve; PCA, principal component analysis; PLS-DA, partial least squares-discriminant analysis; OPLS-DA, orthogonal partial least squares-discriminant analysis; VIP, variable importance in projection; CI, confidence interval; MUFA, monounsaturated fatty acid; TMAO, trimethylamine N-oxide; BCAA, branched chain amino acids; mTORC1, mammalian target of rapamycin complex; TMA, trimethylamine; FMO3, flavin-containing monooxygenase.

## MATERIALS AND METHODS

### Patients

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee. All the participants signed informed consent forms. The surgeons followed the latest guidelines for metabolic surgery (20–23). The inclusion criteria were a T2DM duration ≤ 15 years with adequate islet function (defined by C peptide release test when a fasting C-peptide level > 1 ng/mL and a peak value > 2 ng/mL); age of 16–65 years; and body mass index (BMI) > 35 kg/m$^2$, or BMI of 25–27.5 kg/m$^2$ with poorly controlled T2DM and more than two symptoms of metabolic syndrome, or T2DM complications and a BMI > 27.5 kg/m$^2$ with poorly controlled T2DM. The exclusion criteria were systemic disease, such as pulmonary, renal, liver, cardiovascular, or neurological disease incompatible with surgery; incomplete polysomnography (PSG) data; previous OSA treatment (e.g., surgery, continuous positive airway pressure, oral appliance therapy); current therapy that might affect the clinical and metabolomic results (e.g., hormone replacement therapy); previous open abdominal surgery; acute T2DM complications, type 1 diabetes, or secondary diabetes as a consequence of endocrine disease, hereditary disease or medication (e.g., pancreatectomy, Cushing's syndrome); and a mental disorder or severe alcohol or drug dependency. Mortality and severe complications did not occur in any patients. They were all given nutritional guidance after RYGB surgery and followed-up for 6 months.

### Clinical and Biochemical Measurements

Anthropometric parameters including height, weight, circumference of the waist and hip were recorded. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured by a standard sphygmomanometer. The mean values of two consecutive measurements were recorded before PSG, as previously described (11). BMI was calculated as weight/height$^2$. At 7 AM, fasting blood and urine samples were collected from all patients who underwent PSG. Indices of glycolipid metabolism and the lipid profile, including fasting blood glucose, fasting insulin, insulin (120 min), fasting C-peptide, C-peptide (120 min), glycated hemoglobin (GHb), glycated albumin (GA), cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), apolipoprotein (Apo) A-1, ApoB, ApoE, and lipoprotein (a) (Lp(a)), were measured in the clinical laboratory of our hospital. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting insulin × fasting plasma glucose/22.5 (24).

## Sleep Evaluation

Patients were suspected OSA subjects and underwent portable PSG to assess nocturnal sleep and determine OSA status. Nasal airflow, thoracic/abdominal movement, pulse oximetry, body posture, and snoring were recorded continuously overnight (10 PM–6 AM). Two skilled technicians checked and analyzed the data. The PSG parameters consisted of the apnea-hypopnea index (AHI), oxygen desaturation index (ODI), pulse oxygen saturation (SpO2), lowest pulse oxygen saturation (LSpO2), and microarousal index (MAI). PSG was performed at baseline and 6 months after RYGB surgery. Before the PSG sleep study, daytime sleepiness was assessed using the Epworth Sleepiness Scale (ESS). OSA was classified according to the American Academic Sleep Medicine 2007 criteria (25). Patients with an AHI ≥ 5 events/h were diagnosed with OSA. OSA remission was defined as an AHI > 5 events/h before surgery and an AHI < 5 events/h after surgery. In contrast, patients with AHI > 5 events/h both pre- and postoperatively were classified were classified as OSA non-remission.

## Surgical Procedure

The RYGB was performed laparoscopically by the same team using a standardized method (26). A 25-mL gastric pouch was divided from the distal remnant. The biliopancreatic and alimentary limbs were 100–120 cm in length. After completion of the surgical procedures, the patients were further evaluated and monitored, and were discharged to follow-up once they were stable.

## Statistical Analyses

Normal distributed continuous variables are shown as the mean ± standard deviation and non-normal distributed continuous variables are shown as the median (first to third quartile). Categorical variables are shown as percentages. The Shapiro-Wilk test was performed to assess the normality of the data. According to the results of the normality test, the paired Student's *t*-test or Wilcoxon rank-sum test was performed for comparison of parameters before and after surgery. Analysis of variance or the Kruskal–Wallis test was used to compare multiple groups. We performed logistic regression analysis to identify significantly altered metabolites and confounding variables, controlling for potential confounders such as sex, BMI, fasting glucose, fasting insulin, medication for T2DM, hypertension and dyslipidaemia, ESS, and LSpO2. Using the PSG results as the gold standard, the accuracy of the prediction model was determined by receiver operating characteristic (ROC) curve analysis, i.e., the area under the curve (AUC), with the sensitivity and specificity calculated according to the best diagnostic cut-off points. $p < 0.05$ was considered statistically significant. All analyses were performed using SPSS (version 23.0; SPSS Inc., Chicago, IL, USA, 2015) and R software (version 3.6.1; R Development Core Team, Vienna, Austria). All subjects were required to go to bed at a certain time and to stop smoking and drinking alcohol during the follow-up study period. Two skilled physicians independently entered and analyzed the data to ensure the accuracy thereof. Patients who were lost to follow-up or had incomplete data were excluded from the analysis.

## Metabolomic Analyses

Metabolomics provides detailed information on molecular structure and can detect a wide range of metabolites simultaneously (27). The metabolomics kit used in this investigation was also used in our previous study (28), and provided quantitative data on 52 metabolites, which were mainly fatty acids and amino acids. An unsupervised dimensionality reduction method, namely principal component analysis, was used to investigate the internal characteristics of the dataset and eliminate singularities. Supervised partial least squares-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed to visualize the metabolic differences between the groups. The variable importance in the projection (VIP) score reflects the importance of each variable in the model according to their overall contribution. Significantly altered metabolites ($p < 0.05$) with a VIP score ≥ 2 in OPLS-DA models were selected. Metabolites confirmed by ultra-performance liquid chromatography-mass spectrometry, and those that were statistically significant with a non-zero correlation with OSA biomarkers, were analyzed by logistic regression.

# RESULTS

## Anthropometric and Clinical Information

Of the 86 subjects with obesity, T2DM and suspected OSA who were candidates for RYGB surgery, 22 refused to participate. After 64 subjects who underwent PSG, 27 were excluded due to incomplete PSG data. Finally, 37 subjects (20 men and 17 women) with complete PSG data were included, and they all completed the 6-month follow-up (**Figure 1**). The anthropometric and clinical characteristics at baseline and 6 months after RYGB surgery are shown in **Table 1**. Almost all anthropometric, clinical, and sleep variables improved after RYGB surgery. As shown in **Table 1**, waist circumference, hip circumference, waist-to-hip ratio, BMI, SBP, DBP, fasting glucose, fasting insulin, insulin (120 min), fasting C-peptide, C-peptide (120 min), HOMA-IR, GHb, GA, TC, TG, LDL, ApoB, ApoE, ESS, AHI, and ODI were decreased, whereas HDL, mean SpO2, and LSpO2 were increased. ApoA-1, Lp(a), and MAI showed no significant changes. According to postoperative AHI, OSA patients were then divided into two subgroups: OSA remission and OSA non-remission. The aforementioned parameters were similar between the subgroups showing significant improvement in obesity and OSA severity, except that the postoperative AHI was > 5 events/h in the non-remission group (**Table 2**). In further subgroup analyses with decrease rate of AHI after RYGB surgery, the aforementioned parameters were improved significantly as well. Compared to the group with decrease rate of AHI ≤ 70% after RYGB surgery, the AHI, MAI, fasting glucose, and GA were lower in the group showing a decrease rate of AHI > 70% after RYGB surgery, whereas decrease value of AHI and decrease rate of AHI were higher (**Supplementary Table S1**). Better improvement in glucose metabolism was accompanied with better OSA improvement.

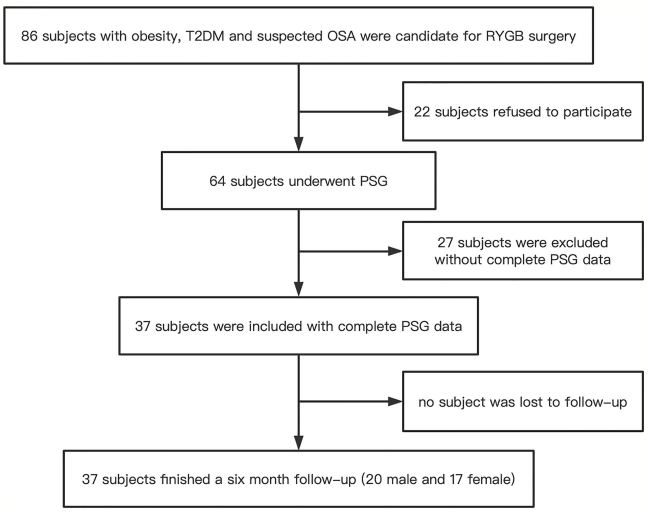


**FIGURE 1** | Flow diagram of the recruitment process. T2DM, type 2 diabetes; OSA, obstructive sleep apnea; RYGB, Roux-en-Y gastric bypass surgery; PSG, polysomnography.

## Differences in Baseline Metabolomic Profiles Between the OSA Remission and Non-Remission Groups

The normalized data of the 52 quantified metabolites are shown in **Supplementary Table S2**. The levels of 38 metabolites were lower, and those of 14 were higher, in the OSA remission group compared to the non-remission group at baseline. Differences in metabolomic profiles between the OSA non-remission and remission groups at baseline were demonstrated by PLS-DA and OPLS-DA (**Figures 2A, B**). Univariate analyses demonstrated that hippurate was a potential biomarker, with a VIP score ≥ 2 and $p < 0.05$ (**Figures 2C, D**).

## Identification of Significantly Altered Metabolites Associated With OSA Remission

We analyzed the metabolite levels of patients with OSA remission before and after surgery (**Figures 3A, B**, and **Supplementary Table S3**). The valine, C24:1(cis-15) and isoleucine levels decreased after surgery (valine: from 58.174 [50.808–62.089] ng/mL to 47.821 [45.342, 49.661] ng/mL [p = 0.003]; C24:1(cis-15): from 0.011 [0.010, 0.013] ng/mL to 0.008 [0.003, 0.010] ng/mL [p = 0.032]; isoleucine: from 4.908 [3.999, 5.794] ng/mL to 3.937 [3.561, 4.613] ng/mL [p = 0.045]), whereas the hippurate, trimethylamine N-oxide (TMAO), and indole-3-propionic acid levels increased (hippurate: from 0.146 [0.091, 0.306] ng/mL to 0.856 [0.503, 1.420] ng/mL [p = 0.002]; TMAO: from 0.303 [0.203, 0.473] ng/mL to 0.972 [0.475, 1.815] ng/mL [p = 0.023]; indole-3-propionic acid: from 0.110 [0.083, 0.159] ng/mL to 0.216 [0.148, 0.270] ng/mL [p = 0.015]) (**Figures 3C, D**, and **Table 3**). Heatmaps based on the Spearman correlation matrix demonstrated clear correlations between the clinical indicators and metabolomic profiles (**Supplementary Figures S1**–**S3**). In the heatmaps, stronger correlations are displayed in darker colors. **Supplementary Figure S1** shows the correlations between clinical indicators and metabolomic profiles in all patients before and after RYGB, whereas **Supplementary Figure S2** shows the correlations in OSA patients. As shown in **Supplementary Figure S3**, changes in clinical parameters were significantly correlated with metabolomic profiles. The correlations among the mean SaO2, LSpO2, MAI, waist circumference, hip circumference, waist-to-hip ratio, BMI, SBP, DBP, fasting glucose, fasting insulin, insulin (120 min), C-peptide (120 min), HOMA-IR, GA, HDL, LDL, TC, ApoE, ESS, and certain metabolites were statistically significant in the OSA remission group.

## Potential Metabolic Biomarkers of RYGB Effect Size for OSA

We performed logistic regression and ROC analyses to determine the metabolites with potential as biomarkers. Compared to the non-remission patients, only hippurate level at baseline was significantly lower in the remission patients (VIP score > 2, $p < 0.05$; **Supplementary Table S2**). For hippurate, the sensitivity and specificity of the ROC curve to distinguish patients with obesity, T2DM and OSA who could versus could not achieve OSA remission were 94.1% and 50%, respectively. The AUC value was 0.700 (95% CI: 0.472–0.928; **Figure 4A**). We further used forward logistic regression to identify baseline clinical parameters predicting likelihood of OSA remission after RYGB surgery, age, AHI, and fasting C-peptide level were statically significant. When we adjusted the confounding factors of BMI, sex, fasting glucose and fasting insulin, the significance still existed (**Supplementary Table S4**). In a model comprising three preoperative clinical parameters (age, AHI, and fasting C-peptide level), the sensitivity and specificity were 88.2% and 90%, respectively. The AUC value was 0.894 (95% CI: 0.755–1.000; **Figure 4B**). We also established a model that included four preoperative variables (age, AHI, fasting C-peptide level, and

**TABLE 1** | Anthropometric and clinical characteristics of the enrolled patients.

| Characteristics | Before RYGB surgery (n = 37) | After RYGB surgery (n = 37) |
|---|---|---|
| **Males, n (%)** | 20 (54.05) | |
| **Age, y** | 43.97 ± 12.70 | |
| **Follow-up time, m** | 6.03 ± 0.10 | |
| **Medication for T2DM, n (%)** | 35 (94.59) | |
| **Medication for hypertension, n (%)** | 22 (59.46) | |
| **Medication for dyslipidaemia, n (%)** | 16 (43.24) | |
| **Waist circumference, cm** | 105.56 ± 12.17 | 86.73 ± 8.41*** |
| **Hip circumference, cm** | 108.19 ± 8.85 | 96.64 ± 6.49*** |
| **Waist-to-hip ratio** | 0.97 ± 0.06 | 0.90 ± 0.05*** |
| **BMI, kg/m$^2$** | 31.96 ± 3.63 | 24.62 ± 2.91*** |
| **Reductions of BMI, kg/m$^2$** | 7.34 ± 2.31 | |
| **SBP, mmHg** | 130 (122-150) | 120 (110-122)*** |
| **DBP, mmHg** | 85 (80-93) | 77 (70-80)*** |
| **Fasting glucose, mmol/L** | 7.66 (6.42-9.07) | 5.43 (4.69-6.06)*** |
| **Fasting insulin, μU/mL** | 16.92 (11.36-30.80) | 8.22 (4.70-9.98)*** |
| **Insulin (120 min), μU/mL** | 75.80 (49.31-157.55) | 17.42 (9.89-30.37)*** |
| **Fasting C peptide, ng/mL** | 3.05 (2.32-3.39) | 2.03 (1.73-2.29)*** |
| **C peptide (120 min), ng/mL** | 6.13 (3.59-11.60) | 4.91 (3.52-7.51)* |
| **HOMA-IR** | 5.60 (3.61-11.06) | 1.86 (1.12-2.92)*** |
| **GHb, %** | 7.60 (6.20-8.60) | 5.80 (5.35-6.20)*** |
| **GA, %** | 17.72 (16.00-19.65) | 14.00 (12.65-15.15)*** |
| **TC, mmol/L** | 4.99 (4.49-5.64) | 4.18 (3.68-4.76)*** |
| **TG, mmol/L** | 1.70 (1.27-2.38) | 0.97 (0.74-1.22)*** |
| **HDL, mmol/L** | 1.02 (0.90-1.14) | 1.21 (1.09-1.32)*** |
| **LDL, mmol/L** | 3.11 (2.49-3.51) | 2.33 (2.05-2.75)*** |
| **ApoA-1, g/L** | 1.05 (0.97-1.11) | 1.07 (1.01-1.13) |
| **ApoB, g/L** | 0.92 (0.86-1.05) | 0.68 (0.61-0.73)*** |
| **ApoE, mg/dL** | 5.02 (4.32-5.51) | 3.58 (3.12-4.02)*** |
| **Lp(a), mg/dL** | 16.54 (12.40-19.90) | 16.60 (10.93-19.72) |
| **ESS** | 8 (6-10) | 3 (2-6)*** |
| **ESS>10, n (%)** | 9 (24.3) | 0 (0)*** |
| **AHI, events/h** | 12.70 (4.35-28.60) | 3 (2.15-7)*** |
| **LSpO2, %** | 81 (76-87) | 89 (87-91)*** |
| **Mean SpO2, %** | 94 (92-95) | 96 (94-97)*** |
| **ODI, events/h** | 16.60 (5.10-29.70) | 5 (1.45-7)*** |
| **MAI, events/h** | 16.80 (12.55-19.05) | 17.10 (10.15-21.65) |

*BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA-IR, homeostasis model assessment of insulin resistance; GHb, glycated hemoglobin; GA, glycated albumin; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; ApoA-I, apolipoprotein A-I; ApoB, apolipoprotein B; ApoE, apolipoprotein E; Lp(a), lipoprotein (a); ESS, Epworth sleepiness score; AHI, apnea–hypopnea index; LSpO2, lowest pulse oxygen saturation; SpO2, pulse oxygen saturation; ODI, oxygen desaturation index; MAI, micro-arousal index. P-value indicates significant differences before and after RYGB surgery. * indicated p-value $< 0.05$ before versus after surgery in subgroup analysis. *** indicated p-value $< 0.001$ before versus after surgery in subgroup analysis.*

hippurate level). The equation used to predict OSA remission is as follows:

$$\ln \frac{p}{1-p} = \exp\left(9.876 - 0.194[age] - 0.064[\text{preoperative AHI}]\right.$$

$$\left.+1.005[\text{preoperative fasting C peptide}] - 3.757[\text{preoperative hippurate}]\right)$$

p is the probability of achieving OSA remission after RYGB surgery. The specificity and sensitivity for distinguishing obese T2DM and OSA patients who could versus could not achieve remission were 82.4% and 100%, respectively. The AUC value was 0.947 (95% CI: 0.866–1) (**Figure 4C**). Patients who were younger, with a lower preoperative AHI and baseline level of hippurate, and a higher preoperative fasting C-peptide level, were more likely to achieve OSA remission after RYGB surgery.

## DISCUSSION

To date, no study has assessed the metabolic profiles of patients with OSA after RYGB surgery in detail. Our study is the first to show that the levels of valine, isoleucine, and C24:1(cis-15) are decreased, whereas those of hippurate, TMAO, and indole-3-propionic acid are increased, after RYGB surgery compared to baseline in OSA patients in remission providing insight for future study on potential mechanisms. A combination of preoperative indices (age, AHI, and fasting C-peptide and hippurate levels) may be highly sensitive and specific for predicting the RYGB effect size for patients with obesity, T2DM and OSA. Since changes in the clinical parameters were significantly correlated with metabolomic profiles, our data also provide insight into the important mechanistic pathways of OSA (29).

Isoleucine and valine, which are branched chain amino acids (BCAAs), were decreased after RYGB surgery in our OSA remission group. Studies have shown that obese patients with T2DM have higher levels of isoleucine and valine than healthy individuals (30, 31). In addition, mitochondrial dysfunction may be caused by downstream accumulation of BCAA metabolites (32). Meanwhile, elevated BCAA levels are associated with

**TABLE 2 |** Clinical characteristics of the OSA remission and non-remission groups before and after RYGB surgery.

| Characteristics | OSA remission (n = 17) | | OSA non-remission (n = 10) | |
|---|---|---|---|---|
| | Before RYGB surgery | After RYGB surgery | Before RYGB surgery | After RYGB surgery |
| Males, n (%) | 10 (58.82) | | 4 (40) | |
| Age, y | 40.06 ± 11.09 | | 54.90 ± 10.60[a] | |
| Follow-up time, m | 6.04 ± 0.14 | | 6.02 ± 0.08 | |
| Medication for T2DM, n (%) | 16 (94.12) | | 10 (100) | |
| Medication for hypertension, n (%) | 13 (76.47) | | 5 (50) | |
| Medication for dyslipidaemia, n (%) | 7 (41.18) | | 4 (40) | |
| Waist circumference, cm | 107.39 ± 14.18 | 86.04 ± 8.84*** | 108.20 ± 12.23 | 88.97 ± 9.33*** |
| Hip circumference, cm | 109.22 ± 9.35 | 96.45 ± 6.50*** | 110.90 ± 9.70 | 98.06 ± 8.22** |
| Waist-to-hip ratio | 0.98 ± 0.07 | 0.89 ± 0.05*** | 0.97 ± 0.05 | 0.91 ± 0.03** |
| BMI, kg/m$^2$ | 32.64 ± 3.88 | 24.76 ± 3.18*** | 32.61 ± 3.79 | 25.10 ± 3.27*** |
| Reductions of BMI, kg/m$^2$ | 7.89 ± 2.25 | | 7.51 ± 1.72 | |
| SBP, mmHg | 136 (120-154) | 120 (110-130)** | 147 (130-154) | 120 (110-126)*** |
| DBP, mmHg | 82 (80-90) | 80 (72-85) | 93 (84-100) | 70 (70-81)*** |
| Fasting glucose, mmol/L | 7.49 (6.03-8.95) | 5.11 (4.58-5.64)*** | 7.60 (6.43-8.87) | 5.84 (5.09-6.41)**[b] |
| Fasting insulin, μU/mL | 16.92 (12.60-30.92) | 6.68 (4.20-9.28)*** | 21.55 (10.29-41.98) | 9.02 (6.52-11.99)** |
| Insulin (120 min), μU/mL | 77.32 (48.98-161.50 | 14.64 (9.44-34.86)*** | 99.84 (52.11-138.58) | 23.17 (12.27-39.74)** |
| Fasting C peptide, ng/mL | 3.17 (3.03-4.37) | 2.01 (1.59-2.46)*** | 2.37 (1.40-3.52)[a] | 2.03 (1.71-2.33) |
| C peptide (120 min), ng/mL | 9.10 (5.75-11.94) | 4.91(3.53-6.70)** | 4.22 (2.27-11.38) | 4.23 (3.67-7.98) |
| HOMA-IR | 5.60 (3.77-11.88) | 1.28 (0.91-2.38)*** | 7.91 (2.99-14.67) | 2.09 (1.51-3.51)** |
| GHb, % | 7.10 (6.00-8.95) | 5.40 (5.05-5.96)*** | 7.55 (6.83-9.45) | 6.20 (5.79-6.70)**[b] |
| GA, % | 17.20 (13.70-19.75) | 13.20 (11.60-14.80)*** | 18.21 (16.75-20.98) | 15.20 (12.38-18.03)**[b] |
| TC, mmol/L | 4.67 (3.97-5.06) | 4.37 (3.79-4.92) | 5.00 (4.32-5.89) | 3.98 (3.60-4.28)*** |
| TG, mmol/L | 1.45 (1.25-3.14) | 0.82 (0.72-1.19)** | 1.66 (1.26-2.53) | 1.05 (0.88-1.31)** |
| HDL, mmol/L | 1.00 (0.90-1.03) | 1.21 (1.11-1.27)*** | 1.14 (0.87-1.16) | 1.13 (1.00-1.46) |
| LDL, mmol/L | 2.96 (2.31-3.14) | 2.62 (2.09-3.16) | 3.27 (1.98-3.63) | 2.19 (2.01-2.27)*** |
| ApoA-1, g/L | 1.06 (0.99-1.13) | 1.07 (1.01-1.09) | 1.13 (1.05-1.29) | 1.07 (0.97-1.12) |
| ApoB, g/L | 0.90 (0.87-0.93) | 0.69 (0.56-0.73)*** | 0.93 (0.77-1.07) | 0.69 (0.59-0.76)** |
| ApoE, mg/dL | 5.10 (4.21-5.91) | 3.66 (2.71-4.12)*** | 5.20 (4.72-5.97) | 3.78 (2.99-4.87)** |
| Lp(a), mg/dL | 16.47 (11.30-21.31) | 16.60 (10.12-20.17) | 16.47 (11.40-23.58) | 16.60 (12.08-22.56) |
| ESS | 9 (6-15) | 3 (2-6)*** | 10 (7-13) | 4 (3-6)** |
| ESS>10, n (%) | 3 (17.65) | 0*** | 3 (30.00) | 0*** |
| AHI, events/h | 19.25 (13.20-21.20) | 2.76 (1.60-2.88)*** | 29.65 (19.25-44.15)[a] | 8.85 (6.94-18.43)***[b] |
| Decrease value of AHI, events/h | 16.48 (8.25-37.36) | | 16.04 (7.13-28.58)[b] | |
| Decrease rate of AHI, % | 85.64 (82.84-88.55) | | 64.86 (26.67-70.40)[b] | |
| LSpO2, % | 81 (78-85) | 88 (87-91)*** | 78 (69-81) | 87 (81-90)**[b] |
| Mean SpO2, % | 94 (92-95) | 96 (95-97)*** | 93 (92-94) | 96 (94-97)** |
| ODI, events/h | 20.55 (12.80-21.90) | 3.00 (1.30-6.02)*** | 33.15 (21.55-44.75) | 7.05 (5.67-17.63)[b] |
| MAI, events/h | 17.83 (13.15-21.42) | 17.82 (11.45-19.25) | 18.32 (14.48-26.38) | 17.82 (10.08-27.40) |

*BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA-IR, homeostasis model assessment of insulin resistance; GHb, glycated hemoglobin; GA, glycated albumin; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; ApoA-I, apolipoprotein A-I; ApoB, apolipoprotein B; ApoE, apolipoprotein E; Lp(a), lipoprotein (a); ESS, Epworth sleepiness score; AHI, apnea–hypopnea index; LSpO2, lowest pulse oxygen saturation; SpO2, pulse oxygen saturation; ODI, oxygen desaturation index; MAI, microarousal index. ** indicated p-value $< 0.05$ before versus after surgery in subgroup analysis. *** indicated p-value $< 0.001$ before versus after surgery in subgroup analysis. [a]indicated the p-value for the difference between the OSA remission and non-remission groups before surgery. [b]indicated the p-value for the difference between the OSA remission and non-remission groups after surgery.*

insulin resistance (33), diabetes (34) and coronary artery disease (35), but the physiological mechanisms underlying the regulation of circulating BCAA concentrations remain unknown (36–39). Whether BCAAs are a causative factor in insulin resistance and T2DM or just a biomarker of impaired insulin action requires further study. Two potential mechanisms explaining how BCAAs might contribute to insulin resistance in obesity and T2DM have emerged (32). First, mammalian target of rapamycin complex 1 (mTORC1) signaling is activated by excess of dietary BCAAs leading to insulin resistance and T2DM. Second, increased levels of BCAAs are a biomarker of impaired metabolism and BCAA dysmetabolism also leads to the accumulation of toxic metabolites that cause mitochondrial dysfunction in pancreatic islet β cells (or elsewhere). These findings indicate that mitochondrial dysfunction may play a role in OSA pathophysiology. When OSA improves, mitochondrial function recovers, causing a decrease in BCAA levels. Further studies are needed to determine the potential regulatory roles of the metabolites identified herein in OSA (40).

C24:1(cis-15), i.e., nervonic acid, is an ultra-long chain monounsaturated fatty acid that was first discovered in the nerve tissue of mammals (41). Increased nervonic acid in the diet improves the metabolic parameters of mice fed a high-fat diet (42). Nervonic acid is negatively associated with HOMA-IR and coronary risk factors, and protects against poor obesity-related metabolic outcomes (43). Martin et al. (44) found that saturated fatty acid and nervonic acid levels in obese adolescents were higher than those in the controls. Also, greater weight loss is

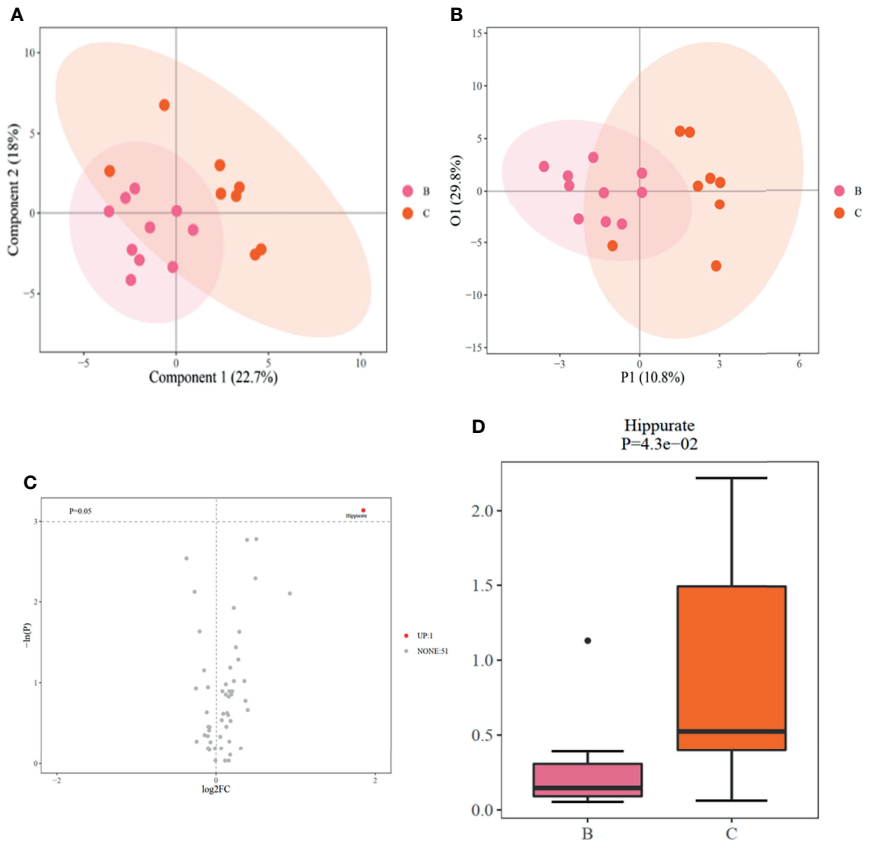


**FIGURE 2** | **(A).** PLSDA scores between OSA non-remission and remission groups at baseline; **(B)**. OPLS-DA scores between OSA non-remission and remission groups at baseline; **(C)**. Volcano plot of differential metabolites generated by univariate analysis between OSA non-remission and remission groups at baseline. UP indicates that the level of the metabolite (hippurate) was higher in the OSA non-remission group than the OSA remission group before RYGB surgery. NONE indicates 51 metabolites with no significant difference between the OSA remission and non-remission groups before RYGB surgery; **(D)**. Boxplot of hippurate generated by univariate analysis compared between OSA non-remission and remission groups. **(B)** indicates the OSA remission group and **(C)** indicates the OSA non-remission group. PLS-DA, partial least squares-discriminant analysis; OPLS-DA, orthogonal partial least squares-discriminant analysis; OSA, obstructive sleep apnea; RYGB, Roux-en-Y gastric bypass surgery.

associated with larger decreases in nervonic acid levels (45). Therefore, after RYGB surgery, obesity improves, and nervonic acid requirements are reduced. In other words, high levels of nervonic acid are not needed to improve obesity and metabolism, and the postoperative nervonic acid level thus decreases. However, the functional roles of nervonic acid in obesity and OSA are not fully understood. Additional research is needed to determine whether changes in nervonic acid expression are involved in the improvement in the pathophysiology, or are simply a consequence of the metabolic and OSA improvements provided by RYGB.

Hippurate is formed by benzoic acid and glycine in the liver. The ability of liver mitochondria to synthesize hippurate is related to the supply of adenosine triphosphate, glycine, and coenzyme A. A significant increase in serum glycine and alanine at 6 months after RYGB surgery was found in this study; thus, hippurate levels increased while BCAA levels decreased (46, 47), leading to improvement in OSA, restoration of mitochondrial function, and increased synthesis of hippurate after RYGB surgery. In our OSA remission group, the remission may have been associated with mitochondrial unction, whereas in the non-remission group, some other factor may have accounted for the high levels of hippurate before surgery, and thus the non-resolution of OSA after surgery.

Lower preoperative TMAO reflects milder inflammation status, making OSA remission more likely. Animal and clinical studies (15) have demonstrated that OSA is correlated with changes in intestinal microbes. Tremaroli et al. (48) found that postoperative TMAO levels were higher than preoperative levels after bariatric surgery, which might be due to shortening of the small intestine and reduced anaerobic metabolism by the gut microbiota. Furthermore, some microbes that increase after RYGB, such as *Pseudomonas*, can convert trimethylamine (TMA) into TMAO *via* the enzyme TMA mono-oxidase, theoretically contributing to the higher TMAO levels (48). Other factors might also increase TMAO levels after RYGB surgery. As the generation of TMAO depends on oxidation by hepatic flavin-containing monooxygenase (FMO3), the improved hepatic steatosis seen after RYGB surgery might result in restored hepatic function and FMO3 activation. Furthermore, an *in vitro* study showed that FMO3 is inhibited by

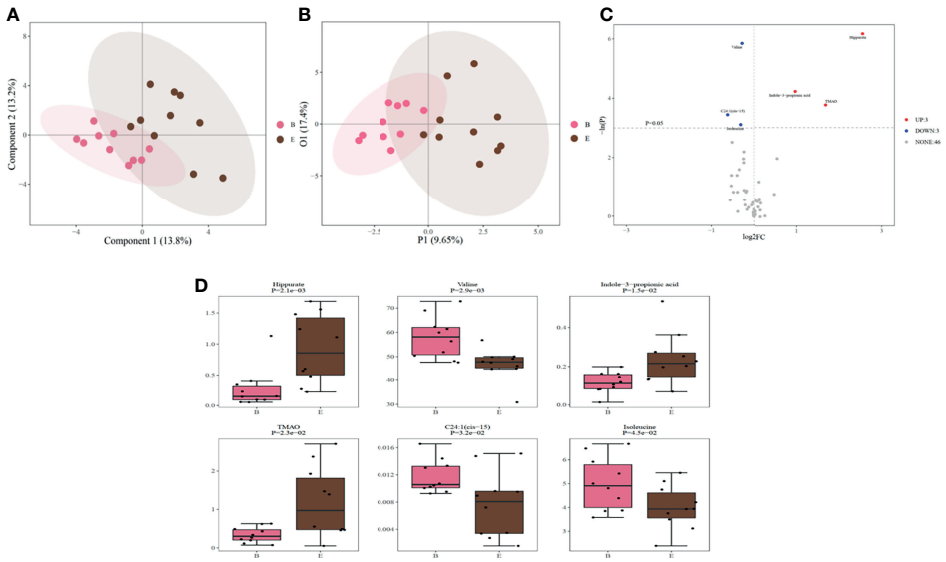


**FIGURE 3** | **(A).** PLS-DA scores reflecting metabolic changes in the OSA remission group before and after RYGB surgery; **(B)**. OPLS-DA scores reflecting metabolic changes in the OSA remission group before and after RYGB surgery. **(C)** Volcano plot of differential metabolites generated by the univariate analysis before and after RYGB surgery; UP indicates the three metabolites that increased after RYGB surgery in the OSA remission group. DOWN indicates the three metabolites that decreased after RYGB surgery in the OSA remission group. NONE indicates the 46 metabolites with no significant expression differences after RYGB surgery in the OSA remission group; **(D)** Boxplot of six differential metabolites with statistical significance generated by univariate analysis. B indicates the preoperative OSA remission group and E indicates the postoperative OSA remission group. PLS-DA, partial least squares-discriminant analysis; OSA, obstructive sleep apnea; OPLS-DA, orthogonal partial least squares-discriminant analysis; RYGB, Roux-en-Y gastric bypass surgery.

insulin (49). Thus, it is possible that, once insulin sensitivity has been restored, insulin levels decrease after RYGB surgery leading to higher expression of FMO3 and TMAO. Finally, dietary changes after RYGB surgery might affect TMAO levels, resulting in increased expression of carnitine and choline after initial diet-related reductions (50). Prospective studies with long-term follow-up are needed on the gut microbiota profile and microbiota-related metabolites in OSA. Altered diversity of the intestinal flora may be the cardinal factor in OSA and its associated cardiovascular, liver, and renal complications.

Indole-3-propionic acid is a bacterial metabolite derived from tryptophan in the gut that helps reduce the weight gain caused by antibiotics and a tryptophan-rich diet (51). The postoperative increase in indole-3-propionic acid promotes sustainable weight loss by attenuating the increased intestinal permeability and reversing the interferon gamma-induced transcriptional increase in expression of fructose transporter SLC2A5 (GLUT5) (52).

The level of C-peptide reflects the function of islet β-cells, and the decline of β-cell function is a prerequisite and core link for the onset of type 2 diabetes (53). With the increase in the severity of OSA, the islet function gradually declines, indicating that OSA is associated with impaired insulin sensitivity and function of islet β-cells (54). So, we hypothesis that a higher level of C-peptide before surgery suggesting a better islet function and a better compensate for the metabolic damage caused by OSA. Therefore, these subjects have better metabolic function and outcomes than those with lower C-peptide after TYGB, which is also of great significance to the improvement of OSA.

With the increasing prevalence of obesity and diabetes in the general population, OSA is also becoming increasingly common. Our results highlight the effect size of RYGB surgery for patients with obesity, T2DM and OSA, and demonstrate the metabolomic changes that occur after RYGB surgery. The main advantage of our procedure is the improvement of obesity and metabolic

**TABLE 3** | Metabolomic changes in the OSA remission group after surgery.

| Class | Metabolite | Before RYGB surgery (ng/mL) | After RYGB surgery (ng/mL) | P |
|---|---|---|---|---|
| Amino acids | Valine | 58.175 (50.808, 62.088) | 47.821 (45.342, 49.661) | 0.003 |
| Amino acids | Isoleucine | 4.908 (3.999, 5.794) | 3.937 (3.561, 4.613) | 0.045 |
| MUFAs | C24:1(cis-15) | 0.011 (0.010, 0.013) | 0.008 (0.003, 0.010) | 0.032 |
| Organic nitrogen compounds | TMAO | 0.303 (0.203, 0.473) | 0.972 (0.475, 1.815) | 0.023 |
| Benzene and substituted derivatives | Hippurate | 0.146 (0.091, 0.306) | 0.856 (0.503, 1.420) | 0.002 |
| Indoles | Indole-3-propionic acid | 0.110 (0.083, 0.159) | 0.216 (0.148, 0.270) | 0.015 |

*MUFA, monounsaturated fatty acid; TMAO, trimethylamine N-oxide. p-value indicated significant differences in metabolomics before versus after RYGB surgery.*

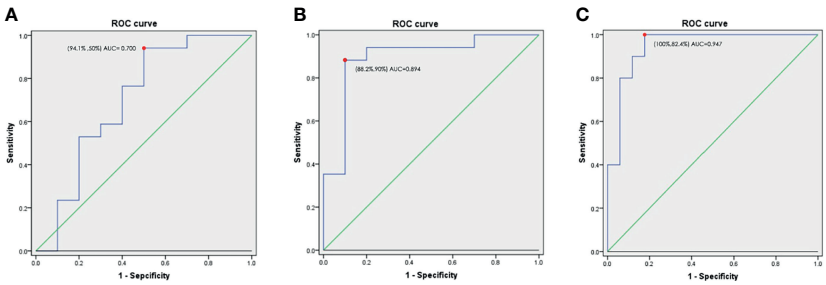


**FIGURE 4** | **(A).** ROC curve and AUC values of hippurate on predicting OSA remission after RYGB surgery; **(B)** ROC curve and AUC values of combined preoperative indices (age, AHI, and fasting C-peptide level) on predicting OSA remission after RYGB surgery; **(C)** ROC curve and AUC values of combined preoperative indices (age, AHI, and fasting C-peptide and hippurate levels) on predicting OSA remission after RYGB surgery. Abbreviations: ROC, receiver operating characteristic; AUC, area under the curve; AHI, apnea–hypopnea index.

status associated with alleviation of OSA. This study is the first to explore the impact of RYGB surgery on the metabolomic profile of OSA patients. We established a simple model to predict the likelihood of OSA remission. However, several limitations of this study should be addressed. First, the study was conducted under limited clinical conditions with a relatively small sample size and an observational design. Further studies including larger populations and more distinct groups are necessary to validate our results, and structured investigations using cell and animal models are necessary to determine the underlying molecular mechanisms. Second, data on the duration of diabetes were not collected, which have affected the outcomes to some extent. Although metabolomics has potential for diagnosing and predicting the prognosis of OSA, studies are still in the preliminary stages. Results are not always consistent and clinical application remains some way off, such that currently used clinical assessment methods, including PSG, cannot yet be replaced. Metabolomic profiling can be affected by several variables such as gender, medications, nutrition, and diurnal variations, and additional studies are needed to clarify the biological functions of the metabolites identified herein.

## CONCLUSIONS

Our study demonstrated that RYGB surgery improved the metabolic status of OSA patients. Several preoperative indices in combination, i.e., age, the AHI and fasting C-peptide and hippurate levels, had high sensitivity and specificity for the prediction of OSA remission. A large population-based, prospective validation study of certain metabolites may help elucidate their potential roles as biomarkers of OSA remission.

## AUTHORS NOTE

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/KmOQLp.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

The corresponding authors are responsible for the authenticity of the data. All authors made a significant contribution to the work reported, i.e., in the conception design or execution of the study, acquisition, analysis or interpretation of the data, or in all of these areas. WH, AZ, HX, XZ, HY, JG, and SY contributed to the study design, manuscript drafting or revision, or critical review of the article. HX, CX, AW, FW, XL, YL, JZ, and HZ took contributed to the data collection. WH, HX, XZ, CX, AW, FW, JZ, and HZ contributed to the statistical analyses. All authors approved the final version of the manuscript to be published, and agreed regarding the journal to which it has been submitted. All authors have agreed to be accountable for all aspects of the work.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.761547/full#supplementary-material

**Supplementary Figure 1 |** Heatmap based on the Spearman correlation matrix demonstrated clear correlations between the clinical indicators and metabolomic profiles of all patients before and after RYGB. RYGB, Roux-en-Y gastric bypass surgery; SaO2, pulse oxygen saturation; BMI, body mass index; AHI, apnea–hypopnea index; ESS, Epworth sleepiness score; ODI, oxygen desaturation index; MAI, micro-arousal index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; GHb, glycated hemoglobin; GA, glycated albumin; HOMA-IR, homeostasis model assessment of insulin resistance; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; ApoA-I, apolipoprotein A-I; ApoB, apolipoprotein B; ApoE, apolipoprotein E; Lp(a), lipoprotein (a).

**Supplementary Figure 2 |** Heatmap based on the Spearman correlation matrix demonstrated clear correlations between the clinical indicators and metabolomic profiles of OSA patients before and after RYGB. OSA, obstructive sleep apnea; RYGB, Roux-en-Y gastric bypass surgery; SaO2, pulse oxygen saturation; BMI, body mass index; AHI, apnea–hypopnea index; ESS, Epworth sleepiness score; ODI, oxygen desaturation index; MAI, micro-arousal index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; GHb, glycated hemoglobin; GA, glycated albumin; HOMA-IR, homeostasis model assessment of insulin resistance; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; ApoA-I, apolipoprotein A-I; ApoB, apolipoprotein B; ApoE, apolipoprotein E; Lp(a), lipoprotein (a).

**Supplementary Figure 3 |** Heatmap based on the Spearman correlation matrix demonstrated clear correlations between the clinical indicators and metabolomic profiles of OSA remission patients before and after RYGB. OSA, obstructive sleep apnea; RYGB, Roux-en-Y gastric bypass surgery; SaO2, pulse oxygen saturation; BMI, body mass index; AHI, apnea–hypopnea index; ESS, Epworth sleepiness score; ODI, oxygen desaturation index; MAI, micro-arousal index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; GHb, glycated hemoglobin; GA, glycated albumin; HOMA-IR, homeostasis model assessment of insulin resistance; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; ApoA-I, apolipoprotein A-I; ApoB, apolipoprotein B; ApoE, apolipoprotein E; Lp(a), lipoprotein (a).

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# Weight and Glycemic Control Outcomes of Bariatric Surgery and Pharmacotherapy in Patients With Melanocortin-4 Receptor Deficiency

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**Background:** Melanocortin-4 receptor (*MC4R*) mutations are the most common of the rare monogenic forms of obesity. However, the efficacy of bariatric surgery (BS) and pharmacotherapy on weight and glycemic control in individuals with MC4R deficiency (MC4R-d) is not well-established. We investigated and compared the outcomes of BS and pharmacotherapy in patients with and without MC4R-d.

**Methods:** Pertinent details were derived from the electronic database among identified patients who had BS with MC4R-d (study group, SG) and wild-type controls (age- and sex-matched control group, CG). Short- and long-term outcomes were reported for the SG. Short-term outcomes were compared between the two groups.

**Results:** Seventy patients were screened for MC4R-d. The SG [six individuals (four females, two males); 18 (10–27) years old at BS; 50.3 (41.8–61.9) kg/m$^2$ at BS, three patients with homozygous T162I mutations, two patients with heterozygous T162I mutations, and one patient with heterozygous I170V mutation] had a follow-up duration of up to 10 years. Weight loss, which varied depending on mutation type [17.99 (6.10–22.54) %] was stable for 6 months; heterogeneity of results was observed thereafter. BS was found superior to liraglutide on weight and glycemic control outcomes. At a median follow-up of 6 months, no significant difference was observed on weight loss (20.8% vs. 23.0%, $p$ = 0.65) between the SG and the CG [eight individuals (four females, four males); 19.0 (17.8–36.8) years old at BS, 46.2 (42.0–48.3) kg/m$^2$ at BS or phamacotherapeutic intervention]. Glycemic control in patients with MC4R-d and Type 2 diabetes improved post-BS.

**Conclusion:** Our data indicate efficacious short-term but varied long-term weight loss and glycemic control outcomes of BS on patients with MC4R-d, suggesting the importance of ongoing monitoring and complementary therapeutic interventions.

**Keywords: bariatric surgery, MC4R deficiency, monogenic obesity, obesity treatment, obesity pharmacotherapy, metabolic surgery**

## INTRODUCTION

Obesity etiology is complex with interplay between genetic and environmental factors. Early twin studies indicate that 40%–70% of obesity is hereditary (1). "Simple obesity" is mostly a polygenic disorder. However, several, albeit rare, monogenic forms are recognized. The commonly reported genetic dysregulations include mutations in the leptin and receptor (*LEP/LEPR*), proopiomelanocortin (*POMC*), proprotein convertase subtilisin/kexin type 1 (*PCSK1*) and melanocortin-4 receptor (*MC4R*) genes (2, 3).

Of those genes, the most common monogenic mutation with a prevalence of up to 6% have been reported to be alterations in the *MC4R* alleles (4–6). Melanocortin-4 receptor (MC4R) is an integral part of the leptin–melanocortin pathway, which is responsible for central energy homeostasis and body weight regulation (7). MC4R is a transmembrane G-protein-coupled receptor expressed in the hypothalamic neurons and follows an autosomal dominant mode of inheritance. Phenotypic characteristics in individuals with homozygous and also heterozygous *MC4R* mutation include excessive hunger and hyperphagia, which is the main driver of the significant and rapid weight gain (8, 9). As this is a genetic effect, it carries a substantial behavioral impact, especially in feeding and satiety (10, 11). In the UK, a recent study has estimated the frequency of heterozygous *MC4R* loss-of-function mutations at 0.30%, a considerably higher rate compared to previous approximations (12).

Treatment of obesity, in general, is a major challenge. Lifestyle interventions and pharmacotherapy may lead to some weight loss, but relapse is common (13, 14). Bariatric surgery (BS) is currently the most efficacious treatment for obesity with good short-term and long-term outcomes (15). However even when BS is part of the treatment algorithm for cases of "simple obesity", there is risk of weight regain in the long term (16).

Treatment of monogenic obesity (MO) may be even more challenging as MO is rare, and data on efficacy of different interventions are also sparse. Failure to lose sufficient weight or weight regain may be more common in patients with MO as their biological drive to eat may be unaffected by the standard interventions, including BS. Previous studies published in 2011 and 2014 suggest that patients with heterozygosity of the *MC4R* gene can be treated effectively in the short term with BS, but the longer-term success rates are not well described (17, 18). Similar observation in a patient with homozygous MC4R deficiency (MC4R-d) was reported in 2015 (19). A systematic review in 2021 synthesized outcomes of BS among patients with monogenic forms of obesity and found that weight loss results were inconsistent for patients with MC4R-d (20). Our group has previously reported short-term effects of BS in three patients with homozygous *MC4R* mutations, the youngest of whom was only 4 years old when a laparoscopic vertical sleeve gastrectomy (LSG) was performed (21). Here, we report longer-term BS efficacy results up to 10 years in these patients with homozygous *MC4R* mutation with a novel report on revisional BS long-term outcomes in a sibling pair. Three additional cases of heterozygous *MC4R* mutations are presented, and pharmacotherapeutic outcomes are also discussed.

## MATERIALS AND METHODS

Patients who have had genetic screening were identified from the Imperial College London Diabetes Centre (ICLDC) electronic database. Genetic screening is performed in patients suspected to have a genetic component for obesity such as morbid obesity and strong family history. *MC4R* mutations were screened through either targeted sequencing for *MC4R* gene, whole exome sequencing, or Diabetes-Obesity New Generation Sequencing panel. Patients who tested positive for MC4R-d (study group, SG) and age- and sex-matched individuals with normal *MC4R* genotype (control group, CG) were identified based on the reported results from the genetic screening. The study flow diagram is presented in **Figure 1**.

Case history and pertinent details such as age, weight, and HbA1c before and after BS and pharmacotherapy were derived from electronic medical records. Weight was measured and recorded as part of vitals assessment. HbA1c test was performed in the in-house laboratory. Medication compliance was assessed based on physicians' or clinic notes. Patients' glycemic control was managed at ICDLC. BS was performed in different centers locally and abroad. For patients who had multiple or revisional BS, the last surgery was used for pertinent analyses. Data were analyzed and presented as median (interquartile range—IQR).

Written informed consent for using medical data for research purpose in an anonymized form was obtained from all participants at the time of first visit. The study was approved by the Research Ethics Committee at ICLDC and followed the Declaration of Helsinki, 1996.

## RESULTS

Genetic screening for *MC4R* mutation(s) was performed on 70 ICLDC patients between 2010 and 2020. Twenty-one patients were tested positive to have a mutation in *MC4R* gene. Three mutations were identified: c.485C>T (T162I), 508A>G (I170V), and 307G>A (V103I). Six individuals composed the SG [four females, two males; 18 (10–27) years old at BS; 50.3 (41.8–61.9) kg/m$^2$ at BS; three patients with homozygous T162I mutations, two patients with heterozygous T162I mutations, and one patient with heterozygous I170V mutation]. Eight individuals composed the CG [four females, four males; 19.0 (17.8–36.8) years old at BS, 46.2 (42.0–48.3) kg/m$^2$ at BS or phamacotherapeutic intervention]. All patients are Emirati.

### Patients With MC4R-d: Presentation of Cases

Patient 1 (P1) was born full term normal delivery (FTND) with a birthweight of 3.5 kg to non-consanguineous parents. He started gaining weight around 3 years of age. He weighed 143 kg (BMI 52 kg/m$^2$; >99$^{th}$ percentile) at the age of 12 years. He underwent LSG in another center in the UAE before a genetic diagnosis in our clinic was made. He lost 18 kg in 6 months and his glycemic control improved (HbA1c 6.1%, off metformin). He started to regain his lost weight, reaching a weight of 177 kg (BMI 60 kg/m$^2$) by 6 years post-surgery. A second operation, RYGB

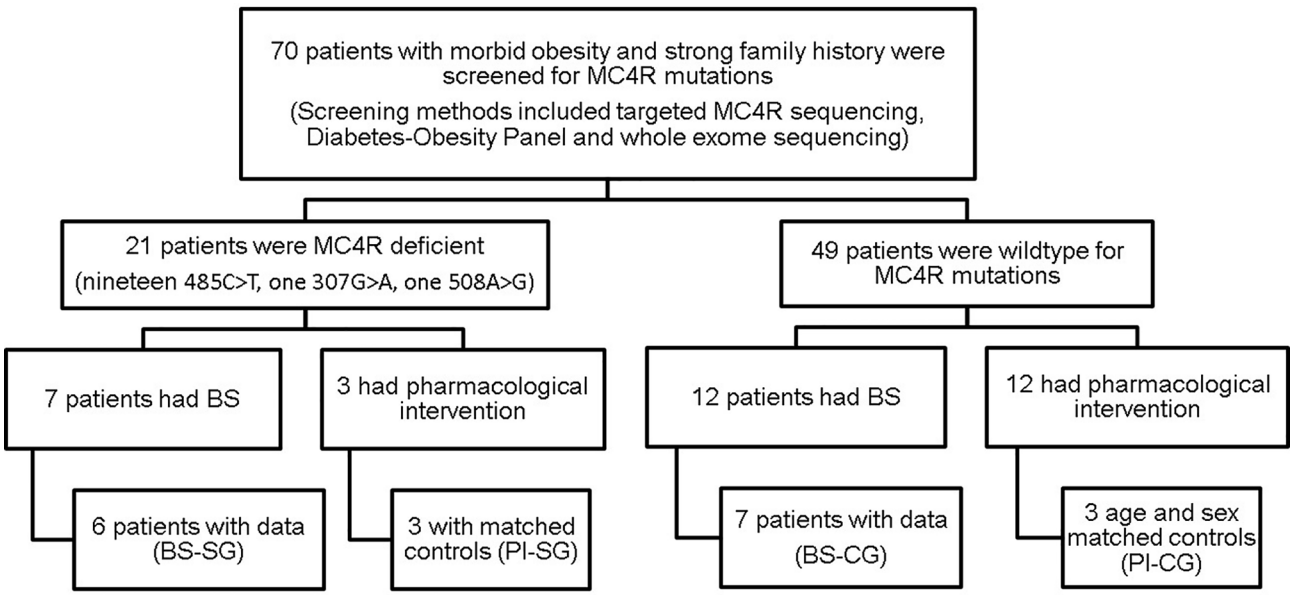


**FIGURE 1** | Flowchart of study population: an overview of patient selection. MC4R, melanocortin-4 receptor; BS, bariatric surgery; PI, pharmacological intervention; SG, study group; CG, control group.

was performed at 18 years of age. He lost 15 kg (BMI 55 kg/m$^2$) in 1 year and had an HbA1c of 5.6% off metformin. In the recent 2 years, he was able to maintain good glycemic control (mean HbA1c 5.5%; range 5.4%–5.7%). However, his weight fluctuated; mean BMI was 52.7 kg/m$^2$ (range 50.0–56.8 kg/m$^2$). Genetic diagnosis was available some months after the operation and showed him to be homozygous for the missense mutation T162I in the *MC4R* allele.

Patient 2 (P2) is the older sister of P1. She was also born FTND with a birth weight of 3.5 kg. She started gaining weight quite early, around the age of 1 year. She weighed 150 kg (BMI 60 kg/m$^2$; >99$^{th}$ percentile) at the age of 14; glycemic control was poor with an HbA1c of 9.7%. She underwent LSG, also prior to genetic diagnosis. She lost 22 kg (BMI 53 kg/m$^2$) in 6 months. Her weight was consistently lower postoperatively with slight fluctuations near 130 kg, but her HbA1c varied widely. She underwent a second operation, RYGB, at the age of 20 years. She lost 9 kg (BMI 47 kg/m$^2$) by 3 months post-RYGB and maintained her weight loss up to 2 years later; glycemic control did improve, but remained inadequate (HbA1c 7.4%). In the subsequent 2 years, there were slight variations in her BMI (mean 46.0; range 43.8–48.2 kg/m$^2$) and wider fluctuations in her glycemic control (mean 8.4%; range 6.9%–9.6%). Most recently, she has been on a diet program and has managed to lose 13 kg in weight, with good improvement in her glycemic control (HbA1c dropping from 9.0% to 5.2%) in a matter of 2 months. She is currently on vidagliptin + metformin. Genetic test results available after LSG have also shown a genotype of *MC4R*-/- with the missense mutation T162I.

Patient 3 (P3) was born full term to consanguineous Emirati parents (second cousins). Weight gain and obesity started at 6 months of age. At the age of 10 months, she was unable to sit normally and was hospitalized to help manage her excessive weight. She had acanthosis nigricans, impaired fasting glycemia, elevated fasting insulin, and HOMA-IR. Other features of note were severe vitamin D deficiency, hypertension, and obstructive sleep apnoea (OSA). At the age of 4, she weighed 67.8 kg (BMI 44 kg/m$^2$). She had a full assessment including a review of management options by the Hospital Ethics Committee and underwent LSG in the United States. Four months post-surgery, her BMI was 9 points lower; she had normal fasting glucose, HbA1c, vitamin D, and cortisol. She lost over 13 kg (BMI 33 kg/m$^2$) 9 months after sleeve gastrectomy. However, she subsequently gained 2 kg within 2 months. By year 4 post-operatively, her BMI was 35.8 kg/m$^2$ with an HbA1c of 6.0%. One year later (5 years after her surgery), her BMI was 39.3 kg/m$^2$, close to her pre-operative BMI; HbA1c was 6.3% and she was prescribed metformin. At 6.5 years post-BS, her BMI is 42.1 kg/m$^2$. Her genetic testing (done at age 4) showed her to be homozygous for an *MC4R* mutation (T162I). P3 has a younger brother with the same *MC4R* mutation who recently had BS at 5 years of age and presented with non-alcoholic steatohepatitis (stage 3–4), a condition not previously seen in P3. P3 also has two other siblings with *MC4R* null genotype and rare *ACBD5* mutation, which affects peroxisomal oxidation of very long chain fatty acids.

Patient 4 (P4), aunt of P3, started to become overweight at age 15 years. She had impaired fasting glucose (IFG) with normal HbA1c levels. Other history of relevance included a strong familial history of diabetes and hyperlipidemia. She was able to lose 20 kg of weight through diet but regained this same amount. At age 33 years, she was severely obese (weight 114 kg, BMI

49 kg/m$^2$). She underwent sleeve gastrectomy and had lost 20 kg (BMI 40 kg/m$^2$) in 4 months. Over a year after surgery, her BMI was 32.9 kg/m$^2$ (total weight loss of approximately 27 kg) and her HbA1c was 5.4%. Two years post-BS, her BMI increased to 36.1 kg/m$^2$ and her HbA1c was slightly lower at 5.1%. Genetic testing (performed and available before surgery) revealed that she was heterozygous for T162I mutation in the *MC4R* gene.

Patient 5 (P5), the sister of P4, was seen in our clinic at the age of 21 years. At the time, she weighed 181 kg (BMI 71 kg/m$^2$). Comorbidities included hypothyroidism, IFG and impaired glucose tolerance (IGT), dyslipidemia, fatty liver, OSA, hypertension, and deficiencies in vitamin B12 and D. A trial of dietetic intervention and medical treatment with liraglutide, with a target BMI of lower than 60 kg/m$^2$ were unfortunately unsuccessful. She proceeded with sleeve gastrectomy (pre-BS BMI 68 kg/m$^2$) at the age of 24 years and had a drastic weight loss of 18 kg (BMI 60 kg/m$^2$) in 1.5 months, with a further 16 kg (BMI 54 kg/m$^2$) weight loss in the subsequent 2 months and a reduction in her HbA1c to 4.9%. She is currently on vitamins B12 and D3, iron, and thyroxine supplementation. Genetic testing (done pre-operatively) showed her to be heterozygous for T162I *MC4R* mutation.

Patient 6 (P6) is a 16-year-old male with a strong family history of diabetes who first came to the center for pediatric obesity management. He was noted to have acanthosis, and by age 17, he developed Type 2 diabetes (T2D). He had poor glycemic control, at least in part due to non-compliance to medication. He was also hypertensive, and had hypertriglyceridemia, proteinuria, hyperuricemia, and joint pain. By age 23, he weighed 110 kg (BMI 35 kg/m$^2$) and had an HbA1c of 9.6%. He underwent sleeve gastrectomy and lost 23 kg (BMI 28 kg/m$^2$) in 7 months with excellent glycemic control on metformin only. He is recently off metformin, has normal blood pressure, and is taking vitamin D3, multivitamins, and vitamin B12. *MC4R* sequencing revealed heterozygosity with variant I170V, a missense alteration.

## Weight Loss and Glycemic Control Outcomes

### Bariatric Surgery

Patient characteristics and response to BS are summarized in **Table 1**. Genetic testing revealed two *MC4R* genotypes [five (83.3%) T162I, one (16.7%) I170V] in our cohort. LSG was the BS performed for all patients, with two siblings (P1 and P2) who had RYGB as revisional BS procedures.

For all six patients, stable weight loss was observed up to at least 6 months (**Figure 2**), supporting our former observation that MC4R may not be essential for short-term weight loss post-BS. However, our data indicate heterogeneity in response to LSG after 6 months: one patient had continued net weight loss for over 5 years (P2); two patients (P3, P4) continued to lose weight for several months with slight increases observed subsequently; while one patient (P1) regained his initial weight after over a year, with further weight gain until 6.5 years when he had a revisional RYGB procedure. Outcomes for P1 and P2, who are siblings with the same mutation and second BS (Roux-en-Y gastric bypass), were very different.

Glycemic control outcomes of the patients after BS are also dissimilar (**Figure 3**). Although P1 has increasingly gained weight post-LSG, his HbA1c levels have lessened and were consistently lower than his pre-surgery HbA1c. In contrast, his sister, P2, who had significant weight reduction post-LSG and RYGB has not seen improvement in her glycemic control; her HbA1c levels were widely fluctuating and were at times much higher than her pre-operative values. Interestingly, her recent diet program (self-reported as composed of low-calorie diet, calorie counting, and no addition of sugar whether white, brown, or artificial sweetener) made her lose 13 kg in less than 3 months, leading to a drastic drop in her HbA1c (from 9.0% to 5.2%). Similar trends were observed for P3 who had a slight increase in HbA1c and a modest weight gain, and P6 who had weight loss and a net reduction in HbA1c levels. Slight variations in glycemic control were observed with considerable weight loss for P4 and P5 who are sisters.

### Pharmacotherapy

Our limited data (**Table 2**) indicate apparent lack of efficacy of liraglutide (1.8 mg OD) in homozygous T162I mutation in short-term use; weight gain and increased HbA1c level were observed in the female patient P2 after 3 months of treatment. In contrast, 14 months use of liraglutide (3.0 mg OD) did benefit patient P5 (heterozygous T162I deficit) with a weight loss of 5.6% (from 179 kg to 169 kg) and a 7.4% improvement (from 5.4% to 5.0% HbA1c) in glycemic control. Short-term (3 months) use of

**TABLE 1** | Patient characteristics and response to bariatric surgery procedures in patients with *MC4R* deficiency.

| Patient | P1 | | P2 | | P3 | P4 | P5 | P6 |
|---|---|---|---|---|---|---|---|---|
| **Sex** | Male | | Female | | Female | Female | Female | Male |
| **Zygosity** | Homozygous | | Homozygous | | Homozygous | Heterozygous | Heterozygous | Heterozygous |
| **Variant** | T162I | | T162I | | T162I | T162I | T162I | I170V |
| **Type of surgery** | LSG | RYGB | LSG | RYGB | LSG | LSG | LSG | LSG |
| **Age (years) at surgery** | 12 | 18 | 14 | 20 | 4 | 33 | 25 | 22 |
| **Weight (kg) before LSG** | 143 | 177 | 150 | 130 | 67.8 | 114 | 171 | 110 |
| **BMI (kg/m$^2$) before LSG** | 52.0 | 60.5 | 60.0 | 50.3 | 44.0 | 49.0 | 67.6 | 35.0 |
| **Lowest weight (kg) after LSG** | 125 | 149 | 124 | 112 | 54.5 | 77 | 137.2 | 88.1 |
| **Lowest BMI (kg/m$^2$) after LSG** | 45.4 | 50.7 | 48.6 | 43.8 | 33.3 | 32.9 | 53.6 | 28.1 |
| **% Weight loss** | 12.6 | 15.8 | 17.0 | 13.8 | 19.6 | 32.5 | 19.8 | 19.9 |
| **HbA1c (%) before LSG** | 8.1 | 6.1 | NA | 8.0 | NA | NA | 5 | 9.6 |
| **HbA1c (%) after LSG** | 5.6–6.6 | 5.4–5.9 | 6.5–11.4 | 6.8–9.6 | 5.5–6.3 | 4.8–5.4 | 4.9 | 5.7–8.3 |

*LSG, laparoscopic sleeve gastrectomy; RYGB, Roux-en-Y gastric bypass; BMI, body mass index; MC4R, melanocortin-4 receptor; NA, not available.*

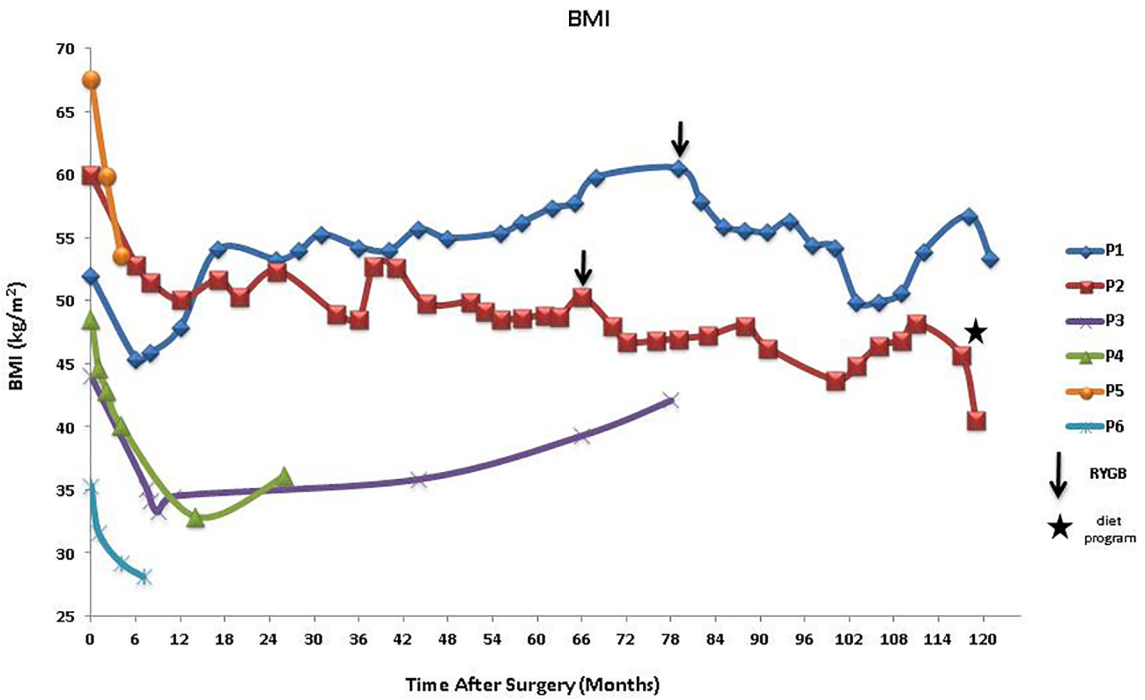


**FIGURE 2** | BMI after LSG in patients with MC4R deficiency showing other interventions, RYGB, and diet program. BMI, body mass index; LSG, laparoscopic sleeve gastrectomy; RYGB, Roux-en-Y gastric bypass; MC4R, melanocortin-4 receptor.

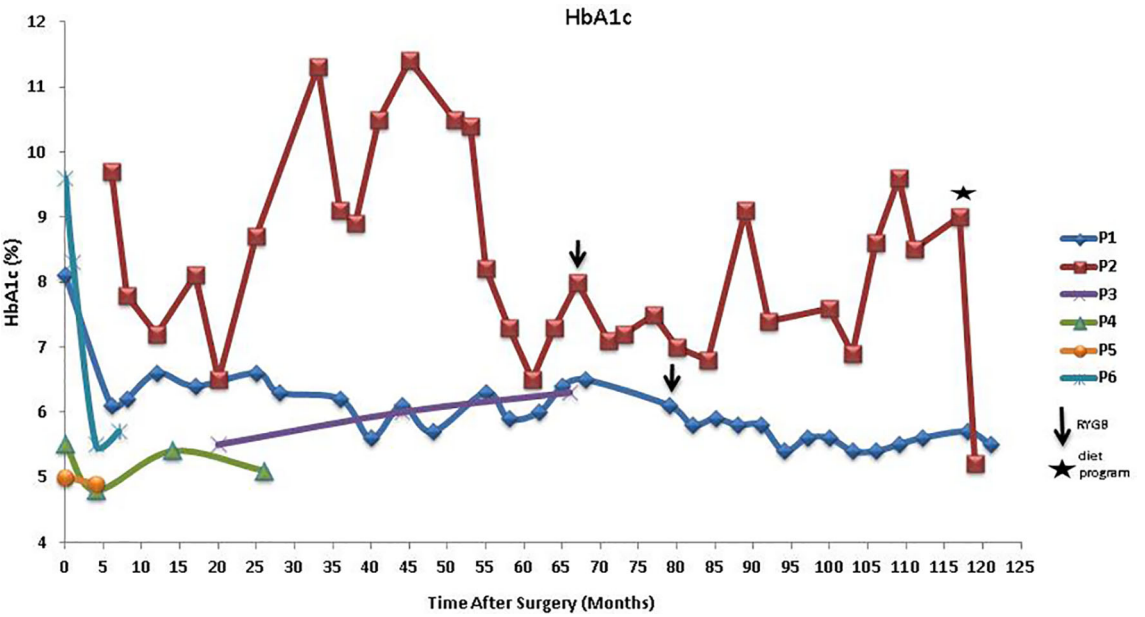


**FIGURE 3** | HbA1c after LSG in patients with MC4R deficiency showing other interventions, RYGB, and diet program. LSG, laparoscopic sleeve gastrectomy; RYGB, Roux-en-Y gastric bypass; MC4R, melanocortin-4 receptor.

**TABLE 2 |** Patient characteristics and response to pharmacotherapy in patients with MC4R deficiency.

| Patient Characteristics | Before BS |
|---|---|
| **P2*** | |
| Pharmacotherapy | Liraglutide |
| Dose | 1.8 mg OD |
| Age (years) at prescription (Rx) | 19 |
| Weight (kg) before Rx | 125 |
| BMI (kg/m$^2$) before Rx | 48.8 |
| Treatment duration (months) | 3 |
| Lowest weight (kg) after Rx | 129 |
| Lowest BMI (kg/m$^2$) after Rx | 50.3 |
| % Weight loss | –3.1% |
| HbA1c (%) before Rx | 7.3 |
| HbA1c (%) after Rx | 8.0 |
| **P5** | |
| Pharmacotherapy | Liraglutide |
| Dose | 3.0 mg OD |
| Age (years) at prescription (Rx) | 22 |
| Weight (kg) before Rx | 179 |
| BMI (kg/m$^2$) before Rx | 70 |
| Treatment duration (months) | 14 |
| Lowest weight (kg) after Rx | 169 |
| Lowest BMI (kg/m$^2$) after Rx | 66 |
| % Weight loss | 5.6 |
| HbA1c (%) before Rx | 5.4 |
| HbA1c (%) after Rx | 5.0–5.1 |
| **P6** | |
| Pharmacotherapy | Orlistat |
| Dose | 120 mg TID |
| Age (years) at prescription (Rx) | 21 |
| Weight (kg) before Rx | 110 |
| BMI (kg/m$^2$) before Rx | 35.1 |
| Treatment duration (months) | 3 |
| Lowest weight (kg) after Rx | 112 |
| Lowest BMI (kg/m$^2$) after Rx | 35.9 |
| % Weight loss | –1.8% |
| HbA1c (%) before Rx | 9.6 |
| HbA1c (%) after Rx | 10.2 |

*MC4R, melanocortin-4 receptor; OD, once daily; TID, three times daily; BMI, body mass index. *Before RYGB.*

orlistat (120 mg TID) did not benefit patient P6 (heterozygous I170V) in both weight loss and glycemic control as the patient, in fact, had small weight and HbA1c increase.

Patients P2, P5, and P6 were 19, 22, and 22 years of age, respectively, when they started the treatments. Patient P2 was treated with liraglutide after LSG due to weight regain. However, due to unsatisfactory outcomes, revisional BS, RYGB, was performed. Both patients P5 and P6 who were treated with liraglutide and orlistat, respectively, were tried on these pharmacotherapeutic interventions prior to LSG. Overall, BS was found superior to liraglutide and orlistat for both weight loss and glycemic control outcomes.

## Weight Loss and Glycemic Control Comparison in Individuals With and Without MC4R-d

Weight loss percentage and glycemic control lowering at a median of 6 months post-intervention were compared between age- and sex-matched MC4R-deficient and wild-type controls (**Table 3**).

Weight loss at median 6 months post BS was not significantly different between patients with and without *MC4R*-d ($p = 0.65$). However, the weight loss post BS in patients with *MC4R*-d showed variation depending on the type of mutation [17.99 (6.1–22.54) %]. Homozygous T162I did not benefit from BS in terms of weight loss compared to their age- and sex- matched controls. Heterozygous T162I and heterozygous I170V benefited similarly from BS compared to the controls. Response to liraglutide treatment was comparable in *MC4R* T162I heterozygous patient and control. Outcomes of orlistat use was inconsistent: patient heterozygous for I170V mutation had a marginal weight gain, while age- and sex-matched wild type had over 10% excess body weight loss; homozygous T162I did not benefit and had small weight gain as much as the control. Weight loss comparison following surgical and pharmacological interventions at median 6 months between the study and control groups is shown in **Figure 4**.

Glycemic control outcomes after sleeve gastrectomy in two *MC4R*-deficient patients (heterozygous I170V and homozygous T162I) who both had T2D were substantial compared to their non-diabetic controls. On liraglutide use, the patient with heterozygous T162I had similar HbA1c lowering with his age- and sex-matched control (0.4% vs. 0.3%).

**TABLE 3 |** Weight loss and glycemic control comparison following surgical and pharmacological interventions at median 6 months between age- and sex-matched individuals with and without *MC4R*-deficiency.

| Type of intervention | Age (MC4R-deficient vs. MC4R wild type | Sex | Mutation | Zygosity | Weight loss % | | HbA1c % lowering | |
|---|---|---|---|---|---|---|---|---|
| | | | | | *MC4R*-deficient | *MC4R* wild type | *MC4R*-deficient | *MC4R* wild type |
| Sleeve gastrectomy | 33.5/32.2 | F | T162I | Heterozygous | 19.05 | 15.24 | NA | 0.5 |
| | 23/18 | M | I170V | Heterozygous | 22.54 | 29.9 | 3.9 | 0.2 |
| | 18/17.8 | M | T162I | Homozygous | 8.08 | 35.27 | 2 | 0.3 |
| | 14/18 | F | T162I | Homozygous | 6.91 | 23.75 | NA | NA |
| | 24.5/18 | F | T162I | Heterozygous | 20.81 | 23.75 | -0.1 | NA |
| Liraglutide (3 mg OD) | 22.9/18.2 | F | T162I | Heterozygous | 4.89 | 5.61 | 0.4 | 0.3 |
| Orlistat (120 mg TID) | 20.5/19.5 | M | I170V | Heterozygous | -0.91 | 10.29 | NA | NA |
| | 12/14.5 | M | T162I | Homozygous | -4.62 | -4.1 | NA | NA |

*MC4R, melanocortin-4 receptor; OD, once daily; TID, three times daily; NA, not available.*

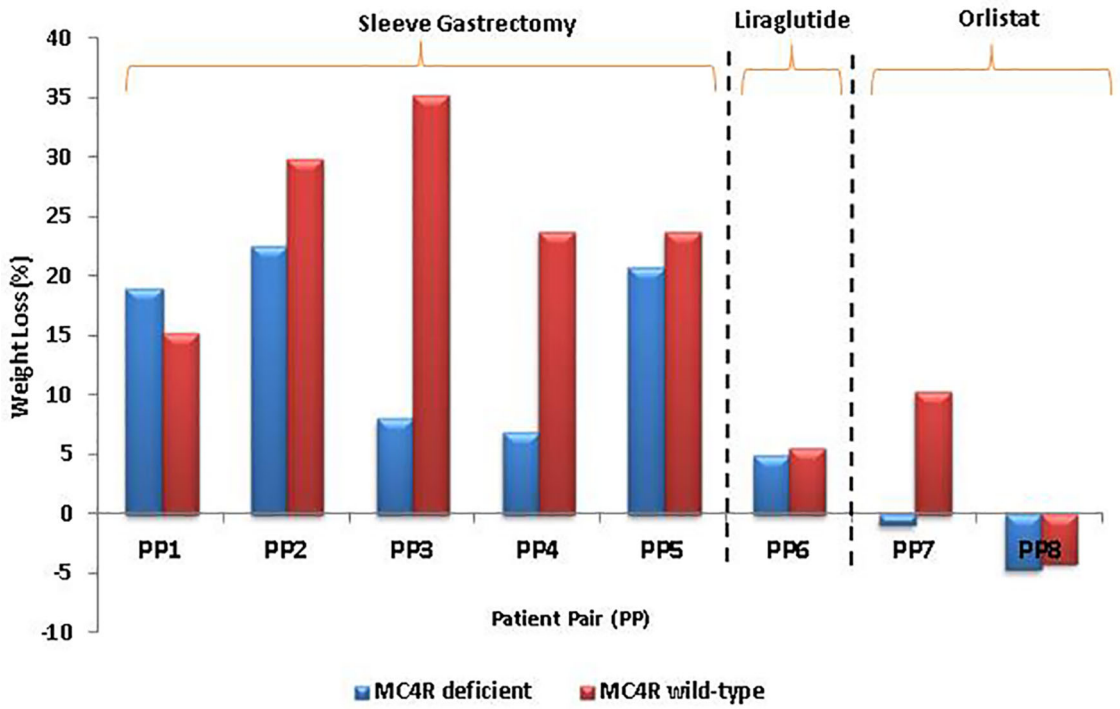


**FIGURE 4** | Comparison of weight loss following surgical and pharmacological interventions at median 6 months between age- and sex-matched individuals with and without MC4R-deficiency, as shown in **Table 3**. MC4R, melanocortin-4 receptor.

## DISCUSSION

The melanocortin system regulates appetite and an intact leptin–melanocortin signaling is required for normal physiological response to calorie restriction (22–24). Lifestyle modification for weight loss is difficult for people with MC4R-d as they are less responsive to diet and exercise. Hence, BS is often considered as the obesity treatment of choice for individuals with MC4R-d. Our previous work has indicated that MC4R signaling may not be needed for short-term response to sleeve gastrectomy, with continuous weight loss observed several months post-BS (21). Here we report the short- and long-term outcomes of BS on weight and glycemic control in Emirati patients with *MC4R*-d, including two siblings (male and female) who had a revision of their previous LSG to a RYGB. The mutations reported in our study, both T162I and I170V, are missense mutations and result in partial (T162I) and complete (I170V) loss of function of *MC4R* gene (25, 26). A recent article reported long-term weight loss outcome on bi-allelic mutation in the *MC4R* gene in a patient who underwent sleeve gastrectomy only (27). The recent systematic review reported that most of the studies on outcomes of BS in MC4R mutation found no association of *MC4R* mutations with weight loss (20). We are reporting, for the first time to our knowledge, long-term outcomes of revisional surgery (RYGB) in both sexes with the *MC4R* null genotype. Our results suggest that longer-term response may be determined by factors other than *MC4R* genotype. These factors may include sex and gut microbiome as well as other environmental factors, as may be the case in patients with "simple obesity" (28–30).

Although the role of genetics in the case of P1 and P2 cannot be delineated, how mutation type and zygosity affect BS outcomes in these patients has been observed. Weight loss 6 months post-BS was not significantly different among patients without and with heterozygous MC4R-d, suggesting that in terms of weight reduction, patients with heterozygous T162I and I170V mutations may benefit from BS as much as MC4R-normal individuals, a finding similar to a previous report (17). Another recent study has reported no significant weight loss on a patient with compound heterozygous mutation (c.105C>A; p.Y35X, c.110A>T; p.D37V) (27). Individuals with homozygous T1621 mutation in *MC4R* and those with compound heterozygous *MC4R*-d might require multiple surgeries or continued pharmacological intervention to maintain weight loss over a longer period of time. It is also noteworthy that our younger patient had a more favorable outcome in terms of weight loss, suggesting that earlier intervention in homozygous T162I is more likely to be beneficial; increasing BMI from 12 months post-BS warrant longer-term monitoring. On glycemic control, mutation type and zygosity do not appear to influence the outcomes suggesting the significance and interplay of other factors (31, 32). Adherence to a strict diet as in the case of P2 may be required for best glycemic control regardless of BS (33). The lack of diet information on the other patients as well as physical activity profile of the cohort including the control group is a

limitation of the study, which may be addressed in prospective studies.

With liraglutide treatment, we have observed that response was comparable in patients with heterozygous T1621 mutation in *MC4R* and controls (34). Liraglutide treatment for 16 weeks has been reported to result in comparable weight loss and decrease in HbA1c in patients with *MC4R*-d and controls (35, 36). However, the difference in % weight loss of BS compared to liraglutide use on individuals with heterozygous T162I may still be vast (14.2% for P5). Again, the outcome of P2's diet program on her weight and glycemic control underscores the importance of diet and lifestyle modification on *MC4R* deficits, in addition to pharmacotherapeutic course of treatment and BS. The effect of dietary and lifestyle interventions may depend on the type of mutation (37). An additional finding here is that short-term orlistat treatment did not seem to have any beneficial effects on weight loss or glycemic control for our patient with heterozygous I170V mutation.

In conclusion, we have described here outcomes of BS with or without selected pharmacotherapy in a cohort of patients with heterozygous and homozygous *MC4R* mutations. Our data indicate beneficial but varied long-term effects of BS on these patients, which suggests that life-long monitoring and additional therapies including adjunct pharmacotherapy and revisional BS should be considered. Further elucidation of the mechanisms behind these results requires a larger cohort of patients in multicenter studies.

## DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the ClinVar repository, accession numbers SCV002014765.1, SCV002014763.1, SCV002014764.1.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee, Imperial College London Diabetes Centre. Written informed consent to participate in this study was provided by the participants and/or participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

EF: study design, data acquisition and interpretation, and manuscript writing; SR: data acquisition, statistical analyses and manuscript writing; TA: study design and manuscript writing; EN: manuscript writing; NL: study design, data interpretation, and manuscript writing.

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# Efficacy and Safety of *Lactobacillus plantarum* K50 on Lipids in Koreans With Obesity: A Randomized, Double-Blind Controlled Clinical Trial

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**Background:** Only few studies have investigated the role of probiotics in the development of obesity. We aimed to determine the efficacy and safety of an intake of *Lactobacillus plantarum* K50 (*LPK*) on body fat and lipid profiles in people with obesity.

**Methods:** This randomized, double-blind, placebo-controlled, clinical trial involved 81 adults with a body mass index of 25–30 kg/m$^2$ who were assigned randomly to a diet including 4 × 10$^9$ colony-forming unit of *LPK* or a placebo. Changes in body fat, anthropometric parameters, and biomarkers of obesity were compared using a linear mixed-effect model.

**Results:** After 12 weeks of treatment, body weight, fat mass, and abdominal fat area did not change significantly in the two groups. However, total cholesterol levels decreased from 209.4 ± 34.4 mg/dL to 203.5 ± 30.9 mg/dL in the *LPK* group, but increased from 194.7 ± 37.5 mg/dL to 199.9 ± 30.7 mg/dL in the placebo group (P = 0.037). Similarly, triglyceride levels decreased from 135.4 ± 115.8 mg/dL to 114.5 ± 65.9 mg/dL in the *LPK* group, with a significant difference between groups. *LPK* supplementation also tended to decrease leptin levels compared with placebo. It also changed the distribution of gut microbiota significantly, with an increase in *L. plantarum* and a decrease in *Actinobacteria*, both of whose changes in abundance were correlated with changes in visceral adiposity, with borderline significance.

**Conclusion:** A 12-week consumption of *LPK* reduced the total cholesterol and triglyceride levels significantly with favorable alterations in microbiota, suggesting potential benefits for controlling blood lipid profiles.

**Keywords: *Lactobacillus plantarum*, probiotics, obesity, body fat, lipid**

## INTRODUCTION

The prevalence of obesity has been increasing worldwide (1). Obesity in Asia is increasing rapidly with rapid environmental changes. Asians have a genetically distinct body composition and are adapted to a high carbohydrate nutritional intake (2). Now, Western-type food intake coupled with reduced physical activity and an urban lifestyle are leading to an increase in obesity in this region (3). South Korea is not an exception to this trend (4). The rates of noncommunicable diseases in Korea, such as coronary heart disease, hypertension, and type 2 diabetes are increasing almost linearly along with increased average body mass index (BMI) (4). Indeed, obesity is associated with a multitude of comorbidities spread across several different organs (5). Comorbidities can be classified into three major domains: the metabolic domain includes cardiovascular diseases and type 2 diabetes; the mechanical domain includes asthma, chronic back pain, and knee osteoarthritis; and the mental domain includes depression and anxiety (6–8).

Of note, dysbiosis of the gut microbiota is now recognized as a major contributor to chronic human diseases including obesity (9, 10). More specifically, people with obesity have larger numbers of *Firmicutes* and fewer *Bacteroidetes* than lean people (9). *Firmicutes* and *Bacteroidetes* are associated with the development of obesity, thereby affecting the host's acquisition of nutrients and energy balance (10, 11). In a study with 68 obese subjects and 47 controls, *Bifidobacterium animalis* tended to be associated with normal weight whereas *Lactobacillus (L.) reuteri* was associated significantly with obesity (12).

From a different perspective, a meta-analysis of 15 randomized controlled trials has found that administration of probiotics, mostly containing $10^9$ to $10^{11}$ colony-forming units (CFU) of *L.* and *Bifidobacterium* species, resulted in a significant reduction in body weight and BMI, compared with placebo (13). A recent proof-of-concept study showed that administration of pasteurized *Akkermansia muciniphila* for 3 months decreased plasma lipopolysaccharide in people with obesity and metabolic impairment (14). Thus, changes in the intestinal microflora have emerged as indicators of obesity and metabolic regulation, indicating that their alteration can be both a cause and treatment target for obesity and metabolic disorders (15).

*L.* species have been studied widely for their metabolic benefits, such as reducing body fat mass, body weight, and cholesterol levels (13). A significant weight loss of 1.5% ($P < 0.0001$) was achieved after 6 months of supplementation with *L.* and *Bifidobacterium* in adults with obesity or overweight status (16). In a study of healthy Japanese subjects, body fat mass decreased significantly after the administration of *L. gasseri* SBT2055 for 12 weeks (17). In one study, there was no significant decrease in the body weight of subjects after the ingestion of soymilk fortified with *L. planetarium* A7 for 8 weeks (18), and another found no significant decrease in body fat after taking *L. gasseri* BNR17 for 12 weeks (19). In our previous study, administration of $5 \times 10^9$ CFU of *L. sakei* resulted in a mean body weight reduction of 2 kg with borderline significance (20).

Thus, the anti-obesogenic effects of probiotics are inconclusive in humans. However, treatment with an *L. plantarum* K50 (*LPK*), which is isolated from Kimchi, was reported to decrease weight in mice (21). Therefore, the aim of this study was to examine whether the administration of probiotics containing *LPK* would have beneficial effects on body fat, body weight, and related metabolic factors in people with obesity.

## MATERIALS AND METHODS

### Subjects and Study Design

We enrolled healthy men and women aged 20 to 65 years with a BMI of 25–30 kg/m$^2$ who understood the content of the study and agreed to participate in this clinical trial. Individuals were excluded if they reported any of the following criteria: had continuously taken drugs that affect weight, lipid, blood glucose metabolism, immune and inflammatory reactions within one month prior to screening; had continuously consumed foods that could affect intestinal health within 1 month before screening; had uncontrolled hypertension or diabetes mellitus requiring treatment; had abnormal liver function with increased aspartate aminotransferase (AST), or alanine aminotransferase (ALT) levels more than 3 times the upper limit of normal; or abnormal kidney function (serum creatinine levels > 1.4 mg/dL); had thyroid dysfunction; had participated in a commercial antiobesity program or had been treated with a calorie-restricted diet within 3 months; had a weight change of 5% or more within 3 months; or had undertaken surgery such as gastroplasty or enterectomy for weight loss.

In all, 92 patients were screened for this study; 81 were eligible to participate and were randomized to either the *LPK* or placebo groups (**Figure 1**). Participants received two daily allocations of $2 \times 10^9$ CFU of *LPK* (total $4 \times 10^9$ CFU/day) or the equivalent placebo for 12 weeks (**Supplementary Figure S1**). The participants were instructed by a trained research coordinator to follow the standard instructions for a healthy lifestyle from the run-in period to the end of the study. They were advised to exercise regularly at least three times per week for ≥30 min for each session. The nutritional instructions were developed based on the Dietary Reference Intakes for Koreans (KDRIs) published by the Ministry of Health and Welfare and The Korean Nutrition Society (http://kns.or.kr/English/Publication.asp). Detailed information about these instructions is provided in the **Supplementary Material**. Good compliance with the treatment was defined as taking over 80% of the allocation.

During the study period, six participants in the *LPK* group and four in the placebo group dropped out (**Figure 1**). Three did not visit for the follow-up measurement and one did not meet the criteria for medication compliance, and the others withdrew from the study without specific reasons. The overall compliance in both groups was >94%.

This study was conducted according to our management standards with approval of the Ethics Committee of Seoul

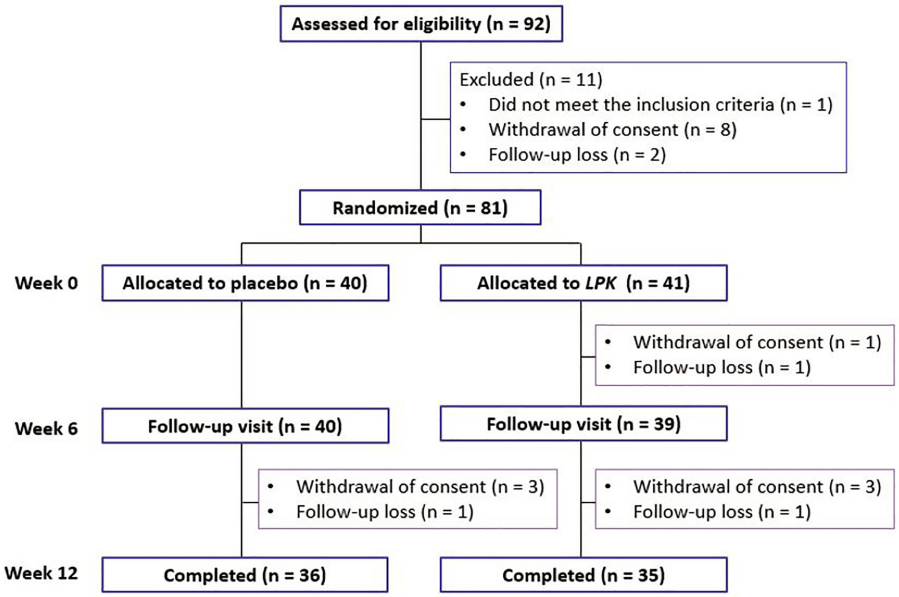


**FIGURE 1** | Distribution of the study participants during the study period. LPK, *Lactobacillus plantarum* K50.

National University Bundang Hospital (SNUBH; B-1901-516-002). The subjects provided written informed consent after listening to detailed explanations from the researchers. This study was registered at the Clinical Research Information Service of the Republic of Korea (KCT0003944; https://cris.nih.go.kr/cris/en/use_guide/cris_introduce.jsp).

## Study Materials

The *LPK* strain was isolated from Kimchi incubated in a modified MRS medium (21). It was cultured in MRS broth at 37°C for 24 h. After that, it was inoculated into a fermenter with the optimized medium. Fermentation was performed under constant pH (6.0 ± 0.5) and agitation at 37°C for 16 h. After fermentation, the culture medium was removed, and cells were harvested, concentrated, and lyophilized. Lyophilized *LPK* was ground and packaged in polyethylene and aluminum bags. Packaged probiotics were stored at 4°C before dispatch. Placebo and probiotic capsules were provided by CKDBiO Corp. (Ansan, South Korea). Probiotic capsules were composed of *LPK* and microcrystalline cellulose powder, named CKDB156. Microcrystalline capsules with texture, color, and odor identical to those of the probiotic were used as the placebo vehicle. Quality checking of both products including coliform group bacteria, heavy metals, residual pesticides, and nutrients was performed and approved by the Korea Advanced Food Research Institute of the Korea Food Industry Association (Uiwang, South Korea).

## Primary and Secondary Outcomes

The primary outcome of this study was any change in the subjects' body fat mass from the baseline to 12 weeks after beginning treatment. The key secondary outcomes were changes in BMI, body weight, waist circumference, and abdominal adipose tissue area from baseline to 12 weeks. Other secondary outcomes were changes in metabolic parameters from baseline to 12 weeks.

## Assessment of Body Composition

Body weight and height were measured using standard methods with the subject in light clothing. BMI was calculated as weight (kg) divided by height (in meters) squared. Waist circumference was measured at the umbilical level. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured with an electronic blood pressure meter while the subjects were seated. Blood pressure was measured twice at a 5-min interval, and the mean value was used in the analysis.

To measure whole-body fat mass, muscle mass, and percent body fat in the study participants, dual-energy X-ray absorptiometry (DXA; Horizon W, Hologic Inc., Bedford, MA, USA) was used. Scanning was performed with the subject supine, and all scans were completed within 15 min.

Abdominal adipose tissue areas were quantified by a single scouting view of a computed tomography (CT) scan (Somatom Sensation 16; Siemens, Munich, Germany). Subjects were examined in the supine position with their arms outstretched overhead to decrease beam hardening or streak artifacts. Scanning was performed at 90-kV exposure. The exposure time was 0.1 s, and the scanning time was 0.5 s. A 10-mm CT slice scan was acquired at the umbilical level to measure the total abdominal and visceral fat areas. Adipose tissue attenuation was determined by measuring the mean value of all pixels within the range of −190 to −30 Hounsfield units. The images were converted into files compatible with a commercial software program (Rapidia; 3DMED, Seoul, South Korea). To assess the visceral adipose tissue (VAT) area, each abdominal image was

edited by erasing the image exterior to the innermost abdominal wall muscles with a mouse-driven cursor, and the resulting images were saved in separate files.

## Collection of Lifestyle Information and Measurement of Biochemical Parameters

Alcohol consumption, smoking status, and menstruation status were investigated through questionnaires. The eating habits of the participants were investigated using a recommended food score questionnaire.

The subjects fasted for 12 h overnight, and venous blood samples were taken for biochemistry assays. Plasma glucose concentration was measured using the glucose oxidase method (747 Clinical Chemistry Analyzer, Hitachi, Tokyo, Japan). Fasting plasma insulin level was measured using a radioimmunoassay (Linco, St. Charles, MO, USA). AST and ALT (NADH-UV method) and creatinine (Jaffe's kinetic method) levels were measured using an Architect Ci8200 Analyzer (Abbott Laboratories, Abbott Park, IL, USA). The estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI creatinine equation (22). Serum high-sensitivity C-reactive protein (hsCRP) level was measured *via* a high-sensitivity automated immunoturbidimetric method (CRP-Latex [II]X2; Denka Seiken Co., Tokyo, Japan). Concentrations of total cholesterol, TG, HDL-C, low-density lipoprotein cholesterol (LDL-C), and free fatty acids were measured using a 747 Clinical Chemistry Analyzer (Hitachi).

Adiponectin levels were measured using an enzyme-linked immunosorbent assay (ELISA; Otsuka Pharmaceutical Co., Tokyo, Japan). Levels of tumor necrosis factor-α (TNF-α) were measured in duplicate in serum samples using a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA, intra-assay coefficient of variation [CV] < 6.2%, inter-assay CV < 11%, detection sensitivity 20 pg/mL). Levels of interleukin (IL)-6 were measured in duplicate in serum samples using the ELISA method (R&D Systems, intra-assay CV < 8.4%, inter-assay CV < 9.6%, detection sensitivity 0.003–0.014 ng/mL). Plasma glucagon concentrations were determined using a validated ELISA (Mercodia AB, Uppsala, Sweden). Serum concentrations of total ketones were measured using enzymatic immunoassay kits (Nittobo Medical Co. Ltd., Tokyo, Japan). Analyses of lipopolysaccharide-binding protein (LBP), resistin, and soluble cluster of differentiation 14 (sCD14) were performed using commercially available ELISA kits (AdipoGen Life Sciences, San Diego, CA, USA) according to the manufacturer's instructions in a certified laboratory (GCCL, Yongin, South Korea). Assays for leptin were performed in duplicate by ELISA (R&D Systems).

## Microbial Sequencing

Metagenomic DNA extraction, library construction, and sequencing for microbiome analysis were all performed by Macrogen, Inc. (Seoul, South Korea). Fecal samples were collected from subjects within 3 days of the study visit date and stored at −80°C until analyzed. Metagenomic DNA was extracted from the fecal samples using DNeasy PowerSoil Pri kits (QIAGEN, Hilden, Germany) and underwent quality control inspection. Qualified samples were subjected to tagmentation that combines the fragmentation of DNA, and 5′ and 3′ adapter-ligation processes. Adapter-ligated DNA fragments were subjected to polymerase chain reaction amplification of the V3 and V4 regions of 16s rRNA genes and purified by gel electrophoresis. Prepared libraries were loaded into flow cells and amplified to generate clusters. Then, sequencing was performed using the MiSeq system (Illumina Inc., San Diego, CA, USA). Sequencing data were converted to FASTQ files for analysis. These FASTQ data were processed for data trimming and taxonomic classification using the Greengenes reference database (http://greengenes.secondgenome.com).

## Statistical Analysis

Based on a previous study using two strains of *L* (23)., the sample size for this study was calculated as 35 per group; this would give 90% power to detect differences in the abdominal fat area. Considering the relatively high follow-up loss rate in obesity studies in general, we planned to enroll 50 participants in each group, assuming a maximum 30% loss rate. The complete randomized intention-to-treat population of this study was used for evaluating efficacy and safety. Baseline demographics and clinical data are reported for all subjects as the number and (percentage) and the mean ± standard deviation (SD). Comparisons between mean subject values at baseline were analyzed using Student's t test for continuous variables and chi-square tests for categorical variables. Anthropometric and biochemical parameters at 12 weeks were compared with their baseline values using a linear mixed-effect model with fixed effects of treatment, time, treatment-by-time interaction, and subject-specific random intercepts. We used IBM SPSS Statistics for Windows version 22.0 (IBM Corp., Armonk, NY, USA) and $P < 0.05$ was considered significant.

For microbiota, relative abundance was used for analysis, including α-diversity calculated using the Shannon index. Gut microbial dissimilarities between groups at the genus level were visualized by principal coordinates analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA) using Bray–Curtis dissimilarities at the genus level. The linear relationship between the changes in clinical values and relative abundance of microbiota before and after dietary supplementation were subjected to Pearson's correlation analysis. Paired Student's t tests or Wilcoxon signed-rank tests were applied to detect mean differences in the gut microbial features at baseline and posttreatment measurements. P-values were adjusted using the Benjamini–Hochberg method for multiple comparisons. The microbiome analysis was conducted using R software version 4.1.0 (R Development Core Team, Vienna, Austria) and RStudio version 1.4.1103 (RStudio, Boston, MA, USA).

# RESULTS

## Baseline Characteristics

**Table 1** lists the baseline characteristics of study participants. The ratio of men to women was similar between the two groups.

**TABLE 1** | Baseline characteristics of study participants.

| Variables | Placebo (*n*=40) | | | *LPK* (*n*=41) | | | P |
|---|---|---|---|---|---|---|---|
| Age, year | 45.5 | ± | 10.0 | 47.8 | ± | 11.7 | 0.335 |
| Sex, male/female | | 16/24 | | | 16/25 | | 0.930 |
| Body weight, kg | 74.0 | ± | 8.8 | 74.6 | ± | 9.9 | 0.752 |
| Body mass index, kg/m$^2$ | 27.3 | ± | 1.6 | 27.1 | ± | 1.5 | 0.559 |
| Waist circumference, cm | 93.0 | ± | 5.1 | 93.2 | ± | 5.6 | 0.886 |
| Systolic blood pressure, mmHg | 125.5 | ± | 10.2 | 124.0 | ± | 10.9 | 0.548 |
| Diastolic blood pressure, mmHg | 78.6 | ± | 9.3 | 76.7 | ± | 10.1 | 0.376 |
| Fasting glucose, mg/dL | 98.5 | ± | 11.8 | 97.5 | ± | 10.8 | 0.698 |
| Total ketone, μmol/L | 214.1 | ± | 121.5 | 244.5 | ± | 164.8 | 0.404 |
| Free fatty acid, μEq/L | 590.6 | ± | 245.4 | 614.9 | ± | 208.2 | 0.661 |
| AST, IU/L | 32.6 | ± | 9.5 | 26.0 | ± | 6.5 | 0.001 |
| ALT, IU/L | 41.2 | ± | 22.2 | 29.2 | ± | 11.6 | 0.004 |
| Creatinine, mg/dL | 0.74 | ± | 0.16 | 0.72 | ± | 0.14 | 0.606 |
| eGFR, mL/min/1.7 m$^2$ | 105.1 | ± | 11.4 | 104.2 | ± | 10.7 | 0.714 |
| hsCRP, mg/L | 0.87 | ± | 0.85 | 0.48 | ± | 0.31 | 0.018 |
| TNF-α, pg/mL | 3.5 | ± | 1.1 | 2.9 | ± | 0.8 | 0.005 |
| Total cholesterol, mg/dL | 194.7 | ± | 37.5 | 209.4 | ± | 34.4 | 0.070 |
| Triglyceride, mg/dL | 119.0 | ± | 44.1 | 135.4 | ± | 115.8 | 0.418 |
| HDL-cholesterol, mg/dL | 55.9 | ± | 11.2 | 59.2 | ± | 13.3 | 0.242 |
| LDL-cholesterol, mg/dL | 125.2 | ± | 30.7 | 129.3 | ± | 24.0 | 0.505 |
| Alcohol drinker, Yes/No | | 20/20 | | | 26/15 | | 0.223 |
| Smoker, Yes/No | | 6/34 | | | 7/34 | | 0.799 |
| Smoking amount, cigarette/day | 13.7 | ± | 11.9 | 11.8 | ± | 5.6 | 0.651 |
| Menstruation, Yes/No/NA | | 14/10/16 | | | 14/11/16 | | 0.983 |
| Recommended food score | 21.1 | ± | 7.6 | 20.3 | ± | 8.2 | 0.658 |

*The data are presented as the mean ± standard deviation or as the number of subjects. Student's t test for continuous variables and chi-square test for categorical variables were used to compare differences between groups. LPK, Lactobacillus plantarum K50; AST, aspartate aminotransferase; ALT, alanine aminotransferase; eGFR, estimated glomerular filtration rate calculated by CKD-EPI creatinine equation; hsCRP, high-sensitivity C-reactive protein; NA, not applicable.*

The mean age was 47.8 ± 11.7 years in the *LPK* group and 45.5 ± 10.0 years in the placebo group. The baseline BMI values were 27.1 ± 1.5 kg/m$^2$ in the *LPK* group and 27.3 ± 1.6 kg/m$^2$ in the placebo group. Most baseline parameters were not significantly different between the two groups except for the levels of liver enzymes, hsCRP, and TNF-α.

## Changes in Body Fat Mass and Other Anthropometric and Body Composition Parameters

Total body fat mass—the primary outcome measure of this study—did not change significantly in either group (**Table 2**). BMI, body weight, and abdominal adipose tissue area also did not change in either group. For waist circumference, the *LPK* group showed a nonsignificant tendency to decrease from 93.2 ± 5.6 cm to 91.3 ± 4.9 cm (P = 0.093) (**Table 2**).

## Changes in Blood Pressure and Biochemical Parameters

The SBP/DBP and other metabolic parameters and their changes are listed in **Table 3**. Most parameters at baseline were not different between the two groups except for the levels of liver enzymes, hsCRP, and TNF-α. The SBP tended to decrease in the *LPK* group and tended to increase in the placebo group, but these differences were not significant (P = 0.059).

The total cholesterol level decreased from 209.4 ± 34.4 mg/dL to 203.5 ± 5.3 mg/dL in the *LPK* group but increased from 194.7 ± 37.5 mg/dL to 199.9 ± 30.7 mg/dL in the placebo group (P = 0.037). Similarly, the TG level decreased from 135.4 ± 115.8 mg/dL to 114.5 ± 65.9 mg/dL in the *LPK* group but increased significantly from 119.0 ± 44.1 mg/dL to 143.0 ± 73.1 mg/dL in the placebo group (P = 0.009), indicating significant differences between the groups (P = 0.014).

Leptin levels decreased from 2.8 ± 1.8 ng/mL to 2.6 ± 1.7 ng/mL in the *LPK* group but increased from 2.4 ± 1.5 ng/mL to 3.3 ± 2.6 ng/mL in the placebo group, showing a marginal difference between the two groups (P = 0.092). Serum creatinine, AST and ALT levels, and other biochemical parameters were not different between the groups (**Table 3**). There was no significant difference between the two groups in terms of changes in other biomarkers such as LBP, resistin, and sCD14.

## Changes in Gut Microbiota

The changes in the gut microbiota are shown in **Figure 2**. At the phylum level, *LPK* supplementation significantly decreased *Actinobacteria* abundance compared with placebo, which was positively correlated with VAT area ($r$ = 0.24, P = 0.051) (**Figure 2A**). Overall α- and β-diversity values were not different between the two groups although *LPK* supplementation significantly increased the abundance of *L. plantarum* (0.05% ± 0.18% vs –0.01% ± 0.05%) (P < 0.05) (**Figures 2B, C**). Changes in *L. plantarum* raw counts were inversely correlated with changes in the abdominal adipose tissue area before and after therapy, with borderline significance ($r$ = –0.25, P = 0.073, **Supplementary Figure S2**). Among the genera in the order *Lactobacillales*, abundance in *Enterococcus*, which was inversely related to

**TABLE 2 |** Changes in body composition in the LPK and placebo groups after 12 weeks.

| Variables | Placebo (*n*=40) | | | | | | | *LPK* (*n*=41) | | | | | | | P† |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Baseline | | | 12 weeks | | | P* | Baseline | | | 12 weeks | | | P* | |
| Body weight, kg | 74.0 | ± | 8.8 | 74.8 | ± | 9.1 | 0.208 | 74.6 | ± | 9.9 | 74.2 | ± | 10.0 | 0.726 | 0.440 |
| BMI, kg/m$^2$ | 27.3 | ± | 1.6 | 27.5 | ± | 1.9 | 0.227 | 27.1 | ± | 1.5 | 27.0 | ± | 1.7 | 0.572 | 0.521 |
| Waist circ., cm | 93.0 | ± | 5.1 | 92.4 | ± | 6.1 | 0.461 | 93.2 | ± | 5.6 | 91.3 | ± | 4.9 | 0.093 | 0.495 |
| Fat mass, kg | 27.3 | ± | 3.8 | 27.9 | ± | 4.6 | 0.892 | 28.0 | ± | 4.3 | 27.1 | ± | 3.3 | 0.257 | 0.380 |
| Lean mass, kg | 43.1 | ± | 8.0 | 43.6 | ± | 8.1 | 0.053 | 43.3 | ± | 8.3 | 42.9 | ± | 8.5 | 0.567 | 0.064 |
| Body fat, % | 38.2 | ± | 5.6 | 38.1 | ± | 5.7 | 0.303 | 38.5 | ± | 5.7 | 38.7 | ± | 5.7 | 0.984 | 0.434 |
| TAT, cm$^2$ | 379.3 | ± | 95.3 | 362.4 | ± | 112.6 | 0.266 | 373.1 | ± | 75.8 | 369.2 | ± | 72.8 | 0.923 | 0.420 |
| VAT, cm$^2$ | 130.9 | ± | 55.7 | 127.4 | ± | 55.1 | 0.686 | 113.9 | ± | 45.1 | 111.6 | ± | 43.2 | 0.977 | 0.753 |
| SAT, cm$^2$ | 248.4 | ± | 76.2 | 235.1 | ± | 85.8 | 0.158 | 247.5 | ± | 70.1 | 245.9 | ± | 62.7 | 0.978 | 0.334 |

*Presented as the mean ± standard deviation. LPK, Lactobacillus plantarum K50; BMI, body mass index; TAT, total adipose tissue; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue. *P-values for the differences between the groups for 12 weeks were obtained from a linear mixed-effect model. †P-values for differences within each group were obtained from a linear mixed-effect model.*

**TABLE 3 |** Changes in blood pressures and biomarkers in the LPK and placebo group after 12 weeks.

| Variables | Placebo (*n*=40) | | | | | | | *LPK* (*n*=41) | | | | | | | P† |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Baseline | | | 12 weeks | | | P* | Baseline | | | 12 weeks | | | P* | |
| SBP, mmHg | 125.5 | ± | 10.2 | 127.9 | ± | 9.6 | 0.057 | 124.0 | ± | 10.9 | 121.9 | ± | 10.1 | 0.436 | 0.059 |
| DBP, mmHg | 78.6 | ± | 9.3 | 77.1 | ± | 8.0 | 0.247 | 76.7 | ± | 10.1 | 74.7 | ± | 7.2 | 0.285 | 0.977 |
| Glucose, mg/dL | 98.5 | ± | 11.8 | 97.3 | ± | 7.9 | 0.824 | 97.5 | ± | 10.8 | 94.5 | ± | 6.3 | 0.154 | 0.400 |
| Insulin, μIU/mL | 9.2 | ± | 4.7 | 10.0 | ± | 4.9 | 0.250 | 6.8 | ± | 3.7 | 6.7 | ± | 3.1 | 0.658 | 0.273 |
| Glucagon, pg/dL | 144.2 | ± | 77.6 | 145.1 | ± | 69.8 | 0.674 | 109.6 | ± | 44.2 | 108.6 | ± | 33.7 | 0.964 | 0.783 |
| Total ketone, μmol/L | 214.1 | ± | 121.5 | 185.7 | ± | 80.6 | 0.109 | 244.5 | ± | 164.8 | 210.4 | ± | 117.6 | 0.358 | 0.891 |
| Free fatty acid, μEq/L | 590.6 | ± | 245.4 | 578.6 | ± | 152.2 | 0.780 | 614.8 | ± | 208.2 | 647.4 | ± | 206.4 | 0.475 | 0.479 |
| Leptin, ng/mL | 2.4 | ± | 1.5 | 3.3 | ± | 2.6 | 0.175 | 2.8 | ± | 1.8 | 2.6 | ± | 1.7 | 0.383 | 0.092 |
| Adiponectin, μg/mL | 20.0 | ± | 12.0 | 20.2 | ± | 11.7 | 0.958 | 21.4 | ± | 12.3 | 23.0 | ± | 13.4 | 0.796 | 0.914 |
| TNF-α, pg/mL | 3.5 | ± | 1.1 | 3.6 | ± | 0.9 | 0.911 | 2.9 | ± | 0.8‡ | 2.8 | ± | 1.0 | 0.818 | 0.795 |
| IL-6, pg/mL | 2.2 | ± | 4.7 | 1.5 | ± | 2.5 | 0.389 | 0.6 | ± | 0.2 | 0.7 | ± | 0.4 | 0.639 | 0.380 |
| sCD14, ng/mL | 1493 | ± | 191 | 1326 | ± | 312 | 0.003 | 1454 | ± | 334 | 1398 | ± | 356 | 0.427 | 0.098 |
| LBP, ng/mL | 14304 | ± | 2226 | 11094 | ± | 1781 | <0.001 | 13699 | ± | 2566 | 11008 | ± | 1789 | <0.001 | 0.356 |
| Resistin, ng/mL | 26.4 | ± | 12.7 | 26.3 | ± | 12.6 | 0.277 | 26.3 | ± | 12.4 | 26.6 | ± | 14.8 | 0.725 | 0.280 |
| AST, IU/L | 32.6 | ± | 9.5 | 29.9 | ± | 7.8 | 0.178 | 26.0 | ± | 6.5‡ | 24.6 | ± | 6.1 | 0.130 | 0.733 |
| ALT, IU/L | 41.2 | ± | 22.2 | 37.0 | ± | 20.1 | 0.171 | 29.2 | ± | 11.6‡ | 24.1 | ± | 10.9 | 0.001 | 0.996 |
| Creatinine, mg/dL | 0.74 | ± | 0.16 | 0.70 | ± | 0.16 | 0.088 | 0.72 | ± | 0.14 | 0.68 | ± | 0.13 | 0.035 | 0.885 |
| eGFR, mL/min/1.7 m$^2$ | 105.1 | ± | 11.4 | 108.9 | ± | 9.7 | 0.068 | 104.2 | ± | 10.7 | 108.1 | ± | 10.2 | 0.04 | 0.759 |
| hsCRP, mg/L | 0.87 | ± | 0.85 | 0.65 | ± | 0.40 | 0.122 | 0.48 | ± | 0.31‡ | 0.46 | ± | 0.25 | 0.867 | 0.168 |
| Total cholesterol, mg/dL | 194.7 | ± | 37.5 | 199.9 | ± | 30.7 | 0.159 | 209.4 | ± | 34.4 | 203.5 | ± | 30.9 | 0.113 | 0.037 |
| Triglyceride, mg/dL | 119.0 | ± | 44.1 | 143.0 | ± | 73.1 | 0.009 | 135.4 | ± | 115.8 | 114.5 | ± | 65.9 | 0.784 | 0.014 |
| HDL-C, mg/dL | 55.9 | ± | 11.2 | 55.4 | ± | 11.4 | 0.798 | 59.2 | ± | 13.3 | 59.6 | ± | 13.3 | 0.609 | 0.861 |
| LDL-C, mg/dL | 125.2 | ± | 30.7 | 129.1 | ± | 27.2 | 0.212 | 129.3 | ± | 24.0 | 128.7 | ± | 25.6 | 0.847 | 0.268 |

*Data are presented as the mean ± standard deviation. LPK, Lactobacillus plantarum K50; SBP, systolic blood pressure; DBP, diastolic blood pressure; TNF-a, tumor necrosis factor-a; IL-6, interleukin-6; sCD14, soluble cluster of differentiation 14; LBP, lipopolysaccharide-binding protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; eGFR, estimated glomerular filtration rate calculated by CKD-EPI creatinine equation; hsCRP, high-sensitivity C-reactive protein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. *P-values for the differences within each group were obtained from a linear mixed-effect model. †P-values for differences between groups after 12 weeks were obtained from a linear mixed-effect model. ‡Significant difference between the two groups at baseline.*

obesity status (fat mass: $r$ = –0.41, P = 0.063; body weight: $r$ = –0.22, P = 0.070), increased significantly in the *LPK* group compared with the placebo group (0.70% ± 2.32% vs 0.09% ± 0.28%) (P < 0.05) (**Figure 2D**). The relative abundance of the order *Lactobacillales* was similar between the groups. However, the composition of genera changed differently resulting in a significant between-group difference (PERMANOVA = 0.003) (**Figure 2E**). Specifically, the abundance of *Enterococcus hirae* was significantly increased in the *LPK* group (0.70% ± 2.33% vs 0.09% ± 0.28%) compared with the placebo group (P < 0.05) (**Figure 2F**). This might have been caused by *LPK* supplementation because it showed a positive correlation with the abundance of *L. plantarum* ($r$ = 0.22, P = 0.047).

## Adverse Events

There were no significant differences between the groups in terms of the occurrence, type, degree of symptoms, and relevance to intervention (**Table 4**). The symptom severities of adverse reactions were all mild, and any relationship with the

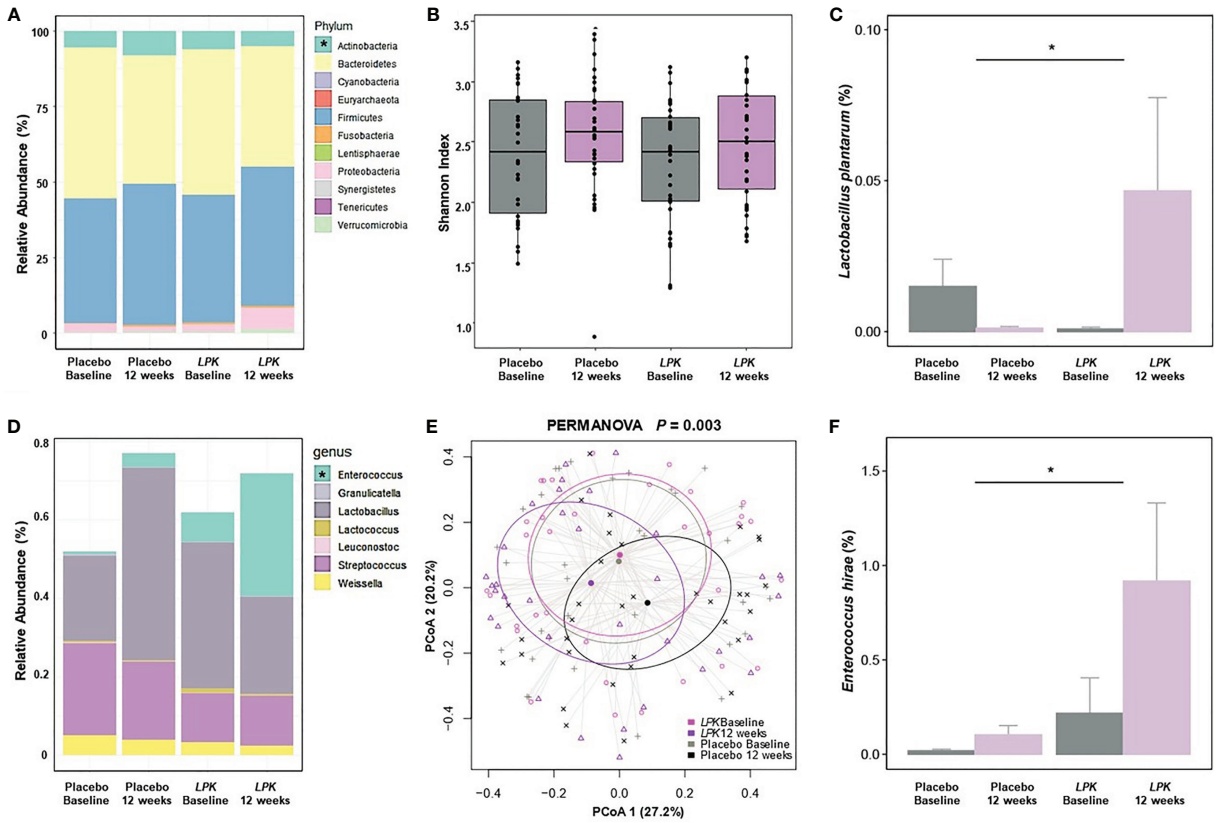


**FIGURE 2** | Changes in gut microbiota after a 12-week dietary supplementation with *LPK* or placebo. **(A)** Relative abundance of the phyla. **(B)** The alpha diversity is shown as a Shannon index. **(C, F)** Comparison of logarithmic mean changes of rarefied counts of *Lactobacillus plantarum* and *Enterococcus hirae*. **(D)** Relative abundance of the genera in the order *Lactobacillales*. **(E)** The beta diversity as PCoA of the genus in the order *Lactobacillales*. *LPK*, *Lactobacillus plantarum* K50; PCoA, principal coordinates analysis. Asterisks show between-group differences for changes after 12-week supplementation. with a Benjamini–Hochberg adjusted P-value < 0.05.

**TABLE 4** | Adverse events.

| | Placebo | *LPK* | P |
|---|---|---|---|
| Adverse event | 1/2 | 2/3 | 1.000 |
| Serious adverse event | 0/0 | 0/0 | – |
| *Type* | | | |
| Pruritus | 0/0 | 1/1 | 1.000 |
| Facial laceration | 1/1 | 0/0 | 0.494 |
| Low back pain | 0/0 | 1/1 | 1.000 |
| Insomnia | 0/0 | 1/1 | 1.000 |
| Vasovagal syncope | 1/1 | 0/0 | 0.494 |
| *Relevance to intervention* | | | |
| Definitely related | 0/0 | 0/0 | – |
| Probably related | 0/0 | 0/0 | – |
| Possible related | 0/0 | 0/0 | – |
| Probably not related | 1/2 | 2/3 | 1.000 |
| Definitely not related | 0/0 | 0/0 | – |
| Unknown | 0/0 | 0/0 | – |

*Presented as the number of subjects/number of cases. Fisher's exact test was used to compare the differences in the numbers of subjects between groups. LPK, Lactobacillus plantarum K50.*

intervention was not considered relevant. There were no deaths or serious adverse events requiring hospitalization.

## DISCUSSION

In this study, the subjects' body weight, fat mass, and abdominal adipose tissue area did not change significantly after a 12-week administration of *LPK*, resulting in no significant differences compared with the placebo group. However, the total cholesterol level decreased in the *LPK* group and increased in the placebo group, resulting in a significant between-group difference of 11.1 mg/dL. Similarly, the TG level decreased in the *LPK* group and increased in the placebo group, resulting in a significant between-group difference of 44.9 mg/dL.

*L. plantarum* is a versatile lactic acid-producing bacterium found in many fermented foods (24). *L. plantarum* is widely employed in the industrial fermentation and processing of raw foods; it is generally recognized as safe and has received a Qualified Presumption of Safety status from the European Food Safety Authority (25, 26). Oral administration of the live *L. plantarum* strain K21 alleviated high-fat diet-induced obesity in a mouse model (27). In another study with high-fat-fed mice, *L. plantarum* supplementation reduced obesity-induced metabolic abnormalities and adipose tissue inflammation (28).

In a study with Indonesian subjects, consumption of indigenous probiotic *L. plantarum* Dad-13 powder in overweight adults decreased the body weight and BMI significantly (29). In a Japanese study, the ingestion of heat-treated *L. plantarum* OLL2712 reduced body fat accumulation, glycemic deterioration, and chronic inflammation in overweight, healthy adults (30). Thus, the effects of *L. plantarum* on altering the metabolic status differ among studies, suggesting that it depends on the study subjects and the strain used (31, 32).

In our study, the administration of $4 \times 10^9$ CFU of *LPK* to people with a baseline BMI ≥ 25 kg/m$^2$ led to reductions in total cholesterol and TG levels. During a prior clinical trial, twelve weeks of consumption of a mixture of *L. plantarum* strains CECT7527, CECT7528, and CECT7529 decreased total cholesterol levels as observed (33). The cholesterol-lowering efficacy of lactic acid-producing bacteria is reported to be a result of producing short chain fatty acids (SCFAs) (9). Of note, in a study with diet-induced obese mice, treatment with $1 \times 10^9$ CFU *LPK* increased the concentrations of SCFAs significantly (21). SCFAs, especially acetate, lowered fat accumulation in metabolic tissues as well as serum TG and total cholesterol levels in an animal study (34). Other SCFAs such as butyrate and propionate protected against diet-induced obesity and regulated gut hormones such as glucagon-like peptide-1, glucose-dependent insulinotropic polypeptide (GIP), peptide YY, and ghrelin (35). GIP regulates TG turnover and promotes TG clearance from the blood by increasing the deposition of fat in adipocytes (36, 37). Based on these findings, the reductions in total cholesterol and TG levels by administration of *LPK* in our study might have been caused by increases in SCFAs and changes in the composition of the gut microbiota.

Dietary supplementation with *LPK* in our study changed the gut microbiota favorably with a decrease in the abundance of *Actinobacteria* and an increase in *Enterococcus*. The decrease in *Actinobacteria* species might be linked with a decrease in obesity, which was increased in obese compared with lean twin subjects (38). Administration of a specific strain of *Bifidobacteria* (M13-4), belonging to the *Actinobacteria*, was reported to be associated with weight gain in a high-fat diet study in rats (39). *Enterococcus* species abundance was reported to be correlated negatively with excessive weight gain and increased leptin (40). We also found that *LPK* supplementation increased the abundance of *Enterococcus hirae* in the order *Lactobacillales* to which *LPK* belongs. In a study of rats fed a high-fat diet, 24-h supplementation of *Enterococcus hirae* (2 mg/$10^{10}$ CFU) decreased total cholesterol and TG levels and alleviated insulin resistance (41). A recent study with hypercholesterolemic rats, administration of specific strains of *Enterococcus faecium* decreased total cholesterol, LDL-C, and TG levels by regulating the genes involved in lipid metabolism, such as *CYP8B1*, *CYP7A1*, *SREBP-1*, *SCD1*, and *LDL-R* (42). Thus, the benefit of *LPK* on lipid profiles might be driven by favorable alterations in the distribution of gut microbiota.

Human gut microbiota can be substantially diverse between individuals and alter over time, changing according to age, genetics, and environment (43). In addition, ethnicity is another important factor affecting the type and abundance in gut microbiota (44, 45). A multi-omics study of 46 East Asian and White participants living in the San Francisco Bay Area revealed marked differences between ethnic groups in bacterial richness and community structure; White individuals were enriched for the mucin-degrading *Akkermansia muciniphila* whereas East Asian subjects had increased levels of multiple bacterial genera including *Blautia, Bacteroides*, and *Streptococcus* (44). In another study with multi-ethnic groups, ethnicity exhibited the largest effect size across participants; notably, the influence of ethnicity on the gut microbiota was retained even after controlling for all demographic, dietary factors, and other covariates (45). In this context, the effects of probiotics, even originating from the same strain, may be different according to the subject's ethnicity.

Food consumption alters the gut microbiome profile in humans and such dietary patterns are important for the link between gut microbiota and body composition changes (46–48). Conversely, changes in the composition and activity of the gut microbiota might affect body weight and its composition (49, 50). However, as previous studies have reported (14, 51), the intake of probiotics intervention did not change the overall gut microbiotic diversity in the present study. Therefore, instead of showing *all* microbiota, only the changes in specific microbiota at the genus or species level that seem to be clinically meaningful are presented. In this study, the abundances of *L. plantarum* and *Enterococcus hirae* increased significantly in the *LPK* group compared with the placebo group. Such compositional changes might play important roles in changes in lipid profiles.

Even though the abundance of *L. plantarum* was negatively correlated with abdominal fat area with borderline significance, no significant reduction in body fat mass was observed in the *LPK* group. There are several possible reasons for this. First, there might be a large variation in the amount of bioavailable *LPK*. Also, despite the significant changes of a few gut microbiota after *LPK* administration, their clinical effects might be small at very low levels. Second, the moderate baseline BMI of 27.3 kg/m$^2$ of our study participants might not have been high enough to confirm the effect of *LPK*. Although the study group was allocated randomly, the uneven baseline levels of the markers of liver function and inflammation might have affected the study results. As our study was conducted in Korean population, further studies may help to generalize these effects to other ethnic populations.

## CONCLUSIONS

In this randomized, double-blind clinical trial, administration of *LPK* for 12 weeks did not lead to significant reductions in total fat mass or in body weight. However, significant decreases were found in total cholesterol and TG levels after *LPK* treatment. These data suggest that *LPK* might be a good auxiliary candidate as a microbiome-targeted therapy for treating dyslipidemia.

## DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Sequence Read Archive (SRA) repository, accession number (PRJNA777658).

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Seoul National University Bundang Hospital. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

SL contributed to conception, acquisition, analysis, or interpretation of data, drafting the work or revising and final approval of the manuscript. MS and GN contributed to the acquisition, analysis, or interpretation of data, drafting the work or revising and Final approval of the manuscript. JC, HJ, and B-KK participated in the acquisition, analysis, or interpretation of data and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

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# NAD+-Increasing Strategies to Improve Cardiometabolic Health?

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Depleted nicotinamide adenine dinucleotide (NAD+) is a common hallmark of metabolic disorders. Therefore, NAD+-increasing strategies have evolved as a potential therapeutic venue to combat cardiometabolic diseases. Several forms of vitamin B3, i.e., nicotinamide and nicotinamide mononucleotide, and especially nicotinamide riboside, have attracted most interest as potentially safe and efficacious candidates for NAD+ restoration. Herein, we dissected the characteristics of the latest clinical trials testing the therapeutic potential of different vitamin B3 molecules to improve cardiometabolic health, with a special focus on randomized, placebo-controlled clinical trials performed in the context of obesity or other pathologies, mainly linked to cardiovascular system and skeletal muscle functionality. The favorable outcomes *via* NAD+-increasing strategies found in the different studies were quite heterogeneous. NAD+-increasing interventions improved capacity to exercise, decreased blood pressure, increased the anti-inflammatory profile and insulin-stimulated glucose disposal, and reduced the fat-free mass. Except for the decreased blood pressure, the significant results did not include many hard clinical end points, such as decreases in weight, BMI, fasting glucose, or HbA1c percentage. However, the analyzed trials were short-term interventions. Overall, the accumulated clinical data can be interpreted as moderately promising. Additional and long-term studies will be needed to directly compare the doses and duration of treatments among different vitamin B3 regimes, as well as to define the type of patients, if any, that could benefit from these treatments. In this context, a major point of advancement in delineating future clinical trials would be to identify subjects with a recognized NAD+ deficiency using novel, appropriate biomarkers. Also, confirmation of gender-specific effect of NAD+-increasing treatments would be needed.

**Keywords: vitamin B3, clinical trials, obesity, diabetes mellitus, nicotinamide**

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## INTRODUCTION

Nicotinamide adenine dinucleotide (NAD+) is a dual molecule. Apart from its well-established role as a cofactor for redox reactions and, in its reduced form, as an electron donor to the mitochondrial oxidative phosphorylation system for the synthesis of ATP, NAD+ has also been described as a signaling molecule. Indeed, NAD+ is also a substrate for NAD+-consuming enzymes involved in the metabolic adaptations that govern cell metabolism and survival.

It is known that low levels of NAD+ result from altered NAD+ homoeostasis. Impaired NAD+-mediated signaling and concurrent alterations in dysfunctional mitochondria commonly underlie cardiometabolic disorders, such as type 2 diabetes, non-alcoholic fatty liver, and aging.

NAD+ precursors are naturally found in food, and their use has emerged as a strategy for NAD+ replenishment and hence to favorably influence NAD+ dependent pathways and rescue tissues from the adverse consequences of aging and metabolic diseases (1). NAD+ content in animal and human tissues can be increased *via* the supplementation of different precursors (2–4). Although NAD+ can be synthesized directly from tryptophan (*de novo* pathway), it is more efficiently generated from other precursors, such as nicotinic acid (NA) (Preiss-Handler pathway) and nicotinamide (NAM) (Salvage pathway). Nicotinamide phosphoribosyltransferase (NAMPT), which converts NAM into nicotinamide mononucleotide (NMN), is the key step for NAD+ synthesis (5). Another source of NAD+ comes from nicotinamide riboside (NR).

NAD+ precursors that are used to elevate NAD+ availability in target tissues have demonstrated efficiency in improving insulin sensitivity and reducing diabetes burden and associated metabolic derangements in preclinical models. Actually, a growing number of studies have successfully used different vitamin B3 forms to boost NAD+ production and positively influence aging and cardiometabolic diseases in experimental *in vivo* models, including our studies performed in the context of accelerated atherosclerosis or obesity (4, 6, 7). However, less consistent results have been obtained in clinical studies.

This review analyzes the accumulating evidence from the latest clinical studies that used different NAD+-increasing strategies to improve cardiometabolic health, with a special focus on randomized, double-blind, placebo-controlled clinical trials, since they are considered the "gold standard" for testing clinical intervention-based studies (8). Particularly, a Pubmed search on clinical reports published in the last 5 years on this topic using "nicotinamide" and "randomized clinical trials" as keywords was performed.

## RESULTS OF THERAPEUTIC INTERVENTIONS WITH DIFFERENT FORMS OF VITAMIN B3

A plethora of pathways require NAD+ as a coenzyme. The easiest strategy to increase NAD+ *in vivo* is *via* the provision of NAD+ precursors, which include NA (also termed niacin), NAM, NR, or NMN (5, 6). However, these NAD+ intermediates can exhibit a distinct behavior due to the different tissue distribution and relative abundance of enzymes or transporters involved in NAD+ metabolism.

### NA

NA has been the most widely used form of vitamin B3 in clinical practice due to its hypolipemic properties, i.e., triglyceride-reducing and high-density lipoprotein cholesterol (HDL-C)-raising effects (5). Besides such lipid effects, NA also interacts with the GPR109A receptor expressed in immune cells, thus blunting immune activation (5). Although such NA properties are beneficial to treat hyperlipidemia, NA administration is frequently associated with some serious adverse effects, i.e., flushing, deterioration of insulin resistance, and hepatic and gastrointestinal toxicity (5), which reduce medication adherence. Moreover, early reports supporting efficacy of NA in secondary cardiovascular prevention were not confirmed by more recent, large prospective trials, including 29,087 subjects, the AIM-HIGH (9), and the HPS2-THRIVE (10), which used extended-released NA and laropiprant, a selective antagonist of prostaglandin D2 receptors, to control adverse effects (5). Since NA also ameliorated wound healing and cardiac function after myocardial infarction *via* prostaglandin D2 receptor subtype 1-mediated M2 macrophage polarization, the inhibition of prostaglandin D2 signaling could have attenuated NA cardiovascular benefits (11). The latter finding would be consistent with data from earlier clinical trials using NA, showing a reduction of mortality in subjects nine years after stopping NA therapy (12). It is also possible that the protective effect of NA is no longer significant in the context of previous statin treatments (9). However, NA may also be a potential source for NAD+ synthesis (13), and could present beneficial effects raising tissue NAD+ levels in case of deficiency. In this regard, the effect of NA in 5 subjects (4 females) with adult-onset mitochondrial myopathy subjects and systemic NAD+ deficiency and 10 healthy controls (8 females) was recently assessed (14). NA (up to 750–1,000 mg/day) or placebos were administered in a non-randomized, open-able parallel assignment to subjects and their matched controls for 10 or 4 months, respectively. The NA administration resulted in blood elevations of NAD+ in all subjects, up to eightfold, also restoring the muscle NAD+ levels of the subjects (14). Noteworthy was that muscle strength and mitochondrial biogenesis increased in all subjects. Furthermore, the muscle metabolome in the subjects with adult-onset mitochondrial myopathy shifted toward that of the healthy controls, whereas liver fat accumulation decreased by 50%. Adiponectin concomitantly increased, a finding consistent with previous studies in subjects treated with a high dose of NA (i.e., 1,500 mg/day) (15, 16). Important, the blood analysis was revealed as a useful sample to identify NAD+ deficiency in subjects with myopathy (14).

### NAM

Unlike NA, NAM is not considered a GPR109A agonist, thus avoiding prostaglandin-related vasodilatory side effects. NAM is a widely available dietary supplement, which, at doses of no more

than 3 g/day, has been proven to be safe (17). In the ENDIT study, 276 subjects with type 1 diabetes mellitus were administered with NAM in daily doses of 1.2 g/m$^2$ (25–50 mg/kg), up to a maximum of 3 g/day for 5 years, with minimal side effects (17). However, this clinical trial with NAM failed to prevent the progression to overt of autoimmune type 1 diabetes (18). Importantly, higher doses of NAM have been reported to produce severe but reversible hepatotoxicity (19). Effects on glucose kinetics and insulin sensitivity are inconsistent, but minor degrees of insulin resistance have also been reported (17).

NAD+ deficiency is also a risk factor for acute kidney injury (20). Recently, a phase 1 placebo-controlled study of oral NAM demonstrated that a dose-related increase in circulating NAD+ was associated with less acute kidney injury (20). Similarly, although minimal, NAD+ biosynthesis from tryptophan, at the expense of the quinolinate phosphoribosyltransferase, also prevents renal NAD+ depletion and mediates resistance to acute kidney injury. Overall, these data support the concept that increasing NAD+ levels could be beneficial for the treatment of some forms of kidney disease.

Consistent experimental observations have suggested similar NAD+-increasing benefits by NAM for the treatment of heart failure with preserved ejection fraction (21). In the former study, it was found that the dietary intake of NAD+ precursors was negatively associated with decreased blood pressure and cardiovascular mortality in a 20-year follow-up of the Bruneck Study, and persisted after adjusting for caloric intake, age, BMI, sex, smoking, diabetes, alcohol intake, and categories of food items.

## NMN

The safety of NMN administration (100, 250, and 500 mg) was recently investigated in 10 healthy men, aged from 40 to 60, in a single-arm, non-randomized intervention clinical trial (22). In this study, single oral administrations of NMN did not cause any significant clinical symptoms or changes in heart rate, blood pressure, oxygen saturation, or body temperature 5 h after NMN administration. The plasma concentrations of NAM metabolites (N-methyl-2-pyridone-5-carboxamide and N-methyl-4-pyridone-5-carboxamide) were dose-dependently increased in treated subjects.

In relation to the impact of NMN intervention on dynamic metabolic adaptations, NMN supplementation enhanced aerobic capacity in amateur runners in a 6-week randomized, double-blind, placebo-controlled, four-arm clinical trial (**Table 1**) (23). In this study, up to 48 young and middle-aged recreational runners were included (40 males and 8 females, aged between 27–50 years, with a previous history of 1–5 years of regular exercise). The participants were randomized into three groups (with a ratio of 10 males to 2 females per group), taking a low (300 mg/day), medium (600 mg/day), and a high dosage group (1,200 mg/day). A control group (placebos) followed the same male to female ratio. The runners trained 5–6 times/week in sessions of 40–60 min. The participants did not show any obvious side effects. Exercise combined with NMN intake did not change the body composition or BMI, but analysis of the change from baseline over the 6-week treatment showed that the oxygen uptake ($VO_2$), the percentage of maximum oxygen uptake ($VO_2$ max), and the power at the first and second ventilatory thresholds increased in the medium- and high-dose NMN groups compared with the placebo.

In a very recent report (24), 25 overweight or obese women (BMI ranged from 25.3 to 39.1 kg/m$^2$, mean age of 61.5) and prediabetes completed a randomized, double-blind, parallel-assigned treatment with 250 mg of NMN or placebos over 10 consecutive weeks (**Table 1**). There were no major adverse side effects. Plasma concentrations of N-methyl-2-pyridone-5-carboxamide and N-methyl-4-pyridone-5-carboxamide metabolites of NMN and NAD+ content in peripheral blood mononuclear cells (PBMC) increased in subjects with NMN supplementation, but not in those taking placebo. Neither body weight nor body composition differed on NMN treatment. In contrast, insulin-stimulated glucose disposal, as assessed by hyperinsulinemic-euglycemic clamp, and skeletal muscle insulin signaling (AKT and mTOR total protein and the phosphorylated forms) increased with NMN, thus revealing improved insulin signaling in treated subjects. In line with this, NMN also upregulated the expression of platelet-derived growth factor receptor β (which enhances insulin-stimulated AKT phosphorylation and glucose transport) and other genes in skeletal muscle that are involved in tissue remodeling. However, no changes in muscle mitochondrial oxidative capacity or muscle function were observed. NMN could

**TABLE 1** | Recently (from 2018) published randomized, double-blind, placebo-controlled clinical trials that tested NMN administration on healthy individuals or in individuals with overweight and obesity.

| Country, NCT code | Dose, duration of treatment, number, and main clinical characteristics | Main results | Reference |
|---|---|---|---|
| China 2000035138 | NMN or placebos (300, 600, and 1,200 mg) were administered during 6 weeks to 48 subjects (8 women), aged 27–50. The recreational runners, training 5–6 times a week, were divided into four groups. | No major adverse effects. Evidence of increased plasma metabolites. Medium and high doses presented increased exercise capacity (i.e., increased oxygen uptake). No effect on cardiac function, BMI, or fat-free mass. | (23) |
| USA 03151239 | NMN or placebo per day (250 mg) over 10 weeks, given to 25 women with mean age of 61, BMI of 33.5 kg/m$^2$, plasma glucose of 5.65 mmol/L, and HbA1c of 5.55%, | No major adverse effects. Increased insulin-stimulated glucose disposal and skeletal muscle insulin signaling. Up-regulated expression of platelet-derived growth factor receptor β and other genes related to muscle remodeling. No change in body weight or composition. | (24) |

*BMI, body mass index; NMN, nicotinamide mononucleotide; NCT, clinical trial identifier (ClinicalTrials.gov).*

therefore be a useful intervention approach in obese, prediabetic subjects, which often present muscle loss with impaired glucose metabolism (25). Further studies are warranted to clarify the potential contribution of NMN in the latter condition.

## NR

NR is also a source for NAD+ synthesis. Consistently, human blood $NAD^+$ levels rose 2.7-fold in a pilot study of one healthy 52-year-old male individual, receiving an oral daily dose of NR (1,000 mg/day) for 7 consecutive days (26). More recently, the different daily doses of NR, administered orally (i.e., 100, 300, and 1,000 mg) in 12 healthy subjects (6 male and 6 female) with ages from 30–55 and BMI 18.5–29.9 $kg/m^2$, were investigated in the context of a randomized, double-blind, three-arm crossover pharmacokinetic study design. The NR produced concomitant dose-dependent elevations in the blood $NAD^+$ metabolome. The rise in nicotinic acid adenine dinucleotide (NAAD) was a highly sensitive biomarker of effective NAD+ replenishment (26).

In an independent, non-randomized, open-label pharmacokinetic study made in 8 healthy volunteers (6 female, 2 male, age range 21–50 years), 250 mg of NR was orally administered on days 1 and 2, and then increased to 1,000 mg twice daily on days 7 and 8. On the morning of day 9, subjects completed a 24-h pharmacokinetics study after receiving 1,000 mg of NR (27). The treatment was well tolerated with no apparent adverse side events. Significant increases from baseline to mean NR blood concentrations at a steady state were observed for both NR and NAD+, with a 100% increase in the NAD+ levels. Absolute changes from baseline to day 9 in NR and NAD+ levels were highly correlated (27).

In another study, a 2- × 6-week randomized, double-blind, placebo-controlled, crossover clinical trial, 24 healthy lean (average BMI = 24 ± 4 $kg/m^2$) men ($n$ = 11) and women ($n$ = 13), aged 55 to 79, received 500 mg of NR twice a day (**Table 2**) (28). NR supplementation was well tolerated by all participants. The NR increased the NAD+ and related metabolite concentrations in PBMC. Importantly, treatment with NR significantly decreased blood pressure, aortic pulse wave velocity, and carotid artery compliance. There were no changes in total energy expenditure or energy expenditure from fat oxidation under resting conditions, blood glucose control, insulin sensitivity, aerobic exercise capacity, or motor function. It is worth noting that this study was conducted in non-obese, healthy middle-aged, and older adults without baseline metabolic dysfunction (28), and this could have limited the improved metabolic outcomes.

The effect of different doses of NR (100, 300, and 1,000 mg/day) on NAD+ metabolite concentration in urine and blood was investigated in 133 healthy males and females (54%–66%, depending on the group), aged 40–60, and overweight (BMI 25–30 $kg/m^2$), in an independent 8-week randomized, double-blind, placebo-controlled parallel clinical trial (29). The NR increased, dose-dependently and significantly, whole blood NAD+ (from 22% to 142%) and other NAD+ metabolites within the first 2 weeks. The NAD+ elevations were maintained thereafter, without any adverse effects (29).

In another study, 12 healthy old men, aged from 70 to 80, and BMI 20–30 $kg/m^2$ (able to discontinue aspirin for 3 days prior to a muscle biopsy, statins and vitamin D one week before the beginning of the study) were supplemented with 500 mg of NR, twice a day, for 21 days, in a placebo-controlled, randomized, double-blind, crossover trial (**Table 2**) (30). NR supplementation decreased levels of circulating inflammatory cytokines (30) but did not produce favorable changes in body weight, blood pressure, lipid profile, fasting glucose and insulin, or homeostatic model assessment of insulin resistance (HOMA-IR). Although the supplementation of NR elevated the muscle NAD+ metabolome, it did not influence mitochondrial bioenergetics or whole-body glucose homeostasis. Despite the hand-grip strength values in these subjects being consistent with muscle aging, targeted NAD+ metabolome analysis likely revealed NAD+ sufficiency. Overall, these findings suggest that chronological age *per se* may not be a major factor impairing NAD+ metabolism.

In an early phase I study of 5 patients admitted with a class IV New York Heart Failure Classification to demonstrate NR safety and feasibility, blood samples were obtained before and after 5–9 days of oral NR administration (NR was up-titrated over 3 days to a final NR dose of 1,000 mg twice daily) (31). The treatment enhanced PBMC respiration and reduced pro-inflammatory cytokine gene expression in 4 male subjects, as one of the subjects did not complete the study.

Several clinical trials have focused on searching for the beneficial effects of NR in individuals with overweight or obesity. Data from the first of these clinical trials were published in three different reports (32–34). This clinical trial consisted of a 12-week randomized, double-blind, placebo-controlled, parallel-group trial conducted in 40 non-smoking, medication-free middle-aged males (40–70 years) with obesity (BMI mean of 32.85 $kg/m^2$), sedentarism (< 30 min of daily exercise), with a mean fasting glucose of 5.6 mmol/L and HbA1c of 5.7% (**Table 2**) (32). They were administered NR at 1,000 mg, twice a day ($n$ = 20) or a placebo ($n$ = 20). After 12 weeks of treatment, increased concentrations of NAD-derived metabolites were detected in the urine of NR-treated subjects, thereby showing that the oral NR was readily absorbed, metabolized, and excreted. No serious adverse events were observed upon NR supplementation, and the safety blood tests were normal. HbA1c, insulin sensitivity, endogenous glucose production, glucose disposal and oxidation, resting energy expenditure, lipolysis, oxidation of lipids, and body composition did not change with NR supplementation (32). The NR supplementation did not affect fasting, the post-glucose challenge concentrations of glucose, insulin, C-peptide, glucagon, glucagon-like peptide-1 or gastric inhibitor polypeptide, or the beta-cell function (as revealed by the oral glucose test tolerance testing) (33). Protein levels of NAMPT in skeletal muscle decreased by 14% with NR, while NAD+ levels, as well as gene expression and protein abundance of other NAD+ biosynthetic enzymes, remained unchanged between the groups. The respiratory capacity of skeletal muscle mitochondria, abundance of mitochondrial associated proteins, mitochondrial fractional area, or network morphology in the skeletal muscle of NR-treated participants did

**TABLE 2 |** Recently (from 2018) published randomized, double-blind, placebo-controlled clinical trials that tested NR administration on individuals either healthy, aged, or overweight or obese.

| Country, NCT code | Dose, duration of treatment, number, and main clinical characteristics | Main results | Reference |
|---|---|---|---|
| USA, 02921659 | Administered 1,000 mg per day, 6 weeks in a crossover design, $n$ = 24 (13 women), described as 55–79-year-old healthy, aged, lean subjects (BMI 24 ± 4 kg/m$^2$). | No major adverse effects. Increased NAD+ and related metabolites in peripheral blood mononuclear cells. Treatment decreased blood pressure, aortic pulse wave velocity, and carotid compliance. No change in total energy intake and expenditure, BMI, % body fat, glucose and insulin metabolism, or exercise capacity. | (28) |
| USA 0271593 | Administered 100 mg, 300 mg, 1,000 mg per day, 8 weeks parallel study, 133 (85 women); 40–60 years old, healthy overweight (BMI 25–30 kg/m$^2$) in a parallel study. | No major adverse effects. Dose-dependent increase of NAD+ whole blood and urine metabolome. No changes in blood pressure, mean heart rate, weight, or resting energy expenditure. | (29) |
| UK, USA, Australia 02950441 | Single center, double blind, placebo-controlled, and crossover study on 12 male aged volunteers, recruited from the Birmingham 1,000 Elders group (https://www.birmingham.ac.uk/research/activity/mds/centres/healthy-ageing/elders.aspx), age 70–80 years, BMI 20–30 kg/m$^2$ receiving 1,000 mg of NR or placebos during 21 days | Muscle RNA sequencing revealed that NR down-regulated energy metabolism and mitochondria pathways without altering mitochondrial bioenergetics or whole-body glucose homeostasis, decreasing circulatory cytokines (especially IL-6, IL-5 and IL-2). NR did not alter hand-grip strength. | (30) |
| Denmark, 2303483 | 2,000 mg per day, 12 weeks: 40 men, 40–70-year-old, insulin resistant, with mean BMI of 38.5 kg/m$^2$, glucose of 5.6 mmol/L and HbA1c of 5.7%. | No major adverse effects. No changes in carbohydrate metabolism, resting energy expenditure, lipolysis, lipid oxidation, or body composition. No impact on glucose tolerance, β-cell secretory capacity, α-cell function or incretin secretion, or bile acid levels. Moreover, there were no changes in NAD+ metabolite concentration in skeletal muscle. | (32–34) |
| Netherlands 02835664 | 1,000 mg per day, 6 weeks, 13 (7 women), age 59 ± 5, healthy overweight or obese (BMI 30.2 ± 2.6 kg/m$^2$) | No major adverse effects. Increased markers of NAD+ synthesis in skeletal muscle. Minor increase in fat free mass, especially in women (6 out of 7). Minor increase in sleeping metabolic rate that could be related to the change in fat free mass. Acetylcarnitine concentrations in skeletal muscle and the capacity to form acetylcarnitine upon exercise were increased in NR in respect to placebo. No effects of NR were found on insulin sensitivity, mitochondrial function, hepatic and intramyocellular lipid accumulation, cardiac energy status, cardiac ejection fraction, ambulatory blood pressure, plasma markers of inflammation, or energy metabolism. | (37) |

*BMI, body mass index; NAD, nicotinamide adenine dinucleotide; NR, nicotinamide riboside; NCT, clinical trial identifier (ClinicalTrials.gov).*

not differ from the placebo group (34). Overall, these data do not support the hypothesis that dietary NR supplementation has a significant impact on skeletal muscle mitochondria in obese and insulin-resistant men. This could at least partly be explained by the fact that, despite having elevated urine levels of NAD+-derived metabolites, the tissue content of NAD+, NADH, NADP+, or NADPH in the skeletal muscle in men receiving NR did not differ from those receiving placebos (35, 36).

In another randomized, double-blinded, placebo-controlled, crossover intervention study, 13 healthy overweight or obese men and women (age: 59 ± 5; BMI: 30.2 ± 2.6; $n$ = 7 women) receiving either NR (1,000 mg/day) or placebo supplementation during 6 consecutive weeks were followed by broad metabolic phenotyping, including hyperinsulinemic-euglycemic clamps, magnetic resonance spectroscopy, muscle biopsies, *ex vivo* mitochondrial function, and *in vivo* energy metabolism assessment (**Table 2**) (37). NR supplementation resulted in elevations of NAD+ synthesis, such as NAAD and methyl-nicotinamide (me-NAM), in skeletal muscle. In particular, the percentage of fat-free mass (62.65% ± 2.49% compared with 61.32% ± 2.58%), the skeletal muscle acetylcarnitine (4,558 ± 749 compared with 3,025 ± 316 pmol/mg dry weight), and the capacity to form acylcarnitine upon exercise were increased upon NR supplementation (37). To our knowledge, such an effect of NR on body composition in humans has not been reported before. Consistently, the sleeping metabolic rate was also affected by NR supplementation. The NR-treated subjects also showed concomitantly higher metabolic rates than the placebo subjects. In contrast, insulin sensitivity was not improved in NR-treated men. However, NR did increase the fat-free mass in 6 out of 7 women, whereas such an increase was only observed in 1 out of 6 men; thus, there might be a gender-specific response pattern in the effect of NR supplementation.

## DISCUSSION

Thanks to the extensive research already performed with some vitamin B3 forms, its considerable safety has been confirmed. The analyzed clinical trials were built on this concept. However, special consideration should be given to the fact that some enzymes determining NAD+ levels have been related to cancer progression, at least at an experimental level (38).

Data from different clinical trials are somewhat difficult to compare and interpret, mainly due to differences in the vitamin B3 forms, doses, duration of treatment, gender, and cardiometabolic characteristics of the studied subjects.

One of the emerging interpretations is that gender could be a determinant of positive response to treatment, since most reports that obtained significant cardiometabolic differences in vitamin B3 treatment comprised a higher proportion of women (24, 28, 37) than those in which no such differences were found (32–34).

However, this was not without exceptions. Elhassaen et al. (30) found some differences in men only, and Conze et al. (2019) did not find such differences, even though the study featured a majority of women (29). Interestingly, the percentage of men in the AIM-HIGH (9) and HPS-THRIVE (10) trials was around 85%.

Apart from gender-specific differences, cardiometabolic status may also differ among studies. In this regard, subjects with obesity were excluded from these two latter studies (29, 30), whereas the analysis of the effect of NR was conducted in subjects with obesity and prediabetes in other clinical trials (24, 37). In the study of Remie et al. in 2020, basically only the women in the study showed a response to the NR (37). In contrast, the main difference in the clinical trials that failed to find response to vitamin B3 treatment was directed only to men (32–34), even though it should also be considered that it also used twice the dose of the other studies. Furthermore, circadian rhythms influence the action of at least some of the key enzymes involved in the bioavailability of the currently used NAD+ precursors, and this could also be considered to be minimizing the variability among future clinical trials (39). Last, in the clinical trial of Dollerup et al. (32–34) there were some indications of limited NR tissue bioavailability.

As mentioned, the favorable outcomes in response to NAD+ precursor supplementation found in the different studies were quite heterogeneous. Thus, NAD+-increasing interventions increased capacity to exercise (23), decreased blood pressure (28), decreased anti-inflammatory circulating cytokines (30, 31), increased insulin-stimulated glucose disposal (24), or decreased fat-free mass (37). Most of these concrete findings were not reproduced in the rest of the clinical trials. Most of the positive findings were not very hard clinical end points (except the decreased blood pressure). No significant reductions in body weight or its surrogate, BMI, fasting glucose, or %HbA1c were observed in response to NAD+ precursors. This could be due to the short duration of the clinical trials analyzed, which obviously impacted the probability of the change in HbA1c percentage.

The health potential of targeting NAD+ homeostasis is still an underexplored field for future interventions of non-communicable disorders. Given the contribution of NAD+-sirtuin signaling on mitochondrial and metabolic homeostasis, an interesting proposal for future clinical trials could be the study of the effects of vitamin B3 interventions in combination with moderate exercise (40) to directly assess the metabolic adaptations provided by restored NAD+ homeostasis, concurrent with improved mitochondrial function. Moreover, future clinical trials should focus on subjects with selective biomarkers of recognized NAD+ deficiency (26) and analyzing the gender-effect in order to maximize the chances of success.

## CONCLUSION

Although NAD+-increasing strategies have the potential to improve overweight, obesity, and cardiometabolic health *in vivo*, additional larger and long-term studies are needed to shed light on its precise clinical indications, preferred vitamin B3 form, doses, and treatment duration.

## AUTHOR CONTRIBUTIONS

Conceptualization: FB-V, NR, and JJ. Writing-Original Draft Preparation: FB-V and JJ. Writing-Review and Editing: MC, DM, and JE-G. Funding Acquisition: FB-V, NR, JE-G, and JJ. All authors contributed to the article and approved the submitted version.

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# Roles of Gastric Emptying and Gastrointestinal Transit Following Ileal Interposition in Alleviating Diabetes in Goto-Kakizaki Rats

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**Objective:** This study aimed to determine the change of gastrointestinal (GI) emptying time after ileal interposition (IT) and elucidate the role of altered GI peristalsis in diabetic control.

**Materials and Methods:** Twelve male Goto-Kakizaki rats were randomly divided into IT and sham groups. Body weight and food intake were recorded. Oral glucose tolerance test (OGTT), insulin tolerance test (ITT), plasma glucagon-like peptide-1 (GLP-1), and gastric emptying were measured at baseline and 4 and 8 weeks after operation. At 9 weeks postoperatively, the rats in the IT group were given atropine which can suppress the emptying of stomach and upper intestine, while sham rats were given metoclopramide (to expedite gastric emptying) for 1 week. At week 10 postoperatively, OGTT and GLP-1 were detected. The intestinal transit was tested at postoperative 12 weeks.

**Results:** No differences were found between groups at baseline. After operation, the IT rats had lower body weight than sham rats. At 4 and 8 weeks postoperatively, the IT group showed better OGTT and ITT, with significantly elevated GLP-1 relative to sham. After administration of the GI motility drugs, however, the effect of diabetic control for the two groups became similar. The GI transit after IT was significantly slower than sham at all tested time points.

**Conclusions:** Although IT inhibits the GI transit time, the earlier interaction between undigested nutrients and interpositioned ileum promotes gut hormone secretion and thus reduces body weight and alleviates hyperglycemia. A decrease of GI transit of IT rats exacerbates the antidiabetic effects.

**Keywords: ileal interposition, type 2 diabetes, glucagon-like peptide-1, Goto-Kakizaki rat, gastric emptying, gastrointestinal transit**

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## INTRODUCTION

Diabetes is a worldwide health crisis that affects morbidity and mortality (1) where 90% of diabetics suffer from type 2 diabetes mellitus (T2DM) (2). Worse yet, the prevalence of T2DM has been dramatically growing (1, 3, 4). Diabetes is a major risk factor of hypertension, cardiovascular disease, renal disorders, stroke, and other diseases.

In recent years, bariatric surgeries have emerged as recommended treatment methods for obesity and T2DM. Bariatric procedures are considered more effective for T2DM resolution than conventional medical therapy, especially for refractory diabetics (5, 6); however, the precise mechanisms are not fully understood. Among the various hypotheses, gastrointestinal (GI) transit seems to play a role in the antidiabetic effect, but the conclusion is not solid. It has been hypothesized that hyperglycemic control after Roux-en-Y bypass (RYGB) is mediated by the time needed to empty nutrients from the stomach to the distal intestine (7, 8). Vertical sleeve gastrectomy (VSG) is being reconsidered not only a restrictive procedure but also as a promoter of GI motility that enhances glucagon-like peptide-1 (GLP-1) release (7. 9), in agreement with "hindgut hypothesis" (10). On the other side, studies in adjusted gastric banding (AGB) reported that AGB has no influence or even delays gastric emptying (11, 12). Some GI devices were also reported to decrease GI emptying although the bariatric effects are good (13).

In this controversial issue, studies involving GI emptying time changes after other bariatric or metabolic procedures such as ileal interposition (IT) are lacking. IT is also widely used in diabetic research, and the effect of IT surgery also supports the hindgut hypothesis (10, 14), as the anatomic structure after IT causes an earlier contact between luminal nutrients and the interposed distal gut. It is not clear whether the increased gut peptides after IT, however, are also attributed to faster chyme delivery. Decades ago, a study by Ohtani et al. (15) showed that IT (referred to as "ileojejunal transposition") inhibits postprandial gastric emptying because of "ileal break", without affecting intestinal transit in dogs. There have not been any other reports on the change of GI transit after IT. Moreover, the role of GI transit in diabetic control following IT remains unclear.

Here, we tested glucose indexes, insulin tolerance, and GLP-1 concentration, under different GI peristalsis conditions, to study the relationship between glucose homeostasis and changed GI transit, seeking to understand the role of GI transit in alleviating diabetes after IT, in Goto-Kakizaki (GK) rat model (16), the most widely used nonobese animal model of T2DM. The implications and limitations of the studies are enumerated.

# MATERIALS AND METHODS

## Animals

Twelve-week-old male GK rats were purchased from Charles River Laboratories (Massachusetts, USA). All animals were housed in individual cages under constant ambient temperature and humidity in a 12-h light/dark cycle room of the California Medical Innovations Institute (San Diego, CA, USA). Animals were given free access to tap water and fed with a commercial diet (18% protein; Envigo, Livermore, CA, USA) during the period of the study, unless otherwise specified. The animal experiments were approved by the Institutional Animal Care and Use Committee of California Medical Innovations Institute.

## Surgical Procedures

After an acclimation of 2 weeks, twelve GK rats were randomly assigned into the IT group and sham group ($n$ = 6 for each). Before operations, rats were fed 10% Ensure solution (Abbott Laboratories, Des Plaines, IL, USA) for 2 days and fasted for 12 h. Under anesthesia with isoflurane (2% with 1 L/min oxygen), the rats were given IT or sham operations, respectively.

As previously described (17), IT (**Figure 1**) involves removal of 10 cm of ileum at 10–20 cm proximal to the ileocecal junction and a transection of the jejunum 10 cm distal to the ligament of Treitz. The resected ileal segment was then interposed at the transection site in the original peristaltic direction, and three end-to-end anastomoses were performed using 6-0 Prolene suture. Sham procedure included the same transections of small intestine, but reanastomoses were made *in situ*. The operative time of sham was prolonged similar to that of the IT group, in order to normalize the anesthetic and surgical stress in both groups.

During the first 24 h following operation, the rats were only given water, with 2 ml 5% glucose solution and 2 ml normal saline in subcutaneous injection for rehydration. The rats were then fed 10% Ensure for 3 days, followed by regular diet. Body weight and calorie intake were recorded three times per week for 10 weeks following surgery.

## Experimental Protocol

The experimental protocol and timelines are shown as listed in **Table 1**. At baseline as well as at 4 and 8 weeks postoperatively, oral glucose tolerance test (OGTT), insulin tolerance test (ITT), total glucagon-like peptide-1 (GLP-1), and gastric emptying were

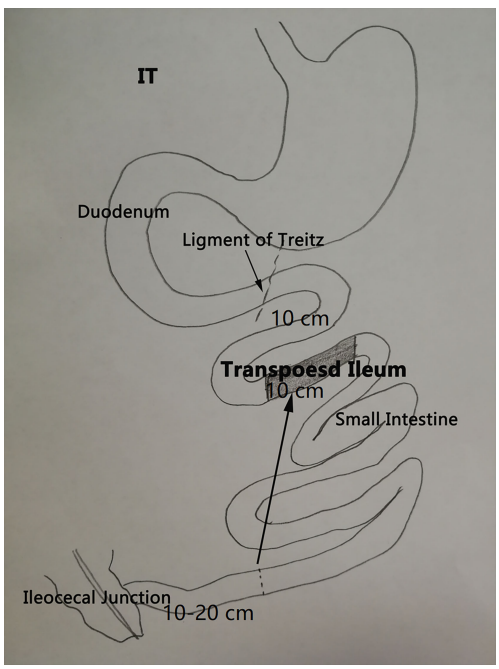


**FIGURE 1** | Schematic diagram of ileal interposition.

**TABLE 1** | Timeline of the experiments.

| Week | IT group (*N* = 6) | Sham-IT group (*N* = 6) |
|---|---|---|
| –2 | Acclimation | |
| 0 | Baseline | |
| | IT surgery | Sham surgery |
| 1 | Postoperative recovery | |
| 2 | Body weight, food intake | |
| 4 | Body weight, food intake, OGTT, ITT, GLP-1, acetaminophen | |
| 8 | Body weight, food intake, OGTT, ITT, GLP-1, acetaminophen | |
| 9 | Atropine, once daily | Metoclopramide, once daily |
| 10 | Stop atropine | Stop metoclopramide |
| | Body weight, food intake, OGTT, GLP-1 | |
| 12 | Measurement of GI transit | |

*If not specified otherwise, sham group followed the same protocol as IT group.*

measured. The detailed specifications of these indexes will be described further below.

To further study the role of GI emptying time, GI motility medications were given. At week 9 postoperation, the IT rats were intraperitoneally injected with atropine (1 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) for 7 days, which can be used to inhibit the movement of stomach and upper intestine. During the same period, sham rats were given an intraperitoneal injection of metoclopramide (1 mg/kg; Sigma-Aldrich, St. Louis, MO, USA), which is known to accelerate gastric emptying. At postoperative week 10 (atropine and metoclopramide had already been used daily for 1 week), OGTT and GLP-1 were also tested.

At postsurgery week 12 (scheduled termination; the administration of medicines had been stopped for 2 weeks), the nutrient transit in GI tract was measured using validated methods (7, 17). Toluidine blue O (Sigma-Aldrich, St. Louis, MO, USA) was mixed with Ensure solution and then orally administered to the fasted rats (0.5 ml/100 g). Thirty minutes later, a laparotomy was performed under isoflurane inhalation. The overall length of small intestine and the distance between the distal end of the dye and the ileocecal junction were measured.

## Glucose Evaluation

The test of blood glucose was always undertaken in duplicate from tail vein by a portable glucometer (Roche Diagnostics, Mannheim, Germany). In OGTT, after an overnight fast, the rats were orally administrated 20% glucose (1 g/kg), and the blood sugar was measured at 0, 15, 30, 60, and 120 min. Meanwhile, blood samples were collected. The serum total GLP-1 was measured using an ELISA kit (Millipore, Burlington, MA, USA). In ITT (18), blood glucose was measured in conscious and fasted rats, and then 10, 30, 60, 90, 120, and 180 min after intraperitoneal injection of insulin solution (0.5 IU/kg; Sigma-Aldrich, St. Louis, MO, USA).

## Gastric Emptying Measurements

The measurement of gastric emptying was based on standard methods (7). Briefly, acetaminophen (100 mg/kg) was added to 3 ml Ensure solution and then perorally infused to overnight-fasted rats. Blood samples were collected at 15, 30, 45, and 60 min after intragastric gavage. The concentration of serum acetaminophen, which is not absorbed in the stomach (thus used as indicator of gastric emptying), was detected by spectrophotometric method under an absorption spectrum at 590 nm (19).

## Statistical Analysis

The results were expressed as mean ± standard deviation (SD). Areas under the curves (AUC) for OGTT ($AUC_{OGTT}$) and ITT ($AUC_{ITT}$) were calculated by trapezoidal integration. Differences between groups over time were evaluated by two-way analysis of variance (ANOVA) or repeated measures ANOVA; differences between the two groups at a specific time point were calculated using Student's *t*-test. Homogeneity of variance was confirmed by levene statistic test, and Friedman test was performed for heterogeneity. All statistical analyses were performed by SPSS19.0 software. $p < 0.05$ was considered statistically significant.

# RESULTS

Before operation, all rats had the same levels of body weight, food intake, serum total GLP-1, and acetaminophen. All procedures were successfully performed, and all rats survived till the scheduled time of termination.

## Body Weight and Food Intake

Postoperatively, rats in the sham group showed greater body weight than IT rats (**Figure 2A**; **Supplementary Material**). After injection of atropine/metoclopramide, however, the body weight of IT and sham rats became similar ($p > 0.05$). No difference was observed in food intake between the two groups, as shown in **Figure 2B**.

## Glucose Homeostasis and Insulin Sensitivity

**Figures 3A–D** shows OGTT data at weeks 0, 4, 8, and 10. There was no significant difference between groups before surgery. At 4 and 8 weeks postoperatively, OGTT was significantly improved in IT rats as compared with sham. These findings were also supported by the data of $AUC_{OGTT}$ (**Figure 3E**). After the use of GI motility pharmacologics (at 10 weeks after surgery), however, the gaps in OGTT curves and $AUC_{OGTT}$ between IT and sham

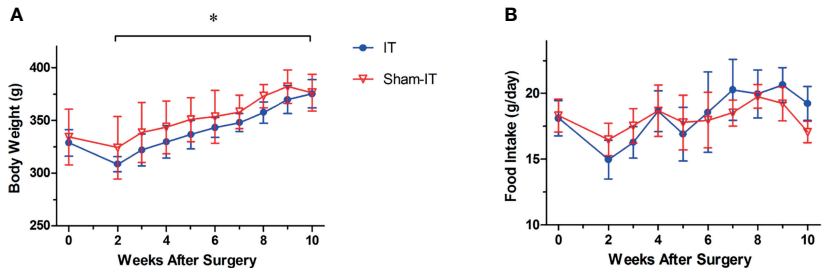


**FIGURE 2** | Body weight and food intake after operation. $N = 6$ in each group. *$p < 0.05$. **(A)** Body weight. IT group exhibited less body weight than sham group after surgery (two-way ANOVA: $p < 0.001$). **(B)** Food intake. No difference was found in food intake between the two groups (two-way ANOVA: $p = 0.454$).

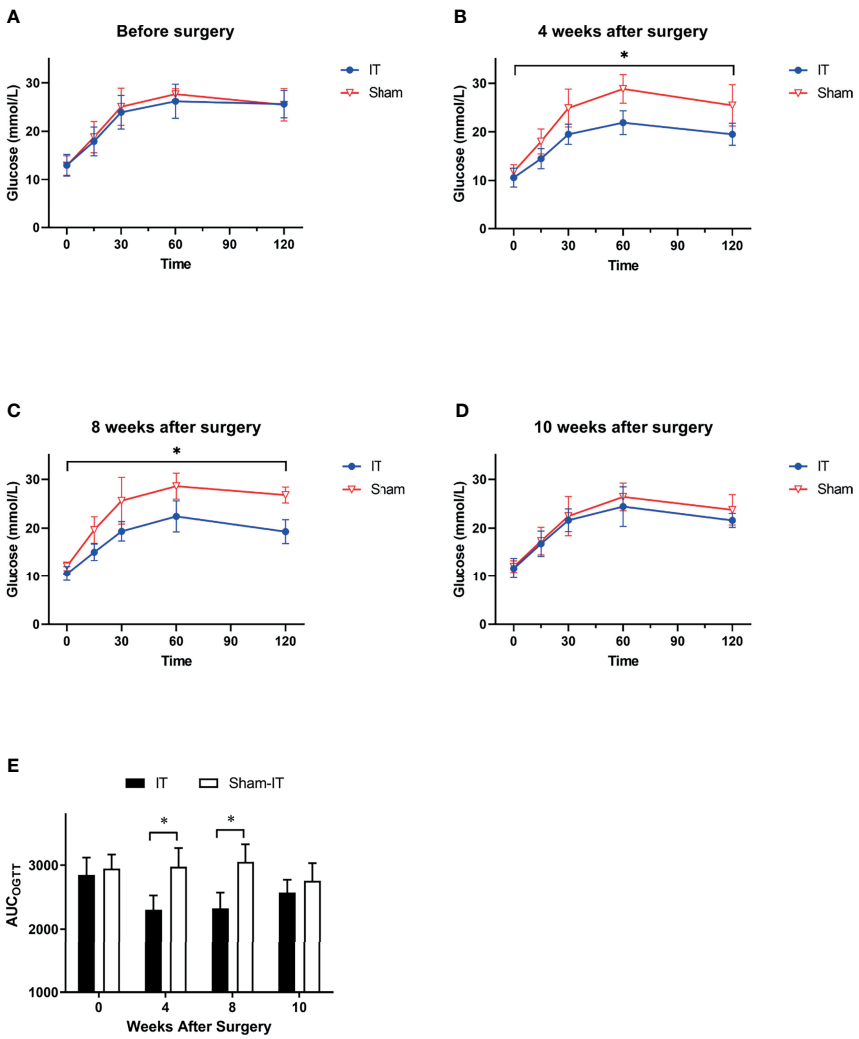


**FIGURE 3** | OGTT results at different time points. $N = 6$ in each group. *$p < 0.05$. **(A)** OGTT before surgery. No difference was shown between IT and sham groups (two-way ANOVA: $p = 0.569$). **(B)** OGTT at 4 weeks after surgery. Glucose level following oral gavage was significantly lower in IT group (two-way ANOVA: $p = 0.002$). **(C)** OGTT at 8 weeks after surgery. IT rats had significantly lower glucose level than sham rats (two-way ANOVA: $p = 0.001$). **(D)** OGTT at 10 weeks after surgery. IT rats and sham rats showed similar OGTT results (two-way ANOVA: $p = 0.269$) after administration of GI motility medications. **(E)** $AUC_{OGTT}$. Before surgery, there was no difference in $AUC_{OGTT}$ between groups ($t$-test, $p = 0.497$). At 4 and 8 weeks after surgery, $AUC_{OGTT}$ was significantly decreased in IT group ($t$-test, $p = 0.001$ of both). At postoperative 10 weeks, however, the difference became not significant ($t$-test, $p = 0.215$).

became smaller, with no statistical differences between the two groups.

As shown in **Figure 4A**, there were no differences in ITT between the IT and sham groups prior to operation. At postoperative 4 and 8 weeks, glucose levels in response to insulin injection were significantly decreased in the IT group as compared with sham, suggesting improvements of glucose tolerance and insulin tolerance (**Figures 4B, C**). The results were consistent with $AUC_{ITT}$ calculation as shown in **Figure 4D**.

## Plasma Total GLP-1

At postoperative weeks 4 and 8, relative to sham, the IT group exhibited significantly higher plasma concentration of GLP-1 (**Figures 5A, B**). At 10 weeks after surgery, following administration of GI dynamic drugs, however, the IT and sham groups showed no difference in GLP-1 concentration (**Figure 5C**). For the IT group, GLP-1 level seemed elevated in week 8 as compared with week 4 postoperatively, but there is no statistically significant difference (**Figure 5D**).

## Gastric Emptying and Intestinal Transit

As shown in **Figure 6A, B**, the serum levels of acetaminophen following IT were significantly lower than that of the sham group at 4 and 8 weeks postoperatively, indicating a slower gastric emptying. For the IT group, the speed of gastric emptying was markedly increased in postoperative week 8, as the concentration of acetaminophen was higher than that of week 4 (**Figure 6C**). In the sham group, the emptying of stomach was not changed over time (**Figure 6D**). **Figure 6E** shows that 30 min after toluidine blue O gavage, the dye travelled significantly shorter in the intestinal tract in IT in comparison with sham (at 12 weeks after surgery).

# DISCUSSION

Studies of IT have been performed in rats for over 30 years (20). In recent years, IT has also been used in patients, with satisfactory outcomes (21–23). Unlike RYGB and VSG, IT does not involve gastrectomy; i.e., it is a "pure" intestinal operation which is suitable for GI research. The hindgut hormones are considered vital mediators of diabetic control following IT (17). Although it is known that the rapid delivery of mixed chyme to transposed ileum promotes the secretion of hindgut hormones, the role of GI emptying in accelerating these hormones and alleviating hyperglycemia post-IT requires further investigations.

To minimize the impact of different body weight on blood glucose and focus on the weight loss independent mechanism (24), we utilized nonobese animal model, i.e., GK rats, for the study. GK diabetic rats are characterized by insulin resistance and insufficient pancreatic beta cell function, which can appropriately simulate human T2DM (18, 25, 26). Using GK rat model, our experiments illustrate the GI transit time to be suppressed after IT, and gastric emptying gradually recovered over time. This represents the first study of the relationship between GI emptying and diabetic control after IT.

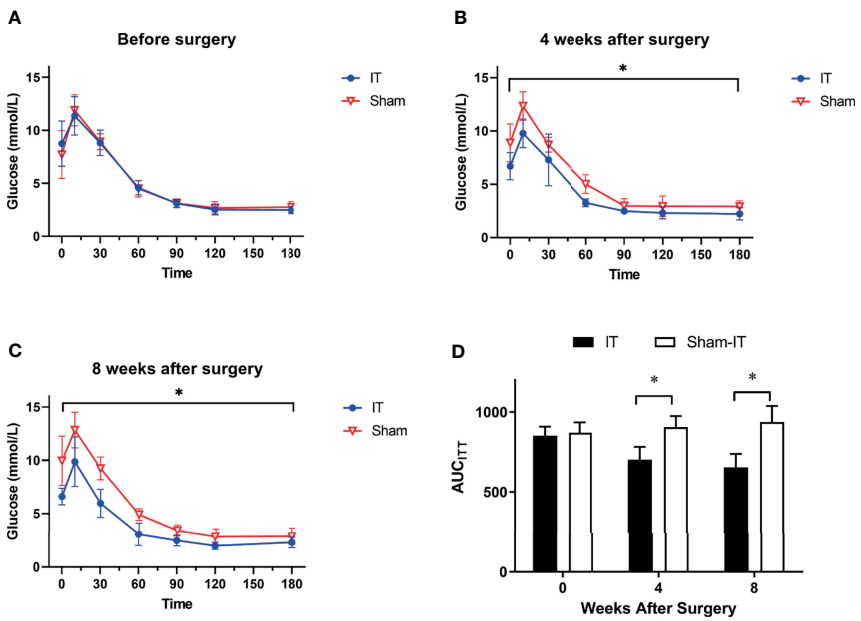


**FIGURE 4** | ITT results at different time points. $N = 6$ in each group. *$p < 0.05$. **(A)** ITT before surgery. No difference was found between groups (two-way ANOVA: $p = 0.993$). **(B)** ITT at 4 weeks after surgery. IT rats showed significantly improved ITT as compared with sham rats (two-way ANOVA: $p = 0.001$). **(C)** ITT at 8 weeks after surgery. ITT data of IT rats was significantly better than sham (two-way ANOVA: $p < 0.001$). **(D)** $AUC_{ITT}$. No difference was observed in $AUC_{ITT}$ between IT and sham groups before operation (*t*-test, $p = 0.605$). At postoperative 4 weeks, $AUC_{ITT}$ were significantly decreased in IT group (*t*-test, $p = 0.001$). At 8 weeks postoperatively, the difference became more significant (*t*-test, $p < 0.001$).

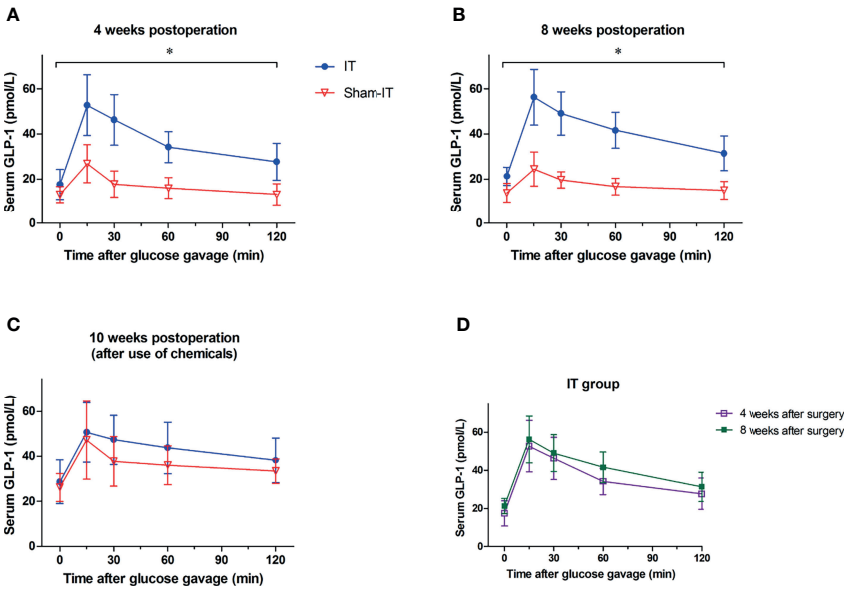


**FIGURE 5** | The levels of serum total GLP-1 after surgery. $N = 6$ in each group. *$p < 0.05$. **(A)** Glucose-stimulated GLP-1 levels at postoperative 4 weeks. As compared with sham, GLP-1 level was significantly higher in IT group at 4 weeks postoperatively (repeated measures ANOVA: $p = 0.001$ IT vs. sham, $p < 0.001$ between time points, $p < 0.001$ of interaction). **(B)** Glucose-stimulated GLP-1 levels at 8 weeks postoperatively. GLP-1 level was significantly higher in IT group at postoperative 8 weeks in comparison with sham group (repeated measures ANOVA: $p < 0.001$ IT vs. sham, $p < 0.001$ between time points, $p < 0.001$ of interaction). **(C)** Glucose-stimulated GLP-1 levels at postoperative week 10. At 10 weeks after operation, because of the use of chemicals, GLP-1 levels were comparable between IT and sham groups (repeated measures ANOVA: $p = 0.309$ IT vs. sham, $p < 0.001$ between time points, $p = 0.680$ of interaction). **(D)** Glucose-stimulated GLP-1 levels of IT group. GLP-1 concentration at 8 weeks postoperatively was not significantly higher than that at 4 weeks postoperatively (repeated measures ANOVA: $p = 0.358$ IT vs. sham, $p < 0.001$ between time points, $p = 0.885$ of interaction).

Although it is believed that sustainable weight loss benefits diabetic alleviation (27), previous data on weight change after IT are not unified. Some literature (14, 28) shows that IT does not affect body weight, while some others (29, 30) found a decrease in weight gain in IT rats. The paradox may be explained by the differences of studied strains, housing environments, operative details, etc. In our study, IT induced reduction of body weight, albeit the amount of food intake was not different. A study by Gaitonde et al. (14) also revealed an unchanged level of food intake after IT. Given that IT does not reduce the absorption area of gut, one reasonable speculation is that IT surgery may augment energy expenditure (31), in agreement with some literature on RYGB (32, 33), resulting in disparity of body weight. Another plausible explanation is that metabolic surgery reprograms the whole-body metabolism to maintain a reduced body weight, rather than lowering caloric intake (34).

Based on our data, glucose tolerance and insulin resistance were both ameliorated after IT, suggesting improved glucose homeostasis. Similarly, Yan et al. (30) and Culnan et al. (35) came to the same conclusion in GK rats and Zucker fatty rats, respectively. We speculate the increased GLP-1 contributes to the remission of glycometabolism (consistent with the hindgut theory), as GLP-1 has effects in improving beta cell function, insulin secretion, and insulin sensitivity (36, 37). In addition, GLP-1 can slow down gastric emptying and intestinal transit (37). Confirmatory to these observations, the movements of both stomach and small intestine were decreased in our IT group. Obviously, the early exposure of chyme is due to the short pathway rather than a rapid transit.

The role of GI emptying and transit was studied at 10 weeks postoperatively. After using atropine in IT rats and metoclopramide in sham rats, it can be speculated that the GI transit of IT decreased while that of sham increased since GI emptying can be inhibited by atropine (7) and promoted by metoclopramide (38). As a result, GLP-1 secretion of the two groups became equalized after the administration of the pharmaceuticals. Consequently, the body weight became comparable, and the differences in glucose tolerance decreased. In other words, increase in GI transit in sham can also be helpful to ameliorate diabetes, while slowing down the GI movement after IT can deteriorate glycemic control. Diabetic rats were found to show gastric hypomotility (39). Furthermore, Bharucha et al. (40) indicated that delayed gastric emptying in diabetes can affect hyperglycemia, which is confirmative of our study. Taken together, the decrease of GI transit following IT may be the result or the negative feedback of increased hindgut hormones, rather than a favorable outcome of diabetic alleviation. The "earlier contact" caused by IT promotes GLP-1 secretion and further changes body weight, glycometabolism, etc., which can be partly blocked by slower GI transit.

One interesting finding is that at 8 weeks postoperatively, the gastric emptying in the IT group was accelerated as compared with postoperative week 4, but it did not lead to further improvement of glucose metabolism or significant increase of

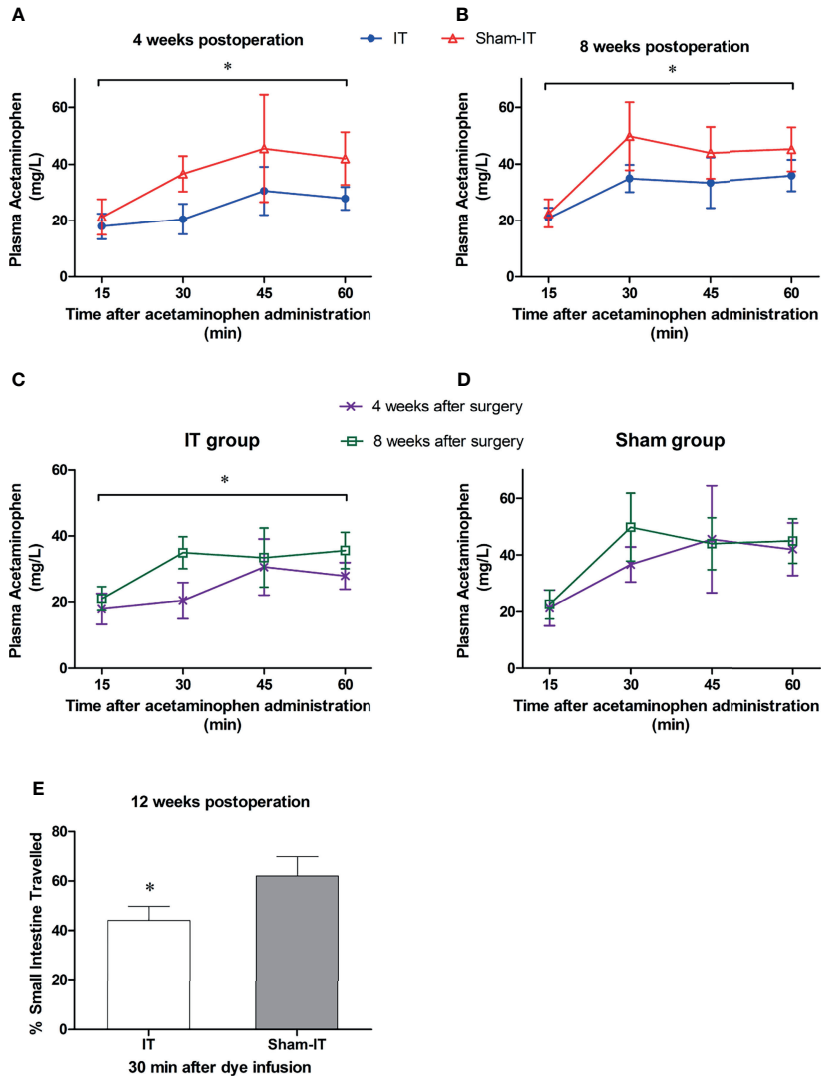


**FIGURE 6** | The tests of GI motility after surgery. $N = 6$ in each group. $^*P < 0.05$. **(A)** Plasma levels of acetaminophen at postoperative 4 weeks. The concentrations of acetaminophen were significantly lower in IT group than in sham group (Friedman test: $p < 0.001$). **(B)** Acetaminophen levels at postoperative 8 weeks. The concentration was significantly higher in sham than in IT group (repeated measures ANOVA: $p = 0.010$ IT vs. sham, $p < 0.001$ between time points, $p = 0.121$ of interaction). **(C)** Plasma levels of acetaminophen in IT group. The concentration of acetaminophen was higher at 8 weeks postoperatively than at 4 weeks (repeated measures ANOVA: $p = 0.021$ IT vs. sham, $p < 0.001$ between time points, $p = 0.015$ of interaction). **(D)** Plasma levels of acetaminophen in sham group. The levels did not differ over time (repeated measures ANOVA: $p = 0.249$ IT vs. sham, $p < 0.001$ between time points, $p = 0.302$ of interaction). **(E)** Intestinal transit (expressed as percentage of intestine travelled) at 30 min after toluidine blue O infusion. The dye travelled shorter in intestinal tract in IT compared to sham group ($t$-test, $p = 0.001$).

GLP-1. This suggests that the acceleration of gastric emptying is not the key factor in diabetic control after IT. Further studies are needed to shed additional light on this issue.

Our study has some limitations. First, we did not test GI motility by scintigraphy, which is more accurate than acetaminophen absorption test and dye measurement, because of the need for complex methodology. Second, the role of other hormones, the measurement of energy expenditure, and the influence of drugs under longer-term application need to be further investigated. Third, gastric emptying and GI transit rate of rats may be different from that of humans, and hence the findings need to be validated in larger animals before translation. Gender-based differences also need to be explored in the future.

In conclusion, IT reduced body weight and alleviated diabetes in GK rats. The increased secretion of GLP-1 may at least partly contribute to the remission of T2DM. However, the inhibition of GI emptying is likely to disable the hypoglycemic effect caused by IT, while the acceleration of GI transit seems advantageous to achieve euglycemia. Following IT, the alteration of GI transit seems secondary to the operation instead of the cause of hyperglycemic control.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of California Medical Innovations Institute.

## AUTHOR CONTRIBUTIONS

YW, XG, and DS performed animal procedures and experiments and data collection. YW and GK designed the study. All authors participated in drafting the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.849923/full#supplementary-material

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