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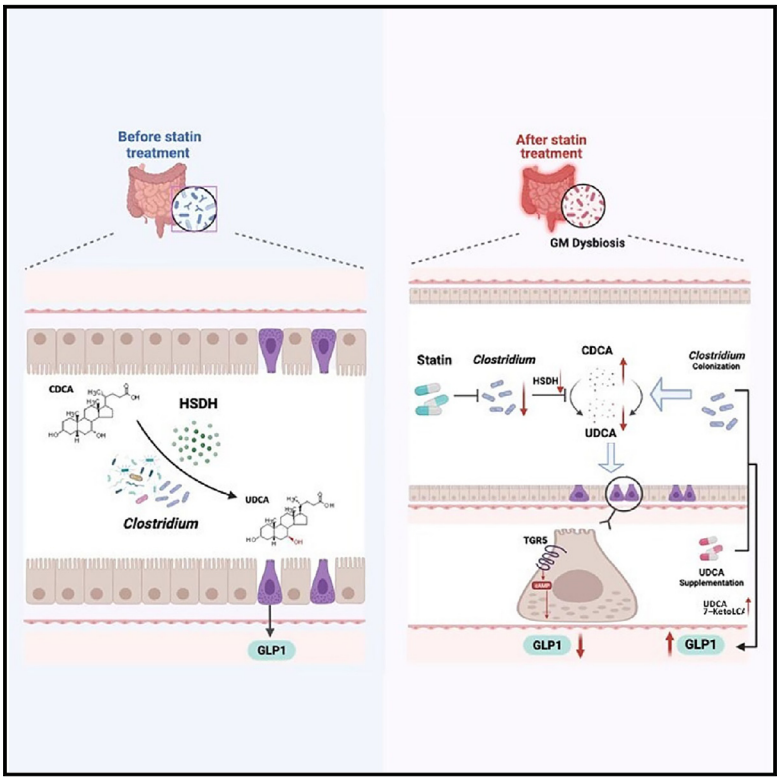
Statins aggravate insulin resistance through reduced blood glucagon-like peptide-1 levels in a microbiota-dependent manner

Graphical abstract



Highlights

- Statin alters gut microbiota and dysregulates bile acid metabolism and glucose homeostasis
- Statin causes dysregulated gut microbiota and decrease of the genus *Clostridium*
- Decreased *Clostridium*-rich microbiota after statin inhibits HSDH and lowers UDCA
- Transplanting *Clostridium sp.* or supplying UDCA ameliorates statin-induced hyperglycemia

Authors

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In brief

Statins are common cholesterol-lowering drugs, but the mechanism of statin-induced hyperglycemia is unclear. Here, She et al. demonstrate the function of gut microbiota-BA axis in this process. Their findings uncover the *Clostridium*-UDCA dependent mechanism in statin-induced hyperglycemia and provide insights into adjuvant therapy of UDCA to lower the adverse risk of statin.

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Statins aggravate insulin resistance through reduced blood glucagon-like peptide-1 levels in a microbiota-dependent manner

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SUMMARY

Statins are currently the most common cholesterol-lowering drug, but the underlying mechanism of statin-induced hyperglycemia is unclear. To investigate whether the gut microbiome and its metabolites contribute to statin-associated glucose intolerance, we recruited 30 patients with atorvastatin and 10 controls, followed up for 16 weeks, and found a decreased abundance of the genus *Clostridium* in feces and altered serum and fecal bile acid profiles among patients with atorvastatin therapy. Animal experiments validated that statin could induce glucose intolerance, and transplantation of *Clostridium sp.* and supplementation of ursodeoxycholic acid (UDCA) could ameliorate statin-induced glucose intolerance. Furthermore, oral UDCA administration in humans alleviated the glucose intolerance without impairing the lipid-lowering effect. Our study demonstrated that the statin-induced hyperglycemic effect was attributed to the *Clostridium sp.*-bile acids axis and provided important insights into adjuvant therapy of UDCA to lower the adverse risk of statin therapy.

INTRODUCTION

Statin is the cornerstone of primary and secondary prevention for atherosclerotic disease by contemporary guidelines. According to statistics, 1 in 4 people over 40 years old on average takes statin as secondary prevention of cardiovascular disease.[1] However, the adverse effects caused by statin utilization have also attracted extensive attention. In addition to severe or common complications such as rhabdomyolysis and liver function injury, the new-onset diabetes mellitus (DM) or glucose intolerance has been reported more and more frequently as a concomitant symptom after statin therapy. According to previous clinical trials, statin treatment was associated with a significant increase in new-onset diabetes. In addition, participants with one or more major diabetes risk factors were at higher risk of developing diabetes than those without.[2–4] Epidemiological studies also suggest that the hazard ratio for developing type 2 diabetes ranges from 1.3 to 3.3 over the follow-up point among patients utilizing statin.[5–8] Statin-caused new-onset diabetes appears to be more pronounced among Asian populations.[7,9] Despite growing concerns about dysregulated glucose homeostasis related to statin therapy, few mechanistic investigations have been conducted.

The concept of "gut dysbiosis" and its association with cardiometabolic diseases have attracted growing attention. An increasing number of studies have shown that changes in the structure and function of gut microbiome can promote the occurrence of obesity and insulin resistance, which is an important pathogenesis for type 2 diabetes and cardiovascular disease. Intestinal bacteria can also ferment dietary fiber, increasing the secretion of intestinal peptides and insulin-like growth factors.[10,11] The gut microbiota modulates host metabolism via regulating various circulating metabolites,

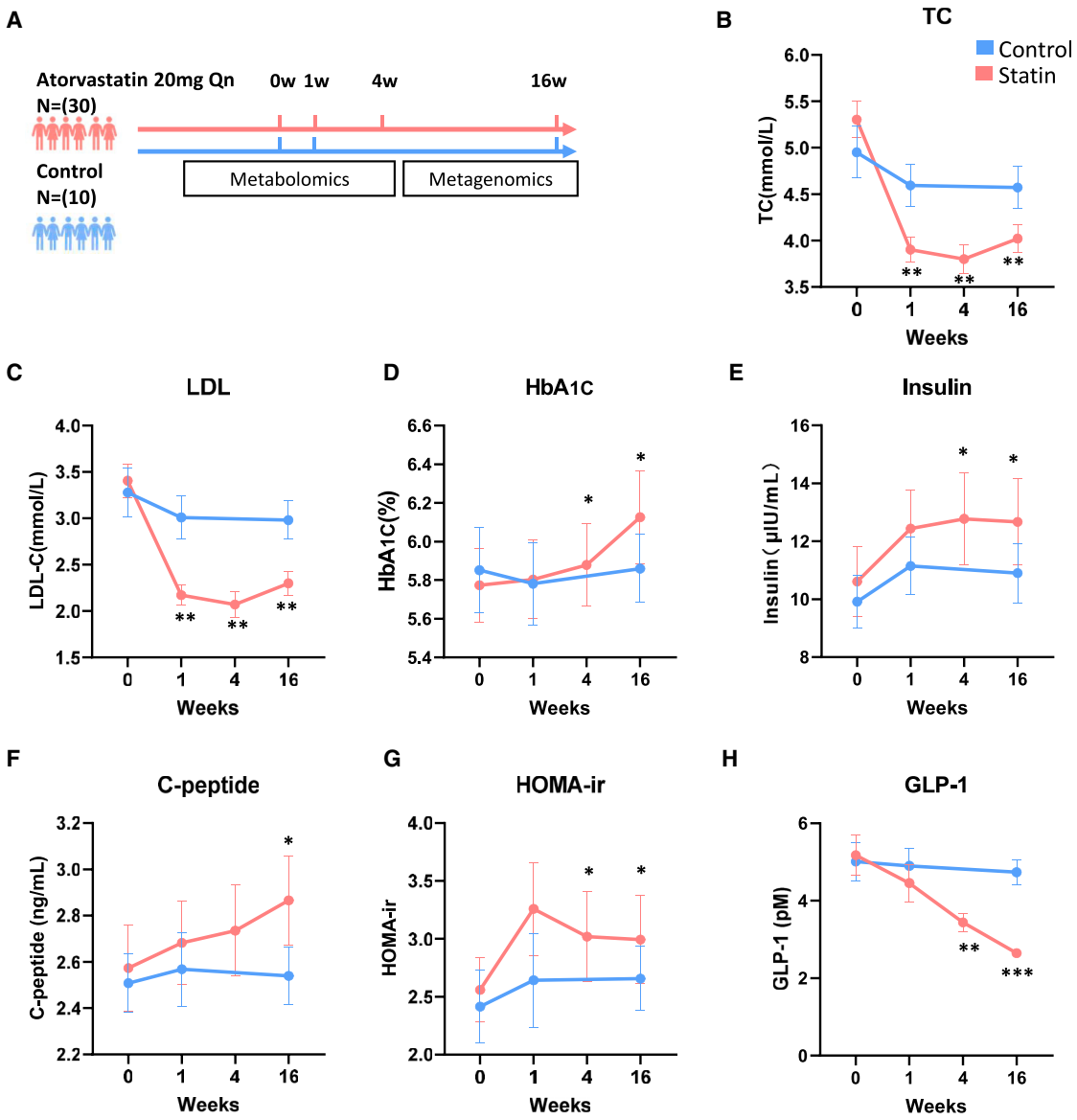


Figure 1. Statin induced glucose intolerance and decreased serum GLP-1 level in hyperlipidemia patients

(A) Clinical study flow chart of patients taking atorvastatin (n = 30) or healthy control (n = 10).

(B and C) Total cholesterol (B) and LDL (C) levels at week 0, 1, 4, and 16.

(D–G) HbA_{1c} (D), insulin (E), C-peptide (F), and HOMA-ir (G) levels at week 0, 1, 4, and 16.

(H) GLP-1 at week 0, 1, 4, and 16. *p < 0.05, **p < 0.01, ***p < 0.001, as determined by one-way ANOVA. Data are shown as means ± SEM.

See also Methods S1.

including bile acids and indoles. Previous studies have analyzed the remodeling effect of metformin on gut microbiota and its metabolites and revealed that glycoursodeoxycholic acid (GUDCA) and intestinal farnesoid X receptor (FXR) are new targets for treating metabolic diseases associated with obesity.[10,12,13] A recent study has further proposed that microbial host isoenzymes are widely present in the intestine. Specifically, bacterial dipeptidyl peptidase-4 (mDPP4) isoenzymes have been identified as capable of degrading the host active glucagon-like peptide-1 (GLP-1), which leads to the disruption of the integrity of the intestinal barrier and the impaired glucose metabolism in mice.[14]

In addition, more and more evidence shows that the therapeutic effects of various drugs are related to the different functions of gut microbiota.[15] Besides the influence of gut microbes on drug metabolism and toxicity, the complex relationship between the gut microbiota and host also interferes with the therapeutic effect of drugs.[15] A recent population-based cross-sectional gut microbiota analysis has shown that statin therapy is associated with lower gut microbiota dysbiosis, with the predominant difference in the distribution of *Bacteroides*, *Ruminococcaceae*, *Prevotella*, etc.[16] Meanwhile, prior work has demonstrated that individual responses to statins, both in terms of on-target low-density lipoprotein (LDL) lowering and in terms of off-target insulin resistance, can

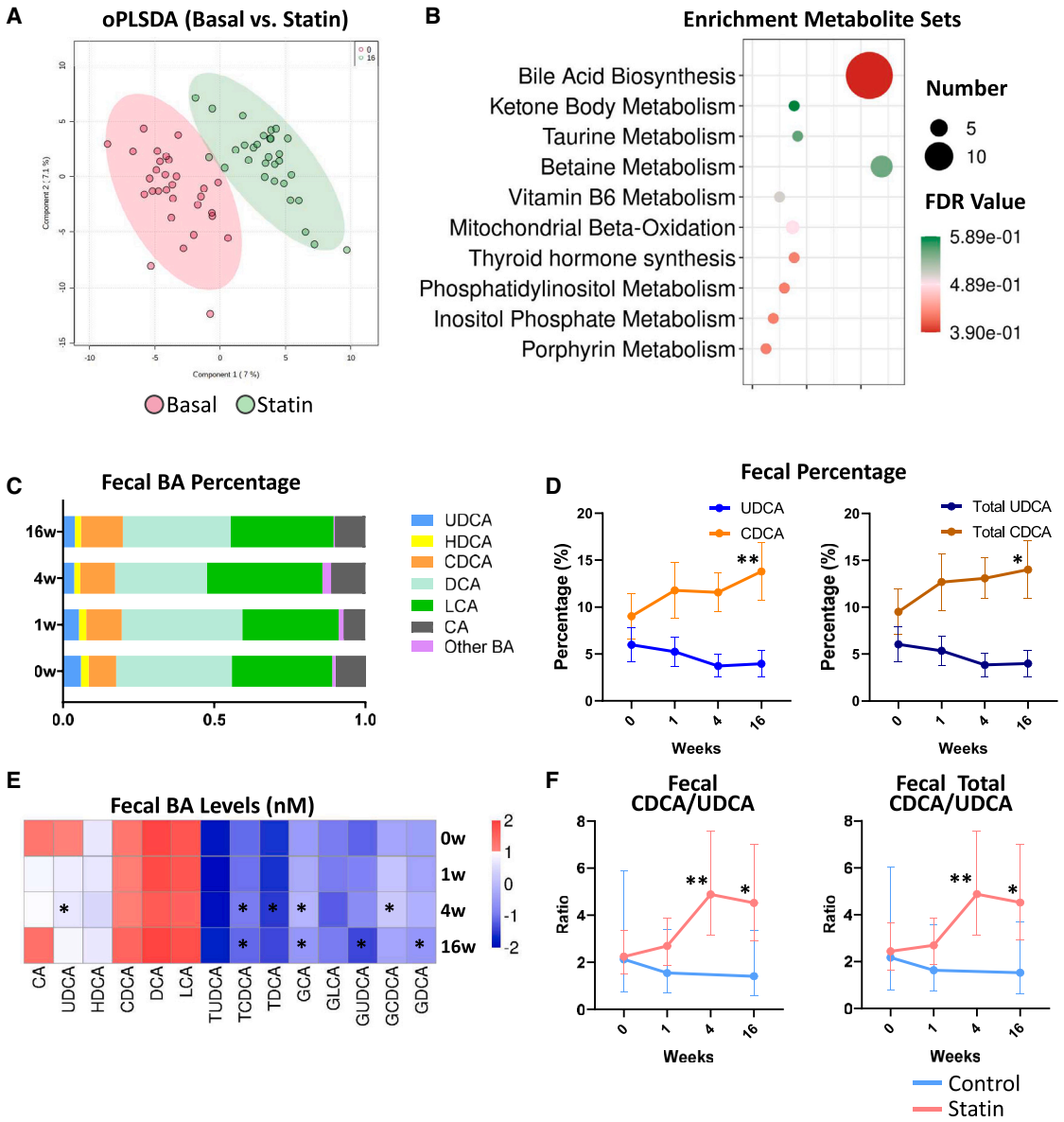


Figure 2. Statin increased fecal CDCA but decreased UDCA among patients taking atorvastatin

(A) Orthogonal partial least squares discriminant analysis (oPLS-DA) showing the metabolites of patients before and 16 weeks after taking atorvastatin. The basal statin is shown in red, and the 16 weeks after is shown in green. n = 30 individuals/group.

(B) KEGG enrichment analysis showing marked enrichment in bile acids biosynthesis pathway.

(C) Proportions of fecal dominant bile acids.

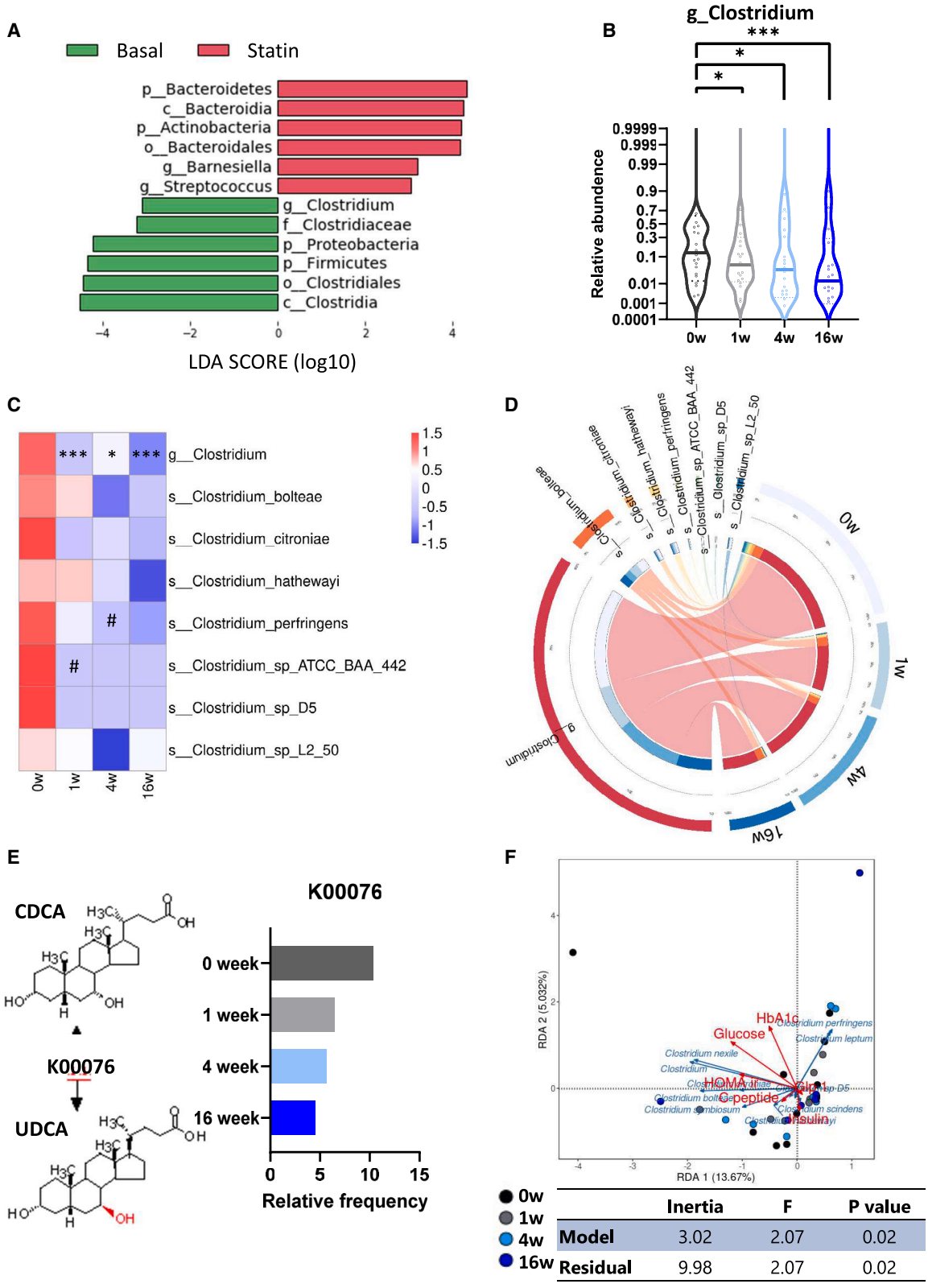
(D) Proportions of fecal UDCA (blue); CDCA (yellow); total UDCA (dark blue, the sum levels of UDCA and its derivatives including UDCA, TUDCA, and GUDCA); and total CDCA (dark yellow, the sum levels of CDCA and its derivatives including CDCA, TCDCA, and GCDCA) at week 0, 1, 4, and 16 after atorvastatin therapy.

(E) Heatmap of the fecal bile acid levels of participants in response to atorvastatin treatment.

(F) Ratios of fecal CDCA to UDCA and total CDCA to UDCA at week 0, 1, 4, and 16 after atorvastatin therapy. *p < 0.05, **p < 0.01 as determined by one-way ANOVA. Data are shown as means ± SEM.

be explained by baseline variation in the microbiome.[17] Our previous investigation has pointed out that the alteration of fecal bacteria also affects the individual response to statin therapy.[18] However, it remains to be elucidated whether the gut microbiome exerts an influence on glucose homeostasis after statin therapy.

Given the vital role of the gut microbiome in glucose homeostasis, we hypothesize that statin utilization may contribute to the gut microbiome dysbiosis, which potentially affects circulating metabolites and host glucose homeostasis. In this study, by performing metabolomic and metagenomic analyses in a cohort of atherosclerotic patients receiving atorvastatin, the *Clostridium* species were identified to be closely associated with bile acid synthesis and excretion in a time-dependent manner. Through a series of animal and cell culture experiments,



	Inertia	F	P value
Model	3.02	2.07	0.02
Residual	9.98	2.07	0.02

Figure 3. Statin reduced the abundance of fecal *Clostridium* species, which correlated to UDCA transformation and glucose homeostasis among patients taking atorvastatin

(A) The LDA score showing the most differentially significant abundant taxa enriched in microbiota at week 16 (red) and week 0 (green) after atorvastatin.

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we also clarified that the *Clostridium*-rich microbiota could influence bile acid excretion, which regulated glucose intolerance via targeting GLP-1 secretion. At last, by supplementing ursodeoxycholic acid (UDCA) to individual patients receiving chronic statin therapy who also exhibited elevated hemoglobin A_{1c} (HbA_{1c}), we observed that the combination therapy of statin and UDCA could ameliorate the glucose homeostasis without affecting its lipid-lowering effects.

RESULTS

Statin induced glucose intolerance and decreased serum GLP-1 level in hyperlipidemia patients

To investigate how statin affects glucose levels in patients, we established a cohort of 30 patients with atorvastatin and 10 control subjects without atorvastatin at baseline and followed up for 16 weeks (Figure 1A). The baseline information for the patients enrolled was listed in Tables S1-1 and S1-2. As compared with the control, patients with atorvastatin exhibited a significant decrease in total cholesterol (TC) and LDL levels 1 week after statin initiation (Figures 1B and 1C). In addition, although the fasting glucose levels showed no difference, the HbA_{1c} increased significantly at 4 weeks and prolonged to 16 weeks (Figure 1D), together with increased insulin, C-peptide, and homeostatic model assessment for insulin resistance (HOMA-ir) (Figures 1E–1G). Previous studies identified that GLP-1 can promote insulin secretion and inhibit glucagon secretion.[19,20] We then tested the effect of atorvastatin on serum level of GLP-1 and found that the active GLP-1 concentration decreased significantly 4 weeks after atorvastatin initiation (Figure 1H), suggesting that atorvastatin could inhibit GLP-1 secretion to affect glucose homeostasis.

Statin increased fecal CDCA but decreased UDCA among patients taking atorvastatin

Since atorvastatin could affect glucose homeostasis, possibly through downregulation of circulating GLP-1 levels, we then applied untargeted metabolomics analysis to identify the circulating metabolomic alteration and seek for potential key metabolites that are associated with the dysregulated glucose homeostasis. As shown by orthogonal partial least squares discriminant analysis (oPLS-DA), substantiaUl altered fecal metabolites were identified after statin treatment (Figures 2A and S1A). The metabolites were enriched to a great extent in the bile acid biosynthesis pathway (Figure 2B). We then compared the fecal and serum bile acids profile at different follow-up points among patients taking statin (Figures 2 and S1B–S1F). Fecal bile acid percentage indicated a gradual increase in chenodeoxycholic acid (CDCA) percentage and a decrease in UDCA percentage (Figures 2C and 2D). The decreased UDCA levels correlated with decreased total UDCA levels (the sum levels of UDCA and its derivatives, including UDCA, TUDCA, and GUDCA) in a time-dependent manner (Figure 2D). The fecal UDCA level was significantly decreased (Figure 2E), and the CDCA-to-UDCA ratio was significantly increased 4 weeks after statin treatment (Figure 2F). This tendency of fecal bile acids was not observed in the control cohort (Figures S2A–S2C). Previous studies have revealed that, in human, CDCA, a classic primary bile acid, was converted to UDCA by gut microbial $7_{\alpha/\beta}$-hydroxysteroid dehydrogenases (HSDHs).[21,22] The alteration of the CDCA-to-UDCA ratio after statin therapy implied that statins might affect bile acid metabolism by regulating the gut microbiota.

Statin reduced the abundance of fecal *Clostridium* species, which correlated to UDCA transformation and glucose homeostasis among patients taking atorvastatin

To further uncover how statin might control the gut microbiota to affect bile acid metabolism, we performed metagenomics profiling of stool samples in the aim of seeking specific gut microbiota that potentially affects host metabolism. Although the Shannon diversity index (α-diversity) and PLS-DA of gut microbiota did not indicate significant alteration of gut microbiota after statin treatment (Figures S3A–S3C), linear discriminant analysis (LDA) indicated decreased abundance of the phylum *Firmicutes*, the class *Clostridia*, and the genus *Clostridium* after taking atorvastatin as compared with baseline (Figure 3A). Among the genus *Clostridium*, the abundances of multiple species were markedly decreased after statin treatment starting from week 1 till week 16 (Figures 3B–3D and S3D), which was not observed in the gut microbiota of the control cohort (Figures S2D–S2F). Furthermore, by taking an untargeted approach to identify other taxa in the microbiome among the clinical cohort, the species from the genus *Clostridium*, which is involved in the secondary bile acid biotransformation, showed a stronger correlation to the bile acid metabolism than other bacteria gena (Figures S3E and S3F). Recently, multiple species belonging to the genus *Clostridium* were observed to mediate pathways toward HSDH[21]; thus, we adopted the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to search for the functional pathways related to secondary bile acid metabolism of the gut microbiota. Notably, the 7-alpha-HSDH (K00076) from KEGG database, the orthology gene of the enzyme crucial for CDCA-to-UDCA transformation, was decreased in a time-dependent manner (Figure 3E). Correlation analysis further identified a negative correlation between *Clostridium* species and blood glucose indicators (Figures 3F, S3F, and S3G).

Taken together, the human cohort study revealed that atorvastatin treatment resulted in decreased genus *Clostridium* after week 1 and then dysregulated bile acid metabolism as well as glucose homeostasis at week 4. These results suggested that statin might induce a reduction of *Clostridium*-rich microbiota, expressing genes for CDCA-to-UDCA transformation. The association between *Clostridium* and blood glucose indicators further implied that the decreased *Clostridium*-rich microbiota might

(B) Abundance of *Clostridium* genus in feces at week 0, 1, 4, and 16 after atorvastatin therapy. *$p < 0.05$, ***$p < 0.001$, as determined by paired Wilcoxon rank-sum test.

(C and D) Heatmap (C) and circus plot (D) of *Clostridium* genus and species at week 0, 1, 4, and 16 after atorvastatin. *$q < 0.05$, ***$q < 0.001$, as determined by two-way ANOVA after false discovery rate (FDR) adjustment. #$p < 0.05$ as determined by two-way ANOVA.

(E) Levels of the K00076 from KEGG database at weeks 0, 1, 4, and 16.

(F) RDA analysis showing the significant correlation between *Clostridium* species and clinical glucose indicators based on different time points ($p = 0.022$). Data are shown as means ± SEM.

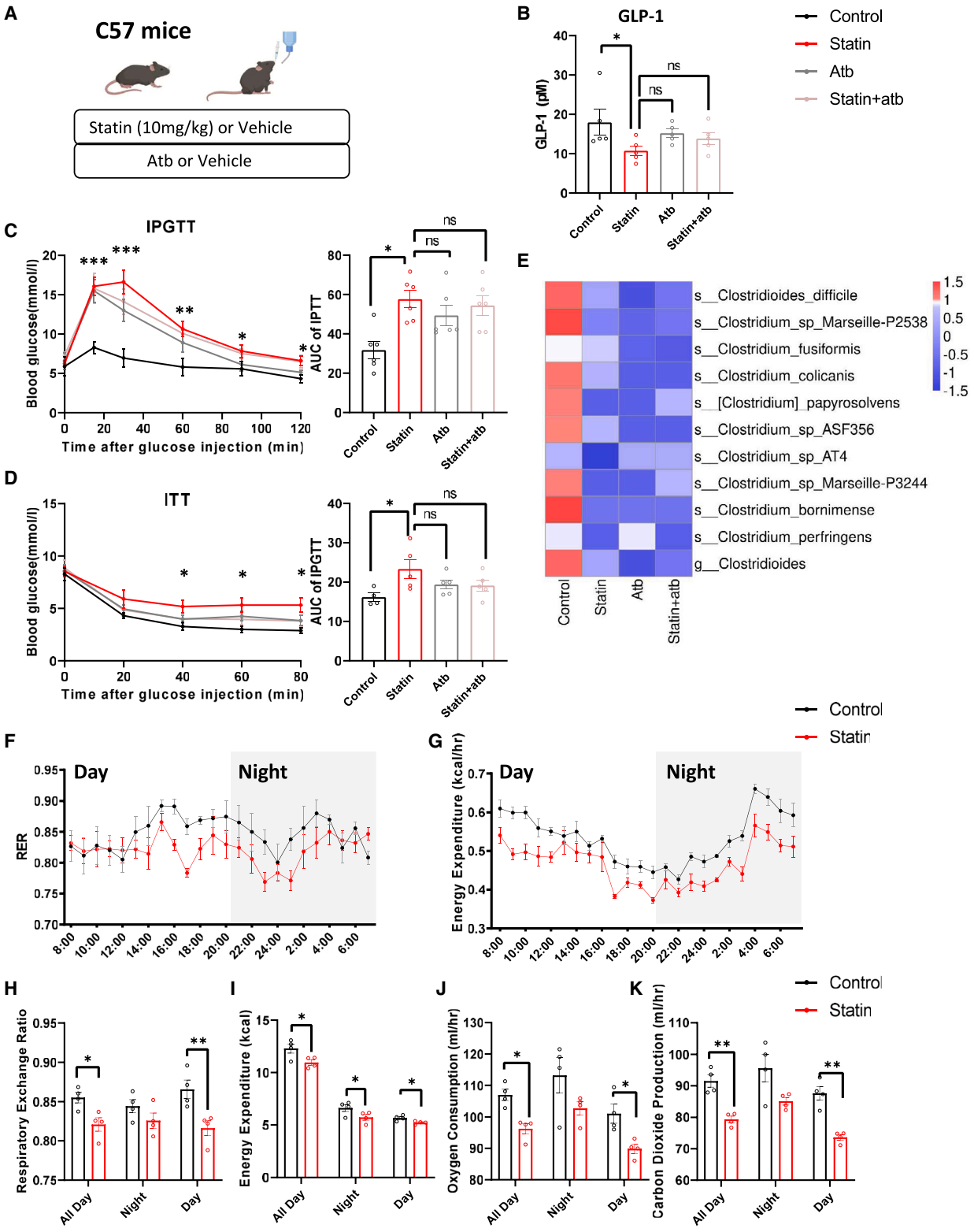

Figure 4. Statin-induced glucose intolerance in mice was not observed in pseudo-germ-free condition

(A) Experimental procedure for atorvastatin (10 mg/kg) initiation in an antibiotic cocktail-induced (Atb) pseudo-germ-free mice or control. Mice that received atorvastatin were grouped as statin mice as compared with control, and mice treated with an antibiotic cocktail with or without statin were classified as Atb or statin + Atb, respectively.

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influence bile acid synthesis and excretion and impair glucose metabolism.

Statin-induced glucose intolerance in mice was not observed in pseudo-germ-free condition

To further evaluate the effect of atorvastatin on glucose metabolism and energy expenditure (EE), we fed C57 mice with or without atorvastatin for 12 weeks. To identify whether atorvastatin affects glucose level via regulating the gut microbiota, we concurrently conducted experiments using the antibiotic-treated, microbiota-depleted pseudo-germ-free mice (Figure 4A). The atorvastatin treatment significantly decreased the serum levels of GLP-1 compared with the control (Figure 4B). Glucose intolerance and insulin resistance were substantially impaired in mice after atorvastatin treatment, but not in pseudo-germfree conditions (Figures 4C and 4D). In keeping with the identification from patients, a reduction of *Clostridium* upon atorvastatin treatment was confirmed in the mouse model receiving atorvastatin as compared with the control mice (Figures 4E and S4A–S4C). Similar to the human metagenomic analysis, the 7-alpha-HSDH (K00076) was also decreased in mice after statin treatment in a time-dependent manner, but the bile salt hydrolase (K01442), showed no significant alteration (Figure S4D). In addition, we then housed the mice in fully automated metabolic cages to evaluate the energy metabolism after atorvastatin. Atorvastatin substantially reduced the respiratory exchange ratio (RER) (Figures 4F and 4H) and EE (Figures 4G and 4I). This was accompanied with a significant reduction in oxygen consumption (Figure 4J) and carbon dioxide production (Figure 4K), implying atorvastatin promoted fat oxidation and impaired glucose utilization, which caused glucose intolerance.

We then performed the fecal microbiota transplantation experiment by transplanting gut microbiota from patients who had been taking statins for more than 6 months to C57BL/6 mice (Figure S5A). Compared with control, insulin intolerance phenotypes were observed among mice transplanted with gut microbiota from patients with long-term utilization of statin and increased HbA_{1c} (Figures S5B and S5C). In addition, we have also identified decreased UDCA and increased CDCA in the fecal bile acids (Figures S5D–S5F). Interestingly, the statin-related dysglycemia was not identified when mice received high-fiber diet (Figures S5G–S5J), suggesting that statin-induced hyperglycemia could be influenced by metabolic dysfunction.

Colonization of *Clostridium sp.* reversed the glucose intolerance induced by statin in mice

Since fecal microbiota transplantation from patients taking statins induced glucose intolerance, we then tried to identify the specific gut microbiota putatively responsible for regulating the dysglycemia after statin utilization. Taking an untargeted approach, the *Clostridium* genus showed a strong correlation to bile acid metabolism (Figure S3E). In addition, various species of *Clostridium sp.* (*C. sp.*) were identified to be inhibited in patients taking atorvastatin (Figure 3C). To further clarify whether *Clostridium sp.* participated in statin-induced hyperglycemia, transplantation of *C. sp.* to pseudo-germ-free mice was conducted. We gave *C. sp.* (10^8 colony-forming unit [CFU]/mouse) to statin-treated mice on a high-fat diet (HFD) and pseudo-germ-free pretreatment for 4 weeks (Figure 5A). After *C. sp.* colonization (Figures 5B and S6A–S6C), reduced serum GLP-1, glucose intolerance, and insulin resistance induced by atorvastatin were all significantly restored to the levels found in the control animals (Figures 5C–5E). The 7-alpha-HSDH (K00076) was also significantly increased after *C. sp.* colonization (Figure S6D). Fecal profiles of bile acids exhibited significantly increased αMCA and UDCA (Figure 5F), in line with the serum metabolomics results (Figure S6E). In addition, colonization of *C. sp.* increased RER and EE (Figures 5G–5J), indicating that *C. sp.* could promote glucose utilization to improve glucose intolerance. Increased adipose browning factors were also identified in the white adipose tissue in the subcutaneous white adipose tissue of the mice colonized with *C. sp.* (Figure S6F). To identify whether the inhibiting effect of statin on *C. sp.* was dose-dependent, we treated *C. sp.* with atorvastatin at concentrates of 0–500 μM *in vitro* and found that atorvastatin directly inhibited *C. sp.* growth in a dose-dependent manner (Figures 5K and 5L). These results demonstrated that *C. sp.* could rescue statin-related glucose intolerance, enhance bile acid synthesis, and increase carbohydrate oxidation.

UDCA supplementation improved glucose tolerance induced by statin in mice

Since the bile acid metabolomics profile in humans suggested a gradual decrease of UDCA after atorvastatin utilization, we then inquired whether UDCA produced therapeutic effects on statin-induced hyperglycemia. To this end, mice fed with HFD-atorvastatin for 12 weeks were supplied with 50 mg/kg UDCA for 4 weeks (Figure 6A). Oral administration of UDCA increased GLP-1 secretion (Figure 6B) while substantially restoring glucose intolerance and insulin resistance (Figures 6C and 6D). The RER was markedly enhanced in UDCA-treated mice, together with carbon dioxide production and EE during night (Figures 6E–6J). Alteration of the liver bile acid synthesis gene and liver and ileum FXR target gene expression was also identified after UDCA supplementation (Figures S7A–S7C). These results demonstrated that UDCA increased GLP-1 secretion and thus ameliorated insulin resistance.

(B) GLP-1 levels of mouse recipients. n = 5 per group. *$p < 0.05$ as determined by one-way ANOVA; ns, not significant.
(C) Intraperitoneal glucose tolerance test (IPGTT) and area under the curve (AUC). n = 6 per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, as determined by one-way ANOVA; ns, not significant.
(D) Insulin tolerance test (ITT) and AUC. n = 4–5 per group. *$p < 0.05$ as determined by one-way ANOVA; ns, not significant.
(E) Heatmap of *Clostridium* genus and species.
(F and G) Curves of respiratory exchange ratio (RER) (F) and energy expenditure (EE) (G) monitored by metabolic cage in statin (red) and control (black) mice during a 24-h cycle (08:00–08:00) (n = 4/group).
(H–K) AUC of the RER (H), EE (I), oxygen consumption (J), and carbon dioxide proportion (K) in statin and control mice calculated for day, night, and all day. n = 4 per group. *$p < 0.05$, **$p < 0.01$ as determined by simple t test or Mann-Whitney U test. ns, not significant. Data are shown as means ± SEM.

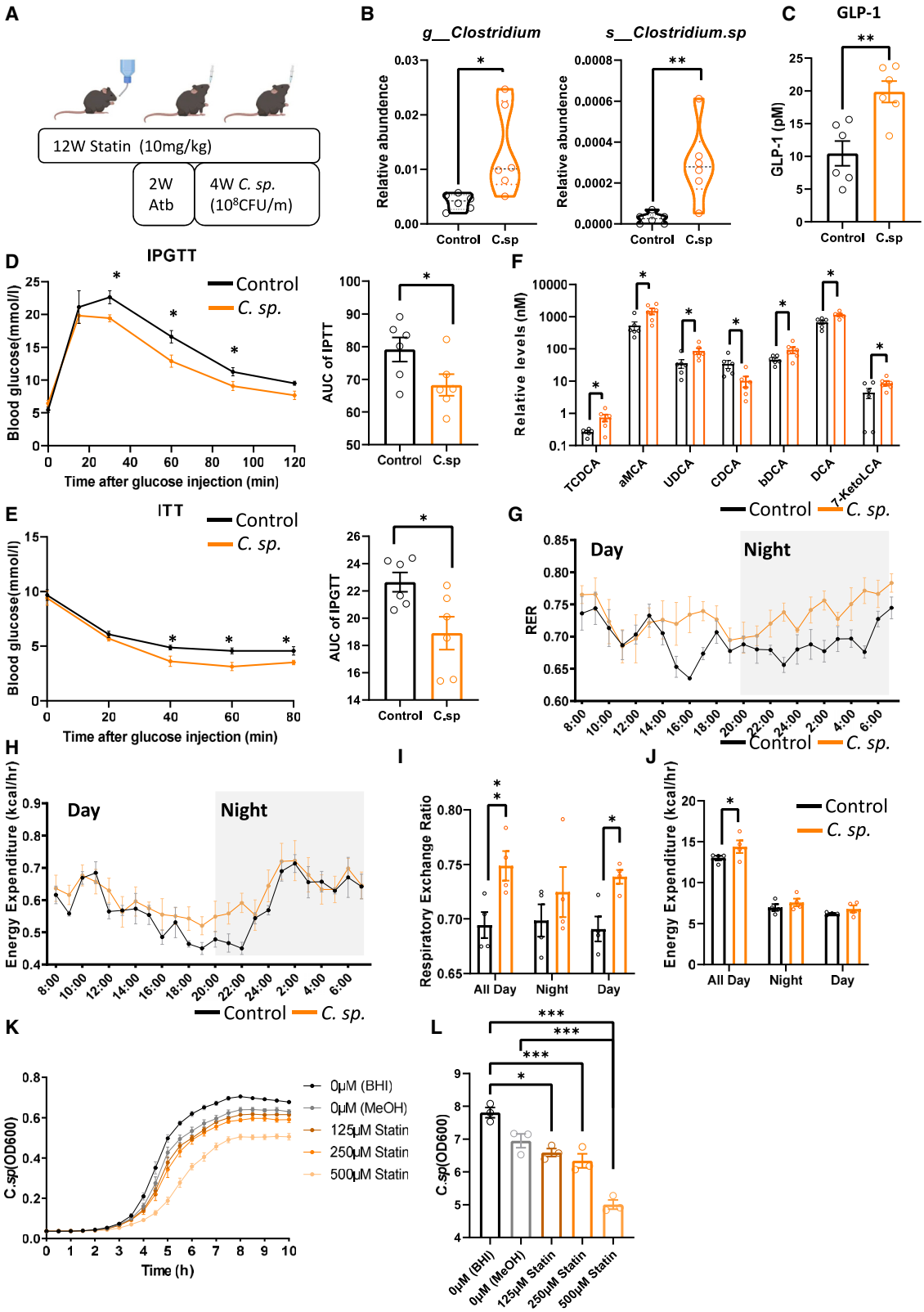

Figure 5. Colonization of *Clostridium sp.* reversed the glucose intolerance induced by statin in mice

(A) Experimental procedure for manipulation of *Clostridium* species in C57 mice supplied with *C. sp.* (10^8 CFU/mouse) or saline, respectively (*C. sp.* and control groups) after 12 weeks atorvastatin and 2 weeks antibiotic cocktail induction.

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UDCA reversed the HbA_{1c} increase in patients utilizing statin without affecting lipid levels

Since UDCA was clinically commonly utilized in liver and gallbladder disease, we then tested whether UDCA treatment had targeted therapeutic effects in glucose intolerance among patients receiving statin treatment. To this end, 5 participants utilizing statin for more than 6 months and having increased HbA_{1c} as compared with 6 months before by screen were recruited. UDCA 500 mg per day (10–13 mg/kg) was administered together with continued statin therapy (Figures 7A and S7D; Table S2). After a 2-month follow-up, the HbA_{1c} levels, as well as the serum insulin, C-peptide, and GLP-1, were substantially decreased individually (Figures 7B and 7C). The levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also lower with ALT reaching significance, indicating that there was no liver injury after the combination therapy (Figure 7D). Serum lipids profile was generally unaltered except that high-density lipoprotein (HDL) showed a significant increase, which was beneficial for lipids regulation (Figure 7E). This suggested that UDCA restored impaired glucose homeostasis without limiting the lipid-lowering effect of statin, indicating the potential therapeutic target that statin and UDCA could be utilized clinically as a compound preparation for dyslipidemia treatment.

DISCUSSION

In this study, we have investigated the gut microbiota and bile acid profile among patients utilizing statin. By comprehensive metagenomics and metabolomics analysis, we have identified a decreased abundance of *Clostridium* upon atorvastatin utilization, which represses fecal CDCA-to-UDCA transformation. Decreased UDCA down-regulates circulating GLP-1 and results in disturbed glucose homeostasis. Animal experiments and clinical validations further indicate that UDCA supplementation is a potentially promising strategy to improve glucose metabolism after statin therapy (Figure 7F). The major contribution of this study is that adjuvant therapy of UDCA in statin treatment could be a potential novel lipid-lowering approach in the future.

Firstly, this study further demonstrates that statin administration could induce glucose intolerance in humans and mice. The human study reveals that after statin administration, glucose homeostasis is disturbed as indicated by increased HbA_{1c}; the gradual increase of insulin and HOMA-ir further suggests insulin resistance. The glucose intolerance observed in clinical cohorts is confirmed by animal experiments following previous publications. Notably, the metabolic cage data show that the RER decreases after statin use, which indicates that the utilization of fat and fatty acid oxidation is increased, leading to glucose accumulation and intolerance. This is also accompanied by a reduction of EE. These findings suggest that statin could cause glucose metabolism disorder. In agreement with our study, a recent study has identified that the gut microbiome composition could potentially modulate the impact of statins influence on off-target physiological effects, particularly with regard to glucose homeostasis.[17] It is noteworthy that, in our study, we have fed the mice with HFD and then administered the statin to induce the dysglycemia phenotype. When we administered statin to mice taking high-fiber diet, we did not identify glucose intolerance. This indicates that the gut microbiota alteration and glucose intolerance induced by statin in the present analysis might be influenced by the obese background.

Our clinical cohort investigation has shown a simultaneous decrease in GLP-1 and UDCA levels following statin treatment. Previous studies have identified bile acids as endogenous signaling molecules that activate the bile acid receptors, FXR and TGR5, to regulate bile acid metabolism and maintain glucose and energy homeostasis.[10,11,23–25] The therapeutic effects of UDCA in liver and metabolic diseases have been reported before. Among those, UDCA has been used clinically to treat cholelithiasis, cholestatic liver disease, fatty liver disease, etc.[26,27] In addition, UDCA acts as a ligand for the TGR5/Gpbar-1 receptor to improve insulin sensitivity by stimulating the secretion of GLP-1.[28,29] It has also been demonstrated previously that UDCA therapy can ameliorate the weight gain and metabolic disorders induced by a HFD.[30,31] In the diet-induced NAFLD mouse model, UDCA supplementation increased Cyp7b1 mRNA expression level and improved fasting blood glucose level and liver steatosis.[32,33] In our animal study, with supplementation of UDCA in statin-treated mice, the altered levels of GLP-1 are rapidly attenuated, together with restored RER. The glucose tolerance and insulin tolerance are also significantly improved. The above results further confirm the improvement effect of UDCA on blood glucose and suggest that UDCA might be a target for improving the glucose intolerance caused by statin.

We have further clarified a causal relationship between *Clostridium* and intestinal UDCA and GLP-1 deficiency after statin utilization. *In vitro*, we have demonstrated that statin could inhibit *Clostridium* growth, which could explain decreased *Clostridium* observed by metagenomics in humans and 16S in mice after statin treatment. Moreover, by the colonization of *C. sp.* in statin-treated mice, glucose and insulin tolerance get improved, as well as RER and oxygen consumption. In addition, UDCA and GLP-1 also increase after *Clostridium* colonization in mice,

(B) *Clostridium* genus and *C. sp.* levels. n = 6 per group. *$p < 0.05$ as determined by simple t test or Mann-Whitney U test.
(C) GLP-1 levels of mouse recipients. n = 6 per group. **$p < 0.01$ as determined by simple t test or Mann-Whitney U test.
(D) Intraperitoneal glucose tolerance test (IPGTT) and area under the curve (AUC). n = 6 per group. *$p < 0.05$ as determined by simple t test or Mann-Whitney U test.
(E) Insulin tolerance test (ITT) and AUC. n = 6 per group. *$p < 0.05$ as determined by simple t test or Mann-Whitney U test.
(F) Significantly altered fecal bile acids after *C. sp.* colonization. n = 6 per group. *$p < 0.05$ as determined by simple t test or Mann-Whitney U test.
(G and H) Curves of respiratory exchange ratio (RER) (G) and energy expenditure (EE) (H) monitored by metabolic cage in *C. sp.* (yellow) and control (black) mice during a 24-h cycle (08:00–08:00).
(I and J) AUC of the RER (I) and EE (J) in *C. sp.* and control mice calculated for day, night, and all day. n = 4 per group. *$p < 0.05$, **$p < 0.01$ as determined by simple t test or Mann-Whitney U test.
(K) Growth curves of *C. sp.* after control and 125, 250, and 500 μM statin colonization (n = 3).
(L) AUC of (K). *$p < 0.05$, ***$p < 0.001$, as determined by one-way ANOVA. Data are shown as means ± SEM.

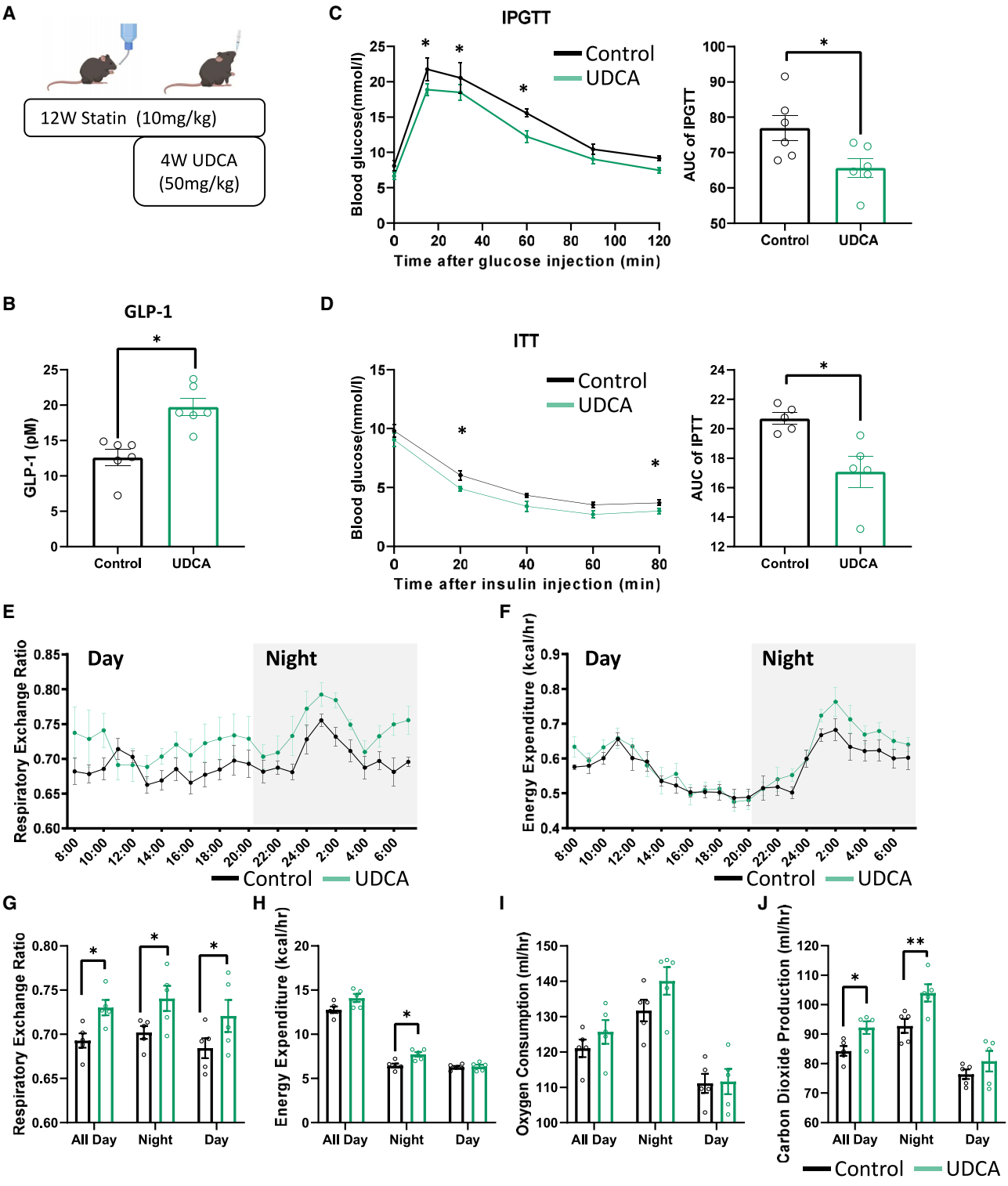

Figure 6. UDCA supplementation improved glucose tolerance induced by statin in mice

(A) Experimental procedure for UDCA or control treatment in mice after 12-week atorvastatin induction.

(B) GLP-1 levels of mouse recipients. n = 6 per group. *p < 0.05 as determined by simple t test or Mann-Whitney U test.

(C) Intraperitoneal glucose tolerance test (IPGTT) and area under the curve (AUC). n = 6 per group. *p < 0.05 as determined by simple t test or Mann-Whitney U test.

(D) Insulin tolerance test (ITT) and AUC. n = 5 per group. *p < 0.05 as determined by simple t test or Mann-Whitney U test.

(legend continued on next page)

suggesting decreased gut *Clostridium* contributes to the decreased UDCA and GLP-1.

We have applied metagenomics analysis and further confirmed reduced abundance of HSDHs, which are key biocatalysts for the biotransformation of UDCA from CDCA.[22,34,35] The CDCA/UDCA ratio gradually increased after statin initiation among the human cohort. This result partially provides evidence that the down-inhibition of the gut microbiota expressing HSDHs by statin may cause the decrease of UDCA and lead to dysregulated glucose metabolism. In turn, the change of bile acid composition mediated by gut microbiota might affect the bile acid signaling pathway and restore the glucose homeostasis.

Our results provide evidence that targeting the UDCA-*Clostridium* axis provides protective benefits against the glucose intolerance induced by statin therapy. Since UDCA has been a commonly used drug for treating liver disease patients, it is reasonable to expect that the combined UDCA and statin therapy could become the new standard of care for lipid-lowering therapy. In our clinical investigation, we found that UDCA administration of patients on statin treatment significantly improved insulin resistance markers over a 2-month intervention window. Of note, the combination treatment did not affect the lipid-lowering effects by statin and significantly reduced the serum ALT levels. The long-term cardiovascular benefits of this treatment need to be further confirmed by large-scale multicenter clinical studies.

Limitations of the study

This study has several limitations. Firstly, 16S amplicon sequencing, and not shotgun metagenomic sequencing, was used for the gut microbiota analysis in mice. Secondly, the relationship between the *Clostridium* and UDCA-related phenotypes has been insufficiently validated in patients; we would expect more sufficient validations in the future if we could realize the gut microbial modulation in patients taking statin. Lastly, among the cohort taking atorvastatin, 20% of participants have a diabetes history, and 10% of participants have a metformin medication history. The previous disease and medication history might cause deviation in the gut microbiota analysis. However, the proposed mechanism was further validated in follow-up mouse and human experiments.

Conclusions

In conclusion, this study demonstrates the changes in the gut microbiota and metabolomics profile among patients and mice receiving statin. We have revealed for the first time that statin causes glucose homeostasis dysregulation through affecting *Clostridium sp.*-bile acids axis to reduce serum UDCA and GLP-1. Adjuvant therapy of UDCA in statin treatment is a potential novel lipid-lowering strategy in the future.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cmet.2023.12.027.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Y.W., Z.Y., and J.Y. conceptualized and designed this study. J.S., G.T., J.L., and N.L. performed the experiments. J.S., G.T., M.G., J.L., X.H., L.G., W.X., Y.Z., F.G., and Y.X. collected and analyzed data. J.S., G.T., T.Z., B.D., B.L., X.G., X.Y., X. Zhuo., Y.Y., X. Zhang., and Y.W. did the revision work. J.S., Y.W., and G.T. wrote the manuscript with input from all of the authors. Y.W., Z.Y., and J.Y. reviewed and edited this manuscript. All of the authors approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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(E and F) Curves of respiratory exchange ratio (RER) (E) and energy expenditure (EE) (F) monitored by metabolic cage in UDCA (green) and control (black) mice during a 24-h cycle (08:00–08:00) (n = 4–5/group).
(G–J) AUC of the RER (G), EE (H), Oxygen consumption (I), and carbon dioxide proportion (J) in UDCA (green) and control (black) mice calculated for day, night, and all day. n = 4 per group. *p < 0.05, **p < 0.01 as determined by simple t test or Mann-Whitney U test. Data are shown as means ± SEM.

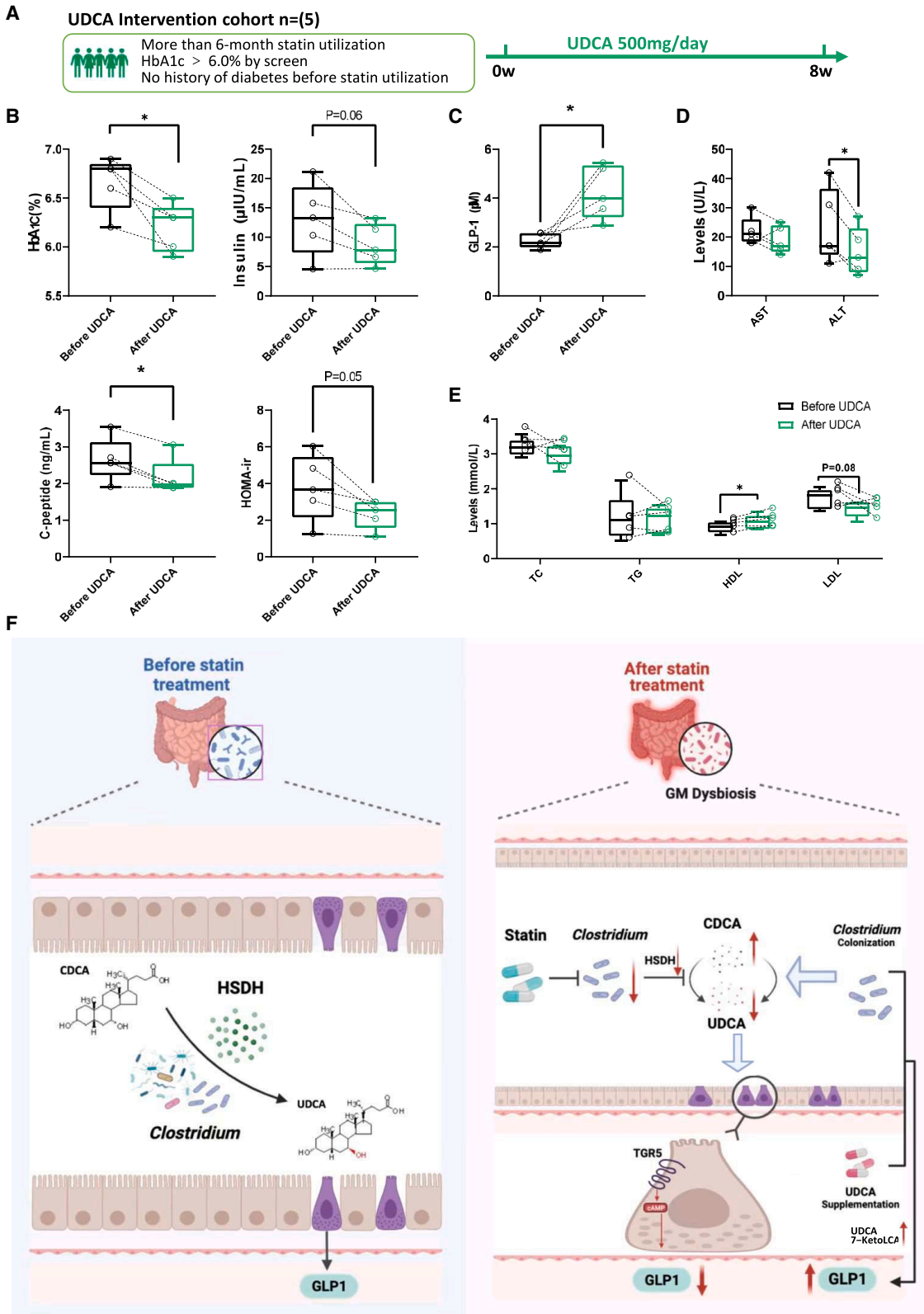

Figure 7. UDCA reversed the HbA_{1c} increase in patients utilizing statin without affecting lipid levels

(A) Clinical study flow chart of patients who received statin therapy and showed glucose intolerance taking UDCA (n = 5).

(B) Levels of HbA_{1c}, insulin, C-peptide, and HOMA-ir of the patients before and 2 months after taking UDCA.

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(C and D) Levels of GLP-1 (C), AST, and ALT (D) of the patients before and 2 months after taking UDCA.
(E) Levels of TC, TG, HDL, and LDL of the patients before and 2 months after taking UDCA. *$p < 0.05$ as determined by simple t test or Mann-Whitney U test. Data are shown as means ± SEM.
(F) Schematic diagram of a potential mechanism by which the atorvastatin decreases the *Clostridium*-rich microbiota and UDCA levels and impairs glucose homeostasis via lowering GLP-1.
See also Methods S1.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Clostridium.sp	Isolated from the feces of the participants	N/A
Biological samples		
Serum from humans	This paper	N/A
Serum from mice	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Ursodeoxycholic acid	MCE, Losan Pharma GmbH	Cat#HY-13771; CAS: 128-13-2
Sitagliptin	MCE	Cat#MK0431; CAS: 486460-32-6
Corn oil	MCE	Cat#HY-Y1888; CAS: 8001-30-7
Dimethyl sulfoxide	MCE	Cat#HY-Y0320; CAS: 67-68-5
Glucose	Baxter Healthcare	H9994062
Biosynthetic Human Insulin Injection	Novo Nordisk	J20171005
Atorvastatin	Pfizer	N/A
Critical commercial assays		
GLP-1 Active ELISA Kit	FineTest	EM1076
RNA Extraction Kit	Takara	9767
PrimeScript RT Master Mix	Takara	RR036A
FastStart Essential DNA Green Master	Roche	06402712001
GLP-1 Active ELISA Kit	FineTest	EM1076
Experimental models: Organisms/strains		
C57BL/6J	Beijing Vital River Laboratory Animal Technology Co., Ltd..	N/A
Oligonucleotides		
β-actin-FOR: ACCTGACAGACTACCT CATGAAGA	Tsingke Biotech	N/A
β-actin-REV: TCATGGATGCCACAG GATTCCATA	Tsingke Biotech	N/A
Fxr-FOR: TGGGCTCCGAATCCTCTTAGA	Tsingke Biotech	N/A
Fxr-REV: TGGTCCTCAAATAAGATCC TTGG	Tsingke Biotech	N/A
Shp-FOR: TCTGCAGGTCGTCCGAC TATTC	Tsingke Biotech	N/A
Shp-REV: AGGCAGTGGCTGTGAGATGC	Tsingke Biotech	N/A
Fgf15-FOR: GCCATCAAGGACGTCAGCA	Tsingke Biotech	N/A
Fgf15-REV: CTTCCTCCGAGTAGCG AATCAG	Tsingke Biotech	N/A
TGR5-FOR: TCCTGTCAGTCTTGGC CTATGA	Tsingke Biotech	N/A
TGR5-REV: GGTGCTGCCCAATGAGATG	Tsingke Biotech	N/A
CYP7A1-FOR:AGCAACTAAACAACCT GCCAGTACTA	Tsingke Biotech	N/A
CYP7A1-REV: GTCCGGATATTCAAG GATGCA	Tsingke Biotech	N/A
CYP27A1-FOR: GCCTCACCTATGGG ATCTTCA	Tsingke Biotech	N/A
CYP27A1-REV: TCAAAGCCTGACGCAGATG	Tsingke Biotech	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CYP8B1-FOR: GGCTGGCTTCCTGA GCTTATT	Tsingke Biotech	N/A
CYP8B1-REV: ACTTCCTGAACAGCT CATCGG	Tsingke Biotech	N/A
CYP7B1-FOR: TAGCCCTCTTTCCTC CACTCATA	Tsingke Biotech	N/A
CYP7B1-REV: GAACCGATCGAACCTA AATTCCT	Tsingke Biotech	N/A
ASBT-FOR: GTGGGCTTCCTCTGTCAGTT	Tsingke Biotech	N/A
ASBT-REV: GCATCATTCCAAGGGCAAGC	Tsingke Biotech	N/A
BSEP-FOR: ACGAAAGGCCACTG ACACTAC	Tsingke Biotech	N/A
BSEP-REV: GCGCACACACTTCCC ATAAAC	Tsingke Biotech	N/A
OSTα-FOR: CTGAAGGACACCCC GATGAG	Tsingke Biotech	N/A
OSTα-REV: CCTGGGTCATAGATGCCGTC	Tsingke Biotech	N/A
OSTβ-FOR: ATCGAAAGAAGCAGC CACAAG	Tsingke Biotech	N/A
OSTβ-REV: ATGGGGTACTCTCAACGCTC	Tsingke Biotech	N/A
Ucp1-FOR: GCCTCTACGACTCAGTCCAA	Tsingke Biotech	N/A
Ucp1-REV: CATTAGATTAGGGGTCGTCC	Tsingke Biotech	N/A
Elovl3-FOR: TTCTCACGCGGGTTA AAAATGG	Tsingke Biotech	N/A
Elovl3-REV: GAGCAACAGATAGA CGACCAC	Tsingke Biotech	N/A
Cox8b-FOR: GAACCATGAAGCCAAC GACT	Tsingke Biotech	N/A
Cox8b-REV: GCGAAGTTCACAGTGG TTCC	Tsingke Biotech	N/A
Tmem26-FOR: ATGGTGCATTTCAAGA AGCC	Tsingke Biotech	N/A
Tmem26-REV: GCTCACCCTCAAGT TCAAGC	Tsingke Biotech	N/A
Prdm16-FOR: CCACCAGCGAGGAC TTCAC	Tsingke Biotech	N/A
Prdm16-REV: GGAGGACTCTCGTA GCTCGAA	Tsingke Biotech	N/A
Pgc1a-FOR: TATGGAGTGACATAGA GTGTGCT	Tsingke Biotech	N/A
Pgc1a-REV: CCACTTCAATCCACCC AGAAAG	Tsingke Biotech	N/A
Deposited data		
Demographics, clinical, and metabolomics data	This paper	Tables S1-1, S1-2, and S2
Software and algorithms		
GraphPad Prism 9 software (GraphPad)	http://www.graphpad.com/	N/A
R project (version 3.5.3)	https://cran.r-project.org/	N/A
QIIME 2 version 2019.10	https://qiime2.org	N/A
IBM SPSS software (V22.0)	https://www.ibm.com/analytics/spss-statistics-software	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Diet	Research Diets	D12108C
Metabolic cage system	Sable promethion core system	N/A
Glucose meter	Roche	ACCU-CHEK Performa
Liquid chromatography	Eclipse	XDB-C18

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yue Wu (yue.wu@xjtu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The datasets generated in this study are available from the lead contact.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Recruitment of Patients

For the atorvastatin cohort, all participants were enrolled from the First Affiliated Hospital of Xi'an Jiaotong University, China. 30 participants taking atorvastatin (Pfizer™) upon enrollment and 10 controls were recruited for the cohort study (Figure 1A). The participants were selected during community medical examination. The inclusion criteria were as follows: patients aged 18-80 yrs.; will to participate; diagnosed of dyslipidemia or atherosclerotic disease, which were evaluated by cardiovascular specialists. The indications for statin initiation included dyslipidemia and atherosclerotic disease. 10 participants who refused statin therapy upon identification of dyslipidemia were enrolled for the control group. The subjects were excluded if they: had been taking statin during the past 6 months; had chronic endocrine diseases, renal failure, or acute or chronic hepatitis with increased transaminase activities; had malignant tumor; or refused to participant in the study. Serum and fecal samples were collected from all patients recruited. None of the patients were lost during follow-up. However, the fecal samples were achieved in 30, 26, 28, and 28 participants during the week 0, 1, 4, and 16 respectively. Group sample sizes of 30 in the treatment group and 10 in the control group achieve 80.0% power to detect a difference between the group proportions of 20. The proportion in the treatment group is assumed to be 0.01 under the null hypothesis and 0.22 under the alternative hypothesis according to previous clinical studies and observations with regard to glucose intolerance after statin treatment.[5–8] The significance level of the test is 0.05.

For the UDCA supplement study, we used the pilot data from the registered ongoing clinical trial (Effect of UDCA on the New Onset Diabetes and Glucose Intolerance Induced by Statin, XJTU1AF2018LSK-92, NCT05500937). The interim analysis of the study was outlined in the trial protocol. 5 participants were recruited when they met the following criteria: had more than 6-month statin utilization; $HbA_{1c} > 6.0\%$ by screen; no history of diabetes before statin utilization; had no renal, liver or other chronic disease; had no malignancy (Table S2). UDCA (Losan Pharma GmbH™) 500mg per day was given beside the routine statin utilization. HbA_{1c}, liver function, and circulating lipids were evaluated after 2-month follow-up. A daily 500mg/day dose of UDCA was administered to the patients according to the Chinese Pharmacopoeia and recommendations.[36] (Figures 7A and S14.) Because no clinical trials of statin and UDCA as a combination therapy had been conducted previously, no sample size calculation related to the pilot data was performed.

Written informed consent was obtained according to the Declaration of Helsinki, and was approved by the ethics committee, Xi'an Jiaotong University. Both clinical investigations were registered on the ClinicalTrials.gov (NCT04215237, NCT05500937).

Mouse models

Experimental protocols and procedures in our study conformed to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and local guidelines of the Laboratory Animals Center at Xi'an Jiaotong University, Xi'an Shaanxi, China. The manipulation of animals was performed in the Animal Center of Xi'an Jiaotong University. The animal protocol number is XJTU2018252. All mice were male and maintained in Specific Pathogen Free (SPF) environment. Mice aged 8 weeks were used

and were maintained under a 12 h light/12 h dark cycle at 23 ± 1°C and humidity (45–55%) with free access to food and water. Adult male C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd..

METHOD DETAILS

Metagenomic sequencing and analysis

Stool samples from study participants and mice were collected and stored at −80°C until further processing. DNA concentration was measured using Qubit® dsDNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies™). A total amount of 1μg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform and paired-end reads were generated. For metagenomic sequencing and analysis, adaptor contamination and low-quality reads were discarded from the raw sequencing reads, and the remaining reads were filtered to eliminate human host DNA based on the human genome reference (hg19). All high-quality reads from each sample were assembled to contigs by using metaSPAdes from SPAdes v3.10.0 package with default settings, and a nonredundant gene catalog was constructed by MetaGeneMark and CD-HIT. Gene profile was calculated according to the high-speed quantification tool Kallisto and normalized by gene length. Genes were annotated by blasting against the NCBI database using Diamond. The taxonomic composition was acquired by MEGAN and KO composition obtained using the KEGG Orthology-Based Annotation System (KOBAS) tool. The sum of relative abundance of all genes involved in a KEGG pathway was defined to be pathway abundance. Gene counts were calculated by counting the number of genes in each sample. Alpha diversity was performed on the basis of the gene profile of each sample according to the Shannon index.

16s RNA sequencing

The abundance and diversity of intestinal flora in mice were determined using Illumina HiSeq sequencing (Novogene, Beijing, China), after amplification and purification of the V3-V4 region of bacterial 16s rRNA genes. The QIIME software package was used to conduct the bioinformatic analyses of the sequences. QIIME was used to carry out alpha, beta diversity and Principal Coordinates Analysis depending on the unweighted unifrac distances as previously described.[18]

Untargeted metabolomics and bile acids analysis

Quantitation of serum and fecal metabolites were performed by Metabo-Profile Inc. (Shanghai, China) using the previously published methods with minor modifications.[11,37,38] Briefly, the untargeted metabolomics was performed on XploreMET platform-based Agilent 7890B gas chromatograph. The bile acids spectrum in the study samples was analyzed with the bile acids detection kit BAP ultra. Sample detection was based on ultra-high performance liquid chromatography tandem mass spectrometry (uplcms/MS). The raw data files exported by UPLC-MS/MS were processed in QuanMET (v1.0, Metabo profile, Shanghai, China) to obtain quantitative metabolomics data and achieve actual concentrations by comparing metabolites in samples of unknown concentrations with a set of standard samples (Quantitative curves).

GLP-1 detection

For clinical samples, blood samples from human subjects were collected after overnight fast. For GLP-1 measurement, DPP-4 inhibitor was added to the blood immediately after collection, and the blood was kept on ice prior to centrifugation. Serum active GLP-1 levels were measured by an active GLP-1 assay kit (EZGLPHS-35K, Millipore) as previously described. For in vivo studies, mice were fasted for 12 hours and gavage with DPP-4 inhibitor 1 hour before sacrifice, sitagliptin (MCE, MK0431, 3mg/kg) dissolved in 10% DMSO and 90% corn oil, and then serum samples were collected. All samples were stored in -80°C until analysis. Glp-1 peptide analyses were performed by using GLP-1 ELISA Kit (FineTest, EM1076) according to the manufacturer's instructions.

Mouse Experiments

In the atorvastatin intervention study, 12-week-old-mice were acclimated for two weeks. Mice were divided into four groups randomly according to whether atorvastatin or antibiotics were given or not. All mice were treated with high-fat diet (HFD) (Research Diets, D12108C, USA). Control group were treated with HFD and distilled water. Atorvastatin group were treated with HFD and atorvastatin (10mg/kg) dissolved in distilled water. Antibiotics group were treated with HFD and antibiotics cocktail dissolved in sterilized water. Atorvastatin plus antibiotics group were treated with HFD and atorvastatin (10mg/kg) plus antibiotics cocktail dissolved in sterilized water. After adaption for environmental changes, HFD was given until sacrifice. After 3-week HFD treatment, 17-week-old-mice were treated with atorvastatin or 2-week antibiotics cocktail (ampicillin 1g/L in drinking water, metronidazole 100mg/kg, neomycin 100mg/kg and vancomycin 50mg/kg) or both. After 12 weeks treatment, we performed metabolic tolerance tests (IPGTT and IPTT) within one-week interval.

In the *C. sp.* colonization study, mice fed with HFD-atorvastatin for 12 weeks were supplied with *C. sp.* (10^8CFU/mouse) or saline respectively (*C. sp.* and control groups) twice a week for 4 weeks by gavage after 2-week antibiotics cocktail treatment. After 4 weeks treatment, we performed metabolic tolerance tests (IPGTT and IPTT) within one-week interval.

In the UDCA treatment study, mice fed HFD-atorvastatin for 12 weeks were supplied with 50mg/kg UDCA or saline respectively (UDCA and control groups) for 4 weeks by gavage. After 4 weeks treatment, we performed metabolic tolerance tests (IPGTT and IPTT) within one-week interval.

In the fecal microbiota transplantation experiment, gut microbiome from patients with more than 6-month utilization of statin with increased HbA_{1c} was transplanted to high fat diet-fed C57BL/6 mice. Gut microbiome from patients without previous statin utilization was collected as control. The gut microbiota was transplanted to HFD diet mice after antibiotic treatment. IPGTT and ITT were performed after 8 weeks of transplantation.

Metabolic Tolerance Tests

Glucose tolerance tests (IPGTT) were performed after fasting 12 hours by using glucose meter (Roche, ACCU-CHEK Performa) and compatible Accu-Chek Performa test strips. After intraperitoneal injection of glucose (2 g/kg body weight), blood samples were collected from the tail at 0, 15, 30, 60, 90, 120 minutes.

Insulin tolerance tests (IPTT) were performed after fasting for 2 hours. Insulin (0.6 U/kg body weight) was injected intraperitoneally, blood samples were collected from the tail at 0, 20, 40, 60, 80 minutes.

C. sp. culture, verification and growth curve

C. sp. culture and verification could be referred to previous publications.[39–41] Briefly, *C. sp.* were isolated from the feces of participants. Single *C. sp.* colonies were selected, and liquid medium was scaled up. 16s sequencing was then tested for *C. sp.* strains after enlargement, and 7a-HDSH activity was performed to determine specific strains. Mice were given $1x10^8$ CFU *C. sp.* or PBS by gavage twice a week. For the growth curve study, *C. sp.* was cultured in anaerobic tubes in the presence or absence of atorvastatin (125 μM, 250 μM and 500 μM) and OD600 value was measured hourly until platform periods.

Determination of the concentration of atorvastatin

Dilute the solution of atorvastatin with methanol gradient to prepare concentrations of 3.906, 1.953, 0.976, 0.488, and 0.244 μg/mL of atorvastatin respectively. Eclipse XDB-C18 high performance liquid chromatography system was utilized to establish a standard curve by comparing the HPLC peak area and corresponding concentration of atorvastatin standards with different concentrations. For sample concentration determination, dissolve the sample in 300ul of sterile water and centrifuge at 12000 rpm to obtain the supernatant for later use. 100 μL sample with an equal volume of methanol was taken, and the supernatant was detected by HPLC after membrane filtration. Calculate the corresponding concentration using the established standard curve equation.

Metabolic cage

Indirect calorimetry including O_2 consumption, CO_2 production, RER, and energy expenditure, were performed by using automatic metabolic cages (Sable promethion core system, USA). Mice were individually monitored for 3 days after acclimatizing for 2 days. Data were collected in a time interval of 5 mins.

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism version 9.0 (GraphPad Software) and SPSS version 22.0 were used for statistical analysis. Data were presented as frequencies or percentages for categorical variables and mean±SEM. for continuous variables, unless otherwise indicated. One-way ANOVA was used to compare continuous variables of three or more independent (unrelated) groups. Two-way analysis of variance (ANOVA) followed by Holm-Sidak test was used to evaluate multiple comparisons among three or more groups. Simple t-test was used to compare continuous variables between two group which were in the normal distribution; Mann-Whitney U test or Wilcoxon matched-pairs signed rank test was used to compare the non-normally distributed continuous data between two groups. Correlation analysis was investigated by Spearman's test. KEGG Enrichment analysis was performed using R 3.5.1 and iPath 3 (https://pathways.embl.de/). Advanced Diff Redundancy analysis and LEfSe was performed using the OmicStudio tools at https://www.omicstudio.cn/tool. Data were shown as means ± s.e.m. $P<0.05$ was considered statistically significant. Statistical parameters can be found in the respective figure legends.

ADDITIONAL RESOURCES

Clinical investigations were registered on the ClinicalTrials.gov (NCT04215237, https://classic.clinicaltrials.gov/ct2/show/NCT04215237; NCT05500937, https://classic.clinicaltrials.gov/ct2/show/NCT05500937).