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Research Paper

Localized increases in CEPT1 and ATGL elevate plasmalogen phosphatidylcholines in HDLs contributing to atheroprotective lipid profiles in hyperglycemic GCK-MODY

Xiaojing Wang [a,1], Sin Man Lam [b,c,1], Mingjun Cao [b,d,1], Tong Wang [a], Zhixin Wang [a], Miao Yu [a], Bowen Li [c], Huabing Zhang [a], Fan Ping [a], Guangyao Song [e], Kai Feng [a], Qian Zhang [a], Jianping Xu [a], Liyuan Zhou [a], Mingqun Deng [a], Xiao Zhai [a], Xinhua Xiao [a,*], Guanghou Shui [b,d,**]

[a] Department of Endocrinology, NHC Key Laboratory of Endocrinology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100730, People's Republic of China
[b] State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, 100101, People's Republic of China
[c] Lipidall Technologies Company Limited, Changzhou, 213022, Jiangsu Province, People's Republic of China
[d] University of Chinese Academy of Sciences, Beijing, People's Republic of China
[e] Hebei Key Laboratory of Metabolic Diseases, Hebei General Hospital, Shijiazhuang, Hebei, 050051, People's Republic of China

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ABSTRACT

Glucokinase-maturity onset diabetes of the young (GCK-MODY) represents a rare genetic disorder due to mutation in the glucokinase (*GCK*) gene. The low incidence of vascular complications in GCK-MODY makes it a natural paradigm for interrogating molecular mechanisms promoting vascular health under prolonged hyperglycemia. Clinical rate of misdiagnosis has remained high, and a reliable serum lipid biomarker that precedes genetic screening can facilitate correct diagnosis and treatment. Herein, we comprehensively quantitated 565 serum lipids from 25 classes in 105 subjects (42 nondiabetic controls, 30 GC K-MODY patients, 33 drug-naïve, and newly-onset T2D patients). At false-discovery rate (FDR) < 0.05, several phosphatidylcholines (PCs) and plasmalogen PCs were specifically increased in GCK-MODY, while triacylglycerols (TAGs) and diacylglycerols (DAGs) were reduced. Correlation matrices between lipids uncovered coregulation between plasmalogen PCs (PCps) and glycerolipid precursors was distinctly enhanced in GCK-MODY compared to T2D. Strengthened positive correlations between serum PCps and circulating HDLs was specifically observed in hyperglycemic subjects (i.e. T2D and GCK-MODY) compared to normglycemic controls, suggesting that HDL-PCps may elicit distinct physiological effects under hyperglycemia. Amongst GCK-MODY patients, individuals harboring variants of *GCK* mutations with elevated PCps also exhibited higher HDLs. Isolated HDLs displayed localized increases ($p < 0.05$) in very-long-chain PUFA-PCs and PCps in GCK-MODY. Protein analyses revealed elevated levels of HDL-resident ATGL ($P = 0.003$) and CEPT1 ($P < 0.0001$), which mediate critical steps of PCps production along the TAG-DAG-PC axis, in GCK-MODY relative to T2D. A panel of four lipids differentiated GCK-MODY from T2D with AUC of 0.950 (95% CI 0.903–9.997). This study provides the first evidence that enhanced recruitment of CEPT1 and ATGL onto HDLs essentially underlie the atheroprotective profiles associated with GCK-MODY. Resultant increases in the production of HDL-PCps and PUFA-PCs provides an active, circulating form of protection towards the vasculature of GCK-MODY, thereby lowering the incidence of vascular complications despite chronic exposure to hyperglycemia since birth.

* Corresponding author. Department of Endocrinology of Chinese Academy of Medical Sciences, Peking Union Medical College Hospital, Chinese Research Hospitals Society of Diabetes Association, China Association for the Prevention and Treatment of Diabetes, Shuai Fu Yuan No.1, Dong Cheng District, Beijing, China.
** Corresponding author. Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Center for Advanced Technologies, No 1. West Beichen Road, Chaoyang District, Beijing, 100101, China.
E-mail addresses: xiaoxh2014@vip.163.com (X. Xiao), ghshui@genetics.ac.cn (G. Shui).
[1] Xiaojing Wang, Sin Man Lam and Mingjun Cao contributed equally to this work.

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1. Introduction

Glucokinase-maturity onset diabetes of the young (GCK-MODY), also known as MODY2, is a rare genetic disorder that results from inactivating heterozygous mutation in the *GCK* gene [1]. GCK-MODY represents a discrete genetic subgroup characterized by mild, asymptomatic fasting hyperglycemia, which is present from birth and shows only moderate deterioration with age. Generally, fasting hyperglycemia in GCK-MODY does not require treatment outside of pregnancy [1,2]. Thus, it is essential to correctly differentiate GCK-MODY from other types of diabetes to avoid unnecessary treatment [3]. GCK-MODY is often clinically misdiagnosed as type 1 diabetes (T1D) in children or adolescents, while individuals diagnosed later in life may be misclassified as type 2 diabetes (T2D), and incorrectly treated with insulin or oral hypoglycemic agents (OHAs) [4]. In China, clinical misdiagnosis for GCK-MODY was reported at 85.4%, with 58.2% of these patients receiving incorrect treatment [4]. While genetic testing remains a gold standard for diagnosis of GCK-MODY, technical and financial constraints preclude its clinical implementation on a large scale [5]. Developing a good biomarker screening that precedes genetic testing, therefore, is expected to increase the cost-effectiveness of genetic testing for GCK-MODY [5]. .

Apart from mild hyperglycemia, GCK-MODY patients possess normal incretin effect and the regulation of glucagon secretion is preserved [6]. Comparing GCK-MODY to other types of diabetes can potentially confer mechanistic insights contributing to an overall maintenance of metabolic health and protection from pathological complications under hyperglycemia. For example, by comparing insulin sensitivity in GCK-MODY and type 1 diabetes subjects, Moore and colleagues found that iatrogenic hyperinsulinemia, but not hyperglycemia, is a principal driver for insulin resistance in type 1 diabetes [7]. Another study revealed fundamental differences in gut microbiota composition between GCK-MODY and type 1 diabetes, and that an elevated abundance of *Prevotella* in GCK-MODY that may serve to improve glycemic control via regulating intestinal gluconeogenesis [8]. A distinct clinical feature of GCK-MODY is the low occurrence of microvascular and macrovascular complications, which is almost comparable to nondiabetic healthy individuals [1,9]. Cardiovascular complications have emerged as the major cause of mortality in diabetic individuals [10]. GCK-MODY therefore denotes a useful paradigm for interrogating molecular mechanisms beneficial towards vascular health in hyperglycemic individuals. Such mechanisms are expected to contribute towards the ultimate treatment goal of cardiovascular protection in diabetes. While epidemiological studies indicated that elevated glycated hemoglobin (HbA1c) is associated with occurrence of vascular ischemia, interventional studies shown that alleviating hyperglycemia has variable outcomes in lowering vascular complications [11,12]. GCK-MODY individuals were shown to be metabolically normal, with lower levels of serum triacylglycerols (TAGs) than healthy subjects [13]. A comprehensive lipidomic evaluation that defines disease-specific lipid alterations with sufficient resolution to render identification of pathway perturbations, however, is still largely lacking for GCK-MODY. Indeed, collective evidence suggest that circulating lipids possess distinct effects on microvascular complications in diabetes [14]. In particular, GCK-MODY is characterized by strongly cardioprotective lipid profiles of high-density lipoproteins (HDLs) subpopulations, with significantly elevated levels of large HDLs and lower levels of intermediate and small (potentially atherogenic) HDLs compared to controls [15]. Our previous work also shown GCK-MODY exhibited lower total cholesterol (TC) and low-density lipoprotein cholesterol compared to T1D [16]. A lipid-centric investigation of serum metabolic alterations accompanying perturbed glucose homeostasis in GCK-MODY, in comparison to type 2 diabetes, may thus reveal new therapeutic targets of vascular protection under concurrent hyperglycemia.

Herein, we conducted an extensive targeted lipidomic analysis optimized in-house for human serum [17,18] to systematically and quantitatively compare differences in lipidomes between GCK-MODY, T2D and healthy controls. Our aims are (1) to explore a serum lipid biomarker panel that could facilitate genetic screening for GCK-MODY and (2) to investigate lipid pathway changes downstream of *GCK* mutation that may serve to mitigate vascular complications under hyperglycemia by comparing the lipidome differences between GCK-MODY and type 2 diabetes. Our serum-based lipidomics underscores HDL-localized lipids and proteins contributing to the atheroprotective lipid profiles in GCK-MODY. We observed HDL-localized increases in the levels of adipose triglyceride lipase (ATGL) and choline/ethanolamine phosphotransferase 1 (CEPT1) proteins in GCK-MODY, possibly leading to enhanced levels of plasmalogen phosphatidylcholines (PCps) per HDL particle that serve to confer vascular protection against chronic exposure to hyperglycemia since birth.

2. Methods

2.1. Study population

The study cohort comprises 105 subjects, which includes 30 GC K-MODY, 33 newly diagnosed drug-naïve T2D patients and 42 healthy controls. GCK-MODY and T2D patients were recruited from the outpatient clinic of the endocrinology department at the Peking Union Medical College Hospital (PUMCH), Beijing, China, between January 2017 and July 2018. Inclusion criteria of GCK-MODY were as follows: (1) current age $\geq$ 18 years and age at onset of diabetes $\leq$ 45 years; (2) absence of pancreatic islet autoantibodies; (3) family history of diabetes in at least two generations with autosomal dominant mode of inheritance; (4) BMI $\leq$28 kg/m^2; (5) the *GCK* gene mutation were verified by Sanger sequencing and genetic analysis (the Blasted reference sequences were NM_000162.3) (Supplemental Table 1); (6) not receiving antidiabetic treatment. The GCK-MODY subjects were recruited across China and regularly followed up in PUMCH outpatient clinic (Supplemental Fig. 1). To match GCK-MODY better, eligible T2D patients were newly diagnosed (according to the WHO 1999 diagnostic criteria for diabetes) and no medications were taken at the time of recruitment and blood collection. Patients were excluded if they had positive islet antibodies, a fasting C-peptide level $\leq$0.6 ng/ml, serum creatinine and alanine aminotransferase levels greater than the upper limit of the normal range and other complicating diseases. The study protocol was approved by institutional review board of the PUMCH. Written informed consent was provided by all participants. Table 1 summarizes the clinical characteristics of the recruited subjects.

2.2. Lipidomics analysis

Lipids were extracted from 20 μL of serum according to a modified Bligh and Dyer's extraction protocol (double rounds of extraction). Organic extracts were dried in SpeedVac under OH mode and resuspended in chloroform: methanol (1:1, v/v) prior to analyses. Targeted lipidomics analyses were conducted on an Exion UPLC coupled to QTRAP 6500 Plus (Sciex) as described recently [18,19]. Individual lipids were quantitated relative to their respective internal standards, comprising 32 compounds including PC14:0/14:0, d_{31}-PC16:0/18:1, PE14:0/14:0, d_{31}-PE-16:0/18:1, d_{31}-PS-16:0/18:1, PA17:0/17:0, PG14:0/14:0, d_{31}-PG16:0/18:1, C14:0-BMP, d_{31}-PI-16:0/18:1, S1P-d17:1, Sph-d17:1, SM-d18:1/12:0, d_{31}-SM-16:0/18:1, LPC-17:0, LPE-17:1, LPI-17:1, LPA-17:0, LPS-17:1, Cer d18:1/d_7-15:0, GluCer d18:1/16:0, d_5-DAG16:0/16:0 and d_5-DAG18:1/18:1 obtained from Avanti Polar Lipids; d_3-GM3 d18:1/18:0 and d_3-LacCer-d18:1/16:0 from Matreya LLC, d_6-cholesterol, d_6-CE-18:0, d_5-TAG(14:0)$_3$, d_5-TAG(16:0)$_3$ and d_5-TAG(18:0)$_3$ from CDN isotopes, d_8-FFA-20:4 from Cayman Chemicals and d_{31}-FFA-16:0 from Sigma-Aldrich. In order to elucidate the differential distribution of lipid substrates between clinical groups, quantitated serum lipid levels in mol/L were normalized to sum of total lipids i.e. MFT (molar fractions of total lipids) for statistical analyses

[18–20]. Nontargeted lipidomics were conducted on an Agilent 1290 UPLC-6546 QTOF system as previous described [21]. Lipid extracts from six samples were randomly selected from GCK-MODY and T2D group, respectively, to create a mix sample representative of each disease condition, and injected into the Agilent LC-MS system for comparative analyses of high-resolution MS profiles.

2.3. Isolation of high-density lipoproteins (HDLs)

Lipoproteins from 500 μL of serum were prestained with Sudan Red 7B to facilitate visualization, and VLDL, LDL and HDL fractions were isolated in a sequential manner based on differences in densities as previously described [22]. In brief, fixed volumes of solutions of different densities ($\rho = 1.006$ g/mL for VLDL isolation; $\rho = 1.182$ g/mL for LDL isolation; $\rho = 1.478$ g/mL for HDL isolation), adjusted by dissolving differing masses of NaBr and NaCl in MilliQ water, were layered sequentially onto serum samples and centrifuged at 180, 000 g for 12 h using a P65A rotor on a CP80WX ultracentrifuge (Hitachi) to successively remove VLDL and LDL in preceding isolations, finally obtaining purified HDLs.

2.4. Western blot analyses

HDL, LDL and VLDL apolipoprotein concentrations were measured by using the Pierce™ BCA Protein Assay Kit (Thermo Fisher). Thereafter, proteins were denatured at 99 °C for 12 min, and 40 μg of HDL and 20 μL of serumwere individually resolved by SDS-PAGE (10% acrylamide gel). After transferring proteins onto PVDF membranes (0.45-μm pore size, Merck Millipore) followed by incubation with 5% nonfat milk in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) for 1 h at room temperature, the membranes were incubated with primary antibodies diluted in 5% bovine serum albumin in TBST at 4 °C overnight before detection with HRP-conjugated secondary antibodies (ZSGB-BIO). Immunoreactive bands were revealed using "SuperSignal™ West Dura Extended Duration Substrate" purchased from ThermoFisher, and gray scale analysis was performed with ImageJ (National Institutes of Health, Java image processing software). Primary antibodies used for western blotting included *anti*-CEPT1 (1:500, abcam, ab133625), *anti*-ATGL (1:500, Cell Signaling Technology, 2138S), and *anti*-CPT1 (1:500, Sigma, SAB2108104).

2.5. Statistical analyses

Serum clinical indices were compared using ANOVA and chi-square test for numeric and categorical variables, respectively. Concentrations of lipid species were normalized by total lipid sum prior to statistical analyses. Multivariable linear regression was conducted to estimate mean difference and 95% confidence intervals (CIs), adjusting for age, sex and BMI. False discovery rate (FDR) was controlled using FDR < 0.05. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) was performed to distinguish between serum lipidomes of GCK-MODY and T2D. To assess the validity of the OPLS-DA model, permutation analysis was employed to obtain pR2Y and pQ2 statistics, which are empirical p-values indicating how likely a model obtained using randomly permutated group labels would outperform the one using their true labels. OPLS-DA was used to generate a list of top 30 lipid candidates for segregating GCK-MODY and T2D subjects (Table 2). To investigate the performance of serum lipid panel in classifying GCK-MODY and T2D subjects, a logistic regression model was built with a panel of four lipids having the highest VIP scores from OPLS-DA analysis, including PC34:0p, PI 36:2(18:1/18:1), PC40:6(18:1/22:5), TAG52:4(16:0). The logistic regression model was built with auto-scaled absolute concentration (mol/L) of the four lipids. Model performance was evaluated on the basis of area under the receiver operating characteristic curve (ROC). Correlation between serum lipids were analysed by Pearson's correlation, and only associations with FDR<0.05 were indicated. Agglomerative hierarchical clustering was built with 1-spearman correlation as the distance measure and complete linkage. Association between clinical characteristics and blood lipids was assessed by Spearman correlation, where pairwise deletion was carried out if missing values occur in the clinical data. All the analyses were performed in 64-bit R 3.6.2 on a windows 10 operating system.

3. Results

3.1. Baseline characteristics differences between nondiabetic, GCK-MODY and T2D subjects

No significant difference in age was observed between controls, GCK-MODY and T2D. Sex and BMI were significantly different across the three groups, and were adjusted for, together with age, in our multivariable linear regression analysis. In accordance with disease phenotypes, FBG and glycated albumin (GA) progressively increased from

Table 1
Baseline characteristics of nondiabetic control, GCK-MODY and T2D patients.

	Control	GCK-MODY	T2D	ANOVA P value	Pairwise *t*-test P value		
n	42	30	33		Control vs GCK-MODY	Control vs T2D	GCK-MODY vs T2D
Age, years	40.81 ± 10.87	40.50 ± 10.94	43.00 ± 8.97	0.563	0.906	0.353	0.323
Female sex, %	19 (45.2)	21 (70.0)	8 (24.2)	0.001	0.065	0.101	0.001
BMI, kg/m^2	23.19 ± 2.87	21.12 ± 3.13	25.50 ± 3.29	<0.001	0.005	0.002	<0.001
FBG, mmol/L	4.93 ± 0.58	6.84 ± 0.60	10.02 ± 2.96	<0.001	<0.001	<0.001	<0.001
GA, %	13.27 ± 1.01	17.69 ± 1.66	23.19 ± 7.58	<0.001	<0.001	<0.001	<0.001
UA, μmol/L	295.79 ± 67.07	256.18 ± 78.57	344.10 ± 93.70	<0.001	0.027	0.014	<0.001
TC, mmol/L	4.46 ± 0.60	4.52 ± 0.87	5.30 ± 1.16	<0.001	0.732	<0.001	0.004
TG, mmol/L	1.02 ± 0.39	0.71 ± 0.32	2.21 ± 1.52	<0.001	0.001	<0.001	<0.001
HDL, mmol/L	1.28 ± 0.25	1.48 ± 0.45	1.10 ± 0.25	<0.001	0.019	0.004	<0.001
LDL, mmol/L	2.64 ± 0.58	2.52 ± 0.65	3.35 ± 0.96	<0.001	0.426	<0.001	<0.001
CRP, mg/L	0.84 ± 0.88	0.67 ± 0.83	2.59 ± 4.01	0.006	0.467	0.028	0.015
2PBG, mmol/L	5.54 ± 1.05	9.15 ± 2.26	16.07 ± 6.12	<0.001	<0.001	<0.001	<0.001
ACR, mg/mmol	6.03 ± 9.54	5.93 ± 3.66	38.27 ± 81.71	0.006	0.957	0.013	0.038
HbA1c, %	5.19 ± 0.63	6.42 ± 0.32	8.86 ± 1.72	<0.001	<0.001	<0.001	<0.001
FCP, ng/mL	1.49 ± 0.50	1.09 ± 0.46	1.96 ± 0.64	<0.001	0.001	0.001	<0.001
FINS, mU/L	7.59 ± 3.88	9.03 ± 4.22	10.07 ± 4.81	0.068	0.234	0.023	0.49

Data are mean ± SD or median (interquartile) for the continuous variables, or number (%) for categorical variables. DBP, diastolic blood pressure. BMI: body mass index; FBG: fasting blood glucose; GA: glycated albumin; UA: uric acid; TC: total cholesterol; TG: triacylglycerol; HDL: high-density lipoproteins; LDL: low-density lipoproteins; CRP: C reactive protein; 2PBG: 2-h postload plasma glucose; ACR: urinary albumin to creatinine ratio; HbA1c: glycated hemoglobin; FCP: fasting C-peptide; FINS: fasting plasma insulin.

Table 2
Table of top 30 lipids ranked by descending VIP scores from OPLS-DA analysis classifying GCK-MODY and T2D. Fold-changes of lipid levels in GCK-MODY relative to T2D were also presented.

Rank	Lipids	VIP score	*Fold Change: GCK-MODY/T2D*
1	PC34:0p	2.002	1.40
2	PI 36:2(18:1/18:1)	1.913	2.15
3	PC40:6(18:1/22:5)	1.908	1.62
4	TAG52:4(16:0)	1.872	0.42
5	TAG52:4(18:2)	1.857	0.44
6	DAG36:4(18:2/18:2)	1.837	0.57
7	PC40:3p(20:0p/20:3)	1.827	1.41
8	PC36:2(18:1/18:1)	1.818	1.57
9	SM d18:1/20:1	1.817	1.41
10	PC36:3(18:1/18:2)	1.809	1.42
11	TAG52:3(18:1)	1.808	0.41
12	PC36:1	1.803	1.25
13	TAG54:4(18:0)	1.803	0.44
14	PC40:5(20:1/20:4)	1.802	1.58
15	PC40:3	1.800	1.30
16	TAG52:3(18:2)	1.797	0.39
17	DAG36:3(18:2/18:1)	1.792	0.54
18	PC38:1	1.787	1.24
19	Medium-chain TAG	1.787	0.47
20	TAG52:3(16:0)	1.771	0.40
21	PC38:4p(18:0p/20:4)	1.764	1.34
22	SM d18:0/26:0	1.762	1.34
23	PC32:0p	1.758	1.32
24	DAG34:2(16:0/18:2)	1.756	0.63
25	PC38:5(18:1/20:4)	1.749	1.44
26	TAG54:3(18:2)	1.743	0.49
27	PC38:3p(18:0p/20:3)	1.738	1.38
28	TAG58:9(22:6)	1.725	0.42
29	TAG56:8(22:6)	1.722	0.45
30	PC40:7(18:2/22:5)	1.714	1.43

controls to GCK-MODY to T2D ($p < 0.001$). Relative to controls, uric acid (UA) was higher in T2D ($p = 0.014$), but significantly lower ($p < 0.001$) in GCK-MODY. C-reactive protein (CRP) was significantly elevated ($p < 0.05$) in T2D relative to both GCK-MODY and controls. Concordant with previous studies [13,15], baseline characteristics indicated that the blood metabolic profiles of GCK-MODY were normal i.e. lower triglycerides (TG) ($p = 0.001$) and similar levels of TC to controls; and cardioprotective i.e. elevated HDLs ($p = 0.019$) and comparable levels of LDLs relative to nondiabetic subjects (Table 1, Supplemental Fig. 2).

3.2. Changes in serum lipidomes between nondiabetic, GCK-MODY and T2D subjects

Multivariable linear regression adjusting for age, sex, and BMI revealed that short-and medium-chain TAGs were significantly reduced in GCK-MODY relative to T2D (FDR < 0.05) (Fig. 1A). Of particular interest, choline-containing lipids, including phosphatidylcholines (PCs), lyso-PCs (LPCs) and sphingomyelins (SMs) were specifically elevated in GCK-MODY relative to T2D (FDR<0.05) (Fig. 1A). Notably, several PCs and PCps were also appreciably increased (FDR < 0.05) in GCK-MODY compared to nondiabetic controls (Fig. 2). These observations imply that the overall biosynthesis of PCs and PCps may be preferentially enhanced in GCK-MODY compared to both healthy controls and T2D.

3.3. Lipid biomarker panel for classification of GCK-MODY and newly-onset T2D

OPLS-DA was conducted to investigate top lipid candidates for segregating GCK-MODY and T2D subjects (Fig. 1B). Score plot shown that GCK-MODY and T2D subjects were well-separated. The top thirty lipids responsible for discrimination between GCK-MODY and T2D were presented in Table 2. Discrimination between GCK-MODY and T2D can be principally attributed to choline-containing lipids including PCs and SMs that were elevated in GCK-MODY, as well as neutral lipids such as TAGs and DAGs that were reduced (Table 2). Logistic regression based on a panel of top four lipids including PC34:0p, PI 36:2(18:1/18:1), PC40:6(18:1/22:5) and TAG52:4(16:0), selected on the basis of highest VIP scores from OPLS-DA analysis, was able to distinguish between GCK-MODY and T2D with an AUC of 0.950 (95% CI 0.903–9.997) after adjusting for age, sex and BMI (Fig. 1C). Our lipidomics investigation thus shown that a panel of four serum lipids exhibited satisfactory performance in differentiating GCK-MODY from T2D subjects, illustrating the potential of serum lipid markers as a preceding test to provide added grounds for implementing genetic testing to facilitate the correct diagnosis of GCK-MODY under clinical settings.

3.4. Co-regulation between PCps and neutral glycerolipid precursors distinct to GCK-MODY

In line with previous reports [13,15], our comparisons based on serum metabolic indices and lipidomic profiles confirmed that GCK-MODY individuals displayed metabolically normal neutral lipid profiles associated with enhanced cardiovascular protection (i.e. lower TG, lower TC, higher HDLs, lower LDLs) compared to T2D subjects (Supplemental Fig. 2). Correlation matrices (Fig. 3A) of lipids along the TAG-DAG-PC pathway shown that PCs, particularly PCps, were specifically and negatively correlated with several TAGs and DAGs in GCK-MODY. The lipid co-regulation distinct to GCK-MODY was lost in T2D subjects (Fig. 3A, cyan box). High resolution MS profiling of serum lipidomes further confirmed findings from targeted lipidomics analyses (Fig. 3B). Extracted ion chromatograms (EICs) for representative PCp and TAG species illustrated increases in serum PCps and reductions in TAGs in GCK-MODY relative to T2D.

3.5. HDLs were distinctly associated with altered profiles of PCps under hyperglycemia

We observed particularly strong positive associations between serum PCps and total HDLs in hyperglycemic individuals (i.e. T2D and GCK-MODY) compared to normglycemic controls (Fig. 4A), suggesting that HDLs content may account for the differing levels of serum PCps in hyperglycemic subjects, or that HDL-PCps may elicit specific physiological properties under a hyperglycemic metabolic micro-milieu. Indeed, an inverse association between HDL-PCps with both stable and acute coronary artery disease have been previously reported, and levels of PCps in HDLs were found to correlate positively with their capacity to inhibit apoptosis of endothelial cells [23]. In another study, it was found that plasmalogen phospholipids were reduced in the HDL2 fraction (density 1.063–1.125 g/mL) of T1D patients [24]. T1D-HDLs were shown to be dysfunctional, with impaired anti-atherogenic and anti-oxidative properties relative to nondiabetic subjects. The anti-inflammatory and anti-apoptotic properties of HDL-PCps are, therefore, expected to contribute positively in maintaining the health of vascular endothelial cells. In addition, several serum PCps were negatively correlated ($p < 0.05$) with urinary albumin to creatinine ratio (ACR), FBG, 2-h postload plasma glucose (2PBG), TG and CRP in GCK-MODY, suggesting that enhanced levels of serum PCps is beneficial towards overall metabolic health under hyperglycemia.

3.6. Within-group heterogeneity in GCK-MODY metabolic phenotypes

Although serum PCs were significantly elevated (FDR<0.05) in GCK-MODY than T2D (Figs. 1A and 2), we noticed an appreciable degree of heterogeneity in total PC levels amongst individual GCK-MODY subjects (Supplemental Fig. 3). In line with this observation, Fendler and colleagues had also previously reported an appreciable degree of heterogeneity in HDL-cholesterol profiles in GCK-MODY, and postulated that interaction between specific *GCK* mutations and other genetic or clinical

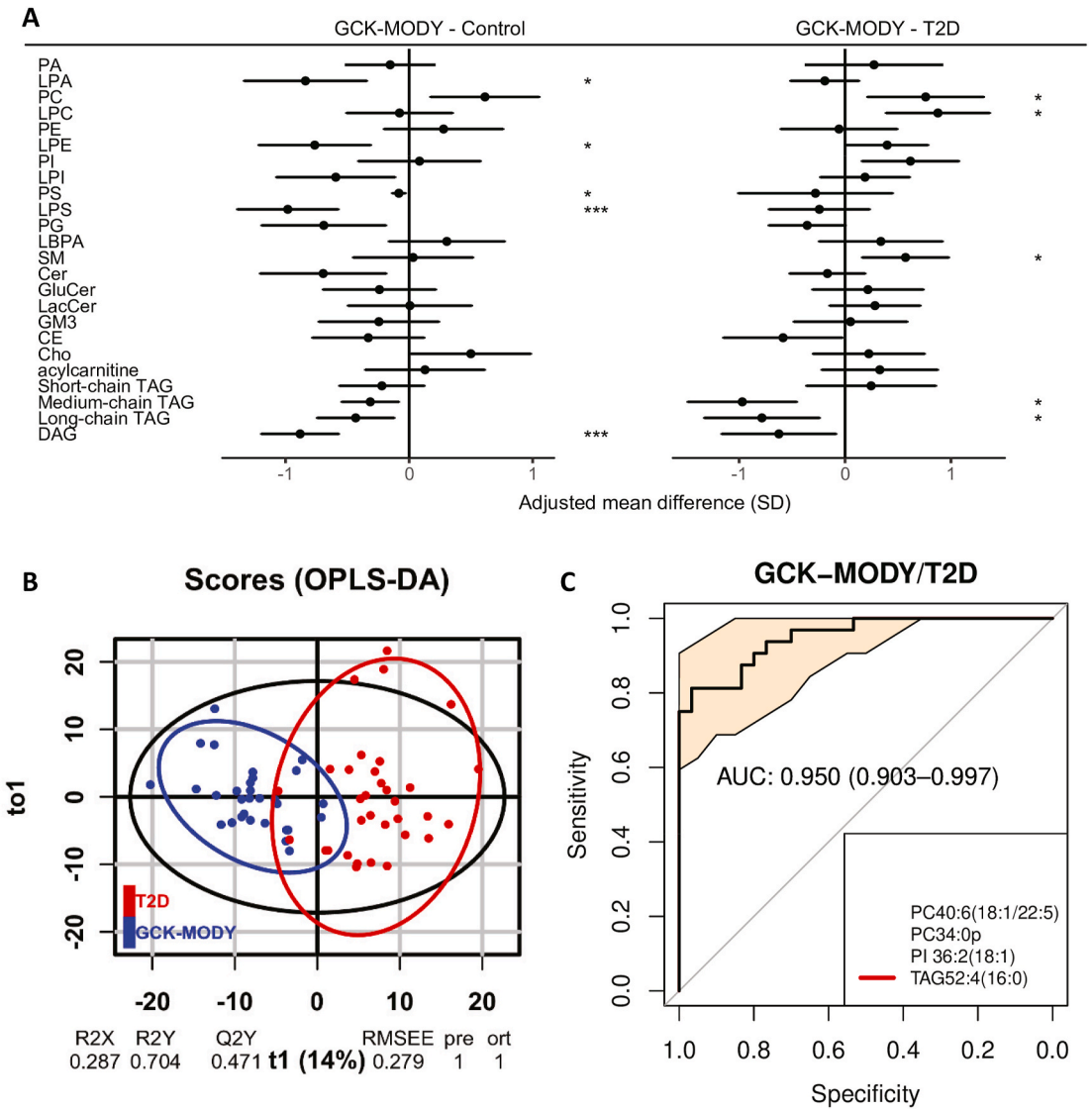

Fig. 1. Serum lipid biomarker panel for distinguishing between GCK-MODY and T2D subjects. A. Forest plot illustrates mean difference and 95% confidence intervals in serum lipid classes between nondiabetic control, GCK-MODY and drug-naïve T2D subjects using multivariable linear regression adjusted for age, sex and BMI. Left panel plots mean difference in GCK-MODY relative to control; right panel plots mean difference in GCK-MODY relative to T2D. **B.** OPLS-DA results shown that GCK-MODY and T2D subjects were separated based on serum lipidomes. **C.** Receiver operating characteristic (ROC) curve from logistic regression using top five lipids ranked by highest OPLS-DA VIP scores separated GCK-MODY and T2D with an AUC of 0.965. Shaded region indicates the 95% confidence interval of AUC. *FDR<0.05; **FDR<0.01; ***FDR<0.001. Short-chain triacylglycerols (TAGs) refer to species with carbon atom number = 44–46); Medium-chain TAGs refer to species total carbon atom number = 48–56; Long-chain TAGs refer to species with total carbon atom number ≥ 58.

factors may account for such heterogeneous phenotypes [15]. Indeed, genetic variations among MODY genes were found to differentially influence patients' response to insulin-sensitizing interventions [25]. Therefore, we further stratified GCK-MODY subjects based on amino acid mutations on the GCK protein, and performed agglomerative clustering analyses based on serum PC profiles and clinical indices of GCK-MODY subjects (Fig. 4B). Notably, we observed a cluster comprising specific *GCK* mutations that were associated with enhanced levels of both serum PCps and HDLs (Fig. 4B, boxed), which includes F195S, C252Y, C364F, and G261R. In particular, subjects harboring F195S and C252Y mutations consistently resided in the PCp-/HDL-enriched cluster, while T168A, I159 N and L430_P431del were found both inside and outside this specific cluster, suggesting that other interactive factors determining serum levels of PCps may be present. We went on to map these *GCK* mutations onto the three-dimensional structure of GCK protein (Supplemental Fig. 4), but found that F195S and C252Y were not located in proximity to each other. Thus, enhanced levels of PCps in individuals harboring these specific *GCK* mutations is probably mediated via signaling events downstream of GCK activity, rather than protein physical interaction *per se*.

3.7. Enhanced CEPT1 and ATGL in HDLs underlie increases in HDL-localized PCps in GCK-MODY

We then isolated the HDLs from a subset of GCK-MODY and T2D patients to investigate HDL-specific changes in diacyl-PCs and PCps. When lipid levels were normalized to total serum volume used for HDL isolation (Fig. 5A), GCK-MODY subjects displayed significant increases in both diacyl-PCs and PCps relative to T2D, which might be attributed to the higher number of circulating HDLs per unit volume of serum in GCK-MODY (Table 1). After normalizing lipid levels to total HDL protein content (Fig. 5B), specific increases in very-long-chain PUFA-PCs, including PC 42:6(22:6/20:0), PC 42:5(22:5/20:0) and PC 40:7, as well as several PCps, were observed in GCK-MODY relative to T2D. These

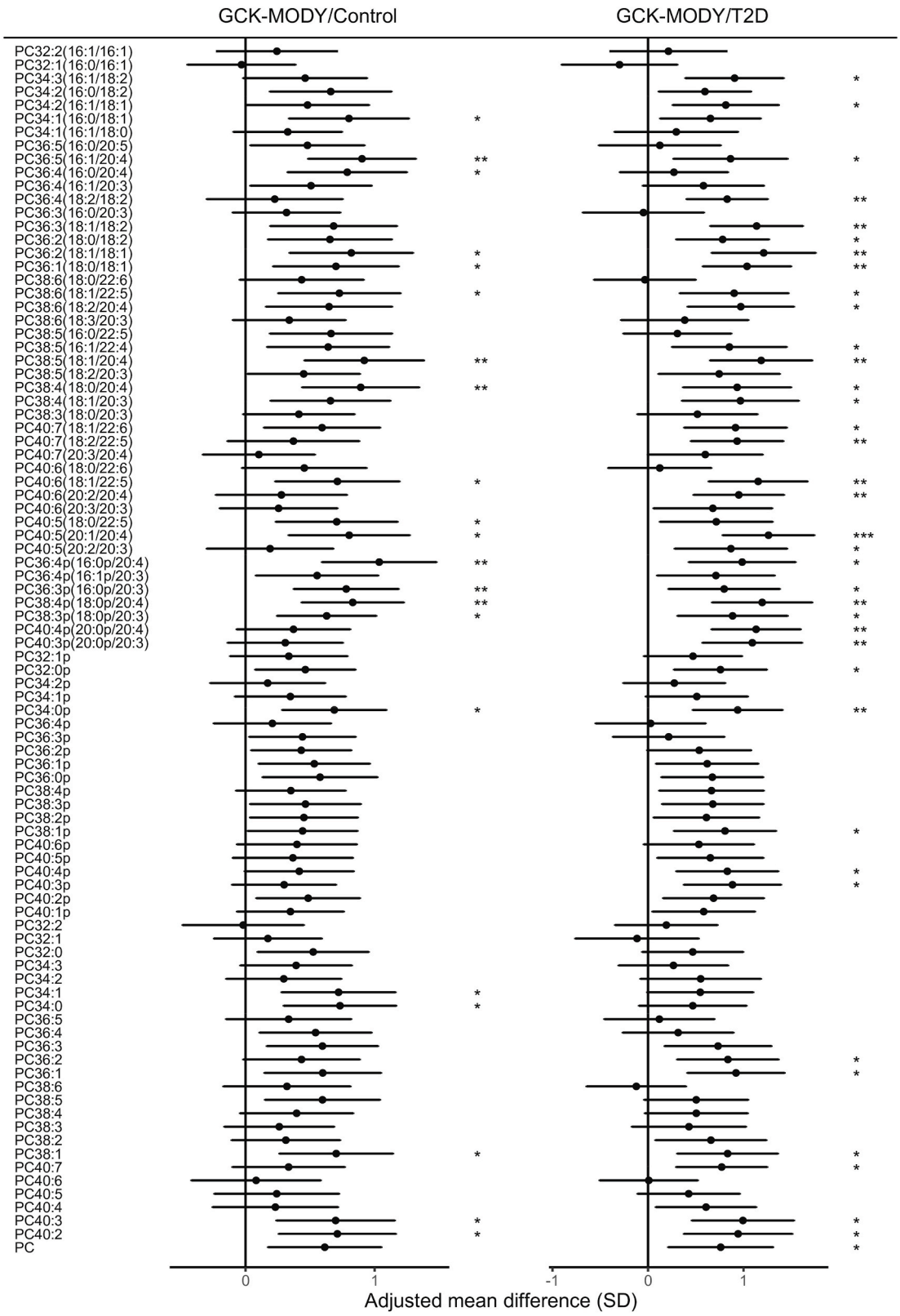

Fig. 2. Changes in serum PCs between GCK-MODY and T2D. Forest plot illustrates mean difference and 95% confidence intervals in serum individual phosphatidylcholines (PCs) nondiabetic control, GCK-MODY and drug-naïve T2D subjects using multivariable linear regression adjusted for age, sex and BMI. Left panel plots mean difference in GCK-MODY relative to controls; right panel plots mean difference in GCK-MODY relative to T2D. *FDR<0.05; **FDR<0.01; ***FDR<0.001.

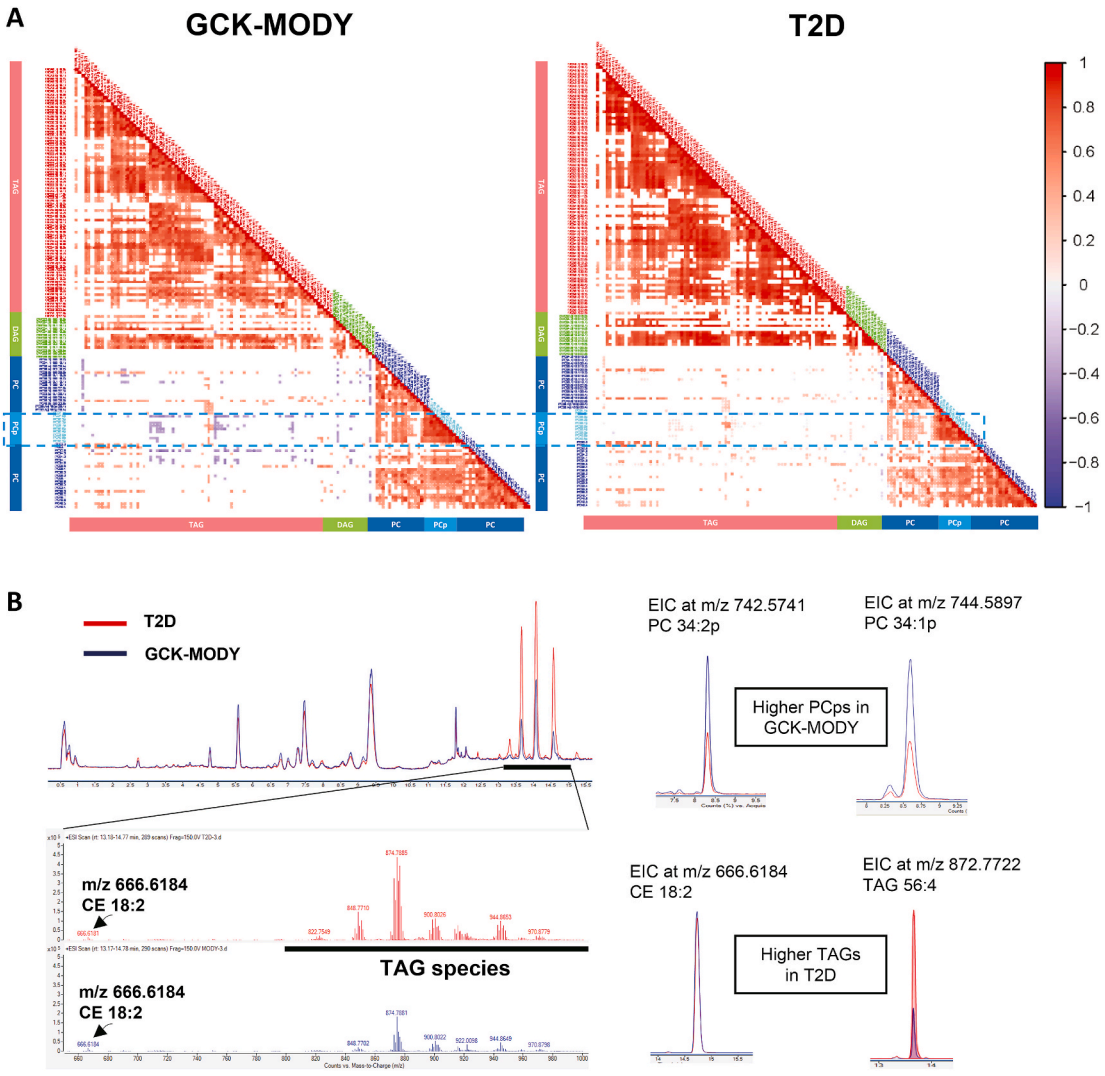

Fig. 3. Plasmalogen PCs (PCps) regulation was specifically altered in GCK-MODY relative to T2D. A. Correlation matrices of lipids along the TAG-DAG-PC axis in GCK-MODY and T2D. Shaded circles indicate Pearson's correlations with FDR<0.05. Colors of circle indicate correlation coefficient (positive correlation: red; negative correlation: blue). Several PCs and PCps were negatively correlated several triacylglycerols (TAGs) and diacylglycerols (DAGs) specifically in GCK-MODY (blue circles), indicating enhanced fluxes of glycerolipid precursors for downstream biosynthesis of PCs and PCps (cyan box). The negative co-regulation between PCps and glycerolipid precursors in GCK-MODY was lost in T2D. **B.** Representative chromatogram and mass spectra from untargeted serum lipidomic analyses of GCK-MODY and T2D patients. Extracted ion chromatograms illustrate increases in PCps and reductions in TAGs in GCK-MODY (blue line) relative to T2D (red line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

observations indicated that the levels of PCps per HDL particle were specifically increased in GCK-MODY. We then investigated changes in the protein levels of key enzymes along the TAG-DAG-PC pathway mediating the biosynthesis of PCs and PCps from glycerolipid precursors using Western Blot (Fig. 5C–D). ATGL mediates the breakdown of TAGs to DAGs. CEPT1 and CPT1 catalyze the final step in the *de novo* synthesis of PCs from DAG precursors, which involves transferring a phosphocholine moiety from cytidine diphosphate (CDP)-choline to DAGs [26]. Notably, CEPT1, but not CPT1, is capable of utilizing DAGs containing ether linkages as substrates [27], rendering CEPT1 as the primary enzyme catalyzing the transfer of phosphocholine moiety to alkenyl-DAGs in the formation of plasmalogen phospholipids. A representative gel-image from eight subjects shown that bands specific to ATGL and CEPT1 were notably more intense in GCK-MODY than T2D subjects (Fig. 5C). We quantitated band intensities from gel-images of 12 GC K-MODY and 12 T2D subjects, and found that HDL levels of ATGL ($p = 0.0034$) and CEPT1 ($p < 0.0001$) were both significantly increased in GCK-MODY relative to T2D, while no significant changes were observed for CPT1 (Fig. 5D). Furthermore, we observed that numerous DAGs that serve as precursors for the biosynthesis of PCs via CEPT1/CPT1 were significantly reduced (FDR<0.05) in GCK-MODY relative to T2D (Supplemental Fig. 5), implying an enhanced utilization of DAGs for the *de novo* biosynthesis of PCs and PCps in GCK-MODY.

4. Discussion

A serum-based lipid biomarker panel that precedes genetic screening for GCK-MODY can enhance diagnostic efficiency and facilitate treatment optimization. Treatment optimization is particularly critical, in view of the appreciable heterogeneity in metabolic phenotypes (i.e. serum PCs and lipoprotein profiles) amongst individuals harboring different *GCK* mutations. Furthermore, specific *GCK* mutations exhibited enhanced increases in both PCps and HDLs amongst others. As PCs represent the most abundant phospholipid component in lipoproteins that constitutes approximately 60–80 M percent of total phospholipids [28], augmented levels of PC biosynthesis from glycerolipid precursors in GCK-MODY can alter both the overall production as well as composition of HDLs. Indeed, relative to T2D, we observed both increases in

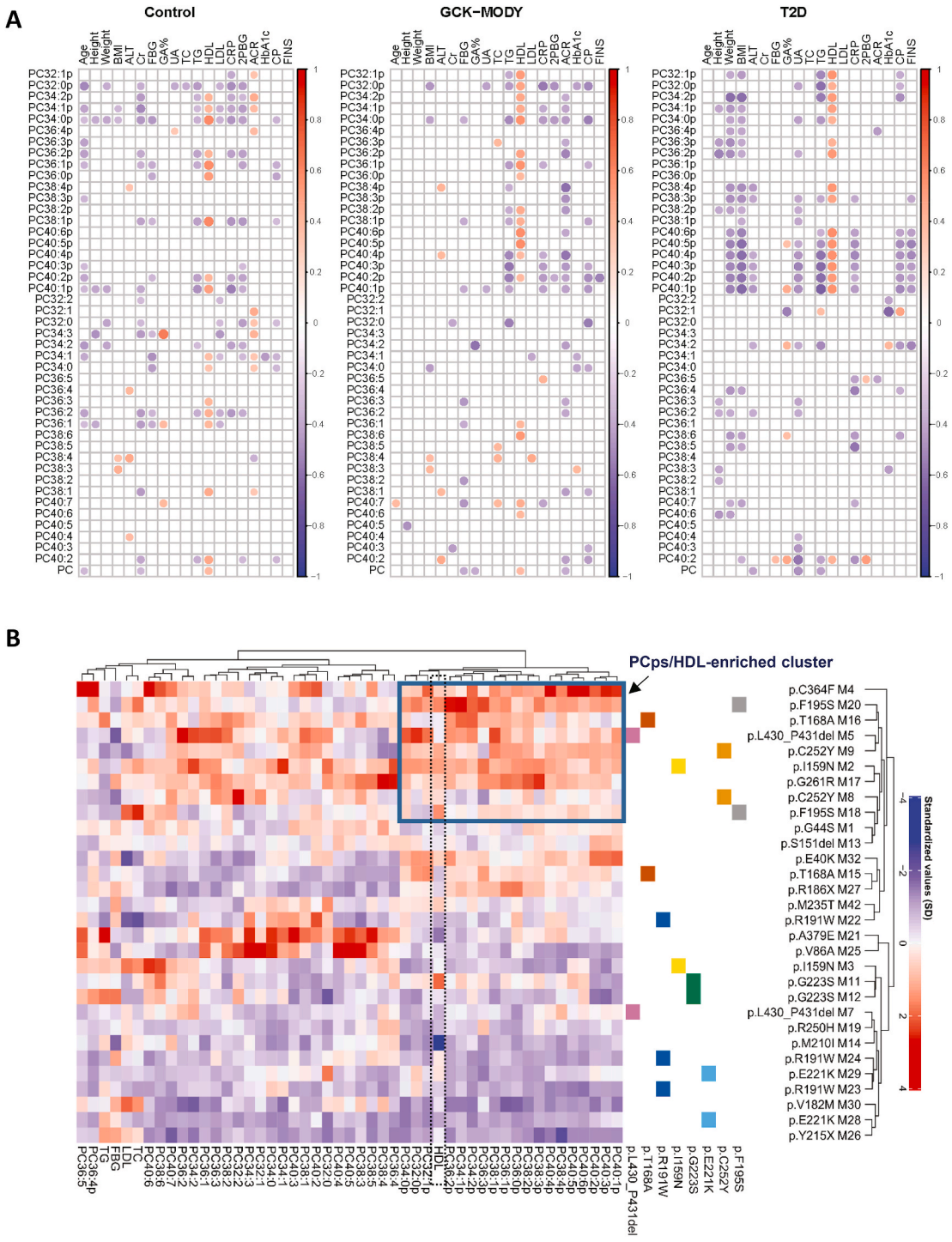

Fig. 4. Serum PCps were specifically correlated with circulating HDLs in hyperglycemic individuals. A. Strengthened positive correlations were observed between serum PCps and HDLs in hyperglycemic individuals (GCK-MODY and T2D) compared to healthy controls. Spearman correlation was used to investigate correlations between individual serum PCs and blood metabolic indices. Color of circles indicates direction of correlations (red: positive correlations; blue: negative correlations), and sizes and color intensities of circles indicate strengths of correlation (Bigger and darker circle indicates stronger correlation). Vertical scale indicates values of correlation coefficients. Only correlations with $P < 0.05$ were illustrated. ALT: alanine transaminase; Cr: creatinine; FBG: fasting blood glucose; GA %: glycated albumin; UA: uric acid; TC: total cholesterol; TG: serum total triglycerides; HDL: high-density lipoproteins; LDL: low-density lipoproteins; CRP: C-reactive proteins; 2PBG: 2-h postload plasma glucose; ACR: urinary albumin to creatinine ratio; HbA1c: glycated hemoglobin; CP: C-peptides; FINS: fasting plasma insulin. **B.** Agglomerative hierarchical clustering of individual serum choline-lipids and laboratory indices with different *GCK* mutations. *GCK*-mutations associated with enhanced serum PCps corresponded with those displaying increased circulating HDLs. Lipid levels were standardized across samples and Euclidean distances were used to visualize differences between different *GCK* mutations. Color legends on the right refer to *GCK* mutations that were repeated with frequency >1 in the cohort, with the same color corresponding to the same mutation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

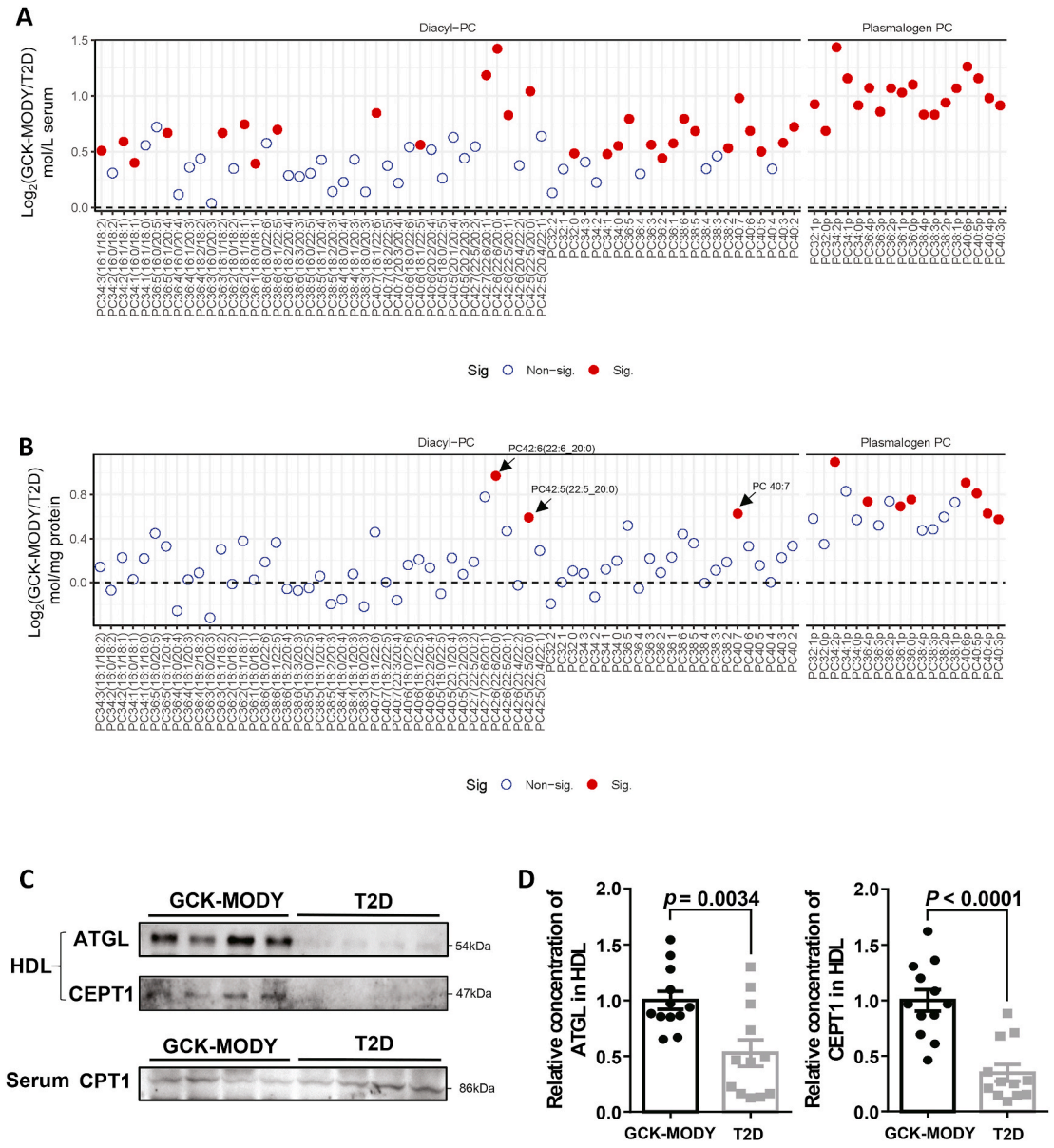

Fig. 5. Changes in lipids and protein levels in HDLs of GCK-MODY relative to T2D. A. Significant increases ($P < 0.05$) in several HDL diacyl-PCs and plasmalogen PCs (PCps) in GCK-MODY (n = 6) relative to T2D (n = 6) when lipids were expressed in μmol/L of serum, indicating elevated HDL numbers in GCK-MODY. **B.** Specific increases in HDL PUFA-PCs and PCps in GCK-MODY (n = 6) relative to T2D (n = 6) when lipids were expressed as μmol/mg of HDL protein, indicating localized and preferential increases in these lipids on per HDL particle basis in GCK-MODY. Shaded red circles indicates statistically significant ($P < 0.05$) from Mann-Whitney *U* test. **C.** Representative gel-images of HDL levels of adipose triglyceride lipase (ATGL) and choline/ethanolamine phosphotransferase 1 (CEPT1), and serum level of choline phosphotransferase 1 (CPT1) in GCK-MODY and T2D subjects. Equal amounts of total proteins were loaded into each lane. **D.** Quantified levels of ATGL and CEPT1 in HDL particles. Relative protein levels in each samples were calculated by normalizing to the mean protein level of GCK-MODY. P-values from Student's *t*-test was indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the number of circulating HDLs, as well as HDL-localized elevations in both PCs and PCps of GCK-MODY. Vascular endothelial cells are expected to benefit from elevated HDL-PCs, particularly PCps, given the anti-apoptotic and anti-inflammatory effects of HDL-PCps towards endothelial cells [29,30].

We also shown that HDL-localized increases in PCps (and PCs) might be primarily attributed to elevated levels of ATGL and CEPT1 proteins in GCK-MODY. Enhanced level of HDL-ATGL serves to increase the availability of DAG precursors by mediating TAG hydrolysis, facilitating the downstream biosynthesis of phospholipids (Fig. 6C). In particular, enhanced levels of CEPT1, but not CPT1, accompanied elevated PCps in the HDLs of GCK-MODY. Although both CEPT1 and CPT1 catalyze the final step in the *de novo* synthesis of PCs, essential differences exist between their tissue and subcellular distributions [26,31]. First, CEPT1 mRNAs are expressed in similar levels in major organs including the heart, liver, spleen, intestines and prostate, while CPT1 expression is highest in the testis (almost a hundred-fold higher than other tissues) [32]. Second, CEPT1 is localized to the endoplasmic reticulum and nuclear envelope, the predominant sites of PC synthesis in mammalian cells. ERs also denote the site for the final stages of plasmalogen phospholipid biosynthesis; while CPT1 is localized to the Golgi apparatus [33]. It was found that only human CEPT1, but not CPT1, was able to affect cell growth in yeast mediated by SEC14 [34], a protein shown to regulate vesicle trafficking and phospholipid transfer between membrane layers [35]. In mammals, PC-transfer protein functions to promote extracellular transport of PCs and cholesterol to produce nascent

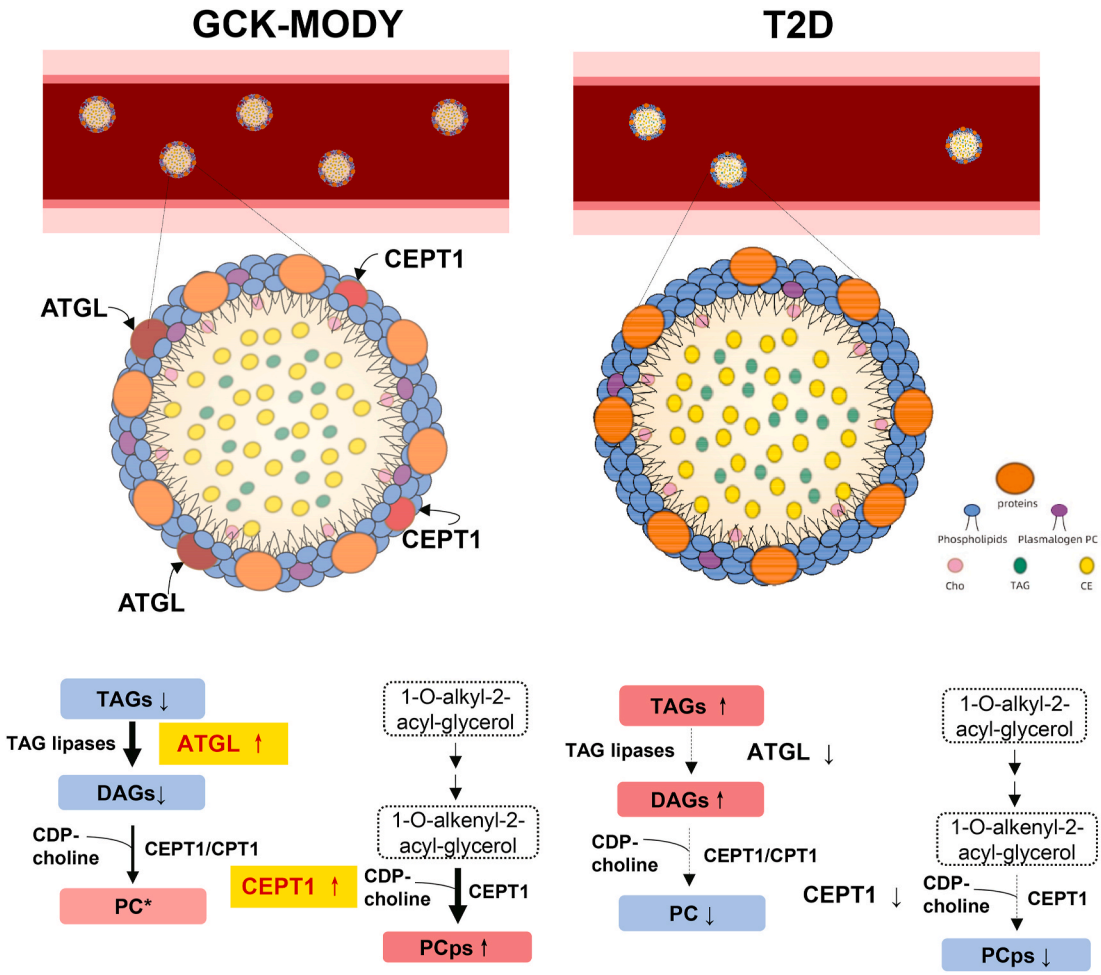

Fig. 6. Schematic overview on the molecular basis underlying atheroprotective properties of HDLs in GCK-MODY relative to T2D. GCK-MODY displays higher circulating levels of HDLs compared to T2D. Considering per HDL particle, GCK-MODY exhibits increased recruitment of ATGL and CEPT1 onto the surface of HDLs, which promotes the breakdown of neutral lipid precursors i.e. diacylglycerols (DAGs) and triacylglycerols (TAGs) and enhances the production of plasmalogen phosphatidylcholines (PCps) and polyunsaturated PCs (PUFA-PCs). Localized increases in PCps are expected to contribute positively towards the cardioprotective properties of HDLs, including elevated capacity of cholesterol efflux from endothelial cells (ECs), anti-apoptotic effects on ECs, as well as enhanced capacity to halt the propagation of phospholipid hydroperoxides (PCOOH) radicals in the surrounding vasculature. These unique atheroprotective properties of HDLs offer GCK-MODY added protection against adverse vascular events, despite prolonged exposure of vascular endothelium to a hyperglycemic milieu since birth. *increases were confined to very-long-chain PUFA-PCs.

pre-β-HDL particles, mediated by the ATP-binding cassette protein AI [36]. Furthermore, enhanced expression of CEPT1 and increased production of polyunsaturated PCs were associated with elevated level of liver X receptor in mice, giving rise to more HDLs and less LDLs that protected the animals from atherosclerosis and steatohepatitis [37]. Thus, PCs derived from CEPT1 and CPT1 may elicit varied metabolic functions and possess distinct metabolic fates. Using *in vitro* mixed micellar assays, substrate specificity for diradylglycerol containing ether linkages was previously demonstrated only CEPT1, but not for CPT1 [27]. CEPT1 is thus able to mediate the biosynthesis of PCp precursors given its ability to transfer CDP-choline/CDP-ethanolamine moieties to ether-DAGs. Thus, in addition to producing more diacyl-PCs and giving rise to a greater number of circulating HDLs (Table 1), localized increases in CEPT1 on HDLs might also lead to enhanced production of PCps. Enhanced levels of ATGL and CEPT1 on the HDLs of GCK-MODY increase localized production of PCps from glycerolipid precursors (Fig. 6), which may be metabolically beneficial in a few ways, such as (1) promoting the clearance of glycerolipid precursors (i.e. TAGs, DAGs) that form the basis of hyperlipidemia; (2) positively modulating HDL secretion by increasing the overall production of PCss; and (3) contributing favorably to the anti-atherogenic properties of HDLs, including cholesterol-efflux capacity [38], anti-apoptotic activity [23] and capacity to accept phospholipid hydroperoxides (PCOOH), which cumulatively create an atheroprotective lipid microenvironment for the vascular endothelium.

Pharmalogical activators of GCK used for treating T2D possess a caveat in lowering blood glucose at the expense of inducing unfavorable serum lipid profiles (i.e. elevated TAGs) [13]. Our current work puts forth an alternative, lipid-centric therapy for mitigating vascular complications under hyperglycemia, stemming from a systems investigation of lipid pathway perturbations in GCK-MODY. Indeed, hepatic PC biosynthesis had been previously proposed as an important pharmaceutical target for metabolic diseases, as it was found that impeding PC biosynthesis leads to reduced HDL formation and secretion into the circulation [28]. Our current findings also highlight that serum and HDL-associated PCs may denote important criteria, in addition to blood glucose, for evaluating the performance and clinical utility of various *GCK* activators in the treatment of diabetes. Our results also indicated that individuals harboring different *GCK* mutations may have varying risk of adverse cardiovascular outcomes, and it may be meaningful to investigate if serum PCs or HDL-PCps could predict the risk of cardiovascular complications associated with different *GCK* mutations in future studies.

Our study has limitations. The current observations were based

solely on GCK-MODY cohort across China. Albeit GCK-MODY represents a rare genetic disorder, cross-validation in other racial and ethnic groups is essential, especially in view of the heterogeneous metabolic phenotypes associated with distinct *GCK* mutations. In addition, how individual *GCK* mutations mediates downstream changes in the activities or localization of ATGL and CEPT1 proteins onto HDLs awaits further mechanistic elucidation. While GCK-MODY is a useful, natural paradigm for probing cardioprotective molecular mechanisms under a hyperglycemic milieu in human subjects, the role of enhanced HDL-ATGL and HDL-CEPT1 with regards to cardiovascular protection, as well as the predictive power of HDL-PCps in determining cardiovascular outcome under hyperglycemia, remain to be validated across different subtypes of diabetes. In support of our hypothesis, statins, which are commonly used to lower cardiovascular risk and morbidity [39], were shown to selectively enrich PUFA-plasmalogens in HDL3 of atherogenic mixed dyslipidemia [40]. Furthermore, a preceding study conducted on a case-cohort of T2D patients (n = 3779) reported that plasma levels of PUFA-PCps, including PC36:5p and PC36:4p, were negatively associated with future cardiovascular events (n = 698) and cardiovascular death (n = 355) [41]. Also, PCps are protective against oxidative stresses by their capacity to accept PCOOH radicals, thereby lowering oxidative stress and lipid peroxidation that denote prominent features in coronary heart disease [42].

5. Conclusions

To summarize, we demonstrated HDL-localized increases in ATGL and CEPT1 catalyzing the production of PCs and PCps from glycerolipid precursors along the TAG-DAG-PC biosynthetic pathway in GCK-MODY (Fig. 6). Localized increases in the levels of biosynthetic enzymes indicated that the higher levels of HDL-PCps observed in GCK-MODY cannot be simply attributed to secondary effects resulting from lower oxidative stress in the circulation, but an ongoing, enhanced biosynthesis. To our knowledge, this study provides the first evidence that enhanced recruitment of CEPT1 and ATGL onto HDLs essentially underlie the antiatherogenic profiles associated with GCK-MODY, i.e., the specific and localized increases in PCps in circulating HDLs. Elevated HDL-PCps contribute positively to the atheroprotective properties of HDLs, which serve as an active, circulating form of protection towards the vasculature of GCK-MODY, thereby lowering the incidence of vascular complications despite extended exposure to hyperglycemia since birth.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.101855.

Conflicts of interest

The authors declare that they have no conflict of interest.

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