Carbohydrate and fat intake associated with risk of metabolic diseases through epigenetics of *CPT1A*

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ABSTRACT

Background: Epigenome-wide association studies identified the cg00574958 DNA methylation site at the carnitine palmitoyltransferase-1A (*CPT1A*) gene to be associated with reduced risk of metabolic diseases (hypertriglyceridemia, obesity, type 2 diabetes, hypertension, metabolic syndrome), but the mechanism underlying these associations is unknown.

Objectives: We aimed to elucidate whether carbohydrate and fat intakes modulate cg00574958 methylation and the risk of metabolic diseases.

Methods: We examined associations between carbohydrate (CHO) and fat (FAT) intake, as percentages of total diet energy, and the CHO/FAT ratio with *CPT1A*-cg00574958, and the risk of metabolic diseases in 3 populations (Genetics of Lipid Lowering Drugs and Diet Network, $n = 978$; Framingham Heart Study, $n = 2331$; and REgistre GIroní del COR study, $n = 645$) while adjusting for confounding factors. To understand possible causal effects of dietary intake on the risk of metabolic diseases, we performed meta-analysis, *CPT1A* transcription analysis, and mediation analysis with CHO and FAT intakes as exposures and cg00574958 methylation as the mediator.

Results: We confirmed strong associations of cg00574958 methylation with metabolic phenotypes (BMI, triglyceride, glucose) and diseases in all 3 populations. Our results showed that CHO intake and CHO/FAT ratio were positively associated with cg00574958 methylation, whereas FAT intake was negatively correlated with cg00574958 methylation. Meta-analysis further confirmed this strong correlation, with $\beta = 58.4 \pm 7.27$, $P = 8.98 \times 10^{-16}$ for CHO intake; $\beta = -36.4 \pm 5.95$, $P = 9.96 \times 10^{-10}$ for FAT intake; and $\beta = 3.30 \pm 0.49$, $P = 1.48 \times 10^{-11}$ for the CHO/FAT ratio. Furthermore, *CPT1A* mRNA expression was negatively associated with CHO intake, and positively associated with FAT intake, and metabolic phenotypes. Mediation analysis supports the hypothesis that CHO intake induces *CPT1A* methylation, hence reducing the risk of metabolic diseases, whereas FAT intake inhibits *CPT1A* methylation, thereby increasing the risk of metabolic diseases.

Conclusions: Our results suggest that the proportion of total energy supplied by CHO and FAT can have a causal effect on the risk of metabolic diseases via the epigenetic status of *CPT1A*. Study registration at https://www.clinicaltrials.gov/: the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN)—NCT01023750; and the Framingham Heart Study (FHS)—NCT00005121. *Am J Clin Nutr* 2020;00:1–12.

Keywords: *CPT1A*, epigenetics, carbohydrate and fat intake, obesity, diabetes, hypertension, dyslipidemia, metabolic syndrome

Introduction

Diet is a key daily environmental factor to which each person is exposed. As such, habitual diet modifies epigenetic status to contribute to the health of the individual, and such epigenetic-health links can be passed to descendants (1). Thus, epigenetic marks can be considered a "signature" of communication between the environment and the genome. Such signatures can be reversed and transmitted to future generations (2). Furthermore, epigenetic changes can be regarded as an adaptation and feedback to environmental exposures, including diet and lifestyle habits (3–5).

Carnitine palmitoyltransferase-1 (CPT1) converts long-chain acyl-CoAs into long-chain acylcarnitines, which facilitates their translocation across the mitochondrial membrane into the mitochondrial matrix, where fatty acid β-oxidation takes place. Based on its role in determining the total rate of fatty acid oxidation and cytosolic concentration of long-chain acyl-CoA esters, CPT1A functions in energy metabolism and is involved

in many physiological processes (6). *CPT1A* expression and activity in multiple tissues regulate physiological functions ranging from insulin-mediated inhibition of glucose production, insulin secretion, and glycogen synthesis, to appetite control (6). High level of methylation at cg00574958 of *CPT1A* is associated with reduced risk of obesity (7, 8) and type 2 diabetes (T2D) (9), low fasting triglyceride (TG) (10), and attenuated postprandial TG response to a high-fat meal (11). Early studies in rodents demonstrated that high fructose consumption induced DNA methylation at *CPT1A* promoter regions (12, 13), and high-fat diets suppressed such methylation (13). Thus, we hypothesize that total dietary carbohydrate (CHO) and fat (FAT) intakes affect methylation of *CPT1A,* leading to altered *CPT1A* expression and risk of metabolic diseases, including obesity, T2D, hypertriglyceridemia, hypertension, and metabolic syndrome (MetS).

Central to this hypothesis is the balance between dietary CHO and FAT as energy sources, and activation (or inhibition) of those transport and enzymatic processes that convert fuel to ATP. In this regard, CPT1A is key. It is a rate-limiting enzyme (RLE) for fatty acid entry into mitochondria for β-oxidation (14) and is inhibited by malonyl-CoA (15). Paralleling this is the glucose- and insulin-induced accumulation of triacylglycerides (16). Thus, CPT1A enzymatic action and its RLE status put it at a nexus of CHO and FAT intakes along with consequences that a chronic imbalance in these macronutrients have on MetS outcomes (16). This study was then conducted to characterize the impact of the energy balance on the relation between *CPT1A* epigenetics and CHO and FAT as affecting the risk of metabolic diseases.

In this study, we determined the correlation between CHO and FAT intake and methylation at cg00574958 in the *CPT1A* promoter in the Genetics of Lipid Lowering Drugs and Diet Network study (GOLDN). We then replicated the analysis in 2 additional cohorts: the Framingham Heart Study (FHS) and the REgistre GIroní del COR (REGICOR) study. To investigate whether CHO and FAT intake act by changing methylation status to influence cardiometabolic disease risk, we conducted a meta-analysis, and examined the correlation of CHO and FAT intake with *CPT1A* transcription, and then performed mediation analysis with *CPT1A*-cg00574958 as the mediator.

Methods

Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study

The GOLDN study was a part of the Family Heart Study sponsored by the NIH National Heart, Lung, and Blood Institute (17). The main objective of the GOLDN study was to identify genetic variants that account for an individual's lipid responses to a high-fat meal and 3-wk treatment with fenofibrate, a TG-lowering drug (11). A total of 1327 men and women aged ≥18 y were enrolled from 2 study centers: Minneapolis, MN, and Salt Lake City, UT. The study protocol was approved by the Institutional Review Boards at Tufts University, the University of Minnesota, University of Utah, and the University of Alabama at Birmingham (United States). In the present study, 978 participants (468 men and 510 women) with whole-genome DNA methylation and dietary data were included for analysis.

The Framingham Heart Study

The FHS was established in 1948, in the town of Framingham, MA, as a prospective longitudinal study over 3 generations of participants. The main goal of the study was to identify the key factors that determine cardiovascular diseases (18). The Offspring Cohort began in 1971 with the recruitment of 5124 participants consisting of the children and spouses of the original cohort (19). In-person physical and clinical examination was conducted every 4–8 y. In the eighth examination cycle of the Offspring Cohort (Exam 8, 2005–2008), peripheral blood samples were collected, from which 2741 were available for whole-genome DNA methylation and transcriptome analysis (20). In the current study, 2331 participants (1056 men and 1275 women) with DNA methylation and transcriptome, and dietary data were included for analysis.

The REGICOR study

The REGICOR study was initially conducted in 1995 with an enrollment of participants from 6 districts of Girona Province, Spain, with the initial goal of investigating risk factors of cardiovascular disease (21). In the follow-up visit performed during 2009–2013, 4980 participants who enrolled in the study during 2003–2005 and continued to live in the same towns were surveyed. For the present study, a subset of 645 participants (316 men and 329 women) was selected randomly, all of European descent, for DNA methylation analysis (22). The study procedure met the requirement of the Declaration of Helsinki and was approved by the local ethics committee. All participants provided informed written consent prior to enrollment in the studies.

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Data described in the manuscript, code book, and analytic code will be made available upon request.

Supplemental Tables 1–7 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviation used: BP, blood pressure; CHO, total carbohydrate intake/total energy intake; *CPT1A,* carnitine palmitoyltransferase-1A; dbGaP, Database of Genotype and Phenotypes; FAT, total fat intake/total energy intake; FHS, Framingham Heart Study; FPG, fasting plasma glucose; GOLDN, Genetics of Lipid Lowering Drugs and Diet Network; HbA1c, glycated hemoglobin; MetS, metabolic syndrome; OGTT, oral-glucose-tolerance test; PBMC, peripheral blood mononuclear cell; PC, principal component; REGICOR, REgistre GIroní del COR study; RLE, rate-limiting enzyme; TG, triglyceride; T2D, type 2 diabetes.

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DNA extraction

In REGICOR (22) and FHS (20), DNA for methylation was extracted from whole peripheral blood (buffy coat) using the QIAamp DNA Blood mini kit or Gentra Puregene DNA extraction kit (Qiagen) (23), whereas in GOLDN, DNA was isolated from $CD4^+$ T-cells from frozen buffy coat samples using DNeasy kits (Qiagen) (11).

Methylome analysis

Genome-wide DNA methylation of isolated DNA samples in all cohorts (GOLDN, REGICOR, and FHS) was determined using Infinium human methylation 450K arrays (Illumina) as described (20, 22–24). The proportion of total methylation-specific signal at each CpG site was calculated as a β score using the Bioconductor minfi package with correction for background while excluding sites with detection $P > 0.01$ and 1.5% of samples having missing data, or >10% of samples lacking sufficient signals (11). The β scores were further controlled for batch effects and normalized using the ComBat function in the ChAMP R package (25) or DASEN (26). To account for the heterogeneity of different cell types across all samples, in each cohort, principal components (PCs) were calculated based on the β score of all CpG sites that passed quality control.

FHS DNA methylome data were requested from the Database of Genotype and Phenotypes (dbGaP) under accession phg000492.v2. Methylome profile was conducted on DNA isolated from the whole blood samples from 2741 participants at Exam 8 of the Offspring Cohort using Illumina Infinium human methylation 450K arrays (23). Methylation signals were processed and normalized as for REGICOR and GOLDN.

Transcription analysis

In FHS, *CPT1A* mRNA expression was extracted from the transcriptome data requested from dbGaP under the accession phe00002.v6. The mRNAs from whole blood samples were collected from FHS Offspring participants at Exam 8 after overnight fasting. The transcriptome analysis was conducted using Affymetrix Human Exon 1.0 ST array (27). The quality control and normalization of the initial gene expression data were as described previously (28, 29). To account for the heterogeneity of different cell types across all samples, PCs were calculated based on the normalized expression of all the genes that passed quality control. The first 10 PCs were used in all subsequent statistical analyses.

Assessment of dietary intake

In GOLDN, dietary intake was assessed using the Diet History Questionnaire (17), and the Willett semiquantitative FFQ was applied in Exam 8 of the FHS (18). For the REGICOR study, participants were requested to complete a validated 137-item semiquantitative survey in Spanish (30). The dietary intake in GOLDN and FHS (16–18) was estimated based on the Harvard University food composition database, the USDA database, and the Minnesota Nutrient System. Energy, and nutrient intakes in REGICOR were estimated using software designed for a Spanish food composition database (31). Total macronutrient intakes per day, including CHO, FAT, PUFAs, MUFAs, SFAs, and total protein were normalized to the total energy intake per day in all 3 populations. CHO intake was further divided into simple carbohydrates and complex carbohydrates intake. Total sugar intake was estimated as simple carbohydrates, whereas complex carbohydrate intake was estimated by subtracting the total sugar intake from the CHO intake (32).

Metabolic disease definitions

Metabolic syndrome (MetS) was defined in the same manner in 3 cohorts (GOLDN, FHS, and REGICOR) as the presence of ≥3 of the following: elevated blood pressure (BP) (≥130 mmHg systolic, ≥85 mmHg diastolic) or treatment for high BP; hypertriglyceridemia (≥150 mg/dL) or treatment with lipid-lowering treatment; low HDL cholesterol (<40 mg/dL in men, <50 mg/dL in women); hyperglycemia (fasting glucose ≥100 mg/dL) or treatment with oral hypoglycemic agents or insulin; and increased waist circumference (≥102 cm for men, ≥88 cm for women). Hypertension was defined as: systolic BP ≥140 mmHg or diastolic BP ≥90 mmHG or taking medication for hypertension. T2D: defined as either fasting plasma glucose (FPG) ≥126 mg/dL, a 2-h postload glucose concentration [2-h oral-glucose-tolerance test (OGTT)] ≥200 mg/dL, glycated hemoglobin (HbA1c) concentration ≥6.5%, or use of hypoglycemic agents (self-reported). PreT2D was defined as either FPG between 100 and 125 mg/dL, HbA1c concentration between 5.7% and 6.4%, or 2-h OGTT between 140 and 199 mg/dL.

Statistical analysis

The primary outcomes of this study were metabolic diseases (hypertriglyceridemia, obesity, T2D, MetS) and related traits, whereas the secondary outcome was hypertension. All association analyses were conducted based on linear or logistic regression models using SAS 9.4 (SAS Inc), R (https://www.r-project.org/), or SVS 8.7 (GoldenHelix Inc). The missing data were omitted for all statistical analyses.

Association between dietary intake, *CPT1A*-cg00574958, and metabolic diseases

We determined the association between macronutrient intake normalized to total energy and *CPT1A*-cg00574958 using a linear regression model while adjusting for age, gender, cell-type heterogeneity, and family relationship (FHS and GOLDN) if applicable. A similar linear or logistic regression model was used to examine the association between *CPT1A*-cg00574958 methylation and metabolic diseases (T2D, hypertension, and MetS) or metabolic traits (BMI, TG, glucose).

Association between dietary intake, *CPT1A*-cg00574958, *CPT1A* expression, and plasma lipids

To determine whether identified CHO/FAT-associated *CPT1A*-cg00574958 methylation was correlated with *CPT1A* expression and plasma lipids, we examined the correlation between CHO/FAT intake and *CPT1A* mRNA expression and plasma lipids in Exam 8 of FHS Offspring. *CPT1A* mRNA expression

TABLE 1 General characteristics of 3 study populations[1]

	GOLDN	FHS	REGICOR
n	978	2331	645
Men/women, n	468/510	1056/1275	316/329
Age, y	48.3 ± 16.43[3]	66.2 ± 8.9	63.2 ± 11.7
Smoker, *n* (%)	265 (27.1%)[3]	170 (7.3%)	107 (16.6%)
Drinker, *n* (%)	476 (48.7%)	1810 (77.8%)	NA
BMI, kg/m^2	28.3 ± 5.6	28.2 ± 5.3	26.9 ± 4.1
Fasting triglyceride, mg/dL	136.5 ± 94.9	117.8 ± 67.0	100 ± 48.6
Fasting glucose, mg/dL	100.9 ± 16.2	106.1 ± 22.2	97.7 ± 20.4
Metabolic syndrome, *n* (%)	265 (27.1%)[3]	1108 (47.5%)	232 (36.1%)
Type 2 diabetes, *n* (%)	67 (6.9%)[3]	346 (14.8%)	63 (9.81%)
Hypertension, *n* (%)	249 (25.5%)[3]	1387 (59.5%)	302 (47.0%)
Physical activity score[2]	34.2 ± 6.3	35.3 ± 5.3	1800 (900, 3225)
Lipid-lowering medication, *n* (%)	149 (15.2%)[3]	1048 (43.0%)	154 (24.0%)
Type 2 diabetes medication, *n* (%)	46 (4.7%)[3]	270 (11.5%)	44 (6.85%)
Hypertension medication, *n* (%)	204 (20.9%)[3]	1144 (49.2%)	200 (31.2%)
Carbohydrate intake (% of total energy)	48.9 ± 2.13	46.6 ± 8.6	41.4 ± 7.51[4]
Total sugar (% of total energy)	12.3 ± 6.4	21.5 ± 7.3	NA
Complex carbohydrate (% of total energy)	36.7 ± 7.3	25.1 ± 5.3	NA
Total fat intake (% of total energy)	35.4 ± 6.9	33.0 ± 6.6	40.9 ± 6.87[4]
Saturated fat (% of total energy)	11.8 ± 2.7	11.1 ± 2.7	11.90 ± 2.58
MUFAs (% of total energy)	13.3 ± 2.8	12.4 ± 2.8	20.50 ± 4.52[4]
PUFAs (% of total energy)	7.7 ± 2.1	6.3 ± 1.8	5.90 ± 1.52
Ratio of carbohydrate and total fat intake	1.47 ± 0.56	1.51 ± 0.57	1.07 ± 0.40[4]
Protein intake (% of total energy)	15.7 ± 2.8	18.0 ± 3.6	17.7 ± 2.74
Total energy intake, kcal	2131 ± 1208	1874 ± 636	2461 ± 805
CPT1A-cg00574958 methylation	0.104 ± 0.030	0.060 ± 0.020	0.10 ± 0.02

[1] All continuous variables were expressed as mean ± SD. *CPT1A,* carnitine palmitoyltransferase-1A; FHS, Framingham Heart Study; GOLDN, Genetics of Lipid Lowering Drugs and Diet Network; NA, not available; REGICOR, REgistre GIroní del COR study.

[2] Physical activity score in REGICOR was estimated as energy expenditure (MET∗min/week =total energy expenditure in PA in the same units: MET ∗ min per week, median, and IQR).

[3] Significant differences ($P \leq 0.05$) in comparison of GOLDN with FHS or REGICOR.

[4] Significant differences ($P \leq 0.05$) in comparison of REGICOR with GOLDN or FHS.

was treated as the dependent variable, CHO/FAT intake, and *CPT1A*-cg00574958 methylation sites were tested as predictors while controlling for age, sex, and cell-type heterogeneity, and family relationship.

Meta-analysis

A meta-analysis was conducted to examine the combined results across 3 populations (GOLDN, REGICOR, and FHS) with fixed-effect models using the meta R package (https://cran.r-project.org/web/packages/meta/index.html).

Mediation analysis

Mediation analysis was designed to determine the total, direct, and indirect effect of exposures in relation to the outcome by a specific causal mechanism—the mediator. It enables the causal interpretation of these effects under the assumption of the counterfactual framework (33). To determine whether CHO and FAT intake mediate metabolic phenotypes through *CPT1A*-cg00574958, we conducted a mediation analysis using Proc CAUSALMED of SAS 9.4. Here CHO and FAT intake were treated as exposures, *CPT1A*-cg00574958 as the single mediator, and metabolic traits and diseases as outcomes while controlling for covariates, sex, age, smoking, drinking, physical activity, and cell type. We estimated the total effect, direct effect, and indirect effect of dietary CHO and FAT on the metabolic traits or diseases from these models. The indirect effect measures the mediation effect of the exposure on the outcome through the mediator, independent of the direct effect of exposure (34).

Results

Characteristics of participating studies

Comparing the 3 study cohorts (**Table 1**) showed that GOLDN was a slightly younger population (mean age = 48.3 y) than FHS and REGICOR (66.2 y and 63.2 y, respectively). Thus, the prevalence of cardiometabolic diseases, hypertension, T2D, and MetS, were slightly higher in FHS and REGICOR than in GOLDN. Although all the cohorts were composed of participants of European ancestry, GOLDN and FHS were located in the United States, whereas REGICOR was a Spanish population. For dietary intake, US cohorts (GOLDN and FHS) tended to consume more total carbohydrate (CHO) (48.9% and 46.6%) than the Spanish (REGICOR) population (41.4%). In contrast, REGICOR consumed more total fat (FAT) (40.9% of total energy) than GOLDN and FHS (35.4% and 33.0%, respectively).

cg00574958 methylation at *CPT1A* is associated with CHO and FAT intake

As shown in **Supplemental Table 1**, we confirmed that *CPT1A*-cg00574958 methylation was consistently and negatively

associated with TG, glucose, BMI, T2D, hypertension, and MetS in all 3 populations. To test the hypothesis that CHO intake can induce epigenetic changes in *CPT1A*, we investigated the association between *CPT1A*-cg00574958 methylation and CHO intakes compared with the intake of other macronutrients. In all 3 populations (**Table 2**), *CPT1A*-cg00574958 methylation was positively and significantly associated with CHO intake normalized to total energy intake, with *P* values ranging from 1.56×10^{-4} (REGICOR) to 3.30×10^{-8} (FHS). With meta-analysis of the 3 populations, the association was even stronger with $\beta = 58.40 \pm 7.27$, *P* value $= 8.98 \times 10^{-16}$. However, FAT intake normalized to the total energy was significantly and negatively correlated with the *CPT1A*-cg00574958 methylation level, which was further evident ($\beta = -36.35 \pm 5.95$, *P* value $= 9.96 \times 10^{-10}$) from the meta-analysis (Table 2).

Because CHO and FAT intake are usually negatively correlated, we also examined the association between *CPT1A*-cg00574958 methylation and the ratio of CHO/FAT. As expected, *CPT1A*-cg00574958 methylation was positively correlated with the ratio of CHO/FAT. However, there was no significant association between *CPT1A*-cg00574958 methylation and protein intake in all 3 populations, nor based on a meta-analysis (Table 2). To determine if different types of carbohydrate intake show stronger association than total CHO, we divided the total CHO intake into simple and complex carbohydrate intake. Although *CPT1A*-cg00574958 methylation showed a stronger association with complex carbohydrate intake ($\beta = 35.48 \pm 9.64$ and *P* value $= 2.47 \times 10^{-4}$) than simple carbohydrate intake ($\beta = 20.9 \pm 8.77$, *P* value $= 0.017$), total CHO intake remained the strongest association with *CPT1A*-cg00574958 methylation. A similar and consistent association was also observed in FHS (Table 2).

Similarly, for the FAT intake, when dividing total FAT intake into MUFA, PUFA, and SFA, total FAT intake still exhibited the strongest association with *CPT1A*-cg00574958 methylation, either based on individual population or meta-analysis over 3 populations (Table 2) when compared with MUFA, PUFA, or SFA. Given this observation, from this point on, we continued our analyses with CHO and FAT intake.

CHO and FAT intake associated with *CPT1A* expression

It was demonstrated previously that *CPT1A*-cg00574958 methylation was negatively associated with *CPT1A* mRNA expression in whole blood in GOLDN and FHS (10). Here we examined the correlation between CHO and FAT intake and *CPT1A* mRNA expression in FHS. As shown in **Figure 1**, CHO was negatively associated with *CPT1A* expression ($\beta = -0.003 \pm 0.001$, $P = 0.0007$), whereas FAT intake was marginally and positively associated with *CPT1A* expression ($\beta = 0.003 \pm 0.002$, $P = 0.0169$). Similar to CHO intake, the ratio of CHO/FAT was negatively associated with *CPT1A* expression ($\beta = -0.039 \pm 0.015$, $P = 0.0083$).

CPT1A expression and metabolic traits

Because *CPT1A*-cg00574958 methylation was strongly associated with TG, glucose, BMI, and hypertension, we then examined whether *CPT1A* expression was correlated with these metabolic traits (**Figure 2**). We found a strong positive correlation

TABLE 2 Association between *CPT1A* cg00574958 methylation and carbohydrate and fat intake normalized to total energy[1]

	GOLDN ($n = 978$)			FHS ($n = 2331$)			REGICOR ($n = 645$)			Meta-analysis		
Dietary intake	β	SE	*P* value	β	SE	*P* value	β	SE	*P* value	β	SE	*P* value
Carbohydrate (% of total energy)	57.44	12.66	5.73×10^{-6}	54.36	9.81	3.30×10^{-8}	79.22	20.82	1.56×10^{-4}	58.40	7.27	8.98×10^{-16}
Total sugar (% of total energy)	20.9	8.77	0.017	39.68	8.47	2.97×10^{-6}	NA	NA	NA	NA	NA	NA
Complex carbohydrate (% of total energy)	35.48	9.64	2.47×10^{-4}	14.78	6.42	0.0220	NA	NA	NA	NA	NA	NA
Total fat (% of total energy)	−34.23	10.07	6.79×10^{-4}	−36.1	7.97	6.19×10^{-6}	−45.78	19.38	0.0185	−36.35	5.95	9.96×10^{-10}
Carbohydrate:total fat ratio	3.01	0.89	7.18×10^{-4}	3.54	0.69	3.12×10^{-7}	3.11	1.10	5.08×10^{-3}	3.30	0.49	1.48×10^{-11}
MUFA (% of total energy)	−12.3	3.89	1.56×10^{-3}	−14.2	3.45	3.91×10^{-5}	−21.69	12.86	0.0923	−13.70	7.27	6.37×10^{-8}
PUFA (% of total energy)	−6.43	2.88	0.025	−4.88	2.21	0.028	−7.98	4.36	0.0681	−5.81	1.38	4.00E-04
SFA (% of total energy)	−11.04	3.69	2.86×10^{-3}	−13.98	3.31	2.45×10^{-5}	−14.24	7.10	0.0452	−12.84	2.33	3.48E-08
Protein (% of total energy))	−5.54	3.85	0.151	3.79	3.76	0.314	−1.66	7.72	0.830	−0.86	2.54	0.735

[1] *CPT1A*, carnitine palmitoyltransferase-1A; FHS, Framingham Heart Study; GOLDN, Genetics of Lipid Lowering Drugs and Diet Network; NA, not available; REGICOR, REgistre GIroní del COR study.

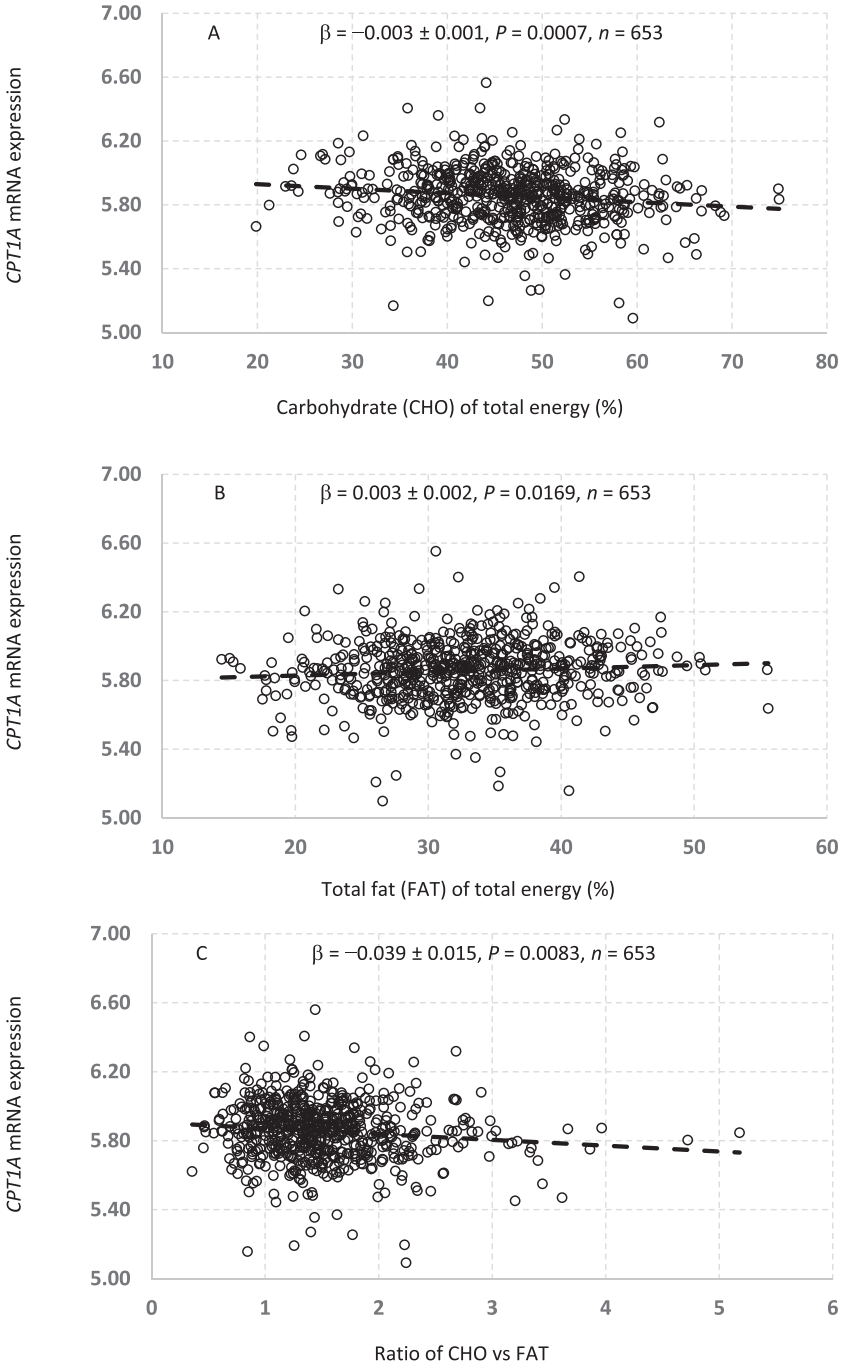

FIGURE 1 Association between carnitine palmitoyltransferase-1A (*CPT1A*) mRNA expression and (A) total carbohydrate intake (CHO), (B) total fat intake (FAT), and (C) ratio of CHO:FAT normalized to total energy. *CPT1A* mRNA expression was measured in participants ($n = 653$) of Framingham Heart Study Offspring at Exam 8 who were not taking medication for dyslipidemia, diabetes, or hypertension. Regression β and P values were calculated using mixed linear models while adjusting for age, sex, cell-type heterogeneity, and family relationships. Dashed lines represent trendlines of the association between dietary intake (CHO, FAT, or ratio) and *CPT1A* mRNA expression.

between *CPT1A* mRNA expression and TG ($\beta = 0.131 \pm 0.031$, P value $= 2.04 \times 10^{-5}$), fasting glucose ($\beta = 0.029 \pm 0.007$, P value $= 8.20 \times 10^{-6}$), and BMI ($\beta = 1.525 \pm 0.689$, P value $= 2.24 \times 10^{-4}$) in participants not taking medication for diabetes, hypertension, or dyslipidemia in FHS while adjusting for sex, age, smoking, drinking, and family relationships. Interestingly, for glucose concentration, men and women showed a distinct pattern with *CPT1A* expression, with men having, on average, 6.3 mg/dL higher glucose than women given the same amount of *CPT1A* mRNA expression (data not shown).

Direct and indirect effects of CHO and FAT intake on metabolic traits through *CPT1A* methylation as a mediator

Considering the strong association of *CPT1A*-cg00574958 methylation with CHO intake, FAT intake and metabolic

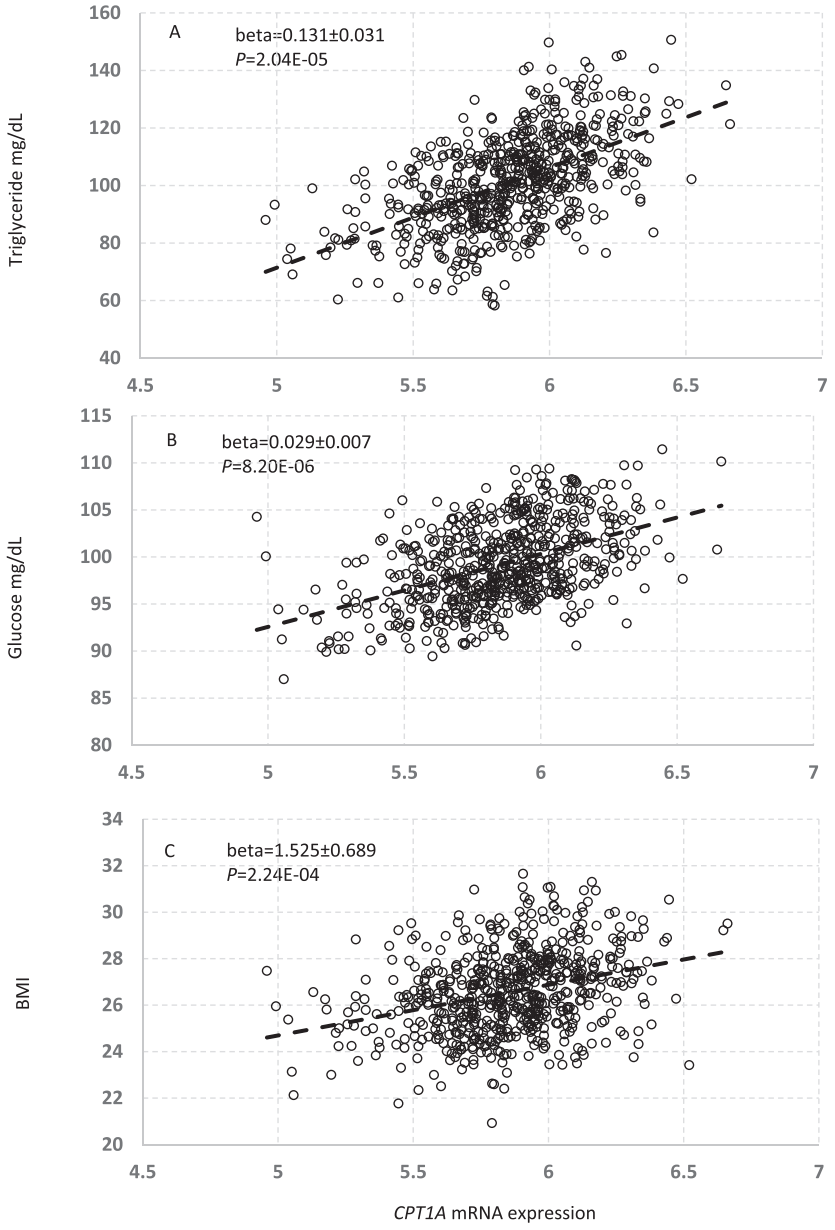

FIGURE 2 The association between metabolic traits and carnitine palmitoyltransferase-1A (*CPT1A*) mRNA expression in whole blood. *CPT1A* mRNA expression was measured in participants ($n = 653$) of Framingham Heart Study Offspring at Exam 8 who were not taking medication for dyslipidemia, diabetes, or hypertension. β and P values were estimated based on a linear regression model while adjusting for potential confounding factors: sex, smoking status, alcohol use, physical activity, family relationship, and heterogeneity of different cell types. The *y*-axis designates metabolic measures (triglyceride, glucose, BMI) after adjusted for confounding factors, and the *x*-axis is for *CPT1A* mRNA expression. Dashed lines represent trendlines of the association between metabolic traits and *CPT1A* mRNA expression. (A) Fasting triglyceride; (B) fasting glucose; and (C) BMI.

measures, in addition to the report that high fructose intake in rats induced *CPT1A* methylation (12, 13), we have a compelling rationale that methylation of the *CPT1A* gene is a mediator of CHO intake affecting metabolic phenotypes. Next, we conducted a mediation analysis to examine *CPT1A*-cg00574958 methylation as a mediator of CHO intake on metabolic outcomes in GOLDN and FHS.

As evident in **Table 3**, CHO intake showed a negative direct effect on glucose, BMI, and T2D, but not hypertension and MetS in GOLDN. Interestingly, CHO intake had a positive direct effect (i.e., not through the mediator, cg00574958) on TG in both GOLDN and FHS ($\beta = 0.0027$ and 0.0018, respectively). Importantly, in both GOLDN and FHS, CHO intake had a significant and negative indirect effect (through the mediator) on all metabolic traits we examined: TG, glucose, BMI, T2D, hypertension, and MetS, with P values ranging from 0.0289 to 1.35E-08. This observation strongly suggests that CHO intake decreases the risk of metabolic diseases mediated through *CPT1A*-cg00574958 methylation.

Similarly, in **Table 4**, FAT intake had a significant negative direct effect on TG only in GOLDN, but showed a significant and positive direct effect on glucose, BMI, and T2D, in both

TABLE 3 Direct and indirect effect of carbohydrate intake on metabolic traits with *CPT1A* cg00574958 as mediator in GOLDN and FHS[1]

		GOLDN ($n = 978$)						FHS ($n = 2331$)					
Outcome	Effect	Estimate	SE	Wald 95% CI		z	P value	Estimate	SE	Wald 95% CI		z	P value
$\log_{10}$ TG[2]	Total effect	0.0012	0.0009	−0.0006	0.0029	1.26	0.207	0.0007	0.0007	−0.0005	0.0021	1	0.3169
	NDE	0.0027	0.0009	0.0010	0.0044	3.12	1.80×10^{-3}	0.0018	0.0007	0.0005	0.0032	2.61	8.90×10^{-3}
	NIE	−0.0016	0.0003	−0.0022	−0.0009	−4.61	4.03×10^{-6}	−0.0011	0.0002	−0.0016	−0.0006	−4.59	4.43×10^{-6}
$\log_{10}$ Glucose[3]	Total effect	−0.0006	0.0002	−0.0009	−0.0002	−3.18	1.50×10^{-3}	−0.0007	0.0001	−0.0009	−0.0004	−5.09	3.58×10^{-7}
	NDE	−0.0004	0.0002	−0.0008	−0.0001	−2.51	0.0121	−0.0006	0.0001	−0.0008	−0.0003	−4.21	2.55×10^{-5}
	NIE	−0.0001	0.0000	−0.0002	−0.0001	−3.22	1.30×10^{-3}	−0.0001	0.0000	−0.0002	−0.0001	−4.46	8.20×10^{-4}
BMI	Total effect	−0.0671	0.0208	−0.1078	−0.0264	−3.23	1.20×10^{-3}	−0.0973	0.0134	−0.1236	−0.0710	−7.25	4.17×10^{-13}
	NDE	−0.0477	0.0206	−0.0882	−0.0072	−2.31	0.0209	−0.0793	0.0133	−0.1053	−0.0532	−5.96	2.52×10^{-9}
	NIE	−0.0194	0.0051	−0.0293	−0.0095	−3.83	1.00×10^{-4}	−0.0180	0.0032	−0.0244	−0.0117	−5.58	2.41×10^{-8}
Type 2 diabetes	Total excess RR	−0.0515	0.0145	−0.0799	−0.0231	−3.56	4.00×10^{-4}	−0.0423	0.0075	−0.0569	−0.0277	−5.66	1.51×10^{-8}
	Excess RR due to NDE	−0.0451	0.0144	−0.0734	−0.0169	−3.13	1.80×10^{-3}	−0.0334	0.0075	−0.0481	−0.0187	−4.46	8.20×10^{-6}
	Excess RR due to NIE	−0.0064	0.0029	−0.0121	−0.0007	−2.19	0.0289	−0.0089	0.0018	−0.0124	−0.0053	−4.85	1.23×10^{-6}
Hypertension	Total excess RR	−0.0210	0.0105	−0.0415	−0.0004	−2	0.0454	−0.0186	0.0058	−0.0300	−0.0072	−3.19	1.40×10^{-3}
	Excess RR due to NDE	−0.0162	0.0107	−0.0371	0.0047	−1.52	0.129	−0.0145	0.0058	−0.0258	−0.0032	−2.51	0.0122
	Excess RR due to NIE	−0.0048	0.0019	−0.0086	−0.0010	−2.5	0.0126	−0.0041	0.0010	−0.0060	−0.0021	−4.08	4.50×10^{-5}
MetS	Total excess RR	−0.0154	0.0099	−0.0349	0.0040	−1.55	0.120	−0.0318	0.0063	−0.0441	−0.0195	−5.05	4.42×10^{-7}
	Excess RR due to NDE	−0.0053	0.0098	−0.0244	0.0139	−0.54	0.589	−0.0234	0.0061	−0.0354	−0.0114	−3.81	1.00×10^{-4}
	Excess RR due to NIE	−0.0102	0.0027	−0.0154	−0.0049	−3.81	1.00×10^{-4}	−0.0084	0.0015	−0.0113	−0.0055	−5.68	1.35×10^{-8}

[1]*CPT1A,* carnitine palmitoyltransferase-1A; FHS, Framingham Heart Study; GOLDN, Genetics of Lipid Lowering Drugs and Diet Network; MetS, metabolic syndrome; NDE, natural direct effect; NIE, natural indirect effect; TG, triglycerides.

[2]TG was log10 transformed and analyzed only in participants ($n = 1259$ for FHS, $n = 978$ for GOLDN) not taking antidyslipidemia medication.

[3]Glucose was log10 transformed and analyzed only in participants ($n = 2025$ for FHS and $n = 932$ for GOLDN) not taking antidiabetes medication.

TABLE 4 Direct and indirect effect of total fat intake on metabolic traits with *CPT1A*- cg00574958 as mediator in GOLDN and FHS[1]

		GOLDN ($n = 978$)						FHS ($n = 2331$)					
Outcome	Effect	Estimate	SE	Wald 95% CI		z	P value	Estimate	SE	Wald 95% CI		z	P value
$\log_{10}$ TG[2]	Total effect	−0.0028	0.0011	−0.0050	−0.0006	−2.53	0.0115	−0.0001	0.0009	−0.0018	0.0016	−0.08	0.9354
	NDE	−0.0041	0.0011	−0.0061	−0.0020	−3.83	0.0001	−0.0013	0.0008	−0.0029	0.0004	−1.51	0.1321
	NIE	0.0012	0.0004	0.0004	0.0020	3.08	0.0021	0.0012	0.0003	0.0006	0.0018	4.17	3.05×10^{-5}
$\log_{10}$ Glucose[3]	Total effect	0.0005	0.0002	0.0001	0.0009	2.47	0.0135	0.0007	0.0002	0.0004	0.0010	4.37	1.24×10^{-5}
	NDE	0.0004	0.0002	0.0000	0.0008	2.03	0.042	0.0006	0.0002	0.0003	0.0009	3.71	0.0002
	NIE	0.0001	0.0000	0.0000	0.0002	2.43	0.0152	0.0001	0.0000	0.0001	0.0002	3.84	0.0001
BMI	Total effect	0.0663	0.0257	0.0160	0.1167	2.58	0.0098	0.1043	0.0166	0.0719	0.1368	6.29	3.17×10^{-10}
	NDE	0.0502	0.0253	0.0005	0.0999	1.98	0.0477	0.0864	0.0163	0.0544	0.1184	5.29	1.22×10^{-7}
	NIE	0.0162	0.0057	0.0050	0.0273	2.85	0.0044	0.0179	0.0039	0.0103	0.0255	4.64	3.48×10^{-6}
Type 2 diabetes	Total excess RR	0.0544	0.0208	0.0135	0.0952	2.61	0.0091	0.0560	0.0102	0.0360	0.0761	5.48	4.25×10^{-8}
	Excess RR due to NDE	0.0481	0.0206	0.0077	0.0884	2.33	0.0196	0.0469	0.0100	0.0273	0.0666	4.68	2.87×10^{-6}
	Excess RR due to NIE	0.0063	0.0030	0.0004	0.0122	2.08	0.0373	0.0091	0.0022	0.0048	0.0134	4.16	3.18×10^{-5}
Hypertension	Total excess RR	−0.0024	0.0127	−0.0273	0.0226	−0.19	0.8507	0.0136	0.0073	−0.0007	0.0280	1.86	0.0628
	Excess RR due to NDE	−0.0066	0.0126	−0.0313	0.0181	−0.52	0.6012	0.0094	0.0072	−0.0047	0.0236	1.31	0.1919
	Excess RR due to NIE	0.0042	0.0019	0.0004	0.0080	2.17	0.0301	0.0042	0.0011	0.0020	0.0064	3.73	0.0002
MetS	Total excess RR	0.0126	0.0124	−0.0117	0.0369	1.01	0.3111	0.0384	0.0084	0.0219	0.0548	4.57	4.88×10^{-6}
	Excess RR due to NDE	0.0046	0.0120	−0.0189	0.0280	0.38	0.7027	0.0297	0.0080	0.0139	0.0454	3.69	0.0002
	Excess RR due to NIE	0.0080	0.0030	0.0021	0.0139	2.64	0.0083	0.0087	0.0019	0.0050	0.0124	4.62	3.84×10^{-6}

[1]*CPT1A,* carnitine palmitoyltransferase-1A; FHS, Framingham Heart Study; GOLDN, Genetics of Lipid Lowering Drugs and Diet Network; MetS, metabolic syndrome; NDE, natural direct effect; NIE, natural indirect effect; TG, triglycerides.

[2]TG was log10 transformed and analyzed only in participants ($n = 1259$ for FHS, $n = 978$ for GOLDN) not taking antidyslipidemia medication.

[3]Glucose was log10 transformed and analyzed only in participants ($n = 2025$ for FHS and $n = 932$ for GOLDN) not taking antidiabetes medication.

GOLDN and FHS. Strikingly, for the indirect effect, that is, the mediated effect through the mediator, *CPT1A*-cg00574958, FAT intake showed a consistent and positive effect on all the metabolic traits (TG, glucose, T2D, BMI, hypertension, and MetS) with *P* values varying from 0.0373 to 3.48 x 10^{-6}. This observation indicates that FAT intake increases the risk of metabolic diseases mediated through *CPT1A*-cg00574958 methylation.

Similar to CHO, the ratio of CHO/FAT (**Supplemental Table 2**) showed a negative indirect effect on all metabolic traits, with *P* values in the range of 0.154 (T2D in GOLDN) to 5.11 x 10^{-6}, regardless of its negative or positive direct effect.

Overall, despite their differences in direct effects (positive or negative), CHO, FAT, or ratio of both, showed very consistent indirect effects: the mediated effect through the mediator, *CPT1A*-cg00574958, on all the metabolic outcomes examined in both GOLDN and FHS.

Discussion

In this study, we demonstrated that CHO intake and the CHO/FAT ratio were consistently and positively associated, and FAT intake was negatively associated with *CPT1A*-cg00574958 methylation level in 3 populations. Meta-analysis further confirmed these strong associations. In addition, CHO intake and the CHO/FAT ratio were negatively correlated, and FAT intake was positively correlated with *CPT1A* gene expression, thereby linking methylation and gene expression. Based on mediation analysis, CHO, FAT, or their ratio, had significant mediation effects on all metabolic traits we examined in both GOLDN and FHS through the mediator, *CPT1A*-cg00574958 methylation. Given the published observation that both high fructose intake or a low-fat diet induced *CPT1A* methylation in rodents (13, 35), our findings support that CHO intake induces *CPT1A* methylation, and hence lowers risk of metabolic diseases (obesity, T2D, hypertension, MetS). In contrast, FAT intake, by inhibiting *CPT1A* methylation, increases that risk.

Dietary nutrients can induce DNA methylation and thus change gene expression in animals and humans (35–37). High sucrose consumption increased DNA methylation of *CPT1A* promoter regions and *CPT1A* expression in the rat liver (13, 35). In this study, our analysis demonstrated that CHO intake is positively associated with *CPT1A* methylation in 3 cohorts, and negatively correlated with *CPT1A* expression in peripheral blood mononuclear cells (PBMCs) in FHS, which could contribute to a reduced risk of metabolic diseases in humans (see below). Contrasting a diet high in CHO is the high-fat diet; a study in rats showed that high-fat diets upregulated *Cpt1a* gene expression in PBMCs (13), which is highly correlated with *Cpt1a* expression in the liver (38). High-fat–fed rats developed an increased risk of MetS (insulin resistance, atherogenic lipid profile, and hepatic fat deposition) in 4 mo. Hence, *Cpt1a* gene expression in PBMCs was proposed as an early biomarker of MetS that is associated with a high-fat and high-protein diet (38). In the present study, we showed that FAT intake is negatively associated with *CPT1A* methylation in 3 populations, and positively correlated with *CPT1A* expression in PBMCs in FHS. *CPT1A* mRNA expression was positively associated with TG, glucose, and BMI in participants not taking medication for diabetes, hyperlipidemia, or hypertension. Hence, our findings that CHO and FAT intake affect *CPT1A* methylation, then *CPT1A* expression, leading to reduced or increased risk of metabolic diseases, are supported by evidence in animal studies. However, the exact mechanisms by which CHO and FAT modify *CPT1A*-cg00574985 methylation remain to be revealed in future studies.

The question then can be posed: why does reduced *CPT1A* expression by CHO-induced methylation lead to protective effects against metabolic diseases? CPT1A facilitates the translocation of long-chain fatty acids across the mitochondrial membrane into the mitochondrial matrix, where β-oxidation takes place (6). Mitochondrial dysfunction induced by a high-fat diet is associated with insulin resistance in muscle, implying that excessive CPT1A activity overloads the mitochondria resulting from incomplete oxidation of long-chain fatty acids (39). Furthermore, glucose oxidation can drive malonyl-CoA levels sufficiently high to inhibit the mitochondrial fatty acid transporter CPT1. This leads to diminished β-oxidation of free fatty acids and an increase in the cytosol of long-chain acyl-CoA esters (40–42). Conversely, inhibition of CPT1A activity in pancreatic β-cells is associated with increased insulin secretion through the potentiation of the rise in cytosolic Ca^{2+} induced by the depolarization through the inhibition of the K^+ ATP channel caused by the increase in cellular ATP associated with glucose metabolism (40, 43). These observations support that CHO-induced *CPT1A* methylation and decreased *CPT1A* expression and activity lead to decreased risk of insulin resistance and other MetS-related traits.

Supported by the evidence that CHO intake induced *CPT1A* methylation and reduced gene expression in animal studies (12, 13), our mediation analysis suggests that CHO and FAT intake could have causal effects on the risk of metabolic diseases (TG, T2D, hypertension, MetS) mediated through *CPT1A* epigenetic changes. CHO intake had a positive direct effect on TG and negative direct effects on metabolic traits and diseases through *CPT1A* methylation. CHO intake showed consistent and negative indirect effects on all metabolic traits or diseases through the *CPT1A*-cg00574958 mediator. The direct effect of CHO (not through *CPT1A* methylation) on TG could imply that CHO can affect the risk of metabolic diseases through other mechanisms besides the CPT1A enzyme.

CHO can be divided into simple and complex carbohydrates, which displayed different strengths of association with *CPT1A*-cg00574958 methylation, but neither was stronger than CHO intake (total carbohydrate, Table 2). Similarly, based on mediation analysis, neither simple carbohydrates nor complex carbohydrates have stronger effects on the risk of metabolic diseases than total CHO (**Supplemental Tables 3** and **4**). In contrast, FAT intake, although having a direct effect on MetS in FHS, showed consistent positive indirect effects on metabolic traits/diseases through *CPT1A*-cg00574958 methylation in both cohorts. Subdividing FAT intake into SFA, MUFA, and PUFA showed that SFA and MUFA intake represents the strongest effect mediated through *CPT1A* methylation on the risk of metabolic diseases (**Supplemental Tables 5–7**). In comparison with FAT (total fat intake), the mediated effects (indirect effect) through *CPT1A* cg00574958 of 3 fat types can be ranked based on *P* values and β values: FAT > SFA > MUFA > PUFA (Supplemental Table 6), even though the composition of the diet for these dietary fats (in the percentage of total energy) is in a different order: FAT > MUFA > SFA > PUFA (Table 1). This can

imply less risk of increased MUFA intake in relation to metabolic diseases.

Although our observation supports that CHO intake decreased metabolic risk, and FAT intake increased the risk of metabolic diseases through regulation of CPT1A, energy metabolism is regulated by many pathways, whereas CPT1A is the rate-limiting enzyme of fatty acid oxidation (6). In animal studies, mitochondrial dysfunction is associated with insulin resistance in muscle, implying that excessive CPT1A activity leads to mitochondrial overload (39). However, inhibition of CPT1A activity in β-cells also promotes the development of insulin resistance (44). These observations imply that balanced CPT1A activity is highly correlated with metabolic outcomes. This is well supported by recent epidemiological studies based on 432,179 participants in 18 countries, demonstrating that both low (<40% of total energy intake) and high (>70% of total energy intake) carbohydrate consumption were associated with increased mortality when compared with moderate intake (45).

There are some limitations to the present study. First, our mediation analysis was based on observational studies. Although there is strong evidence from animal studies that high-sucrose or high-carbohydrate diets induce methylation at promotors of *CPT1A*, future intervention studies in humans are needed to confirm causal effects. Thus, conclusions based on findings from the current study need to be interpreted with caution. Second, *CPT1A*-cg00574958 methylation and gene expression were based on PBMCs rather than the most physiologically relevant tissue (liver). Yet, *Cpt1a* expression in PBMCs was highly correlated with that in the liver based on rat data (33).

In summary, we demonstrated that CHO and FAT intake are strongly associated with *CPT1A*-cg00574958 methylation and *CPT1A* expression. Our mediation analysis supports that dietary CHO and FAT intake mediate methylation at *CPT1A*, influencing the risk of metabolic diseases through energy metabolic homeostasis.

The authors' responsibilities were as follows—C-QL, JMO: study concept and design; C-QL, DMA, LDP, CES, SS-B, SA, HKT, MRI, PNH: acquisition of data; C-QL, TG, SS-B, CES, DF, CB: analysis and interpretation of data; C-QL, TG, SS-B, DF: statistical analysis; C-QL: drafting of the manuscript; C-QL, LDP, CES, BH, LDP, DKA, JMO: critical revision of the manuscript for intellectual content; JMO, DKA, RE: funding and supervision; and all authors: read and approved the final manuscript.

The authors report no conflicts of interest.

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