Early Prediction of Developing Type 2 Diabetes by Plasma Acylcarnitines: A Population-Based Study

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OBJECTIVE

Acylcarnitines were suggested as early biomarkers even prior to insulin resistance in animal studies, but their roles in predicting type 2 diabetes were unknown. Therefore, we aimed to determine whether acylcarnitines could independently predict type 2 diabetes by using a targeted metabolic profiling approach.

RESEARCH DESIGN AND METHODS

A population-based prospective study was conducted among 2,103 community-living Chinese individuals aged 50–70 years from Beijing and Shanghai with a mean follow-up duration of 6 years. Fasting glucose, glycohemoglobin, and insulin were determined at baseline and in a follow-up survey. Baseline plasma acylcarnitines were profiled by liquid chromatography–tandem mass spectrometry.

RESULTS

Over the 6-year period, 507 participants developed diabetes. A panel of acylcanitines, especially with long chain, was significantly associated with increased risk of type 2 diabetes. The relative risks of type 2 diabetes per SD increase of the predictive model score were 2.48 (95% CI 2.20–2.78) for the conventional and 9.41 (95% CI 7.62–11.62) for the full model including acylcarnitines, respectively. Moreover, adding selected acylcarnitines substantially improved predictive ability for incident diabetes, as area under the receiver operator characteristic curve improved to 0.89 in the full model compared with 0.73 in the conventional model. Similar associations were obtained when the predictive models were established separately among Beijing or Shanghai residents.

CONCLUSIONS

A panel of acylcarnitines, mainly involving mitochondrial lipid dysregulation, significantly improved predictive ability for type 2 diabetes beyond conventional risk factors. These findings need to be replicated in other populations, and the underlying mechanisms should be elucidated.

The escalating global epidemic of type 2 diabetes has contributed considerably to socioeconomic burdens in both developed and developing countries (1). To understand biological mechanisms and improve clinical predictions, it is essential to identify novel biomarkers to enhance the capability to predict early pathophysiological changes (2). As a largely preventable disease, early prediction is the key to control the epidemic trend of type 2 diabetes, particularly in those countries with

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large populations with prediabetes or undiagnosed diabetes, such as China, which has two-thirds of the patients with undiagnosed diabetes (3).

The concept of "mitochondrial stress" induced by mitochondrial lipid overload and increased incomplete fatty acid oxidation (FAO) has been proposed to have an important impact on the pathogenesis of insulin resistance and diabetes (4). This hypothesis postulates that emerging FAO dysregulating prior to insulin resistance and glucose deterioration may provide a unique window to discover novel biomarkers for early detection of diabetes. Carnitine is known to play a pivotal role in transporting long-chain fatty acids across the mitochondrial inner membrane for β-oxidation (5). As intermediates of carnitine metabolism, acylcarnitines were previously used to screen for genetic defects in FAO (6). Recent studies in obese and diabetic animal models showed that acylcarnitines might reflect mild FAO dysregulation and "mitochondrial stress" (4). Moreover, different profiles of acylcarnitines were detected comparing cases of obesity, insulin resistance, metabolic syndrome, or diabetes with relevant controls (7–10). However, it remains to be evaluated whether acylcarnitines are able to identify high-risk individuals for future development of type 2 diabetes.

With the rapid development of advanced metabolomic technology, omics-based biomarkers are expected to be used for earlier prediction and prevention (2,11). Therefore, by utilizing a targeted metabolomic profiling approach to accurately quantify acylcarnitine profiles, we aimed to investigate the predictive role of certain acylcarnitines, individually and collectively, for the incidence of type 2 diabetes and also to determine the extent that including certain acylcarnitines could enhance the predictive ability for the incidence of type 2 diabetes beyond conventional risk factors in a prospective cohort of middle-aged and elderly Chinese people.

RESEARCH DESIGN AND METHODS

Nutrition and Health of Aging Population in China Study

The Nutrition and Health of Aging Population in China (NHAPC) study, conducted in Beijing and Shanghai community-living adults aged 50–70 years at baseline, was a prospective study aimed to investigate the effects of environmental and genetic factors and their interactions in the development of metabolic diseases (12). In 2005, 3,289 residents were recruited by a multistage sampling method (12). Data on demographic variables, lifestyle, and health information were collected by a standardized questionnaire. Family history of diabetes was positive if a parent or sibling had diabetes. Body weight, height, and blood pressure were measured in a physical examination by standardized procedures. In 2011, all participants were invited to the 6-year follow-up survey. The questionnaires and anthropometric procedures in the baseline survey were applied with minor modifications in the follow-up survey (13). Peripheral venous blood samples were collected after overnight fasting. The measurements of glucose, glycohemoglobin (HbA_{1c}), and insulin were described previously (12,14).

Among the 3,289 participants enrolled at baseline, 760 of them (23.1%) either lost contact (n = 554) or refused to participate (n = 206). Therefore, a total of 2,529 eligible subjects were successfully enrolled in the follow-up survey. After excluding 331 individuals with diabetes at baseline, and 95 people without a baseline acylcarnitine profile, the current analysis consisted of 2,103 participants.

Ethical Approval

The study protocol was approved by the institutional review board of the Institute for Nutritional Sciences. Written informed consent was provided by all participants.

Acylcarnitine Profile Measurements

Plasma acylcarnitine profiles were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS). High-performance liquid chromatography grade of acetonitrile and methanol were obtained from Merck KGaA (Darmstadt, Germany). Carnitine hydrochloride (C0), acetylcarnitine hydrochloride (C2), and acetyl chloride were purchased from Sigma-Aldrich. 1-Butanol was obtained from CNW Technologies GmbH (Düsseldorf, Germany). Deuterium-labeled carnitine and acylcarnitines (NSK-B Set; Cambridge Isotope Laboratories, Inc., Tewksbury, MA) (Supplementary Table 1) were initially redissolved in 1 mL methanol as stock solution of internal standards (IS) for quantification. The aliquots of quality control (QC) plasma, by pooling the representative plasma samples of the NHAPC study, were adopted throughout sample test and calibration curve preparation. Sample preparation was modified from the method developed by Vreken et al. (15). Mixtures of 10 μL thawed plasma or QC plasma and 90 μL methanol containing 360-fold diluted IS were vibrated and centrifuged (15 min, 16,000 rcf, 4°C), and 80 μL supernatants were transferred and evaporated under nitrogen flow (45°C). Dried residues were redissolved in freshly prepared 1-butanol/acetyl chloride (9:1, volume for volume) for butyl ester derivatization (65°C, 15 min). Consequently, the derivatives were dried under nitrogen flow (45°C), reconstituted in acetonitrile/water (4:1, volume for volume), and centrifuged (15 min, 16,000 rcf, 4°C) prior to performing LC-MS/MS analysis.

Chromatographic separation of free carnitine and acylcarnitines was performed on the 1260 HPLC system (Agilent Technologies) with an HSS T3 column (3.0 × 100 mm, 3.5 μm; Waters Corp.). The sampler and column temperatures were maintained at 4°C and 40°C, respectively. The injection volume was 5 μL. The mobile phases contained 0.1% formic acid in ultrapure water (A) and 0.1% formic acid in acetonitrile (B). The flow rate of the mobile phase was 0.35 mL/min. A gradient elution program was performed as follows: 0–1 min, hold at 50% B; 9 min, 80% B; 11–16.5 min, 100% B; and 17–23 min, 50% B to re-equilibrate the column. Mass spectrometric analysis was performed on an Agilent 6410B QQQ mass spectrometer in a positive ESI mode with the following settings: capillary voltage 4,000 V, source gas temperature 300°C, drying gas (pure nitrogen) flow 10 l/min, and nebulizer 40 ψ. The fragmentor was 120 V, and the collision energy was optimized for carnitine and each acylcarnitine (20–50 eV). All targets were monitored by transition precursor and product (m/z = 85) in dynamic MRM detection mode. MassHunter software (version B.03.01; Agilent Technologies) was used for instrument control and data acquirement.

For calibration curves, the serial dilution (18-, 36-, 72-, 180-, 360-, 720-, 1,800-, 3,600-, 7,200-, and 18,000-fold dilution) of stock IS in methanol (90 μL)

and the aliquot of QC plasma (10 μL) were mixed, prepared, and determined according to the above protocol. The calibration curves were obtained by plotting the derivative's peak area of serially diluted IS to the corresponding injection amount (pmol) on column. Supplementary Table 1 showed good linearity within wide range, lower limit of quantitation, and precision. The acylcarnitines with a big retention time shift (>0.1 min) or signal-to-noise ratio <10 were checked by hand. The acylcarnitines below the lower limit of quantitation in >50% of samples were excluded. Finally, a total of 34 acylcarnitines with high quality were included in the current study. Endogenous concentrations of acylcarnitines were quantified in μmol/L by the calibration curves of appropriate IS. Those acylcarnitines without corresponding IS were quantified with the calibration curve of chromatographically neighboring or structurally similar IS.

Ascertainment of Diseases

Incidence of type 2 diabetes during the 6-year follow-up period in the NHAPC study was defined as presenting at least one of the following components: *1*) self-reported doctor-diagnosed diabetes, *2*) taking antidiabetic medications, and *3*) fasting glucose ≥7.0 mmol/L in the follow-up survey. Metabolic syndrome was defined based on the updated National Cholesterol Education Program Adult Treatment Panel III criteria for Asian Americans (12). Impaired fasting glucose was defined as 5.6≤ fasting glucose <7.0 mmol/L.

Statistical Analysis

Baseline plasma acylcarnitine concentrations and metabolic features were compared between incident diabetes case subjects and noncase subjects by ANCOVA in eligible participants. Plasma acylcarnitines and insulin were log transformed to approximate normality. *Z* scores of log-transformed acylcarnitines were used to summarize short-, medium-, and long-chain acylcarnitines, defined as carbon chains ≤6, 7–14, and ≥16, respectively. Partial correlation analysis on ranks (Spearman correlation) was used to calculate correlation coefficients among acylcarnitines, as well as of acylcarnitines with fasting glucose, HbA_{1c}, and insulin in the baseline and follow-up surveys, and their 6-year changes, after adjusting for age, sex, region, and residence. Relative risks (RRs) of type 2 diabetes per SD increase of each acylcarnitine were calculated by sequential logistic regression models after controlling for age, sex, geographical region (Shanghai or Beijing), residence (urban or rural), current smoking (yes or no), drinking (yes or no), physical activity (active or inactive), family history of diabetes (yes or no), BMI, systolic blood pressure, fasting glucose, and HbA_{1c} at baseline.

The elastic net regression model (16) (implemented in the R package glmnet [17]) was used to build a predictive model for incident diabetes. Elastic net regression is a regularized regression combining the Lasso and Ridge penalties to avoid overfitting and improve prediction performance. The prediction accuracy based on parameters of lambda.1se (more conservative) and lambda.min (optimal output from the model) were both examined. Since the results using these two parameters were similar, only the results for lambda.min (if unspecified) were presented. The predictive model scores were computed as the weighted sum of all covariates with weights equal to the regression coefficients from the predictive models. The RRs of type 2 diabetes per SD increase of predictive model score were calculated by logistic regression models. The RR attributed to acylcarnitines selected by the model was computed by taking the acylcarnitine score as the weighted sum of selected acylcarnitines, with weights equal to the coefficients from the model, and then estimate the RR due to the acylcarnitine score. Joint classification analyses were also conducted to examine whether the baseline status of impaired fasting glucose and metabolic syndrome could modify the associations. The likelihood ratio tests were used to assess statistical significance of the interactions.

To obtain an unbiased estimate of prediction accuracy, 10-fold cross-validation was used. In each run, the elastic net method was applied to 90% of the samples and the model obtained was applied to the remaining 10% of samples. The area under the receiver operator characteristic (ROC) curve (AUC) was computed using the predicted probability of type 2 diabetes and the true status of type 2 diabetes for each sample. Moreover, a cross-region prediction for the subpopulations living in Beijing and Shanghai was used to examine the robustness of our prediction approach. Analyses were performed using SAS version 9.3 (SAS Institute) and R version 3.0 (http://R-project.org/). Two-sided $P < 0.05$ was considered as statistical significance. Bonferroni correction was used for multiple testing adjustment when analyzing individual acylcarnitines in relation to incident diabetes risk.

RESULTS

Baseline Characteristics

During the 6-year follow-up, 507 (24.1%) of the 2,103 eligible participants developed type 2 diabetes. Among them, 83 (16.4%) reported doctor-diagnosed diabetes, 5 (0.99%) reported taking antidiabetic medications, and 419 (82.6%) had fasting glucose ≥7.0 mmol/L in the follow-up survey. The mean ± SD of glucose and HbA_{1c} values in incident diabetes and nondiabetes were 7.93 ± 1.48 mmol/L and 6.91 ± 1.06% (52 mmol/mol), and 6.01 ± 0.58 mmol/L and 6.34 ± 0.40% (46 mmol/mol), respectively. Baseline characteristics and plasma acylcarnitine concentrations were compared between incident diabetes case subjects and noncase subjects (Table 1). Compared with the noncase subjects, the case subjects were more likely to be Beijing residents and have a family history of diabetes. They also exhibited higher levels of BMI, systolic blood pressure, fasting glucose, HbA_{1c}, and insulin (all $P < 0.05$). With respect to baseline plasma acylcarnitines, the case subjects showed significantly higher concentrations of C0, C3DC, C4, C5, C5OH, C8:1, C10, C14OH, C14:1OH, C16:1, C16:2, C18, C18OH, C18:1, C18:2, C20, and C20:4 acylcarnitines, but lower levels of 3-dehydroxycarnitine, 3-dehydrocarnitine, C7DC, C10DC, C12, C12OH, and C12:1 acylcarnitines when compared with their counterparts without diabetes after Bonferroni correction. Differences were also observed in several acylcarnitines compared those with and without impaired fasting glucose, as well as those with and without metabolic syndrome at baseline (Supplementary Table 2).

Acylcarnitines and Metabolic Traits

The correlations were calculated among baseline acylcarnitines (Fig. 1). Although most acylcarnitines were correlated

Table 1—Baseline characteristics and acylcarnitines between incident diabetes case subjects and noncase subjects

	Nondiabetes (n = 1,596)	Diabetes (n = 507)	P for difference
Male, n (%)*	673 (42.2)	225 (44.4)	0.51
Age (years)*	58.4 (6.1)	58.3 (5.9)	0.75
Beijing residents, n (%)*	687 (43.1)	306 (60.4)	<0.001
Rural residents, n (%)*	937 (58.7)	294 (58.0)	0.69
Current smoker, n (%)	456 (28.6)	145 (28.6)	0.12
Current drinker, n (%)	432 (27.1)	153 (30.2)	0.48
Family history of diabetes, n (%)	148 (9.3)	63 (12.4)	0.033
Low physical activity, n (%)	806 (50.5)	242 (47.7)	0.57
BMI (kg/m^2)	23.9 (3.4)	25.5 (3.8)	<0.001
Systolic blood pressure (mmHg)	137 (21.7)	144 (22.8)	<0.001
Fasting glucose (mmol/L)	5.26 (0.53)	5.64 (0.57)	<0.001
RBC HbA_{1c}, % (mmol/mol)	5.65 (0.40) (38)	5.86 (0.46) (41)	<0.001
Fasting insulin (μU/mL)†	12.9 (12.6–13.3)	13.7 (13.1–14.4)	0.004
Acylcarnitines (μmol/L)			
C0	58.1 (14.8)	61.7 (14.9)	<0.001‡
3-Dehydroxycarnitine†	0.41 (0.40–0.42)	0.27 (0.26–0.29)	<0.001‡
3-Dehydrocarnitine†	2.74 (2.66–2.81)	2.09 (1.98–2.21)	<0.001‡
C2†	11.0 (10.8–11.3)	11.2 (10.8–11.5)	0.06
C3†	0.45 (0.44–0.46)	0.47 (0.45–0.48)	0.020
C3DC† (10^{-2})	4.23 (4.14–4.32)	4.82 (4.66–4.99)	<0.001‡
C4†	0.20 (0.19–0.20)	0.21 (0.20–0.22)	<0.001‡
C5† (10^{-2})	5.96 (5.84–6.08)	6.58 (6.34–6.82)	<0.001‡
C5OH† (10^{-2})	0.52 (0.51–0.53)	0.56 (0.54–0.57)	<0.001‡
C5:1† (10^{-2})	0.98 (0.96–1.01)	0.93 (0.90–0.96)	0.12
C6† (10^{-2})	3.66 (3.59–3.74)	3.58 (3.48–3.69)	0.68
C6OH† (10^{-2})	1.28 (1.25–1.30)	1.21 (1.16–1.25)	0.11
C6DC† (10^{-2})	0.55 (0.53–0.57)	0.52 (0.50–0.56)	0.17
C7DC† (10^{-2})	0.25 (0.25–0.26)	0.21 (0.20–0.22)	<0.001‡
C8†	0.20 (0.20–0.21)	0.21 (0.20–0.22)	0.14
C8:1†	0.44 (0.43–0.45)	0.53 (0.50–0.56)	<0.001‡
C10†	0.26 (0.25–0.27)	0.30 (0.28–0.31)	<0.001‡
C10DC† (10^{-2})	0.49 (0.48–0.50)	0.46 (0.45–0.47)	<0.001‡
C12† (10^{-2})	8.18 (7.97–8.40)	7.44 (7.13–7.75)	<0.001‡
C12OH† (10^{-2})	0.73 (0.71–0.75)	0.66 (0.64–0.69)	<0.001‡
C12:1† (10^{-2})	12.3 (11.9–12.6)	8.89 (8.46–9.33)	<0.001‡
C12DC† (10^{-2})	0.61 (0.58–0.64)	0.50 (0.47–0.54)	0.015
C14† (10^{-2})	2.20 (2.15–2.25)	2.16 (2.07–2.24)	0.41
C14OH† (10^{-2})	0.84 (0.81–0.87)	1.67 (1.54–1.81)	<0.001‡
C14:1OH† (10^{-2})	1.06 (1.02–1.10)	2.06 (1.90–2.23)	<0.001‡
C16† (10^{-2})	8.46 (8.32–8.60)	8.79 (8.55–9.04)	0.002
C16:1† (10^{-2})	3.47 (3.33–3.62)	6.64 (6.18–7.13)	<0.001‡
C16:2† (10^{-2})	1.44 (1.39–1.50)	2.93 (2.70–3.17)	<0.001‡
C18† (10^{-2})	4.55 (4.37–4.75)	11.3 (10.5–12.3)	<0.001‡
C18OH† (10^{-2})	0.17 (0.17–0.18)	0.23 (0.22–0.24)	<0.001‡
C18:1† (10^{-2})	4.05 (3.82–4.30)	9.90 (8.52–11.5)	<0.001‡
C18:2†	0.14 (0.13–0.14)	0.31 (0.29–0.33)	<0.001‡
C20† (10^{-2})	0.38 (0.36–0.39)	0.73 (0.68–0.79)	<0.001‡
C20:4† (10^{-2})	0.37 (0.35–0.38)	0.80 (0.74–0.87)	<0.001‡

Values are the arithmetic means (SD) unless otherwise stated. P values were calculated after adjustment for sex, age, region (Beijing/Shanghai), and residence (urban/rural). Percentages may not sum to 100 because of rounding. *Data not adjusted for its category. †Values are geometric mean (95% CI). ‡Bonferroni–corrected statistical significance.

with each other, the highest correlation coefficients ($r > 0.7$) were clustered among medium- and long-chain acylcarnitines. Interestingly, when assessing the correlations between baseline acylcarnitines and metabolic traits in the baseline and the follow-up surveys, much higher correlation coefficients with fasting glucose were noticed in the follow-up rather than in the baseline survey, especially for the long-chain acylcarnitines (Supplementary Table 3). Stronger correlations were also observed between baseline acylcarnitines and the change of fasting glucose than between baseline acylcarnitines and baseline fasting glucose. In contrast, the correlation coefficients of acylcarnitines with HbA_{1c} and insulin were similar in the baseline and the follow-up surveys for most acylcarnitines.

Acylcarnitine Selection and Prediction of Incident Type 2 Diabetes

At the 6-year follow-up, risk of type 2 diabetes was positively associated with baseline free carnitine (C0) and C3DC, medium-chain (C8:1, C10, C14OH, and C14:1OH), and long-chain acylcarnitines (C16:1, C16:2, C18, C18OH, C18:1, C18:2, C20, and C20:4), and inversely associated with carnitine precursors (3-dehydroxycarnitine and 3-dehydrocarnitine) and medium-chain dicarboxylic (C10DC and C12DC) and C12 acylcarnitines (C12, C12OH, and C12:1), after adjusting for age, sex, region, residence, smoking, drinking, physical activity, family history of diabetes, systolic blood pressure, BMI, fasting glucose, and HbA_{1c} with Bonferroni correction (Supplementary Table 4). When acylcarnitines were classified as categories, only long-chain ones, but not those of short- and medium-chain species, were significantly associated with high incident diabetes risk ($P < 0.001$), in accordance with the consistent effects for individual acylcarnitines (all $P < 0.001$; except C16, $P = 0.10$).

The predictive models were constructed with the selected variables by elastic net model. The RRs of type 2 diabetes per SD increase were 2.48 (95% CI 2.20–2.78) for the conventional model and 9.41 (95% CI 7.62–11.62) for the full model (conventional risk factors + acylcarnitines), with RR = 6.94 (95% CI 5.73–8.41) attributed to the selected acylcarnitines (Table 2). In joint classification analyses examining whether baseline status of impaired fasting glucose and metabolic syndrome could modify the associations (Supplementary Table 5), none of the interactions were statistically significant (all P-interaction > 0.05).

The coefficients of full models after running the elastic net model 10 times were presented in Supplementary Table 6. To explore the predictive ability of established models, ROC curve analyses were performed (Fig. 2). The AUC was

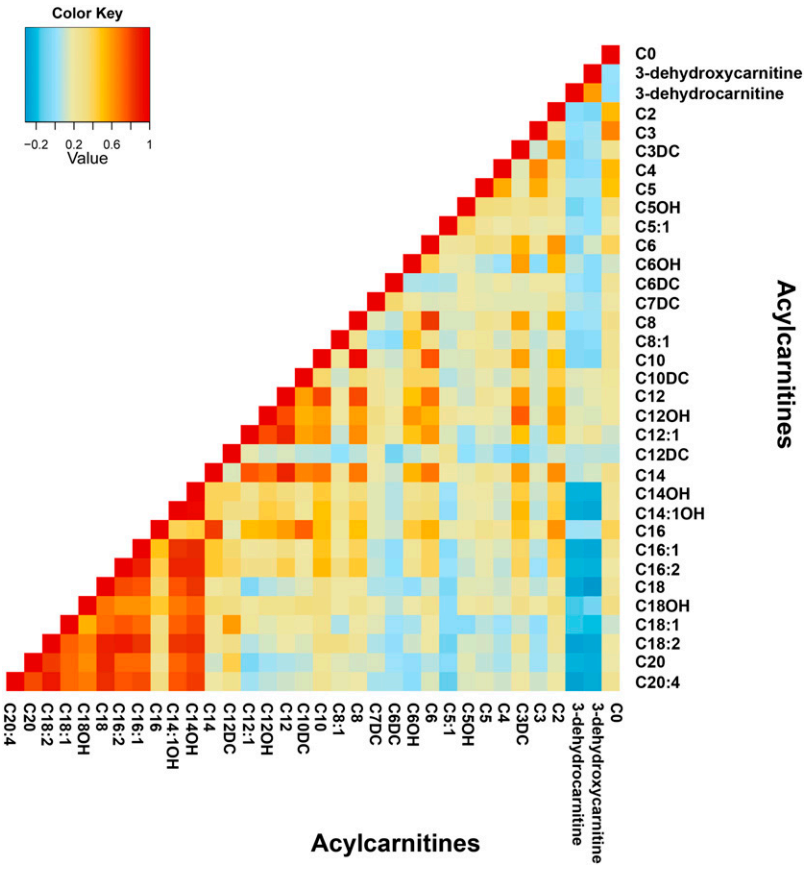

Figure 1—The correlations among baseline concentrations of acylcarnitines.

0.73 (95% CI 0.70–0.76) in the conventional model and significantly improved to 0.89 (95% CI 0.87–0.90) in the full model estimated based on 10-fold cross-validation.

Cross-Region Prediction

Cross-region predictions were applied to examine model robustness when applying to other regions in China. After being stratified by region, the elastic net models derived from Beijing and Shanghai participants separately were cross-validated. ROC curves were presented in Supplementary Fig. 1, in which the AUCs were 0.82 (95% CI 0.79–0.84) for the Shanghai model to predict Beijing participants and 0.87 (95% CI 0.85–0.90) for the Beijing model to predict Shanghai participants in the full models (s = lambda.min). The slight dropping of AUC compared to the 10-fold cross-validation result is not unexpected due to the great reduction in sample size of the training dataset for either region. When comparing the parameter coefficients of predictive models, the magnitude and direction of coefficients estimated based on the Beijing or Shanghai subpopulation or the entire population were consistent, as well as among lambda.min and lambda.1se models (Supplementary Fig. 2).

CONCLUSIONS

By applying a mass spectrometry–based acylcarnitine profiling platform, a panel of acylcarnitines, especially long-chain acylcarnitines, was found to be significantly associated with future risk of type 2 diabetes and also substantially improved predictive ability for incident diabetes beyond conventional risks, including BMI and fasting glucose.

To the best of our knowledge, the current study was the first study showing that acylcarnitines could effectively predict incident diabetes, by an accurate quantification of relevant metabolites (18). Previously, studies in obese and diabetic animal models demonstrated the links of acylcarnitines with mild FAO dysregulation and "mitochondrial stress," which led to or worsened insulin resistance and glucose deterioration (4). Moreover, treating myotube with acylcarnitines resulted in decreased Akt phosphorylation and glucose uptake in response to insulin stimulation (19). Indeed, suppression of mitochondrial fatty acid import or acylcarnitine synthesis could alleviate lipid-induced insulin resistance (4,19), supporting the postulation that lipid-induced "mitochondrial stress" predates the emergence of glucose deterioration and diabetes. To date, only a few small case-control studies and a weight loss trial showed that acylcarnitine profiles were associated

Table 2—Selected models and risk of incident diabetes

	Variables in model	RR per SD increase of predictive model score	*P*-trend
Model 1	Age, sex, region, residence, smoking, drinking, physical activity, family history of diabetes, BMI, fasting glucose, HbA_{1c}, and systolic blood pressure	2.48 (2.20–2.78)	<0.001
Model 2 (full model)	Age, sex, region, residence, smoking, drinking, physical activity, family history of diabetes, BMI, fasting glucose, HbA_{1c}, systolic blood pressure, and 3-dehydroxycarnitine, 3-dehydrocarnitine, C0, C3, C3DC, C4, C5, C5OH, C6OH, C6DC, C7DC, C8:1, C10, C10DC, C12:1, C12DC, C14:1OH, C16, C16:1, C16:2, C18, C18OH, C18:1, C18:2, and C20:4	9.41 (7.62–11.62), among which 6.94 (5.73–8.41) was attributed to the 25 acylcarnitines	<0.001

The predictive model scores were computed as the weighted sum of all covariates with weights equal to the regression coefficients from the predictive models built by the elastic net regression model.

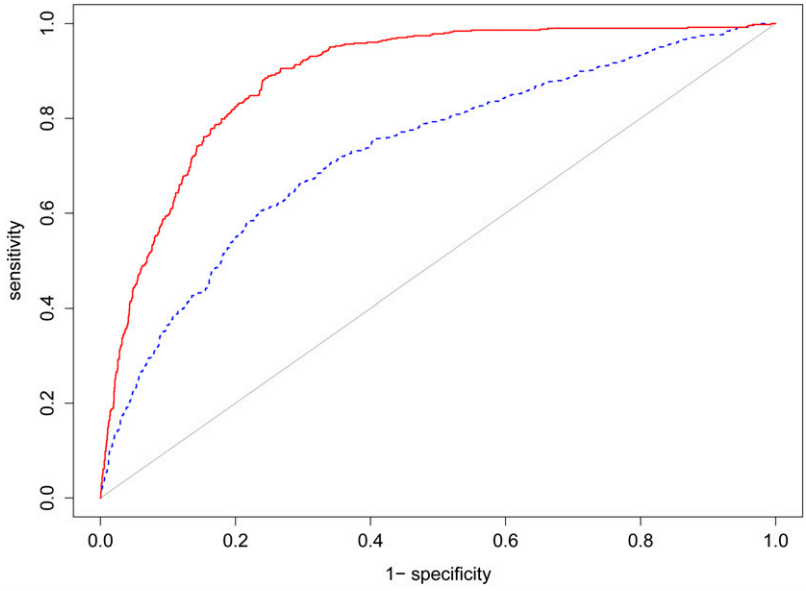

Figure 2—ROC curves for prediction of incident diabetes. Blue curve, conventional model including age, sex, region, residence, smoking, drinking, physical activity, family history of diabetes, BMI, fasting glucose, HbA_{1c}, and systolic blood pressure, AUC = 0.73 (95% CI 0.70–0.76); red curve, conventional model + acylcarnitines selected by elastic net model with s = lambda.min, AUC = 0.89 (95% CI 0.87–0.90).

with insulin sensitivity (20,21). Moreover, significantly different acylcarnitine profiles were observed among subjects with normal glucose tolerance, prediabetic states, or type 2 diabetes in a German study (22). Recently, several prospective studies indicated that medium- and/or long-chain acylcarnitines predicted cardiovascular events or mortality in people aged >85 years or selected patients undergoing cardiac catheterization or dialysis (23–25). However, previous European studies using the targeted BIOCRATES metabolomics platform failed to demonstrate a significant association between acylcarnitines and incident diabetes (26,27). Notably, the differences in study design, methodology (LC-MS/MS vs. flow injection analysis without chromatographic separation), and statistical analyses, as well as ethnic differences in genetic background and lifestyle might also explain, to a certain extent, the discrepancy between our study and others. In the current study, introducing selected acylcarnitines to the predictive models substantially improved the predictive ability for type 2 diabetes beyond conventional risk factors such as BMI and fasting glucose (AUCs: 0.89 vs. 0.73) (Fig. 2). Therefore, as a relatively simple alternative approach to assess mitochondria FAO dysregulation in vivo, specific acylcarnitine patterns may be considered promising early biomarkers for diabetes prediction.

It was noticed that most of the long-chain acylcarnitines were consistently associated with increased risk of incident diabetes. Long-chain fatty acids are oxidized predominately inside mitochondrion (28). They are transported across the mitochondrion inner membrane as long-chain acylcarnitines after being esterified from activated long-chain acyl-CoA by carnitine palmitoyl transferase I (CPT1), and consequently de-esterified by CPT2 to acyl-CoA for β-oxidation (5). As initial metabolites of β-oxidation, the accumulation of long-chain acylcarnitines might reflect relatively severe conditions of FAO dysregulation and mitochondrial overload with an impaired tricarboxylic acid cycle (4). Previously, obese individuals and individuals with diabetes were shown to have higher nonesterified fatty acids and long-chain acylcarnitines than those of noncase subjects (7). Although the underlining mechanism is unclear, studies in vitro showed that treatment with long-chain acylcarnitines induced insulin resistance and oxidative stress in human myotubes (19), as well as regulated calcium efflux in cardiac tissue and human ether-à-go-go-related gene (hERG) channels in renal cells (29,30). Likewise, the potential functional roles of these long-chain fatty acid derivatives might go beyond biomarkers of mitochondrial dysfunction.

Unlike those of long-chain acylcarnitines, the associations of medium-chain acylcarnitines with diabetes remain uncertain. One plausible explanation might be that different precursors or metabolic compartments are involved in the generation of medium-chain acylcarnitines, which could be later β-oxidation metabolites of long-chain fatty acids, or directly esterified derivatives from medium-chain fatty acids (31). In fact, controversial results of medium-chain fatty acids were reported as proinflammatory agents in some studies in vitro (32) but as antimicrobial, anti-inflammatory agents in other studies in vivo and in vitro (33,34). Certainly, the role of medium-chain acylcarnitines in metabolic disorders remains to be elucidated. On the other hand, elevated circulating 3-dehydroxycarnitine and 3-dehydrocarnitine, the precursors of L-carnitine productions (35), were shown to be associated with lower risk of incident diabetes. Previously, carnitine was suggested to improve insulin-stimulated glucose utilization and type 2 diabetes–related metabolic disorders in humans and rodents (36–38). Although the mechanistic interpretation remains unclear, the administration of 3-dehydroxycarnitine in a mouse model lacking L-carnitine transporter improved fatty acid metabolism (39), implicating the potential benefits of these carnitine precursors on maintaining energy hemostasis.

Other interesting findings were that dicarboxylic acid acylcarnitines with odd chains or even chains exerted different impacts on the development of type 2 diabetes. Malonylcarnitine (C3DC) was suggested to be an indicator to reflect malonyl-CoA levels (40). Malonyl-CoA is one of the key regulators in mammalian energy homeostasis by inhibiting mitochondrial CPT1 and FAO (41). Notably, insulin-resistant patients with obesity and type 2 diabetes had high muscle malonyl-CoA concentrations (42). However, it remains unclear whether malonyl-CoA accumulation, indicated by accumulated C3DC, serves as a feedback signal to suppress excessive β-oxidation and mitochondria stress by blocking CPT1 activity, thereby to switch fuel utilization from fatty acids to glucose in order to counteract lipid-induced

insulin resistance (4). Nevertheless, the positive C3DC-diabetes association in our study highlighted the role of malonyl-CoA in diabetes pathogenesis. Unlike odd-chain dicarboxylic acids, even-chain dicarboxylic acids are derived from ω-oxidation of fatty acids and thereafter undergo β-oxidation (43). Intervention studies indicated that sebacic acid (C10DC fatty acid) and dodecanedioic acid (C12DC fatty acid) had favorable impacts on glycemic control and energy utilization in patients with type 2 diabetes (43). In accordance with these findings, the observed inverse associations of C10DC and C12DC acylcarnitines with incident diabetes in our cohort supported potential benefits of sebacic acid and dodecanedioic acid in type 2 diabetes.

The elastic net model, performed successfully in both predictive accuracy and sparsity for the high-dimensional data sets (16), was used in the variable selection and model construction. Furthermore, our results were cross-region validated between Beijing and Shanghai, two megalopolises in Northern and Southern China with different lifestyles and environmental exposures. However, our study also has some limitations. First, only 77% of participants were successfully followed up, and a few baseline characteristics were found different comparing those lost to follow-up with all eligible participants, including more urban residents, and high levels of educational attainment, family income, and plasma triglycerides (13). Nevertheless, the follow-up rate was comparable to other prospective studies conducted in comparable age-groups (44,45). Second, the diagnosis of diabetes in the current study was based on either self-report, taking diabetes medications, or a single fasting glucose, which did not constitute a rigorous evaluation for the presence or absence of diabetes. Third, our participants are limited to middle-aged and elderly Chinese adults, who might have a higher risk of developing diabetes than Caucasians and younger adults. Thus, it is unknown whether the results could be extended to other age, racial, and ethnic groups. Finally, although our results were internally cross-validated, it is critical that they are independently replicated in different prospective cohorts.

In summary, the current study suggested that acylcarnitines significantly improved the predicting power for type 2 diabetes when compared with conventional risk factors, suggesting the potential utility of acylcarnitines as novel early predictors in diabetes risk assessment. These findings need to be replicated in other populations, and underlying biological mechanisms should be elucidated in future studies.

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