IJC
International Journal of Cancer

A prospective evaluation of serum methionine-related metabolites in relation to pancreatic cancer risk in two prospective cohort studies

Joyce Y. Huang[1,2], Hung N. Luu[1,2], Lesley M. Butler[1,2], Øivind Midttun[3], Arve Ulvik[4], Renwei Wang[1], Aizhen Jin[5], Yu-Tang Gao[6], Yuting Tan[6], Per M. Ueland[3,7], Woon-Puay Koh[5,8] and Jian-Min Yuan[1,2]

[1]Division of Cancer Control and Population Science, UPMC Hillman Cancer Center, University of Pittsburgh Medical Center, Pittsburgh, PA
[2]Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA
[3]Bevital A/S, Bergen, Norway
[4]Department of Clinical Science, University of Bergen, Bergen, Norway
[5]Health Service and Systems Research, Duke-NUS Medical School Singapore, Singapore, Singapore
[6]Department of Epidemiology, Shanghai Cancer Institute/Shanghai Jiaotong University, Shanghai, China
[7]Laboratory of Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway
[8]Saw Swee Hock School of Public Health, National University of Singapore, Singapore, Singapore

Deficiencies in methyl donor status may render DNA methylation changes and DNA damage, leading to carcinogenesis. Epidemiological studies reported that higher dietary intake of choline is associated with lower risk of pancreatic cancer, but no study has examined the association of serum choline and its metabolites with risk of pancreatic cancer. Two parallel case–control studies, one nested within the Shanghai Cohort Study (129 cases and 258 controls) and the other within the Singapore Chinese Health Study (58 cases and 104 controls), were conducted to evaluate the associations of baseline serum concentrations of choline, betaine, methionine, total methyl donors (i.e., sum of choline, betaine and methionine), dimethylglycine and trimethylamine *N*-oxide (TMAO) with pancreatic cancer risk. In the Shanghai cohort, odds ratios and 95% confidence intervals of pancreatic cancer for the highest quartile of choline, betaine, methionine, total methyl donors and TMAO were 0.27 (0.11–0.69), 0.57 (0.31–1.05), 0.50 (0.26–0.96), 0.37 (0.19–0.73) and 2.81 (1.37–5.76), respectively, compared to the lowest quartile. The corresponding figures in the Singapore cohort were 0.85 (0.23–3.17), 0.50 (0.17–1.45), 0.17 (0.04–0.68), 0.33 (0.10–1.16) and 1.42 (0.50–4.04). The inverse associations of methionine and total methyl donors including choline, betaine and methionine with pancreatic cancer risk in both cohorts support that DNA repair and methylation play an important role against the development of pancreatic cancer. In the Shanghai cohort, TMAO, a gut microbiota-derived metabolite of dietary phosphatidylcholine, may contribute to higher risk of pancreatic cancer, suggesting a modifying role of gut microbiota in the dietary choline-pancreatic cancer risk association.

Introduction

Worldwide pancreatic cancer is the 12th most common cancer in men and 11th most common cancer in women with an estimated number of new cases of 460,000 in 2018.[1] Pancreatic cancer is the third leading cause of cancer death in the US with an estimated 55,440 deaths due to pancreatic cancer in 2018,[2] with only 8% of patients survive 5 years after diagnosis.[3] Established risk factors for pancreatic cancer include chronic pancreatitis, obesity and type 2 diabetes.[4] Collectively, these risk factors are attributable to less than half of pancreatic cancer burden in the US.[5] The underlying causes are still controversial and unknown for majority of pancreatic cancer

Additional Supporting Information may be found in the online version of this article.

Key words: pancreatic cancer, risk factors, choline, betaine, methionine, trimethylamine N-oxide, DNA methylation, microbiota

Abbreviations: CI: confidence interval; CV: coefficient of variation; DMG: dimethylglycine; eGFR: estimated glomerular filtration rate; ICD: International Classification of Diseases-Oncology; LC–MS/MS: liquid chromatography–tandem mass spectrometry; MAPK: mitogen-activated protein kinase; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells or nuclear factor kappa B; NHANES: US National Health and Nutrition Examination Survey; OR: odds ratio; SAM: *S*-adenosylmethionine; SCH: Shanghai Cohort Study; SCHS: Singapore Chinese Health Study; SD: standard deviation; sTNF-R p55: human soluble TNF receptor *p55*; sTNF-R p75: human soluble TNF receptor *p75*; TLR: Toll-like receptor; TMA: trimethylamine; TMAO: trimethylamine N-oxide; TNF-alpha: tumor necrosis factor-alpha

DOI: 10.1002/ijc.32994
History: Received 30 May 2019; Accepted 11 Mar 2020; Online 29 Mar 2020

Correspondence to: Hung N. Luu, E-mail: luuh@upmc.edu

What's new?

Over half of all pancreatic cancers aren't associated with known risk factors. In this prospective study, the authors examined serum levels of three nutrients (choline, methionine, and betaine) that have been associated with reduced oxidative DNA damage and epigenetic changes such as methylation. They found that, as predicted, higher serum levels of these nutrients were correlated with lower pancreatic cancer risk. They also found that certain compounds associated with gut microbiota increased this risk. These results identify novel etiological factors that may guide future prevention strategies for pancreatic cancer.

cases. Therefore, it is an urgent need to identify novel etiological factors that would help the development of primary prevention strategies for pancreatic cancer.

Choline is involved in lipid transportation, methylation reaction and synthesis of neurotransmitters (i.e., glycine or glutamine)[6] and shapes the composition of gut microbiota. Abnormalities of choline metabolism were reported to be associated with oncogenesis and tumor progression.[6] In rats, choline deficiency altered composition of mitochondria membranes and induced excessive production of reactive oxygen species,[7] which can induce oxidative DNA damage, in turn promote carcinogenesis.[8] Choline may also influence DNA epigenetic change by its one-carbon metabolism to produce methionine and dimethylglycine (DMG).[9] Methionine is the precursor of *S*-adenosylmethionine (SAM), an important methyl donor in histone methylation.[10] Deficiency in methyl donors may result in global DNA hypomethylation, leading to genetic instability[11] and loss of heterozygosity.[12] In addition, hypomethylation was reported to induce endogenous retroviral elements, leading to the activation of proto-oncogenes, such as *c-myc*.[12]

Dietary restriction in choline and/or methionine was found to alter genome methylation pattern and enhanced incidence of carcinogen-induced pancreatic cancer.[13] Our group recently found a novel inverse association between intake of dietary choline and pancreatic cancer risk in a prospective cohort of Singapore Chinese men and women.[14] In a similar cohort study of a Swedish population, higher dietary intake of methionine was associated with lower risk of pancreatic cancer.[15]

Besides betaine, choline can be metabolized to trimethylamine (TMA) in the gut.[16] The metabolism is catalyzed by the bacteria enzyme choline TMA-lyase.[17] Two different phyla *Firmicutes* and *Proteobacteria* and eight genera (i.e., *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Escherichia fergusonii*, *Proteus penneri*, *Providencia rettgeri*, *and Edwardsiella tarda*) were reported to be involved in this metabolism process.[16] TMA, after absorbed and transported to the liver, is converted to trimethylamine N-oxide (TMAO) by the liver enzyme flavin monooxygenase (FMO).[18] TMAO has been shown to disturb cholesterol transport and bile acid synthesis and promote atherosclerosis.[19] Prior studies have showed that trimethylamine N-oxide (TMAO) was associated with the risk of cardiovascular events,[20] type 2 diabetes,[21] and colorectal cancer.[22] Similar to other gastrointestinal cancers,[23] the risk of pancreatic cancer has been found to be associated with oral and intestinal microbiota such as *Neisseria elongate*, *Streptococcus mitis*, and *Granulicatella adiacens*.[24] The exact mechanism for microbiota promoting pancreatic cancer development in humans, however, remains unknown.

To the best of our knowledge, no epidemiologic study has investigated the associations for serum levels of choline and its metabolites including betaine, DMG and TMAO with risk of pancreatic cancer. The objective of our study was to comprehensively examine these associations in two prospective cohorts of more than 80,000 participants.

Methods

Study population

Subjects were drawn from two population-based cohorts—the Shanghai Cohort Study and the Singapore Chinese Health Study.[25,26] All study participants from both cohorts provided written informed consent. Both cohort studies have been continuously approved by the Institutional Review Boards of the respective institutions—the Shanghai Cancer Institute, the National University of Singapore and the University of Pittsburgh.

The Shanghai Cohort Study[25] consisted of 18,244 male residents in four communities in Shanghai, China (representing 80% of eligible subjects), 45–64 years of age at enrollment during January 1986–September 1989. In addition to in-person interviews for information on use of tobacco and alcohol, usual diet and medical history, a 10-ml nonfasting blood sample and a single-void urine specimen were collected from each participant at baseline. Serum and urine samples were stored at −72°C until analysis.

The Singapore Chinese Health Study[26] enrolled 63,257 Chinese men and women 45–74 years of age at enrollment during April 1993–December 1998, in Singapore. At baseline each participant was interviewed in person by a trained interviewer using a structured questionnaire that requested information on demographics, lifetime use of tobacco, current consumption of alcoholic beverages, current physical activity, menstrual and reproductive histories (women only), occupational exposure, medical history, family history of cancer and dietary habits during the past 12 months using a validated[27] semi-quantitative food frequency questionnaire. A nonfasting blood (20 ml) and single-void urine specimens were requested from a random 3% sample of cohort participants during April 1994–December 1999. Beginning in January 2000, request for biospecimens was extended to all surviving cohort participants. Overall,

biospecimens were obtained from 32,535 participants, representing approximately 60% of eligible subjects by April 2005. Blood components (i.e., plasma, serum, buffy coat and red blood cells) were separated and stored at −80°C until analysis.

Alcohol consumption definition

We used a structured questionnaire for collection of information on subject's alcohol consumption for both cohorts. In Singapore Chinese Health Study,[28] participant was asked drinking frequency during the past year of four types of alcoholic beverages (i.e., beer, wine, Western and Chinese hard liquors), using eight predefined categories response: never or hardly, once a month, 2–3 times a month, once a week, 2–3 times a week, 4–6 times a week, once a day and ≥2 times a day. The portion size was defined as: (*i*) Beer: 1 small bottle (375 ml), 2 small bottles or 1 large bottle (750 ml), 2 large bottles or ≥3 large bottles; (*ii*) Wine: 1 glass (118 ml), 2, 3 or ≥4 glasses; (*iii*) hard liquor or Chinese rice wine: 1 shot (30 ml), 2, 3 or ≥4 shots. In Shanghai Cohort Study,[29] participant was asked if he had ever alcoholic beverages at least once a week for ≥6 months continuously. If the answer was "yes", he was asked to provide information about age he began drinking regularly and typical amount of beer, wine, or spirits consumed per week, separately. One drink was defined as 360 g of beer (12.6 g ethanol), 103 g of wine (12.3 g ethanol) or 30 g of spirits (12.9 g ethanol).[28,29]

Identification of pancreatic cancer cases

The incident cancer cases and deaths among participants of the Shanghai Cohort Study were identified through annual contacts with surviving study participants or next of kin for deceased participants, and through record linkage analyses with the databases of the population-based Shanghai Cancer Registry and the Shanghai Municipal Vital Statistics Office. The incident cancer cases and deaths among participants of the Singapore Chinese Health Study were identified *via* linkage analyses with the databases of the nationwide Singapore Cancer Registry and the Birth and Death Registry. Pancreatic cancer was defined as cancer cases with the International Classification of Diseases-Oncology, 9th edition (ICD-9) code 157 and ICD 10th edition (ICD-10) 2nd revision code C25. The follow-up for cancer incidence and death is virtually complete. To date, 612 (3.4%) participants of the Shanghai Cohort Study and 56 (<0.1%) participants of the Singapore Chinese Health Study were lost in annual follow-ups.

Nested case–control studies

The present study included two nested case–control studies. We identified 129 incident pancreatic cancer cases among participants of the Shanghai Cohort Study by December 31, 2009. For each case, two controls were randomly chosen among the cohort participants who were free of cancer and alive during the time from blood draw to pancreatic cancer diagnosis of the index case. The selected controls were individually matched to the index case by age (±2 years), date of blood draw (±1 month) and residence location at study enrollment.

For the Singapore Chinese Health Study, we identified 58 incident pancreatic cancer cases among participants who provided a prediagnostic blood sample as of December 2013. Similarly, we randomly selected up to two control subjects for each cancer case among all eligible participants who provided a baseline blood sample and were alive and free of cancer during the time from blood collection to cancer diagnosis of the index case. The controls were individually matched to the index case by gender, dialect group (Hokkien, Cantonese), age at enrollment (±3 years), date of baseline interview (±2 year) and date of biospecimen collection (±6 months).

To be consistent and comparable for biomarkers in serum for the nested case–control study from two different cohorts, we restricted the study subjects who provided baseline serum samples only. We excluded 12 control subjects from the Singapore cohort whose blood samples provided plasma but not serum sample. Thus, we included 104 control subjects of the Singapore cohort. The present study included a total of 187 incident pancreatic cancer cases and 362 individually matched controls.

Assessment of serum biomarkers

Serum specimens of cases and their matched controls were processed, aliquoted, shipped in frozen state and assayed together at Bevital A/S (www.bevital.no), Bergen, Norway. The serum samples of each matched case–control set (1 case and up to 2 controls) were placed next to each other in random order and tested in the same batch. The case/control status of all test samples was blind to laboratory personnel. We used liquid chromatography–tandem mass spectrometry (LC–MS/MS) to quantify serum choline, betaine, methionine, DMG, TMAO, creatinine and cotinine,[30] metabolites of choline,[31] and serum pyridoxal 5′-phosphate.[32] We additionally measured methionine sulfoxide, an oxidative metabolite of methionine formed during prolonged storage of serum samples.[30] Serum creatinine was used for the calculation of glomerular filtration rate (eGFR), an indicator of renal function.[33] Serum cotinine is a metabolite of nicotine, an indicator of recent exposure to tobacco smoking and use of other nicotine-containing products.[34] For quality control purpose, 14 duplicated samples derived from a pool of serum samples collected from cohort participants at the same time period of the study sample collection were dispersed in seven batches of test sample (two per batch). The within-batch coefficients of variation (CV) for all biomarkers tested ranged from 3.07% and 4.90% while the between-batch CV ranged from 2.29% to 11.24% (Supporting Information Table S1).

Statistical analysis

In the current analysis, the sum of methionine and methionine sulfoxide for each sample was used to represent total

amount of methionine. Total methyl donors were the sum of choline, betaine and methionine. We used natural logarithmic transformation of original values for statistical analysis to reduce their skewness and improve normal distribution. The analysis of covariance (ANCOVA) was used to examine the difference in concentrations among controls according to demographic characteristics and lifestyles, as well as between cases and controls.

Conditional logistic regression method was used to examine the associations for serum concentrations of individual choline metabolites and total methyl donors with pancreatic cancer risk. Study subjects were divided into quartiles according to the distribution of individual biomarkers among control subjects of each cohort. The magnitude of the association between levels of serum biomarkers and pancreatic cancer risk was evaluated using odds ratios (ORs) and their 95% confidence intervals (CIs). Ordinal values of quartile (i.e., 1, 2, 3 and 4) for each of the studied biomarkers were used for linear trend test in the biomarker-pancreatic cancer risk association.

Multivariable conditional logistic regression models for pancreatic cancer risk included the following covariates as potential confounders: body mass index (BMI) categories (<18.5, 18.5–<23 or ≥23 kg/m^2), level of education (no formal schooling, primary school, secondary school or above), smoking status (never, former or current smokers), serum cotinine (nmol/l), alcohol consumption (number of drinks per week), history of physician-diagnosed diabetes (yes, no), estimated glomerular filtration rate (eGFR),[32] and serum pyridoxal 5′-phosphate (nmol/l).[32] The analyses were conducted separately for the two cohorts and combined.

Stratified analysis by smoking status (never or ever smokers) and alcohol intake (nondrinkers or drinkers) were performed on the two cohorts combined. Potential effect modification of these risk factors on the biomarker-pancreatic cancer risk associations was assessed by adding to the model a product term of the biomarker of interest and alcohol intake (or smoking). We also conducted a sensitivity analysis by excluding all pancreatic cancer cases diagnosed within the first 2 years after blood draw (n = 13 incident cases which were excluded) and their matched controls. Statistical analyses were carried out using SAS software version 9.3 (SAS Institute, Cary, NC). All p values reported are two-sided. $p \leq 0.05$ was considered being statistically significant.

Data availability

De-identified data relevant to the report can be shared and is available upon request through the University of Pittsburgh for researchers who meet the criteria for access to confidential data. Data is accessible to the corresponding author and also is available from the University of Pittsburgh Institutional Data Access/Ethics Committee with the following contact information: 3500 Fifth Avenue, Hieber Building Main Office, Suite 106 Pittsburgh, PA 15213. Tel.: +1-412-383-1480. Fax: +1-412-383-1508. E-mail: ude.ttip@briksa.

Results

The mean (standard deviation [SD]) age at blood draw for study participants of the Shanghai and Singapore cohorts was 56.4 (5.5) and 64.4 (7.3) years, respectively. The average (range) time from blood draw to cancer diagnosis was 12.5 years (3 months-23.2 years) for cases of the Shanghai cohort, and 6.8 years (5 months–13.0 years) for cases of the Singapore cohort.

The characteristics of pancreatic cancer cases and matched controls of the Shanghai and Singapore cohorts were described previously.[32] There was a higher proportion of current smokers in pancreatic cancer cases than controls of the Shanghai cohort only at baseline (68.2% *vs.* 50.0%, p = 0.003) whereas no statistically significant differences between cases and controls in both Shanghai and Singapore cohorts, respectively, were observed for the distributions of age, sex, alcohol intake, history of diabetes, use of multivitamins and eGFR (Supporting Information Table S2).

Serum concentrations of choline, betaine, methionine and DMG were moderately correlated with each other. The Spearman correlation coefficient ranged from 0.202 (between choline and DMG) to 0.323 (between betaine and DMG). TMAO was positively associated with choline, betaine, methionine and DMG whereas eGFR was inversely associated with serum concentrations of all biomarkers except for betaine (Supporting Information Table S3).

We examined the potential effect of demographic and lifestyle factors on serum concentrations of all measured biomarkers among control subjects of the Shanghai and Singapore cohorts, separately. Age was positively associated with serum concentration of choline in both cohorts. Women had significantly lower serum levels of choline, DMG, betaine and methionine. BMI and alcohol consumption were inversely related to TMAO in the Singapore cohort. Former smokers had elevated level of choline in the Shanghai cohort whereas higher number of cigarettes was positively associated with choline and methionine in the Singapore cohort. Diabetic patients had significantly lower level of betaine in the Singapore cohort. Higher eGFR was associated with significantly lower serum concentrations of choline, DMG and TMAO in both controls of Shanghai and Singapore cohorts (Supporting Information Table S4).

Pancreatic cancer cases had significantly lower concentrations of choline and methionine in the Shanghai cohort, lower methionine and total methyl donors in Singapore cohort and lower levels of betaine, choline, methionine and total methyl donors in both cohorts combined, than their respective control subjects. Between the two cohorts, circulating levels of choline, betaine and DMG were significantly higher whereas total methyl donors and TMAO were significantly lower in both cases and controls of the Shanghai cohort than their counterparts of the Singapore cohort (all p values < 0.05; Table 1). Comparing with control subjects, pancreatic cancer cases had significantly lower levels of choline in the Shanghai cohort, methionine in both Shanghai and Singapore cohorts and total methyl donors in the Singapore cohort. When both

Table 1. Geometric means of serum concentrations of betaine, choline, methionine, total methyl donors, dimethylglycine (DMG) and TMAO among pancreatic cancer cases and control subjects (both Cohorts), the Shanghai Cohort Study and the Singapore Chinese Health Study

	Combined Cohorts			Shanghai Cohort			Singapore Cohort		
Biomarkers, μmol/l	Controls, *n* = 362	Cases, *n* = 187	*p*-value	Controls, *n* = 258	Cases, *n* = 129	*p*-value[1]	Controls, *n* = 104	Cases, *n* = 58	*p*-value[1]
Choline	19.0	18.0	0.05	22.2	20.5	0.03	15.8	15.9	0.91
Betaine	50.2	48.0	0.03	60.0	57.6	0.12	48.6	46.1	0.19
Methionine	33.3	31.2	0.002	33.6	31.6	0.01	32.4	30.0	0.04
Total methyl donors[2]	108.6	103.2	0.003	98.0	93.2	0.11	117.6	111.8	0.01
DMG	5.9	5.9	0.65	6.2	6.1	0.39	5.4	5.5	0.71
TMAO	3.7	4.1	0.16	3.1	3.6	0.12	4. 6	4.6	0.99

[1]*p*-Value to compare geometric means adjusted for age and gender.
[2]Total methyl donors: the sum of betaine, choline and methionine.
Abbreviations: DMG, dimethylglycine; eGFR, estimated glomerular filtration rate; TMAO, trimethylamine N-oxide.

cohorts combined, cases had significantly lower serum levels of choline, betaine, methionine and total methyl donors. While the difference in serum betaine concentration between cases and controls was not statistically significant for the two cohorts separately, such a difference in both cohorts combined reached statistical significance ($p = 0.03$; Table 1).

Given the difference in the serum concentrations of biomarkers between the Shanghai and Singapore cohorts, we used cohort-specific cut-off values of each biomarkers for grouping of study subjects into quartiles (Supporting Information Table S5). compared to the lowest quartile, higher quartiles of choline (the Shanghai cohort and the two cohorts combined), betaine (the two cohorts combined), methionine (two cohorts separately and combined) and total methyl donors (the Shanghai cohort and the two cohorts combined) were associated with significantly lower risk of pancreatic cancer, whereas higher TMAO was associated with higher risk of pancreatic cancer in the Shanghai cohort and in the two cohorts combined (Table 2). DMG was not associated with risk of pancreatic cancer in either of the two cohorts separately or combined (Table 2).

The inverse association between choline and pancreatic cancer risk was present in alcohol drinkers, but not in nondrinkers (Table 3). The ORs (95% CIs) for pancreatic cancer in the highest *versus* lowest quartile of choline was 0.26 (0.09–0.72) in alcohol drinkers ($p_{trend} = 0.01$) and 0.80 (0.39–1.64) in nondrinkers. The heterogeneity in the choline-pancreatic cancer risk association was statistically significant between alcohol drinkers and nondrinkers ($p_{interaction} = 0.03$). In contrast, the inverse association was presented in nondrinkers for betaine ($OR_{Q4\ vs.\ Q1} = 0.50$, 95% CI: 0.26–0.96, $p_{trend} = 0.02$). The methionine and total methyl donors were inversely associated with risk of pancreatic cancer in both alcohol drinkers and nondrinkers. Higher quartiles of TMAO were associated with elevated risk of pancreatic cancer in both alcohol drinkers and nondrinkers although the linear trend test in nondrinkers was not statistically significant ($p_{trend} = 0.19$).

The risk estimates for choline metabolites in relation to pancreatic cancer risk were similar for never smokers as those in ever smokers (all $p_{interaction} > 0.05$; Supporting Information Table S6). Higher levels of choline and methionine were significantly associated with lower risk of pancreatic cancer in ever smokers only whereas the inverse association between total methyl donors and risk of pancreatic cancer was present in both never smokers and ever smokers. The multivariable-adjusted ORs (95% CIs) for the highest *versus* lowest quartile of total methyl donors in never and ever smokers were 0.30 (0.11–0.83) and 0.41 (0.20–0.81), respectively (both $p_{trend} < 0.05$). On the other hand, higher levels of TMAO were associated with elevated risk of pancreatic cancer in both never and ever smokers, although none of the linear trend test was statistically significant (Supporting Information Table S6).

We also examined the associations between the biomarkers measured and pancreatic cancer risk by gender and did not find discernable difference in the association between male and female study participants in the Singapore cohort (all $p_{heterogeneity} > 0.05$; all participants were male in the Shanghai cohort). For example, the ORs (95% CIs) of pancreatic cancer for the highest *versus* lowest quartile of choline, betaine, methionine and total methyl donors among men were 2.05 (0.48–8.85), 0.33 (0.11–0.98), 0.25 (0.07–0.94) and 0.31 (0.09–1.11), respectively. The corresponding figures among women were 0.49 (0.08–2.97), 0.51 (0.09–2.83), 1.16 (0.24–5.69) and no risk estimate obtained for total methyl donors. The large variation in risk estimates by gender was due to small sample size, especially for women.

The sensitivity analysis after excluding incident cases of pancreatic cancer diagnosed within the first 2 years after blood draw and their matched controls provided the similar results as those observed in the entire dataset. Compared to the lowest quartiles, ORs (95% CIs) of pancreatic cancer in the highest quartiles of choline, methionine and total methyl donors, and TMAO were 0.38 (0.18–0.80), 0.41 (0.23–0.73), 0.38 (0.21–0.70), and 2.36 (1.26–4.36), respectively (all

Table 2. Associations between serum concentrations of serum choline, betaine, methionine, total methyl donors, DMG and TMAO and pancreatic cancer risk in pooled analysis of both cohorts

	Combined cohorts		Shanghai cohort OR (95% CI)[1]	Singapore cohort OR (95% CI)[1]
	Controls/cases	OR (95% CI)[1]		
Choline				
Q1	91/61	1.00	1.00	1.00
Q2	90/43	0.65 (0.39–1.08)	0.58 (0.31–1.06)	0.88 (0.29–2.65)
Q3	91/47	0.70 (0.40–1.23)	**0.42 (0.21–0.83)**	2.22 (0.68–7.24)
Q4	90/36	**0.42 (0.20–0.85)**	**0.27 (0.11–0.69)**	0.85 (0.23–3.17)
p_{trend}		**0.03**	**0.003**	0.98
Continuous scale (nmol/l)		0.98 (0.95–1.01)	0.97 (0.94–1.01)	1.00 (0.90–1.13)
Betaine				
Q1	91/67	1.00	1.00	1.00
Q2	90/41	**0.54 (0.32–0.93)**	**0.42 (0.21–0.82)**	0.94 (0.36–2.48)
Q3	91/37	**0.51 (0.30–0.87)**	0.59 (0.32–1.11)	**0.29 (0.09–0.95)**
Q4	90/42	**0.59 (0.35–0.98)**	0.57 (0.31–1.05)	0.50 (0.17–1.45)
p_{trend}		**0.04**	0.11	0.12
Continuous scale (nmol/l)		**0.99 (0.98–1.00)**	0.99 (0.98–1.01)	0.98 (0.95–1.01)
Methionine				
Q1	91/72	1.00	1.00	1.00
Q2	90/33	**0.48 (0.28–0.79)**	0.58 (0.32–1.04)	**0.33 (0.11–0.98)**
Q3	91/50	0.66 (0.41–1.08)	0.59 (0.32–1.07)	0.77 (0.29–2.10)
Q4	90/32	**0.40 (0.23–0.70)**	**0.50 (0.26–0.96)**	**0.17 (0.04–0.68)**
p_{trend}		**0.004**	**0.03**	**0.05**
Continuous scale (nmol/l)		**0.96 (0.94–0.99)**	**0.97 (0.94–1.00)**	**0.94 (0.89–0.99)**
Total methyl donors				
Q1	91/74	1.00	1.00	1.00
Q2	90/41	**0.48 (0.28–0.81)**	0.56 (0.30–1.02)	**0.28 (0.08–0.93)**
Q3	91/38	**0.44 (0.26–0.74)**	**0.37 (0.19–0.71)**	0.69 (0.25–1.87)
Q4	90/34	**0.38 (0.21–0.68)**	**0.37 (0.19–0.73)**	0.33 (0.10–1.16)
p_{trend}		**<0.001**	**0.001**	0.18
Continuous scale (nmol/l)		**0.99 (0.98–1.00)**	**0.99 (0.98–1.00)**	**0.98 (0.95–1.00)**
DMG				
Q1	91/53	1.00	1.00	1.00
Q2	90/41	0.75 (0.43–1.29)	0.81 (0.42–1.56)	0.63 (0.22–1.78)
Q3	91/44	0.74 (0.43–1.26)	0.86 (0.46–1.61)	0.50 (0.17–1.46)
Q4	90/49	0.93 (0.54–1.60)	0.96 (0.50–1.82)	0.94 (0.31–2.88)
p_{trend}		0.76	0.94	0.69
Continuous scale (nmol/l)		0.99 (0.92–1.07)	0.97 (0.88–1.07)	1.02 (0.92–1.13)
TMAO				
Q1	91/31	1.00	1.00	1.00
Q2	90/52	**1.92 (1.09–3.39)**	**2.00 (0.98–4.08)**	1.52 (0.58–4.00)
Q3	91/45	1.46 (0.83–2.59)	1.60 (0.79–3.25)	1.09 (0.40–2.93)
Q4	90/59	**2.36 (1.30–4.26)**	**2.81 (1.37–5.76)**	1.42 (0.50–4.04)
p_{trend}		**0.02**	**0.01**	0.71
Continuous scale (nmol/L)		1.01 (0.99–1.04)	1.01 (0.99–1.04)	0.99 (0.91–1.08)

[1]Odds ratios were calculated from conditional logistic regression models; adjusted for level of education (no formal schooling, primary school and secondary school or above), body mass index (<18.5, 18.5–<23.0, ≥23.0 kg/m^2), smoking status (never, former and current smokers), serum cotinine concentration (nmol/l), number of alcoholic drinkers per week (continuous), history of diabetes (no, yes), serum pyridoxal 5′-phosphate concentration (nmol/L) and estimated glomerular filtration rate ((ml/min/1.73 m^2). For $p < 0.05$, the values are in bold.
Abbreviations: DMG, dimethylglycine; TMAO, trimethylamine N-oxide.

Table 3. Associations between serum concentrations of choline, betaine, methionine, total methyl donors, DMG and TMAO and pancreatic cancer risk, stratified by alcohol drinking status

	Nondrinkers		Drinkers	
	Controls/Cases	OR (95% CI)[1]	Controls/Cases	OR (95% CI)[1]
Choline				
Q1	64/32	1.0	27/29	1.0
Q2	56/30	0.93 (0.49–1.75)	34/13	**0.29 (0.11–0.73)**
Q3	59/35	1.07 (0.57–2.02)	32/12	**0.38 (0.15–0.97)**
Q4	53/24	0.80 (0.39–1.64)	37/12	**0.26 (0.09–0.72)**
p_{trend}		0.69		**0.01**
$p_{interaction}$	**0.033**			
Betaine				
Q1	61/45	1.0	30/22	1.0
Q2	58/31	0.67 (0.36–1.23)	32/10	0.41 (0.15–1.10)
Q3	55/19	**0.43 (0.22–0.85)**	36/18	0.83 (0.34–1.98)
Q4	58/26	**0.50 (0.26–0.96)**	32/16	0.85 (0.34–2.09)
p_{trend}		**0.02**		0.97
$p_{interaction}$	0.269			
Methionine				
Q1	63/41	1.0	28/31	1.0
Q2	53/25	0.73 (0.38–1.38)	37/8	**0.17 (0.06–0.46)**
Q3	64/38	0.82 (0.46–1.48)	27/12	**0.36 (0.14–0.96)**
Q4	52/17	**0.44 (0.22–0.90)**	38/15	**0.38 (0.15–0.94)**
p_{trend}		**0.05**		**0.05**
$p_{interaction}$	0.664			
Total methyl donors				
Q1	55/30	1.0	36/44	1.0
Q2	47/16	**0.45 (0.20–1.00)**	43/25	**0.47 (0.24–0.93)**
Q3	39/15	0.68 (0.29–1.56)	52/23	**0.36 (0.18–0.70)**
Q4	35/8	**0.30 (0.11–0.83)**	55/26	**0.41 (0.20–0.81)**
p_{trend}		**0.04**		**0.005**
$p_{interaction}$				
DMG				
Q1	56/32	1.0	35/21	1.0
Q2	58/30	0.87 (0.46–1.65)	32/11	0.45 (0.17–1.19)
Q3	56/32	0.87 (0.45–1.67)	35/12	0.51 (0.20–1.34)
Q4	62/27	0.67 (0.34–1.35)	28/22	1.80 (0.71–4.55)
p_{trend}		0.29		0.29
$p_{interaction}$	0.145			
TMAO				
Q1	63/18	Ref.	28/13	Ref.
Q2	55/40	**2.52 (1.26–5.02)**	35/15	0.52 (0.19–1.46)
Q3	52/31	**2.17 (1.07–4.42)**	39/14	0.70 (0.25–1.92)
Q4	62/32	1.89 (0.93–3.85)	28/27	2.53 (0.94–6.85)
p_{trend}		0.19		**0.03**
$p_{interaction}$	0.546			

[1]Odds ratios were calculated from conditional logistic regression models adjusted for level of education (no formal schooling, primary school and secondary school or above), body mass index (‹18.5, 18.5–‹23.0, ≥23.0 kg/m^2), smoking status (never, former and current smokers), serum cotinine concentration (nmol/l), number of alcoholic drinkers per week (continuous), history of diabetes (no, yes), serum pyridoxal 5′-phosphate concentration (nmol/l) and estimated glomerular filtration rate ((ml/min/1.73 m^2). For p ‹ 0.05, the values are in bold.
Abbreviations: DMG, dimethylglycine; TMAO, trimethylamine N-oxide.

p_{trend} < 0.05). The inverse association between betaine and pancreatic cancer risk was not linear, but the risk estimates were significantly below one; the ORs (95% CIs) of pancreatic cancer for the second, third and fourth quartile of betaine were 0.57 (0.32–0.99), 0.49 (0.28–0.87) and 0.63 (0.37–1.06), respectively, compared to the lowest quartile (p_{trend} = 0.07). The null association between DMG and pancreatic cancer risk remained in this subset analysis (p_{trend} = 0.69; Supporting Information Table S7).

Discussion

To the best of our knowledge, this is the first study investigating the associations between serum concentrations of individual and total methyl donors including choline and its metabolites with pancreatic cancer risk. We found that highest quartiles of choline and total methyl donors in Shanghai cohort and of methionine and total methyl donors in both the Shanghai and Singapore cohorts in serum samples collected on average about 10 years prior to cancer diagnosis were associated with a statistically significant 40–70% lower risk of pancreatic cancer than the lowest quartile. We also found a significant interaction between choline and alcohol intake on pancreatic cancer risk. Our study also showed a significant association between high risk of pancreatic cancer and high concentration of TMAO in the Shanghai cohort, a gut microbiota-derived metabolite from choline and L-carnitine, which is abundant in red meat, and a mediator in chronic kidney disease patients.[35]

Our finding on the inverse association between serum methionine and pancreatic cancer risk is consistent with the results from a prior study in a Swedish population,[15] suggesting the association between methionine and pancreatic cancer risk is robust across different study populations.

Choline is obtained mainly from food sources such as animal liver, eggs, fish and milk[36] or through the *de novo* synthesis of phosphatidylethanolamine N-methyltransferase (PEMT)-related pathway. Choline is required for synthesizing phospholipids, particularly phosphatidylcholine and sphingomyelin, which are important components of cell membranes.[9] Experimental studies in rats showed that choline deficiency altered the composition of mitochondrial membranes, impaired mitochondria function and induced overproduction of reactive oxygen species.[37] Furthermore, rats deprived of dietary choline had accumulated oxidized purines and oxidative DNA damage.[38] In mammals, including humans, prolonged dietary choline deficiency can lead to cell death and functional disorders in the pancreas, liver, muscle and lymphocytes.[39]

Choline also plays a significant role in one-carbon metabolism (Fig. 1). Choline is converted to betaine, a reaction that is catalyzed by the choline dehydrogenase.[10] Betaine donates a methyl group to the re-methylation of homocysteine, forming methionine and DMG.[9] Methionine is the precursor of SAM, a key methyl donor in methylation of DNA and histones.[9] DNA methylation and histone modification lead to aberrant gene expression and increased cell growth and survival, potentially contributing to the development of pancreatic cancer.[40] In rodents, a diet depleted with choline and methionine resulted in decreased concentration of SAMs in tissues,[41] leads to hypomethylation and increased expression of oncogenes, including c-Ha-ras, c-Ki-ras and c-fos,[42] and increases the incidence of carcinogen-induced pancreatic carcinomas.[43] These experimental data suggest choline may play an important role in the development pancreatic cancer in humans.

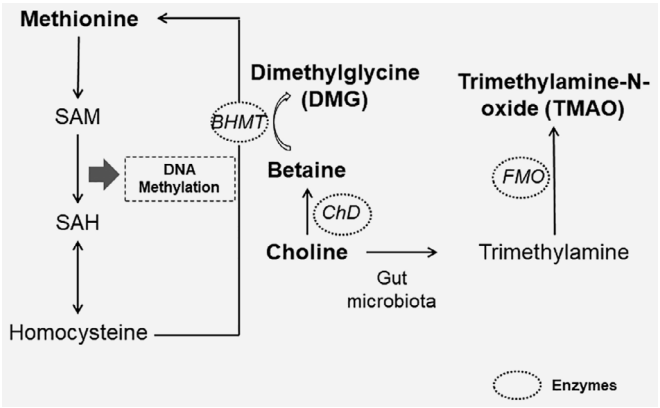

Figure 1. Schematic diagram of choline metabolism pathway.

Alcohol intake has significant impact on the metabolism and uptake of choline.[44] Our study demonstrated a statistically significant interaction between serum choline and alcohol drinking on pancreatic cancer risk. The disturbing effect of alcohol on absorption and transport of nutrients involved in one-carbon metabolism in the pancreas has been well documented.[45] Chronic ingestion of alcohol increased requirement of choline,[10] which may explain why the inverse association between serum choline and pancreatic cancer risk was stronger for alcohol drinkers than nondrinkers in our study. The similar inverse association between total methyl donors and pancreatic cancer in both alcohol nondrinkers and drinkers could be explained by the fact that betaine and methionine may compensate the decreased level of choline. In animal studies, betaine could partially replace dietary methionine[46] and betaine supplementation can reduce the dietary requirement for choline.[47] The lack of linear dose–response relationship between total methyl donors and pancreatic cancer risk among nondrinkers might be due to the small sample size. Future studies with larger samples are, therefore, warranted to replicate our findings.

The apparent inverse association between choline concentration and pancreatic cancer risk in Shanghai cohort might be explained by the fact that the Shanghai cohort included only men, younger age at enrollment and consumed more alcohol than the subjects of the Singapore cohort. Furthermore, the inverse association of choline with pancreatic cancer risk was seen only in drinkers and not in nondrinkers (Table 3). Our ability to detect the association between the

biomarkers studied and risk of pancreatic cancer among women was limited due to a small sample size (23 cases and 41 controls). These differences in the exposure to risk factors for pancreatic cancer and their impact on the circulating levels of choline may explain the more apparent choline-pancreatic cancer risk association in the Shanghai cohort than the Singapore cohort.

Our findings of an inverse association for methionine with lower risk of pancreatic cancer in both the Shanghai and the Singapore cohorts are inconsistent with those of a similarly designed case–control study involved 170 incident pancreatic cancer cases and 340 controls from a Japanese population.[48] In that study, Nakagawa *et al.*[48] reported a statistically nonsignificant reduced risk of pancreatic cancer (OR = 0.77, 95% CI: 0.17–3.44) for the highest *versus* the lowest quartile of serum methionine. Differences between our analysis and Nakagawa *et al.*[48] may due to the difference in study populations and assay platform. We used a validated liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay[31] in our study whereas the gas chromatography/MS/MS in the Japanese.[48] The mean concentrations of methionine among controls of the Shanghai and Singapore cohorts were 33.6 and 32.4 μmol/l, respectively, compared to the corresponding figure at 27.2 μmol/l, which was approximately 20% lower in healthy individuals of the Japanese study.[48] The difference in the method and study population may explain the different results between ours and the Japanese study.

Our study demonstrated a positive association between TMAO and pancreatic cancer risk in Shanghai cohort only. TMAO can be generated *via* TMA from choline and L-carnitine, nutrients that are abundant in red meat,[20] or provided by bacteria in the human oral cavity and gut.[49] No previous epidemiological study has investigated the association between TMAO and pancreatic cancer risk. However, a matched case–control study (n = 835 pairs) within the Women's Health Initiative Observational Study[22] found an increased risk of rectal cancer at high plasma TMAO. Although mechanistic studies directly linking TMAO to pancreatic cancer is lacking, TMAO may contribute to pancreatic cancer development through several plausible mechanisms. Recent experimental evidence suggests that trimethylamine (TMA)-producing microbiota in the gut could reduce the bioavailability of choline.[16] Alternatively, TMAO could contribute to low-grade chronic inflammation that is known to promote pancreatic cancer.[50] In human study, plasma concentration of TMAO was positively associated with pro-inflammatory biomarkers, including TNF-alpha, sTNF-R p55 and sTNF-R p75.[51] Microbial dysbiosis plays a role in the development of gastrointestinal cancers, including pancreatic cancer.[52] Accordingly Toll-like receptors (TLRs), which recognize compounds derived from microbes, are upregulated in human pancreatic cancers.[53] In mice studies, TLR activation promotes inflammation and accelerates carcinogenesis in the pancreas by stimulating the NF-kB and MAPK pathways.[50,53] Additionally, epidemiological studies suggest periodontal pathogens, in particular *P. gingivalis*, to be associated with increased risk of pancreatic cancer.[54] Experimental studies in mice showed that oral administration of *P. gingivalis* leads to an increase in *Bacteroidetes*, a TMA-producing bacteria phylum in the gut, and to increased expression of genes related to pro-inflammatory cytokines.[55] Mechanistic studies are warranted to reveal the exact mechanisms by which TMAO or the TMAO-producing bacteria influences pancreatic cancer development.

In the current study, lower eGFR, a test to measure kidney function, was significantly associated with high serum DMG and TMAO in both cohorts. This observation is consistent with a recent experimental study by Johnson *et al.*[56] in which they showed that mice fed a diet supplemented with 0.2% adenine to induced chronic kidney disease, resulting in increased serum TMAO concentration and that accumulation of circulating TMAO was accompanied by a decrease in renal clearance. Thus, it is important for the adjustment for kidney function in any study that examines the association for serum TMAO with disease risk. In the present study, we adjusted for eGFR in the statistical analysis, thus our results were less likely to be confounded by decreased renal function.

TMAO has a very long stability. Many dietary sources including fish and seafood contain TMAO. Dietary TMAO contributes to the increase of serum TMAO concentration.[57,58] In humans, long-term dietary L-carnitine can alter the production of TMAO from dietary precursors *via* the gut microbial composition; studies have shown that meat-eaters had higher concentration of circulating TMAO than vegetarians.[19] TMAO could be a link underlying the association between red meat intake and pancreatic cancer.[59] Interventions modulating gut microbiota, such as probiotics, may have beneficial effect on choline metabolism by shifting the bacterial production of TMAO from choline to the synthesis of membrane phospholipids and one-carbon groups by the host and thereby have a potential as primary prevention strategy against pancreatic cancer.

In the current study, we found that DMG is not associated with pancreatic cancer risk. One possible reason for this null association is that DMG is not a methyl donor, thus would not alter gene expression through epigenetic changes.[9]

The strengths of our study include prospective study design, long-term follow-up, inclusion of two independent cohorts and comprehensive assessment of methionine and their related metabolites using a validated state-of-the-art LC–MS/MC assay.[31] In addition, we adjusted for many potential confounders, especially eGFR, an important determinant of circulating concentration of choline and TMAO.[60] Serum concentrations of choline reflected both dietary intake and *de novo* synthesis of choline. No previous epidemiological study has assessed the associations for serum concentrations of various choline metabolites and total methyl donors with risk of pancreatic cancer. The main limitation of our study is modest sample size, especially for women. However, given a plausible biological mechanism for the role of one-carbon metabolism in the development of pancreatic cancer, our sample size with

187 cases and 362 controls would have at least 80% power to detect a minimal OR of 2 or 0.5 for the two extreme quartiles of biomarker concentrations (https://dceg.cancer.gov/tools/design/power). Thus, the present study provided a reasonable effect size of the biomarkers studied on the risk of developing pancreatic cancer.

Our finding on serum choline (in the Shanghai cohort alone and both cohorts combined), betaine (two cohorts combined), methionine (both the Shanghai and Singapore separately and combined) and TMAO (the Shanghai cohort alone and the two cohorts combined) in relation to pancreatic cancer risk has several implications and warrants future mechanistic studies. The adequate intake of choline is recommended at 550 mg for men and 425 mg for women.[36] According to the US National Health and Nutrition Examination Survey (NHANES) 2007–2008, the mean daily intake of choline was at 332 mg in men and 294 mg in women, 30–40% below the recommended daily intake levels, respectively. Foods high in choline include beef liver, eggs, fish and milk.[36] Strict vegetarians and vegans may be at greater risk for inadequate dietary intake of choline. Studies on the interaction between gene and nutritional status of choline may provide an evidence-based approach for the development of personalized nutrition strategy. On the other hand, epidemiological studies in general support a positive association between intake of red meat and pancreatic cancer risk,[61,62] although the mechanisms remain unclear.

In summary, our study found novel inverse associations between individual and total methyl donors and risk of pancreatic cancer, supporting a protective role of metabolites in the choline pathway against the development of pancreatic cancer in humans. Our study also showed an interaction effect between choline and alcohol drinking on pancreatic cancer risk. A statistically significant positive association between TMAO and pancreatic cancer suggests that gut microbiota may play a contributing role in the development of pancreatic cancer through their function on TMAO production.

Acknowledgements

We thank Xue-Li Wang of the Shanghai Cancer Institute for assistance with data collection and management and the staff of the Shanghai Cancer Registry for their assistance in verifying cancer diagnoses in study participants. We thank the Singapore Cancer Registry for the identification of incident cancer cases among participants of the Singapore Chinese Health Study. We also thank Siew-Hong Low of the National University of Singapore for supervising the field work of the Singapore Chinese Health Study. The Shanghai Cohort Study and the Singapore Chinese Health Study were supported by the National Institutes of Health (NIH) of the United States (grants # R01 CA144034 and UM1 CA182876). W-P Koh is supported by the National Medical Research Council, Singapore (NMRC/CSA/0055/2013). HN Luu is partially supported by the University of Pittsburgh Medical Center Hillman Cancer Center start-up grant.

Conflict of interest

Dr L.M.B. is currently a full-time employee of F. Hoffmann–La Roche LTD.

References

1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018;68:394–424.
2. American Cancer Society. *Cancer Facts Figures 2018*. Washington, DC: ACS, 2018.
3. Noone A, Howlader N, Krapcho M, et al. *SEER cancer statistics review, 1975–2015*. Bethesda, MD: National Cancer Institute, 2018 Based on November 2017 SEER data submission, posted to the SEER web site, April 2018. Available from: https://seer.cancer.gov/csr/1975_2015/.
4. Diet, Nutrition, Physical Activity and Cancer: a Global Perspective. *Continuous Update Project Expert Report 2018*. London: WCRF/AICR, 2018.
5. Maisonneuve P, Lowenfels AB. Risk factors for pancreatic cancer: a summary review of meta-analytical studies. *Int J Epidemiol* 2015;44:186–98.
6. Glunde K, Bhujwalla ZM, Ronen SM. Choline metabolism in malignant transformation. *Nat Rev Cancer* 2011;11:835–48.
7. Petrosillo G, Portincasa P, Grattagliano I, et al. Mitochondrial dysfunction in rat with non-alcoholic fatty liver involvement of complex I, reactive oxygen species and cardiolipin. *Biochim Biophys Acta* 1767;2007:1260–7.
8. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;420:860–7.
9. Zeisel SH. Dietary choline deficiency causes DNA strand breaks and alters epigenetic marks on DNA and histones. *Mutat Res* 2012;733:34–8.
10. Zeisel SH. Choline: critical role during fetal development and dietary requirements in adults. *Annu Rev Nutr* 2006;26:229–50.
11. Omura N, Goggins M. Epigenetics and epigenetic alterations in pancreatic cancer. *Int J Clin Exp Pathol* 2009;2:310–26.
12. Gaudet F, Hodgson JG, Eden A, et al. Induction of tumors in mice by genomic hypomethylation. *Science* 2003;300:489–92.
13. Sato N, Maitra A, Fukushima N, et al. Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. *Cancer Res* 2003;63:4158–66.
14. Huang JY, Butler LM, Wang R, et al. Dietary intake of one-carbon metabolism-related nutrients and pancreatic cancer risk: the Singapore Chinese health study. *Cancer Epidemiol Biomarkers Prev* 2016;25:417–24.
15. Larsson SC, Giovannucci E, Wolk A. Methionine and vitamin B6 intake and risk of pancreatic cancer: a prospective study of Swedish women and men. *Gastroenterology* 2007;132:113–8.
16. Romano KA, Vivas EI, Amador-Noguez D, et al. Intestinal microbiota composition modulates choline bioavailability from diet and accumulation of the proatherogenic metabolite trimethylamine-N-oxide. *MBio* 2015;6:e02481.
17. Craciun S, Balskus EP. Microbial conversion of choline to trimethylamine requires a glycyl radical enzyme. *Proc Natl Acad Sci USA* 2012;109:21307–12.
18. Krueger SK, Williams DE. Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. *Pharmacol Ther* 2005;106:357–87.
19. Koeth RA, Wang Z, Levison BS, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med* 2013;19:576–85.
20. Tang WHW, Wang Z, Levison BS, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N Engl J Med* 2013;368:1575–84.
21. Dambrova M, Latkovskis G, Kuka J, et al. Diabetes is associated with higher Trimethylamine N-oxide plasma levels. *Exp Clin Endocrinol Diabetes* 2016;124:251–6.
22. Bae S, Ulrich CM, Neuhouser ML, et al. Plasma choline metabolites and colorectal cancer risk in the Women's Health Initiative observational study. *Cancer Res* 2014;74:7442–52.
23. Schwabe RF, Jobin C. The microbiome and cancer. *Nat Rev Cancer* 2013;13:800–12.
24. Farrell JJ, Zhang L, Zhou H, et al. Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer. *Gut* 2012;61:582–8.
25. Yuan JM, Ross RK, Wang XL, et al. Morbidity and mortality in relation to cigarette smoking in Shanghai, China. A prospective male cohort study. *JAMA* 1996;275:1646–50.
26. Yuan J-M, Stram DO, Arakawa K, et al. Dietary cryptoxanthin and reduced risk of lung cancer: the Singapore Chinese health study. *Cancer Epidemiol Biomarkers Prev* 2003;12:890–8.
27. Hankin JH, Stram DO, Arakawa K, et al. Singapore Chinese health study: development, validation, and

calibration of the quantitative food frequency questionnaire. *Nutr Cancer* 2001;39:187–95.
28. Tsong WH, Koh W-P, Yuan J-M, et al. Cigarettes and alcohol in relation to colorectal cancer: the Singapore Chinese health study. *Br J Cancer* 2007; 96:821–7.
29. Yuan JM, Ross RK, Gao YT, et al. Follow up study of moderate alcohol intake and mortality among middle aged men in Shanghai, China. *BMJ* 1997; 314:18–23.
30. Midttun Ø, Hustad S, Ueland PM. Quantitative profiling of biomarkers related to B-vitamin status, tryptophan metabolism and inflammation in human plasma by liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2009;23:1371–9.
31. Midttun Ø, Kvalheim G, Ueland PM. High-throughput, low-volume, multianalyte quantification of plasma metabolites related to one-carbon metabolism using HPLC-MS/MS. *Anal Bioanal Chem* 2013;405:2009–17.
32. Levey AS, Stevens LA, Schmid CH, et al. CKD-EPI (chronic kidney disease epidemiology collaboration). A new equation to estimate glomerular filtration rate. *Ann Intern Med* 2009;150:604–12.
33. Benowitz NL, Hukkanen J, Jacob P. Nicotine chemistry, metabolism, kinetics and biomarkers. *Handb Exp Pharmacol* 2009;192:29–60.
34. Huang JY, Butler LM, Midttun Ø, et al. Serum B6 vitamers (pyridoxal 5′-phosphate, pyridoxal, and 4-pyridoxic acid) and pancreatic cancer risk: two nested case-control studies in Asian populations. *Cancer Causes Control* 2016;27:1447–56.
35. Tomlinson JAP, Wheeler DC. The role of trimethylamine N-oxide as a mediator of cardiovascular complications in chronic kidney disease. *Kidney Int* 2017;92:809–15.
36. Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline. *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline*. Washington, DC: National Academies Press, 1998.
37. Vrablic AS, Albright CD, Craciunescu CN, et al. Altered mitochondrial function and overgeneration of reactive oxygen species precede the induction of apoptosis by 1-O-octadecyl-2-methyl-rac-glycero-3-phosphocholine in p53-defective hepatocytes. *FASEB J* 2001;15: 1739–44.
38. Banni S, Corongiu FP, Dessi MA, et al. Free radicals and lipid peroxidation in liver of rats kept on a diet devoid of choline. *Free Radic Res Commun* 1989;7:233–40.
39. Zeisel SH, Blusztajn JK. Choline and human nutrition. *Annu Rev Nutr* 1994;14:269–96.
40. Vincent A, Herman J, Schulick R, et al. Pancreatic cancer. *Lancet* 2011;378:607–20.
41. Zeisel SH, Zola T, daCosta KA, et al. Effect of choline deficiency on S-adenosylmethionine and methionine concentrations in rat liver. *Biochem J* 1989;259:725–9.
42. Zapisek WF, Cronin GM, Lyn-Cook BD, et al. The onset of oncogene hypomethylation in the livers of rats fed methyl-deficient, amino acid-defined diets. *Carcinogenesis* 1992;13:1869–72.
43. Mizumoto K, Tsutsumi M, Denda A, et al. Rapid production of pancreatic carcinoma by initiation with N-nitroso-bis(2-oxopropyl)amine and repeated augmentation pressure in hamsters. *J Natl Cancer Inst* 1988;80:1564–7.
44. Halsted CH, Villanueva JA, Devlin AM, et al. Metabolic interactions of alcohol and folate. *J Nutr* 2002;132:2367S–72S.
45. Said HM, Mee L, Sekar VT, et al. Mechanism and regulation of folate uptake by pancreatic acinar cells: effect of chronic alcohol consumption. *Am J Physiol Gastrointest Liver Physiol* 2010;298: G985–93.
46. Fernández-Fígares I, Wray-Cahen D, Steele NC, et al. Effect of dietary betaine on nutrient utilization and partitioning in the young growing feed-restricted pig. *J Anim Sci* 2002;80:421–8.
47. Dilger RN, Garrow TA, Baker DH. Betaine can partially spare choline in chicks but only when added to diets containing a minimal level of choline. *J Nutr* 2007;137:2224–8.
48. Nakagawa T, Kobayashi T, Nishiumi S, et al. Metabolome analysis for pancreatic cancer risk in nested case-control study: Japan public health center-based prospective study. *Cancer Sci* 2018; 109:1672–81.
49. Benavides MA, Bosland MC, da Silva CP, et al. L-methionine inhibits growth of human pancreatic cancer cells. *Anticancer Drugs* 2014;25:200–3.
50. Fennema D, Phillips IR, Shephard EA. Trimethylamine and trimethylamine N-oxide, a flavin-containing monooxygenase 3 (FMO3)-mediated host-microbiome metabolic axis implicated in health and disease. *Drug Metab Dispos* 2016;44:1839–50.
51. Zambirinis CP, Pushalkar S, Saxena D, et al. Pancreatic cancer, inflammation, and microbiome. *Cancer J Sudbury Mass* 2014;20:195–202.
52. Rohrmann S, Linseisen J, Allenspach M, et al. Plasma concentrations of trimethylamine-N-oxide are directly associated with dairy food consumption and low-grade inflammation in a German adult population. *J Nutr* 2016;146:283–9.
53. Pushalkar S, Hundeyin M, Daley D, et al. The pancreatic cancer microbiome promotes Oncogenesis by induction of innate and adaptive immune suppression. *Cancer Discov* 2018;8: 403–16.
54. Ochi A, Graffeo CS, Zambirinis CP, et al. Toll-like receptor 7 regulates pancreatic carcinogenesis in mice and humans. *J Clin Invest* 2012;122:4118–29.
55. Michaud DS, Joshipura K, Giovannucci E, et al. A prospective study of periodontal disease and pancreatic cancer in US male health professionals. *J Natl Cancer Inst* 2007;99:171–5.
56. Nakajima M, Arimatsu K, Kato T, et al. Oral Administration of *P. gingivalis* induces dysbiosis of gut microbiota and impaired barrier function leading to dissemination of enterobacteria to the liver. *PLoS One* 2015;10:e0134234.
57. Johnson C, Prokopienko AJ, West RE, et al. Decreased kidney function is associated with enhanced hepatic flavin monooxygenase activity and increased circulating trimethylamine N-oxide concentrations in mice. *Drug Metab Dispos* 2018; 46:1304–9.
58. Maitre L, Lau C-HE, Vizcaino E, et al. Assessment of metabolic phenotypic variability in children's urine using 1H NMR spectroscopy. *Sci Rep* 2017; 7:46082.
59. Maitre L, Robinson O, Martinez D, et al. Urine metabolic signatures of multiple environmental pollutants in pregnant women: an exposome approach. *Environ Sci Technol* 2018;52:13469–80.
60. Rohrmann S, Linseisen J, Nöthlings U, et al. Meat and fish consumption and risk of pancreatic cancer: results from the European prospective investigation into cancer and nutrition. *Int J Cancer* 2013;132:617–24.
61. Mueller DM, Allenspach M, Othman A, et al. Plasma levels of trimethylamine-N-oxide are confounded by impaired kidney function and poor metabolic control. *Atherosclerosis* 2015;243:638–44.
62. Larsson SC, Wolk A. Red and processed meat consumption and risk of pancreatic cancer: meta-analysis of prospective studies. *Br J Cancer* 2012;106:603–7.