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Diabetes mellitus is associated with elevated urinary pyrrole markers of γ-diketones known to cause axonal neuropathy

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ABSTRACT

Introduction Progressive distal symmetrical axonal neuropathy, a complication of diabetes mellitus (DM), has an unknown cause. Normal physiological metabolism and diabetic dysmetabolism are associated with the generation of γ-diketones. γ-Diketones form pyrroles with protein amines, notably with axonal proteins required for the maintenance of nerve fiber integrity, especially elongate, large-diameter peripheral nerve fibers innervating the extremities. We tested the hypothesis that neuropathy-associated γ-diketone pyrroles are elevated in DM.

Research design and methods We measured the urinary concentration of γ-diketone pyrroles in age-matched and gender-matched elderly (60–84 years) persons with (n=267) or without (n=267) indicators of DM based in a community population (9411 community older adults aged ≥60 years) in Shenzhen city, Guangdong, China. We used statistical methods, including a generalized linear model, multivariate logistic regression analysis and restricted cubic splines, to assess linear and nonlinear relationships between urinary γ-diketone pyrroles and indicators of DM.

Results Compared with healthy controls, those with DM had significantly higher levels of fasting blood glucose, glycated hemoglobin A1c, urinary ketone bodies and urinary γ-diketone pyrroles. The median concentration of urinary γ-diketone pyrrole adducts was significantly higher ($p<0.0001$) in individuals with DM (7.5 (5.4) μM) compared with healthy controls (5.9 (4.3) μM). Both linear and non-linear relations were found between urinary γ-diketone pyrroles and indicators of DM.

Conclusions Diabetic dysmetabolism includes increased generation and excretion of neuropathy-associated γ-diketone pyrroles. These findings form the foundation for studies to test whether γ-diketone pyrrole concentration correlates with quantitative sensory (vibration and temperature) and electrodiagnostic testing.

Significance of this study

What is already known about this subject?

- Distal symmetrical large-fiber polyneuropathy frequently occurs in diabetes mellitus (DM), but the metabolic cause is unknown. Diabetic dysmetabolism increases levels of 2-hexanone, which has neurotoxic potential via oxidation to the corresponding γ-diketone and formation of pyrroles with amines of axonal and other proteins.

What are the new findings?

- We found the concentration of urinary γ-diketone pyrroles, which are formed from the established reaction between protein amines and γ-diketones with potential to induce distal symmetrical axonal neuropathy, are significantly elevated in elderly Chinese persons with DM.
- This finding is consistent with the hypothesis that diabetic dysmetabolism is linked to the increased risk for large-fiber diabetic polyneuropathy.

How might these results change the focus of research or clinical practice?

- If future studies show that elevated urinary γ-diketone pyrroles correlate with quantitative measures of stocking-and-glove sensory deficit in DM, this may provide a non-invasive biological indicator of risk for distal symmetrical large-fiber diabetic polyneuropathy.

INTRODUCTION

Diabetes mellitus (DM) has been described as a global epidemic[1] and peripheral neuropathy its most common complication.[2] The prevalence rates of diabetic neuropathy in a large study of Chinese subjects with type 1 and type 2 DM were approximately 22% and 35%, respectively.[2] Comparable rates of diabetic neuropathy have been recently reported in Barbados, Libya, Qatar and South Korea.[3–6] Approximately half of all individuals with DM develop a distal symmetrical and slowly progressive axonal polyneuropathy, with a stocking-and-glove distribution of sensory abnormalities.[7 8]

While the most common form of diabetic neuropathy is a central-peripheral distal axonopathy,[9] which can be modeled in rodents by repeated systemic treatment with

the γ-diketone 2,5-hexanedione (2,5-HD),[10] the molecular mechanisms underlying progressive axonal degeneration in DM are unknown.[1] 2,5-HD reacts with the amino groups of axonal (and other tissue) proteins to form 2,5-dimethylpyrrole monomers that result in covalent cross-linking of derivatized proteins, including neurofilament and microtubule-associated proteins. This disrupts longitudinal axonal transport and eventually results in distal axonal degeneration of elongate large-diameter myelinated nerve fibers.[11–14]

Since the serum of individuals with and without DM has been documented to contain 2-hexanone and 3-heptanone,[15] both of which can undergo *ω*-1 oxidation to form the neurotoxic γ-diketones 2,5-HD and 3,6-heptanedione, respectively, that form amine pyrroles,[15–17] we tested the novel hypothesis that elevated concentrations of γ-diketone pyrroles are present in diabetic urine.

Data reported here are consistent with this hypothesis and form the foundation for future studies to determine if there is a relationship between elevated levels of γ-diketone pyrroles and quantitative measures of stocking-and-glove neuropathy.

RESEARCH DESIGN AND METHODS

Study participants

The study included 534 participants: 267 persons with DM and 267 individuals serving as the control group. The participants were all from the baseline of the Shenzhen Aging-related Disorder Cohort established in Luohu district of Shenzhen City, Guangdong, China, which consists of 9411 older community members.[18] The participants were all older individuals (aged ≥60 years) who responded to a questionnaire and were given a physical examination during the period from July 2017 to October 2018. Figure 1 shows the strict inclusion criteria of cases were: self-reported DM diagnosed by a physician plus evidence of the therapeutic use of insulin or other glucose-lowering agents, a fasting glucose level >7.0 mM and a glycated hemoglobin A1c (HbA1c) level of >6.5%. Inclusion criteria for healthy controls included: no self-reported diabetes plus no use of insulin or other glucose-lowering agents, a fasting blood level of <7.0 mM and an HbA1c level of <6.5%. Among the 9411 participants of the original cohort, 521 individuals fell in the group of those with DM and 4571 in the group of healthy controls. Among the participants with diabetes, those with missing samples (n=200) or missing information on cognitive functions (n=54) were excluded from the study. Case and control persons were matched at 1:1 by age and gender.

Questionnaire and physical examination

A general questionnaire administered by face-to-face interview was applied to all study individuals on the day of the physical examination. Information was collected on demographic characteristics (gender, birth date, occupation before retirement), lifestyle (active and passive smoking status), individual histories of chronic

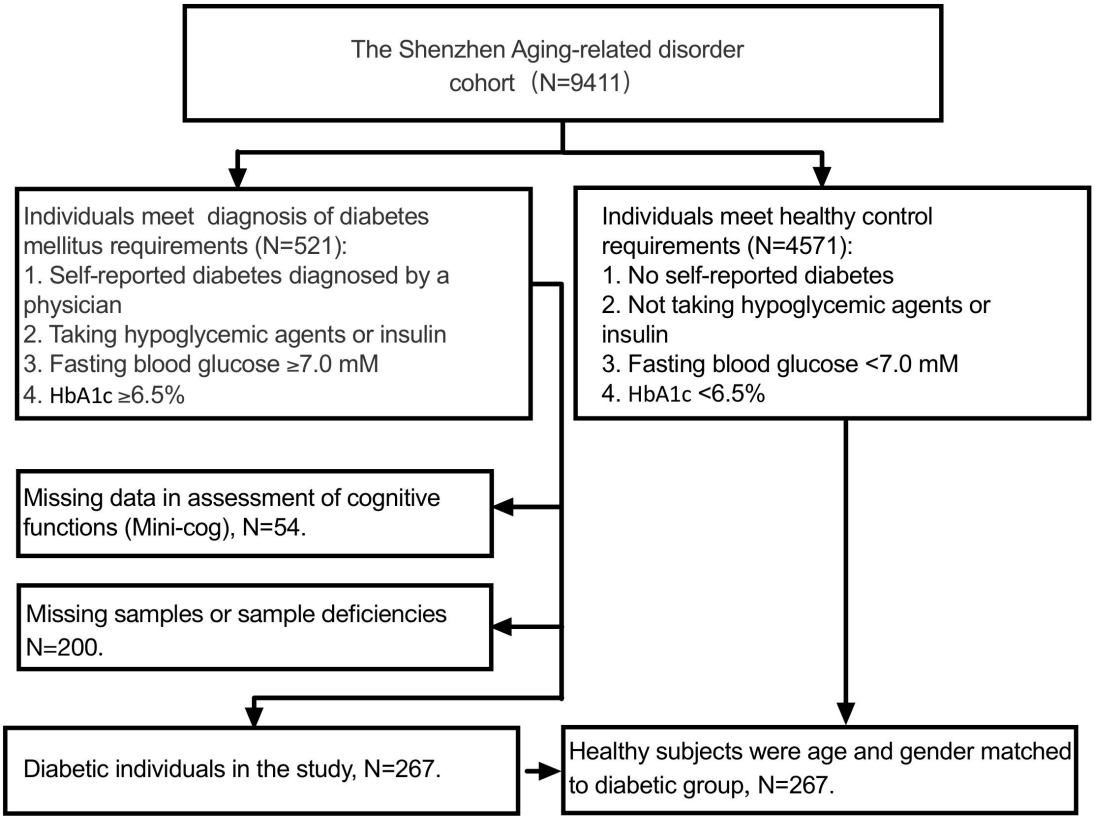

Figure 1 Subject selection. HbA1c, glycated hemoglobin A1c.

diseases (DM but not DM-type, hypertension and coronary disease) and medication history. The physical examination included measurements of height, weight, glucose level (coagulated blood) and HbA1c (EDTA-anticoagulated whole blood) of fasting venous blood, and routine urinalysis (urobilinogen and ketone bodies) of an early-morning sample. Body weight with light clothing and height without shoes were measured with an ultrasonic electronic height-and-weight scale (Omron HNH-219, Kyoto, Japan). The plasma glucose level of fasting venous blood was determined by a biochemistry autoanalyzer (Hitachi 7600-010, Hitachi, Tokyo, Japan). The HbA1c of fasting venous blood was determined by an HbA1c Analyzer (Premier Hb9210, Trinity Biotech, Bray, Ireland). Urinalysis used a urine analyzer (URIT-500B, URIT Medical Electronic Group, Shenzhen, China).

Pyrrole analysis

Urine samples from the participants were collected in the early morning at the time of physical examination. Samples were stored at −20°C prior to use. Analysis of urinary pyrroles employed minor modifications of published methods.[19–21] Pyrrole adducts were measured spectrophotometrically after reaction of 0.08 mL urine with 0.08 mL guanidine hydrochloride (70%) and 0.08 mL of Ehrlich's reagent 3% 4-dimethylaminobenzaldehyde (DMBA) in the solution of 40% vol/vol methanolic 14% boron trifluoride and 60% vol/vol ethanol.[22] Absorption values were measured at 526 nm with an automatic microplate reader (Infinite 1000, Tecan, Switzerland). Calculations were based on a standard curve prepared with different concentrations of 2,5-dimethylpyrrole.

Data analysis

The Kolmogorov-Smirnov test was used to examine the distribution of variables. Continuous variables that distributed normally were expressed as a mean±SD. Non-normally distributed variables were presented as medians and interquartile range (IQR) and were compared between two groups by the Mann-Whitney U test. Categorical variables were reported as frequencies and proportions; these were compared between two groups using the χ^2 test and Fisher's exact test if at least one cell had an expected count <5. Multiple linear regression analysis was used to determine the independent predictors of concentration of pyrrole adducts. Non-linearity in dose-response relationships between log-transformed diabetic indices (fasting blood glucose or HbA1c) and pyrrole adducts were assessed in the restricted cubic splines functions in linear models. Three knots (a term used in cubic spline functions) were set at the 5th, 50th and 95th percentiles of the distributions of pyrrole adducts. The covariates adjusted in the non-linearity in dose-response relationships included: smoking, body mass indices (BMI), coronary disease, hypertension. Participants were stratified into two gender subgroups in dose-response analyses. The statistical analyses were performed using SPSS (V.26.0; SPSS, Chicago, Illinois, USA) and SAS V.9.4 statistical software (SAS Institute, Cary, North Carolina, USA). P <0.05 was considered to be statistically significant.

RESULTS

Characteristics of persons with and without DM are summarized in table 1. No significant difference was observed between cases and controls with respect to age, gender, urinary urobilinogen or occupational exposure to organic solvents prior to retirement. The DM group had significantly higher (p<0.01) levels of fasting blood glucose, HbA1c, urinary ketone bodies and pyrrole adducts than the control group. Of those with DM, the mean age of males (69.5 years, n=129) was higher (p=0.038) than females (68.5 years, n=138); no significant sex difference (p=0.80) was seen in fasting blood glucose levels (7.6 (4) mM and 7.5 (3) mM, respectively) or percentage (7.1 (2)) and of HbA1c (p=0.80).

The distribution of pyrrole adducts for both DM and controls is plotted in figure 2 and the key characteristics are summarized in table 2.

Of those with DM, mean levels of pyrrole adducts were higher (p<0.0001) in males (8.1 (4.5) µM) than females (6.7 (4.6) µM). The median concentration of pyrrole adducts was significantly higher and the maximum value was double in the DM group versus the control group (tables 1 and 2). Outliers (kurtosis) in the DM group exceeded those for controls (table 2). Higher skewness was also observed in the DM group (figure 2 and table 2).

The univariate linear regression model (table 3) showed pyrrole adduct levels were positively associated with age, DM, fasting blood glucose level, HbA1c and urinary ketone bodies. Pyrrole adduct concentration was also significantly associated with gender adduct concentration (p<0.0001), DM (p<0.0001), fasting blood glucose level (p<0.0001), HbA1c (p<0.0001) and urinary ketone bodies (p=0.088).

The multivariate linear regression model reflects that pyrrole adducts remained independently associated with DM, fasting blood glucose and HbA1c after adjustment for age, gender, smoking status, hypertension and coronary disease.

The dose-response relationships between pyrrole adducts and log-transformed diabetic indices (fasting blood glucose or HbA1c) were evaluated by restricted cubic spline analysis (figure 3). Strong overall association was observed for both fasting blood glucose (figure 3A, p for overall association <0.0001) and HbA1c (figure 3D, p for overall association <0.0001). Non-linear associations were found for both fasting blood glucose (p <0.0001) and HbA1c (p for non-linearity=0.0149). In the subgroup analysis, males and females showed an equivalent overall association in the dose-response relationships between pyrrole adducts and diabetic indices. Males showed higher nonlinearity (p=0.0005) than females (p=0.083) in the concentration-response relationship between pyrrole adducts and fasting blood glucose (figure 3B,C), while

Table 1 Descriptive statistics of the study population

	Healthy control	Diabetes mellitus	P value
Number of participants (F/M)	267 (138/129)	267 (138/129)	1.000*
Age of participants (years)	68.3±4.9	68.3±4.9	1.000*
Disease duration	–	13.0±8.4	–
Taking glucose-lowering agent (N)	–	255	–
Taking insulin (N)	–	42	–
Fasting blood glucose (mM)	5.4 (0.7)	8.9 (2.5)	<0.0001†
HbA1c (%)	5.9 (0.5)	7.8 (1.9)	<0.0001†
Urinary ketone bodies (negative/weak positive/positive)	267 (265/2/0)	252/13/2	0.002*
Urobilinogen (negative/weak positive/positive/strong positive)	267 (265/0/1/1)	267 (265/1/1/0)	1.000*
Occupational exposure to organic solvent (N/Y)	267 (259/8)	267 (261/6)	0.788
Smoking (never/former and current)	266 (204/62)	267 (202/65)	0.839*
BMI (<24 kg/m^2/=24 kg/m^2/>24 kg/m^2)	265 (136/107/22)	266 (119/105/42)	0.024*
Hypertension (negative/positive)	267 (122/145)	267 (86/181)	0.002*
Coronary disease (negative/positive)	267 (156/11)	263 (236/27)	0.007*
Pyrrole adduct (μM)	5.9 (4.3)	7.5 (5.4)	<0.0001†

*Differences between groups were examined by the χ^2 test.
†Differences between groups were examined by Mann-Whitney U test.
BMI, body mass index; HbA1c, glycated hemoglobin A1c.

females showed higher non-linearity (p=0.0291) than males (p=0.1407) in the relationship between pyrrole adducts and HbA1c (figure 3E,F).

DISCUSSION

We show that γ-diketone pyrrole adducts in urine samples of elderly Chinese individuals with DM are significantly elevated relative to healthy age-matched and gender-matched individuals. Healthy Chinese persons aged 21–50 years had urine pyrrole adduct levels approximately half those in serum.[19] In rodents exposed to *n*-hexane, the highest concentration of pyrrole adducts was observed in kidney, followed by liver, brain, spinal cord, urine, sciatic nerve and serum.[20]

We observed significant linear associations between urinary pyrrole adduct concentrations and diabetic indices (DM status, fasting blood glucose levels or HbA1c levels). We also found significant non-linear associations between urinary pyrrole adduct concentrations and two indices of DM (fasting blood glucose or HbA1c). Moreover, male persons showed a significant non-linear association between pyrrole adduct and fasting blood glucose levels, while females showed a non-linear association between pyrrole adducts and HbA1c. The generalizability of these findings is unknown.

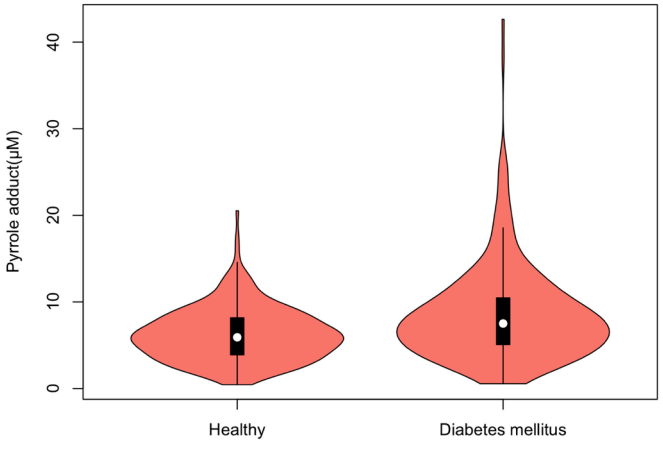

Figure 2 Violin plot of the distribution of pyrrole adducts. The thick black bar in the center represents the IQR and the white dot in the middle represents the median value. The thin black line extended from the thick black bar represents the upper (max) and lower (min) adjacent values of the data.

Table 2 Concentration of pyrrole adduct in urine samples

		Healthy control	Diabetes mellitus
Geometric mean (μM)		5.5	7.2
Kurtosis		1.8	8.4
Skewness		0.9	2.2
Percentile (μM)	25	3.9	5.1
	50	5.9	7.5
	75	8.2	10.5
Maximum (μM)		20.5	42.6

Table 3 Multiple linear regression analysis of independent predictors of pyrrole

	B	SE	P value	Adjust R^2
Univariate analysis				
Age	0.021	0.041	0.633	−0.001
Gender	−0.159	0.395	<0.0001	0.024
Diabetes mellitus	0.252	2.318	<0.0001	0.062
Fasting blood glucose	0.167	0.071	<0.0001	0.026
HbA1c	0.172	0.117	<0.0001	0.028
Urinary ketone bodies	0.074	0.974	0.088	0.004
Multivariate analysis				
Diabetes mellitus*	0.265	0.392	<0.0001	0.107
Fasting blood glucose*	0.169	0.073	<0.0001	0.067
HbA1c*	0.168	0.120	<0.0001	0.067

*Adjusted by age, gender, smoking status, BMI, hypertension, and coronary disease.
BMI, body mass index; HbA1c, glycated hemoglobin A1c.

While the three DM-associated classical ketone bodies (acetoacetate, β-hydroxybutyrate and their breakdown product acetone) are well known, a much larger number of ketones is found in human serum, including 2-hexanone, 3-heptanone and 2-butanone.[15] 3-Heptanone and 2-hexanone undergo *w*-1 oxidation (potentiated by 2-butanone—also reported in diabetic urine) to form 3,6-heptanedione and 2,5-HD, respectively,[15–17] repeated treatment with which results experimentally in axonal neuropathy.[23] Noteworthy is that classical ketone bodies were found in only 0.92% of the total cohort from which the present study population was drawn.

Since the serum of individuals with and without DM has been documented to contain 2-hexanone and 3-heptanone,[15] both of which can undergo *w*-1 oxidation to form the neurotoxic γ-diketones 2,5-HD and 3,6-heptanedione, respectively, that form amine pyrroles,[15–17] we tested the novel hypothesis that elevated concentrations of γ-diketone pyrroles are present in diabetic urine.

Demonstration that 2,5-HD induces central-peripheral distal axonopathy evolved from outbreaks of peripheral neuropathy among persons occupationally exposed to the solvent chemicals *n*-hexane or 2-hexanone in the presence or absence of 2-butanone.[23] Since *n*-hexane is an inexpensive and widely used commercial and industrial solvent,[24] low levels are likely to be present in ambient air. Given that *n*-hexane is metabolized to 2,5-HD, an exogenous source might contribute to levels in human biofluids. One study found that 1.3% of 1200 normal Americans with no known occupational exposure to *n*-hexane had blood levels of the neurotoxic alkane.[25] A Japanese study of 31 individuals with no known *n*-hexane exposure found low levels (<0.006 mg/L) of free 2,5-HD in urine.[26] A third reported that healthy Italians without occupational exposure to *n*-hexane had detectable levels of 2,5-HD in urine (0.17–0.98 mg/L), only a minimal part of which was considered to have derived from exposure to hydrocarbon-polluted air.[27] Subsequent study of urine samples from 123 healthy Italians recorded a 2,5-HD reference value of 0.795 mg/L for men and 0.627 mg/L for women.[28] A fifth, very large investigation of healthy Chinese people (n=8235) with no occupational exposure to *n*-hexane or 2-hexanone, showed a median urine 2,5-HD concentration of 0.171 mg/L for males and 0.147 mg/L for females, with increasing 2,5-HD excretion with the advance of age.[29] A sixth study of 227 Swedish persons randomly selected from the general population found that men had higher levels of 2,5-HD excretion than women (0.48 and 0.38 mg/L, respectively).[30] In a seventh study, investigations of 208 male and female subjects aged 18–24 years revealed a median level of urinary pyrrole adducts of unstated origin of 0.91 nmol/mL, which corresponds to a concentration of 0.91 µM.[31] This compares with the present findings of a median urinary pyrrole adduct level of 5.9 (4.3) µM for elderly healthy subjects, a result that might indicate pyrrole levels increase with age and correlate with the advance of sensory loss (notably vibration perception) with age.[32] Importantly, age-matched subjects with DM in the present study had significantly higher (p=0.0001) urinary pyrrole levels and with greater variance, that is, 7.5 (5.4) µM.

Given our study involves an elderly residential population with no known current or recent exposure to exogenous 2,5-HD or its precursors, the present findings suggest the pyrrole-forming γ-diketone (or γ-diketones) arise from endogenous metabolism, in agreement with a previous study,[27] with elevated levels associated with aging and in particular with diabetic dysmetabolism. This tentative conclusion should be strengthened by examination of a larger population over a wider age range, preferably in settings where airborne concentrations of *n*-hexane and 2-hexanone and serum/urine levels of γ-diketones are contemporaneously measured. If, as we hypothesize, the elevated levels of γ-diketone pyrrole adducts are associated with an increased risk for sensory neuropathy, these may be able to serve as molecular markers of axonopathy-associated diabetic dysmetabolism. More importantly, since the enzymatic mechanisms responsible for the P450 oxidation of 2-hexanone to neurotoxic 2,5-HD are understood,[33] it may be possible to control this process and, in this way, prevent or arrest the progress of diabetic neuropathy.

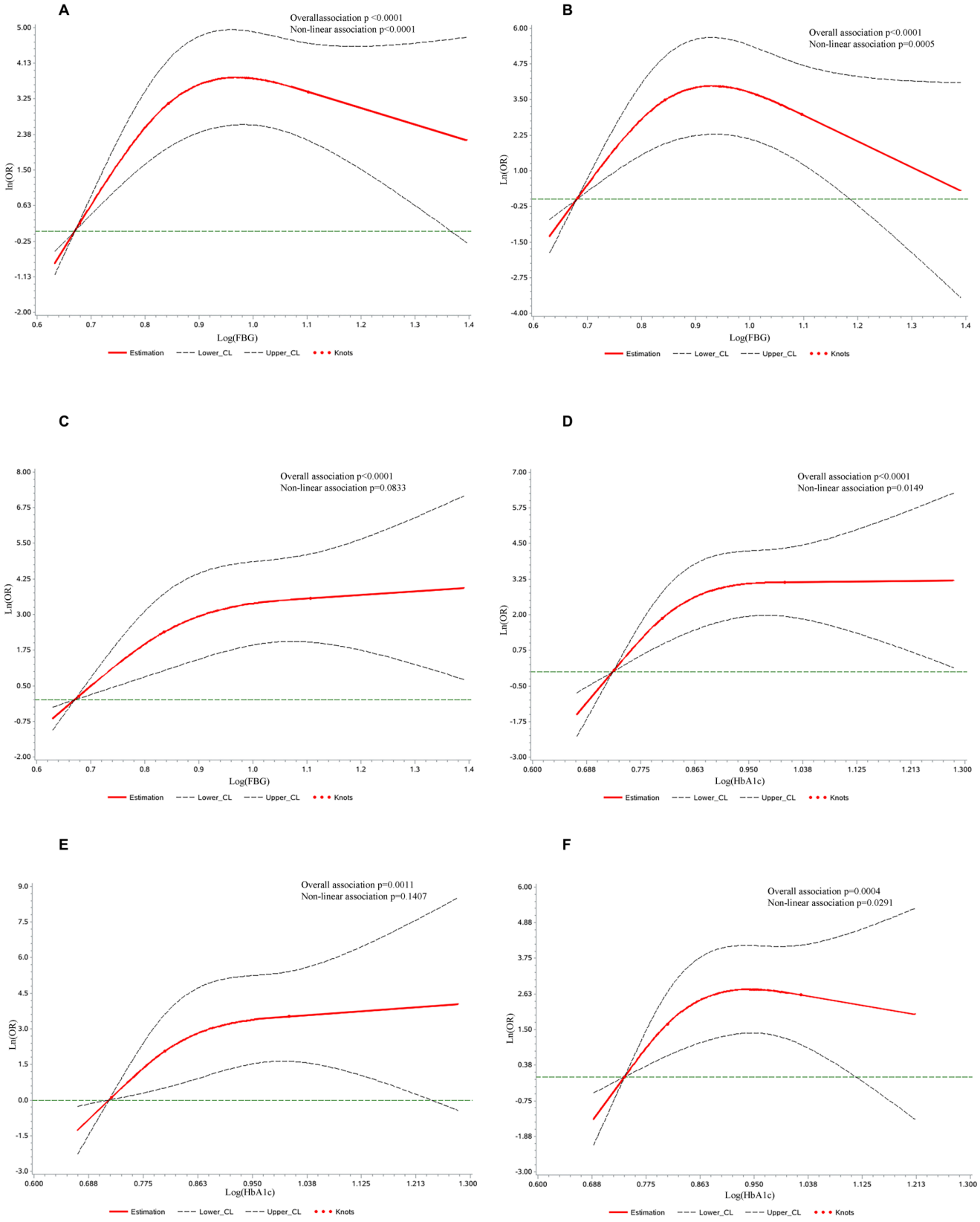

Figure 3 The restricted cubic spline for associations between indices of diabetes and concentration of pyrrole adducts. Dose-response curve between log-transformed fasting blood glucose and concentration of pyrrole adducts in the overall study population (A), male only (B) and female only (C). Dose-response curve between log-transformed glycated hemoglobin A1c (HbA1c) and concentration of pyrrole adducts in the overall population (D), male only (E) and female only (F). The lines represent adjusted ORs (solid lines) and 95% CIs (long dashed lines). The reference values were set at 5th percentiles, and the knots were set at 20th, 5th, 50th and 95th percentiles of the log-transformed concentrations, respectively. Adjusted factors were consistent with the multivariate analysis of multiple linear regression analysis. FBG, fasting blood glucose.

The strengths and weaknesses of this study include the use of a sample nested in a very large and well-characterized cohort of elderly individuals. The pyrrole assay procedure employed a method that has been shown in experimental studies to reflect 2,5-HD concentrations in urine, although the DMBA method is semi-quantitative and might underestimate total pyrrole adducts.[15] While the method cannot identify the specific amino targets of pyrrolization,[34] the pyrrole-forming mechanism is a required step for induction of 2,5-HD axonopathy.[35 36] Urinary urobilinogen could potentially interfere with the pyrrole assay but detectable levels were found in only 1.52% of study subjects in the total cohort of 9411 individuals, and there was no difference between subjects with and without DM. There was also no difference in the small number of persons in each group who reported prior occupational exposure to organic solvents, and chemicals and their metabolites arising in the workplace would have long before disappeared from the elderly retirees in this study. Follow-up studies are now needed to determine if urinary pyrrole adducts correlate with the results of quantitative sensory (vibration and temperature) and electrodiagnostic testing. Correlation of elevated pyrrole adduct levels with sensory loss would support (but not prove) an etiological role for γ-diketones in the induction of DM-associated stocking-and-glove neuropathy, which occurs more often in males than females.[37–39] We found that levels of urinary 2,5-dimethylpyrrole adducts were somewhat higher in males than females with DM, as reported in healthy Chinese persons aged 31–50 years but not those aged 18–24 years.[19 31]

Summary

We compared the γ-diketone pyrrole content of urine samples drawn from elderly Chinese individuals with and without DM. Urinary pyrrole levels were significantly elevated in individuals with DM (males>females). Both linear and non-linear relations were found between urinary pyrroles and indicators of DM. This provides indirect evidence that diabetic dysmetabolism generates neurotoxic γ-diketones with potential to induce distal symmetrical polyneuropathy. This hypothesis can be tested by comparing urinary γ-diketone pyrrole levels with indices of sensory dysfunction.

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Contributors PS developed the hypothesis and proposed the study. XC designed the study and performed the analyses of pyrrole markers. Wei L, LW, LN, and KH did the survey and managed the human sample. XC, LW and DL researched the dataset. WF and DL did the routine analyses of serum and urine. XC and PS drafted the manuscript. Weimin L, FZ, XY, PS and JL revised the paper. PS and JL (guarantor) supervised the study.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The study was approved (R2018020) by the research ethics committee of Shenzhen Center for Disease Control and Prevention and informed consent was obtained from all recruited individuals.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. While the data sets generated and/or analyzed in the current study are not publicly available at this time, they are available to researchers on reasonable request. Specific ideas and proposals for potential collaboration are welcome and should be directed to the corresponding authors, primarily Professor Jianjun Liu (junii8@126.com).

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