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Title page

Title: Dietary copper and selenium are associated with insulin resistance in overweight and obese Malaysian adults

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Abbreviations

ANCOVA; analysis of covariance, ATP; adenosine triphosphate, BMI; body mass index; BMR; basal metabolic rate, CI; confidence interval, COX; cytochrome c oxidase, Cu; copper, DSM-BIA; direct segmental multi-frequency bioelectrical impedance analysis, FFQ; food frequency questionnaire, Gpx; glutathione peroxidases, HbA1c; glycated hemoglobin, HDL; high-density lipoprotein, HOMA-IR; homeostatic model assessment-insulin resistance, hsCRP; high sensitivity C-reactive protein, IL; interleukin; IR; insulin resistance, LDL; low-density lipoprotein, MET; metabolic equivalent, MsrB; methionine sulfoxide reductase B, ORs; odds ratio, RNI; recommended nutrient intake, ROC; reactive oxygen species, Se; selenium, SEM; standard error of mean, Sepp1; selenoprotein P plasma 1, SOD; superoxide dismutase, TE; total energy intake, T2DM; type 2 diabetes mellitus, UNM; University of Nottingham in Malaysia, US; United State, VIF; variance inflation factor, WC; waist circumference, β; beta coefficient.

Abstract

Imbalance in or inadequate intake of micronutrients may impair insulin synthesis, secretion, and its signaling pathways. This study aimed to investigate the associations between dietary copper (Cu) and selenium (Se) with insulin resistance (IR), in overweight/obese adults. We hypothesized that dietary Cu and Se are associated with IR in a non-linear trend. A cross-sectional study was conducted on 128 non-diabetic overweight and obese Malaysian adults aged ≥18 years with a body mass index ≥23kg/m^2. Dietary intake was assessed using food frequency questionnaire. IR was defined as homeostatic model assessment-insulin resistance (HOMA-IR) ≥1.7. Locally weighted scatterplot smoothing (LOESS) regression was performed to detect non-linearity and piecewise regression models were computed to examine the trend of the associations at different cut off points. In this study, 45% (n=57) of the study participants were found to be insulin resistant. A U-shaped non-linear relation between Se and HOMA-IR was observed. Three-piecewise regression models (β coefficient) revealed positive association between Se and HOMA-IR in individuals with relatively low (<0.3µg/kg/day) and high (≥1.01µg/kg/day) intake of Se, 3.835 (CI=-12.216-19.886; p=0.614) and, 0.785 (CI=0.386-1.185; p=0.014), respectively. Significant positive association was only found between dietary Cu and HOMA-IR with intake of Cu ≥13.4µg/kg/day, 0.276 (CI=0.025-0.526; p=0.033). In conclusion, this study provides evidence on a U-shaped non-linear relationship between dietary Se and HOMA-IR, and a positive association between Cu with HOMA-IR with intake of Cu ≥13.4µg/kg/day. Thus, an appropriate intake of dietary Cu and Se is crucial to improve insulin sensitivity and reduce the risk of diabetes mellitus.

Keywords: Dietary copper; dietary selenium; insulin resistance; HOMA-IR; non-linear relationships; overweight and obese Malaysian adults.

65 **Manuscript Text**

66

67 **1. Introduction**

68

69 Insulin resistance is characterized by impaired glucose tolerance, dyslipidemia,
70 hyperglycemia and hyperinsulinemia, which precedes the development of Type 2 Diabetes
71 Mellitus (T2DM) [1] [2]. Earlier studies have reported that dietary trace minerals such as
72 copper (Cu) and selenium (Se) play a vital role in the regulation of enzymes catalyzing
73 antioxidant metabolism, glucose metabolism, pancreatic β-cell function and insulin signaling.
74 These findings have rekindled the interest of researchers in the role of these essential dietary
75 trace minerals in oxidative stress and in glucose metabolism [3]–[5].

76

77 Selenium is known to play a significant role in the pathophysiology of obesity and insulin
78 resistance due to its anti-inflammatory and anti-oxidant functions through selenium-
79 dependent glutathione peroxidases and other selenoproteins [3]. Thus, it is expected that
80 higher level of Se may have a protective effect against T2DM, and its deficiency could be
81 responsible for impaired insulin sensitivity [6]. Few longitudinal prospective cohort studies
82 reported that high levels of serum Se were significantly associated with decreased risk of
83 dysglycemia and diabetes in various populations including US and French [7], [8]. However,
84 positive associations have been found between the Se status and risk of diabetes in other
85 studies [9], [10]. A recent systematic review and meta-analysis concluded that the levels of
86 Se, assessed by dietary intake and various biological samples, were significantly associated
87 with increased risk of diabetes from both nonexperimental and experimental studies [11]. The
88 authors reported that subjects receiving Se supplementation of 200 µg/day were associated
89 with increased risk of diabetes compared to placebo in trials, but this effect was largely

limited to subjects with high baseline Se levels. Similar finding was reported in a population in Newfoundland, where higher dietary Se was associated with lower insulin resistance [12]. However, this beneficial effect on insulin sensitivity was only found in individuals with total dietary Se intake below 1.6μg/kg/day, and disappeared above this threshold value [12]. These findings suggest a non-linear relationship between Se levels and diabetes and deserve further investigation.

Copper is an important trace element involved in redox reactions. It is necessary for the catalytic activity of enzymes such as Cu/Zinc superoxide dismutase (SOD), which are involved in the protection of cells from superoxide radicals. Copper deficiency may lead to cardiac hypertrophy and blood vessel abnormalities [13], whereas copper overload is associated with dyslipidemia, increased oxidative stress and kidney dysfunction in diabetic rats [14]. Animal experiments have reported that dietary copper supplementation may restore β-cell function of Cohen diabetic rats [15]. However, there is increasing evidence that Cu acts as a pro-oxidant. A systematic review and meta-analysis of 15 observational studies also reported that subjects with diabetes mellitus have significant higher plasma or serum levels of Cu compared to healthy individuals [16]. Kim & Song 2014 also reported positive association between Cu levels and insulin resistance in visceral-obese adults [17]. Higher plasma Cu level was associated with increased levels of glycated hemoglobin in patients with diabetes mellitus [18].

Given that the pathological changes in metabolism contributing to hyperglycemia and insulin resistance occur at a preclinical phase, it may be expected that there is a metabolic imbalance in free radical production and in dietary antioxidants in clinically overt diabetics [19]. Thus, it is essential to determine the relationship between the dietary intake of Cu and Se and insulin resistance in the non-diabetic overweight and obese population in order to implement dietary

strategies for the prevention and management of diabetes at an early stage. The authors hypothesize that dietary Cu and Se are associated with insulin resistance in a non-linear trend. Therefore, the authors aimed to explore the associations between dietary nutrients, particularly Se and Cu and insulin resistance (IR), in non-diabetic overweight and obese Malaysian adults.

2. Methodology

2.1. Ethics approval

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were reviewed and approved by the Science and Engineering Research Ethics Committee, University of Nottingham Malaysia (UNM) (ID: SM190614). This study was also registered under the Medical Research and Ethics Committee (MREC) of National Medical Research Registry (Registration number-25110), Ministry of Health Malaysia (MOH). Written informed consent was requested and obtained from each individual participant.

2.2. Participant selection

This study is nested in a broader study investigating the effect of dietary nutrients on obesity-related phenotypes. Detailed information of the study design and method has been described in our previous publication [20]. Briefly, this cross-sectional study was conducted to assess the associations between dietary intake including Cu and Se and insulin resistance (IR). A total of 128 non-diabetic (as reported by self-reported questionnaire and later confirmed by serum glucose levels) overweight and obese Malaysian (Malaysian Malays, Chinese and

Indians) adults aged 18 years and above with a body mass index (BMI) ≥23kg/m^2 [21] were recruited at random through advertisements and flyers, distributed at the University of Nottingham in Malaysia (UNM) campus and schools in the vicinity of UNM (Semenyih and Kajang areas of Selangor state in Malaysia). Interested individuals attended an initial screening to determine whether the participants met the specified inclusion and exclusion criteria. Participants were requested to complete a health and lifestyle questionnaire which included questions on past diseases, family history of past diseases, physical activity level and substance abuse. Smoking and alcohol consumption status were reported as i) never, ii) former and iii) current. The exclusion criteria included pregnant woman, individuals who were diagnosed with cardiovascular diseases, stroke, diabetes, renal or endocrine disorders such as hypothyroidism, and subjects on vitamin/micronutrient supplementations or drugs (e.g. cholesterol lowering, hypoglycemic and psychiatric medications).

2.3. Measurement of anthropometric parameters

Height of the individual with barefoot was measured with a standard height rod. Anthropometric parameters including weight (kg), fat mass (kg), skeletal muscle mass (kg), fat free mass (kg) and percent body fat (%) were measured using a body composition analyzer direct segmental multi-frequency bioelectrical impedance analysis (DSM-BIA) (InBody 230, Seoul, Korea). Body mass index (BMI) was defined as weight in kilograms divided by the square of height in meters (kg/m^2). Waist circumference (WC) was measured at the midpoint between the top of iliac crest and the lower margin of the last palpable rib [22].

2.4. Assessment on dietary intake

Energy, macro- and micronutrient intake were assessed by an interviewer-administered validated food frequency questionnaire (FFQ). This FFQ consisting 156 food items which were listed according to 12 categories (grain, meat and poultry, fish and seafood, egg and eggs products, legumes, milk and milk products, vegetables, fruits, drinks, confectionary, bread spread and flavorings), was collected from each participant [23]. For each item, participants indicated the frequency of consumption for the past week (never, once a week, 2–4 times a week, 5–6 times a week, once per day, 2–3 times per day, 4–5 times per day). The number of standard portions consumed per sitting was recorded with the aid of photographs of standard portion sizes [24]. Detailed information related to the brands, methods of cooking, supplementation of vitamins and minerals and oil consumption were collected and documented to avoid under-reporting and to capture macro- and micronutrient intake as accurately as possible. Total daily intake of macro- and micronutrients including Cu and Se were calculated by multiplying the nutrient content of unit portion of each food item, by the frequency of consumption of each food, times the number of portions consumed per sitting, and finally summed across all food items to give an estimate of the per day intake. The per day consumption data were entered into an energy and nutrient assessment software, Dietplan7 (Forestfield Software Ltd., UK) to compute energy, macro and micro-nutrient intake. All the Malaysian food items not found in the Dietplan7 database were keyed in from the "Recommended Nutrient Intakes (RNI) Malaysia 2017" [25] and "Nutrient Composition of Malaysian Foods" [26]. The intakes of macronutrients were expressed as a percentage of total energy consumed, while micronutrients (e.g. Cu and Se) were adjusted to unit/kg of body weight/day. TE/basal metabolic rate (BMR) ratio <1.2 was used to exclude under-reporters [27]. In the current study, none of the participant was found to be under-reporting. Therefore, all the participants were included for analysis.

187

188 **2.5. Assessment of physical activity**

189 Physical activity data were collected through a structured questionnaire including a list of
190 physical activities with corresponding metabolic equivalent (MET) values [28]. Details of
191 activities asked included (1) the type and intensity of the activity; (2) the time and duration
192 (mins) of each activity performed per day; and (3) the number of days each activity was
193 performed in a week. The reported physical activity was then categorized into light (<3
194 METs), moderate (3-6 METs) and vigorous (>6 METs) intensity physical activity, according
195 to Ainsworth et al., 2000 [28]. The total time (mins) spent on sedentary, moderate and
196 vigorous intensity physical activity in a week was computed for each participant. Physically
197 active was defined as accumulation of at least 150 minutes/week of moderate intensity
198 physical activity or 60 minutes/week of vigorous intensity physical activity, according to the
199 Ministry of Health Malaysia, or else they are considered as physically inactive [29].
200 Participants of the current study were then categorized into two groups; physically active and
201 physically inactive.

202

203 **2.6. Blood biochemical analysis**

204 Fasting blood samples were collected by an experienced phlebotomist, from the antecubital
205 vein into grey vacutainer tubes containing fluoride oxalate (Becton Dickinson, Oxford, UK)
206 for plasma glucose analysis, and yellow vacutainer tube with clot activator and gel (Becton
207 Dickinson, Oxford, UK) for serum insulin, lipid profile including total cholesterol,
208 triglyceride and high-density lipoprotein (HDL) cholesterol, and high sensitivity C-reactive
209 protein (hsCRP) analysis. For serum preparation, tube was left to clot immediately at room
210 temperature for 30 minutes and centrifuged at 1500x g at 4°C for 15 minutes within 2 hours.
211 The supernatant (serum) was kept frozen at -80°C until analysis. For plasma preparation, tube

was inversed several times to ensure a proper mixing of the additive with the blood. Plasma was then separated by centrifugation at 2000x g at 4 °C for 15 minutes within 2 hours. Plasma was kept frozen at -80°C until analysis.

All blood biochemical analyses were carried on the Abbott Architect CI8200 Automatic Analyzer. Serum hsCRP level was quantified by immunoturbidimetric method using ARCHITECT C-Reactive Protein Reagent Kit (8G65) (Abbott Laboratories, IL, USA). Serum insulin was analyzed by chemi-luminescent micro-particle immunoassay (CMIA) using ARCHITECT Insulin Reagent Kit (8K41) (Abbott Laboratories, IL, USA). Plasma glucose was measured by hexokinase method using ARCHITECT Glucose Reagent Kit (3L82-20) (Abbott Laboratories, IL, USA). Homeostatic model assessment-insulin resistance (HOMA-IR) was calculated as = [fasting plasma glucose (mmol/L) x fasting serum insulin (uU/ml)]/22.5 according to homeostatic model assessment (HOMA) [30]. Insulin resistance (IR) was defined as HOMA-IR≥1.7 [31]. Serum total cholesterol and triglyceride were determined employing enzymatic-colorimetric methods using ARCHITECT Cholesterol Reagent Kit (7D62) and ARCHITECT Triglyceride Reagent Kit (7D74) (Abbott Laboratories, IL, USA), respectively. HDL-cholesterol was measured by enzymatic-direct determination method using ARCHITECT Commercial HDL Cholesterol Liquid Reagent Kit (Abbott Laboratories, IL, USA). LDL cholesterol was determined using the Friedewald formula: LDL cholesterol = Total cholesterol – [(Triglyceride/5) + HDL cholesterol] [32].

2.7. Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (IBM SPSS statistic, Chicago, IL, USA, version 22). Data were expressed as mean ± standard error of mean (SEM) or number (percentage). Normality was checked using Shapiro-Wilk Test, and log transformation was performed to transform nonnormally distributed data into

normally distributed data. Independent-t test and Chi square test were performed to assess the differences in general characteristics for continuous and categorical variables, respectively, between the insulin resistant and non-insulin resistant groups. One-way ANCOVA was performed to determine the differences in anthropometric, blood biochemical and dietary parameters between the insulin resistant and non-insulin resistant groups, after adjusting for covariates using different models.

Associations between dietary intake and HOMA-IR were evaluated by using multiple linear regression, beta coefficient (β) and 95% of confidence interval (CI) were computed. Adjustments for covariates such as age, social-demographic, dietary, anthropometric and blood biochemical parameters were applied using different models (models 1, 2 and 3). Variance inflation factor (VIF) was taken as a measure for testing multicollinearity among related parameters [33], with VIF value ≥10 indicates high collinearity. The VIF values for dietary parameters were close to 1.0, indicating lack of multicollinearity.

Locally weighted scatterplot smoothing (LOESS) regression was constructed to detect non-linear relationship between dietary micronutrient (e.g. Cu and Se) and HOMA-IR using STATGRAPHICS Centurion version 19 (Statepoint Technologies Inc.Warrenton, Va, Virginia, USA). When a non-linear association was observed, piecewise regression analysis was performed to examine how the associations differed by cut off points. A two- and three piecewise linear regression model was constructed for dietary Se and Cu, respectively. The cut-off points were determined by trying all possible values along a predefined interval and choosing the turning point that gave the highest likelihood. Then, log likelihood ratio test for the linear regression model and two piecewise linear regression model was conducted to determine the best fit for the model.

2.8. Power and sample size calculation

To determine the association between dietary micronutrients and insulin resistance, sample size was computed using the formula $n = \frac{r+1}{r} \frac{SD^2(Z_\beta + Z_{1-\alpha/2})2.}{d^2}$, for case-control study, according to Charan & Biswas, 2013 [34]. $Z_{1-\alpha/2}$ is the value from the standard normal distribution holding 1- α/2 below it, which is 1.96 and Z_β is the standard normal variate for a power of 80%, which is 0.84. SD is the standard deviation of dietary Cu in the Malaysian population which was previously reported, 0.6mg [35]. d is the expected mean difference in dietary Cu between the insulin resistant and non-insulin resistant groups, which is 0.25mg. r is the ratio of the two groups, which is 1. Thus, $n = \frac{1+1}{1} \frac{0.6^2(0.84+1.96)2.}{0.25^2}$, =90. Assuming an attrition rate of 20%, at least a total of 108 participants should be enrolled in this study.

3. Results

A total of 128 participants were recruited after screening for inclusion criteria. Of the total, 45% (n=57) of the study participants were found to be insulin resistant (HOMA-IR≥1.7). General characteristics of the insulin resistant and non-insulin resistant groups are reported in Table 1. No significant difference was found in age, sex, ethnicity, physical activity status, smoking and alcohol consumption between the two groups.

3.1. Differences in the average values of the anthropometric, blood biochemical and dietary parameters between the insulin resistant and non-insulin resistant groups

The differences in the mean values of the anthropometric, blood biochemical and dietary parameters between the insulin resistant and non-insulin resistant groups are reported in Table 2. Insulin resistant group had significantly higher body weight, BMI, WC , muscle mass, fat mass, percent body fat, fasting glucose, fasting insulin, HOMA-IR, triglyceride and total cholesterol/HDL cholesterol and significantly lower HDL cholesterol compared to the

non-insulin resistant group, even after adjusting for covariates ($p<0.05$). These variables were adjusted as covariates in the subsequent regression analysis. The correlations between HOMA-IR and other dietary parameters are reported in the Table S1.

3.2. Non-linear relationship between dietary copper and HOMA-IR

Locally weighted scatterplot smoothing (LOESS) regression was conducted to detect the relationship between dietary Cu and Se and HOMA-IR. As shown in the Figure 1, non-linear relationship between dietary Cu and HOMA-IR was identified. The result from two-piecewise linear regression analysis revealed that the inflection point was at 13.4µg/kg/day (Table 3). Interestingly, on the right side of the inflection point, significant positive association was found between dietary Cu and HOMA-IR, with an effect size (β) of 0.276 (CI=0.025-0.526; p=0.033). Significance remained even after adjusting for covariates. On the left side of the inflection point, an inverse association was detected between dietary Cu and HOMA-IR, -0.025 (CI=-0.137-0.086), however such association did not reach statistical significance (p=0.601). Further, a stratified analysis between dietary Cu and HOMA-IR was conducted based on sex and age to observe the effect of sex (females and males) and age (using median age 41y as cut off) on these associations (Table S2). No significant effect of sex and age was found on these associations after adjusting for covariates.

3.3. Non-linear relationship between dietary selenium and HOMA-IR

A U-shaped non-linear relationship between dietary Se and HOMA-IR was observed (Figure 2). When linear regression model analysis was performed (including adjusting for covaristes), an positive association was found between dietary Se and HOMA-IR (Table 4) which did not achieve statistical significance. However, the results from the three-piecewise linear regression analysis revealed that the inflection points were at 0.3 and 1.01µg/kg/day (Table

4). Positive associations between dietary Se and HOMA-IR were found in individuals with relatively low (≤0.3µg/kg/day) and high (≥1.01µg/kg/day) intake of dietary Se, with an effect size (β coefficient) of 3.835 (-12.216-19.886; p=0.614) and 0.785 (CI=0.386-1.185; p=0.014), respectively, after adjusting for covariates. However, such positive association was found significant only with intakes of Se ≥1.01µg/kg/day. Whereas an inverse association was found between dietary Se and HOMA-IR with intakes of Se between 0.3-1.01µg/kg/day, -1.064 (CI=-4.486-2.357; p=0.536). However, such associations did not reach statistical significance. When participants were stratified based on sex and age, similar findings were found in female participants (Table S3). However, due to the small sample size of male participants, the regression coefficient and p value could not be computed. Age showed no significant effect on these assocation. No significant association was found between other dietary micronutrients and HOMA-IR using multiple linear regression (Table S1), thus the results are not discussed.

4. Discussion

This is a cross sectional study assessing the associations between dietary Cu and Se with HOMA-IR in non-diabetic overweight and obese Malaysian adults. Locally weighted scatterplot smoothing (LOESS) regression revealed a non-linear relationship in both dietary Cu with HOMA-IR and dietary Se with HOMA-IR. Two-piecewise regression analysis identified significant positive association between dietary Cu and HOMA-IR in individuals with dietary Cu intake ≥13.4µg/kg/day.

Copper is a dietary essential trace element. It serves as a cofactor for the catalytic activity of many metalloenzymes. Such enzymes play a critical role in the function of cytochrome c

oxidase (COX) at the terminal end of the mitochondrial electron transport chain [36]. Efficient COX activity is crucial for the generation of adenosine triphosphate (ATP) via the mitochondrial electron transport chain, necessary to mediate insulin secretion by the pancreatic β-cells. However, excess Cu can increase the production of reactive oxygen species (ROS) resulting in heightened oxidative stress. It is reported that higher level of oxidative stress can trigger the onset and progression of T2DM [4]. Further, it has been reported that decrease in serum Cu is associated with decreased production of ROS in experimental animal models of diabetes mellitus.

Our finding is in line with a systematic review and meta-analysis which reported that subjects with diabetes mellitus have significantly higher plasma or serum levels of Cu compared to healthy individuals [16]. Similar finding was reported in a prospective cohort study which found that dietary Cu was associated with increased odds of T2DM in the Japanese population [37]. Higher serum Cu levels were associated with hyperinsulinemia and decreased insulin sensitivity in the prediabetes individuals [38]. So and so reported that glycated hemoglobin A1c (HbA1c) levels were positively correlated with Cu, in the patients with T2DM from a couple of observational studies [39] [40]. The studies referred to support the role of copper in the pathogenesis of diabetes mellitus. However, there is limited data available from clinical trials in human populations, raising concerns on the appropriate quantity of dietary or supplemental intake of Cu per day for normal cellular metabolism. Linear regression analysis of the current study (Table 4) shows that dietary Se was not significantly correlated to HOMA-IR. However, a U-shaped non-linear relationship between dietary Se and HOMA-IR was observed (Figure 2). The 3-piecewise regression analysis indicated that the correlation between dietary Se and HOMA-IR had a segmental trend at different inflection points. An inverse association was found in intakes of Se between 0.3 and

1.0µg/kg/day, and a positive association were found in intakes of <0.3 µg/kg/day and ≥1.0µg/kg/day.

Earlier studies in the Chinese population have reported positive association between dietary Se and type 2 diabetes [41], [42]. However, yet others have reported conflicting evidence. Ozkaya et al., 2009 reported that in a Turkish population, HOMA-IR was significantly higher in diabetic patients with lower levels of serum Se (<80µg/dl) compared to those with higher levels of serum Se (≥80µg/dl) [43]. Supplementation of Se (200 µg/d) in patients with diabetic nephropathy had demonstrated positive impact on insulin sensitivity [44]. However, a 3-year Se supplementation trial reported no protective effect of Se on pancreatic β-cell function and insulin sensitivity [45].

A recent meta-analysis supports our findings, reporting a U-shaped non-linear dose–response relationship between serum selenium and diabetes mellitus [46]. The authors reported that positive association between Se and risk of type 2 diabetes exists in individuals with relatively low (<100µg/l) and high (>130µg/l) levels of serum selenium. Another study reported non-linear relationship between serum Se and diabetes in the US population, with high levels of Se (≥137.66 ng/ml) being significantly associated with increased odds of diabetes compared to low levels of Se (<111.62 ng/ml) [9]. A 7.7 years of clinical trial reported that selenium supplementation in effect may increase the risk of diabetes mellitus and not alleviate it. The authors suggested that individuals with higher baseline serum or plasma selenium levels of ≥122µg/L should not be supplemented with selenium (200 µg/d) [47], as supplemental intake of Se above the adequate level may potentially raise the risk of type 2 diabetes mellitus, whereas those who have deficient levels of selenium may benefit from Se supplementation.

386

387 The anti-oxidant action of seleno-proteins selenoprotein P plasma 1 (Sepp1) and glutathione
388 peroxidases (GPX) in the β-pancreatic cells, is expected to inhibit the excess production of
389 ROS including hydrogen peroxide that impairs the insulin signaling pathway [48]. However,
390 animal studies demonstrated that both over expression of GPx1 and methionine sulfoxide
391 reductase B (MsrB) selenoproteins caused by Se supplementation and Se deficiency, could
392 lead to the development of diabetes mellitus [49]. Excess GPx1 may stimulate reductive
393 stress due to lack of oxidants or excess reducing equivalents [50], which prevent normal
394 hydrogen peroxide signaling, leading to hyperinsulinemia and decreased insulin sensitivity.
395 However, decreased expression of GPx1 and other selenoproteins may result in increased
396 production of ROS and lead to oxidative stress-induced insulin resistance [51]. Therefore,
397 through measured intake of dietary and supplemental Se an appropriate redox balance/ROS
398 flux must be maintained to limit negative effects of oxidative or reductive stress on glucose
399 homeostasis.

400

401 Our findings suggest that dietary intake of Cu ≥13.4 µg/kg/day, as well as both low
402 (<0.3µg/kg/day) and high (≥1.01µg/kg/day) intake of dietary Se may increase the risk of
403 insulin resistance and diabetes. These findings are in line with the Malaysia's dietary
404 guidelines which suggest that the recommended nutrient intake (RNI) for Se for males and
405 females are 0.42µg/kg/day and 0.37µg/kg/day respectively. The RNI for Cu is 900µg/day
406 which is equivalent to 13.8µg/kg/day [25]. Finally, caution needs to be practiced in Cu and/or
407 Se supplementation if any, as excess or deficiency may disturb the balance in cellular
408 metabolism in both normal and diabetic individuals.

409

410 **4.1. Strengths and limitations**

411

412 Insulin resistance is a complex metabolic dysregulation of glucose homeostasis. In this study
413 the authors adjusted for a number of the covariates such as age, height, weight, smoking,
414 alcohol consumption, physical activity level, total energy intake, and other macro and micro-
415 nutrients which are all potentially important covariates of insulin resistance [52], [53]. Even
416 after statistical adjustments the associations between dietary Cu, Se and insulin resistance
417 remained significant. Therefore, systematic control of the confounding factors is one of the
418 major strengths of the current study. Given that the metabolic requirements of Cu and Se is
419 most likely to be different in pre and overt diabetics, this study can pave the way for similar
420 large scale studies to draw up recommended guidelines for dietary trace mineral intake in the
421 prevention of diabetes mellitus in overweight and obese Malaysian adults.

422

423 A few limitations exist in the current study. The study design limits causal inference of the
424 pathology of insulin resistance from dietary intake. As in any observational study,
425 measurement errors may occur in self-reported variables (e.g. dietary intake and physical
426 activity data). There is also the possibility of unmeasured or residual confounding, although
427 we have carefully controlled several lifestyle and dietary factors which are potentially
428 associated with insulin resistance.

429

430 Further, dietary Cu and Se may not completely reflect its actual nutritional status in our study
431 participants. Future studies investigating the link between the nutritional status of Se/Cu,
432 should include analysis of blood biomarkers of Se/Cu. However, it is also noteworthy to
433 highlight that this is an exploratory study which aimed to generate initial insight on the
434 associations between dietary micronutrients and insulin resistance to identify serum
435 micronutrients of interest to be tested in future research.

436 The Cu and Se content of the food items may vary depending on the sources or the soil
437 characteristics of farmlands. The lack of speciation analysis and of the measurement of
438 bioavailability of micronutrients may have limited the derivation of dietary recommendation
439 from the current findings. Besides, the interactions between Se and Cu and other
440 micronutrients should be taken into consideration, particularly micronutrients with high
441 antioxidant properties, as well as the genetic influences on the inter-individual variability in
442 dietary absorption and metabolism.
443
444 Our study participants were recruited from a specific geographical location and they were
445 predominantly women. These factors may have influenced the study outcome and hence the
446 findings from the current experiments may not be generalizable to the Malaysian population.
447 Therefore, large scale studies from other geographical locations are required to generalize the
448 findings of the current study to the Malaysian population.
449
450 **5. Conclusion**
451
452 Our results reveal significant positive association between dietary Cu and HOMA-IR in
453 individuals with intake of dietary Cu ≥13.4µg/kg/day. Positive associations between dietary
454 Se and HOMA-IR were found in individuals with relatively low (<0.3µg/kg/day) and high
455 (≥1.01µg/kg/day) intake of dietary Se, indicating a U-shaped non-linear relationship. The
456 authors opine that dietary intake of trace minerals such as Cu and Se may play a critical role
457 in the pathogenesis of insulin resistance and T2DM and deserve further investigation in larger
458 populations.
459
460 **6. Declarations**

6.1. Acknowledgement

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6.3. Conflicts of interest

The authors declare no conflict of interest.

6.4. Author contributions

Pui Yee Tan: Investigation, Project Administration, Data Curation, Formal Analysis, Visualization, Writing-Original Draft and Writing-Review & Editing. Soma Roy Mitra: Conceptualization, Methodology, Supervision, Validation, Writing-Review & Editing and Funding Acquisition. Both authors read and approved the final manuscript.

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Figure legends:

Figure 1: Non-linear relationship between dietary copper and HOMA-IR

Figure 2: Non-linear relationship between dietary selenium and HOMA-IR

Table 1: General characteristics-difference between the insulin resistant and non-insulin resistant groups

General characteristics	Total (n=128)	Non-insulin resistant (n=71)	Insulin resistant (n=57)	p value
Age (y)	44.0 ± 10.9	44.0 ± 1.2	44.5 ± 1.5	0.791[a]
Sex				
Female	108 (84.4%)	62 (89.9%)	44 (77.2%)	0.053[b]
Male	20 (15.6%)	7 (10.1%)	13 (22.8%)	
Physical activity status				
Physically inactive	36 (94.7%)	66 (95.7%)	53 (93.0%)	0.515[b]
Physically active	2 (5.3%)	3 (4.3%)	4 (7.0%)	
Smoking Status				
Never	125 (97.6%)	68 (98.6%)	55 (96.5%)	0.537[b]
Current	1 (0.8%)	0	1 (18%)	
Former	2 (1.6%)	1 (1.4%)	1 (1.8%)	
Alcohol consumption				
Never	126 (98.4%)	68 (98.6%)	56 (98.2%)	0.361[b]
Current	1 (0.8%)	0	1 (1.8%)	
Former	1 (0.8%)	1 (1.4%)	0	

Data were expressed as mean (±SEM) or number (percentage).
[a]p value based on independent-t test.
[b]p value based on chi square test.

Table 2: Anthropometric, blood biochemical and dietary parameters-difference in means between the insulin resistant and non-insulin resistant groups

	Total (n=128)	Non-insulin resistant (n=71)	Insulin resistant (n=57)	p value
Anthropometric parameters				
[1]Height (cm)	157.9 ± 0.7	157.4 ± 0.8	158.6 ± 1.1	0.825
[1]Weight (kg)	73.9 ± 1.4	69.0 ± 1.1	79.8 ± 2.6	<0.001
[1]BMI (kg/m^2)	29.5 ± 0.4	27.8 ± 0.4	31.5 ± 0.7	<0.001
[1]WC (cm)	93.8 ± 1.1	89.0 ± 1.1	99.9 ± 1.7	<0.001
[1]Muscle mass (kg)	23.4 ± 0.4	22.2 ± 0.4	25.0 ± 0.8	0.005
[1]Fat mass (kg)	31.1 ± 0.9	28.1 ± 0.8	34.6 ± 1.7	<0.001
[1]Percent Body Fat (%)	41.6 ± 0.6	40.6 ± 0.7	42.6 ± 1.0	0.002
Blood biochemical parameters				
[2]Fasting glucose (mmol/L)	5.2 ± 0.2	4.8 ± 0.1	5.8 ± 0.4	0.015
[2]Fasting insulin (uU/mL)	10.1 ± 0.9	4.8 ± 0.3	16.3 ± 1.5	<0.001
[2]HOMA-IR	2.5 ± 0.3	1.0 ± 0.1	4.3 ± 0.5	<0.001
[2]Total cholesterol (mmol/L)	5.5 ± 0.1	5.5 ± 0.1	5.5 ± 0.2	0.900
[2]Triglyceride (mmol/L)	1.4 ± 0.1	1.2 ± 0.1	1.6 ± 0.1	0.010
[2]HDL cholesterol (mmol/L)	1.5 ± 0.4	1.7 ± 0.1	1.4 ± 0.1	0.001
[2]LDL cholesterol (mmol/L)	3.4 ± .1.0	3.3 ± 0.1	3.5 ± 0.1	0.998
[2]Total cholesterol/HDL cholesterol	3.7 ± 1.0	3.5 ± 0.1	4.1 ± 0.1	0.013
[2]hsCRP (mg/L)	5.8 ± 0.7	4.8 ± 0.7	7.1 ± 0.4	0.744
Dietary parameters (intake per day)				
[2]Total energy intake (kcal)	2009 ± 30	1950 ± 40	2075 ± 46	0.103
[3]Protein intake (g)	74.8 ± 2.1	74.0 ± 2.5	73.5 ± 3.2	0.513
[3]Fat intake (g)	82.8 ± 1.9	81.7 ± 2.3	84.3 ± 3.1	0.958
[3]Carbohydrate intake (g)	253.7 ± 4.7	242.1 ± 7.4	268.3 ± 8.6	0.685
[3]Percent energy from protein (% of TE)	14.8 ± 0.4	15.2 ± 0.5	14.0 ± 0.5	0.369
[3]Percent energy from fat (% of TE)	36.7 ± 0.6	37.4 ± 0.7	36.0 ± 1.1	0.735
[3]Percent energy from carbohydrate (% of TE)	47.0 ± 0.7	45.8 ± 1.0	48.7 ± 1.1	0.275
[3]Cu intake (µg)	793.5 ± 45.1	686.1 ± 45.0	891.4 ± 78.6	0.126
[3]Se intake (µg)	45.5 ± 3.0	46.1 ± 3.5	39.9 ± 3.7	0.479
[3]Weight adjusted Cu intake (µg/kg)	11.0 ± 0.6	10.1 ± 0.7	11.7 ± 1.1	0.150
[3]Weight adjusted Se intake (µg/kg)	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.472

Data were expressed as mean (±SEM) or number (percentage).
One-way ANCOVA was performed to determine the differences in the mean values of anthropometric, blood biochemical and dietary parameters after adjusting for covariates in different models [1]age, height, sex, physical activity status, smoking status and alcohol consumption; [2] model[1] + weight; [3] model[2]+ total energy intake.
BMI, body mass index; WC, waist circumference; HOMA-IR, homeostatic model assessment -insulin resistance; hsCRP, high sensitivity C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TE, total energy intake; Cu, copper; Se, selenium.

Table 3: Non-linear relationship between dietary copper and HOMA-IR addressing by two-piecewise regression model

	Unadjusted		Model 1[a]		Model 2[b]		Model 3[c]	
	β (95% CI)	p value	β (95% CI)	p value	β (95% CI)	p value	β (95% CI)	p value
Linear regression model	0.161 (0.089-0.232)	<0.001	0.145 (0.074-0.217)	<0.001	0.135 (0.064-0.205)	<0.001	0.139 (0.071-0.207)	<0.001
Two-piecewise linear regression model								
Inflection point (13.4µg/kg/day)								
<13.4	-0.025 (-0.137-0.086)	0.651	-0.016 (-0.127-0.096)	0.781	-0.001 (-0.121-0.119)	0.986	-0.032 (-0.155-0.090)	0.601
≥13.4	0.341 (0.106-0.577)	0.006	0.326 (0.104-0.547)	0.005	0.274 (0.034-0.513)	0.027	0.276 (0.025-0.526)	0.033
p for log likelihood ratio test				0.011				

[a]Model 1: adjusted for age, BMI, sex, ethnicity, physical activity status, smoking status, alcohol consumption and total energy intake.
[b]Model 2: adjusted for model 1 + height, weight, WC, fat mass, muscle mass and percent body fat.
[c]Model 3: adjusted for model 2 + triglyceride, HDL cholesterol and total cholesterol per HDL cholesterol.
CI; confidence interval, β; beta coefficient.

Table 4: Non-linear relationship between dietary selenium and HOMA-IR addressing by three-piecewise regression model

	Unadjusted		Model 1[a]		Model 2[b]		Model 3[c]	
	β (95% CI)	p value	β (95% CI)	p value	β (95% CI)	p value	β (95% CI)	p value
Linear regression model	-0.720 (-1.831-0.391)	0.202	0.101 (-1.040-1.243)	0.861	0.206 (-0.886-1.298)	0.710	0.270 (-0.795-1.335)	0.617
Three-piecewise linear regression model								
Inflection point (0.3 and 1.0µg/kg/day)								
≤0.3	9.204 (-4.686-23.095)	0.186	7.845 (-8.565-24.256)	0.331	2.494 (-13.654-18.642)	0.748	3.835 (-12.216-19.886)	0.614
0.31-1.0	-3.431 (-6.946-0.083)	0.056	-2.544 (-5.849-0.761)	0.129	-1.712 (-5.006-1.582)	0.303	-1.064 (-4.486-2.357)	0.536
≥1.01	0.669 (-0.235-1.573)	0.136	0.885 (0.426-1.345)	0.002	0.729 (0.093-1.366)	0.032	0.785 (0.386-1.185)	0.014
P for log likelihood ratio test				0.022				

[a]Model 1: adjusted for age, BMI, sex, ethnicity, physical activity status, smoking status, alcohol consumption and total energy intake.
[b]Model 2: adjusted for model 1 + height, weight, WC, fat mass, muscle mass and percent body fat.
[c]Model 3: adjusted for model 2 + triglyceride, HDL cholesterol and total cholesterol per HDL cholesterol.
CI; confidence interval, β; beta coefficient.