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Effects of a genetic variant rs13266634 in the zinc transporter 8 gene (*SLC30A8*) on insulin and lipid levels before and after a high-fat mixed macronutrient tolerance test in U.S. adults

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ABSTRACT

Background: The common C-allele of rs13266634 (c.973C>T or p.Arg325Trp) in *SLC30A8* (*ZNT8*) is associated with increased risk of type 2 diabetes. While previous studies have examined the correlation of the variant with insulin and glucose metabolism, the effects of this variant on insulin and lipid responses after a lipid challenge in humans remain elusive. The goal of this study was to determine whether the C-allele had an impact on an individual's risk to metabolic syndromes in U.S. adults.
Method: We studied the genotypes of rs13266634 in 349 individuals aged between 18 and 65 y with BMI ranging from 18.5 to 45 kg/m^2. The subjects were evaluated for insulin, glucose, HbA1c, ghrelin, and lipid profiles before and after a high-fat mixed macronutrient tolerance test (MMTT).
Results: We found that the effects of variants rs13266634 on glucose and lipid metabolism were sex-dimorphic, greater impact on males than on females. Insulin incremental area under the curve (AUC) after MMTT was significantly decreased in men with the CC genotype ($p < 0.05$). Men with the CC genotype also had the lowest fasting non-esterified fatty acid (NEFA) concentrations. On the other hand, the TT genotype was associated with a slower triglyceride removal from the circulation in men after MMTT. The reduced triglyceride removal was also observed in subjects with BMI $\geq$ 30 carrying either the heterozygous or homozygous T-allele. Nevertheless, the SNP had little effect on fasting or postprandial blood glucose and cholesterol concentrations.
Conclusion: We conclude that the CC genotype negatively affects insulin response after MMTT while the T-allele may negatively influence lipolysis during fasting and postprandial blood triglyceride removal in men and obese subjects, a novel finding in this study.

1. Introduction

Zinc is an essential trace mineral as it play important roles in regulation of gene expression, enzymatic activity of many metalloenzymes, and signal transductions in cells. Dietary zinc deficiency results in stunted growth, skin lesions, hypogonadism, and frequent infections in humans. If not treated, severe zinc deficiency in infants and children can results in death [1] while in adults zinc deficiency increases risk for sepsis mortality in elderly, alcoholic, and hospitalized patients [2].

Crystallization of insulin with zinc ions is a critical step in insulin maturation and glucose-induced insulin secretion in pancreatic β-cells. The majority of zinc ions (~70%) in the β-cell are in the insulin-containing secretory vesicles [3,4] where two zinc ions are bound to 6 insulin monomers forming a crystalized structure that increases insulin

Abbreviations: SNP, single nucleotide polymorphism; ZNT8, zinc transporter 8; SLC30A8, Solute Carrier Family 30 Member 8; MMTT, Mixed macronutrient tolerance test; TG, triglycerides; NEFA, non-esterified free fatty acid; TC, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; T2D, type 2 diabetes; R325W or p. Arg325Trp, substitution of the amino acid at position 325, arginine → tryptophan; c.973 C>T, substitution of the nucleotide at position 973, C→T.

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storage capacity in β-cells [5]. SLC30A8 or ZNT8, a zinc transporter, is predominantly expressed in β-cells where it delivers zinc ions into the secretory granule for insulin crystallization [6].

Genome-wide association studies (GWAS) in humans have identified a single nucleotide polymorphism (SNP, rs13266634) in *ZNT8* (c.973 C>T or p.Arg325Trp or p.R325W) that confers increased risk of type 2 diabetes (T2D) [7]. The risk C-allele is the major allele with a carrier frequency at 75–92%, which is associated with ~12% and ~23% increase in T2D risk in European [8] and Asian populations [9,10], respectively. Case-control studies of rs13266634 in T2D patients indicated that the carrier rate of the T-allele in T2D patients was significantly lower than the control group [11,12]. Soltanian et. al. reported that the CC, CT, and TT genotypes were 49.6%, 50.4%, and 0%, respectively, in the case group (n = 125) while it was 50.4%, 41.6%, and 8.0%, respectively, in the control group (n = 125) in which the CT or TT carrier rate was significantly lower than the case group [12]. Other reports have shown that the risk C-allele carriers have decreased proinsulin to insulin conversion rates during oral glucose tolerance tests [13,14] and glucose-stimulated insulin secretion was reduced accompanied by an increased fasting blood glucose level and impaired β-cell function [14–17]. Moreover, Merriman et al. showed that the R325 ZNT8 protein exhibits better functionality in transporting zinc into the insulin-containing granules than the W325 form [18] resulting in an unbalanced intracellular zinc distribution within the β-cell [19] and a lower proinsulin to insulin conversion rate in granules of the β-cell in diet-induced obesity animals [20]. Nevertheless, peripheral insulin sensitivity seems to not be affected by the risk C-allele of rs13266634 [13,15–17].

Due to its negative effect on insulin secretion and β-cell dysfunction during T2D development, rs13266634 has drawn attention to the development of insulin resistance and T2D. Dysregulation of lipid metabolism is also an important risk factor for T2D and cardiovascular disease [21]. However, the contribution of this SNP to the abnormal changes in lipid profiles is still not well understood in humans. Thus it is important to understand the interplay between rs13266634-associated insulin metabolism and blood lipid markers in humans, especially in healthy adults. Hence, the goal of this study was to investigate the effects of the risk variant of rs13266634 on blood insulin, glucose, lipid profiles, among other factors that linked the risks of T2D before and after MMTT in a cohort of healthy men and women with different ages and BMIs (kg/m^2) living in Northern California of the United States. We show that insulin incremental area under the curve after MMTT was significantly reduced in men carrying the homozygous C-allele ($p < 0.05$). Men with the CC genotype also had the lowest fasting non-esterified fatty acid (NEFA) concentrations. On the other hand, the TT genotype was associated with a slower triglyceride removal from the circulation in men after MMTT. The decreased triglyceride removal was also observed in subjects with BMI ≥ 30 carrying the T-allele.

2. Materials and methods

2.1. Study subjects and dietary challenge

Three hundred ninety-three healthy U.S. adults aged 18–66 years old with BMI ranges of 18–45 kg/m^2 were enrolled for a cross-sectional nutritional phenotyping study (*ClinicalTrials.gov*, ID: NCT02367287) conducted in the USDA-ARS-Western Human Nutrition Research Center (WHNRC), Davis, California, USA [22]. The exclusion criteria for participation in this study was described previously [22]. For each sex group, 9 sampling bins were established to recruit subjects evenly among these bins. Three age bins, 18–33, 34–49, and 50–65 y were established, each with BMI 18–24.9, 25–29.9, or 30–45 kg/m^2. The sample size calculation for each study bin was described previously [22]. Upon completion of the study, 19–24 subjects were recruited for each sampling bin with the exception of these with fewer subjects: men with BMI 30–45 kg/m^2 at 18–33 y (n = 13), 34–49 y (n = 17), and 50–65 y (n = 8) and women with BMI 30–45 kg/m^2 at 18–33 y (n = 17) and 50–65 y (n = 17) as well as women with BMI 25–29.9 kg/m^2 at 34–49 y (n = 15). Details of recruitment and demographic characteristics of the overall population are contained within another report on this cross-sectional nutritional phenotyping study [23]. Specially, the ethnicities of subjects involved in this study were White 212 (60.74%), Hispanic 59 (16.91%), Asian 55 (15.75%), Black 17 (4.87%), and mixed races 3 (0.86%) with race unknown, 3 (0.86%).

The study included two visits to the WHNRC scheduled within a period of 10–14 days. Visit 1 included the informed consent procedure and a screening of vital signs to ensure the volunteers fell within designed ranges for the study. Visit 2 was the challenge meal test day. The night before the test day, subjects were provided a high carbohydrate meal (17% kcal from fat, 77% kcal from carbohydrate, and 7.5% kcal from protein) and asked to eat it by 19:00 h. Subjects arrived fasted (12 h) the next morning and blood was collected before a high-fat liquid mixed macronutrient tolerance test (MMTT; 60% kcal from fat (~60 g palm oil), 27% kcal from carbohydrates (~60 g sucrose), and 13% kcal from protein (~30 g egg white)) was given. Blood draws were performed before (0 h) and after MMTT at 0.5, 3, and 6 h. Heights (m) and weights (kg) were recorded in both Visit 1 and Visit 2. BMI (kg/m^2) was then calculated from the averages of weights and heights from Visit 1 and Visit 2.

2.2. SNPs used in the study

We studied a T2D-associated *ZNT8* SNP rs13266634 [8,24]. This SNP causes a missense amino acid change (R325W) in the C-terminal end of the ZNT8 protein. Table 1 lists allele frequencies of the SNP in global and American populations as well as in the population of the current study.

2.3. Genomic DNA purification and quantification

Whole blood (8 mL) was collected in a PAXgene Blood DNA Tube (Qiagen, Germantown, MD) at 0.5 h after the challenge meal was given. Collected blood was gently inverted in the PAXgene blood DNA tube and immediately stored at − 80 °C until use. Genomic DNA was then purified using a PAXgene Blood DNA Kit according to the manufacturer's instructions (Qiagen). DNA concentrations were determined by a NanoPhotometer™ P300 (Implen, Westlake Village, CA, USA). All DNA samples had a A260/A280 ratio greater than 1.6, indicating the purity of nucleic acids was suitable for genotyping. DNA was then diluted to 25 ng/μL in sterilized double distilled water for subsequent TaqMan SNP genotyping assays.

2.4. SNP genotyping

The TaqMan SNP probe set for rs13266634 was purchased from ThermoFisher Scientific (Carlsbad, CA, USA). TaqMan genotyping reactions were performed using a TaqMan SNP assay-based PCR in an

Table 1
Allelic frequency of rs13266634 in *ZNT8* in humans.

Gene	SNP ID	Nucleotide Change	Codon change	Reference frequency[a]	Frequency in the current study
ZNT8	rs13266634	C>T	R325W	C= 74.5% (global) C= 73.3% (American)	C= 71.7%

Data are presented by frequency for the major allele. Frequencies of alleles are presented as percentages. No significant Hardy–Weinberg equilibrium deviation was found (Chi-square, $p = 0.08$) in the current study cohort for the genotypes of rs13266634.

[a] global allelic frequency from the 1000 genome project (https://www.ncbi.nlm.nih.gov/snp/rs13266634)

Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System according to the manufacturer's instructions (ThermoFisher Scientific). Genomic DNA (50 ng) was used for each PCR reaction. Allelic discrimination assays were performed using QuantStudio™ Real-Time PCR software (ThermoFisher Scientific). All ambiguous genotypes were repeated in independent PCR reactions.

2.5. Serum zinc analysis

Fasting blood were collected from subjects. Serum was purified by centrifugation at 1300x g at 4 °C for 10 min and stored at − 80°C until use. Two hundred microliters of serum were transferred in to a 15-mL polypropylene tube and 0.25 mL of Optima 70% HNO_3 (trace element grade) was added to the serum sample (all serum samples were acid-extracted in duplicate). Samples were mixed and incubated overnight (16–18 h) at 60 °C degree with shaking (250 rpm). Duplicate samples were subjected to acid-digestion. Next day, triple distilled water (3.05 mL) was added to dilute nitric acid to 5%. Samples were subjected to centrifugation at 3,000x g at RT for 10 min and the supernatant was collected into a new polypropylene tube. The dilution factor was determine by the formula: ((3.05 mL + 0.25 mL+ sample weight g)/ sample weight g). Digested serum samples, blanks, digestion batch quality controls, and standards (Inorganic Ventures multi-element standard mix from SPEX CertiPrep (Metuchen, NJ, USA) were analyzed in four batches using an Agilent 8900 Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent Technologies, Palo Alto, CA, USA). The standards were prepared in 7% HNO_3 (vol:vol; trace-metals grade with MilliQ water) and blanks were 7% HNO_3. The Seronorm™ Trace Elements Serum L-1 RUO (SERO, Billingstad, Norway; Ref# 201413; Lot#1309438) was used as quality control as described per the manufacturer's instructions. The raw data was processed using MassHunter ICP-MS software (G7201C, Version C.01.03, Agilent). The % of difference from the batch quality controls was 1.11 ± 2.13 (mean ± S.E.) and the % recovery of Seronorm™ serum (48 tested) were 0.27 ± 0.04 (mean ± S.E.).

2.6. Clinical measures

Fasting blood was collected and serum or plasma was obtained by centrifugation at 1300x g at 4 °C for 10 min. Lipid-related markers of cardiovascular diseases, including triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol, and non-esterified fatty acids (NEFA) were measured using a Cobas Integra 400/800 kit (Roche, Indianapolis, IN), a Cobas CHOL2 kit (Roche), a Cobas HDL-C plus 3rd generation kit (Roche), a Cobas LDLC3 kit (Roche), and a Wako HR Series NEFA-HR (2) kit (Wako), respectively. All assays were completed on an auto-analyzer, Cobas Integra 400 + instrument (Roche). Percent of HbA1c in the whole blood sample was determined using Tina-quant Hemoglobin A1c Gen.3 kits from Roche. Glucose concentrations in plasma samples were measured using Glucose HK Gen.3 kits (Roche). Both HbA1c and glucose assays were conducted on the Cobas Integra 400 + instrument (Roche). Serum insulin levels were determined by Elecsys Insulin kits on a Cobas e411 analyzer (Roche) and total ghrelin levels were determined using U-PLEX Human Ghrelin (total) Assay purchased from MSD (Rockville, MD).

Homeostatic model assessment (HOMA) is a method for assessing β-cell function and insulin resistance (IR) from fasting glucose and insulin concentrations. Thus, we calculated insulin resistance score (HOMA-IR) with the following formula: fasting plasma glucose (mg/dL) *fasting serum insulin (mU/L)/405, with HOMA-IR > 2 = insulin resistant.

2.7. Statistical analysis

Allele frequency was determined by direct counting. Differences in general characteristics among genotypic groups were assessed for significance using a Tukey's test. Outcome variables were assessed for conformance to the normal distribution and transformed if needed; insulin, glucose, ghrelin, and triglycerides were transformed using natural logarithm, and HbA1c, total cholesterol, HDL-C, LDL-C, and NEFA did not require transformation. Zinc data were transformed by negative inverse. The association between variables and genotypes was tested with analysis of variance (ANOVA) or Student *t*-test with or without adjusting for sex, age, and BMI as indicated. Two-factor interactions between SNP and sex, age, or BMI category were also examined. The outcome variables are presented as mean ± S.E. with $p < 0.05$ being significant (two-tailed). Significant difference in genotype frequencies among the subgroups were analyzed in a Hardy-Weinberg equilibrium test by chi-square test (2 degrees of freedom).

3. Results

The frequency for the C-allele of rs13266634 is around 74.5% in global populations and 73.3% in Americans (1000 Genome Project) (Table 1). In the current study cohort, the C-allele frequency was found at 71.7%, similar to that in the global and American populations. No sign of linkage disequilibrium of the SNP genotypes was revealed using the Hardy-Weinberg equilibrium test among all subjects ($\chi 2$ test, $p = 0.08$).

3.1. Genetic and clinical data of the study subjects

Among 393 subjects recruited for the study, 349 of them gave the consent for this targeted SNP study and their DNA was also available for SNP genotyping. The genotypes of rs13266634 were successfully determined in 348 subjects resulting in a call rate of the tested SNP at 99.7%. Thus, all subsequent results were analyzed based on the SNP genotyping results obtained from these 348 subjects. The allelic frequency of the risk C-allele was also calculated based on sex, age, and BMI subgroups (Table 2). It was 74.3% for men and 69.3% for women ($p > 0.05$ between sexes); 70.8% for 18–33 years old, 75.4% for 34–49 years old, and 68.8% for 50–65 years old ($p > 0.05$ among age subgroups); and 66.8% for BMI 18–24.9 (normal weight), 74.6% for BMI 25–29.9 (overweight), 75.3% for BMI 30–45 (obese) ($p > 0.05$ among BMI subgroups). In addition, among all subjects, 53.4% of them had the homozygous CC genotype and 36.5% had the CT heterozygous genotype. The remaining of 10.1% subjects had the homozygous TT genotype.

The characteristics of the participants, including age, height, weight, BMI, waist circumference, waist to hip ratio, and total % body fat categorized by genotypes (CC, CT, and TT) are summarized in Table 3. Subjects with either the CC or CT genotype showed higher height and weight than those with the TT genotype. However, it was not significant ($p > 0.05$) using ANOVA analysis after controlling for sex. Additionally, subjects with the CC genotype had higher BMI than those with the CT genotype that reached significance ($p < 0.05$). For all other characteristics, no significant difference was detected among the three genotypes ($p > 0.05$).

To examine whether the tested *ZNT8* SNP would influence blood zinc levels, we determined fasting serum zinc levels in the study subjects. As shown in Table 4, no statistical significance was detected among the three genotypic groups stratified in all subjects, sex, age, or BMI subgroups. Table 5 summarized the clinical parameters measured in this study including insulin, glucose, HbA1c, total cholesterols (TC), high-density lipoprotein cholesterols (HDL-C), low-density lipoprotein cholesterols (LDL-C), triglycerides (TG), and non-esterified fatty acids (NEFA), in overnight-fasted participants prior to MMTT on the test day. We found no significant difference for any of these indicators examined after adjusting for sex, age, and BMI among the three genotypes ($p > 0.05$). Moreover, the circulating levels of total ghrelin, a peptide hormone with the regulatory effects on food intake, glucose hemostasis, fat deposition, energy expenditure, growth hormone release and among

Table 2
The risk C allele frequency of rs13266634 in subgroups of the study.

Group		Sex		Age (year)			BMI (kg/m^2)		
Subgroup	All	Men	Women	18–33	34–49	50–65	18–24.9	25–29.9	30–45
N	348	167	181	120	116	112	135	126	87
C allele frequency (%)	71.7	74.3	69.3	70.8	75.4	68.8	66.8	74.6	75.3

Data are presented by frequency for the major allele. Frequency of alleles is presented as a percentage.

Table 3
General characteristics of subjects carrying variants of rs13266634 in *ZNT8*.

Genotype	CC	CT	TT
N (man/women)	186 (90/96)	127 (68/59)	35 (9/26)
Age (years)	40.0 ± 1.0	41.0 ± 1.2	41.5 ± 2.6
Height (cm)	170.7 ± 0.7	171.1 ± 1.0	166.3 ± 1.2
Weight (kg)	81.3 ± 1.4	78.1 ± 1.5	74.1 ± 2.8
BMI (kg/m^2)	27.7 ± 0.4	26.6 ± 0.4 *	26.7 ± 0.9
Waist circumference (cm)	86.1 ± 1.0	84.1 ± 1.1	83.6 ± 2.3
Waist to hip ratio	0.82 ± 0.01	0.82 ± 0.01	0.81 ± 0.01
Total % body fat	30.1 ± 0.8	28.9 ± 0.9	31.8 ± 1.7

BMI, body mass index; Data represent unadjusted means ± S.E.M. Tukey's test (2-tails) was used for analysis of variables including age, height, weight, waist circumference, and BMI. * , $p = 0.02$ between the CC and CT genotypes.

Table 4
Fasting serum zinc levels in subjects carrying variants of rs13266634 in *ZNT8*.

Genotype Zinc (mg/L)	CC	CT	TT
All subjects	0.930 ± 0.013	0.949 ± 0.022	0.880 ± 0.026
(n = 347)	(n = 185)	(n = 127)	(n = 35)
Sex			
Men	0.964 ± 0.019	0.928 ± 0.020	0.849 ± 0.048
(n = 166)	(n = 89)	(n = 68)	(n = 9)
Women	0.859 ± 0.015	0.900 ± 0.020	0.863 ± 0.029
(n = 181)	(n = 96)	(n = 59)	(n = 26)
Age (y)			
18–33	0.922 ± 0.021	0.919 ± 0.025	0.888 ± 0.042
(n = 119)	(n = 63)	(n = 42)	(n = 14)
34–49	0.922 ± 0.020	0.904 ± 0.025	0.921 ± 0.058
(n = 116)	(n = 67)	(n = 41)	(n = 8)
50–66	0.874 ± 0.020	0.910 ± 0.024	0.828 ± 0.039
(n = 112)	(n = 55)	(n = 44)	(n = 13)
BMI (kg/m^2)			
18.5–24.9	0.914 ± 0.021	0.918 ± 0.022	0.853 ± 0.037
(n = 134)	(n = 60)	(n = 58)	(n = 16)
25–29.9	0.901 ± 0.019	0.902 ± 0.025	0.888 ± 0.04
(n = 126)	(n = 73)	(n = 42)	(n = 11)
30–45	0.907 ± 0.022	0.908 ± 0.031	0.904 ± 0.057
(n = 87)	(n = 52)	(n = 27)	(n = 8)

Zinc data were transformed by negative inverse (+0.2). Fasting zinc levels (mg/L) in three genotypic groups separated by sex, age, and BMI, respectively. Data are means ± SEM adjusted for other confounding variables (sex, age, and BMI). Subject numbers (n) is indicated. No statistical significance was detected among the genotypic groups.

others [25], were comparable among the three genotypes ($p > 0.05$).

3.2. Effect of rs13266634 on insulin and glucose metabolism before and after MMTT

To evaluate the impact of rs13266634 on glucose and insulin responses to MMTT, we examined blood insulin and glucose concentrations at 0, 0.5, 3, and 6 h after the challenge. All values were adjusted for age and BMI in sex subgroups. Likewise, values in age and BMI subgroups were adjusted for sex and BMI and for sex and age, respectively. We detected statistical significance in the interaction of SNP variants with fasting insulin levels in sex subgroups ($p < 0.05$) (Fig. 1A) while such interaction was not detected in BMI or age subgroups (Suppl. Fig. 2). Women carrying the TT genotype tended to have higher fasting insulin levels than those carrying the CC genotype whereas the opposite was observed in men (Fig. 1 A). The insulin incremental area under the curve post MMTT was significantly decreased by ~20% in men with the CC genotype compared to those with the CT or TT genotype ($p < 0.05$) (Fig. 1B). However, no difference in the insulin incremental area under the curve was observed in women across the three genotypes (Fig. 1B) although women generally had higher insulin levels than men at 3 ($p < 0.001$) and 6 h ($p < 0.01$) post MMTT (Fig. 1C). Again, the interaction of genotype-sex was significant for the insulin incremental area during MMTT ($p < 0.05$). Taken together, these results indicate that the common risk C-allele of rs13266634 influences fasting insulin levels as well as insulin response post MMTT in men but not in women, suggesting sex-dependent effect of the SNP.

Table 5
Fasting clinical parameters in subjects carrying variants of rs13266634 in *ZNT8*.

Genotype	CC	CT	TT
N (man/women)	186 (90/96)	127 (68/59)	35 (9/26)
Glucose (mg/dL)	95.7 ± 1.4	93.7 ± 0.7	93.1 ± 1.3
HbA1c (%)	5.29 ± 0.02	5.26 ± 0.02	5.33 ± 0.05
Insulin (pmol/L)	65.9 ± 4.6	56.0 ± 3.1	68.8 ± 8.7
Ghrelin (pg/mL)	103.3 ± 5.6	112.1 ± 7.0	98.6 ± 12.5
Triglycerides (mg/dL)	96.9 ± 3.80	98.5 ± 3.9	92.9 ± 7.2
NEFA (mEq/L)	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.01
Total cholesterol (mg/dL)	172.0 ± 2.4	177.7 ± 3.4	179.8 ± 5.1
HDL-C (mg/dL)	54.9 ± 1.2	55.0 ± 1.4	57.8 ± 3.1
LDL-C (mg/dL)	107.1 ± 2.2	112.8 ± 3.1	113.8 ± 4.3

LDL-C, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; For ANOVA analysis, datasets for total cholesterol, triglycerides, LDL-C, HDL-C, glucose, insulin, HbA1c, and ghrelin were adjusted for BMI, age, and sex. Data for triglyceride, insulin, and ghrelin were transformed by natural logarithm.

Homeostatic model assessment (HOMA) is a measurement for assessing β-cell function and insulin resistance (IR) from fasting glucose and insulin concentrations. Thus, we calculated insulin resistance score (HOMA-IR) in subgroups adjusted for sex, BMI, and age. We noticed that HOMA-IR scores were significantly higher in the BMI 30–45 subgroup than the other two BMI subgroups (Suppl. Fig. 1C), which is consistent with previous studies that high BMI or obesity is a risk for insulin resistance and type 2 diabetes [26,27]. Moreover, subjects with BMI higher than or equal to 30 had increased insulin levels at all-time points examined before and after MMTT compared to those with BMI below 30 (Suppl. Fig. 2A). Likewise, the insulin AUC (area under the curve) was also elevated as BMI increased (Suppl. Fig. 2B). However, this BMI-associated insulin incremental increase was not correlated to the genotypes of rs13266634. Moreover, we show that there was no association of the genotypes with age in the postprandial insulin response after MMTT (Suppl. Fig. 2C & D).

The physiology of glucose homeostasis is primarily regulated by insulin together with its counterpart, glucagon. We, therefore, determined blood glucose concentrations categorized by sex, age, and BMI before and after MMTT. The results showed that fasting glucose levels and the AUC of glucose concentrations were associated with sex, age and BMI. Men, elder subjects, as well as the subjects with BMI ≥ 30 had higher glucose levels before and after the challenge than women, young subjects, and the subjects with BMI below 30 ($p < 0.0001$) (Suppl. Fig. 3).

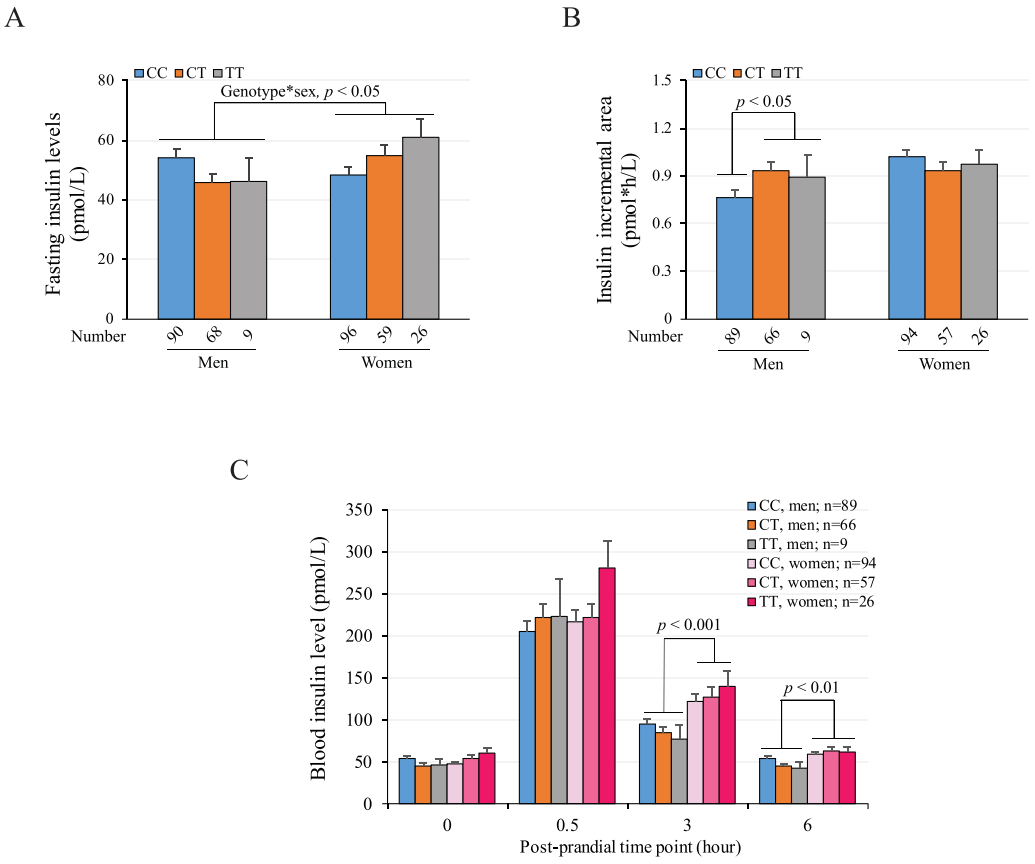

Fig. 1. Serum insulin levels in the study subjects before and during a 6-h protocol for an oral high-fat MMTT. (A) Fasting insulin levels in men and women. The interaction of genotypes with sex was significantly different (genotype*sex, $p < 0.05$). (B) The insulin incremental area during MMTT. Data were summarized from 341 participants who completed all four time points during the course of the protocol (CC = 183; CT = 123; and TT = 35). Men with the CC genotype had a lower insulin incremental area under the curve than those with the CT or TT genotype ($p < 0.05$). (C) Time course of insulin levels in men and women carrying the CC, CT, or TT genotype. Data are means ± SEM adjusted for age and BMI. n is indicated in the panels. CC, subjects carrying the homozygous C-allele of the SNP; CT, subjects carrying one C and one T allele; TT, subjects carrying the homozygous T-allele.

However, these changes were not associated with the SNP genotypes. We also measured blood glucagon levels. However, the MSD multiplex assay that we used for detecting glucagon did not have sufficient sensitivity for reliably determining the concentrations of glucagon. Thus, an association analysis of glucagon levels with the rs13266634 genotypes could not be determined in this study.

3.3. *Effect of rs13266634 on lipid profiles during MMTT*

To evaluate the impact of the genotypes of rs13266634 on lipid metabolism, we sought to investigate the associations between lipid markers, including TG, NEFA, total cholesterol, HDL-C, and LDL-C, and the genotypes during MMTT. All statistical analyses were carried out separately in three subgroups: sex (adjusting for age and BMI), age (adjusting for sex and BMI), and BMI (adjusting for sex and age). As shown in Fig. 2, among the five investigated lipid markers, the mean concentration of fasting NEFA was the only one that displayed a significant genotype-sex interaction ($p < 0.01$) (Fig. 2A). Men with the CC genotype had a lower level of fasting NEFA (0.27 ± 0.01 mEq/L) than men with the CT (0.31 ± 0.02 mEq/L) or TT genotype (0.37 ± 0.04 mEq/L) ($p < 0.001$) (Fig. 2A). On the other hand, the opposite was found for women. The mean NEFA levels for women carrying the CC, CT, and TT genotypes were 0.37 ± 0.01, 0.33 ± 0.02, and 0.31 ± 0.03 mEq/L, respectively ($p < 0.05$) (Fig. 2A). In addition, a trend for a genotype-sex interaction in fasting triglyceride levels was observed; but it was not significant ($p = 0.06$) (Fig. 2D). Circulating NEFA levels were significantly increased in subjects with BMI ≥ 30 ($p < 0.001$) (Fig. 2C) and TG levels were associated with sex, age or BMI (Fig. 2D, E & F). However, these changes were not associated with the tested genotypes. Finally, we found no evidence of confounding effects of age and BMI on the interaction of rs13266634 genotypes with cholesterol levels, including TC, LDL-C, and HDL-C, albeit these lipid levels increased due to aging or increased BMI except for HDL-C which decreased as BMI increased (data not shown).

We showed that during MMTT NEFA levels decreased by 21–25% at 0.5 and 3 h compared to the baseline ($p < 0.0001$) and the mean NEFA level recovered at 6 h to a level 73% higher than the baseline (Suppl. Fig. 4A). However, these changes were independent on the genotypes (Suppl. Fig. 4A), sex (Suppl. Fig. 4B), age, and BMI subgroups (data not shown). Moreover, the AUC of NEFA remained similar among the genotypic groups within the sex, age, or BMI subgroups (Suppl. Fig. 4C and data not shown).

We also showed that MMTT elevated circulating triglycerides at 3 h by 8–10% ($p < 0.001$) and recovered towards to the baseline at 6 h post MMTT (Fig. 3A). In general, men had higher postprandial TG levels than women did at 3 and 6 h ($p < 0.01$) (Fig. 3A). The AUC of TG concentrations was also higher in men than women during the challenge (Fig. 3B). Most importantly, we found that men with the TT genotype presented the slowest postprandial TG removal from the circulation as determined by the change between 3- and 6-h time points than men with the CC or CT genotype ($p < 0.05$) while this genotype-triglyceride correlation was not noted in women (Fig. 3A). However, the AUC of TG in the CT genotypic group was different from the CC genotypic group in women ($p < 0.05$) (Fig. 3B). The slower postprandial TG removal was also observed in the subjects, both men and women, with BMI ≥ 30 and having the TT or CT genotype ($p < 0.05$) (Fig. 3C). Subjects with BMI ≥ 30 and carrying the T-allele (CT or TT genotype) had higher postprandial TG at 3 h (24–30%) or 6 h (19–34%) than those with the same BMI but carrying the CC genotype ($p < 0.05$; Fig. 3C). Moreover, in the BMI ≥ 30 subgroup, the AUC of TG concentrations was significantly higher (8–11%) in the subjects carrying the CT or TT genotype (11%) than those carrying the CC genotype ($p < 0.05$; Fig. 3D).

In agreement with previously reported data [28], age-induced changes in TG metabolism were also observed in this study. Aging

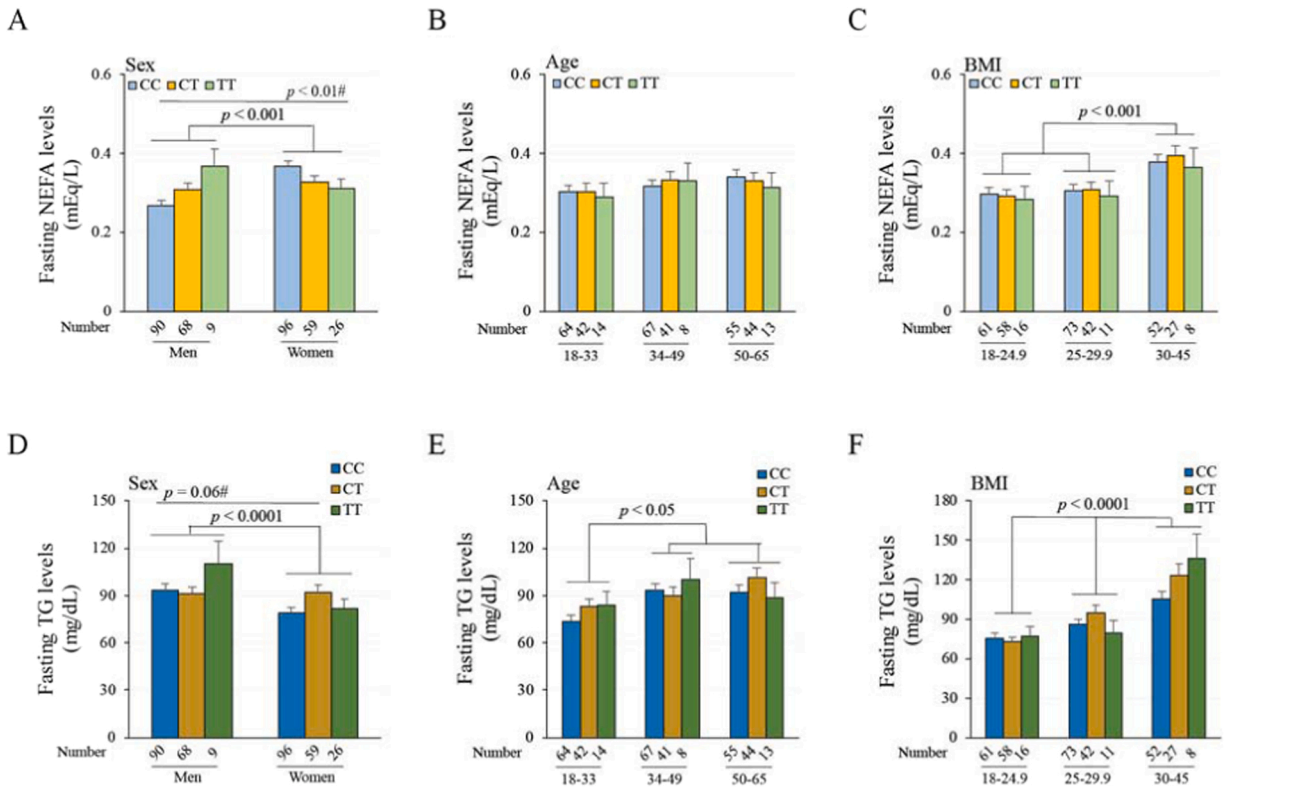

Fig. 2. Effect of rs13266634 on the fasting NEFA and TG levels. (A) & (D) Fasting NEFA and TG levels in men and women in three genotypic groups, respectively. Data were mean ± S.E. adjusted for age and BMI. (B) & (E) Fasting NEFA and TG levels in three genotypic groups separated by age. Data were mean ± S.E. adjusted for sex and BMI. (C) & (F) Fasting NEFA and TG levels in three genotypic groups separated by BMI, respectively. Data were mean ± S.E. adjusted for sex and age. *p*-values are shown in each panel. #, significant difference in the genotype-sex interaction. *, significant difference in sex, age, or BMI subgroups regardless of genotypes.

increased postprandial blood TG levels at 0.5 and 6 h after MMTT. However, these changes were independent on the SNP genotypes ($p > 0.05$) (Fig. 3E). The AUC of TG concentrations also tended to increase by aging regardless of genotypes (Fig. 3F). Nevertheless, it is worth noting that postprandial TG levels were elevated (~25%) at the 3-h time point post MMTT in the middle-aged subjects (34–49 years old) carrying the TT genotype compared to those with the CC genotype ($p < 0.05$) (Fig. 3E). Taken together, these results suggest that the minor T-allele of rs13266634 may negatively affect TG removal from the circulation after a lipid-rich mixed macronutrient meal challenge in obese and middle-aged people.

Lastly, the consumption of the high-fat mixed macronutrient meal slightly increased serum levels of total cholesterol, HDL-C, and LDL-C at 0.5 h and then the levels recovered to the baselines (TC and LDL-C) or lower than the baseline (HDL-C) at 3 or 6 h post MMTT (Suppl. Fig. 5). However, no association of the SNP genotypes with the postprandial TC, LDL-C and HDL-C levels was detected in all examined three subgroups (sex, age, and BMI; data not shown).

4. Discussion

We studied the effect of a *ZNT8* SNP (rs13266634) on glucose and lipid metabolism by investigating clinical markers related to these metabolic pathways in healthy adults recruited for a cross-sectional nutritional phenotyping study conducted in the USDA-Agriculture of Research Service-Western Human Nutrition Research Center. The novelty of the current study was to investigate the response of insulin, glucose, and lipids together after a high-fat mixed macronutrient meal challenge in healthy human subjects in the United States.

We performed a detailed clinical study for the association of the SNP with glucose and insulin metabolism. We showed an association of the risk C-allele of the SNP with higher fasting insulin levels, consistent with previous findings [29]. Importantly, we report that this association was sex-dependent, a unique study finding. We found that the C-allele influenced fasting insulin levels and insulin response after MMTT in men but not in women (Fig. 1). In addition, men with the CC genotype had decreased insulin incremental area under the curve after MMTT compared to those of the CT or TT genotype. These results agree with previous reports indicating that non-diabetic individuals carrying the risk C-allele had reduced glucose-stimulated insulin levels [30], and the CC genotype favors a higher risk of insulin impairment and glucose intolerance in diabetic patients [31].

Postprandial blood glucose levels are maintained by many factors, including increased insulin secretion from the pancreas, inhibition of glucose production from the liver and insulin-trigged glucose uptake in the peripheral tissues to remove glucose from the circulation. Therefore, impaired function in these factors or disruption of their interactions would contribute to the development of overt hyperglycemia [32]. In the current study, we revealed that men with the CC genotype had decreased incremental area under the curve of insulin post MMTT by ~20% compared to those with the CT or TT genotype ($p < 0.05$) whereas postprandial glucose concentrations at each examined time point remained similar among the three genotypes. This could be explained by the fact that we had recruited healthy subjects to participate in this cross-sectional nutritional phenotyping study. Thus, the functional interaction was maintained between postprandial insulin secretion, inhibition of glucose production, and peripheral insulin action in these subjects resulting in a normal glucose tolerance.

Sex difference in glucose and energy metabolism is known due, at least in part, to the action of sex-specific hormones, including estrogens and progesterone in females and androgens in males [33–35]. Female sex hormones usually have favorable effects on insulin sensitivity [36,

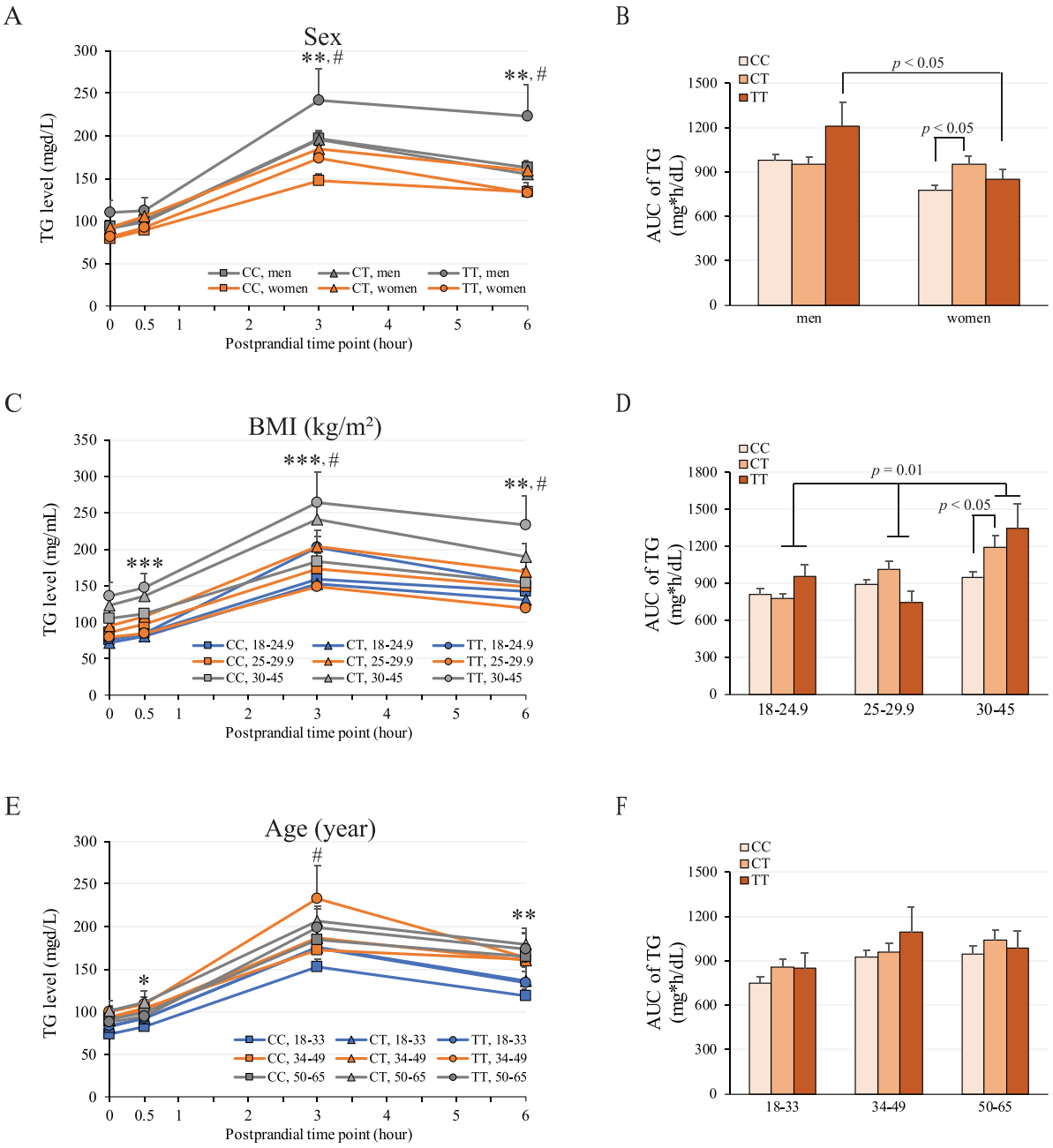

Fig. 3. Effect of rs13266634 on the TG responses after MMTT. (A), (C), & (E) TG responses after MMTT in three genotypic groups separated by sex, age, and BMI, respectively. (B), (D), & (F) The AUC of TG during MMTT in three genotypic groups separated by sex, age, and BMI, respectively. Data are mean ± S.E. adjusted for other two confounding factors. n is the same as shown in Fig. 1. #, significant difference ($p < 0.05$) in the genotype-sex interaction at the indicated time points. * ($p < 0.05$), * * ($p < 0.01$), or * ** ($p < 0.001$), significant difference in sex, age, or BMI subgroups at the indicated time points regardless of genotypes.

37]. Thus, an interaction of the risk C-allele with sex hormones in the determination of fasting insulin level is expected. Further research into the mechanisms underlying the interactions of sex hormones with R325 ZNT8 in regulation of insulin secretion, insulin resistance, and energy metabolism is needed.

Given that obesity is a significant risk factor for T2D development [38] and R325 ZNT8 increases the risk of T2D [31], it would be essential to evaluate the effect of R325 on lipid metabolism in humans. In the current study, we showed for the first time that men with the risk CC genotype had the lowest level of NEFA in the fasting state relative to those with the CT or TT genotype (Fig. 2). However, the NEFA levels during MMTT seemed to not be significantly affected by the risk genotype (Suppl. Fig. 4). In the fasting state, a major source of NEFA in the circulation is from the mobilization of stored fat from the adipose tissue. Adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) are the two major lipases in this process of fatty acid mobilization in adipocytes when energy is demanded. ATGL is an adipose-enriched enzyme with triglyceride-specific lipase activity, which catalyzes the first reaction of lipolysis, where triglycerides are hydrolyzed to diacylglycerols [39–41]. HSL hydrolyzes triglycerides, diglycerides, and monoglycerides freeing fatty acids in the adipocytes. The first step of cleaving fatty acids from triglycerides is rate limiting as it is controlled by epinephrine, catecholamines, adrenocorticotropic hormone (ACTH), and insulin. Both HSL and ATGL enzymatic activities are induced by epinephrine, catecholamines, and ACTH during fasting, while they are inhibited by the increased insulin level after a meal [39,42]. Our findings that the CC genotype affected fasting NEFA levels but not postprandial NEFA concentrations in men during the 6-h course of MMTT

suggest that the activation of adipose ATGL or HSL was suppressed, likely due to increased fasting insulin. Given that ZNT8 is expressed in many other endocrine and neuroendocrine cells, albeit in a very low level compared to its expression in β-cells [43], the effect of ZNT8 on the synthesis and secretion of epinephrine, catecholamines, and ACTH cannot not be excluded. The CC genotype might also negatively influence these hormone levels during fasting leading to reduced lipolysis in the adipocytes. Nevertheless, the current study's results warrant future studies addressing the role of ZNT8 in HSL and ATGL activities during fasting in humans.

Surprisingly, in this study, we found that the TT genotype of rs13266634, a beneficial genotype against T2D development, exhibited a negative effect on triglyceride removal from the circulation in men and obese individuals after MMTT without significantly affecting postprandial removal of total cholesterol, LDL-cholesterol, and HDL-cholesterol. Epidemiological studies have shown postprandial hypertriglyceridemia constitutes an independent risk for atherosclerotic vascular disease [7,21,44]. Generally, after a lipid-rich meal, a small elevation of blood TG can be detected around 10–30 min, which usually represents the release of the stored TG from the enterocyte into the circulation before the absorption of the meal fat [45,46]. The second postprandial peak of blood TG, which generally occurs 3–4 h after a meal, signifies the primarily absorbed TG in the circulation and the rise of blood TG and it is a result of increases in both chylomicron particles and TG content [47]. At around 4–6 h post meals, blood TG mainly from the liver-derived very low-density lipoprotein (VLDL) particles, tends to slowly return towards the baseline [48,49]. In this study, we showed that the TT genotype was associated with higher postprandial total TG levels at 3 and 6 h in men and obese subjects after MMTT, suggesting that both chylomicron and VLDL removal may be negatively affected by the TT genotype. This negative association was also observed in obese subjects carrying only one T-allele of the SNP, indicating obesity is a confounding factor associated with postprandial hypertriglyceridemia. Inverse relationships of genetic traits affecting dyslipidemia with glucose metabolism have been reported previously [50,51]. For example, Li et al. reported that genetic risk scores related to a higher triglyceride level correlated to lower levels of fasting glucose, HbA1c, and HOMA-IR [50]. Thus, our findings support the fact that there is a complex genetic and metabolic interplay between glucose and lipid metabolism.

TG in chylomicrons and VLDL is removed by lipoprotein lipase (LPL), an enzyme attached to the luminal side of endothelial cells in capillaries [52]. LPL is expressed in adipose, heart, and skeletal muscle tissues to hydrolyze TG to release free fatty acids from lipoprotein particles for energy storage or use after food intake. In adipocytes, insulin stimulates LPL presentation on the luminal surface of the capillary endothelium and activates its enzymatic activity after meals. On the other hand, insulin decreases the expression and activation of LPL in skeletal and myocardial muscle post meals. Thus, postprandial TG levels generally depend on the LPL activity in the adipose tissue where TG is stored and the liver where chylomicron remnants are removed from the circulation and VLDL is released into the bloodstream. Thus, owing to the role of ZNT8 in delivery of zinc in the secretory granule of the β-cell essential for insulin crystallization and storage, one plausible explanation for the slower TG removal from the circulation in men or obese people carrying the T-allele after a mixed meal challenge is that the insulin-mediated activation of LPL may be somewhat inhibited in the presence of non-modifiable factors, such as male sex hormones or modifiable factors, such as obesity.

Although novel findings in the effect of ZNT8 SNP rs13266634 on human lipid metabolism are demonstrated in this study, many limitations exist in the current study. For example, the research findings of this study might be limited by relatively lower numbers of TT genotype carriers in the study cohort. Therefore, further studies in the exploration of lipid metabolism in subjects with the TT genotype are warranted to reveal a potential role of ZNT8 or zinc in bridging glucose and lipid metabolism.

In summary, the observations of the current study suggest ZNT8 is involved, either directly or indirectly, in insulin, NEFA, and postprandial TG metabolism. We confirmed that the C-allele of the SNP conferred the risk for abnormal insulin levels in humans, especially in men. On the other hand, we found that the T-allele negatively affected fasting NEFA levels as well as postprandial TG removal from the circulation in men and obese individuals. Our findings suggest that the genotypes of rs13266634 interact with sex hormone and/or adiposity leading to an opposite risk effect on insulin and TG metabolism. More studies are needed to identify the mechanisms underlying this complex interplay between glucose and lipid metabolism mediated by the function of ZNT8.

Ethics approval and consent to participate

The study was registered on ClinicalTrials.gov (ID: NCT02367287) and received ethical approval from the University of California Davis Institutional Review Board. This study was carried out at the USDA/Agriculture Research Service/Western Human Nutrition Research Center at Davis, CA. Generally healthy people living near Davis, CA were invited to participate in this cross-sectional study. Details of the study were explained to and discussed with participants, and those who agreed to the terms of the study provided informed consent during the first study visit.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

LH conceived the project, designed scientific objectives, analyzed the data, participated in drafting the manuscript, and final approval of the manuscript. ZY drafted the manuscript; YEW performed genomic DNA

purification and quantification and edited the manuscript. CPK optimized protocols for genomic DNA purification and was involved in blood DNA purification, quantification, SNP genotyping, and edited the manuscript. CBS was the project administrator and supervised the project, reviewed and edited the manuscript. JWN supervised the measurements of clinic lipid markers and participated in manuscript editing. NLK supervised the measurements of glucose metabolism-related clinical makers, reviewed and edited the manuscript. YC participated in manuscript drafting and editing the manuscript.

Impact statement

The C-allele of rs13266634 in *ZNT8* is associated with increased risk of type 2 diabetes (T2D) as a result of impaired zinc homeostasis in the insulin-containing granule in the islets. While previous studies have examined the correlation of the variants with insulin and glucose metabolism, the effects of this SNP on lipid responses after a lipid challenge remain elusive in humans. Thus, the current study investigated the association of the risk C-allele with fasting and postprandial lipid levels in adults in the U.S. Our results suggest ZNT8 is involved, either directly or indirectly, in insulin, NEFA, and postprandial triglyceride metabolism. We found for the first time that the minor T-allele negatively affected fasting NEFA levels as well as postprandial triglyceride removal from the circulation in men and obese individuals, suggesting that the variant of the SNP had an opposite risk effect on T2D and dyslipidemia. From this study, we identified zinc might be an important regulatory factor for bridging glucose and lipid metabolism. Our study highlights that zinc may be involved in dual co-regulation of lipid and glucose levels, which provides a direction to study the mechanism underlying this complex interplay mediated by zinc or ZNT8.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jtemb.2023.127142.

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