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Association between serum vitamin C and body mass index in adolescents aged 12–19 years

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Abstract

Background Evidence on the association between serum vitamin C (sVC) levels and obesity is limited. This study aimed to explore the relationship between sVC and body mass index (BMI) in adolescents aged 12 to 19 years.

Methods We analyzed data from the National Health and Nutrition Examination Survey (NHANES) 2003–2006, with 3952 participants. sVC and BMI were independent variables and dependent variables, respectively. The associations of sVC with BMI were examined using multivariable linear regression models. Age, sex, and race/ethnicity were analyzed as subgroups. Then, we devised smooth curve fittings and saturation threshold analysis to address the nonlinear relationship.

Results sVC had a negative correlation with BMI after adjusting for all covariates (β: -1.020, 95% CI: -1.359, -0.680). In the subgroup analysis by age, sex, and race/ethnicity, there was still a negative correlation between sVC and BMI ($p < 0.05$). The analysis of saturation effects of sVC and BMI showed the relationship between sVC and BMI in female adolescents followed an N-shaped curve, whereas the relationship between sVC and BMI in adolescents aged 12–15 years and Mexican Americans followed a U-shaped curve.

Conclusion Based on the results, proper vitamin C supplementation may be beneficial to weight loss. However, considering the threshold effect, large-scale and good-quality randomized controlled trials are required to obtain the optimal vitamin C level for weight control.

Keywords Obesity, Body mass index, Serum vitamin C, Adolescents, NHANES

Introduction

In recent decades, obesity has become a global public health issue. Obesity is associated with a higher risk of cardiovascular and metabolic diseases, resulting in a serious social and economic burden [1, 2]. It has been reported that obesity in adolescence was significantly associated with an increased risk of coronary artery disease and diabetes in adulthood [3, 4]. Therefore, it was necessary to take effective measures to prevent overweight and obesity early in life. Lifestyle changes, such as controlling energy intake and increasing physical activity, could delay the progression of obesity. In addition,

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adequate vitamin C could boost the immune system and improve body metabolism [5, 6]. However, more than 30% of American adolescents have insufficient vitamin C intake (<45 mg/d) [7]. A low intake of vitamin C (<10 mg/d) could lead to metabolic disorders [8]. Previous studies have evaluated the potential relationship between serum vitamin C (sVC) and obesity [9, 10], but have focused mainly on adults and paid little attention to adolescents. However, studies [11] have also shown that age makes a difference in vitamin C concentrations in the body. Therefore, it is important to assess the relationship between sVC and obesity in adolescents, which may provide guidance for longer-term weight management.

Vitamin C is an essential micronutrient and has many important biological functions, the most important of which is as an antioxidant and cofactor [12]. Vitamin C can facilitate the uptake of iron, improve immune function, collagen synthesis, etc. [13, 14]. Given that vitamin C regulates metabolism and enhances immune function, it could be beneficial for weight reduction. Previous studies have demonstrated the relationship between sVC and metabolism. Researchers have found that low sVC concentrations are commonly associated with metabolic syndrome [15]. Vitamin C deficiency can promote endotoxemia, which leads to metabolic dysfunction [16]. Metabolic dysfunction affects the consumption of excess nutrients, causing them to be converted into fat and stored in adipose tissue, which eventually leads to the occurrence of obesity [17–19]. Thus, vitamin C potentially contributes to reducing weight and obesity.

However, there is a lack of valid evidence that taking vitamin C helps with weight loss. Therefore, whether vitamin C is recommended (45 ~ 200 mg/d) for adolescents to aid in weight management requires further study. In this study, we aimed to address the research gaps mentioned earlier. Specifically, we examined the association between sVC and body mass index (BMI) in an adolescent population using data from the NHANES, a representative sample of the US population, from 2003 to 2006.

Materials and methods

Study population

The study population was adolescents aged 12 to 19 years from the NHANES between 2003 and 2006. The NHANES is a population-based, cross-sectional survey conducted by the National Center for Health Statistics of the Centers for Disease Control and Prevention. An extensive collection of demographic information, lifestyle and dietary data, and anthropometric and laboratory measurements are available in the NHANES data set. The National Center for Health Statistics (NCHS) Institutional Review Board approved the NHANES protocol, and a signed informed consent form was obtained. The target population was adolescents aged 12–19 years (n=4591) in America, including Mexican Americans (n=533), other Hispanic (n=219), non-Hispanic White (n=2901), non-Hispanic Black (n=697), other races (n=241). After excluding those with missing sVC (n=607) and those with missing BMI (n=32), 3952 participants were finally included in the analysis sample. Figure 1 illustrates the inclusion process.

Independent and dependent variables

The blood vitamin C level was designed as an independent variable. sVC is measured using isocratic high-performance liquid chromatography (HPLC) with electrochemical detection at 650 mV1. Testing for vitamin C began in 2003. Detailed instructions on specimen collection and processing can be found on the NHANES website.

BMI was designed as a dependent variable. BMI is a component of body measurement. The body measurement data were collected by trained health technicians. The health technician was accompanied by a recorder during each body measurement examination. Height and weight were measured while wearing light indoor clothing and without shoes. The World Health Organization classifies BMI as: (i) Underweight: BMI < 18.5 kg/m^2, (ii) Normal range: 18.5 ~ 24.9 kg/m^2, (iii) Overweight: 25 ~ 29.9 kg/m^2, (iv) Obesity: BMI ≥ 30 kg/m^2, (v) Grade I obesity: 30.0 ~ 34.9 kg/m^2, (vi) Degree II obesity: 35.0 ~ 39.9 kg/m^2, and (vii) Degree III obesity: BMI > 40.0 kg/m^2. BMI was calculated by dividing the weight by the square of the height (kg/m^2).

Covariates

In addition, it also included the following covariables: age, sex, race/ethnicity, poverty-income rate (PIR), serum cotinine, total cholesterol, high density lipoprotein (HDL)-cholesterol, glycohemoglobin, systolic blood pressure (SBP), and diastolic blood pressure (DBP). Information about age, sex, race/ethnicity, and PIR were obtained from demographic data. Laboratory tests determined serum cotinine, total cholesterol, HDL-cholesterol, and glycohemoglobin. Blood pressure was taken three times, including SBP and DBP, and averaged for analysis.

Statistical analysis

All analyses were performed using EmpowerStats (http://www.empowerstats.com). Based on NHANES sample weights, all estimates were calculated. Means (standard deviations, SD) were calculated for continuous variables, and frequencies (percentages) were calculated for categorical variables. sVC showed a continuous variable and was divided into quartiles. Weighted multiple regression models were used to estimate linear relationships between sVC and BMI after adjusting for potential

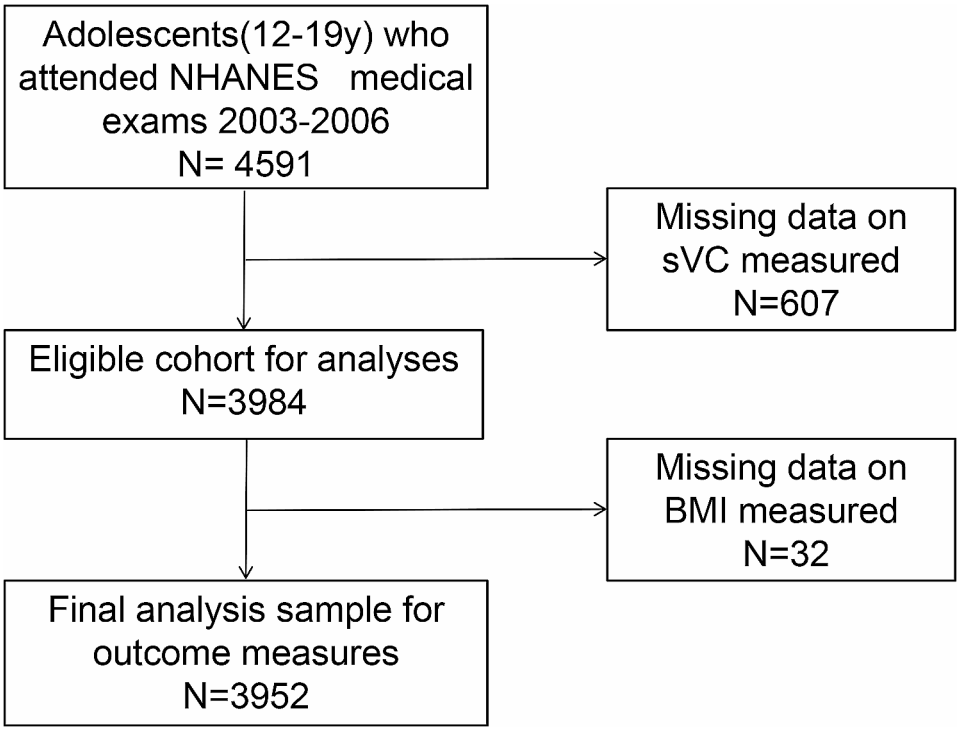

Fig. 1 Eligible participants and those included in the analyses of the association between serum vitamin C and body mass index in adolescents

confounders. In this study, three weighted linear regression models were constructed: model 1, unadjusted; model 2, age, sex, race/ethnicity were adjusted; model 3, age, sex, race/ethnicity, serum cotinine, PIR, total cholesterol, HDL-cholesterol, glycohemoglobin, SBP, DBP were adjusted. For the construction of these three models, we refer to several other researchers' published articles [20–22] which followed a similar pattern of the analyses. A smoothed curve fitting and saturation threshold analysis were used to further examine the linear or nonlinear relationship sVC and BMI. When nonlinearity was detected, we calculated the inflection point using a recursive algorithm and then made two-piecewise linear regressions around the inflection. *P* values < 0.05 were considered statistically significant.

Results

Characteristics of participants

A total of 3952 participants from NHANES 2003–2006 were finally included in this study. The baseline characteristics of the study population are listed in Table 1. The weighted characteristics of the subjects were subdivided into sVC quartiles (Q1: 0.01–0.77 mg/dL, Q2: 0.78–1.04 mg/dL, Q3: 1.05–1.29 mg/dL, and Q4: 1.30–3.75 mg/dL). Among all participating adolescents, the sample comprised 51.52% males and 48.48% females, along with 11.6% Mexican Americans, 4.78% other Hispanic, 63.19% non-Hispanic White, 15.18% non-Hispanic Black, and 5.25% other races. The average (SD) values of sVC and BMI were 1.02 ± 0.46 mg/dL and 23.58 ± 5.77 kg/m^2, respectively. Among the different groups of sVC (quartiles, Q1-Q4), we found significant differences in age, sex, race/ethnicity, serum cotinine, PIR, HDL-cholesterol, SBP, and BMI. (All $p < 0.001$). sVC levels decreased with age ($p < 0.001$). SBP decreased with the increasing sVC levels ($p < 0.001$). HDL-cholesterol increased linearly with the increasing sVC levels ($p < 0.001$).

Association between sVC and BMI

Using a multivariate regression analysis, we found a negative association between sVC and BMI in all three models (model 1: β= -1.935, 95% CI: (-2.319 to -1.550); model 2: β = -1.731, 95% CI: (-2.108 to -1.354); model 3: β= -1.020, 95% CI: (-1.359 to -0.680)). Figure 2 shows that sVC was negatively correlated with BMI in the fully adjusted model.

Furthermore, we also performed subgroup analysis stratified by age, gender, and race/ethnicity. We found that this negative correlation remained in male adolescents (β= -2.460, 95% CI: -3.020 to -1.900), female adolescents (β= -1.017, 95% CI: -1.493 to -0.541), adolescents aged 12–15 years (β= -1.184, 95% CI: -1.631 to -0.737), and adolescents aged 16–19 years (β= -0.933, 95% CI: -1.452 to -0.415). This negative correlation also remained mainly in Mexican Americans (β= -1.076, 95% CI: -1.771 to -0.381), non-Hispanic White (β= -0.978, 95% CI: -1.578 to -0.379), and non-Hispanic Black (β= -1.169,

Table 1 Description of 3952 participants included in the present study

Serum vitamin C (mg/dL)	All	Q1(0.01–0.77)	Q2(0.78–1.04)	Q3(1.05–1.29)	Q4(1.30–3.75)	*P* value
Age (years)	15.46 ± 2.24	15.78 ± 2.22	15.50 ± 2.24	15.35 ± 2.23	15.18 ± 2.23	< 0.001
Age (%)						< 0.001
12–15 years	50.27	44	51.11	52.86	54.25	
16–19 years	49.73	56	48.89	47.14	45.75	
Sex (%)						< 0.001
Male	51.52	52.66	59.53	49.94	44.76	
Female	48.48	47.34	40.47	50.06	55.24	
Race/ethnicity (%)						< 0.001
Mexican American	11.6	9.78	13.78	12.62	10.8	
Other Hispanic	4.78	3.22	6.74	5.56	4.1	
Non-Hispanic White	63.19	70.2	56.01	60.1	64.38	
Non-Hispanic Black	15.18	10	18.58	16.79	16.61	
Other Race-Including Multi-Racial	5.25	6.79	4.89	4.93	4.11	
PIR	2.56 ± 1.60	2.53 ± 1.58	2.39 ± 1.52	2.57 ± 1.63	2.74 ± 1.65	< 0.001
Serum cotinine (%)						< 0.001
<=0.011	15.8	10.69	14.87	14.84	23.44	
0.011-10	68.79	65.87	70.74	74.05	65.34	
> 10	15.41	23.45	14.39	11.11	11.22	
Total cholesterol (mg/dL)	161.01 ± 31.71	161.99 ± 32.94	158.63 ± 30.14	161.71 ± 32.14	161.30 ± 31.06	0.090
HDL-cholesterol (mg/dL)	52.41 ± 12.71	50.25 ± 13.16	52.08 ± 11.46	53.26 ± 12.17	54.35 ± 13.34	< 0.001
Glycohemoglobin	5.12 ± 0.40	5.12 ± 0.32	5.12 ± 0.33	5.13 ± 0.42	5.13 ± 0.50	0.791
Systolic blood pressure (mmHg)	109.77 ± 9.97	110.56 ± 9.90	110.12 ± 10.25	109.84 ± 9.53	108.48 ± 10.10	< 0.001
Diastolic blood pressure (mmHg)	59.74 ± 11.44	60.16 ± 11.02	59.52 ± 11.80	59.78 ± 11.37	59.41 ± 11.63	0.438
BMI (kg/m^2)	23.58 ± 5.77	24.82 ± 6.61	23.40 ± 5.36	23.16 ± 5.23	22.71 ± 5.31	< 0.001

Description of 3952 participants included in the present study

Mean ± SD for continuous variables: *P*-value was calculated by weighted linear regression model

% for categorical variables: *P*-value was calculated by weighted chi-square test

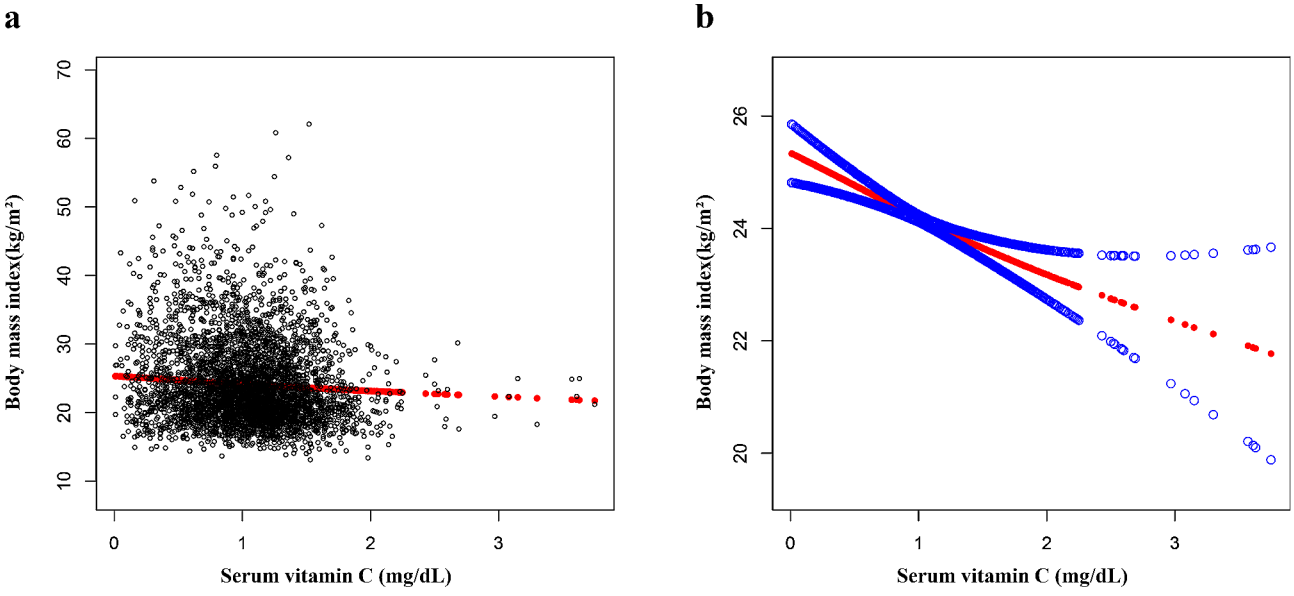

Fig. 2 The association between serum vitamin C and body mass index. (**a**) Each black point represents a sample. (**b**) The area between two blue dotted lines is expressed as a 95% CI. Each point shows the magnitude of the serum vitamin C and is connected to form a continuous line. Age, sex, race/ethnicity, serum cotinine, PIR, total cholesterol, HDL-cholesterol, glycohemoglobin, SBP, and DBP were adjusted

Table 2 Association of serum vitamin C with body mass index

	Model 1 β (95% CI) *P* value	Model 2 β (95% CI) *P* value	Model 3 β (95% CI) *P* value
Serum vitamin C	-1.935 (-2.319, -1.550) < 0.00001	-1.731 (-2.108, -1.354) < 0.00001	-1.020 (-1.359, -0.680) < 0.00001
Stratified by age			
12–15 years	-1.473 (-1.974, -0.972) < 0.00001	-1.554 (-2.051, -1.058) < 0.00001	-1.184 (-1.631, -0.737) < 0.00001
16–19 years	-1.915 (-2.485, -1.344) < 0.00001	-2.109 (-2.684, -1.535) < 0.00001	-0.933 (-1.452, -0.415) 0.00043
Stratified by sex			
Male	-2.460 (-3.020, -1.900) < 0.00001	-2.029 (-2.588, -1.470) < 0.00001	-2.460 (-3.020, -1.900) < 0.00001
Female	-1.587 (-2.120, -1.053) < 0.00001	-1.625 (-2.141, -1.109) < 0.00001	-1.017 (-1.493, -0.541) 0.00003
Stratified by race/ethnicity			
Mexican American	-2.442 (-3.252, -1.633) < 0.00001	-2.068 (-2.868, -1.268) < 0.00001	-1.076 (-1.771, -0.381) 0.00247
Other Hispanic	-2.224 (-4.141, -0.308) 0.02471	-2.141 (-4.075, -0.208) 0.03198	-0.387 (-2.087, 1.312) 0.65602
Non-Hispanic White	-2.002 (-2.661, -1.343) < 0.00001	-1.680 (-2.333, -1.027) < 0.00001	-0.978 (-1.578, -0.379) 0.00142
Non-Hispanic Black	-1.992 (-3.021, -0.963) 0.00015	-1.908 (-2.902, -0.915) 0.00017	-1.169 (-2.058, -0.279) 0.01014
Other Race - Including Multi-Racial	-1.574 (-3.477, 0.328) 0.10668	-1.052 (-2.888, 0.784) 0.26296	-2.086 (-3.630, -0.542) 0.00898

Model 1, no covariates were adjusted

Model 2, age, sex, race/ethnicity were adjusted

Model 3, age, sex, race/ethnicity, serum cotinine, PIR, total cholesterol, HDL-cholesterol, glycohemoglobin, SBP, and DBP were adjusted

In the subgroup analysis stratified by age, sex or race/ethnicity, the model is not adjusted for the stratification variable itself

Table 3 Association of serum vitamin C with body mass index. Threshold effect analysis of serum vitamin C on body mass index using two-piecewise linear regression

Body mass index	Adjusted β (95% CI), *P* value
12–15 years	
Serum vitamin C <1.53(mg/dL)	-1.965 (-2.544, -1.386) < 0.0001
Serum vitamin C >1.53(mg/dL)	1.110 (-0.064, 2.285) 0.0641
Female	
Serum vitamin C <1.74(mg/dL)	-1.477 (-2.052, -0.901) < 0.0001
Serum vitamin C >1.74(mg/dL)	1.041 (-0.504, 2.585) 0.1869
Mexican American	
Serum vitamin C <1.42(mg/dL)	-1.790 (-2.631, -0.949) < 0.0001
Serum vitamin C >1.42(mg/dL)	2.125 (-0.239, 4.489) 0.0784

Age, sex, race/ethnicity, PIR, serum cotinine, total cholesterol, HDL-cholesterol, glycohemoglobin, SBP, and DBP were adjusted

In the subgroup analysis for 12–15 years adolescents, female, Mexican Americans, the model is not adjusted for age, sex or race/ethnicity, respectively

95% CI: -2.058 to -0.279). All the above results are presented in Table 2.

Non-linearity and saturation effect analysis between sVC and BMI

In addition, to delineate the non-linear relationship between sVC and BMI in adolescents, we used smooth curve fittings and saturation threshold analysis. The adjusted smooth curve fittings showed that BMI decreased with increasing sVC up to the turning point (turning point: sVC 1.53 mg/dL) in adolescents aged 12–15 years (Table 3; Fig. 3). Similarly, there was a turning point in female adolescents (turning point: sVC 1.74 mg/dL) (Table 3; Fig. 4) and in Mexican Americans (turning point: sVC 1.42 mg/dL) (Table 3; Fig. 5). In conclusion, the association between sVC and BMI in female adolescents followed an N-shaped curve, whereas the association between sVC and BMI in adolescents aged 12–15 years and Mexican Americans followed a U-shaped curve.

Discussion

The study aimed to investigate the relationship between sVC and BMI in adolescents. We investigated the relationship between sVC and BMI by analyzing large population data from the NHANES database. We observed that serum levels of vitamin C were lower in participants with a higher BMI. The most significant finding of this study was that sVC was negatively correlated with BMI. After adjustment for confounders, this negative correlation remained.

Subsequently, we performed a subgroup analysis of the effects of sVC on BMI in several categories, and these results showed that the effects of sVC on BMI differed across groups. The effects of sVC on BMI indicated a threshold effect among female adolescents, whereas a substantial linear association may occur among male adolescents. Females seem to have higher vitamin C levels than males [11, 23]. The significant difference in vitamin C levels between males and females may be the result of the volume dilution effect due to the higher fat-free

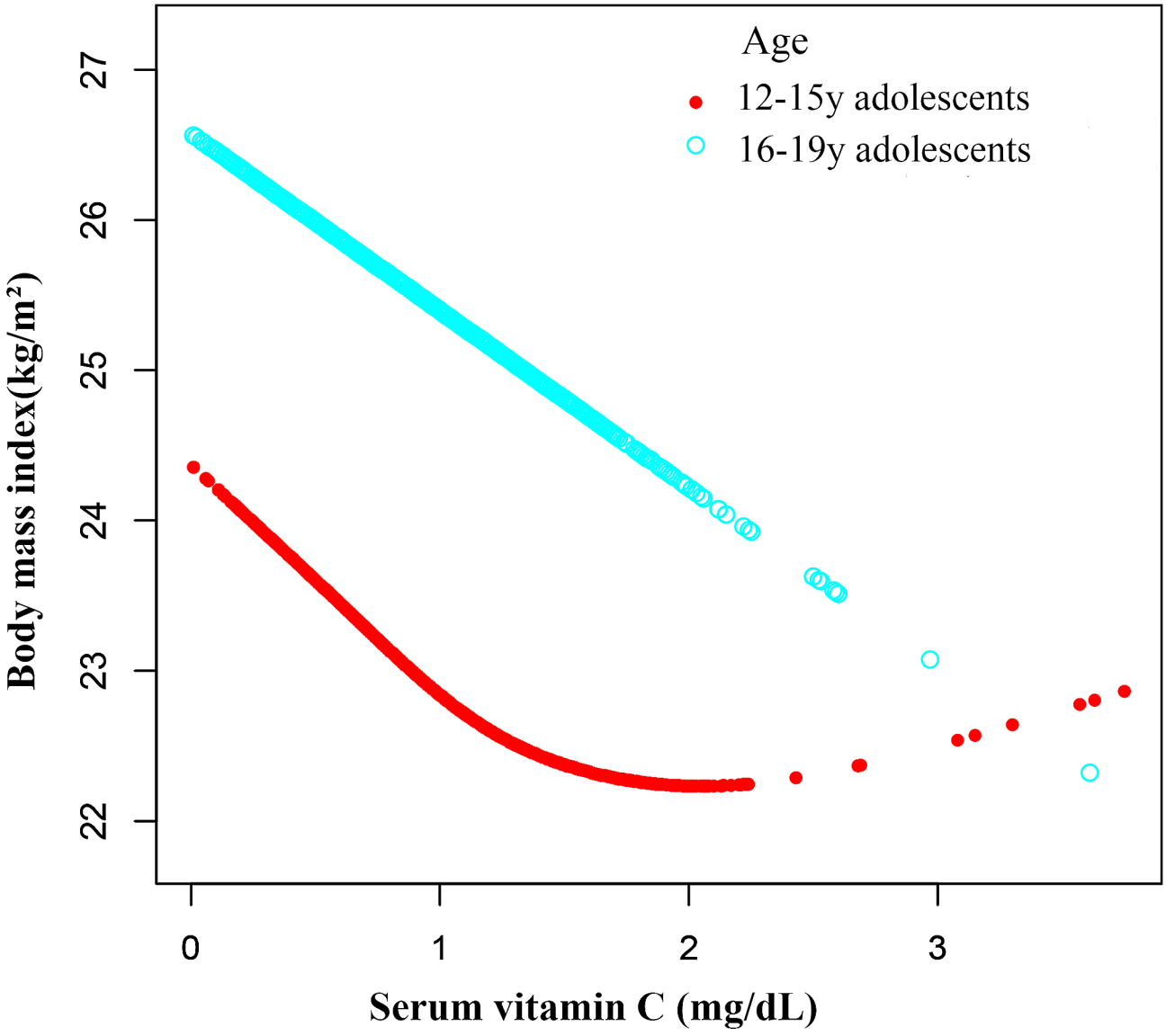

Fig. 3 The association between serum vitamin C and body mass index by age. Sex, race/ethnicity, serum cotinine, PIR, total cholesterol, HDL-cholesterol, glycohemoglobin, SBP, and DBP were adjusted

mass of males [23]. At the same time, the prevalence of obesity is higher in females than in males because of gender differences in endocrine and metabolic disorders [24, 25]. Females are also more susceptible to hormones than males. Gender may have a greater effect on BMI than sVC at the time, and thus, this may explain why there is a threshold effect.

In addition, the influence of sVC on the development of obesity has a threshold effect between 12 and 15 years old, whereas there may be a substantial linear correlation between 16 and 19 years old. The onset of puberty, characterized by the appearance of secondary sexual characteristics, begins at the age of 8 to 13 for girls and 9 to 14 for boys [26]. Adolescents aged 12 to 15 have just begun to have secondary sexual characteristics, and their weight changes in early adolescence vary widely, while their weight changes in late adolescence are relatively stable [27, 28]. For another, the demand for vitamin C is different among different age groups in adolescence [7, 29]. Therefore, the reason for the threshold effect can be understood.

Finally, we found that the relationship between sVC and BMI has a threshold effect among Mexican Americans, and it decreased dramatically in whites and blacks, which suggests that the impact of various races on the association between sVC and BMI is also significantly different. Our results are comparable to those of previous studies [30]. This possible reason is that Mexican-American young people have a higher prevalence of obesity [31]. Earlier studies [32] have also shown that

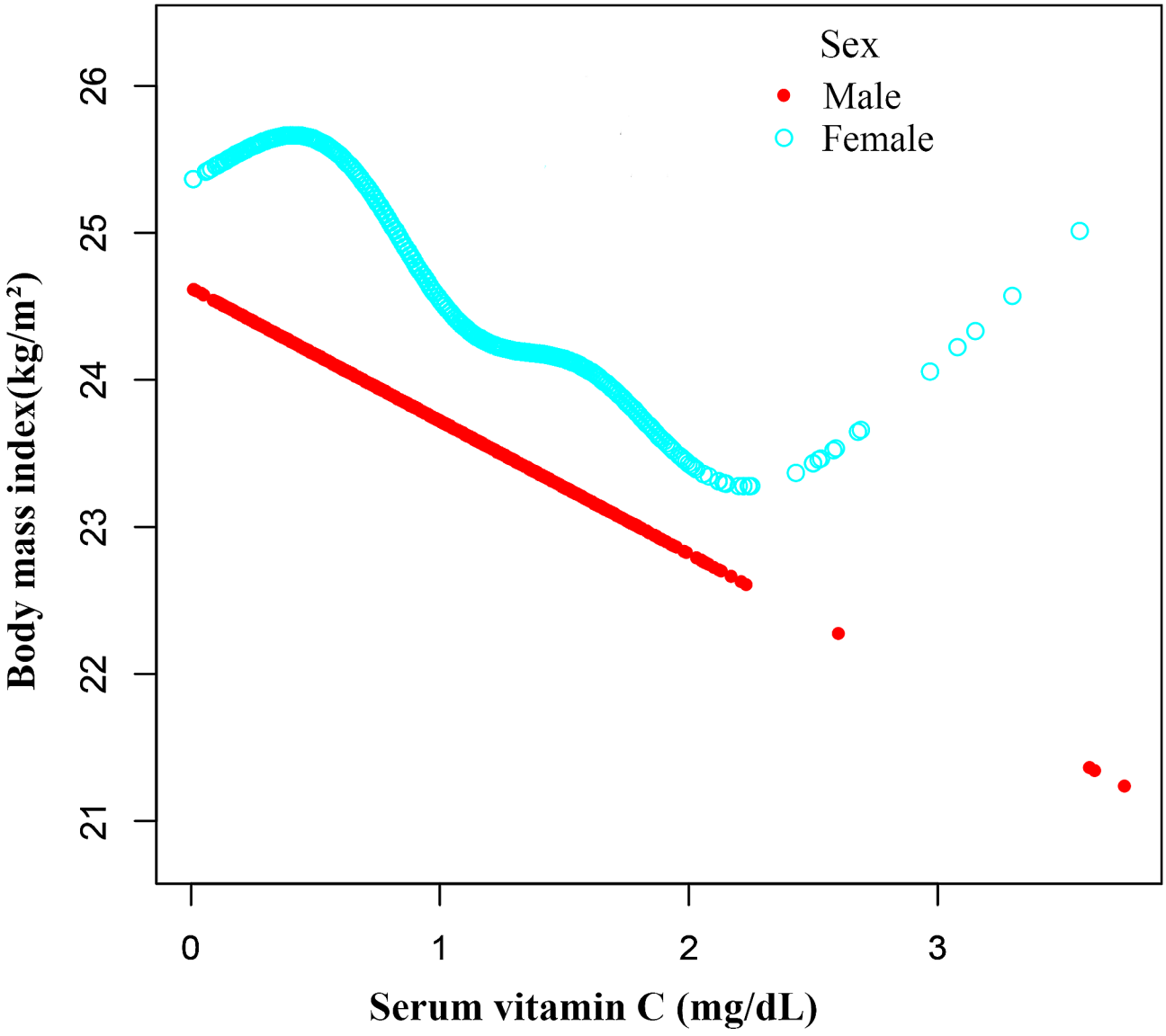

Fig. 4 The association between serum vitamin C and body mass index by sex. Age, race/ethnicity, serum cotinine, PIR, total cholesterol, HDL-cholesterol, glycohemoglobin, SBP, and DBP were adjusted

Mexican-American teenagers are more likely to be overweight or obese than teenagers of other races. Studies have also indicated that vitamin C status varies by race. The reason for this phenomenon may be the joint action of social, cultural, and environmental factors. Socioeconomic status affects diet quality. Studies have also confirmed the association of high vitamin C status with high socioeconomic status [11, 33]. Further research is needed to find out the potential intervention measures that these factors may take to eliminate racial differences in adolescent obesity.

Adolescents with severe obesity may be more likely to have a risk of metabolic disease and cardiovascular complications [34], including diabetes, hypertension, and metabolic syndrome [35]. These complications can easily lead to cardiovascular and cerebrovascular rupture, even sudden death, and the prognosis is poor. sVC is an endogenous marker to evaluate the severity of nutritional metabolic syndrome [36]. Because sVC can be reliably detected, especially when the level of sVC in obese patients is significantly reduced [37–39], it is very important to determine the influence of sVC on obesity. Unfortunately, the evidence linking sVC to BMI formation is very limited. In a cross-sectional study, Rodríguez et al. [40]. observed a significant difference in the level of vitamin C between normal-weight and overweight or obese young people and showed that vitamin C is an independent predictor of overweight or obesity. Carlos et al. [41]. also found that among overweight and obese adolescents, the prevalence distribution of insufficient intake of trace

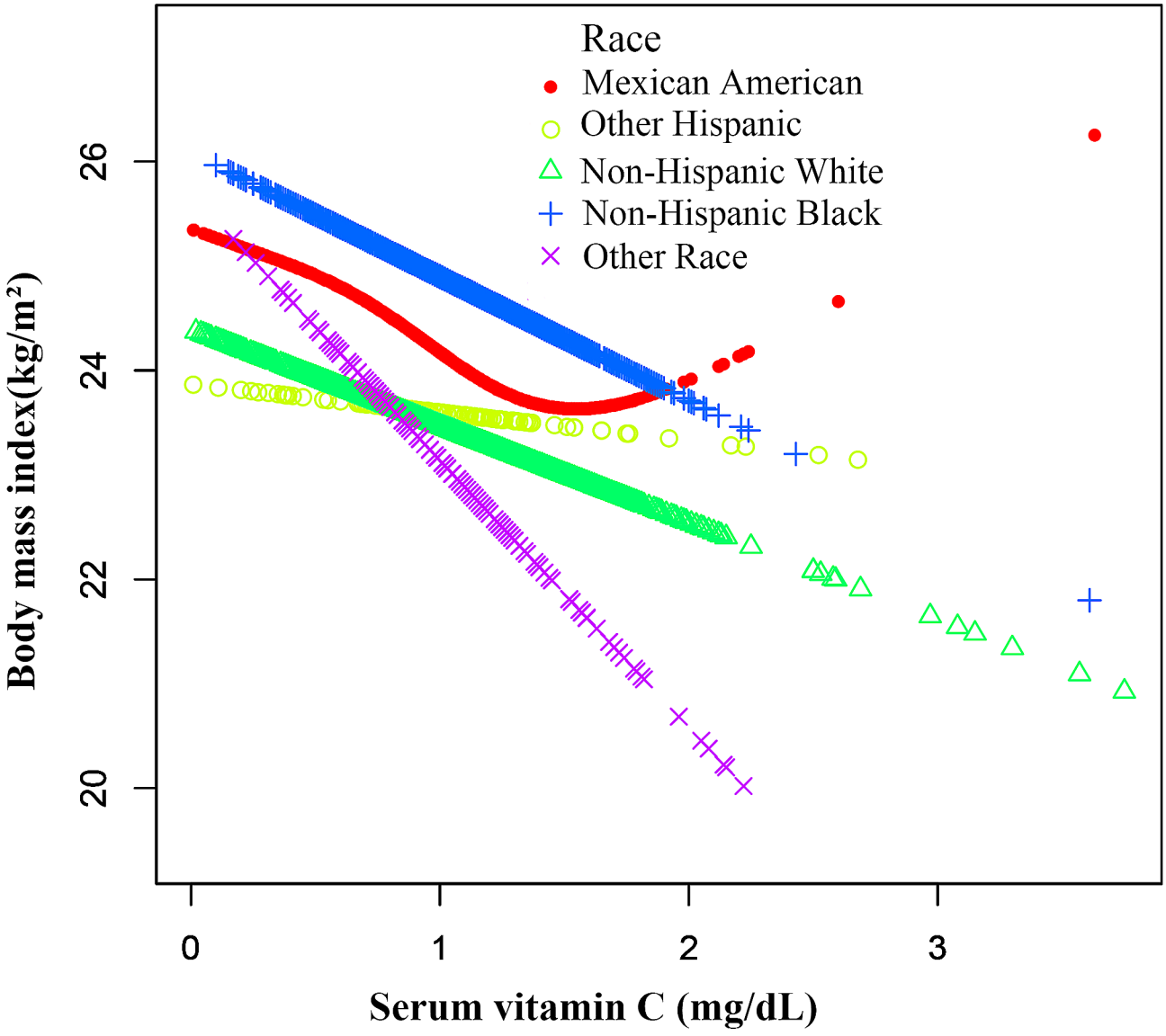

Fig. 5 The association between serum vitamin C and body mass index by race/ethnicity. Age, sex, serum cotinine, PIR, total cholesterol, HDL-cholesterol, glycohemoglobin, SBP, and DBP were adjusted

elements, and insufficient intake of vitamin C is the most common. In a case-control study, Maryam et al. [42]. shown that a higher intake of vitamin C may be protective against adolescent obesity. The relative mechanism, however, is as yet unknown. Vitamin C supplementation modulates adipocyte lipolysis [43] and protects adipocytes from inflammation, hypoxia, and endoplasmic reticulum stress [44]. Vitamin C is closely related to lipid metabolism and plays a major protective role in metabolic disorders [45]. Our study found that sVC was negatively correlated with BMI. Adolescents with a higher BMI have a lower concentration of sVC, while adolescents with a lower BMI have a higher concentration of sVC. The reason may be that adolescents with a low BMI have a relatively high metabolism. Therefore, we boldly speculate that vitamin C supplementation is beneficial to improve metabolism and lose weight. Obviously, further research is needed to verify this view.

To our knowledge, this is the first attempt to assess the influence of vitamin C on adolescent obesity. A large sample size allowed us to perform multiple subgroup analyses. We used smooth curve fittings and two-piecewise linear regression to explore a non-linear relationship between sVC and BMI. In addition, the negative correlation between sVC and BMI may have an incomplete linear relationship in different stratifications, indicating that it may exist in special populations, a similar approach followed by previous studies [46, 47]. Therefore, we performed a non-linearity and saturation effect analysis between sVC and BMI. However, some shortcomings

also need to be pointed out. Firstly, because this study is a cross-sectional study, the causal relationship is unclear. People with overweight or obese may prefer to consume high-fat foods [48] and eat less vitamin-containing foods [49, 50]. Thus, the potential effects of reverse causality cannot be excluded. Secondly, we excluded data with missing values in independent and dependent variables, hence potential selection bias may exist. Thirdly, although we controlled for many potential confounding factors, residual confounding by unmeasured potential confounding factors, such as non-communicable diseases and infectious diseases, remains possible. Fourthly, there are many indicators related to obesity, such as waist circumference and waist-hip ratio, but we only analyzed the most common index of body mass index. Finally, dietary vitamins could influence adolescent obesity. We statistically analyzed the vitamin C level in serum, while dietary vitamin C data were not acquired [51]. Moreover, the data of this popultion based study limitedly ranges between 2003 and 2006, because the recent data of vitamins in children is not publicly avilable on the NHANES website.

In conclusion, sVC data is easy to collect, and the detection cost is relatively inexpensive. The present study demonstrated that sVC was significantly negatively correlated with obesity in adolescents. There were saturation and threshold effects in this connection, and its impacts also took on diverse forms in various subcategories. Further research is needed to investigate the causal mechanism between sVC and obesity. Our findings also provide a biomarker for early identification of obesity, which is conducive to better weight control.

Abbreviations

sVC	Serum vitamin C
BMI	Body Mass Index
NHANES	National Health and Nutrition Examination Survey
NCHS	National Center For Health Statistics
HPLC	High performance liquid chromatography
PIR	Poverty-income rate
SBP	Systolic blood pressure
DBP	Diastolic blood pressure

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Not applicable.

Author contributions
Xiaoqi Su, Nishant Patel, and Xuming Mo contributed to the study's design. Ye Chen and Xin Zhou were responsible for data collection and data analysis. Xiaoqi Su, Nishant Patel and Jun Chen wrote the original draft. Xuming Mo and Shanliang Zhu reviewed and edited the final manuscript.

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Data availability
The datasets generated and analyzed for the current study are available in the NHANES database. More information about the NHANES can be obtained at: https://wwwn.cdc.gov/nchs/nhanes/Default.aspx.

Declarations

Ethics approval and consent to participate
This study analyzed the data from the public database of the National Health and Nutrition Examination Survey. Ethical review committee of the National Health Statistics Center gave ethical approval. The methods involved in this study are carried out in accordance with relevant guidelines and regulations (Helsinki Declaration). All subjects provided written informed consent before participating in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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