Genetically high plasma vitamin C, intake of fruit and vegetables, and risk of ischemic heart disease and all-cause mortality: a Mendelian randomization study

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ABSTRACT

**Background:** High intake of fruit and vegetables as well as high plasma vitamin C concentrations have been associated with low risk of ischemic heart disease in prospective studies, but results from randomized clinical trials have been inconsistent.

**Objective:** We tested the hypothesis that genetically high concentrations of plasma vitamin C, such as with high intake of fruit and vegetables, are associated with low risk of ischemic heart disease and all-cause mortality.

**Design:** We used a Mendelian randomization approach and genotyped for solute carrier family 23 member 1 (SLC23A1) rs33972313 in the sodium-dependent vitamin C transporter 1 in 97,203 white individuals of whom 10,123 subjects had ischemic heart disease, and 8477 subjects died. We measured plasma vitamin C in 3512 individuals and included dietary information on 83,256 individuals.

**Results:** The SLC23A1 rs33972313 G allele was associated with 11% higher plasma vitamin C. The multivariable adjusted HRs for highest compared with lowest fruit and vegetable intakes were 0.87 (95% CI: 0.78, 0.97; \( P = 0.01 \)) for ischemic heart disease and 0.80 (95% CI: 0.73, 0.88; \( P < 0.001 \)) for all-cause mortality. Corresponding HRs for rs33972313 GG (93%) compared with AA plus AG (7%) genotypes were 0.95 (95% CI: 0.88, 1.02; \( P = 0.21 \)) and 0.96 (0.88, 1.03; \( P = 0.29 \)), respectively. In an instrumental variable analysis, the OR for genetically determined 25% higher plasma vitamin C concentrations was 0.90 (95% CI: 0.75, 1.08; \( P = 0.27 \)) for ischemic heart disease and 0.88 (0.72, 1.08; \( P = 0.22 \)) for all-cause mortality.

**Conclusions:** High intake of fruit and vegetables was associated with low risk of ischemic heart disease and all-cause mortality. Although the 95% CI for genetically high plasma vitamin C concentrations overlapped 1.0, which made certain statistical inferences difficult, effect sizes were comparable to those for fruit and vegetable intake. Thus, judging by the effect size, our data cannot exclude that a favorable effect of high intake of fruit and vegetables could in part be driven by high vitamin C concentrations. *Am J Clin Nutr* 2015;101:1135–43.

**Keywords:** genetic variants, ischemic heart disease, Mendelian randomization, mortality, vitamin C

INTRODUCTION

High intake of fruit and vegetables has been associated with low risk of ischemic heart disease (1–3) and all-cause mortality (4). Although the exact mechanism behind these associations remains unknown, low risk could be mediated through an advantageous effect on blood pressure (5, 6), plasma cholesterol concentrations (7), or lung function (8), or they may be due to the contents in fruit and vegetables of important antioxidants such as vitamin C. Vitamin C potentially protects biological components such as low-density lipoproteins against oxidative modifications and, thereby, could possibly take on an atheroprotective role (9).

The discrepancy between epidemiologic findings and results from large randomized studies on the role of vitamin supplementation on cardiovascular disease exists (10, 11); however, such discrepancy could be explained by the limited duration of clinical trials (12) or the proneness of observational studies to confounding and reverse causation. Thus, an alternative way to evaluate the association between plasma vitamin C concentrations and cardiovascular disease, such as the Mendelian randomization approach, is needed (13–15). The Mendelian randomization approach is based on the assumption that the inheritance of a genetic variant from parents to offspring is independent of the environment; thus, genetic variants that either alter or are markers of alterations in plasma vitamin C concentrations provide an ideal system to assess consequences of lifelong high vitamin C concentrations independently of other risk factors and not prone to reverse causation (16). In the current study, we used a genetic variant in solute carrier family 23 member 1 (SLC23A1) rs33972313 G allele was associated with 11% higher plasma vitamin C.
(SLC23A1)$^5$ rs33972313 that encodes the sodium-dependent vitamin C transporter 1 involved in the maintenance of whole-body vitamin C homeostasis through dietary absorption and renal reabsorption (16–18).

We tested the hypothesis that genetically high concentrations of plasma vitamin C, such as with high intake of fruit and vegetables, are associated with low risk of ischemic heart disease and all-cause mortality. First, we tested whether high consumption of fruit and vegetables was associated with low risk of ischemic heart disease and all-cause mortality in 83,256 individuals from the Copenhagen General Population Study (CGPS). Second, we investigated whether the genetic variant rs33972313 in SLC23A1 was associated with high concentrations of plasma vitamin C in 3512 individuals from the CGPS as previously shown (18). Third, we tested whether genetically high concentrations of plasma vitamin C were associated with low risk of ischemic heart disease and all-cause mortality in 97,203 individuals from the CGPS and the Copenhagen City Heart Study (CCHS).

METHODS

Study population

We used the CGPS, which was initiated in 2003 with ongoing enrollment, and the CCHS, which was initiated in 1976–1978 with follow-up examinations in 1981–1983, 1991–1994, and 2001–2003 (19, 20). DNA was available in the CGPS and 1991–1994 and 2001–2003 examinations of the CCHS. For both studies, individuals aged 20–100 y were invited randomly from the Danish Civil Registration System to reflect the Danish general population. The participation rate was 45% in the CGPS and 61% and 50% in the CCHS 1991–1994 and 2001–2003 examinations, respectively. The study was approved by Herlev Hospital and Danish Ethical Committees and conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants. No subject was lost to follow-up. We included only white individuals of Danish descent with DNA available, which included a total of 87,030 individuals for the CGPS and 10,173 individuals from the CCHS. In addition to this, to measure plasma vitamin C, we included the first 3512 individuals enrolled into the CGPS after the beginning of the current study in 2013.

Fruit and vegetable intake

Information on fruit and vegetable intake was available in the CGPS where it was ascertained by using the following questions: “How often do you eat a whole fruit or a portion of fruit?” and “How often do you eat vegetables as a snack, as part of breakfast or lunch, or as a larger part of a hot meal?” with 8 possible answers ranging from almost never to >3 times/d. In the analyses, answers were grouped into 4 categories of almost never, <1 time/d, 1/d, and ≥2 times/d. A combined fruit and vegetable intake score was also calculated by adding the score from fruit intake (1–4, with 4 being the highest intake) and vegetable intake (1–4, with 4 being the highest intake) to create a score from 2 to 8, which was further grouped into 3 groups of approximately the same size for maximal statistical power. A total of 83,256 individuals with complete information on fruit and vegetable intake were included in these analyses. However, the 3774 individuals with missing information on fruit and vegetable intake were included in subsequent genotype analyses to obtain a maximal statistical power. Of the 3512 individuals in whom we measured plasma vitamin C, information on fruit intake and vegetable intake was available in 2109 and 2105 individuals, respectively, and information on both intakes was available in 2102 individuals.

Plasma vitamin C

We measured plasma vitamin C in the first 3512 individuals who were enrolled into the CGPS after the beginning of the current study in 2013. Thus, these participants contributed to only the study with plasma vitamin C and SLC23A1 genotype. Because of the instability of vitamin C in plasma, we did not wish to measure plasma vitamin C in plasma samples that had been stored for several years. Thus, we measured plasma vitamin C in only newly recruited participants and, thus, in only short-time–stored plasma samples. Unfortunately, because of the large setup of the CGPS, information from the questionnaire and physical examination from these latest recruited participants was not yet finally organized in the database for all 3512 individuals at the time of manuscript preparation. For this reason, information on fruit or vegetable intake was available only in a subset of the 3512 participants. Likewise, full information from the physical examination, questionnaire, and laboratory analyses for the 3512 participants where only available in 1007 of these participants (Supplemental Figure 1).

Laboratory analyses

In the CGPS and CCHS, we genotyped 87,030 and 10,173 individuals, respectively, for the SLC23A1 rs33972313 variant by using a TaqMan-based assay (Applied Biosystems). We included lipoprotein lipase genetic variants from the CCHS and the lactose intolerance minichromosome maintenance complex component 6 (MCM6) genetic variant from the CGPS that had already been genotyped in our laboratory before this study for other projects by also using TaqMan-based assays (Supplemental Figure 1). A total of 10,042 participants from the CCHS were successfully genotyped for lipoprotein lipase genetic variants, and a total of 74,211 participants were genotyped for the MCM6 genetic variant. The different number of participants was due to differences in genotyping call rates and the number of enrolled participants at the time of genotyping.

For all genetic analyses, we used DNA extracted from leukocytes in peripheral blood by using a blood kit (Qiagen) for DNA extraction. The genotype distribution was in Hardy-Weinberg equilibrium in both studies ($P = 0.53$ and $P = 0.06$, respectively). Lipoprotein lipase genetic variants, the lactose intolerance variant, and the vitamin C genetic variant were genotyped in our laboratory for different projects at different time periods over some years, which explained why the number of participants with DNA available differed between different variants.

Plasma vitamin C concentrations were measured consecutively in 3512 nonfasting CGPS participants by using a fluorometric assay (BioAssay Systems). Venous blood samples were drawn into standard heparin-coated evacuated tubes and immediately stored on ice. Within 3 hours of blood sampling, the evacuated tubes were centrifuged at 3500 rpm for 10 min at 4°C, plasma pipetted into a clean tube, and immediately stored at −80°C. Samples

$^5$Abbreviations used: CGPS, Copenhagen General Population Study; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; MCM6, minichromosome maintenance complex component 6; SLC23A1, solute carrier family 23 member 1.
were collected Monday through Thursday and were thawed and immediately analyzed every Friday so that the storage time was ≤4 d. This procedure was validated through pilot projects that indicated little or no loss of vitamin C compared with samples analyzed immediately after blood draw. The intra-assay CV was 8% on the basis of 42 duplicate measurements that covered the entire concentration range. Individuals in whom plasma vitamin C concentrations were measured were used only for the association between plasma vitamin C, genotype, and fruit and vegetable intake because no additional information was available for these individuals at the time of analysis (these individuals were the latest recruited participants into the CGPS). Plasma concentrations of C-reactive protein were measured by using latex-enhanced turbidimetry (Dako) or nephelometry (Dade Behring). Plasma concentrations of triglycerides, LDL cholesterol, and HDL cholesterol were measured by using standard hospital assays (21).

Ischemic heart disease and all-cause mortality

Information on the diagnosis of ischemic heart disease (WHO International Classification of Diseases, Eighth Revision codes 410-414 and Tenth Revision codes I20-I25) were collected from 1977 through April 2013 from the national Danish Patient Registry and the national Danish Causes of Death Registry, whereas the date of death was obtained from the Danish Civil Registration System, as done previously (19, 22, 23).

Covariates

Baseline characteristics were recorded from a self-administered questionnaire, physical examination, and blood samples. Participants reported on smoking status (never, former, or current) and, if relevant, the number of years of smoking and daily tobacco consumption from which cumulative tobacco consumption in pack-years was calculated; a pack-year was 20 cigarettes or equivalent smoked per day for 1 y. Self-reported weekly alcohol intake was in units of ~12 g alcohol. Furthermore, information on weekly physical activity (highest one-half compared with lowest one-half of physical activity groups), level of income (highest one-third compared with lowest two-thirds), and any use of vitamin supplements were obtained from the questionnaire. Systolic blood pressure was measured. BMI (in kg/m²) was calculated from measured weight divided by measured height squared. The forced expiratory volume in 1s (FEV₁) and forced vital capacity (FVC) were measured as described (20). Algorithms for the calculation of the percentage predicted of FEV₁ and FVC were measured imprecision to a minimum. The strength of measurement imprecision to a minimum. The strength of variation explained by genotype. To examine the association between fruit and vegetable intake and plasma vitamin C concentrations and risk of ischemic heart disease because we are not aware of studies that have reported on the direct relation between plasma vitamin C concentrations and risk of ischemic heart disease.

In genetic analyses, to examine the association of SLC23A1 rs33972313 genotype with plasma vitamin C, we used multiple linear regression adjusted for lot number of assay to reduce measurement imprecision to a minimum. The strength of SLC23A1 rs33972313 as an instrument of plasma vitamin C was assessed by using the F statistic, where F > 10 indicates sufficient strength to carry out statistically reliable instrumental variable analyses (14), and R² as a measure of variation explained by genotype. To examine the association between fruit and vegetable intake and plasma vitamin C, we used multiple linear regression, which was likewise adjusted for the lot number of assay.

For the Cox proportional hazards regression model to evaluate the association between SLC23A1 rs33972313 genotype and ischemic heart disease and all-cause mortality, we grouped the AA (0.1%) and AG (7%) genotypes together and used this group with the lowest plasma vitamin C concentrations as the reference group; sensitivity analyses used AG as the reference only. Because all measured confounders were evenly distributed between genotypes, we adjusted only for age. For ischemic heart disease, we used both prevalent and incident cases because the genotype is present throughout life. These genetic analyses were conducted in the CCHS and CGPS separately and combined to maximize statistical power by using fixed effects meta-analyses.
Potential causal relations between genetically high plasma vitamin C and risk of ischemic heart disease and all-cause mortality were assessed by instrumental variable analyses using the ratio estimator. Genetically determined ORs were calculated by using the Wald-type estimator, which involved taking the ratio of the mortality-allele–score log OR to the exposure-allele–score coefficient and exponentiating to express this value as an OR with SEs being derived by using the δ method (28).

As a positive control, we included instrumental variable analyses of the effect of genetically low plasma triglycerides in the CCHS on risk of ischemic heart disease and all-cause mortality as shown in previous studies (29–31). We used the genotypes S447× (rs3328), D9N (rs1801177), N291S (rs268), and G188E (rs118204057), which are all well-known variants in the lipoprotein lipase gene that are important for triglyceride hydrolysis in plasma, and combined these genotypes to a triglyceride-decreasing allele score from 0 to 4 (29). The ratio estimator was used as previously described, whereby the genetic instrument was the triglyceride-decreasing allele score from 0 to 4, and the exposure was plasma triglyceride concentrations ($F = 6.8$, $R^2 = 0.7\%$). However, note that, although the effects of genetically lower triglyceride concentrations were investigated before in the CCHS, a combination of these 4 variants in the lipoprotein lipase allele score has not previously been used for ischemic heart disease to our knowledge. Furthermore, although we repeated the study by Thomsen et al. (29), we used an updated version of the database with a longer follow-up time.

For sensitivity analyses, we divided participants into low or high 10-y risk of fatal cardiovascular disease; high risk was defined as a 10-y risk $\geq$5% according to the Systematic Coronary Risk Evaluation algorithm, which is based on sex, age, smoking status, systolic blood pressure, and plasma total cholesterol concentrations (32).

## RESULTS

Characteristics of 83,256 individuals from the CGPS according to fruit and vegetable intake, ischemic heart disease, and all-cause mortality are shown in Table 1 and Supplemental Tables 1 and 2. In the CGPS, 2823 individuals developed ischemic heart disease, and 3940 individuals died during follow-up. All potential confounders were associated with either fruit and vegetable intake or endpoints. In contrast, baseline characteristics did not differ according to SLC23A1 rs33972313 genotype in the CGPS or CCHS (Table 1, Supplemental Tables 3 and 4), which illustrated that the genotype can be used as a largely unconfounded instrument of plasma vitamin C concentrations for risk of ischemic heart disease and all-cause mortality. In the CGPS, genotyping for SLC23A1 rs33912313 identified 113 AA homozygotes (0.1%), 6226 AG heterozygotes (7%), and 80,691 GG homozygotes (93%); corresponding numbers in the CCHS were 7 AA homozygotes (0.01%), 741 AG heterozygotes (7%), and 9425 GG homozygotes (93%) (Supplemental Tables 3 and 4). We showed no evidence of population stratification (chi-square = 5.94, $P = 0.20$; Supplemental Table 5).

### TABLE 1

CGPS baseline characteristics according to self-reported fruit intake and the association with SLC23A1 genotype, ischemic heart disease, and all-cause mortality

<table>
<thead>
<tr>
<th>Self-reported fruit intake</th>
<th>SLC23A1$^2$</th>
<th>IHD</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P^3$</td>
<td>$P^4$</td>
<td>$P^4$</td>
</tr>
<tr>
<td>Participants, n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Almost never</td>
<td>6369</td>
<td>17,576</td>
<td>28,517</td>
</tr>
<tr>
<td>$&lt;1$ time/d</td>
<td>71</td>
<td>59</td>
<td>45</td>
</tr>
<tr>
<td>1 time/d</td>
<td>58 (46, 67)</td>
<td>58 (47, 67)</td>
<td>59 (48, 68)</td>
</tr>
<tr>
<td>2 times/d</td>
<td>29 (15, 45)</td>
<td>21 (9, 35)</td>
<td>15 (6, 30)</td>
</tr>
<tr>
<td>3 times/d</td>
<td>26.2 (23.7, 29.1)</td>
<td>26.0 (23.6, 28.8)</td>
<td>25.6 (23.2, 28.4)</td>
</tr>
<tr>
<td>4 times/d</td>
<td>11 (4, 21)</td>
<td>10 (4, 17)</td>
<td>9 (4, 15)</td>
</tr>
<tr>
<td>High, %</td>
<td>28</td>
<td>36</td>
<td>40</td>
</tr>
<tr>
<td>High physical activity (leisure), %</td>
<td>36</td>
<td>43</td>
<td>49</td>
</tr>
<tr>
<td>High physical activity (work), %</td>
<td>27</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>140 (130, 156)</td>
<td>140 (128, 155)</td>
<td>140 (126, 155)</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.3 (2.6, 4.0)</td>
<td>3.2 (2.6, 3.9)</td>
<td>3.2 (2.6, 3.8)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.5 (1.1, 1.8)</td>
<td>1.5 (1.2, 1.9)</td>
<td>1.6 (1.3, 2.0)</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.6 (1.1, 2.5)</td>
<td>1.5 (1.1, 2.3)</td>
<td>1.4 (1.0, 2.1)</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>1.6 (1.1, 3.0)</td>
<td>1.5 (1.1, 2.6)</td>
<td>1.4 (1.0, 2.4)</td>
</tr>
<tr>
<td>FEV₁, % predicted</td>
<td>93 (81, 104)</td>
<td>96 (86, 106)</td>
<td>98 (88, 108)</td>
</tr>
<tr>
<td>FEV₁:FVC, %</td>
<td>78 (72, 82)</td>
<td>78 (73, 83)</td>
<td>79 (74, 83)</td>
</tr>
<tr>
<td>Current use of vitamins, %</td>
<td>40</td>
<td>44</td>
<td>51</td>
</tr>
</tbody>
</table>

1Continuous variables are summarized as medians; IQRs in parentheses. Self-reported fruit intake is the intake of a piece or portion of fruit. CGPS, Copenhagen General Population Study; CRP, C-reactive protein; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; IHD, ischemic heart disease; SLC23A1, solute carrier family 23 member 1.
2SLC23A1: rs33972313 from Supplemental Table 3.
3Calculated by using Cuzick’s nonparametric trend test from Supplemental Table 3.
4Calculated by using Kruskal-Wallis test from Supplemental Table 2.
5One to 4 times/wk.
6In pack-years summarized for current smokers only, not the whole population.
7Not significant after Bonferroni correction for 17 multiple tests.
Fruit and vegetable intake and risk of ischemic heart disease and all-cause mortality

For ischemic heart disease, HRs for highest compared with lowest fruit and vegetable intakes were 0.63 (95% CI: 0.57, 0.70; \( P < 0.001 \)) after age adjustment, 0.87 (95% CI: 0.78, 0.97; \( P = 0.01 \)) after multivariable adjustments, and 0.90 (95% CI: 0.81, 1.00; \( P = 0.06 \)) after additional adjustments for the possible mediators of systolic blood pressure, LDL cholesterol, HDL cholesterol, triglycerides, \( \text{FEV}_1 \) in percentage of predicted, and \( \text{FEV}_1 : \text{FVC} \) (Figure 1). For all-cause mortality, corresponding HRs were 0.50 (95% CI: 0.46, 0.55; \( P < 0.001 \)), 0.80 (95% CI: 0.73, 0.88; \( P < 0.001 \)) and 0.84 (95% CI: 0.76, 0.93; \( P = 0.001 \)), respectively. We showed similar results when we examined fruit intake and vegetable intake separately (Supplemental Figures 2 and 3). When we examined these associations stratified into low and high 10-y risk of fatal cardiovascular disease, results for both ischemic heart disease and all-cause mortality were similar in the 2 strata (Supplemental Figure 4).

**SLC23A1 rs33972313, fruit and vegetable intake, and vitamin plasma C**

In the plasma vitamin C substudy, baseline characteristics according to SLC23A1 rs33972313 genotype were similar to those of the remaining CGPS (compare Supplemental Table 3 and Supplemental Table 6). The G variant in SLC23A1 rs33972313 was associated with 19% higher plasma vitamin C (4.1 \( \mu \text{mol/L} \)) in AG heterozygotes and 25% higher plasma vitamin C (7.3 \( \mu \text{mol/L} \)) in GG homozygotes than in AA homozygotes (Figure 2A) (\( P \)-trend < 0.001); the per–G-allele higher vitamin C was 11% (3.1 \( \mu \text{mol/L} \)). SLC23A1 rs33972313 had an \( F \) value of 30 as an instrument of plasma vitamin C concentrations and an \( R^2 \) of 0.9%. In contrast, the SLC23A1 rs33972313 genotype was not associated with intake of fruit and vegetables (\( P \)-trend = 0.46) (Figure 2B, Supplemental Figure 5).

We showed stepwise higher plasma vitamin C concentrations with higher fruit intake, whereby mean plasma vitamin C ranged from 24.0 \( \mu \text{mol/L} \) (95% CI: 22.9, 25.9 \( \mu \text{mol/L} \)) for the lowest fruit intake group to 33.1 \( \mu \text{mol/L} \) (95% CI: 32.4, 33.7 \( \mu \text{mol/L} \)) for the highest fruit intake group (\( P \)-trend < 0.001 across 4 groups) (Figure 2C). These results were similar for vegetable intake (\( P \)-trend < 0.001) and for the combined fruit and vegetable intake score (\( P \)-trend < 0.001) (Figure 2D, Supplemental Figure 6).

**Plasma vitamin C, fruit and vegetable intake, and all-cause mortality**

With the use of an HR for the association between plasma vitamin C and all-cause mortality from Khawt et al. (27), we estimated that the higher plasma vitamin C concentrations associated with high intake of fruit and vegetables would theoretically predict an HR for all-cause mortality of 0.96 (95% CI: 0.95, 0.98) for fruit and vegetable intake scores of 5–6 and 0.93 (95% CI: 0.90, 0.96) for scores 7–8 compared with scores 2–4. Corresponding observed HRs were 0.81 (95% CI: 0.75, 0.87) and 0.80 (95% CI: 0.73, 0.88) (Supplemental Figure 7).

**SLC23A1 rs33972313 and risk of ischemic heart disease and all-cause mortality**

The SLC23A1 rs33972313 genotype GG compared with AA plus AG was associated with an HR of 0.95 (95% CI: 0.88, 1.02) for ischemic heart disease and 0.96 (95% CI: 0.88, 1.03) for all-cause mortality in the meta-analyses of the combined studies (Figure 3). Corresponding HRs in the CGPS and CCHS separately were similar. Findings were also similar in analyses in which the AG genotype alone served as the reference group (Supplemental Figure 8). The

### Table 1

<table>
<thead>
<tr>
<th>Fruit- and vegetable intake</th>
<th>No. of participants</th>
<th>No. of events</th>
<th>Adjusted for age</th>
<th>Multivariable adjusted</th>
<th>Further adjusted for possible mediators</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ischemic heart disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score 2-4</td>
<td>15,673</td>
<td>820</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Score 5-6</td>
<td>31,254</td>
<td>1,171</td>
<td>0.73 (0.66, 0.79)</td>
<td>0.86 (0.78, 0.94)</td>
<td>0.87 (0.80, 0.96)</td>
</tr>
<tr>
<td>Score 7-8</td>
<td>31,600</td>
<td>832</td>
<td>0.63 (0.57, 0.70)</td>
<td>0.87 (0.78, 0.97)</td>
<td>0.90 (0.81, 1.00)</td>
</tr>
<tr>
<td><strong>All-cause mortality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score 2-4</td>
<td>16,909</td>
<td>1,369</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Score 5-6</td>
<td>33,338</td>
<td>1,701</td>
<td>0.64 (0.59, 0.68)</td>
<td>0.81 (0.75, 0.87)</td>
<td>0.83 (0.76, 0.90)</td>
</tr>
<tr>
<td>Score 7-8</td>
<td>33,009</td>
<td>870</td>
<td>0.50 (0.46, 0.55)</td>
<td>0.80 (0.73, 0.88)</td>
<td>0.84 (0.76, 0.93)</td>
</tr>
</tbody>
</table>

**FIGURE 1** HRs (95% CIs) of fruit and vegetable intake score in the Copenhagen General Population Study and risk of ischemic heart disease and all-cause mortality. The score is the sum of the self-reported vegetable intake score and self-reported fruit intake score. Multivariable adjustment was done for age, sex, smoking, alcohol intake, BMI, income, use of vitamin supplementation, physical activity at work and in leisure time, and C-reactive protein. Additional adjustment done for possible mediators was for the mentioned variables and systolic blood pressure, LDL cholesterol, triglycerides, HDL cholesterol, \( \text{FEV}_1 \) in percentage of predicted, and \( \text{FEV}_1 : \text{FVC} \). For ischemic heart disease, 4729 participants who received a diagnosis of ischemic heart disease before the examination date are not included in the Cox regression. HRs were derived from Cox proportional hazards regression. \( \text{FEV}_1 \), forced expiratory volume in 1 s; \( \text{FVC} \), forced vital capacity.
division of participants into low and high 10-y risk of fatal cardio-
vascular disease gave similar results (Supplemental Figure 9).

Plasma vitamin C and risk of ischemic heart disease and all-
cause mortality: instrumental variable analyses

In an instrumental variable analysis, the OR given a genetically
determined 25% higher plasma vitamin C was 0.90 (95% CI:
0.75, 1.08; \( P = 0.27 \)) for ischemic heart disease and 0.88 (95%
CI: 0.72, 1.08; \( P = 0.22 \)) for all-cause mortality (Figure 4). As
a positive control in the instrumental variable analysis, we
examined the association between plasma triglyceride–
creasing lipoprotein lipase genotypes S447X, D9N, N291S,
and G188E and risk of ischemic heart disease and all-cause
mortality. This allele score was associated with a 0.200-
mmol/L lower plasma triglyceride concentration per allele
(\( P < 0.001 \)). A genetically determined 25% lower plasma
triglycerides gave an OR of 0.83 (95% CI: 0.65, 1.07) for
ischemic heart disease and 0.76 (95% CI: 0.59, 0.99) for
all-cause mortality (Figure 4).

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**FIGURE 2** Plasma vitamin C and fruit and vegetable intakes and SLC23A1 genotype in the Copenhagen General Population Study. (A) Plasma vitamin C is shown as means (±SEs) and as the increase in percentage compared with that for the AA genotype. (B) Fruit and vegetable intake score is shown as medians (IQRs). (C and D) Plasma vitamin C is shown as means (±SEs). \( P \) values were calculated by using Cuzick’s nonparametric trend test.

**FIGURE 3** HRs (95% CIs) of risk of ischemic heart disease and all-cause mortality as a function of SLC23A1 genotype in the Copenhagen General Population Study, Copenhagen City Heart Study, and both studies combined. HRs were derived from Cox regression with age as the underlying time scale unless otherwise indicated. \( P \) values were derived from Cox regression. Weight denotes cohort weights from meta-analyses. Note that weights should be read horizontally. \(^1\)HRs were derived from meta-analyses.
DISCUSSION

In this study of 97,203 individuals from the Danish general population, we showed that high intake of fruit and vegetables was associated with low risk of ischemic heart disease and all-cause mortality with similar effect sizes for genetically high plasma vitamin C concentrations; however, the 95% CI for the latter overlapped 1.0. Thus, our data could not exclude that a favorable effect of high intake of fruit and vegetables could be driven, at least in part, by high vitamin C concentrations. To our knowledge, our study is the first study to date to examine the association between genetically high plasma vitamin C concentrations and risk of ischemic heart disease and all-cause mortality.

Mechanistically, fruit and vegetables are rich in minerals, vitamins, antioxidants, micronutrients, and phytochemicals, and it is plausible that one or a combination of several of these constituents might confer cardiovascular protection through an effect on vascular function (33), a reduction of blood pressure (5, 6), lower plasma concentrations of LDL cholesterol (7), or a reduction in oxidative stress (34). The latter effect could be mediated through vitamin C, which is abundantly present in fruit and vegetables and considered a powerful antioxidant (34); in vitro studies showed vitamin C protected against the oxidation of low-density lipoproteins (35), which is a step that was hypothesized to be important in the formation of atherosclerotic plaques (12).

Previous observational studies likewise showed an inverse relation between intake of fruit and vegetables and risk of cardiovascular disease and all-cause mortality (36–38). However, randomized clinical trials on the effect of fruit and vegetable intake have been scarce, with short follow-up times and inconsistent results (6, 39). Also, although high concentrations of plasma vitamin C have been associated with low risk of hypertension (40), myocardial infarction (41), heart failure (42), and mortality (27), randomized clinical trials of vitamin C’s effect on cardiovascular disease prevention have been disappointing (11, 43–46). Likewise, a recent study that applied the SLC23A1 rs33972313 genotype as a proxy for vitamin C in ~10,000 European individuals showed no association between the genetic variation and cardiometabolic outcomes (47).

The discrepancy between observational studies and randomized clinical trials can be explained by social or behavioral factors confounding the positive findings in observational studies (13, 48) or by randomized clinical prevention trials suffering from a limited follow-up time and an intervention placed too late in life, i.e., after the formation of atherosclerotic plaques has begun. Therefore, we used the alternative Mendelian randomization approach to address the question of the role of vitamin C in disease prevention and showed that we could not exclude an effect of elevated plasma vitamin C of similar size as that of reduced triglycerides on risk of ischemic heart disease and all-cause mortality. The use of the SLC23A1 genotype to largely circumvent confounding and effectively exclude reverse causation allowed us to assess the causal effect of lifelong high plasma vitamin C on risk of ischemic heart disease and all-cause mortality. The SLC23A1 rs33972313 genotype seems like a valid instrument for this assessment because 1) it is robustly associated with plasma vitamin C, 2) the gene codes for the sodium vitamin C transporter 1, which makes it believable that it does not have pleiotropic effects and only asserts its effect through plasma vitamin C, and 3) we showed that it is not associated with any of the recorded potential confounding factors for the association of vitamin C with outcomes.

Strengths of this study included a large sample size from a homogeneous general population and no losses to follow-up. Also, we were able to adjust for a large number of potential confounders. Finally, we used genetically high plasma vitamin C, excluding reverse causation and largely circumventing confounding.

However, potential limitations to our study should also be considered. First, the genetic estimate was based on a single genetic variant that explained only 0.9% of the variation in plasma vitamin, and thus, an even larger sample size and more and stronger genetic instruments for plasma vitamin C may be desired to increase statistical power. Second, all participants were of Danish descent, and thus, our results may not necessarily apply to other races; however, this feature also minimized risk of population stratification affecting instrumental variable analyses, and we are not aware of results that indicate that our findings should not be applicable to other ethnic groups. Third, we used

| Risk estimates for ischemic heart disease for 25% higher vitamin C or 25% lower triglyceride concentrations |
|---------------------------------------------------------------|-----------------|-----------------|------|
| N | OR (95% CI) | P |
| Genetically higher vitamin C in CCHS+CGPS | 97,150 | 0.90 (0.75, 1.08) | 0.27 |
| Genetically lower triglycerides in CCHS | 10,042 | 0.83 (0.65, 1.07) | 0.15 |

FIGURE 4 For genetic risk estimates [ORs (95% CIs)] for ischemic heart disease and all-cause mortality for 25% higher vitamin C or 25% lower triglyceride concentrations. Instrumental variable analyses were used to estimate genetically derived ORs adjusted for age. P values were derived from logistic regression. CCHS, Copenhagen City Heart Study; CGPS, Copenhagen General Population Study.
self-reported dietary intake, which has limited accuracy. Fourth, we measured plasma vitamin C only in a smaller group of newly recruited CGPS participants; however, we showed no evidence that this group differed from the remaining CGPS subjects. Fifth, we tested for population stratification in an indirect way, and we could not rule out that more-direct ways of examining for population stratification could have yielded a different result; however, such data were not available in the current study. Finally, we showed an attenuation of our observational risk estimates after the inclusion of possible confounders, and it is possible that the remaining relation between fruit and vegetable intake and outcomes could have been attributed to measurement errors in confounders and/or to an influence from unmeasured confounders.

In conclusion, we observed an association between high intake of fruit and vegetables and low risk of ischemic heart disease and all-cause mortality. A genetic variant leading to lifelong high plasma vitamin C concentrations gave comparable effect sizes, although the 95% CI overlapped 1.0, which made certain statistical inferences difficult. Thus, judging by the effect size, our data cannot exclude that a favorable effect of high intake of fruit and vegetables could in part be driven by high vitamin C concentrations. In addition, when we compared risk estimates from genetically high plasma vitamin C with genetically low plasma triglycerides, which are known to cause reduced cardiovascular disease and reduced all-cause mortality (29, 30, 49), we cannot exclude an effect of high plasma vitamin C of a similar size as that of low triglycerides on risk of ischemic heart disease and all-cause mortality.

The authors’ responsibilities were as follows—CJK: analyzed data; BGN: had primary responsibility for the final content of the manuscript; and all authors: designed the research, interpreted data and wrote the manuscript, and read and approved the final manuscript. None of the authors reported a conflict of interest related to the study.

REFERENCES


