Genetic variation at the *SLC23A1* locus is associated with circulating levels of L-ascorbic acid (Vitamin C). Evidence from 5 independent studies with a total of 15087 participants.


**Supplementary Online Material**

**Additional methodological details for discovery study and replication studies**

**Discovery study – British Women’s Heart and Health Study (BWHHS)**

**Measurement of circulating L-ascorbic acid**

Venous blood samples were taken after a minimum 6-hour fast onto EDTA. The sample was centrifuged within 2h of removal from the participant. 0.5ml of plasma was removed and mixed with 0.5ml 100g metaphosphoric acid/L and assays were then snap frozen on dry ice and on return to the laboratory frozen at -80° C before analysis.

The plasma samples were vortexed after thawing and spun in a microcentrifuge for 30min at 4° C at 5000g. The centrifugation was repeated and the supernatant passed through a 40,000 molecular filter at 2000g to remove traces of protein. Standards of pure ascorbate were treated in a similar manner. All samples and standards were used for analysis of ascorbate by high performance liquid chromatography(1, 2). This was undertaken using a Jasco UK (Great Dunmow, Essex) liquid chromatograph fitted with a reverse phase column. The mobile phase consisted of 839ml acetonitrile/ 16ml15mM KH₂PO₄ buffer/ 0.1 glacial acetic acid and the peaks were detected using UV absorption at 254m. The amount of ascorbate per sample was calculated against the diluted solutions of stock ascorbate and an internal standard of uric acid.
Measurement of potential confounding factors

Binary confounding factors included the questionnaire responses to ‘current smoker’ (derive from categories of smoking regularity), adherence to moderate drinking habits (no more than two alcoholic drinks per day), use of HRT (yes/no), less than two hour’s vigorous activity per week (derived from a detailed physical activity questionnaire(3)) and parental cardiovascular disease (derived from parental experience of heart attack or stroke). The continuous confounding factors life course socioeconomic position score(4), longitude and latitude were organized into tertiles for assessment of differences in mean adjusted circulating L-ascorbic acid.

BWHHS ethical approval

Local ethics committee approvals were obtained for the British Women’s Heart and Health Study along with UK multicentre ethical approval. Participants were asked for informed consent to review their medical records and for permission to perform anonymised genetic tests relating to cardiovascular disease on stored blood. Eight women declined to give consent and have not been included in this study.

Replication study details

*European Prospective Investigation of Cancer Norfolk Study (EPIC-Norfolk)*

We analysed data from a random sub-study of 5000 participants (EPIC5000) from the Norfolk arm of the European Prospective Investigation on Cancer (EPIC-Norfolk) study. Described in detail previously(5), EPIC-Norfolk is an ongoing prospective study of men and women aged between 40 and 79 years, resident in Norfolk, UK. The participants in the current analysis (EPIC5000 sub-set) were a random sub-subset without cancer, coronary heart disease or diabetes at baseline, and had completed arrayed
DNA samples, and complete plasma vitamin C and HbA1c measures. The study includes a largely ethnically homogeneous white European population.

Genotyping of three single nucleotide polymorphisms (SNPs) in the \textit{SLC23A1} vitamin-C-transporter gene which corresponded to those scored in the BWHHS (rs33972313, rs10063949 and rs6596473) was undertaken. These SNPs were genotyped using Custom TaqMan assays (Applied Biosystems, Warrington, UK) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Warrington, UK). The genotyped SNPs had a genotyping call rate of ≥96.8%. With complete phenotypic and genotypic data for basic analyses \(n=4501\) participants were available.

Plasma L-ascorbic acid concentration was measured in blood samples drawn into citrate bottles, placed in dark boxes, and refrigerated overnight at 4-7°C. The blood was then spun at 2,100 g for 15 min at 4°C. The plasma was stabilised in a standardised volume of metaphosphoric acid and stored at -70°C. Within one week of sampling a fluorometric assay was used to estimate the plasma L-ascorbic acid concentration(6).

\textit{MIDSPAN family study}

The name MIDSPAN is given to 4 separate occupational and general population cohort studies based in Scotland (almost entirely white European). The 3 original studies took place between 1964 and 1976. Twenty years later in 1996 the next generation was studied when offspring of couples in the original Renfrew/Paisley Study were recruited into the Family Study(7). This latter group is the subject of the present analysis, \(n=1814\) with complete data taken from 1040 sons and 1298 daughters from 1477 families who participated in the 1996 study (response prevalence of 73% for individuals, 84% for families). They completed a questionnaire and attended a screening examination for
measurement of a range of established and novel risk factors for vascular disease including measurement of a range of vitamins. The study was approved the relevant Local Research Ethics Committees and all subjects provided written informed consent.

Genotyping was performed on a ABI PRISM 7900HT sequence detection System using a Taqman assay developed by Applied Biosystems followed by allelic discrimination using software from Applied Biosystems (SDS V2.0)(8, 9). Specific SNP Taqman Assays (Assay ID: C__25986101_10) were obtained from Applied Biosystems. All genotyping errors were manually resolved by checking raw genotype data, individuals were either blanked (zeroed) or corrected prior to analysis.

L-ascorbic acid status was assessed by measuring ascorbic acid in plasma using HPLC with electrochemical detection. The measurement in plasma was based on the method of Margolis and Davis(10). The limit of sensitivity of the method is 0.5umol/L and the intra and inter-assay CV less than 2% and 4% respectively.

**10 Towns**

The Ten Towns Study was a longitudinal study of the development of cardiovascular risk among children and adolescents in ten British towns, five with high and five with low adult cardiovascular mortality rates(11, 12). The study includes children predominantly of white European origin.

In the third phase of the study, ~1500 adolescents aged 13-16 years were examined and provided whole blood and plasma samples from which DNA was later extracted and L-ascorbic acid analysed. Genotyping of SNP rs33972313 was carried out by KBiosciences
using essentially identical methods to those used in the BWHHS study and describe above. Complete phenotypic and genotypic data were available on 1359 participants.

L-ascorbic acid was assayed in a similar way to that for BWHHS. It was measured in plasma treated with metaphosphoric acid at the point of collection and then snap-frozen with dry ice and maintained at -70°C until analysis within 3 months of collection. It was estimated using a fluorimetric assay(13). The relevant Local Research Ethics Committees gave permission for the original data collection while the genetic studies were approved by the Welsh Research Ethics Committee.

**British Regional Heart Study (BRHS)**

In a study which gave rise to that used in the BWHHS 7735 men aged 40-59 were recruited in 1978-80 from a single general practice in each of 24 towns across Great Britain, and have been followed ever since(14). In 1998-2000, when the subjects were aged 60-79 years, 4252 were re-examined and most provided a whole blood sample. The study includes a largely ethnically homogeneous white European population.

Genotyping of SNP rs33972313 was carried out in the same laboratories and using the same methods as those used for the BWHHS and describe above. 3740 men who gave consent for genetic testing and had complete data on genotype and vitamin C levels were included in the analyses.

Measurement of L-ascorbic acid was also carried out in the same laboratories and using the same methods as those used for the BWHHS and describe above.
Table S1.
Study specific results for the association between rs33972313 and L-ascorbic acid in each of the four replication studies.

<table>
<thead>
<tr>
<th></th>
<th>EPIC</th>
<th>10 Towns</th>
<th>BRHS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
</tr>
<tr>
<td>Sex (%M)</td>
<td>44.1</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Genotype count</td>
<td>4208</td>
<td>287</td>
<td>3609</td>
</tr>
<tr>
<td>Hardy Weinberg</td>
<td>0.6</td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>Mean (95% CI) L-ascorbic acid (μmol/L)</td>
<td>(56.08, 57.24)</td>
<td>(45.99, 50.43)</td>
<td>(29.42, 31.21)</td>
</tr>
<tr>
<td>Per allele effect of rs33972313 variation</td>
<td>-8.31 (1.12)</td>
<td>-6.26 (2.40)</td>
<td>-2.87 (1.72)</td>
</tr>
<tr>
<td>Mean (95% CI) L-ascorbic acid (μmol/L)</td>
<td>56.66 (56.08, 57.24)</td>
<td>48.21 (45.99, 50.43)</td>
<td>30.312 (29.42, 31.21)</td>
</tr>
<tr>
<td>Per allele effect of rs33972313 variation</td>
<td>8.31 (1.12)</td>
<td>10 Towns</td>
<td>6.26 (2.40)</td>
</tr>
<tr>
<td>Sex (%M)</td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>Genotype count (youngest singletons)</td>
<td>1223</td>
<td>99</td>
<td>12</td>
</tr>
<tr>
<td>Hardy Weinberg</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (95% CI) L-ascorbic acid (µmol/L)</td>
<td>45.02 (40.63, 49.42)</td>
<td>39.30 (24.08, 54.52)</td>
<td>48.74 (43.16, 54.31)</td>
</tr>
<tr>
<td>Per allele effect of rs33972313 variation</td>
<td>-7.51 (1.82)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Effects are adjusted for age and sex.

MIDSPAN – diagnostics from youngest singletons and effects derived from the whole sample employing a mixed model.
**Supplementary Table S2.**
Meta-analysis sensitivity analysis for association of rs33972313 with circulating L. ascorbic acid from discovery and replication studies stratified by assay type.

<table>
<thead>
<tr>
<th>Meta-analysis group</th>
<th>Contributing studies</th>
<th>N</th>
<th>Vitamin C assay</th>
<th>Study effect estimates (95%CI)</th>
<th>Pooled effect (95%CI)</th>
<th>I² (95%CI)</th>
<th>phet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BWHHS</td>
<td>3425</td>
<td>Type one</td>
<td>-4.15 (-7.81, -0.49)</td>
<td>-4.07 (-6.26, -1.87)</td>
<td>0 (0-90)</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>BRHS</td>
<td>3740</td>
<td>(as described for BWHHS)</td>
<td>-2.87 (-6.24, 0.49)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ten Towns</td>
<td>1359</td>
<td></td>
<td>-6.26 (-10.97, -1.55)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>EPIC</td>
<td>4501</td>
<td>Other</td>
<td>-8.31 (-10.51, -6.11)</td>
<td>-8.09 (-9.97, -6.22)</td>
<td>0 (0-90)</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>MIDSPAN</td>
<td>1814</td>
<td>(as described for EPIC and MIDSPAN)</td>
<td>-7.51 (-11.09, -3.94)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Overall a meta-analysed pooled estimate of per allele effect (random effects).
Supplementary references:


8. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of


