Genetic evidence of the causal relationship between serum micronutrients and Graves' disease: A Mendelian randomization and cross-sectional observational study

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ABSTRACT

Background and Objectives: Exploring the effects of circulating micronutrients on Graves' disease (GD) through observational research or randomized controlled trials has drawn more attention. In order to investigate the putative causal inference, we provide an illustrative estimate of two-sample Mendelian randomization (MR) study. **Methods and Study Design:** Inverse-variance weighted (IVW) method was employed as the primary approach to determine the causal relationships between micronutrients level and GD. Several complementary sensitivity analyses were also undertaken to evaluate the impact of potential violations of MR assumptions. In addition, we utilized cross-sectional data from the National Health and Nutrition Examination Survey (NHANES) to analyze the differences in the prevalence of GD among participants with different levels of trace nutrient concentrations. **Results:** In terms of vitamins, IVW MR analysis revealed a suggestive relationship between each standard deviation decrease in vitamin D level and increased risk of GD (OR=1.28, 95% CI: 1.04-1.59, p = 0.0212). A nominally significant association was also noted for genetically predicted vitamin B-6 concentration and higher risk of GD (OR=1.56, 95% CI: 1.08-2.25, p = 0.0171). Genetically predicted concentrations of other vitamins level and 6 minerals levels were not in association with GD susceptibility. The causal estimates from other complementary MR approaches were consistent with these findings. Additionally, we found that participants from NHANES with vitamin D and VB-6 deficiency had a higher prevalence of GD. **Conclusions:** Our study provides an obvious unidirectional causality of circulating vitamin B-6 and vitamin D with GD. Dietary supplementation with micronutrients may be a complement to classical therapies for preventing and treating GD.

Key Words: Graves' disease, micronutrient, mendelian randomization, single nucleotide polymorphism, casual relationship

INTRODUCTION

Graves' disease (GD), also known as the leading cause of hyperthyroidism condition, is an autoimmune symptom characterized by the formation of thyroid receptor autoantibodies (TRAbs), which directed against the thyroid-stimulating hormone receptor (TSHR) on a diffusely enlarged and overactive thyroid gland.[1-3] Several epidemiological investigations have shown an annual incidence of 20–25 cases per 100000 and 5-10 times greater in women than in men.[4,5] Although the alternative administrations for relapsing GD include medical therapy, radioactive iodine, and surgery, the continuous-treatment cases often impose an enormous financial and psychological burden on public health care.[6]

The precise etiology and pathogenesis of GD still remains a difficult issue. It is considered to be a very complex multifactorial and polygenic disease arising from a combination of genetic susceptibility, hormonal and environmental factors, resulting breakdown of immune tolerance towards the thyroid antigens and the initiation of immune reaction against the thyroid.[3,7] The associations of increased GD susceptibility with certain human leukocyte antigen (HLA) genes (HLA*DR3 and DQA*10501), the immune-related genes (CTLA4, FOXP3, and CD40) and the thyroid-specific genes (Thyroglobulin, and TSHR) were well known and widely investigated.[8,9] Additionally, the strength of the immune response and environmental or structural factors (for example, infection, iodine, iodine-containing drugs, and major stress) lead to an immunopathogenic process of GD risk.[8,10] However, to date, a causal relationship between distinct genetic or environmental risk factors and the development of GD has not been fully established.

In the past decades, the potential association between serum micronutrient levels (including vitamins and minerals) and GD have drawn a great deal of attention.[11-13] Several published observational studies have assessed the connection of micronutrients levels, including plasma selenium, calcium, and vitamin D, with the risk of GD.[14,15] Previous studies have demonstrated that serum vitamin D levels are significantly lower in GD patients as compared to those in healthy individuals,[16,17] while other studies did not identify the decreased vitamin D level with the risk of GD.[18,19] Moreover, it has been proposed that serum selenium level was considerably lower in patients with newly diagnosed GD compared to randomly selected controls.[14] However, a randomized, double-blind, placebo-controlled trial revealed that supplemental selenium was not related to the patients’ response or recurrence rates in GD.[20]

As mentioned above, the causal relationships between serum micronutrient levels and GD susceptibility remained inconsistent and conflicting. The relatively small sample size and inescapable confounding factors may be to blame for this potential bias. In order to overcome the limitations of conventional observational approaches, mendelian randomization (MR) analysis could be utilized to discreet the causal inferences between exposure factors and GD outcome using genetic variants (for example single nucleotide polymorphisms, SNPs) as instrumental variables (IVs).[21] As individual germline genetic variants are randomly allocated to different genotypes from parents to offspring, those IVs would not be controlled by potential confounding factors that influence exposure-outcome relationship.[22] To the best of our knowledge, we performed a two-sample MR (2SMR) analysis to determine whether serum micronutrient levels are crucial in GD risk.

MATERIALS AND METHODS

Study design and data sources

For the validity of each IV, three key assumptions in MR analysis have to be fulfilled: (1) the IVs (SNPs) would be strongly correlated with exposure factor; (2) the IVs should not be linked to any conceivable confounding variables; (3) a valid instrument that is associated only with outcome through the exposure of interest.[23] Diagram of the basic principles of MR model is presented in Figure 1.

A total of 14 common circulating micronutrients (including vitamins A, B-6, B-12, C, D, and E, folate, calcium, magnesium, zinc, selenium, copper, iron, and phosphorus) associated with GD risk have been previously described in electronic literature. A structured literature search was carried out using the OpenGWAS, GWAS catalog, FinnGen study, and PubMed databases for all available studies referring to micronutrient levels and GD from inception to August 2022. Vitamin E, folate, and copper were subsequently excluded because no relative GWAS research has been conducted or genome-wide significant findings have been provided. Finally, recently published GWAS for 11 micronutrients were retrieved, namely 5 vitamins (vitamins A, B-6, B-12, C, and D) and 6 minerals (calcium, magnesium, zinc, selenium, iron, and phosphorus).[24-36] This MR investigation covered all participants with European ancestry, and Table 1 summarizes the specific details of GWASs connected to selected exposures. Regarding GD outcome, the summary genetic data restricted to the participants of East Asian descent were derived from a number of 2176 patients with GD and 210277 healthy individuals, which was obtained from OpenGWAS project (dataset: bbj-a-123).

Besides, IVs associated with vitamin D levels were also derived from another 2 previously published GWASs in individuals of European ancestry (SUNLIGHT and UK Biobank),[37,38] and were used to further validate the MR estimate. For GD, we next sought to detect any potential associations in the latest publicly available FinnGen data release (freeze 8), which included 2575 cases and 339924 controls of European ancestry.[39] The IVW method is applicable to multiple IVs. It performs a weighted average based on the effect estimates and their standard errors for each SNP to obtain an overall causal effect estimate. MR-Egger regression is suitable in the presence of directional pleiotropy, providing a framework to detect and adjust for potential horizontal pleiotropy. The maximum likelihood approach accounts for sample overlap in two-sample MR studies, offering a more accurate estimation under such conditions. The weighted median method can provide an unbiased causal effect estimate as long as at least 50% of the SNPs are valid IVs. Lastly, the MR-RAPS method incorporates weak instruments, enabling robust statistical estimation for Mendelian randomization even when weaker instruments are included.[40]

Finally, we collected data from 2001 to 2011 (excluding 2003-2006, due to thyroid function was not collected). Participants were classified into three groups based on serum vitamin D (VD) levels: VD deficiency group (VD $\leq$25 nmol/L) with 427 participants, VD insufficiency group (25 nmol/L < VD < 75 nmol/L) with 5,986 participants, and VD sufficiency group (VD $\geq$75 nmol/L) with 2,480 participants. Similarly, participants were categorized based on serum PLP (pyridoxal-5'-phosphate, a marker for vitamin B6) levels into three groups: VB-6 deficiency group (PLP $\leq$25 nmol/L) with 1,028 participants, VB-6 insufficiency group (25 nmol/L <PLP <100 nmol/L) with 5,326 participants, and VB-6 sufficiency group (PLP $\geq$100 nmol/L) with 2,092 participants. Finally, participants were defined as GD patients if they had TSH $\leq$0.1 mIU/L or were taking methimazole or propylthiouracil.[41] Then, we calculate the prevalence of GD across different concentration groups of micronutrients and use the chi-square test to determine statistically significant differences.

Defining genetic instruments

Selecting at a genome-wide association threshold of 5×10^{-8} and excluding any linkage disequilibrium (LD) by defining at a r^2 value of 0.01, total available candidate genetic variations related to micronutrients levels were achieved independently. Besides, all putative IVs were adjusted for age, sex, assessment center as a proxy for latitude, and body mass index. SNPs with secondary phenotypes other than micronutrients were identified using the PhenoScanner V2 and were subsequently removed to exclude the possible pleiotropic effects.[42] Potential SNPs related to high-risk confounding factors of outcome would be excluded. Additionally, the selected SNPs that could not be identified from outcome dataset would be replaced by proxy SNPs with a high LD (r^2 $\geq$0.8) using the LDlinkR package. To avoid potential weak instrumental bias, we treated R2, power analysis (https://sb452.shinyapps.io/power/) and F-statistic as the characteristics of the GWAS traits.[43,44] F was deemed strong enough to counteract any bias in the causative IV estimate if it was more than 10. The flowchart of inclusion and exclusion of valid SNPs into the MR analysis is shown in Figure 2.

Statistical analysis

The available IVs for exposure and outcome statistics were harmonized to ensure the reference alleles from both datasets match. In the MR analysis, five common statistical approaches including primary inverse variance weighted (IVW),[45] MR-Egger regression,[46] maximum likelihood,[47] weighted median,[48] and MR-robust adjusted profile score (MR-RAPS),[49] were used to determine the robustness of the causal inference. The odds ratios (OR) with 95% confidence intervals (95% CIs) per standard deviation (SD) decrease in the exposure were used to quantitatively determine the relationship between circulating micronutrients and GD susceptibility.

The MR-Egger method and Cochran's Q statistics were used to perform additional verifications including sensitivity analysis and pleiotropy test, respectively. A random-effects model was applied to the IVW results of the MR if the *p* value of Q test was less than 0.05; otherwise, a fixed-effects model was adopted. Regarding MR-Egger test, a *p* value for intercept less than 0.05 is indicative of an overall directional pleiotropy. A leave-one-out sensitivity analysis was then conducted to determine whether any specific SNPs had an impact on the IVW causal estimate. Notably, 4 genetically predicted exposures (vitamin A, vitamin B-6, zinc, and iron) associated with less than 3 SNPs were not considered to conduct sensitivity analysis. Possible pleiotropic outliers were also detected using the MR-pleiotropy residual sum and outlier (MR-PRESSO) global and outlier test.[50] In addition, two robust radial IVW and MR-Egger estimate approaches were also performed to address the issue of potential pleiotropy.[51]

The statistical analyses were performed using R software (version 4.1.0, using the “TwoSampleMR”, “mr.raps”, and “MR-PRESSO” R packages; R Foundation for Statistical Computing, Vienna, Austria). A Bonferroni-corrected *p* value of 4.55×10^{-3} (0.05/11 putative risk exposure factors) was considered significant, and $4.55\times10^{-3} < p$ value <0.05 was deemed as suggestive significance.

RESULTS

Characteristics of the selected instrumental variables

After strict confounding instrument removal, clumping for the available SNPs, and data harmonization using the TwoSample MR package, there are a total of 46 SNPs that met the inclusion criteria for the core assumption (Supplementary Table 1). Briefly, SNPs for vitamin A, B-6, B-12, C, and D, calcium, magnesium, zinc, selenium, iron, and phosphorus levels could explain 5.47%, 3.87%, 1.81%, 2.05%, 1.13%, 4.73%, 30.9%, 4.09%, 0.552%, 0.663%, and 2.37% of the variance, respectively. The F-values of the IVs were all greater than 10, and suggested no weak instrument bias.

Causal estimates of serum micronutrient levels on GD

There was no any significant heterogeneity measured between SNPs by Cochran's Q test (p >0.05), thus a fix-effects model of MR analysis would be applied. As shown in Table 2, each 1-SD reduction in genetically predicted vitamin D was suggestively associated with higher odds of GD risk using the primary IVW model (OR = 1.28, 95% CI: 1.04-1.59, p = 0.0212, Supplementary Figure 1). However, 2SMR results based on currently available data indicated that predicted circulating concentrations of other vitamins and minerals had no causal effect on GD susceptibility (p >0.05, Supplementary Figure 2). Moreover, the results from complementary approaches such as MR-Egger, weighted median, maximum likelihood, and MR-RAPS methods were in line with these findings (Table 2).

In order to facilitate the classification of the evidence for a causal relationship between the exposures and the outcomes of interest, we evaluated the MR estimates based on population and dataset heterogeneity. As shown in Table 3, only a genetically predicted higher vitamin B-6 level was found to be suggestively associated with GD in IVW analysis (OR=1.56, 95% CI: 1.08-2.25, p = 0.0171, Supplementary Figure 3). The genetic predisposition to high circulating levels of other remaining serum micronutrients showed no significant associations with the risk of GD (p >0.05, Supplementary Figure 4). In addition, the causal inference between two additional GWAS related to vitamin D levels and GD susceptibility was further examined. According to 2SMR study based on 62, 87 distinct SNPs, genetically decreased vitamin D level per SD was causally linked to an increased risk of GD (IVW: OR=1.53, 95% CI: 1.21-1.93, p <0.001; OR=1.26, 95% CI: 1.00-1.59, p = 0.0462, respectively, Figure 3).

Pleiotropy and sensitivity analysis

Based on MR-Egger regression intercept and MR-PRESSO global test, we did not find any conclusive evidence of horizontal pleiotropy or outliers between the micronutrient levels and GD risk (p >0.05, Supplementary Table 2). Also, the visual inspection of the funnel plot did not show any asymmetry or potential heterogeneity (Supplementary Figure 5). Regarding the leave-one-out analysis, the results of IVW MR analysis demonstrated that a single SNP had little influence on the overall effect of causal estimates except for those in serum vitamin C level (Supplementary Figure 6). In the subsequent analysis applying for radial IVW and MR-

Egger models, there did not appear to be any signs of outlying genetic variants among selected IVs.

Prevalence of GD across different micronutrients concentration groups

We found statistically significant differences in the prevalence of GD across the vitamin D concentration groups (VD deficiency group: 1.2%, VD insufficiency group: 0.7%, VD sufficiency: 1.1%, p = 0.0453) (Supplementary Table 3). Similarly, there were statistically significant differences in the prevalence of GD among the vitamin B6 concentration groups (VB-6 deficiency group: 1.5%, VB-6 insufficiency group: 0.7%, VB-6 sufficiency group: 0.8%, p = 0.0472) (Supplementary Table 4).

DISCUSSION

It has been widely recognized that any individual patient with GD harbors a cluster of genetic, immune, environmental susceptibility factors, and interactions. Despite much research, the etiological basis of GD has remained elusive. To gain insight into the causal associations among a set of serum micronutrients, we attempted to elucidate the potential inference of 5 vitamins and 6 minerals levels in GD susceptibility using a comprehensive 2SMR analysis. In our present research, we found evidence that a lower genetically determined vitamin D level was suggestively linked to a 28.4% higher risk of GD from Asian population. Besides, our findings suggest that the circulating vitamin B-6 concentration is potentially causally associated with risk of GD from European population. Based on the NHANES database, we found that the prevalence of GD in the Vitamin D and vitamin B-6 deficiency group was higher than that in the micronutrients insufficient and sufficient groups. This difference was statistically significant and, to some extent, supports our MR results. Furthermore, there was no obvious evidence to support a causal relationship between other circulating micronutrient levels and GD.

These results corroborate those of other studies that found a connection between serum vitamin D levels and GD susceptibility. Recent case-control research indicated that the lower serum vitamin D level may be involved in the occurrence and development of GD, and vitamin D supplements may prevent the production of TRAbs molecules.[52] Additionally, results from a randomized controlled trial (RCT) suggested that reaching optimal vitamin D level could increase the early efficacy of antithyroid methimazole treatment for GD.[53] Several meta-analyses concluded that low vitamin D status (such as vitamin D deficiency or insufficiency) can greatly increase the rate of GD.[54-56] Additionally, a cross-sectional study demonstrated that vitamin D supplementation might have a protective effect against GD recurrence with a borderline significant recurrence rate reduction.[57] However, there have also been some reports contradicting such relationships.[58]

In the current MR study, we observed a positive relationship between genetically predicted concentrations of vitamin B-6 and risk of GD, but sensitivity analyses could not be performed. There were only 2 genetic variants available for vitamin B-6; thus, we cannot preclude the presence of a potential causal association. In patients with autoimmune hyperthyroidism, reduced levels of vitamin B6 can be observed.[59] Sakakeeny et al.[60] tested PLP levels in 2,229 patients and found that individuals with chronic inflammation had the lowest levels of this vitamin. Conversely, individuals with higher levels of this vitamin exhibited lower degrees of inflammation. Vitamin B6 has therapeutic potential for various inflammatory diseases. It has been reported that Vitamin B6 participates in T1-T2 immune regulation, and its deficiency can lead to elevated circulating TNF-α levels.[61] PLP also influences the formation of gut microbiota, which in turn affects human immunity.[62,63] Additionally, patients with GD exhibit a marked TH1 immune dominance, with TNF-α playing a significant role in GD pathogenesis.64 and anti-TNF-α therapy (Etanercept) has already been used in the treatment of GD. This suggests that Vitamin B6 may be involved in GD by modulating immune responses. Additionally, the observational molecular epidemiology literature for vitamin B-6 concentration and risk of GD is relatively sparse, larger GWASs and RCTs are urgent to better understand the genetic regulation of vitamin B-6 and to better define instrumental variables for MR analysis.

There have been several underlying mechanisms explaining the advantages of greater vitamin D levels on GD outcome. By suppressing the excessive activity of CD4+, Th1, Th2, and Th17 cells and the production of their associated cytokines by activating the vitamin D receptor, vitamin D could play a crucial role in preventing over-activation of pro-inflammatory responses.[65] Also, vitamin D could affect the differentiation and maturation of dendritic cell through a reduced expression of the major histocompatibility complex class II molecules and IL-12 level.[55] Additionally, vitamin D supplement also makes the induction of T regulatory cells simpler to weaken T cell-dependent immune responses in common autoimmune disorders.[66]

As the primary factors promoting the pathogenesis of GD, oxidative stress and inflammatory response have been identified. Smoke-induced increased generation of reactive oxygen species may be implicated.12 GD related ocular fibroblasts have exaggerated response to cigarette smoke extract challenge along with increased oxidative stress.[67] Treating vitamin D deficiency in those who are most at risk for developing GD would be a logical step toward investigating the effect of vitamin D therapy in postponing the development or severity of GD. Animal experiment suggested that BALB/cJ mice given a vitamin D-deficient diet posed lower preimmunization T4 levels and were more likely to develop persistent hyperthyroidism as opposed to those receiving regular chow.[68] Additionally, vitamin D treatment successfully prevented disease development in mice with experimental autoimmune thyroiditis.[69]

There have been many observational studies examining the causal relationships between the other micronutrient levels analyzed in our study and GD susceptibility. However, up until this point, the findings of these investigations were contradictory and ambiguous. For instance, in a case-control study with 124 participants, Lin et al.[70] demonstrated that higher serum phosphorus was positively associated with euthyroid GD susceptibility. Nevertheless, in another observational study,[71] this causal inference cannot be replicated. The underlying reasons should be further elucidated. Thus, we have conducted the MR analysis, aiming to exhibit a broader and more reliable perspective on the causal relationship between 11 micronutrient levels and GD risk. Here, our findings showed that circulating vitamin D levels rather than the other 10 micronutrients were suggestively associated with GD susceptibility. Additionally, our MR analyses using another two large-scale GWAS significant vitamin D variants showed consistent effect sizes, which would mean that our results are generalizable to comparable clinical populations.

Our analysis has several strengths. Firstly, the current study is the first to use a MR approach to investigate the causal relationship between common micronutrients, including 5 vitamins and 6 minerals, and GD risk. Secondly, compared with conventional observational or RCT research, the MR method is more likely to reduce the concerns about confounding variables and reverse causation because genetic variants are fixed at conception and less related to confounders than directly measured environmental exposures. Thirdly, no obvious heterogeneity was discovered between SNPs by Cochran's Q test. Five common MR statistical approaches were used to strengthen the consistency of the MR estimates. In addition, we also employed the MR-Egger intercept, MR-PRESSO, and radial IVW MR models to control horizontal pleiotropy bias and find aberrant SNPs. Lastly, our data provide significant evidence in support of a potential role of low vitamin D levels in GD susceptibility, despite the lack of large-scale, high-quality, and long-term RCT.

Nevertheless, some limitations should be highlighted in our present MR analysis. Due to the relative low proportion of variance in the micronutrient levels explained by genetic variants (ranging from 0.55% to 30.92%), we would not completely rule out the possibility that our MR analysis may have poor power for detecting a weak association. Additionally, there were only a small number of SNPs that were regarded as IVs, which means that only a limited causality could be explained. Moreover, we mainly synthesized the extracted population-based datasets from individuals of European and Asian descent. Thus, racial differences may lead to confusing bias between exposure and outcome factors. Lastly, total individual-level datasets cannot be systematically incorporated in current MR study. In the meantime, we did not infer whether the genetic association between circulating micronutrient levels and GD was a non-linear causal manner.

Conclusion

In summary, using a comprehensive MR study, our present results provided strongly suggestive evidence to establish a causative correlation between vitamin D and GD from Asian individuals, as well as vitamin B-6 and GD from European populations. Future research involving large-scale populations is required to confirm our findings and clarify the underlying mechanisms.

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CONFLICT OF INTEREST AND FUNDING DISCLOSURE

The authors declare no conflict of interest.

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Table 1. Summary of details on GWASs and related datasets involving 11 micronutrients

Micronutrients	Sample size	Ethnicity	Publicly available websites	PMID	Reference
Vitamins					
Vitamin A	8902	European	NA	21878437	24
Vitamin B-6	4763	European	NA	19744961	25
Vitamin B-12	45576	European	NA	23754956	26
Vitamin C	52018	European	doi.org/10.6084/m9.figshare.13227443.v1	33203707	27
Vitamin D	79366	European	gwas.mrcieu.ac.uk	29343764	28
Minerals					
Calcium	39400	European	NA	24068962	29
Magnesium	15366	European	NA	20700443	30
Zinc	2603	European	NA	23720494	31
Selenium	56166	European	NA	25343990	32
Iron	48972	European	NA	25352340	33
Phosphorus	21708	European	NA	20558539	34

NA, not available

Table 2. The characteristics of participants

Exposure	nSNP	OR	95% CI	*p* value
Vitamin A				
IVW	2	0.487	0.081-2.943	0.433
Maximum likelihood	2	0.483	0.079-2.937	0.429
MR-RAPS	2	0.483	0.077-3.013	0.436
Vitamin B-6				
IVW	2	0.961	0.537-1.721	0.895
Maximum likelihood	2	0.961	0.536-1.724	0.895
MR-RAPS	2	0.961	0.532-1.736	0.896
Vitamin B-12				
IVW	6	1.076	0.783-1.478	0.653
MR Egger	6	1.268	0.764-2.104	0.409
Weighted median	6	1.059	0.739-1.517	0.755
Maximum likelihood	6	1.075	0.783-1.478	0.654
MR-RAPS	6	1.076	0.781-1.482	0.655
Vitamin C				
IVW	7	0.652	0.419-1.016	0.059
MR Egger	7	1.227	0.443-3.399	0.71
Weighted median	7	0.79	0.447-1.398	0.418
Maximum likelihood	7	0.649	0.415-1.016	0.058
MR-RAPS	7	0.649	0.413-1.020	0.061
Vitamin D				
IVW	5	1.284	1.039-1.587	0.021
MR Egger	5	1.521	1.002-2.308	0.143
Weighted median	5	1.295	1.026-1.636	0.029
Maximum likelihood	5	1.284	1.039-1.588	0.021
MR-RAPS	5	1.284	1.038-1.589	0.021
Calcium				
IVW	6	2.73	0.512-14.57	0.239
MR Egger	6	0.267	0.003-24.03	0.596
Weighted median	6	1.264	0.159-10.02	0.825
Maximum likelihood	6	2.779	0.514-15.05	0.235
MR-RAPS	6	2.78	0.499-15.50	0.243
Magnesium				
IVW	5	0.422	0.002-82.54	0.749
MR Egger	5	0.013	3.09e-10-5.66e+05	0.663
Weighted median	5	0.19	3.66e-04-99.02	0.603
Maximum likelihood	5	0.418	0.002-84.17	0.748
MR-RAPS	5	0.419	0.002-95.90	0.753
Zinc				
IVW	2	1.052	0.879-1.259	0.58
Maximum likelihood	2	1.053	0.878-1.263	0.579
MR-RAPS	2	1.053	0.876-1.265	0.582
Selenium				
IVW	6	0.974	0.818-1.160	0.767
MR Egger	6	1.062	0.438-2.576	0.899
Weighted median	6	0.963	0.779-1.189	0.727
Maximum likelihood	6	0.973	0.817-1.161	0.766
MR-RAPS	6	0.974	0.813-1.167	0.773
Iron				
IVW	2	0.997	0.713-1.394	0.986
Maximum likelihood	2	0.997	0.713-1.394	0.986
MR-RAPS	2	0.997	0.713-1.395	0.986
Phosphorus				
IVW	3	1.295	0.264-6.350	0.751
MR Egger	3	0.094	2.61e-4-335.58	0.672
Weighted median	3	1.579	0.270-9.219	0.612
Maximum likelihood	3	1.296	0.264-6.363	0.749
MR-RAPS	3	1.296	0.251-6.682	0.757

IVW, inverse variance weighted; MR-RAPS, MR-robust adjusted profile score; nSNP, number of SNPs; OR, odds ratio; 95% CI, 95% confidence interval.

Table 3. Mendelian randomization estimations between genetically predicted micronutrient levels and GD outcomes from European population

Exposure	nSNP	OR	95% CI	*p* value
Vitamin A				
IVW	2	1.649	0.414-6.574	0.478
Maximum likelihood	2	1.654	0.412-6.648	0.478
MR-RAPS	2	1.654	0.399-6.843	0.487
Vitamin B-6				
IVW	2	1.559	1.079-2.251	0.017
Maximum likelihood	2	1.562	1.070-2.279	0.021
MR-RAPS	2	1.561	1.062-2.296	0.023
Vitamin B-12				
IVW	9	0.948	0.830-1.082	0.426
MR Egger	9	0.871	0.705-1.074	0.235
Weighted median	9	0.906	0.768-1.069	0.241
Maximum likelihood	9	0.948	0.831-1.081	0.425
MR-RAPS	9	0.939	0.821-1.075	0.363
Vitamin C				
IVW	9	1.125	0.795-1.593	0.505
MR Egger	9	0.876	0.461-1.663	0.697
Weighted median	9	0.973	0.598-1.584	0.913
Maximum likelihood	9	1.128	0.795-1.599	0.501
MR-RAPS	9	1.021	0.793-1.603	0.505
Vitamin D				
IVW	5	1.021	0.834-1.251	0.839
MR Egger	5	1.045	0.711-1.537	0.836
Weighted median	5	1.021	0.816-1.278	0.855
Maximum likelihood	5	1.021	0.834-1.251	0.839
MR-RAPS	5	1.021	0.833-1.252	0.841
Calcium				
IVW	6	0.922	0.366-2.325	0.864
MR Egger	6	0.594	0.092-3.837	0.614
Weighted median	6	0.784	0.268-2.291	0.656
Maximum likelihood	6	0.921	0.364-2.335	0.863
MR-RAPS	6	0.921	0.360-2.356	0.864
Magnesium				
IVW	5	7.761	0.161-373	0.299
MR Egger	5	389.38	0.003-5.53e+07	0.397
Weighted median	5	7.261	0.079-6.65e+02	0.389
Maximum likelihood	5	8.084	0.161-4.06e+02	0.296
MR-RAPS	5	8.077	0.153-4.25e+02	0.302
Zinc				
IVW	2	0.957	0.790-1.159	0.653
Maximum likelihood	2	0.957	0.790-1.160	0.653
MR-RAPS	2	0.957	0.786-1.165	0.661
Selenium				
IVW	6	1.113	0.945-1.312	0.201
MR Egger	6	1.493	0.499-4.469	0.525
Weighted median	6	1.085	0.884-1.332	0.436
Maximum likelihood	6	1.115	0.945-1.316	0.199
MR-RAPS	6	1.115	0.943-1.318	0.203
Iron				
IVW	2	0.978	0.755-1.267	0.867
Maximum likelihood	2	0.978	0.755-1.267	0.867
MR-RAPS	2	0.978	0.755-1.267	0.867
Phosphorus				
IVW	3	0.176	7.49e-03-4.133	0.281
MR Egger	3	2.36E-05	3.63e-07-0.002	0.126
Weighted median	3	0.662	0.126-3.473	0.626
Maximum likelihood	3	0.176	7.49e-03-4.133	0.281
MR-RAPS	3	0.176	7.49e-03-4.134	0.281

IVW, inverse variance weighted; MR-RAPS, MR-robust adjusted profile score; nSNP, number of SNPs; OR, odds ratio; 95% CI, 95% confidence interval.

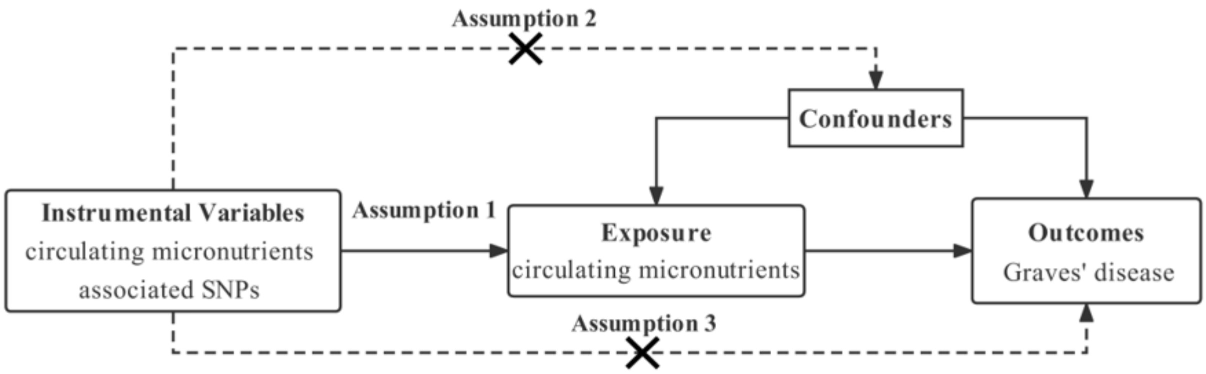

Figure 1. Schematic diagram of the two-sample Mendelian randomization analysis in the present study. SNP, single-nucleotide polymorphism

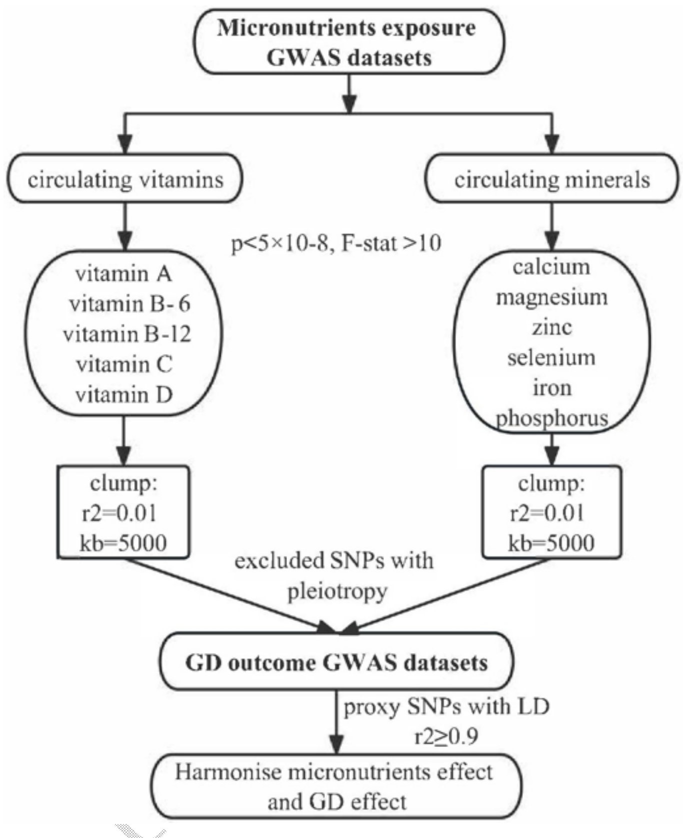

Figure 2. A flowchart of the inclusion and exclusion of SNPs. GWAS, genome-wide association study; LD, linkage disequilibrium

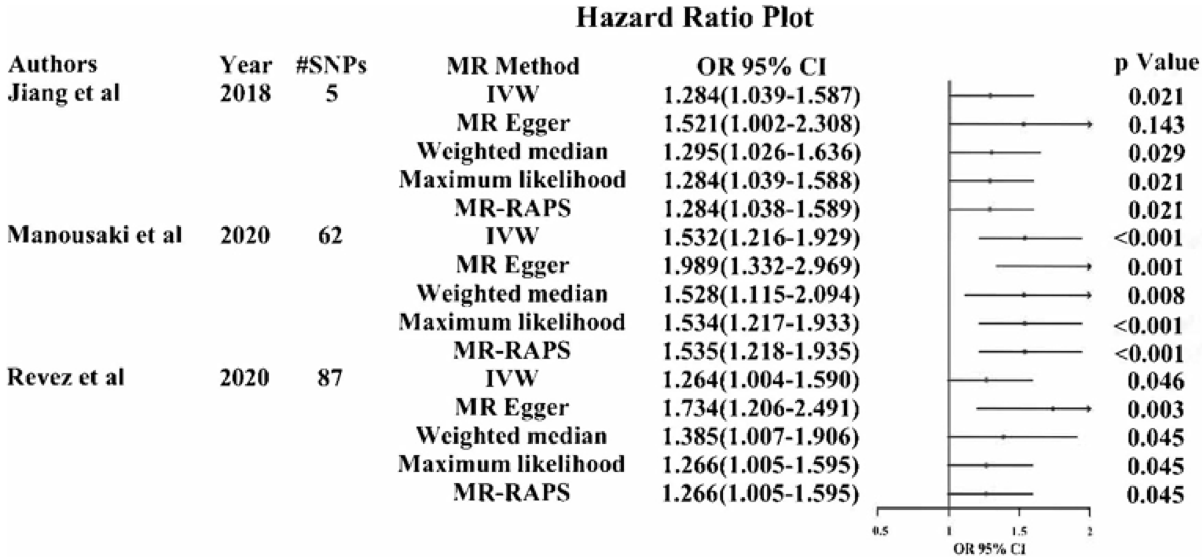

Figure 3. Mendelian randomization estimations between genetically predicted vitamin D levels and GD outcomes. IVW, inverse variance weighted; MR-RAPS, MR-robust adjusted profile score; #SNPs, number of SNPs; OR, odds ratio; 95% CI, 95% confidence interval

Supplementary Table 1. Characteristics of the SNPs associated with circulating micronutrients and their association with GD

Micronutrients	SNP	Chr	BP	EA	EAF	Micronutrient levels			GD			R^2	F
						beta	SE	p value	beta	SE	p value		
Vitamin A													
1	rs10882272	10	95348182	C	0.35	-0.03	0.004	7.80E-12	-0.039485	0.0498064	0.427907	0.0257666	56.3
2	rs1667255	18	29187279	C	0.31	0.03	0.004	6.35E-14	-0.048408	0.0330244	0.142698	0.0289567	56.3
Vitamin B-6													
1	rs1256335	1	21890386	A	0.79	0.14	0.02	6.35E-14	-0.241347	0.168414	0.151841	0.0136994	49.0
2	rs4654748	1	21786068	T	0.52	0.1	0.01	7.80E-12	0.0070095	0.0306602	0.819165	0.0250024	100.0
Vitamin B-12													
1	rs1141321	6	49412433	C	0.627	0.061	0.007	1.40E-16	-0.017854	0.0368068	0.627632	0.0035287	75.9
2	rs1801222	10	17156151	G	0.593	0.11	0.007	1.10E-52	-0.004475	0.042715	0.916554	0.0038906	246.9
3	rs2336573	19	8367709	T	0.031	0.32	0.021	1.10E-51	0.0719261	0.0671672	0.284237	0.0019776	232.2
4	rs3742801	14	74759006	T	0.294	0.045	0.008	3.50E-08	-0.001778	0.036017	0.960636	0.002992	31.6
5	rs41281112	13	100518634	C	0.948	0.17	0.016	9.60E-27	-0.049894	0.0809515	0.537668	0.001923	112.9
6	rs602662	19	49206985	A	0.596	0.16	0.008	4.10E-96	1.72028	1.63719	0.293373	0.0037628	400.0
Vitamin C													
1	rs10051765	5	176799992	C	0.342	0.039	0.007	3.64E-09	-0.058277	0.0326725	0.0744766	0.0029603	31.0
2	rs13028225	2	220031255	T	0.857	0.102	0.009	2.38E-30	-0.066001	0.0407448	0.105262	0.0026125	128.4
3	rs174547	11	61570783	C	0.328	0.036	0.007	3.84E-08	-0.042821	0.0315499	0.174702	0.0029426	26.4
4	rs2559850	12	102093459	A	0.598	0.058	0.006	6.30E-20	0.0011051	0.0328208	0.973141	0.0035852	93.4
5	rs33972313	5	138715502	C	0.968	0.36	0.018	4.61E-90	0.0849632	0.249713	0.733674	0.0021893	400.0
6	rs6693447	1	2330190	T	0.551	0.039	0.006	6.25E-10	-0.044692	0.0358066	0.211972	0.003452	42.3
7	rs9895661	17	59456589	T	0.817	0.063	0.008	1.05E-14	0.0029216	0.0308216	0.924482	0.0027183	62.0
Vitamin D													
1	rs10741657	11	14914878	A	0.4	0.094	0.007	2.05E-46	-2.03E-05	0.0319981	0.999494	0.0021676	180.3
2	rs10745742	12	96358529	T	0.4	0.052	0.007	1.88E-14	-0.029121	0.0313372	0.352748	0.0019933	55.2
3	rs12785878	11	71167449	T	0.75	0.109	0.007	3.8E-62	0.0688953	0.0331526	0.0376973	0.0022334	242.5
4	rs17216707	20	52732362	T	0.79	0.079	0.008	8.14E-23	-0.024454	0.0652841	0.70798	0.0018412	97.5
5	rs3755967	4	72609398	C	0.28	0.27	0.007	1E-200	0.0720803	0.0343001	0.0356008	0.0030793	1487.8
Calcium													
1	rs10491003	10	9328651	T	0.09	0.027	0.005	5.00E-09	-0.089665	0.193748	0.643514	0.0053292	29.2
2	rs1570669	20	52774427	G	0.66	0.018	0.003	9.00E-12	0.002882	0.030969	0.925856	0.0086941	36.0
3	rs17711722	7	65271197	T	0.47	0.015	0.003	8.00E-09	0.0466709	0.0562958	0.407088	0.0086425	25.0
4	rs1801725	3	122003757	T	0.15	0.071	0.004	9.00E-86	-0.020775	0.123384	0.866287	0.0072602	315.1
5	rs7481584	11	3029089	G	0.3	0.018	0.003	1.00E-10	0.005826	0.0323637	0.857141	0.0086941	36.0
6	rs780094	2	27741237	T	0.42	0.017	0.003	1.00E-10	0.065593	0.0309738	0.0342011	0.0086769	32.1

GD, Graves' disease; SNP, single nucleotide polymorphism; Chr, chromosome; BP, base pair; EA, effect allele; EAF, effect allele frequency; SE, standard error
R2, variance in exposure explained by the SNPs.

Supplementary Table 1. Characteristics of the SNPs associated with circulating micronutrients and their association with GD

Micronutrients	SNP	Chr	BP	EA	EAF	Micronutrient levels			GD			R^2	F
						beta	SE	p value	beta	SE	p value		
Magnesium													
1	rs13146355	4	77412140	A	0.56	0.005	0.001	6.30E-13	0.0418901	0.0372364	0.260598	0.0616785	25.0
2	rs3925584	11	30760335	T	0.45	0.006	0.001	5.20E-16	-0.023257	0.0328826	0.47939	0.0617943	36.0
3	rs4072037	1	155162067	T	0.46	0.01	0.001	2.00E-36	-0.01525	0.0407771	0.708409	0.0622598	100.0
4	rs448378	3	169100899	A	0.47	0.004	0.001	1.30E-08	-0.01265	0.0440637	0.774048	0.0615629	16.0
5	rs7965584	12	90305779	A	0.29	0.007	0.001	1.10E-16	-0.008865	0.0516992	0.863845	0.0619104	49.0
Zinc													
1	rs1532423	8	86268313	A	0.43	0.178	0.026	9.00E-12	0.0466341	0.0314928	0.138664	0.0206583	46.9
2	rs2120019	15	75334184	T	0.81	0.287	0.033	1.50E-18	-0.007761	0.0308194	0.801181	0.0202494	75.6
Selenium													
1	rs11951068	5	78304314	A	0.06	0.21	0.04	1.86E-11	-0.031096	0.0341258	0.362176	0.000677	27.6
2	rs1789953	21	44482936	T	0.16	0.12	0.03	3.40E-08	0.0116167	0.0510469	0.81998	0.0007539	16.0
3	rs3797535	5	78300397	T	0.1	0.21	0.04	2.05E-15	-0.026929	0.0942586	0.775115	0.000677	27.6
4	rs567754	5	78416416	C	0.67	0.17	0.02	8.38E-20	-0.012122	0.0331247	0.714412	0.0012491	72.3
5	rs705415	5	78291960	C	0.88	0.23	0.04	4.64E-10	0.0270256	0.0515298	0.599954	0.0007046	33.1
6	rs921943	5	78316476	T	0.29	0.25	0.02	1.90E-39	0.0156642	0.0450355	0.727978	0.0014656	156.3
Iron													
1	rs1800562	6	26098474	A	0.067	0.328	0.016	2.72E-97	-0.913591	1.793	0.610378	0.0024534	420.3
2	rs855791	22	37462936	G	0.554	0.181	0.007	1.32E-139	-5.1E-05	0.0309437	0.998684	0.0041721	668.6
Phosphorus													
1	rs17265703	3	122048644	A	0.15	0.036	0.006	4.32E-09	0.0099599	0.132541	0.940099	0.0081833	36.0
2	rs9469578	6	33706479	C	0.08	0.059	0.009	1.11E-11	-0.036724	0.0943896	0.697225	0.0057265	43.0
3	rs947583	6	136133659	C	0.29	0.035	0.005	3.45E-12	0.0204192	0.0340771	0.549035	0.0097845	49.0

GD, Graves' disease; SNP, single nucleotide polymorphism; Chr, chromosome; BP, base pair; EA, effect allele; EAF, effect allele frequency; SE, standard error
R2, variance in exposure explained by the SNPs.

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Supplementary Table 2. Pleiotropy test of the genetically predicted micronutrients levels with GD GWAS dataset

Exposure	MR-egger			MR-PRESSO	
	Intercept	SE	p value	SD	p value
Vitamin B-12	-0.026	0.032	0.459	0.119	0.566
Vitamin C	-0.042	0.032	0.235	0.221	0.103
Vitamin D	-0.016	0.064	0.134	0.14	0.099
Calcium	0.049	0.046	0.337	0.771	0.249
Magnesium	0.024	0.059	0.713	1.862	0.668
Selenium	-0.018	0.09	0.854	0.047	0.601
Phosphorus	0.103	0.16	0.637	NA	NA

SE, standard error; SD, standard deviation; NA, not available

Supplementary Table 3. Prevalence of GD with different concentrations of serum vitamin D levels, NHANES

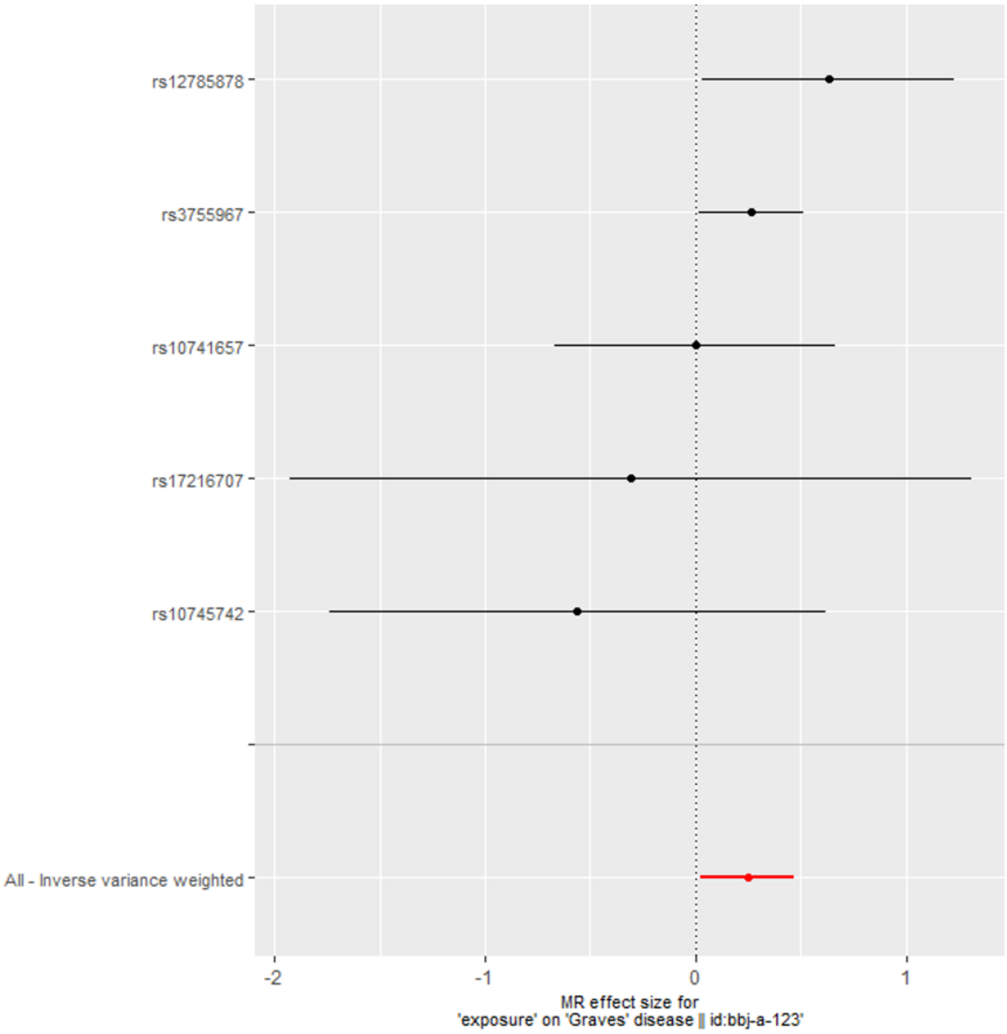
	Vitamin D deficiency[†] N = 427[‡]	Vitamin D insufficiency[†] N = 5,986[‡]	Vitamin D sufficiency[†] N = 2,480[‡]	p value[§]
GD Status				0.0453
No GD	422 (99%)	5,947 (99%)	2,452 (99%)	
GD	5 (1.2%)	39 (0.7%)	28 (1.1%)	

[†]Vitamin D dificiency: Serum 25-hydroxyvitamin D ≤25nmol/l; Vitamin D insufficiency: 25nmol/L<Serum 25-hydroxyvitamin D ≤25nmol/l; Vitamin D sufficiency: Serum 25-hydroxyvitamin D ≥75nmo/l
[‡]n (%)
[§]Fisher's exact test

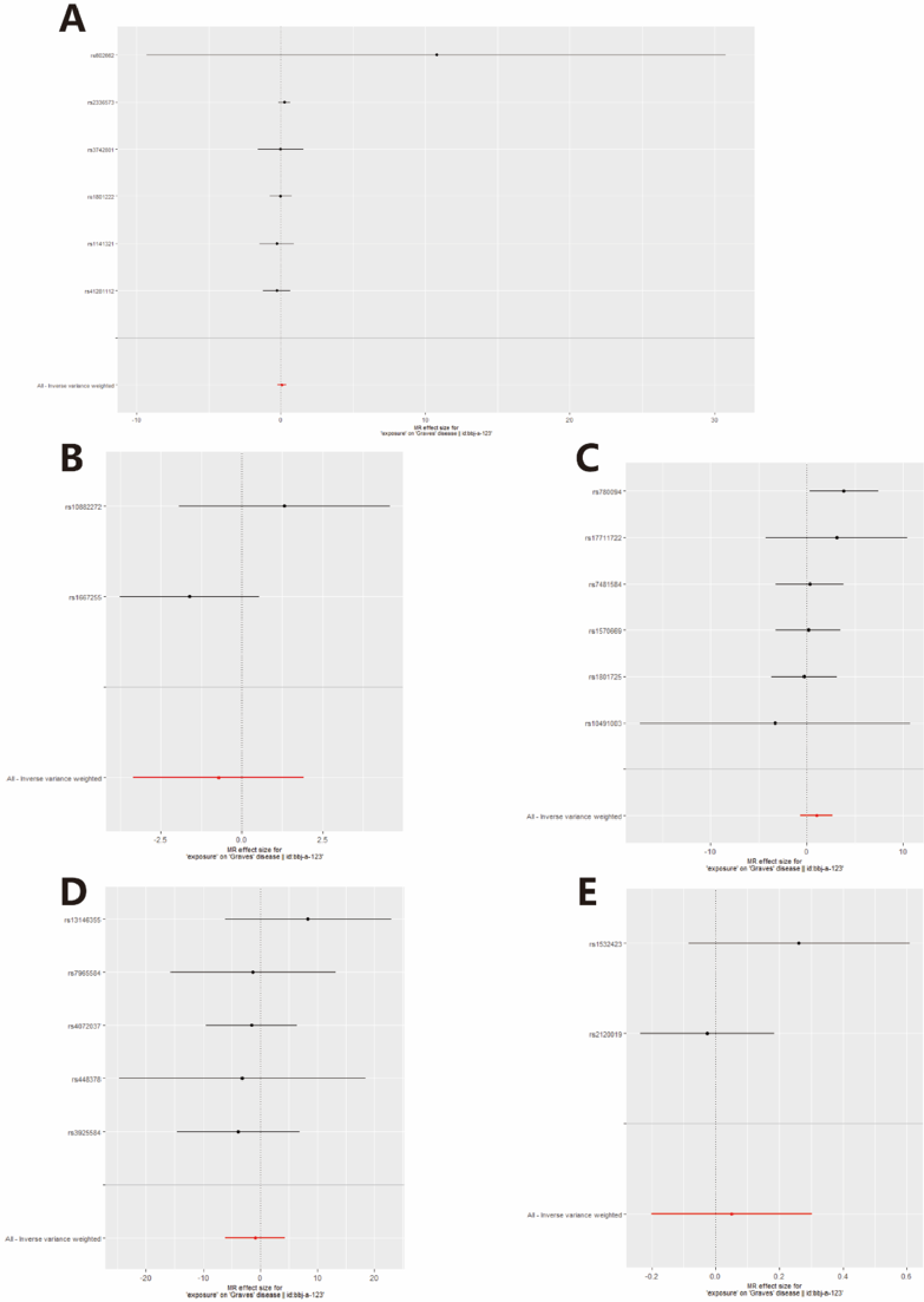
Supplementary Table 4. Prevalence of GD with different concentrations of serum VB-6 levels, NHANES

	Vitamin D deficiency[†] N = 1,028[‡]	Vitamin D insufficiency[†] N = 5,364[‡]	Vitamin D sufficiency[†] N = 2,018[‡]	p value[§]
GD Status				0.0472
No GD	1013 (99%)	5,326 (99%)	2092 (99%)	
GD	51 (1.5%)	38 (0.7%)	16 (0.8%)	

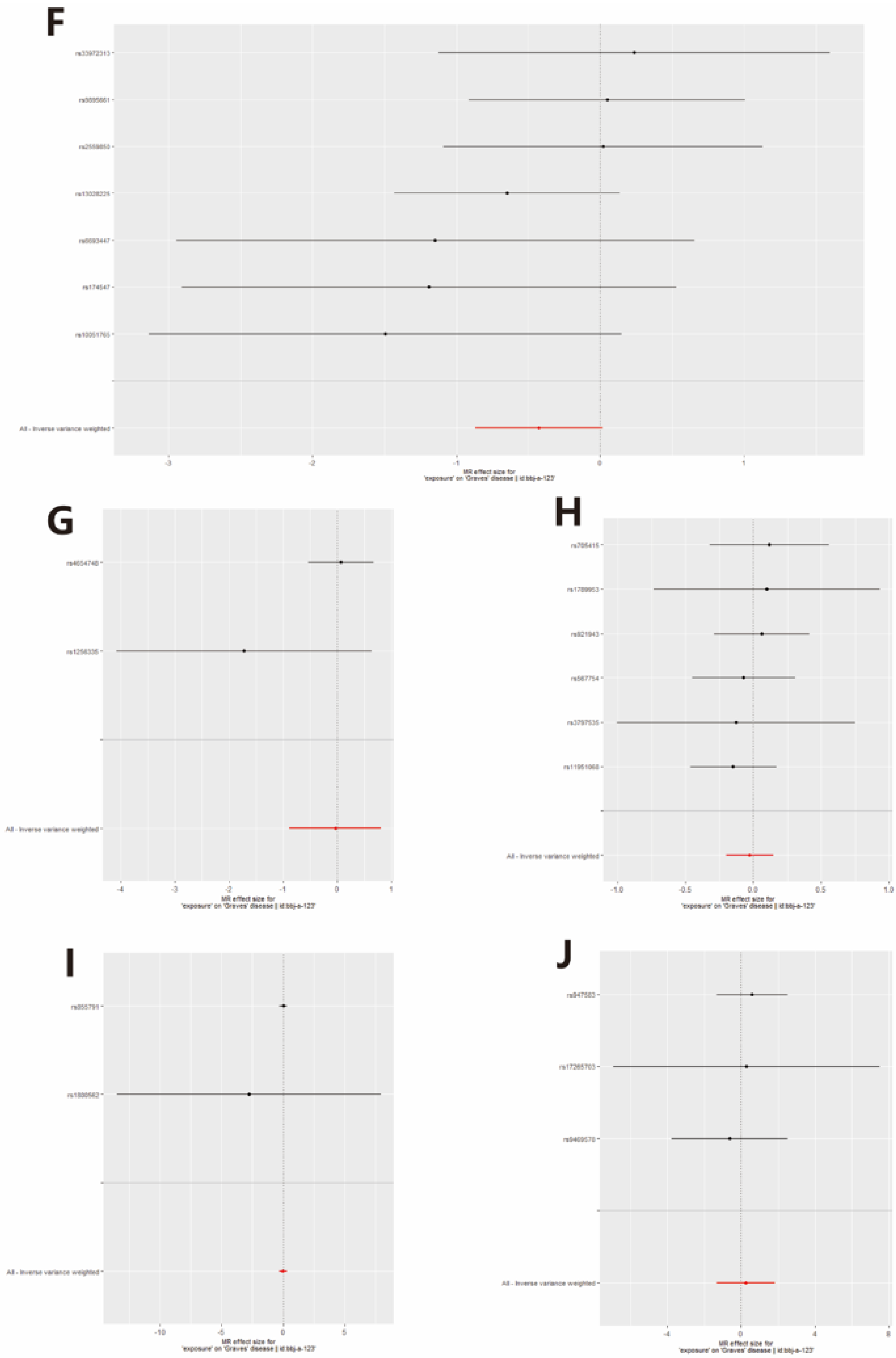
[†]VB-6 dificiency: Serum pyridoxal 5'-phosphate D ≤25nmol/l; Vitamin D insufficiency: 25nmol/L<Serum pyridoxal 5'-phosphate D < 100nmol/l; Vitamin D sufficiency: Serum pyridoxal 5'-phosphate≥100nmo/l
[‡]n (%)
[§]Pearson's Chi-squared test



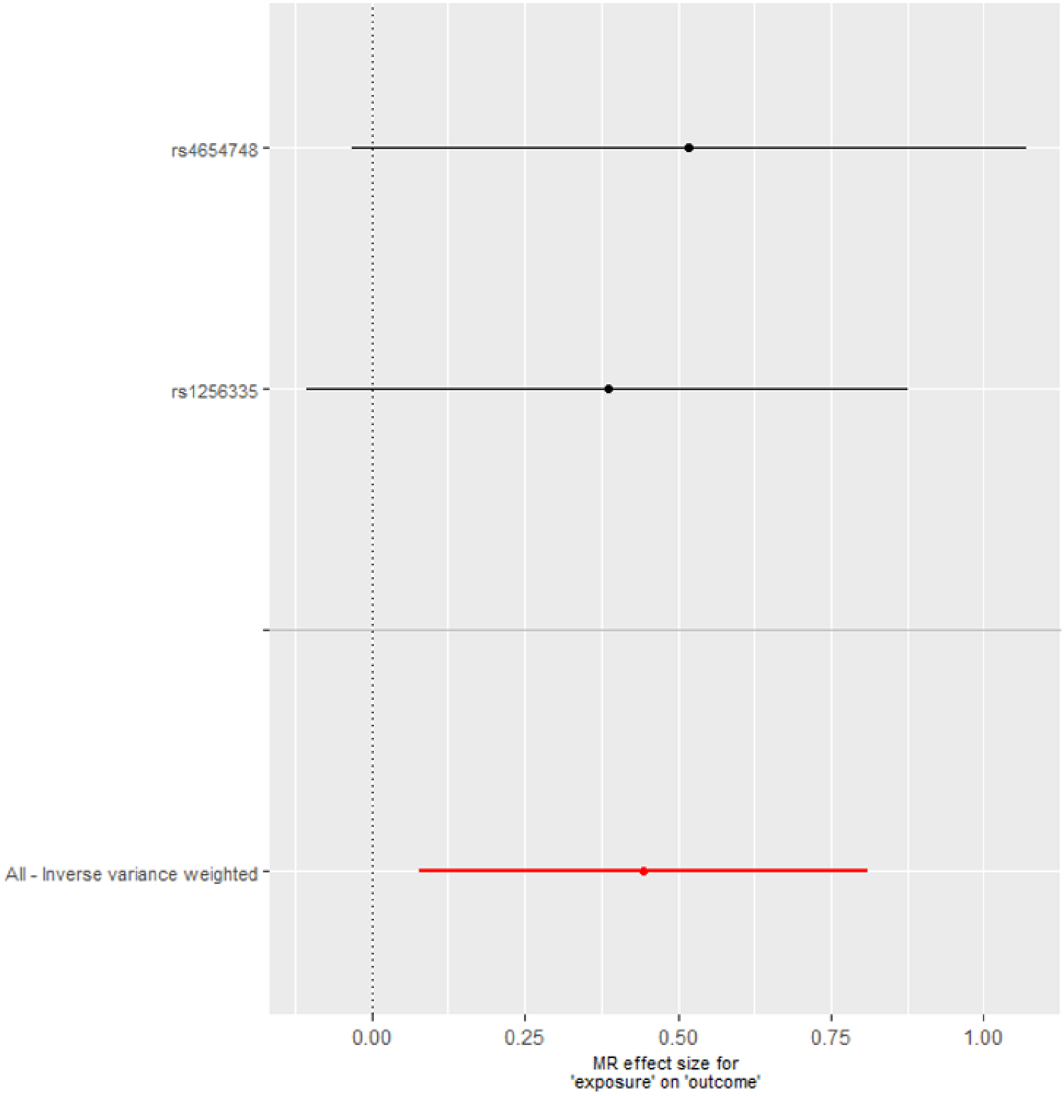
Supplementary Figure 1. Forest plots of the causal effects of vitamin D associated SNPs on GD risk



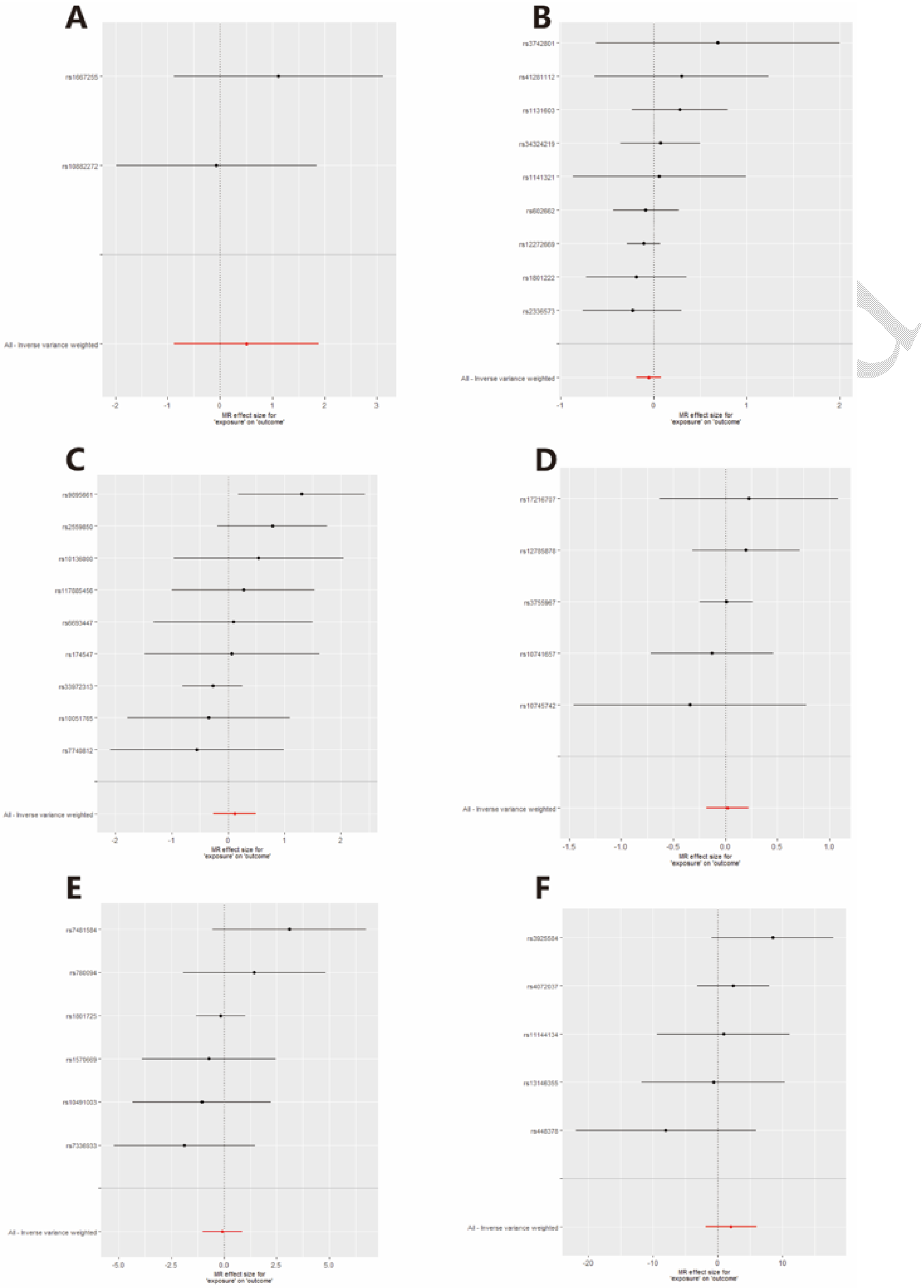
Supplementary Figure 2. Forest plots of the causal effects of micronutrients associated SNPs on GD risk. (A) vitamin A, (B) vitamin B-12, (C) Calcium, (D) Magnesium, (E) Zinc, (F) vitamin B-6, (G) vitamin C, (H) Selenium, (I) Iron, (J) Phosphorus.



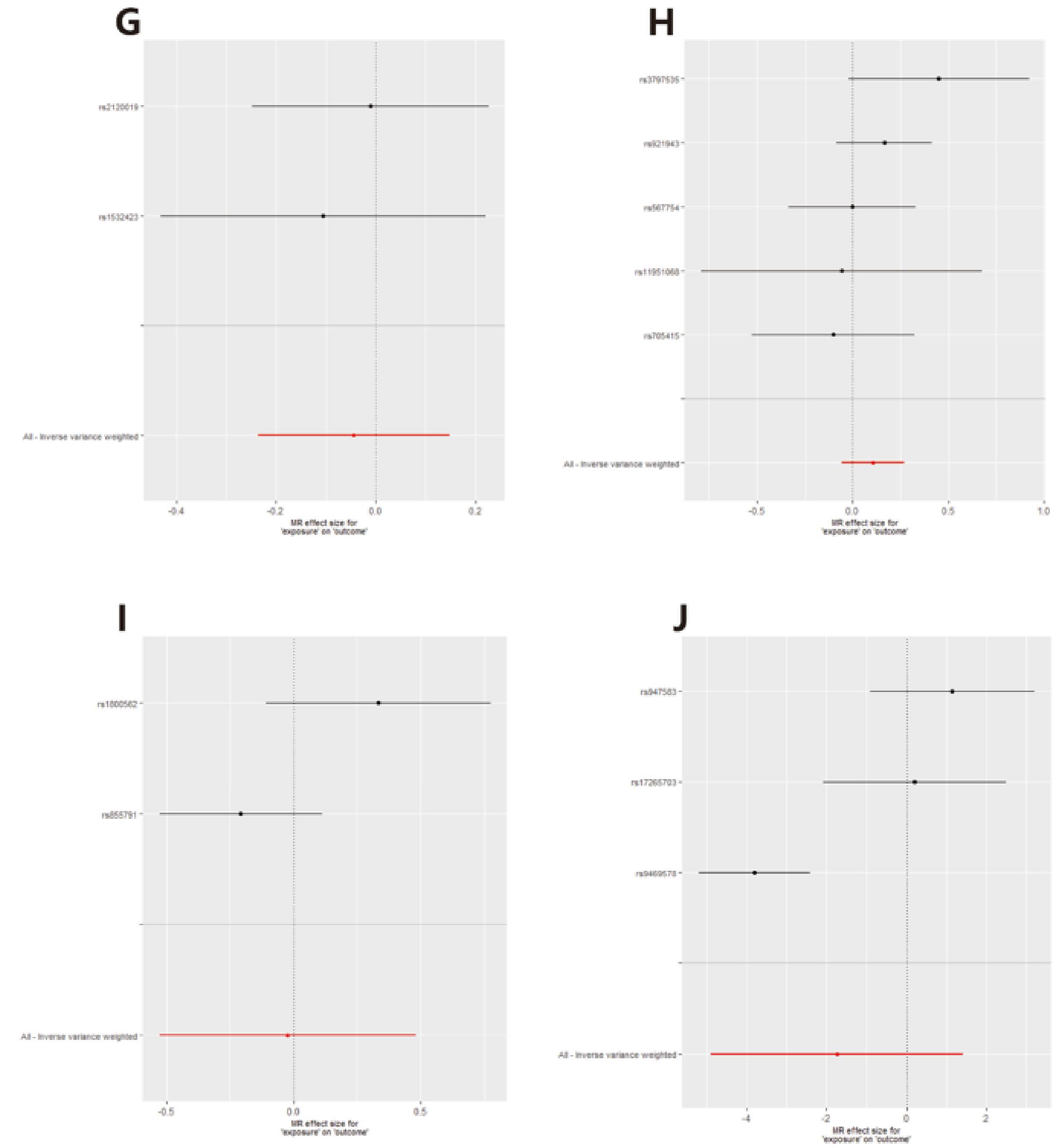
Supplementary Figure 2 (cont.). Forest plots of the causal effects of micronutrients associated SNPs on GD risk. (A) vitamin A, (B) vitamin B-12, (C) Calcium, (D) Magnesium, (E) Zinc, (F) vitamin B-6, (G) vitamin C, (H) Selenium, (I) Iron, (J) Phosphorus.



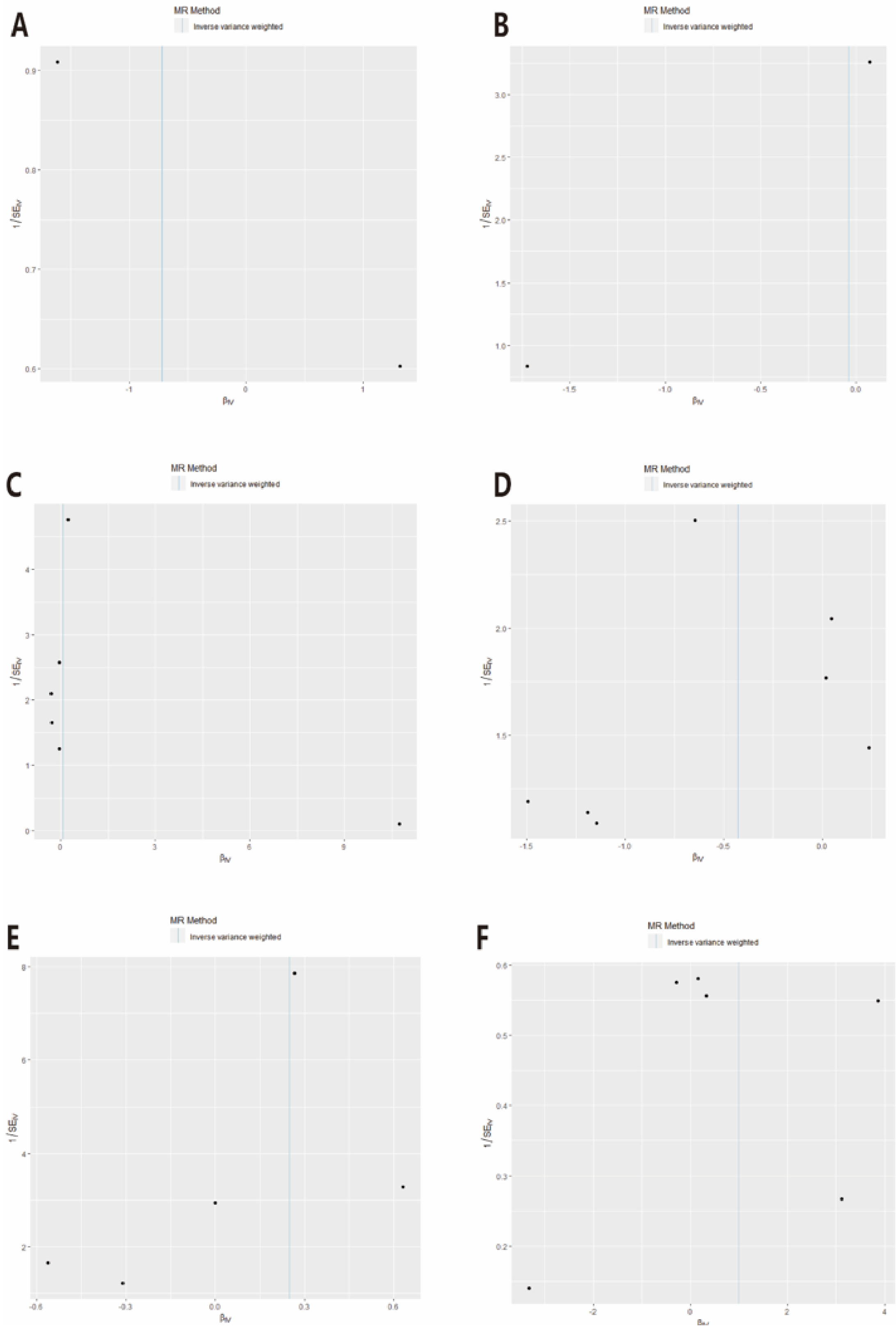
Supplementary Figure 3. Forest plots of the causal effects of vitamin B-6 associated SNPs on GD risk



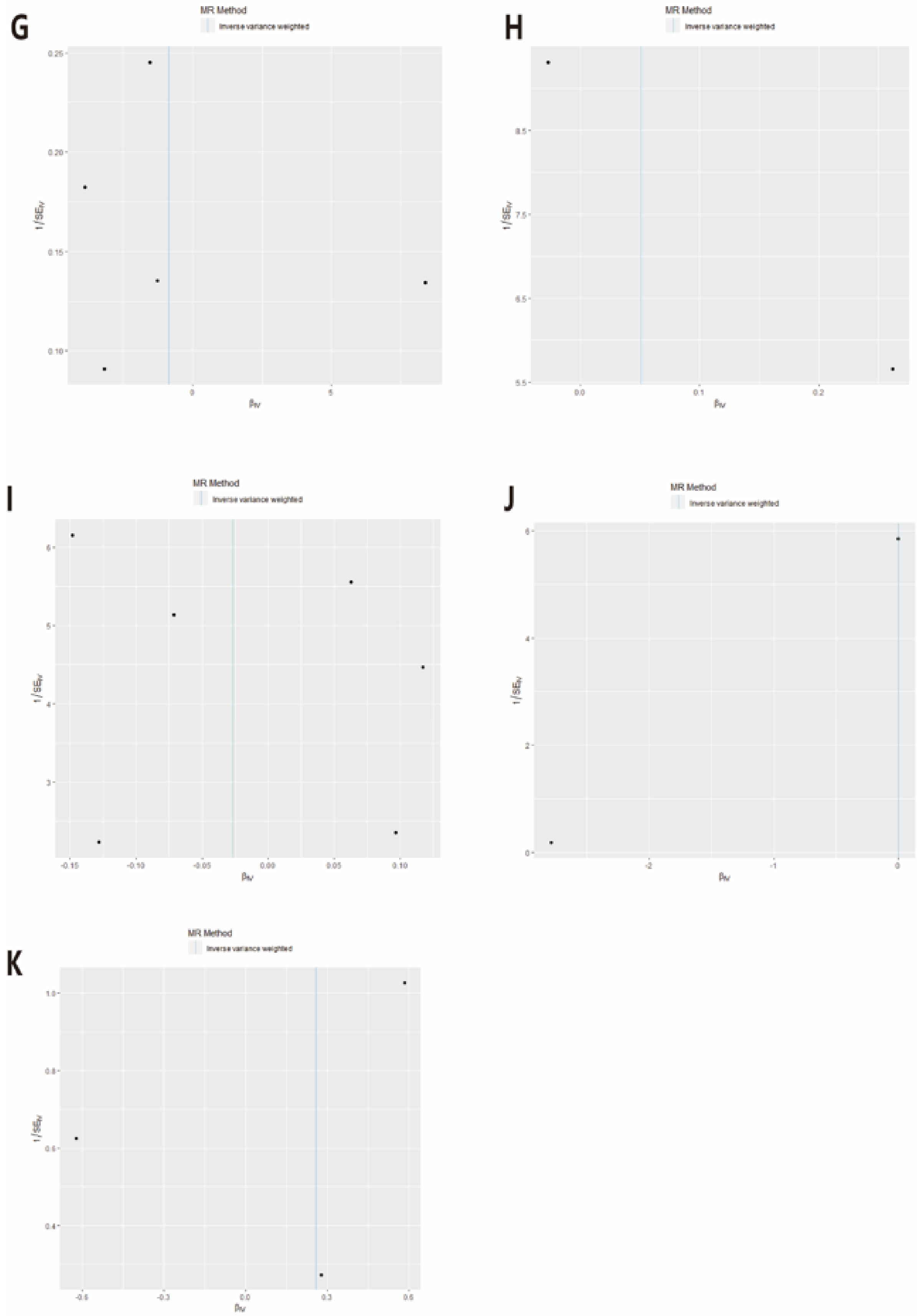
Supplementary Figure 4. Forest plots of the causal effects of micronutrients associated SNPs on GD risk. (A) vitamin A, (B) vitamin B-12, (C) vitamin C, (D) vitamin D, (E) Calcium, (F) Magnesium, (G) Zinc, (H) Selenium, (I) Iron, (J) Phosphorus



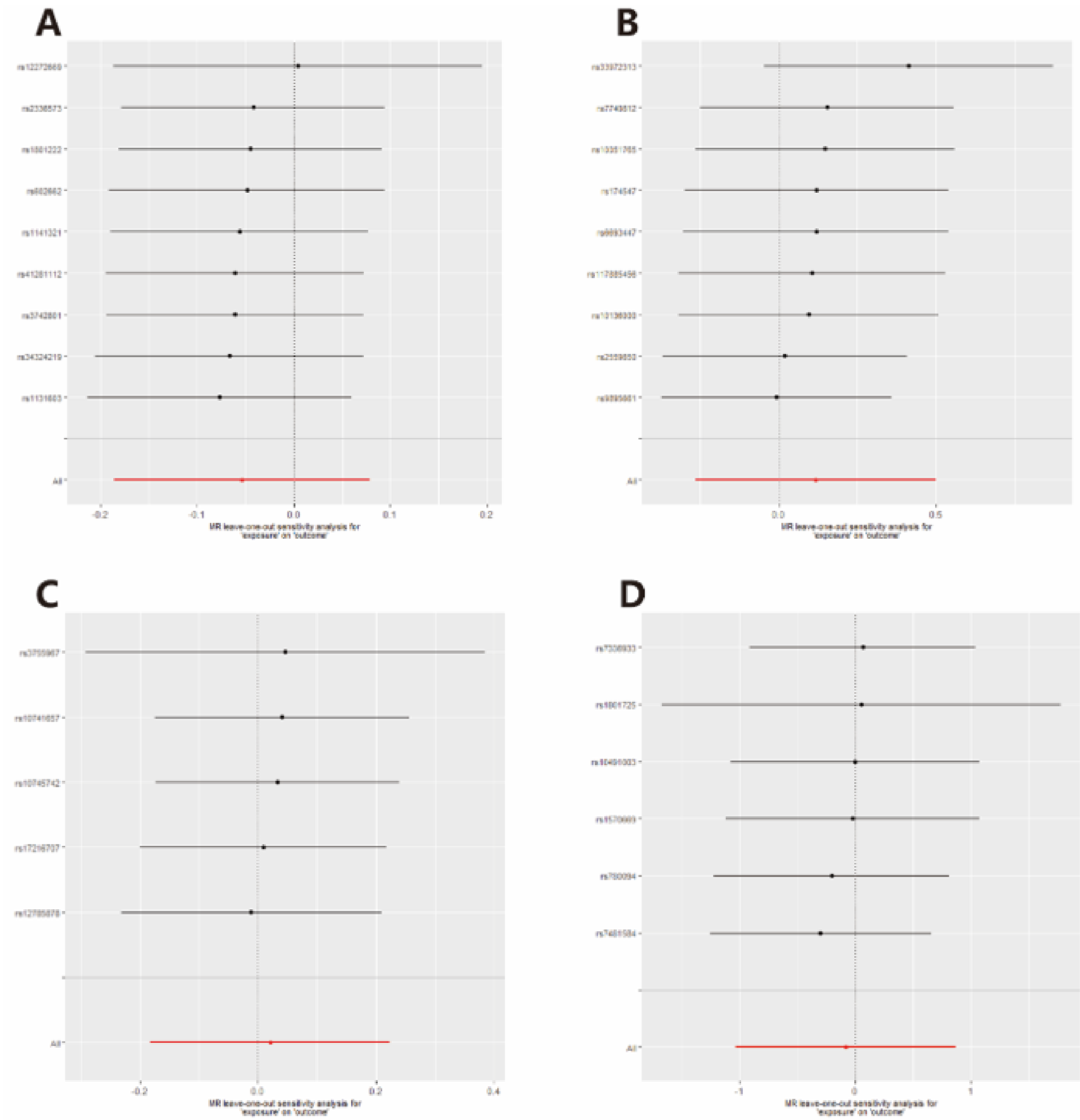
Supplementary Figure 4. (cont.) Forest plots of the causal effects of micronutrients associated SNPs on GD risk. (A) vitamin A, (B) vitamin B-12, (C) vitamin C, (D) vitamin D, (E) Calcium, (F) Magnesium, (G) Zinc, (H) Selenium, (I) Iron, (J) Phosphorus



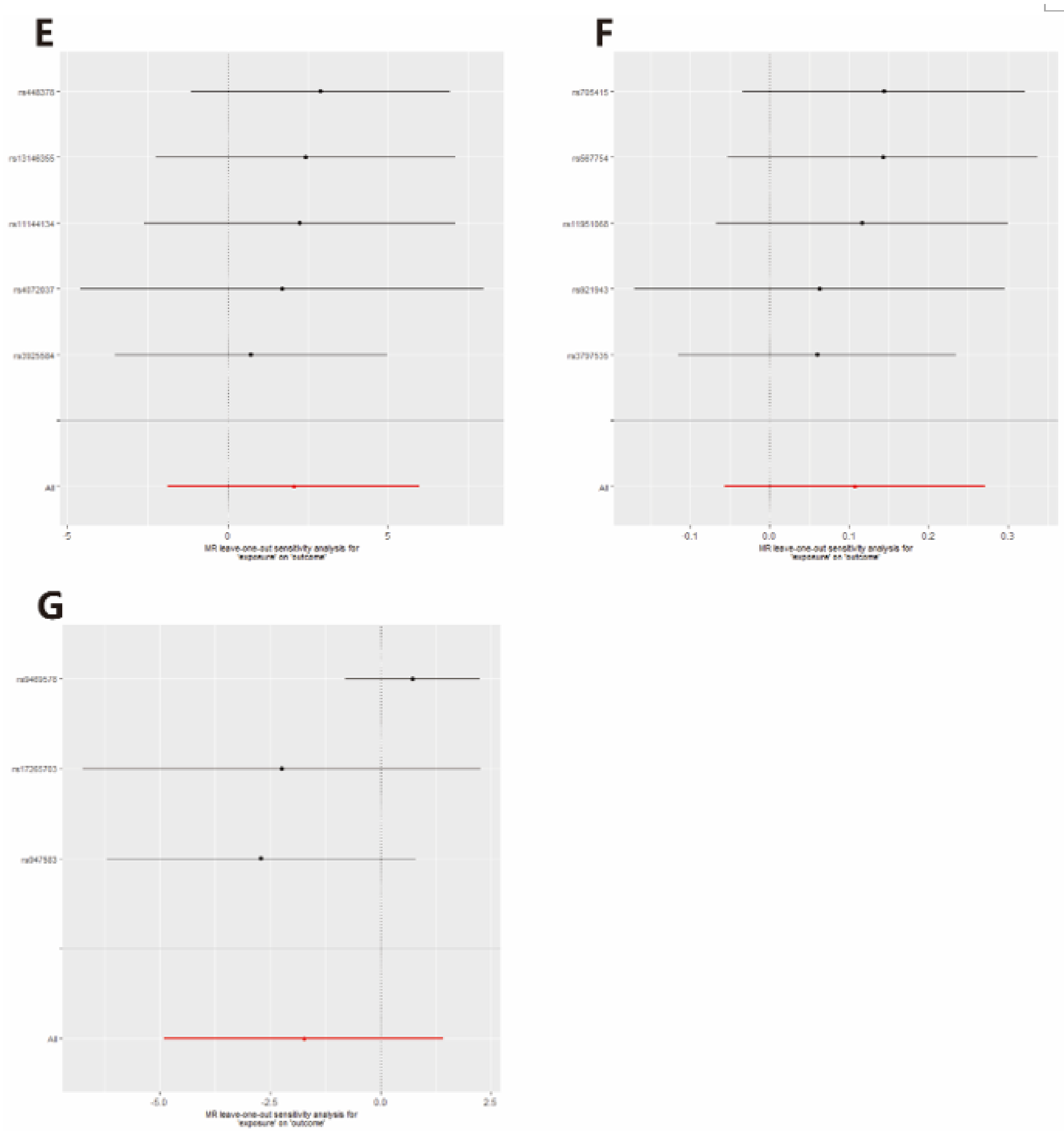
Supplementary Figure 5. Funnel plots of the causal effects of micronutrients related SNPs on GD risk. (A) vitamin A, (B) vitamin B-6, (C) vitamin B-12, (D) vitamin C, (E) vitamin D, (F) Calcium, (G) Magnesium, (H) Zinc, (I) Selenium, (J) Iron, (K) Phosphorus



Supplementary Figure 5. (cont.) Funnel plots of the causal effects of micronutrients related SNPs on GD risk. (A) vitamin A, (B) vitamin B-6, (C) vitamin B-12, (D) vitamin C, (E) vitamin D, (F) Calcium, (G) Magnesium, (H) Zinc, (I) Selenium, (J) Iron, (K) Phosphorus



Supplementary Figure 6. Sensitivity analyses of the causal effects of micronutrients-associated SNPs on GD risk. (A) vitamin B-12, (B) vitamin C, (C) vitamin D, (D) Calcium, (E) Magnesium, (F) Selenium, (G) Phosphorus



Supplementary Figure 6. (cont.) Sensitivity analyses of the causal effects of micronutrients-associated SNPs on GD risk. (A) vitamin B-12, (B) vitamin C, (C) vitamin D, (D) Calcium, (E) Magnesium, (F) Selenium, (G) Phosphorus