Genetic variation in PEAR1, cardiovascular outcomes and effects of aspirin in a healthy elderly population

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ABSTRACT

The platelet endothelial aggregation receptor-1 (*PEAR1*) rs12041331 variant has been identified as a genetic determinant of platelet aggregation in response to antiplatelet therapies, including aspirin. However, association with atherothrombotic cardiovascular events is less clear, with limited evidence from large trials. Here, we tested association of rs12041331 with cardiovascular events and aspirin use in a randomized trial population of healthy older individuals. We undertook post-hoc analysis of N=13,547 participants of the ASPirin in Reducing Events in the Elderly (ASPREE) trial, median age 74 years. Participants had no previous diagnosis of atherothrombotic cardiovascular disease at enrolment, and were randomized to either 100 mg daily low-dose aspirin or placebo for median 4.7 years follow-up. We used Cox proportional hazard regression to model the relationship between rs12041331 and the ASPREE primary cardiovascular disease endpoint (CVD), and composites of major adverse cardiovascular events (MACE) and ischaemic stroke (STROKE); and bleeding events; major hemorrhage (MHEM) and intracranial bleeding (ICB). We performed whole-population analysis using additive and dominant inheritance models, then stratified by treatment group. Interaction effects between genotypes and treatment group were examined. We observed no statistically significant association ($P<0.05$) in the population, or by treatment group, between rs12041331 and cardiovascular or bleeding events in either model. We also found no significant interaction effects between rs12041331-A and treatment group, for CVD (P=0.65), MACE (P=0.32), STROKE (P=0.56), MHEM (P=0.59) or ICB (P=0.56). The genetic variant *PEAR1* rs12041331 is not associated with cardiovascular events in response to low-dose aspirin in a healthy elderly population.

INTRODUCTION

Interindividual variability in response to antiplatelet medications including aspirin can result in inadequate platelet inhibition and subsequent cardiovascular and cerebrovascular events. Clinical and anthropometric factors are known to contribute to variable aspirin efficacy including age, gender, body mass index (BMI), diabetes mellitus (DM), compliance, and certain drug-drug interactions(1-3). In addition, heritability estimates, as assessed by *ex vivo* agonist-stimulated platelet aggregation, suggest that a large proportion of variation in response to aspirin may be attributable to genetic factors(4). Indeed, multiple pharmacogenetic investigations ranging from candidate gene reports to genome-wide association studies have been conducted to identify novel genetic determinants of aspirin response. Identification of genetic polymorphisms that lead to suboptimal platelet control may have important implications in primary and secondary prevention of adverse clinical events in patients with an indication for this medication.

The platelet endothelial aggregation receptor 1 (*PEAR1*) gene encodes a type 1 membrane protein, that is highly expressed in platelets and endothelial cells(5) and critical in haematological processes including megakaryopoiesis, thrombopoiesis, and αIIbβ3-mediated platelet aggregation(6, 7). Genetic variants in the *PEAR1* gene have been associated with platelet aggregation(8, 9) and in response to antiplatelet agents including aspirin(10-12), clopidogrel(13), ticagrelor(14), and prasugrel(15). Most notably, an intronic variant rs12041331 in the *PEAR1* gene is among the strongest genetic modifiers of platelet aggregation, particularly in those treated with aspirin, and results in allele-specific differences in H3K4Me1 methylation leading to differential gene and protein expression(11, 16, 17). Despite the evidence demonstrating the influence of *PEAR1* rs12041331 on agonist-stimulated platelet aggregation in aspirin-treated patients, the impact of this variant on thrombotic and bleeding events remains unclear.

In this investigation, we assessed the interaction between *PEAR1* rs12041331 and randomized aspirin use in 13,547 participants of the Aspirin in Reducing Events in the Elderly (ASPREE) trial. Specifically, individuals with no previous diagnosis of atherothrombotic cardiovascular disease were randomized to either 100 mg daily low-dose aspirin or placebo for median 4.7 years and relationships between rs12041331 genotypes and cardiovascular events, as well as clinically significant bleeding events, were examined. Moreover, gene x environment (i.e. aspirin treatment) interaction models were used to test whether the effect of rs12041331 genotypes on clinical endpoints varied based on aspirin use.

METHODS

Study population

Details of the ASPREE trial are described previously(18, 19). Briefly, ASPREE was a double-blind, randomized (1:1), placebo-controlled trial to examine the effect of daily low-dose aspirin on disability-free survival in healthy older individuals(19-21). At enrolment, the 19,114 participants of the ASPREE trial had no current symptoms or prior history of cardiovascular events, including: myocardial infarction, heart failure, stroke, transient ischemic attack, atrial fibrillation or high blood pressure. Participants also had no dementia diagnosis, physical disability, or illness likely to cause death within 5 years at enrolment. Participants were followed prospectively, monitored for a range of primary and secondary outcomes, including cardiovascular events, dementia, persistent disability and cancer. Participants were enrolled from March 2010 through December 2014, with follow-up data collected until June 2017 (median follow-up of 4.7 years). All investigational protocols were approved by local Institutional Review Boards across Australia and the United States and conformed to the principles outlined in the Declaration of Helsinki. Each participant gave informed consent prior to enrolment.

Outcomes

We analysed the relationship between aspirin use and the common variant *PEAR1* rs12041331, in relation to clinically adjudicated cardiovascular and bleeding events in ASPREE. Endpoints analysed included the primary cardiovascular disease endpoint in the ASPREE study (CVD), and composites of major cardiovascular events considered most likely to be impacted by aspirin; including major adverse cardiovascular events (MACE) and ischaemic stroke (STROKE); and clinically significant bleeding events, including major hemorrhage (MHEM) and intracranial bleeding (ICB). Details of ASPREE endpoints are described previously(21).

Genetic analysis

Ethical approval for genetic analysis was obtained from the Alfred Hospital Human Research Ethics Committee (390/15). Genome-wide SNP genotyping of N=14,177 samples collected by the ASPREE Healthy Ageing Biobank was performed using the Axiom 2.0 Precision Medicine Diversity Research Array (Thermo Fisher Scientific, CA, USA) following standard protocols, at the Ramaciotti Centre for Genomics, University of New South Wales, Australia. Samples were excluded based on poor quality control (QC) metrics or genotyping performance (N=426) and other filters including gender discordance (N=80), and relatedness (N=124). In the final dataset, a total of N=13,547 samples from individuals of predominantly Non-Finish European ancestry were available for genetic analyses. Individual variants

were excluded based on genotyping rate (<95%) and Hardy-Weinberg equilibrium. Imputation was performed on European-ancestry samples using the haplotype reference consortium (HRC) panel on the University of Michigan imputation server(22). Post-imputation QC removed all variants with <0.3 imputation quality scores. The imputation quality score of the variant rs12041331 was R^2=0.99.

Statistical analysis

We use Cox proportional hazards regression models to analyze the relationship between rs12041331-A allele and cardiovascular events, adjusting for variables related to cardiovascular risk, including: age, gender, smoking status, alcohol intake, high density lipoproteins (HDL), low density lipoprotein (LDL)-cholesterol, triglycerides (TG), total cholesterol (TC), hypertension, body mass index (BMI), diabetes mellitus (DM) and statin use prior to enrolment. Analysis of the entire cohort (N=13,547) was performed using an additive model of inheritance (GG vs AG vs AA) and dominant model (GG vs AG/AA), followed by stratified analysis of the placebo (N=6,806) and aspirin (N=6,741) groups, separately. Then the modifying effect of the rs12041331-A genotype on association between incident CVD events and aspirin treatment was analysed using an interaction term in a multivariable Cox regression model using participants from both groups, under both the additive and dominant genetic models. We calculated the minimal detectable effect for all endpoints under both models. All analyses were performed with Stata 15.1 (Stata Corp., Tex., USA) and R (R Core Team, 2014). P-values < 0.05 were considered as statistically significant (two-sided). Figures were produced using the package ggplot2.

RESULTS

The 13,547 genotyped participants had a median age of 73.9 years and were 54.7% female. The distributions of age, sex, plasma lipids and BMI variables were balanced between the groups (Table 1). Genotype distribution for rs12041331 was also balanced between the placebo and aspirin groups, with GA heterozygotes found at 16.3% (N=1,112) and 15.3 % (N=1,034) respectively, and AA homozygotes at 0.9% (N=64) and 0.9% (N=60) respectively (Figure 1, Table 2).

We did not observe evidence of association between *PEAR1* rs12041331 and cardiovascular or bleeding events when stratifying the cohort by treatment effect (Table 3), based on using an additive (Figure 2) or dominant (Figure 3) models of inheritance. In the placebo group, when comparing major allele homozygotes to heterozygotes or minor allele homozygotes using the additive model, no difference in event rate was noted for CVD (P=0.27 and P=0.62, respectively), MACE (P=0.66 and P=0.73, respectively), STROKE (P=0.92 and P=0.95, respectively), MHEM (P=0.57 and P=0.61, respectively) or ICB (P=0.92 and P=0.95, respectively). Hazard ratio [HR] point estimates (Table 3) were generally in the same direction as observed when evaluating the entire cohort (see below) (Table S1).

Similarly, there was no evidence to suggest that *PEAR1* rs12041331 genotype impacted cardiovascular or bleeding event rate in participants treated with aspirin (Table 3). In individuals randomized to aspirin, rs12041331 A-allele carrier status did not result in statistically significant differences in atherothrombotic event rates (CVD, MACE and STROKE) or bleeding events (MHEM and ICB) regardless if individuals carried 1 (CVD P=0.14, MACE P=0.11, STROKE P=0.51, MHEM P=0.92, and ICB P=0.51) or 2 (CVD P=0.78, MACE P=0.85, STROKE P=0.76, MHEM P=N/A, and ICB P=0.76) copies of the A-allele (Table 3). We repeated these analyses using a dominant model of inheritance (wild-type GG vs all AA/AG carriers) and also found no significant associations (Table S2).

In the combined cohort (both aspirin- and placebo-treated groups), no association was observed between rs12041331 A-allele carrier status and atherothrombotic events for either the additive (Table S1) or dominant (Table S2) genetic models. Specifically, no statistical difference in event rates for CVD, MACE and STROKE were seen when comparing *PEAR1* rs12041331 major allele homozygotes to either heterozygotes (HR=0.81; 95% confidence interval [CI] 0.64 to 1.02; P=0.07, HR=0.84, 95% CI 0.64 to 1.09, P=0.19, and HR=0.94, 95% CI 0.64 to 1.38, P=0.74, respectively) or minor allele homozygotes (HR=1.10; 95% CI 0.50 to 2.44; P=0.50, HR=1.22, 95% CI 0.51 to 2.90, P=0.65, and HR=1.13, 95% CI 0.28 to 4.53, P=0.86, respectively). Similarly, no correlation between genotype and MHEM or ICB was observed when comparing rs12041331 major allele homozygotes to either heterozygotes (HR=0.96; 95% CI 0.73 to 1.26; P=0.74 and HR=0.94, 95% CI 0.64 to 1.38, P=0.74, respectively) or minor allele

homozygotes (HR=0.27; 95% CI 0.04 to 1.93; P=0.19 and HR=1.13, 95% CI 0.28 to 4.53, P=0.28, respectively).

Using a multivariable Cox regression model, adjusting for age, gender, smoking status, alcohol intake, HDL, LDL-cholesterol, TG, TC, hypertension, BMI, diabetes mellitus and statin use prior to enrolment, we found no evidence of an interaction effect between rs12041331 genotypes and aspirin treatment under the additive or dominant inheritance model, for incident CVD, MACE, ischemic stroke, major haemorrhage and intracranial bleeding (Table 4, Tables S3-8). For calculations of minimal detectable effects, under both models for all endpoints, see Tables S9-10.

DISCUSSION

There is considerable evidence that the *PEAR1* receptor is a critical part of platelet aggregation in response to several agonists, and that rs12041331 is a strong genetic determinant of platelet aggregation(5, 6, 12, 23, 24). However, there is less data regarding the impact of this variant on cardiovascular event risk, in untreated individuals or in patients prescribed antiplatelet medication. In the current study, we sought to evaluate the impact of *PEAR1* SNP rs12041331, a well-described genetic variant implicated in aspirin-related platelet function, on MACE and clinically significant bleeding events in a large healthy cohort of elderly individuals randomized to either aspirin or placebo in the ASPREE trial. In analyses of the entire cohort or when stratifying by treatment effect (i.e. aspirin vs. placebo), using either an additive or dominant model of inheritance, we observed no evidence of association for rs12041331 with increased risk of experiencing a thrombotic or bleeding event. Furthermore, we observed no significant interaction effects between *PEAR1* genotype, aspirin use and clinical outcomes using either an additive or dominant model.

Since its identification in 2005, multiple investigations have been published highlighting the role of *PEAR1* in several important biological processes(5). The most extensively studied of these processes has been platelet aggregation; however, other proposed functions of this receptor include maintenance of endothelial cell function(13, 25), megakaryopoiesis and thrombopoiesis(7), neoangiogenesis(26), leukocyte function(17), and neuronal phagocytosis(27, 28). These and other publications have prompted numerous genetic studies attempting to correlate *PEAR1* polymorphisms with related phenotypes and diseases. From these investigations, rs12041331 has been among the most thoroughly studied, with the strongest effect on platelet-related phenotypes, and established functional effect on *PEAR1* expression. Previously, rs12041331 A-allele carriers have been shown to have significantly lower DNA methylation in a CpG-island which contains binding sites for the methylation-sensitive transcription factor CTCF compared to GG homozygotes, presumably leading to differences in gene and protein expression observed by others at this locus(11, 16).

Given the effect of rs12041331 on *PEAR1* expression and platelet-related processes, recent investigations have focused on the impact of this polymorphism on cardiovascular-related diseases with mixed results. Initial reports in percutaneous coronary intervention patients treated with aspirin and clopidogrel showed that A-allele carriers of this variant experienced MACE (HR=2.62; 95% CI 0.96 - 7.10; P=0.06) and cardiovascular-related death (HR=3.97; 95% CI 1.10-14.31; P=0.04) more frequently compared to GG homozygotes (29). However, these results were not confirmed in a Flemish population, which reported no association with several different cardiovascular and cerebrovascular phenotypes when using rs12566888 as a proxy SNP for rs12041331 (R^2 = 0.99; D' = 1.00 with each other)(30). These latter

findings were consistent with a subsequent publication showing no association between *PEAR1* rs12041331 and ischemic events in Chinese patients with an acute coronary syndrome treated with aspirin and clopidogrel (HR=1.30, 95% CI 0.74 - 2.29, P=0.07), although it should be noted that statistical power was likely quite low in this study given a limited sample size of 196 patients(31).

Most recently, Xu *et al*. prospectively assessed rs12041331 in over 2,400 Chinese patients with acute coronary syndrome or stable coronary artery disease undergoing stent implantation and treated with aspirin and clopidogrel(32). Based on *PEAR1* genotype, they observed significantly different ADP-induced platelet aggregation (29.1, 25.4, and 22.9 for genotypes GG, GA, and AA, respectively, P=0.0005) and detected differences in the 30-day incidence of a composite cardiovascular phenotype consisting of cardiovascular death, nonfatal myocardial infarction, and ischemic stroke (HR=2.78, 95% CI 1.13 - 6.82, P=0.03). Positive associations with deep vein thrombosis in those with sticky platelet syndrome as well as coronary artery aneurysm in individuals with Kawasaki disease have also been shown by *PEAR1* rs12041331 genotype(33, 34).

Further studies have suggested a genotype x aspirin interaction exists whereby aspirin-treated carriers of rs12041331 minor (A) allele have enhanced risk of experiencing a clinical event compared to carriers of the same allele who are not treated with aspirin(29). Specifically, it was observed in stable coronary artery disease patients of European descent that rs12041331 A-allele carriers treated with aspirin have significantly increased risk of myocardial infarction (odds ratio [OR] = 2.08, 95% CI 1.01 - 4.09; P=0.048) compared to those who were not exposed to aspirin (OR = 0.25, 95% CI 0.03-2.03, P=0.19). Similar trends were observed using a composite cardiovascular outcome consisting of death, non-fatal stroke, and non-fatal myocardial infarction (OR = 1.62, 95% CI 0.91-2.90, P=0.10 and OR = 0.54, 95% CI 0.22 - 1.31, P=0.16, respectively)(29). Evidence also suggests that the *PEAR1* receptor may be able to exert cardiovascular-related effects through processes that are independent of platelet function (e.g. endothelial-related effects). Investigation of other molecular processes that are regulated by PEAR1 would likely allow for more targeted investigation of effects that may modify cardiovascular risk.

It is noteworthy that all previous studies discussed above have been undertaken in populations known to be affected by cardiovascular disease. The present study, however, has been undertaken in a highly selected population of healthy older individuals with no previous diagnosis of atherothrombotic cardiovascular disease, and in the context of primary prevention. Our study results, therefore, must be interpreted accordingly.

In our investigation, which is the largest study of *PEAR1* rs12041331 and cardiovascular outcomes conducted to date, we did not observe significant association between rs12041331 and cardiovascular

events or clinically significant bleeding in the entire cohort, regardless of aspirin use (Table 3). We also found no significant effects in interaction analyses using either the additive (Table 4, Tables S3-7) or dominant (Table S8) genetic model. Given that the minor allele of this variant resulted in lower agonist-stimulated platelet aggregation in prior investigations, we also assessed whether aspirin-dependent effects could modify the relationship between *PEAR1* genotype and clinically significant bleeding events. Consistent with our analyses pertaining to thrombotic outcomes, no association was observed between rs12041331 and bleeding in both placebo- or aspirin-randomized individuals and no statistically significant interaction was observed between aspirin treatment and genotype on occurrence of bleeding events.

While our data suggests that this polymorphism is not an important contributor to clinical events in a healthy older population, in the setting of primary prevention, additional studies are warranted, investigating the effect of rs12041331 on MACE in other populations, and including the general population, and patients with higher cardiovascular burden, such as those following a coronary intervention procedure, and/or on a dual antiplatelet therapy regimen.

Strengths of our study include the sample size, being the largest evaluation of *PEAR1* rs12041331 conducted to date, with regards to cardiovascular outcomes and aspirin use. To our knowledge, it is also the only study to examine the effect of rs12041331 on clinically significant bleeding events. In addition, the prospective, randomized nature of the study design implemented in the ASPREE trial allowed us to confidently assess the interaction between *PEAR1* genotype and aspirin use on adverse events in cohorts that were well-matched for clinical and anthropometric characteristics known to influence cardiovascular and bleeding risk(18). Central adjudication of cardiovascular and bleeding events allowed for blinded evaluation of suspected outcomes and ultimately allowed us to maximize data consistency while minimizing potential biases.

Limitations of our study include the highly selected nature of the ASPREE population, and the predominance of European ancestry, which limit the generalizability of our results to other populations. Participants enrolled into the ASPREE trial were elderly, generally healthy individuals with no known atherothrombotic cardiovascular disease and were randomized to aspirin in the context of primary prevention. Therefore, our study results may not be indicative of data obtained in patients with more severe cardiovascular burden and/or in the context of secondary prevention. Furthermore, ethnicity-specific analyses were not performed in the current investigation. While prior investigations in European-, African- and Asian-derived populations have shown consistent effects of *PEAR1* rs12041331 genotype with regards to platelet aggregation, the vast majority of ASPREE participants were Caucasian (~93%). Therefore, caution should be used when extrapolating these results to populations of other ethnic/racial

origins especially given that the minor allele frequency of rs12041331 varies significantly among such groups. Our study had very few events in the AA homozygote group, which limited the interpretation of results from the additive genetic model used in the primary analysis. To account for this, we conducted analysis using a dominant model, and found similar results. Finally, it is important to note that participants were randomized to low dose aspirin in this study (i.e. 100 mg/daily). If a true interaction between *PEAR1* genotype and aspirin use exists, it is possible that such an interaction may be dependent on aspirin dose.

It is also noteworthy that low-dose aspirin did not significantly decrease CVD, MACE, or stroke events versus placebo in the overall ASPREE trial (21). The lack of an aspirin treatment effect in the ASPREE healthy elderly population must be considered when interpreting of the lack of associations found in genetic analysis of *PEAR1* rs12041331 in this cohort. It is possible that the selection bias that occurred as a result of the strict ASPREE enrolment criteria, excluding any individuals with a history of diagnosed cardiovascular events, has resulted in ascertainment of a unique population where genetic effects are diminished or attenuated. This is indeed consistent with others studies that have shown a diminished effect of genetic risk factors as a function of age in other contexts (35, 36).

Based on our calculations of minimal detectable differences between rs12041331 genotypes and the endpoints analysed in the ASPREE trial (Tables S9-10), it is possible that larger study populations or meta-analyses may uncover significant effects of *PEAR1* and aspirin use on cardiovascular or bleeding events. However, it is also possible that in such larger study populations, investigations may find the effect of rs12041331 moving closer towards zero. Further post-hoc analysis of other large aspirin trials is therefore warranted, as new datasets from other large trials become available.

In conclusion, we could not replicate previous findings suggesting that *PEAR1* rs12041331 was an important genetic determinant of cardiovascular outcomes. Furthermore, no effects of this variant on clinically significant bleeding was observed in the ASPREE study. While differences in study designs and participant populations may, in part, explain these divergent results, our data do provide robust evidence that genetic variation in *PEAR1* do not contribute to cardiovascular disease risk in relatively healthy older individuals regardless of aspirin treatment. Additional well-powered investigations in patients with high-risk clinical indications would likely be helpful to determine the role, if any, *PEAR1* has on the occurrence of adverse clinical events.

STUDY HIGHLIGHTS:

o What is the current knowledge on the topic?

Identification of genetic factors that influence aspirin response may facilitate more personalized antiplatelet treatment, and targeted prevention of cardiovascular disease. Yet identification of pharmacogenomic variants of high clinical effect for aspirin has been challenging. Previous studies suggest that genetic variation in the platelet endothelial aggregation receptor 1 (PEAR1) gene results in altered aspirin efficacy. However, association of PEAR1 genotypes with cardiovascular events is less clear.

o What question did this study address?

Our study, the largest study of *PEAR1* and cardiovascular outcomes to date, examined the effect of rs12041331 genotypes and aspirin use on cardiovascular and bleeding events in a population of 13,547 healthy older individuals without a history of atherothrombotic cardiovascular disease enrolled in the ASPirin in Reducing Events in the Elderly (ASPREE) trial.

o What does this study add to our knowledge?

We observed no association between genotypes and these outcomes, and no interaction with aspirin use in the ASPREE population. Our study indicates *PEAR1* genetic variation does not influence aspirin primary prevention in healthy older individuals without a history of cardiovascular events.

o How might this change clinical pharmacology or translational science?

Our study represents an important finding that contributes to the evolving pharmacogenomics evidence-base for aspirin, one of the world's most widely used drugs.

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AUTHOR CONTRIBUTIONS

P.L and J.P.L wrote the manuscript. J.P.L, A.R.S, J.J.M and P.L made substantial contributions to the conception and design of the work. M.R, S.X, G.P, R.W, M.N, A.M.T, C.M.R and A.M.M made substantial contributions to the acquisition, analysis, or interpretation of data, and drafting and revising of the manuscript. J.J.M, A.M.M and P.L are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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FIGURE LEGENDS

Figure 1. Schematic Overview (Graphical Abstract). We examined the relationship between rs12041331 genotypes and cardiovascular and bleeding events in a randomised placebo-controlled study population of N=13,547 participants of the ASPirin in Reducing Events in the Elderly (ASPREE) trial. Participants had no previous diagnosis of atherothrombotic cardiovascular disease at enrolment, and were randomized to either 100 mg daily low-dose aspirin or placebo for a median 4.7 years of follow-up. Endpoints analysed included the primary cardiovascular disease endpoint in the ASPREE study (CVD), and composites of major adverse cardiovascular events (MACE) and ischaemic stroke; and bleeding events including major hemorrhage and intracranial bleeding. Details of ASPREE endpoints are described previously (21). The modifying effect of the rs12041331-A genotype on association with cardiovascular and bleeding events and aspirin treatment was analysed using an interaction term in a multivariable Cox regression model.

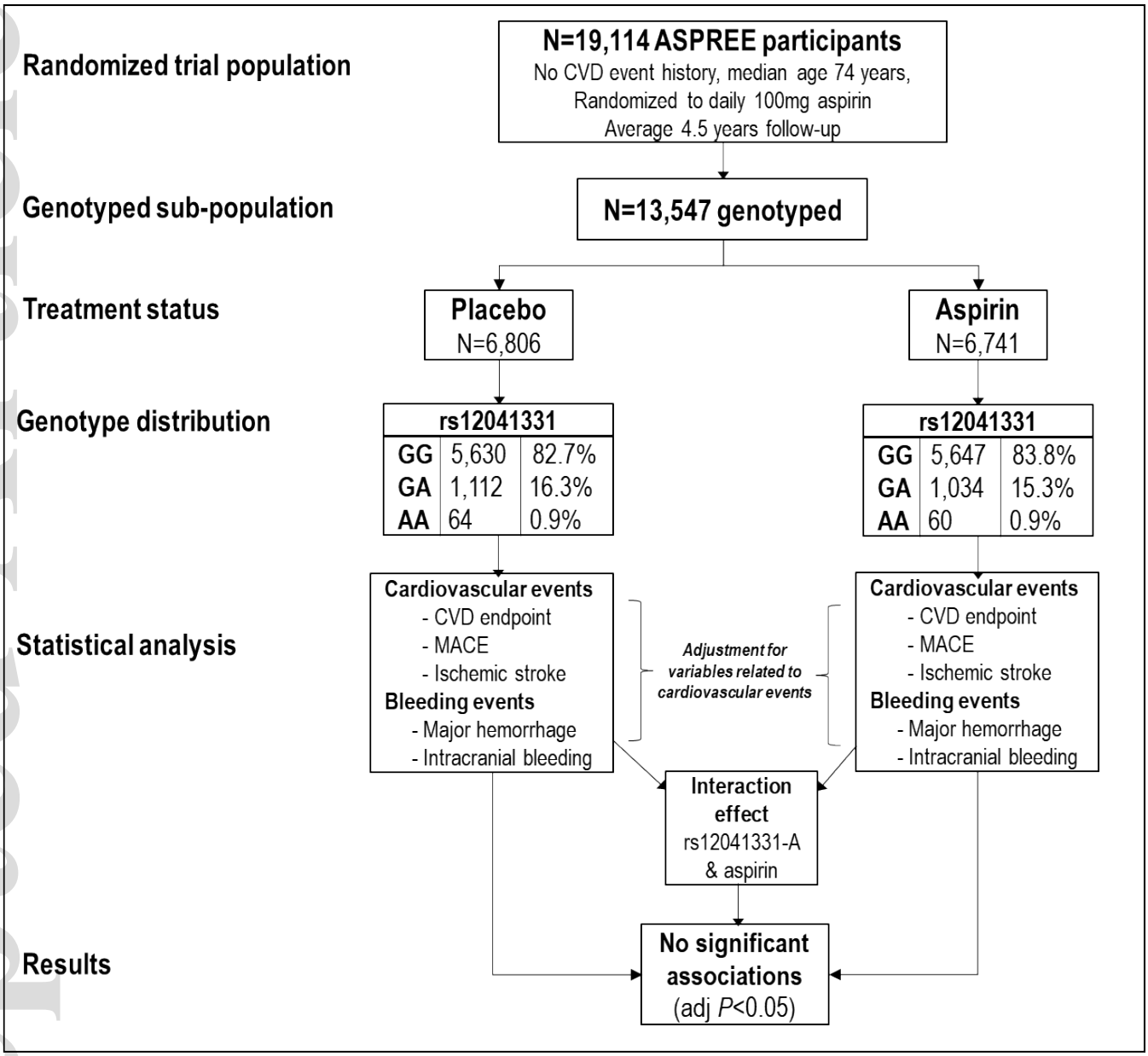

Figure 2: Cumulative incidence curves (additive genetic model). We undertook cumulative incidence analysis of cardiovascular and bleeding events in aspirin and placebo groups, stratified by rs12041331 genotypes using an additive model of genetic inheritance; GG wild type (blue curves), AG heterozygotes (red curves), and AA homozygotes (yellow curves). Events analysed included the primary cardiovascular disease endpoint in the ASPREE study (CVD) (**a-b**), and composites of major adverse cardiovascular events (MACE) (**c-d**) and ischaemic stroke (STROKE) (**e-f**); and the ASPREE clinically significant bleeding endpoint sub-compo[illegible] **-h**) and intracranial bleeding (ICB) (**i-j**).

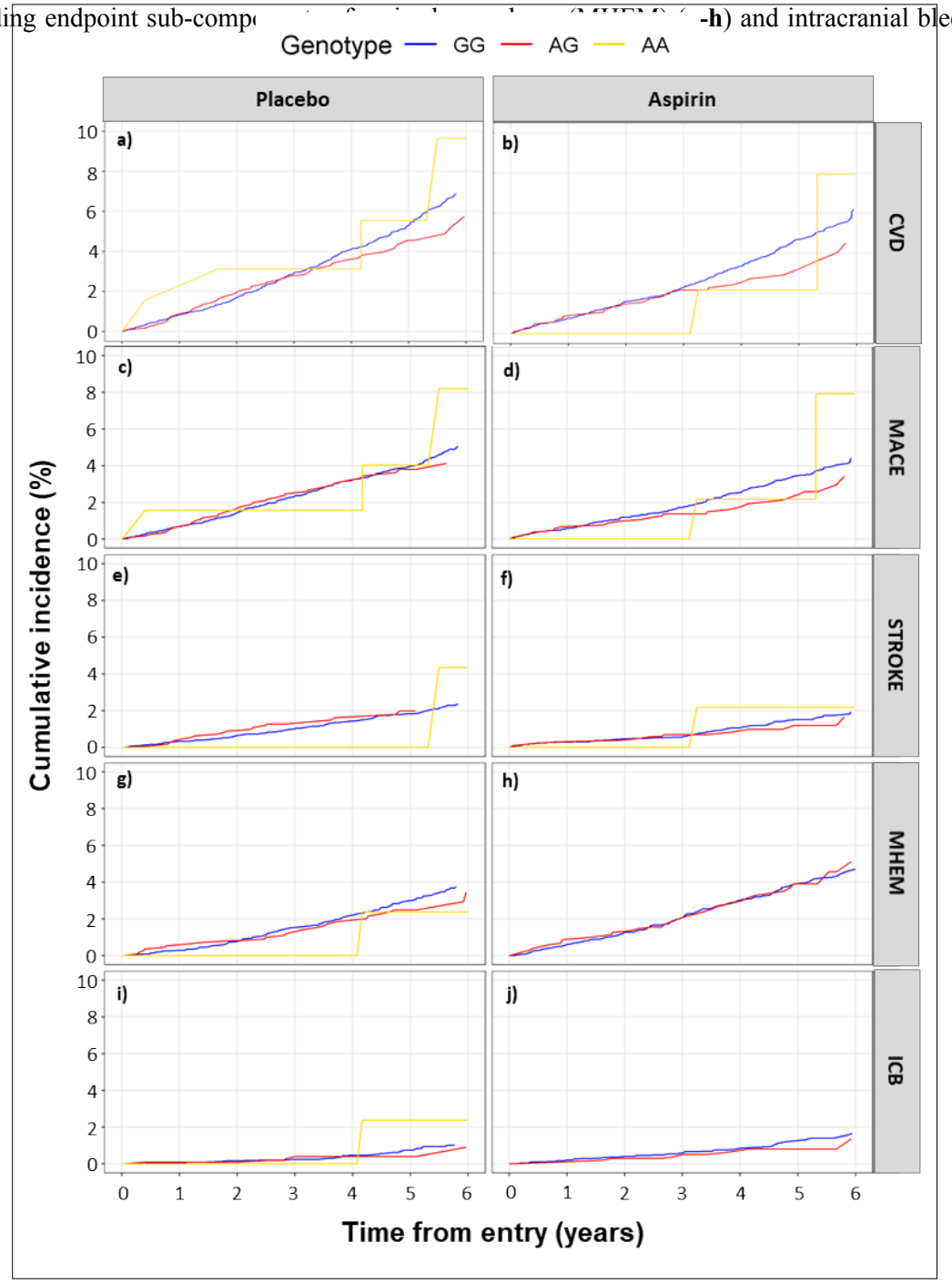

Figure 3: Cumulative incidence curves (dominant genetic model). We undertook cumulative incidence analysis of cardiovascular and bleeding events in aspirin and placebo groups, stratified by rs12041331 genotypes using a dominant model of genetic inheritance; GG wild type (blue curves) versus AG/AA carriers (red curves). Events analysed included the primary cardiovascular disease endpoint in the ASPREE study (CVD) (**a-b**), and composites of major adverse cardiovascular events (MACE) (**c-d**) and ischaemic stroke (STROKE) (**e-f**); and the ASPREE clinically significant bleeding endpoint sub-components of major hemorrhage (MHEM) (**g-h**) and intracranial bleeding (ICB) (**i-j**).

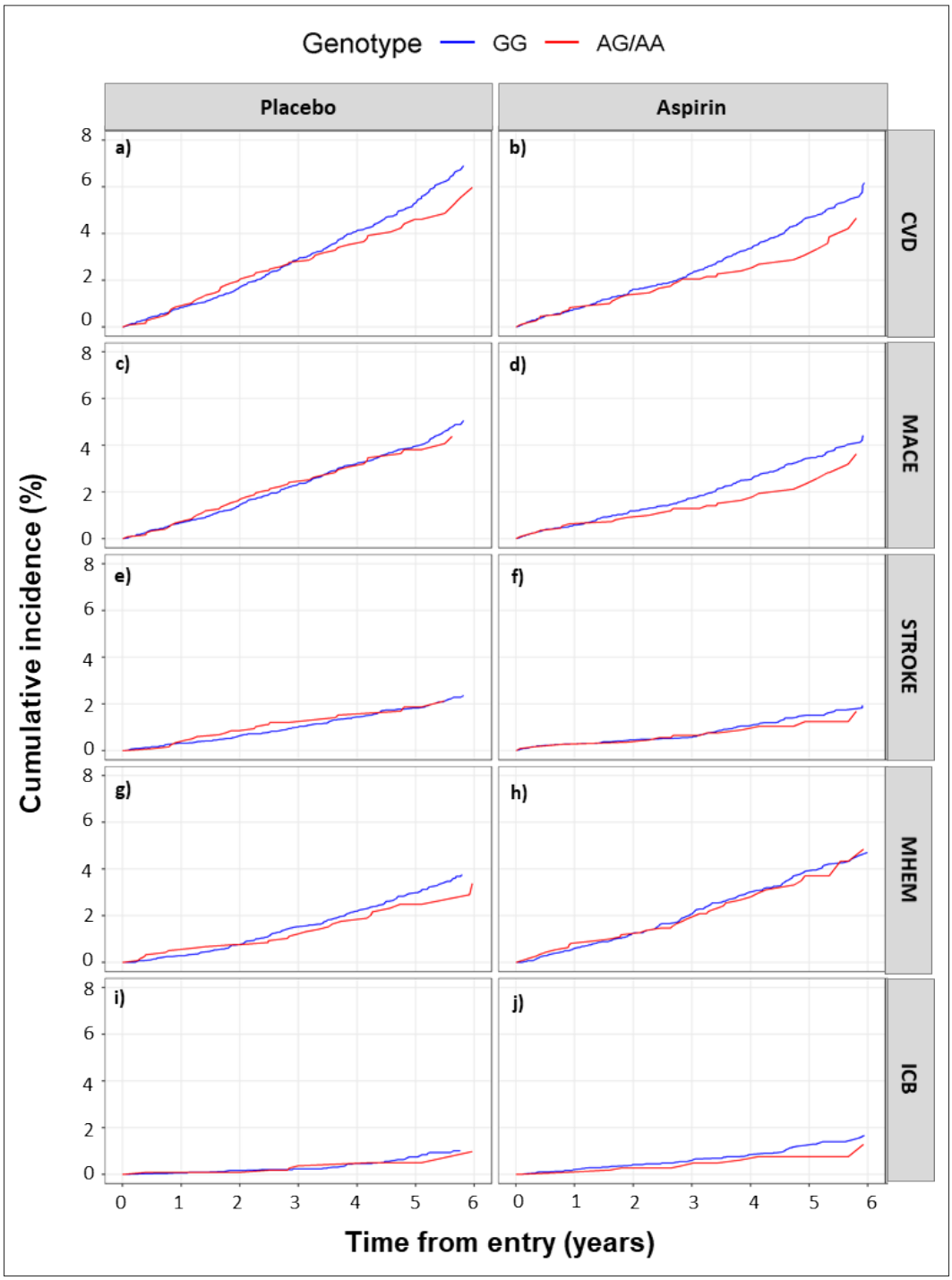

Table 1: Characteristics of Genotyped Participants by Treatment Status. Data summaries are median (IQR) for continuous measures, and n (%) for categorical measures. *Self-report

	Placebo N=6,806	**Aspirin N=6,741**	**Total N=13,547**
Age at randomization (yrs)	73.9 (71.7, 77.3)	73.9 (71.7, 77.3)	73.9 (71.7, 77.3)
65-73.9 yr	3,485 (51.2%)	3,446 (51.1%)	6,931 (51.2%)
≥74 yr	3,321 (48.8%)	3,295 (48.9%)	6,616 (48.8%)
Gender			
Female	3,716 (54.6%)	3,691 (54.8%)	7,407 (54.7%)
Male	3,090 (45.4%)	3,050 (45.2%)	6,140 (45.3%)
Blood lipid levels (mg/DL at baseline)			
LDL-C	119.9 (96.7, 140.0)	116.0 (96.0, 139.2)	116.0 (96.7 to 139.2)
HDL-C	58.0 (50.3, 69.6)	58.0 (50.0, 69.6)	58.0 (50.3 to 69.6)
TG	106.3 (79.7, 141.7)	106.3 (79.7, 141.7)	106.3 (79.7 to 141.7)
TC	201.1 (177.9, 228.2)	201.1 (177.9, 228.2)	201.1 (177.9 to 228.2)
BMI kg/m^2 (baseline)	27.4 (24.9-30.5)	27.4 (24.9-30.5)	27.4 (24.9 to 30.5)
Underweight, <20	112 (1.7%)	119 (1.8%)	231 (1.7%)
Normal, 20-24.9	1,635 (24.1%)	1,643 (24.5%)	3,278 (24.3%)
Overweight, 25-29.9	3,112 (45.9%)	3,037 (45.2%)	6,149 (45.6%)
Obese, ≥30	1,917 (28.3%)	1,914 (28.5%)	3,831 (28.4%)
Statin use*	2,369 (34.8%)	2,384 (35.4%)	4,753 (35.1%)
Hypertension*	5,056 (74.3%)	4,928 (73.1%)	9,984 (73.7%)

Table 2. Genotype Distribution by Treatment Status. Genome-wide SNP genotyping of N=14,177 samples collected by the ASPREE Healthy Ageing Biobank was performed using the Axiom 2.0 Precision Medicine Diversity Research Array (Thermo Fisher Scientific, CA, USA) following standard protocols. A total of N=13547 samples passed post-genotyping quality control (QC) filters. Samples were excluded based on filters related to gender discordance, genotyping rate (<95%), relatedness, Hardy-Weinberg equilibrium, and ethnicity. Imputation was performed on European-ancestry samples using the haplotype reference consortium (HRC) panel on the Michigan imputation server (22). Post-imputation QC removed all variants with <0.3 imputation quality scores. The imputation quality score of the variant rs12041331 was R^2=0.99.

rs12041331	PLACEBO	ASPIRIN	TOTAL
GG	5,630 (82.7%)	5,647 (83.8%)	11,277 (83.2%)
GA	1,112 (16.3%)	1,034 (15.3%)	2,146 (15.8%)
AA	64 (0.9%)	60 (0.9%)	124 (0.9%)
	6,806	**6,741**	**13,547**

Table 3: Association of rs12041331 Genotypes with ASPREE Cardiovascular Disease and Bleeding Events in Aspirin and Placebo Treatment Groups. We used Cox proportional hazard regression to model the relationship between rs12041331 genotypes with cardiovascular and bleeding events. Endpoints analysed included the ASPREE cardiovascular disease endpoint (CVD), and sub-components of major adverse cardiovascular events (MACE) and ischaemic stroke (STROKE); and the ASPREE clinically significant bleeding endpoint (CSB), and sub-components of major hemorrhage (MHEM) and intracranial bleeding (ICB). Details of ASPREE endpoints are described previously (21). HR adj: Hazard ratio, adjusted for age, gender, smoking status, alcohol intake, high density lipoproteins, low density lipoprotein cholesterol, triglycerides, total cholesterol, hypertension, body mass index, diabetes mellitus and statin use prior to enrolment (multivariable Cox regression analysis).

ASPREE Cardiovascular Disease Endpoint (CVD)									
PLACEBO					**ASPIRIN**				
	NO CVD n (%)	**CVD n (%)**	**HR adj (95% CI)**	***p*-value**		**NO CVD n (%)**	**CVD n (%)**	**HR adj (95% CI)**	***p*-value**
GG	5351 (95.0)	279 (5.0)	1	-	**GG**	5406 (95.7)	241 (4.3)	1	-
GA	1065 (95.8)	47 (4.2)	0.84 (0.61, 1.15)	0.27	**GA**	1001 (96.8)	33 (3.2)	0.76 (0.53, 1.09)	0.14
AA	60 (93.8)	4 (6.3)	1.28 (0.48, 3.40)	0.62	**AA**	58 (96.7)	2 (3.3)	0.82 (0.21, 3.21)	0.78
Major Adverse Cardiovascular Events (MACE)									
PLACEBO					**ASPIRIN**				
	NO MACE n (%)	**MACE n (%)**	**HR adj (95% CI)**	***p*-value**		**NO MACE n (%)**	**MACE n (%)**	**HR adj (95% CI)**	***p*-value**
GG	5418 (96.2)	212 (3.8)	1	-	**GG**	5469 (96.8)	178 (3.2)	1	-
GA	1073 (96.5)	39 (3.5)	0.93 (0.66, 1.31)	0.66	**GA**	1011 (97.8)	23 (2.2)	0.70 (0.46, 1.09)	0.11
AA	61 (95.3)	4 (6.3)	1.22 (0.40, 3.71)	0.73	**AA**	58 (96.7)	2 (3.3)	1.15 (0.29, 4.54)	0.85
Ischemic Stroke (STROKE)									
PLACEBO					**ASPIRIN**				

	NO STROKE n (%)	STROKE n (%)	HR adj (95% CI)	*p*-value		NO STROKE n (%)	STROKE n (%)	HR adj (95% CI)	*p*-value
GG	5534 (98.3)	96 (1.7)	1	-	**GG**	5773 (98.7)	74 (1.3)	1	-
GA	1093 (98.3)	19 (1.7)	1.02 (0.63, 1.68)	0.92	**GA**	1023 (98.9)	11 (1.1)	0.81 (0.43, 1.52)	0.51
AA	63 (98.4)	1 (1.6)	0.94 (0.13, 6.63)	0.95	**AA**	59 (98.3)	1 (1.7)	1.36 (0.19, 9.83)	0.76

Major Hemorrhage (MHEM)

PLACEBO					ASPIRIN				
	NO MHEM n (%)	MHEM n (%)	HR adj (95% CI)	*p*-value		NO MHEM n (%)	MHEM n (%)	HR adj (95% CI)	*p*-value
GG	5479 (97.3)	96 (1.7)	1		**GG**	5448 (96.5)	199 (3.5)	1	-
GA	1093 (98.3)	19 (1.7)	0.88 (0.57, 1.34)	0.57	**GA**	998 (96.5)	36 (3.5)	1.02 (0.71, 1.45)	0.92
AA	63 (98.4)	1 (1.6)	0.60 (0.08, 4.25)	0.61	**AA**	60 (100.0)	0 (0)	NA	-

Intracranial Bleeding (ICB)

PLACEBO					ASPIRIN				
	NO ICB n (%)	ICB n (%)	HR adj (95% CI)	*p*-value		NO ICB n (%)	ICB n (%)	HR adj (95% CI)	*p*-value
GG	5594 (99.4)	36 (0.6)	1	-	**GG**	5585 (98.9)	62 (1.1)	1	-
GA	1106 (99.5)	6 (0.5)	1.03 (0.63, 1.68)	0.92	**GA**	1026 (99.2)	8 (0.8)	0.81 (0.43, 1.52)	0.51
AA	63 (98.4)	1 (1.6)	0.94 (0.13, 6.63)	0.95	**AA**	60 (100.0)	0 (0)	-	-

Table 4. Tests of Interaction Effect between treatment group and the rs12041331-A Genotype on ASPREE Cardiovascular Disease and Bleeding Events. The modifying effect of the rs12041331-A genotype on association between incident CVD events and aspirin treatment was analysed using an interaction term in a multivariable Cox regression model using participants from both groups, adjusted for age, gender, smoking status, alcohol intake, high density lipoproteins, low density lipoprotein cholesterol, triglycerides, total cholesterol, hypertension, body mass index, diabetes mellitus and statin use prior to enrolment. Endpoints analysed included the ASPREE cardiovascular disease endpoint (CVD), and sub-components of major adverse cardiovascular events (MACE) and ischaemic stroke (STROKE); and the ASPREE clinically significant bleeding endpoint (CSB), and sub-components of major hemorrhage (MHEM) and intracranial bleeding (ICB). Details of ASPREE endpoints are described previously (21).

rs12041331	**CVD**		**MACE**		**STROKE**		**MHEM**		**ICB**	
	z	***P***	**z**	***P***	**z**	***P***	**z**	***P***	**z**	***P***
GA # Aspirin	-0.46	0.649	-0.99	0.321	-0.58	0.561	0.54	0.591	-0.58	0.561
AA # Aspirin	-.052	0.606	-0.09	0.928	0.25	0.802	-35.2	-	0.25	0.802

Supplementary Information

Supplemental Material