Assessing the Causality between Blood Pressure and Retinal Vascular Caliber through Mendelian Randomisation

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We aimed to determine the association between blood pressure (BP) and retinal vascular caliber changes that were free from confounders and reverse causation by using Mendelian randomisation. A total of 6528 participants from a multi-ethnic cohort (Chinese, Malays, and Indians) in Singapore were included in this study. Retinal arteriolar and venular caliber was measured by a semi-automated computer program. Genotyping was done using Illumina 610-quad chips. Meta-analysis of association between BP, and retinal arteriolar and venular caliber across three ethnic groups was performed both in conventional linear regression and Mendelian randomisation framework with a genetic risk score of BP as an instrumental variable. In multiple linear regression models, each 10 mm Hg increase in systolic BP, diastolic BP, and mean arterial BP (MAP) was associated with significant decreases in retinal arteriolar caliber of a 1.4, 3.0, and 2.6 μm, and significant decreases in retinal venular caliber of a 0.6, 0.7, and 0.9 μm, respectively. In a Mendelian randomisation model, only associations between DBP and MAP and retinal arteriolar narrowing remained yet its significance was greatly reduced. Our data showed weak evidence of a causal relationship between elevated BP and retinal arteriolar narrowing.

Microvascular abnormalities, in particular retinal arteriolar narrowing, are consistently associated with elevated blood pressure (BP) in clinical studies1–4. Since the haemodynamic auto-regulation is provoked by persistently elevated BP, vasospasm with its myogenic tone may lead to arteriolar remodelling, which results in increased arteriolar resistance and further decompensation of peripheral BP elevation5–8. However, the role of small veins in such pathophysiological process is still not clear, due to the lack of muscular coating in the venular walls. As a window for studying microvasculature in vivo, retinal venular caliber has been investigated on its relation with either elevated BP or incident hypertension, yet the findings are not consistent2,9–12. The difference in estimates might lie in a few aspects, such as the nature of the study design, potential selection and information bias (e.g. only taking one-time clinical BP into account yet neglecting masked and white coat hypertension), or environmental confounding factors embedded in the studies themselves that could not be corrected or accounted for completely.

Mendelian randomisation provides a method for assessing the causal nature of exposures with health outcomes or diseases, by using genetic variants as robust proxies (i.e. instrumental variables [IVs]) for environmentally modifiable exposures13–16. As part of the genetic epidemiological methodology, this approach applied in our study is based on three assumptions14,17. First, genetic variants have been identified to be lifetime exposure to affect BP. Second, the BP variants will not be affected by confounders such as lifestyle, socioeconomic status and environmental risk factors, and reverse causation. Third, the association between BP genotype and retinal vascular caliber is only dependent on BP levels.

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Given that multiple genetic variants influencing BP have been repeatedly identified through genome-wide association studies (GWAS) in Europeans and/or Asian population\(^1\text{-}^\text{23}\), our study aimed to use the genetic variants as IV to determine the potential causal effect of BP on retinal vascular caliber in the Singapore Epidemiology of Eye Diseases (SEED) Study, a multi-ethnic, population-based study of Singaporean adults.

### Results

The demographic and clinical characteristics of the study participants are shown in Table 1. The mean ($\pm$ standard deviation) age of the study participant was 58.1 $\pm$ 10.0 years, and 50.7% were males. Among the middle-to-old age population, 62.5% had hypertension while 23.4% had diabetes at the time they were recruited.

In conventional multiple linear regression models, the relationship between elevated BP and retinal arteriolar narrowing was consistent in both age, gender-, and ethnic-group-adjusted model (Model 1, Table 2) and a more sophisticated model additionally adjusting for household income, body mass index (BMI), total cholesterol, blood glucose, blood creatinine level, presence of hypertension, presence of diabetes, smoking history, and alcohol drinking history (Model 2, Table 2). Compared to Model 1, the effect size became smaller after additional confounders were taken into account in Model 2, where each 10 mm Hg increase in systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) was significantly associated with a 1.4, 3.0, and 2.6 mm Hg decrease, respectively, in central retinal arteriolar equivalent (CRAE) ($P < 0.001$). For central retinal venular equivalent (CRVE), each 10 mmHg increase in SBP, DBP, and MAP was significantly related to a 4.5, 9.0, and 6.6 mm decrease ($P < 0.001$), respectively.

In the Mendelian randomisation model adjusted for age, gender and principal components (PCs) (Model 3, Table 2), each 10 mm Hg increase in SBP and MAP was associated with a 4.5, 9.0 mmHg decrease ($P = 0.01$) in CRAE, and the effect size of MAP on CRAE remained similar ($\beta = -2.6$, $P = 0.05$). There was no significant association between SBP and CRAE ($\beta = -1.3$, $P = 0.10$). None of the three BP phenotypes remained significantly associated with CRVE.

The possibility of pleiotropic effect tested insignificant in our study (Table 3). No significant association was found between BP genetic risk scores (GRSs) and traditional confounders. Therefore, we believe that the possibility of pleiotropic effect in our Mendelian randomisation model was minimal and it would not affect our findings.

### Discussion

In this population-based cohort study of multi-ethnic Asians aged 40 years and above, we provided weak evidence of a possible causal effect of elevated BP (DBP and MAP) with retinal arteriolar narrowing but not with retinal venular narrowing, by using a Mendelian randomisation approach with BP genetic risk scores as the instrument variable.

As a widely recognised surrogate for general microcirculation in vivo, retinal vascular morphology has been suggested to mirror BP variation in several population-based, cross-sectional epidemiological studies\(^24\text{-}^\text{26}\). Yet
on the contrary, the role of retinal venular caliber is still unknown, evidenced by the inconsistent findings in a number of epidemiological and clinical studies. In cross-sectional studies, the relationship between BP and retinal venular narrowing was reported in Multi-ethnic Study of Atherosclerosis (MESA)37, Sydney Children Eye Study (SCES)38 and Wisconsin Epidemiological Study of Diabetic Retinopathy (WESDR)39 to be a 1.8–2.1 mm change in retinal venular narrowing with each 10 mm Hg increase in MAP. However, the direction was null in the major drawback of the epidemiologic study design; that is, the presence of measured and unmeasured confounders in observational observation. Controlling for confounders has proven to be difficult when retinal vascular caliber is related to many other factors such as age, gender, lifestyle (smoking and alcohol drinking), and chronic conditions (e.g. type 2 diabetes), which greatly influence BP levels and even hypertension. The drawback of using ordinary linear regression in epidemiological studies is that it generally produces biased (e.g. selection bias and information) and inconsistent estimates with changes to population and study design13–15. Associations between modifiable exposures and disease seen in observational epidemiology are sometimes confounded and thus misleading, despite our best efforts to improve the design and analysis of the studies.

Mendelian randomisation is a well-known statistical tool for epi-genetic analysis, akin to a randomised controlled trial without the longitudinal design. If all assumptions of the Mendelian randomisation model are valid, such bias or systemic errors would be very much prevented compared with running an ordinary linear regression13–15. Mendelian randomisation uses genetic polymorphisms (e.g. BP genetic variants) that are known to have effects equivalent to those produced by modifiable exposures (e.g. BP). Associations between genetic variants (e.g. BP genetic risk score) and outcome (e.g. retinal vascular caliber) are generally not confounded by behavioural or environmental exposures13. Therefore, cognizant of the limitation in observational association between BP and retinal microvasculature, we adopted the principle of Mendelian randomisation and also used BP single nucleotide polymorphisms (SNPs)-derived BP genetic risk score as an IV.

Our findings of observational and IV estimates on the association between BP and retinal arteriolar caliber were consistent; however, this was not the case for the association between BP and venular caliber. After taking

| Blood Pressure | CRAE | CRVE |
|----------------|------|------|
| **Systolic blood pressure, per 10 mm Hg ↑** | | |
| Model 1* | −1.4 (−1.6, −1.3) | < 0.001 |
| Model 2† | −1.4 (−1.6, −1.2) | < 0.001 |
| Model 3: Mendelian randomisation‡ | −1.3 (−2.8, 0.3) | 0.10 |
| **Diastolic blood pressure, per 10 mm Hg ↑** | | |
| Model 1* | −3.1 (−3.5, −2.8) | < 0.001 |
| Model 2† | −3.0 (−3.4, −2.6) | < 0.001 |
| Model 3: Mendelian randomisation‡ | −4.5 (−7.9, −1.0) | 0.01 |
| **Mean arterial pressure, per 10 mm Hg ↑** | | |
| Model 1* | −2.6 (−2.9, −2.3) | < 0.001 |
| Model 2† | −2.6 (−2.9, −2.3) | < 0.001 |
| Model 3: Mendelian randomisation‡ | −2.6 (−5.2, 0.01) | 0.05 |

Table 2. Assessing the association between blood pressure and retinal vascular caliber by using conventional multiple linear regression model vs. Mendelian randomisation analysis. Abbreviations: CRAE, central retinal arteriolar equivalent; CRVE, central retinal venular equivalent; CI, Confidence Interval. *Model 1: adjusting for age and gender, and ethnicity. †Model 2: adjusting for age, gender, ethnicity, household income, body mass index, total cholesterol, blood glucose, blood creatinine level, anti-hypertension medication, diabetes history, smoking history and alcohol drinking history. ‡Model 3: adjusting for age, gender and the first 5 genetic principal components. β: effect size or correlation coefficient of the linear regression model.

| Traditional confounders for elevated blood pressure | SBP GRS, per 1 score increase | DBP GRS, per 1 score increase | MAP GRS, per 1 score increase |
|-------------------------------------------------|-----------------------------|-----------------------------|-----------------------------|
|                                                 | β, SE | P | β, SE | P | β, SE | P |
| Total cholesterol, mmol/L | 0.01, 0.02 | 0.62 | −0.02, 0.01 | 0.19 | 0.001, 0.01 | 0.94 |
| LDL, mmol/L | 0.05, 0.03 | 0.06 | −0.002, 0.01 | 0.90 | 0.006 | 0.50 |
| Serum glucose, mmol/L | 0.02, 0.01 | 0.06 | 0.004, 0.004 | 0.28 | 0.30 | 0.35 |
| Serum creatinine, mg/dl | 0.04, 0.06 | 0.53 | 0.01, 0.03 | 0.63 | −0.15 | 0.72 |

Table 3. Assessing the associations between blood pressure genetic risk scores and traditional biomarkers as potential confounders for elevated blood pressure. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; GRS, genetic risk score; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HbA1C, glycated haemoglobin; β, beta, SE, standard error.
all major confounders into account, each 10 mm Hg increase in SBP, DBP, and MAP was significantly associated with a 1.4, 3.0, and a 2.6 μm decrease (all P values < 0.001) in retinal arteriolar caliber in our conventional linear regression model, respectively, and a relatively larger reduction in retinal arteriolar caliber in Mendelian randomisation as 1.3 μm (P = 0.10), 4.5 μm (P = 0.01), and 2.6 μm (P = 0.03), respectively. For CRVE, each 10 mmHg increase in SBP, DBP, and MAP was significantly related to a 0.6 μm (P < 0.001), 0.7 μm (P < 0.01), and 0.9 μm (P < 0.001) decrease in conventional linear regression model, respectively, yet the reduction in retinal venular caliber in Mendelian randomisation was insignificant. Considering the interaction between BP and hypertension, we further stratified our significant associations into non-hypertensive and hypertensive groups. The results remained consistently significant within two groups both in the observational model and the Mendelian randomisation model (supplementary material).

Our study has several strengths. All study participants followed the same standardised protocols and were examined at the same clinic, thus the measurements are comparable and vary consistently in terms of clinical performance. However, there was some potential bias to be considered in our analysis. First, we used the semi-automated vessel grading software for retinal vessel assessment; however, the manual input during the grading process might cause misclassification of the vessel parameters. Second, our sample size might not carry enough power to detect the true association between blood pressure and retinal vascular caliber, as most of the significant associations shown in conventional linear regression are attenuated in Mendelian randomisation. Third, even though our genetic risk scores were calculated based on individual weighted allele, there might still be a misspecification in our genetic mode, which requires further cross-validation validity. Unfortunately, we cannot identify any other cohort with BP GWAS data together with similar genetic background for one-third of our study participants (i.e., Malays). Therefore, such validation of our genetic risk scores approach may also be required in other samples of the same and different ethnicities to test for generalisation.

In summary, our findings showed weak association that genetically determined BP influences retinal arteriolar caliber but not retinal venular caliber to the same degree as the observed epidemiological associations. This suggests that elevated DBP and MAP and retinal arteriolar narrowing, but not retinal venular narrowing, are likely to represent a causal relationship. Changes in retinal venular caliber in accordance with elevated BP in conventional linear regression might be influenced by other major confounders such as dyslipidaemia or hyperglycaemia. Further elucidation of the role of retinal venules should provide insights into the pathophysiological mechanism of other chronic condition such as obesity or type 2 diabetes.

Materials and Methods

Study Population. The SEED Study is a population-based study, comprising three major ethnic groups in Singapore: Malays (the Singapore Malay Eye Study, 2004–2006), Indians (the Singapore Indian Eye Study, 2007–2009), and Chinese (the Singapore Chinese Eye Study, 2009–2011). All studies were conducted the same research team followed by the same protocol at the start in terms of recruitment and clinic examination. The detailed methodology of the SEED study has been published elsewhere, and the three ethnic group participants came to the same clinic and were examined by the same team with the same standardised protocol28,29. Briefly, SEED were launched with an invitation to all Singapore citizens or permanent residents aged 40–80+ years, residing in the South-East Singapore Ministry of Home Affairs database by using an age-stratified random sampling process. At the completion of recruitment, 3280 Singapore Malays, 3353 Singapore Chinese, and 3400 Singapore Indians had participated the clinical examination with a response rate of more than 75.6%. Participants who had both fundus photos available for retinal vascular caliber measurement, DNA sample for genetic analysis and were included in the present analysis. The final number of participants was 6528: 2375 Malays, 2308 Indians, and 1845 Chinese. This study was approved by both Singhealth Centralized Institutional Review Board and was conducted according to the tenets of the Declaration of Helsinki. Informed consents were obtained from participants prior to any examination.

Clinical Examinations. Retinal photography and vessel assessment. SEED participants underwent a complete ocular examination, including a dilated fundus photography. Digital retinal photographs centered on the optic disc and macula were taken using a Canon 45° digital retinal camera (Model CR-DGi, Canon Inc). Measurement of retinal vascular caliber from the retinal photographs was performed at the Singapore Eye Research Institute, following a standardised protocol as described in previous reports in adults and children21,22,28,29. The computer imaging program (IV AN, University of Wisconsin, Madison, WI) was used to measure the caliber of all retinal arterioles and venules located in zone 0.5 to 1-disc diameter from the optic disc margin in the retinal photograph (zone B). By using the revised Knudtson-Parr-Hubbard formula to compute retinal vascular caliber, only the largest six arterioles and venules were used in calculating average vascular caliber, and estimates of the average diameters of arterioles and venules were summarised as CRAE and CRVE30. A single grader, masked to BP measurements and participant characteristics, performed all of the retinal vascular caliber measurements for this cohort. Intra-grader reliability was assessed in 70 randomly selected retinal photographs, and the intra-class correlation coefficient was 0.98 for CRAE and 0.99 for CRVE.

Blood pressure measurement. BP was taken with the participant seated and after five minutes of rest. SBP and DBP and pulse rate were measured with a digital automatic BP monitor (Dinamap model Pro Series DP110X-RW, 100V2; GE Medical Systems Information Technologies, Inc., USA) by following methods used in the Multi-Ethnic Study of Atherosclerosis (MESA)28,29. BP was measured on two occasions five minutes apart. If the BP differed by more than 10 mmHg systolic and 5 mmHg diastolic, a third measurement was made. The BP of the individual was then taken as the mean between the two closest readings. Mean arterial pressure (MAP) was calculated as one-third of SBP plus two-thirds of DBP.
Genetic Risk Scores of Blood Pressure. First, we identified 10 index SNPs from 10 genetic loci of SBP and DBP (Table 4) discovered from a few GWAS studies both in Europeans and East Asians. All 10 genetic loci were identified with meta-analysis in Asian population while 4 of them were repeated in European population. Due to the various genetic inheritances among Chinese, Malays, and Indians, we also included the 4 loci previously identified in Europeans to avoid selection bias. Candidate variants were searched based on a genomic region plus/minus 100 kb around each of the 10 index SNPs. Genotypes of SNPs were coded as 0, 1, and 2 for carrying 0, 1, and 2 copies of the risk alleles, respectively. Each SNP was tested for association with SBP, DBP, and MAP in the study participants using linear regression models. We then selected the most significantly associated SNP from each loci for each of the three BP phenotypes.

Second, we constructed SBP, DBP, and MAP-specific multi-locus GRSs for each individual by summing the number of risk alleles of the 10 selected SNPs, weighted by their estimated effect sizes (β from linear regression models) on each of the three BP phenotypes. This GRS method was adopted in previous literature. The final GRS of SBP, DBP, and MAP were treated as the IV of SBP, DBP, and MAP, respectively, for subsequent Mendelian randomisation analyses.

Statistical Analysis. The associations between BP phenotypes and retinal vascular caliber were assessed in two ways: 1) conventional linear regression, and 2) Mendelian randomisation analysis. All analyses were performed with the Statistical Analysis System (SAS) version 9.4. P-values were two-sided, and a value of 0.05 was considered to be statistically significant.

Figure 1. The theory of Mendelian randomisation by using BP genotype as an instrumental variable.
and serum creatinine) were further analysed. The Mendelian randomisation model, associations between BP GRSs and traditional confounders (e.g. cholesterol, serum glucose) were performed using STATA (version 11.1, StataCorp, College Station, Texas). Two-tailed P values < 0.05 were considered significant.

In the conventional model, multiple linear regression was performed with retinal vascular caliber as outcome variables and BP as independent variable, adjusting for for age and gender and ethnicity (Model 1). Additional adjustment was made to account for potential confounders, including household income, BMI, total cholesterol level, blood glucose, blood creatinine level, presence of hypertension, presence of diabetes, cigarette smoking history, and alcohol drinking history (Model 2).

In the Mendelian randomisation model (Model 3), analysis was performed using a two-stage least squares approach with the STATA package ivreg2. The first stage was a linear regression assessing the association between BP GRSs and BP phenotypes (SBP, DBP, and MAP). The predicted value of BP phenotypes from the model was saved and used as an independent variable in the second stage, where the dependent variable was retinal vascular caliber (CRAE and CRVE). This effect size, or the IV estimate reflects an un-confounded effect of genetically determined BP level on retinal vascular caliber. Considering the potential pleiotropic effect in our Mendelian randomisation model, associations between BP GRSs and traditional confounders (e.g. cholesterol, serum glucose and serum creatinine) were further analysed.

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| no | Chromosome | SNP* | Nearby Gene | Starting BP | Ending BP | Ethnicity |
|----|------------|------|-------------|-------------|-----------|-----------|
| 1  | 1          | rs1703613 | ST7L-CAPZA1  | 112767664   | 113115764 | Asians    |
| 2  | 1          | rs880315  | CASZ1       | 10596666    | 10956733  | Europeans |
| 3  | 2          | rs16849225| FIGN-GRB14  | 164074308   | 165286606 | Asians    |
| 4  | 4          | rs16998073| FGF5        | 81084091    | 81312171  | Europeans |
| 5  | 4          | rs6825911 | ENPEP       | 111516678   | 111803942 | Asians    |
| 6  | 5          | rs1173766 | NPR3        | 32610743    | 32904778  | Asians    |
| 7  | 10         | rs1119548 | CNNM2-NTSC2-CYP17A1 | 104490288 | 105053064 | Europeans |
| 8  | 12         | rs11066280| RPL6-PTPN11-ALDH2 | 112104691 | 113024727 | Asians    |
| 9  | 12         | rs35444   | TRX1        | 115008059   | 115652687 | Asians    |
| 10 | 12         | rs17249754| ATP2B1      | 89881826    | 90168386  | Europeans |

Table 4. Blood pressure single nucleotide polymorphisms (SNPs) selected for this study. Abbreviations: BP, blood pressure. Candidate variants were searched based on a genomic region plus/minus 100 kb around each of the 10 index SNPs, based on a series of published GWAS studies either in European or Asian population.
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Author Contributions
L.-J.L. directed the study’s implantation, including retinal photography examination, grading process and participants’ clinical interview, and prepared the whole manuscript, including data analysis and paper writing. J.L. helped in performing data analysis. C.Y.-L.C and M.K.I helped supervise the field activities and literature review and revise the manuscript. T.E.S. and T.-Y.W. helped supervise the field activities and manuscript writing. C.-Y.C. helped design the study and directe its implementation, including quality assurance and control, and he also helped conduct the literature review and manuscript amendment.

Additional Information
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