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Special Issue: Gene-environment interactions in human health

Guest Editor: Aifric O'Sullivan

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OBESITY AND WEIGHT MANAGEMENT

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Role of the *rs10401670* variant in the resistin gene on the metabolic response after weight loss secondary to a high-fat hypocaloric diet with a Mediterranean pattern

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Abstract

Background: The single nucleotide polymorphism (SNP) (*rs10401670*) of the *RETN* gene has been associated with metabolic disorder in obese subjects and has scarcely been evaluated after dietary interventions. The present study aimed to analyse the effects of the *rs10401670 RETN* gene polymorphism on metabolic changes secondary to weight loss and secondary to a high-fat hypocaloric diet with a Mediterranean dietary pattern.

Methods: A Caucasian population comprising 284 obese patients without diabetes mellitus was analysed. Before and after 3 months of a high-fat hypocaloric diet with a Mediterranean pattern, an anthropometric evaluation, an assessment of nutritional intake and a biochemical analysis were performed. A statistical analysis was conducted for the combined *CT* and *TT* as a group and for wild-type *CC* as a second group.

Results: Decreases in weight, body mass index (BMI), fat mass, systolic blood pressure and waist circumference were similar in both genotypes groups. In *T* allele carriers, insulin, homeostatic model assessment for insulin resistance (HOMA-IR), triglycerides and C-reactive protein levels were decreased. The decrease in these parameters was statistically significant for triglycerides (-22.3 ± 9.3 mg dl^{-1}: $p = 0.03$), C-reactive protein (-2.8 ± 0.5 mg dl^{-1}: $p = 0.03$), insulin (-7.4 ± 2.9 mUI L^{-1}: $p = 0.03$) and HOMA-IR (-2.4 ± 1.0: $p = 0.02$). Leptin levels were decreased in both genotypes groups after the hypocaloric diet, as well as the anthropometric parameters BMI, weight, waist circumference and fat mass. Resistin and adiponectin levels remained unchanged in both groups.

Conclusions: In the present study, we have detected a significant association between the *T* allele of this SNP and a better response of insulin resistance, triglycerides and C-reactive protein compared to non *T* allele carriers after weight loss with a high-fat hypocaloric diet and a Mediterranean diet.

KEYWORDS

high-fat hypocaloric diet, insulin resistance, resistin, *rs10401670* gene variant

Key points

- In *T* allele carriers of the *rs10401670* variant, insulin, homeostatic model assessment for insulin resistance (HOMA-IR), triglycerides and C-reactive protein levels were decreased.

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- Leptin levels were decreased in both genotypes groups (carriers and non *T* allele carriers) after the hypocaloric diet, as well as the anthropometric parameters body mass index, weight, waist circumference and fat mass.
- Resistin and adiponectin levels remained unchanged in both groups.

INTRODUCTION

Resistin is an adipokine that produces insulin resistance in rodent models,[1] and it is secreted by adipocytes and macrophages in adipose tissue and liver. In humans, its role in insulin resistance remains controversial. Resistin was identified as a gene for which the expression is induced by adipocyte differentiation and inhibited by peroxisome proliferator-activated receptor ligands in 3T3-L1 cells.[2] Serum resistin levels are associated with increased obesity, visceral fat,[3] metabolic syndrome[4] and type 2 diabetes mellitus,[5] whereas other studies have failed to observe such metabolic associations.[6,7]

The gene encoding resistin (*RETN*) is located on chromosome 19p13 and many studies have reported genetic variants in *RETN*.[8–12] In Caucasians, up to 70% of the variations in serum resistin can be explained by genetic factors.[8] For example, some single nucleotide polymorphisms (SNPs) in *RETN* have been associated with indices of insulin resistance.[9,10] The SNP 3′UTR C/T (*rs10401670*) is a genetic variant that has been associated with diabetes mellitus in the Framingham Offspring Study.[11] Ortega et al.[12] have described an association between *rs10401670* and low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-C levels. Despite the obvious relationships with metabolic parameters of this polymorphism in obese subjects, few studies have evaluated the effect of weight reduction in these variables and related it to the *rs10401670* variant of *RETN*. One study has shown,[13] after a bariatric intervention with biliopancreatic diversion, an average weight loss of 41 kg in 1 year, with resistin levels changing depending on genotypes and also on the improvement of insulin and homeostatic model assessment for insulin resistance (HOMA-IR). Moreover, after a dietary intervention with a standard hypocaloric diet for 3 months, there were no differences in resistin levels but, again, there were differences in the modifications of insulin and HOMA-IR levels as a function of genotype after an average weight loss of 3.5 kg.[14]

The present study aimed to analyse the effects of the *rs10401670 RETN* gene polymorphism on metabolic changes secondary to weight loss and secondary to a high-fat hypocaloric diet with a Mediterranean dietary pattern.

METHODS

Subjects

We enrolled, in a prospective way, a sample of 284 adults comprising obese, non-diabetic Caucasian outpatients. Adult obesity was defined by a body mass index (BMI) ≥ 30 kg m^{-2}. The recruitment of subjects was carried out with a consecutive method of sampling among patients with obesity who were sent from primary care physicians. All participants provided their written informed consent to a protocol that had been approved by the local ethical review board. The study was conducted in accordance with the guidelines laid down in the Declaration of Helsinki. The local ethics committee approved all of the procedures involving patients.

Inclusion criteria were age >18 years, BMI > 30 kg m^{-2} and the absence of dieting during the 6 months previous to the study. Exclusion criteria included: history of cardiovascular disease or stroke during the previous 24 months, total cholesterol > 200 mg dl^{-1}, triglycerides > 250 mg dl^{-1}, blood pressure > 140/90 mmHg and fasting plasma glucose > 126 mg dl^{-1}, as well as the use of metformin, sulphonilurea, dypeptidil type IV inhibitor drugs, thiazolidinedions, insulin, glucocorticoids, antineoplasic agents, angiotensin receptor blockers, angiotensin-converting enzyme inhibitors, psychoactive medications, statins and other lipid drugs.

Biochemical and anthropometric procedures

Ragarding biochemical parameters, fasting (12 h) venous blood samples were obtained by venipuncture and collected in Vacutainer tubes (Becton Dickinson). Basal fasting glucose, C-reactive protein (CRP), insulin, insulin resistance (HOMA-IR), and the lipid profile composed of total cholesterol, LDL-cholesterol, HDL-cholesterol, plasma triglycerides concentration and basal serum adipokines levels (leptin, adiponectin and resistin) were measured within the basal time of the trial and repeated after 3 months of follow-up. The *rs10401670* variant of *RETN* gene was evaluated.

Regarding anthropometric parameters, a bioimpedance analysis was conducted to measure fat mass. Weight, height and blood pressure measures were measured at the start of the trail and repeated after 3 months of intervention. These measurements were carried out at

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same time of the day (morning). Systolic and diastolic blood pressure were also measured.

Genotyping of the *rs10401670 RETN* gene polymorphism

Genomic DNA was obtained from peripheral mononuclear blood cells. A real-time-polymerase chain reaction (PCR) was carried out with 50 ng of genomic DNA, 0.5 µl of 100 µM of each oligonucleotide primer (primer forward: 5′-ACGTTGGATGGCTGTTGACGTGCTA ATGAG-3′ and reverse 5′-ACGTTGGATGAGCCA CCCTCAGCGATCTAA-3′), 0.25 µl of 10 µM probes (wild probe: 5′-Fam-TAT ACA CAC GGG CTG ACC TGA-Tamra-3′ and mutant probe: 5′-Hex-CTT ATA CAC ACA GGC TGA CCT GA- Tamra-3′), 0.5 µl of iScript reverse transcriptase, 6.25 µl of nuclease free water and 5 µl of nucleic acid extract. The oligonucleotide primers and probes were designed with Beacon Designer 5.0 (Premier Biosoft International). During the PCR, DNA was denaturated at 95°C for 3 min; this was followed by 45 cycles at 95°C for 15 s, and annealing at 59.3°C for 45 s, with an extension step of 60°C for 5 min with hot start Taq DNA polymerase. Thermocycler software (CFX Opus Real-time PCR System [Biorad, CA, LA, USA]) was used to classify each patient as wild-type homozygous (*CC*), heterozygous (*CT*) and risk homozygous (*TT*).

Biochemical parameters

Regarding lipid profile, serum total cholesterol and triglyceride concentrations were determined by an enzymatic colorimetric assay (Technicon Instruments, Ltd), whereas HDL cholesterol was determined enzymatically in the supernatant after precipitation of other lipoproteins with dextran sulfate-magnesium. LDL cholesterol was calculated using the Friedewald formula.[15]

Fasting plasma glucose levels were determined using an automated glucose oxidase method (Glucose Analyser 2; Beckman Instruments). Insulin was measured using a radioimmunoassay (RIA Diagnostic Corporation) with a sensitivity of 0.5 mUI L^{-1} (normal range 0.5–30 mUI L^{-1})[16] and the homeostasis model assessment for insulin sensitivity (HOMA-R) was calculated using these values[17] and the equation: fasting plasma insulin (mU L^{-1}) × glucose (mmol L^{-1})/22.5.

Regarding adipokine determination, resistin was measured using an enzyme-linked immunosorbent assay (ELISA) (Biovendor Laboratory, Inc.) with a sensitivity of 0.2 ng ml^{-1} and a normal range of 4–12 ng ml^{-1}.[18] Leptin was measured using an ELISA (Diagnostic Systems Laboratories, Inc.) with a sensitivity of 0.05 ng ml^{-1} and a normal range of 10–100 ng ml^{-1}.[19] Adiponectin was measured using an ELISA (R&D Systems, Inc.) with a sensitivity of 0.246 ng ml^{-1} and a normal range of 8.65–21.43 ng ml^{-1}.[20]

Adiposity parameters and blood pressure

For body composition, body weight was measured to an accuracy of 0.1 kg and BMI was computed as body weight in kg/height in m^2 (kg m^{-2}). Waist circumference was determined at the narrowest diameter between the xiphoid process and iliac crest. Bioimpedance was used to measure body composition with an accuracy of 50 g[21] (BIA 101 EFG; Akern). Blood pressure was determined three times after a 10-min rest with a random zero mercury sphygmomanometer (Omrom) and then averaged.

Nutritional intervention

The present study was designed to achieve a calorie reduction of 500 calories daily compatred to the usual intake. The subjects during this interventional study (12 weeks) received individualised counselling on a high-fat hypocaloric diet with a Mediterranean profile and physical exercise. At baseline, the dietary habits of subjects were determined using 7-day food records. Tables and images comprising color photographs of food were used to illustrate the intervention with a Mediterranean dietary pattern, including legumes, vegetables, poultry, whole grains, fish, fresh fruit and olive oil, and limiting unhealthy fats such as margarines, fatty meats, snacks and industrially made pastries.[22] The prescribed percentage of macronutrients was 35% carbohydrates, 41% fats and 24% proteins. Percentage of fats was 60.0% monounsaturated fats, 25.0% saturated fats and 15.0% polyunsaturated fats. All participants had three educational sessions (90 min with diet sheets and example menu plans) at the start of the trial to explain the dietary intervention and solve doubts. The same dietitian assessed the completion of the diet each 10 days via a phone call. All enrolled subjects received instruction to record their daily dietary intake for 7 days including a weekend day, before the dietary intervention and after 3 months. Records were analysed with a computer-based data evaluation system (Dietosource®) with national composition food tables being used as a reference.[22] The recommended physical exercise program consisted of an aerobic exercise at least three times per week (60 min each, reaching a total of 180 min each week) and the patient recorded this using a self-reported questionnaire

Statistical analysis

Sample size was assesed to detect differences over 4 kg in body weight after diets with 90% power and 5% significance ($n = 280$). The Kolmogorov–Smirnov test was used to determine variable distribution. The data are reported as the mean ± SD. Numerical variables with a

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normal distribution were analysed with a two-tailed Student's *t* test. Categorical variables were evaluated with a chi-squared test, with Yates correction as necessary. Non-parametric variables were analysed with the Mann–Whitney *U* test. The differences in anthropometric and biochemical variables between the genotype groups were tested with analysis of the covariance, adjusting for age and sex. The statistical analysis was performed for the combined C*T* and *TT* genotypes as a group (risk genotype) and the *CC* genotype as a second group (wild genotype) in a dominant model. $p < 0.05$ was considered statistically significant. All analysis was conducted using SPSS, version 23.0 (IBM Corp.).

RESULTS

Two hundred and eighty-four patients provided their informed consent and were included in the study. All patients completed the 3-month follow-up period without dropping out. The mean ± SD age was 52.9 ± 8.3 years and the mean ± SD BMI was 36.5 ± 4.2, with 76 males (26.8%) and 208 females (73.2%). Ninety-nine patients (34.9%) had the genotype *CC* (major allele group) and 185 (65.1%) patients had the other genotypes: *CT* (148 patients, 52.1%) or *TT* (37 patients, 13.0%) (minor allele group). Hardy–Weinberg equilibrium was assessed with a chi-squared test to compare our expected and observed counts. This genetic variant was in Hardy–Weinberg equilibrium ($p = 0.21$).

Average ages were similar in both genotypes groups (major allele group: 53.1 ± 9.0 years vs. minor allele group: 52.7 ± 8.1 years; not significant). The sex distribution was similar in both groups, males (21.2% vs. 29.7%) and females (78.7% vs. 70.3%).

Following the food recommendations and sessions of the dietitian, both groups (as indicated in the Methods) reached the dietary recommendations. The total caloric amount was similar in both genotypes groups (*CC* vs. *CT* + *TT*) (1550.2 ± 191.9 calories vs. 1497.8 ± 220.2; not significant). The distribution of macronutrients in both groups (*CC* vs. *CT* + *TT*) was also similar for carbohydrates (35.9 ± 3.3% vs. 36.0 ± 2.5%; $p = 0.28$), fats (41.1 ± 3.3% vs. 40.9 ± 2.9%; $p = 0.41$) and proteins (23.0 ± 2.6% vs. 22.1 ± 2.3%; $p = 0.13$). Finally, the distribution of dietary fats in both groups (*CC* vs. *CT* + *TT*) was similar for monounsaturated fats (60.0 ± 4.1% vs. 59.5 ± 4.9%; $p = 0.41$), saturated fats (24.9 ± 3.1% vs. 25.3 ± 2.9%; $p = 0.31$) and polyunsaturated fats (16.1 ± 1.3% vs. 15.2 ± 1.9%; $p = 0.43$).

The modifications in anthropometric parameters and blood pressure are shown in Table 1. After a high-fat hypocaloric diet with a Mediterranean pattern, weight, BMI, fat mass, systolic blood pressure and waist circumference decreases were similar in both genotypes groups, without any statistical differences. In the *CC* group, the decrease in weight was −3.1 ± 1.2 kg (decrease in *T* allele carriers −3.5 ± 1.5 kg; $p = 0.49$), the decrease in BMI was −2 to 0 ± 0.5 kg m^{-2} (decrease in *T* allele carriers −1.9 ± 0.6 kg m^{-2}; $p = 0.39$), the decrease in fat mass was −3.0 ± 1.1 kg (decrease in *T* allele carriers −2.9 ± 1.2 kg; $p = 0.31$) and the decrease in waist circumference was −5.6 ± 2.4 cm (decrease in *T* allele carriers −5.9 ± 2.1 cm; $p = 0.39$). In non *T* allele carriers, the decrease in systolic blood pressure was −6.6 ± 3.9 mmHg (decrease in non *T* allele carriers −6.1 ± 2.9 mmHg; $p = 0.24$). No differences were detected in diastolic blood pressure after dietary intervention. Finally, no differences were detected among basal and post-treatment values of anthropometric parameters between both genotypes groups *CC* vs. *CT*/*TT*.

We report the biochemical parameters in Table 2. The decrease in biochemical variables was not significant in patients with the *CC* genotype. In *T* allele carriers, insulin, HOMA-IR, triglycerides and CRP levels decreased. The decrease of these parameters was statistically significant for triglycerides (−22.3 ± 9.3 mg dl^{-1}; $p = 0.03$), CRP (−2.8 ± 0.5 mg dl^{-1}; $p = 0.03$), insulin −7.4 ± 2.9 mUI L^{-1}; $p = 0.03$) and HOMA-IR (−2.4 ± 1.0; $p = 0.02$). Finally, no statistical differences were detected among the basal and post-treatment values of variables between major allele genotype *CC* and minor allele genotype (*CT* + *TT*).

Table 3 shows the levels of serum adipokines. Leptin levels decreases in both genotypes groups after the hypocaloric diet (−23.3 ± 9.5 ng dl^{-1} in non T allele carriers vs. −19.0 ± 8.2 ng dl^{-1} in T allele carriers; $p > 0.05$). Resistin and adiponectin levels remained unchanged in both groups. No differences were detected among the basal and post-treatment values of adipokines between both genotypes groups *CC* vs. *CT*/*TT*.

DISCUSSION

Despite the importance of adipose tissue, as a result of its secretion of adipocytokines with multiple biological actions, and especially resistin,[1,2] studies that evaluate the effects of weight loss in obese patients and different polymorphisms of the *RETN* gene are rare. In our design analysing the *rs10401670* variant of the *RETN* gene, we detected a significant association between the *T* allele of this SNP and a better response of insulin resistance, triglycerides and CRP compared to non-carriers after weight loss with a high-fat hypocaloric diet and a Mediterranean diet.

The previous investigations into the role of resistin on cardiovascular parameters are contradictory. Some studies have reported that resistin levels were related to obesity and insulin resistance[2,23] and other studies did not detect associations between resistin levels and metabolic parameters.[24,25] Furthermore, the effect of weight loss on resistin levels is also an area with conflicting results. Santoro et al.[26] reported a reduction in resistin levels after weight loss secondary to an omentectomy

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TABLE 1 Basal and post-intervention antropometric parameters of obesity and blood pressure measurement (mean ± SD)

Parameters	*CC* (*n* = 99)		*CT* + *TT* (*n* = 148)		*p* values
	Basal	3 months	Basal	3 months	− Time *CC* − Basal genotype − Time *CT* + *TT* − 3 months genotype
BMI	36.6 ± 6.0	34.6 ± 5.2[a]	36.5 ± 5.4	34.6 ± 5.2[a]	$p = 0.01$ $p = 0.36$ $p = 0.02$ $p = 0.39$
Weight (kg)	92.2 ± 11.6	88.1 ± 9.9[b]	93.9 ± 6.2	90.4 ± 6.3[b]	$p = 0.02$ $p = 0.40$ $p = 0.03$ $p = 0.49$
Fat mass (kg)	40.8 ± 8.2	37.8 ± 7.1[c]	39.8 ± 6.2	36.9 ± 5.9[c]	$p = 0.02$ $p = 0.28$ $p = 0.02$ $p = 0.31$
WC (cm)	108.4 ± 12.1	102.8 ± 10.1[d]	109.2 ± 9.9	103.3 ± 7.1[d]	$p = 0.03$ $p = 0.41$ $p = 0.04$ $p = 0.49$
SBP (mmHg)	127.9 ± 9.2	121.3 ± 8.1[e]	128.7 ± 5.2	122.6 ± 5.8[e]	$p = 0.01$ $p = 0.37$ $p = 0.01$ $p = 0.42$
DBP (mmHg)	83.3 ± 7.1	81.0 ± 4.1	81.9 ± 6.1	80.4 ± 5.2	$p = 0.51$ $p = 0.60$ $p = 0.61$ $p = 0.52$

Note: First *p*, significance of dietary intervention after 12 weeks in *CC* genotype, second *p*, significance between *CC* genotypes vs. *CT* + *TT* baseline values, third *p*, significance of dietary intervention after 12 weeks in *CT* + *TT* genotype, fourth *p*, significance between *CC* genotypes vs. *CT* + *TT* post-treatment values. Statistical differences: $p < 0.05$, in each genotype group ([a]BMI; [b]weight; [c]fat mass; [d]WC; [e]SBP).

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; WC, waist circumference.

plus enterectomy. Similar results were reported after a biliopancreatic diversion[13] and gastric bypass.[27] Whithson et al.[28] reported an increase of resistin levels after weight loss with bariatric techniques. In addition, Moschen et al.[29] demonstrated a biphasic response after adjustable gastric banding, with a decrease in resistin levels initially and an increase at 12 months of follow-up. Lastly, studies have been conducted with caloric restrictions[14] in which, after a weight loss that was not as high as that previously achieved with bariatric surgery, the levels of resistin were not changed. Some dietary intervention studies have demonstrated a decrease of resistin levels after weight loss[30] or a contradictory increase of serum levels after weight loss.[31] Moreover, Cabrera et al.[32] showed that, in the general population, the resistin level is positively associated with saturated fat intake and inversely associated with monounsaturated fat intake.

To the best of our knowledge, this is the second study to analyse the effects of a caloric restriction and the *RETN* gene variant *rs10401670* on body weight loss and subsequent changes of metabolic parameters. In a previous study[14] with a standard low-calorie diet of 1500 calories and a macronutrient distribution of 52% of calories in the form of carbohydrates, 25% in the form of lipids (50% monounsaturated fats) and 23% in the form of proteins, the presence of the *T* allele was shown to produce a better response in insulin and HOMA-IR levels. These results are similar to those obtained in our present study with a diet that reaches the same caloric restriction (approximately 1500 calories), but with a fat percentage of 41% with 60% monounsaturated fats. Moreover, in the present study, we also show a greater decrease in triglyceride and CRP levels in *T* allele carriers. Our study did not reveal a relationship between this polymorphism and resistin levels. Moreover, a previous study that analysed the effect of the *rs10401670*[11] polymorphism reported a strong association between the minor allele and higher resistin levels. These contradictory results regarding the relationship of resistin levels

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TABLE 2 Basal and post-intervention levels biochemcial parameters (mean ± SD)

Parameters	*CC* (*n* = 99)		*CT* + *TT* (*n* = 148)		
	Basal	3 months	Basal	3 months	*p* values
					− Time *CC* − Basal genotype − Time *CT* + *TT* − 3 months genotype
Glucose (mg dl^{-1})	100.2 ± 9.1	97.1 ± 8.1	100.7 ± 8.2	96.1 ± 9.3	$p = 0.12$ $p = 0.31$ $p = 0.19$ $p = 0.49$
Total cholesterol (mg dl^{-1})	197.1 ± 20.7	189.2 ± 13.2	205.2 ± 23.1	190.4 ± 16.2	$p = 0.13$ $p = 0.50$ $p = 0.01$ $p = 0.36$
LDL-cholesterol (mg dl^{-1})	123.6 ± 18.3	112.1 ± 12.1	121.2 ± 9.1	110.1 ± 8.0	$p = 0.12$ $p = 0.49$ $p = 0.11$ $p = 0.16$
HDL-cholesterol (mg dl^{-1})	53.7 ± 4.1	52.1 ± 6.2	54.6 ± 5.0	53.8 ± 3.1	$p = 0.22$ $p = 0.45$ $p = 0.53$ $p = 0.44$
Triglycerides (mg dl^{-1})	110.6 ± 21.9	109.7 ± 16.4	119.1 ± 13.2	98.8 ± 10.2[a]	$p = 0.12$ $p = 0.61$ $p = 0.03$ $p = 0.45$
Insulin (mUI L^{-1})	14.2 ± 6.1	13.5 ± 4.1	18.4 ± 3.2	11.0 ± 4.7[b]	$p = 0.22$ $p = 0.31$ $p = 0.03$ $p = 0.41$
HOMA-IR	3.6 ± 1.1	3.4 ± 1.0	5.4 ± 1.0	3.0 ± 0.9[c]	$p = 0.33$ $p = 0.35$ $p = 0.02$ $p = 0.49$
CRP	6.5 ± 2.1	6.9 ± 1.1	6.6 ± 1.8	3.8 ± 1.1[d]	$p = 0.22$ $p = 0.39$ $p = 0.03$ $p = 0.05$

Note: First *p*, significance of dietary intervention after 12 weeks in *CC* genotype, second *p*, significance between *CC* genotypes vs. *CT* + *TT* baseline values, third *p*, significance of dietary intervention after 12 weeks in *CT* + *TT* genotype, fourth *p*, significance between *CC* genotypes vs. *CT* + *TT* post-treatment values. Statistical differences: $p < 0.05$, in each genotype group ([a]triglicerides; [b]insulin; [c]HOMA-IR; [d]CRP).

Abbreviations: CRP, C-reactive protein; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment; LDL, low-density lipoprotein.

with this polymorphism may be a result of different genetic disequilibrium linkages or other unknown genetic and environmental variables.

An interesting result of our work is the best response for HOMA-IR and insulin levels in *T* allele carriers. These results were already observed in a previous study,[14] although, in the present study, the improvement is still greater. This may be a result of the greater amount of monounsaturated fats in our diet and the effect of olive oil consumption on these markers.[33] This better metabolic response in *T* allele carriers could be secondary to a potential interaction of transcriptors of the *rs10401670* variant in the *RETN* gene and glucose pathways because this genetic variant is located in an intron of *MCEMP1* (mast cell expressed membrane protein 1). This gene encode a protein with a single transmembrane domain expressed mainly by mast cells lines and monocytes.[34] This could also explain our findings for inflammatory markers such as CRP. Because resistin is mainly expressed by macrophages that evolve from monocytes in adipose tissue, it would be interesting to determine whether MCEMP1 and its product are functionally influenced by *rs10401670* or the SNPs in its 5_region and whether this protein is

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TABLE 3 Basal and post-intervention levels of serum adipokines (mean ± SD)

Parameters	*CC* (*n* = 99)		*CT* + *TT* (*n* = 148)		*p* values
	Basal	3 months	Basal	3 months	− Time *CC* − Basal genotype − Time *CT* + *TT* − 3 months genotype
Resistin (ng dl^{-1})	3.6 ± 1.5	3.7 ± 1.3	3.9 ± 1.1	2.8 ± 0.9	$p = 0.41$ $p = 0.69$ $p = 0.16$ $p = 0.49$
Adiponectin (ng dl^{-1})	33.1 ± 9.1	34.2 ± 8.1	30.1 ± 7.9	32.3 ± 3.2	$p = 0.21$ $p = 0.52$ $p = 0.21$ $p = 0.51$
Leptin (ng dl^{-1})	93.5 ± 11.6	70.2 ± 12.5[a]	93.8 ± 10.1	64.8 ± 9.1[a]	$p = 0.02$ $p = 0.41$ $p = 0.02$ $p = 0.46$

Note: First *p*, significance of dietary intervention after 12 weeks in *CC* genotype; second *p*, significance between *CC* genotypes vs. *CT* + *TT* baseline values; third *p*, significance of dietary intervention after 12 weeks in *CT* + *TT* genotype; fourth *p*, significance between *CC* genotypes vs. *CT* + *TT* post-treatment values. Statistical differences: $p < 0.05$ in each genotype group ([a]leptin).

involved in glucose metabolism and the inflammatory processes. For example, there are indications that resistin is involved in the pathogenesis of other inflammatory states such as rheumatoid arthritis. Resistin has been found in the plasma and synovial fluid of rheumatoid arthritis patients.[35] Qi et al.[36] reported an association of resistin with inflammatory markers and fibrinolytic markers such as fibrinogen, CRP and plasminogen activator inhibitor.

The observed improvement in triglyceride levels may also be related to the improvement in the inflammatory status of patients observed after weight loss in *T* allele carriers.[37] Previously, Ortega et al.[12] reported an association between *rs10401670* and LDL-cholesterol levels in 12–16-year-old boys, as well as between the polymorphism and HDL-cholesterol levels in girls. The relationship with both types of lipids has different pathophysiological bases.

There are some limitations to the present study. First, only one SNP in the *RETN* gene has been evaluated, whereas several others could be related to the metabolic parameters. Second, there is the lack of a control group without dietary intervention with which to compare the effect of weight loss. Finally, the short duration of the clinical trial does not allow us to observe what would happen to the resistance levels over a longer period.

In conclusion, we describe an association of the *rs10401670T* allele with a better metabolic response (insulin, HOMA-IR, trygliceride and CRP) secondary to weight loss after a high-fat hypocaloric diet with a Mediterranean pattern. However, further studies are necessary to confirm our results and to explore the effect of new dietary interventions,[37] taking into account a possible functional variant of the *RETN* gene.[38,39]

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (HVUVA committee 2/2018) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

AUTHOR CONTRIBUTIONS

Daniel Antonio de Luis designed the study and wrote article. Rocío Aller and Olatz Izaola conducted the nutritional intervention. David Primo conducted the laboratory analysis.

TRANSPARENCY DECLARATION

The lead author affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted.

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Variation in cardiovascular disease risk factors among older adults in the Hunter Community Study cohort: A comparison of diet quality versus polygenic risk score

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Abstract

Background: The interplay between cardiovascular disease (CVD) genetic risk indexed by a polygenic risk score (PRS) and diet quality still requires further investigation amongst older adults or those with established or treated CVD. The present study aimed to evaluate the relative contribution of diet quality, measured using the Australian Recommended Food Score (ARFS) and PRS, with respect to explaining variation in plasma lipids CVD outcomes in the Hunter Cohort.

Methods: The study comprised a secondary analysis of cross-sectional data from the Hunter Cohort study. Single-nucleotide polymorphisms from previously derived polygenic scores (PGSs) for three lipid classes were obtained: low-density lipoprotein, high-density lipoprotein and triglycerides, as well as PRS for coronary artery disease (CAD) from the PGS catalogue. Regression modelling and odds ratios were used to determine associations between PRS, ARFS and CVD risk.

Results: In total, 1703 participants were included: mean ± SD age 66 ± 7.4 years, 51% female, mean ± SD total ARFS 28.1 ± 8 (out of 74). Total diet quality and vegetable subscale were not significantly associated with measured lipids. By contrast, PGS for each lipid demonstrated a markedly strong, statistically significant correlation with its respective measured lipid. There was a significant association between CAD PRS and 5/6 CVD phenotypes (all except atrial fibrillation), with the largest effect size shown with coronary bypass. Adding dietary intake as a covariate did not change this relationship.

Conclusions: Lipid PGS explained more variance in measured lipids than diet quality. However, the poor diet quality observed in the current cohort may have limited the ability to observe any beneficial effects. Future research should investigate whether the diet quality of older adults can be improved and also the effect of these improvements on changes in polygenic risk.

KEYWORDS

cardiovascular disease, cohort, diet quality, Hunter Community Study, polygenic risk

Key points

- The Australian Recommended Food Score (ARFS) had little association with lipid and cardiovascular disease (CVD) endpoints.

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- Lipid polygenic score (PGS) explained more variance in measured lipids than diet quality.
- The lipid PGS was associated with all three lipid parameters and some CVD endpoints, especially high cholesterol.
- Coronary artery disease polygenic risk score was associated with CVD endpoints angina, coronary bypass, heart attack, high cholesterol and hypertension and some lipid parameters (high-density lipoprotein cholesterol).

INTRODUCTION

Dietary intake is a modifiable, lifestyle factor contributing to the global burden of disease.[1,2] Higher diet quality, characterised by higher intakes of fruits, vegetables, wholegrains and lean sources of protein, as well as lower intakes of energy-dense, nutrient-poor foods including confectionery, biscuits, sugar sweetened beverages and take-away foods (e.g., pizza, chips or deep-fried foods) is associated with a lower risk of developing cardiovascular disease (CVD).[3,4] A recent systematic review of cohort studies identified that higher diet quality, measured by dietary indexes, was associated with significantly lower CVD incidence and mortality risk (relative risk = 0.80; 95% confidence interval [CI] = 0.78–0.82).[4]

Emerging evidence shows that there is a relationship between genetic predisposition, dietary intake and CVD.[5] Although mRNA expression is partially controlled by genetic factors, it is also impacted directly by environmental effects such as dietary intake.[6,7] For example, associations between healthier dietary patterns such as the Prudent and Mediterranean diets and gene expression of inflammatory, immune response and cardiovascular pathways have been identified.[7,8] Other nutrient specific interactions include the ratio of omega-6 to omega-3 fatty acids consumed, which impacts on genes such as cytosolic PLA_2 alpha or 5-lipoxygenase, thus affecting inflammation and inflammatory related disease or conditions.[9,10] High-density lipoprotein (HDL) and low-density lipoprotein (LDL) concentrations can be influenced by variants in genes, such as vanin 1, as well as dietary intake.[11] Identification of individual variation in these pathways is termed nutrigenomics and these data can be used to inform personalised dietary advice targeting the biology of individuals.[9]

Polygenic risk scores (PRSs) predict the likelihood of a specific outcome, such as CVD, by including known DNA variants into a statistical model.[12] In other words, the effect of variants on CVD are summed genome wide to derive an individual's genetic liability to the disorder. This allows development of models that stratify individuals by risk of developing specific diseases over time. PRSs have been used in observational studies to measure associations between lifestyle risk factors and genetic markers for disease.[13–16] These studies have shown promise, having identified that healthier lifestyles, including healthful dietary patterns, are associated with a lower risk of disease, including type 2 diabetes, CVD, CVD mortality and all-cause mortality, even among those with a high PRS.

The majority of genome-wide association studies (GWAS) evaluation to date have been in populations of European descent and, although studies in other ethnicities are underway, including Japanese and African-American,[17] findings are not yet able to be used routinely in clinical settings.[12]

The UK Biobank cohort has been a major resource for analysing the interplay between diet and lifestyle influences and genetics on CVD liability. A recent longitudinal analysis in middle aged (40–69 year old) adults with no history of CVD ($n = 77{,}004$) not only demonstrated a significant enrichment of coronary artery disease (CAD) PRS amongst participants who went on to develop CVD, but also identified that, among those with high PRS, a better diet quality was protective to some degree.[16] For example, for those with a high PRS, every one-point higher Healthy Diet Indicator (higher diet quality) score was associated with a reduction in myocardial infarction risk (hazard ratio = 0.93, 95% CI = 0.88–0.99, $p = 0.017$).[16] Another study identified that participants without CVD, but with a familial predisposition to CVD and therefore higher PRS, had a lower risk of CVD among those with higher intakes of fish and a higher risk among those with higher processed-meat intakes.[18] This study also identified a protective effect within those with a familial predisposition to CVD, and higher PRS showing a lower risk of CVD among those with higher intakes of cheese.[18] Although cheese consumption has historically been associated with an increased risk of CVD because of its high saturated fatty acid intake,[19] recent meta-analyses of observational studies have identified an association between higher intakes of cheese and lower risk of CHD,[20,21] supporting the findings of a protective effect with higher cheese consumption.[18]

The interplay between CVD genetic risk indexed by a PRS and diet quality still requires further investigation amongst older adults or those with established or treated CVD. Moreover, other dietary related CVD risk factors such as a measured lipids also have a strong genetic basis, although the extent to which diet modifies genetic predisposition to higher plasma lipids is also still

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relatively uncharacterised. The present study therefore aimed to evaluate the relative contribution of diet quality, measured using the Australian Recommended Food Score (ARFS) and PRS with respect to explaining variation in plasma lipids and liability to CVD phenotypes in the Hunter Community Study cohort.

METHODS

Study cohort

The present study comprises a secondary analysis of cross-sectional baseline data from the Hunter Cohort study. The Hunter Community Study (HCS) cohort is a population-based study comprised of men and women aged between 55 and 85 years at recruitment and who resided in the Newcastle region of New South Wales, Australia. Briefly, the participants were recruited by random selection from the electoral role between 2004 and 2007, with those agreeing providing their written informed consent for collection of a blood sample, attending a clinic visit, and answering a series of health-related questionnaires on demographics, dietary intake, physical activity, morbidity, mental health and quality of life. Clinical measures were conducted by a trained nurse and included anthropometry, respiratory function, cardiovascular function, cognition, bone mineral density, functional capacity routine blood biomarkers, with DNA also extracted for genotyping. In total, 9784 individuals were sent invitation letters, 7575 responded (77.4%), 3877 agreed to participate via written informed consent and 3253 (response rate 44.5%) completed the study (47% men and 53% women). The full details of this cohort and the measures collected have been outlined elsewhere.[22] Although non-response can affect bias, characteristics of the included population such as age and marital status, are reflective of the profiles of population of the Hunter, NSW and Australian population[22]; however, the mean age is marginally younger.

The HCS protocol and all procedures involving study participants were approved by the University of Newcastle Human Research Ethics committee (H-820–0504) with informed consent obtained from all participants. The current analysis was approved by the HCS data custodians and a 'Memorandum of Understanding' and 'Confidentiality Statement' signed by all authors.

Genotyping and imputation

Extracted DNA was genotyped using the Axiom Kaiser array (Affymetrix), with variants excluded with low call rate (<0.95), significant deviations from Hardy–Weinberg equilibrium ($p < 1 \times 10^{-6}$) and minor allele frequency less than 1%. Imputation and quality control of this cohort was described previously in Reay *et al.*,[23] where imputation was performed using the Haplotype Reference Consortium panel via the Michigan Imputation Server, with high confidence ($R^2 > 0.8$) common variants retained post-imputation that exhibited missingness <2%.[23,24] Unrelated individuals of European ancestry were selected for these analyses to account for the effect of population stratification and relatedness on the distribution of polygenic scores (PGSs). Physically genotyped common variants in relative linkage equilibrium, excluding known regions of long-range linkage disequilibrium (LD), were the input for relatedness testing and principal component analysis (PCA) using PLINK, version 1.9 (https://www.cog-genomics.org/plink) to define population outliers via clustering the first two eigenvectors with that of each of the 1000 genomes phase 3 superpopulations via *k*-means clustering.[25,26] PCA was then repeated using the same parameters in the defined European ancestry HCS cohort subset such that the principal components could be used as covariates in downstream analyses

PGSs

We obtained single nucleotide polymorphism (SNP) weights from previously derived PGS for three lipid classes: LDL, HDL and triglycerides (TGs), as well as PRSs for CAD from the PGS catalogue.[27] PGS is utilised hereafter to denote genetic scores related to the continuous lipid traits, whereas PRS relates to a disease phenotype, and thus, is more appropriate to designate the CAD scores. Two PGSs for each trait constructed using different methods were selected from the catalogue to profile in the HCS cohort. For the three lipid traits, two classes of PGS were considered: (i) clumping and thresholding (C + T) of established lipid loci in relative linkage equilibrium (GWAS, $p < 5 \times 10^{-8}$, $r^2 < 0.1$) trained using a GWAS meta-analysis of up to 331,368 individuals (PGS catalogue accession IDs: PGS000064, PGS000065 and PGS000066)[28] and (ii) penalised regression (batch screening iterative LASSO–BASIL) for SNP selection trained in a subset of the UK Biobank ($N = 255{,}256$, PGS catalogue accession IDs: PGS000688, PGS000686, PGS000699).[29] The two CAD PRS selected were an LDPred derived score trained in the UK Biobank using an external CAD GWAS which is a Bayesian method that shrinks effect sizes to account for LD (PGS catalogue accession ID: PGS000013), with 0.001 selected as the optimal fraction of non-zero effect sizes variants, and a 'metaGRS' approach that combined three pre-existing CAD PRS (PGS catalogue accession ID: PGS000018).[30,31] In all instances, the PGS/PRS in the HCS were calculated by summing the j SNP weights ($\hat{\beta}_j$) profiled in each individual i multiplied by their genotype dosage ($G_{ij} \epsilon \quad G = 0, 1, 2$) under an additive model (Equation 1).

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$$PGS_i = \sum_{i=1} \hat{\beta}_j G_{ij} \quad (1)$$

We used the *--score* flag in PLINK 1.9 to calculate the scores in the HCS cohort, with each score then averaged by the number of non-missing alleles in the score.

Dietary information

The ARFS was previously calculated in this cohort as a subscale of responses to a self-administered 145-item semiquantitative food frequency questionnaire (FFQ) completed by HCS participants.[32] The FFQ uses the NUTTAB90 nutrition database[33]; however this nutrient database is not needed for the calculation of ARFS. The ARFS was scored directly from the frequency response data from the included questions on the FFQ. ARFS was previously been shown to be a valid measure of diet quality.[32,34–36] The ARFS sums seven categories: vegetables, fruit, protein foods, grains, dairy, fats and alcohol, with a maximum score of 74 points and higher ARFS indicative of a dietary pattern more closely aligned with the Australian Dietary Guidelines[37] and variety with food groups recommended in the Australian Guide to Health Eating.[38] One point was given for consumption of once or more per week for all foods, with upper limits on consumption of red meat and processed dairy foods such as flavoured milk and ice cream. Additional points were awarded for choosing whole grain bread, low fat milk and greater consumption of vegetables. ARFS scoring has been detailed further elsewhere.[39] We also considered the vegetable subscale of the ARFS, given the established association between vegetable intake, cardiovascular outcomes[40] and health usage.[41,42]

CVD phenotypes

Total, HDL and TG were all measured from the fasting blood samples, whereas LDL was calculated using Friedewald's equation (LDL [mmol L^{-1}] = total cholesterol − HDL − TG/2.2). Samples were obtained at baseline in the HCS, and thus were chosen as outcome phenotypes in assessing the relationship between diet quality and PGSs. Moreover, we investigated six CVD phenotypes that individuals self-reported they had been previously diagnosed with at baseline: angina, atrial fibrillation, coronary bypass, heart attack, high cholesterol and hypertension.

Statistical analysis

All statistical analyses were conducted using R, version 3.6.0.[43] We derived four main subsets from the European, unrelated HCS cohort with genotype data available: (i) non-missing cholesterol and ARFS; (ii) non-missing cholesterol and ARFS, as well as non-missing additional covariates of interest (ever smoked, educational attainment and statin usage); (iii) non-missing self-reported CVD diseases (the six described above) and ARFS; and (iv) non-missing self-reported CVD diseases and ARFS, as well as the covariates outlined in the second cohort. First, ARFS was regressed against each lipid class in the first cohort using linear regression covaried for age and sex, whereas each PGS/PRS was also regressed against the lipids, with the first-five SNP derived principal components as additional covariates. A model with ARFS as a covariate was then utilised with the genetic scores. We calculated the variance explained in the lipids by the PGS for that same lipid using the ΔR^2 ($\Delta R^2 = R^2_{\text{Full}} - R^2_{\text{Null}}$), where R^2 was the adjusted R^2, the coefficient of determination adjusted for the number of predictors in the model. As result, the null model included age, sex, five principal components and the ARFS, whereas the full model included the PGS for each lipid trait. The PGS and ARFS were both scaled to have a mean of zero and unit variance. We also considered the impact of natural log transformation of the lipid outcomes on ΔR^2. An interaction term was the included between the PGS and ARFS in these models to test for a departure from additivity. The association of the PGS with each lipid was then also tested including the additional covariates of smoking status, educational attainment and self-reported statin usage. Bonferroni correction was applied to the genetic results, with a significant association designated as $p < 1.04 \times 10^{-3}$ ($0.05/[2x(3 \times 8)]$).

The CVD binary disease phenotypes were assessed for association with each PGS in a similar fashion, with these phenotypes the outcome variable in binomial logistic regression models. First, we tested the association of the ARFS and the eight PGS/PRS separately with each of the CVD phenotypes, using the entire rest of the cohort as controls, which would include other CVD cases. As a result, we then repeated these models by only retaining individuals who did not self-report any of the six CVD phenotypes as controls for each model. Models with both ARFS and the genetic scores were also the constructed as in the lipid analyses, with the additional smoking, education and statin covariates included in additional models. The variance explained by the CAD PRS in each of these CVD binary phenotypes was estimated using Nagelkerke's R^2, which was the converted to the liability scale such that this metric was not biased by sample composition and population prevalence of the CVD trait, assuming the following population prevalences of 5%, 10%, 15% and 20%.[44] We used the most significantly associated CAD PRS from the two tested for each trait, with Nagelkerke's R^2 derived by subtracting the full model with the CAD PRS from the null covaried for age, sex, five principal components and

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the ARFS. In total, 192 genetic models were tested, and thus the threshold for significance was set as $p < 2.60 \times 10^{-4}$. Finally, as heart attack was the most severe CVD phenotype recorded, we tested whether people who had experienced this phenotype had an enrichment of CAD PRS relative to the remaining CVD traits.

RESULTS

Baseline characteristics of study population

Of the 3318 HCS cohort participants, there were 1703 unrelated, European ancestry individuals who survived genotyping quality control and had a non-missing record of measured blood lipids (LDL, HDL and TG) and the ARFS diet quality score. The cohort mean ± SD age was 66.1 ± 7.4 years (51.32% female), whereas the mean ± SD total ARFS, vegetable subscale ARFS and lipids were: ARFS = 28.12 ± 8; ARFS vegetable subscale = 9.9 ± 3.6; LDL = 3.08 ± 0.91 mmol L^{-1}; HDL = 1.35 ± 0.36 mmol L^{-1}; and TG = 1.30 ± 0.70. A subset of these 1703 individuals ($N = 1486$) had smoking status, educational attainment and self-reported statin usage recorded, and thus were retained for the sensitivity analyses that included those additional covariates. The sex and age profile of this subset was very similar (see Supporting information, Table S1). Furthermore, of the six binary self-reported CVD phenotypes, there were 1678 individuals with non-missing status for all of these traits. The number of cases ranged from 129 for heart attack to 758 for hypertension (see Supporting information, Table S2). There were 556 individuals who did not self-report any of these conditions. Analogous to the lipid analyses, the majority of the cohort had non-missing educational attainment, smoking status and statin usage ($N = 1508$) for those sensitivity analyses (see Supporting information, Table S3).

Lipid PGSs explain more variance in measured cholesterol than diet quality

We found that overall diet quality as measured by total ARFS score was not significantly associated with measured LDL or HDL, either in the baseline model adjusted for age and sex, or the full sensitivity model covaried for educational attainment, smoking and statin usage (see Supporting information, Table S4). However, there was a weak association between better diet quality and decreased plasma TG, with each SD increase (scaled to be one) in ARFS associated with a −0.035 mmol L^{-1} (95% CI = −0.001 to −0.069; $p = 0.042$) lower TG level (Figure 1a). This remained relatively consistent in the full sensitivity model. By contrast, the PGS for each lipid demonstrated a markedly stronger, statistically significant correlation with its respective measurement (Figure 1; see also Supporting information, Table S5). The The association between the best performing PGS for LDL, HDL, and triglycerides, respectively, with each measured lipid was as follows per standard deviation increase in PGS-LDL: $\beta = 0.177$ mmol L^{-1} ($p = 9.35 \times 10^{-17}$); HDL, $\beta = 0.134$ mmol L^{-1} ($p = 2.70 \times 10^{-67}$); and TG, $\beta = 0.234$ mmol L^{-1} ($p = 7.89 \times 10^{-47}$). Penalised regression (BASIL) scores performed best for HDL and TG, whereas the C + T approach was most significantly associated for LDL. Diet quality as measured by ARFS was then added as a covariate, with only a minute effect on estimated correlation between the score and measured lipids (Figure 1b). The variance explained (ΔR^2) by each of the scores in the respective lipids relative to the null model covaried for age, sex, five SNP-derived principal components, and the ARFS was 3.37%, 13.6% and 11.14% for LDL, HDL and TG, respectively. Natural log transformation of the outcome did not impact these estimates in any notable way (see Supporting information, Table S6). There was no significant interaction between ARFS and TG PGS on measured TG. The same sensitivity models applied to diet quality in this subset of the cohort did reduce the effect sizes for HDL and TG, however the PGS correlations with lipids all remained highly significant ($p < 1 \times 10^{-16}$) (Figure 1b). Interestingly, the addition of these other covariates resulted in an elevated correlation between the LDL PGS and measured LDL, likely because the large effect of statins was not accounted for in the baseline model (see Supporting information, Table S5). Genetic risk for CAD (CAD PRS) was associated with lower HDL but was not significantly correlated with measured TG or LDL (see Supporting information, Table S5). We next investigated whether the ARFS vegetable specific subscale would have differing effects than the total score. There were no statistically significant effects of vegetable subscale on plasma lipids levels (see Supporting information, Table S4). Moreover, adjusting PGS for ARFS vegetable subscale yielded very similar effects to that observed with the total ARFS (see Supporting information, Table S5).

CAD PRS is associated with a broad range of CVD phenotypes independent of diet quality

We first tested the association of diet quality measured by total ARFS with six binary self-reported CVD phenotypes and found that there were no strong relationships between diet and odds of any phenotypes (Table 1). These analyses were repeated after removing other CVD cases from the 'control' cohort. However, this did not reveal any diet related associations. The only exception was a potentially counterfactual positive relationship between ARFS and self-reported atrial

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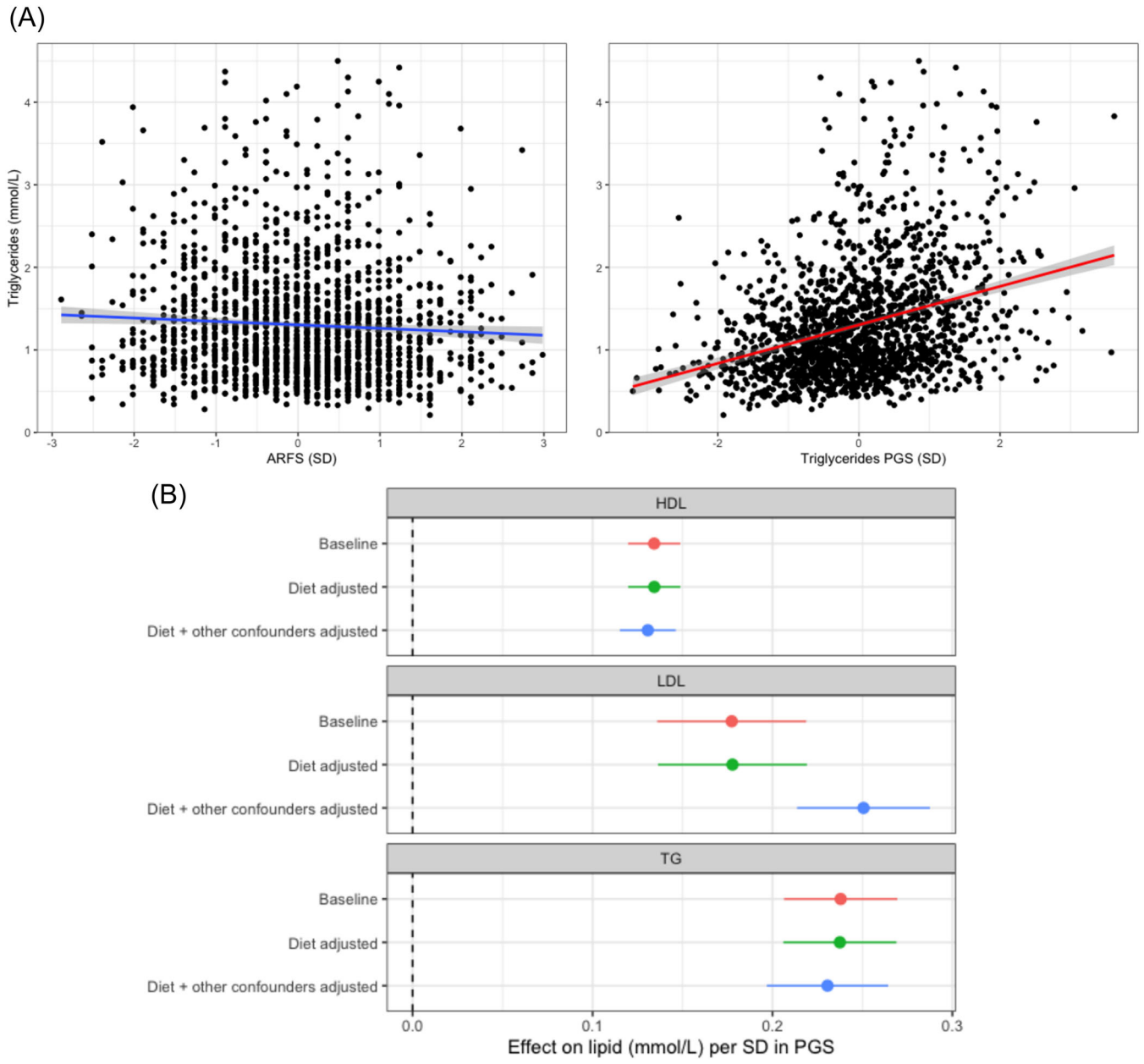

FIGURE 1 The relationship between diet, polygenic scores (PGSs) of lipids and measured lipids. (a) Scatterplots of distribution of measured triglycerides (TGs) relative to diet quality (Australian Recommended Food Score [ARFS]) and PGS of TGs. Grey shading around trend line denotes the 95% confidence interval (CI) from the linear model regressing either trait on the outcome of measured TGs. (b) Forest plot of the effect of a SD increase in the best performing polygenic score (PGS) for that lipid in three different models: (i) baseline = adjusted for age, sex, and five single nucleotide polymorphism derived principal components; (ii) diet adjusted = ARFS total score as an additional covariate to the first model; and (iii) diet + other confounders adjusted = smoking status, educational attainment, and statin usage in addition to covariates in the second model. Error bars represent 95% CIs of the beta coefficient

fibrillation, although this was very nominal ($p = 0.03$) and does not pass multiple-testing correction. The ARFS vegetable subscale was then tested, with the same lack of association as with the total ARFS score. By contrast, genetic risk for CAD expressed as a PRS was strongly enriched amongst each class of CVD cases, with the exception that the CAD PRS was only associated with self-reported atrial fibrillation in the reduced cohort where other CVD cases were removed as controls (Figure 2 and Table 1; see also Supporting information, Table S7).

The largest effect size observed for a CAD PRS was between the 'metaGRS' CAD score and odds of a coronary bypass, odds ratio (OR) per SD = 2.01 (95% CI = 1.79–2.23; $p = 3.74 \times 10^{-10}$). Notably, the CAD PRS were still associated with the relevant CVD outcomes after the inclusion of total ARFS as a covariate, as well as the ARFS vegetable subscale score. The mean

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TABLE 1 Association between diet and polygenic risk for coronary artery disease and self-reported cardiovascular disease phenotypes

CVD phenotype	ARFS (log odds per SD)[a]	CAD PRS (log odds per SD)[b]
Unselected controls[c]		
Angina	−0.01(0.01)	0.37 (0.09)***
Atrial fibrillation	0.02 (0.01)*	0.11 (0.08)
Coronary bypass	0.01 (0.01)	0.47 (0.09)***
Heart attack	−0.01 (0.01)	0.42 (0.10)***
High cholesterol	−0.002 (0.01)	0.25 (0.05)***
Hypertension	−0.001 (0.01)	0.19 (0.06)***
Selected controls		
Angina	−0.01 (0.11)	0.60 (0.12)***
Atrial fibrillation	0.20 (0.09)*	0.29 (0.09)**
Coronary bypass	0.14 (0.10)	0.69 (0.11)***
Heart attack	0.003 (0.11)	0.51 (0.12)***
High cholesterol	−0.001 (0.06)	0.31 (0.06)***
Hypertension	−0.01 (0.06)	0.30 (0.06)***

Abbreviations: ARFS, Australian Recommended Food Score; CAD, coronary artery disease; CVD, cardiovascular disease; PRS, polygenic risk score.

[a]Log odds (SE) of each CVD phenotype per SD increase in diet score (ARFS).

[b]Log odds (SE) of each CVD phenotype per SD increase in best performing CAD PRS.

[c]Cases who self-reported the CVD phenotype in question were compared to all other participants (unselected controls), and participants who did not self-report any CVD phenotypes (selected controls).

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

phenotypic variance explained by these CAD PRS on the liability scale when excluding other CVD cases as controls assuming a series of population prevalences was 9.5% for angina, 2.3% for atrial fibrillation, 11.2% for coronary bypass, 9% for heart attack, 2.6% for high cholesterol and 2.4% for hypertension (see Supporting information, Table S8). The lipid PGS were also associated with self-reported high blood cholesterol, as expected, along with several other CVD phenotypes. Addition of smoking status, educational attainment and statin use as covariates did reduce the effect size of these associations, although most remained significant after multiple-testing correction (see Supporting information, Table S9). For example, the association between the CAD PRS described above and coronary bypass was weakened upon adjustment for these additional factors but were still significant (OR per SD = 1.29; 95% CI = 1.16–1.43; $p = 6.89 \times 10^{-4}$). As a result, these genetic scores were independently associated with CVD phenotypes from diet quality, however, there was potentially some inflation of effect size because of factors such as smoking. We then tested whether heart attack would have a higher burden of CAD associated genetic risk relative to the five other CVD phenotypes considered, and we found some evidence that this was the case in this cohort. Specifically, each SD in CAD PRS was associated with a 42.70% (95% CI = 20.41%–63.93%) increase in the odds of a participant self-reporting a heart attack relative to individuals who self-reported at least one of the following: angina, atrial fibrillation, coronary bypass, high cholesterol or hypertension. We visualise the odds ratio of self-reported attack for each quartile of CAD PRS and diet quality (total ARFS and vegetable subscale) in the three cohorts considered: (i) heart attack cases vs. participants without any other CVD phenotype (Figure 3a); (ii) heart cases vs. the remaining participants (Figure 3b); and (iii) heart attack cases vs. other CVD cases who did not self-report a heart-attack (Figure 3c). In all three instances, the odds of heart attack monotonically increased with each quartile of CAD PRS relative to the reference quartile, as expected, whereas there was no discernible relationship with diet quality.

The key findings are summarised in Figure 4.

Investigation of the effect of energy intake

Given that total energy intake is known to be related to diet quality,[45] the influence of total energy intake in this cohort was evaluated in a sensitivity analysis. Total energy intake was positively correlated with total ARFS in this sample ($R^2 = 0.186$), with each SD increase in ARFS associated with a 0.432 (95% CI = 0.389–0.475) SD elevation in total energy intake (scaled such that SD = 1 in both instances). The association between energy intake and vegetable ARFS subscale was comparatively smaller ($R^2 = 0.107$) but still highly statistically significant. Covariation for total energy intake did not change the association between ARFS and lipids, with TGs still the only nominally significant association with ARFS. Similarly, adding total energy intake to the model along with ARFS did not impact the association between each lipid PGS and its respective lipid in any notable capacity (see Supporting information, Table S10). Total energy intake also did not ablate the association between CAD PRS and any of the CVD phenotypes (see Supporting information, Table S11). In summary, the additional covariate of total energy intake does not alter any of the conclusions drawn from these analyses.

DISCUSSION

We found that overall diet quality as measured by total ARFS score was not significantly associated with measured LDL or HDL, either in the baseline model adjusted for age and sex, or the full sensitivity model covaried for educational attainment, smoking and statin usage. There were also no strong relationships between

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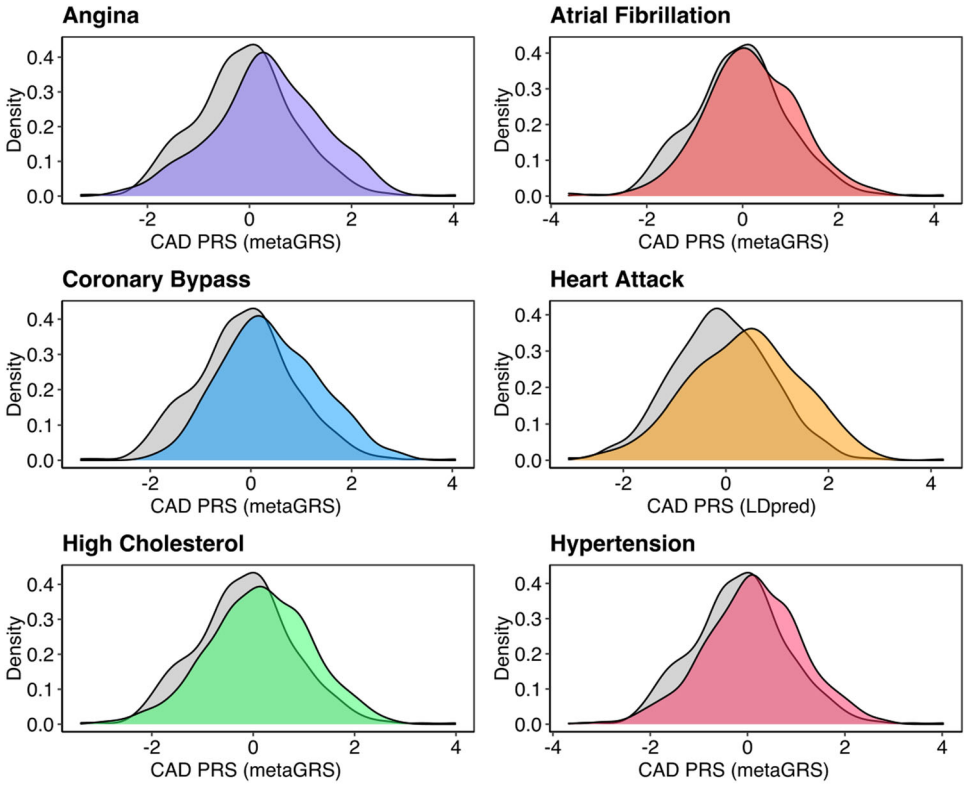

FIGURE 2 Distribution of coronary artery disease (CAD) polygenic risk score (PRS) in self-reported cardiovascular disease cases and health controls. Kernel density estimation plot of the most significantly associated CAD PRS (metaGRS or LDpred, standardised to have SD units) for six cardiovascular disease phenotypes relative to participants who did not self-report any of the phenotypes. For each plot, the cases are coloured, and the controls are grey

diet and the odds of any of the six phenotypes. Of note is that the mean diet quality at 28 points was very low in this population, and considered poor in comparison to a sample of over 93,000 in a cross-sectional population sample where diet quality was calculated using the ARFS via the publicly available website, the Healthy Eating Quiz[46] where the mean was 34, with the subset of 2111 adults aged 65–74 years scoring an average of 37.[47] A SD of eight within this study also highlights the narrow range in diet quality with relatively few considered to have dietary patterns reflecting that which would reduce disease risk. Previous research has shown that ARFS scores higher than those scores seen in the present study are associated with a lower risk of hypertension and non-fatal CVD,[48] and ARFS-vegetable sub-scores were associated with higher HDL cholesterol.[49] The overall poor diet quality observed in the current cohort and the small SD in ARFS scores, may have limited the ability to observe any beneficial effects of diet quality in those with higher PRS.

Currently, over 27,500 Australians die each year from preventable deaths secondary to poor diet quality,[50] whereas 7 million live with at least one diet-related chronic disease.[51] Surprisingly, the current study found that lipid PGSs explained more variance in measured lipids and CAD phenotypes than did diet quality. Considering the current cohort were older adults who, in some cases, had existing CVD and, furthermore, that the mean diet quality scores were relatively low, this may have reduced the ability to detect relationships between diet quality and PRS. Additionally, although genetics did explain more of the variance in the lipid levels, genetics are not amendable to change, whereas diet is. It is possible that diet interventions need to occur earlier, before CVD progression, and this is where Accredited Practising Dietitians (APDs) may make an important contribution in delivery of medical nutrition targeting diet related risk factors. Evidence-based health guidelines recommend medical nutrition therapy (MNT) interventions as first-line treatment for CVD.[52,53] Yet, in 2018–2019, only 1% of eligible Australians had a Medicare (the Australian public health care insurance scheme) funded MNT consult from an APD.[54] Receiving MNT interventions not only improves individual health outcomes, but also counselling by APDs confers annual healthcare savings of $830 to $1893 per patient, with patients taking fewer medications and hospital admissions.[55] This suggests that personalised dietary interventions, targeted to specific lifestage and diet related risk factors, commencing earlier in life and/or triggered by lifestage or risk factor screening programs, are required to cost-effectively reduce diet-related CVD risk.

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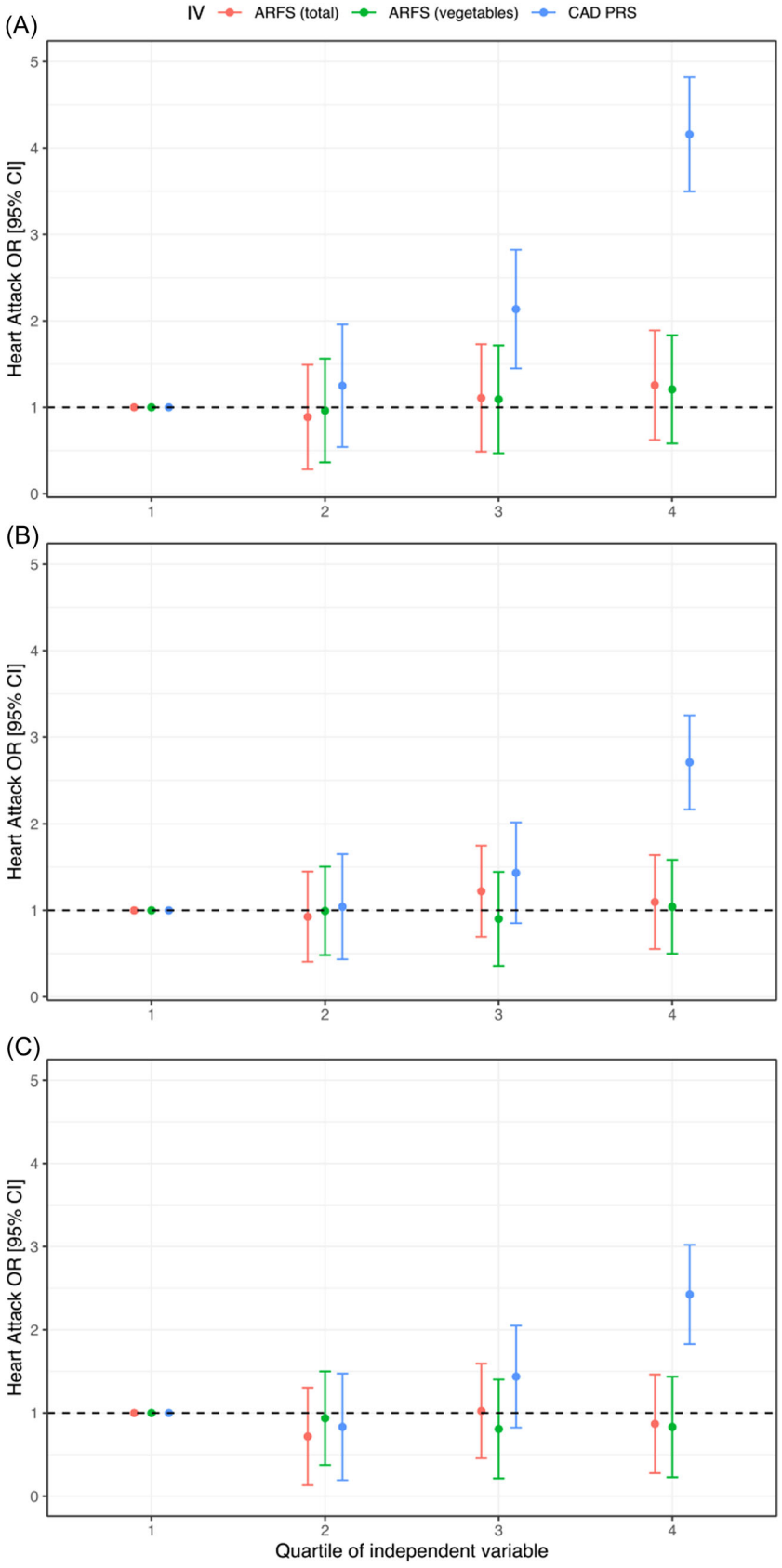

FIGURE 3 Effect size of diet and coronary artery disease polygenic risk score (PRS) per quartile of variable. Points denote the odds ratio (OR) estimate of that quartile relative to the lowest quartile (1, reference category), error bars denote 95% confidence intervals (CIs). Heart attack cases were compared to the following three cohorts: (a) participants with no self-reported binary cardiovascular disease (CVD) phenotypes; (b) all other participants with relevant dietary data available; and (c) participants who self-reported one or more other CVD phenotypes but not heart attack. ARFS, Australian Recommended Food Score, with the total and vegetable specific subscale tested

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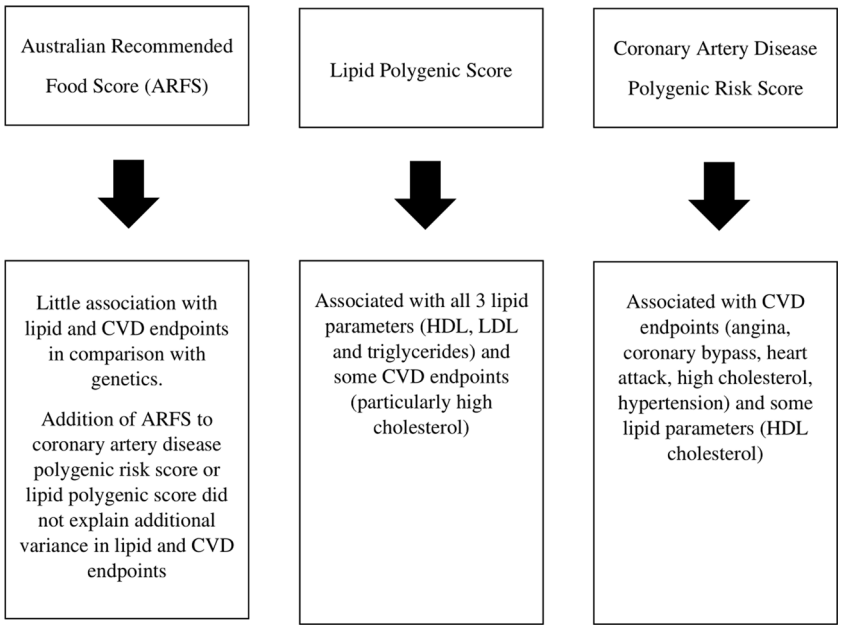

FIGURE 4 Summary of findings. ARFS, Australian Recommended Food Score; CVD, cardiovascular disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein

In the present study, we found that a penalised regression lipid PGS, explained more phenotypic variance in measured HDL and TG levels, whereas a more restricted clumping and thresholding (C + T) approach was optimal for measured LDL. The C + T approach only retains the most confidently associated genetic variants with each lipid biomarker and filters out variants that are statistically likely to be inherited together, such that each variant is independent. By contrast, a penalised regression approach includes more variants from throughout the genome and models, rather than explicitly excluding, co-inherited variants. Both approaches were significantly associated with all three lipid subsets, demonstrating that different methodological approaches for polygenic scoring are useful to investigate, and further work is required to define the most effective types of scores.

Interestingly, the addition of smoking status, educational attainment and statin usage as covariates in addition to age, sex, five SNP derived principal components and ARFS, resulted in an elevated correlation between the LDL PGS and measured LDL, likely because the large effect of statins was not accounted for in the baseline model.

Genetic risk for CAD (CAD PRS) was associated with lower HDL but was not significantly correlated with measured TG or LDL. This further highlights that there is genetic overlap between CAD and plasma lipids. Previous evidence obtained using summary-based data from GWAS supports this relationship, as represented by a genetic correlation between the effect of variants on lipids and on CAD.[56] Further work is required to refine all potential mechanisms that could contribute to this overlap. However, it has been shown in CAD genetic studies that the genetic signal associated with CAD is disproportionately enriched amongst pathways related to lipid transport, metabolism and signalling.[57] Genetic risk for CAD expressed as a PRS was strongly enriched amongst each class of CVD cases, with the exception of atrial fibrillation, indicating that genetic risk for CAD was not as strongly associated as for atrial fibrillation, which may reflect the differing pathophysiology in terms of dietary risk factors. CAD PRS demonstrated the strongest statistical association with coronary bypass, which is not unexpected because it is a more severe CVD phenotype. Additionally, adjusting for diet did not alter the strong enrichment of CAD PRS amongst those with self-reported CAD. The effect size observed when comparing the different CVD phenotypes is partially related to statistical power. Hence, bypass having the largest effect size is a function of that.

Each SD in CAD PRS was associated with a 42.7% increase in the odds of a participant self-reporting a heart attack relative to individuals who self-reported either angina, atrial fibrillation, coronary bypass, high cholesterol or hypertension. This highlights that individuals who self-reported a heart attack appear to carry a higher burden of CAD associated genetic risk, as indexed by a PRS, compared to other participants with a CVD phenotype without heart attack. This supports the idea that a higher genetic risk may also be a marker of severity. Therefore, further clinical research investigating the feasibility of CAD PRS together with traditional risk factors to stratify risk of heart attack is warranted.

Strengths, limitations and future directions

This cohort study included over 3000 individuals, providing a strong sample size to investigate the relative

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contribution of diet quality and PRS with respect to explaining variation in plasma lipids and CAD outcomes. Future research may determine whether providing feedback on genetic predisposition, measured by PRS, leads to improved diet quality, potentially by increasing an individual's motivation to make healthful dietary changes, or not, and, additionally, evaluate whether any strategies to improve diet quality have greater effectiveness if utilised as a preventative strategy for those at highest genetic risk. There are a number of limitations that should be acknowledged. Despite the ARFS previously having a association with reduced medicare costs, and fewer medications and hospital admissions, the diet quality score was not designed to detect negative nutrients, namely nutrients that likely increase CAD risk. A final limitation is the small variability in diet quality score and therefore few individuals at the highest and lowest ends of the score. This may limit our ability to detect a relationship between diet quality and measured lipids or CAD phenotypes.

CONCLUSIONS

Lipid PGSs explained more variance in measured lipids and CAD phenotypes than diet quality. However, the less than average diet quality observed in the current cohort, along with small variance, may have limited the ability to observe any beneficial effects. Future research should investigate whether the diet quality of older adults can be improved, as well as the effect of these improvements in relation to polygenic risk.

AUTHOR CONTRIBUTIONS

Clare Elizabeth Collins, Murray J. Cairns, William R. Reay, John Attia and Rebecca Haslam contributed to project conception. William R. Reay and Murray J. Cairns analysed the data. All authors contributed to data interpretation. William R. Reay and Clare Elizabeth Collins drafted the initial paper. All authors revised and approved the final version of the manuscript submitted for publication.

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ETHICAL STATEMENT

University of Newcastle Human Research Ethics committee (H-820–0504) approved the present study.

TRANSPARENT PEER REVIEW

This paper has undergone a Transparent Peer review.

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internal medicine (MD) and obtained his fellowship with the Royal College of Physicians of Canada and the Royal Australasian College of Physicians. He also obtained a BSc in Physiology (Faculty scholar at McGill University), an MSc in Epidemiology (McMaster University) and a PhD in Molecular Genetics (University of Toronto). From 2008 to 2018, he was founding academic director of general medicine at John Hunter Hospital responsible for the advanced training program, as well as the founding director of the Clinical Research Design, IT, and Statistical Support (CReDITSS) Unit, a unit consisting of two epidemiologists, 10 statisticians, two bioinformaticians and a clinical informatics officer, which provides epidemiological and statistical methodological advice to clinical researchers across the university and health district. He has straddled the university and the health system for his entire academic life; he took up the role of Assistant Dean Research for the Faculty of Health in 2019, and is still active in clinical medicine providing a consult service in Palliative Medicine. He has been awarded > $36 million in competitive research grants in his career to date, and has published > 670 scientific papers. He brings expertise in clinical, molecular and genetic epidemiology with a strong methodological bent, particularly around observational epidemiology and the use of directed acyclic graphs.

Clare Elizabeth Collins, Laureate Professor, is a Fellow of the Australian Academy of Health and Medical Sciences, Nutrition Society of Australia and Dietitians Australia. L/Prof Collins research focusses on personalised nutrition technologies and programs evaluating impact on diet-related health across life stages and chronic disease.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Impact of the *MTHFR C677T* polymorphism on blood pressure and related central haemodynamic parameters in healthy adults

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Abstract

Background: The C677T polymorphism in the gene-encoding methylenetetrahydrofolate reductase (MTHFR) is associated with an increased risk of hypertension and cardiovascular disease. Riboflavin, the MTHFR cofactor, is an important modulator of blood pressure (BP) in adults homozygous for this polymorphism (TT genotype). The effect of this genetic variant on BP and related central haemodynamic parameters in healthy adults has not been previously investigated and was examined in this study.

Methods: Brachial BP, central BP and pulse wave velocity (PWV, SphygmoCor XCEL) were measured in adults aged 18–65 years prescreened for *MTHFR* genotype. Riboflavin status was assessed using the erythrocyte glutathione reductase activation coefficient assay.

Results: Two hundred and forty-two adults with the *MTHFR* 677TT genotype and age-matched non-TT (CC/CT) genotype controls were identified from a total cohort of 2546 adults prescreened for *MTHFR* genotype. The TT genotype was found to be an independent determinant of hypertension ($p = 0.010$), along with low-riboflavin status ($p = 0.002$). Brachial systolic and diastolic BP were higher in TT versus non-TT adults by 5.5 ± 1.2 and 2.4 ± 0.9 mmHg, respectively (both $p < 0.001$). A stronger phenotype was observed in women, with an almost 10 mmHg difference in mean systolic BP in TT versus non-TT genotype groups: 134.9 (95% confidence interval [CI] 132.1–137.6) versus 125.2 (95% CI 122.3–128.0) mmHg; $p < 0.001$. In addition, PWV was faster in women with the TT genotype ($p = 0.043$).

Conclusion: This study provides the first evidence that brachial and central BP are significantly higher in adults with the variant *MTHFR* 677TT genotype and that the BP phenotype is more pronounced in women.

KEYWORDS

blood pressure, haemodynamics, hypertension, methylenetetrahydrofolate reductase, personalised nutrition, riboflavin

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Key points

This is the first study to show that central blood pressure is significantly higher in *younger* adults with the variant *MTHFR* 677TT genotype compared to those with the CC/CT genotypes providing further evidence *for the role* of this genetic risk factor in hypertension. Furthermore, the BP phenotype and pulse wave velocity appear to be more pronounced in women compared to men. Riboflavin, the cofactor for the MTHFR enzyme, was found to be an important modulator of blood pressure, with suboptimal riboflavin status almost doubling the risk of hypertension. These data have relevance for public health given that riboflavin has been shown to lower blood pressure specifically in individuals with the *MTHFR* TT genotype. Given the high prevalence of the *MTHFR* C677T polymorphism globally, these findings have important implications for the prevention and treatment of hypertension in adults worldwide with this genetic risk factor.

INTRODUCTION

Hypertension continues to be a leading contributor to global mortality,[1] with the prevalence estimated to have doubled between 1990 and 2019.[2] Despite a trend towards improved blood pressure (BP) control in high-income countries, control rates remain at 37% in the United Kingdom compared to approximately 50% in the United States and up to 69% in Canada, suggesting that other factors which are currently not being targeted, are at play.[3] Genome-wide association studies have identified a number of genetic loci associated with hypertension, including a locus close to the gene encoding the folate-metabolising enzyme, methylenetetrahydrofolate reductase (MTHFR).[4,5] In addition, strong evidence from epidemiological and clinical studies shows that the common C677T polymorphism in this gene is associated with a 24–87% increased risk of hypertension and up to 40% increased risk for cardiovascular disease (CVD), particularly stroke.[6] Of note, however, randomised controlled trials (RCTs) from this centre conducted in premature CVD patients and in hypertensive adults without overt CVD indicate that this phenotype can be significantly modulated by intervention with low-dose riboflavin, the MTHFR cofactor.[7–9] The influence of other factors such as age and sex on this phenotype is unknown but is relevant given that the homozygosity for the polymorphism (TT genotype) affects 2–32% of the global population[10] and 10–12% of the UK and Irish populations.[11] Recent observational analysis of 6076 adults demonstrated that carrying the variant TT genotype combined with riboflavin deficiency (prevalent in 30% of the cohort) tripled the likelihood of being classed as hypertensive (systolic BP ≥140 and/or diastolic BP ≥ 90 mmHg).[11]

To date, the evidence linking this polymorphism with BP is based mainly on brachial BP as the primary outcome. Central pressure and haemodynamic parameters are additional and potentially superior prognostic markers for CVD risk compared to clinic BP, as surrogate markers of large-artery stiffness.[12,13] Central haemodynamic parameters can be noninvasively, reliably measured and have been widely reported in clinical studies; their use, however, in nutrition research is limited. The influence of the *MTHFR* genotype on central BP and haemodynamics has been confined to a small number of studies in specific patient cohorts, with limited data reported.[14–16] Thus, a comprehensive profile of central haemodynamic parameters in healthy adults stratified by *MTHFR* genotype remains to be investigated.

Male sex is a well-established risk factor for both hypertension and CVD. Hypertension-related mortality is however reported to be higher in women compared to men across all age groups,[17] a finding that may be explained, in part, by the steeper BP trajectories reported in women aged 30 years and persisting throughout the life course.[18] Furthermore, cardiovascular physiology appears to be influenced by sex, and male–female differences in response to hypertension treatment have been reported.[19] There is also some evidence suggesting sex differences in the influence of genetic variance on BP, with reports that genes are differentially expressed or contribute differently to diseases in men versus women.[20] Differential regulation of genes may have important ramifications for health outcomes of pregnancy, with an estimated 10–15% of pregnancies affected by hypertension, which can lead to serious hypertensive disorders of pregnancy, including pre-eclampsia.[6] Although the *MTHFR* 677TT genotype has been linked with an increased risk of hypertension in pregnancy,[6,21] the effect of sex on the role of this common polymorphism in BP has been largely ignored to date.

The primary aim of this observational study, therefore, was to investigate BP and related central haemodynamic parameters in healthy adults aged 18–65 years stratified by the *MTHFR* C677T genotype, and the secondary aim was to consider the effect of sex on this association.

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METHODS

Study design and participants

This study consisted of an observational investigation of brachial, or office, BP, central BP, pulse wave analysis (PWA) and pulse wave velocity (PWV) in healthy adults stratified by the *MTHFR* C677T genotype. Apparently healthy adults aged 18 years and older were recruited from workplaces and the wider community across Northern Ireland as part of the Riboflavin And Folic Acid (RAFA) study. The study has been registered at ClinicalTrials.gov (identifier NCT04948086), and ethical approval was granted by the Ulster University Research and Ethics Committee (UREC/11/0081). All participants provided written informed consent. Inclusion criteria were individuals aged 18 years and older and prescreened for the *MTHFR* genotype, and those who were identified as B-vitamin supplement users, were pregnant or planning a pregnancy were excluded. DNA from 2546 participants was collected using buccal swabs and screened for the *MTHFR* C677T genotype at LGC Genomics using KASP technology. The study design is shown in Figure 1.

BP measurements

Brachial BP was assessed using an Omron 705IT BP monitor and appropriately sized cuff (Cardiac Services, Belfast, UK). A fully trained researcher conducted the measurement consistent with a standard operating procedure and in accordance with NICE Guidelines for Hypertension in the United Kingdom.[22] Briefly, after 10 min at rest, with the participant seated and the arm resting on a table, the reference arm was identified (the arm with the highest BP), and two BP readings taken 5 min apart were used to determine mean BP. If a difference of >5 mmHg was observed between readings, subsequent readings to a maximum of six were taken. Pulse pressure (PP) was calculated as systolic BP minus diastolic BP (mmHg) and mean arterial pressure (MAP) as 1/3 systolic BP plus 2/3 diastolic BP (mmHg).

PWA and PWV

PWA and PWV were assessed noninvasively using the SphygmoCor XCEL device (AtCor Medical, NSW, Sydney, Australia) with the participant in the supine position on a flat examination couch after 5–10 min at rest. Speaking was avoided during the measurements, and participants were asked to abstain from caffeine, smoking, exercise and alcohol for 4 h before the assessment. The same researcher conducted all the measurements.

The central pressure waveform was derived from cuff pulsations recorded at the brachial artery. A brachial cuff was initially inflated to measure brachial systolic and diastolic BP and was reinflated to a subdiastolic pressure to acquire a volumetric displacement signal that automatically captured the PWA waveform for 5 s. A generalised transfer function, built in the manufacturer's software, calculated the aortic waveform. Augmentation pressure was calculated as the difference between the early and late systolic peaks of the estimated central pressure waveform. Augmentation index (AIx), normalised to a heart rate of 75 bpm by the internal software, was calculated as the augmentation pressure as a percentage of central PP. PP amplification was determined as the difference between central PP and brachial PP, and PP ratio was the brachial PP divided by the central PP.

Carotid-femoral PWV was obtained by simultaneous acquisition of the carotid pulse by applanation tonometry (high-fidelity transducer, Millar Instruments, Houston, Texas, USA) and the femoral pulse by volumetric displacement within a cuff placed around the upper

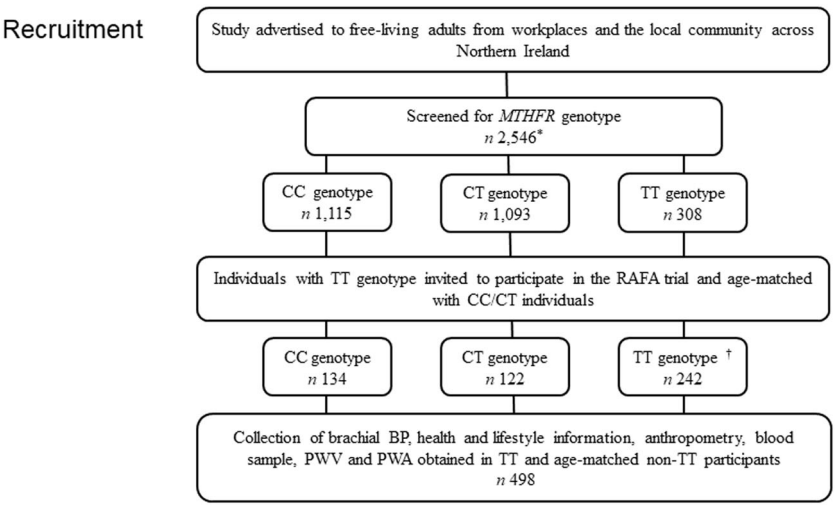

FIGURE 1 Flow chart of study population. *30 swabs deemed unviable and no genotype result returned. †Lost to follow-up; uncontactable (n = 40); declined to participate (n = 26). CC (wild type), CT (heterozygous), TT (homozygous) genotypes for the 677 C → T polymorphism in *MTHFR*. BP, blood pressure; *MTHFR*, methylenetetrahydrofolate reductase; PWA, pulse wave analysis; PWV, pulse wave velocity; RAFA, Riboflavin And Folic Acid trial.

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thigh. PWV was calculated as the distance travelled over the time taken. Distance between the recording sites, in millimetres, was calculated using the subtraction method, that is, the distance between the suprasternal notch and the carotid pulse palpation point subtracted from the distance between the suprasternal notch and the proximal edge of the femoral cuff, using a nonstretch measuring tape. Transit time was determined using the 'foot-to-foot' method. Calibration was performed using the systolic and diastolic BP obtained by the brachial cuff in PWA.

Questionnaire and anthropometric measurements

Details of the participant's health, including medications and family history of CVD, were obtained. Dietary information on habitual intake of specified foods (sources of B-vitamins, including milk and fortified breakfast cereals) was collected using a researcher-assisted food frequency questionnaire, previously validated for B-vitamin intake against B-vitamin biomarkers.[23] Participants also had their weight (kg; SECA 770 scales), height (m) and waist circumference (cm) measured.

Blood sampling and laboratory analysis

A 25-ml nonfasting blood sample was collected from participants during the appointment by a trained phlebotomist into four separate blood collection tubes: 9- and 4-ml EDTA vacutainers and 8- and 4-ml serum vacutainers as described elsewhere.[24] All blood tubes were placed on ice packs, processed within 4 h and stored at −80°C for batch analysis at the end of the study. Riboflavin status was determined at Ulster University by erythrocyte glutathione reductase activation coefficient (EGRac) assay. Oxidised glutathione was added and converted to reduced glutathione using NADPH as the reducing agent. This conversion was catalysed by glutathione reductase and mediated by flavin adenine dinucleotide (FAD), with the rate of absorbance measurable at a wavelength of 340 nm. EGRac was calculated by comparing the rate of absorbance change with added FAD compared to the rate of absorbance change without added FAD, with an EGRac ratio ≤1.26 indicating optimal riboflavin status, >1.26–<1.4 indicating suboptimal status and ≥1.4 signifying riboflavin deficiency.[25] Analysis was conducted on a Daytona+ clinical chemistry analyser (Randox Laboratories Ltd, Antrim, Northern Ireland). Quality controls (QCs) were provided by repeated analysis of pooled samples covering a wide range of values. The inter-assay variation was 2% for the high-riboflavin QC and 3% for the low-riboflavin QC.

Power calculation

An estimation of sample size was based on observational differences in mean value and variability of 24-h ambulatory BP between the *MTHFR* genotype groups from research previously conducted at this centre (McMahon et al., unpublished). An estimated sample size per group (i.e., 245 individuals with the TT genotype and 245 with the non-TT group) was estimated with a power of 80% and $\alpha = 0.05$ using G* power.[26]

Statistical analysis

All statistical analysis was performed using SPSS Statistical Package for Social Sciences (version 25.0). Variables were tested for normality before analysis, and data were log transformed before analysis where appropriate. Differences between general characteristics were analysed using independent samples *t* tests. For outcome measures, differences between groups (non-TT vs. TT) for continuous data were analysed by ANCOVA, controlling for age, sex, body mass index (BMI), use of antihypertensive medications and consumption of fortified breakfast cereals. Differences between categorical groups were analysed by χ^2 analysis. Logistic regression analysis was performed to examine the association between *MTHFR* genotype and the risk of hypertension after adjustment for established risk factors. $p < 0.05$ was considered statistically significant.

RESULTS

General characteristics

The study was advertised to an estimated 11,000 free-living individuals from workplaces and the local community across Northern Ireland, from which 2546 individuals were recruited and provided a buccal swab to collect DNA to enable screening for the *MTHFR* genotype. This analysis yielded a prevalence of 44.3% with the CC genotype, 43.4% with the CT genotype and 12.2% with the TT genotype for the C677T polymorphism. Of those identified ($n = 308$) as having the TT genotype, 242 agreed to participate in this observational study (40 individuals with the TT genotype were uncontactable, and 26 declined to participate in the study) and were age matched with 134 CC and 122 CT genotype adults, yielding a total available sample of 498 study participants (Figure 1). The general characteristics of the *MTHFR* genotype are presented in Table 1. Participants had a mean age of 45.6 years and mean BMI in the overweight category, with 22.7% classified as normal weight, 43% as overweight and 34.3% as obese. The majority of participants (>90%) were regular milk consumers and reported to consume B-vitamin-fortified

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TABLE 1 General characteristics of study population by *MTHFR* genotype (n = 498)

	***MTHFR* genotype**		
	Non-TT (n = 256)	**TT (n = 242)**	***p*-value***
Age (years)	45.5 (9.8)	44.8 (11.1)	0.311
Male sex *n* (%)	162 (63)	138 (57)	0.182
BMI (kg/m^2)	28.9 (5.3)	29.1 (5.7)	0.902
Diabetes mellitus *n* (%)	3 (1)	9 (4)	0.119
Smoker *n* (%)	28 (11)	32 (13)	0.519
Alcohol consumer *n* (%)	192 (75)	195 (81)	0.165
Fortified food consumer[a] *n* (%)	197 (77)	172 (71)	0.213
Milk consumer *n* (%)	233 (91)	227 (94)	0.317
Family history CVD *n* (%)	119 (47)	89 (37)	0.035
Statin use *n* (%)	20 (8)	15 (6)	0.597
Hypertension[b] *n* (%)	76 (30)	97 (40)	0.019
Antihypertensive medication use *n* (%)	41 (16)	35 (15)	0.721
Treated and controlled[c]	26 (63)	16 (46)	0.188
Riboflavin status			
EGRac (biomarker)	1.33 (0.17)	1.36 (0.17)	0.057
Optimal (<1.26) *n* (%)	126 (52)	103 (44)	0.203
Suboptimal (1.26–1.40) *n* (%)	52 (21)	54 (23)	
Deficient (≥1.40) *n* (%)	65 (27)	77 (33)	

Note: Values are mean (SD) unless otherwise stated.

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; EGRac, erythrocyte glutathione reductase activation coefficient (a marker of riboflavin status where lower EGRac values indicate better riboflavin status); *MTHFR*, methylenetetrahydrofolate reductase (CC; wild type, CT; heterozygous, TT homozygous); SD, standard deviation.

[a]Participants who consumed foods fortified with B-vitamins at least once per week.

[b]Hypertension classified as systolic BP ≥ 140 mmHg and/or diastolic ≥90 mmHg.[39]

[c]Those taking antihypertensive medications and achieving BP <140/90 mmHg.

*p-Values refer to differences between genotype groups compared using an independent samples t test. χ^2-test is used for comparison between categorical variables. $p < 0.05$ is considered significant.

foods (>70%). There was a higher prevalence of hypertension (BP ≥140/90 mmHg) in those with the TT genotype. Also, despite similar rates of antihypertensive medication use across genotype groups, the TT genotype group was less likely to achieve BP control on treatment, albeit this failed to reach statistical significance. Riboflavin status, determined by the functional biomarker EGRac, was similar across the groups. Overall, the riboflavin status of 48.0% of the cohort was classed as optimal, 22.2% as suboptimal and 29.8% as deficient. There were no other significant differences in general characteristics between genotype groups. A detailed breakdown of antihypertensive drug use and drug combinations by genotype group is provided in Supporting Information, Table 1.

Determinants of hypertension

In this study cohort, the TT genotype was associated with a 71% increased risk of hypertension (odds ratio [OR], 1.71, 95% confidence interval [CI]: 1.14–2.56, $p = 0.010$) after adjustment for significant predictors of BP, namely age (OR: 1.04, 95% CI: 1.02–1.07, $p < 0.001$), male sex (OR: 2.10, 95% CI: 2.36–3.23, $p = 0.001$), BMI (OR: 1.06, 95% CI: 1.02–1.10, $p = 0.005$), antihypertensive drug use (OR: 1.07 95% CI: 0.61–1.87, $p = 0.818$) and a family history of CVD (OR: 1.13, 95% CI: 0.74–1.72, $p = 0.577$; Table 2). Suboptimal riboflavin status, as indicated by EGRac >1.26,[25] was also associated with an increased risk of hypertension in the same model, OR: 1.97, 95% CI: 1.27–3.05, $p = 0.002$, independent of the *MTHFR* genotype. Regression analysis split by sex revealed that the genotype effect on hypertension appeared to be driven by women (OR: 2.57, 95% CI: 1.24–5.32, $p = 0.011$) compared with a nonsignificant effect in men (OR: 1.51, 95% CI: 0.91–2.50, $p = 0.110$) (Table 2).

BP and central haemodynamic parameters

Brachial systolic and diastolic BP were significantly higher in the TT compared to the non-TT genotype groups (CT, CC) by 5.5 ± 1.2 and 2.4 ± 0.9 mmHg, respectively (Table 3). In addition, brachial MAP was significantly higher in the TT relative to the non-TT genotype groups by 3.5 ± 0.9 mmHg. This pattern of elevated brachial pressure in the TT genotype group was evident across the age range of the study (18–65 years), as shown in Figure 2. Similar patterns of elevated pressure in the TT compared to the non-TT groups were observed, with significant differences between genotype groups in central systolic BP (by 3.1 ± 1.0 mmHg), central diastolic BP (by 1.9 ± 0.8 mmHg), MAP (by 2.5 ± 0.9 mmHg) and PP (by 1.4 ± 0.6 mmHg) observed (Table 3). No genotype effect was evident on measures of PWA or PWV in the cohort as a whole (n = 498). When the cohort was split according to normotensive/hypertensive status, the phenotype of elevated BP was still evident. Systolic BP was significantly higher in the normotensive and hypertensive TT individuals when compared to the non-TT genotype groups (by 1.8 ± 1.3 and 4.1 ± 2.9 mmHg, respectively). In addition, brachial MAP was still significantly higher in the normotensive TT individuals relative to the non-TT genotype groups. None of the other parameters were statistically significant.

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TABLE 2 Factors associated with risk of hypertension in study cohort as a whole and stratified by sex

	Cohort as a whole ($n = 498$)				Men ($n = 300$)				Women ($n = 198$)			
	β	OR	95% CI	p*	β	OR	95% CI	p*	β	OR	95% CI	p*
Age (years)	0.041	1.04	1.02–1.07	<0.001	0.030	1.03	1.00–1.06	0.028	0.068	1.07	1.03–1.11	0.001
Male sex	0.740	2.10	2.36–3.23	0.001								
Body mass index (kg/m^2)	0.055	1.06	1.02–1.10	0.005	0.066	1.07	1.01–1.13	0.019	0.038	1.04	0.98–1.10	0.176
Antihypertensive drug use	0.066	1.07	0.61–1.87	0.818	0.244	1.28	0.64–2.53	0.484	−0.453	0.64	0.22–1.84	0.403
Family history of CVD	0.120	1.13	0.74–1.72	0.577	0.294	1.34	0.80–2.23	0.271	−0.277	0.76	0.37–1.57	0.455
Low riboflavin status[a]	0.677	1.97	1.27–3.05	0.002	0.647	1.91	1.13–3.23	0.016	0.664	1.94	0.88–4.31	0.102
MTHFR TT genotype[b]	0.535	1.71	1.14–2.56	0.010	0.411	1.51	0.91–2.50	0.110	0.945	2.57	1.24–5.32	0.011

Note: Hypertension is defined as systolic BP ≥140 and/or a diastolic BP ≥90 mmHg.

Abbreviations: BP, blood pressure; CI, confidence interval; CVD, cardiovascular disease; OR, odds ratio.

[a]Riboflavin biomarker status is determined by the functional assay, erythrocyte glutathione reductase activation coefficient (EGRac). Participants were arbitrarily classed as having 'lower' or 'higher' riboflavin status using an EGRac value of 1.26 as a cut-off point: lower riboflavin status (EGRac <1.26) was compared to higher riboflavin status (EGRac <1.26; reference category).

[b]Non-TT (CC, wild type; CT, heterozygous), TT (homozygous), genotypes for the 677 C → T polymorphism in MTHFR; reference category is non-TT genotype.

*Data analysed by multiple logistic regression with adjustment for other factors in the model. $p < 0.05$ is considered significant.

TABLE 3 Blood pressure and central haemodynamic profile of study population by *MTHFR* genotype (n = 498)

	MTHFR genotype		
	Non-TT ($n = 256$)	TT (n = 242)	p-value*
Brachial pressure			
Systolic BP (mmHg)	130.6 (128.9, 132.3)	136.1 (134.4, 137.8)	<0.001
Diastolic BP (mmHg)	79.6 (78.5, 80.7)	82.1 (80.9, 83.2)	0.003
MAP[a] (mmHg)	96.6 (95.4, 97.8)	100.1 (98.8, 101.3)	<0.001
Pulse pressure[b] (mmHg)	51.0 (49.7, 52.3)	54.0 (52.7, 55.4)	0.002
Central pressure			
Systolic BP (mmHg)	117.8 (116.4, 119.1)	120.9 (119.6, 122.3)	0.001
Diastolic BP (mmHg)	78.2 (77.1, 79.2)	80.1 (79.0, 81.2)	0.011
MAP[b] (mmHg)	93.3 (92.1, 94.5)	95.8 (94.5, 97.0)	0.004
Pulse pressure[b] (mmHg)	39.0 (38.2, 39.8)	40.4 (39.5, 41.2)	0.064
Pulse wave analysis			
Augmentation pressure (mmHg)	9.0 (8.3, 9.6)	9.6 (9.0, 10.2)	0.112
Augmentation index (%)	22.5 (21.3, 23.7)	23.2 (21.9, 24.4)	0.448
PP amplification	14.6 (14.2, 15.1)	14.7 (14.2, 15.2)	0.791
PP ratio	1.38 (1.37, 1.40)	1.38 (1.36, 1.39)	0.332
Pulse wave velocity			
PWV (m/s)[c]	7.47 (7.32, 7.61)	7.59 (7.44, 7.74)	0.201

Note: Data are presented as adjusted means (95% CI). All units given as mmHg, unless otherwise stated.

Abbreviations: ANCOVA, analysis of covariance; BP, blood pressure; MAP, mean arterial pressure; PP, pulse pressure; PWV, pulse wave velocity.

[a]Mean arterial pressure is calculated as 1/3 systolic BP plus 2/3 diastolic BP.

[b]Pulse pressure is calculated as systolic BP minus diastolic BP.

[c]For PWV: CC, n = 131; CT, n = 120; TT, n = 236; it was not possible to obtain a measurement of adequate quality in 11 subjects due to attenuation of the pulse signal by subcutaneous fat or difficulty in accessing the position of the artery.

*One-way ANCOVA adjusting for age, sex, BMI, use of antihypertensive medications and fortified breakfast cereal consumption with Bonferroni post hoc analysis. $p < 0.05$ is considered significant.

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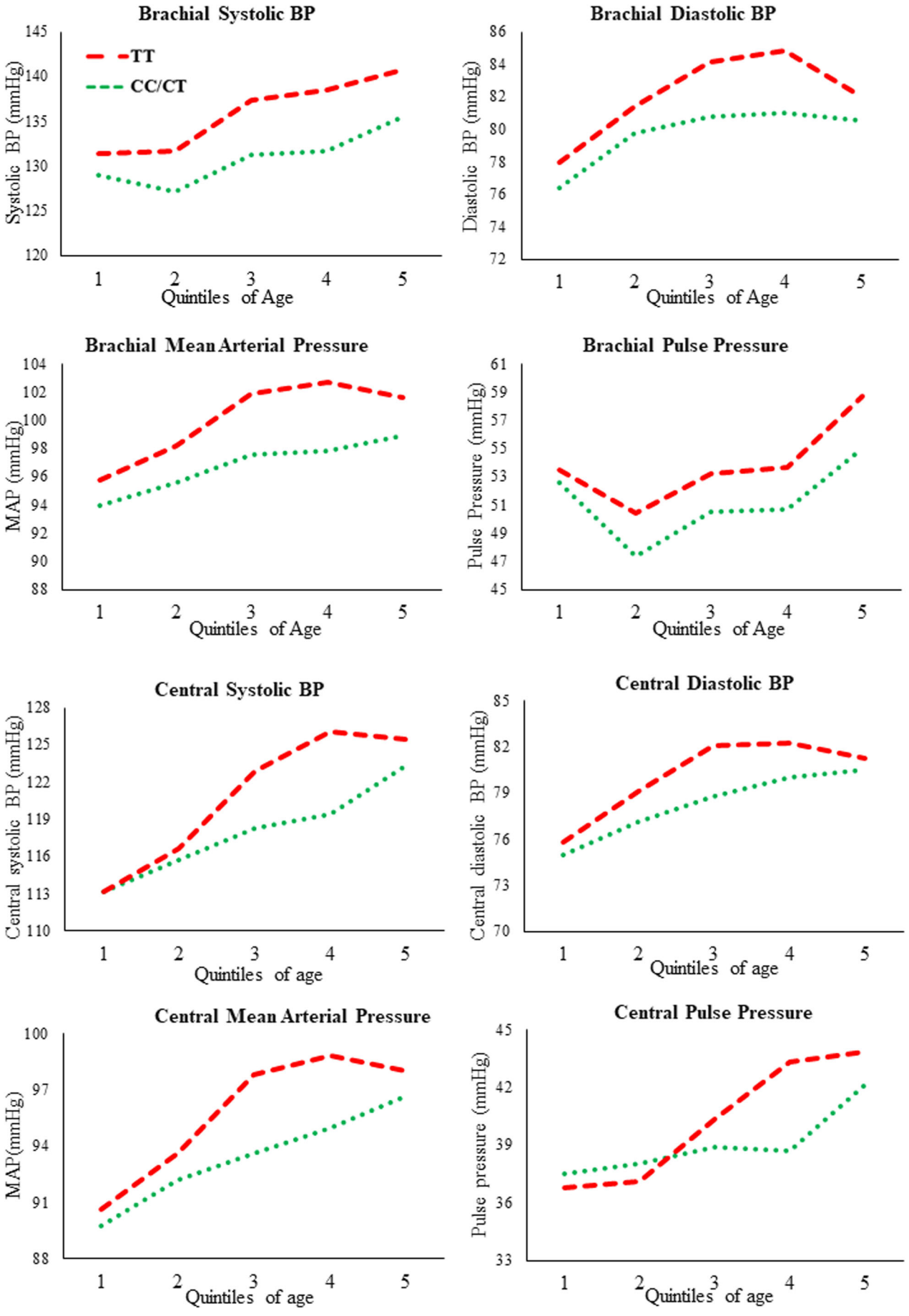

FIGURE 2 (See caption on next page)

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Effect of sex on BP and central haemodynamic parameters

When stratified by sex, the effect of the TT genotype on brachial systolic BP remained evident in both sexes (Supporting Information, Table 2). The phenotype was, however, more pronounced among women, with 9.7 ± 2.0 mmHg higher systolic BP ($p < 0.001$) and 3.4 ± 1.2 mmHg ($p = 0.005$) higher diastolic BP in women with the TT compared to the non-TT genotypes. Women with the TT genotype were also twice as likely to be classed as hypertensive (i.e., BP ≥ 140/90 mmHg) compared to non-TT women, 37 versus 18%, respectively ($p = 0.006$, data not shown). For central pressure, the genotype effect was again most pronounced in women, with central systolic BP higher by 5.5 ± 1.7 mmHg ($p = 0.002$) and central diastolic BP higher by 3.7 ± 1.3 mmHg ($p = 0.004$) in the TT compared to the non-TT genotype women (Supporting Information, Table 2).

When stratified by sex, a genotype effect was observed in some, but not all, PWA measures. Both augmentation pressure and AIx were significantly higher in men with the TT compared to the non-TT genotypes by 1.2 ± 0.6 mmHg ($p = 0.034$) and $2.3 \pm 1.2\%$ ($p = 0.049$), respectively. PWV was significantly faster in women with the TT compared to the non-TT genotypes by 0.36 ± 0.16 m/s ($p = 0.043$).

DISCUSSION

We investigated the influence of the *MTHFR* C677T polymorphism on BP and related central haemodynamic parameters in adults aged 18–65 years and observed elevated brachial BP in individuals with the variant *MTHFR* 677TT genotype when compared to those with CC/CT genotypes. Our study provides the first evidence of higher central BP in apparently healthy adults with the TT genotype. Furthermore, our findings indicate that the BP phenotype (both brachial and central BP) associated with this polymorphism was more pronounced in women compared to men. Importantly, suboptimal status of the B-vitamin riboflavin, the MTHFR cofactor, led to an almost doubling in the risk of hypertension.

In the current study, we report consistently higher brachial systolic and diastolic BP, in addition to MAP and PP, in those homozygous for the common *MTHFR* C677T polymorphism. The higher systolic BP observed in this sample of healthy adults is clinically relevant[27] and is consistent with our recent observational evidence from over 6000 adults, where we demonstrated that the variant *MTHFR* 677TT genotype is associated with a 42% increased risk of hypertension and predisposes an individual to higher systolic and diastolic BP across adulthood.[11] The only other study to have investigated the interaction between this polymorphism and BP in a healthy cohort (as opposed to hypertensive or CVD patients) was a study of Japanese men aged 40–59 years; however, the study was limited by a small sample size, with only 14 of the 129 participants carrying the TT genotype.[28] To the best of our knowledge, this is the first study to report an association between the *MTHFR* C677T polymorphism and PP. PP has recently been associated with all-cause and CVD mortality in a 6-year follow-up of 13,223 Chinese adults aged <65 years,[29] and although the *MTHFR* genotype was not considered, there is a high frequency of the *MTHFR* 677TT genotype in the Chinese population. Of particular interest in the current study of healthy younger adults, central pressure, as assessed using a range of measures (systolic, diastolic, MAP and PP), was also found to be higher in the variant TT relative to the non-TT genotype groups, with a significant 3-mmHg genotype difference observed in central systolic BP. To put this in context, one meta-analysis of older participants found that a 10-mmHg increase in central systolic BP was associated with a 9% increased risk of total cardiovascular events.[13] Furthermore, in one of the studies included in the latter meta-analysis, the Strong Heart Study involving 2403 participants, a 6-mmHg higher central PP was found to predict cardiovascular events more strongly than brachial PP, leading the authors to propose central BP as a treatment target for future studies.[30]

PWV, the gold standard measurement of arterial stiffness for predicting future cardiovascular events and mortality[12,13] and previously associated with BP,[31] did not differ significantly between the *MTHFR* groups in the overall sample in the current study. Although this finding is contrary to what we had hypothesised, it is consistent with results from three earlier studies in healthy younger[14,32] and older adults[15] stratified by the *MTHFR* genotype. When split by sex, however, our results showed for the first time significantly faster PWV among women with the TT compared to the non-TT genotypes. Because PWV is strongly related to future cardiovascular events and mortality,[12,13] the findings of the current study provide preliminary evidence that women with the TT genotype may be at particular risk

FIGURE 2 Brachial blood pressure profiles of study participants across quintiles of age,[1] stratified by *MTHFR* genotype (n = 498; CC/CT genotype, n = 256; TT genotype, n = 242). Data are presented as mean values. (a) Systolic BP; (b) diastolic BP; (c) mean arterial pressure (MAP); and (d) pulse pressure.[1] Quintiles of age are as follows: <35 years (youngest), 35–42 years, 43–49 years, 50–54 years, >54 years (oldest). MAP is calculated as 1/3 systolic BP plus 2/3 diastolic BP. Pulse pressure is calculated as systolic BP minus diastolic BP.

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of CVD. Given that the current analysis is based on a relatively small sample of women ($n = 198$), further investigations in larger cohorts of women are required before firm conclusions can be drawn. The largest study conducted to date investigating PWV with respect to the *MTHFR* genotype is a substudy of the China Stroke Primary Prevention Trial (CSPPT),[16] an RCT investigating the effect of 0.8-mg folic acid plus enalapril versus enalapril alone in reducing the occurrence of first stroke in hypertensive Chinese patients, stratified by the *MTHFR* genotype.[33] Similar to the findings of this study, a substudy analysis in CSPPT of 2529 participants reported no difference in PWV between the genotype groups at baseline; however, after the 5-year intervention period, folic acid was found to reduce PWV, an effect that was greatest in the CC genotype group.[16] The authors suggested that those with the TT genotype had a higher mechanistic requirement for folic acid; however, the CSPPT did not consider the status of the MTHFR cofactor, riboflavin, which could arguably have had a greater phenotype effect or influenced the response to intervention with folic acid.

Male sex is an important risk factor for both hypertension and CVD; however, it is not widely appreciated that CVD-related mortality rates are, in fact, higher in women compared to men.[17,19] A secondary objective of the current study was to examine the effect of sex on BP parameters as this area is largely under-investigated. In the present study, sex was found to influence both brachial and central pressure, with the greatest phenotype for both measures observed in women. A marked difference of 9.4-mmHg higher systolic BP was observed in the TT compared to the non-TT women; a BP difference of this magnitude can be estimated to equate with a 50% higher risk of CVD.[27] Brachial and central systolic BP are reported to be higher in men compared to women until age 60 years[34]; however, in the current study, both brachial and central BP in the female TT genotype group were similar to BP values measured in the TT and non-TT genotype men. Although not a primary outcome of the Cardiovascular Risk in Young Finns Study,[32] the authors reported BP by the *MTHFR* genotype in 1400 young Finnish adults and observed no significant genotype effect; however, only 5.4% of the cohort carried the variant TT genotype, and thus, the study was likely underpowered to detect significant differences. If the phenotype is indeed stronger in women with the variant genotype, it may have important implications for pregnancy. Meta-analyses have previously linked this polymorphism with an increased risk of hypertension in pregnancy[35] and pre-eclampsia,[36] which affects up to 15% of pregnancies globally and can lead to increased CVD risk in later life for the mother.[6] Further studies should thus aim to consider the influence of the *MTHFR* genotype on BP and vascular health in pregnancy and in women of reproductive age generally.

Riboflavin was found to be an important modulating factor for BP in this cohort of younger, healthy adults, as suboptimal riboflavin status almost doubled the risk of hypertension after adjustment for other well-established risk factors. We previously reported an exacerbated genetic risk of hypertension in adults with low or deficient biomarker riboflavin, with a three-fold risk of hypertension observed for the TT genotype in combination with deficient riboflavin status.[11] Furthermore, RCTs conducted at this centre have demonstrated that BP is highly responsive to riboflavin supplementation at doses within the dietary range, specifically in adults with the *MTHFR* 677TT genotype. Our trials demonstrated significant reductions of 6–14 mmHg in systolic and 3–8 mmHg in diastolic BP in response to riboflavin intervention, indicating a novel, personalised nutrition approach for the treatment for hypertension in these genetically at-risk individuals.[7–9] None of these RCTs considered the effect on PWV or alternative measures of vascular health; therefore, further trials to examine the effect of riboflavin intervention on PWV in adults with the TT genotype are needed. The precise mechanism linking the common *MTHFR* C677T polymorphism with hypertension and the modulating effect of riboflavin on BP in these genetically at-risk individuals remains unexplained but is likely to involve the potent vasodilator, nitric oxide (NO).[6,37] Both the *MTHFR* genotype and 5-methyltetrahydrofolate (5-MTHF), the folate co-factor generated by the MTHFR-catalysed reaction, have previously been associated with NO bioavailability in biopsy samples from patients undergoing coronary artery bypass graft (CABG) surgery.[38] In CABG patients, vascular tissue levels of 5-MTHF were shown to be lower in adults with the TT genotype (likely as a result of reduced MTHFR activity), which would subsequently affect NO bioavailability and BP. Although not considered in the current study, a noninvasive assessment of endothelial function, such as flow-mediated dilation, would provide valuable information to help further our understanding of this potential mechanism.

Strengths and limitations

This study has a number of strengths. It is the largest study of its kind to investigate the effect of the common *MTHFR* C677T polymorphism on both brachial and central pressure. Recruitment was genotype driven and investigated an apparently healthy cohort aged 18–65 years. A comprehensive noninvasive investigation of vascular health, including PWV, considered to be the gold standard noninvasive measurement for arterial stiffness, was conducted; to the best of our knowledge this is the first time these measures have been reported in a sample stratified by *MTHFR* genotype and sex. Furthermore, all vascular measures were conducted by

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the same researcher to minimise inter-operator variability. The study was, however, not without limitations. Although the study was powered to investigate the effect of genotype on BP parameters, it was not powered for the secondary analysis for sex effects, and therefore, future studies are required to confirm the current findings with regard to sex differences. In addition, further biomarker data, in particular measures of folate and other B-vitamins and intermediates of the one-carbon network of pathways, were not available but could provide further insights into the role of one-carbon metabolism and this gene–nutrient interaction in BP. Future studies with additional measures of endothelial function, such as flow-mediated dilation, could provide additional insights into the relationship between the TT genotype and vascular function and related effects on NO bioavailability.

CONCLUSION

In conclusion, this is the first study to show that both brachial and central BP are significantly higher in healthy adults aged up to 65 years with the variant *MTHFR* 677TT genotype and that the BP phenotype and effect on PWV are more pronounced in women. Moreover, low biomarker status of riboflavin was found to be independently associated with hypertension. Given the high prevalence of the *MTHFR* C677T polymorphism globally, these findings have important implications for the prevention and treatment of hypertension in adults worldwide with this genetic risk factor.

AUTHOR CONTRIBUTIONS

All authors contributed equally.

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CONFLICTS OF INTERESTS

The authors declare that they have no conflict of interest.

PEER REVIEW

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