THE EFFECT OF A SINGLE NUCLEOTIDE POLYMORPHISM OF THE *CYP4F2* GENE ON BLOOD PRESSURE AND 20-HYDROXYEICOSATETRAENOIC ACID EXCRETION AFTER WEIGHT LOSS

Natalie C WARD^a, Kevin D CROFT^a, Ian B PUDDEY^a, Michael PHILLIPS^c, Frank VAN BOCKXMEER^B, Lawrence J BEILIN^a, and Anne E BARDEN^a

^aUniversity of Western Australia, School of Medicine & Pharmacology, Royal Perth Hospital, and ^bSchool of Surgery and Pathology and ^cWestern Australian Institute for Medical Research, University of Western Australia, Perth, Australia

Short Title: Genetic differences in BP responses to weight loss

Funding: This study was funded from a National Health and Medical Research Council of Australia Project Grant #634359

Conflict of Interest: None of the authors has any conflict of interest

Word Count: 4765

Tables=5

Figures=2

Author for Correspondence: Professor Anne Barden

School of Medicine & Pharmacology,

University of Western Australia

GPO Box X2213

Perth, 6847, Australia

Phone 618 9224 0272

Fax 618 9224 0246

Email: anne.barden@uwa.edu.au

ABSTRACT

Background: Genetic background partly determines the efficacy of interventions to lower BP. The CYP4F2 and CYP4A11 enzymes are renal 20-HETE synthases that regulate BP. Gene variants of *CYP4F2* and *CYP4A11* associate with hypertension and stroke. We showed that a gene variant of CYP4F2 but not *CYP4A11* associated with increased 20-HETE excretion and BP.

Aim: To compare BP and 20-HETE responses in carriers of the *CYP4F2* 1347 G/A polymorphism and controls *CYP4F2*-GG (wildtype), during weight loss.

Methods: Volunteers genotyped as *CYP4F2*-GA/AA (n=26) and controls genotyped as *CYP4F2* GG (n=27), were counselled to reduce weight for 12 weeks, followed by 4 weeks weight stabilisation. Weight, 24 hour BP, pulse pressure, and urinary 20-HETE were measured at baseline, 12 and 16 weeks.

Results: At baseline, SBP was (+1.7mmHg, $P=0.047$) in the *CYP4F2* GA/AA genotype. Compared with baseline, weight fell by 3.9kg, $P=0.0001$, in both genotypes, and was maintained to 16 weeks. SBP fell by (-7.6mmHg, $P=0.004$) in both genotypes after 12 weeks. However, after weight stabilisation SBP was +3.6mmHg, $P=0.004$ in *CYP4F2* GA/AA genotype. DBP and heart rate changed similarly over time. Pulse pressure fell with weight loss ($P<0.001$), but was elevated in the *CYP4F2* GA/AA genotype at all time-points (+3.1mmHg, $P<0.001$). Urinary 20-HETE was similar at baseline and 12weeks but elevated in the *CYP4F2* GA/AA genotype ($P=0.017$) after weight stabilisation.

Conclusion: Maintenance of lower blood pressure after weight loss is more difficult for carriers of the *CYP4F2* G1347A polymorphism and may be related to increased arterial stiffness and increased 20-HETE synthesis.

Key words: Cytochrome P450, *CYP4F2* V433M, 20-HETE, weight loss, weight stabilisation, pulse pressure

INTRODUCTION

Arachidonic acid (AA) can be metabolised by a number of different enzyme systems including cyclooxygenase, lipoxygenase and the cytochrome P450 to a range of bioactive metabolites. Enzymes of CYP4A, 4B and 4F families catalyse ω -hydroxylation of fatty acids. Several isoforms produce 20-hydroxyeicosatetraenoic acid (20-HETE) when incubated with arachidonic acid. There is accumulating evidence that 20-HETE is involved in the pathogenesis of hypertension [1]. 20-HETE is a vasoconstrictor but also inhibits renal sodium reabsorption causing a natriuresis [2]. Therefore the effect of 20-HETE on blood pressure can be complicated and dependent on the site of synthesis. The efficacy of interventions to lower blood pressure varies significantly between individuals and is partly determined by genetic background.

The CYP4F2 and CYP4A11 enzymes function as renal 20-HETE synthases in humans [3, 4]. In human renal microsomes the CYP4F2 isoform accounts for ~70% of the 20-HETE production [3]. A guanine to-adenine missense transition at nucleotide (nt) 1347 on the *CYP4F2* gene results in a nonsynonymous valine-to-methionine amino acid substitution at residue 433. In an *in vitro* study transfection of this single nucleotide polymorphism into Sf9 insect cells using recombinant baculoviruses resulted in a 50% reduction in 20-HETE production [3]. A functional polymorphism has also been described for *CYP4A11* gene. The polymorphism is a thymidine-to-cytosine missense transition at nucleotide 8590 that results in a nonsynonymous phenylalanine-to-serine amino acid substitution at residue 434. This substitution has little effect on the enzyme's affinity for arachidonic acid but does reduce the enzymatic activity by ~50% *in vitro* and has been associated with hypertension in a white, but not African American, population [4].

These two common functional polymorphisms *CYP4A11* T8590C (also referred to as *CYP4A11* F434S) and the *CYP4F2* G1347A (*CYP4F2* V433M) have been associated with increased blood pressure in population studies and have been postulated to act by altering 20-HETE levels [3, 5]. In a study of more than 200 normotensive and hypertensive men and women, we showed that the A allele of the *CYP4F2* G1347A polymorphism was associated with increased urinary 20-HETE excretion and elevated systolic blood pressure while the C-allele of the *CYP4A11* T5890C polymorphism was associated with reduced urinary 20-HETE. We have previously shown that overweight subjects with features of the metabolic syndrome had higher plasma and urinary 20-HETE excretion compared with their case controls [6]. This study examined the effect of the G1347A polymorphism of *CYP4F2* on blood pressure and plasma and urinary 20-HETE in response to weight loss in overweight men and women. Although the *CYP4A11* 5890 T/C polymorphism has been associated with elevated blood pressure, we observed that the C-allele for the *CYP4A11* 5890 T/C had opposing effects on urinary 20-HETE compared with the A-allele for *CYP4F2* 1347 G/A. For this reason C-allele carriers of the *CYP4A11* 5890 T/C polymorphism were excluded from the study. We hypothesised that a similar degree of weight loss in carriers of the G1347A polymorphism of *CYP4F2* would result in a greater fall in blood pressure and urinary 20-HETE than non-carriers.

STUDY DESIGN AND METHODS

Participant Recruitment

Men aged between 20 and 70 years and post-menopausal women less than 70 years were recruited from the general population by newspaper advertisement. They were telephone screened and invited to attend our research unit for further screening if they reported being overweight, had no known chronic diseases and were not taking medication for hypertension or lipid disorders.

Screening

At the first screening visit height, weight and waist circumference were measured. Volunteers completed a lifestyle questionnaire, which included details of medical history, any medication usage and alcohol consumption. A fasting blood sample was obtained for measurement of glucose, lipids (total cholesterol, HDL cholesterol, triglycerides and derived LDL cholesterol) creatinine and liver function. Blood pressure was measured in the seated position using a Dinamap 1846 SX P (Critikon; USA). Six BP readings were obtained at 1 min intervals, after 5min seated rest, on 2 separate days. Participants completed 3-day diet records to estimate their caloric intake. Volunteers whose waist circumference fitted the criteria of the metabolic syndrome according to the National Cholesterol Education Program Adult Treatment Panel III [7], >102cm for men and >88cm for women, and whose SBP was ≥ 120 mmHg were referred for a physical examination to ensure they qualified for the study. The volunteers were then screened for the *CYP4F2* G1347A and *CYP4A11* T8590C polymorphisms prior to being studied.

Exclusion Criteria

Volunteers were further excluded if they were smokers, carriers of the C-allele for the *CYP4A11* T8590C polymorphism, consuming > 30g alcohol/day or > 40g alcohol in a single session, or if they had a history of cardiovascular or peripheral vascular disease, diabetes, renal disease or liver disease.

The study was approved by the University of Western Australia Human Ethics Committee and volunteers provided written informed consent to participate. The weight loss intervention was registered with the Australian Clinical Trials Registry # *ACTRN12610000714088*.

Weight reduction intervention

Two groups were studied in parallel:- those genotyped as carrying the A-allele (GA/AA) for the *CYP4F2* G1347A polymorphism and those homozygous for the G-allele (GG, wildtype) who acted as controls. Both groups participated in a 16 week study. The first 12 weeks was a weight loss program followed by a 4 week program designed to stabilise weight. After baseline measurements, all volunteers received individualised diet counselling every two weeks that aimed to restrict calories by ~2000-6500 kJ/day (depending on baseline caloric intake), leading to a reduction in weight of ~4-5kg. This was followed by 4 weeks of counselling with a view to stabilising weight. All participants were instructed not to change their level of physical activity or alcohol consumption during the study and completed a questionnaire at baseline, 12 and 16 weeks relating to their level of physical activity and alcohol consumption. At baseline, 12 and 16 weeks, all participants had BP monitored for 24hr, weight and waist circumference was measured, and they provided a fasting blood sample and 24hr urine collection. A three-day diet record was completed at baseline and during week 12 and week 16 and the major nutrients were examined overtime using Foodworks.

Measurements

Ambulatory BP Monitoring

Ambulatory BP was monitored for 24hr with a Spacelabs 90207 monitor (Spacelabs Healthcare, Issaquah, WA, USA) set to record at 20-minute intervals while awake and 30-minute intervals while asleep. The monitor was fitted to the non-dominant arm by a trained researcher. The correct cuff size was determined by measuring mid-arm circumference. Volunteers were instructed to continue their normal routine and maintain a diary while awake. A valid 24hr recording was accepted as a minimum of 80% successful readings. Aggregated readings for each hour, were used to determine average 24hr SBP, DBP and MAP. Awake and asleep periods were determined from the subject's diary.

Blood collection and biochemical measurements

A fasted venous blood sample was collected into cold tubes containing EDTA and reduced glutathione and centrifuged immediately at 1000 x g for 10 min at 4°C for measurement of plasma 20-HETE and F₂-isoprostanes. The plasma was protected from oxidation by the addition of butylated hydroxytoluene at a final concentration of 20µg/ml plasma and stored at –80°C until analysis. Aliquots from the 24hr urine collection were stored frozen at –80°C until analysis. Fasting serum glucose, total and HDL cholesterol, triglycerides, creatinine and liver function tests and urinary sodium and creatinine were analysed at the PathWest Laboratory Medicine Unit at Royal Perth Hospital by routine methods.

***CYP4F2* V433M and *CYP4A11* F434S Genotyping**

The *CYP4F2* V433M (rs2108622) and *CYP4A11* F434S polymorphisms (rs1126742) were detected by PCR amplification of 20 to 50 ng of genomic DNA with 5 pmol of primer (*CYP4F2*: forward: 5'-GTCTCCTGGGTAGGAAGAG-3' and reverse: 5'-GTTGTGTGTGTCTTTGAG-3' and for *CYP4A11*: forward: 5'-GGGCCTTCTTTTGTGCCTGCT-3' and reverse: 5'-GCCCACACCAGGTGCCTGACT-3' in a 25µL reaction mixture containing 1 U of Hotstar Tag (Qiagen) and reaction buffer as supplied by the manufacturer. Because of expected close homology between the *CYP4F2* and *CYP4F3*, forward and reverse primers bracketing the *CYP4F2* V433M locus were designed to optimize amplicon specificity with verification using BLAST software (National Center for Biotechnology Information). The reaction mixture was subjected to an initial 15-minute denaturation period at 95°C followed by 35 cycles of 95°C for 45 seconds, 56°C for 45 seconds, 72°C for 1 minute, and a final 1-minute extension at 72°C. Identification of G1347A and T8590C was by sequence analysis of 0.5 mL of PCR product using BigDye Terminator V3.1 chemistry (Applied Biosystems). Chromatograms were viewed with Chromas Lite 2.01 (Technelysium Pty Ltd) and analyzed using the Bioedit Sequence Alignment Editor. Both

polymorphisms were in Hardy-Weinberg equilibrium in this study population.

Urinary and plasma 20-HETE

Urinary 20-HETE was measured by gas chromatography mass spectrometry (GCMS) using electron capture negative chemical ionization. The assay was a modification of the method previously described [8] that extracted a smaller volume of urine (0.5ml) and used 2ng of d₆-20-HETE (2ng, Cayman Chemical Co.) as an internal standard. Plasma free 20-HETE was measured in 125µl of plasma. After the addition of 1ng of d₆-20-HETE internal standard the plasma was acidified with 0.1M sodium acetate buffer pH-4.6 and applied under vacuum to Bond-Elut Certify II columns (200mg) (Varian, Australia), that were pre-washed with methanol and 0.1M sodium acetate pH 7.0 with 5% MeOH. The column was then washed with methanol/water (50:50) and dried under vacuum (6mmHg for 2 mins) prior to elution of 20-HETE with hexane/ethyl acetate/acetic acid (75:25:1). The sample was dried and derivatised as previously described for urine [8].

STATISTICAL ANALYSIS

The study was powered on the basis of using a dominant-effect model for the *CYP4F2* G1347A polymorphism as previously described [5]. This model compares the combined effect of the *CYP4F2* AA/GA genotypes with the *CYP4F2* GG (wildtype genotype). From our previous studies, we estimated that if we excluded carriers of the C-allele for the *CYP4A11* T8590C polymorphism and compared 21 volunteers genotyped *CYP4F* AA/GA with 21 volunteers genotyped *CYP4F2* GG, we would have 80% power to detect a 20% difference in urinary 20-HETE and plasma 20-HETE at $P<0.05$.

All analysis used the STATA 12 statistical package. Blood pressure and heart rate analysis used the average hourly readings at baseline, 12 and 16 weeks. Pulse pressure (SBP-DBP) was calculated from the hourly readings at baseline, 12 and 16 weeks. Between genotype

differences in SBP, DBP heart rate and pulse pressure; urinary 20-HETE, body weight, lipids and glucose were compared over time using linear mixed models with restricted maximum likelihood estimation that adjusts for differences at baseline and the effect of time. The influence of the *CYP4F2* allele status over time was assessed by incorporating interaction terms for the effect of allele status and time for each outcome variable. Linearity assumptions were assessed using restricted cubic splines. A *p* value less than 0.05 was regarded as statistically significant. The models were adjusted for the effects of gender, dietary sodium and potassium intake as assessed from urine collection and major dietary components assessed from 3-day diet records.

RESULTS

Four hundred and nineteen men and women were telephone screened, 137 underwent screening at the research unit that included measurement of anthropometry, blood pressure, biochemistry and genotyping. Fifty-seven were suitable for study but 4 declined to participate. Fifty-three volunteers entered the study. Twenty six were carriers of the A-allele of the *CYP4F2* G1347A polymorphism and 27 were homozygous for the G-allele. Forty-two completed the study with equal numbers in each group. Baseline characteristics for the two genotypes are described in Table 1. There were 21 volunteers who were heterozygotes (GA) and 5 who were homozygous (AA) for the A-allele of the *CYP4F2* G1347A polymorphism. At baseline, the two groups were similar with respect to age, gender distribution, BMI, waist circumference, fasting glucose total and HDL cholesterol. Triglycerides were higher in the A-allele carriers.

The rate of weight loss was similar between the GG genotype and A-allele carriers (Figure 1). Compared with baseline, body weight was reduced by -3.9 kg (CI -6.2,-1.7kg, *P*=0.0001) after 12 weeks weight loss and was not different between the genotypes *P*=0.932. At 16 weeks after

4 weeks weight maintenance, weight loss was maintained at -4.1kg (CI -6.5,-1.7kg, $P=0.0001$) compared with baseline, and was not different between the genotypes ($P=0.717$). Compared with baseline waist circumference was reduced after 12 weeks weight loss by -5.8cm (CI -7.3,-4.3cm, $P=0.0001$) and maintained at 16 weeks at -6.1cm (-7.7,-4.6cm, $P=0.0001$). Waist circumference did not differ between the genotypes at 12 or 16 weeks, ($P=0.345$ and $P=0.559$), respectively. BMI for the two genotypes at baseline, 12 weeks and 16 weeks are presented in Table 2.

Major dietary components (total fat, protein and carbohydrate) assessed from 3-day diet records were not different between the genotypes over time.

The effect of *CYP4F2* genotype on 24 hour blood pressure and heart rate and pulse pressure responses to weight loss

The mean 24 hour, awake, and asleep SBP and DBP at baseline, 12weeks and 16 weeks are shown in Table 2. Figure 2(A-C) shows the unadjusted hourly SBP in A-allele carriers compared with the GG genotype at baseline (Figure 2A), and after 12 weeks (Figure 2B) and 16 weeks (Figure 2C). At baseline, the A-allele carriers had 24 hour ambulatory SBP that was on average higher +1.7mmHg (CI 0.1, 3.35 mmHg, $P=0.047$) compared with the GG genotype (Table 3). When the data was analysed according to whether the volunteers were awake or asleep SBP in the A-allele carriers tended to be higher at baseline but not significantly so during both awake and asleep periods (Table 2). SBP fell in response to weight reduction by an average of -7.6mmHg, (CI -12.4,-2.7 mmHg, $P=0.004$), and was not different between the two groups after 12 weeks ($P=0.383$) (Table 3). Awake and asleep SBP at 12 weeks are shown in Table 2. However, there was a significant difference in the SBP response to weight stabilisation with the A-allele carriers having a higher SBP +3.6mmHg, (CI 1.1, 6.1mmHg, $P=0.004$) after 4 weeks of weight stabilisation compared with the GG genotype (Table 2). SBP at 16 weeks in the A-allele carriers was significantly higher during both awake hours ($P< 0.05$) and asleep

periods ($P<0.05$), Table 2.

Figure 2(D-F) shows the unadjusted hourly DBP in A-allele carriers compared with the GG genotype at baseline (Figure 2D), and after 12 weeks (Figure 2E) and 16 weeks (Figure 2F). At baseline, the A-allele carriers average 24 hour DBP was lower -1.4 mmHg (CI -2.6, -0.3mmHg, $P=0.014$) than the GG genotype (Table 3). The difference in 24hr DBP was mainly due to lower DBP in the A-allele carriers during awake hours ($P<0.05$) with little difference between the genotypes observed while asleep (Table 2). Compared with baseline, DBP fell to a similar extent in both groups after 12 weeks of weight reduction -5.4mmHg (CI -9.4, -1.4 mmHg, $P=0.008$) and was not different between the groups (Table 2). Awake and asleep DBP at 12 weeks are shown in Table 2. The A-allele carriers had significantly higher DBP after weight stabilisation +3.2mmHg (CI 1.6, 5.0 mmHg, $P=0.0001$) compared with the GG genotype (Table 3). The rebound increase in DBP in A-allele carriers was mainly due to a rebound in DBP during awake hours ($P<0.05$).

Heart rate was lower in the A-allele carriers at baseline -1.8beats/min, (CI -2.9, -0.7 beats/min, $P=0.0001$). Heart rate fell in to the same extent in both groups with weight reduction -5.4beats/min, (CI -9.0, -1.8 beats/min, $P=0.004$) and was not different between the groups (Table 3). After weight stabilisation heart rate in the A-allele carriers was significantly elevated compared with the GG genotype +2.6 beats/min, (CI 0.9, 4.3 beats/min, $P=0.0001$) (Table 3).

The differential effect of the A-allele to increase SBP, DBP and heart rate after weight stabilisation was not affected by changes in weight, sodium excretion, fasting glucose or differences in nutrient intake.

Pulse pressure was significantly higher in the A-allele carriers +3.1mmHg, (CI 2.3, 3.8mmHg, $P=0.0001$) (Table 3). Compared with baseline, there was a fall in pulse pressure after 12 weeks weight loss -2.5mmHg (-3.6,-1.4 mmHg, $P=0.0001$) and at 16 weeks -2.0mmHg (CI -3.1,-0.9 mmHg, $P=0.0001$) that was similar for both genotypes (Table 3).

The effect of *CYP4F2* genotype on urinary 20-HETE in response to weight loss.

Urinary 20-HETE was not different between the genotypes at baseline and was not significantly different after 12 weeks weight loss. However, the response to weight maintenance differed between the two genotypes with the A-allele carriers having significantly higher urinary 20-HETE compared with the GG genotype after weight maintenance $P=0.017$ (Table 4). The genotype differences in urinary 20-HETE during weight maintenance were still significant ($P=0.012$) after adjusting for changes in sodium intake as assessed from 24h sodium excretion and after adjusting for gender ($P=0.016$). Plasma 20-HETE did not differ significantly between the genotypes at baseline, after weight reduction or after weight stabilisation. Plasma 20-HETE was 827 pmol/L (95% CI 726, 941 pmol/L) and 808 pmol/L (95% CI 682, 957 pmol/L) for the GG genotype and A-allele carriers, respectively, at baseline; 915pmol/L (95% CI 739, 1132 pmol/L) and 909 pmol/L (95% CI 762, 1082 pmol/L) for the GG genotype and A-allele carriers; respectively, after 12 weeks of weight reduction and 860 pmol/L (95% CI 702, 1055 pmol/L) for the GG genotype and 910 pmol/L (95% CI 728, 1136 pmol/L) for the A-allele carriers, respectively, after weight stabilisation. Adjusting the model for the effects of gender did not significantly affect the results.

The effect of *CYP4F2* genotype on glucose and blood lipids in response to weight loss

In regression models that included the effect of genotype and time, there was no significant difference between the genotypes with respect to fasting levels of glucose, cholesterol, or HDL cholesterol at any time point (Table 5). All of these parameters fell significantly in both groups

with weight reduction and remained lower at 16 weeks. Triglycerides levels were significantly higher ($+0.28\pm0.12\text{mmol/L}$, $P=0.021$) in A-allele carriers at all time-points. Triglycerides levels were reduced to a similar extent in both groups with weight reduction and remained lower at 16 weeks (Table 5).

DISCUSSION

In a parallel controlled trial of overweight volunteers, we have shown for the first time that blood pressure and urinary 20-HETE responses to weight maintenance following weight loss are different in carriers of the *CYP4F2* G1347A polymorphism. This is an important observation given that approximately 30% of the population carry the A-allele for the *CYP4F2* G1347A polymorphism independent of the *CYP4A11* T8590C polymorphism. The differences are not apparent until the period of weight stabilisation after weight loss suggesting that the mechanisms to maintain a lower blood pressure after weight loss are less robust in carriers of this polymorphism. The associated increase in urinary 20-HETE in carriers of the polymorphism after weight stabilisation suggest that 20-HETE synthesis may in part mediate the elevated blood pressure.

Our baseline data in overweight volunteers confirm previous reports of higher SBP in carriers of the polymorphism [5, 9]. In contrast, baseline DBP was comparatively lower in the A-allele carriers. The fall in SBP and DBP in response to weight reduction was similar between the genotypes, and the rebound increase in blood pressure in A-allele carriers after weight maintenance could not be explained by genotype differences in weight, glucose, sodium or nutrient intake. The mechanism related to the increase in blood pressure is unknown. However, differences in plasma renin activity, muscle sympathetic nerve activity and baroreflex sensitivity have been described in humans during active weight loss compared with weight

maintenance following weight loss. A small study in humans showed that weight loss of ~10kg resulted in reduced noradrenaline spillover rate, muscle sympathetic nerve activity and plasma renin activity, and increased baroreflex sensitivity while weight maintenance associated with a rebound increase in plasma renin activity, muscle sympathetic nerve activity with reduced baroreflex sensitivity [10]. Although these parameters were not measured in our study it is possible that A-allele carriers may be particularly sensitised to these mechanisms during weight maintenance with a resultant increase in blood pressure.

Heart rate changes in the two groups after weight reduction and weight maintenance paralleled that in systolic blood pressure suggesting an autonomic adrenaline mediated or cardiac effect.

We found that A-allele carriers had significantly elevated pulse pressure compared to the GG genotype over the 16 weeks. An increased pulse pressure associates with stiffer arteries and has been reported to confer an increased risk for cardiovascular mortality and stroke [11, 12]. Our findings agree with the report by Hu et al. who showed that carriers of this polymorphism had elevated peripheral and central augmentation index and urinary 20-HETE [13]. The fall in pulse pressure with weight loss in our study, was similar for both genotypes and in accord with reports of improvements in arterial stiffness after weight loss [14-16]. However, the fact that A-allele carriers still had higher pulse pressure may be of relevance to the increase in blood pressure after weight maintenance. In this regard, a recent study in a mouse model of diet induced obesity demonstrated that arterial stiffening preceded the development of hypertension [17]. In that study, arterial stiffening was associated with increased oxidative stress and reduced nitric oxide bioavailability both of which have been linked to increased 20-HETE synthesis [6, 18]. Therefore, it is possible that the consistently higher pulse pressure in A-allele carriers in our study may cause undue stress on the resistance vessels that manifests as

increased BP during weight maintenance. The precise mechanism by which BP is increased is not known but could relate to increased 20-HETE that has been associated with reduced nitric oxide and increased oxidative stress.

This study found a significant increase in urinary 20-HETE in A-allele carriers after weight stabilisation that coincided with the increase in BP. Altered urinary 20-HETE excretion may be relevant to regulation of renal function, vascular tone and BP control. Plasma 20-HETE responses were not affected by genotype suggesting that altered synthesis of 20-HETE may be confined to the kidney. In humans, the CYP4F2 isoform accounts for a large proportion of 20-HETE synthesis in the kidney [19, 20]. 20-HETE is excreted in the urine as the glucuronide conjugate [21] and may reflect renal synthesis although the site of glucuronidation is not known. 20-HETE can affect blood pressure differently depending on whether it is synthesised in the pre-glomerular vasculature where it causes vasoconstriction [22] or in the renal tubules where it causes a natriuresis [23]. There were no differential effects of the A-allele on sodium excretion after weight stabilisation. Therefore any effect of renal 20-HETE to raise blood pressure during weight stabilisation would likely be due to effects on the vasculature that may occur via multiple mechanisms including increased PRA and/or renal sympathetic nerve activity that are potent stimuli for 20-HETE synthesis [24, 25] and/or reduced nitric oxide availability [26].

There are numerous polymorphisms of *CYP4F* and *CYP4A* genes and both *CYP4F2* G1347A and *CYP4A11* T8590C polymorphisms have been associated with elevated blood pressure. We previously showed that the *CYP4F2* G1347A polymorphism and *CYP4A11* T8590C polymorphisms had opposite effects on urinary 20-HETE excretion [5]. Our study did not confirm our previous report of elevated 20-HETE excretion in carriers of the A allele. This

may be because the population was overweight with the majority of volunteers post-menopausal women and therefore less heterogeneous in terms of BMI and gender, that have been shown to be strong predictors of urinary 20-HETE [5, 18].

Interestingly confining our study population to exclude the effects of the *CYP4A11* T8590C polymorphism also showed that carriers of *CYP4F2* G1347A polymorphism had raised triglyceride levels compared with the GG genotype. A large Swedish population study also reported increased triglycerides in male but not female carriers of this polymorphism, however, that study did not exclude carriers of *CYP4A11* T8590C polymorphism [27]. In our study, triglyceride responses to weight reduction and weight stabilisation were similar between the genotypes. Cholesterol, HDL cholesterol, and glucose did not differ between the genotypes and were reduced to a similar extent by weight reduction.

In conclusion, our study showed that overweight volunteers who were carriers of the A-allele for the *CYP4F2* G1347A polymorphism had higher SBP, pulse pressure and triglycerides. They also have more difficulty maintaining a lower blood pressure during weight stabilisation after weight loss. This may be related to genotype differences in arterial stiffness and increased 20-HETE synthesis during weight stabilisation.

ACKNOWLEDGMENTS

We wish to thank Helen Bevelaqua who provided diet counselling for the study participants, and Sarah Simpson who assisted with nursing duties for the study. NC Ward acknowledges the support of a MRF/UWA Postdoctoral Fellowship

REFERENCES

1. Roman RJ. P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiological Reviews*. 2002; 82 (1):131-85.
2. Hoagland KM, Flasch AK, Roman RJ. Inhibitors of 20-HETE formation promote salt-sensitive hypertension in rats. *Hypertension*. 2003; 42 (4):669-73.
3. Stec DE, Roman RJ, Flasch A, Rieder MJ. Functional polymorphism in human CYP4F2 decreases 20-HETE production. *Physiol Genomics*. 2007; 30 (1):74-81.
4. Gainer JV, Bellamine A, Dawson EP, Womble KE, Grant SW, Wang Y, et al. Functional variant of CYP4A11 20-hydroxyecosatetraenoic acid synthase is associated with essential hypertension. *Circulation*. 2005; 111 (1):63-9.
5. Ward NC, Tsai IJ, Barden A, van Bockxmeer FM, Puddey IB, Hodgson JM, et al. A single nucleotide polymorphism in the CYP4F2 but not CYP4A11 gene is associated with increased 20-HETE excretion and blood pressure. *Hypertension*. 2008; 51 (5):1393-8.
6. Tsai IJ, Croft KD, Mori TA, Falck JR, Beilin LJ, Puddey IB, et al. 20-HETE and F2-isoprostanes in the metabolic syndrome: the effect of weight reduction. *Free Radic Biol Med*. 2009; 46 (2):263-70.
7. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA*. 2001; 285 (19):2486-97.
8. Rivera J, Ward N, Hodgson J, Puddey IB, Falck JR, Croft KD. Measurement of 20-hydroxyecosatetraenoic acid in human urine by gas chromatography-mass spectrometry. *Clin Chem*. 2004; 50 (1):224-6.
9. Fava C, Montagnana M, Almgren P, Rosberg L, Lippi G, Hedblad B, et al. The V433M variant of the CYP4F2 is associated with ischemic stroke in male swedes beyond its effect on blood pressure. *Hypertension*. 2008; 52 (2):373-80.
10. Straznicky NE, Grima MT, Eikelis N, Nestel PJ, Dawood T, Schlaich MP, et al. The Effects of Weight Loss Versus Weight Loss Maintenance on Sympathetic Nervous System Activity and Metabolic Syndrome Components. *Journal of Clinical Endocrinology & Metabolism*. 2011; 96 (3):E503-E8.
11. Domanski MJ, Mitchell GF, Norman JE, Exner DV, Pitt B, Pfeffer MA. Independent prognostic information provided by sphygmomanometrically determined pulse pressure and mean arterial pressure in patients with left ventricular dysfunction. *Journal of the American College of Cardiology*. 1999; 33 (4):951-8.

12. Zheng L, Sun Z, Li J, Zhang R, Zhang X, Liu S, et al. Pulse pressure and mean arterial pressure in relation to ischemic stroke among patients with uncontrolled hypertension in rural areas of China. *Stroke*. 2008; 39 (7):1932-7.
13. Hu BC, Li Y, Li FH, Zhang Y, Sheng CS, Fan HQ, et al. Peripheral and central augmentation indexes in relation to the CYP4F2 polymorphisms in chinese. *J Hypertension*. 2011; 29:501-8.
14. Dengel DR, Kelly AS, Olson TP, Kaiser DR, Dengel JL, Bank AJ. Effects of weight loss on insulin sensitivity and arterial stiffness in overweight adults. *Metabolism-Clinical and Experimental*. 2006; 55 (7):907-11.
15. Goldberg Y, Boaz M, Matas Z, Goldberg I, Shargorodsky M. Weight loss induced by nutritional and exercise intervention decreases arterial stiffness in obese subjects. *Clinical Nutrition*. 2009; 28 (1):21-5.
16. Dengo AL, Dennis EA, Orr JS, Marinik EL, Ehrlich E, Davy BM, et al. Arterial Destiffening With Weight Loss in Overweight and Obese Middle-Aged and Older Adults. *Hypertension*. 2010; 55 (4):855-61.
17. Weisbrod RM, Shiang T, Al Sayah L, Fry JL, Bajpai S, Reinhart-King CA, et al. Arterial Stiffening Precedes Systolic Hypertension in Diet-Induced Obesity. *Hypertension*. 2013; 62 (6):1105-10.
18. Ward NC, Rivera J, Hodgson J, Puddey IB, Beilin LJ, Falck JR, et al. Urinary 20-hydroxyeicosatetraenoic acid is associated with endothelial dysfunction in humans. *Circulation*. 2004; 110 (4):438-43.
19. Lasker JM, Chen WB, Wolf I, Bloswick BP, Wilson PD, Powell PK. Formation of 20-hydroxyeicosatetraenoic acid, a vasoactive and natriuretic eicosanoid, in human kidney - Role of CYP4F2 and CYP4A11. *J Biol Chem*. 2000; 275 (6):4118-26.
20. Schwartzman ML, Martasek P, Rios AR, Levere RD, Solangi K, Goodman AI, et al. Cytochrome-P450-dependent arachidonic-acid metabolism in human-kidney. *Kidney International*. 1990; 37 (1):94-9.
21. Prakash C, Zhang JY, Falck JR, Chauhan K, Blair IA. 20-hydroxyeicosatetraenoic acid is excreted as a glucuronide conjugate in human urine. *Biochem Bioph Res Co*. 1992; 185 (2):728-33.
22. Imig JD, Zou AP, Stec DE, Harder DR, Falck JR, Roman RJ. Formation and actions of 20-hydroxyeicosatetraenoic acid in rat renal arterioles. *Am J Physiol*. 1996; 270 (1 Pt 2):R217-27.

23. Schwartzman M, Ferreri NR, Carroll MA, Songumize E, McGiff JC. Renal Cytochrome-P450-related arachidonate metabolite inhibits (Na⁺ + K⁺)atpase. *Nature*. 1985; 314 (6012):620-2.
24. Croft KD, McGiff JC, Sanchez-Mendoza A, Carroll MA. Angiotensin II releases 20-HETE from rat renal microvessels. *Am J Physiol Renal Physiol*. 2000; 279 (3):F544-51.
25. Kalyankrishna S, Malik KU. Norepinephrine-induced stimulation of p38 mitogen-activated protein kinase is mediated by arachidonic acid metabolites generated by activation of cytosolic phospholipase A(2) in vascular smooth muscle cells. *Journal of Pharmacology and Experimental Therapeutics*. 2003; 304 (2):761-72.
26. Cheng J, Ou JS, Singh H, Falck JR, Narsimhaswamy D, Pritchard KA, et al. 20-hydroxyeicosatetraenoic acid causes endothelial dysfunction via eNOS uncoupling. *Am J Physiol-Heart C*. 2008; 294 (2):H1018-H26.
27. Fava C, Montagnana M, Danese E, Sjogren M, Almgren P, Guidi GC, et al. The functional variant V433M of the CYP4F2 and the metabolic syndrome in Swedes. *Prostag Oth Lipid M*. 2012; 98 (1-2):31-6.

FIGURE LEGENDS

FIGURE 1. Comparison of weight loss recorded every 2 weeks in GG genotype (closed circles ●) and A-allele carriers (open squares □). There were no significant differences in weight loss between the genotypes.

FIGURE 2.

Panel A. Comparison of systolic blood pressure over 24h at baseline (solid black lines) in volunteers with the GG genotype (closed circles ●) and A-allele carriers (open squares □), $P=0.041$ for effect of genotype using mixed model.

Panel B. Comparison of systolic blood pressure over 24h after 12 weeks weight reduction GG genotype (closed circles ●) and A-allele carriers (open squares □) $P=0.002$ for effect of weight loss, $P=0.38$ for genotype differences at after weight reduction using mixed model.

Panel C Comparison of systolic blood pressure over 24h at 16 weeks (solid black lines) in volunteers with the GG genotype (closed circles ●) and A allele carriers (open squares □) $**P=0.004$ for genotype differences after weight stabilisation using mixed model.

Panel D Comparison of diastolic blood pressure over 24h at baseline (solid black lines) in volunteers with the GG genotype (closed circles ●) and A allele carriers (open squares □), $P=0.014$ for effect of genotype using mixed model.

Panel E Comparison of diastolic blood pressure over 24h after 12 weeks weight reduction (solid black lines) in volunteers with the GG genotype (closed circles ●) and A allele carriers (open squares □), $P=0.008$ for effect of weight loss, $P=0.06$ for genotype differences after weight reduction using mixed model.

Panel F Comparison of diastolic blood pressure over 24h at 16 weeks (solid black lines) in volunteers with the GG genotype (closed circles ●) and A allele carriers (open squares □), $P=0.0001$ for genotype differences after weight stabilisation using mixed model.

TABLE 1. Baseline characteristics for the two genotypes

	GG genotype	A allele Carriers (GA/AA)
	(n=27)	(n=26)
Age (yrs)	58.2±9	56.7±8
Gender (men/women)	7/20	8/18
BMI (Kg/m²)	33.0±2.9	32.9±3.2
Waist circumference (cm)	104.9±10.3	106.1±9.3
<u>Fasting:</u>		
Glucose (mmol/L)	5.3±0.5	5.4±0.4
Total Cholesterol (mmol/L)	5.7±1.0	6.1±0.8
HDL cholesterol (mmol/L)	1.4±0.4	1.3±0.3
Triglycerides (mmol/L)	1.3±0.5	1.6±0.4*

Values are means and Standard Deviation. * p < 0.05 compared with GG genotype.

Table 2. Average BMI and 24 hour, awake and asleep SBP, DBP and Heart Rate for A-allele carriers and the GG genotype at baseline, after 12weeks of weight reduction and at 16 weeks, after 4 weeks of weight maintenance.

	Baseline	Week 12	Week 16
BMI (kg/m ²) GG genotype	32.7 (31.4, 34.0)	31.3 (29.8,32.7)	31.2 (29.8,32.7)
A-allele	32.9 (31.4, 34.5)	31.4 (29.9,33.0)	31.2 (29.7,32.7)
24hr SBP GG genotype	125.3 (121.8,128.8)	117.7 (114.2,121.2)	118.5 (114.2,122.0) ^c
A-allele	127.0 (123.6,130.5) ^a	120.5 (117.0,124.0)	123.8 (120.3,127.3)
SBP Awake GG genotype	131.9 (130.3,133.4)	122.6 (121.0,124.2)	123.2 (121.5,124.9)
A-allele	133.0 (131.5,134.6)	126.4 (124.7,128.0)	129.7 (128.0,131.4) ^c
SBP Asleep GG genotype	114.2 (111.8,116.5)	108.7 (106.1,111.2)	109.3 (106.2,111.2)
A-allele	116.4 (114.0,118.7)	109.3 (106.6,111.9)	112.2 (109.6,114.9) ^c
24hr DBP GG genotype	74.0 (71.2,76.9)	68.6 (65.8,71.5)	69.4 (66.5,72.2)
A-allele	72.6 (69.7,75.4) ^a	68.9 (66.0,71.8)	71.2 (68.3,74.0) ^c
DBP Awake GG genotype	79.5 (78.2,81.0)	72.4 (71.0,73.9)	73.4 (71.9,74.8)
A-allele	77.2 (75.8,78.6) ^a	73.1 (71.6,74.5)	75.4 (73.9,76.9) ^c
DBP Asleep GG genotype	65.1 (63.3,67.0)	61.0 (59.0,63.0)	61.1 (59.0,63.2)
A-allele	65.1 (63.2,66.9)	60.3 (58.2,62.3)	62.4 (60.2,64.5)

Values are mean and 95% CI. ^a ($P<0.05$) for A-allele compared with GG genotype at

baseline. ^c ($P<0.05$) for differences in the A-allele compared with GG genotype at 16 weeks

TABLE 3. Mixed models examining the effect of genotype on SBP, DBP, Heart rate, and pulse pressure after weight loss at 12 weeks, and 4 weeks of weight stabilisation at 16 weeks

	Coefficient (95% CI)	P value
<u>SBP (mmHg)</u>		
<u>Genotype effect at baseline</u>		
A-allele v's GG	1.7 (0.1, 3.35)	0.041
<u>Time effect</u>		
12 weeks	-7.6 (-12.4,-2.7)	0.002
16 weeks	-6.9 (-11.2,-2.0)	0.006
<u>Genotype *Time interaction</u>		
A-allele X 12 weeks	1.1 (-1.4, 3.5)	0.383
A-allele X 16 weeks	3.6 (1.1, 6.1)	0.004
Constant	125.3 (121.9, 128.7)	< 0.0001
<u>DBP (mmHg)</u>		
<u>Genotype effect at baseline</u>		
A-allele v's GG	-1.4 (-2.6,-0.3)	0.014
<u>Time effect</u>		
12 weeks	-5.4 (-9.4,-1.4)	0.008
16 weeks	-4.7 (-8.6,-0.7)	0.022
<u>Genotype *Time interaction</u>		
A-allele X 12 weeks	1.7 (-0.02, 3.4)	0.060
A-allele X 16 weeks	3.2 (1.6, 5.0)	0.0001
Constant	74.0 (71.2, 76.8)	< 0.0001
<u>Heart rate (beat/min)</u>		
<u>Genotype effect at baseline</u>		
A-allele v's GG	-1.8 (-2.9,-0.7)	0.001
<u>Time effect</u>		
12 weeks	-5.4 (-9.0,-1.8)	0.004
16 weeks	-5.3(-8.9,-1.6)	0.004
<u>Genotype *Time interaction</u>		
A-allele X 12 weeks	1.0 (-0.6, 2.7)	0.212
A-allele X 16 weeks	2.6 (0.9, 4.3)	0.002
Constant	74.3 (71.7, 76.8)	< 0.0001
<u>Pulse pressure (beat/min)</u>		
<u>Genotype effect</u>		
A-allele v's GG	3.1 (2.3, 3.8)	0.0001
<u>Time effect</u>		
12 weeks	-2.5 (-3.6,-1.4)	0.0001
16 weeks	-2.0 (-3.1,-0.9)	0.0001
Constant	51.4 (50.5, 52.2)	< 0.0001

TABLE 4. Mixed model examining the effect of genotype on urinary 20-HETE after weight loss at 12 weeks, and 4 weeks of weight stabilisation at 16 weeks.

Ln Urinary 20-HETE (mmol/mmol creatinine)

Genotype effect at baseline

A-allele v's GG	-0.18 (-0.47, 0.10)	0.204
-----------------	---------------------	-------

Time effect

12 weeks	0.12 (-0.06, 0.29)	0.180
----------	--------------------	-------

16 weeks	-0.13(-0.30, 0.05)	0.165
----------	--------------------	-------

Genotype *Time interaction

A-allele X 12 weeks	-0.11.0 (-0.34, 0.16)	0.471
---------------------	-----------------------	-------

A-allele X 16 weeks	0.31 (0.05, 0.56)	0.017
---------------------	-------------------	-------

Constant	4.81 (4.61, 5.01)	< 0.0001
----------	-------------------	----------

Data was corrected for creatinine excretion and log transformed for analysis

TABLE 5. Mixed models examining the effect of genotype on fasting glucose, cholesterol, HDL cholesterol, and triglycerides after weight loss at 12 weeks and 4 weeks of weight stabilisation at 16 weeks

	Coefficient (95% CI)	<i>P</i> value
<u>Glucose (mmol/L)</u>		
<u>Genotype effect</u>		
A-allele v's GG	0.06 (-0.15, 0.27)	0.60
<u>Time effect</u>		
12 weeks v's baseline	-0.14 (-0.25,-0.033)	0.010
16 weeks v's baseline	-0.14 (-0.25,-0.036)	0.009
Constant	5.28 (5.12, 5.44)	< 0.0001
<u>Cholesterol (mmol/L)</u>		
<u>Genotype effect</u>		
A-allele v's GG	0.31 (-0.14, 0.76)	0.182
<u>Time effect</u>		
12 weeks v's baseline	-0.19 (-0.33,-0.038)	0.014
16 weeks v's baseline	-0.10 (-0.25, 0.05)	0.180
Constant	5.71 (5.39, 6.03)	< 0.0001
<u>HDL cholesterol (mmol/L)</u>		
<u>Genotype effect</u>		
A-allele v's GG	-0.10 (-.24, 0.04)	0.171
<u>Time effect</u>		
12 weeks v's baseline	-0.10 (-0.14,-0.06)	0.0001
16 weeks v's baseline	-0.07 (-0.11,-0.02)	0.002
Constant	1.45 (1.35, 1.55)	< 0.0001
<u>Triglycerides (mmol/L)</u>		
<u>Genotype effect</u>		
A-allele v's GG	0.28 (0.04, 0.52)	0.021
<u>Time effect</u>		
12 weeks v's baseline	-0.13 (-0.24,-0.03)	0.015
16 weeks v's baseline	-0.12 (-0.23,-0.013)	0.028
Constant	1.31 (1.13, 1.48)	<0.0001

Figure 1

Figure 2