GENETIC EPIDEMIOLOGY

The C-532T polymorphism of the angiotensinogen gene is associated with pulse pressure: A possible explanation for heterogeneity in genetic association studies of AGT and hypertension

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Accepted 26 September 2007

Background

Many previous studies have investigated whether there is an association between genotypes at the angiotensinogen (AGT) gene and hypertensive status, but few have incorporated quantitative data. Although meta-analyses support a possible effect of AGT variants on blood pressure (BP), substantial unexplained between-study heterogeneity has been observed. We hypothesized that a primary effect of AGT variants on arterial stiffness (and thus pulse pressure) might explain such heterogeneity, and tested for such an effect in a family study.

Methods

We studied 1425 individuals from 248 families ascertained through a proband with essential hypertension. BP was measured using 24 h ambulatory monitoring, and polymorphisms of the AGT gene that had been previously associated with hypertension and/or plasma angiotensinogen levels were typed. Pulse pressure was used as a measurement of arterial stiffness.

Results

We observed a highly significant association between genotypes at the AGT C-532T polymorphism and pulse pressure ($p=0.00006$). Each T allele was associated with a 5% lower pulse pressure (that is, an additive effect). This resulted from opposing genotypic effects to (slightly) lower systolic BP and (slightly) elevate DBP.

Conclusions

These results suggest that genetic variation at the angiotensinogen locus may primarily affect arterial stiffness, and therefore pulse pressure. The heterogeneity between previous genetic studies of AGT and hypertension status could in part be explained by this finding, since case selection criteria based on systolic BP, diastolic BP, or both would result in different levels of selection for the −532T allele.

Keywords

Hypertension, arterial stiffness, angiotensinogen, polymorphism, heritability

Background

Susceptibility to hypertension is clearly determined by certain environmental and lifestyle factors, but numerous studies have also shown a significant genetic component, estimated to account for between 20 and 50% of the population variability.
in blood pressure.1–4 Progress in identifying these genetic determinants has thus far been slow, probably because the individual genetic effects present are of small size. Among the most studied genes is angiotensinogen (AGT), the substrate for the action of renin and a key molecule in the renin–angiotensin system. The first study to suggest that polymorphisms of AGT were associated with the risk of hypertension appeared in 1992; since then over 45,000 subjects have been studied and a number of meta-analyses of these data have been conducted.5–12 These analyses have shown strong support for association between the T allele of the AGT M235T polymorphism originally described by Jeunemaitre et al.12 and plasma levels of AGT in Caucasian subjects; however, it is much less clear whether or not the M235T variant is associated with high blood pressure (BP). The most recent meta-analysis of 25 studies of hypertension status in Caucasians showed highly significant evidence for increased risk of hypertension with the TT genotype of M235T compared with the MM genotype [Odds Ratio (OR) 1.21; 95% Confidence interval (CI): 1.11–1.32; P < 0.0001]; and was concordant with previous meta-analyses of this question.8 However, there was substantial unexplained heterogeneity among the contributing studies (heterogeneity chi-square 58.9; df = 24; P = 0.0001; I² = 59.3). It has been shown for other genetic associations that such heterogeneity may arise because of publication bias or differences in the methodological quality between included studies.13,14 Although such considerations often do not explain all between-study heterogeneity.

Components of the renin–angiotensin system are expressed in vascular smooth muscle cells. Local production of AGT occurs in these cells as part of their proliferative response to insulin or insulin-like growth factor 1.15 Recently, AGT was shown to have direct effects on vascular wall remodelling in vivo in a rat model.16 We, therefore, hypothesized that the principal effect of polymorphisms in AGT is on arterial stiffness, and tested this in a large family study using pulse pressure as the measure of arterial stiffness. Although the majority of studies thus far have only typed the M235T polymorphism, other polymorphisms with larger effects on plasma angiotensinogen levels (the presumed mechanism of action on blood pressure) have been identified. In a comprehensive haplotype-based analysis of the AGT gene in Caucasians, Brand et al.18 showed that the C-532T polymorphism in the promoter region had the strongest effect on plasma AGT levels, accounting for around 4% of the total variability of plasma AGT. Patients carrying one or two copies of the -532T allele had higher plasma levels of AGT. In the present study, we therefore typed both the M235T and C-532T variants.

Methods
The collection strategy of this family study has been described previously.19 Briefly, families were ascertained between 1993 and 1997 through a proband with essential hypertension. In order to be eligible, a proband had to fulfil at least one of the following three criteria for hypertension. First, an ambulatory BP monitor (either on or off treatment) with mean daytime systolic pressure >140 mmHg and mean daytime diastolic pressure >90 mmHg. Second, multiple clinic BP readings >160 mmHg systolic and 95 mmHg diastolic. Third, treatment with two or more drugs for the control of BP. Secondary hypertension was excluded using the standard screening protocol applied in the hypertension clinic, reinforced by further investigations if required. In order to be suitable for the study, families were required to consist of at least three siblings (including the proband) clinically assessable for BP if at least one parent of the sibship was available to give blood for DNA analysis, and to consist of at least four assessable siblings (including the proband) if no parent was available for DNA analysis. This was to maximize the power to assign identity-by-descent (IBD) in the genetic analyses. Qualifying sibships could be either in the generation of the proband or his/her offspring, and there was no requirement for the sibship to contain additional members affected with hypertension (though this was not an exclusion criterion). Where members of the sibship were found to be hypertensive (using identical criteria to those applied in the probands), families were extended and the spouses and offspring of hypertensive sibs collected. Thus, the majority of the individuals in the family collection have BPs within the normal range, and the family collection includes some extended families, though most are nuclear families. The study received ethical clearance from the appropriate review committees, and corresponded with the principles of the Declaration of Helsinki; all participants gave informed consent.

Blood pressure was measured using ambulatory monitoring for a period of 24 h in all subjects willing to undergo monitoring, using the A&D TM2421 monitor (Takeda Medical, Japan) according to a previously described protocol.20 In brief, the monitor was programmed to record BP every half-hour during the daytime and every hour during the night, and a recording was considered of satisfactory technical quality if greater than 20 daytime ambulatory data points were available for analysis. The principal analyses focused a priori on the daytime mean BPs, since the largest number of data points were available to contribute to these means. The daytime pulse pressure was defined as the mean daytime systolic pressure minus the mean daytime diastolic pressure. A full clinical history was taken, which included the subject’s medical history and lifestyle factors including consumption of alcohol and tobacco and habitual physical exercise. Anthropometric measurements including height, weight, waist and hip were available to contribute to these means. The daytime pulse pressure was defined as the mean daytime systolic pressure minus the mean daytime diastolic pressure. A full clinical history was taken, which included the subject’s medical history and lifestyle factors including consumption of alcohol and tobacco and habitual physical exercise. Anthropometric measurements including height, weight, waist and hip were available to contribute to these means. The daytime pulse pressure was defined as the mean daytime systolic pressure minus the mean daytime diastolic pressure. A full clinical history was taken, which included the subject’s medical history and lifestyle factors including consumption of alcohol and tobacco and habitual physical exercise. Anthropometric measurements including height, weight, waist and hip were available to contribute to these means. The daytime pulse pressure was defined as the mean daytime systolic pressure minus the mean daytime diastolic pressure.

Plasma levels of angiotensinogen were measured on heparinized samples using a previously described radioimmunoassay protocol.21 DNA was extracted from blood and genotyping was carried out at the C-532T and M235T polymorphisms of the AGT gene using a SEQUENOM MassArray system. Control individuals of known genotype (determined by restriction fragment length polymorphism assays) were included in each plate, and 10% of the samples were randomly selected for re-genotyping, with an estimated genotype error rate of <1%. Genotypes were checked for Hardy–Weinberg equilibrium and Mendelian inheritance within families using PEDSTATS.22 Additional checks to detect genotyping errors not producing
Our previous described. We tested the 'total association' dual polymorphisms was performed using the QTDT program, MERLIN and quantitative trait association analysis for individual treatment). In subsidiary analyses, we adjusted on-treatment defined phenotypes (that is, ambulatory monitors recorded off fore, we included only those individuals who had quantitatively treatment when they were monitored. Initially, there- families.

Mendelian inconsistencies were carried out using the errorchecking option in MERLIN. Phenotypic variables were assessed for Normality. Variables that did not follow a Normal distribution were logarithmically transformed to approximate Normality prior to analyses. Significant covariates were then determined, and adjustment for those covariates carried out, by regression analyses performed using MINITABv14. Haplotype frequencies were estimated using FUGUE. IBD vectors for each marker were calculated in MERLIN and quantitative trait association analysis for individual polymorphisms was performed using the QTDT program, as previously described. We tested the 'total association' model (which tests both between-family and within-family components of association), incorporating non-shared environmental, polygenic, and major-gene effect components of variance in the model. We tested for stratification by evaluating the differences between the within-family and the between-family components of association using the option provided in QTDT, to check that any significant result did not arise from unsuspected ethnic heterogeneity between the families.

Nineteen per cent of our population was taking antihyper- tensive treatment when they were monitored. Initially, therefore, we included only those individuals who had quantitatively defined phenotypes (that is, ambulatory monitors recorded off treatment). In subsidiary analyses, we adjusted on-treatment BPs using the method suggested by Tobin et al., adding an empirical 5 mmHg systolic and 2.5 mmHg diastolic for each antihypertensive agent prescribed to treated patients (but without taking any account of agent dose).

Results

Characteristics of the 1425 individuals from 248 families who participated in the study are summarized in Table 1. Females comprised 52% of the sample and 36% of participants were classified as hypertensive. As expected, given the selection of families through a hypertensive proband, family members tended to be overweight and ambulatory BPs tended to be higher than would be expected in a community-based population.

Quantitative recordings of ambulatory BP were available in 958 people, with on-treatment recordings available in a further 224. The distribution of the BP phenotypes was skewed, so log-transformation was undertaken before covariate adjustment. Covariates (age, sex, smoking and alcohol consumption) accounted for 14–48% of the observed variability in the phenotypes. The heritability of covariate-adjusted, logtransformed pulse pressure was 26.3% (P < 0.00001). The median family size was five people, with 60% of families comprising between four and six genotyped and phenotyped members. Families consisting of two generations comprised 71% of the families sampled, and the remainder consisted of three generations.

Genotyping was successful in over 95% of individuals for both polymorphisms. Both markers were in Hardy–Weinberg equili- brium at the 0.05 level in founders of the families. At the C-532T SNP, the frequency of the -532 C allele was 0.887 and of the -532T allele 0.113. At the M235T SNP, the frequency of the -532C/235M 58.6%; -532C/235T 30.2%; -532T/ 235M 11.2%; -532T/235T 0.1%. These findings are concordant with previously published data in European Caucasian cohorts. Table 2 shows the values of systolic, diastolic and pulse pressures in family members with different genotypes at the typed polymorphisms. Values in Table 2 have been log-transformed, covariate-adjusted and standardized (thus, each phenotype has a mean of zero and an SD of one). There was a trend towards lower SBD and higher DBP in carriers of the polymorphism, but these trends did not reach statistical significance (P = 0.07 and P = 0.768, respectively). There was no difference in the mean BP (calculated as the sum of the diastolic pressure and one-third of the pulse pressure) between genotypes.

### Table 1 Characteristics of 1425 members of 248 families studied

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Median</th>
<th>Interquartile range</th>
<th>( \hat{\rho}^{2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>1425</td>
<td>50.9</td>
<td>35.7–60.9</td>
<td>–</td>
</tr>
<tr>
<td>Alcohol consumption (Units/week)</td>
<td>1008</td>
<td>413 non-drinkers</td>
<td>7</td>
<td>3–16</td>
</tr>
<tr>
<td>Smoking (current/former/never)</td>
<td>313/376/734</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Regular exercise habit (None/once/twice/three times or more per week)</td>
<td>609/302/305/197</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>1402</td>
<td>25.4</td>
<td>23.1–28.2</td>
<td>15.2</td>
</tr>
<tr>
<td>Waist–Hip Ratio</td>
<td>1357</td>
<td>0.85</td>
<td>0.78–0.91</td>
<td>48.7</td>
</tr>
<tr>
<td>Daytime systolic BP (mmHg)</td>
<td>958a</td>
<td>131</td>
<td>121.1–144.1</td>
<td>20.4</td>
</tr>
<tr>
<td>Daytime diastolic BP (mmHg)</td>
<td>958a</td>
<td>78.6</td>
<td>72.0–88.0</td>
<td>17.9</td>
</tr>
<tr>
<td>Daytime pulse pressure (mmHg)</td>
<td>958a</td>
<td>51.4</td>
<td>45.0–58.4</td>
<td>14.2</td>
</tr>
</tbody>
</table>

1Proportion of variability explained by adjustment for environmental variables (i.e. age, sex, alcohol consumption, smoking behaviour, exercise taken). All variables were log-transformed before adjustment except Waist-hip ratio, to approximately normalize the distributions.

2Of whom 52.4% were females and 36.1% were classified as hypertensive.

3Quantitative recordings.
There was a highly significant association of the -532T allele with lower pulse pressure \((p = 0.0002)\) in those family members with quantitative recordings available. To validate our findings, we repeated the analyses including those individuals who had had ambulatory monitors on known anti-hypertensive therapy, adding an empirical 5 mmHg to systolic and 2.5 mmHg to diastolic BP per agent taken.\(^{27}\) In these analyses, that included 1136 people, a highly significant association was also present \((p = 0.00006)\). The association between the AGT C-532T polymorphism and pulse pressure is represented graphically in Figure 1. Only 19 people carried the homozygous T/T genotype at C-532T. We, therefore, conducted subsidiary analyses under a dominant genetic model, comparing pulse pressure in those with C/C genotype against those with either C/T or T/T genotype. In those analyses, there remained highly significant evidence of association \((p = 0.0002)\). Back-calculation by exponentiation of the between-genotype differences in the adjusted, standardized, log-transformed values used in the analysis, and calibration to the mean pulse pressure in the population, was carried out to provide an approximate value for the difference in the untransformed pulse pressure between genotypes. This suggested that pulse pressure was on average lower by 2.88 mmHg per T allele carried \((95\% \text{ CI } 1.26–4.23)\) under the additive model in this population. Genotypes at AGT C-532T accounted for 1.6% of the total variability of pulse pressure observed.

Previous meta-analyses have suggested an additive effect of the AGT M235T polymorphism on plasma AGT levels and BP, so an additive model was tested first for this polymorphism. Under an additive model, there was no significant association between genotypes at AGT M235T and SBP, DBP or pulse pressure in those subjects with quantitative recordings available. There was a borderline significant association \((p = 0.014)\) between the 235T allele and lower pulse pressure when individuals with ambulatory monitors recorded on treatment were included in the analyses (Table 2). However, this was much weaker than the association observed with the C-532T polymorphism. There was no significant association between the M235T polymorphism and systolic or diastolic BP in that larger group, or between the M235T polymorphism and hypertension status (data available on request). Examination of the trend of the mean pulse pressures by M235T genotype, however, (Table 2) suggested a recessive model for an association of M235T with pulse pressure. When such a

<table>
<thead>
<tr>
<th>Table 2 Association of BP phenotypes with AGT polymorphisms</th>
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</thead>
<tbody>
<tr>
<td><strong>AGT C-532T</strong></td>
</tr>
<tr>
<td>Genotype</td>
</tr>
<tr>
<td>C/C</td>
</tr>
<tr>
<td>No. of subjects (untreated only, (n = 921))</td>
</tr>
<tr>
<td>Systolic BP</td>
</tr>
<tr>
<td>(1.014)</td>
</tr>
<tr>
<td>Diastolic BP</td>
</tr>
<tr>
<td>(0.9789)</td>
</tr>
<tr>
<td>Mean BP</td>
</tr>
<tr>
<td>(1.001)</td>
</tr>
<tr>
<td>Pulse pressure</td>
</tr>
<tr>
<td>(1.007)</td>
</tr>
<tr>
<td>No. of subjects (treated and untreated, (n = 1136))</td>
</tr>
<tr>
<td>Pulse pressure (including drug-adjusted values)</td>
</tr>
<tr>
<td>(0.9980)</td>
</tr>
</tbody>
</table>

\(^{a}\)Additive genetic model.

Values are means of log-transformed, standardized, blood pressures adjusted for age, sex, alcohol consumption, smoking and exercise for each genotype, together with SD.

![Figure 1](https://academic.oup.com/ije/article-abstract/36/6/1356/822529)
model was tested, there was a significant association of TT genotype (vs MT and MM genotypes) with pulse pressure in the quantitatively studied individuals \( (P = 0.004) \) which was somewhat stronger in all individuals \( (P = 0.002) \). This association was considerably less strong than that observed for C-532T, and it was non-significant in models incorporating the effect of the C-532T SNP.

Plasma levels of AGT were available on 548 individuals. Each -532T allele was associated with an ~10% higher plasma angiotensinogen level \( (r^2 = 1.0\%; \ P = 0.024 \text{ under an additive genetic model}) \). The mean plasma AGT in those of C/C genotype was 1.53 nMol/ml (with SD 0.74), in those of C/T genotype was 1.70 nMol/ml (0.74), and in those of T/T genotype 1.79 nMol/ml (0.83). Each 235T allele was associated with a 7% higher plasma angiotensinogen level \( (r^2 = 1.0; \ P = 0.027 \text{ under an additive genetic model}) \). The mean plasma AGT in those of C/C genotype was 1.53 nMol/ml (0.75), in those of M/T genotype was 1.56 nMol/ml (0.21), and in those of M/M genotype was 1.70 nMol/ml (0.75). Addition of M235T genotype to a regression model incorporating C-532T genotype did not significantly improve the model’s fit.

### Discussion

We have shown a small but highly significant effect of the C-532T polymorphism of the AGT gene on pulse pressure in 1136 members of 248 families. The T allele was associated with a 2.8 mmHg lower pulse pressure in carriers, under an additive model. There was evidence for association of the T allele at the M235T polymorphism with lower pulse pressure (under a recessive genetic model), but this was much less strong than for the C-532T polymorphism. Our results, therefore, support the argument of Brand and colleagues \(^{18}\) that the functional polymorphism at AGT is either C-532T itself or strongly correlated with C-532T. Genotype at the C-532T polymorphism accounted for just under 2% of the total population variability in pulse pressure. We found no strong evidence for a primary association of either the C-532T or the M235T polymorphism with systolic or diastolic BP—there were small differences in opposite directions for SBP and DBP between different C-532T genotypes, but these were non-significant even in this large study. This suggests that the principal physiological effect of genetic variation at the AGT locus is on arterial stiffness. Increased arterial stiffness is recognized as a risk factor for cardiovascular and cerebrovascular events.\(^{28}\) Several methods exist to measure arterial stiffness, of which pulse pressure is the simplest. Although pulse pressure is not the most sensitive method to measure arterial stiffness, it has the advantage over other methods that it has been strongly associated with mortality from cardiovascular disease and stroke in prospective studies involving very large numbers of participants.\(^{29}\)

Few previous studies have investigated the association between AGT polymorphisms with BP as a quantitative trait, and support for such an association is presently lacking. As part of their 2003 meta-analysis Sethi et al.\(^{10}\) collated information on eight quantitative studies. These showed no evidence for association of the AGT M235T genotype with systolic or diastolic BPs in a total of 8774 Caucasian subjects. However, only two studies had included greater than 500 subjects, and neither of these larger studies typed the C-532T variant. Why meta-analysis tends to support the association of the 235T allele with hypertension status, but not with BP measured as a quantitative trait, remains an open question. Few studies thus far have examined the relationship between AGT genotypes and pulse pressure, even though, as here, individually non-significant differences in systolic and diastolic BPs can when taken together result in a highly significant difference in pulse pressure. The large study performed by Sethi et al.\(^{10}\) found no significant differences in pulse pressures between individuals with different M235T genotypes, but those investigators did not type the C-532T polymorphism.

Substantial between-study heterogeneity has been observed in meta-analyses of the association between the AGT M235T polymorphism and hypertension status. Our results could in part account for these observations. There is strong linkage disequilibrium between the AGT M235T and C-532T polymorphisms; the -532T allele occurring almost exclusively on a subset of the chromosomes bearing the 235T allele. Because of the lower pulse pressure associated with the -532T allele (in the absence of significant differences in mean BP between genotypes), studies which had ascertained cases based on an individual exceeding particular criteria for both systolic and diastolic BP would tend to have a higher proportion of -532T allele carriers (and therefore of 235T allele carriers) in the case group than studies which had ascertained cases based on SBP criteria alone. This would give rise to an apparently larger effect of the 235T allele on hypertension status in studies ascertaining on both systolic and diastolic BP criteria than in studies ascertaining on systolic pressure alone (regardless of whether there was, in fact, any true association).

It is perhaps surprising that the T allele of the C-532T polymorphism, which is associated in this and in other studies with higher levels of plasma AGT, is also strongly associated with lower pulse pressure in the present study. We suggest two possible explanations. First, we note that the genetic factors influencing the production of AGT, and the local consequences of differences in AGT levels, are little studied in most tissues. They may differ from those factors principally responsible for regulating AGT production in hepatocytes (the source of most AGT present in plasma). Such tissue-specific differences accounting for our result cannot at this stage be ruled out. Further studies that investigate the production and effects of AGT in those cell types potentially most relevant to arterial stiffness (such as vascular smooth muscle cells and perivascular adipocytes) would be necessary to investigate this possibility. Second, it is possible that genetically mediated differences in particular physiological factors during development (here, a slightly higher level of AGT production) could be ‘buffered’ by epigenetic changes in other components of the affected physiological system and alter the expected relationship in later life between a particular SNP, plasma levels of a corresponding quantitative trait, and a final physiological phenotype—the phenomenon of canalization.\(^{30}\) With respect to this system, Hopkins and colleagues \(^{31}\) investigated in hypertensive patients the effect of genotype at an AGT promoter variant (G-6A) in linkage disequilibrium with the C-532T variant and similarly associated with plasma levels of AGT. In that study, the ‘high angiotensinogen’ allele (-6A) was
associated with a reduced renal vascular response and production of aldosterone by the adrenal cortex in response to angiotensin II infusion, consistent with downregulation of the angiotensin II receptor or other downstream components of the signalling pathway in those genetically predisposed to higher AGT levels.

Strengths of this study include its large size, the use of ambulatory monitoring and its family-based design which enables additional checks on genotyping accuracy (based on Mendelian inheritance) and alleviates concerns regarding population stratification as a spurious source of association. The result was reinforced by the addition of individuals with ambulatory monitors on treatment to those with quantitative records. Selection of the families through a proband with essential hypertension is a potential theoretical limitation of the study’s generalizability; however, some selection is necessary in such genetic studies to achieve power to detect effects, and two-thirds of the cohort were normotensive. Pulse pressure is a relatively unsophisticated measurement of arterial stiffness, therefore we may have underestimated the size of the genetic effect attributable to AGT C-532T. Future studies involving similarly large numbers of patients characterized using more specific techniques (such as magnetic resonance imaging of the central arteries) will be needed to increase the accuracy with which the effect we have described can be quantified.

Conclusions

We have shown a significant association between the C-532T polymorphism of the AGT gene and pulse pressure. This association could be a genetic source of heterogeneity between studies of the neighbouring M235T polymorphism and hypertension. Genotyping of the C-532T polymorphism in published cohorts genotyped for M235T, together with a detailed examination of study inclusion criteria, may enable the substantial between-study heterogeneity apparent in meta-analyses of the association between M235T and BP to be to some extent resolved.

Acknowledgement

The Sources of Funding are British Heart Foundation, UK Medical Research Council and Wellcome Trust.

Conflicts of interest: None declared.

KEY MESSAGES

- The C-532T SNP in the promoter region of the AGT gene is associated with arterial stiffness as measured by the pulse pressure.
- Each T allele is associated with about a 2.9 mmHg lower pulse pressure despite no significant association being found in this study with systolic or diastolic blood pressure alone.
- This primary effect on arterial compliance could in part account for unexplained heterogeneity observed in previous meta-analyses of AGT polymorphisms and risk of hypertension.

References


