

ARTICLE

# Measured haplotype analysis of the aldosterone synthase gene and heart size

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Gene-association studies of heart size and the aldosterone synthase (CYP11B2) gene have produced inconsistent results, possibly because of limitations in the sample size and/or the number and location of the polymorphisms. An analysis of six polymorphisms spanning 6 kb of the CYP11B2 gene in Caucasian British families revealed a limited number of haplotypes because of strong linkage disequilibrium over this small region. The genotype and haplotype information was used in an association study involving 955 members of 229 families phenotyped for echocardiographic measures of heart size. In a mixed effects linear modelling analysis, the G5937C polymorphism was associated with cardiac wall thickness ( $P=0.02$ ), and the intron conversion and A4550C polymorphisms were associated with left ventricular cavity size ( $P=0.02$  and  $0.002$ , respectively). Measured haplotype analyses confirmed the association of alleles at the intron conversion and G5937C polymorphisms with cardiac wall thickness ( $P=0.02$ ), and alleles at the intron conversion polymorphism with left ventricular cavity size ( $P=0.04$ ). The polymorphisms contributed to 2.0–3.4% of the variability in these traits. In summary, genetic polymorphisms at the CYP11B2 gene make a small contribution to quantitative variation in echocardiographic measures of heart size. These results point to the importance of analysing the full extent of genetic variation that captures the haplotype structure of a locus in gene association studies.

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## Introduction

Measures of heart size, including left ventricular (LV) mass, LV cavity size, and cardiac wall thickness are multifactorial traits that are influenced by environmental and genetic factors. The importance of the genetic contribution to the variability of these traits is reflected in heritability estimates of 28% for echocardiographic LV mass, 48% for cardiac wall thickness, and 93% for LV cavity size.<sup>1–3</sup> Therefore, the motivation to identify specific genes that influence quantitative variation in these phenotypes is well

founded, with the prospect that such findings would generate insights into the pathogenesis of cardiac hypertrophy. Association studies that focus on genes with a plausible role in cardiac remodelling are one way of addressing this question.

A number of lines of evidence suggest that genetic variation in the aldosterone synthase (CYP11B2) gene may, through its effects on aldosterone metabolism, influence variation in cardiac structure.<sup>4</sup> Rare mutations of the CYP11B2 gene, which encodes a key enzyme in aldosterone synthesis, cause either markedly elevated or suppressed aldosterone levels, while common polymorphisms may be associated with variability in plasma aldosterone in the general population.<sup>5</sup> Significant positive correlations have been observed between serum aldosterone and measures of heart size in humans.<sup>6</sup> Furthermore, aldosterone has direct

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cardiac cellular effects, including stimulation of fibroblast proliferation and collagen deposition by activation of local mineralocorticoid receptors.<sup>7</sup> The direct effects of aldosterone on cardiac structure, coupled with the association of CYP11B2 gene variants with plasma aldosterone levels, has led to the hypothesis that common polymorphisms in the CYP11B2 gene may influence cardiac size.

Kupari *et al*<sup>8</sup> reported a possible association between echocardiographic LV mass and the T-344C polymorphism in the promoter region of the CYP11B2 gene in 84 healthy Finnish individuals aged 36–37 years; CYP11B2 promoter genotype predicted statistically significant variation in LV mass and cavity size, independently of the covariates of sex, body size, and blood pressure. Three other studies have subsequently investigated the association of the same CYP11B2 polymorphism with heart size.<sup>9–11</sup> Although the study by Delles *et al*<sup>11</sup> of 120 young men with mild hypertension seemed to confirm the association, two larger studies that enrolled a total of 2613 participants from the MONICA project did not.<sup>9,10</sup>

There may be several reasons to account for these conflicting results. The positive association studies involved small sample sizes of unrelated individuals.<sup>8,11</sup> Results of small studies should be treated with caution, as they may not have the power to detect the small effects likely to be attributable to genetic variants that influence multifactorial traits. Furthermore, it has been shown that small genetic studies that observed an extreme result are more likely to be publicly available than those that did not, leading to an exaggeration of the real effect.<sup>12</sup> The possibility of publication bias in this case is supported by the fact that the larger studies were negative.<sup>9,10</sup> However, these larger studies only studied associations with one polymorphism in the 5' end of the CYP11B2 locus, and the failure to examine other polymorphisms in the CYP11B2 locus could lead to false-negative findings.

To clarify the uncertainty concerning the association of polymorphisms at the CYP11B2 locus with quantitative measures of heart size, we have typed several markers that span the CYP11B2 gene and combined these into haplotypes, resulting in greater power to resolve loci that influence quantitative traits.<sup>13</sup> We have determined the contribution of individual variants and haplotypes to the variability in echocardiographic measures of heart size in a large panel of extended families specifically ascertained for fine mapping of quantitative cardiovascular trait loci.<sup>14</sup>

## Methods

### Families and phenotypes

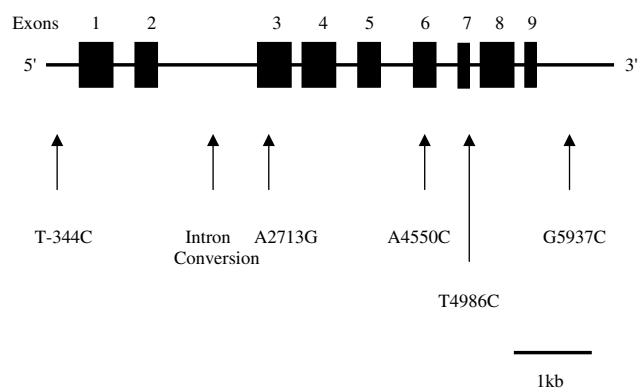
In all, 248 British Caucasian extended families (1425 individuals) were recruited through hypertensive probands for genetic studies of blood pressure and other quantitative cardiovascular risk factors.<sup>15–17</sup> The local institutional review committee approved the study, and all subjects

gave written consent. A subset of 955 members of 229 families attended for echocardiography and acceptable studies were obtained in 826 members of 222 families.<sup>17</sup> Here we report data on septal wall thickness, LV cavity size, and LV mass.

The traits were adjusted for the significant covariate effects of age, systolic blood pressure, height, weight, waist–hip ratio, and presence of diabetes by stepwise linear regression (SPSS for Windows 9.0.0, SPSS Inc., 1999); sex-specific standardised residuals (mean 0, variance 1) were used in the genetic analyses.<sup>17</sup> The distributions of the residuals were tested for normality; it was necessary to eliminate several (ie, septal wall thickness, 3; LV cavity size, 1; LV mass, 2) extremely high outliers and log transform septal wall thickness and LV mass prior to the regression analysis. Analysis of the data with or without the outliers did not change the results of the study.

### Genotyping

Six bi-allelic polymorphisms spanning the CYP11B2 gene were genotyped. The location of each polymorphism is shown with respect to CYP11B2 exonic sequences in Figure 1. The method of Davies *et al*<sup>18</sup> was used to type the intron conversion polymorphism located in the second intron of CYP11B2. A further five single nucleotide polymorphisms, T-344C, A2713G, A4550C, T4986C, and G5937C, originally described by others,<sup>5,19–21</sup> were genotyped following PCR amplification and digestion by restriction enzymes. Full PCR experimental details are available at [http://www.cardiov.ox.ac.uk/data\\_supp.html](http://www.cardiov.ox.ac.uk/data_supp.html). Genotyping was performed blind to the phenotypic information. Mendelian inheritance within families was confirmed using the PedCheck program<sup>22</sup> and inconsistencies resolved by re-examination of the raw data, and re-genotyping where necessary.



**Figure 1** Schematic diagram of the aldosterone synthase (CYP11B2) gene illustrating the location of the six-biallelic polymorphisms. Polymorphisms are numbered in base pairs relative to the start of transcription of the CYP11B2 gene. Exons 1–9 are indicated with vertical bars. The X-axis represents the physical size of the gene in kilobase (kb) Scale 1 cm=1 kb).

## Haplotyping

Haplotypes of the biallelic polymorphisms of the CYP11B2 gene were studied in 1425 members of 248 extended pedigrees by an approach that has been previously described in detail.<sup>23</sup> Briefly, incomplete genotypes were reconstructed using a modified version of the UNKNOWN program, which takes known genotypes in relatives into account.<sup>24</sup> The program HAPLOTRY (available on request from the authors) was used to compile a complete list of alternative phase assignments (assuming no intragenic recombination) for most families. For two families, each with more than 14 genotyped members, SIMWALK2 was repeatedly run (using different pseudo-random number generator seeds) to sample from the total space of alternative haplotype vectors<sup>14</sup> until this list converged. Haplotype frequencies in the British Caucasian population were estimated in the founders (assuming no recombination) by maximum likelihood analysis using the Merlin and fugue programs.<sup>23</sup>

## Association analysis

A two-stage approach was used to investigate association between CYP11B2 polymorphisms and measures of heart size. Firstly, each trait was tested for association in a mixed effects linear model to identify phenotypes with significant genetic associations for detailed study by measured haplotype analysis. In the mixed effects linear model, genotypes for all six markers were specified as fixed effects, and a sequential integer to identify each pedigree was specified as a random effect. This allowed us to model (at least partially) the residual correlations within each family that were not accounted for by shared genotypes at the CYP11B2 locus as well as additive and dominance effects for each genotype. We implemented a step-wise, backward selection procedure, with a retention threshold  $P < 0.05$  based on type III sums-of-squares tests, in order to select a parsimonious model that adequately fits the data.

In the second stage of analysis, the alternative phase assignments were entered into a modified version of the Pedigree Analysis Package (PAP) to undertake a measured haplotype analysis in which a codominant quantitative trait locus (QTL) model is fitted to measure the influence of each haplotype on the traits of interest. The model assumes homoscedasticity of the means associated with each haplotype. Likelihoods were maximised numerically and standard errors were calculated for parameter estimates. Intranuclear family correlations not accounted for by shared haplotype effects were modelled using the PAP *papwgfc* subroutine.<sup>25</sup>

In order to summarise the impact of the variants that showed evidence of association with a trait by the mixed effects linear models, we fitted an additive, allele substitution model within the measured haplotype analysis framework.<sup>25</sup> In this model, the average substitution effect of each polymorphism was estimated relative to the com-

monest or 'reference' haplotype. Parameters were specified to model the effects of substituting alleles at the polymorphic sites relative to their state in the 'reference' haplotype. For example, the haplotype TIATG differs from the 'reference' haplotype TCATG at a single site (IC polymorphism). Thus, the mean associated with TIATG was specified as the sum of the mean for TCATG plus the substitution effect C → I. Maximisation of the likelihood of the additive, allele substitution model provides a numerical solution to a set of simultaneous equations.

## Results

The clinical characteristics of the 826 members of 222 families with echocardiographic measurements are shown in Table 1. The prevalence of echocardiographic LV hypertrophy of 34.3% was similar to the rates found in other populations with hypertension.<sup>26</sup> Almost all the subjects with hypertension were receiving antihypertensive treatment, but no participant was receiving spironolactone, a diuretic that acts by antagonising the effect of aldosterone. The average family size and number of generations per family was seven members (range 3–27) and two generations (range 2–4), respectively.

## Haplotyping

Haplotype frequencies were estimated in the founders of 248 extended families initially for six biallelic polymorphisms of the CYP11B2 gene. The T-344C and A2713G were found to be in complete linkage disequilibrium (LD) on haplotypes with frequencies >0.2% and the haplotype frequencies were re-estimated after omitting A2713G from the analysis. Table 2 shows the 11 most frequent five-locus haplotypes, which cumulatively account for more than 95% of the founders' haplotypes (a complete listing of the haplotypes is available at [http://www.cardiov.ox.ac.uk/data\\_supp.html](http://www.cardiov.ox.ac.uk/data_supp.html)). Pairwise LD extended to the 3' untranslated region of the CYP11B2 gene, although some variation in the strength of LD was present, with the T-344C and A2713G polymorphisms in tightest linkage disequilibrium ( $D'$  0.89), and T4986C and G5937C having the weakest linkage disequilibrium ( $D'$  0.04) (Table 3).

**Table 1** Clinical characteristics (mean, standard deviation) of the participants

Variable	Echo study (n=826)
Male (%)	43.8
Age (years)	52.4 (13.5)
Hypertension (%)	42.3
Systolic blood pressure (mmHg)	137.4 (21.0)
Left ventricular hypertrophy (%)	34.3
Body mass index (kg/m <sup>2</sup> )	27.1 (4.8)
Diabetes (%)	2.8

**Table 2** Ranked haplotype frequencies for five CYP11B2 markers

Rank	Frequency (%)	T-344C	IC	A4550C	T4986C	G5937C
1	38.0	T	C	A	T	G
2	22.3	C	I	C	T	C
3	13.8	C	I	C	T	G
4	6.7	C	C	A	C	G
5	3.3	C	I	C	C	C
6	3.1	T	I	A	T	G
7	2.0	C	C	A	T	G
8	1.9	T	C	C	T	G
9	1.7	C	C	C	T	G
10	1.6	C	C	C	T	C
11	0.9	C	I	C	C	C

IC, intron conversion polymorphism.

**Table 3** Standardised linkage disequilibrium coefficients,  $D'$  (Delta squared values are given in brackets)

Marker	IC	A2713G	A4550C	T4986C	G5937C
T-344C	0.72 (0.59)	0.89 (0.89)	0.78 (0.64)	0.61 (0.21)	0.74 (0.43)
IC		0.72 (0.58)	0.67 (0.66)	0.26 (0.09)	0.68 (0.49)
A2713G			0.77 (0.62)	0.66 (0.23)	0.74 (0.43)
A4550C				0.34 (0.12)	0.80 (0.57)
T4986C					0.04 (0.01)

IC, intron conversion polymorphism.

**Table 4** Mixed linear model analysis of single marker associations with echocardiographic measures of heart size

Factor	Major allele frequency	Degrees of freedom		Septal wall thickness		LV cavity size		Echo LV mass	
		Numerator df	Denominator df	F-statistic	P-value	F-statistic	P-value	F-statistic	P-value
T-344C	0.52	2	381	0.14	0.87	0.49	0.61	0.08	0.92
IC	0.53	2	381	1.50	0.23	3.96	0.02*	0.89	0.41
A2713G	0.52	2	381	0.11	0.90	0.11	0.89	0.29	0.75
A4550C	0.54	2	381	1.74	0.18	6.38	0.002*	1.64	0.20
T4986C	0.82	2	381	0.36	0.70	0.58	0.56	0.13	0.88
G5937C	0.64	2	381	4.05	0.02*	0.96	0.38	0.49	0.62
Family		194	381	1.28	0.02*	1.12	0.18	1.48	0.001*

IC, intron conversion; LV, left ventricular; df, degrees of freedom were the same for all phenotypes; echo, echocardiogram; \*, significant  $P$ -values.

### Association analysis

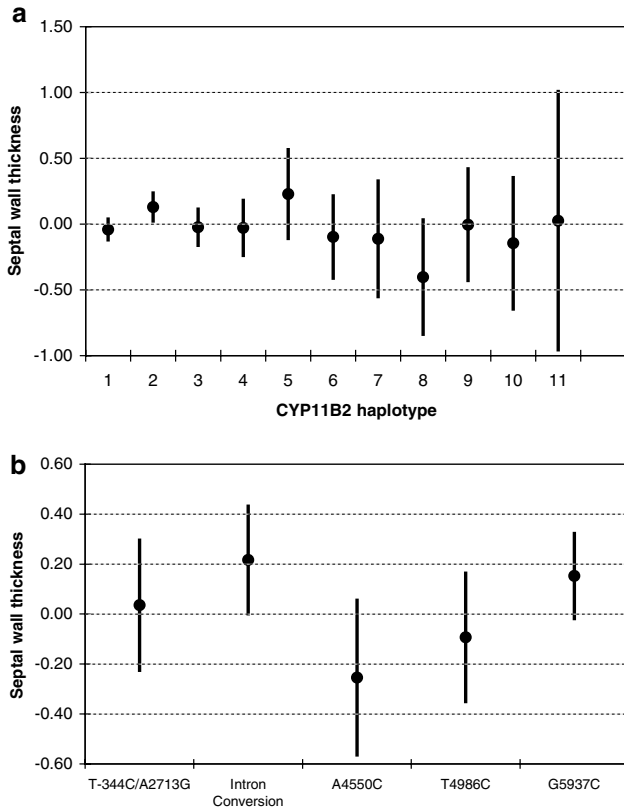
In the mixed linear model analysis (Table 4), the G5937C polymorphism was associated with variability in septal wall thickness ( $P=0.02$ ), and the intron conversion and A4550C polymorphisms were associated with variability in LV cavity size ( $P=0.02$  and  $0.002$ , respectively). A stepwise, backwards elimination model improved the fit of the mixed effects linear model (Table 5). The polymorphisms contributed to 2.4 and 2.0–3.4% of the variability in septal wall thickness and LV cavity size, respectively. There was no evidence for association between polymorphisms at the CYP11B2 locus and echocardiographic LV mass. Significant residual familial effects were present for septal wall thickness and echocardiographic LV mass (Table 4).

In measured haplotype analyses, haplotype 2 was associated with a significant increase in septal wall thickness (mean, 0.13; 95% CI, 0.02–0.24) (Figure 2a). Analysis of the average effect of the alleles relative to the commonest haplotype in an allele substitution model provided support for association of alleles at the intron conversion and G5937C polymorphisms with septal wall thickness ( $\chi^2=7.94$ ,  $df=2$ ,  $P=0.02$ ) (Figure 2b). Haplotypes 8 (mean, 0.68; 95% CI, 0.24–1.11) and 11 (mean, 1.03; 95% CI, 1.06–1.94) were associated with a significantly greater LV cavity size (Figure 3a). The allele substitution model provided evidence for a significant association between alleles at the intron conversion polymorphism and LV cavity size ( $\chi^2=4.34$ ,  $df=1$ ,

**Table 5** Mixed linear model analysis of the three CYP11B2 markers that are associated with measures of heart size in a stepwise, backward elimination procedure: parameter estimates of the final model

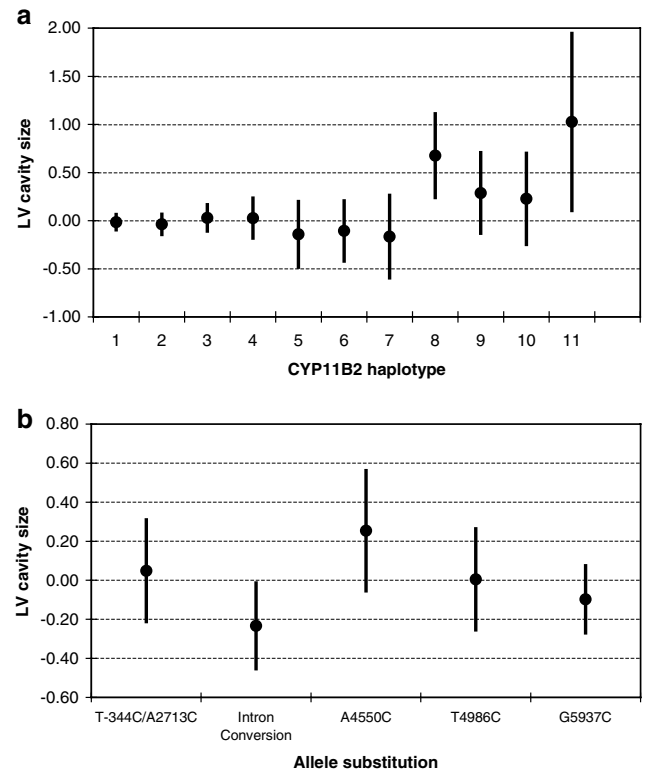
Trait	Marker	P-value on entry	F-statistic (final model)	Numerator df	Denominator df	P-value (final model)
Septal wall thickness	G5937C	0.02	4.23	2	489	0.015
LV cavity size	IC	0.02	4.88	2	443	0.008
LV cavity size	A4550C	0.002	5.71	2	443	0.003

IC, intron conversion; LV, left ventricular; df, degrees of freedom; Echo, echocardiogram; \*, significant P-values.



**Figure 2** (a) Additive influence of each CYP11B2 haplotype on covariate-adjusted standardised residual of the natural logarithm of septal wall thickness (mean 0, variance 1). The solid circles represent the mean effect of each haplotype on the trait, and the bars show the 95% confidence interval of the mean. A significant haplotype effect on the trait is present if the confidence interval does not overlap the mean value for the trait of zero, that is, haplotype 2. (b) Analysis of the average substitution effect of marker alleles relative to the commonest 'reference' haplotype on septal wall thickness. Substitution of alleles at the intron conversion and G5937C polymorphisms on a haplotype 1 background were associated with a significant raising effect on septal wall thickness ( $P=0.02$ ).

$P=0.04$ ), but no significant substitution effect was detected with alleles at the A4550C polymorphism ( $\chi^2=2.61$ ,  $df=1$ ,  $P=0.11$ ).



**Figure 3** (a) Additive influence of each CYP11B2 haplotype on covariate-adjusted standardised residual of LV cavity size (mean 0, variance 1). The solid circles represent the mean effect of each haplotype on the trait and the bars show the 95% confidence interval of the mean. A significant haplotype effect on the trait is present if the confidence interval does not overlap the mean value for the trait of zero, that is, haplotypes 8 and 11. (b) Analysis of the average substitution effect of marker alleles relative to the commonest haplotype on LV cavity size. Substitution of alleles at the intron conversion polymorphism on a haplotype 1 background was associated with a significant lowering effect on LV cavity size ( $P=0.04$ ).

### Discussion

We found that polymorphisms and haplotypes at the CYP11B2 locus are associated with a small but significant effect on the quantitative variation in cardiac septal wall thickness and LV cavity size. No significant association

was detected of genetic variation at the CYP11B2 locus with echocardiographic LV mass, a compound phenotype derived from septal wall thickness, LV internal dimension, and posterior wall thickness. This suggests that the effect of genetic variation on LV mass is insufficient to outweigh the relative inaccuracy of this estimated measure even in this large family study.

The results of the study support the hypothesis that genetic variability at the CYP11B2 locus has a modest effect on cardiac structure.<sup>8</sup> The mechanism of the association is not known, but may involve an effect of genotype on the activity of the CYP11B2 gene and consequent differences between genotypes on plasma and tissue aldosterone activity. However, the data relating CYP11B2 variants and plasma or urine aldosterone levels are conflicting, with some observing opposite effects of the T-344C polymorphism,<sup>5,27,28</sup> while others have found no effect.<sup>10</sup> Thus, the mechanism of the association between variants at the CYP11B2 locus and anatomical measures of cardiac size remains to be established. It is also possible that these polymorphisms are merely markers for neighbouring variant(s) in linkage disequilibrium that actually mediate the observed effects on heart size. More extensive measured haplotype analysis, including, for example, the adjacent steroid 11  $\beta$ -hydroxylase (CYP11B1) gene, will be necessary to resolve this issue.

Our study adds to the previous studies of the association between polymorphisms at the CYP11B2 locus and measures of cardiac size in several respects. Firstly, we have studied the linkage disequilibrium and haplotype structure of several polymorphisms that span the CYP11B2 gene, and assessed the relation of the genetic variation with quantitative measures of cardiac size. Our findings show association of polymorphisms in the hitherto incompletely studied 3' end of the gene with heart size, and may explain why some previous studies, which only studied 5' polymorphisms, failed to detect any effect. Also, all the previous studies were conducted in unrelated individuals from the general population.<sup>8–11</sup> In our study, the availability of family data acts as a check on genotyping accuracy and the study is less susceptible to the potential confounding issue of population stratification.<sup>29</sup>

It has been suggested that genetic variability at the CYP11B2 locus may influence blood pressure levels in the general population.<sup>18,20</sup> Since blood pressure is an important determinant of cardiac hypertrophy, the selection of the families by a hypertensive index case could confound the results of the study. Several steps were taken to address this potential bias in our study. Firstly, the echocardiographic phenotypes used in the genetic analyses were adjusted by regression analysis for the covariate effects of blood pressure. Secondly, the effect of ascertainment by hypertension on the results

was explored by performing additional family-based association analyses using the Quantitative Transmission Disequilibrium Test (QTDT) program incorporating an ascertainment correction procedure.<sup>30</sup> The results of the QTDT analysis were similar to the findings of the measured haplotype analysis, suggesting no evidence of bias introduced by the ascertainment scheme (data not shown). Finally, a preliminary measured haplotype analysis showed no evidence for significant association of the CYP11B2 locus and blood pressure phenotypes in these families (data not shown). It is likely, therefore, that the effect of the variants at the CYP11B2 locus on the measures of heart size is independent of blood pressure in this family study.

In this study we have examined three correlated traits and six polymorphisms in moderate/strong linkage disequilibrium, which raises the possibility of false-positive results because of multiple testing. A simple statistical adjustment for multiple testing with the three traits ignoring the correlations would be anticonservative. However, even if statistical adjustment equivalent to two independent tests is carried out as a compromise in this study, the association of septal wall thickness with polymorphism at the CYP11B2 remains significant. Furthermore, the haplotype-based analysis to some extent, provides a natural solution to the issue of multiple testing with several markers.

These results may be useful for the future design of studies of the genetic basis of multifactorial traits of relatively modest heritability, which include the majority of traits associated with heart disease. Of particular importance is the need to explore the structure of genetic variability present within candidate genes. In addition, the study illustrates the utility of haplotype-based approaches to study human QTLs.<sup>23</sup> Finally, the haplotype architecture that has been established in this study, and the potentially informative variants implicated within these haplotypes, will be of use in other genetic association studies of the CYP11B2 locus in Caucasian populations.

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