

GWAS of butyrylcholinesterase activity identifies four novel loci, independent effects within *BCHE* and secondary associations with metabolic risk factors

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Received July 6, 2011; Revised and Accepted August 18, 2011

Serum butyrylcholinesterase (BCHE) activity is associated with obesity, blood pressure and biomarkers of cardiovascular and diabetes risk. We have conducted a genome-wide association scan to discover genetic variants affecting BCHE activity, and to clarify whether the associations between BCHE activity and cardio-metabolic risk factors are caused by variation in BCHE or whether BCHE variation is secondary to the metabolic abnormalities. We measured serum BCHE in adolescents and adults from three cohorts of Australian twin and family studies. The genotypes from ~2.4 million single-nucleotide polymorphisms (SNPs) were available in 8791 participants with BCHE measurements. We detected significant associations with BCHE activity at three independent groups of SNPs at the *BCHE* locus ($P = 5.8 \times 10^{-262}$, 7.8×10^{-47} , 2.9×10^{-12}) and at four other loci: *RNPEP* ($P = 9.4 \times 10^{-16}$), *RAPH1-ABI2* ($P = 4.1 \times 10^{-18}$), *UGT1A1* ($P = 4.0 \times 10^{-8}$) and an intergenic region on chromosome 8 ($P = 1.4 \times 10^{-8}$). These loci affecting BCHE activity were not associated with metabolic risk factors. On the other hand, SNPs in genes previously associated with metabolic risk had effects on BCHE activity more often than can be explained by chance. In particular, SNPs within *FTO* and *GCKR* were associated with BCHE activity, but their effects were partly mediated by body mass index and triglycerides, respectively. We conclude that variation in BCHE activity is due to multiple variants across the spectrum from uncommon/large effect to common/small effect, and partly results from (rather than causes) metabolic abnormalities.

INTRODUCTION

Genetic variation affecting plasma butyrylcholinesterase (pseudo-cholinesterase, BCHE) activity was initially recognized through study of succinylcholine apnoea and its association with BCHE deficiency (1,2). A more recently appreciated aspect of BCHE activity is its association with plasma lipid and lipoprotein concentrations, obesity,

components of the metabolic syndrome and type 2 diabetes (3–13). Despite numerous pharmacogenetic and disease association studies, the physiological functions of BCHE and the reasons for associations between BCHE activity and components of the metabolic syndrome are unknown.

The BCHE enzyme is coded by the *BCHE* locus at 167 Mb on chromosome (Chr) 3. A common single-nucleotide polymorphism (SNP) (rs1803274, Ala539Thr, K-variant) is

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associated with ~7% reduction in enzyme activity in heterozygotes and 14% in homozygotes (14). Many less common variants, with greater effects on enzyme activity, are known. Homozygotes for the dibucaine-resistant atypical enzyme (rs1799807, Asp70Gly) have 40% of usual activity (2) and the deficient allele frequency is ~1–2% in Europeans. Other deficiency variants [MIM 177400] are rare in the general population.

Several lines of evidence connect genetic variation in *BCHE* with obesity and obesity-related disease. Studies on *BCHE* knockout mice have shown unexpected effects on body weight. These animals are viable in the homozygous state and show few effects of the enzyme deficiency, but they become obese when fed a high fat diet despite similar food intake to control wild-type mice (13). Conversely, people with high *BCHE* activity and an additional electrophoretic *BCHE* band (15) may have lower body weight than controls (16). There are also reports that the K-variant in *BCHE* is associated with type 2 diabetes (17) and with coronary artery disease (18,19).

Other evidence connects *BCHE* activity, rather than genotype, with obesity and its metabolic consequences. We have previously shown that higher *BCHE* activity is associated with higher body mass index (BMI), higher triglycerides, lower high-density lipoprotein (HDL) and lower insulin sensitivity (10). We also reported highly significant *BCHE* associations with serum total and low-density lipoprotein (LDL) cholesterol, apolipoproteins B and E, urate, gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT) and blood pressure. Each of these is related to metabolic syndrome and fatty liver. These associations with *BCHE* activity have been confirmed, and extended to measures of visceral, subcutaneous and hepatic fat stores (11,12), with higher *BCHE* being associated with less favourable lipid and adiposity status. These results are consistent with plasma or serum *BCHE* activity being a marker of metabolic syndrome and fatty liver, but previous studies have not combined comprehensive genotyping with measurement of both *BCHE* activity and metabolic variables. Advances in SNP genotyping and imputation of untyped SNPs, and discovery of loci affecting lipids and/or metabolic syndrome, allow us to investigate causality in the associations between *BCHE* activity and variables related to obesity.

This paper presents a genome-wide association study to investigate the genetic causes of variation in serum *BCHE* activity in the general population, and the causes of its covariation with markers of metabolic syndrome and cardiovascular risk. The results of these tests for genotype–phenotype associations improve our understanding of the relationship between *BCHE* and cardiovascular and diabetes risk factors.

RESULTS

Phenotypic associations between *BCHE* activity and metabolic risk factors

There were highly significant ($P < 10^{-9}$) phenotypic correlations between *BCHE* activity and BMI, HDL- and LDL-cholesterol, triglycerides, glucose, insulin, C-reactive protein, urate, ferritin, GGT and ALT in the 2000–2005

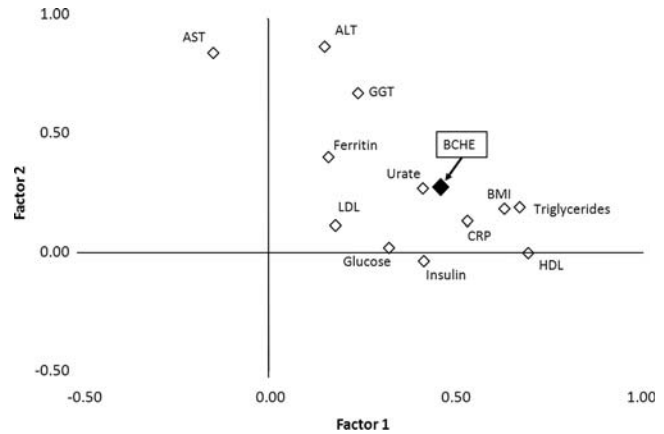


Figure 1. Clustering of phenotypes related to metabolic risk for cardiovascular disease and type 2 diabetes and *BCHE* activity, measured in the 2000–2005 Adult Studies. X- and Y-axes show the factor loadings from PCs analysis for each phenotype for Factor 1 and Factor 2.

Adult Studies. The strongest correlation ($R = 0.32$) was with triglycerides. Significant correlations were also found between *BCHE* activity and BMI, LDL-C, triglycerides and ALT in the Adolescent Study, but the correlations were less than in the adults and there was evidence of significant heterogeneity of the correlations between adults and adolescents. Details are shown in Supplementary Material, Table S1.

Factor analysis using data from the 2000–2005 Adult Studies illustrated the relationships between *BCHE* activity and other variables related to metabolic syndrome and/or cardiovascular risk. The clustering of factor loadings for *BCHE* activity and these correlated variables for two unrotated principal components (PCs) are shown in Figure 1; *BCHE* is located close to the lipids, BMI, glucose and insulin, urate and C-reactive protein.

Heritability of *BCHE* activity

Heritability in the 1993–1996 Adult Study was 0.65 (10). The sex-corrected repeatability in adults, across occasions up to 10 years apart, was $r = 0.71$. Heritability was estimated from the 2000–2005 Adult Studies as 0.63, and for the adolescents as 0.95. As well as the difference in additive genetic variance estimates between adolescents and adults, the total *BCHE* variance in the 2000–2005 Adult Studies was almost twice that in the Adolescent Study (s^2 4 383 970 against 2 323 141, $F_{15642,770} = 1.887$, $P = 8.56 \times 10^{-29}$). The differing distributions for *BCHE* activity in adults and adolescents are shown in Supplementary Material, Figure S1.

Genome-wide association analysis for *BCHE* activity

Genome-wide significant allelic association results for the discovery and two replication samples and the combined results are summarized in Table 1 and Figure 2. More detailed results are provided in Figures 3 and 4 and in Supplementary Material, Table S3. The genomic inflation factors (lambdas) for the 2000–2005 Adult, 1993–1996 Adult and Adolescent cohorts were 1.029, 1.016 and 1.016, respectively.

Table 1. Summary of independently genome-wide significant SNPs, showing beta, SE and *P* for each of the three studies (two groups of adults and one of adolescents) and overall

SNP, loci	Allele	2000–2005 Adult Studies (N = 6879)			Adolescents Study (N = 652)			1993–1996 Adult Study (N = 1260)			Overall (N = 8791)						
		Freq.	Beta	SE	R ²	<i>P</i> -value	Beta	SE	R ²	<i>P</i> -value	Beta	SE	<i>P</i> -value				
(A)																	
rs1803274, <i>BCHE</i>	T	0.21	-0.705	0.023	16.25	6.7×10^{-204}	-0.831	0.076	19.84	8.8×10^{-28}	-0.651	0.055	13.68	7.5×10^{-32}	-0.707	0.02	5.8×10^{-262}
rs4950806, <i>RNPEP</i>	T	0.40	-0.157	0.020	1.17	1.3×10^{-15}	-0.087	0.066	0.32	0.19	-0.09	0.047	0.39	0.052	-0.142	0.018	9.4×10^{-16}
rs11675251, <i>ABI2</i>	A	0.45	-0.134	0.019	0.88	2.5×10^{-12}	-0.291	0.066	3.72	9.7×10^{-6}	-0.159	0.047	1.23	0.0006	-0.148	0.017	4.1×10^{-18}
(B)																	
rs2668196, <i>BCHE</i>	A	0.20	-0.248	0.021	2.40	2.9×10^{-31}	-0.489	0.073	8.36	1.7×10^{-11}	-0.303	0.053	3.46	1.5×10^{-8}	-0.271	0.019	7.8×10^{-47}
rs7592624, <i>UGT1A1</i>	G	0.44	0.083	0.018	0.41	2.2×10^{-6}	0.154	0.056	1.32	0.0057	0.074	0.044	0.31	0.09	0.088	0.016	4.0×10^{-8}
rs6601299, <i>Chr-8</i>	T	0.11	-0.126	0.029	0.34	1.5×10^{-5}	-0.276	0.093	1.54	0.003	-0.200	0.073	0.83	0.006	-0.147	0.026	1.4×10^{-8}
(C)																	
rs2034445, <i>BCHE</i>	G	0.39	-0.127	0.018	0.95	4×10^{-13}	-0.003	0.056	0	0.96	0.088	0.043	0.45	0.040	-0.111	0.016	2.9×10^{-12}

(A) initial results, (B) additional loci found after adjusting for effects of rs1803274 in *BCHE*, (C) lead SNP for an independent group of SNPs in *BCHE* found after adjustment for effects of both rs1803274 and rs2668196.
Freq., allele frequency; SE, standard error of beta; R², percentage of phenotypic variance accounted for by the SNP.

In the discovery samples (Cohort 1: 2000–2005 Adult studies), we found 359 genome-wide significant SNPs ($P \leq 5 \times 10^{-8}$). These SNPs were at or near the *BCHE* gene (Chr 3), *RNPEP1* (Chr 1) and *ABI2* (Chr 2). The top SNPs in these loci were rs1803274 ($P = 6.7 \times 10^{-204}$), rs4950806 ($P = 1.3 \times 10^{-15}$), and rs11675251 ($P = 2.5 \times 10^{-12}$), respectively. The allelic effects of these SNPs were consistent with those in the replication samples (the Adolescent and Adult 1993–1996 cohorts), with the combined *P*-values of 5.8×10^{-262} , 9.4×10^{-16} and 4.0×10^{-18} , respectively (Table 1).

To find other SNPs independently associated with *BCHE* levels, we next conducted association analysis after adjusting for the substantial effect of the *BCHE* K-variant (rs1803274) in each cohort. In the discovery samples, we found additional SNPs within the *BCHE* locus independently and significantly associated with *BCHE* activity, with rs2668196 being the most significant at $P = 2.9 \times 10^{-31}$. This independent association was confirmed in the two replication cohorts, with a combined *P*-value of 7.8×10^{-47} . The meta-analysis of these conditional analysis results revealed two other loci significantly associated with *BCHE* activity, i.e. *UGT1A* (rs7592624; $P = 4.1 \times 10^{-8}$) and intergenic region of Chr 8 between *PPP1R3B* and *TNKS* (rs6601299; $P = 1.4 \times 10^{-8}$). Repeating this process by adjusting for rs2268196 as well as rs1803274 revealed a further set of independent SNPs near *BCHE*, with the most significant association at rs2034445 ($P = 4 \times 10^{-13}$), some 250 kbp from the *BCHE* gene (Fig. 3). This SNP was not replicated in the replication cohorts (Table 1). Significant and independent SNP associations accounted for ~22% of total variation in *BCHE* activity in the combined data.

Apart from rs1803274 (the K-variant), which showed a minor allele frequency of 0.21 in our data, *BCHE* deficiency variants were uncommon. The atypical or dibucaine-resistant variant (rs1799807, Asp70Gly) was neither genotyped nor imputed in the genome-wide typing because of its low minor allele frequency. As part of another project, it had been selectively genotyped in the 1993–1996 Adult Study samples, where it had a minor allele frequency of 0.02 and was associated with a 33% per-allele decrease in activity compared with the wild-type homozygous state.

Effects of genes associated with *BCHE* activity on cardiovascular and metabolic risk factors, and vice versa

We tested for associations between the most significant SNPs at the five identified loci and the metabolic syndrome-related phenotypes. Except for rs6601299 and HDL-C ($P = 2.4 \times 10^{-5}$), the distribution of *P*-values can be accounted for by chance (Supplementary Material, Table S4). To check whether the associations between *BCHE* activity and BMI or triglycerides were both caused by the K variant, we compared the effect size of *BCHE* activity on BMI or triglycerides before and after adjusting for the effect K variant. In the 2000–2005 Adult Studies, we found that the effect sizes (beta) of *BCHE* activity on BMI or triglycerides were 0.234 ± 0.012 and 0.313 ± 0.012 , respectively. When we adjusted *BCHE* activity for the effect of K variant, the effect sizes increased to 0.268 ± 0.013 and 0.353 ± 0.013 , respectively. This showed that the associations between *BCHE* activity and BMI or triglycerides were not

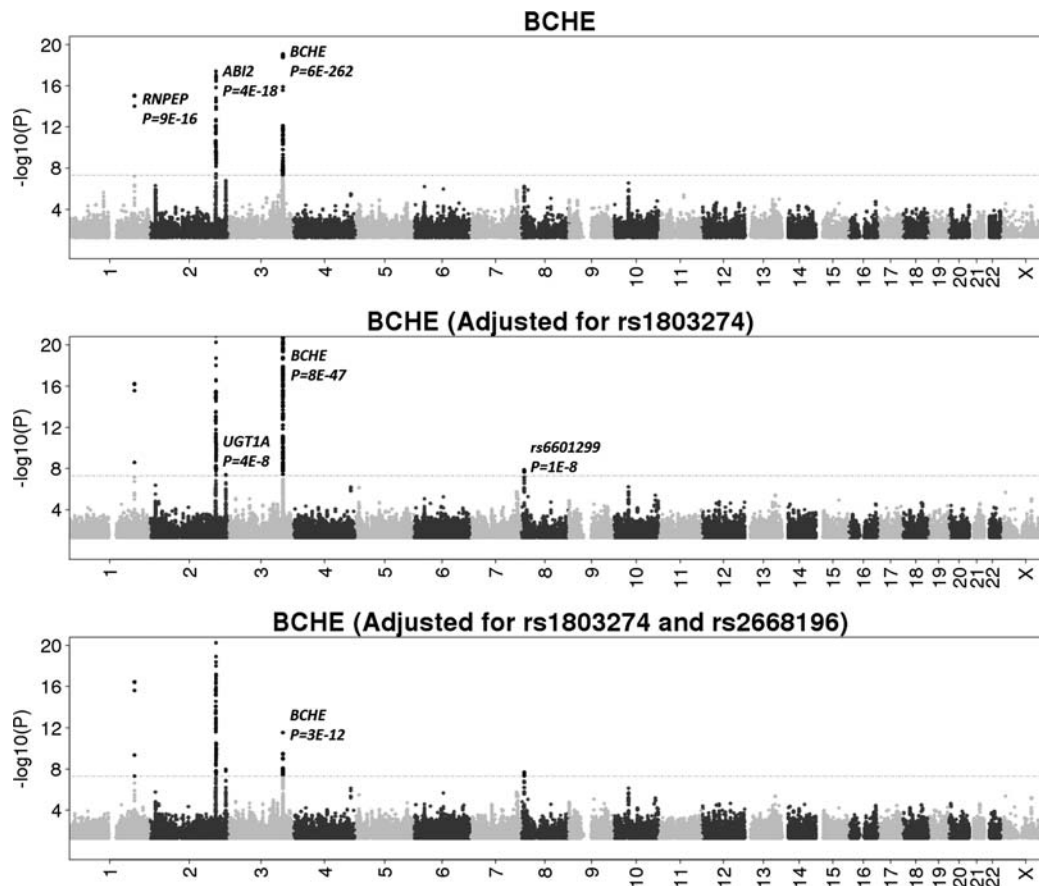


Figure 2. Manhattan plots for initial association analysis, and after adding rs1803274 (BCHE K variant) and then rs2668196 genotype as covariates.

attenuated by adjustment for the K variant; this is the outcome expected if the K variant affects BCHE activity but not BMI or triglycerides.

Conversely, we tested the effects of loci previously reported to affect other metabolic syndrome components on BCHE activity. We compiled a list of 213 reported SNPs (203 of which we had typed or imputed) at 97 distinct loci, from published papers and from the NHGRI database at <http://www.genome.gov/gwastudies/>, accessed 29 August 2011. Nineteen SNPs at eight of the loci (see Supplementary Material, Table S5) showed associations with BCHE at $P < 0.01$ (*CDH13*, *CELSR2*, *FADS2*, *FTO*, *GCKR*, *MC4R*, *MTNR1B* and *NCAN*), which is more than would be expected by chance. The divergence between the observed and expected P -values is illustrated as a Q–Q plot in Figure 5.

The traits most strongly correlated with BCHE activity are triglycerides and BMI. Other studies have reported that variants within *GCKR* (rs1260326) and *FTO* (rs9939609) are associated with triglyceride and BMI, respectively. We found that these variants were also associated with BCHE activity (P -values from the combined data were 0.0003 and 0.0025, respectively). To test whether the associations between these variants and BCHE activity were mediated by triglycerides or BMI, respectively, we performed a Mendelian randomization method (20). In the largest cohort, 2000–2005 Adult Studies, rs9939609 and rs1260326 were associated with BMI and triglycerides

($P = 0.0074$ and 4.5×10^{-7} , respectively). Each copy of the A allele of rs9939609 was associated with 0.054 ± 0.019 SD higher level of triglycerides and each T allele of rs1260326 was associated with 0.097 ± 0.019 SD higher BMI. In this cohort, these SNPs were associated with BCHE activity ($P = 0.006$ and 0.004 , respectively). Each copy of the minor allele of rs9939609 (A) and rs1260326 (T) was associated with 0.053 ± 0.019 and 0.055 ± 0.019 SD increase in BCHE activity, respectively (Supplementary Material, Table S6). Given the *FTO*-BMI and BMI-BCHE activity associations, the expected effect of *FTO* variant on BCHE activity was 0.012 ± 0.004 SD. Similarly, the expected *GCKR* variant effect on BCHE activity was 0.028 ± 0.006 SD. We then compared the observed effects of *FTO* or *GCKR* variants on BCHE activity with those expected given the *FTO*-BMI and BMI-BCHE associations, or the *GCKR*-triglycerides and triglycerides-BCHE associations. The results showed that the difference in effect size was marginally significant ($P = 0.036$) for *FTO* variant and not significant ($P = 0.181$) for *GCKR* variant.

These results were confirmed by the allelic association tests, when BMI or triglycerides were used as a covariate. In the 2000–2005 Adult Studies, the P -value for rs9939609 in *FTO* was 0.006 originally, but 0.051 with BMI as a covariate; and the P -value for rs1260326 in *GCKR* was 0.004 without, and 0.168 with, triglycerides included as a covariate.

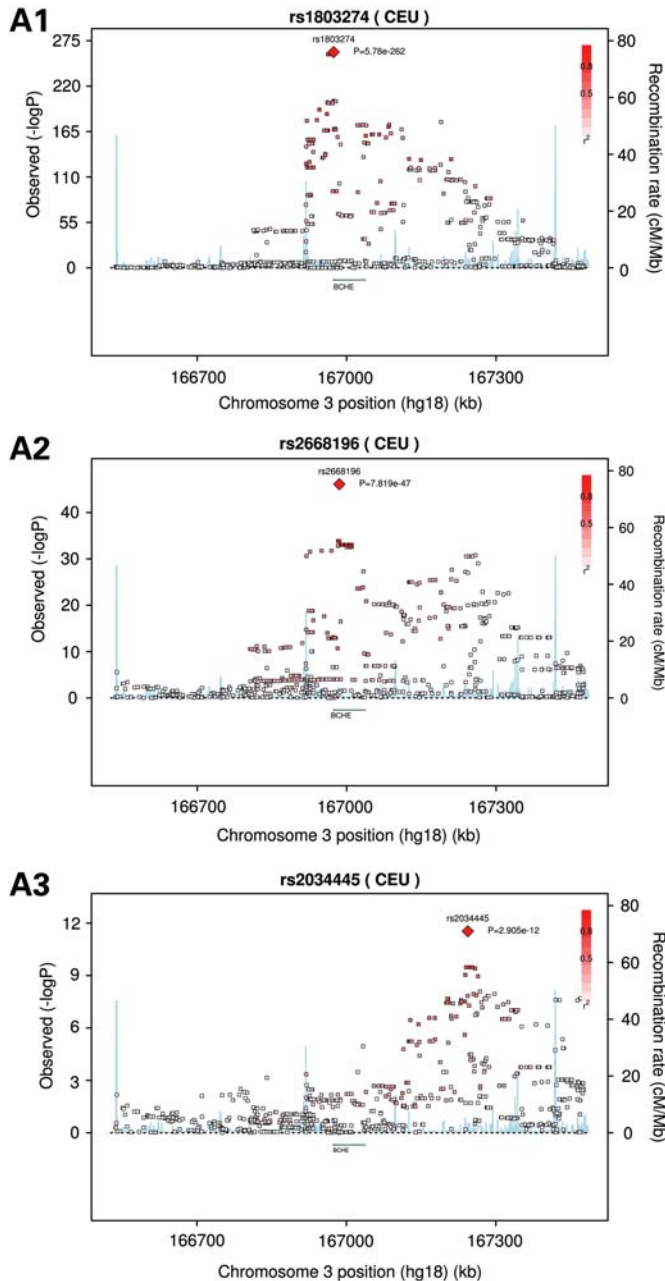


Figure 3. Detailed plots of associations with SNPs at the *BCHE* locus. (A1) Initial results; (A2) after covariate adjustment for rs1803274 genotype; (A3) after covariate adjustment for rs1803274 and rs2668196 genotypes.

Accordingly, the effects of *FTO* and *GCKR* variants on *BCHE* activity were reduced to 0.037 ± 0.019 and 0.025 ± 0.018 SD, respectively (Supplementary Material, Table S6). These results showed that the associations between *FTO* or *GCKR* variants and *BCHE* activity were partly mediated by the associations between triglycerides or BMI and *BCHE* activity.

DISCUSSION

We found seven independent genome-wide significant loci ($P < 5 \times 10^{-8}$) affecting *BCHE* activity; three in or near

BCHE, and one each at *ABI2-RAPH1*, *RNPEP*, *UGT1A* and an intergenic region on Chr 8. In view of the phenotypic correlations between *BCHE* activity and the quantitative risk factors which define or accompany the metabolic syndrome, we can consider the genetic sources of variation in plasma *BCHE* activity in the population in two categories. Direct effects act on the expression of the *BCHE* gene or the amino acid sequence of the encoded protein, or potentially on the processing, secretion or circulation lifetime of the *BCHE* protein. Indirect allelic effects on *BCHE* activity are those which primarily affect the cluster of characteristics associated with cardiovascular and metabolic risk such as obesity, dyslipidaemia, insulin resistance and fatty liver, for which *BCHE* activity is a biomarker. These indirect allelic effects (together with non-genetic causes of covariation) contribute to the correlations between *BCHE* and the biochemical markers of metabolic risk. The concept of genetic effects on *BCHE* activity mediated through metabolic risk is supported by the suggestive associations between loci previously reported to affect obesity, glucose homeostasis or plasma lipids and *BCHE* activity, and by the results of Mendelian randomization analysis.

Genetic variation at *BCHE* and other significant loci

The most significant association between typed or imputed SNPs and *BCHE* activity was for rs1803274, the well-recognized K-variant in *BCHE*. A large number of other SNPs in or near the *BCHE* gene also achieved genome-wide significance (see Supplementary Material, Table S3). Most but not all of these were in linkage disequilibrium with rs1803274, and may not represent independent effects. However, when we adjust for the effects of rs1803274 as a covariate in the association analysis, then a second set of SNPs within *BCHE* shows significant association (minimum $P = 7.8 \times 10^{-47}$, for rs2668196). This SNP is in intron 3 of the *BCHE* gene and has no known functional significance. A further iteration revealed another independent group of SNPs about 250 kbp from *BCHE*, but not near any other gene; the strongest association ($P = 2.9 \times 10^{-12}$) is for rs2034445, which like rs2668196 has no known function. This region presumably contains variation which affects the expression of *BCHE*; there are many examples of SNPs discovered through genome-wide association studies which regulate genes at some distance from their location (21,22). Our findings illustrate the complexity of the *BCHE* locus, with multiple influences on gene expression or protein sequence and stability. These groups of SNPs identified through genome-wide association are in addition to the uncommon or rare variants with known clinical significance, such as the atypical dibucaine-resistant enzyme resulting from rs1799807. This SNP, with a minor allele frequency of 0.02, did have a detectable effect in the 1993–1996 Adult Study subjects for whom it was genotyped, and it would have contributed to the effects associated with the K variant in our meta-analysis because it is in linkage disequilibrium with it (23). For the purposes of examining whether *BCHE* SNPs affect the metabolic variables correlated with *BCHE* enzyme activity, the K variant and all other variants associated with it were taken as a group and

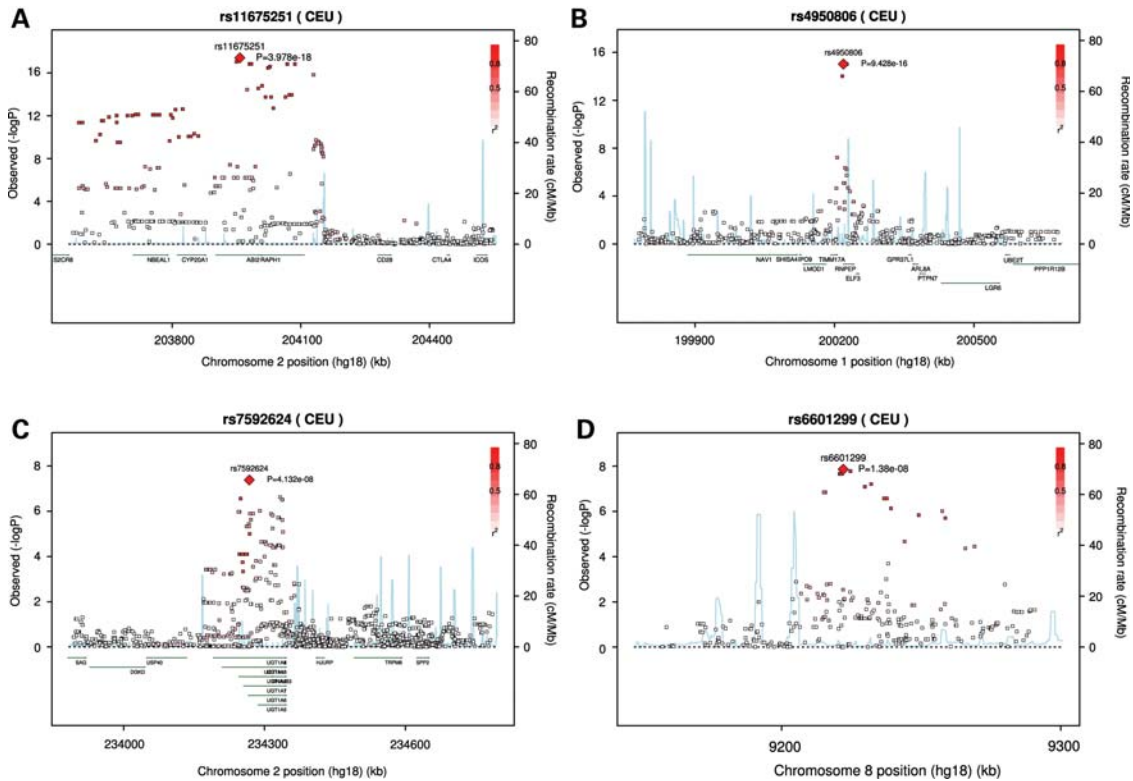


Figure 4. Detailed plots of associations with SNPs at other loci. (A) *RAPH1-ABI2* locus on chromosome 1; (B) *RNPEP* locus on chromosome 2; (C) *UGT1A1* locus on chromosome 2 after covariate adjustment for rs1803274 genotype; (D) chromosome 8 locus after covariate adjustment for rs1803274 genotype.

the absence of positive results makes it unnecessary to determine which is or are causal.

Four other loci showed genome-wide significant associations with BCHE activity, as summarized in Table 1. The *ABI2* gene has no obvious connection with cholinesterase activity, coding for a tumour suppressor and cell migration inhibitor. It has been implicated in vascular disease through a near-significant association ($P = 3.8 \times 10^{-7}$ at rs1376877) with carotid intima-medial thickness (24), but did not affect the metabolic syndrome components in our data (see Supplementary Material, Table S4). Although the most significant SNP association in this region is within the *ABI2* gene, BCHE associations cover a substantial region including *WDR12*, *ALS2CR8*, *CYP20A1*, *ABI2* and *RAPH1* (Fig. 4). Among these genes, *RAPH1* codes for lamellipodin, which gives rise to proline-rich peptides which promote the tetramerization and stability of BCHE (25). It is possible that the biological effect is produced by variation in *RAPH1*, and some *ABI2* SNPs are in LD with this. Variation at this locus may be the cause of the high-molecular-weight electrophoretic variant of BCHE, through effects on BCHE polymerization, but we have not tested this.

RNPEP codes for aminopeptidase B, which is an exopeptidase selectively removing arginine or lysine residues from the amino-terminal end of peptides. It may be involved in post-translational modification of proteins (26) and since BCHE is synthesized as a precursor protein which needs to be processed before secretion, there may be a necessary role for aminopeptidase B. However, there is no experimental information to supplement our association finding.

Finally, among our genome-wide significant results, and only revealed by re-analysis of the data with K-variant status included as a covariate, are SNPs within *UGT1A1* and an intergenic region of Chr 8 at 9.23 Mbp. *UGT1A1* is best known for the effect of its variants on bilirubin conjugation, leading to increased serum bilirubin concentration (Gilbert syndrome [MIM 143500]). The relevance for BCHE activity is obscure, though it has been claimed that *UGT1A1* variants may decrease oxidative stress and fatty liver through the antioxidant effect of increased bilirubin concentration (27). The intergenic region of Chr 8 at 9.23 Mbp showed a minimum P -value of 1.4×10^{-8} at rs6601299. This SNP also showed association with HDL-C (see Supplementary Material, Table S4, $P = 1.4 \times 10^{-5}$; the Bonferroni-corrected P for 77 tests is 6.5×10^{-4}). The two nearest genes are *PPP1R3B* and *TNKS*; although the former controls phosphorylation of proteins important in glycogen synthesis and thus provides a possible connection with carbohydrate metabolism, it is some 180 kbp from rs13265179 and not in linkage disequilibrium with it.

Associations between BCHE activity and cardiovascular or metabolic factor variables

Using the data from all 2000–2005 Adult Study participants with relevant measurements, we confirmed substantial and highly significant phenotypic correlations between BCHE activity and the cluster of characteristics related to obesity, dyslipidaemia, insulin resistance and fatty liver (see

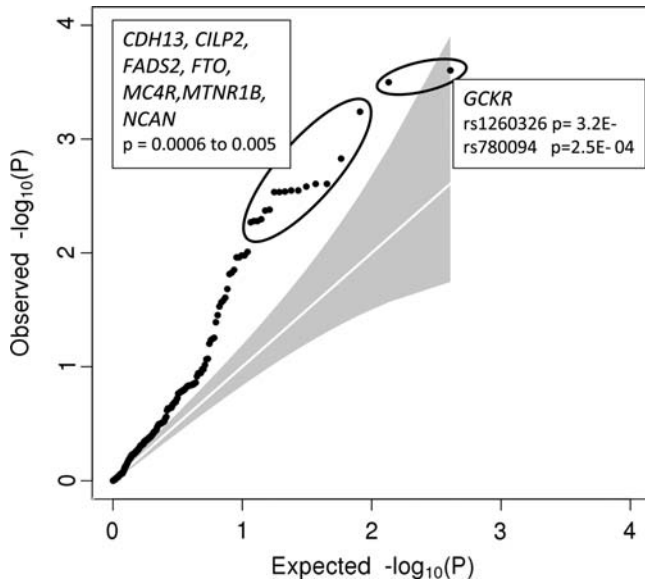


Figure 5. Quantile–quantile plot of observed against expected P -values for allelic associations with BCHE activity, for 203 SNPs previously reported as showing associations at $P < 5.0 \times 10^{-7}$ with one or more components of the metabolic syndrome. The grey shaded area represents the 95% confidence interval.

Supplementary Material, Table S1). Factor analysis showed that BCHE activity clustered with the measured metabolic and cardiovascular risk factors, as seen from the factor loadings for phenotypes which are known to predict risk of cardiovascular disease or type 2 diabetes (Fig. 1).

These correlations were notably stronger in adults than in adolescents, suggesting age-dependent development of inter-individual differences affecting both BCHE activity and the other components of an adverse metabolic risk profile. Perhaps related to this, the estimated heritability of BCHE activity declined from an extremely high value of 0.95 in the adolescents to a still substantial 0.65 in the adults. We have previously noted high heritability of plasma lipids, urate, ALT and GGT in the adolescents (28,29). This is compatible with the concept that age-related ‘wear and tear’ of mainly random or environmental origin contributes to the covariation between BCHE and the other metabolic abnormalities, which is present to a greater extent in adults than adolescents. Also relevant to the idea of novel influences in the adult group, the total variance was almost twice as great in the 2000–2005 Adult Study data compared with the Adolescents, even though BCHE activity was measured using the same method, in the same laboratory and over the same time period. The cumulative frequency distributions for BCHE activity in adults and adolescents, and the estimated genetic (A) and non-shared environmental (E) components of variance, are compared in Supplementary Material, Figure S1 and both the dispersion of results and the E component are substantially greater in adults.

Turning to common genetic effects on BCHE activity and metabolic syndrome components, the largest genetic influence on BCHE activity was associated with the non-synonymous coding SNP rs1803274, the K variant. This SNP was used to

test whether a genetic effect on BCHE leads to variation in the other clinically important variables which showed strong phenotypic correlations with BCHE activity. The negative results for this and for the other significant loci (Supplementary Material, Table S4), consistent with those previously reported for glucose and insulin-related measures in Danish subjects (30), show that variation in these metabolic risk factors is not caused by variation in BCHE activity.

Conversely, we tested whether the gene polymorphisms which have been shown to affect the lipid and metabolic syndrome-related variables also have effects on BCHE activity. Out of 203 SNPs at 97 loci, 19 SNPs at eight loci (*CDH13*, *CELSR2*, *FADS2*, *FTO*, *GCKR*, *MC4R*, *MTNR1B* and *NCAN*) showed associations with BCHE at $P < 0.01$ (see Supplementary Material, Table S5). This is more than would be expected by chance (Fig. 5). The strongest of these associations was with *GCKR*, which is already known to affect serum triglycerides (31), urate (32), glucose (33) and C-reactive protein (34). The gene product, glucokinase regulatory protein, plays a key role in glucose homeostasis through binding to glucokinase and controlling glycogen synthesis, glycolysis and glucose uptake by hepatocytes (35). Other genes with significant BCHE associations included *MC4R*, related to melatonin and with reported associations with obesity, insulin sensitivity and glucose concentration (36–41), and *MTNR1B* (42) coding for a melatonin receptor implicated in glucose homeostasis (43,44); the cluster of *FADS* genes (particularly *FADS2*) coding for fatty acid desaturases; *FTO*, which is strongly implicated in obesity (39,40,45); and members of the cadherin family (*CDH13*, *CELSR2*) (46). Most of these genes are at the interface between carbohydrate and lipid metabolism. However, it is also notable that there are gene variants with strong effects on phenotypes correlated with BCHE activity which do not show effects on BCHE. Examples are the highly significant variants rs734553 in *SLC2A9* [$P = 5.22 \times 10^{-201}$ for urate (32), but $P = 0.953$ for BCHE in our data] and rs1532624 in *CETP* [$P = 9.40 \times 10^{-94}$ for HDL (31), but $P = 0.556$ for BCHE].

Out of these associations with BCHE activity, we chose *GCKR* and *FTO* to test whether results are consistent with the direction of causation being SNP \rightarrow metabolic \rightarrow BCHE activity. Both the comparison of these SNP-BCHE associations with and without inclusion of the postulated intermediate (triglycerides and BMI), and the comparison of observed associations with those expected from the *GCKR*-triglycerides and triglycerides-BCHE associations, or the *FTO*-BMI and BMI-BCHE associations, were consistent with the model of indirect effects of these SNPs on BCHE activity.

Conclusions

The main conclusion from our results is that BCHE activity is a biomarker of the events and processes which lead to dyslipidaemia and metabolic syndrome and increase the risk of cardiovascular disease and diabetes, and genes which affect this metabolic risk have indirect effects on BCHE activity. Although we have not assessed metabolic syndrome by the published diagnostic criteria (47) in our subjects, the associations with quantitative components of the syndrome are strong and for our purposes it is more useful to consider the syndrome as a continuum of risk than as a binary diagnosis. Further

studies to evaluate BCHE activity (adjusted for at least K-variant status) as a marker of clinically diagnosed metabolic syndrome, and as a predictive risk factor for cardiovascular disease and diabetes, would be justified.

In a different context, genetic variation in BCHE activity is relevant to its use as a diagnostic test. The BCHE enzyme is inhibited by organophosphate compounds including insecticides and nerve agents, and activity measurements on plasma or serum can be used for the assessment of exposure to these (48,49). When pre-exposure BCHE activity of a patient is not known, knowledge of genotype would allow use of narrower genotype-specific reference intervals and potentially better diagnosis of organophosphate poisoning.

In relation to genetic architecture, variation in serum BCHE activity results from a combination of rare Mendelian, reasonably common oligogenic and polygenic gene effects, together with secondary effects from other phenotypes which are in turn subject to both genetic and environmental influences. The oligogenic effect of the K variant in *BCHE* is substantial, accounting for ~16% of variance in the phenotype through a combination of high prevalence and moderate effect size. The prevalence of this variant is notably similar and moderately high throughout human populations. Data from NCBI [http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1803274, accessed 29 August 2011; Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/, accessed 29 August 2011] shows minor allele frequencies of rs1803274 as 0.15–0.17 in Africans, 0.08–0.21 in Asians and 0.17–0.18 (similar to our value of 0.21) in Europeans. Maintenance of a similar level of heterozygosity across human populations which separated some 2000 generations ago may imply a balance between advantages and disadvantages associated with a moderate decrease in activity of this poorly understood enzyme.

MATERIALS AND METHODS

Subjects

The subjects are twins and their families who participated in one of the studies conducted at the Genetic Epidemiology Unit, Queensland Institute of Medical Research, Australia (Table 2). Participants gave informed consent and the studies were approved by appropriate institutional review boards. The studies can be grouped into three cohorts:

Cohort 1 (2000–2005 Adult Studies). This cohort includes a study of anxiety and depression (50); a study on genetic risk factors for endometriosis (51); a study of alcohol and nicotine dependence and metabolic risk factors for alcoholic liver disease (52). Participants for the alcohol and nicotine study were not selected for any disease but were over-sampled from families with alcohol- or nicotine-dependent members. From these 2000–2005 Adult Studies, 14 527 people had serum BCHE results and 6879 of them had both BCHE results and GWAS genotyping. To avoid overlap, results for 458 of them were excluded from the association analysis because of inclusion in the data analysis for the 1993–1996 Adult Study.

Table 2. Subject characteristics (mean \pm SD, *N*)

Sex	2000–05 Adult Studies		Adolescent Study		1993–1996 Adult Study	
	Male	Female	Male	Female	Male	Female
<i>N</i> (total with BCHE data)	5463	9064	383	387	706	1500
<i>N</i> (with BCHE and GWA markers)	3137	3742	331	321	239	1021
Age	51.72 \pm 13.88 (5463)	47.80 \pm 14.32 (9064)	13.8 \pm 1.68 (383)	13.7 \pm 1.80 (387)	44.81 \pm 10.92 (706)	46.6 \pm 12.02 (1500)
BCHE, units/l	9675 \pm 1923 (5463)	8712 \pm 1963 (9064)	8952 \pm 1500 (383)	8334 \pm 1487 (387)	8.97 \pm 2.09 (706)	7.80 \pm 2.04 (1500)
BMI, kg/m ²	27.03 \pm 3.85 (2141)	26.06 \pm 5.19 (2047)	20.13 \pm 3.68 (377)	20.20 \pm 3.44 (385)	25.72 \pm 3.35 (699)	24.92 \pm 4.57 (1481)
HDL-C, mmol/l	1.355 \pm 0.369 (5463)	1.676 \pm 0.424 (9064)	1.365 \pm 0.303 (383)	1.401 \pm 0.294 (387)	1.221 \pm 0.292 (668)	1.554 \pm 0.390 (1452)
LDL-C, mmol/l	3.376 \pm 0.929 (5116)	3.169 \pm 0.915 (8880)	2.372 \pm 0.641 (380)	2.497 \pm 0.613 (386)	3.552 \pm 0.915 (625)	3.392 \pm 0.988 (1427)
Triglyceride (log), mmol/l	0.683 \pm 0.529 (5461)	0.420 \pm 0.494 (9061)	0.100 \pm 0.211 (383)	0.081 \pm 0.170 (387)	0.599 \pm 0.588 (704)	0.281 \pm 0.533 (1498)
Urate, μ mol/l	0.342 \pm 0.076 (5463)	0.255 \pm 0.069 (9063)	0.283 \pm 0.072 (383)	0.250 \pm 0.047 (387)	0.386 \pm 0.077 (572)	0.284 \pm 0.072 (1084)
Ferritin (log), mg/l	2.271 \pm 0.364 (5460)	1.837 \pm 0.412 (9058)	1.663 \pm 0.211 (383)	1.584 \pm 0.256 (387)	2.256 \pm 0.345 (695)	1.793 \pm 0.423 (1084)
GGT (log), units/l	1.472 \pm 0.287 (5463)	1.266 \pm 0.263 (9062)	1.102 \pm 0.141 (383)	1.017 \pm 0.153 (387)	1.377 \pm 0.293 (660)	1.165 \pm 0.273 (1360)
ALT (log), units/l	1.434 \pm 0.207 (5463)	1.270 \pm 0.191 (9064)	1.214 \pm 0.167 (383)	1.147 \pm 0.167 (387)	1.289 \pm 0.240 (660)	1.125 \pm 0.218 (1360)
C-reactive protein (log), mg/l	0.621 \pm 1.043 (5411)	0.793 \pm 1.143 (8947)	—	—	0.928 \pm 0.520 (475)	1.067 \pm 0.525 (970)
Insulin (log), pmol/l ^a	1.819 \pm 0.435 (1087)	1.797 \pm 0.363 (1145)	—	—	1.143 \pm 0.299 (451)	1.119 \pm 0.292 (863)
Glucose, mmol/l ^a	5.34 \pm 1.83 (3106)	4.92 \pm 1.43 (3915)	—	—	5.29 \pm 1.30 (449)	5.04 \pm 0.88 (862)

^aAdjusted for time between last meal and blood collection (fasting time).

Cohort 2 (1993–1996 Adult Study). This cohort was collected for a study of genetic effects on risk factors for common psychiatric and metabolic diseases in adults, conducted in 1993–1996 and described in reference (10). All participants were Australian adult twins, aged 29–92 years, not selected for any disease. Results were obtained for 2237 samples and 1260 of these had both BCHE results and GWAS genotyping. The 1993–1996 Adult Study was grouped separately from the 2000–2005 Adult Studies because of the difference in BCHE methods.

Cohort 3 (Adolescent Study). This cohort is an ongoing study of adolescent twins and their siblings living in Brisbane, described in reference (28). Three thousand twenty-three twins or siblings had biochemical results from participation in these studies between 1992 and 2006, mostly on more than one occasion, but BCHE activity was only measured on serum samples from 767 participants (3 of whom had two BCHE measurements from different visits) from this ‘Adolescent Study’. Six hundred fifty two of these had both BCHE results and GWAS genotyping.

There was no overlap in participation between the Adolescent and Adult studies, nor in the allelic association analysis between the 1993–1996 and 2000–2005 Adult Studies. Seven hundred fifty four of the adults participated in two or more of the 2000–2005 Adult Studies and had repeated measures of serum BCHE activity, using the same method, from blood samples obtained at different times. Where multiple measurements were available for any participant, the mean value was used in the genetic association analysis.

Measurements

BCHE activity was measured in serum or plasma samples. For the Adolescent Study and the 2000–2005 Adult Studies, BCHE activity was measured in the laboratory of JBW, using either a Roche 917 or Modular P analyser with butyrylthiocholine as substrate (Roche Diagnostics, Castle Hill, NSW Australia). For the 1993–1996 Adult Study, BCHE activity was measured in the laboratory of DTO'C, using acetylthiocholine as substrate and the specific acetylcholinesterase inhibitor BW284c51 to measure BCHE (53). Other biochemical measurements were performed with Roche methods using the 917 or Modular P analysers, except for insulin which was measured on an Abbott Architect (Abbott Laboratories, Abbott Park, IL, USA) for the alcohol-nicotine study and by radioimmunoassay (Diagnostic Products, Los Angeles, CA, USA) for the 1993–1996 Adult Study.

Genotyping and exclusions

Genotyping was performed on DNA extracted from blood samples by standard methods, as previously described (54). rs1799807, which codes for the dibucaine-resistant atypical BCHE enzyme, was genotyped in the 1993–1996 Adult Study participants only, using primer extension mass spectrometry (Sequenom Inc., San Diego, CA, USA).

Genome-wide SNP data used in this study were derived from eight genotyping projects at the Genetic Epidemiology Laboratory, Queensland Institute of Medical Research (See

Supplementary Material, Table S2). We genotyped a total of 15 925 individuals using either Illumina 317, 370 or 610 K chips at CIDR, deCode Genetics or the Finnish Genome Centre. We integrated the data after performing separate quality controls (QC) for each genotyping project as previously described (55). Basically, SNPs were included in the analyses if they met the following conditions: Hardy–Weinberg equilibrium test $P \geq 10^{-6}$, minor allele frequency $\geq 1\%$, call-rate ≥ 0.95 and the mean value of BeadStudio GenCall score ≥ 0.7 .

We used principal components analysis (EIGENSTRAT) of the genotyping data to exclude subjects of non-European ancestry. We used a set of $\sim 275\,000$ autosomal SNPs that were common to Australian samples, HapMap 3 (11 global populations) and 5 Northern European (Denmark, Finland, the Netherlands, the UK and Sweden) populations from the GenomeEUtwin Consortium. We excluded 277 individuals (1.7% of the total genotyped individuals) who were >6 standard deviations from the mean of PC 1 and 2 derived from the European populations.

Imputation

We imputed non-typed HapMap SNPs using the program MACH (<http://www.sph.umich.edu/csg/abecasis/mach/index.html>, accessed 29 August 2011). To avoid bias due to both missingness and SNP density, we used a set of SNPs common to the genotyping chips ($N = 274\,604$). The best guess genotypes were used for the association analyses if they met the same conditions as mentioned for the genotyped SNPs as well as an imputation quality score, R^2 -sq-hat (the squared correlation between imputed and true genotypes) of 0.3 or greater. After QCs, 2 383 238 SNPs were available for the association analyses.

Statistical analyses

We tested each SNPs for association with the phenotypes under an additive model using family-based score test implemented in Merlin (56, <http://www.sph.umich.edu/csg/abecasis/Merlin/>, accessed 29 August 2011). This test takes into account family relationships, where both within- and between-family components were used to produce a total association estimate. Prior to association analyses, the phenotypes were adjusted for the effects of sex, age, age*age, sex*age and sex*age*age. Data from the 2000–2005 Adult Studies served as a discovery sample and the data from the Adolescent Study and the 1993–1996 Adult Study were used as replication samples. We meta-analysed the SNP-phenotype associations from the discovery and association cohorts by weighting the effect size estimates using the inverse of the corresponding standard errors in Metal (57, <http://www.sph.umich.edu/csg/abecasis/Metal/index.html>, accessed 29 August 2011). The heterogeneity between the estimates in three cohorts was assessed using Cochran's Q statistic implemented in Metal.

We performed a Mendelian randomization approach following the example of Freathy *et al.* (20). The expected effect sizes for the associations between the genotypes of rs9939609 (*FTO*) or rs1260326 (*GCKR*) with BCHE activity were calculated by multiplying the effect sizes of the

genotypes-trait association and BCHE activity-trait associations. To test for difference between the observed and expected effect sizes of the association between the genotypes and BCHE activity, we computed a Z-statistic = $[\beta_1 - \beta_2] / SE[\beta_1 - \beta_2]$. β_1 and β_2 are the observed and expected effect sizes, respectively, and SE is the standard error of its difference.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We acknowledge the contributions of many staff in the Genetic Epidemiology Unit, Queensland Institute of Medical Research, in interviewing study participants, sample processing and DNA extraction, and data management; and of Jole Bojovic in phenotype measurement. We also thank David Duffy for discussions regarding Mendelian randomization analysis of family-based data.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the Australian National Health and Medical Research Council (241944, 339462, 389927, 389875, 389891, 389892, 389938, 442915, 442981, 496739, 552485, 552498), the Australian Research Council (A7960034, A79906588, A79801419, DP0770096, DP0212016, DP0343921), the EU 5th Framework Programme GenomEUtwin Project (QLG2-CT-2002-01254) and the U.S. National Institutes of Health (AA07535, AA10248, AA11998, AA13320, AA13321, AA13326, AA14041, AA17688, DA12854, MH66206 to co-authors; and R37-GM18360 and P42-ES010337 to Palmer Taylor). A portion of the genotyping on which this study was based was carried out at the Center for Inherited Disease Research, Baltimore (CIDR) (Illumina 370 K scans on 4300 individuals), through an access award to our late colleague Richard Todd. Parts of the statistical analyses were carried out on the Genetic Cluster Computer, which is financially supported by the Netherlands Scientific Organization (NWO 480-05-003). B.B., R.P.M., D.R.N., S.E.M., P.M.V. and G.W.M. are supported by National Health and Medical Research Council (NHMRC) Fellowship Schemes.

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