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Evidence of age-dependent genetic influences on plasma total cholesterol

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Background **Causes of variation in cardiovascular risk factors include biological variation within individuals, and more permanent differences between individuals, which are at least partly genetic in origin. We have compared the magnitude of genetic and non-genetic factors within and across occasions through repeated measures of plasma cholesterol in twin subjects, and have also determined how far the same genes affect cholesterol levels at different ages.**

Methods **Data on plasma total cholesterol were extracted for 208 twin pairs who had provided blood on up to six occasions across a period of 17 years. They were aged 18–30 years at the time of first study and 30–47 at the time of the last. Multivariate models of variation due to genetic, shared environmental and unique environmental factors were fitted to the multi-occasion data and the proportions of variation due to these factors were estimated.**

Results **One genetic factor influenced plasma cholesterol on all occasions and a second genetic factor only influenced cholesterol results on the fifth and sixth occasions 10–17 years after the first. Environmental factors did not have significant long-term effects.**

Conclusions **We conclude that individuals' long-term mean plasma cholesterol values are strongly genetically determined, but that some of these genes are age-specific in their effects.** ***Eur J Cardiovasc Prev Rehabil*** **12:380–386 © 2005 The European Society of Cardiology**

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Introduction

Plasma cholesterol concentration is a well-known risk factor for cardiovascular disease. Although other lipids and lipoproteins or their ratios, and some inflammatory markers, may give slightly better risk predictions than total cholesterol [1,2], it is clear that there is a continuous increase in risk across the range of total cholesterol values found in the population [3,4]. Therefore variation in plasma total cholesterol is relevant to the causes of cardiovascular disease, and can be used to explore or illustrate a number of epidemiological principles.

Variation in a continuously distributed variable such as plasma cholesterol concentration may be categorized as biological variation within an individual (including measurement error), and genetic or non-genetic (environmental) causes of variation between individuals. In addition, specific causes (such as allelic variation at known loci or measured pre- or post-natal environmental factors) can be tested for their effects. If only a single measurement is made on each individual, then within-individual variation contributes to environmental variation; but repeated measurements within a twin or family design can distinguish it from long-term effects of differences in environment. However, if repeated measurements are made on the same subjects across years or decades, the causes of variation may change and in particular there may be age-specific expression of

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different genes. This would complicate the search for genes affecting for example, cholesterol levels, and would also have implications for screening or case finding because risk assessments would need to be repeated at intervals.

Many studies have estimated the relative importance of genetic and non-genetic effects in determining plasma cholesterol, and summaries of results from multiple studies [5] or from several populations within a co-ordinated study [6] are available. The median estimate of heritability for plasma total cholesterol is approximately 60%. The review by Snieder *et al.* [5] paid particular attention to possible age-related effects, and concluded that although heritability estimates from twin studies are not strongly dependent on the age of the subjects, results of family studies and longitudinal studies support the hypothesis that the relative importance of different genes may change with age.

We have analyzed data from Australian adult male and female twins who have had plasma cholesterol measured on more than one occasion, with the aim of determining: (a) how much of the repeatable between-individual variation in cholesterol is due to genetic differences between individuals; and (b) whether the same genes affect cholesterol across time or whether there are age-specific genetic effects.

Methods

Subjects

The data used in the present paper are derived from four different studies, all based on twins who were recruited from the Australian Twin Register, a volunteer register begun in 1978. The first study (AC), conducted during 1979–1980, was mainly directed towards the effects of an alcohol challenge on intoxication and alcohol metabolism [7]. Questionnaire data and a pre-alcohol blood sample were obtained for 412 young twins (206 pairs who were born 1944–1963) who completed the alcohol challenge protocol. Some months after their initial participation 82 individuals returned for repeat testing (Alcohol Challenge Repeat Study, ACR). Analysis of genetic and environmental effects on plasma lipids from this study has been published previously [8]. The second study contained 106 monozygotic (MZ) twin pairs who participated in a co-twin control study of the effects of vitamin C (VC) [9]; blood was taken before commencement of vitamin C supplementation. Three months later, these twins came back for a second measurement (VCR). Thirdly, a follow-up study on twins from the alcohol challenge study was conducted in 1990–1992 (SY) [10]. A total of 334 out of the 412 original twins had their blood collected. The final study (SB), which started in 1993, included blood collection from twins who took part in a study to assess genetic influences on psychological and social manifestations of alcoholism and related disorders [11]. Overall 3375 twins provided blood samples for the SB study, but most had not participated in the earlier studies listed.

Blood samples were collected and serum was separated from the blood, and either stored at 4°C until analysis on the next working day or stored at −70°C until analyzed. Total cholesterol was measured enzymatically by Technicon methods on a SMAC analyser (for the AC, ACR, VC and VCR studies) and by Boehringer Mannheim methods on a Hitachi 747 analyzer (for the SY and SB studies). In each case the calibration materials supplied by the reagent manufacturer were used.

In all, blood samples and results were available on up to six occasions, although few subjects participated in all six. For each of these studies, informed consent had been obtained from participants and appropriate Ethics Committees approved the studies.

Data analysis

Because the emphasis in this paper is on repeatability of cholesterol measurements and on changes in genetic and non-genetic causes of variation over time, only those subjects who participated in more than one study were included in the data analysis. Participants were included if they participated in either the AC or VC studies and were aged between 18 and 30 years at that time. Within that group, 17 twins who were taking lipid-lowering drugs and six twins considered to be outliers (extreme high or low cholesterol values, $> 3 \times$ SD from the mean) were excluded from the analysis. Table 1 gives information on the six occasions of study. The final dataset used in this analysis consists of 208 twin pairs, comprising 50 MZ female pairs (MZF), 41 MZ male (MZM), 42 dizygotic (DZ) female (DZF), 37 DZ male (DZM) and 38 DZ opposite sex (DOS).

Table 1 **Information on the subjects and studies**

Study	Study code	Year	Potential number of participants*	Actual number used**	Age **mean (min, max)
Alcohol Challenge	AC	1979–1981	412	350	22.3 (18, 29)
Alcohol Challenge repeats	ACR	1979–1981	82	67	22.7 (18, 29)
Vitamin C	VC	1980	212	70	22.7 (18, 29)
Vitamin C repeats	VCR	1980	212	68	22.6 (18, 29)
Sydney	SY	1990–1992	412	333	34.2 (27, 43)
SSAGA Blood	SB	1993–1996	3347	235	37.14 (30, 47)

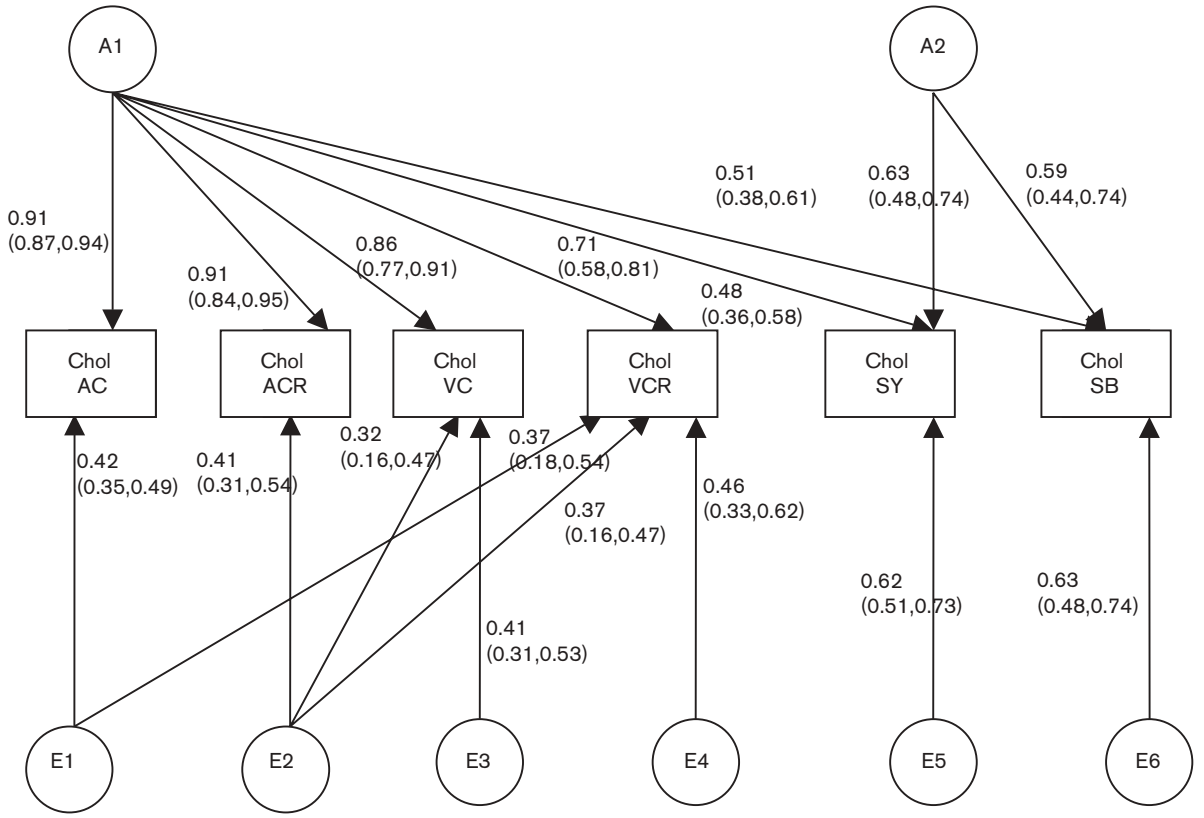
*Total number of twins in each of the original studies; **twins whose data were used in this analysis, after applying the inclusion/exclusion criteria.

Quantitative genetic analyses were used to identify and quantify genetic and environmental influences on variation in total cholesterol. Prior to genetic modelling analyses, distribution of total cholesterol and twin assumptions (i.e., that all zygosity groups have the same mean and variance, after adjusting for sex effects) were performed. Initially, phenotypic correlations between cholesterol results from the six measurements were examined. As there is significant correlation between studies, sources of covariation between them were modelled using multivariate analysis. In addition, repeatability within-individual at repeat visits $(S_b^2/(S_b^2 + S_w^2))$ was obtained using an analysis of variance. This measure estimates the repeatable component of variance, and generally sets an upper limit for heritability.

Models were fitted by the method of maximum likelihood estimation using raw data. The Mx program [12] was used for all quantitative genetic model fitting. Each model comprises a set of simultaneous linear equations, which specify the expected degree of additive genetic (A), shared-environment (C) and non-shared environment (E) effects. The significance of A, C and E was tested by removing each sequentially in specific submodels. If variance components did not give a significant contribution (i.e., $P > 0.05$), they were dropped from the model. To compare the nested models, the likelihood ratio chi-squared test (i.e., a difference between -2 log likelihood of the full model from that of a restricted model) was used. In comparing the non-nested models, the best model was chosen on the basis of the lowest value of Akaike's information criterion, reflecting the best balance between goodness of fit and parsimony. Effects of age and sex were also included as covariates in all models.

To investigate the contribution of genetic and environmental factors to covariation between total cholesterol measurements across the six time points, the Cholesky model, which may explain the pattern of correlations among time points, was fitted. In the Cholesky model, the number of factors equals the number of observed variables. The first factor contributes to all six assessments; the second factor influences the subsequent five assessments and so on (see Fig. 1). This model provides a saturated (but not parsimonious) description of the contribution of genetic and environmental factors to the covariance.

Fig. 1



Standardized path coefficients (and 95% confidence intervals) for the final model of sources of covariation between the six sets of plasma total cholesterol results. A1 and A2 are genetic factors, and E1 to E6 are non-shared environmental factors, influencing cholesterol results in the six studies AC to SB.

Results

Means and standard deviations of total cholesterol by sex and study are listed in Table 2. Despite the aging of the subjects, possible changes in the diet of the population, and possible analytical effects arising from method or calibrator changes, mean plasma cholesterol did not change greatly between studies. The within-person variance (Table 3) was greater at older ages. Total cholesterol seems to be more variable within men than women in the twins at younger ages but the opposite is true when they were older.

Overall, phenotypic correlations between studies were highest among the group of earlier studies (AC, ACR, VC, VCR) and lowest between them and the more distant studies (SY, SB) (Table 4). This suggested that a developmental model might best fit the data. An ACE model that included age and sex effects on the means was first fitted to the data (see Methods). Standardized path coefficients and variance component results were obtained. To test the significance of common environmental effects, covariation due to this source was excluded from the model and the likelihood of the reduced model tested against that of the full model to give a likelihood ratio chi-square (Table 5). This showed that C could be excluded from the models without significant deterioration in the fit. The A component could not be dropped (as seen from comparison of model 6 with model 1 or model 4 in Table 5) but the number of additive genetic (A) factors could be reduced to two (compare model 7 with model 4 in Table 5).

Tests for path coefficients showed that some non-shared environmental (E) paths could be dropped from the model (see the comparison of model 9 with model 7 in Table 5). Figure 1 and Table 6 illustrate the final model, which shows that covariation in total cholesterol across time is largely influenced by additive genetic factors. All variable loadings were strongly significant on the first genetic factor, suggesting that the same set of genes (i.e., those comprising factor A1) influences total cholesterol in all studies at all time points. However, we were unable to drop the independent second genetic factor (A2 in Fig. 1). This loads only on the last two studies, suggesting that in addition to a common set of genes influencing cholesterol variation at all ages, there are other genes which come into play only later in life.

Table 2 Means and standard deviations of plasma total cholesterol for male and female twins included in the data set for this study

	Females ($n=222$)	Males ($n=189$)
Study	Mean ± SD (n)	Mean ± SD (n)
AC	5.45 ± 1.11 (186)	5.39 ± 0.98 (164)
ACR	5.39 ± 1.05 (34)	4.95 ± 0.87 (33)
VC	5.47 ± 1.05 (42)	5.52 ± 0.79 (28)
VCR	5.44 ± 0.90 (38)	5.42 ± 0.63 (30)
SY	5.16 ± 1.01 (179)	5.35 ± 0.99 (154)
SB	5.08 ± 0.84 (135)	5.55 ± 0.83 (100)

Table 3 Intra-class correlation, r_i, and within-individual, S_w^2, and between-individual S_B^2, components of variance for males, females and both sexes, calculated for variation among the earlier studies (AC, ACR, VC and VCR) and the later studies (SY and SB)

	Males	Females	Both sexes
Between and within the earlier studies:			
Number of results	225	300	555
Variance, S^2	1.377	1.783	1.598
S_B^2	1.178	1.614	1.415
S_w^2	0.199	0.169	0.183
r_i	0.86	0.91	0.89
Between and within the later studies:			
Number of results	254	314	568
Variance, S^2	1.504	1.580	1.579
S_B^2	1.211	1.196	1.235
S_w^2	0.293	0.385	0.344
r_i	0.81	0.76	0.78

The findings with respect to the additive genetic factors are in sharp contrast to the effects of unique environment, which are largely specific to the different occasions. There is modest covariation for the four earlier studies but no significant covariation between the earlier and later studies. This implies that environmental influences (E) on cholesterol are largely ephemeral. These estimates of E include measurement error, including day-to-day biological variation, any collection and processing effects, and analytical errors, but they exclude (in this design) any genetic effects on choice of diet, lifestyle or other broadly 'environmental' influences.

Discussion

We know from post-mortem studies on young accident victims or combat casualties that atherosclerosis starts early in life [13,14], although it is not usually clinically manifested until the sixth or seventh decades. Therefore it is a chronic process and risk factors must exert their effects over a long period. Understanding the temporal evolution of genetic (or indeed non-genetic) risk over time is important for scientific understanding and also for clinical or public health risk assessments. It has sometimes been assumed that genetic factors must have enduring or even lifelong effects, but it is quite possible that gene × age interactions contribute to changes in cholesterol (or other risk factors) as we grow older.

We had two aims in performing this analysis: to determine whether non-genetic influences on plasma cholesterol persist across time, or are occasion-specific and hence represent only measurement error and biological within-individual variation; and secondly to determine whether the same genes affect cholesterol across time, or whether the effects of some genes are age-specific (developmental expression).

Because data for this analysis were extracted from several of our studies, and cholesterol measurements were performed at several times and with two different reagent

Table 4 Phenotypic correlations (and the number of observations) of total cholesterol between different studies according to sex group. Results for women are shown below the diagonal, and for men above. Correlations within the earlier group of studies, and within the two later studies, are shown within the dotted lines while correlations which cross the intervening period are outside these lines

	AC	ACR	VC	VCR	SY	SB
				MEN		
AC	– (186)	0.81 (33)	0.74 (26)	0.56 (26)	0.45 (133)	0.41 (85)
ACR	0.83 (34)	– (34)	0.71 (8)	– 0.33 (8)	0.70 (29)	0.63 (17)
VC	0.82 (28)	0.50 (6)	– (42)	0.49 (28)	0.46 (23)	0.25 (18)
VCR	0.90 (26)	0.42 (6)	0.86 (38)	– (38)	0.49 (23)	0.03 (18)
SY	0.43 (157)	0.45 (33)	0.43 (25)	0.44 (23)	– (179)	0.62 (94)
SB	0.57 (116)	0.67 (23)	0.45 (18)	0.48 (15)	0.53 (121)	– (135)
				WOMEN		

Table 5 Model fitting results of a Cholesky model for variation in total cholesterol (age and sex differences in all models)

Model	– 2ll	*df*	Compared to model	$\Delta\chi^2$	Δdf	*P*
1. Saturated model, ACE	2508.936	1052	–	–	–	–
2. ACE + drop factors C3 to C6	2508.967	1065	1	0.031	13	1.000
3. ACE + drop factors C2 to C6	2509.946	1067	2	0.979	2	0.613
4. AE only, all C factors dropped	2514.173	1073	3	4.227	6	0.646
5. CE only, all A factors dropped	2544.252	1073	1	35.316	21	0.026
6. E only, all C and A factors dropped	2702.553	1094	1	193.617	42	0.000
7. AE + drop factors A3 to A6	2535.406	1086	4	21.233	13	0.068
8. AE + drop factors A2 to A6	2588.474	1088	7	53.068	2	0.000
9. Final model (AE + drop factors A3 to A6 & 12 coefficients for E, as shown in Fig. 1)	2553.241	1098	7	17.835	12	0.121

Table 6 Proportion of variation in plasma total cholesterol at each time of study (AC to SB) due to (a) additive genetic (A1, A2) and (b) unique environmental (E1 to E6) factors. The 95% confidence intervals are given in parentheses

(a)	A1	A2
1. (AC)	83% (76–88%)	
2. (ACR)	83% (71–90%)	
3. (VC)	74% (59–83%)	
4. (VCR)	50% (34–66%)	
5. (SY)	23% (13–34%)	40% (23–55%)
6. (SB)	26% (14–37%)	35% (19–55%)

(b)	E1	E2	E3	E4	E5	E6
1. (AC)	18% (12–24%)					
2. (ACR)		17% (10–29%)				
3. (VC)		10% (3–22%)	17% (10–28%)			
4. (VCR)	14% (3–29%)	14% (3–22%)		21% (11–38%)		
5. (SY)					38% (26–53%)	
6. (SB)						40% (23–55%)

and instrument systems, it is possible that cholesterol values are affected by technical differences between occasions. However, this would only affect the mean values for each study and not the sources of variation between people within each study, nor between studies. The results in Table 2 show that for the men, differences in mean between the first four studies and the last two are minimal. For women, mean results were lower in the two later studies; and women also have lower values than men in these two studies.

Persistence of the cholesterol status of individuals across time can be seen at the phenotypic level, from the correlations between cholesterol values observed on different occasions (Table 4). It can also be seen that the correlations across shorter periods of time (one or two years at most between the AC, ACR, VC and VCR data) were greater than those across a gap of around 12 years (between the earlier studies and the SY or SB data). This might represent gradual or abrupt change in environmental similarity as people's circumstances and lifestyles change, particularly between their early twenties and late thirties; or it might represent a developmental change in the genes affecting cholesterol. This has been explored using data from twin pairs assessed on multiple occasions, and the results are presented in Table 6 and Figure 1. These show that only a negligible proportion of variance in later cholesterol is due to long-term E effects, compared to about 25% due to A1 and 35–40% to A2. The remainder of the variation is

due to occasion-specific or short-term E effects, including day-to-day biological variation and measurement error. There is some cross-occasion persistence of E effects across the early AC, ACR, VC and VCR occasions but not between these and the later SY and SB studies.

Therefore, in general, environmental effects are specific to the occasion of measurement, whereas genetic effects persist across time. This implies that the risk of cardiovascular disease attributable to differences in cholesterol level is much more genetic than previously thought, because only effects which persist over the long term can affect the chronic process of atherogenesis and its eventual clinical manifestation. Just as it has recently been emphasized that short-term biological variation in risk factors, and measurement errors, lead to substantial underestimation of the effect of cholesterol or blood pressure on cardiovascular risk [15,16], so too the use of single measurements of a phenotype in a genetic study may cause underestimation of the long-term importance of genetic effects.

Complex (or multifactorial) diseases are the result of the additive or interactive effects of multiple genes and environmental influences, and there is little reason to suppose that their relative importance is constant. The results of this exploratory analysis suggest that there are 'new' genes, which act at the later ages, so that there are new genetic influences affecting total cholesterol in the older age group. This has obvious implications for linkage or association studies searching out the genes, which affect cholesterol in the general population, and also for risk prediction for individual people. In the first case, study participants need to be stratified by age group as a preliminary step in data analysis and investigators should be aware that results might differ between such groups. A possible example of such an age-related effect can be found in the report of linkage between total or low-density lipoprotein (LDL) cholesterol and a locus on chromosome 19 [17]; four groups of subjects were studied and although evidence of linkage was found for three samples of adults from Australia (including twins from the present study), the Netherlands and Sweden, no such linkage was found for a fourth sample of adolescent subjects.

Secondly, risk assessment by phenotypic measurements of (for example) cholesterol will need to be repeated at intervals, and any genetically based risk assessment may need to be based on different loci or alleles depending on the age of the subject and the time-span for which the assessment is to be useful.

Twin studies alone cannot exclude gene × environment interaction as an explanation for apparent genetic effects, and these may be relevant to our finding of different genes affecting plasma total cholesterol in the later studies. The period between the twenties and thirties does not appear to be one in which major physiological events such as growth and puberty, or menopause or the onset of aging, occur but it is often a time of change in the individual's circumstances and environment. Our results show that different genetic sources of variation are appearing, but this may in part reflect genetically determined responses to changing environments. Further work is needed to explore this possibility, and also the issue of whether similar age-related genetic effects are significant for other cardiovascular risk factors.

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