Mendelian Randomization: Genetic variants as instruments for strengthening causal influence in observational studies

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Introduction

The incorporation of biomarkers into population – based health surveys is generally intended to improve categorisation of exposures or health outcome measures. (Finch, Vaupel and Kinsella 2001) An unintended consequence of the growing use of biomarkers, for example, in assessing nutritional status, is that investigators are less aware of the continued threats to validity of their findings caused by measurement error, confounding and reverse causality which affect biomarkers in the same way as exposures and outcomes measured using less precise methods. Here we will develop these ideas, namely that of inferring causal associations between exposures and health outcomes within population based health surveys. A clear limitation of observational studies is that it has been difficult to reliably identify causal processes through conventional methods. This chapter will briefly outline when and why conventional observational approaches have been misleading and then introduce the Mendelian randomization approach. The variety of inferences that can be drawn from this approach will be illustrated and then potential limitations and ways to address these limitations outlined. The chapter will conclude by summarising the ways in which Mendelian randomization approaches differ from other methodologies that depend upon the use of genetic markers within population-based research.

Limits of observational epidemiology

To investigators interested in the health consequences of a modifiable environmental exposure – say, a particular aspect of diet – the obvious approach would be to directly study dietary intake and how this relates to the risk of disease. Why, then, should an alternative approach be advanced? The impetus for thinking of new approaches is that conventional observational study designs have yielded findings that have failed to

be confirmed by randomised controlled trials. (Davey Smith and Ebrahim 2002) Observational studies demonstrated that beta carotene intake was associated with a lower risk of lung cancer mortality, and this stimulated an already active market for vitamin supplements that was based on the notion that they substantially influenced chronic disease risk (figure 1). The scientists involved in conducting the observational studies have advocated taking supplements in material intended for the general public . (Willett 2001). Large numbers of people took beta carotene supplements, for example, justification for which could be found in reports relying on observational data such as the 1990 review of this issue that concluded "Available data thus strongly support the hypothesis that dietary carotenoids reduce the risk of lung cancer" (Willett 1990). However when large-scale randomised controlled trials reported their findings, disappointingly for all concerned, beta carotene supplementation produced no reduction in risk of lung cancer (Alpha-Tocopherol 1994).

With respect to cardiovascular disease, observational studies suggesting that beta carotene, (Manson et al 1991) vitamin E supplements, (Rimm et al 1993; Stampfer et al 1993) vitamin C supplements, (Osganian et al 2003) and hormone replacement therapy (HRT) (Stampfer and Colditz 1991) were protective were followed by large trials showing no such protection. (Omenn et al 1996; Alpha-tocopherol 1994; Dietary supplementation 1999; Heart Protection Study 2002; Beral et al 2002) In each case special pleading was advanced to explain the discrepancy – were the doses of vitamins given in the trials too high or too low to be comparable with the observational studies? Did HRT use start too late in the trials? Were differences explained by duration of follow up or other design aspects? Were interactions with other factors such as smoking or alcohol consumption key? Rather than such

particular explanations being true (with the happy consequence that both the observational studies and the trials had got the right answers, but to different questions) it is likely that a general problem of confounding – by lifestyle and socioeconomic factors, or by baseline health status and prescription policies – is responsible. Indeed in the vitamin E supplements example the observational studies and the trials tested precisely the same thing. The figures (fig 2a and b) show the findings from observational studies of taking vitamin E supplements (Rimm et al 1993; Stampfer et al 1993) and a meta-analysis of trials of supplements (Eidelman et al 2004). The point here is that the observational studies specifically investigated the effect of taking supplements for a short period (2-5 years) and found an apparent robust and large protective effect, even after adjustment for confounders. The trials tested randomization to essentially the same supplements for the same period, and found no protective effect. Importantly, the trial findings cannot be attributed to confounding or self-selection of healthier people into a vitamin-taking group as taking or not taking vitamin E was determined randomly, which providing it is done properly, avoids these sources of bias.

In 2001 the Lancet published an observational study demonstrating an inverse association between circulating vitamin C levels and incident coronary heart disease (Khaw et al 2001). The left hand side of figure 3 summarises these data, presenting the relative risk for 15.7μmol/l higher plasma vitamin C level, assuming a log-linear association. As can be seen, adjustment for confounders had little impact on this association. However a large-scale randomised controlled trial, the Heart Protection Study, examined the effect of a supplement that increased average plasma vitamin C

levels by 15.7μmol/l. In this study randomization to the supplement was associated with no decrement in coronary heart disease risk (Heart Protection Study 2002).

What underlies the discrepancy between these findings? One possibility is that there is considerable confounding between vitamin C levels and other exposures that could increase the risk of coronary heart disease. In the British Women's Heart and Health study (BWHHS), for example, women with higher plasma vitamin C levels were less likely to be in a manual social class, have no car access, be a smoker or be obese and more likely to exercise, be on a low fat diet, have a daily alcoholic drink, and be tall (Lawlor et al 2004). Furthermore for these women in their 60s and 70s those with higher plasma vitamin C levels were less likely to have come from a home 50 years or more previously in which their father was in a manual job, or had no bathroom or hot water, or within which they had to share a bedroom. They were also less likely to have limited educational attainment. In short, a substantial amount of confounding by factors from across the lifecourse that predict elevated risk of coronary heart disease was seen. Table 1 illustrates how four simple dichotomous variables from across the lifecourse can generate large differences in cardiovascular disease mortality (Table 1) (Davey Smith and Hart 2002).

In the BWHHS 15.7 mmol/l higher plasma vitamin C level was associated with a relative risk of incident coronary heart disease of 0.88 (95% CI 0.80 to 0.97), in the same direction as the estimates seen in the observational study summarised in figure 2. When adjusted for the same confounders as were adjusted for in the observational study reported in figure 2 the estimate changed very little—to 0.90 (95% CI 0.82 to 0.99). When additional adjustment for confounders acting across the life course was

made considerable attenuation was seen, with a residual relative risk of 0.95 (95% CI 0.85 to 1.05) (Lawlor et al 2005). It is obvious that given inevitable amounts of measurement imprecision in the confounders, or a limited number of missing unmeasured confounders, the residual association is essentially null and close to the finding of the randomised controlled trial. Most studies have more limited information on potential confounders than is available in the BWHHS, and in other fields we may be even more ignorant of the confounding factors we should measure. In these cases inferences drawn from observational epidemiological studies may be seriously misleading. As the major and compelling rationale for doing these observational studies is to underpin public health prevention strategies, their repeated failures are a major concern for public health policy makers, researchers and funders.

Other processes in addition to confounding can generate robust, but non-causal, associations in observational studies. Reverse causation – where the disease influences the apparent exposure, rather than *vice versa*, may generate strong and replicable associations. For example, many studies have found that people with low circulating cholesterol levels are at increased risk of several cancers, including colon cancer. If causal, this is an important association as it might mean that efforts to lower cholesterol levels would increase the risk of cancer. However, it is possible that the early stages of cancer may, many years before diagnosis or death, lead to a lowering in cholesterol levels, rather than low cholesterol levels increasing the risk of cancer. Similarly in studies of inflammatory markers such as C-reactive protein and cardiovascular disease risk it is possible that early stages of atherosclerosis – which is an inflammatory processes – leads to elevation in circulating inflammatory markers, and since people with atherosclerosis are more likely to experience cardiovascular

events a robust, but non-causal, association between levels of inflammatory markers and incident cardiovascular disease is generated. Reverse causation can also occur through behavioural processes – for example, people with early stages and symptoms of cardiovascular disease may reduce their consumption of alcohol, which would generate a situation in which alcohol intake appears to protect against cardiovascular disease. A form of reverse causation can also occur through reporting bias, with the presence of disease influencing reporting disposition. In case-control studies people with the disease under investigation may report on their prior exposure history in a different way than do controls—perhaps because the former will think harder about potential reasons that account for why they have developed the disease.

In observational studies associations between an exposure and disease will generally be biased if there is selection according to an exposure–disease combination in case-control studies, or according to an exposure–disease risk combination in prospective studies. Such selection may arise through differential participation in research studies, conducting studies in settings such as hospitals where cases and controls are not representative of the general population, or study of unusual populations (e.g. vegetarians). If, for example, those people experiencing an exposure but at low risk of disease for other reasons were differentially excluded from a study the exposure would appear to be positively related to disease outcome, even if there were no such association in the underlying population. This is a form of 'Berkson's bias', well known to epidemiologists (Berkson 1946). A possible example of such associative selection bias relates to the finding in the large American Cancer Society volunteer cohort that high alcohol consumption was associated with a reduced risk of stroke (Thun et al 1997). This is somewhat counter-intuitive as the outcome category

included haemorrhagic stroke (for which there is no obvious mechanism through which alcohol would reduce risk) and because alcohol is known to increase blood pressure— a major causal factor for stroke. Population-based studies have found that heavy alcohol consumption tends to increase stroke risk, particularly haemorrhagic stroke (Hart et al 1999, Reynolds et al 2003). Heavy drinkers who volunteer for a study known to be about the health effects of their lifestyle are likely to be very unrepresentative of all heavy drinkers in the population, in ways that render them to be at low risk of stroke. Moderate and non-drinkers who volunteer may be more representative of moderate and non-drinkers in the underlying population. Thus the low risk of stroke in the heavy drinkers who volunteer for the study could erroneously make it appear that alcohol reduces the risk of stroke.

The problems of confounding and bias discussed above relate to the production of associations in observational studies that are not reliable indicators of the true direction of causal associations. A separate issue is that the strength of associations between causal risk factors and disease in observational studies will generally be underestimated due to random measurement imprecision in indexing the exposure. A century ago Charles Spearman demonstrated mathematically how such measurement imprecision would lead to what he termed the 'attenuation by errors' of associations.(Spearman 1904, Davey Smith and Phillips 1996) This has more latterly been renamed 'regression dilution bias'.

Observational studies can and do produce findings that either spuriously enhance or downgrade estimates of causal associations between modifiable exposures and disease. This has serious consequences for the appropriateness of interventions that

aim to reduce disease risk in populations. It is for these reasons that alternative approaches — including those within the Mendelian randomization framework — need to be applied.

Mendelian randomization

The basic principle utilised in the Mendelian randomization approach is that if genetic variants either alter the level of, or mirror the biological effects of, a modifiable environmental exposure that itself alters disease risk, then these genetic variants should be related to disease risk to the extent predicted by their influence on exposure to the risk factor. Common genetic polymorphisms that have a well-characterized biological function (or are markers for such variants) can therefore be utilized to study the effect of a suspected environmental exposure on disease risk (Davey Smith and Ebrahim 2003;2004; 2005; Davey Smith 2006). The exploitation of situations in which genotypic differences produce effects similar to environmental factors (and *vice versa*) clearly resonates with the concepts of phenocopy and genocopy in developmental genetics (Box 1).

It may seem illogical to study genetic variants as proxies for environmental exposures rather than measure the exposures themselves. However there are several crucial advantages of utilising functional genetic variants (or their markers) in this manner, that relate to the problems with observational studies outlined above. First, unlike environmental exposures, genetic variants are not generally associated with the wide range of behavioural, social and physiological factors that, for example, confound the association between vitamin C and coronary heart disease. This means that if a

genetic variant is used to proxy for an environmentally modifiable exposure it is unlikely to be confounded in the way that direct measures of the exposure will be. Further, aside from the effects of population structure (see Palmer and Cardon 2005 for a discussion of the likely impact of this) such variants will not be associated with other genetic variants, excepting those with which they are in linkage disequilibrium. This latter assumption follows from the law of independent assortment (sometimes referred to as Mendel's second law); hence the term "Mendelian randomization" (see Box 2).We illustrate this powerful aspect of Mendelian randomization in Table 2, showing the strong associations between a wide range of variables and blood C reactive protein (CRP) levels, but no association of the same factors with genetic variants in the *CRP* gene. The only factor related to genotype is the expected, biological, influence of the genetic variant on CRP levels.

Second, we have seen how inferences drawn from observational studies may be subject to bias due to reverse causation. Disease processes may influence exposure levels such as alcohol intake, or measures of intermediate phenotypes such as cholesterol levels and C-reactive protein. However germline genetic variants associated with average alcohol intake or circulating levels of intermediate phenotypes will not be influenced by the onset of disease. This will be equally true with respect to reporting bias generated by knowledge of disease status in case-control studies, or of differential reporting bias in any study design.

Third, associative selection bias in which selection into a study is related to both exposure level and disease risk and can generate spurious associations (as illustrated above with respect to alcohol and haemorrhagic stroke above) are unlikely to occur

with respect to genetic variants. For example empirical evidence supports a lack of association between a wide range of genetic variants and participation rates in three separate case-control studies: breast cancer, non-Hodgkins lymphoma, and lung cancer (Bhatti et al 2005). Comparisons of genetic variants concerned with DNA repair, growth factors, immune responses, and oxidative stress (over 100 SNPs and 15 tandem repeats) were compared in participants who had responded early or with minimal effort and participants who required incentives or increased time and contact to respond. Odds ratios for differences in prevalence of genetic variants between those willing and less willing to participate were generally null, showing no strong evidence to support any associations between genotype and willingness to participate in research (Bhatti et al, 2005). As these investigators noted, it is important that researchers test this assumption in their own data, as it is possible that other genotypes than those tested here, particularly those associated with health relevant behaviours (e.g. alcohol consumption), may show associations.

Finally, a genetic variant will indicate long-term levels of exposure and if the variant is taken as a proxy for such exposure it will not suffer from the measurement error inherent in phenotypes that have high levels of variability. For example, groups defined by cholesterol-level related genotype will, over a long period, experience the cholesterol difference seen between the groups. For individuals, blood cholesterol is variable over time, and the use of single measures of cholesterol will under-estimate the true strength of association between cholesterol and, say, coronary heart disease. Indeed use of the Mendelian randomization approach predicts a strength of association that is in line with randomised controlled trial findings of effects of cholesterol lowering when the increasing benefits seen over the relatively short trial

period are projected to the expectation for differences over a lifetime (Davey Smith and Ebrahim 2004) and discussed further below.

Categories of Mendelian randomization

There are several categories of inference that can be drawn from studies utilising the Mendelian randomization paradigm. In the most direct forms genetic variants can be related to the probability or level of exposure ("exposure propensity") or to intermediate phenotypes believed to influence disease risk. Less direct evidence can come from genetic variant-disease associations that indicate that a particular biological pathway may be of importance, perhaps because the variants modify the effects of environmental exposures. Several examples from of these categories have been given elsewhere (Davey Smith and Ebrahim 2003; 2004; Davey Smith 2006); here a few illustrative cases are briefly outlined.

Exposure propensity

Milk intake and bone health .

Osteoporosis may be related to habitual low calcium intake, but measuring this exposure accurately is difficult. It has been suggested that assessing the association between calcium exposure and bone health may be done by comparing people with and without lactase persistence, as this may provide a better index of long-term low calcium intake (Honkanen et al 1996). Lactase persistence is an autosomal dominant condition and a LCT polymorphism, -13910 T/C near the lactase phlorizin hydrolase gene has been found. In post-menopausal women, the CC genotype is strongly

associated with low dietary intake of calcium from milk, a 10% lower bone mineral density at hip and spine, and a greater risk of non-vertebral fractures (Obermayer-Pietsch et al 2004). (See figure 4). This provides strong evidence that milk drinking improves bone health, especially since directly studying milk intake is potentially beset with problems of confounding, reverse causation (people with bone problems may be told to drink more milk) and measurement error. Indeed in another field claims of associations between milk drinking and reduced risk of coronary heart disease (Elwood 2001; Ness et al 2001) have been criticised for inadequately dealing with confounding and reverse causation (Shaper et al 1991). (check date of Elwood paper)

Alcohol intake and health

The possible protective effect of moderate alcohol consumption on CHD risk remains controversial (Marmot 2001; Bovet and Paccaud 2001; Klatsky 2001). Non-drinkers may be at a higher risk of CHD because health problems (perhaps induced by previous alcohol abuse) dissuade them from drinking (Shaper 1993). As well as this form of reverse causation, confounding could play a role, with non-drinkers being more likely to display an adverse profile of socioeconomic or other behavioural risk factors for CHD (Hart et al 1999). Alternatively, alcohol may have a direct biological effect that lessens the risk of CHD – for example by increasing the levels of protective high density lipoprotein (HDL) cholesterol (Rimm 2001). It is, however, unlikely that an RCT of alcohol intake, able to test whether there is a protective effect of alcohol on CHD events, will be carried out.

Alcohol is oxidized to acetaldehyde, which in turn is oxidized by aldehyde dehydrogenases (ALDHs) to acetate. Half of Japanese people are heterozygotes or homozygotes for a null variant of ALDH2 and peak blood acetaldehyde concentrations post alcohol challenge are 18 times and 5 times higher respectively among homozygous null variant and heterozygous individuals compared with homozygous wild type individuals (Enomoto et al 1991). This renders the consumption of alcohol unpleasant through inducing facial flushing, palpitations, drowsiness and other symptoms. As Figure 5a shows, there are very considerable differences in alcohol consumption according to genotype (Takagi et al 2002). The principles of Mendelian randomization are seen to apply – two factors that would be expected to be associated with alcohol consumption, age and cigarette smoking, which would confound conventional observational associations between alcohol and disease, are not related to genotype despite the strong association of genotype with alcohol consumption (Figure 5b).

It would be expected that ALDH2 genotype influences diseases known to be related to alcohol consumption, and as proof of principle it has been shown that ALDH2 null variant homozygosity – associated with low alcohol consumption – is indeed related to a lower risk of liver cirrhosis (Chao et al 1994). Considerable evidence, including data from randomized controlled trials, suggests that alcohol increases HDL cholesterol levels (Haskell 1984; Burr 1986) (which should protect against CHD). In line with this, ALDL2 genotype is strongly associated with HDL cholesterol in the expected direction (figure 5c). Given the apparent protective effect of alcohol against CHD risk seen in observational studies possession of the ALDH2 allele - associated with lower alcohol consumption - should be associated with a greater risk of myocardial infarction, and this is what was seen in a case-control study (Takagi et al 2002). Men either homozygous or heterozygous for null ALDH2 were at twice the risk of myocardial infarction. Supporting reasoning that the HDL cholesterol elevating effects of alcohol are what renders it protective against coronary heart disease, statistical adjustment for HDL cholesterol greatly attenuated the association between ALDH2 genotype and CHD.

Alcohol intake has also been postulated to increase the risk of oesophageal cancer, however some have questioned the importance of its role (Memik 2003). Figure 6 presents findings from a meta-analysis of studies of ALDH2 genotype and oesophageal cancer risk (Lewis and Davey Smith 2005), clearly showing that people who are homozygous for the null variant, who therefore consume considerably less alcohol, have a greatly reduced risk of oesophageal cancer. Indeed this reduction in risk is close to that predicted by the joint effect of genotype on alcohol consumption

and the association of alcohol consumption on oesophageal cancer risk in a meta-analysis of such observational studies (Gutjahr et al 2001). When the heterozygotes are compared with the homozygous functional variant, an interesting picture emerges – the risk of oesophageal cancer is higher in the heterozygotes who drink rather less alcohol than those with the homozygous functional variant. This suggests that it is not alcohol itself that is the causal factor but acetaldehyde, and that the increased risk is only apparent in those who drink some alcohol but metabolise it inefficiently, leading to high circulating levels of acetaldehyde.

Intermediate phenotypes

Genetic variants can influence circulating biochemical factors such as cholesterol, homocysteine, or fibrinogen levels. This provides a method for assessing causality in associations observed between these measures (*intermediate phenotypes*) and disease, and thus whether interventions to modify the intermediate phenotype could be expected to influence disease risk.

Cholesterol and coronary heart disease

Familial hypercholesterolaemia is a dominantly inherited condition in which many rare mutations (over 700 DNA sequence variations (LDL Receptor 2003;) of the low density lipoprotein receptor gene (about 10 million people affected world-wide, a prevalence of around 0.2%), lead to high circulating cholesterol levels (Marks et al 2003). The high risk of premature CHD in people with this condition was readily appreciated, with an early UK report demonstrating that by age 50 half of men and

12% of women had suffered from CHD (Slack 1969). Compared with the population of England & Wales (mean total cholesterol 6.0mmol/l), people with familial hypercholesterolaemia (mean total cholesterol 9 mmol/l) suffered a 3.9 fold increased risk of CHD mortality, although very high relative risks among those aged less than 40 years have been observed (Scientific Steering Committee 1991) . These observations, regarding genetically determined variation in risk, provided strong evidence that the associations between blood cholesterol and CHD seen in general populations reflected a causal relationship. The causal nature of the association between blood cholesterol levels and coronary heart disease has historically been controversial (Steinberg 2004). As both Daniel Steinberg (Steinberg 2005) and Ole Færgeman discuss (Færgeman 2003), many clinicians and public health practitioners rejected the notion of a causal link for a range of reasons. However from the late 1930s onwards evidence that people with genetically high levels of cholesterol had high risk for coronary heart disease should have been powerful and convincing evidence of the causal nature of elevated blood cholesterol in the general population.

With the advent of effective means of reducing blood cholesterol through statin treatment, there remains no serious doubt that the cholesterol-CHD relationship is causal. Among people without CHD, reducing total cholesterol levels with statin drugs by around 1 to 1.5 mmol/1 reduces CHD mortality by around 25% over 5 years (– this was secondary prevention – best to cite recent meta-analysis. Baigent C, Keech A, Kearney P et al for Cholesterol Lowering Treatments Collaboration. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins .Lancet. 2005 Oct 8;366:1267-78). Assuming a linear relationship between blood cholesterol and CHD risk, and given

the difference in cholesterol of 3.0 mmol/l between people with familial hypercholesterolaemia and the general population , the randomized controlled trial evidence on lowering total cholesterol and reducing CHD mortality would predict a relative risk for CHD of around 2, as opposed to 3.9, for people with familial hypercholesterolaemia. However the trials also demonstrate that the relative reduction in CHD mortality increases over time from randomization – and thus time with lowered cholesterol - as would be expected if elevated levels of cholesterol operate over decades to influence the development of atherosclerosis. People with familial hypercholesterolaemia will have had high total cholesterol levels throughout their lives and this would be expected to generate a greater risk than that predicted by the results of lowering cholesterol levels for only 5 years. Furthermore, ecological studies relating cholesterol levels to CHD demonstrate that the strength of association increases as the lag period between cholesterol level assessment and CHD mortality increases (Rose 1982), again suggesting that long-term differences in cholesterol level are the important aetiological factor in CHD. As discussed above, Mendelian randomization is one method for assessing the effects of long-term differences in exposures on disease risk, free from the diluting problems of both measurement error and of only having short-term assessment of risk factor levels. This reasoning provides an indication that cholesterol – lowering efforts should be lifelong rather than limited to the period for which RCT evidence with respect to CHD outcomes is available.

More recently, mutations in the gene coding for apolipoprotein B (apoB) have been found to produce a syndrome phenotypically indistinguishable from familial hypercholesterolaemia –Familial Defective ApoB (Soria et al 1989; Tybjærg-Hansen

et al 1992; Myant 1993). In a recent study of the Arg3500Gln mutation of the *APOB* gene, the basic principle behind Mendelian randomization can be demonstrated, in that Arg3500Gln heterozygotes had higher levels of total cholesterol but other CHD risk factors (including triglycerides, fibrinogen, glucose, body mass index and waist-hip ratio) did not differ from non-heterozygotes in the general population (Tybjaerg-Hansen et al 1998). The Arg3500Gln heterozygotes had a median 2.6 mmol/l higher blood cholesterol level and a high (but imprecise) odds ratio for CHD of 7.0 (95% CI 2.2 to 22) compared with the general population. As in the case of familial hypocholesterolaemia this is greater than that predicted by the randomized controlled trial data, but again the differences in cholesterol by genotype will have been life-long, and the elevated CHD risk probably reflects the effects of long-term differences in cholesterol level.

Recently sequence variations in *PCSK9* associated with levels of LDL-cholesterol between 15-23% lower than levels in people without the mutant variants have been evaluated in the Atherosclerosis Risk in Communities study (ARIC), and considerably lower risks of CHD - between 47-88% lower – have been observed, depending on the level of LDL-cholesterol associated with each sequence variant. (Cohen et al 2006) Despite participants in ARIC having substantial burdens of other cardiovascular risk factors, these data indicate that *life-long* exposure to low levels of LDL-cholesterol (consistent with those achieved by statin treatment) is associated with markedly reduced risks of CHD, greater than the reductions observed for short-term cholesterol lowering in the statin trials.As other commentators have observed this is not surprising as atherosclerosis begins early in life whereas statin treatment in later life would not be expected to achieve the same benefit (Brown & Goldstein 2006).

C-reactive protein (CRP) and coronary heart disease

Strong associations of C-reactive protein (CRP), an acute phase inflammatory marker, with hypertension, insulin resistance and coronary heart disease have been repeatedly observed (Danesh et al 2004; Wu et al 2002; Pradhan et al 2001; Han et al 2002; Sesso et al 2003; Hirschfield and Pepys 2003, Hu et al 2004), with the obvious inference that CRP is a cause of these conditions (Ridker et al 2005; Sjöholm and Nystöm 2005, Verma et al 2005). A Mendelian randomization study has examined the association between polymorphisms of the *CRP* gene and demonstrated that while serum CRP differences were highly predictive of blood pressure and hypertension, the CRP variants – which are related to sizeable serum CRP differences – were not associated with these same outcomes (Davey Smith et al 2005a). It is likely that these divergent findings are explained by the extensive confounding between serum CRP and outcomes (as shown in Table 2). Current evidence on this issue, though statistically underpowered, also suggests that CRP levels do not lead to elevated risk of insulin resistance (Timpson et al 2005) or coronary heart disease (Casas et al 2006). Again, confounding, and reverse causation – where existing coronary disease or insulin resistance may influence CRP levels – could account for this discrepancy. Similar findings have been reported for serum fibrinogen, variants in the beta fibrinogen gene and CHD (Davey Smith et al 2005b; Keaveney et al 2006). The CRP and fibrinogen examples demonstrate that Mendelian randomization can both increase evidence for a causal effect of an environmentally modifiable factor (as in the cases of milk, alcohol and cholesterol levels discussed earlier) and also provide evidence

against causal effects, that can help direct efforts away from targets of no preventative or therapeutic relevance.

Identifying biological pathways for disease

The suggestion that taking aspirin reduces the risk of colon cancer originated from a case-control study exploring a large number of potential risk factors (Kune 1988), but has been replicated in other studies (Sandler et al 1998). Taking a Mendelian randomization approach provides one way of strengthening inference regarding the causal nature of this association. When examining variants in the gene coding for prostaglandin H synthase 2 (PTGS2), an enzyme involved in conversion of arachidonic acid to prostaglandin H_2 which is inhibited by aspirin (Lin et al 2002), an association was found with reduced colon cancer risk. The investigators hypothesised that naturally occurring *PTGS2* variants might mimic long-term aspirin use. A larger study is required to confirm these exciting preliminary data. The data do, however, provide supportive evidence that aspirin (and other *PTGS2* inhibitors) protects against colon cancer, and that this protection is due to inhibition of the conversion of arachidonic acid to prostaglandin. Positive findings have been reported from two small randomised trials of aspirin in high risk patients, providing further evidence in support of a causal role for aspirin (Sandler et al 2003; Baron et al 2003). Combining data from observational epidemiological studies, Mendelian randomization designs and RCTs provides a powerful basis for causal inference.

Mendelian randomization studies using the *PTGS2* variant would have considerable utility in resolving one of the remaining questions about the value of antiplatelet

therapy in people without evidence of cardiovascular disease but neither at very low risk (where the harms of antiplatelet treatment outweigh the risks) or very high risk (where the benefits of treatment are established). Currently, there is clinical uncertainty that would require a very large, long duration, expensive trial to resolve but an examination of the association between *PTGS2* variants and cardiovascular disease incidence in large observational cohorts stratified by predicted cardiovascular risk (high, intermediate, low), would, if a lower incidence in those at intermediate risk who had the aspirin mimicking variant was found, support use of antiplatelet treatments.

Modifiers of environment exposure

Sheep dip may be hazardous because of the organophosphates contained in it, but the vague symptoms farm workers attribute to exposure are not considered to be causal, but motivated by secondary gain from compensation (**http://news.bbc.co.uk/1/hi/health/383003.stm & http://news.bbc.co.uk/1/hi/health/537549.stm**). Conducting trials would be unethical and valid observational studies impossible, as reporting bias would be likely. Variants of the paraoxonase gene that produce forms of the enzyme paraoxonase with varying ability to detoxify organophosphates can be used to indicate the effects of different levels of sheep dip exposure. If organophosphates in sheep dip truly cause ill-health then among people exposed to sheep dip a higher proportion of those with symptoms would be expected to carry the genetic variants related to less efficient detoxification, and this is what has been found (Cherry et al 2002). Since it is unlikely that possession of the detoxification genotype is related to the tendency to

report symptoms differentially, or to the desire for compensation, these findings provide evidence that sheep dip, and not compensation neurosis, is the cause of farm workers' symptoms. However the lack of association of reporting tendency with genotype cannot be assumed and should be explicitly examined, and in this case the Mendelian randomization approach would be formulated in terms of an expected gene-environment interaction, an issue that will be discussed later.

Intergenerational influences – Methyl-tetrahydrofolate reductase (MTHFR) polymorphisms and neural tube defects.

Examining the effects of mother's genotype (independent of genotype of offspring) on the health outcomes of their offspring is a form of "intergenerational" Mendelian randomization, providing evidence on the role of intrauterine environment on the health of children. For example, peri-conceptual and early pregnancy folate deficiency is now known to be a cause of neural tube defects (NTDs), an effect confirmed by randomized controlled trial evidence (MRC Vitamin Study 1991; Czeizel and Dudás 1992). The *MTHFR 677C→T* polymorphism can be considered to mimic reduced folate and in a meta-analysis of case-control studies of NTDs, TT mothers had a 2-fold risk of having an infant with a neural tube defect compared with CC mothers (Botto and Yang 2000). The relative risk of a neural tube defect associated with the TT genotype in the infant was less than that observed with respect to maternal genotype, and there was no effect of paternal genotype on offspring neural tube defect risk. This suggests that it is the intra-uterine environment – influenced by maternal TT genotype– rather than the genotype of offspring that increases the risk of NTD (Davey

Smith and Ebrahim 2003), and that higher maternal folate intake would reduce the risk of offspring NTDs, as found in the trials.

Implications of Mendelian randomization study findings

Establishing the causal influence of environmentally modifiable risk factors from Mendelian randomization designs informs policies for improving population health through population-level interventions. They do not imply that the appropriate strategy is genetic screening to identify those at high risk and application of selective exposure reduction policies. For example, the implications of studies on maternal MTHFR genotype and offspring NTD risk is that population risk for NTDs can be reduced through increased folate intake peri-conceptually and in early pregnancy. It does not suggest that women should be screened for MTHFR genotype; women without the TT genotype but with low folate intake are still exposed to preventable risk of having babies with NTDs. Similarly establishing the association between genetic variants (such as familial defective ApoB) associated with elevated cholesterol level and CHD risk strengthens causal evidence that elevated cholesterol is a modifiable risk factor for CHD for the whole population. Thus even though the population attributable risk for CHD of this variant is small it usefully informs public health approaches to improving population health. It is this aspect of Mendelian randomization that illustrates its distinction from conventional risk identification and genetic screening purposes of genetic epidemiology.

Mendelian randomization and randomised controlled trials

Randomised controlled trials are clearly the definitive means of obtaining evidence on the effects of modifying disease risk processes. There are similarities in the logical structure of RCTs and Mendelian randomization, however. Figure 7 illustrates this, drawing attention to the unconfounded nature of exposures proxied for by genetic variants (analogous to the unconfounded nature of a randomised intervention), the lack of possibility of reverse causation as an influence on exposure-outcome associations in both Mendelian randomization and randomised controlled trial settings and the importance of intention to treat analyses – i.e. analysis by group defined by genetic variant, irrespective of associations between the genetic variant and the proxied for exposure within any particular individual.

The analogy with randomised controlled trials is also useful with respect to one objection that has been raised with respect to Mendelian randomization studies. This is that the environmentally modifiable exposure proxied for by the genetic variants (such as alcohol intake or circulating CRP levels) are influenced by many other factors in addition to the genetic variants. (Jousilahti et al 2004). This is of course true. However consider a randomised controlled trial of blood pressure lowering medication. Blood pressure is mainly influenced by factors other than taking blood pressure lowering medication – obesity, alcohol intake, salt consumption and other dietary factors, smoking, exercise, physical fitness, genetic factors and early-life developmental influences are all of importance. However the randomization that occurs in trials ensures that these factors are balanced between the groups that receive the blood pressure lowering medication and those that do not. Thus the fact that many other factors are related to the modifiable exposure does not vitiate the power of RCTs; neither does it vitiate the strength of Mendelian randomization designs.

A related objection is that the genetic variants often explain only a trivial proportion of the variance in the environmentally modifiable risk factor that is being proxied for. (Glynn 2006) Again consider a randomised controlled trial of blood pressure lowering medication, where 50% or participants receive the medication and 50% received a placebo. If the antihypertensive therapy reduced blood pressure by a quarter of a standard deviation, which is approximately the situation for such pharmacotherapy, then within the whole study group treatment assignment (i.e. antihypertensive use versus placebo) will explain 1.54%? of the variance in blood pressure – i.e. SD (BP) approximately 20mmHg, treatment effect of 5mmg (quarter of 1 SD), variance (BP) = $SD^2 = 20^2$; treatment will explain $5/20^2 = 1.25\%$ In the example of CRP haplotypes used as instruments for CRP levels, these haplotypes explain 1.66% of the variance in CRP levels in the population (Lawlor et al 2007 in press). As can be seen the quantitative association of genetic variants as instruments can be similar to that of randomised treatments with respect to biological processes that such treatments modify. Both logic and quantification fail to support criticisms of the Mendelian randomization approach based on either the obvious fact that many factors influence most phenotypes of interest or that particular genetic variants only account for a small proportion of variance in the phenotype.

// Any other Nitsch objections that need to be considered?

Mendelian randomization and instrumental variable approaches

As well as the analogy with randomised controlled trials, Mendelian randomization can also be likened to instrumental variable approaches that have been heavily utilised in econometrics and social science, although rather less so in epidemiology. In an

instrumental variable approach the instrument is a variable that is only related to the outcome through its association with the modifiable exposure of interest. The instrument is not related to confounding factors nor is its assessment biased in a manner that would generate a spurious association with the outcome. Furthermore the instrument will not be influenced by the development of the outcome (i.e. there will be no reverse causation). Figure 8 presents this basic schema, where the dotted line between genotype and the outcome provides an unconfounded and unbiased estimate of the causal association between the exposure that the genotype is proxying for and the outcome. The development of instrumental variable methods within econometrics, in particular, has led to a sophisticated suite of statistical methods for estimating causal effects, and these have now been applied within Mendelian randomization studies (e.g. Davey Smith et al 2005a, 2005b; Timpson et al 2005). The parallels between Mendelian randomization and instrumental variable approaches are discussed in more detail elsewhere (Thomas and Conti 2004, Didelez and Sheehan, 2007 in press; Lawlor et al 2007 in press).

Mendelian randomization and gene by environment interaction

Mendelian randomization is one way in which genetic epidemiology can inform understanding about environmental determinants of disease. A more conventional approach has been to study interactions between environmental exposures and genotype (Perera et al 1997; Mucci et al 2001). From epidemiological and Mendelian randomization perspectives several issues arise with gene-environment interactions.

The most reliable findings in genetic association studies relate to the main effects of polymorphisms on disease risk.(Clayton and McKeigue 2001) The power to detect meaningful gene-environment interaction is low (Wright et al 2002), with the result being that there are a large number of reports of spurious gene-environment interactions in the medical literature (Colhoun et al 2003). The presence or absence of statistical interactions depends upon the scale (e.g. linear or logarithmic with respect to the exposure-disease outcome) and the meaning of observed deviation from either an additive or multiplicative model is not clear. Furthermore the biological implications of interactions (however defined) is generally uncertain (Thompson 1991). Mendelian randomization is most powerful when studying modifiable exposures that are difficult to measure and/or considerably confounded, such as dietary factors. Given measurement error – particularly if this is differential with respect to other factors influencing disease risk – interactions are both difficult to detect and often misleading when, apparently, they are found (Clayton and McKeigue 2001).

The situation is perhaps different with exposures that differ qualitatively rather than quantitatively between individuals. Consider the issue of the influence of smoking tobacco on bladder cancer risk. Observational studies suggest an association, but clearly confounding and a variety of biases could generate such an association. The potential carcinogens in tobacco smoke of relevance to bladder cancer risk include aromatic and heterocyclic amines, which are detoxified by *N*-acetyl transferase 2 (NAT2). Genetic variation in NAT2 enzyme levels leads to slower or faster acetylation states. If the carcinogens in tobacco smoke do increase the risk of bladder cancer then it would be expected that slow acetylators, who have a reduced rate of

detoxification of these carcinogens, would be at an increased risk of bladder cancer if they were smokers, whereas if they were not exposed to these carcinogens (and the major exposure route for those outside of particular industries is through tobacco smoke) then an association of genotype with bladder cancer risk would not be anticipated. Table 3 tabulates findings from the largest study to date reported in a way that allows analysis of this simple hypothesis (Gu et al 2005). As can be seen the influence of the *NAT2* slow acetylation genotype is only appreciable among those also exposed to heavy smoking. Since the genotype will be unrelated to confounders it is difficult to reason why this situation should arise unless smoking is a causal factor with respect to bladder cancer. Thus the presence of a sizable effect of genotype in the exposed group but not in the unexposed group provides evidence as to the causal nature of the environmentally modifiable risk factor, in this example, smoking. It must be recognised, however, that gene by environment interactions interpreted within the Mendelian randomization framework as evidence regarding the causal nature of environmentally modifiable exposures are not protected from confounding to the extent main genetic effects are. In the *NAT2* / smoking / bladder cancer example any factor related to smoking – such as social class – will tend to show a greater association with bladder cancer within NAT2 slow acetylators than within NAT2 rapid acetylators. Because there is not a 1- to-1 association of social class with smoking this will not produce the qualitative interaction of essentially no effect of the genotype in one exposure stratum and an effect in the other, as in the *NAT2*/smoking interaction, but rather a qualitative interaction of a greater effect of *NAT2* in the poorer social classes (amongst whom smoking is more prevalent) and a smaller (but still evident) effect in the better off social classes, amongst whom smoking is less prevalent. Thus situations in which both the biological basis of an expected

interaction is well understood and in which a qualitative (effect versus no effect) interaction may be anticipated are the ones that are most amenable to interpretations related to the general causal nature of the environmentally modifiable risk factor. Further discussion of gene by environment interaction as interpreted within the Mendelian randomization framework is available elsewhere (Davey Smith, in press, 2007).

Problems and limitations of Mendelian randomization

We consider Mendelian randomization to be one of the brightest current prospects for improving causal understanding within population-based studies. There are, however, several potential limitations to the application of this methodology (Davey Smith and Ebrahim 2003; Little and Khoury 2003), which we discuss below.

Failure to establish reliable genotype-intermediate phenotype or genotype-disease associations

If the associations between genotype and a potential intermediate phenotype, or between genotype and disease outcome, are not reliably estimated, then interpreting these associations in terms of their implications for potential environmental causes of disease will clearly be inappropriate. This is not an issue peculiar to Mendelian randomization, rather the non-replicable nature of perhaps most apparent findings in genetic association studies is a serious limitation to the whole enterprise. In Table 4 we summarize possible reasons for the non-replication of findings (Colhoun et al 2003; Cardon and Bell 2001). Population stratification – i.e. confounding of

genotype-disease associations by factors related to subpopulation group membership within the overall population in a study – is unlikely to be a major problem in most situations (Wacholder et al 2000; Wacholder et al 2002; Palmer and Cardon 2005). Genotyping errors can, of course, lead to failures of replication of genotype-disease associations. Where intermediate phenotypes can be measured, as is the case of CRP, a demonstration of the expected relationship between genotype and intermediate phenotype in such studies indicates that genotyping errors are not to blame. For example, in the study demonstrating a lack of association between CRP genotypes and coronary heart disease risk (Casas et al 2006) the report of a lack of association between genotype and CHD risk could be claimed to reflect genotyping errors (and thus could not be taken to provide evidence against a causal role for CRP). However since within this study the investigators also demonstrated that their genotyping data did predict CRP levels to the same degree as in other studies, this interpretation is not tenable.

Regarding failure to replicate results in genetic epidemiology, true variation between studies is clearly possible – for example, people heterozygous for familial hypercholesterolaemia only seem to experience increased mortality in populations with substantial dietary fat intake and the presence of other CHD risk factors (Sijbrands et al 2001; Pimstone et al 1998). Nevertheless, the major factor for non-replication is probably inadequate statistical power (generally reflecting limited sample size), coupled with publication bias (Colhoun et al 2003)

Interestingly, Gregor Mendel appreciated the need for adequate sample size when he carried out his experiments on pea crosses, stating that "with a small number of plants

... very considerable fluctuations may occur" and that the "true ratio of the numbers can only be ascertained by an average deduced from the sum of as many single values as possible; the greater the number, the more are merely chance effects eliminated" (Mendel 1866)

In the case of quantitative approaches to Mendelian randomization, sample size calculations need to consider the magnitude of both the effect of genotype on the modifiable risk factor that is being proxied for and the predicted effect of the modifiable risk factor on disease outcome. This often leads to very large studies being required, and failure to recognize this can lead to studies being uninformative. For example, in a report of a case-control study entitled "Elevated plasma fibrinogen. Cause or consequence of cardiovascular disease?" (Van der Bom et al 1998), the relative risk of coronary heart disease for a 1 g higher fibrinogen level was 1.45 (95% CI 1.12-1.88), while the association between genotype and CHD risk was essentially null (relative risk 1.08 95% CI 0.71-1.65 for GA and AA genotypes compared with GG genotype). The authors interpreted these results as indicating that fibrinogen was not a cause of CHD. However given the strength of the association between genotype and fibrinogen, with GA plus AA individuals having 0.17 g/l higher fibrinogen than GG individuals, the predicted risk according to genotype, given the observational association between fibrinogen and CHD, would be around 1.07. This is clearly not different from the estimated relative risk – indeed the point estimates are close, although there is a very wide confidence interval around the relative risk for genotype. Thus the authors' claim that their study suggests that fibrinogen is not causally related to the risk of CHD is not supported by evidence from their own study, although later

larger studies and meta-analysis suggest their conclusion was correct (Davey Smith et al 2005b, Keavney et al 2006).

The small genotype-associated relative risks predicted by knowledge of intermediate phenotype in the case of CRP and ß-fibrinogen, mean that very large studies are required; in other cases it may be that even smaller relative risks would be expected. If polymorphisms at more than one locus influence an intermediate phenotype then it may be possible to explore combinations of polymorphisms at different loci that produce differences in intermediate phenotype that are substantial enough to generate detectable effects on disease outcome. If the loci are not in linkage disequilibrium and thus segregate independently this could be termed "factorial Mendelian randomization", with interest being in the groups in which the combination of polymorphisms produce the most extreme difference in intermediate phenotype. Alternatively, haplotypes that produce more extensive phenotypic differences than single SNPs could be studied, as they have been in the case of CRP and insulin resistance (Timpson et al 2005).

The problems in establishing reliable genotype-disease associations are, of course, a general issue in genetic epidemiology. Tabor and colleagues have emphasized the advantages of candidate-gene approaches in which plausible links between the functional effects of candidate polymorphisms and disease outcomes exist (Tabor et al 2002). Such studies are less likely to produce false-positive findings than are investigations relating non-functional genetic variants to disease risk. Mendelian randomization clearly depends upon studying genetic variants that have a defined biological effect, and therefore the relevant studies fit within this model. Tabor and

colleagues extend their reasoning on candidate-gene association studies to suggest that researchers carry out initial sequencing work on the functional regions of a gene to identify new SNPs, then determine the population frequency of these SNPs and their functional relevance, before performing the epidemiological analyses. The need for epidemiologists to work closely and collaboratively with laboratory scientists to take forward Mendelian randomization is made clear in this exposition.

Confounding of genotype - environmentally modifiable risk factor – disease associations

The power of Mendelian randomization lies in its ability to avoid the often substantial confounding seen in conventional observational epidemiology. However confounding can be reintroduced into the Mendelian randomization studies and when interpreting the results it needs to be considered whether this has arisen.

Linkage disequlibrium

It is possible that the locus under study is in linkage disequilibrium – i.e. is associated – with another polymorphic locus, with the effect of the polymorphism under investigation being confounded by the influence of the other polymorphism. It may seem unlikely - given the relatively short distances over which linkage disequilibrium is seen in the human genome - that a polymorphism influencing, say, CHD risk would be associated with another polymorphism influencing CHD risk (and thus producing confounding). There are, nevertheless, cases of different genes influencing the same metabolic pathway being in physical proximity. For example, different

polymorphisms influencing alcohol metabolism appear to be in linkage disequilibrium (Osier MV 2002).

Pleiotropy and the multi-function of genes

Mendelian randomization is most useful when it can be used to relate a single intermediate phenotype to a disease outcome. However polymorphisms may (and probably often will) influence more that one intermediate phenotype and this may mean they proxy for more than one environmentally modifiable risk factor. This can be the case through multiple effects mediated by their RNA expression or ?immediate? protein coding, through alternative splicing, where one polymorphic region contributes to alternative forms of more than one protein (Glebart 1998), or through other mechanisms. The most robust interpretations will be possible when the functional polymorphism appears to directly influence the level of the intermediate phenotype of interest (as in the CRP example), but such examples are probably going to be less common in Mendelian randomization than cases where the polymorphism can influence several systems, with different potential interpretations of how the effect on outcome is generated.

The association of possession of the *APOE* ε2allele with cholesterol levels and CHD might be an example of pleiotropic effects, since carriers of this allele have lower cholesterol levels but do not have the degree of protection against CHD that would be anticipated from this (Keavney et al 2004; Song et al 2004). In addition to lower cholesterol levels the ε2 allele is associated with less efficient transfer of very low density lipoproteins and chylomicrons from the blood to the liver, greater postprandial

lipaemia, and an increased risk of type III hyperlipidaemia (Smith 2002; Eichner et al 2003). These differences go alongside the lower cholesterol levels and may counterbalance the predicted benefits.

Multiple instruments as an approach to confounding within Mendelian randomization

Linkage disequilibrium and pleiotropy can reintroduce confounding and vitiate the power of the Mendelian randomization approach. Genomic knowledge may help in estimating the degree to which these are likely to be problems in any particular Mendelian randomization study, through, for instance, explication of genetic variants that may be in linkage disequilibrium with the variant under study, or the function of a particular variant and its known pleiotropic effects. Furthermore, genetic variation can be related to measures of potential confounding factors in each study, and the magnitude of such confounding estimated. Empirical studies to date suggest that common genetic variants are largely unrelated to the behavioural and socioeconomic factors considered to be important confounders in conventional observational studies. However, relying on measuring of confounders does, of course, remove the central purpose of Mendelian randomization, which is to balance unmeasured as well as measured confounders (as randomization does in RCTs).

It may be possible to identify two separate genetic variants, that are not in linkage disequilibrium with each other, but which both serve as proxies for the environmentally modifiable risk factor of interest. If both variants are related to the outcome of interest and point to the same underlying association then it becomes

much less plausible that reintroduced confounding explains the association, since it would have to be acting in the same way for these two unlinked variants. This can be likened to RCTs of different blood pressure lowering agents, which work through different mechanisms and have different potential side-effects, but lower blood pressure to the same degree. If the different agents produce the same reductions in cardiovascular disease risk then it is unlikely that this is through agent-specific effects of the drugs, rather it points to blood pressure lowering as being key. The use of multiple genetic variants working through different pathways has not been applied in Mendelian randomization to date, but represents an important potential development in the methodology.

Canalization and developmental stability

Perhaps a greater potential problem for Mendelian randomization than reintroduced confounding arises from the developmental compensation that may occur through a polymorphic genotype being expressed during fetal or ealy post-natal development, and thus influencing development in such a way as to buffer against the effect of the polymorphism. Such compensatory processes have been discussed since C.H. Waddington introduced the notion of canalization in the 1940s (Waddington CH 1942). Canalization refers to the buffering of the effects of either environmental or genetic forces attempting to perturb development, and Waddingtion's ideas have been well developed both empirically and theoretically (Wilkins 1997; Rutherford 2000; Gibson and Wagner 2000; Hartman et al 2001; Debat and David 2001; Kitami and Nadeau 2002; Gu et al 2003; Hornstein and Shomron 2006). Such buffering can be achieved either through genetic redundancy (more than one gene having the same or

similar function) or through alternative metabolic routes, where the complexity of metabolic pathways allows recruitment of different pathways to reach the same phenotypic endpoint. In effect a functional polymorphism expressed during fetal development or post-natal growth may influence the expression of a wide range of other genes, leading to changes that may compensate for the influence of the polymorphism. Put crudely, if a person has developed and grown from the intra-uterine period onwards within an environment in which one factor is perturbed (e.g. there is elevated CRP due to genotype) then they may be rendered resistant to the influence of lifelong elevated circulating CRP, through permanent changes in tissue structure and function that counterbalance its effects. In intervention trials – for example, RCTS of cholesterol lowering drugs - the intervention is generally randomized to participants during their middle-age; similarly in observational studies of this issue, cholesterol levels are ascertained during adulthood. In Mendelian randomization, on the other hand, randomization occurs before birth. This leads to important caveats when attempting to relate the findings of conventional observational epidemiological studies to the findings of studies carried out within the Mendelian randomization paradigm.

The most dramatic demonstrations of developmental compensation come from knockout studies – where a functioning gene is essentially removed from an organism. The overall phenotypic effects of such knockouts have often been much lower than knowledge of the function of the genes would predict, even in the absence of others genes carrying out the same function as the knock-out gene (Morange 2001; Shastry 1998; Gerlai 2001; Williams and Wagner et al 2000). For example, pharmacological inhibition demonstrates that myoglobulin is essential to maintain

energy balance and contractile function in the myocardium of mice, yet disrupting the myoglobulin gene resulted in mice devoid of myoglobulin with no disruption of cardiac function (Garry et al 1998) .

In the field of animal genetic engineering studies – such as knockout preparations or transgenic animals manipulated so as to over-express foreign DNA – the interpretive problem created by developmental compensation is well recognized. (Morange 2001; Shastry 1998; Gerlai 2001; Williams and Wagner 2000). Conditional preparations – in which the level of transgene expression can be induced or suppressed through the application of external agents – are now being utilized to investigate the influence of such altered gene expression after the developmental stages during which compensation can occur (Bolon et al 2002). Thus further evidence on the issue of genetic buffering should emerge to inform interpretations of both animal and human studies.

Most examples of developmental compensation relate to dramatic genetic or environmental insults, thus it is unclear whether the generally small phenotypic differences induced by common functional polymorphisms will be sufficient to induce compensatory responses. The fact that the large gene-environment interactions that have been observed often relate to novel exposures that have not been present during the evolution of a species (e.g. drug interactions) (Wright et al 2002) may indicate that homogenization of response to exposures that are widely experienced – as would be the case with the products of functional polymorphisms or common mutations – has occurred; canalizing mechanisms could be particularly relevant in these cases. Further work on the basic mechanisms of developmental stability and how this relates

to relatively small exposure differences during development will allow these considerations to be taken forward. Knowledge of the stage of development at which a genetic variant has functional effects will also allow the potential of developmental compensation to buffer the response to the variant to be assessed.

In some Mendelian randomization designs developmental compensation is not an issue. For example, when maternal genotype is utilised as an indicator of the intrauterine environment then the response of the fetus will not differ whether the effect is induced by maternal genotype or by environmental perturbation, and the effect on the fetus can be taken to indicate the effect of environmental influences during the intrauterine period. Also in cases where a variant influences an adulthood environmental exposure – e.g. *ALDH2* variation and alcohol intake – developmental compensation to genotype will not be an issue. In many cases of gene by environment interaction interpreted with respect to causality of the environmental factor the same applies. However in some situations there remains the somewhat unsatisfactory position of Mendelian randomization facing a potential problem that cannot currently be adequately assessed. The parallels between Mendelian randomisation in human studies and equivalent designs in animal studies are discussed in Box 3.

Complexity of associations and interpretations

The interpretation of findings from studies that appear to fall within the Mendelian randomisation remit can often be complex, as has been previously discussed with respect to *MTHFR* and folate intake (Davey Smith and Ebrahim 2003). As a second example consider the association of extracellular superoxide dismutase (EC-SOD) and CHD. EC-SOD is an extracellular scavenger of superoxide anions and thus

genetic variants associated with higher circulating EC-SOD levels might be considered to mimic higher levels of antioxidants. However findings are dramatically opposite to this – bearers of such variants have an increased risk of CHD (Juul et al 2004). The explanation of this apparent paradox is that the higher circulating EC-SOD levels associated with the variant may arise from movement of EC-SOD from arterial walls; thus the in situ antioxidative properties of these arterial walls is lower in individuals with the variant associated with higher circulating EC-SOD. The complexity of these interpretations – together with their sometimes speculative nature – detracts from the transparency that otherwise makes Mendelian randomisation attractive.

Lack of suitable genetic variants to proxy for exposure of interest

An obvious limitation of Mendelian randomization is that it can only examine areas for which there are functional polymorphisms (or genetic markers linked to such functional polymorphisms) that are relevant to the modifiable exposure of interest. In the context of genetic association studies more generally it has been pointed out that in many cases even if a locus is involved in a disease related metabolic process there may be no suitable marker or functional polymorphism to allow study of this process (Weiss and Terwillger 2000). In an earlier paper on Mendelian randomization (Davey Smith and Ebrahim 2003) we discussed the example of vitamin C, since one of our examples of how observational epidemiology appeared to have got the wrong answer related to vitamin C. We considered whether the association between vitamin C and coronary heart disease could have been studied utilizing the principles of Mendelian randomization. We stated that polymorphisms exist that are related to lower

circulating vitamin C levels—for example, the haptoglobin polymorphism (Langlois et al 1997; Delanghe et al 1999)— but in this case the effect on vitamin C is at some distance from the polymorphic protein and, as in the apolipoprotein E example, the other phenotypic differences could have an influence on CHD risk that would distort examination of the influence of vitamin C levels through relating genotype to disease. SLC23A1—a gene encoding for the vitamin C transporter SVCT1, vitamin C transport by intestinal cells— would be an attractive candidate for Mendelian randomization studies. However, by 2003 (the date of our earlier paper) a search for variants had failed to find any common SNP that could be used in such a way (Erichsen et al 2001). We therefore used this as an example of a situation where suitable polymorphisms for studying the modifiably risk factor of interest – in this case vitamin C – could not be located. However, since the earlier paper was written, functional variation in SLC23A1 has been identified that is related to circulating vitamin C levels (Timpson et al, personal communication). We use this example not to suggest that the obstacle of locating relevant genetic variation for particular problems is observational epidemiology will always be overcome, but to point out that rapidly developing knowledge of human genomics will identify more variants that can serve as instruments for Mendelian randomization studies.

Conclusions: Mendelian randomization, what it is and what it isn't

Mendelian randomization is not predicated on the presumption that genetic variants are major determinants of health and disease within populations. There are many cogent critiques of genetic reductionism and the over-selling of "discoveries" in genetics that reiterate obvious truths so clearly (albeit somewhat repetitively) that

there is no need to repeat them here (e.g. Berkowitz 1996; Baird 2000; Holtzman 2001; Strohman 1993; Rose 1995). Mendelian randomization does not depend upon there being "genes for" particular traits, and certainly not in the strict sense of a gene "for" a trait being one that is maintained by selection because of its causal association with that trait (Kaplan and Pigliucci 2001). The association of genotype and the environmentally modifiable factor that it proxies for will be like most genotype-phenotype associations, one that is contingent and cannot be reduced to individual level prediction, but within environmental limits will pertain at a group level (Wolf 1995). This is analogous to an RCT of antihypertensive agents, where at a collective level the group randomised to active medication will have lower mean blood pressure than the group randomised to placebo, but at an individual level many participants randomised to active treatment will have higher blood pressure than many individuals randomised to placebo. Indeed in the phenocopy / genocopy example of pellagra and Hartnup disease discussed in box 1, only a minority of the Hartnup gene carriers develop symptoms, but at a group level they have a much greater tendency to such symptoms and a shift in amino acid levels that reflect this (Scriver et al 1987; Scriver 1988). These group level differences are what creates the analogy between Mendelian randomization and RCTs, outlined in figure 7.

Finally, the associations that Mendelian randomization depend upon do need to pertain to a definable group at a particular time, but do not need to be immutable. Thus *ALDH2* variation will not be related to alcohol consumption in a society where alcohol is not consumed, the association will vary by gender, by cultural group and may change over time (Higuchi et al 1994; Hasin et al 2002). Within the setting of a study of a well defined group, however, the genotype will be associated with group-

level differences in alcohol consumption and group assignment will not be associated with confounding variables.

Mendelian randomization and genetic epidemiology

Critiques of contemporary genetic epidemiology often focus on two features of findings from genetic association studies: that the population attributable risk of the genetic variants is low, and that in any case the influence of genetic factors is not reversible. Illustrating both of these criticisms, Terwilliger and Weiss suggest as reasons for considering that many of the current claims regarding genetic epidemiology are hype, (1) that alleles identified as increasing the risk of common diseases 'tend to be involved in only a small subset of all cases of such diseases' and (2) that in any case 'while the concept of attributable risk is an important one for evaluating the impact of removable environmental factors, for non-removable genetic risk factors, it is a moot point' (Terwilliger and Weiss 2003). These evaluations of the role of genetic epidemiology are not relevant when considering the potential contributions of Mendelian randomization. This approach is not concerned with the population attributable risk of any particular genetic variant, but the degree to which associations between the genetic variant and disease outcomes can demonstrate the importance of environmentally modifiable factors as causes of disease, for which the population attributable risk is of relevance to public health prioritization. Consider, for example, the case of familial hypercholesterolaemia or familial defective apo B. The genetic mutations associated with these conditions will only account for a trivial percentage of cases of CHD within the population—i.e. the population attributable risk will be low. For example, in a Danish population, the frequency of familial defective apo B is 0.08% and despite its 7-fold increased risk of CHD, will only

generate a population attributable risk of 0.5% (Tybjaerg H et al 1998). However, by identifying blood cholesterol levels as a causal factor for CHD the triangular association between genotype, blood cholesterol and CHD risk identifies an environmentally modifiable factor with a very high population attributable risk—assuming that 50% of the population have raised blood cholesterol above 6.0 mmol/l and this is associated with a relative risk of 2-fold, a population attributable risk of 33% is obtained. The same logic applies to the other examples discussed above—the attributable risk of the genotype is low, but the population attributable risk of the modifiable environmental factor identified as causal through the genotype–disease associations is large. The same reasoning applies when considering the suggestion that since genotype cannot be modified, genotype–disease associations are not of public health importance (Terwilliger and Weiss 2003). The point of Mendelian randomization approaches is not to attempt to modify genotype, but to utilize genotype–disease associations to strengthen inferences regarding modifiable environmental risks for disease, and then reduce disease risk in the population through applying this knowledge.

Mendelian randomization differs from other contemporary approaches to genetic epidemiology in that its central concern is not with the magnitude of genetic variant influences on disease, but rather on what the genetic associations tell us about environmentally modifiable causes of disease. As David B Abrams, Director of the Office of Behavioural and Social Sciences Research at the U.S. National Institutes of Health has said, "The more we learn about genes the more we see how important environment and lifestyle really are". Many years earlier the pioneering geneticist Thomas Hunt Morgan articulated a similar sentiment in his Nobel Prize acceptance

speech, when he contrasted his views with the then popular genetic approach to disease – eugenics. He thought that "through public hygiene and protective measures of various kinds we can more successfully cope with some of the evils that human flesh is heir to. Medical science will here take the lead – but I hope that genetics can at times offer a helping hand" (Morgan 1935). More than 7 decades later it might now be time that genetic research can directly strengthen the knowledge base of public health.

Table 1: Cardiovascular mortality according to cumulative risk indicator (father's social class, screening social class, smoking, alcohol use). From Davey Smith and Hart 2002

	N	CVD deaths	Relative risk
4 favourable (0 unfavourable)	517	47	1
3 favourable (1 unfavourable)	1299	227	1.99 (1.45 - 2.73)
2 favourable (2 unfavourable)	1606	354	2.60 (1.92 - 3.52)
1 favourable (3 unfavourable)	1448	339	2.98 (2.20 - 4.05)
0 favourable (4 unfavourable)	758	220	4.55 (3.32 - 6.24)

Table 2a: Means or proportions of blood pressure, pulse pressure, hypertension and potential confounders by quarters of C-reactive protein (CRP) N = 3,529 (from Davey Smith et al 2005)

	Means or proportions by quarters of C-reactive protein (Range mg/L)				P trend across categories
	1 (0.16-0.85)	2 (0.86-1.71)	3 (1.72-3.88)	4 (3.89-112.0)	
Hypertension (%)	45.8	49.7	57.5	60.	< 0.001
BMI (kg/m^2)	25.2	27.0	28.5	29.7	< 0.001
HDLc (mmol/l)	1.80	1.69	1.	1.53	< 0.001
Lifecourse socioeconomic position score	4.08	4.37	4.46	4.75	< 0.001
Doctor diagnosis of diabetes (%)	3.5	2.8	4.1	8.4	< 0.001
Current smoker (%)	7.9	9.6	10.9	15.4	< 0.001
Physically inactive (%)	11.3	14.9	20.1	29.6	< 0.001
Moderate alcohol consumption (%)	22.2	19.6	18.8	14.0	< 0.001

Table 2b: Means or proportions of CRP systolic blood pressure, hypertension and potential confounders by 1059G/C genotype (from Davey Smith et al 2005)

	Means or proportionsby genotype		P
	GG	GC or CC	
CRP (mg/L log scale)[a]	1.81	1.39	< 0.001
Hypertension (%)	53.3	53.1	0.95
BMI (kg/m^2)	27.5	27.8	0.29
HDLc (mmol/l)	1.67	1.65	0.38
Lifecourse socioeconomic position score	4.35	4.42	0.53
Doctor diagnosed diabetes (%)	4.7	4.5	0.80
Current smoker (%)	11.2	9.3	0.24
Physically inactive (%)	18.9	18.9	1.0
Moderate alcohol consumption (%)	18.6	19.8	0.56

[a] Geometric means and proportionate (%) change for a doubling of CRP

CRP: C-reactive protein; OR: odds ratio; FEV_1: forced expiratory volume expiratory in one second; HDLc: high density lipoprotein cholesterol; CVD: cardiovascular disease (stroke or coronary heart disease)

Table 3: Reasons for inconsistent genotype-phenotype associations

True variation

Variation of allelic association between subpopulations: (1) disease causing allele in linkage disequilibrium with different marker alleles in different populations; or (2) different variants within the same gene contribute to disease risk in different populations

Effect modification by other genetic or environmental factors that vary between populations

Spurious variation

Genotyping errors
Misclassification of phenotype
Confounding by population structure
Lack of power
Chance
Publication bias

Adapted from Cardon and Bell 2001 and Colhoun et al 2003.

Table 4: Calculated from data in Gu et al. Mutation Research 2005; 581 97-104

NAT2 (slow versus fast acetylator), smoking and bladder cancer

Overall	Never/light smokers	Heavy smokers
1.35 (1.04-1.75)	1.10 (0.78-1.53)	2.11 (1.30-3.43)

Figure 1 – Advert from the Boston Globe

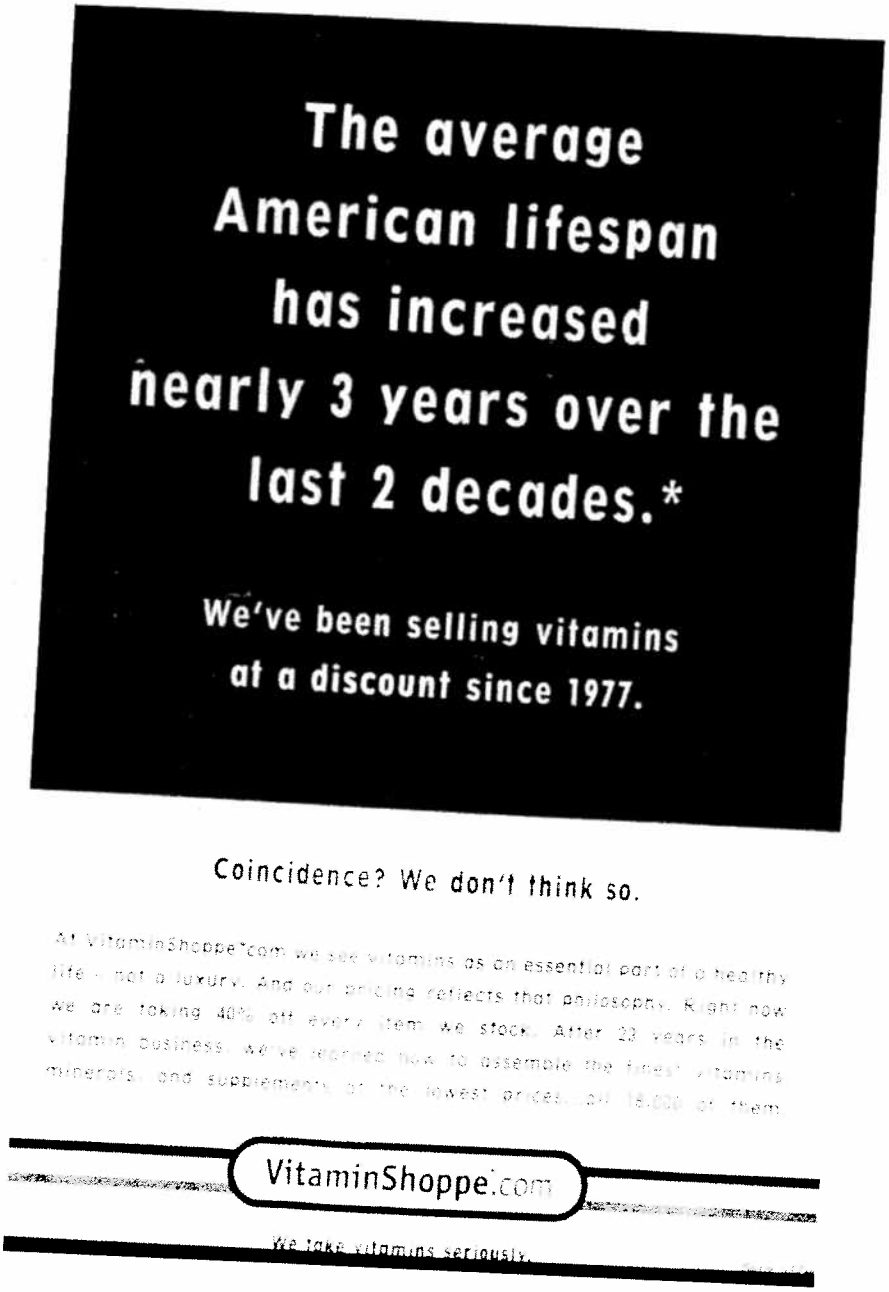
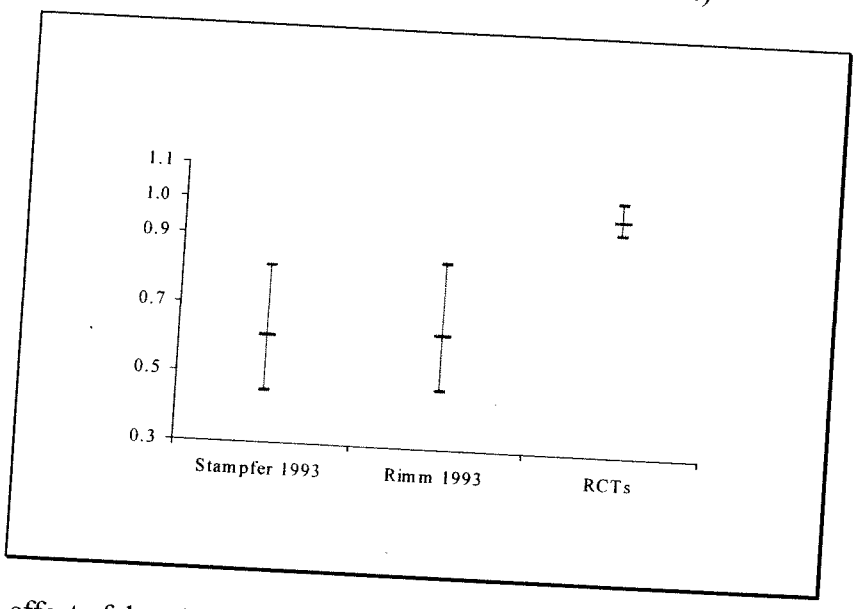


Figure 2a) and Figure 2b)

Vitamin E supplement use and risk of CHD in two observational studies (Rimm 1993; Stampfer 1993) and in a meta-analysis of RCTs (Eidelman 2004)



Observed effect of duration of Vitamin E use compared to no use on CHD events in the Health Professional Follow-up Study (Rimm 1993)

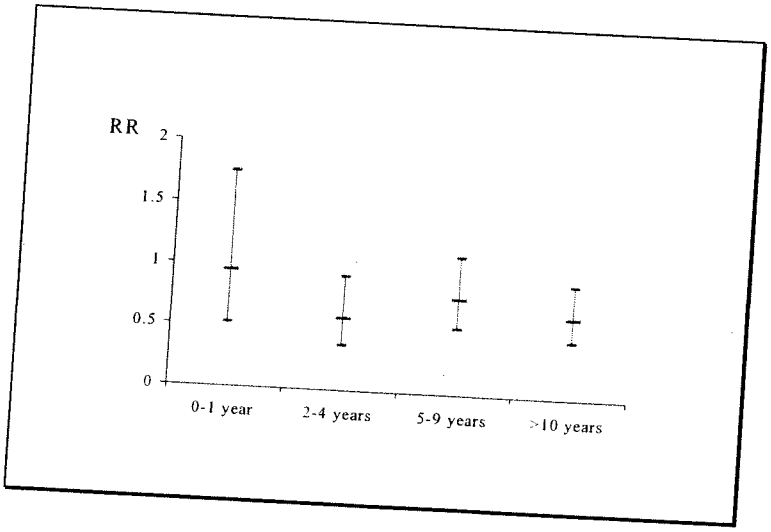


Figure 3: Estimates of the effects of an increase of 15.7μmol/l plasma vitamin C on CHD 5-year mortality estimated from observational epidemiological EPIC (Khaw 2001) and randomised controlled Heart Protection Study.(Heart Protection Study Collaborative Group 2002) (EPIC m = men, age-adjusted; EPIC m* = men, adjusted for systolic blood pressure, cholesterol, BMI, smoking, diabetes and vitamin supplement use; EPIC f = women, age-adjusted; EPIC f* = women, adjusted for systolic blood pressure, cholesterol, BMI, smoking, diabetes and vitamin supplement use)

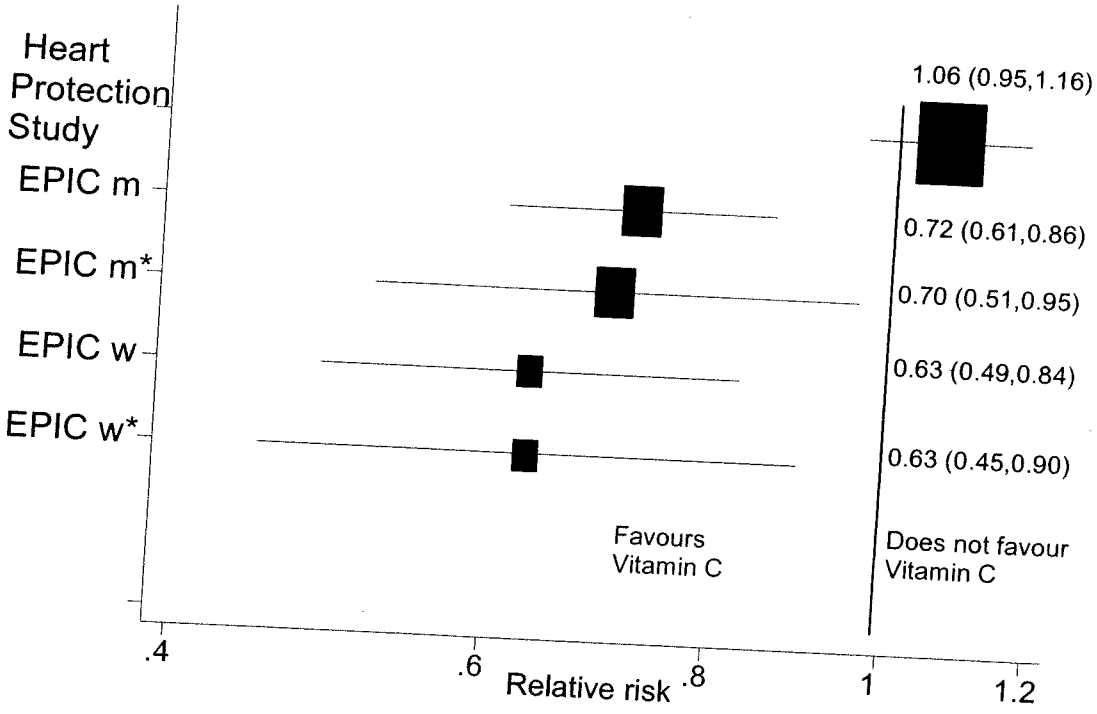


Figure 4a: Milk drinking and fracture risk according to lactose persistence genotype. Individuals with genotype CC (dark bars) had lower calcium intake from milk (*p = 0.004) compared with TT (dashed bars), and TC (shaded bars) genotypes. From Obermayer Petsch et al 2004.

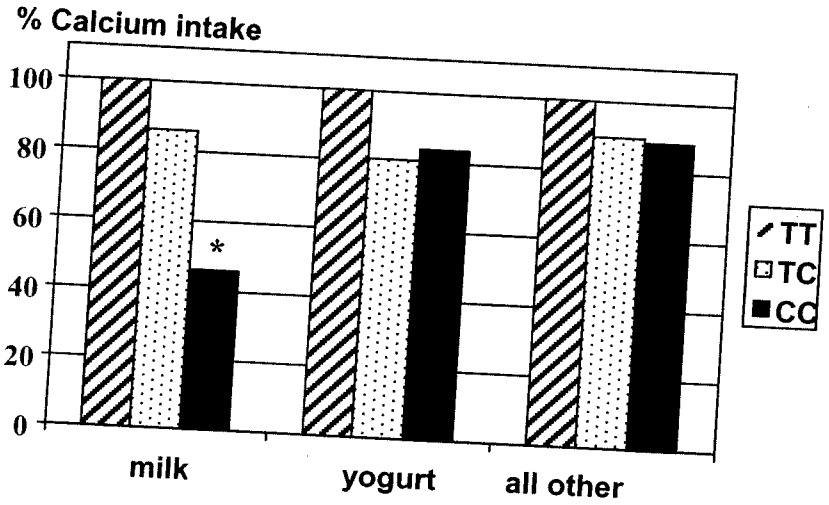


Figure 4b. Fracture incidence per 100 subjects in postmenopausal women according to LCT genotypes. Individuals with genotype CC (dark bars) had a higher nonvertebral fracture incidence (*$p = 0.001$) than TC (shaded bars) and TT (dashed bars) genotypes, showing an increasing gene-dose effect towards these genotypes.

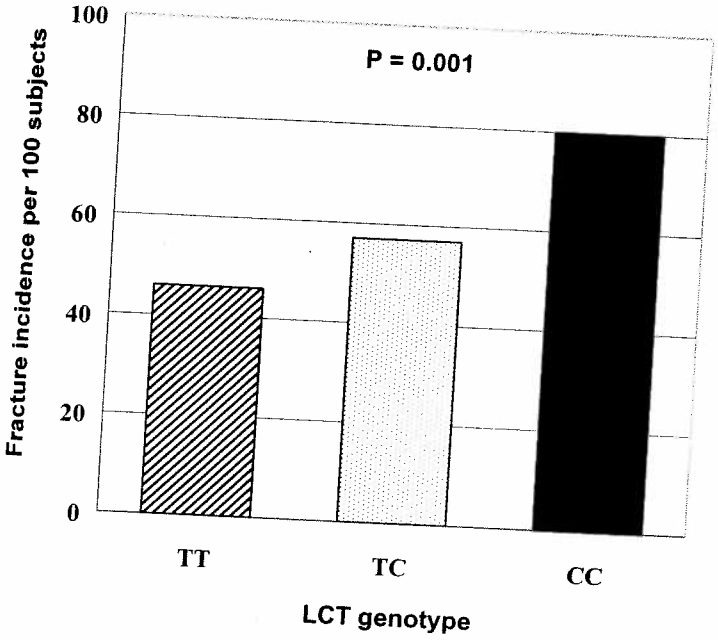


Figure 4c Bone Mineral Density z-score in post menopausal women according to LCT genotypes. Indviduals with genotypes CC (dark bars) had a lower BMD score than TC (shaded bars) and TT (dashed bars) genotypes.

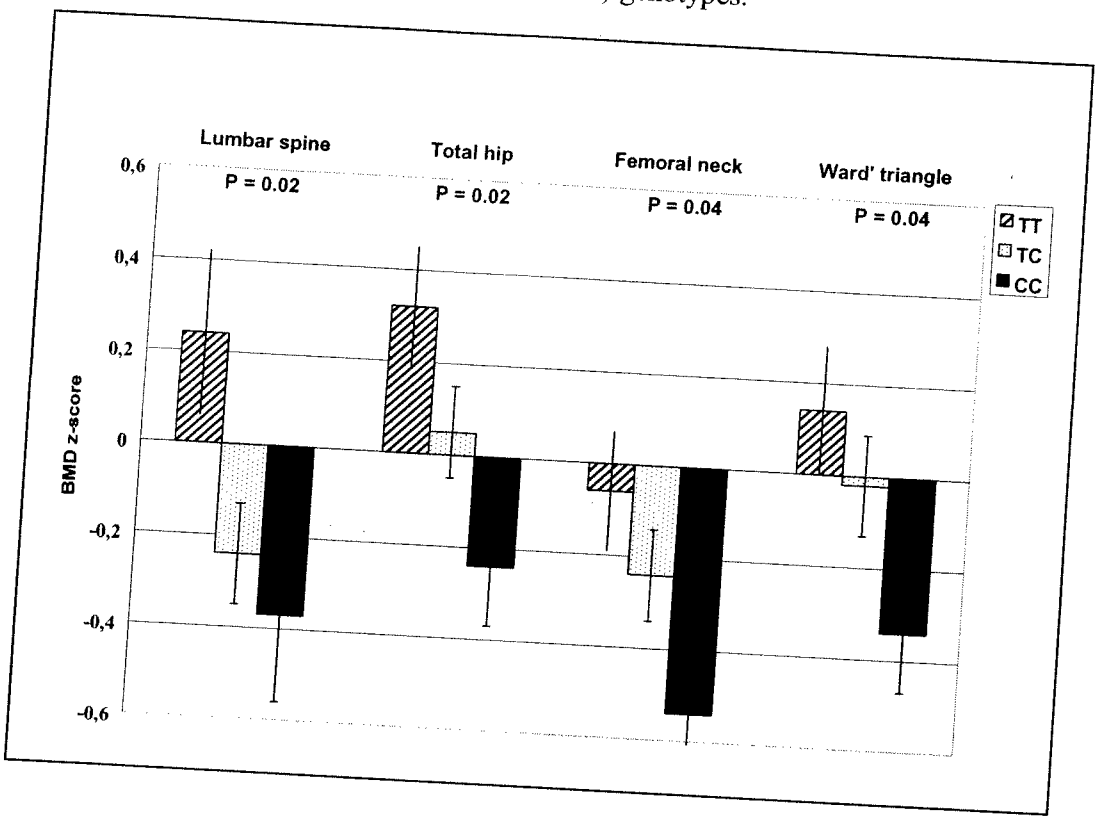
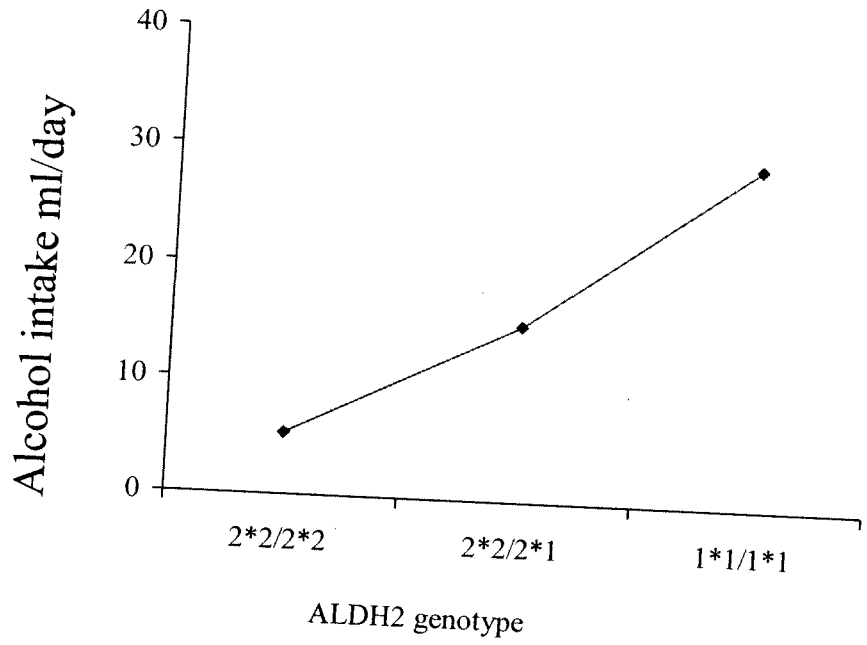
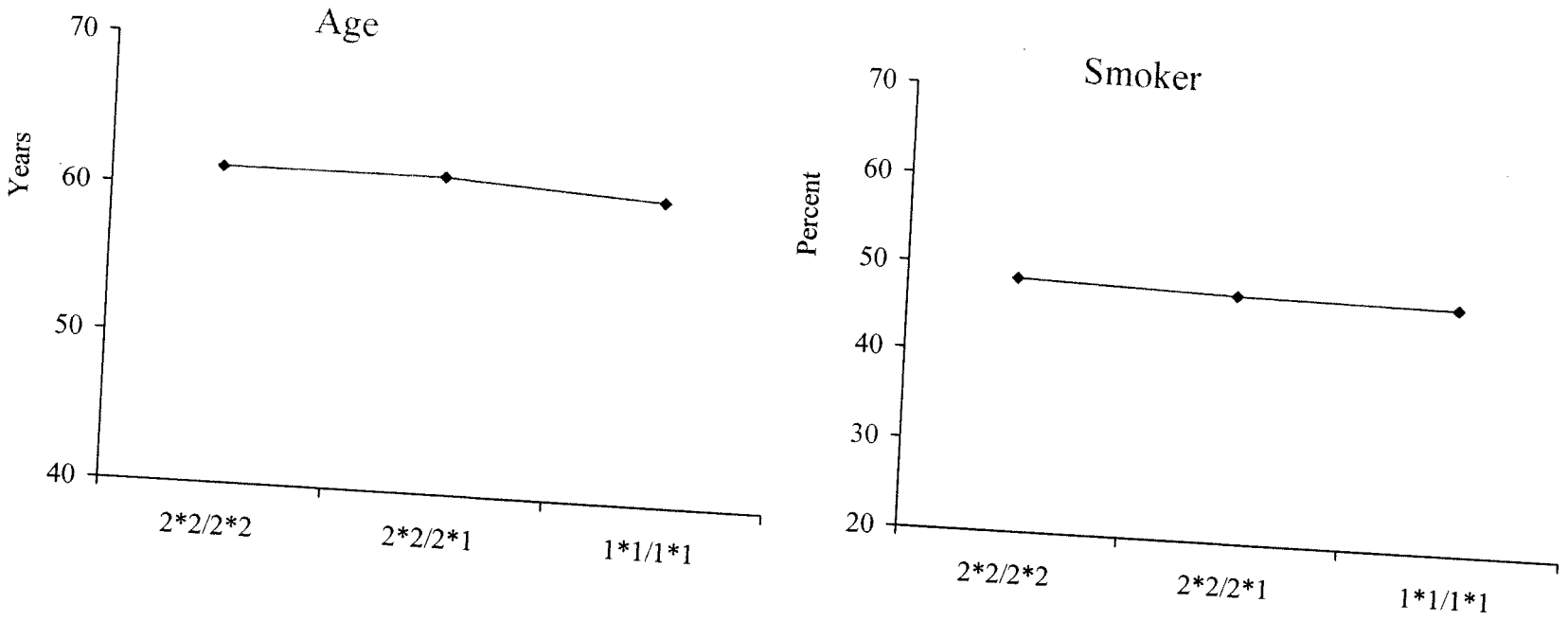


Figure 5a. Relationship between alcohol intake and ALDH2 genotype



Data from Takagi et al 2002.

Figure 5b Relationship between characteristics and ALDH2 genotype



Data from Takagi et al, 2002

Figure 5c. Relationship between HDL cholesterol and ALDH2 genotype
Data from Tagaki et al, 2002

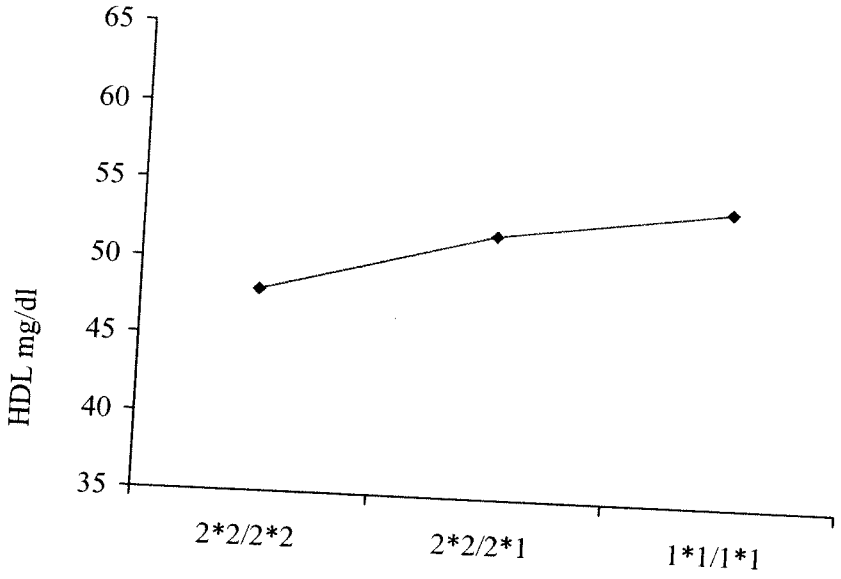
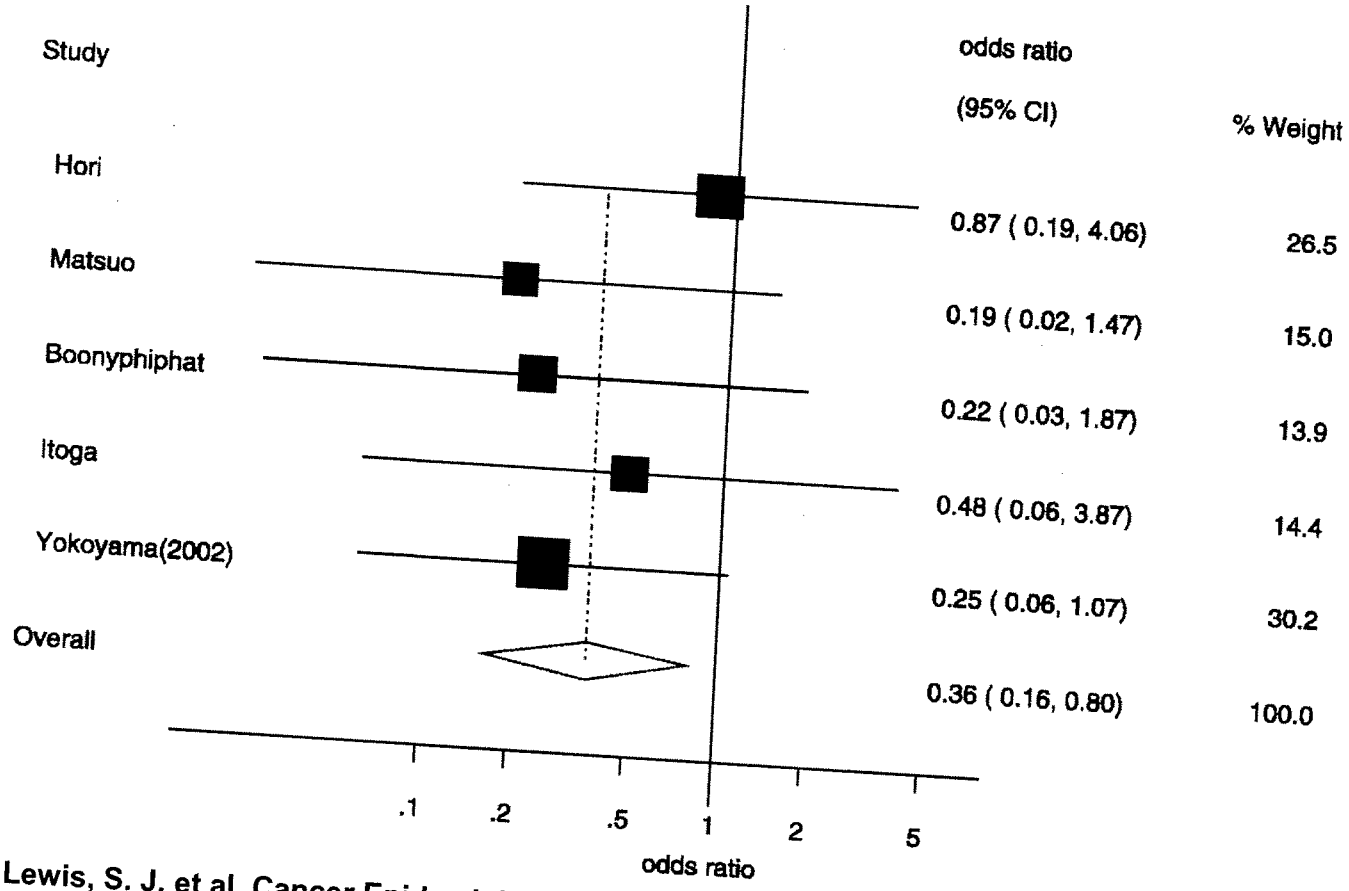


Figure 6. Risk of esophageal cancer in individuals with the ALDH2*2*2 versus ALDH2*1*1 genotype



Lewis, S. J. et al. Cancer Epidemiol Biomarkers Prev 2005;14:1967-1971

Figure 7. Mendelian randomization and randomized controlled trial designs compared

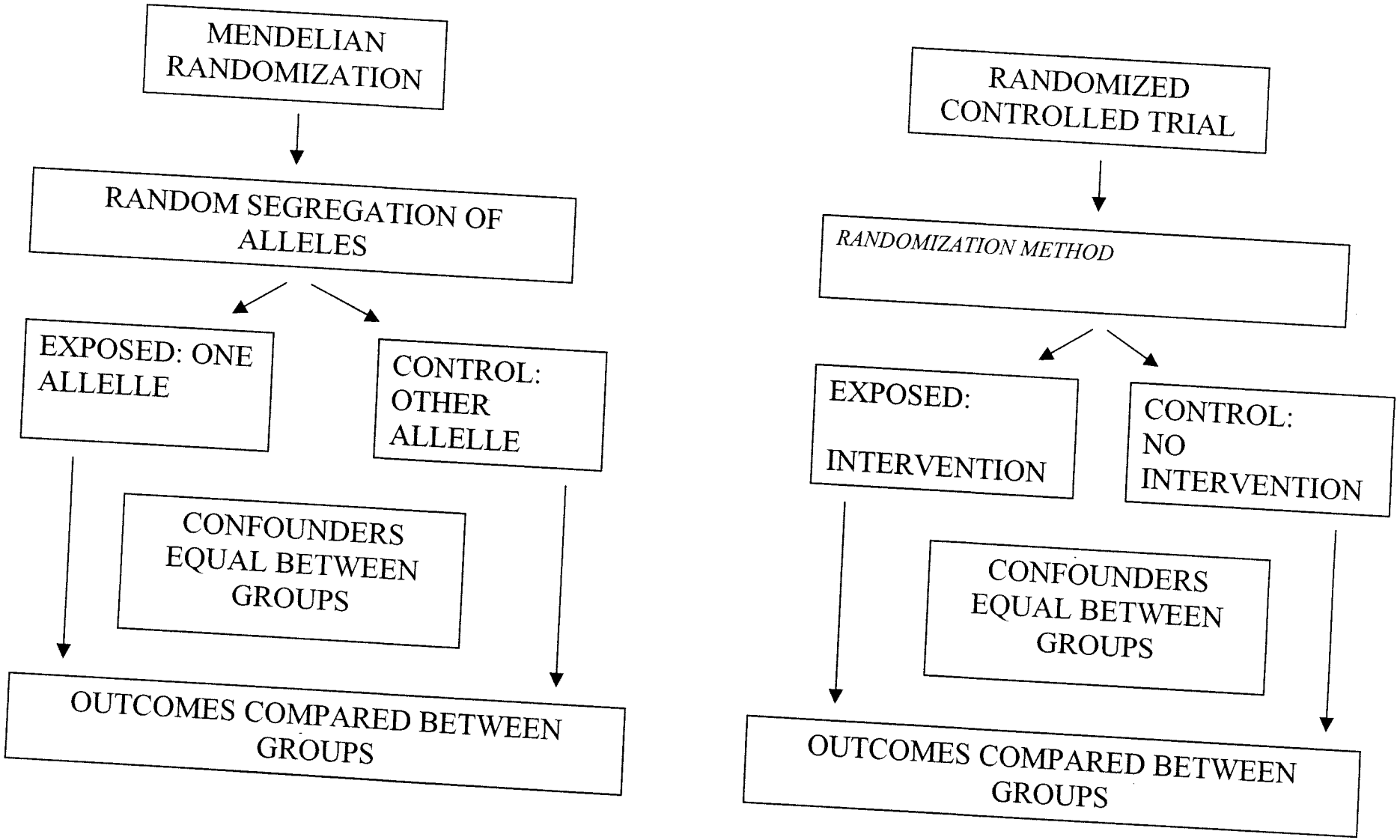
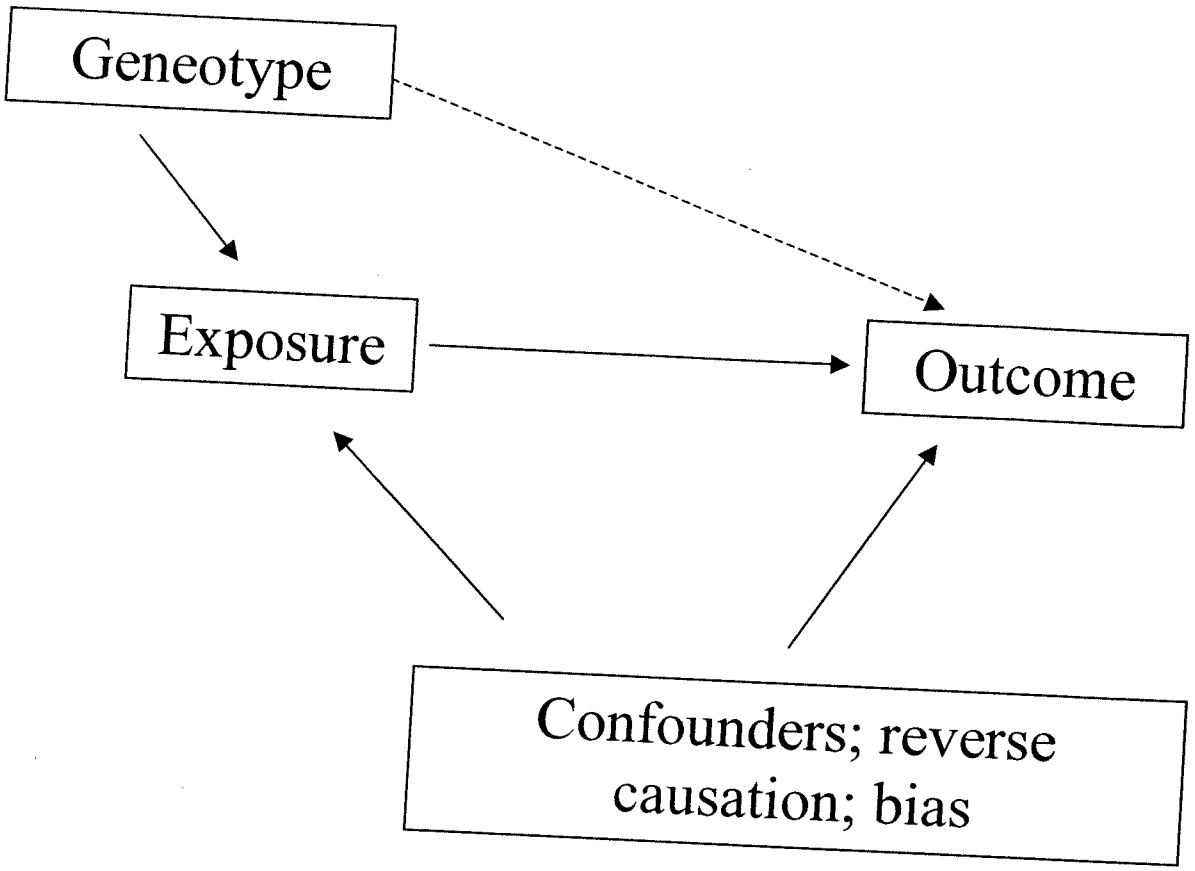


Figure 8 Mendelian randomization as an instrumental variables approach …



Box 1: Phenocopy, genocopy and Mendelian randomization

The term phenocopy is attributed to Goldschmidt (Goldschmidt 1938) and is used to describe the situation where an environmental effect could produce the same effect as was produced by a genetic mutation. As Goldschmidt explicated "different causes produce the same end effect, presumably by changing the same developmental processes in an identical way". (Goldschmidt 1938) In human genetics the term has generally been applied to refer to an environmentally produced disease state that is similar to a clear genetic syndrome. For example the niacin-deficiency disease pellagra is clinically similar to the autosomal recessive condition Hartnup disease (Baron et al 1956), and pellagra has been referred to as a phenocopy of the genetic disorder (Snyder 1959, Guy 1993). Hartnup disease is due to reduced neutral amino acid absorption from the intestine and reabsorption from the kidney, leading to low levels of blood tryptophan which in turn leads to a biochemical anomaly which is similar to that seen when the diet is deficient in niacin (Kraut and Sachs 2005; Bröer et al 2004). Genocopy is a less utilised term, attributed to Schmalhausen (Schmalhausen 1938, cited by Gause 1942) but has generally been considered to be the reverse of phenocopy – i.e. when genetic variation generates an outcome that could be produced by an environmental stimulus (Jablonka-Tavory 1982). It is clear that, even when the term genocopy is used polemically (e.g. Rose 1995) the two concepts are mirror-images reflecting differently motivated accounts of how both genetic and environmental factors influence physical state. For example Hartnup disease can be called a genocopy of pellagra, while pellagra can be considered a phenocopy of Hartnup disease. Mendelian randomization can, therefore, be viewed as an appreciation of the phenocopy-genocopy nexus that allows causation to be separated from association.

Phenocopies of major genetic disorders are generally rarely encountered in clinical medicine, but as Lenz (1973) comments, "they are, however, most important as models which might help to elucidate the pathways of gene action". Mendelian randomization is concerned with less major (and thus common) disturbances and reverses the direction of phenocopy → genocopy, to utilize genocopies, of known genetic mechanism, to inform us better about pathways through which the environment influences health.

The scope of phenocopy - genocopy has been discussed by Zuckerkandl and Villet (1988), who advance mechanisms through which there can be equivalence between environmental and genotypic influences. Indeed they state that "no doubt all environmental effects can be mimicked by one or several mutations". The notion that genetic and environmental influences can be both equivalent and interchangeable has received considerable attention in developmental biology (e.g. West-Eberhard 2003; Leimar et al 2006). Furthermore, population genetic analyses of correlations between different traits suggest there are common pathways of genetic and environmental influences, with Cheverud concluding that "most environmentally caused phenotypic variants should have genetic counterparts and vice versa" (Cheverud 1988).

Box 2 : Why "Mendelian randomization"?

Gregor Mendel (1822-1884) concluded from his hybridisation studies with pea plants that "the behaviour of each pair of differentiating characteristics [such as the shape and colour of seeds] in hybrid union is independent of the other differences between the two original plants" (Mendel 1866). This formulation was actually the only regularity that Mendel referred to as a "law" and in Carl Correns' 1900 paper (one of a trio appearing that year that are considered to represent the rediscovery of Mendel) he refers to this as Mendel's Law (Correns 1900; Olby 1966). Morgan (1913) discusses independent assortment and refers to this process as being realised "whenever two pairs of characters freely Mendelize". Morgan's use of Mendel's surname as a verb did not catch on, but Morgan later christened this as Mendel's second law (Morgan 1918) and it has been known as this, or as "The Law of Independent assortment" since this time. The law suggests that inheritance of one trait is independent of – that is, randomized with respect to – the inheritance of other traits. The analogy with a randomized controlled trial will clearly be most applicable to parent-offspring designs investigating the frequency with which one of two alleles from a heterozygous parent is transmitted to offspring with a particular disease. However, at a population level, traits influenced by genetic variants are generally not associated with the social, behavioural and environmental factors that confound relationships observed in conventional epidemiological studies. Thus while the 'randomization' is approximate and not absolute in genetic association studies, empirical observations suggest that it applies in most circumstances (Davey Smith et al. 2005a; Bhatti et al. 2005).

The term "Mendelian randomization" itself was first introduced in a somewhat different context, in which the random assortment of genetic variants at conception is utilised to provide an unconfounded study design for estimating treatment effects for childhood malignancies (Gray and Wheatley 1991; Wheatley and Gray 2004). The term has recently become widely used with the meaning we ascribe to it in this chapter.

The notion that genetic variants can serve as an indicator of the action of environmentally modifiable exposures has been expressed in many contexts. For example, since the mid-1960s various investigators have pointed out that the autosomal dominant condition of lactase persistence is associated with milk drinking. Associations of lactase persistence with osteoporosis, bone mineral density or fracture risk thus provide evidence that milk drinking protects against these conditions (Birge et al. 1967; Newcomer et al. 1978). In a related vein, it was proposed in 1979 that as N-acetyltransferase pathways are involved in the detoxification of arylamine, a potential bladder carcinogen, the observation of increased bladder cancer risk among people with genetically determined slow acetylator phenotype provided evidence that arylamines are involved in the aetiology of the disease (Lower et al. 1979).

Since that time various commentators have pointed out that the associations of genetic variants of known function with disease outcomes provides evidence about aetiological factors (McGrath et al. 1999; Ames 1999; Rothman et al. 2001; Brennan 2002; Kelada et al. 2003). However, these commentators have not emphasised the key strengths of Mendelian randomization – the avoidance of confounding, bias due to reverse causation or reporting tendency, and the underestimation of risk

associations due to variability in behaviours and phenotypes (Davey Smith and Ebrahim 2004).

These key concepts were present in Martijn Katan's 1986 Lancet letter in which he suggested that genetic variants related to cholesterol level could be used to investigate whether the observed association between low cholesterol and increased cancer risk was real (Katan 1986) and by Honkanen and colleagues in their understanding of how lactase persistence could better characterise the difficut-to-measure environmental influence of calcium intake than could direct dietary reports (Honkanen et al 1969). Since 2000 there have been several reports using the term 'Mendelian randomization' in the way it is used here (Youngman et al. 2000; Fallon et al 2001; Clayton and McKeigue 2001; Keavney 2002; Davey Smith and Ebrahim 2003), and its use is becoming widespread. (17,900 Google hits on November 22nd 2006).

Box 3 Meiotic Randomization in Animal Studies.

The approach to causal inference underlying Mendelian randomization is also utilised in non-human animal studies. For instance in investigations of the structural neuroanatomical factors underlying behavioural traits in rodents there has been use of genetic crosses that lead to different on-average structural faatures have been carried out (Roderick et al 1976; Weimer 1973, Lipp et al 1989). Lipp et al refer to this as "meiotic randomization" and consider that the advantages of this method are that the brain morphology differences that are due to genetic difference occur before any of the behavioural traits develop and therefore the brain morphology differences cannot be a feedback function of behaviour (which is equivalent to the avoidance of reverse causality in human Mendelian randomization studies) and that other difference between the animals are randomized with respect to the brain morphology differences of interest (equivalent to the avoidance of confounding in human Mendelian randomization studies). Li and colleagues (2006) apply this method to the dissection of adiposity and body composition in mice and point out that in experimental crosses "meiosis serves as a randomization mechanism that distributes naturally occurring genetic variation in a combinatorial fashion among a set of cross progeny. Genetically randomized populations share the properties of statically designed experiments that provide a basis for causal inference. This is consistent with the notion that causation flows from genes to phenotypes. We propose that the inference of causal direction can be extended to include relationships among phenotypes" (Li et al 2006). Mendelian randomization within epidemiology reflects similar thinking among transgenic animal researchers. Williams and Wagner consider that 'A properly designed transgenic

experiment can be a thing of exquisite beauty in that the results support absolutely unambiguous conclusions regarding the function of a given gene or protein within the authentic biological context of an intact animal. A transgenic experiment may provide the most rigorous test possible of a mechanistic hypothesis that was generated by previous observational studies. A successful transgenic experiment can cut through layers of uncertainty that cloud the interpretation of the results produced by other experimental designs (Williams 2000). The problems of interpreting some aspects of transgenic animal studies may also apply to Mendelian randomization within genetic epidemiology, however, and linked progress across the fields of genomics, animal experimentation and epidemiology will better define the scope of Mendelian randomization in future.

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