

Commentary

Understanding environmental causes of disease: what can we expect from new technologies?

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ABSTRACT

In spite of decades of epidemiological research, the etiology and causal patterns for many common diseases, such as breast and colon cancer or neurodegenerative diseases, are still largely unknown. The observation of incidence rates in migrant populations unequivocally showed that migrants rapidly acquire the risk of disease that is typical of the populations where they move, suggesting that such diseases have a largely environmental origin. However, “environmental” risks have been often elusive in epidemiological studies. This is a conundrum for current epidemiological research. On the other side, the relative contribution of genes to chronic diseases seems to be small (15-50% increase in disease risk). In spite of that, genetic associations – e.g. as emerging from GWAS - are perceived as more exciting and more credible than environmental associations, probably because genes are stable in the course of life (while exposures are changing over time), and associations are more easily replicated in independent genetic studies. A further recent revolution is the application of biomarkers in human observational studies, particularly with the introduction of “-omic” high-throughput technologies, such as transcriptomics, proteomics and metabonomics. These techniques are still largely immature. However, they are likely to provide, in the next years, powerful tools to investigate early effects of environmental exposures and understand the etiology of common diseases better. What is yet to be explored extensively is a potential model for the effects of low doses in the absence of thresholds, incorporating genetic and acquired susceptibility (“clinical vulnerability”) and the cumulative effects of different exposures. We propose a disease model that might allow to overcome both the limitations (weak associations) of genetic data coming from GWAS and the still elusive role of many (low-level) environmental exposures as measured by traditional epidemiological tools.

INTRODUCTION

We are still far from understanding the etiology and causal patterns for many common diseases, such as breast and colon cancer, or neurodegenerative diseases. In spite of decades of epidemiological research, the etiology of such conditions is still largely unknown.

For a long time it has been claimed that most chronic diseases have an environmental origin (using the term “environment” in a broad sense, to indicate essentially what is not due to genetic predisposition). This claim was based on descriptive data showing the broad range of incidence rates in different parts of the world, the rapid temporal changes – such as those currently occurring in China and India -, and the crucial observation of incidence rates in migrant populations. The latter unequivocally showed that migrants rapidly acquire – sometimes already in the first generation after migration - the risk of disease that is typical of the population where they move.

However, “environmental” risks have been largely elusive in epidemiological research, and there is still much debate on the real impact of the environment, whose role tends to be overshadowed in recent times by the successes of genetics, in particular Genome-wide association studies (GWAS). Clearly, we need a disease model that allows us to encompass both the mass of genetic data coming from GWAS and the still elusive role of many environmental exposures.

Genes vs. environment

The technological revolution in genetics, which in 2007-2008 led to the identification of a number of novel genes for several common diseases, is exciting but at the same time a little disappointing. Most highly-penetrant genes already had been uncovered by linkage studies, so that “genome-wide scans” could mainly identify low-penetrant genes. It is a fact that – with a few exceptions – what has been found is genetic variants weakly associated with chronic diseases, with relative risks of 1.15-1.5. These risks are much lower than some known environmental exposures,

and of the same order of magnitude of other environmental exposures that are usually rejected as too weakly associated with disease to be meaningful. For example, Hung *et al* have found an allelic relative risk of 1.3 for a gene variant associated with lung cancer, in the nicotinic acetylcholine receptor subunit on chromosome 15q25 [1]. The genotype nicely combines with the habit of smoking, increasing the probability of developing lung cancer in smokers (Figure 1) [1]. However, it is clear from the figure that the effect of smoking is by far greater than the effect of the genotype. The former increases the cumulative risk in life from less than 1% (never smokers) to about 15-20% (current smokers), while the effect of the genotype is a small additional amount (~1 to 2%) that does not seem to contribute much to the Public Health problem of lung cancer. Of course genetic research is not tailored for Public Health purposes. It can be extremely rewarding, instead, (a) for the investigation of disease mechanisms, (b) to shed light on potential therapies, or (c) to suggest clues for unidentified etiological agents in the case of diseases whose etiology is still unknown.

It is unclear, however, how goals (b) and (c) can be met if the relative contribution of genes is so small (15-50% increase in disease risk) which is in the same order of magnitude of relative risks found for unclear and controversial environmental exposures, such as electromagnetic fields and leukemia, or some pesticides and prostate cancer. It is therefore useful to reflect on the potential reasons why weak genetic associations (generally) are perceived as much more exciting than environmental associations.

1. One reason – perhaps the main one - is certainly the greater robustness of technical detection of gene variants compared to environmental exposures. It is estimated that the error rate with common genotyping techniques is less than 10%, while it can be as high as 50% for environmental exposure assessment, at least for dichotomous exposure variables (for quantitative measures it could be much more than 50%) . Moreover genes are stable in the course of life, while environmental exposures are changing over time

(let us think of diet) leading to more inherent measurement error in environmental exposure than in gene variance assessment.

2. A second obvious reason is replication. In the study mentioned above on lung cancer, the association was found in five independent studies. This usually is not the case for environmental exposures, and is not easily achievable. For example, a cohort study from Belgium suggested that high levels of cadmium in urine are associated rather strongly with lung cancer [2]. The study is strong (though small), because exposure assessment is based on a biomarker reflecting long-term exposure (i.e. with much higher accuracy than usually available in epidemiology). However, the study is not easily reproducible, because there are not many other populations with similar levels of environmental exposure to cadmium, and there might be several confounders or effect modifiers that either obscure the association with cadmium or change the strength of the association. This is commonly observed in environmental epidemiology, i.e. a number of small-medium size associations that are difficult to reproduce in different settings, both because of methodological difficulties and because of variable study contexts.

Alternatives to epidemiology: research in animals, biomarkers and omics

For the second reason described above, Lorenzo Tomatis, former Director of the International Agency for Research on Cancer (IARC) had proposed already in the 70's that prevention of human cancer could not rely on epidemiology alone, and promoted research in animals as a surrogate for research in humans. The IARC Monographs are the most prestigious instrument for cancer prevention, thanks to their sound scientific methodology, and rely besides human epidemiological evidence heavily on research in animals for the categorization of carcinogens. This is illustrated in the case of 1,3-butadiene, whose carcinogenicity has been confirmed recently by a review of the Working Group of the IARC Monographs in 2007 [3]. More than 20 years ago, experiments in rodents showed that this widely used chemical induced cancers at

multiple organ sites, including a very high incidence of otherwise extremely rare cancers (e.g. heart hemangiosarcomas). Also an excess of lymphopietic cancers was found in animals. There was no doubt that the chemical was a potent animal carcinogen, given the consistency of the observations, the presence of a dose-response relationship, the unusual type of tumours induced, and the very high incidence. Even today, we still lack a satisfactory number of sound epidemiological studies capable of confirming these observations in humans. The results from the available studies, however, are remarkably consistent with animal studies, at least for lymphopietic tumours, given the considerable difficulties encountered in such investigations.

However, a few new developments occurred after the foundation of the IARC Monographs (the early seventies) of which one is the genetic revolution. This revolution is exciting, will probably contribute to the unraveling of mechanisms underlying cancer, and may contribute to the identification of some environmental carcinogenic exposures. But it is not likely to be the main avenue to prevent cancer. The second revolution is the application of biomarkers in human observational studies, which more recently has been revolutionized with the introduction of “-omic” high-throughput technologies, such as transcriptomics, proteomics and metabolomics. These techniques are still largely immature, the measurement error per analyte is unknown and large intraindividual variability is to be expected, thus blurring any association with environmental exposures and/or disease. However, they are likely to provide, in the next years, powerful tools to investigate early effects of environmental exposure and understand the etiology of common diseases better [4]. An exciting example is the recent paper by Holmes and others on the metabonomic profiles associated with hypertension [5].

Low doses and acquired susceptibility to disease

There is also an unsung revolution which has taken place recently within epidemiology, which complements those referred to above, but which has attracted less attention. This is the increasing ability of epidemiological tools to consistently unravel the effects of low environmental

exposures. The cases of air pollution and environmental tobacco smoke (ETS) are exemplar. In the seventies and eighties many researchers thought it was impossible to detect plausible causal associations with such low-level exposures. The underlying idea was that “noise” (bias, confounding) was larger than the signal. However, a large number of well-conducted studies have been published since: in the case of ETS more than 60 studies show (with few exceptions) increased risks of lung cancer in the order of 1.25, i.e. the same magnitude as the gene for the nicotine acetylcholine receptor (1, 6, 7). For air pollution, an association with lung cancer has been reported in six cohort studies. One of these studies is sufficiently large as to show the association in non-smokers [6,7]. Again, the relative risk is around 1.25. In both cases biomarkers, such as cotinine or NNK for ETS and DNA adducts for air pollution, have contributed to make the association more plausibly causal.

Recent studies have also been able to identify clear associations between ETS, air pollution, cardiovascular disease and possibly some neurological diseases. Again, these are well conducted prospective studies, and biomarkers have substantiated the epidemiological observations. We have thus to admit that ETS and air pollution (mainly due to traffic exhaust) are able to induce, after long-term exposure, chronic diseases such as cancer and coronary artery disease. The interesting observation is that this happens at dose levels that are much lower than those of tobacco smoke or “classical” carcinogens. For example, exposure to ETS occurs at levels that are 1/100 compared to active smoking.

One potential explanation for the effects of extremely low doses and the absence of a threshold is based on acquired susceptibility and the cumulative effects of different exposures. As defined by Rothman and Greenland, “the cause of a disease event is an antecedent event, condition or characteristic that was necessary (given that all other conditions are fixed) for the occurrence of the disease at the moment it occurred” [8]. Said in other words, a cause can also be viewed as something that “completes an incomplete causal chain” [9] or precipitates a chain of events which

creates a state of vulnerability. This concept of “clinical vulnerability” is expressed in Figure 2. Exposure to low levels of e.g. ETS or air pollution is not a “cause” of cancer in itself (like an accident is the cause of a death), but because it occurs on top of pre-existing vulnerability. This could well explain why small changes in environmental exposures can have big effects, if they occur in a population of vulnerable subjects.

Vulnerability can be acquired or genetically-based. The concept of acquired “clinical vulnerability” is related to previous insults/pathophysiological changes that predispose to disease, as expressed in Figure 2. An example is the finding of a greater effect of ETS among ex-smokers compared to never smokers in a large prospective investigation [10]. It is plausible that ex-smokers have a greater vulnerability because of already existing mutations or epigenetic changes, so that further exposure to ETS leads to selection and clonal expansion of mutated cells.

Another type of vulnerability (more often called susceptibility) is genetically determined. Many years ago we showed that subjects with the genetically based NAT2 slow acetylator genotype could have greater susceptibility to being damaged by tobacco smoke related arylamines at lower levels of exposure rather than at higher levels [11]. Our reasoning was that on very rare occasions, e.g. among people exposed to extremely high doses of potent carcinogens, the whole population or a vast majority develops cancer. This is what happened among British chemical workers exposed to 2-naphthylamine in the 50s and before. For example, all 15 workers exposed to 2-naphthylamine in a plant developed bladder cancer, probably the only example of a “sufficient” exposure in the history of carcinogenesis [12]. It is clear that in that case genetic susceptibility was totally irrelevant.

Mendelian randomization tests

If we believe in the hypothesis of a cumulative effect of exposures that increase vulnerability, so that even low doses of exposure to carcinogens can lead to the effect (like in Figure

2), how can we design better studies to test the hypothesis? One possibility is to include in questionnaires sections about the past history of exposure, so to identify vulnerabilities. However, this is a relatively weak approach that has not given clear results. For example, it is still not clear what the joint effect of exposures such as smoking and asbestos is on lung cancer, after many years of research, mainly because of exposure misclassification that leads to underestimates particularly when addressing interaction.

An elegant way to address such a sophisticated hypothesis is through Mendelian Randomization, i.e. exploiting the random assortment of genes from parents to offspring. Since gene variants are randomly assorted from parents to the offspring during conception (and following meiotic recombination), then the gene variants one has do not depend on her exposures or social class (with some caveats and exceptions). If one can show that a gene involved in a process that increases vulnerability to disease is also associated with cancer or CVD, then this is additional support to the theory that exposures increasing vulnerability are really causally involved. An example might be exposure to air pollutants or ETS, inflammatory processes, cardiovascular disease and cancer. A Mendelian Randomization test would be looking at variants of genes in the NF- κ B pathway (IL1, IL6) or COX-2. If these variants are associated with CVD or lung cancer, then it is more likely that vulnerability induced by ETS and air pollution is causally involved in disease etiology.

The contribution of intermediate biomarkers and 'omics'

Intermediate Biomarkers

Intermediate biomarkers directly or indirectly represent events on the continuum between exposure and disease. Intermediate biomarkers can provide important mechanistic insight into the pathogenesis of environmental diseases including perhaps clinical vulnerability. As such, they complement classic epidemiological studies that use disease endpoints. Examples of early biologic

effect biomarkers include measures of cellular toxicity, chromosomal alterations, DNA, RNA and protein expression, and early non-neoplastic alterations in cell function (e.g., altered DNA repair, altered immune function). For maximum utility, an intermediate biomarker must be shown to be predictive of developing disease. The criteria for validating intermediate biomarkers have been discussed by Schatzkin and colleagues [13,14] and focus on the calculation of the etiologic fraction of the intermediate endpoint, which varies from 0 to 1. The closer the etiologic fraction is to 1, the greater the biologic marker reflects events, either directly or indirectly, on the causal pathway to disease. For instance, bone-marrow toxicity has been linked to future risk of leukemia. Therefore, environmental exposures that lead to bone-marrow toxicity are potentially leukomogens. Bone-marrow toxicity can be measured by studying peripheral blood cell counts including circulating progenitor cells. Studies based on such endpoints have the advantage that the biological marker can be measured quantitatively and therefore increasing the power as compared to dichotomous (rare) disease endpoints. Furthermore, these studies can be carried out using cross-sectional or semi-longitudinal study designs in which exposure can be accurately assessed. An exciting example of the use of intermediate markers is a recent study on hematological effects of benzene at low levels of exposure [15]. In this study peripheral blood cells and progenitor cell colony formation were significantly decreased among low exposed individuals in a dose-dependent matter. Moreover, these effects occurred at exposure levels lower than previously thought safe.

Omics

The rapid development of several fields in molecular biology is described with the term ‘omics’ – that generally refers to the study of a complete set of biological molecules with high-throughput techniques [16]. One of the main hallmarks of omics is the departure from traditional molecular biology focusing on a single biological structure (e.g. a single gene or protein) to a more comprehensive analysis of biological systems.

Cells and tissues alter their metabolism or gene expression in response to exposure as indicated by for example, the induction of specific groups of genes in response to heat shock, hypoxia, or osmotic stress [17-19]. These responses can produce patterns of specific changes in gene expression, proteins, or metabolic profiles that reflect exposure to a particular agent or class of agents. Complex sets of gene expression changes and corresponding changes in protein networks and metabolite profiles have been beyond the reach of older technologies. Toxicogenomic technologies offer new opportunities to detect and quantify these changes and apply them as new classes of biomarkers. Genomics, proteomics and metabolomics enable investigators to broadly explore biologic responses to exogenous and endogenous exposures, to evaluate potential modification of those responses by variants in essentially the entire genome, and to define disease at the chromosomal, DNA, mRNA and protein levels.

The field of genomics can be divided into three areas: genotyping, which focuses on gene sequence; gene expression profiling, focusing on gene expression; and epigenomics, which focuses on epigenetic regulation of gene expression. Proteomics is the field that studies the complete set of proteins present in a sample collected at a specific point in time, while metabolomics studies the complete set of metabolites in a sample. Proteomics and metabolomics are promising for discovering new markers. Both have recently been adapted to high throughput, highly sensitive technologies, like SELDI-TOF/MS, LC-MS/MS, and NMR, enabling the screening of large numbers of samples for a large number of potential markers. Although there are still limitations in the current techniques and in their applications in epidemiological studies which are characterized by small sample sizes and generally poor study designs, it is merely a matter of time before these techniques produce new leads in the discovery of novel intermediate markers. This will be largely attributable to a better understanding and consideration of methodological issues [4].

Application of omics to investigate human toxicological effects from environmental insults

Gene expression profiling

Gene expression profiling can be used to determine which genes are differently expressed as a result of changes in environmental conditions. For example, a self-controlled study design, which involved taking measurements before and after individual exposure, was used to overcome the problems of large interindividual variations compared with the small changes caused by environmental exposure. The group was stratified according to smoking status (which profoundly affected the whole blood expression profiles), and nonsmokers after exposure to welding fumes exhibited altered gene expression in 35 genes from eight functional pathways, including processes related to oxidative stress, proinflammatory responses, phosphate metabolism, cell proliferation, and apoptosis (reference missing).

Another study [20] used microarrays to analyze transcriptomic profiles of blood lymphocytes of Chinese shoe factory workers chronically exposed to relatively high levels of benzene (mean = 47 parts per million) and matched nonexposed controls. This analysis identified 29 genes with highly altered expression in the benzene-exposed group. Real-time polymerase chain reaction confirmed changes in four of six selected genes. These transcripts may provide reliable molecular indicators of benzene exposure and its biological effect.

Epigenomics

Epigenomics involves the study of epigenetic processes, which are independent of DNA sequence and are involved in the inhibition of gene expression (gene silencing), on a large scale [21-23]. Altered gene silencing plays a causal role in human diseases [24-27]. Epigenomics is mainly based on two comprehensively studied mechanisms – DNA methylation and histone modification, and more recently on RNA interference by non-coding RNAs such as microRNA and siRNA. It is believed that interactions exist between these epigenetic processes. A study by Bollati *et al* demonstrated that there was a significant reduction in markers of genome-wide methylation and gene-specific hypermethylation of genes p15 and MAGE-1 among individuals exposed to relatively low levels of benzene [28].

Proteomics

Whereas transcriptional profiling is a powerful method, proteins are generally the functional “business end” of genomic expression affecting cellular metabolism and regulation [29]. Proteins are active agents in cells that execute the biological functions encoded by genes. The proteome consists of all proteins present in specific cell types or tissue and, in contrast to the genome, it is highly dynamic over time, between cell types and in response to the environment. Although interesting, the following examples represent only a few published studies describing proteomic approaches to identify biomarkers of exposure and effect. Whether agent-specific changes can be identified is a key question to be answered about proteomic approaches (note, the same issue applies to other –omics, such as metabonomics) and the issue will be resolved only after a much larger body of proteomic data on exposed cells, tissues, and biofluids is collected and analyzed [29]. Joo *et al* performed proteomic analysis of plasma proteins, among workers exposed to benzene and matched unexposed controls and associated up-regulation of specific proteins with lymphocyte DNA damage [30]. Using MS and Western blot, they found statistically significant differences in protein profiles between the exposed and unexposed subjects. The specific proteins that were found to be up-regulated in benzene exposed workers were TCR beta, FKBP51 and MMP13 [30]. Vermeulen *et al* examined the impact of benzene on the human serum proteome in a study of benzene- exposed workers and unexposed controls, where protein-expression patterns were analysed by SELDI-TOF MS. They found that three proteins were consistently down-regulated in the exposed group compared to controls and all proteins were highly inversely correlated with individual estimates of benzene exposure ($r > 0.75$) [31].

Metabolomics

Changes in gene and protein expression can alter metabolism in particular ways that can provide distinct signatures. The metabolome is the biochemical network consisting of small molecules (e.g. lipids, vitamins) also known as metabolites [32]. Metabolites are involved in energy

transmission in cells by interacting with other biological molecules following metabolic pathways. The metabolome is highly variable and time dependent and consists of a wide range of different chemical structures. The most important challenge in this field is to acquire qualitative and quantitative information on metabolites that occur under normal circumstances in order to be able to detect perturbations in the complement of metabolites due to changes in environmental factors.

Global metabolomic profiling might be a simpler task than transcriptomic or proteomic profiling and more amenable to high-throughput screening, suggested by studies showing that the main yeast metabolome consists of fewer than 600 low-molecular-weight compounds, but significant metabolomic analysis of exposure effects will be needed to address the possibility of high-throughput screening [29,33]. Global metabolomic analysis in exposure studies in animals has just begun, but the low-level exposures of interest in large populations, has not yet been addressed [29].

Metabolomic studies can readily be performed with noninvasive samples such as biofluids and breath condensate as well as on tissues in vivo. Profiles within a tissue or cell can be compared with profiles in biologic fluids or with cell secretion products to understand the metabolic consequences of xenobiotic-induced toxicity. Examples of this approach are the studies by Waters and colleagues that used NMR and pattern recognition analysis to investigate time-related metabolic effects of α -naphthylisothiocyanate on liver, urine, and plasma in animals [34,35]. Waters and colleagues found that hepatic lipidosis was associated with the increased urinary excretion of taurine and creatine. In addition, there was reduced urinary excretion of intermediates in the tricarboxylic acid cycle and increased excretion of plasma ketone bodies. These studies enabled a clearer understanding of key metabolic effects during development of and recovery from a toxic lesion.

The role of omics

The ultimate goal of using ‘omics’ technologies to identify environmental causes of disease is to derive an integrated view of the biological processes involved in the continuum from exposure to disease. Although high-sensitivity and data-rich toxicogenomic approaches may already be feasible for analyzing human responses to environmental stressors in some settings, applying these technologies to human populations exposed to low-level environmental contaminants will require considerably more development. In this context, the integration of information from multiple toxicogenomic approaches may provide considerably more analytical power than any one approach alone.

For successful implementation, validation of markers, appropriate research questions, and careful interpretation of results are of paramount importance. ‘Omics’ has already played an important role in generation of new insights into the etiology of disease and gene-environment interactions. With the development of validated technologies, appropriate study designs and advanced statistical methods for data interpretation, ‘omics’ could potentially contribute significantly to the identification of environmental causes of disease and will help the field progress towards an integrated view of the interaction between environment and human health.

Conclusions - a model of disease

The prevailing model of “environmental disease” for decades has been influenced by toxicology, with single agents evaluated at a time, and dose as a key issue. A more recent model, which was not overall very successful, was that of “gene-environment” interactions, in which environmental agents at low doses interact with individual genetic susceptibility. What has not been explored extensively yet is another potential model for the effects of extremely low doses and the absence of thresholds, represented by acquired susceptibility and the cumulative effects of different exposures. The concept of “*clinical vulnerability*” could explain why small changes in exposure can have big effects, if they occur in a population of vulnerable subjects. The concept of acquired “*clinical vulnerability*” is related to previous insults/pathophysiological changes that predispose to

disease, and in this sense - as we have tried to suggest - the new omics can be extremely useful in tracing the “history” of such insults.

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Figure 1 Cumulative risk of lung cancer by rs8034191 genotype. Relevance of smoking and of rs8034191 genotype to lung cancer mortality in men aged 45-75 years. Cumulative risk (in the absence of other causes of death) based on national lung cancer death rates for men in Poland in the year 2000, assuming that the prevalence of current smoking, former smoking, and never smoking are as in this study and that the relative risks for lung cancer incidence and mortality are similar (Hung *et al* 2008) [1].

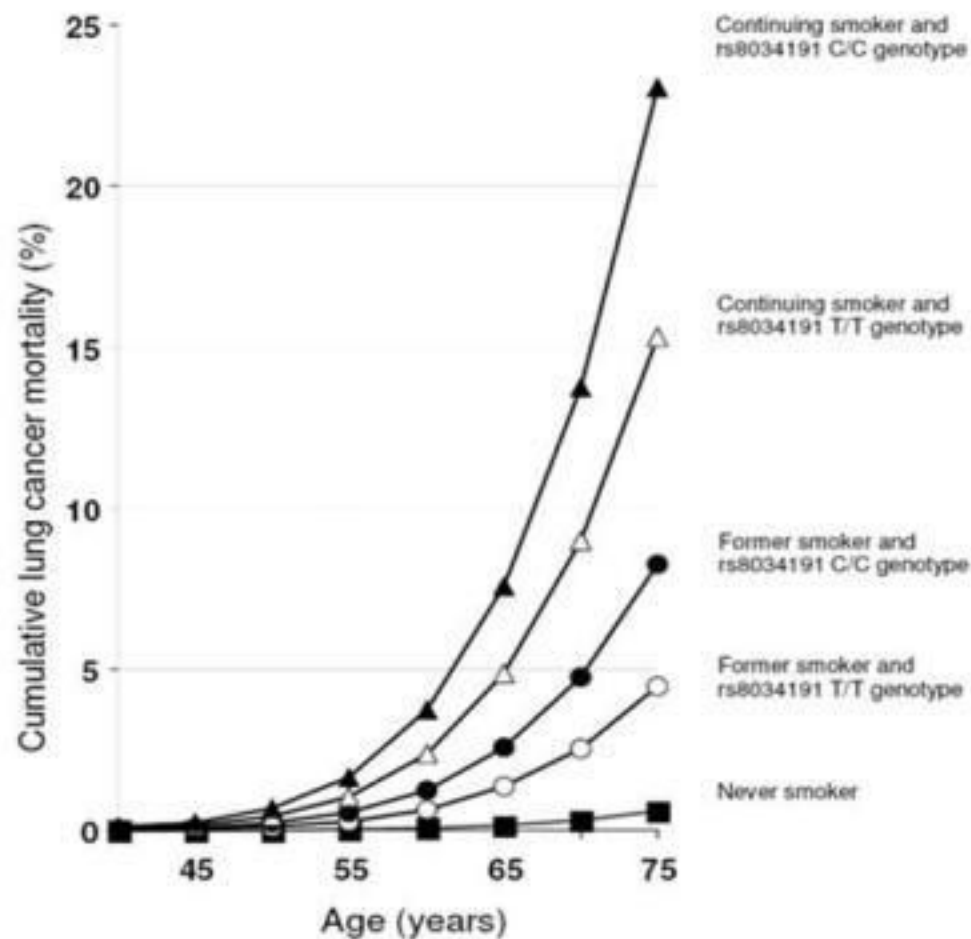
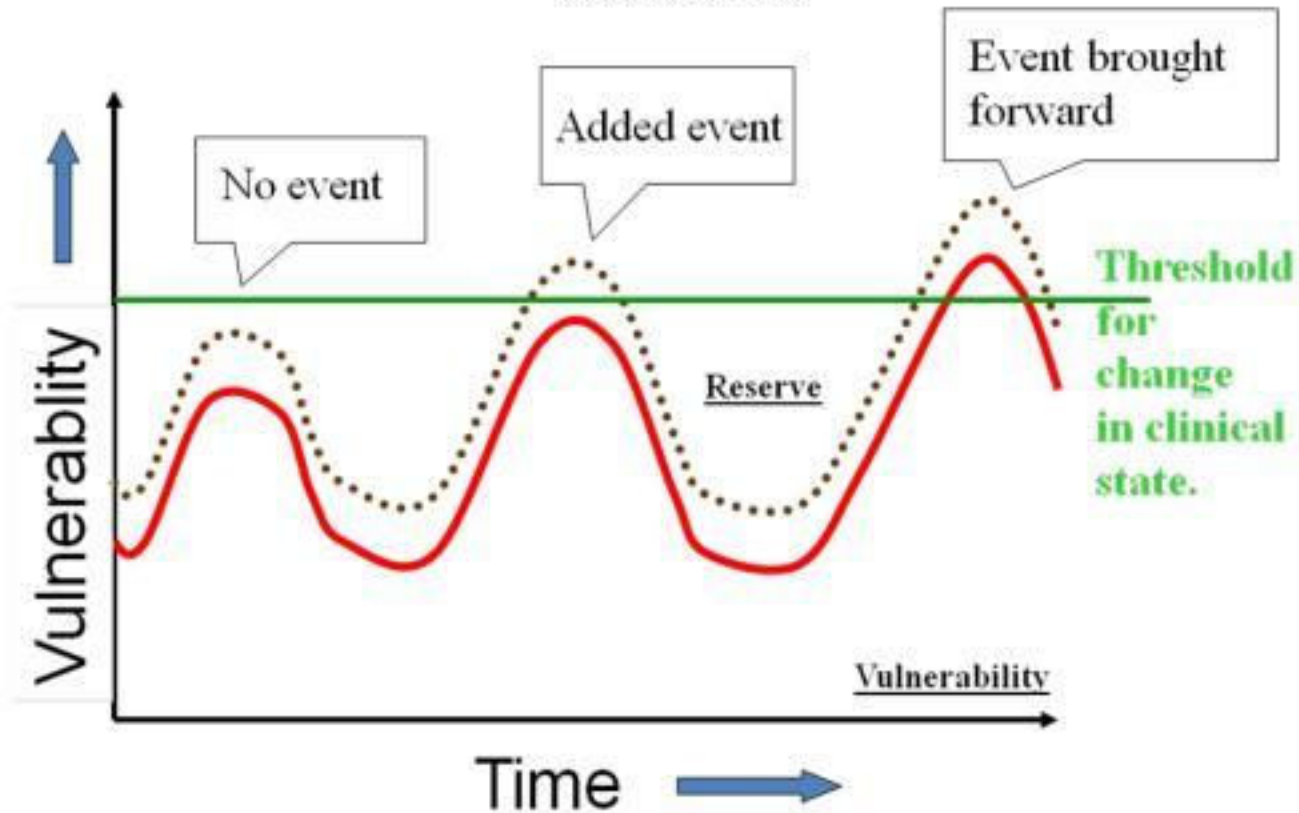


Figure 2. Vulnerability plus exposure events change the physiological state, but a reserve is present. When the reserve is overtaken, clinical manifestations appear. From http://www.sahsu.org/jubilee_presentations/Anderson_ppt#392_21, courtesy of R Anderson.



Interaction between air pollution and clinical vulnerability