Future Research Directions for Evaluating Human Genetic and Cancer Risk from Environmental Exposures

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The utility of biomarkers for evaluating the genotoxicity of environmental exposures is well documented. Biomarkers of both exposure and effect provide bases for assessing human-genotoxic interactions and may be indicative of future disease risk. At present, there is little information on the predictive value of these assays for either a population or the individuals tested. This paper describes some aspects of biomarker assays, the possible use of susceptibility measures in biomonitoring protocols, and the need for evaluation of disease relevance. A population study involving epidemiologists, geneticists, toxicologists, statisticians, and physicians is proposed to determine the disease relevance of these biomarkers. — Environ Health Perspect 104(Suppl 3):503–510 (1996)

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Introduction

The number of biomarkers available for evaluating genetic and cancer risks in humans is quite large (1). Their utility for human monitoring is suggested by a well-known paradigm of environmentally induced cancer (Figure 1) (2), which presents end points for assessing the entire spectrum of human-genotoxic interactions. These biomarkers begin with exposure and include absorption, metabolism, distribution, critical target interaction (i.e., DNA damage and repair), genetic changes and, finally, disease. Disease is the province of traditional epidemiology; the development of biomarkers has given rise to the field of molecular epidemiology, which uses these biomarkers rather than disease to assess the risks of environmental exposures. However, even though massive efforts have been expended to develop biomarker assays and define their sensitivities, much remains to be done. For example, the central question of disease relevance, widely assumed but never proven, is only beginning to be explored.

The tools of molecular epidemiology may be divided into several categories. There are biomarkers of exposure/dose that detect genotoxic agents at any level of body penetration, including the target DNA. There are biomarkers of susceptibility, which measure interindividual variability in the response to a given level of exposure. Both imply disease relevance, the former at the population level and the latter at the individual level. The disease relevance of these biomarkers is, of course, in the toxicity (carcinogenicity) of the agent of concern, or in unusual susceptibility to the agent, not in the measured biomarker end point per se. An important characteristic for exposure/dose biomarkers is sensitivity, i.e., the ability to detect exposures at levels that exist in real human populations.

Biomarkers of effect, which measure processed genetic damage, i.e., chromosome aberrations or gene mutations, are more complicated. The end points they measure, although defining consequences, are sometimes also used to define exposures, much in the manner discussed above. Because of this, the terminology of exposure/dose and effect biomarkers is somewhat ambiguous, and more mechanistic terms such as reversible (transient) genotoxic responses (exposure/dose) and irreversible (permanent) genotoxic responses (effect) may be better. Effect or irreversible genotoxic end points require host processing of DNA lesions into informational changes in the cell (e.g., mutations) and therefore may be relatively insensitive when used as dosimeters. Nonetheless, there are aspects of exposure assessment that are best accomplished by irreversible genotoxic end points. By recording permanent DNA damage, they provide memory for dose reconstructions of past exposures in terms of cumulative effect. In other instances there may be no specific assay for assessing exposure to a given genotoxic agent, or the agent or agents may be unknown. Irreversible genotoxic responses are then used to determine exposure to an undefined genotoxic agent. Finally, there may be a large database for the use of a particular marker for assessing some specific exposure, which is best illustrated by the use of chromosome

Figure 1. The paradigm of environmental cancer. From the Committee on Biological Markers of the National Research Council (2).
aberrations for quantifying acute ionizing radiation exposures (3,4).

When biomarkers of effect are used to assess exposures, some of the disease relevance again derives from the genotoxicity of the agents of concern. However, because the irreversible genotoxic end points actually measured are the kind that are also responsible for genotoxic diseases, i.e., chromosomal and gene mutations, the question of disease relevance assumes an additional dimension. Although the irreversible genotoxic end points actually measured occur in noncritical targets, they presumably occur in the same manner, in response to the same influences, and at the same frequencies as disease-related irreversible genotoxic responses. This assumes that the nonpathogenic reporter genotoxic responses are valid surrogates for their pathogenic but unmeasurable counterparts. To the extent that these responses are surrogates, it becomes reasonable to raise several important questions. Do effect biomarkers have disease implications per se? Does what has occurred in one portion of the genome, as measured by the biomarker, also occur in another? Is there added disease relevance for effect biomarkers not related to why and how they occur, but to that they occur? Also, because the genotoxic effects are measured in individuals, is there an implication of disease relevance for that individual?

There would be, of course, great utility for biomarkers that identify individuals at increased risk for developing cancer or having children with birth defects. A variety of steps could be taken including increased surveillance, early diagnoses, or other direct interventions. Preventive measures could be directed at individuals most likely to benefit from them, and the real goal of human mutagenicity monitoring could be within reach, i.e., the prevention of genotoxic disease.

Future research for evaluating genetic and cancer risks from accidental exposures then should be directed to questions of the disease relevance of the tools of molecular epidemiology. This paper considers some aspects of the assays themselves and discusses approaches to optimize the value of these assays for human preventive medicine.

The Assays

Biomarkers of Exposure/Dose
(Reversible Genotoxic Responses)

Currently available measures of exposure/dose for detecting in vivo penetrations of genotoxic agents to various levels are depicted in Figure 2. These include measurements of carcinogens/mutagens in body tissues. Greater penetration, at least in a functional sense, is afforded by measuring the active metabolites of the agent(s) of concern. Steady states of exposure to carcinogenic/mutagenic electrophiles are assessed by measuring protein or DNA adducts (5-8). Most electrophiles will adduct to the amino acids of proteins. Hemoglobin is the preferred protein for monitoring amino acid adducts because it is available in large quantities in small blood samples (9). Because the life span of circulating red blood cells is approximately 120 days and the adducts are stable, cumulative doses of genotoxicants over a 4-month interval can be determined (10-13).

DNA adducts are better representations of penetration of the agent to the target molecules of genotoxic concern than are protein adducts; however, the fact that DNA molecules are repaired, which must be considered when using DNA adducts as in vivo dosimeters. DNA adducts are assessed by several means, the most sensitive and specific being immunological assays (5,14); however, these assays have the disadvantage of requiring precise knowledge of the adduct of concern. In addition to direct detection in DNA, metabolized DNA adducts can be detected in urine (15,16).

One of the most important features of exposure/dose biomarkers for human biomonitoring is sensitivity. In our future research, we must continue to improve sensitivities, with new assays if necessary, and document this sensitivity by human studies where exposure levels are precisely known. The development of more generic exposure/dose biomarkers is also needed. Researchers have long awaited a marker for exposure to ionizing irradiations that will be more sensitive than chromosome aberrations for acute exposures and will reliably detect low-dose chronic exposures. A promising start was made a decade ago in using serum antibodies to DNA adducts to detect chronic exposures to genotoxic electrophiles (15,17,18). This reverses the usual assay that used antibodies formed in animals to detect in situ DNA adducts in cells from humans and could permit serological studies in humans to yield information regarding cumulative exposures to carcinogens/mutagens.

Biomarkers of Susceptibility

As used here, biomarkers of susceptibility refer to measurements that reveal interindividual differences in response to genotoxic influences (usually taken to mean genotypic markers). Individuals who are susceptible to various environmental carcinogens/mutagens have greatly heightened genotoxic responses to exposure levels of agents that induce little or no response in nonsusceptible individuals. Figure 3 indicates several kinds of biomarkers of susceptibility.

Genetic screening, another term for evaluating human populations using biomarkers of susceptibility, evolves from the concept that human populations are heterogeneous, i.e., made up of individuals who are genetically and, therefore, inherently susceptible or resistant to various environmental agents. There are many ethical issues associated with the application of biomarkers of susceptibility to genetic screening, including the right to work and insurance and job discrimination.

Metabolic genotypes reveal interindividual differences in ability to activate or detoxify genotoxic agents (19-24), which influence internal and biologically effective doses of the reactive forms of deleterious agents that penetrate to in vivo targets. Important genes of this type include those for P450 and other enzymes that convert inactive carcinogens/mutagens to their genotoxic forms, i.e., the class I reactions, and those that conjugate and thereby detoxify these reactive forms, i.e., the class II
reactions (22, 23, 25, 26). Many of these genes are polymorphic in human populations and they potentially explain much of the interindividual differences observed both in genotoxic responses revealed by biomarkers and by disease outcomes (21, 22, 25, 26). It appears to be the combined genotype at these loci rather than that at a single gene that governs individual susceptibility or resistance.

There are also genetically determined interindividual differences in the ability to repair damage to the DNA (27–36). Individuals with impaired repair capabilities have an increase in irreversible genotoxic damage resulting from the processing of reversible DNA lesions such as adducts. It is now well recognized that persons with such defects have increased susceptibilities to cancer and, indeed, many of the heritable cancer syndromes are due to inherited mutations at such loci (29, 37–42). The genes of interest here are those that encode DNA repair enzymes, polymerases, cell-cycle, or check-point proteins (32–34, 43, 44). The biomarkers of susceptibility include identification of relevant mutations in the critical genes, identification of mutant proteins, or loss of a critical repair or cellular function. As indicated in Figure 3, susceptibility due to impaired DNA repair capacity will influence the occurrence of early biological effects in either reporter or disease genes.

One class of genetically determined biomarkers of susceptibility usually not considered part of human biomonitoring includes genetically defined levels of host resistance to the emergence of disease—usually cancer. As shown in Figure 3, susceptibility factors will operate after the early biological effects have been induced. At some point, factors such as immunological competence will have to be considered in assessing risk for developing a genotoxic disease such as cancer in the context of given levels of exposure, dose, and early biological effects. For example, a human leukocyte antigen association (HLA-DPB1 glutamate 69) has been shown for a lung disorder related to beryllium exposure (45).

To include biomarkers of susceptibility in human biomonitoring protocols in any practical way, these biomarkers will have to be developed as reliable and simplified assays and then validated as to their disease relevance. Recent studies in this regard show that variability in biomonitoring results, especially using effect biomarkers, is often attributable to null genotypes for one of the genes of importance in metabolism (34). Similarly, individuals who are homozygous deficient for DNA repair functions have grossly elevated frequencies of chromosome aberrations or gene mutations (46–49). Clearly, it is important to consider inter-individually determined susceptibility and resistance factors when making assessments of genetic and cancer risks.

Other kinds of indicators of susceptibility will have to be considered (50). Nutrition factors are important in animal in vivo mutagenesis studies and have established relevance in the development of cancer (51). Studies of in vivo genotoxicity in humans have shown that nutrition factors such as folate deficiency can enhance the occurrence of chromosome aberrations and gene mutations (52). Future research will have to define the factors and nutrients of relevance to genotoxicity, devise simplified means for their measurements in human populations, and incorporate such measurements into human monitoring protocols. Again, accurate risk assessments require such information.

Finally, the unit of susceptibility or resistance to carcinogens/mutagens may not be the individual per se, but the cell. For genotoxic diseases such as cancer, which are cellular disorders that are usually clonal in origin, the genotoxic event that initiates the disease occurs in a single cell. As noted above, individuals with inherited DNA repair defects have increased frequencies of in vivo chromosome aberrations, somatic mutations, and cancers. These disorders are often referred to as genetic instability syndromes and, at the constitutional level, are functionally homozygous recessive conditions. In affected individuals, all cells in the body are deficient for the necessary stability factor, and the unit of susceptibility is considered to be the individual.

One of these disorders, ataxia-telangiectasia, is also suspected of having heterozygous effects in female carriers, causing breast cancer (53, 54). The ataxia-telangiectasia gene has recently been cloned (30); this will allow clear genotyping of heterozygous individuals and the evaluation of possible increased cancer risk.

Undoubtedly, the cancer-prone characteristic of the genetic instability syndromes results from a genetic alteration in a single cell. Because every cell in the body of a homozygous individual is homozygous deficient, the probability that such oncogenic alterations will occur by mutation is very high, i.e., almost one. However, an individual who is a constitutional heterozygote for one of these conditions will almost certainly be a mosaic at the cell level, with a majority population of heterozygous cells and several minority populations of cells that have lost function of the active copy of the relevant gene by somatic mutation or loss of heterozygosity of the single wild-type allele. Actually, calculations reveal that there can be many mutations of the originally wild-type allele, with the extent of the minority cell populations in the individual being determined by the number of these somatic events and, more importantly, the time during fetal development that they occurred. The earlier the inactivating mutation of the intact allele occurs, the larger the resultant deficient clonal cell fraction will be. These cells now homozygous for a genetic instability gene will in effect have acquired a mutator phenotype.

While homozygosity for DNA repair diseases such as ataxia-telangiectasia are very rare, the heterozygotes occur at measurable frequency by Hardy-Weinberg expectations. When the large number of DNA repair, DNA stability, cell cycle, and checkpoint genes are considered, a large proportion of the human population will
be constitutionally heterozygous for one or more of these mutator genes. There is also the possibility that dominant negative mutations may occur in these genes, thereby not requiring the individual to be constitutionally heterozygous at those loci. In toto, interindividual differences in susceptibility to cancer could be due to intraindividual interclonal differences in genetic stability. Colon cancer patients who are constitutionally heterozygous for mutations in mismatch repair genes have shown the replication error (RER) phenotype indicating mismatch repair deficiency in somatic cells that are not part of their cancer; this suggests that such individuals are true somatic mosaics for this function (55). Future research should be devoted to characterizing this phenomenon and its extent in human populations. If this kind of mosaicism is not rare and if it does account for a substantial portion of interindividual variability in cancer susceptibility, then appropriate biomarkers of susceptibility will have to be devised and incorporated into human monitoring protocols.

**Biomarkers of Effect (Irreversible Genotoxic Responses)**

As indicated in Figure 4, biomarkers of effect can be divided into two classes: those that measure genotoxic events in reporter genomic regions and those that measure genotoxic effects in disease-critical regions. Traditionally it has been the former that have constituted the effect biomarkers used for human monitoring. More recently, genotoxic events are also being measured in disease-critical genes. These genotoxic events would certainly be expected to have disease relevance, but it must be determined whether they are biomarkers of effect or early indicators of disease.

As shown in Figure 4, biomarkers of effect include measurements at both the chromosome and the gene levels for both reporter and disease-critical events. Among the chromosome-level reporter events are the traditional nonspecific chromosome aberrations (56,57), micronuclei (58-62), sister chromatid exchanges (SCE) (56,63-65), and more recently, single-cell electrophoretic determinations of chromosome fragmentation (comet assay) (66,67). It has become possible over the last decade to directly measure and characterize gene level somatic mutations arising in vivo in humans. Four or five assays are in use (68), but a significant database has been developed for only two, i.e., hypoxanthine guanine phosphoribosyltransferase (hprt) mutations in T lymphocytes (69-71) and glycosylphosphatidylinositol A (gpa) mutations in red blood cells (72,73). Of these, the assay for hprt mutations allows recovery of mutant isolates for molecular and other characterizations, such as the discovery of mutational spectra (74), while the assay for gpa mutations allows phenotypic scoring only.

As indicated in Figure 4, all of the specific chromosome aberrations or gene mutations that characterize the various human malignancies can, in theory, be used as biomarkers of effect. Polymerase chain reaction (PCR) or ligase chain reaction amplification-based technologies can be used to define any of these chromosome aberrations or gene mutations in blood or other samples, allowing determination of chromosome changes that characterize the leukemias or lymphomas or specific mutations in oncogenes or tumor suppressor genes. Using these changes as biomarkers for human monitoring holds great promise in terms of disease relevance; however, future research, in addition to developing rapid and reliable assays, will have to deal with issues such as clonal amplifications when attempting to ascertain the frequency of the monitored event. Nonetheless, assay development and validation for this class of biomarkers of effect are important future research directions.

For surrogate biomarkers to have disease predictability, it must be demonstrated that the genotoxic events actually measured really mimic disease-causing genotoxic events. Indications exist that pathogenic mutagenic mechanisms that presumably occur elsewhere in the genome are faithfully reproduced in the reporter events. At the gene level, somatic recombination, an important event for loss of heterozygosity in malignancies, is recorded in HLA mutations in T lymphocytes (75-77) and presumable gpa mutations in red blood cells (73). Specific cancer-relevant mutagenic mechanisms such as DNA breaks at topoisomerase II and V(D)J recombinase consensus cleavage sequences have been demonstrated for hprt mutations (78,79). Future studies must extend the pathogenic mechanisms detected at reporter loci.

The issue of surrogate of tissue must also be addressed in future research. At present, assays of genotoxic effects all use blood cells, i.e., T lymphocytes or red blood cells. That specific lymphoma-related mutations, such as those mediated by the V(D)J recombinase, are captured in hprt mutations in T lymphocytes implies that this genetic event in this cell type is a good surrogate for lymphocyte malignancies (80-82). However, future research must devise methods to assess irreversible genotoxic effects in other cell types. In this regard, our laboratory is devising a method for assessing in vivo hprt mutations that arise in CD34+ myeloid stem cells, the precursors of most nonlymphocytic leukemias, to determine if these cells are a better surrogate for this kind of malignancy. We need assays of in vivo-arising irreversible genotoxic effects in epithelial cells, as well as simple, inexpensive, and reliable molecular assays for specific in vivo mutations.

**Population Studies**

Future research will improve the current assays used by molecular epidemiology, making them simpler, cheaper, and more reliable, but these are only the tools needed for human monitoring. What will be of paramount importance for this field will be studies of the meaning of human monitoring. Specifically, the disease relevance of the biological end points measured, separately or together, must be established in quantitative terms. Disease relevance must be determined at the population level in order to permit and defend public health decisions made at that level, e.g., removal of sources of contamination. If it can be documented that biomarkers have disease
predictability at the individual level, a wide variety of early intervention strategies will be possible. Because disease relevance at the population level usually depends on the toxicity of the agents of concern, it will not be discussed further here. The determination of disease relevance at the individual level is something that must be initiated by those involved in molecular epidemiology. The determination must be expanded beyond the laboratory and encompass field studies of real human populations and the cooperative efforts of epidemiologists, geneticists, toxicologists, and physicians.

Recent Studies
Reports that at least one of the effect biomarkers, i.e., chromosome aberrations, has disease relevance per se are beginning to appear in the literature. The first concerns a large Nordic cohort study of cancer incidence in individuals who had determinations of chromosome aberrations, micronuclei, or SCE between 1970 and 1988 (83). Cyogenetic studies were carried out with populations with environmental exposures and with unexposed referents. (Individuals with cancer diagnosed before the study were not, of course, included in the cohort.) Interlaboratory variation was standardized by trichotomizing the data. There was a statistically significant linear trend in that the upper tertile of chromosome aberrations had a greater than 2-fold increase in cancer incidence over the lower tertile; however, there was no positive association of cancer incidence with SCE results (which are biomarkers of exposure). Data with micronuclei were too limited for firm conclusions (83).

A similar study has recently been reported from Italy in which chromosome aberration results obtained from 17 laboratories, again trichotomized, were correlated with standardized mortality ratios (SMR) for cancer (84). Statistically significant increases in SMR were found in individuals in the middle and upper tertile of chromosome aberrations for all cancers and in individuals in the upper tertile for respiratory tract, lymphatic, and hematopoietic malignancies. These results of SMR analysis were confirmed by multivariate Poisson regression analysis after adjusting for potential mutagen/carcinogen exposures, which indicates that the disease predictability was not due entirely to the fact that a genotoxic exposure had been measured.

A third study also compared chromosome aberrations and cancer outcomes. The results of cytogenetic analyses performed many years after pelvic irradiation were compared for two groups of women, i.e., those with benign and those with malignant gynecological disease (85). Frequencies of stable aberrations were found to be only slightly higher in the cancer survivors, despite that group having received more than a 10-fold higher bone marrow dose of ionizing radiation than the group of benign disease survivors. This finding correlated with the earlier observation that the excess leukemia risk is also approximately equal for patients receiving radiotherapy for benign or for malignant disease despite the dose difference. Kleinerman et al. (85) conclude that, for patients receiving partial-body radiotherapy, stable chromosome aberrations measured a long time after exposure appear to serve as biomarkers of effective risk rather than as biomarkers of radiation dose received.

The results of these three studies suggest that biomarkers of effect may have disease relevance per se, independent of their reflecting exposures to carcinogens/mutagens. This should encourage future research to fully document and quantify this potential predictive value of these assays.

Future Studies
Both the Nordic and Italian studies above (83,84) were prospective studies in which cancer incidence or mortality, respectively, were ascertained several decades after the cytogenetic analysis. Although these findings are exciting, it is not practical to base large-scale studies of disease relevance on this design, and multiple biomarkers cannot be assessed in the same general analysis. Rather, a source of blood and, if possible, other tissue samples should be obtained from individuals at the time the carcinogenic/mutagenic influences that initiate the malignancy are operative. That is, the surrogate tissues for detection of biomarkers must be obtained at the time when the disease-causing genotoxic events are arising, which is well before the clinical detection of cancer. The surrogate tissues must then be cryopreserved and the individual from whom they were obtained followed medically to ascertain final disease outcome. Only at that time should the tissues be analyzed for biomarkers.

The individuals from whom tissue samples are obtained should have a high risk of developing cancer from a known carcinogen/mutagen exposure. If at all possible, the exposure level or dose of the deleterious agent(s) should be known. This situation describes cancer patients receiving chemo-radiotherapy or individuals who are exposed in environmental disasters. Blood or tissue samples should be collected early and, if possible, at several intervals following the exposures. (For cancer patients, there should also be pretreatment samples.) Ideally, blood and tissue sampling should be performed by experts disinterested in the final outcome of the study, e.g., multi-institution cooperative cancer treatment groups for treated cancer patients or the International Red Cross for environmental disasters. These samples should be preserved in a repository especially established for that purpose by specialists trained in maintaining tissue samples and information for later retrieval. Medical follow-up is critical in this scheme, and clinical information must be linked to the tissue samples. Testing should not be done until there have been a sufficient number of cases of the disease of interest to allow statistically valid determinations.

After a sufficient number of cases of, for example, acute nonlymphocytic leukemia (ANLL) have occurred, a panel of epidemiologists, geneticists, toxicologists, statisticians, and physicians should design an appropriate nested case-control study for determining the disease relevance of biomarkers. Biomarkers chosen for study should include appropriate measures of susceptibility and effect certainly, but perhaps others also. Expert laboratories should be selected to conduct the biomarker analyses blindly. In the case of somatic mutations, mutational spectra may be determined to ascertain if particular molecular kinds of events are more disease relevant than others. Biomarkers of effect that measure reporter events and those that measure disease genotoxic events should be tested. Assay results should be returned to the panel for unblinding and final analyses.

Over a period of 5 to 10 years, studies such as this should provide answers to questions of disease relevance and individual predictability. ANLL was chosen as the first example because, unfortunately, the incubation period for such malignancies is quite short. Another advantage of choosing this disease for initial correlations is that PCR-based methods for detecting the specific chromosome changes in these leukemias can give added confidence to the diagnosis of no disease. The information to be obtained in this type of study should be the relative risk (odds ratio) for developing disease (ANLL) among exposed individuals with abnormal biomarker
results compared to exposed individuals who do not show such abnormal results. This is the beginning of developing quantitative data for using biomonitoring data to assess genetic and cancer risks from accidental exposures.

Conclusions

There are a number of biomarkers available for carcinogenicity/mutagenicity monitoring in humans. The assays themselves are becoming more and more sophisticated and are allowing basic questions about carcinogenic and mutagenic mechanisms to be approached in humans. This progress in assays development has occurred in laboratories; however, the availability of biomarkers has resulted in their use in the field for assessing human environmental health risks and has allowed development of the field of molecular epidemiology. As biological rather than disease end points find greater and greater application for assessing human health risks, more attention must be paid to the health relevance and disease predictability of the end points measured. This will require studies of the scope and complexity outlined above and cannot be accomplished in laboratories alone or in the confines of a single discipline. Furthermore, these studies will require a level of national and international cooperation that has not thus far been shown by the genetic toxicology community. Samples and information must be shared, one or more repositories must be created, commitments must be made to continue the research over time, and perhaps wet workshops will have to be conducted. Studies will be group efforts, and the research will be cumulative and develop over time. The scheme suggested above is for an initial disease assessment; others must follow. Also, new and better biomarkers will continually become available, and these also will require validation of this sort. The effort will be great, but so will be the potential benefits.

Traditional epidemiology has been and remains the gold standard for studying disease outcomes in human populations; however, such approaches to human genetic and cancer risks from environmental exposures is impractical because of the infrequency of the disease events, the number of individuals therefore required for study, and the long incubation periods of many of the resultant diseases. In any event, the purposes of monitoring the population is defeated because the diseases that are to be prevented must first occur.

The use of biomarkers in molecular epidemiology holds the promise of disease prevention. If successful, monitoring in this way allows one to buy time and initiate measures aimed at true disease prevention. For this to be accomplished, the tools with which human populations are studied must provide information that is interpretable in terms of health risks. Calibrating these tools for this use should become our major research goal.

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