Proceedings of a Joint U.S. Food and Drug Administration (FDA), National Cancer Institute (NCI), Office of Dietary Supplements (ODS) Workshop on the Use and Misuse of Biomarkers as Indicators of Cancer Risk Reduction Following Dietary Manipulation

Lister Hill Auditorium, NIH Campus
Bethesda, MD
July 12–13, 2005

Proceedings Summary Report

TUESDAY, JULY 12, 2005

WELCOME AND OPENING REMARKS

Peter Greenwald, Division of Cancer Prevention (DCP), National Cancer Institute (NCI), National Institutes of Health (NIH); Paul Coates, Office of Dietary Supplements (ODS), NIH; and Kathleen Ellwood, U.S. Food and Drug Administration (FDA)

Peter Greenwald, MD, DrPH, Director, DCP, NCI, welcomed participants and described the importance of biomarkers for use across the cancer continuum, particularly for cancer prevention. Historically, biomarkers for other disease states (e.g., blood pressure for cardiovascular disease) have been used effectively to direct recommendations for intervention and treatment. Cancer biomarkers should be identified as early as possible in the cancer process, ideally in the pre-initiation phase of carcinogenesis, reflecting the progression of disease and/or improvement in outcomes. Cancer biomarkers must be based on sound science and include reproducible validation.

Christine Swanson, PhD, Director, Botanical Research Center Program, ODS, NIH, welcomed participants and thanked the organizers for addressing this important area of research. She noted that nutritional interventions developed through rigorous scientific study have the potential to significantly reduce the disease burden and stressed that the ODS is solidly committed to supporting investigations of dietary supplements for the prevention and treatment of chronic diseases, including cancer.

Kathleen Ellwood, Ph.D., Director, Division of Nutrition Programs and Labeling, Center for Food Safety and Applied Nutrition, FDA, welcomed participants and described the use of biomarkers for FDA's scientific review of health claims. She stated how identifying and validating nutrition-related biomarkers for use in determining populations at risk for developing various types of cancer was important for understanding the disease process.

Research Opportunities and Needs for Study of Dietary Modification and Cancer Risk Reduction: The Role of Biomarkers

Ross Prentice, Fred Hutchinson Cancer Center

Ross Prentice, Ph.D., Professor, Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, presented a keynote address on the role of biomarkers in cancer research with dietary modification. The basic premise of a vigorous diet/nutrition and chronic disease research agenda should include investigating a set of changes that lead to an outcome. These changes should be evaluated in observational studies in carefully selected populations, with nutrient/dietary pattern exposure data calibrated using specific validated exposure biomarkers. This would develop into a substantial intervention development/initial testing research enterprise, including small scale human feeding studies with biomarkers of disease risk as outcomes; and a forum comprised of basic, clinical, and population scientists to stimulate needed research and to assess readiness for dietary intervention randomized controlled trials (RCTs).

There are significant challenges to nutrition-related prevention research, including extending the relevance of animal feeding trials to human nutrition. Additional challenges include the understanding of confounding and bias in ecologic, time trend, and migrant studies, as well as determining measurement error in analytical epidemiologic studies, and the reliability or relevance of intermediate outcomes in RCTs compared to clinical outcomes. An example of the challenges for nutrition-related prevention research is seen in the history of the changing association between breast cancer and dietary fat intake. Ecologic, case-control, and cohort studies suggested that dietary fat intake and breast cancer risk were positively associated; subsequent methodological studies, however, indicated significant under reporting and measurement errors that equivocated these findings. This highlights an area of nutrition research that would benefit from specific and validated exposure biomarkers.

In the Women's Health Initiative (WHI), a large clinical and observational trial that assessed the effect of dietary modification on cancer and cardiovascular risk, authors of a substudy of nutrient biomarkers reported that younger women and women with a high body mass index (BMI) under reported energy intake by approximately 27 percent on food frequency questionnaires compared to that assessed by double-labeled water (DLW). This example points to the need for more accurate biomarkers of exposure based on new measurement models for nutritional epidemiology. Areas that need additional research are the development of additional recovery-type biomarkers and new methodological approaches to facilitate the use of concentration biomarkers.

The future approach to prevention research must be based on results from therapeutic research; post marketing epidemiologic surveillance of drugs, supplements, botanicals, or other agents; and observational studies of lifestyle factors and clinical and laboratory parameters. A key strategy to consider is a substantial multidisciplinary discovery effort that can identify and investigate initial testing of nutrient , chemopreventive and lifestyle modification interventions. Biomarkers can have an important role in this effort in conjunction with association studies that identify determinants of disease risk and disease pathways. In the WHI, for example, a genome-wide scan of single nucleotide polymorphisms (SNPs) associated with breast cancer, cardiovascular disease, and stroke is such a multidisciplinary effort. Results from this scan—which include 250,000 tag

SNPs to be assessed in a case-control investigation—could identify promising areas of research to target for larger-scale human trials. To assist in the creation of a large-scale effort, it was suggested that the NCI consider forming a multidisciplinary group with representation across health outcomes (e.g., cancer, cardiovascular disease, diabetes, and osteoporosis) and research foci (e.g., basic, clinical, population) to stimulate needed research and to identify preventive interventions for testing in RCTs. One important outcome of such a focus would be the development of reliable chronic disease prevention information.

Discussion

Various participants asked Dr. Prentice to provide additional information on the WHI SNP investigation. Dr. Prentice responded that the 250,000 tag SNPs were chosen from the approximate 15 million SNPs in the human genome because they have a 5 percent or greater prevalence in the human population. The cost of this project, at approximately $0.01/SNP, is still considerable. It was pointed out that the current HapMap project may preclude the need for such a WHI SNP project.

In response to a question about the type of forum needed to focus on nutritional research, Dr. Prentice said he would leave it to coordinating committees to provide the direction and momentum for this effort. Dr. Greenwald added that DCP has experience in providing opportunities for multidisciplinary researchers to come together to develop initiatives that address current and future program needs. Among the most pressing needs at this point is to determine how to establish a biorepository to take advantage of samples collected in clinical trials, such as the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial. There are issues of ownership and cost. Dr. Prentice commented that the National Heart, Lung, and Blood Institute (NHLBI) will be issuing a program announcement in 2006 inviting proposals on the use of the 17 to 35 million specimens from the WHI.

A participant asked if it is possible to extract RNA from biorepository tissues, as promising research is focusing on gene expression patterns. Dr. Greenwald recognized the growing interest in RNA. Dr. Milner added that some of the technology for gene expression analysis is emerging, but it is easier to investigate SNPs than to investigate proteomics and metabolomics.

As to the applicability and acceptability of measurement error equations presented by Dr. Prentice, a participant commented that the equations add to nutritional epidemiology, which has been data-poor in the past. Many of these equations have not been tested fully in trials to determine whether they can be applied to biomarker research. A participant expressed concern about the equations identifying interrelationships between nutrients that would make it difficult to identify the specific nutrient responsible for biologic changes.

Padma Maruvada, DCP, NCI

Padma Maruvada, Ph.D., Program Director, Cancer Biomarkers Research Group (CBRG), DCP, NCI, NIH, discussed the report summarizing the "Research Strategies, Study Designs, and Statistical Approaches to Biomarker Validation for Cancer Diagnosis and Detection Workshop" held in July 2004. There has been a precipitous decline in applications to the FDA for new protein biomarkers in the past decade; only two biomarkers have been approved, which implies that efficient research translation is not occurring. It is likely that inadequate tools and validation are currently available for biomarker research. Reasons for inadequate biomarker discovery include: 1) a proof-of-principle is not well established in biomarker research; 2) bias exists in study designs; 3) previous research lacks statistical power; and 4) results from biomarker studies are non-reproducible at this time due to lack of standardized laboratory practices and sample collection.

The July 2004 NCI workshop tried to address some of these issues. The full report may be found on the NCI Web Site at http://www3.cancer.gov/prevention/biomarkers2004/. Recommendations included in the summary report offer specific suggestions for characterizing biomarkers, handling of high-dimensional data, and study designs. Based on suggestions and recommendations from the July 2004 workshop participants, an appropriate and sound biomarker study would include:

X Developing various statistical study designs and methodological approaches for pre - and post-processing of data, especially high dimensional data;

X Developing more facile approaches to biomarker validation for clinical utility, including RCT- based and non-RCT-based validation designs;

X Exploring the potential for piggybacking validation studies with ongoing prevention and treatment trials and case-control study designs based on completed trials;

X Establishing criteria and standards that can be applied to study designs for consideration for approval (PMA and 510[k]) by the FDA; and

X Evaluating the suitability of samples for high-throughput molecular assays for the validation study.

Discussion

A participant commented that Dr. Maruvada mentioned that cut-off points should be based on biology rather than technology, but that biomarkers can indicate changes that occur over a long period of time before cancer appears. The participant also inquired whether, with changes so subtle, biomarkers have the potential to be useful in sporadic cancers. Dr. Maruvada responded that subtle differences might be seen with the correct biomarker assessment panels. Dr. Seifried added that cut-off points historically have been frequently set at the lowest level of sensitivity for an analytical technique, but

action levels or cut-offs must be based on biology even if the technology can measure orders-of-magnitude below the level that indicates significantly increased cancer risk.

Promises and Perils of Validating Biomarkers for Cancer Risk
Arthur Shatzkin, NCI

Arthur Shatzkin, M.D., Dr.P.H., Chief, Nutritional Epidemiology Branch, NCI, NIH, reviewed the potential contributions that biomarkers can make for understanding the etiology and prevention of cancer. He also reviewed the problems of using surrogate endpoints and validating them for cancer research. Existing biomarkers relevant to diet and disease are known for tissues (adenomas, CIN3), cells (proliferation, apoptosis), molecules (DNA adducts, strand breaks, allelic variants of genes encoding metabolizing enzymes), infection (HPV infection, *H. pylori* antibodies), blood analytes (estrogen, PSA), and imaging (mammographic density, ovarian ultrasound abnormalities). It is important to remember that biomarkers should enhance the biologic plausibility of the diet-disease relationship by clarifying causal pathways, increasing the strength of the diet-disease associations, and serving as surrogate endpoints. Design options to confirm biologic plausibility include: 1) metabolic studies to show the effect of an intervention (e.g., dietary change or supplement use) on biomarkers and 2) nutrition-gene studies to unravel the apparent synergistic effects of dietary mixtures and to gain a better understanding of Mendelian randomization (e.g., polymorphisms that mimics high or low dietary exposure) to help understand confounding and measurement error.

Increasing the strength of exposure-disease associations can sharpen the determination of the changes in relative risk through the determination of intake biomarkers and diet-gene interactions. For example, for the N-acetyltransferase (NAT) genotype, individuals can have a rapid or non-rapid acetylation status. High red meat intake among rapid acetylators (NAT 1 genotype) results in an approximately four-fold greater risk of colorectal cancer in this group compared with non-rapid acetylators (NAT 2 genotype). Because of numerous nutritional factors, enzyme receptors, and allelic combinations, discovery of useful nutrition-gene biomarkers and the ability to successfully intervene in the cancer processes is a significant challenge.

The use of surrogate endpoint biomarkers (SEBs) allows the conduct of clinical and intervention studies that are smaller, faster, and cheaper than large RCTs. There are, however, significant challenges in identifying and validating SEBs. Dr. Schatzkin described the conditions needed to validate SEBs for nutritional exposure and cancer. They include the biomarker being associated with cancer, the exposure being associated with the biomarker, and the biomarker mediating the association between exposure and cancer. For example, a recent study of BMI and postmenopausal breast cancer found that relative risk unadjusted for free estradiol is 1.19 However, the relationship between BMI and estrogen is well established, total estrogen levels in the obese are 1.8 times higher than normal levels and free estradiol is 2.5 higher. As a result, when this group data comparing BMI with breast cancer risk is adjusted for free estradiol, the relative risk falls to 1.02, supporting a more direct relationship between estrogen and breast cancer among these women and indirectly to BMI.

A challenge for understanding SEBs and causal pathways to disease may also be illustrated by proliferation markers as surrogates for colorectal cancer. Interventions that address one causal pathway, such as hyperproliferation, may also affect an alternative causal pathway that counterbalances the effect of the intervention. Understanding the totality of causal connections is a key to developing validated SEBs and is a research area that should be considered for further investigation.

Dr. Shatzkin concluded that SEBs may be particularly valuable in Phase II studies, but there would be a cost in lower predictive certainty associated with using surrogates instead of cancer outcomes. Ideally, SEB studies would be used to enhance results determined from RCTs and this should be encouraged.

Discussion

A discussion of issues about confounding ensued and included the conclusion that SEBs will be useful for understanding cancer processes and may lead to valid prevention strategies. Conducting clinical trials with frank cancer endpoints, however, may be unavoidable for some cancer sites, at least when first validating the SEBs. The genes or metabolites that should be studied have not been identified, and an important issue is making sure dual mechanisms, such as apoptosis and proliferation are studied together. A participant asked about the most worrisome aspect of using large data sets. Dr. Shatzkin responded that individual variation "noise" is often very worrisome, but information can still be gained from the data sets affected by "noise."

A discussion of whether biomarkers can be used without completely understanding the disease process took place. Dr. Shatzkin commented that SEBs have been used for a long time, but not all of them offer the perfect solution for understanding cancer risk. This leads to the conclusion that there still is a need for animal and population research to identify and validate SEBs. Dr. Greenwald added that biomarker research has led to many theories but few clinical trials in humans. It is important to determine which Phase II trials need further consideration due to their complexity and cost..

SESSION 1: WHAT ARE THE STRENGTHS AND WEAKNESSES OF CYTOLOGICAL AND HISTOPATHOLOGICAL INDICATORS OF CANCER RISK?

Session Chair: *Christine Swanson, ODS*

Dr. Swanson commented that since histopathology is the final arbiter of whether or not a tissue is cancerous, the first area selected was pathology and a very distinguished expert chosen to put the topic in perspective.

Histological Markers

Bob Cardiff, University of California–Davis

Bob Cardiff, M.D., Ph.D., Professor of Pathology, Department of Pathology and Laboratory Medicine, Center for Comparative Medicine, University of California, Davis, reviewed a slide of the cervical carcinoma *in situ* that exemplified histopathology of cancer as an endpoint. Early lesions, represented by abnormal foci, represent early changes in the cervix and other organs that indicate a high risk of carcinoma *in situ*. There are, however, other cofactors (i.e., infection with human papilloma virus for cervical cancer) that also play an important role in predicting a greater risk of cervical cancer from carcinoma *in situ*. In breast cancer, abnormalities in histopathology also indicate which lesions (e.g. ductal carcinoma in situ- DCIS) will lead to invasive disease. There is a morphological continuum which implies a sequential acquisition of traits leading to cancer. The basic question is, "How early is it possible to detect events that are predictive of future invasive cancer, and are these changes able to become useful biomarkers?" There is no clearly discernible morphological difference between similarly appearing premalignant cells with different potential for progression and metastasis. Individual cell lines derived from premalignant lesions do have very consistent latency period and the same metastasis rate in the each generation

Predictive models have been developed to identify disease progression using histopathologic methods. The linear sequential model is commonly used, but a newer model known as the "parallel" branch model has also been developed. Both models describe progenitor cells that are altered molecularly or genetically to begin a progression to precancer and cancer; the branch model, however, includes the concept that there is divergent evolution in the progenitor cells after the initial changes.

Experiments in genetically-engineered mice (GEM) are being used to validate the biology of lesions through test-by-transplantation, which provides an operational definition of each stage of disease and offers an opportunity to study the biology and molecular biology of the transitions between normal and pre-malignant, pre-malignant and invasive, and invasive and metastatic disease. An advantage of the GEM model is that the tumors look much like human tumors, morphologically and both in molecular mechanisms and outcomes; serial transplantation of small fragments of a given tumor line results in the same latency period and the same metastatic rate in the each generation However, "What you get is *not* (necessarily) what you see". Dr. Cardiff described an experiment in mammary intraepithelial neoplasia (MIN) mice to illustrate test-by-transplantation. GEM have biologically premalignant foci of mammary intraepithelial neoplasia (MIN) that can be identified in situ, isolated, and serially transplanted in gland-free fat pads. Three samples from the same pre-malignant lesion, initiated by the same oncogenes, isolated from the same animal and the same mammary gland, had different morphologies and different biological potentials when transplanted. This confirmed that there are progenitor tumor cells, but what happens to them afterwards involves complimentary changes rather than a primary genetic change. In analyzing what is occurring, it was determined that there are inherited genes of susceptibility that are influenced by epigenetic factors to produce different outcomes. To determine the biomarkers in the MIN model, Dr. Cardiff suggested that critical events on a morphological level must occur early in the life of the cell, although it is not clear what the critical events are.

Can Indole-3-Carbinol (I3C)-Induced Changes in Cervical Intraepithelial Neoplasia (CIN3) Be Extrapolated to Other Food Components?
Karen Auborn, Albert Einstein College of Medicine

Karen Auborn, Ph.D., Head, Phytochemical Research, Associate Professor, Microbiology and Immunology, Albert Einstein College of Medicine, North Shore Long Island Jewish Institute for Medical Research, Manhasset, New York, presented information on I3C and CIN3, with a focus on information that can be extrapolated to other bioactive food components (BFCs). I3C is found in cruciferous vegetables, which have been shown in epidemiological studies to reduce the risk of breast and prostate cancers. Studies in transgenic mice have shown that I3C reduces breast, endometrial, and cervical cancer. In a mouse model for cervical cancer, for example, I3C reduced cervical abnormalities and ultimately cervical cancer. In a small RCT in women, complete regression of CIN3 occurred after 12 weeks with I3C intervention at doses of 200 and 400 mg/day. Currently, a large RCT is being conducted in England with more than 3,000 women who are receiving I3C for cervical abnormalities.

Risk factors for CIN include HPV, estradiol exposure, and cyclo-oxygenase-2 (COX-2), which have been confirmed in transgenic mouse studies. For example, the COX-2 inhibitor Celecoxib reduces the incidence of cervical cancer in HPV transgenic mice. Possible I3C-affected mechanisms that reduce CIN and prevent cervical cancer include altered expression in more than 100 genes; induction of phase I and II enzymes; modulation of estrogen metabolism; induction of G1 cell cycle arrest; induction of apoptosis; alterations in estrogen signaling; down-regulating or preventing activation of NF6B; and inducing an endoplasmic reticulum stress response.

Various other BFCs, besides I3C and its metabolite di-indolylmethane (DIM), reduce the risk of CIN or cervical cancer alone and in combination. For example, DIM and genistein synergistically increase apoptosis and decrease estrogen signaling in C33A cell lines. I3C also increases the ratio of the estrogen metabolites 2-hydroxyestrone and 16∀-hydroxyestrone (16∀-OHE) favorably by reducing the amount of 16∀-OHE, which is present in high levels in cancers of the cervix. In addition, genistein and omega-3 polyunsaturated fatty acids (n-3 PUFAs) increase the conversion of androgens to estrogens through different mechanisms, and with the addition of I3C, the ratios become more favorable.

Other mechanisms of BFCs activity that may be important in reducing the risk of CIN3 include the reduction of NF6B by DIM and genistein, and the reduction of COX-2 and other inflammatory processes by n-3 PUFA. The addition of I3C to genistein, DIM, or n-3 PUFA positively impacts multiple processes and reduces CIN3 risk.

What Do Diet-Induced Alterations in Colorectal Polyps and Aberrant Crypts Indicate for Risk?
Mike Wargovich, University of South Carolina

Michael Wargovich, Ph.D., Director, Chemoprevention Program, South Carolina Cancer Center, School of Medicine, University of South Carolina, Columbia, described the importance of testing future diet-derived chemopreventive agents in high-risk groups, with colorectal cancer as the focus. One of the current problems is that the biomarkers used to determine efficacy in chemoprevention trials are not the same biomarkers that may be used to identify individuals at high risk of colorectal cancer. Dr. Wargovich described a model for the continuum of colorectal cancer, which includes the sequential progression of normal crypts, aberrant crypt foci (ACF), Beta-catenin Accumulated Crypt Foci (BCACF) to adenomas, and stressed the rarity of this progression (i.e., from 10^7 normal crypts to 1-2 adenomas).

Aberrant crypt foci (ACFs) have been defined as "focal areas of dysplasia", evidenced by methylene-blue staining of fresh or whole mounted colonic mucosa. In favor of recognizing these ACFs as preneoplastic or precancerous lesions are the following: 1) they are induced by most colonic carcinogens, 2) they exhibit some, but not all, of the common mutations in genes identified as central to colon cancer, 3) they show good overlap with anatomic sections of the colon at risk for polyps and cancer, 4) they have proven to be markedly efficient markers for dietary agents and pharmaceuticals that have cancer preventive activity.

The strongest chemopreventive agents for the inhibition of ACFs in the colon are the non-steroidal anti-inflammatory drugs (NSAIDS) sulindac, indomethacin, and ibuprofen. Among dietary-derived products, the garlic compound diallyl sulfide (DAS) and curcumin have shown some promise, as have the dietary supplements quercetin, silymarin, and ginseng.

ACFs are histologically similar in humans and animals. Two recent studies have described a technique known as high-magnification chromoendoscopy that allows viewing and intervention of ACFs during colonoscopy. Studies in Japan have been investigating BCACFs as a potential biomarker for adenoma. Dr. Wargovich described studies in his laboratory that show proliferation and dysplasia are increased in BCACFs more than in ACFs; paneth cells are involved; BCACFs respond to COX-2 inhibitors; and they have mutations in the beta-catenin gene. A study of green tea intervention in AOM APC^{Min}-treated mice indicated that green tea suppressed beta-catenin, and hence, tumor formation.

For maximum utilization of biomarkers in prevention studies, it would be ideal to find in a panel of biomarkers, a biomarker that is increased while another is decreased. RXR∀-depleted lesions and beta-catenin accumulated lesions may meet this parameter and be a subset of AFC. Green tea compounds re-regulate the expression of RXR∀ in the colon or increase the appearance of lesions that are more regulated.

Dr. Wargovich described a Web site that lists all agents that have been tested in an ACF panel. (See www.inra.fr for more information) The list contains dietary components and chemopreventive agents from industry, and includes information on efficacy, potency, and the effect on large and small ACFs. Conclusions from a review of studies on dietary

factors and adenoma recurrence have shown little effect among dietary factors except for tea, fruits and vegetables, and the highest level of intake of the Mediterranean diet. These population studies, however, have not been confirmed in RCTs, where modest declines in adenoma recurrence have been shown for calcium (for large polyps) and ursodeoxycholic acid (UCDA). This points out the need to conduct more studies on any adenomas or cancers that occur in prevention trials.

Discussion Session 1
Steve Clinton, Ohio State University
Discussion Leader

Steven K. Clinton, M.D., Ph.D., Director, Prostate and Genitourinary Oncology, Department of Internal Medicine, Division of Hematology and Oncology, The James Cancer Hospital and Solove Research Institute, The Ohio State University Comprehensive Cancer Center, Columbus, led the discussion for Session 1. During the discussion period, participants questioned the continued use of the mouse model rather than other animals that may reflect disease in humans more adequately. It was noted that cancer in the same organ or tissue evolves differently, even in genetically-identical animals, which makes intervention at the earliest stages much more important.

A participant asked how to increase statistical power in human prevention trials to reflect what is being found in animal studies. Dr. Wargovich responded that if researchers can identify groups that are at high risk, this would be equivalent to the mouse models that are constructed to reflect a high risk of lesions and cancer.

Dr. Milner commented that experimentally some BFCs and BFC-related drugs have been shown to be efficacious for cancer prevention, but only smaller subset have been tested in trials. There may be many more that can be identified and tested, and the issue of synergy among BFCs is still an open area of research. Dr. Auborn said that testing nutrients synergistically would require large numbers of groups of animals, and this would be a significant barrier.

SESSION 2: WHAT IS THE RELATIONSHIP BETWEEN ALTERATIONS IN XENOBIOTIC METABOLISM AND CANCER RISK?
Session Chair:
Claudine Kavanaugh, FDA

Claudine Kavanaugh, Ph.D., Office of Nutrition Products, Labeling, and Dietary Supplements, FDA, College Park, Maryland, introduced the session with a short presentation on the stages of cancer and related it to the science of chemoprevention, which focuses on the stages of initiation and promotion.

Clinical Relevance: What Do Diet-Induced Changes in Phase I and II Enzymes Tell Us About Prevention?

James Felton, Lawrence Livermore National Laboratory

James Felton, Ph.D., Senior Biomedical Scientist, Bioscience Program, Lawrence Livermore National Laboratory, California, presented information on heterocyclic amines (HAs) in overcooked food and the chemoprevention efforts, both in humans and animals, which have focused on them.

Well-done protein containing foods derived from muscle contain 1-200 parts-per- billion of mutagenic/carcinogenic heterocyclic amines depending on cooking preference. The most abundant of these compounds, PhIP, has been recently classified by the National Toxicology Program (NTP) to be: "A Reasonably Anticipated Human Carcinogen". Human exposure to these carcinogens can be reduced by not cooking meat well-done, using temperatures below 200ºC, pre-microwaving the food, turning often on the grill, and/or marinating the food. Once ingested the heterocyclic amines are metabolized by cytochrome P4501A2 to N-hydroxy or ring-hydroxy intermediates that can then be either detoxified by conjugation to phase II enzymes like UDP-glucuronosyltransferase or activated by other phase II enzymes which presumably act as leaving groups. The reactive free radicals formed during the activation bind almost exclusively to the C-8 of guanine causing DNA adducts, mutations, chromosomal abnormalities, and cancer.

There are many HAs in cooked food and there are other foods that can be eaten with HA-laden cooked food that can modulate cancer risk. HA formation is dependent on the type of food, length of cooking, temperature of cooking, and the type of food eaten with meats. Human differences in response to ingestion and uptake of these compounds most likely depend on 2 major things. One, individual genotypic differences in the activities of the Phase I and II enzymes, and DNA repair enzymes, and two, the dietary modulation of the enzyme levels by natural inducers in our food. Studies have also shown that absorption is important for calculating the internal dose. For example, eating well-cooked chicken with pasta reduces the excreted urinary mutagens compared to eating well-cooked chicken without pasta.

Flavonoids reduce the mutagenic activity of HAs, possibly by inhibition at the P450 active site, as well as the mutagenic activity of 2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Studies in Wistar and Gunn rats that do not have UDP-glucuronyltransferase (UGT) have different PhIP profiles. Gunn rats also have more DNA adducts in the liver and colon than Wistar rats when UGT is lacking, which suggests the importance of UGTs in detoxification of PhIP. This interpretation is supported by metabolism studies in mammalian cells transfected with human genes. Accelerator Mass Spectrometry-based dosimetry indicates that PhIP preferentially binds to rat prostate tissue and DNA, as well as activating the estrogen receptor making the overall picture more complex.

Xenobiotic Metabolism Relevance to Cancer

Roderick Dashwood, Linus Pauling Institute–Oregon State University

Roderick Dashwood, Ph.D., Professor, Linus Pauling Institute, Oregon State University, Corvallis, reviewed xenobiotic metabolism's relevance to cancer and the history of research that helped develop an understanding of mechanisms and processes that have been applied to cancer prevention. Some of the early studies focused on "blocking" agents that alter xenobiotic metabolic activation and protect against DNA damage. For example, indoles and cruciferous vegetables modulate Phase 1 enzymes, such as cytochrome P450; whereas compounds such as isothiocyanates are able to induce Phase 2 enzymes to detoxify carcinogens and eliminate them from the body. A third class of compounds, the "suppressing agents," prevented initiated cells from progressing to neoplasia. Historically, some of these compounds were considered a "double-edged sword" because it was known that some blocking agents that act on cyctochrome P450 can protect against some procarcinogens, but enhance the metabolic activation of other more direct acting compounds. To illustrate, rats treated with green tea have induction of cytochrome P4501A2, which metabolically activates HAs such as PhIP but can slow the detoxification of some drugs.

Animals treated with I3C also can illustrate the "double-edged sword" of nutritional compounds. In animals fed I3C along with aflatoxin B-1, a potent carcinogen, I3C blocked aflatoxin B-1 in the liver and prevented hepatocarcinogenesis. If the animals were treated with aflatoxin B-1 and discontinued use before treating with I3C, it was found that I3C acted as a potent cancer promoter in the liver. There are many more instances of dietary chemopreventive "blocking" agents acting as a "double-edged sword."

"Suppressing" agents are now some of the most promising agents being studied for chemoprevention. For example, the retinoic acid receptor (RAR) is bound to the retinoic acid response element in the promoters of the genes that are modulated by "suppressing" agents. It binds with various enzymes, including histone deacetylase (HDAC), to prevent access to transcription factors. This is over-simplified, but the key element here is that the binding of agonists causes a conformational change. It is known that acetylation and deacetylation of histones is disturbed in cancer cells, and that some HDAC inhibitors can turn on repressed genes and induce apoptosis in cancer cells. HDAC inhibitors in the diet include butyrate, diallyl disulfide (DADS), and sulforaphane and are the focus of promising chemoprevention research. Studies in colon and prostate cell lines and animal studies with sulforaphane have shown increased inhibition of HDAC and high levels of acetylated histones associated with the *p21* promoter.

It may be important for cancer prevention researchers to rethink weak ligands, such as HDAC inhibitors, in their research. Cancer cells seem to be more responsive to these agents than are normal, non-transformed cells. The basis of this is unknown, but some evidence indicates that oxidative stress may be a key factor. These compounds in the diet may be allowing normal cells to respond to external stimuli appropriately.

"Suppressing" agents are now some of the most promising agents being studied for chemoprevention, due to their actions *post*-initiation. There is growing interest in the epigenetic mechanisms, including possible effects on histone acetylation. It is known that acetylation and deacetylation of histones is disturbed in cancer cells, and that some histone deacetylase (HDAC) inhibitors can turn on repressed genes such as *P21* and *bax* and induce apoptosis in cancer cells. HDAC inhibitors in the diet include butyrate, diallyl disulfide (DADS), and sulforaphane. Studies in colon and prostate cell lines and animal studies with sulforaphane have shown increased inhibition of HDAC and higher levels of acetylated histones associated with the *P21* promoter.

It may be important to rethink the significance of 'weak ligands', such as the dietary HDAC inhibitors sulforaphane and DADS, and their impact on cancer chemoprevention via chromatin remodeling. Cancer cells seem to be more responsive to these agents than are normal, non-transformed cells. The basis of this apparent selectivity is unknown, but some evidence indicates that oxidative stress may be a key factor. These compounds, through modifying HDAC activity, might allow normal cells to respond most effectively to external stimuli and toxic insults (see M.C. Myzak et al *Carcinogenesis* Nov 2, 2005).

Discussion Session 2
Chung S. Yang, Rutgers, The State University of New Jersey
Discussion Leader

Chung Yang, Ph.D., Professor II and Associate, Chair, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, presented a few slides to reinforce the purpose of this session. The key point of the session was that the inhibition of carcinogen activation or enhancement of carcinogen elimination is expected to reduce cancer risk. Different carcinogens, however, may be affected differently by a specific dietary chemical, and dietary chemicals may affect many other factors that could decrease or increase cancer risk. A caution to these statements is that biomarkers developed based on inadequate or inappropriate studies could be misleading.

Dr. Yang used the example of epigallocatechin gallate (EGCG) to illustrate that chemoprevention research has done well in showing some of the mechanisms-of-action for dietary factors, but does not help one to understand clearly, which are generally relevant in animal and human models. In addition, it is not clear which *in vitro* study results can be extrapolated to animal or human situations. Issues of dose and mixtures of bioactive food components (BFCs) also are questions that need to be addressed in the future.

A participant added that there may be differences between acute administration of chemopreventive agents and chronic administration. Other participants commented on the complication of determining the frequency of eating in humans, which occurs in "waves" rather than through constant intake during the day, and understanding the number of compounds that could modify the same protein or mechanism.

Dr. Yang asked Dr. Dashwood if there is a bioassay for histone acetylation status that can assess modifications after a meal. Dr. Dashwood responded that these compounds are just being considered for human trials and there are some assays that can be used, for example, immunoblotting of white blood cells to assess histone acetylation status.

A consideration of microbial influence in colonic digestion was discussed regarding potential interference with HA excretion. This could affect the results of HA research because of the variability seen in human and animal trials with the same intake of HAs. This concept will have an impact, especially in the study of colon cancer. In studies of phytochemicals, it is known that what is eaten can affect the bacterial metabolism; it may be useful to know what effect phytochemicals have on bacteria.

A participant commented on butyrate and its ability to stimulate the growth of normal epithelial cells. Studies have shown that a specific *ras* mutation determines the response of the cells to butyrate. Also, multiple HDACs are expressed in the colon (e.g., HDAC1 and HDAC3) and are expressed as the cells migrate through the colonic mucosa surface and are inhibited by HDAC inhibitors.

SESSION 3: WHAT DOES DNA (OXIDATIVE) DAMAGE AND REPAIR INDICATE ABOUT CANCER RISK?

Session Chair:
Harold Seifried, DCP, NCI

DNA (Oxidative) Damage and Repair?

Lynnette Ferguson, University of Auckland, New Zealand

Lynette Ferguson, D.Phil. (Oxon), D.Sc., Head, Discipline and Nutrition, Department of Nutrition, University of Auckland, New Zealand, mentioned the large number of potential anti-carcinogens in the diet and the possible synergy that occurs; testing these will not be a trivial exercise. She reviewed the evidence for alcohol as a factor in reducing cardiovascular disease, and related results from a recent study on methods of serving alcohol (shaken or stirred martinis) that showed shaking a martini decreases the amount of oxidants in the mixture. Her presentation focused on the effect of oxidative stress and reactive oxygen species (ROS) produced through normal metabolic processes in the body and consequent DNA damage. The signature issue in ROS research is how to interfere in this process, and what biomarkers can be identified and measured to show that an intervention has been successful.

Oxidative damage occurs in lipids, proteins, and DNA, and there are many causes for the increasing levels of damage, including aging, exercise, protein and lipid peroxidation, signal transduction, proliferation, and apoptosis. In addition, inflammation has long been known to be associated with cancer and recent studies have identified ROS as participants in the inflammatory process. There is, however, no effective validation study on antioxidants and cancer.

Dr. Ferguson reviewed the role of exogenous dietary antioxidants (i.e., free radical scavengers) and endogenous enzymatic cellular defense mechanisms that inhibit pro-oxidant enzymes and inducers of endogenous defenses. Dietary sources of anti-oxidants have been studied by measuring plasma levels, although it is unclear what levels are protective against cancer and whether plasma measurements are indicative of tissue levels. Timing of ingestion of dietary anti-oxidants also appears to affect their protective value. Measurement of excretory products as biomarkers is used to imply cellular levels, although this may not be an accurate assumption; high urinary levels could indicate either higher levels of DNA damage or lower levels of DNA repair. It may be more accurate to measure at the cell or tissue level, but the question remains how to get as close to the biologic event as possible to have the biomarker accurately predict cancer.

Determining tissues that could be used as biomarkers needs to be relatively noninvasive, such as exfoliated cells from the oral cavity, peripheral blood lymphocytes, or exfoliated cells from bodily fluids. For example, the use of buccal swabs has shown some promise although it is difficult to capture good cells with this method. Newer assays (e.g., single cell gel electrophoresis assay [also known as the COMET assay] and 24 Color fluorescent *in situ* hybridization [FISH] assay) are being applied to biomarker studies because they show DNA breakage clearly. The COMET assay also is being used for peripheral blood lymphocytes to identify high-risk individuals in a study in New Zealand being conducted by Dr. Ferguson, although there is no validation included in the study (on selenium supplementation). It is hoped that validation can occur as a follow-up study at the end of this trial. [Editors note: The OECD is actively pursuing an international effort to standardize and validate the COMET assay]

Discussion

Following the presentation, Dr. Ferguson expanded her description of the selenium supplementation trial in New Zealand. At present, there are approximately 100 trial participants, 25 of whom may develop prostate cancer. This would give enough statistical power to make some definitive statements about selenium and cancer, and biomarker status. She described promising results from the study of buccal swabs regarding biomarkers. She also said that it is important to identify different biomarkers for different stages of pre-neoplasia and cancer. A participant noted that in his experience, the COMET assay is very good for showing DNA damage, and caspase-3 is appropriate for showing apoptosis; terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL) assay appears to indicate both occurrences. This makes the point that a panel of biomarkers which detect many endpoints with numerous dietary factors may be the study goal.

What Is the Clinical Significance of Diet and Changes in Oxidative Markers?
Robert Russell, Tufts University

Robert Russell, M.D., Director, Jean Mayer USDA Human Nutrition Research, Center on Aging, Boston, Massachusetts, presented information on the clinical significance of diet and changes in oxidative markers on cancer risk. The apparent paradox of a protective

effect of dietary antioxidants against oxidative DNA damage versus the procarcinogenic effects of antioxidants seen in certain intervention studies could indicate that DNA damage is not a good biomarker. However, it is noteworthy that there have been no prospective epidemiologic or intervention studies performed showing that modification of oxidative DNA damage by antioxidants correlates with a lower incidence of invasive cancer.

Other biomarkers used for antioxidant purposes include 8-hydroxyguanine in urine, comet assay in lymphocytes, serum levels of antioxidant nutrients, and "total" antioxidant capacity. To illustrate, the COMET assay has been used in a study of DNA strand breaks in peripheral lymphocytes after carotenoid intake; it shows that there is a substantial reduction of DNA strand breaks. This does not necessarily mean that there has been a decrease in cancer risk.

Dr. Russell reviewed the epidemiologic studies of ∃-carotene and lung cancer that show positive outcomes for cancer risk. Intervention trials, however, have raised doubts about these outcomes. For example, the Alpha-tocopherol, Beta-carotene (ATBC) cancer prevention study, which was one of the first intervention trials using ∃-carotene, showed that high-dose intake of ∃-carotene increased cancer risk, although follow-up studies showed that this higher risk disappeared over time. An RCT of antioxidants to prevent second primary cancers in head and neck cancer patients also saw increased cancers among users of ∀-tocopherol supplements; risk was reduced after withdrawal of the supplement. Studies in humans on supplementation with antioxidants provide evidence that reductions in DNA base damage may be dose related and transient.

Dr. Russell described biochemical and structural characteristics that may explain some of the pro-oxidant activities of ∃-carotene. In a ferret model of smoke-exposed animals, there was an increase in squamous metaplasia in animals exposed to smoke and ∃-carotene, but also in animals only exposed to ∃-carotene. Subsequent studies comparing the lung cells of smoke-exposed ferrets to those not exposed showed that the formation of carotenoid breakdown products was three-fold higher in the lung cells of smoke-exposed ferrets. This indicates that the free radical-rich environment in the lungs of the exposed animals leads to conditions that cause the destruction of the ∃-carotene and the production of retinoid-like compounds that interfere with the metabolism of retinoic acid and retinoid signaling. In addition, it causes increases in P450 enzymes, c-*fos*, c-*jun*, cell proliferation, and decreases in retinoic acid and RAR∃. Further studies also indicated that the dose was very important in creating the environment for metaplasia in the lungs of the ferrets, with evidence of metaplasia at all dose levels, but more significant metaplasia as the dose of ∃-carotene increased.

Dr. Russell concluded that there are a lot of areas of antioxidant research that have produced inconclusive results. It may be that biomarkers for DNA damage and repair have not been validated or tested for predictive value for human disease. Many of the assays used currently are not appropriately standardized and validated and those that are, have not been tested for predictive value. The take home message is clearly that the dose is instrumental in understanding the actions of antioxidants. More research is needed in

designing prospective and intervention studies, standardized assays, studies on cancer that are site specific, a better understanding of genetic variations, and mechanistic studies.

Discussion

Following the presentation, Dr. Russell clarified that in the ATBC study, the incidence of lung cancer fell in the follow-up period, although the participants continued to smoke. This was not seen in the Carotene and Retinol Efficacy Trial (CARET) study, which strongly suggests that the follow-up should continue in order to determine what may have caused the decline seen in ATBC. He reiterated that his group is studying Vitamin E, Vitamin C, and Selenium to try and quantify at what dose they may become pro-oxidants. It is evident that this phenomenon occurs with both ∃-carotene and Vitamin C, but results are not specific for the other supplements.

A participant commented that polyphenols given with a large dose of vitamin C increase the anti-oxidant properties of the polyphenols. Dr. Russell responded that his lipid-based assay shows total anti-oxidant capacity and can be adjusted to determine what dose remains anti-oxidant and what dose becomes a pro-oxidant. Part of the problem of assessing whether antioxidants play a chemopreventive role in cancer is that there has not (until recently) been an appropriate assay for measuring true antioxidant capacity or performance *in vivo.* The assays being used to test total antioxidant capacity in blood have shown almost no contribution of fat soluble nutrients, such as vitamin E and carotenoids. This is due to the fact that these assays are performed in water soluble systems; thus the contribution of fat-soluble antioxidants, such as carotenes or vitamin E would be minimal.

Common Sites of Action in Oxidative Damage
Henry Thompson, Colorado State University

Henry Thompson, Ph.D., Professor and Director, Cancer Prevention Laboratory, Colorado State University, Fort Collins, reviewed the common sites in oxidative damage, including preclinical biomarkers. Considerable evidence exists indicating that oxidation of nucleic acids can play a causal role in the carcinogenic process. Because of its propensity for attack by reactive oxygen species, products of guanine oxidation have been the most extensively investigated and the mutagenic potential of guanine oxidation products has been characterized. Of particular interest is evidence that 8-hydroxy-2-deoxguanosine (8-oxodG), the most prevalent promutagenic oxidation product of guanine, can give rise to G to T transversion mutations in key genes known to be involved in the development of cancer. Collectively, these observations provide a basis for the hypothesis that the concentration of 8-oxodG in genomic DNA is a biomarker for cancer risk.

If looking at oxidative damage as a marker of cancer risk, it is important to understand what to measure; oxidation products seem the most logical as compared to antioxidant levels, ROS, or downstream events. The most important issue is causality rather than

association. 8-oxo-2'-deoxyguanosine (8-oxodG) is an oxidation product that can be measured and it has a potential relationship to carcinogenesis. In addition, it appears to predict causality. Other predictive measures of causality include increased cancer incidence, oncogene activation or loss of activity of tumor-suppressor genes (TSGs), and guanine:thymine transversion mutations in the codons that are relative to the oncogenes or TSGs having a change in function.

Dr. Thompson reviewed enzymes associated with 8-oxodG and animal studies that show the association of lung cancer, adenomas, and increased levels of 8-oxodG. In fact, tumorigenesis increases at multiple cancer sites with increased levels of 8-oxodG. While the appeal of measuring concentrations of 8-oxodG is strong, unanswered issues related to sample processing, lingering problems with adventitious oxidation of guanine, and the amount of sample required for analysis indicate that there is need for additional methodological work before widespread use of this analyte as a cancer risk biomarker can be considered. There are at least two protocols—one enzymatic and one chromatographic procedure—recommended by the European Standards Committee on Oxidative DNA Damage (ESCODD) to measure 8-oxodG levels. ESCODD protocols take into consideration that the half-life of 8-oxodG is approximately 11 minutes. It is likely that past experimental studies have not met this time limit, which may have led to some of the equivocal findings. There is, however, a need for more research on the measurement of 8-oxodG and other biomarkers.

Assessment of who would benefit from anti-oxidant supplements is one of the key issues in biomarker research. Dr. Thompson discussed a study in his laboratory on 270 women at risk for breast cancer with a range of 8-oxodG levels. The study will try to assess the level of 8-oxodG that reduces or increases the risk of breast cancer. Another study is trying to assess whether anti-oxidant effects are conditional (the anti-oxidant conundrum). He suggested that it would be important to know an individual's steady state level of anti-oxidants or whether it is more important to know how the individual responds to episodic exposures of oxidative stress. This can be investigated using the COMET assay, single cell gel electrophoresis analyses of DNA damage. The comet assay, while lacking in specificity, does offer the advantage of reduced levels of adventitious oxidation of DNA, the requirement for small amounts of sample, rapid sample processing, and the ability to measure both steady state levels of DNA oxidation as well as DNA damage sensitivity and DNA damage repair capacity. Despite existing questions about comet analysis methodology, as well as assay calibration and validation, the application of this approach for cancer risk assessment merits serious consideration, which could result in a useful panel of biomarkers for answering some of these questions

Dr. Thompson summarized his presentation as showing that strong evidence is emerging about a causal relationship between specific oxidative damage products and carcinogenesis, and that the comet analysis approach has advantages, but needs to be validated. In addition, more attention should be given to determining abnormal rather than basal levels of anti-oxidants, to considering the conditionality of anti-oxidant effects, and to investigating how to gauge risk relative to the response to episodic oxidative exposures.

Discussion

A participant commented that he is having trouble with the comet assay because the reagents being used do not appear to be standardized. He suggested the establishment of a repository for comet assay reagents. Another participant added that it is important to have standardized reagents and a standardized scoring method for comet assay results. ESCODD has published a few papers on these topics. [Editor note: The OECD is actively pursuing an international effort to standardize and validate the COMET assay]

Discussion Session 3
Steven Zeisel, University of North Carolina–Chapel Hill
Discussion Leader

Steven Zeisel, M.D., Ph.D., Chairman and Professor, Associate Dean for Research, American Institute for Cancer Research/World Cancer Research Fund International, Distinguished Professor, Department of Nutrition, University of North Carolina at Chapel Hill, chaired the discussion session and asked "What can scientists say about biomarkers and how can research needs be identified for biomarkers that the NCI can focus on in the future?" He asked for comments on these issues.

A participant commented that for example, in a knock-out mouse system, if an anti-oxidant was used that decreased levels of 8-oxodG and a carcinogenic response, this would be a valuable step toward validation of 8-oxodG as a biomarker At this point, the system is not good enough to satisfy surrogate biomarker needs. Dr. Zeisel responded that it may be possible to further develop the COMET assay and a related biomarker panel that could be useful. Dr. Milner asked if it was possible to have a panel of markers that can be applied to more than one cancer site. Another participant commented that it is unlikely that lymphocytes will be exact replicas of the target tissue of interest in all cases. If researchers are going to continue to rely on RCTs to answer questions that may result in a health claim, a different approach must be developed.

A participant asked if repositories of blood or lymphocytes from past cohort or prospective trials could be used to test biomarkers. Dr. Mayne said that none of the samples have been collected for oxidative biomarkers specifically and for that reason would not likely be very useful. A collection method that allows stable biomarkers to be collected from future clinical trials is needed and a few past trials have done this. Samples have been collected from the Selenium and Vitamin E Cancer Prevention Trial (SELECT), but doing this has been very expensive.

Dr. Prentice added that it would be easier to make a claim about properties of an agent (e.g., it has anti-oxidant properties), than make a specific claim for reducing risk for a disease. Dr. Zeisel agreed and noted that it would be easier to claim that something lowers the existence of DNA adducts rather than decreases cancer risk. Dr. Dashwood added that the lessons of the past have led researchers to understand that bioassay of urine and DNA damage are potentially flawed and it is better to use protein changes, such

as small changes in redox-sensitive thiols and proteins that are regulating signaling pathways rather than bioassays. This tends to lead to the conclusion that the oxidative stress area, rather than the DNA damage area, may be more important.

Dr. Zeisel responded to a participant requesting clarification about the contradictory research results on 8-oxodG-knockout enhanced carcinogenesis, which suggest that oxidative stress leads to cancer. Although intervention studies with anti-oxidant nutrients suggest that this is not confirmed, he said that the balance is to find an intervention that keeps DNA from being damaged to protect against cancer in the long term, versus affects that have to do with cells that already have been initiated and the repression of those initiated cells. Anti-oxidants may use different processes to push initiated cells toward cancer, but simultaneously, protecting uninitiated cells from DNA damage. This means that help is needed to do a better job of identifying those individuals with no pre-existing DNA damage, if such individuals exist. Determining a panel of gene changes that can identify those individuals is a key research strategy. Such a panel should be able to pick up the effects of both low dose and high dose interventions.

A discussion of what tests would be on a panel to measure oxidative biomarkers resulted in several suggestions from participants. These included the COMET and TUNEL assays, measuring isoprostanes, and lipophilic-hydrophilic anti-oxidant capacity tests to establish the background baseline of individuals. Dr. Milner asked panel members to further explain specificity in responses and the anti-oxidants. Dr. Zeisel replied that different anti-oxidants protect different compartments of the cells and that it is unlikely that one anti-oxidant will protect all of them. A system that includes hydrophilic and lipophilic anti-oxidants may be best. Dr. Milner questioned whether the anti-oxidant panel being suggested could measure across cellular compartments. Dr. Zeisel responded that a panel could be developed for each. A participant added that serum or urine samples cannot tell the researchers the origin (i.e., compartment) that the biomarker came from.

WEDNESDAY, JULY 13, 2005

SESSION 4: WHAT DO SHIFTS IN INDICATORS OF PROLIFERATION, DIFFERENTIATION, AND APOPTOSIS INDICATE ABOUT THE CANCER PROCESS?

Session Chair:
John Milner, DCP, NCI

John Milner, Ph.D., Chief, Nutritional Sciences Research Group, DCP, NCI, NIH, Bethesda, Maryland, welcomed participants to the second day of the workshop. He introduced the topics for the day and reviewed the following main points from the previous day's presentations and discussions:

X There are 25,000 bioactive food components (BFCs) in the diet and all must be considered for a comprehensive nutritional strategy to reduce cancer risk.

X A few of the most promising BFCs include the 5,000 plus flavonoids and the isothiocyanates. Components of the diet other than plants, such as conjugated linoleic acid and fungi, should be considered, but were not discussed.

X Metabolism in microbes in the human body as well as the human cells themselves should be considered in the study of metabolomics.

For the Session 4 agenda, Dr. Milner listed the fundamental questions as: 1) determining which of the fundamental processes associated with cancer—cell proliferation, differentiation, and apoptosis—is the most important for explaining the response to BFCs; 2) which of these processes occurs first; and 3) what concentrations of BFCs are needed to lead to these changes.

Cell Proliferation

Len Augenlicht, Albert Einstein Cancer Center

Leonard Augenlicht, Ph.D., Professor and Associate Director, Department of Oncology, Albert Einstein Cancer Center, Montefiore Medical Center, Bronx, New York, presented information on pathways involved in cell proliferation. Because diet is so important for proliferation, it can be used as a probe to understand the cancer process. In the colon, the colonic mucosa will undergo 10^{12} cell divisions and only one of them will be involved in producing cancer. Geographic distribution of colorectal cancer shows the effect of diet and lessens the possibility that it is primarily a genetically-determined disease. Dr. Augenlicht reviewed genetic models (e.g., APC with *p21* or *p27* knock-out mice) of colon cancer that indicate a Western diet is a stronger risk factor for colon cancer than genetics. One key factor in these mouse models is that the phenotype of tumors in mouse models is dependent on diet. In addition they develop phenotypic lesions heretofore only observed in humans and at incidence and temporal rates more similar to the human case.

The recently developed "new Western" diet increases colon tumor formation in every mouse model investigated. When calcium and vitamin D are added to this diet, suppression of tumors occurs and there is a shift in gene expression toward normal. On the gene expression chip used for these experiments, there are 28,000 genes with 136 functional groups. Of particular interest are the functional groups that are involved in transcription and the regulation of translation. Changes that occur with changes in diet indicate that biologic pathways are changed by changes in diet. For example, changes in lipid pathways in mice on the "new Western" diet, which is high in fat, occur with the addition of calcium and vitamin D, although the fat content of the diet remains the same. This indicates an interaction of calcium and vitamin D with lipid metabolism. The same is true for calcium homeostasis, glucose pathways, and apoptosis pathways as reflected by increases in NF6B, as well as other pathways associated with cell cycling. The key pathway regulated by APC is Wnt-signaling, which is the main cause of tumor formation. When the "new Western" diet is compared to the control; the risk for tumor formation increases, as ∃-catenin and TCF-4 expression levels go up; when calcium and vitamin D are added to the diet, ∃-catenin and TCF-4 expression levels go down.

As colonic cells migrate to the top of the colonic epithelium, *c-myc* is important because it regulates *p21* expression and changes as the migration occurs. This influences the maturation pathways of colon cells. Sulindac, which has shown promise as a colon cancer chemopreventive agent, requires *p21*, but not *p27*, for its action. Further studies will need to be conducted to determine the exact association between Sulindac and *p21*, but it appears that a better understanding of this mechanism may give a better picture of the interactions among dietary and genetic components of colon carcinogenesis.

Discussion

A participant asked what the folate content was for the "new Western" diet. Dr. Augenlicht responded that the levels were relatively equivalent to normal human dietary intake as determined for humans without additional supplementation. Dr. Milner asked if maturation rate of colon crypt cells is a better biomarker for what is occurring than differentiation in the colon. Dr. Augenlicht said that this is true for colon cancer, and it has been shown experimentally in a variety of mouse models.

Differentiation
Leena Hilakivi-Clarke, Georgetown University

Leena Hilakivi-Clarke, Ph.D., Professor of Oncology, Georgetown University, Washington, DC, discussed mammary gland differentiation as a potential marker of diet-induced alterations in breast cancer risk. She reviewed morphological and epidemiological findings associated with breast cancer. It has been proposed that high estrogen levels during pregnancy may increase the risk of breast cancer, especially in women over 30 years of age.

Studies in rats have shown that high leptin levels and obesity also increase breast cancer risk. In a 7,12-dimethylbenz[a]anthracene (DMBA) rat model, dams exposed *in utero* or prepubertally to estradiol show opposite mammary tumor incidence; *in utero* exposure to DMBA increases risk and prepubertal exposure reduces risk. In addition, *in utero* exposure to DMBA increases the number of terminal end buds (TEBs) while prepubertal exposure reduces the number of TEBs. The same is true for cell proliferation in the mammary gland; *in utero* exposure to DMBA reduces apoptosis but prepubertal exposure induces it. Caveolin-1 is a trans-membrane protein expressed in differentiated cells and acts as a tumor suppressor in breast tissue. This protein down regulates several genes associated with breast cancer risk, including *ER-∀, Src, Akt, ras, ERK1/2*, and *ErbB2*. *In utero* exposure to DMBA reduced caveolin-1 expression while prepubertal exposure increased caveolin-1 expression; *in utero* exposure appears to have no effect on *BRCA-1* or *p53* expression in postnatal mammary glands.

Diet can modify estrogen levels early in life. For example, a high-fat diet and obesity increases circulating estrogen levels; alcohol stimulates aromatase; and genistein, other phytochemicals, and some heavy metals can increase circulating estrogens. Studies in humans have shown that high soy intake during childhood and adolescents can reduce

breast cancer risk. Animal studies have also shown that genistein exposure during the same life stage reduces tumorigenesis. Animal studies on vitamin A have indicated that either an excess or deficiency increases mammary tumorigenesis. Studies on flaxseed are equivocal. In the DMBA rat model, prepubertal genistein exposure reduces mammary tumorigenesis, possibly through increased caveolin-1 expression; it also increases *BRCA-1* expression.

Dr. Hilakivi-Clarke described an investigation of prepubertal dietary exposure to n-3 polyunsaturated fatty acids (PUFAs) and the response to mammary tumorigenesis in rats. Results indicate that prepubertal exposure to low or high n-3 PUFA reduces the number of TEBs; exposure to a low fat fish oil diet reduced later breast cancer risk; and exposure to a high fat fish oil diet increased later breast cancer risk. These findings also suggested that prepubertal exposure to the low fish oil diet reduced cell proliferation and induced apoptosis in TEBs, increased the expression of caveolin-1, and reduced the expression of *BRCA-1*. The conclusion of these investigations suggests that differentiation *per se* is not a consistent biomarker of reduced breast cancer risk in TEBs.

Discussion

During the discussion period, Dr. Hilakivi-Clarke responded to a question on whether the use of a different strain of rat could have yielded different results since Fisher rats, for example, are resistant to DMBA. She said Sprague-Dawley rats, which are more susceptible to breast cancer than some other strains, were used in her experiments, but she thinks similar results would have been seen in comparable rat strains. Dr. Milner commented that this brings up the issue of timing and dose in differentiation as well. Dr. Hilakivi-Clarke responded that it would be better to use a panel of markers to understand the full effects of exposure to BFCs or other agents.

Apoptosis
Priscilla Furth, Georgetown University

Priscilla Furth, M.D., Professor of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, discussed biomarkers of apoptosis. To measure apoptosis, one must look at the cellular and biochemical processes that govern apoptosis and histological characteristics of apoptotic cells such as condensation and fragmentation. Apoptotic cells are sloughed off into the lumen or are engulfed by phagocytic cells. Genetic pathways that govern apoptosis also can be used as biomarkers. Extrinsic apoptotic pathways include death receptor families on the outside of the cells; intrinsic, or mitochondrial-driven pathways are regulated by a family of proteins known as the *bcl* family and are inside the cells. Both pathways coalesce on the caspase family to complete the apoptotic process.

The most useful techniques supply both detection and quantification of apoptosis as it is not the presence or absence of apoptosis that must be determined in studies of cancer progression, but rather the change in the relative amounts of apoptosis. Historically, biomarkers of apoptosis have been identified visually by electron microscopy. Current

biomarkers are identified by hemotoxilin and eosin (H&E) staining, which can indicate the rate of apoptosis, but may be less accurate because it is observer dependent. Techniques for tissue culture may be less laborious than staining and counting apoptotic cells. There are techniques that can distinguish between apoptotic and necrotic cells, although this is not currently available for use in biopsy tissue.

A new technique, reflectance confocal microscopy (RCM), uses laser light to image the nuclear structure and allows one to identify specifically stained apoptotic cells and fix them in formalin, or use them for gene expression assays. Dr. Furth described experiments in a mouse model using RCM to determine the rate of apoptosis. She also described laboratory research techniques for examining human tissue to identify apoptosis using 3'-end labeling and gel electrophoresis in conjunction with the. **T**DT-mediated d**U**TP **N**ick-**E**nd **L**abeling (TUNEL) assay to derive an apoptotic index that could be useful as a biomarker.

These techniques allow us to see gene expression changes in small samples and can focus on the *bcl* family or other extrinsic or intrinsic pathways of interest. Because there are 20 to 30 *bcl* family members, it is important to identify specific changes in each member of the family and find the family member most relevant for the cell type and situation being examined. The caspase family is a logical target for biomarker assessment because this family is downstream from both the extrinsic and intrinsic pathways. Dr. Furth said that there are inhibitors of caspases (e.g., X-Linked Inhibitor of Apoptosis Protein - XIAP) that modulate apoptosis that must be considered in any assessment of caspase activity. For example, XIAP expression levels give some prognostic information for determining if apoptosis is occurring in a tissue. Dr. Furth commented that the most useful biomarkers of apoptosis would be ones that could be measured in serum. Some progress is occurring in this area. For example, cytochrome C and cytokeratin 18 are serum biomarkers being investigated for apoptosis although the assays for these markers would need to be sensitive enough for use in cancer prevention.

There is little clinical trial data on apoptosis biomarkers in humans and those researchers who have measured apoptosis indicate there is no significant increase in apoptosis for studies of solid tumors. The conclusions from all data suggest that apoptosis may not be the only pathway to cell death that could be used as a biomarker.

Discussion

A participant commented that in relation to tumors, apoptosis may not correlate to the response to chemotherapeutic agents. It is unclear whether the cancer stem cell is undergoing apoptosis and is being specifically detected, rather than measuring apoptosis in the general tumor cell population, which can be high in tumors. Another factor to be considered is that there is a distribution of mitochondrial membrane potentials in tumors,. Dr. Furth agreed that only measuring rates of apoptosis in many cases will not be a sufficient measure of therapeutic response as non-apoptotic pathways of cell death may be activated as well.

A discussion of the amount of cytochrome C or other markers of apoptosis in the serum resulted in clarification of the possible origins of such markers that may be related to apoptosis or nutritional exposures. Timing also was discussed and Dr. Furth commented that timing issues are an important area that can be addressed in animal models.

Discussion Session 4
Clinton Grubbs, University of Alabama–Birmingham
Discussion Leader

Clinton Grubbs, Ph.D., Director, Chemoprevention Center, Department of Surgery, University of Alabama at Birmingham, led the discussion for Session 4. He commented that it is important to determine the effect of the dose of a therapeutic agent or BFC on biomarkers, and whether biomarkers can be used in the clinic to monitor the intake and exposure level of a dietary supplement. His final point was that trials are not designed just to measure a biomarker related to a cancer. In studies of dietary supplements, different pathways, cancer sites, and organs are going to be affected; this means that researchers should look at the whole organism while they investigate biomarkers.

A participant commented that it may be relevant to consider the difference in apoptosis in tumor tissue treatment and in prevention. Also, the time to assess apoptosis may be shortly after the carcinogenic process begins, rather than later in the process. Dr. Furth responded that apoptosis is greater in therapeutic events than in prevention. In prevention one might see much smaller rates of apoptosis and a researcher would have to look for small changes, but this is still meaningful.

Dr. Milner asked if a biomarker panel that measured each of the three processes discussed in this session—proliferation, differentiation, and apoptosis—might have better than a 70 percent rate of success, the rate currently seen for any of them individually. Dr. Augenlicht responded that, at least for the intestinal mucosa, apoptosis is at a low level and may not indicate tumor formation. A discussion ensued about the need to investigate as many situations and biomarkers as possible to obtain as accurate an assessment as possible and not limit the focus to the markers we expect to be important.

Dr. Furth commented that there is a need for protein and gene expression markers for differentiation that fundamentally examine differentiation. Dr. Hilakivi-Clarke added that markers of normal tissue may be different from markers of early or later changes in the cancer process. Dr. Augenlicht said that identifying and measuring signaling pathways that are regulating and coordinating processes may be beneficial.

A participant asked if genetic or other biomarkers of early detection, such as ACF or polyps in the colon, can be used in the clinic. Dr. Augenlicht responded that the technology is available so it is unnecessary to look for only one marker. Current gene expression profiling allows the identification of patterns that could be used for early detection. They are not yet widely available in the clinic, but may be in the future. The question is, from what point of view of polyp or crypt origin in the past, can it be measured with some assurance that future disease can be predicted.

SESSION 5: EMERGING NEW AND PROMISING BIOMARKERS

Session Chair:
Sudhir Srivastava, DCP, NCI

Sudhir Srivastava, Ph.D., M.P.H., Branch Chief, Cancer Biomarkers Research Group, NCI, NIH, Bethesda, Maryland, introduced the session by describing the state of biomarkers research. Ho commented that since 1998, only two protein biomarkers have been listed by the FDA. Many biomarkers are being discovered, but very few have been validated, and even fewer have demonstrated clinical applications. He called for renewed efforts in this area.

PSA and Prostate Cancer: A Case Study

Ian Thompson, University of Texas Health Science Center

Ian Thompson, M.D., Chairman, Department of Urology, Health Science Center, The University of Texas at San Antonio, said that more then 75 percent of men in the United States have had a PSA test, with almost 50 percent having one yearly. Interestingly, as far back as 1971, publications discounted PSA as a biomarker for early diagnosis of prostate cancer due to poor specificity. Originally, PSA was used to monitor patients during treatment for prostate cancer. In the 1990s, the Prostate Cancer Prevention Study (PCPT) tested finasteride and found it prevented some prostate cancer and decreased PSA values in treated patients. Interestingly, the original report from PCPT investigators indicated that the incidence of high-grade prostate cancer was higher in the finasteride group. The recent update reviewed the PCPT data and showed that this effect disappeared over time. Dr. Thompson felt this was due to the fact that the earlier results were based on for cause biopsies but the later end of study results were more randomly distributed through out the patient population

He also noted that in a recent update of the PCPT of 5,587 men with a prostate biopsy, a Gleason score higher than 7 was correlated with a negative prognosis and response to therapy. This update also showed that PSA had a sensitivity of approximately 25 percent; to obtain a sensitivity of over 90 percent, a PSA less than 1.0 would have to be used. In this case, biopsy using the Gleason scale would be the only way to predict prostate cancer accurately and the number of biopsies required for this would be prohibitive.

Dr. Thompson provided unpublished results of the analysis that suggested PSA distorts prostate cancer detection and grade of disease. A summary of results from PCPT suggests that for predicting prostate cancer, absolute PSA, family history, digital rectal exam (DRE), and a prior negative biopsy are independent variables; there is no predictive value for age or PSA velocity. For predicting high-grade prostate cancer, absolute PSA, DRE, prior negative biopsy, and age are predictive; family history and PSA velocity are not predictive.

A study from the San Antonio Early Detection Research Network (NCI) cohort found that obesity, as determined by BMI quintile increases PSA levels. Dr. Thompson described the Southwest Oncology Group (SWOG) 8794 study from 1987 that compared observation only to adjuvant radiotherapy in men with prostate cancer who underwent radical prostatectomy. The trial used metastasis-free survival as an endpoint. Very little difference was seen between the intervention and control groups. In addition, quality of life indicators in the adjuvant radiotherapy group were significantly worse than those of the observation group. Dr. Thompson showed data to indicate that if PSA response alone had been used as an endpoint in SWOG 8794, it would have shown a 50 percent greater increase in metastasis-free survival, which would have been a significant finding. This illustrates one of the most significant problems with PSA as an endpoint; if the data on PSA had been used for clinical decision-making (50 percent increase in survival), clinicians may have dismissed the differences in quality of life although survival was not positively affected.

The conclusions from the presentation are that PSA is not a good biomarker for prostate cancer, it should not be used for population screening, more clinically-meaningful endpoints should be used, and without these endpoints, actual harm may be occurring to patients depending on solely on PSA for medical decision making.

Discussion

During the discussion period, Dr. Thompson offered an explanation of why biopsies on men taking finasteride showed more high-grade disease. Of the 600 radical prostatectomy patients in PCPT, biopsy found about 50 percent of the high-grade disease; finasteride, which shrinks the prostate; this shrinkage allowed 70 percent of high grade disease to be detected. This is an ascertainment bias. Another participant asked about the usefulness of the free-to-bound PSA ratio as a biomarker. Dr. Thompson responded that in patients with a high PSA who have a biopsy, there is little advantage to the ratio, although there is some improvement in detection.

Dr. Srivastava asked whether PSA doubling time or velocity are good biomarkers. Dr. Thompson responded that neither is very useful. Dr. Allan Kristal, NCI, added that in dietary studies, high-dose calcium supplement users have a much lower PSA velocity than nonusers, but epidemiologic studies associate high-dose calcium supplementation with prostate cancer risk. This is another example of how using PSA as a biomarker can give researchers an indicator of possible outcome that is the opposite of actual occurrence.

What Are the Links Between Mammary Density and Cancer Risk?
Carol Fabian, University of Kansas Medical School

Carol Fabian, M.D., Director, Breast Cancer Prevention Center, Professor, Medicine, Division of Hematology/Oncology, University of Kansas Medical Center, Kansas City, discussed mammographic breast density (MBD) as a biomarker in prevention trials. She

reviewed the Gail and Tyrer-Cuzick breast cancer risk models. She noted while discriminatory accuracy is higher for the Tyrer-Cuzick model than for Gail, discriminatory accuracy, useful for individual risk prediction, is suboptimal for both models. Risk biomarkers may improve discriminatory accuracy. For clinicians, there are four well-defined reversible biomarkers in use for breast cancer: 1) serum bioavailable estradiol (E2) in postmenopausal women; 2) serum IGF-1 and IGF-1/IGFBP-3 (i.e. binding protein) in premenopausal women; 3) MBD; and 4) breast interepithelial neoplasia (IEN).

MBD is a risk biomarker for both ER+ and ER- cancers in pre- and post-menopausal women. It can be obtained at no additional risk and minimal extra expense from women age 40-70 currently undergoing regular screening mammography, and in addition it is directly reflective of breast events. Breast density in excess of 75% is associated with up to a five-fold increase in risk of developing breast cancer compared with no risk. MBD also is positively associated with other risk factors or risk biomarkers, including IEN, serum IGF-1 and growth hormone in premenopausal women, and family history of breast cancer. It is negatively associated with the protective factors of IGFBP3 in premenopausal women, early pregnancy and multiparity. MBD, however, may not be an optimal risk biomarker in BRCA-1 and BRCA-2 mutation carriers, elderly, or obese women. Tice et al have recently published a study in which mammographic density in combination with the Gail model modestly improved discriminatory accuracy compared to Gail risk alone.

Dr. Fabian described random periareolar fine-needle aspiration (RPFNA) that is used to sample breast tissue for cancer risk assessment. Adequate cytology is achieved in 95 percent of high-risk women, which makes this a useful procedure for procuring samples. Atypia by RPFNA indicates a five-fold increase in risk of breast cancer, and RPFNA cytomorphology in combination with the Gail model improves discriminatory accuracy over that observed with the Gail model alone.

It has been shown that tamoxifen modulates various breast cancer risk biomarkers, including reducing the incidence of hyperplasia, atypical hyperplasia, proliferation, serum IGF-1, and mammographic density (especially in premenopausal women). Tamoxifen does not decrease levels of serum E2 and actually increases it in premenopausal women. Results from the International Breast Cancer Intervention Study (IBIS) indicates tamoxifen reduces both breast cancer incidence and mammographic density, but reduction in risk is more than expected from reduction in density. Six months of letrozole, an aromatase inhibitor which is known to be effective in reducing risk of contralateral breast cancer in adjuvant trials, was not associated with reduction in breast density in a Phase II trial. There was, however, a decrease in Ki-67. Studies of breast density and dietary factors have found that a low-fat diet may reduce breast density in premenopausal women, but not postmenopausal women, despite the observation that a low fat diet appears to reduce the incidence of contralateral breast cancer. Studies of soy and genistein, have produced mixed results.

Dr. Fabian concluded by summarizing findings on breast density that suggest reductions in density in premenopausal women may signal a reduction in breast cancer risk. However, not all effective agents reduce density or reduce density in postmenopausal as well as premenopausal women. When evaluating a new risk reduction strategy in a Phase II trial, change in breast density should not be used as the sole response endpoint. If mammographic density is the primary endpoint, it is important that studies be conducted on women with an existing density of 25 percent or more.

Discussion

A participant asked about the relationship between breast density and the use of mammography. Dr. Fabian responded that the higher the density, the higher the chance of a false negative mammography finding because high density makes more difficult to see small cancers. In addition, biopsy rates are higher in women with high breast density than in women with low breast density.

Genomics, Proteomics, and Metabolomics
Stephen Barnes, University of Alabama–Birmingham

Stephen Barnes, Ph.D., Pharmacology/Toxicology, University of Alabama at Birmingham, discussed genomics, proteomics, and metabolomics regarding biomarkers. He suggested that a significant challenge in biomarker research is to use and interpret the overwhelming amount of data provided by genomics, proteomics, and metabolomics research effectively. There needs to be an integrated model to take advantage of the research, and researchers may need significant help from engineers. He reviewed the differences between "classical" and "quantum" analysis and how quantum phenomena emerge from the analysis of complex systems. Approaches to using data should be multi-dimensional, spatially resolved, and have strict standards for collection and use. Systems biology may be a useful tool for associating data with the information that is needed.

The Heisenberg Uncertainty Principle in physics can be extrapolated to data by analogy: The more features of microarray genomic and metabolomic methods one has, the less one can be certain that what one sees has meaning. In the past, only one protein could be studied at a time and a researcher could know everything about it after years of study. Today, with methods such as multidimensional protein identification technology (MudPIT), it is possible to study as many as 60,000 proteins at a time. This allows the collection of large data sets that create a problem for analyses because multiple conclusions can be drawn from the data set. For progress to continue in proteomics, the proteome needs to be defined, a better understanding of differential expression of proteins in different cell compartments must be developed, and one must measure the proteome with a high degree of quantitative accuracy.

The study of metabolites and metabolomics has been made possible by identification and understanding of the chemical nature of proteins and enzymes in cellular processes. Technologies for assessing metabolites include nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography mass spectrometry (LC/MS). NMR can

measure approximately 100 metabolites simultaneously. Newer technologies such as LC/MS, can assess more than 5,000 metabolites simultaneously. LC/MS assessment has led to problems with handling large amounts of data. Mathematical models are being developed to use these large amounts of data more effectively.

Dr. Barnes concluded that the integration of genomics, proteomics, and metabolomics will be essential for predictive interpretations in translational research. A major challenge will be to understand more complex time- and spatial-resolved matters. For integration to occur there must be collaborations with mathematicians, engineers, and experts on control systems.

Discussion

A participant asked how often samples should be taken to address the time issue, and what sensitivity and specificity issues exist. Dr. Barnes responded that these issues are yet to be resolved. Dr. Srivastava asked what the NIH should be doing to address collection procedures and timing. Dr. Barnes replied that it is time to collect enough data to eliminate the uncertainty that will increase if researchers continue to focus on new technology. A participant added that technology is moving very fast and it is difficult to be able to stop to ask questions about the biology that is needed to make a difference in applying knowledge to the clinic. Dr. Barnes said it would be nice to have customized arrays that target the questions being asked. There is a need to target biologic pathways of interest for whatever research agenda is followed. Additional studies increase the difficulty of interpreting the data.

The Influence of Genetic Polymorphism
David Hunter, Harvard University

David Hunter, Sc.D., Professor and Director, Program in Molecular and Genetic Epidemiology, Department of Epidemiology and Nutrition, School of Public Health, Harvard University, Boston, Massachusetts, discussed the influence of genetic polymorphisms in nutrient metabolism. The major issues in nutrition research are: understanding variance in disease risk, identifying subpopulations at higher risk, and translating findings to the public. The major challenges are that many dietary constituents are hard to measure observationally, biomarkers of intake are not always available, and significant between-person differences in metabolism exist.

Dietary studies have produced some consistent findings, such as the consistent association between the intake of red meat and colorectal cancer. He reviewed some of the polymorphism research on N-acetyl transferase (NAT) phenotypes that may account for part of the increase in colorectal cancer associated with the intake of red meat. Because there are other substances in the mixture of compounds interacting with colonic cells, and polymorphisms for many of the genes that determine individual susceptibility, it is prudent to understand the substrates of the enzymes as key factors in determining cancer risk.

Polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene have been studied with regard to diet. MTHFR is involved in one-carbon metabolism pathways involving methionine and folic acid, which affects DNA methylation. Dr. Hunter reviewed the pathways and presented information on MTHFR polymorphisms and odds ratios for colorectal cancer with alcohol and folic acid intake. With alcohol exposure, depending on the MTHFR polymorphisms present, colorectal cancer risk is increased or decreased based on resulting homocysteine and folate levels. Implications for these findings are that alcohol and folate influences on colorectal neoplasia risk are altered by a common functional MTHFR polymorphism, uracil mis-incorporation and/or genome-wide methylation which may be integrated markers of both dietary exposure and susceptibility. He felt that studies are needed to link these with risk directly. In addition, implications for dietary advice for disease prevention should include information about genotype. Diet-gene interactions play an important role in determining cancer risk with dietary factors.

Other issues for nutrition research are consistent with what has been discussed in previous presentations. These include the refinement of biostatistical methods for pathway analysis that will be needed to cope with the inherent complexity of pathways, as well as the increasing ability to measure multiple polymorphisms related to biomarkers.

Discussion

A participant commented that if a dietary factor or other agent has a toxic effect, people can identify their genotype (e.g., flushing with alcohol intake). In relating this to cancer, people do not know their genotype and researchers may not know what the effect is until many years into the future. Dr. Hunter recognized that chronic exposure and cancer have a long latency. This speaks to the need to have better biomarkers of exposure. A participant asked if there are privacy issues regarding biomarker clinical trials. Dr. Hunter responded that this is not an issue if the data are anonymized, even if placed on a Web site.

Dr. Milner reviewed a study on PPAR(and fatty acids that shows some individuals benefit from a higher intake of n-3 PUFAs and some individuals benefit from a higher intake of n-6 PUFAs. He asked whether it is possible that this is related to a SNP or polymorphism. Dr. Hunter agreed that this hypothesis is probable and is being investigated for cardiovascular disease, but he did not know if this could be translated into individual advice. Another participant asked whether it is now possible to determine risk related to genotype without having to do clinical trials. Dr. Hunter thought this may be possible in the future, but no area of research can do this at present.

Discussion Session 5
Bill Go, University of California–Los Angeles
Discussion Leader

Vay Liang (Bill) Go, M.D., Professor of Medicine, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, provided context for the study of biomarkers for clinical nutrition from the 1970s to the present. Over time, the focus has shifted from monitoring therapy to diagnosis to screening. There now is a better understanding of the role of diet and exercise in modifying disease. The use of genomic and proteomic methods is making biomarker research more accessible. In the discussion period, Dr. Go asked the speakers to focus on how the biomarkers can be used, the type of tissue to be assessed, and what analyses and technology can be used and standardized. He asked members of the panel to focus on breast cancer first, and colon cancer second.

Dr. Fabian stated that for breast cancer, it is critical to understand the nature of cancerous tissue and the unique morphology that can be assessed to assure that one is seeing the tissue that will become cancerous. Studies should investigate how breast density and serum change over time, along with the effects of multiple polymorphisms to get a clear idea of normal and high-risk individuals. Re-education of NIH study sections might allow the submitting of grant applications that propose to study this issue which is more observational and not hypothesis driven.

Dr. Hunter commented that it is possible to do a study on a limited number of focused genetic variants that are well characterized on the same pathway, but it is difficult to obtain enough statistical power to assure validity of the data. He stated that the reasonable costs of assaying SNPs make it alluring to include them in studies, but it is not clear if there are enough solid hypotheses to rationalize this inclusion. It is unlikely that one biomarker will tell everything about a specific cancer.

Dr. Barnes emphasized that the goal is cancer prevention. The question has not been able to be answered regarding what happens to the cancer between initiation and overt disease. Signals being sent out by tumors can be modified by diet and these might be used to assess how well the pre-tumor or tumor is being modified.

Dr. Fabian added the perspective of the patient, who wants to know what he or she should do to avoid getting breast cancer, heart disease, or other diseases. At this time, the issues are: 1) whether biomarkers exist that can be used to inform a patient that he or she is at high risk, and 2) reinforcement of good eating habits to prevent (or lessen) disease in general. It also is important that clinicians understand what they should be telling their patients. A participant responded that some tests on patients are very aggressive, such as fine-needle aspiration. Dr. Fabian agreed that women in her study on breast density are willing to make simple changes—take a supplement or exercise more—but may not be willing to take drugs with side effects or undergo invasive tests. Because there are no biomarkers for diet and physical activity in the study of breast density, this becomes a research question.

Dr. Barnes used the example of smokers to show that even after clear evidence exists for negative consequences related to lifestyle, many people will not make changes. It is difficult to see changes in diet even if the evidence for cancer prevention is strong.

Researchers are looking at biomarkers as markers only, rather than as a representation of a mechanism that is undergoing change in the body. This may be a serious problem for acceptance in the wider scientific community. Dr. Srivastava responded that for early detection, biomarkers are accepted in the wider scientific community.

Dr. Srivastava discussed the role of biomarkers in nutritional science and how they can assist in the design of studies. He described the Early Detection Research Network (EDRN), an NCI initiative established in 2000 to develop, test, and validate promising biomarkers. Information on the EDRN may be found at http://www3.cancer.gov/prevention/cbrg/edrn/. EDRN has a 5-phase paradigm to bring biomarkers from development to testing in clinical trials, of which the EDRN is responsible for phases 1 to 3.

LEAD DISCUSSION RELATED TO ENTIRE WORKSHOP/GOALS
John Milner, DCP, NCI
Discussion Leader

Dr. Milner clarified that this meeting is a collaboration among NCI, ODS, and the FDA to determine the state-of-the-science in biomarkers. There has been a lot of progress, but there is a long way to go to make full use of this science. He recognized that few good markers exist to assess exposure to the approximately 25,000 BFCs. There are three types of biomarkers: (1) biomarkers of exposure; (2) biomarkers of effect; and (3) biomarkers of susceptibility. All three types of biomarkers need to be studied in different capacities.

Something that was repeated during the workshop was the need to understand timing and when intervention should begin. In addition, it will be critical to determine what biomarkers to use to indicate whether the intervention has been successful. Many studies of biomarkers and BFCs have focused on therapy rather than prevention. It is likely that these are two different areas for research and will demand different biomarkers and different levels of sensitivity and specificity. Finding out when to intervene, and what type of intervention is needed, are key to successful prevention. Another key point is the need for research on the concentrations of agents that are likely to give maximum benefits and still be dietarily achievable.

Dr. Milner reviewed the six processes that are modified in cancer and there are a lot of nutrients that can modify each of these components.

Dr. Go added that when determining if a biomarker can do what it is purporting to do, it may be beneficial to piggyback a biomarker evaluation onto a clinical trial. Dr. Milner said that there is a U54 program at NCI that is designed to examine genetic pathways that are associated with cancer. NCI needs to find out if these pathways lead to cancer outcomes. Dr. Srivastava added that he has been interacting with the FDA in the areas of treatment and critical pathways for the Investigational New Drug (IND) process. In cancer diagnostics, the FDA is setting up a critical pathway for biomarkers. Dr. Milner

commented that he has been meeting with FDA representatives to try to identify some issues for prevention, especially relevant biomarkers and how they are used and misused.

A participant commented that there is no downside to telling the public to eat a healthy diet and increase the amount of fresh fruits and vegetables, whole grains, less sugar, less salt, and less soda. This is a message everyone agrees may reduce the incidence of heart disease, diabetes, arthritis, osteoporosis, and cancer. Dr. Milner agreed that there is more specificity in response and that broad-based public health messages are exceedingly important. He added there is a need to develop a nutritional preemption model that says certain people are vulnerable and need to increase the levels of one food or another. One size does not fit everyone; there must be multiple messages and they may not be simple.

Dr. Prentice commented that there may need to be a meta-analytic approach where one looks at the agreement between a biomarker and a cancer endpoint across a range of interactions. The issue is how to get to this endpoint. Dr. Milner said this is a good point and there may be a need to convene a group to discuss a systems approach to some of these issues, especially if reducing cancer risk causes an increase in another disease state.

Dr. Swanson, ODS, said that she has been encouraged by what has been presented at this workshop and maybe the approach toward biomarkers has been too simplistic. She felt there may be a need to change the way research is conducted in this area. Dr. Milner said that from what has been discussed at this workshop, there is a need to develop more collaboration, possibly using a systems approach to refine how some of these things can add to the knowledge of predictive value. This will include a substantial effort in bioinformatics.

Dr. Clinton summarized what he saw as the major points from the workshop.

X In the past two decades, under the leadership of Dr. Greenwald and NCI, there have been some important clinical trials that have provided definitive data about cancer prevention. One of the issues made clear by hindsight is that these trials could have provided a greater benefit if samples had been collected that could be used for the "-omics" era.
X There is a need to collect samples, properly store them and create a system that make it easier for partners to use these samples.
X There is a need to develop a cooperative group that has a prevention focus similar to those that are developed for therapeutic trials. Most of the therapeutic groups have established prevention subcommittees, but there is no focus that puts prevention in the forefront.

Dr. Greenwald provided a review of the three types of biomarkers. Research on biomarkers of exposure in the nutrition field has been inadequate for what is needed for substantial progress in the field. Understanding time of exposure (e.g., time of day and time in a person's life cycle) is a largely unexplored area that needs attention. There must be a large investment in the nutrition field to understand intake and exposure. For biomarkers of effect, enough is not known to give advice, although those biomarkers

could be used to prioritize nutrition trials. Biomarkers of susceptibility have probably been approached in too simplistic a manner.

As for the establishment of a new cooperative group on prevention, Dr. Greenwald explained that it is difficult to get approval for groups that focus on more than one disease, which is what biomarker and nutrition research will have to include. This area is open to developing a partnership with the FDA.

Dr. Greenwald discussed two areas that have not been discussed at the workshop, training and peer review. At this point, a system for training scientists may not be adequate for the science of the future. There is so much specialization today that it is difficult to produce enough scientists with an interdisciplinary focus, without losing the benefit of original thinking of individuals. For improving the process of peer review, it is critical that researchers with a biomarker and nutritional focus volunteer for review groups.

SUMMARY AND FINAL DISCUSSIONS FOR PUBLISHING WORKSHOP PROCEEDINGS, INCLUDING FUTURE RESEARCH NEEDS

Harold Seifried, DCP, NCI

Dr. Seifried reviewed the main points from the workshop and reviewed highlights and recommendations from the presentations and discussions. The main points included the following.

- X Animal models are needed for the identification of biomarkers. New models need to be developed and biomarkers from other programs (NCI and NIH-WIDE) should be acquired and evaluated.
- X The use of transgenic and knock-out models needs to be expanded into the nutritional arena, with creation of nutritional models.
- X The selection of agents for study needs a concerted effort based on epidemiologic and clinical studies, with issues of dose response included in any research effort.
- X Alternative animal models need to be developed using other species, such as the ferret, woodchuck, dog, and others as have been used is specific disease models.
- X Although mouse models have given insight into many of areas in biomarker research, there is a need to expand the number of animal models.
- X Genetic and chemical models need more research emphasis in the biomarker arena.
- X Biomarker discovery and evaluation in the area of prevention needs a heightened focus.
- X Most of the foci of current research are on treatment, which is important, but prevention needs attention and can benefit from a close association..
- X Every attempt should be made to identify changes that begin the process of carcinogenesis; identifying changes at their earliest will provide potentially greater benefits.
- X A battery of biomarkers can provide a potential “fingerprint” of disease.

- X Bioinformatics systems are needed that are sophisticated and user-friendly to efficiently handle the size and complexity of the database needed for biomarker research.
- X Centralization of tissue biorepositories is needed, with samples from all applicable NCI clinical and intervention trials included.
- X Data needs to be put into a database that is accessible and contains adequate security to protect trial participant identification.
- X Standardization of laboratory procedures and verification of findings can improve reproducibility.
- X There is a need for specificity markers and whether a change in a preneoplastic lesion is an indication of a general response or whether is it indicative of a specific cancer cell.
- X There is a need to understand what changes identified through biomarkers mean to the overall carcinogenic process.
- X A critical issue is the importance of characterizing the normal state as well as the disease state.
  Biomarkers are temporally variable as the disease progresses. There are biomarkers for monitoring compliance with intervention whether it is lifestyle, behavioral, or pharmaceutical.