November 2003 Issue | Eleanor Rogan, PhD The Eppley Institute

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Welcome to *Functional Medicine Update* for November 2003. I think you will find this an exceptionally interesting issue.

The 11th International Symposium on Functional Medicine will focus on one of the most prevalent of the chronic, age-related disease complexes—type-2 diabetes, insulin resistance, and hyperinsulinemia—the coming pandemic. Mark your calendars for this event, which will take place May 11-15, 2004. You will receive an announcement in the mail soon about this event. We have scheduled world-class plenary speakers, and I believe you will find the workshop materials, the "news-to-use" portion of the program, helpful in integrating the concepts from the plenary sessions into clinical practice.

An article on glycyrrhizin recently appeared in *The 0Lancet*.[1] Glycyrrhizin is a constituent of licorice, whose history of use goes back thousands of years into traditional Chinese medicine. There has probably been more phytopharmacology done on the constituents of licorice than on any other natural product. An active component of licorice root, glycyrrhizin, has pleotrophic effects. One effect, described in the *Lancet* report, is its ability to suppress replication of the SARS-associated coronavirus. You may have heard about this effect, as the story was picked up in the wire services.

Investigators assessed the anti-viral activity of a variety of traditionally used pharmaceutical agents, including ribavirin, 6-azauridine, pyrazofurin, and mycophenolic acid, as well as glycyrrhizin. These *in vitro* tests were done against clinical isolates of coronavirus FFM-1 and FFM-2 from patients admitted with SARS to a university medical center in Frankfurt, Germany. Of all the substances tested, glycyrrhizin was the most active in inhibiting replication of the SARS-associated coronavirus, suggesting it should be assessed for use in the treatment of SARS.

Antiviral Effects of Glycyrrhizin
This virus-inhibiting effect is not without precedent, as previous reports from patient trials indicated glycyrrhizin was effective in improving outcome in HIV-1 and hepatitis C virus infections. The low concentrations of P24 antigens seen in patients with HIV who were given glycyrrhizin, i.e., licorice concentrate, were attributed to its ability to have a gene-regulation effect on the upregulation of tumor-specific or viral-specific cytokines.

In fact, a variety of studies on the mechanism of glycyrrhizin have shown that it can influence cellular signaling pathways such as protein kinase C, and transcription factors such as activator protein-1 and nuclear factor Kappa B (NF-κB) expression. Furthermore, glycyrrhizin and its aglycone metabolite 18β glycyrrhetinic acid have demonstrated the ability to upregulate the expression of inducible forms of

nitric oxide synthase and the production of nitric oxide from macrophages as one of the microbiocidal "killing agents" produced by the immune system.

Glycyrrhizin may not be the preferred method for treating SARS, but the *Lancet* study demonstrates that the plant kingdom contains agents, historically used as herbal medicines, which contain a variety of anti-viral compounds that can suppress replication of viruses. Interestingly, these investigators found glycyrrhizin was effective during the early stages of virus replication in inhibiting absorption and penetration of the virus. It appears that it is better to give it early rather than later. In acute infection, it may not be as effective.

Another bit of information that is relevant to this month's focus in *FMU* is connected with hormone-related cancers, particularly of the breast and prostate. This may appear to be a big step from the topic of viral replication, but we will look at some extraordinary emerging work concerning estrogen metabolism and its relationship to hormone-related cancer of the breast and prostate. This topic cuts across a wide range of activities. It is not just cancer in which these metabolites can play a role. That role may also be related to atherosclerotic risk; it may have effects on insulin resistance and insulin sensitivity. We may be looking at a mechanism that cuts across many chronic, age-related diseases.

In reference to hormone-related cancer of the breast and prostate, a paper recently appeared in the journal *Molecular and Cellular Biology*, titled "Transposable Elements: Targets for Early Nutritional Effects on Epigenetic Gene Regulation."[2] You may recall that the phenotype of the organism, the so-called phenome, describes the way the organism looks, acts, and feels, as well as its health patterns. The phenome is the compilation of the complex interaction between genes and environment. That interaction affects the modulation of genetic expression and proteomic activities. Ultimately, metabolism and the interaction of complex signaling pathways control function. That is the model we have been developing in *FMU* for several years.

Production of Epigenetic Effects
Once a substance is produced (or transcribed) from a gene, it can undergo effects called epigenetic, which means those effects occur after the genetic transcription has occurred. These can be things like phosphorylation, sulfation, oxidation, glycation, and methylation. These epigenetic modifications of things like proteins and nucleic acids can regulate the ultimate function of that system. Outcome is based not simply on what comes off the genes in and of themselves; it is also based on what might occur after the genetic transcription process has taken place through what are called epigenetic relationships.

Why is this of interest clinically? We cannot change the genes of a person who has a gene coding for certain susceptibility factors. Instead, we change the way that gene is expressed, through modification of various environmental factors.

Turning Genes On and Off
Some of those environmental factors may elicit changes in the way the genes can be turned on or off, meaning gene-silencing effects, or gene- activation effects. One epigenetic gene-silencing effect is the process of methylation, which we have frequently discussed in *FMU*. Methylation is the transfer of a carbon with three hydrogens, a methyl group, from S-adenosylmethionine (SAM) to a receiving molecule to create a methylated derivative. We will discuss this further with our Researcher of the Month on Side 2 of this issue.

Methylation is controlled by and interrelated with the folate cycle, through SAM, and through methylating enzymes. One site of recipients of methylation is the genes themselves, which are selectively methylated and silenced so certain genes very specifically are not available to be read. It is like putting bookmarks on the pages that cannot be read in the book of life. One does not want to read all the messages in one's book of life all the time. Some negative messages are locked into that book, and we may prefer to keep them silent. Methylation is an important part of controlling, epigenetically, the way the body regulates expression of function.

Mouse Obesity Study

In the paper on transposable elements, researchers in the Department of Radiation Oncology at Duke University Medical Center used an animal that has been modified to be genetically obese—the "agouti" mouse. This mouse harbors a transposable element of the agouti gene associated with genetic factors that lead to obesity. The researchers wanted to know what would happen if they took the yellow-colored mouse that had a genetic predisposition toward obesity, and gave the pregnant mother high doses of the methylating nutrients—folate, B12, B6, betaine, thiamin, and riboflavin—that would support and stimulate the folate cycle through SAM.

Doses given were 4 to 20 times higher than that contained in normal mouse chow to prevent mice from having insufficiencies. The results showed that high-dose supplementation of these nutrients in the mouse chow administered to these genetically modified animals led to offspring whose phenotypes were totally different. The phenotypes of these offspring were correlated with increased methylation of specific regions of their genomes.

Changing Phenotype through Supplementation

Think of the implications for modifying certain functions we might consider hard-wired into our genes, such as obesity, cancer, or arthritis. If you could silence some of the genes that express the patterns and the phenotype of these disorders by selective supplementation or selective nutrient modulation based on the person's genetic uniqueness, you might have the potential to silence those characteristics. That is an interesting personalized medicine approach toward chemoprevention in this case, which may be an age-related disorder. It is a far cry to jump from the agouti mouse to a human, but it opens a door. From that model system might come a better understanding of the modification of risk for hormone-related cancer. By modifying the environment we might epigenetically modify the outcome of the phenotype.

As specifically related to the folate cycle, the best screening tool for evaluating the potential insufficiency of folate or its family of nutrients in the human clinical situation is plasma level of homocysteine. We have talked at length about this in *FMU*. A review in the *American Journal of Clinical Nutrition* discusses screening for vitamin B12 and folate deficiency in older persons using the methylmalonic acid test or the homocysteine test, or a combination of the two.[3]

Homocysteine appears to be very sensitive. According to the authors, in individuals with borderline vitamin concentrations, the homocysteine test may identify those at high risk for insufficiency of both vitamin B12 and folate.

One wants to look at certain markers for insufficiencies of methylation. At this point homocysteine is probably the most clinically sensitive marker available in the standard laboratory assessment. According to Dr. Kilmer McCully, concentrations in animals of 8 micromoles per liter or above probably indicate

insufficiency. Many labs have normal ranges up to 12, so there is a range of ambiguity between 8 and 12. To take a more aggressive position on homocysteine, you might start to be concerned at levels above 8.

Measuring Alterations in Homocysteine

The best way to pick up alterations in homocysteine levels in certain people is by the methionine challenge. A dose of several grams of methionine is administered orally before looking postprandially or post-challenge at the patient's plasma homocysteine. A baseline is established first, and then a post-challenge evaluation is made. In my opinion, an increase of homocysteine of more than 25 percent suggests an underlying propensity toward hyperhomocysteinemia. In other words, using the model developed by Dr. James Fries, it indicates that pathway may not have much organ reserve. In those cases, patients may benefit from higher levels of the folate-supporting nutrients

At a symposium that addressed the effects of folate cycle activity on the function of methyl transfer reactions, several international experts expressed their opinions. One of those experts was Lionel Poirier from the National Center for Toxicological Research of the FDA, who discussed the effects of aberrant DNA methylation on the risk of cancer. He explained that any individual, animal or human, who is insufficient with regard to the folate-supporting nutrients—methionine, B12, folic acid, and so forth—has significantly increased risk of carcinogen-induced injury to DNA. Methylation improves DNA stability and lowers the risk of carcinogenic injury to the DNA. Hypomethylation, low levels of methylation, has been associated with increasing risk. The article on this symposium appeared in the *Journal of Nutrition*.[4]

Diet, Methyl Donors, and DNA Methylation

Diet, methyl donors, and DNA methylation are closely interrelated. It is possible to study DNA undermethylation in lymphocytes as a way of assessing folate cycle status in that cell type. Examination of DNA methylation in lymphocytes has even been used as a screening tool in some studies with men and women to examine folate status, showing subclinical folate insufficiency is associated with hypomethylation.

Investigators are currently conducting a prospective trial in which they are following DNA hypomethylation relative to cancer incidence. The results should help us understand how this relative risk stacks up against other cancer risk factors. Mihai Niculescu and Steven Zeisel review the cancer and methylation relationship in an article in the *Journal of Nutrition*.[5]

Regulation of Liver Methionine Adenosyltransferase

The DNA methylation process has many regulatory effects on DNA expression, stability, and the folding and unfolding of histones to facilitate access to the DNA message. A lot of this process is controlled through the production of the active methylating agent, S-adenosylmethionine (SAM). That probably explains why SAM has been used in numerous conditions, including cancer prevention, depression, heart disease, arthritis, and insulin sensitivity. The folate cycle is important for signaling processes that cut across many functions.

SAM is part of the pathway of the folate cycle that cuts across the etiology and cellular physiology associated with many disorders. This pathway is described in a review paper by Corrales et. al. from the Division of Hepatology and Gene Therapy, School of Medicine, University of Navarra in Spain. The authors are particularly concerned with the way SAM and the folate cycle in generalrelates to protection

against liver disease and virally- induced liver problems. For example, increasing SAM levels is an active part of the liver protective program in immune defense.[6] SAM is an important methylating agent, and its precursor is S-adenosylhomocysteine (SAH).

Elevation in SAM and DNA Hypomethylation
A buildup of SAH suggests a block in the formation of SAM. In fact, many individuals are now saying that instead of measuring homocysteine, we should measure SAH or SAM in the blood, because SAM reveals more about potential imperfections in the folate cycle. I believe we will see the development in the clinical lab of the SAM and SAH tests, which may be more sensitive for insufficiencies in this pathway. At present, clinical labs are offering the homocysteine test, but look for measurement of SAH, elevation of which is strongly correlated with DNA hypomethylation, as a potentially important new tool in the clinical lab. In an article in the *Journal of Nutrition*, James et al. discuss this topic.[7]

DNA Hypomethylation
It appears that by supplementing with methyl donor nutrients one can increase DNA methylation, better regulate gene expression, and have an epigenetic effect on producing a better outcome in that tissue, organ, organ system, or individual. In cases of low DNA methylation, a number of signs of DNA instability may occur, such as centromeric region instability. In offspring, facial and structural abnormalities in the fetus are common.

Individuals have asked if there is a correlation between folate status and Down syndrome. Could Down syndrome, trisomy 21, be a precursor marker for an environmental factor called low folate status and folic acid cycle capability? Can women who carry the Down's type of precursor marker, or individuals who require much higher levels of these folate nutrients, overcome that genetic susceptibility? In another *Journal of Nutrition* paper, Melanie Ehrlich from Tulane Medical School discusses this question.[8]

Cellular Vitamins, DNA Methylation, and Cancer Risk
The story of gene expression, epigenetic effects, methylation, and the folate cycle is at the forefront of several interesting developments. Clearly, there must be a potential correlation among cellular vitamins, DNA methylation, cancer risk, and the association of certain methyl-dependent cancers. Methyl-dependent cancers may be related to altered patterns of gene methylation. They may also be related to altered patterns of analyte methylation, because methyl groups from SAM are also transported to things like neurotransmitters. The conversion of epinephrine/norepinephrine is a methylation process. Serotonin methylation is another part of the methylation process, with regulation of neurochemistry.

Methylation occurs at phospholipids to form phosphatidylcholine from phosphatidylethanolamine, which is needed to form proper membrane structure/function and used in myelin and cardiolipin. Methylation is important in the conversion of hormones. We will talk specifically about the estrogen metabolites and methylation with the enzyme catechol-O-methyltransferase on Side 2 of this month's *FMU*. It has significant implications for potential breast and prostate cancer risk. An article in the *Journal of Nutrition* features a discussion of cellular vitamin DNA methylation cancer risk.[9]

Folate treatment, unbalanced methylation, and changes of allelic expression were the topic of a recent discussion in *The Lancet*.[10] The results of this study suggest that hyperhomocysteinemia affects control

of gene expression, which can be reverted by folate treatment, completely consistent with the model we have been developing. This study supports the hypothesis that the toxic action of homocysteine could be mediated by hypomethylation and its epigenetic effects on an array of gene expression patterns related to such conditions as cardiovascular disease, cancer, arthritis, diabetes, depression, and Alzheimer's disease.

The question is whether we can overcome this problem in all people by simply giving higher doses of folic acid. That question is still being explored.

MTHFR Polymorphism

A number of genetic polymorphisms are related to folic acid utilization. The one that has been most studied, and which we have discussed extensively, is methylenetetrahydrofolate reductase, or MTHFR, a very common genetic polymorphism. About 12 percent of the mixed-genotype population in the United States seem to have the 677C→T homozygous MTHFR polymorphism, which has characteristics of slower methylation reactions going to 5-methyltetrahydrofolate. That can be a rate-limiting step in the formation of SAM. In those individuals, it is possible that, under certain circumstances, higher levels of folic acid would be required, even greater than would be available in a highly dense diet.

To overcome that block, these individuals might require some of the 5-methyltetrahydrofolate, which is the step beyond methylenetetrahydrofolate. The 5-methyltetrahydrofolate form of folic acid found in cells is the principal, most bioavailable form that is used in methylation reactions directly, and one that does not depend on MTHFR activity for its own activity. Folic acid itself, or 5-formyl folic acid, sometimes cannot elevate cerebral spinal 5-methyltetrahydrofolate, even after intravenous doses. In methotrexate-treated patients, 5-formyl folic acid was unable to raise levels of 5-methyltetrahydrofolate CSF. This is discussed in the *Journal of Neuro-Oncology*.[11]

There may be cases in which the polymorphism 677C→T variant modulates folate responsiveness and increases the need for 5-methyltetrahydrofolate. Considerable current research is being conducted on this topic. One study in the *Journal of Nutrition* talks about the influence of this MTHFR polymorphism on folate status in response to folate intake in women.[12]

Altered Methylation and Prostate Cancer

Let us assume that an individual has altered methylation reactions for this combination of genes and environment. Could that play a role in prostate cancer? A paper in the *Journal of the National Cancer Institute* discussed methylation and inactivation of estrogen, progesterone, and androgen receptors in prostate cancer.[13]

In this study, investigators looked at different receptors for steroid hormones. They found that some steroid receptor genes appeared to be inactivated by the cytosine/guanosine (CpG) methylation patterns in prostate cancer cells. This result suggested that altered selected methylation can influence the hormone sensitivity to things like androgens or estrogens and/or their metabolites in prostate cells.

There are many potential SNPs in the folate cycle. I talked about one—MTHFR 677C→T, but SNPs occur at many places in that pathway. They may be responsive to riboflavin, pyridoxine, cobalamin, or betaine as a methyl donor. It is not just one nutrient that controls this pathway; it is a combination and balance of those nutrients, individualized to the person's genotype

A recent editorial in *The New England Journal of Medicine* is titled “The Prevention of Prostate Cancer—The Dilemma Continues.”[14] We still do not have a handle on the question of how important the suppression of dihydrotestosterone (DHT) is in the prevention of prostate cancer. What role do androgens directly play in prostate cancer?

On side 2 of this issue, we will discuss the role of other substances in the promotion of prostate cancer. Knowing that cancer is a multi-step process, it may be that the relationship between estrogen and estrogen metabolites, and androgens and their metabolites, tips the balance toward potential prostate cancer.

Finasteride and Prostate Cancer
Prevention of prostate cancer may be related to giving a DHT testosterone inhibitor, such as finasteride, which is the classic drug. A report in *The New England Journal of Medicine*concluded, “Finasteride prevents or delays the appearance of prostate cancer, but this possible benefit and a reduced risk of urinary problems must be weighed against sexual side effects and the increased risk of high-grade prostate cancer.”[15]

Finasteride may subdue the risk of early-grade prostate cancer only to have it appear later as a fulminant form of cancer. The most significant problem has not been treated, only the early warning signs of the problem. In other words, you may have put a veil over what is going on until it is much more significant and of clinical concern. That is why the editorial that followed this study talks about the continuing dilemma.

PSA Testing
It is very important for men to be screened with routine PSA testing when they reach age 50, but anyone with prostate-related symptoms should be screened even earlier. Screening should be done serially on a routine basis because the change in relative PSA levels over time seems to be more important than the absolute number. Some numbers that are considered borderline-high stay high and do not change. Other numbers that are initially low may, while still in the normal range when next measured, be double or triple what they were. A change from .5 to 1.5, for example, is still within the range of normal, but that relative change appears to be a greater indication of risk than the absolute value. The important consideration is a combination of the absolute value (in other words, how high the number is) and the relative change. I urge all men to undergo routine serial prostate analyses on their physicals.

Two articles that discuss prostate examination appeared recently in the *Journal of the National Cancer Institute.* One is an editorial titled “Prostate Cancer and Prostate-Specific Antigen: The More We Know, the Less We Understand.”[16] An associated article is titled “Association between Genetic Polymorphisms in the Prostate-Specific Antigen Gene Promoter and Serum Prostate-Specific Antigen Levels.”[17] We do not know the whole story. PSA may not be the only measurement, but at present, it is the best clinical marker we have, and it should be used on a routine basis to get a history of the PSA levels in a specific patient.

Prostate cancer prevention trials are still showing some positive value in suppressing DHT, but we need to pay attention to a few precautions.[18] The story is more than DHT alone.

Vitamin D and Prostate Cancer

We might also be looking at cell signaling in the prostate cell. What controls genomic signaling? What might control suppression of the expression of oncogenes in the prostate gland? These are interesting questions. As we learned from our Clinician/Researcher of the Month last month, vitamin D with its cell signaling capability plays a role in this process. Research related to pathways that mediate the growth actions of vitamin D is ongoing. Vitamin D and its metabolites inhibit prostate cancer growth and prostate cell proliferation. Through 1,25 dihydroxyvitamin D3, vitamin D may play a role in preventing cell replication or keeping it at low levels in prostate cancer.[19]

Hydroxylated Estrogens and Prostate Cancer
Estrogen metabolites, the so-called 4-hydroxycatecholestrogens may be stimulators for cell proliferation in the prostate gland. They may trigger oncogenesis and relate to further amplification of cell growth with dihydrotestosterones. There may be an association between estrogen metabolites in the prostate and androgens. A study in *Carcinogenesis*, titled "Catechol Estrogen Metabolites and Conjugates in Different Regions of the Prostate of Noble Rats Treated with 4-Hydroxyestradiol: Implications for Estrogen-Induced Initiation of Prostate Cancer,"discusses this topic.[20] We will discuss this study further during the second half of this discussion.

The topic of hydroxylated estrogens and prostate cancer has been around for some time and goes back to the research of Dr. Martin Bosland at New York University. We should look not only at androgens such as DHT in prostate cancer, but also at the estrogen component.

Resveratrol and Prostate Cancer
Scientists have identified a number of natural products that favorably modify production of the 4-hydroxycatecholestrogens that may initiate prostate cancer. One compound you have heard about is resveratrol, one source of which is the skin of grapes. A candidate for prostate cancer prevention, resveratrol has a significant effect on modifying hydroxylation patterns of estrogen and it also inhibits aromatase somewhat, preventing excessive estrogen production. The discussion of resveratrol as a candidate nutritional substance for prostate cancer prevention appeared in a recent paper in the *Journal of Nutrition*.[21]

Resveratrol is present in red wine. Amounts vary from wine to wine, from 2 to 40 mmol, or from grape skins, in which it represents 5 to 10 percent of the biomass once the grape skins have been dried. It has a variety of effects related to reduction of prostate cancer risk. In fact, it may be just the tip of the iceberg of a broad class of phytonutrients with a polyphenolic structure that are valuable for inhibiting each of the many stages of carcinogenesis found in prostate cancer.

Polyphenols scavenge incipient populations of androgen-dependent prostate cancer cells through androgen receptor antagonism, and they scavenge incipient populations of androgen-independent prostate cancer cells by short-circuiting the epidermal growth factor-dependent autocrine loops. Finally, they influence the metabolism of androgens to estrogens and influence estrogen metabolism into the hydroxylated estrogens. As I pointed out from the previous study, these substances may be involved in the initiation of prostate cancer.

Resveratrol has multi-factorial influences. It has also proven useful in breast cancer because of its effect on hormonal modulation.[22] Much of the observed activity related to estrogen and its metabolites is not

necessarily focused on the estrogen classic receptor. It is away from the receptor. We will hear about other effects from our Researcher of the Month

The last nutrient I want to focus on is indole-3-carbinol (I3C), which comes from cruciferous vegetables. It is one of the glucosinolates found in the crucifers—broccoli, cauliflower, Brussels sprouts, and cabbage. Masticating the vegetable product breaks up the cell walls, releasing the plant enzyme myrosinase, which reacts with glucosinolates to liberate I3C and a variety of other phytochemicals. As an indication of dietary equivalents, two daily servings of broccoli (which would be four florets twice a day) provides about 300 mg equivalent of I3C. If one were to consume a meal containing a cruciferous vegetable twice a day (a portion of broccoli, cauliflower, Brussels sprouts, or cabbage) he or she would be ingesting upwards of 300 mg of I3C a day. Preliminary human trials indicate that dose modulates pathways that may be related to prostate and breast cancer risk.

I3C causes bax translocation to the mitochondria, inducing selective apoptotic cell death in transformed cells. By helping the cells commit suicide, it may help scavenge cells that would go on to become malignant. An article in the *Journal of Nutrition* discusses this topic.[23]Therefore, vitamin D, resveratrol, green tea catechins, and I3C may all work together.

Another part of the I3C story is the recent recognition that it helps regulate tumor suppressor gene expression.[24] Again, these are anti-proliferative signaling pathways that work away from the traditional estrogen receptor site. We will hear much more from our Researcher of the Month about these estrogen receptor-independent pathways and the risk of prostate and breast cancer.

Based on the research that has been conducted to date, I can say with certainty that if I had elevated PSA levels and was at risk for prostate cancer, I would make changes in my diet. I would eat more soy-based foods and more cruciferous vegetables. I would consume a lot more fluids and eliminate alcohol and high-fat foods from my diet. I would particularly avoid foods containing trans saturated fats. I would consume more omega-3 fatty acids and make sure I got plenty of resveratrol, ellagic acid, polyphenols from green tea, and vitamin D. These are all wise practices in light of what we are learning about cellular mechanisms.

It is time to have our discussion with our researcher of the month.

INTERVIEW TRANSCRIPT

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JB: This month’s FMU focuses on hormone replacement therapy (HRT) and the relationship of hormones to breast and prostate cancer. Just last month on its website, the U.S. Food and Drug Administration posted a release titled, “FDA Launches Collaborative Campaign to Inform Women about Menopausal Hormone Therapy.” This report discusses the advocacy of Congressman Henry Bonilla and

Congresswoman Rosa DeLauro, who have launched a nationwide information campaign to raise awareness about the risk and benefits of menopausal hormone therapy. Included in the information is the following quote from Dr. McClellan of the FDA:

“Postmenopausal hormone therapy is a major, personal decision for women, and they should be armed with the latest facts and useful tools to make the best decision for their needs. It is very important that women realize that this beneficial therapy also carries significant risks. Our recommendation is that if you choose to use hormone therapy for hot flashes or vaginal dryness, or if you prefer it to other treatments to prevent thin bones, take the lowest dose for the least duration required to provide relief.”[25]

A Shift in Official Opinion on ERT

This position concerning menopause management differs from what would have appeared on the FDA website 5 to 10 years ago. It leads to a discussion of relative risk and what the estrogen-related problems are.

We have always thought that perhaps risk from HRT was driven by estradiol, the highly mitogenic form of estrogen that causes changes in cell cycling and cell replication. Now, however, we are learning more about the story of estrogen metabolism and some of the estrogen metabolites.

There is no better person we could discuss this with than our Researcher of the Month, Dr. Eleanor Rogan, who works with a group at the Eppley Institute at the University of Nebraska Medical Center. Dr. Rogan has an impressive publication record. She has published more than 140 articles in this area. She and her group are pioneers looking at this story from an exogenous and endogenous perspective. Dr. Rogan, who is a clinical researcher and medical biochemist, has received the attention of many people in the fields of endocrinology and obstetrics/gynecology concerning the estrogen metabolite story.

Eppley Institute

Welcome to FMU, Dr. Rogan. Tell us about the Eppley Institute, your colleagues, and the evolution of this research over the last 20 years.

ER: Thank you, Dr. Bland. The Eppley Institute has been a cancer research institute for almost 40 years. My collaborator Dr. Cavalieri and I have been here for 30 of those 40 years. That time has been devoted primarily to basic research in cancer and, more recently, into clinical and drug development aspects of cancer research. There are about 25 to 30 primary faculty members here on the University of Nebraska Medical Center campus, and we have lots of interaction with the other parts of this medical center.

Research Beginnings

JB: When I look back at the work you and your colleagues have accomplished during those 30 years, I am impressed by its breadth. How did you begin this journey 30 years ago, and how did you arrive where you are today with your research on the estrogen metabolite catecholestrogen/estrogen quinone?

ER: We’ve always had the view that it was damage to DNA that initiates the multi-stage process that eventually ends up being a malignant tumor. We started out to investigate this DNA damage, and to look specifically at what are called DNA adducts—the idea that a carcinogen chemically attaches itself to DNA to do some kind of damage.

We spent many years looking at a group of carcinogens called polycyclic hydrocarbons, which are found

in all kinds of smoke or any kind of combustion—from cigarette smoke to chimney smoke, or from any kinds of fossil foods that are burned. About 25 years ago, as we studied these DNA adducts, we discovered that adducts were formed that stayed in DNA, and depending on where they attach in DNA, they stay there and are removed by repair—what we call stable adducts. You can also have so-called depurinating adducts, because when you form these adducts, that causes the bond between the base—the adenine or the guanine—to break.

Research into DNA Mutations

The bond between that and the deoxyribose breaks and they fall out, leaving what is called an apurinic site that is highly mutagenic. These apurinic sites, this kind of damage in the DNA, is what we think is generating the mutations. That process can lead to the cell's becoming malignant. We started studying these first with the polycyclic hydrocarbons.

That work with the polycyclic hydrocarbons took us close to 15 or 20 years. Interestingly, when we shifted over into studying estrogens, starting in the early 90s, we were able to accomplish that research in about five years because so much of what we had learned about the polycyclic hydrocarbons was applicable to the estrogens.

We quickly discovered the estrogen metabolites that other groups had already shown to be carcinogenic and cause tumors in laboratory animals, formed these depurinating adducts that leave the DNA. They leave behind these mutagenic, apurinic sites, whereas estrogen metabolites that don't cause tumors in laboratory animals tended to form only the stable adducts. This relationship we had seen earlier held up with the estrogens and has really guided what we've done.

Estrogen Chemistry

JB: When you look at testosterone or androgen conversion to estrogens, the A ring of the steroid nucleus is converted into an aromatic compound. Was it that chemical similarity that originally caused you to wonder about the estrogens?

ER: In part, it's that. It's also the fact that both the polycyclic hydrocarbons and the estrogens are pretty much planar molecules. They are more or less flat like a pancake, and that gives them this chemical similarity. Of course, as you commented, the aromatic ring in the estrogens is critical to their following this pathway of activation.

Pharmacogenomics

JB: That discovery must have led you to move to other areas like pharmacogenomics. For example, how do these compounds, these estradiol and estrone molecules, become hydroxylated? That leads into mixed function oxidases and cytochrome P450 (CYP). You published a number of papers looking at different forms of CYP, the 1B1, 1A1 and the 1A2 forms and their relationship. Would you describe the evolution of the story?

ER: We began looking at the enzymes involved in the activation of estrogens and also the protection of estrogens. We focused on four enzymes. One of them is the CYP 19, which is the aromatase that actually takes androgens to estrogens. That is the enzyme, by the way, that is inhibited by aromatase inhibitors, which are under trial to prevent second breast tumors in women who have already had breast tumors and are showing some effects.

So aromatase is one of the enzymes we're interested in. P450 1B1 very specifically takes estrogens and oxidizes them to a catecholestrogen at the 4 position. They're called 4-catecholestrogens. The 1A family, instead of making 4-catecholestrogens, makes 2-catecholestrogens.

Enzymes and Breast Cancer Prediction
Then an enzyme called catechol-O-methyltransferase puts a methyl group on catecholestrogen. We think of that as a protective enzyme. What we found (that actually hasn't been published yet) is that the two activating enzymes we are studying—the aromatase and P450 1B1 (that we think push activation of estrogen metabolism to make DNA-damaging forms) are higher in the breast tissue of women who have breast carcinoma. And instead, two protective enzymes—the catechol-O-methyltransferase and another enzyme called quinone reductase—are higher in women who don't have breast cancer and vice versa.

It seems that at an enzymatic level, we see that the profiles of estrogens in women's breast tissue relate to whether or not she has gotten breast cancer. We hope in the future to use this information to predict whether or not she will develop breast cancer. These findings go along with the studies of estrogen metabolites that we have published.

Inducible vs. Constitutive Genetic Factors in Cancer
JB: There is a difference between the inducible forms of CYP and the constitutive forms. Is all of this locked into the genes, or are some of these modifiable in their risk, like CYP 1B1? Is it inducible? Are some effects on COMT activities related to environmental factors? Many people share the view that cancer is hard-wired into our genes and there is little we can do about it. On the other hand, there is an emerging understanding that some factors are inducible and environmentally related.

ER: Exactly. For example, CYP 1B1, which we think is one of these activating enzymes, is highly inducible in breast tissue. I can only speculate about this, but dioxin is known to induce CYP 1B1. Dioxin is a very toxic environmental pollutant that is related to PCBs. We think, although we haven't started studying this yet, that perhaps the association that is sometimes seen between breast cancer and exposure to PCBs in the environment, might be through induction of this 1B1.

I don't believe these data are cast in stone, but there seems to be a possible association, at least in some women, between breast cancer and smoking. Certainly, some of the constituents in cigarette smoke may well be inducers of CYP 1B1. We would see that link. So I can think of two environmental links that speak to induction of some of these enzymes that can lead down the road to the development of breast cancer.

Dietary Factors in 1B1 Expression
JB: On the other side, many dietary substances appear to be modifiers or down-regulators of 1B1 expression. It may be, as Ames suggested years ago, in dietary carcinogens and anticarcinogens, an interesting dynamic equilibrium between what we're exposed to as xenoestrogens in the environment. Perhaps some dietary and other factors play roles in suppressing the expression of 1B1.

ER: Exactly. People are studying components in the cruciferous vegetables like broccoli and cauliflower. We think those can definitely play a role in reducing the levels of some of these enzymes and protecting against the metabolic formation of these metabolites of estrogen that damage DNA.

Estradiol

JB: Let's move from that to a discussion of the personality of this 4-hydroxyestradiol, the 3,4 catecholestrogens. You have shown they undergo auto-oxidation into the 3,4 quinones. Using considerable poetic license, I have euphemistically called them the "flame-dancing estrogens," because of their oxidative potential and their redox recycling capability. Would you describe the fate of estradiol as it moves down that pathway?

ER: Estradiol is oxidized enzymatically by either CYP 1B1 into the 4-catecholestrogens, or by CYP 1A1 into the 2-catecholestrogens. Both of these catecholestrogens can then be further metabolized by CYP450s or peroxidases, including peroxidases like prostaglandin H synthase, to the catecholestrogen quinones. These are highly reactive oxidized species that then react with DNA. Or they can react with the cellular scavenger, glutathione. That's a good protective mechanism.

The quinones can also get reduced back to the catecholestrogens and you can set up this situation of redox cycling that I think you were referring to, where you get oxidation and reduction, and that produces a lot of oxygen radicals and species like that. To be honest, we are not convinced that the DNA damage caused by the oxygen radicals is involved in the induction of cancer because it's very nonspecific. We think that much more specific DNA damage is involved. But that's just our opinion at the moment.

Hydroxylation Patterns and Outcome

JB: In your work with these catecholestrogens, you have shown that the 2-hydroxylation patterns going on into the 2,3 catechols, which then can be oxidized to the 2,3 quinones, have a different reactivity with DNA from those that go through the 4-hydroxylation pathway.

ER: They do. It took a post-doc a year or so to work all this up. The different position there in that aromatic A ring of the estrogens of where the dehydroxy groups are, leads to a different kind of reactivity, as you said. For anyone listening who has a chemical background, the 3,4 quinones actually react as quinones. In the 2,3 quinones, there is a little bit of rearrangement and they react as quinone methides.

Probably, for most people, that doesn't mean anything, but that's why they react at a different position. You get these adducts in the 2,3, quinones forming at their 6 position and actually an adjacent ring, whereas the 3,4, quinones react at the first position in the A ring.

Equilinen and Equiline

JB: At the University of Illinois at Chicago, Judith Bolton and her group have been studying the B-unsaturated ring estrogens, the so-called equine estrogens like equilinen and equiline. Would you explain how that research fits in? These are the Premarin compounds.

ER: It's a little hard for me to relate what we're doing to what Judy is doing. Judy and her group have thus far defined, synthesized, and identified a number of adducts of equilinen that are actually stable adducts in DNA. At the moment, they don't fit into the same scheme that we're working on. To be honest, I really can't relate these two subjects.

Clinical Applications

JB: You have recently published some extraordinary work that will help clinicians understand some of your ideas. A Journal of the National Cancer Institute article in a winter 2003 edition asked why this work

hasn't been more supported by the clinical groups. One reason the authors gave was that the investigators, i.e., the Rogan/Cavalieri group, had not yet published a paper showing this model can be operative in humans.

You have now overcome this obstacle with the elegant study recently published in Carcinogenesis, in which you discussed the relative imbalances in estrogen metabolism and conjugation in breast tissue of women with carcinoma.[26] Would you tell us about that study?

ER: Last spring, in Carcinogenesis, we published a small study on breast tissue obtained from women at the time of biopsy. Abby Biopsies did the biopsies, so there was enough material for the pathologists to take what they needed, with a little bit left over for us. I want to point out that this is non-tumor tissue. This is just adjacent tissue in human breast for the women who had carcinoma. This is our first study of tissue like this, from human breast.

We had 28 cases of women with breast carcinoma and 49 controls who turned out to have no obvious problem, although something probably showed up on a mammogram that was suspicious, or they might have had fibrocystic breast tissue. But those were our controls. We analyzed them for 30 estrogen metabolites and conjugates of glutathione.

At the time we did this study, we could not look at depurinating DNA adducts, although we now have that capability. So, this is just adducts and conjugates. We can analyze all of these in one rod on a high-pressure liquid chromatograph using electrochemical detection. The methodology to do this kind of work has just been developed in the last five or six years.

The first thing we were able to show was that we could see the estrogen metabolites and conjugates. Until we published this, the prevailing opinion was that the levels of estrogen would be too low and we couldn't possibly see any estrogen metabolites. But we could readily detect them.

Estrogen Study Results

The findings we obtained were, first of all, that the level of estrogens—estrone and estradiol—were actually about twice as high in the women with breast carcinoma as in the control women. Although it was not a statistically significant difference, we found it an intriguing difference because of the study's clinical use of aromatase inhibitors. We also had an idea that perhaps higher levels of estrogen in the breast, or in a particular location in the breast, might be a factor in the development of tumors. However, when we looked at these catecholestrogens, the 2- and 4-catecholestrogens, we found the 2-catecholestrogens weren't any different between the two groups.

However, the levels of the 4-catecholestrogens increased dramatically, almost four times in the women with breast carcinoma compared to the control women. That fit in with our hypothesis that this is a dangerous metabolite. We looked at the ability of the catechol-O-methyltransferase to methylate the catecholestrogens and protect them from further oxidation to quinones. Again, we saw that in the controls the levels of the methylated catecholestrogens were a little higher than in the cases, but those results are not statistically significant yet either. We will have to do a lot more cases and controls to see if we reach significance.

Catecholestrogen Quinones in Breast Tissue

Most interestingly and satisfyingly, these glutathione conjugates were about three or four times higher in the women with breast cancer compared to the women who did not have breast cancer. That tells us right away, unequivocally, that these catecholestrogen quinones are present in this breast tissue, because that's the only way you can get these glutathione conjugates. It also implies that if there were quinone there to react with glutathione, it also was reacting with the DNA and damaging it, even though we couldn't see that in this study.

These are exciting results we saw. We were able to launch our studies into human breast tissue, with implications for this pathway to initiate the process that will lead to breast tumors down the road. This also gives us some targets to look at in terms of trying to prevent this damage to DNA.

Region-Specific Hydroxylation Pattern
JB: Dr. Rogan, I absolutely concur. This is pioneering and breakthrough work. This is a seminal paper that will stand up for many years. I'll bet we'll see a lot of citations and index references to this article. Many doctors probably don't understand that this 4-hydroxylation pattern you're describing can occur in situ in the breast in the absence of ovarian hydroxylation. Perhaps you can point out that some of these processes are region-specific to the woman's body.

ER: Oh, yes, absolutely. I would first like to make it clear that we're talking about endogenous estrogens in women. This doesn't have anything to do with estrogen they might take, although those estrogens also could get metabolized in these ways. Breast tissue contains all of the enzymes we're talking about—CYP 1B1, the aromatase, and the catecholmethyltransferase. All of this happens in situ in the breast tissue. Circulating estrogens may enhance it, but these processes go on right in the breast tissue.

Hydroxylation Patterns in Breast Cancer Risk Prediction
JB: Let me move to Leon Bradlow's concept of the 2- 16-hydroxylation patterns as a prognostic marker for breast cancer risk. Correct me if I'm wrong, but work seems to suggest that perhaps the action is not just in the 2- 16-, but may be related to this 4-hydroxylation. And because we haven't been able to measure 4-hydroxylation, clinicians thought it wasn't important. Now we may be beginning to reinterpret this 2- 16- story on the basis of the 4- discoveries.

ER: Right. Because the levels of 4-catecholestrogens in normal laboratory animals are much lower than levels of 2-catecholestrogens, they were discovered and analyzed much later. I think that's one of the reasons why this other story began. In our study, we didn't see any difference between the 16a hydroxyestrogens in the cases and the controls.

I know the Bradlow hypothesis about the 2-catecholestrogens and the 16as is probably the best known story in estrogen metabolism. I think it's fair to say, though, that in general, no convincing evidence has been published that this really is a pathway to initiate cancer. We were not surprised that the levels of the 2s and 16as weren't any different between the women without breast cancer and the cases. I think this whole field of estrogen metabolism is moving beyond the Bradlow hypothesis to some new ones that, in my opinion, are going to be cold water as they're tested.

Possible Surrogate Marker of Breast Cancer
JB: Is it possible that the 2- 16- ratio, which in some studies seemed to have a correlation effect with breast cancer, could be a surrogate marker and that what we're really looking at underneath as the

mechanism was the 4, but because it wasn't a study, it just tracks with the 2- 16- ratio?

ER: That might be. The 16as cannot undergo further oxidative metabolism. I suppose they might be a surrogate, but my guess is we're going to find better markers than that.

The 2 as an Inverse Marker
JB: I was actually thinking more of the 2 being the surrogate. Possibly, as the 2 goes up, the 4 is really going down because of partitioning at that central level of metabolism.

ER: It would kind of be an inverse marker. Perhaps it is, I don't know. I just think it would be easier to measure the 4 and do that directly, or to measure quinone conjugates.

Potential Modifiable Factors
JB: I want to discuss the way the body gets rid of these potentially reactive molecules. Methoxylation as contrasted to glutathione conjugation and sulfation appearS to be the principal pathway. That brings the highly polymorphic catecholmethyltransferase into play. That connects with the folate cycle, because you must have S-adenosylmethionine, as you pointed out in your paper. So there could be a 5-methyltetrahydrofolate connection through the folate cycle, through SAM, into COMT into methylation. Now it becomes a much more complex but more interesting story in regard to potentially modifiable factors.

ER: It does. One of the approaches we are trying to take is the idea that we're probably not going to find one enzyme that makes all the difference. It is probably patterns of metabolism in people and combinations of enzyme activities that are high or low, and particular patterns of those with a number of enzymes that lead one to be at risk, let's say, for developing breast cancer.

The Role of 2-Methoxyestradiol
JB: Emerging literature suggests that 2-methoxyestradiol is a counterbalancing estrogen to estradiol and other mitogenic estrogen metabolites. Does that story have validity? Do you think that endogenous 2-methoxyestradiol insufficiency in tissues might cause this expression of DNA in different ways?

ER: That certainly could be, but it's not something I've spent a lot of time studying.

Estrogen and Prostate Cancer
JB: Let's discuss the paper you and your colleagues wrote, which appeared in Carcinogenesis. It is titled "Catechol Estrogen Metabolites and Conjugates in Different Regions of the Prostate of Noble Rats Treated with 4-Hydroxyestradiol: Implications for Estrogen-Induced Initiation of Prostate Cancer."[27] Most men probably believe estrogen has nothing to do with their prostate. Maybe you could tell us a bit about estrogen and the prostate and also about 4-hydroxylation and estrogen and prostate cancer.

ER: As men age, their levels of testosterone go down and their levels of estrogen go up. Of course, the level of estrogen in men is a lot lower than in women, but it's always present, and it does go up as men age.

The idea of prostate cancer developing in response to initiation by estrogen and then promotion by testosterone actually did not originate with us. Martin Bosland, a colleague of ours, who is at New York

University, has worked with prostate cancer in the Noble rat model for many years. It was Martin's idea. We decided to test this idea and look at the profile of estrogen metabolites in the prostate. In fact, we did find that metabolism of estradiol changes in different parts of the rat prostate. It does seem to correlate with the formation of the 4-catecholestrogens. The catecholestrogen quinones and their conjugates do seem to correlate with the areas of the prostate where tumors develop in this model. We are studying the same enzymes that we studied in the breast. We are also studying in rat prostate the aromatase, CYP 1B1, and the catecholmethyltransferase. They are all present in the rat prostate. We are continuing those kinds of studies and will see what happens to both estrogen and testosterone in the prostate. In fact, maybe it could be that in the prostate, a little bit of testosterone is converted into estradiol by the aromatase and that can initiate prostate tumors through DNA damage, and then the testosterone goes along and promotes those cells into forming actual tumors. That would be the model here.

Adduct Formation in Prostate
JB: Have you had any evidence so far that there is adduct formation in the prostate comparable to what you've seen in breast?

ER: We have not analyzed for adducts yet. If we happened to do this interview in about a month, I think I would have a different answer. We have those samples ready to be analyzed, and they're on our books to do very soon.

Environmental and Nutritional Medicine Implications
JB: Your research may have implications for the cultural epidemiological studies that have associated certain lifestyles and diets with lower incidence of breast and estrogen-related cancer in women, and prostate cancer in men. A connection seems to be emerging between your work on estrogen metabolism and the 4-hydroxylation pattern and cultures that have consumed diets and lived in environments that might be commensurate with lowered 4-hydroxylation or greater methylation. Do you see something emerging here from an environmental medicine perspective or a nutritional medicine perspective?

ER: I think what you've described is exactly correct. That is not an area we are yet able to pursue, although it ties in with some of our ideas about prevention of breast cancer. We think that understanding the role of this 4-hydroxylation is key to being able to develop preventive schemes so that we would either not get the quinones formed or they would be scavenged off. It certainly involves some dietary components that are emerging from epidemiological evidence.

Biochemical vs. Medical Research
JB: The JNCI article asked why this extraordinary work with all the hundred plus publications has taken 20 years to get some attention from clinicians. One explanation was that it has come up through biochemists rather than through clinicians in endocrinology or obstetrics/gynecology. Do you feel the biochemical route of discovery has to overcome a barrier in medicine to become accepted and incorporated?

ER: I wouldn't have described it exactly that way. To me, the biggest barrier is that the emphasis to this point has been on reviewing estrogens in terms of their receptor-mediated processes. I think it's fair to say that endocrinologists and scientists have been reluctant to acknowledge that estrogens might be doing something else besides just the estrogen receptor-mediated processes.

We see the initiation of breast cancer as sort of a two-fold process in which you have the DNA damage by estrogen metabolites initiating the process. Then receptor-mediated events participate in the promotion that leads through all those of the multi-stage processes. And then, eventually, you end up with a tumor.

I guess I never really thought about physicians never having heard about any of this research until recently. I have thought that a bigger barrier to overcome is this idea that estrogens only act through receptor-mediated processes, whereas now we can see they also act as more traditionally understood carcinogens.

Advice to Clinicians
JB: Do you have any advice for clinicians as they see patients in their daily lives and have to make decisions about estrogen and exogenous agents and how they influence hormone-sensitive tissues?

ER: I would go along wholeheartedly with the recommendation that women take estrogen replacement therapy at the lowest possible doses for the least amount of time to alleviate unpleasant postmenopausal problems. I think that is a very good recommendation. I think at this point, for clinicians, all of the standard things you can recommend in terms of diet and eating cruciferous vegetables and not smoking and things like that make absolutely good sense. I don't think I could add to them at this point.

Diet and Lifestyle in Cancer Outcome
JB: That is a major piece of advice for clinicians to take away. I think many individuals persist in the belief that diet plays little role, and cancer comes as a consequence of a fixture from our genealogy. That message you just transmitted is very empowering.

Dr. Rogan, I compliment you and your group for excellent science and pioneering work. I think we will hear much more about this over the years to come. I wish you tremendous success in your work. We are all going to benefit from it. I hope we'll be able to check in with you in the future.

ER: Thank you very much.

t's not often in the history of our 21 years of publishing *FMU* that we've dedicated half of an entire issue to our Clinician/Researcher of the month. This interview certainly justifies that dedication, and we have only touched the tip of the iceberg in terms of the impact and the breadth of the work that Dr. Rogan and Dr. Cavalieri have done in the past 30 years.

Dr. Rogan and Dr. Cavalieri have recently written a review paper titled "Initiation of Cancer and Other Diseases by Catechol Ortho-Quinones: a Unifying Mechanism," which appeared in *Cell and Molecular Life Sciences*.[28] This article presents an integrated theme. Environmental xenoestrogens, endogenous estrogens, or exogenous estrogens all travel through these metabolic pathways. They all may induce risk and have synergistic and amplifying factors.

Diet becomes very important. I hope you had the epiphany I had in listening to Dr. Rogan regarding the direction of research in this field. Don't get locked into thinking about either estradiol alone or the 2-16-hydroxylation story. We have much more yet to learn.

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