Methylation and Nutrigenomics of Cancer in Prevention, Treatment and Recovery

Presenter:
Benjamin Lynch, ND
Naturopathic Oncology
Philadelphia, PA 19124
June 6-8, 2014

“Clinicians will be central to helping consumer-patients use genomic information to make health decisions.” – NEJM
Disclaimer & Disclosures

The information presented here is for informational and educational purposes only. Docere, Inc and Benjamin Lynch will not be liable for any direct, indirect, consequential, special, exemplary, or other damages arising from the use or misuse of any materials or information published.

President and CEO of SeekingHealth.com, SeekingHealth.org and founder of MTHFR.Net
# American Cancer Society

## The Lifetime Probability of Developing and Dying from Cancer, 2007-2009*

### MALES

<table>
<thead>
<tr>
<th>All Sites†</th>
<th>Developing %</th>
<th>1 in 2</th>
<th>Dying %</th>
<th>1 in 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Sites†</td>
<td>44.81</td>
<td>2</td>
<td>23.08</td>
<td>4</td>
</tr>
<tr>
<td>Brain &amp; ons</td>
<td>0.70</td>
<td>144</td>
<td>0.50</td>
<td>201</td>
</tr>
<tr>
<td>Breast</td>
<td>0.13</td>
<td>781</td>
<td>0.03</td>
<td>3,142</td>
</tr>
<tr>
<td>Colorectal</td>
<td>5.17</td>
<td>19</td>
<td>2.11</td>
<td>47</td>
</tr>
<tr>
<td>Esophagus</td>
<td>0.81</td>
<td>124</td>
<td>0.78</td>
<td>127</td>
</tr>
<tr>
<td>Hodgkin lymphoma</td>
<td>0.25</td>
<td>401</td>
<td>0.04</td>
<td>2,253</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
<td>2.04</td>
<td>49</td>
<td>0.61</td>
<td>164</td>
</tr>
<tr>
<td>Larynx</td>
<td>0.60</td>
<td>166</td>
<td>0.21</td>
<td>475</td>
</tr>
<tr>
<td>Leukemia</td>
<td>1.59</td>
<td>63</td>
<td>1.02</td>
<td>98</td>
</tr>
<tr>
<td>Liver &amp; intrahepatic bile duct</td>
<td>1.18</td>
<td>85</td>
<td>0.83</td>
<td>120</td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>7.77</td>
<td>13</td>
<td>6.74</td>
<td>15</td>
</tr>
<tr>
<td>Melanoma of skin‡</td>
<td>2.87</td>
<td>35</td>
<td>0.48</td>
<td>209</td>
</tr>
<tr>
<td>Myeloma</td>
<td>0.77</td>
<td>129</td>
<td>0.46</td>
<td>215</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>2.34</td>
<td>43</td>
<td>0.88</td>
<td>114</td>
</tr>
<tr>
<td>Oral cavity &amp; pharynx</td>
<td>1.50</td>
<td>66</td>
<td>0.38</td>
<td>263</td>
</tr>
<tr>
<td>Ovary</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.48</td>
<td>67</td>
<td>1.32</td>
<td>76</td>
</tr>
<tr>
<td>Prostate</td>
<td>16.15</td>
<td>6</td>
<td>2.75</td>
<td>36</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.09</td>
<td>92</td>
<td>0.51</td>
<td>195</td>
</tr>
<tr>
<td>Testis</td>
<td>0.37</td>
<td>268</td>
<td>0.02</td>
<td>5,420</td>
</tr>
<tr>
<td>Thyroid</td>
<td>0.52</td>
<td>191</td>
<td>0.05</td>
<td>1,865</td>
</tr>
<tr>
<td>Urinary bladder§</td>
<td>3.81</td>
<td>26</td>
<td>0.88</td>
<td>114</td>
</tr>
<tr>
<td>Uterine cervix</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Uterine corpus</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

### FEMALES

<table>
<thead>
<tr>
<th>All Sites†</th>
<th>Developing %</th>
<th>1 in 3</th>
<th>Dying %</th>
<th>1 in 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Sites†</td>
<td>38.17</td>
<td>3</td>
<td>19.39</td>
<td>5</td>
</tr>
<tr>
<td>Brain &amp; ons</td>
<td>0.55</td>
<td>181</td>
<td>0.40</td>
<td>252</td>
</tr>
<tr>
<td>Breast</td>
<td>12.38</td>
<td>8</td>
<td>2.76</td>
<td>36</td>
</tr>
<tr>
<td>Colorectal</td>
<td>4.78</td>
<td>21</td>
<td>1.94</td>
<td>51</td>
</tr>
<tr>
<td>Esophagus</td>
<td>0.23</td>
<td>427</td>
<td>0.21</td>
<td>480</td>
</tr>
<tr>
<td>Hodgkin lymphoma</td>
<td>0.21</td>
<td>476</td>
<td>0.04</td>
<td>2,757</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
<td>1.20</td>
<td>83</td>
<td>0.34</td>
<td>291</td>
</tr>
<tr>
<td>Larynx</td>
<td>0.14</td>
<td>736</td>
<td>0.05</td>
<td>1,873</td>
</tr>
<tr>
<td>Leukemia</td>
<td>1.14</td>
<td>88</td>
<td>0.71</td>
<td>140</td>
</tr>
<tr>
<td>Liver &amp; intrahepatic bile duct</td>
<td>0.49</td>
<td>202</td>
<td>0.43</td>
<td>231</td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>6.35</td>
<td>16</td>
<td>5.02</td>
<td>20</td>
</tr>
<tr>
<td>Melanoma of skin‡</td>
<td>1.85</td>
<td>54</td>
<td>0.23</td>
<td>426</td>
</tr>
<tr>
<td>Myeloma</td>
<td>0.57</td>
<td>175</td>
<td>0.37</td>
<td>274</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>1.93</td>
<td>52</td>
<td>0.71</td>
<td>141</td>
</tr>
<tr>
<td>Oral cavity &amp; pharynx</td>
<td>0.68</td>
<td>148</td>
<td>0.18</td>
<td>548</td>
</tr>
<tr>
<td>Ovary</td>
<td>1.38</td>
<td>72</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.45</td>
<td>69</td>
<td>1.30</td>
<td>77</td>
</tr>
<tr>
<td>Prostate</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.38</td>
<td>72</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>Testis</td>
<td>1.15</td>
<td>87</td>
<td>0.34</td>
<td>298</td>
</tr>
<tr>
<td>Thyroid</td>
<td>1.53</td>
<td>65</td>
<td>0.07</td>
<td>1,433</td>
</tr>
<tr>
<td>Urinary bladder§</td>
<td>2.64</td>
<td>38</td>
<td>0.54</td>
<td>185</td>
</tr>
<tr>
<td>Uterine cervix</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Uterine corpus</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* For those free of cancer at beginning of age interval.
† All sites excludes basal and squamous cell skin cancers and in situ cancers except urinary bladder.
‡ Statistics are for whites.
§ Includes invasive and in situ cancer cases.
NA=Not applicable.

**Source:** Software: DevCan: Probability of Developing or Dying of Cancer Software, Version 6.6.1, National Cancer Institute, 2012.
### Leading New Cancer Cases and Deaths – 2013 Estimates

#### Estimated New Cases*

<table>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>Breast</td>
</tr>
<tr>
<td>238,590 (28%)</td>
<td>232,340 (29%)</td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>Lung &amp; bronchus</td>
</tr>
<tr>
<td>118,080 (14%)</td>
<td>110,110 (14%)</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>Colon &amp; rectum</td>
</tr>
<tr>
<td>73,680 (9%)</td>
<td>69,140 (9%)</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>Uterine corpus</td>
</tr>
<tr>
<td>54,610 (6%)</td>
<td>49,560 (6%)</td>
</tr>
<tr>
<td>Melanoma of the skin</td>
<td>Thyroid</td>
</tr>
<tr>
<td>45,060 (5%)</td>
<td>45,310 (6%)</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
<td>Melanoma of the skin</td>
</tr>
<tr>
<td>40,430 (5%)</td>
<td>31,630 (4%)</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>Kidney &amp; renal pelvis</td>
</tr>
<tr>
<td>37,600 (4%)</td>
<td>24,720 (3%)</td>
</tr>
<tr>
<td>Oral cavity &amp; pharynx</td>
<td>Pancreas</td>
</tr>
<tr>
<td>29,620 (3%)</td>
<td>22,480 (3%)</td>
</tr>
<tr>
<td>Leukemia</td>
<td>Ovary</td>
</tr>
<tr>
<td>27,880 (3%)</td>
<td>22,240 (3%)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Kidney &amp; renal pelvis</td>
</tr>
<tr>
<td>22,740 (3%)</td>
<td>10,820 (4%)</td>
</tr>
<tr>
<td>All sites</td>
<td>Urinary bladder</td>
</tr>
<tr>
<td>854,790 (100%)</td>
<td>10,590 (3%)</td>
</tr>
</tbody>
</table>

#### Estimated Deaths

<table>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung &amp; bronchus</td>
<td>Lung &amp; bronchus</td>
</tr>
<tr>
<td>87,260 (28%)</td>
<td>72,220 (26%)</td>
</tr>
<tr>
<td>Prostate</td>
<td>Breast</td>
</tr>
<tr>
<td>29,720 (10%)</td>
<td>39,620 (14%)</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>Colon &amp; rectum</td>
</tr>
<tr>
<td>26,300 (9%)</td>
<td>24,530 (9%)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Pancreas</td>
</tr>
<tr>
<td>19,480 (6%)</td>
<td>18,980 (7%)</td>
</tr>
<tr>
<td>Liver &amp; intrahepatic bile duct</td>
<td>Liver &amp; intrahepatic bile duct</td>
</tr>
<tr>
<td>14,890 (5%)</td>
<td>14,030 (5%)</td>
</tr>
<tr>
<td>Leukemia</td>
<td>Leukemia</td>
</tr>
<tr>
<td>13,660 (4%)</td>
<td>10,060 (4%)</td>
</tr>
<tr>
<td>Esophagus</td>
<td>Non-Hodgkin lymphoma</td>
</tr>
<tr>
<td>12,220 (4%)</td>
<td>8,430 (3%)</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>Uterine corpus</td>
</tr>
<tr>
<td>10,820 (4%)</td>
<td>8,190 (3%)</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>Kidney &amp; renal pelvis</td>
</tr>
<tr>
<td>10,590 (3%)</td>
<td>6,780 (2%)</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
<td>Brain &amp; other nervous system</td>
</tr>
<tr>
<td>8,780 (3%)</td>
<td>6,150 (2%)</td>
</tr>
<tr>
<td>All sites</td>
<td>All sites</td>
</tr>
<tr>
<td>306,920 (100%)</td>
<td>273,430 (100%)</td>
</tr>
</tbody>
</table>

*Excludes basal and squamous cell skin cancers and in situ carcinoma except urinary bladder.

©2013, American Cancer Society, Inc., Surveillance Research
Genetic and Epigenetic Contributions to Human Nutrition and Health: Managing Genome–Diet Interactions

PATRICK J. STOVER, PhD; MARIE A. CAUDILL, PhD, RD

ABSTRACT
The Institute of Medicine recently convened a workshop to review the state of the various domains of nutritional genomics research and policy and to provide guidance for further development and translation of this knowledge into nutrition practice and policy. Nutritional genomics holds the promise to revolutionize both clinical and public health nutrition practice and facilitate the establishment of (a) genome-informed nutrient and food-based dietary guidelines for disease prevention and healthful aging, (b) individualized medical nutrition therapy for disease management, and (c) better targeted public health nutrition interventions (including micronutrient fortification and supplementation) that maximize benefit and minimize adverse outcomes within genetically diverse human populations. As the field of nutritional genomics matures, which will include filling fundamental gaps in knowledge of nutrient–genome interactions in health and disease and demonstrating the potential benefits of customizing nutrition prescriptions based on genetics, registered dietitians will be faced with the opportunity of making genetically driven dietary recommendations aimed at improving human health.

P. J. Stover is a professor and director, and M. A. Caudill is an associate professor, Division of Nutritional Sciences, Cornell University, Ithaca, NY.

Address correspondence to: Patrick J. Stover, PhD, Cornell University, Division of Nutritional Sciences, Ithaca, NY 14853. E-mail: pjs13@cornell.edu

Public health nutrition continues to be challenged by increasing expectations from the food supply on one hand, and fundamental gaps in nutrition knowledge on the other, which can constrain the development and implementation of nutrition and food policy (1). Current demands on the food supply are no longer limited to ensuring general safety and preventing micronutrient deficiencies. Increasingly, there is interest in engineering medicinal qualities into the food supply to enable diets that promote health and “nurture” a sense of well-being that transcends the mere absence of disease by improving biological functions and even increasing life spans.

Unquestionably, nutrition is one of the primary environmental exposures that determines health. Common human chronic diseases, including type 2 diabetes, metabolic syndrome, cardiovascular and neurological disease, and many cancers are initiated and/or accelerated by nutrient/food exposures. However, it is also recognized that chronic diseases are complex in their etiology and include a substantial genetic component; individuals respond differently to foods and even individual nutrients. Investigation in this new field of nutrition research, often referred to as nutritional genomics, focuses on deciphering the biological mechanisms that underlie both the acute and persistent genome-nutrient interactions that influence health.

Nutritional genomics, while centered on the biology of individuals, distinguishes itself from other “omics” fields by its unique focus on disease prevention and healthy aging through the manipulation of gene–diet interactions. Nutritional genomics promises to revolutionize both clinical and public health nutrition practice and facilitate the establishment of (a) genome-informed nutrient and food-based dietary guidelines for disease prevention and healthful aging, (b) individualized medical nutrition therapy for disease management, and (c) better...
Hereditary mutations

Hereditary mutations (also called germline mutations) are gene defects that are passed from a parent to child. Hereditary mutations are present in the egg or sperm that join during fertilization and develop into a fetus. Because the mutation is present at the beginning, it exists in all cells of the body, including reproductive cells (the cells that make sperm in males or the egg cells in females). This means the mutation can be passed from generation to generation.

A hereditary mutation is a major factor in about 5% to 10% of all cancers.

Some people are more likely to develop cancer than others simply because they are born with mutations in their genes. To learn more about this, see our document, Heredity and Cancer.

Acquired mutations

Most cancers are caused by DNA changes that happen during the person's life. These are called acquired, sporadic, or somatic mutations. An acquired mutation can be caused by things in the environment such as exposure to radiation or toxins. But for most acquired mutations, no specific cause can be found.

Unlike the inherited mutations, acquired mutations start in one cell of the body and are found only in the offspring of that cell. They are not in every cell of the body. Because they are not in the reproductive cells, acquired mutations cannot be passed on to the next generation.

It is important to realize that mutations in our cells happen all the time. Usually, the cell detects the change and repairs it. If it can’t be repaired, the cell will get a signal telling it to die in a process called apoptosis. But if the cell doesn't die and the mutation is not repaired, it may lead to a person developing cancer. This is more likely if the mutation affects a gene involved with cell division or a gene that normally causes a defective cell to die.

Definitions

**Cancer**: disease caused by an uncontrolled division of abnormal cells in a part of the body.

**Proto-oncogene**: a normal gene that becomes an oncogene due to mutations or increased expression.

**Oncogene**: a gene that has the potential to cause cancer.

Cancer Risk Factors (some)

The major risk factors for cancer are:
- Tobacco
- Alcohol
- Diet
- Sexual and reproductive behavior
- Infectious agents (immune escape)
- Family history
- Occupation
- Environment (heavy metals / pesticides / herbicides, ...)
- Pollution
Proto-oncogenes

↑ Hypoxia = Shift in Pyruvate Metabolism

Cancer
NASH is widely considered to be the liver expression of the metabolic syndrome—diseases related to diabetes mellitus type 2, insulin resistance, central (truncal) obesity, hyperlipidemia (low high-density lipoprotein cholesterol, hypertriglyceridemia), and hypertension. There is at present a worldwide epidemic of diabetes and obesity. At least 1.46 billion adults were overweight or obese and 170 million of the world’s children were overweight or obese in 2008. In some parts of Africa, obesity afflicts more children than malnutrition. The numbers are continuing to rise, indicating that NASH will become an increasingly common liver problem in both rich and poor countries, increasing the global burden of liver disease.
Mitochondrial dysfunction precedes insulin resistance and hepatic steatosis and contributes to the natural history of non-alcoholic fatty liver disease in an obese rodent model

A critical complication of the obesity epidemic experienced by children and adults in Westernized societies is non-alcoholic fatty liver disease (NAFLD). NAFLD affects ~30% of all US adults and 75–100% of obese and morbidly obese individuals [1,2] and is now considered the hepatic representation of the metabolic syndrome [3]. Even more alarming, as the number of overweight and obese children has doubled in the past 2–3 decades in the US, there is an increasing propensity of NAFLD and non-alcoholic steatohepatitis (NASH) development in younger individuals [4]. In fact, it is estimated that 10% of lean and 38% of obese children have fatty livers [5]. Consequently, clinicians warn that the demand for liver transplants may rise as these children become adults if steps are not taken to reverse this trend.

Day and James [6] have proposed the “two-hit hypothesis” to explain the development of hepatic steatosis and the progression to inflammation (NASH), fibrosis, and cirrhosis. This hypothesis states that factors such as insulin resistance and impaired hepatic fatty acid oxidation contribute to NAFLD development [3] and that once steatosis is present, inflammation and oxidative stress are thought to activate stellate cells and increase collagen deposition and fibrogenesis [6]. However, it has been speculated that mitochondrial abnormalities may be involved in the pathogenesis of NAFLD [7–10]. In fact, we previously demonstrated that heterozygosity for mitochondrial β-oxidation defects causes development of NAFLD in aging mice [10], which raises the possibility that NAFLD may be a mitochondrial disease. However, little is known about the spectrum of changes in mitochondrial content and function and their potential role in the natural history of NAFLD.

Otsuka Long–Evans Tokushima Fatty (OLETF) rats are a commonly studied model of NAFLD.
This study demonstrates for the first time that in an experimental rat model of NASH [10], the combination of two physiologic antioxidants, namely SAMe and DLPC, have a synergistic effect and significantly oppose liver injury generated by the excess of fat. Since the initial proposition by James and Day [1] for the “two hit” model of NASH, the new evolving view is that fat accumulation, inflammation, necrosis of hepatocytes, and cell death occur simultaneously and are responsible for a series of molecular events involving nuclear transcriptional factors leading to insulin resistance, mitochondrial dysfunction, CYP2E1 induction and lipid peroxidation [3]. These features were reproduced in animals fed a high fat liquid diet. After six weeks of strict controlled feeding, only the combination of SAMe+DLPC was effective, whereas SAMe and DLPC alone did not have a significant effect on the parameters measured. Other animal models of NASH have been studied as reviewed by Nanji [24]. Agent-stimulating glutathiones, such as SAMe and 2(RS)-n-propylthiazolidine-4(R)-carboxylic acid, have been found to be hepatoprotective in rats fed a methionine-choline deficient (MCD) diet [25]. However, the combination of two innocuous compounds largely available and used by the general population, such as SAMe and DLPC, has never been tested before in conjunction with a balanced liquid diet able to reproduce all the pathophysiological “hit” characteristics of human NASH. A high saturated fat diet administered to obese rats accelerates liver damage, thereby acting as a second “hit” mechanism [26]. Our study demonstrates that the combination of SAMe + DLPC down-regulates CYP2E1. This effect is possibly attributed to DLPC, which has been shown before to be an effective inhibitor of CYP2E1 induced by chronic alcohol consumption [27]. SAMe, by contrast, is a methyl-donor compound largely studied after chronic alcohol consumption [28;29] because of its capacity to restore in the liver the decreased SAMe and glutathione levels [30], thereby improving mitochondrial functions [29].
Cancer Pathways Affected (some)

The major pathways affected:
- Methylation
- Transsulfuration
- Oxidative phosphorylation
- Kynurenine
Methylation
Functions of Methylation (some):

- Gene regulation: turn on/off genes via SAMe
- Biotransformation: glutathione production
- DNA base formation: uracil $\rightarrow$ thymine
- Cell membrane components: phosphatidylcholine and DHA to membranes
- Mitochondrial support: adenosine as substrate for ATP
- Cell protection: NFKB expression to reduce TNF cytotoxicity

Uracil (RNA) is converted into Thymine (DNA) using the TYMS enzyme. The methyl group for this conversion comes from 5,10 MTHF.
MTHFR snp can reduce one type of cancer risk. Which one?

(Methotrexate Site)

(5-FU site)
**HyperMethylation or HypoMethylation in Cancer?**

**Chicken or the Egg?**
- Global DNA Hypomethylation triggering CpG Island Hypermethylation?
- Depleting methyl donors the right thing to do in cancer treatments?
  - ↓ DNA Base formation and thus ↓ tumor growth?
- Short and long term consequences of global methyl donor depletion?
  - 5-FU
  - Methotrexate
- Short and long term consequences of global oxidative damage?
  - Chemotherapy
  - Radiation
- Secondary recurring cancers are the norm, not the exception.
Figure 1 CpG island DNA hypermethylation and global DNA hypomethylation in colorectal cancer as compared with normal colonic epithelium

Lao, V. V. & Grady, W. M. (2011) Epigenetics and colorectal cancer
"Although both malignant and normal samples display considerable diversity in their DNA’s m5C content, a high percentage of malignant tumors, especially metastases, have DNA with unusually low m5C contents relative to the normal tissues."

"A number of studies have shown significant decreases in global DNA methylation levels with progressing tumor stage, tumor grade, or various other indicators of poor prognosis."

"Studies of cancer-associated DNA hypomethylation sound a note of caution in the current development of cancer therapies aimed at decreasing DNA methylation in a non-targeted manner."

Prostate          B Cell Leukemia
Wilm’s tumor       Liver
Colon              Ovarian
Breast             Cervical
Irreversible global DNA hypomethylation as a key step in hepatocarcinogenesis induced by dietary methyl deficiency

Abstract

Dietary methyl group deprivation is now well recognized as a model of hepatocarcinogenesis in rodents. In the present study, we examined the effects of feeding a methyl-deficient diet followed by a methyl-adequate diet on the extent of methylation of liver DNA and on the formation and evolution of altered hepatic foci. Male F344 rats were fed a methyl-deficient diet for 9, 18, 24, and 36 weeks, followed by re-feeding a methyl-adequate diet for a total of 54 weeks. Similar to previous findings, the methyl-deficient diet resulted in decreased levels of S-adenosylmethionine (SAM), SAM/SAH ratios, and global DNA hypomethylation. Feeding the methyl-adequate diet restored the liver SAM levels and SAM/SAH ratios to control levels in all experimental groups. In contrast, re-feeding the complete diet restored DNA methylation to normal level only in the group that had been fed the methyl-deficient diet for 9 weeks; in animals exposed to methyl deprivation longer, the methyl-adequate diet failed to reverse the hypomethylation of DNA. Liver tissue of rats exposed to methyl deficiency for 9, 18, 24, or 36 weeks was characterized by the persistent presence of placental isoform of glutathione-S-transferase (GST\textsubscript{\pi})-positive lesions despite re-feeding the methyl-adequate diet. The persistence of altered hepatic foci in liver after withdrawal of methyl-deficient diet serves as an indication of the carcinogenic potential of a methyl-deficient diet. Substitution of the methyl-deficient diet with complete diet failed to prevent the expansion of initiated foci and restore DNA methylation in animals exposed to deficiency for 18, 24, or 36 weeks. The association between DNA hypomethylation and expansion of foci suggests that stable DNA hypomethylation is a promoting factor for clonal expansion of initiated cells. These results provide an experimental evidence and a mechanistic basis by which epigenetic alterations may contribute to the initiation and promotion steps of carcinogenesis.

Published by Elsevier B.V.

Keywords: Methyl-deficient diet; Methyl-adequate diet; DNA hypomethylation; Rat hepatocarcinogenesis

only 4.5 months
The evidence accumulated in recent years shows the importance of diet as a major factor in cancer development. It is well documented that deficiencies of the major dietary sources of methyl groups—methionine, choline, folic acid, and Vitamin B₁₂, are sufficient to induce liver tumor formation in male rats and certain mouse strains [1–5]. The methyl-deficient model of endogenous carcinogenesis is unique: in that dietary omission rather than the addition of chemical carcinogens leads to tumor formation [2,5]. The biochemical and molecular events predisposing to cancer in this model result from chronic metabolic stress and may provide an ideal model system to study progressive alterations that occur during carcinogenic process [3,4]. It has been shown that even after a relatively short exposure of rats to a choline-devoid diet (10–12 weeks), the liver contains initiated cells that are capable of full evolution to cancer even in the absence of active promotion [5,6]. Previous experiments of rodent methyl deficiency in vivo have shown that such diets lead to rapid fat accumulation in the liver, increased lipid peroxidation, necrotic and apoptotic cell death, increased cell proliferation, depletion of intracellular methyl group pools, the imbalance of deoxynucleotide pool resulting in uracil incorporation into DNA, DNA strand breakage, and increased genomewide and gene-specific hypomethylation [7–11]. Any or all of these factors may contribute to the hepatocarcinogenic effects of the methyl-deficient diet. Additionally, DNA methyltransferase activity and expression [10,11]. Despite the fact that global DNA hypomethylation was the first epigenetic alteration identified in cancer cells [13,14], most of the research in the field of cancer epigenetic has been focused on the role of increased methylation of specific gene promoters for initiating or enforcing silencing of tumor suppressor genes [15,16]. The mechanisms responsible for loss of DNA methylation and functional importance of DNA hypomethylation during carcinogenesis are unclear. Several possibilities that may contribute to the development of DNA hypomethylation have been proposed, including reduction of methylation capacity because of intracellular depletion of SAM, the inhibitory effect of SAH on DNA methyltransferases, the presence of unrepaired lesions in DNA that interfere with the methylation ability of DNA methyltransferases, and the inability of mammalian maintenance DNA methyltransferase (DNMT1) to methylate double-stranded unmethylated CpG sites [9]. A major gap in the understanding of the role of DNA methylation dysregulation in carcinogenic process is the lack of knowledge about specific alterations in cytosine methylation that may be mechanistically related to neoplastic transformation and the precise timetable of epigenetic alterations occurring between the transitions of a normal cell through intermediate tumorigenic stages to a tumor cell [17,18].

The results of previous studies indicate that feeding of a methyl-deficient diet for as little as 3–4
Methylenetetrahydrofolate reductase C677T and methionine synthase A2756G polymorphisms influence on leukocyte genomic DNA methylation level.

Weiner AS¹, Boyarskikh UA, Voronina EN, Mishukova OV, Filipenko ML.

Author information

Abstract

Methionine synthase (MTR) and methylenetetrahydrofolate reductase (MTHFR) enzymes are involved in the metabolism of methyl groups, and thus have an important role in the maintenance of proper DNA methylation level. In our study we aimed to evaluate the effect of the polymorphism A2756G (rs1805087) in the MTR gene on the level of human leukocyte genomic DNA methylation. Since the well-studied polymorphism C677T (rs1801133) in the MTHFR gene has already been shown to affect DNA methylation, we aimed to analyze the effect of MTR A2756G independently of the MTHFR C677T polymorphism. For this purpose, we collected the groups of 80 subjects with the MTR 2756AA genotype and 80 subjects with the MTR 2756GG genotype, having equal numbers of individuals with the MTHFR 677CC and the MTHFR 677TT genotypes, and determined the level of DNA methylation in each group. Individuals homozygous for the mutant MTR 2756G allele showed higher DNA methylation level than those harboring the MTR 2756AA genotype (5.061 ± 1.761% vs. 4.501 ± 1.621%, P=0.0391). Individuals with wild-type MTHFR 677CC genotype displayed higher DNA methylation level than the subjects with mutant MTHFR 677TT genotype (5.103 ± 1.767% vs. 4.323 ± 1.525%, P=0.0034). Our data provide evidence that the MTR A2756G polymorphism increases the level of DNA methylation and confirm the previous reports that the MTHFR C677T polymorphism is associated with DNA hypomethylation.
Elevated Plasma Vitamin B12 Levels as a Marker for Cancer: A Population-Based Cohort Study

Johan Frederik Berg Arendt, Lars Pedersen, Ebba Nexo, Henrik Toft Sørensen

Manuscript received February 6, 2013; revised September 22, 2013; accepted September 30, 2013.

Correspondence to: JFB Arendt, BSc, Department of Clinical Epidemiology, Aarhus University Hospital, Olof Palme Allé 43–45, DK-8200 Aarhus, Denmark (e-mail: johan.frederik.bergl.arendt@sun.au.dk).

Background A substantial proportion of patients referred for plasma vitamin B12 (cobalamin [Cbl]) measurement present with high Cbl levels, which have been reported in patients with different cancer types. However, the cancer risk among patients with newly diagnosed high Cbl levels has not been adequately examined.

Methods We conducted this cohort study using population-based Danish medical registries. Patients referred for Cbl measurement with levels greater than the lower reference limit (≥200 pmol/L) were identified from the population of Northern Denmark during the period of 1998 to 2009 using a database of laboratory test results covering the entire population. Data on cancer incidence (follow-up 1998–2010), Cbl treatment, and prior diagnoses were obtained from medical registries. Patients receiving Cbl treatment were excluded. Cancer risks were calculated as standardized incidence ratios (SIRs) with 95% confidence intervals (CIs), stratified by plasma Cbl levels. All statistical tests were two-sided.

Results We identified 333,667 persons without prevalent cancer and not receiving Cbl treatment. Six percent had Cbl levels greater than the upper reference limit (≥601 pmol/L). Cancer risk increased with higher Cbl levels and was highest during the first year of follow-up (Cbl 601–800 pmol/L: SIR = 3.44, 95% CI = 3.14 to 3.76; Cbl >800 pmol/L: SIR = 6.27, 95% CI = 5.70 to 6.88; both P < .001). The risks were particularly elevated for hematological and smoking- and alcohol-related cancers for persons with high Cbl levels.

Conclusions High Cbl levels were associated with the risk of subsequently diagnosed cancer, mostly within the first year of follow-up. This may have clinical implications for the interpretation of high Cbl levels.

Histamine and Cancer

Most malignant cells express Histidine decarboxylase (HDC)
(or is it a result of localized low methylation?)

HDC expression = ↑ histamine levels

Histamine triggers:
• Angiogenesis
• Growth factor in malignant melanoma, colon, gastric, leukemias
• Reduced apoptosis in monocytes
• Immunosuppression by NK cell blunting
• Activation of T suppressor cells

On the Sulfation and Methylation of Catecholestrogens in Human Mammary Epithelial Cells and Breast Cancer Cells

Ying Hui, Shin Yasuda, Ming-Yih Liu, Yi-yong Wu, and Ming-Cheh Liu

Department of Pharmacology, College of Pharmacy, The University of Toledo; Toledo, OH 43606, U.S.A.; Graduate School of Peking Union Medical College; Beijing 100730, China; National Synchrotron Radiation Research Center; Hsinchu, Taiwan, R.O.C.; and Gynecology and Obstetric Department, Beijing Hospital; Beijing 100730, China.

Received November 18, 2007; accepted January 27, 2008

Prolonged exposure to high level of estrogen is a known risk factor for breast carcinogenesis. In cells, estrogens, in particular estrone (E1) and 17β-estradiol (E2), can be converted to catecholestrogens (CEs) which may be oxidized to form CE-semiquinones and CE-quinones that are capable of binding to DNA to induce mutations, followed by carcinogenesis. Whether the body is equipped with protective mechanisms against potentially harmful CEs, therefore, is an important issue. The present study was designed to examine the role of sulfation in the metabolism of CEs. MCF-7 breast cancer cells and MCF 10A human mammary epithelial cells were metabolically labeled with [35S]sulfate in the presence of individual CEs. Analysis of the labeling media showed the generation and release of exclusively [35S]sulfated 2-methoxy-E1 or [35S]sulfated 2- or 4-methoxy-E2 by cells labeled in the presence of 2-OH-E1 or 2- or 4-OH-E2. Whereas both [35S]sulfated 4-methoxy-E1 and [35S]sulfated 4-OH-E1 were detected in the labeling media of cells labeled in the presence of 4-OH-E1. These results indicated a concerted action of catechol-O-methyltransferase (COMT) and the cytosolic sulfotransferase (SULT) enzyme(s) in the metabolism of CEs. Enzymatic assays revealed that, five (SULT1A1, SULT1A2, SULT1A3, SULT1C4, and SULT1E1) of eleven known human SULTs tested could use CEs and methoxyestrogens (MEs) as substrates, with SULT1E1 displaying the strongest sulfating activity.

Key words catecholestrogen; sulfation; methylation; methoxyestrogen; cytosolic sulfotransferase
Figure S1: Part of the metabolic pathway of estradiol and the role of various enzymes involved: Estradiol is metabolized into 2-hydroxyestradiol (2-OHE2) and 4-hydroxyestradiol (4-OHE2) by CYP1A1 and CYP1B1 respectively. These catechols undergo further oxidation into semiquinones and quinones that react with DNA to form depurinating adducts leading to mutations associated with breast cancer. NQO1 reduces these quinones back to catechols which are detoxified into methoxy derivatives by the action of COMT. This protects the cells against DNA adducts formation and lowers the potential for mutagenic damage.
### NQO1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>SNP</th>
<th>Versions</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NQO1</td>
<td>69743760</td>
<td>rs10517</td>
<td>A or G</td>
<td>Benjamin Lynch, GG, MATHEW LYNCH, GG, NADIA LYNCH, GG, TASMAN LYNCH, GG, THEODOR LYNCH</td>
</tr>
<tr>
<td>NQO1</td>
<td>69744747</td>
<td>rs1063556</td>
<td>A or G</td>
<td>Benjamín Lynch, AA, MATHEW LYNCH, AA, NADIA LYNCH, AA, TASMAN LYNCH, AA, THEODOR LYNCH</td>
</tr>
<tr>
<td>NQO1</td>
<td>69744774</td>
<td>rs1050873</td>
<td>G or T</td>
<td>TT, Benjamin Lynch, MATHEW LYNCH, TT, NADIA LYNCH, TT, TASMAN LYNCH, TT, THEODOR LYNCH</td>
</tr>
<tr>
<td>NQO1</td>
<td>69744855</td>
<td>rs3191214</td>
<td>A or G</td>
<td>GG, Benjamin Lynch, MATHEW LYNCH, GG, NADIA LYNCH, GG, TASMAN LYNCH, GG, THEODOR LYNCH</td>
</tr>
</tbody>
</table>

**Minus Orientation:** Flip the base. Ex. GG → CC

**Plus Orientation:** Just read it.
Breast Cancer Risk Scenario

TNF G308A = (rs1800629)
CYP1B1 119Ser  = 3x more active (rs1056827)
CYP1A1 Val462Ile  = 2x more active (rs1048943)
COMT Met/Met = 3x less active (rs4680)
NQO1 609C>T = hetero 3x less active // homo only 4% activity (rs1800566)

<table>
<thead>
<tr>
<th>Lower Risk</th>
<th>More Risk</th>
<th>Most Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF -/-</td>
<td>TNF -/+</td>
<td>TNF +/+</td>
</tr>
<tr>
<td>CYP1B1 -/-</td>
<td>CYP1B1 -/+</td>
<td>CYP1B1 +/+</td>
</tr>
<tr>
<td>CYP1A1 -/-</td>
<td>CYP1A1 -/+</td>
<td>CYP1A1 +/+</td>
</tr>
<tr>
<td>COMT -/-</td>
<td>COMT -/+</td>
<td>COMT +/+</td>
</tr>
<tr>
<td>NQO1 -/-</td>
<td>NQO1 -/+</td>
<td>NQO1 +/+</td>
</tr>
</tbody>
</table>

Relative Risk:
- Obesity, Smoking, Alcohol
- Total Years of Estrogen Exposure
- Polycyclic aromatic hydrocarbons (PAH): Fried, broiled, well-done meat

Catechol-O-Methyltransferase (COMT)-mediated Metabolism of Catechol Estrogens Comparison of Wild-Type and Variant COMT Isoforms and Breast Cancer Risk Associated with Genotype Polymorphism of the Estrogen-metabolizing Genes CYP17, CYP1A1, and COMT A Multigenic Study on Cancer Susceptibility and Effect of tumour necrosis factor-alpha on estrogen metabolic pathways in breast cancer cells

(c) 2014: Benjamin Lynch, ND
COMT Downregulation: Symptoms Match SNP?

What is issue? ↑ Dopamine? ↑ Norepi? ↓ Epi? ↑ Estrogen?

Support COMT:
- Molybdenum (prn)
- Magnesium
- Vitamin B6
- Vitamin C
- Niacin
- SAM
- Adaptogens
- Watch Phenylalanine and Tyrosine Intake
- MSM, NAC or sulfur-containing foods (if tolerated)
- Blood sugar stabilization (diet, sleep, exercise, chromium)
Breast cancer is the most common malignancy and a major cause of mortality in women worldwide [1]. Accumulating evidence indicates that an increased risk for breast cancer is associated with dietary factors [2] and a few significant-risk genetic components (e.g., \textit{BRCA1}) [3]. \textit{BRCA1} is a tumor suppressor gene which plays a key role in numerous cellular processes, including transcription regulation, DNA damage repair and protein ubiquitination.\textsuperscript{4} Recent research has confirmed that \textit{BRCA1} is an important transcriptional regulator, and \textit{BRCA1}-depleted breast cancer cells shows changes to approximately 7\% of the mRNAs expressed [4]. Moreover, our recent study also indicated that Phosphatidylethanolamine \textit{N}-methyltransferase (\textit{PEMT}) is a small integral membrane protein that catalyzes the \textit{de novo} synthesis of choline using \textit{S}-adenosylmethionine as a methyl donor [17]. The human \textit{PEMT} gene is located on factor receptor displayed different expression patterns in \textit{BRCA1}-defective cancer cells [5,6], and confirmed that differential epigenetic regulation of transcription exist along with \textit{BRCA1} inactivation [7,8]. Therefore, one can speculate that there are wide ranges of gene expression and regulation differences between \textit{BRCA1} dysfunction and the basal phenotype. To date, choline is among the well-studied essential nutrients that are involved in breast cancer; for example: (i) choline-containing compounds are significantly changed in breast cancer [9,10]; (ii) choline intake is inversely correlated with breast cancer risk [11-13]; and (iii) aberrant choline metabolism is often associated with malignant transformation, invasion, and metastasis of breast cancer [14-16].

\textsuperscript{4} http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4012741/pdf/oncotarget-05-1315.pdf
FIGURE 1. Major reactions involved in transmethylation flux and methylneogenesis. The total transmethylation flux is equivalent to the total flux occurring through reactions that convert S-adenosylmethionine to S-adenosylhomocysteine. The 3 S-adenosylmethionine-dependent reactions thought to contribute quantitatively most to this flux are methylation of guanidinoacetate by guanidinoacetate methyltransferase (GAMT) to form creatine; methylation of phosphatidylethanolamine by phosphatidylethanolamine methyltransferase (PEMT) to form phosphatidylcholine; and methylation of glycine by glycine N-methyltransferase (GNMT) to form sarcosine (N-methylglycine). A large number of additional S-adenosylmethionine-dependent methyltransferases also occur in mammals [see Katz et al (3)], but their collective quantitative contribution to transmethylation flux may be small compared with those mentioned above. The final steps in methylneogenesis are the reduction of a methylene group of 5,10-methylenetetrahydrofolate (methylene-THF) by methylenetetrahydrofolate reductase (MTHFR) to form 5-methyltetrahydrofolate (methyl-THF), followed by transfer by methionine synthase of the newly formed methyl moiety to homocysteine, forming methionine and tetrahydrofolate (THF). Sarcosine is formed not only by GNMT, but also by oxidation of choline to betaine, formation of dimethylglycine by betaine homocysteine methyltransferase (BHMT), and oxidation of dimethylglycine to sarcosine. Sarcosine is oxidized by sarcosine dehydrogenase (SDH). During the reaction, glycine is produced, and a 1-carbon unit is transferred to THF, forming methylene-THF. MAT, methionine adenosyltransferase; CBS, cystathionine β-synthase; CGL, cystathionine γ-lyase; SAHH, S-adenosylhomocysteine hydrolase.
Fig. S5F, P = 0.278), c-erbB-2-positive (Supplementary Fig. S5H, P = 0.244), p53-negative (Supplementary Fig. S5I, P = 0.072), or Ki67-positive (Supplementary Fig. S5J, P = 0.145) breast cancer showed a trend for poor overall survival, although not statistically significant. No significant difference in overall survival was found among patients with different age at diagnosis, premenopausal, tumor size, progesterone receptor status, or PEMT methylation (Supplementary Fig. S5A, B, C, G, and L).

**DISCUSSION**

Promoter methylation, with concurrent changes in histone modifications, is an epigenetic phenomenon that can affect the conformation of chromatin and tissue-specific gene expression[23,24]. In this study, we report that the first-promoter-specific transcript 1 is the major PEMT mRNA species in human breast tissues, and that methylation of the -132 site is a key regulatory element for PEMT transcription in BRCA1-mutated breast cancer. The molecular mechanism may involve the hypermethylated -132 site-mediated loss of active histone marker H3K9ac and an increase of the repressive histone marker H3K9me enrichment, which synergistically inhibit the transcription of PEMT. Interestingly, the synergistic inhibitory effect of hypermethylated -132 site and H3K9ac and H3K9me histone modification were only observed primarily in cells originating from BRCA1-mutated breast cancer; the transformed cell line 293T and non-mutated breast cancer

PEMT-related choline synthesis and choline-related BRCA1 inactivity. Meanwhile, choline plays a critical role in the methionine cycle [29]. Beetstra confirmed that defects in methionine metabolism may be related to breast cancer risk in BRCA carriers [30]. These results, together with our observations, suggest that low levels of PEMT may be involved in the development of BRCA1-mutated breast cancer through choline deficiency.

Promoter hypermethylation is often associated with adverse clinical events [31]. In line with this, clinicopathological data indicated that hypermethylation of the -132 site may be an effective indicator for histological grade and estrogen receptor status in BRCA1-mutated breast cancer tissues (Table 1). Moreover, univariate survival and multivariate analyses indicated that lymph node metastasis was an independent and reliable prognostic factor which is associated with worse outcomes for BRCA1-mutated breast cancer patients.

This study provides new insights into the causes and prognosis of PEMT inactivation in BRCA1-mutated breast cancer. The mechanism involves the synergistic effects of promoter methylation and histone modification. Therefore, a more specific epigenetic therapy could be developed for BRCA1-mutated breast cancer.

**METHODS**

**Ethics Statement**
Arsenic and Cancer

**Conditions related to arsenic-induced toxicity**
- Skin conditions
- CVD
- PVD
- Lung disorders
- Neurological disorders

**Arsenic increases risk of cancer (skin, lung, bladder, liver, kidney) via multiple routes**
- Various enzyme inhibition (SUOX, PDH, etc)
- ↓ DNA repair
- Chromosomal instability
- ↑ ROS (Superoxide – needing SOD & H₂O₂ – needing GSH and CAT)
- ↓ DNA methylation
- Altered gene expression

(c) 2014: Benjamin Lynch, ND

http://nas-sites.org/emergingscience/files/2011/05/10-Session-2b-Graziano_POST.pdf
Arsenic Exposures

Increased arsenic levels due to:

• Drinking water
• Pesticides
• Antibiotics (in chicken)
• Rice
• Apples
• Sea vegetables
• SNPs
Figure 1. The metabolism pathway of inorganic arsenic showing arsenate reduction to arsenite and methylation to pentavalent and trivalent forms.

2. Genotoxicity

The genotoxic role of iAs in the cells has long been controversial. Arsenic is reported to cause DNA modifications such as aneuploidy, micronuclei formation, chromosomal aberrations, deletion mutations, sister chromatid exchange and DNA-protein cross-linking [1]. Several mechanisms have been proposed to explain the genotoxicity of arsenic, as well as induction of oxidative stress and altered patterns of DNA repair [15].
Mitochondria

Oxidative Phosphorylation
Genetics: Mitochondria

Mitochondrial DNA (mtDNA)
- Inherited only from Maternal side (family hx Important)
- Majority of ATP produced in mitochondria
- Require importing nDNA gene products to function
- SNPS/mutations in mtDNA may be pathological
  - Cancer
  - Diabetes
  - Cardiovascular Diseases
  - Neurodegenerative Diseases
  - Aging
  - Degenerative Diseases
- Lack of Histones = high rate of mtDNA mutagenesis (10x nDNA)
- Mitochondrial Transcription Factor A (TFAM) = Protective coating and regulation
- mtDNA copy number ↑ cell survival and function

(c) 2014: Benjamin Lynch, ND
Mitochondrial regions harboring common mutations in different cancer sites

(a) Schematic representation of the mitochondrial genome showing the 16 coding and non-coding regions including the D-loop. The mitochondrial genome showing the various mutations summarized in this review. For mutations in complex I (ND1, 3, ND4, ND5, ND6 and ND4L), refer to Table 1. Mutations in complex III (Cyt B), refer to Table 2. Mutations in complex IV (COX II and COX III), refer to Table 3. Mutations in complex V (ATPase 6), refer to Table 4.
Introduction

Despite advances in clinical therapy, metastasis is still the leading cause of death in breast cancer patients (1). A clearer understanding of molecular mechanisms that drive metastasis will help to develop more effective therapies (2). Our present study focused on metabolism as an essential driver of tumor growth and metastasis, potentially common to all breast cancer types. Normal cells primarily use mitochondrial oxidative phosphorylation (OXPHOS) for energy production, whereas cancer cells depend on aerobic glycolysis (the Warburg effect) to generate energy and glycolytic intermediates for enhanced growth (3, 4). Tumor cells also generate high levels of reduced forms of NAD+, NADH, and NADPH as important cofactors and redox components (4, 5). These altered metabolic activities can be linked to mitochondrial dysfunction that inhibits OXPHOS, increases ROS, promotes uncontrolled growth, and causes DNA damage that further supports a metastatic phenotype (6, 7). Mitochondrial dysfunctions can be caused by mutations in mitochondrial DNA (mtDNA) or nuclear genes encoding mitochondrial proteins (6, 8) that are essential for the respiratory chain/OXPHOS system. Due to the lack of protective histones and limited DNA repair (8), mtDNA mutations occur at high rates and were found in tumors including breast cancer (6, 9–14), which suggests that defects in OXPHOS might contribute to tumorigenesis.

By combining the nuclear genome of a recipient cell with the mitochondrial genome of a donor cell using hybrid technology, mitochondria from the triple-negative aggressive breast cancer cell lines MDA-MB-435 (15) and MDA-MB-231 facilitated tumor progression and metastasis in nonmetastatic tumor cells (7, 10). The donor cell lines harbor mtDNA mutations in tRNAs, in the noncoding D-loop region (9, 10), and in mitochondrial complex I subunit genes (10). These defects suggest a role of mtDNA mutations and complex I in tumor progression. Therefore, these cell lines are excellent models for defining a specific role of complex I activity in tumor growth and metastatic aggressiveness.

Complex I is the gatekeeper of the respiratory chain and catalyzes the first step of NADH oxidation. It elevates the NAD+/NADH ratio and translocates protons across the inner mitochondrial membrane, which ultimately leads to energy production. mtDNA mutations in genes encoding complex I subunits are found in malignancies including breast cancer (6, 11–14, 16). However, it is largely unknown how alterations in complex I and the cellular NAD+/NADH redox balance affect tumorigenesis and metastasis.

We used a unique approach to define contributions of complex I activity to breast cancer progression, based on expression of the yeast NADH dehydrogenase Ndi1 in human tumor cells. Ndi1 encodes a single protein that faces the inner mitochondrial matrix and oxidizes NADH from the Krebs cycle. Unlike mammalian complex I, Ndi1 is rotenone insensitive (17). Ndi1 contains 26 N-terminal residues for mitochondrial import (17), can be functionally expressed in mammalian cells (18, 19), and does not cause an immune response (20). Ndi1 restores complex I function (18) in diseased cells, e.g., in neurons of Parkinson’s disease (21) and optic neuropathy (22); protects cardiomyocytes from ischemic reperfusion injury (23); and increases lifespan in Drosophila (24). Recently, it was shown that Ndi1 expression in complex I-deficient tumor cells can reduce soft agar colony formation (25).

We used Ndi1 to investigate a cause-and-effect relationship between aberrant mitochondrial complex I activity and malignant progression in breast cancer. Moreover, we analyzed metabolic alterations caused by mitochondrial complex I malfunction and translated the information gained into a novel therapeutic
**Oxidative Stress: Degree Is Everything**

“When oxidative stress is severe, or when the function of protective enzymes is deeply compromised, cells may ‘sacrifice themselves’ by apoptosis, which protects the surrounding healthy tissue from further damage. Only under the most severe oxidative stress conditions and when adaptation mechanisms fail will cells then undergo necrotic death, which exposes the surrounding non-damaged cells to immune responses.”

“In contrast to apoptosis, necrosis can result from extensive damage to the plasma membrane with disturbance of ion transport, dissolution of membrane potential, cell swelling and eventual rupturing of the cell. During necrosis, the cell liberates breakdown products, including lipid peroxides, aldehydes and eicosanoids triggering an inflammatory response. Apoptosis is energy dependent and is considered to be a protective mechanism that prevents cells from oncotic necrosis, which often promotes more damage to surrounding cells through the involvement of the immune system.

In the presence of ATP, cell death can proceed by apoptosis, but when mitochondria are de-energized, cell death proceeds by necrosis.”

(c) 2013: Benjamin Lynch, ND

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3704158/
Pathway Planner
Folate, Transsulfuration & Methionine

SeekingHealth.org
Caffeine Inhibits Adenosine-Induced Accumulation of Hypoxia-Inducible Factor-1α, Vascular Endothelial Growth Factor, and Interleukin-8 Expression in Hypoxic Human Colon Cancer Cells

K.V., C.S., P.A.B.); Department of Human Anatomy, Pharmacology, and Forensic Medicine, Human Anatomy Section, University of Parma, Parma, Italy (P.M.); King Pharmaceuticals R&D, Cary, North Carolina (S.M.L., E.L.); Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy (P.G.B.); and Interdisciplinary Center for the Study of Inflammation, Ferrara, Italy (P.A.B.)

Received November 22, 2006; accepted May 8, 2007

http://molpharm.aspetjournals.org/content/72/2/395.full.pdf

ABSTRACT

Frequent coffee consumption has been associated with a reduced risk of colorectal cancer in a number of case-control studies. Coffee is a leading source of methylxanthines, such as caffeine. The induction of vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) is an essential feature of tumor angiogenesis, and the hypoxia-inducible factor-1 (HIF-1) transcription factor is known to be a key regulator of this process. In this study, we investigated the effects of caffeine on HIF-1 protein accumulation and on VEGF and IL-8 expression in the human colon cancer cell line HT29 under hypoxic conditions. Our results show that caffeine significantly inhibits adenosine-induced HIF-1α protein accumulation in cancer cells. We show that HIF-1α and VEGF are increased through A3 adenosine receptor stimulation, whereas the effects on IL-8 are mediated via the A2B subtype. Pretreatment of cells with caffeine significantly reduces adenosine-induced VEGF promoter activity and VEGF and IL-8 expression. The mechanism of caffeine seems to involve the inhibition of the extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and Akt, leading to a marked decrease in adenosine-induced HIF-1α accumulation, VEGF transcriptional activation, and VEGF and IL-8 protein accumulation. From a functional perspective, we observe that caffeine also significantly inhibits the A3 receptor-stimulated cell migration of colon cancer cells. Conditioned media prepared from colon cells treated with an adenosine analog increased human umbilical vein endothelial cell migration. These data provide evidence that adenosine could modulate the migration of colon cancer cells by an HIF-1α/VEGF/IL-8-dependent mechanism and that caffeine has the potential to inhibit colon cancer cell growth.
within 14 days with fatty liver. Adenine nucleotides were decreased and S-adenosylhomocysteine, a potent inhibitor of transmethylation reactions, was increased in the mutant liver. Thus, a deficiency in adenosine metabolism is identified as a powerful contributor to the development of neonatal hepatic steatosis, providing a model for the rapid development of postnatally lethal fatty liver.

http://www.pnas.org/content/99/10/6985.full.pdf

Neonatal hepatic steatosis (OMIM 228100) is a fatal condition characterized by a rapid microvesicular fat infiltration and enlargement of the liver, which shows a pale and yellowish coloration (1). In this condition, microvesicular fat infiltration, liver failure, coma, and finally death, is considered to be a consequence of severe impairment of mitochondrial function (2–4). So far, mitochondrial dysfunction related to hepatic steatosis has been associated (i) with genetic defects in the β-oxidation of fatty acids (5), (ii) with inhibition of β-oxidation by drugs (i.e., valproate) (3, 4), (iii) with defective carnitine-dependent transport of fatty acids (6), and (iv) with an impaired production of ATP (7).

In the present study, a deficit in adenosine-dependent metabolism is proposed as a causative factor for the development of microvesicular hepatic steatosis. A deficiency in adenosine kinase (ADK; EC 2.7.1.20) (Fig. 1), the major adenosine-removing enzyme of postnatal liver, is expected to affect liver function on three different levels: (i) Availability of adenine nucleotides, (ii) disruption of the futile cycle between AMP and adenosine, and (iii) maintenance of transmethylation reactions.

(i) Physiologically, adenosine is constantly recycled into the adenine nucleotide pool by ADK-mediated phosphorylation of adenosine to AMP. Because the metabolic flux rate in liver mitochondria is proportional to the amount of available adenine nucleotides, decreased levels of adenine nucleotides in the liver should lead to an impairment of mitochondrial function. Thus, the transmethylation reactions differ in the nature of their substrates, but have a common end product, namely S-adenosylhomocysteine (SAH). Despite the diversity of the methyl acceptors and the specificity of the catalyzed reactions the mammalian methyltransferases share the property of product inhibition by SAH (11). Removal of SAH by SAH-hydrolase (EC 3.3.1.1) is therefore an essential step leading to the formation of adenosine, an obligatory product of SAM-dependent transmethylation reactions (Fig. 1). The thermodynamic equilibrium of the SAH-hydrolase reaction favors the synthesis of SAH, but physiologically the reaction proceeds in the hydrolytic direction because of the efficient hydrolysis of homocysteine under the formation of adenosine (11). The further metabolism of adenosine follows two pathways. During embryonic development, adenosine is mainly degraded to inosine by adenosine deaminase (ADA, EC 3.5.4.4), but this reaction is not pronounced in postnatal liver where ADA is expressed at very low levels (12). Postnatally, adenosine is converted to AMP by ADK (13).

ADK is present in most tissues with the highest expression level in liver (14), the organ in which 85% of all transmethylation reactions take place (15) (Fig. 1). On the basis of its low affinity for adenosine ($K_m = 0.2–2 \mu M$), the phosphorylation of adenosine mediated by ADK is believed to be the primary route of postnatal adenosine metabolism (16). ADK is therefore an important downstream control point (i) for the recycling of adenine nucleotides, (ii) for regulating adenosine release by means of the AMP/adenosine futile cycle, and (iii) for the maintenance of transmethylation reactions (17). Dysfunction of adenosine metabolism may therefore be associated with a failure of liver function.

So far, no human disease has been linked to mutations in the Adk locus. The murine Adk gene was therefore disrupted to test the potential role of the adenosine futile cycle in the development of fatal neonatal hepatic steatosis.
http://www.nature.com/nrc/journal/v3/n3/box/nrc1013 BX1.html
The image depicts a diagram of mitochondrial electron transport and oxidative stress pathways. Key components include:

- **NADH** and **FADH₂** as electron donors to the respiratory chain.
- **O₂⁻** (superoxide radical) and **H₂O₂** (hydrogen peroxide) production from the respiratory chain.
- **ONOO⁻** (peroxynitrite) formation from the reaction of **O₂⁻** and **NO**.
- **GPX/CAT** enzymes involved in the detoxification of **H₂O₂**.
- **GR** (glutathione reductase) and **GSSG** (glutathione disulfide) in the glutathione cycle.
- **MnSOD** (manganese superoxide dismutase) and **Zn/CuSOD** (zinc/copper superoxide dismutase) for the dismutation of **O₂⁻**.

The diagram illustrates the complex interactions between these molecules, including the formation of reactive oxygen species (ROS) and their role in protein oxidation and lipid peroxidation, contributing to mitochondrial dysfunction.
Mitochondrial complex I activity and NAD⁺/NADH balance regulate breast cancer progression

Antonio F. Santidrian,1,2 Akemi Matsuno-Yagi,1 Melissa Ritland,1,2 Byoung B. Seo,1 Sarah E. LeBoeuf,1,2 Laurie J. Gay,1,2 Takao Yagi,1 and Brunhilde Felding-Habermann1,2

1Department of Molecular and Experimental Medicine and 2Department of Chemical Physiology, The Scripps Research Institute, La Jolla, California, USA.

Despite advances in clinical therapy, metastasis remains the leading cause of death in breast cancer patients. Mutations in mitochondrial DNA, including those affecting complex I and oxidative phosphorylation, are found in breast tumors and could facilitate metastasis. This study identifies mitochondrial complex I as critical for defining an aggressive phenotype in breast cancer cells. Specific enhancement of mitochondrial complex I activity inhibited tumor growth and metastasis through regulation of the tumor cell NAD⁺/NADH redox balance, mTORC1 activity, and autophagy. Conversely, nonlethal reduction of NAD⁺ levels by interfering with nicotinamide phosphoribosyltransferase expression rendered tumor cells more aggressive and increased metastasis. The results translate into a new therapeutic strategy: enhancement of the NAD⁺/NADH balance through treatment with NAD⁺ precursors inhibited metastasis in xenograft models, increased animal survival, and strongly interfered with oncogene-driven breast cancer progression in the MMTV-PyMT mouse model. Thus, aberration in mitochondrial complex I NADH dehydrogenase activity can profoundly enhance the aggressiveness of human breast cancer cells, while therapeutic normalization of the NAD⁺/NADH balance can inhibit metastasis and prevent disease progression.
Kyurenine Pathway
Kyurenine Pathway

Functions (some):

• Immune tolerance
• NAD synthesis

When upregulated leads to immune tolerance and escape
**Figure 2.** IDO is activated inflammation and helps create conditions that favor immune suppression and tolerance. Primary insults create local inflammation and generalized signals that activate immune cells. However, overall immune outcomes depend on the balance of additional signals that promote either effector and regulatory responses. Thus inflammation drives the immune response, but the specific character depends critically on the overall balance of immune stimulatory and regulatory pathways activated in a particular setting. Inflammatory signals that favor regulatory/suppressor outcomes often activate and/or sustain local IDO enzyme activity.
Kyurenine Pathway

“...acute stress exposure causes a rapid upregulation of IDO1 expression. We found that sustained Trp catabolism reduces the anti-bacterial defense.”

“Kynurenines which are generated in stressed mice are rapidly used for NAD+ synthesis because of a high energy demand in stressful situations.” — NAD → mitochondria

“Prolonged activation of the Trp catabolic pathway during repeated stress alters the capacity to cope with infection.”

“IDO1 activation in repeated stress-induced loss of the anti-bacterial defense is underlined by the yet preliminary findings that stressed IDO12/2 mice were capable to cope with an E. coli infection comparable to non-stressed, wild-type mice.”

“Patients undergoing surgery who suffer from increased anxiety, helplessness and social isolation show IDO1 activation…. Acute stress-induced low-grade inflammation induces transient IDO1-mediated tryptophan-catabolism and prolonged Trp shortage due to expanded stressful episodes as well as the formation of kynurenines are immunosuppressive and simultaneously may induce mood alterations.

Psychological stress-induced, IDO1-dependent tryptophan catabolism: implications on immunosuppression in mice and humans
The Kynurenine Pathway in Brain Tumor Pathogenesis

Seray Adams, Nady Braidy, Alban Bessesse, Bruce J. Brew, Ross Grant, Charlie Teo, and Gilles J. Guillemain

Abstract

Brain tumors are among the most common and most chemoresistant tumors. Despite treatment with aggressive treatment strategies, the prognosis for patients harboring malignant gliomas remains dismal. The kynurenine pathway (KP) is the principal route of L-tryptophan catabolism leading to the formation of the essential pyridine nucleotide, nicotinamide adenine dinucleotide (NAD\(^+\)), and important neuroactive metabolites, including the neurotoxin, quinolinic acid (QUIN), the neuroprotective agent, picolinic acid (PIC), the T\(_{H}17\)/Treg balance modulator, 3-hydroxyanthranilic acid (3-HAA), and the immunosuppressive agent, \(\alpha\)-kynurenine (KYN). This review provides a new perspective on KP dysregulation in defeating antitumor immune responses, specifically bringing light to the lower segment of the KP, particularly QUIN-induced neurotoxicity and downregulation of the enzyme \(\alpha\)-amino-\(\beta\)-carboxymuconate-\(\varepsilon\)-semialdehyde decarboxylase (ACMSD) as a potential mechanism of tumor progression. Given its immunosuppressive effects, 3-HAA produced from the KP may also play a role in suppressing antitumor immunity in human tumors. The enzyme indoleamine 2, 3-dioxygenase (IDO-1) initiates and regulates the first step of the KP in most cells. Mounting evidence directly implicates that the induction and overexpression of IDO-1 in various tumors is a crucial mechanism facilitating tumor immune evasion and persistence. Tryptophan 2, 3-dioxygenase (TDO-2), which initiates the same first step of the KP as IDO-1, has likewise recently been shown to be a mechanism of tumoral immune resistance. Further, it was also recently shown that TDO-2-dependent production of KYN by brain tumors might be a novel mechanism for suppressing antitumor immunity and supporting tumor growth through the activation of the Aryl hydrocarbon receptor (AhR). This newly identified TDO-2-KYN-AhR signaling pathway opens up exciting future research opportunities and may represent a novel therapeutic target in cancer therapy. Our discussion points to a number of KP components, namely TDO-2, IDO-1, and ACMSD, as important therapeutic targets for the treatment of brain cancer. Targeting the KP in brain tumors may represent a viable strategy likely to prevent QUIN-induced neurotoxicity and KYN and 3-HAA–mediated immune suppression. Cancer Res; 72(22): 5649–57. ©2012 AACR.

Introduction

Primary brain tumors are the most common form of solid malignancy among children (1), and the second leading cause of cancer death in males under 39 years of age and females under 20 years of age (2). As such, they are among the leading causes of cancer-related morbidity and mortality during childhood. Glioblastoma multiforme (GBM) is, by far, the most prevalent and most malignant type of primary brain tumor in adults (3). It is an aggressive tumor that progresses rapidly; the median survival of patients with GBM is approximately 14.6 months (4). Despite technological advancements on all fronts of neurological practice, the prognosis for patients with malignant gliomas remains dismal (5). The poor prognosis for patients with malignant gliomas and the lack of effective therapies highlight the importance of developing novel pharmacologic therapies with greater clinical efficacy than those that are currently available. During tumor development, malignant cells evolve to a state in which they have the capacity for immune escape (6). Ultimately, mechanisms of immune escape enable the evasion and/or suppression of the immune response that tumor antigens elicit, which is central to tumor survival, growth, local invasion, regional spread, and metastasis.
In a proof of principle clinical trial, Mayo Clinic researchers have demonstrated that virotherapy — destroying cancer with a virus that infects and kills cancer cells but spares normal tissues — can be effective against the deadly cancer multiple myeloma. ...

Oncolytic virotherapy — using re-engineered viruses to fight cancer — has a history dating back to the 1950s. Thousands of cancer patients have been treated with oncolytic viruses from many different virus families (herpes viruses, pox viruses, common cold viruses, etc.). However, this study provides the first well-documented case of a patient with disseminated cancer having a complete remission at all disease sites after virus administration.

“What this all tells us is something we never knew before — we never knew you could do this in people,” Russell said. “It’s a very important landmark because now we know it can happen. It’s a game changer. And I think it will drive a development in the field.”

The Star Tribune explained how it works:

[Viruses] bind to tumors and use them as hosts to replicate their own genetic material; the cancer cells eventually explode and release the virus. Antiviral vaccines that have been rendered safe can produce the same effects and can also be modified to carry radioactive molecules to help destroy cancer cells without causing widespread damage to healthy cells around the tumors. The body’s immune system then attacks any remaining cancer that carries remnants of the vaccine’s genetic imprint.

Russell said the trial taught the medical researchers two things: “No. 1, you need a really big dose and No. 2, the patient needs to not have an antibody to the virus.”
Action Steps
History

- Maternal side = mitochondrial disorders
- Zipcode = www.scorecard.org (for USA)
- Lifestyle / Diet / Environment / Occupation / Hobbies
- Major illnesses / hospitalizations
- ALL symptoms – not chief complaints
Apoptosis

Reinstate Apoptosis

• Radiation
• Chemotherapy
• Mitochondrial support
• Glutathione
• Cell Membrane support
• Immune support
• Mind/Body Exercises
• Fasting
• Exercise
Lab Tests (Routine labs plus...)

- Fasting insulin
- Fatty Liver Index - [http://www.mayoclinic.org/medical-professionals/model-end-stage-liver-disease/alcoholic-liver-disease-nonalcoholic-fatty-liver-disease-index](http://www.mayoclinic.org/medical-professionals/model-end-stage-liver-disease/alcoholic-liver-disease-nonalcoholic-fatty-liver-disease-index)
- Kyurenine
- Uracil / Thymine
- VAP – advanced cholesterol panel
- Oxidative Stress – lipid peroxidation, SOD and GPX expression
- Methylation profile (with adenosine, nitric oxide and nitrotyrosine)
- RBC Fatty Acids
- Pathogens – viral, mold, bacterial, Lyme, parasites
- Lactate
- Electrolytes
- Environmental – heavy metals (unprovoked and provoked – inc post-chemo)
- Genetic Testing (23andMe, et al)
Cancer Prevention, Treatment and Follow Up

Lifestyle, Diet and Other Recommendations

• Caffeine (coffee enemas?)
• Belly breathing
• Restrict carbs and sugars
• Support mitochondrial respiration
• Sauna
• Intermittent fasting
• Meditation
• Exercise
• Earthing
• Raw foods and vegetable/berry/greens juicing with pulp → ↑ e-
## Methylation Profile; plasma

### PRIMARY & INTERMEDIATE METABOLITES

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Result/Unit</th>
<th>Reference Interval</th>
<th>2.5th</th>
<th>16th</th>
<th>50th</th>
<th>84th</th>
<th>97.5th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine</td>
<td>2.0 µmol/dL</td>
<td>1.6-3.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>50 µmol/dL</td>
<td>20-38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-adenosylmethionine (SAM)</td>
<td>205 nmol/L</td>
<td>86-145</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-adenosylhomocysteine (SAH)</td>
<td>46.1 nmol/L</td>
<td>10-22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homocysteine</td>
<td>20.6 µmol/L</td>
<td>&lt;11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystathionine</td>
<td>0.30 µmol/dL</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### METHYLATION INDEX

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Result</th>
<th>Reference Interval</th>
<th>PERCENTILE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM : SAH</td>
<td>4.5</td>
<td>&gt;4</td>
<td>68th 95th</td>
</tr>
</tbody>
</table>
AMINOACIDS IN PLASMA
Nitrotyrosine
Glutathione (oxidised)
Glutathione (reduced)
MISCELLANEOUS
Ammonia (plasma)
NO (Nitric oxide)
Derivates
S-Adenosylmethionine (RBC)
S-Adenosylhomocysteine (RBC)
FOLIC ACID DERIVATIVES
5-CH3-THF
10-Formyl-THF
5-Formyl-THF
THF
Folic Acid
Folinic Acid (WB)
Folic Acid, active (RBC)  
BIOLOGICAL AMINES
CATACHOLAMINES IN PLATELETS
Histamine (whole blood)
NUCLEOSIDE
Adenosine

(c) 2014: Benjamin Lynch, ND

http://www.hdri-usa.com/tests/methylation/
Blood Test Predicts Breast Cancer Recurrence

http://www.cancernetwork.com/breast-cancer/blood-test-predicts-breast-cancer-recurrence

News | April 18, 2014 | Breast Cancer
By Anna Azvolinsky, PhD

As researchers continue to show that cell-free circulating tumor DNA (ctDNA) can be consistently detected in the blood of cancer patients, assays that can provide reliable information about a patient’s treatment progress or potential recurrence by a simple blood draw are becoming increasingly appealing.

A new pilot study demonstrates the ability of a blood-based test to detect metastatic breast cancer recurrence, with high sensitivity and specificity. The ctDNA test “shows great potential for development as a clinical laboratory test for monitoring therapy and disease progression and/or recurrence,” concluded the study authors.

The study was published in Cancer Research, a journal of the American Association for Cancer Research.

The test, called cMethDNA, detects the hypermethylation state of 10 genes through a quantitative, methylation-specific polymerase chain reaction (PCR) assay from ctDNA extracted from a patient’s blood sample. DNA methylation is the addition of methyl groups to either cytosine or adenine DNA nucleotides of a gene. Extensive methylation of a gene typically results in the silencing, or non-expression, of a gene. In breast tumors, for example, the tumor suppressor BRCA1 gene has been found to be either mutated or methylated, inactivating the gene or dampening its expression.

Using a prospective cohort of stage IV metastatic patients enrolled in clinical trials at Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, the cMethDNA test identified a sensitivity of 91.7% and a specificity of 96% for patients with recurrent metastatic breast cancer ($P < .0001$).

“I think we have achieved a very high sensitivity and specificity of detection for metastatic breast cancer [that is] higher than current tests. Our next step will involve testing the assay in a large prospective study through the large cooperative groups,” senior author Saraswati Sukumar, PhD, professor of oncology and pathology at Johns Hopkins University School of Medicine, told Cancer Network.
Stay Informed

Great ways to stay informed:

• Newsletter Available at www.MTHFR.net
• Facebook: https://www.facebook.com/drbenjaminlynch
• October 2013 Nutrigenomics Conference www.SeekingHealth.org

Thank You