American Institute of Integrative Oncology
RESEARCH & EDUCATION

Foundations of Integrative Oncology
COURSE

GRAND ROUNDS CALL
With Dr. Nalini Chilkov
April 4th, 2018

Typically Second Wednesday of Every Month
5:30 PM Pacific / 6:30 PM Mountain / 7:30 PM Central / 8:30 PM Eastern

Clinical Pearl: Ketogenic Diets and Cancer Metabolism: Overview

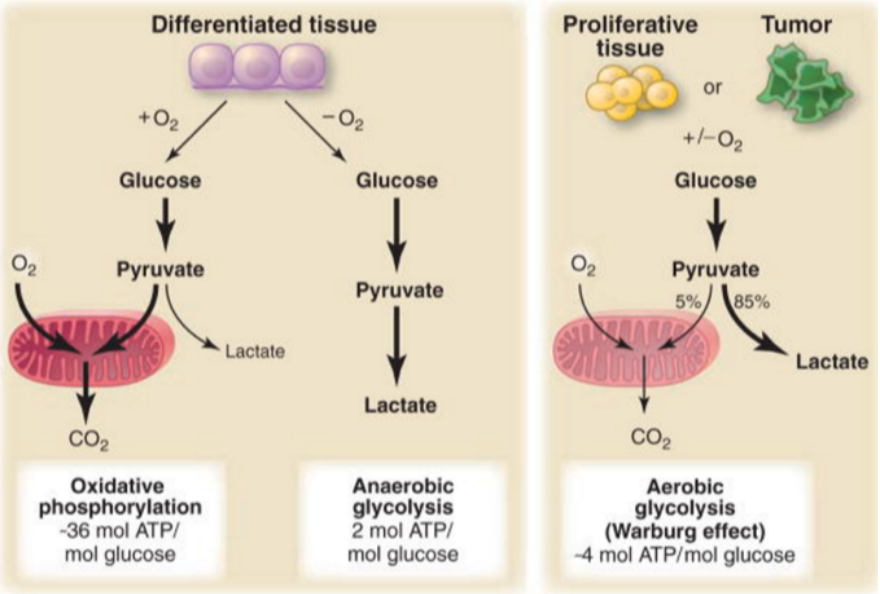
The tumor microenvironment contributes to every aspect of carcinogenesis.

Hallmark of Cancer: Shift in Mitochondrial Energy Metabolism

- Oxidative phosphorylation (OXPHOS) in mitochondria plays a pivotal role in ATP production.
- Pyruvate generated by glycolysis is one of the major fuels of mitochondrial energy production.
- Ketone bodies are generated by fatty acid oxidation and by degradation of ketogenic amino acids.
- Ketone bodies can be used as an alternative fuel for OXPHOS.
- Aerobic energy production via OXPHOS is often impaired in cancer.

Warburg Effect: Shift to AEROBIC Glycolysis

- Increased Dependence on Glucose
- Increased Lactic Acid Production



Article: ***Cancer as a metabolic disease: Implications for novel therapeutics*** (Seyfried et al, 2013)

- When systemic glucose availability becomes limiting, most normal cells of the body will transition their energy metabolism to fats and ketone bodies. Ketone bodies are generated almost exclusively in the liver from fatty acids of triglyceride origin during periods of fasting.
- Most tumor cells are unable to use ketone bodies for energy due to abnormalities in mitochondria structure or function.
- Advanced metastatic cancers can become manageable when their access to fermentable fuels becomes restricted.

Ketogenic Diet (Vidali et al, 2015)

- 75% Fat
- 20% Protein
- 5% CHO
- 4:1 or 3:1 Ratio (Fat : Protein + CHO)

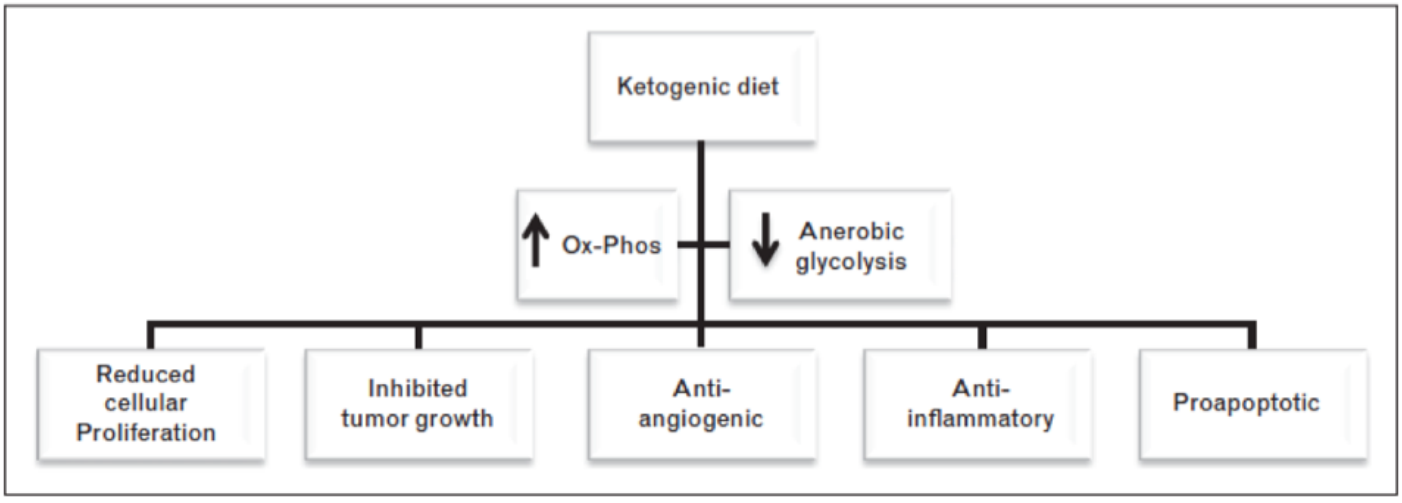
The major substrate for energy production in cancer cells is glucose. Ketone bodies generated by fatty acid oxidation can serve as alternative metabolites for aerobic energy production.

The ketogenic diet, which is high in fat and low in carbohydrates, mimics the metabolic state of starvation, forcing the body to utilize fat as its primary source of energy.

The ketogenic diet is a high-fat, adequate-protein, low-carbohydrate diet that forces the body to burn fats rather than carbohydrates.

Takes advantage of Mitochondropathy and Warburg effect in CA cells and altered use of glucose and oxidation and production of ATP

- Low glycemic
- Low insulin, low IGF-1
- Improved glycemic control
- Increase in LDL cholesterol
- Can be high in arachidonic acid
- May increase CA risk depending on type of fat (saturated animal fats, trans fats)
- Deficient in trace minerals Se, Cu, Zn
- May cause hypoglycemia, nausea, diarrhea
- Depending on robustness of the patient, can be used for 12, 24, or 48 hours prior to IV chemotherapy infusion to stress tumor cells, stimulate immunity



Ketogenic Diets As Adjuvant Cancer Therapy
A ketogenic diet has been shown to impede tumor growth in a variety of cancers through anti-angiogenic, anti-inflammatory, and pro-apoptotic mechanisms (Wright & Simone, 2016).

Dietary CHO Restriction
Article: *A low carbohydrate, high protein diet slows tumor growth and prevents cancer initiation* (Ho et al, 2011)

- Hyperglycemia Enhances Proliferation in some tumors
- Insulin is the primary growth factor and driver of proliferation (Insulin Makes Glucose Available)
- Tumor Size and Growth is Related to Plasma Insulin Levels and Plasma Lactate Levels
- Low CHO diets cause a drop in plasma insulin and lactate
- Low CHO diets can reduce insulin-mediated glucose uptake into tumor cells

Cancer cells rely more heavily on glycolysis than normal cells.
10% or 20% CHO High Protein diet slows tumor growth as effectively as No CHO Ketogenic Diets

Reduces serum glucose, insulin, and glycolysis, slows tumor growth, reduces tumor incidence, and works additively with existing therapies without weight loss or kidney failure.

Intermittent Fasting

- A daily fast of 13 hours or more can tip patient into mild ketosis, improve insulin sensitivity, stimulate immunity
- Integrate as a lifestyle habit
- Note: See the February 2018 Grand Rounds Call for further discussion on intermittent fasting

What to Eat on a Ketogenic Diet

- Meats – fish, beef, lamb, poultry, eggs, etc
- Leafy Greens – spinach, kale
- Above ground vegetables – broccoli, cauliflower, etc
- High Fat Dairy – hard cheeses, high fat cream, butter
- Nuts and seeds –walnuts, sunflower seeds
- Avocado and berries – raspberries, blackberries
- Other fats – coconut oil, high-fat salad dressing, saturated fats
- Drink more water

SUMMARY: Cancer is a Metabolic Disease

- Diet Influences the Tumor Microenvironment
- Diet Influences Onco-Genes & Tumor Suppressor Genes
- Ketogenic Diet Influences Tumor Cell Metabolism
- 10% or 20% CHO High Protein slows growth as effectively as No CHO Ketogenic Diet

References:

Gao, F., Liang, B., T Reddy, S., Farias-Eisner, R., & Su, X. (2014). Role of inflammation-associated microenvironment in tumorigenesis and metastasis. Current cancer drug targets, 14(1), 30-45.

Ho, V. W., Leung, K., Hsu, A., Luk, B., Lai, J., Shen, S. Y., ... & Nelson, B. H. (2011). A low carbohydrate, high protein diet slows tumor growth and prevents cancer initiation. Cancer research, 71(13), 4484-4493.

Seyfried, T. N., Flores, R. E., Poff, A. M., & D'Agostino, D. P. (2013). Cancer as a metabolic disease: implications for novel therapeutics. Carcinogenesis, 35(3), 515-527.

Vidali, S., Aminzadeh, S., Lambert, B., Rutherford, T., Sperl, W., Kofler, B., & Feichtinger, R. G. (2015). Mitochondria: The ketogenic diet—A metabolism-based therapy. The international journal of biochemistry & cell biology, 63, 55-59.

Wright, C., & Simone, N. L. (2016). Obesity and tumor growth: inflammation, immunity, and the role of a ketogenic diet. Current Opinion in Clinical Nutrition & Metabolic Care, 19(4), 294-299.

Recipe: Ketogenic Protein Shake

1 scoop Protein Powder (30 grams)
2 Tablespoons MCT Oil
1 Tablespoon Flaxseed Oil
2 Tablespoons Omega 3 Fatty Acids Liquid
2 Tablespoons Phophatidyl Choline
1 level teaspoon Carnitine Tartrate Powder
200mg CoQ10
2 teaspoons Concentrated Reds-Greens Powder
½ Avocado
1 Tablespoon Pumpkin Seeds
½ teaspoon Acidophilus-Bifidus powder
1 tablespoon Fiber Powder (soluble + insoluble)
½ organic lemon with seeds and peel

Mix with water or green tea

2 capsules Pancreatic Enzymes + Ox Bile.
2 capsules Lipase Digestive Enzyme
Take with iron-free, copper-free multivitamin

Clinical Pearl Questions & Answers on Ketogenic Diet

Judy Pruzinsky: *Too many saturated fats can increase risk of certain types of cancers. Is there any more that you can say about that?*

- Animal fats harbor environmental fat soluble toxins and hormones.

Judy Pruzinsky: *Do you think that there is less concern about eating fat from grass-fed animals?*

- Animals may receive cleaner feed but are living in the same environment as other animals with the same exposures.
- The larger the mammal and the higher up the food chain, the more environmental toxins are concentrated in fat tissue.
- Studies show that plant-based diets are safer because of our modern toxic environment.

Judy Pruzinsky: *Do you have any concern about people getting enough protein if they go a more vegetarian route?*

- More work and education with patient about how to consume adequate protein
- Shake with 30-40-grams of protein (taken with proteases) is a viable alternative to other protein sources

Judy Pruzinsky: *Not clear about adding in exogenous ketones. Thoughts on this?*

- No research on exogenous ketones.
- Ketone supplements designed for athletes using ketones for fuel.
- Ketogenic diet offers ketone production while starving cancer cells of glucose. This cannot be accomplished by adding ketones into the diet.

Cathy: *You discussed the ketogenic diet basically stressing the tumor cells by following the diet 24-48 hours before chemo. And you also talked about intermittent fasting. I'm wondering, if someone is not open to working with the keto diet, does fasting accomplish the same thing? How long of a fast before chemotherapy would you recommend?*

- Yes, fasting is appropriate but not with extreme fasting approaches where there is a risk of depleting the patient of fluids and electrolytes or low body weight
- Fasting duration depends on the robustness of the patient
- Modify caloric intake during fast with bone broth and ketogenic shakes for minerals and nutrients

Questions & Answers

Corey Deacon: *Do you have any experience/recommendations with peptides for cancers? Specifically renal cell carcinoma and metastatic cholangiocarcinoma, dendritic cell vaccines and pro-apoptotic peptides?*

Dr. Nalini:
Peptides and vaccines fall under the rubric of IMMUNOTHERAPY:

- Leveraging innate and adaptive immunity
- The strategy of triggering the immune system to control tumor progression
- Immunotherapy represents an "unconventional" way of treating cancer by targeting the immune system, not the tumor itself
- Tumor microenvironment also generates fibrosis which can coat the surface of tumor cells, like a raincoat, and

hide the cell from the immune system
- Dendritic cell vaccines have historically had a small and not durable response according to Dr. Dwight McKee MD, Integrative Oncologist
- Robust immune competence is required for a sufficient response to a vaccine or immune therapy

Types of Vaccines:
- Dendritic Cell Vaccines
- Peptide based vaccines
- Bacteria based vaccines
- Adenoviral Vaccines
- DNA Vaccines
- Cell based Vaccines
- Virus Based Vaccines

I have no experience or expertise in peptide-based vaccines and/or dendritic cell vaccines.
I am not an expert on this topic.
A review of recent studies is very disappointing. Most "vaccines" increase overall survival by 5-10 months. Only a very few have shown longer efficacy for up to 30 months. Most are typically used WITH conventional therapies (CT and RT) to boost the immune response. Some therapies such as RT are known to trigger a distal immune response. There are studies combining RT and immunotherapies such as vaccines and peptides and combining CT and vaccine therapy.

- There are some clinics in Germany where vaccine therapy is more advanced than in the U.S.
- There was one study at UCLA on vaccines for glioblastoma.

Dendritic Cell Vaccines
Dendritic cells (DCs), capture, process, and present antigens to T cells, have received considerable interest as a basis for cellular vaccines that can be manipulated to induce responses against tumor-associated antigens (TAAs).

Three main approaches have been evaluated in DC vaccination:
- ex vivo antigenic peptide loading followed by autologous infusion of the conditioned DCs,
- gene modification of DCs in vivo through the use of recombinant viruses,
- and ex vivo genetic engineering for antigen presentation with or without enhanced co-signaling.

Peptide vaccines

Another approach to the stimulation of antitumor immune activity involves the use of personalized peptide vaccines (PPVs). These consist of multiple exogenously administered cancer-associated peptides that can be presented on HLA class I molecules for recognition by T cells.
Aided by rapid improvements in next-generation sequencing, these peptides seek to induce robust and rapid cytotoxic T-lymphocyte activation without the costs and cell availability limitations of cell-based approaches.

Corey Deacon: *I also found some information on CIMAvax-EGF. Is this considered a dendritic vaccine?*

Dr. Nalini:
CIMAvax-EGF is a vaccine developed in Cuba for EGF Positive Non Small Cell Lung Cancer.

Epidermal growth factor receptor overexpression by tumor cells is associated with uncontrolled proliferation, angiogenesis, anti-apoptotic signals, metastization, and invasiveness.

This is a combination -- a conjugate, of Epidermal Growth Factor + Meningitis B bacteria >>>injected into patient>>>production antibodies against Epidermal Growth Factor

- Results in the decline of the circulating EGF in sera
- Significantly decreases the probability that the remaining EGF binds to its receptor (EGFR) on the surface of cancer cells.
- EGF withdrawal results in the loss of a key pro-proliferation and pro-survival signal for the neoplastic cells

- The vaccine has demonstrated to be safe and immunogenic in more than 5,000 advanced NSCLC patients

Several clinical trials are currently ongoing to validate EGF as a predictive biomarker of CIMAvax-EGF efficacy.

Reference:
Saavedra, D., & Crombet, T. (2017). CIMAvax-EGF: a new therapeutic vaccine for advanced non-small cell lung cancer patients. Frontiers in immunology, 8, 269.

Corey Deacon: *Gemcitabine vs Gemcitabine + Carboplatin? Single drug vs combination?*

Dr. Nalini:
Because most tumors are heterogeneous, combination therapies generally yield better results. However, they also lead to more rapid development of chemoresistance. There is a trend in oncology for very advanced patients who will always be undergoing treatment to give one agent at a time in lower and more frequent (weekly) doses to reduce toxicity, improve quality of life and lengthen time to resistance. This allows a terminal patient to have a better quality of life and still exert some control over the tumor behavior.

Carboplatin is an alkylating agent: Alkylating agents are so named because of their ability to add alkyl groups to many electronegative groups under conditions present in cells. Alkylating agents are cell cycle-nonspecific.

Mechanism of action: Carboplatin undergoes activation inside cells and forms reactive platinum complexes that cause the intra- and inter-strand cross-linkage of DNA molecules within the cell. This modifies the DNA structure and inhibits DNA synthesis. This may affect a cell in all the phases of its cycle.

Gemcitabine inhibits thymidylate synthetase, leading to inhibition of DNA synthesis and cell death. Inhibits processes required for DNA synthesis.

Gemcitabine is a prodrug so activity occurs as a result of intracellular conversion to two active metabolites, gemcitabine diphosphate and gemcitabine triphosphate by deoxycitidine kinase.

Gemcitabine, sold under the brand name **Gemzar**, among others, is used to treat a number of types of cancer. These cancers include solid tumors such as breast cancer, ovarian cancer, non-small cell lung cancer, pancreatic cancer, and bladder cancer First line of treatment for pancreatic cancer

Chemoresistance is common.

Corey Deacon**:** *Are there any laboratories doing good RNAseq transcriptome analysis on cancer cells currently?*

Dr. Nalini:

Transcriptome RNA Sequencing

Illumina
MD Anderson Illumina Next Generation Sequencing
https://www.mdanderson.org/research/research-resources/core-facilities/sequencing-and-microarray-facility-smf/services-and-fees/illumina-next-generation-sequencing.html

Genomics Products for Cancer Research
https://www.illumina.com/products/by-area/oncology.html

Mays Cancer Center University of Texas MD Anderson Cancer Center
Next Generation Sequencing and BioInformatics
http://www.uthscsa.edu/patient-care/cancer-center/next-generation-sequencing-bioinformatics

NORGEN Biotek Corp Canada

https://norgenbiotek.com/
RNA Isolation
Liquid Biopsies
Cell Free Tumor DNA
Microbiome
DNA RNA sequencing

RNA DIAGNOSTICS. Measure Tumor Response to Chemotherapy
http://rnadiagnostics.com/physicians/

PROTEOMICS-Personalized Medical Decision Making

Consultative Proteomics Services
University of Texas, Houston
Department of Pathology and Laboratory Medicine
Recommended by Dr.Mark Renneker MD (integrative oncologist in San Francisco)

Morphoproteomic ImmunoHistochemistry (matching signalling proteins in the tumor with the current adaptive (immune) state of the tumor)

Consultative Proteomics provides molecular-based medical consults to physicians worldwide who are treating patients with hard-to-treat or recurrent cancers.
https://med.uth.edu/pathology/clinical-services/consultative-proteomics/

Resource:
Panichnantakul, P., Bourgey, M., Montpetit, A., Bourque, G., & Riazalhosseini, Y. (2016). RNA-Seq as a Tool to Study the Tumor Microenvironment. In The Tumor Microenvironment (pp. 311-337). Humana Press, New York, NY.

Abstract
The transcriptome is composed of different types of RNA molecules including mRNAs, tRNAs, rRNAs, and other noncoding RNAs that are found inside a cell at a given time. Analyzing transcriptome patterns can shed light on the functional state of the cell as well as on the dynamics of cellular behavior associated with genomic and environmental changes. Likewise, transcriptome analysis has been a major help in solving biological issues and understanding the molecular basis of many diseases including human cancers. Specifically, since targeted and whole genome sequencing studies are becoming more common in identifying the driving factors of cancer, a comprehensive and high-resolution analysis of the transcriptome, as provided by RNA-Sequencing (RNA-Seq), plays a key role in investigating the functional relevance of the identified genomic aberrations.
PMID: 27581031 DOI: 10.1007/978-1-4939-3801-8_22

Research Paper: Immunotherapy for Prostate Cancer: Where Do We Go From Here? PART 1: Prostate Cancer Vaccines.

Patel, A., & Fong, L. (2018). Immunotherapy for Prostate Cancer: Where Do We Go From Here?-PART 1: Prostate Cancer Vaccines. Oncology (Williston Park, NY), 32(3).

Abstract / Synopsis:
Immunotherapies have emerged as a revolutionary modality for cancer treatment, and a variety of immune-based approaches are currently being investigated in the field of prostate cancer. Despite the 2010 approval of sipuleucel-T, subsequent progress in prostate cancer immunotherapy development has been limited by disappointing results with novel vaccination approaches and by prostate cancer's general resistance to immune checkpoint blockade.

Nevertheless, there remains strong preclinical and clinical evidence to suggest that prostate cancer is a susceptible target for immune therapies. Innovative strategies for vaccine development, adoptive cell transfer, alleviation of immunosuppression in the tumor microenvironment, and combinatorial approaches using existing drugs and novel immune agents hold great promise for improving the treatment of prostate cancer.

The first article in this two-part series will provide an overview of both past and present therapeutic vaccination strategies for the promotion of antitumor immunity against prostate cancer.

Later, in Part 2, we will discuss novel areas of clinical development and identify the trends that may define the future of prostate cancer immunotherapy.

MAIN POINTS:

- Various vaccination approaches have so far failed to show significant clinical benefit in late-stage trials, but the consistent demonstration of antigen-specific immune responses and improvements in surrogate endpoints such as PSA doubling time with many vaccination strategies is reason for optimism about the future.
- Growing evidence suggests that implementation of vaccination strategies earlier in disease and/or in combination strategies may enhance clinical benefit. Future studies will have to investigate vaccination use in localized and low-tumor-burden states, in addition to use of vaccines in synergistic combinations with other immunostimulatory agents.

Research Paper: Effects of cryotherapy on objective and subjective symptoms of paclitaxel-induced neuropathy: Prospective self-controlled trial

Hanai, A., Ishiguro, H., Sozu, T., Tsuda, M., Yano, I., Nakagawa, T., ... & Tsuboyama, T. (2018). Effects of cryotherapy on objective and subjective symptoms of paclitaxel-induced neuropathy: Prospective self-controlled trial. JNCI: Journal of the National Cancer Institute, 110(2).

Abstract and Introduction

Background Chemotherapy-induced peripheral neuropathy (CIPN) is a dose-limiting and disabling side effect of taxane anticancer agents. We prospectively evaluated the efficacy of cryotherapy for CIPN prevention.

Methods

Breast cancer patients treated weekly with paclitaxel (80 mg/m^2 for one hour) wore frozen gloves and socks on the dominant side for 90 minutes, including the entire duration of drug infusion. Symptoms on the treated sides were compared with those on the untreated (nondominant) sides.

The primary end point was CIPN incidence assessed by changes in tactile sensitivity from pretreatment baseline in a monofilament test at a cumulative dose of 960 mg/m^2. We also assessed thermosensory deficits, subjective symptoms (Patient Neuropathy Questionnaire [PNQ]), manipulative dexterity, and the time to events and hazard ratio by PNQ. All statistical tests were two-sided

Results Among the 40 patients, four did not reach the cumulative dose (due to the occurrence of pneumonia, severe fatigue, severe liver dysfunction, and macular edema), leaving 36 patients for analysis. None dropped out due to cold intolerance.

The incidence of objective and subjective CIPN signs was clinically and statistically significantly lower on the intervention side than on the control (hand: tactile sensitivity = 27.8% vs 80.6%, odds ratio [OR] = 20.00, 95% confidence interval [CI] = 3.20 to 828.96, $P < .001$; foot: tacile sensitivity = 25.0% vs 63.9%, OR = infinite, 95% CI = 3.32 to infinite, $P < .001$; hand: warm sense = 8.8% vs 32.4%, OR = 9.00, 95% CI = 1.25 to 394.48, $P = .02$; foot: warm sense: 33.4% vs 57.6%, OR = 5.00, 95% CI = 1.07 to 46.93, $P = .04$; hand: PNQ = 2.8% vs 41.7%, OR = infinite, 95% CI = 3.32 to infinite, $P < .001$; foot: PNQ = 2.8% vs 36.1%, OR = infinite, 95% CI = 2.78 to infinite, $P < .001$; hand: hazard ratio [HR] = 0.13, 95% CI = 0.05 to 0.34; foot: HR = 0.13, 95% CI = 0.04 to 0.38, dexterity mean delay = −2.5 seconds, SD = 12.0 seconds, vs + 8.6 seconds, SD = 25.8 seconds, $P = .005$).

Conclusions: Cryotherapy is useful for preventing both the objective and subjective symptoms of CIPN and resultant dysfunction.

Research Review: Young Adult Cancer: Influence of the Obesity Pandemic

Berger, N. A. (2018). Young Adult Cancer: Influence of the Obesity Pandemic. Obesity, 26(4), 641-650.

Obesity Linked to Increased Cancer Frequency in Young Adults (summary)
https://www.medscape.com/viewarticle/894594#vp_1

Objective: The purpose of this article is to **review the association of the obesity pandemic with appearance of cancers in young adults under age 50 and to define potential mechanisms by which obesity may accelerate the development of malignancy.**

Methods: A comprehensive narrative review was performed to integrate preclinical, clinical, and epidemiologic evidence describing the association of obesity with cancer in young adults based on a search of PubMed and Google databases.

Results: Results from more than 100 publications are summarized. Although they differ in age groups analyzed and incidence of obesity, sufficient data exists to suggest an influence of the obesity pandemic on the increase of cancer among young adults.

Conclusions:
Cancer in young adults is occurring with increasing frequency. Overweight and obesity have become major public health issues reaching pandemic proportions. **Excess weight is associated with increased cancer risk, morbidity, and mortality.** Multiple murine models indicate that **obesity not only increases cancer incidence but also accelerates its development.** Thus, the possibility exists that overweight and obesity may be **contributing to the appearance of specific malignancies at younger ages.**

This prospect, in association with the worldwide expansion of obesity, suggests an impending explosive increase in obesity-associated cancers in young adults.

Clinical Questions & Answers:

***Cathy*:** *I'm wondering if you have supplemental suggestions or other therapies to help patients with severe chemo rash - on the face and head?*

Dr. Nalini:
Important to know exactly which chemotherapy agents are being used. Two general causes:

Detoxification impairment: Liver toxicity or kidney dysfunction
- Support liver and kidney function with milk thistle, NAC, dandelion, burdock (avoid using during time chemo agents are active (days 1-5))

Tyrosine kinase inhibitors trigger Inflammatory response
- Anti-inflammatory support: Omega 3 fatty acids (4,000-5,000mg divided doses daily with lipase), boswellia, curcumin, DFH Inflammatone, oatmeal bath for comfort, acupuncture points: LI4 LI11 Sp10, Liv2, apex of ear

***Cathy*:** *Have you heard of bioresonance therapy and do you think it can be helpful for cancer patients?*

Dr. Nalini:
These are energetic therapies that re-establish balance in the body to help expel toxins in the body.
- Supportive of therapies that will potentially help a patient be more balanced
- Recognize that there are many healing therapies available that may or may not work for an individual

Clinical Pearl

Ketogenic Diet and Cancer Metabolism

American Institute of Integrative Oncology RESEARCH & EDUCATION®

Let the Oncologist be the Disease Expert

You Can be the HEALTH EXPERT Cancer Patients Are Looking For

The Tumor Microenvironment Contributes to Every Aspect of Carcinogenesis

Current Cancer Drug Targets, 2014, 14, 30-45
Role of Inflammation-Associated Microenvironment in Tumorigenesis and Metastasis. Feng Gao, et al

CANCER A Chronic Metabolic Mitochondrial Disease

American Institute of Integrative Oncology RESEARCH & EDUCATION®

HALLMARK OF CANCER SHIFT in Mitochondrial Energy Metabolism

- Oxidative phosphorylation (OXPHOS) in mitochondria plays a pivotal role in ATP production.
- Pyruvate generated by glycolysis is one of the major fuels of mitochondrial energy production.
- Ketone bodies are generated by fatty acid oxidation and by degradation of ketogenic amino acids.
- Ketone bodies can be used as an alternative fuel for OXPHOS.
- Aerobic energy production via OXPHOS is often impaired in cancer.

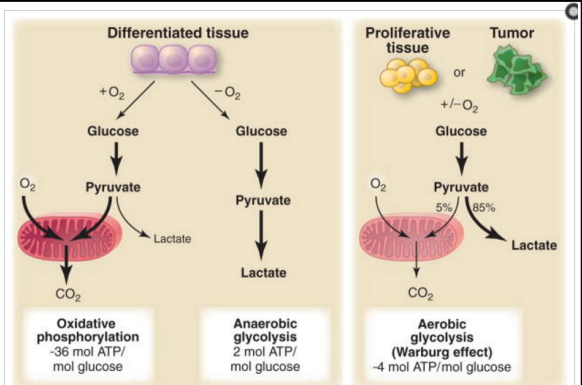
American Institute of Integrative Oncology RESEARCH & EDUCATION®

WARBURG EFFECT

SHIFT to AEROBIC GLYCOLYSIS

Increased Dependence On Glucose

Increased Lactic Acid Production



Thomas Seyfried, et al

"Cancer as a metabolic disease: implications for novel therapeutics"

>When systemic glucose availability becomes limiting, most normal cells of the body will transition their energy metabolism to fats and ketone bodies. Ketone bodies are generated almost exclusively in the liver from fatty acids of triglyceride origin during periods of fasting

>Most tumor cells are unable to use ketone bodies for energy due to abnormalities in mitochondria structure or function

>Advanced metastatic cancers can become manageable when their access to fermentable fuels becomes restricted.

Carcinogenesis vol.35 no.3 pp.515–527, 2014

KETOGENIC DIET

Dr. Thomas Seyfried — Cancer as A Metabolic Disease
Dr. Nasha Wynters — Metabolic Approach to Cancer

KETOGENIC DIET

The International Journal of Biochemistry & Cell Biology, 2015 *63*, 55–59.
Mitochondria: The ketogenic diet—
A metabolism-based therapy Silvia Vidali et al

75% Fat
20% Protein
5% CHO

4:1 or 3:1Ratio
Fat : Protein + CHO

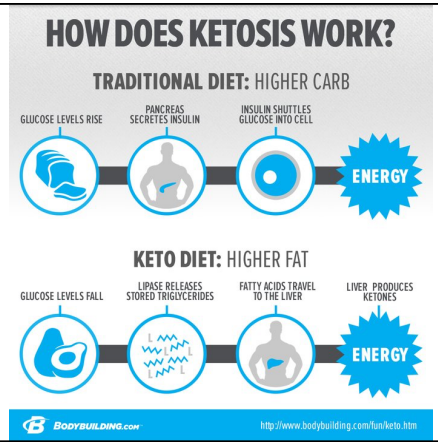
KETOGENIC DIET

The **major substrate** for energy production in cancer cells is **glucose**

Ketone bodies generated by fatty acid oxidation can serve as **alternative metabolites for aerobic energy production**.

The ketogenic diet, which is high in fat and low in carbohydrates, mimics the metabolic state of starvation, **forcing the body to utilize fat as its primary source of energy**.

The Ketogenic Diet is a high-fat, adequate-protein, low-carbohydrate diet that forces the body to burn fats rather than carbohydrates



KETOGENIC DIET

Takes advantage of Mitochondropathy and Warburg effect in CA cells and altered use of glucose and oxidation and production of ATP

Low glycemic

Low insulin, low IGF-1

Improved glycemic control

Increase in LDL cholesterol

Can be high in arachidonic acid

May increase CA risk depending on type of fat (saturated animal fats, trans fats)

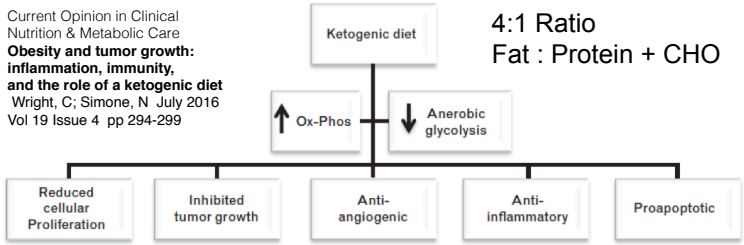
Deficient in trace min Se, Cu, Zn

Hypoglycemia

Ketogenic Diets As Adjuvant Cancer Therapy

A ketogenic diet has been shown to impede tumor growth in a variety of cancers through anti-angiogenic, anti-inflammatory, and pro-apoptotic mechanisms

Current Opinion in Clinical Nutrition & Metabolic Care
Obesity and tumor growth: inflammation, immunity, and the role of a ketogenic diet
Wright, C; Simone, N July 2016
Vol 19 Issue 4 pp 294-299



DIETARY CHO RESTRICTION

- Hyperglycemia Enhances Proliferation in some tumors
- Insulin is the primary growth factor and driver of proliferation (Insulin Makes Glucose Available)
- Tumor Size and Growth is Related to Plasma Insulin Levels and Plasma Lactate Levels
- **Low CHO diets cause a drop in plasma insulin and lactate**
- **Low CHO diets can reduce insulin-mediated glucose uptake into tumor cells**

Cancer Res.2011Jul 1;71(13):4484-93. Ho VW, et al

A low carbohydrate, high protein diet slows tumor growth and prevents cancer initiation.

Cancer cells rely more heavily on glycolysis than normal cells

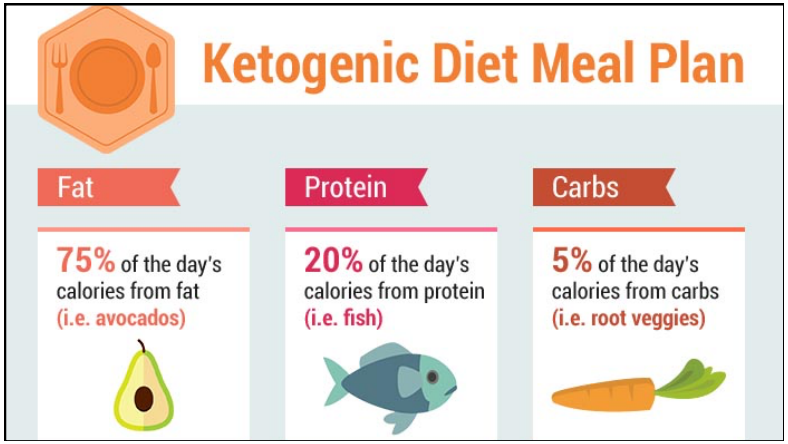
10% or 20% CHO High Protein diet slows tumor growth as effectively as No CHO Ketogenic Diets

Reduces serum glucose, insulin, and glycolysis, slows tumor growth, reduces tumor incidence, and works additively with existing therapies without weight loss or kidney failure

Cancer Res. 2011 Jul 1;71(13):4484-93. Ho VW, et al

What to Eat on a Ketogenic Diet

- **Meats** – fish, beef, lamb, poultry, eggs, etc.
  Leafy Greens – spinach, kale
  Above ground vegetables – broccoli, cauliflower, etc.
  High Fat Dairy – hard cheeses, high fat cream, butter,
  Nuts and seeds –walnuts, sunflower seeds
  Avocado and berries – raspberries, blackberries
  Other fats – coconut oil, high-fat salad dressing, saturated fats.
- **Drink more water**



KETOGENIC PROTEIN SHAKE

Protein Powder (30 grams)
MCT Oil 2 Tablespoons
Flaxseed Oil 1 Tablespoon
Omega 3 Fatty Acids Liquid
2 Tablespoons
Phophatidyl Choline 2 Tablespoons
Carnitine Tartrate Powder
1 level teaspoon
200mg CoQ10
Concentrated Reds-Greens Powder
2 teaspoons

½ Avocado
1 Tablespoon Pumpkin Seeds
½ teaspoon Acidophilus-Bifidus powder
Fiber Powder (soluble + insoluble)
1 tablespoon
½ organic lemon with seeds and peel

Mix with water or green tea.
2 capsules Pancreatic Enzymes + Ox Bile.
2 capsules Lipase Digestive Enzyme
Take with iron free copper free multivitamin

American Institute of Integrative Oncology RESEARCH & EDUCATION

SUMMARY

CANCER IS A METABOLIC DISEASE

Diet Influences the Tumor Microenvironment

Diet Influences Onco-Genes & Tumor Suppressor Genes

Ketogenic Diet Influences Tumor Cell Metabolism

10% or 20% CHO High Protein slows growth as effectively as No CHO Ketogenic Diet

American Institute of Integrative Oncology RESEARCH & EDUCATION

Review
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Young Adult Cancer: Influence of the Obesity Pandemic

Nathan A. Berger

Objective: The purpose of this article is to review the association of the obesity pandemic with appearance of cancers in young adults under age 50 and to define potential mechanisms by which obesity may accelerate the development of malignancy.
Methods: A comprehensive narrative review was performed to integrate preclinical, clinical, and epidemiologic evidence describing the association of obesity with cancer in young adults based on a search of PubMed and Google databases.
Results: Results from more than 100 publications are summarized. Although they differ in age groups analyzed and incidence of obesity, sufficient data exists to suggest an influence of the obesity pandemic on the increase of cancer among young adults.
Conclusions: Cancer in young adults is occurring with increasing frequency. Overweight and obesity have become major public health issues reaching pandemic proportions. Excess weight is associated with increased cancer risk, morbidity, and mortality. Multiple murine models indicate that obesity not only increases cancer incidence but also accelerates its development. Thus, the possibility exists that overweight and obesity may be contributing to the appearance of specific malignancies at younger ages. This prospect, in association with the worldwide expansion of obesity, suggests an impending explosive increase in obesity-associated cancers in young adults.

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Introduction

Cancer in young adults is being reported with increasing frequency and has become a matter of urgent concern (1). At the same time, overweight and obesity have become major public health issues in both children and adults, reaching pandemic proportions worldwide (2,3). While it has been clearly documented that excess weight is associated with both increased risk of occurrence and increased morbidity and mortality for multiple malignancies (4-6), there has been relatively little focus on the impact of overweight and obesity on shifts in timing of cancer appearance to individuals of younger age. However, recent Centers for Disease Control and Prevention data indicate an increase in overweight- and obesity-associated cancers in 20- to 49-year-old individuals (Supporting Information Supplement S1). Importantly, multiple murine models indicate that obesity and obesogenic diets not only increase the incidence of malignancy but also accelerate its development and shift its occurrence to earlier ages (5,7-17).

Thus, the possibility needs to be considered that overweight and obesity may be contributing significantly to the clinical appearance of some malignancies at younger ages. This prospect, in association with the continued worldwide expansion of obesity (2,3), suggests an impending explosive increase in obesity-associated cancers in young adults. Anticipation of the potential dire consequences of this evolution compels careful epidemiologic monitoring; more research on mechanisms by which obesity promotes and accelerates cancer, especially in young adults; development of focused strategies for prevention; and potentially new approaches to screening and care.

The goals of this article are 1) to enhance awareness of the obesity–cancer linkage; 2) to illustrate how both obesity and obesogenic diets may shift appearance of obesity-promoted cancers to younger age groups, especially into the 20- to 50-year-old age group; 3) to examine preclinical murine models and potential mechanisms by which obesity and obesogenic diets accelerate the appearance of malignancy; 4) to review the epidemiologic and clinical evidence indicating where this may already be happening; 5) to identify which obesity-associated cancers are most likely to pose this threat; and 6) to consider approaches to better document and avert the crisis.

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TABLE 1 Relation of obesity-associated cancers to young adult malignancies and murine models

Obesity-associated cancer[a]	US incidence $\times 10^{-3}$[b]	Population attributable fraction %, M/F (21)	Peak age incidence, years[c]	Usual age range[d] of all years with incidence >15%	Percent new cases in 20-44 years age group[e]	DIO and HFD in murine models promoted cancer
Breast	253	–/14	62	55-84	**10.5**	MMTV-TGFα (7)
Colon and rectal	135	32/17	67	45-84	**5.8**	APC^{Min} (8)
Kidney	63	25/34	64	55-74	**7.8**	
Endometrial	61.3	–/48	62	45-74	**7.3**	$Pten^{+/-}$(16)
Thyroid	57	32/5	51	20-64	**23.9**	$Thrb^{PV/PV}$ $Pten^{+/-}$ (13)
Pancreas	54	14/11	70	55-84	2.4	$Kras^{G120}$ conditional (9,10)
Liver	41	NA	63	55-84	**2.5**	C57BL/6J (11) MUP-UPA (12)
Myeloma	30	NA	69	55-84	3.5	KwLwRij (14)
Gastric cardia	28	NA	68	55-84	**6.2**	
Meningioma[f]	27	NA	58	45-74[f]	**16.8**	
Ovary	22	–/7	63	45-84	**10.6**	KpB (15)
Esophageal adenocarcinoma	17	44/48	67	55-84	**2.3**	L2-IL-1β (17)
Gallbladder	7	–/53	85+[g]	65-90[g]		

[a]Obesity-associated cancers identified by 2016 IARC analysis (6).
[b]US incidence of specific cancers from American Cancer Society Cancer Facts & Figures, 2017 (19).
[c]Peak age incidence from SEER Cancer Statistics, 1975-2014 (20).
[d]Usual age range years from SEER Cancer Statistics (20) combining all decades with incidence ≥15% for each malignancy.
[e]Percent new US cases in 20- to 44-year-old age group from SEER Cancer Statistics (20) combining 20- to 34- and 35- to 44-year-old, age groups. Values in bold font indicate malignancies among top 20 invasive cancers in the United States at ages 20 to 39 years (18).
[f]Age range for meningioma provided for all primary brain tumors (19).
[g]Gallbladder age incidence and range from UK data, 2015 (92).
NA, not available.

Obesity–Cancer Linkage

Although adolescent and young adult cancers have been operationally defined as those occurring in the 15- to 39-year-old age group (18), this article is focused on malignancies, most commonly associated with patients over age 50, that have recently been reported with increasing frequency in the younger-than-50-year-old age group. Moreover, because this article examines the impact of obesity on cancers in young adults, it will concentrate on the 13 cancers listed in Table 1, which, based on epidemiologic review by the International Agency for Research on Cancer (IARC), have been identified as having sufficient evidence to be linked to excess body fat (6). It will not include discussion of tumors such as sarcoma, acute leukemia, and others that may occur in young adults but have not been clearly linked to obesity. It also will not consider malignancies such as hematologic malignancies, prostate cancer, and others for which evidence for an association with obesity has not reached the level of significance as those recently reported (6). This article uses the following categories of BMI (weight in kilograms divided by square of height in meters): normal weight, BMI = 18.5-24.9; overweight, BMI = 25.0-29.9; obesity, BMI ≥ 30; and severe or morbid obesity, BMI ≥ 40 (6).

Table 1, column 1 lists the 13 tumors recently reported by the IARC for which there is sufficient evidence to identify an association between obesity and specific malignancies (6). These malignancies are arranged in order of annual incidence of new US cases (column 2) (19,20). Column 3 lists the US population attributable fraction (PAF) as the percentage of each malignancy attributed to obesity for both males and females (21). These data demonstrate an important contribution of obesity to cancers of colon and rectum, thyroid, esophagus, pancreas, and kidney in men and to breast, colon, kidney, endometrium, esophagus, and gallbladder cancers in women. With 253,000 new cases of breast cancer in the United States, a 14% PAF calculates to 35,420 new cases per year attributable to obesity. For colorectal cancer (CRC), adjusting for male/female distribution, the PAF indicates 22,655 new cases of CRC in men and 10,812 new cases in women attributable to obesity. Applying similar calculations to the incidence and PAF data provided in Table 1 indicates that in 2017, more than 144,000 of those cancers occurring in the United States were attributable to obesity. However, this number is probably an underestimate, as PAFs are not available for several obesity-associated malignancies such as liver, myeloma, gastric cardia, or meningioma.

The fourth and fifth columns indicate the peak and usual age range incidence for each of these tumors. The median age at which all cancers are diagnosed in the United States is 66 years. While most are diagnosed in patients older than 50 years (20), the appearance of thyroid, ovarian, endometrial, and CRC cancers and meningiomas is not uncommon in patients younger than 50 (20). Strikingly, as shown in the sixth column of Table 1, of the 13 IARC obesity-associated malignancies, at least 9 (shown in bold) have been reported as occurring in young adults and are in the top 20 adolescent and young adult cancers (18). The last column indicates murine

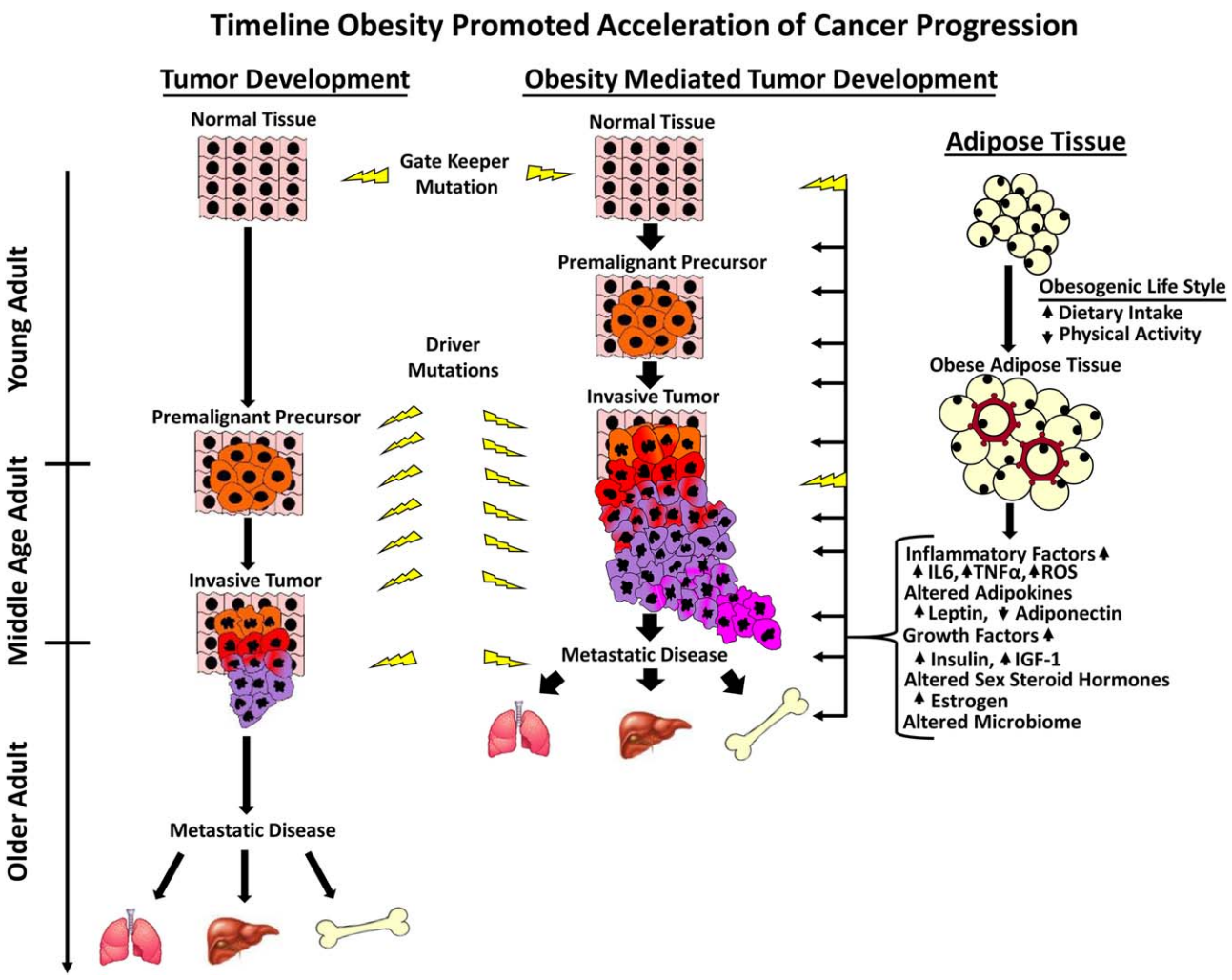

Figure 1 Timeline of obesity-promoted acceleration of cancer progression.

model systems in which nine of the malignancies have been shown to be accelerated and become more aggressive in association with obesity (7-17).

Obesity Accelerates Cancer Development

From a mechanistic viewpoint, overweight and obesity are generally considered to be promoters of cancer progression (5). Thus, overweight and obesity promote cancer by multiple concurrent mechanisms, including 1) stimulation of low-grade inflammation and oxidative stress with increased levels of proinflammatory cytokines such as IL-6, tumor necrosis factor (TNF), and increased reactive oxygen species (ROS), the latter of which may also contribute to mutagenesis; 2) alteration of growth-promoting factor levels, especially insulin and insulinlike growth factor (IGF-1), which increases in association with metabolic syndrome and insulin resistance; 3) altered sex steroid hormones with increased conversion of androgens to estrogens resulting from increased adipose tissue production of aromatase, the enzyme responsible for this conversion; 4) altered adipocytokine proteins, including increased growth-promoting and pro-inflammatory components such as leptin, retinol binding protein 4, resistin, and visfatin and reduced growth-controlling adipokines such as adiponectin; 5) alterations in intestinal microbiome with expansion of tumor-promoting species such as fusobacteria; and 6) mechanical effects of obesity such as those leading to hiatal hernia and gastroesophageal reflux disease, predisposing to esophageal adenocarcinoma (5).

Figure 1 provides a conceptual model, supported by multiple murine studies, of how obesity impacts cancer by accelerating its development (7-17). As postulated for development of colon cancer, and now widely accepted for multiple malignancies, mutations in a gatekeeper gene (13,22,23), sometimes similar to those mutations causing hereditary cancer syndromes, initiate sporadic tumors. Mutated cells then progress through a multistage process in which multiple genetic changes ultimately lead to the development of a benign premalignant, and then to malignant, neoplasm with invasive and subsequently

metastatic properties. For CRC, transformation from normal epithelium to benign adenoma, ultimately leading to frank cancer and metastatic disease, is projected to require at least seven independent genetic events and possibly mutation in as many as 15 driver genes (24) as well as multiple epigenetic alterations (25). In some cases, this process may require long latent periods extending to multiple decades to progress from normal epithelium to frank cancer.

The rate of progression to invasive cancer is determined by multiple factors, including mutation and proliferation rates, which are affected by DNA damage response, DNA repair systems, and a host of growth factors. This rate may vary among different tumors and even among different transformed clones in the same individual. Thus, obesity may enhance mutation rates by generation of increased ROS. Importantly, however, high-fat diets (HFDs) and diet-induced obesity (DIO) have been shown to accelerate tumor growth rates in association with production of increased growth factors, such as insulin, IGF-1, leptin, retinol binding protein 4, and others (8,26,27).

As shown in Figure 1, development of obesity, usually due to a combination of HFD and decreased physical activity, results in expanded fat mass, characterized by increased number and size of adipocytes, some of which undergo necrosis and become surrounded by macrophages to form crown-like structures with a propensity for releasing proinflammatory cytokines such as IL-6 and TNFα (28). In addition, expanded and inflamed adipose tissues may provide increased levels of multiple growth-promoting cytokines, adipokines, and hormones, many of which accelerate the multistage transition from normal tissue to invasive and metastatic cancer. This process not only accounts for the accelerated development of tumors in the presence of adipose tissue excess, but it also explains why patients with obesity-driven cancers may present with more advanced tumors at earlier ages. Thus, the long latent period required for initial presentation of many tumors provides the basis for obesity to impact the process and, in fact, accelerate both the appearance and extent of clinical disease. Accordingly, it is expected that initial mutations for sporadic cancer will occur with similar frequency and at similar ages in both normal patients and those with obesity. In addition, it is possible that obesity-associated inflammation and ROS may further contribute to mutagenesis and cancer initiation. Nonetheless, the metabolic and growth-promoting consequences of concurrent or prior obesity can provide the stimulus for accelerated development of cancer and associated comorbidities, including death.

Systems Demonstrating That Obesity Promotes and Accelerates Development of Malignancies

The shortening of the latent period from benign to malignant disease in association with obesity has been most clearly demonstrated at the clinical level, where disease-associated monoclonal immunoglobulin provides a biomarker for early detection and demonstration that obesity accelerates the conversion of monoclonal gammopathy of unknown significance (MGUS) to multiple myeloma (MM) (29). In further support of this proposal that obesity does not initiate but rather promotes cancer progression, almost all murine models in which obesogenic diets and DIO promote tumors require experimental utilization of genetically modified animals containing cancer-predisposing genes or transplantation of preexisting tumor cell lines (5,7-17).

It is noteworthy that in some murine models, HFD has been shown to promote CRC and breast cancer in mice that are resistant to DIO (7,8,30). These studies indicate that proinflammatory and growth-promoting effects of HFD, even in the absence of DIO, may accelerate tumor progression. Other murine systems have shown that even after HFD-induced DIO and subsequent weight loss, the tumor-promoting effects of obesity may endure for varying time periods, thereby providing a model for promotion of adult tumors by childhood, adolescent, and young adult obesity (7,8,30).

The contribution of proinflammatory and growth-promoting factors as mediators in the HFD- and DIO-accelerated malignancies is further illustrated by the demonstration that tumor-promoting effects of obesity can be abrogated by molecular or pharmacologic interference with proinflammatory and growth-promoting pathways such as pharmacologic inhibition of receptors for insulin or IGF-1 (31), molecular interference with leptin receptor (32), and genetic and pharmacologic interference with proinflammatory activity of complement system (8). It is further noteworthy that not all HFDs are equal in promoting malignancy, as shown by olive oil, an important component of the Mediterranean diet, which was found to protect against HFD acceleration of gastrointestinal neoplasia in APC^{Min} mice (8).

Clinical and Epidemiologic Evidence Indicating That Obesity Shifts Malignancies to Younger Ages

From a clinical and epidemiologic viewpoint, we focus initially on CRC, which has become one of the major adult tumors generating alarm for its increasing appearance in young adults (33). CRC, usually occurring between 45 and 84 years of age with peak incidence at 67 years (20), and uncommonly seen in young adults, is now being increasingly identified in both men and women below age 50 (20,34-39), with greater increase noted for left-sided sigmoid and rectosigmoid CRC then right-sided CRC (35).

Analysis of Surveillance, Epidemiology, and End Results Program (SEER) Population data and multiple Hospital Based Cancer Registries, covering periods from 1973 to 2017, indicates that CRC incidence has remained stable and/or decreased in people over 50 by as much as 3% per year. In contrast, CRC has shown an average 1.5% increase per year among 20- to 40-year-old men and women (40,41). Moreover, younger patients have been noted to present with more advanced, higher-stage, more poorly differentiated disease, and those presenting with stage IV CRC have shown inferior survival.

The overall decrease in incidence of CRC has been attributed to expanded screening programs and removal of early premalignant adenomas. Because of the much higher incidence of CRC in older individuals, these programs have been primarily targeted at patients over 50. Thus, increase in incidence and more advanced stage at presentation among young adults have been attributed, in part, to lack of screening and to tumor promotion by lifestyle factors including obesity, consumption of red and processed meat, and possibly alcohol and tobacco use (35,42).

Many of the above reports point to a concurrent increase of obesity and CRC in the young. Some have documented increased obesity and cancer in the same population; however, few have provided

obesity demographics in the CRC patients (38). In addition, although not specific for young adults, some series indicate an association of obesity with increased risk of sigmoid and rectosigmoid cancers (43). In an important study of more than 1.1 million Israeli Jewish men with 19.5 million person-years of follow up, overweight and obesity in adolescents aged 16 to 19 years were associated with a substantially increased risk for colon cancer (HR = 1.53, 95% CI: 1.17-2.0) but not for rectal cancer in adult years. The median age for patients with newly diagnosed colon cancer was 43.3 ± 8.7 years, thereby supporting an association of adolescent-detected obesity with young adult colon cancer (37) and suggesting effects of obesity over a long latent period of cancer development.

In addition to its association with increased risk for CRC, obesity is also associated with a twofold increase in risk for colorectal adenoma (CRA), a premalignant precursor to CRC (44). CRAs have been commonly reported in patients younger than age 55 (45) and have been noted to be more advanced in patients with obesity (46). In a study of people examined across an age range from 30 to greater than 70 years, high BMI was identified as a risk factor for CRA in 30- to 39-year-old men and 40- to 49-year-old women (36). Thus, subjects with overweight or obesity are at increased risk for developing CRC and its precursor, CRA, during young adulthood. Moreover, obesity has been shown to precede the diagnosis of CRA and CRC by long latent periods (47).

In summary, the clinical development of CRA and CRC fits well with the model provided in Figure 1, including increased and early development of obesity-associated benign adenoma preceding cancer with a long latent period, providing time for the impact of obesity-stimulated growth factors to accelerate tumor development. Moreover, in addition to decreased screening in young adults, obesity-promoted progression may help explain why CRC in the young is more advanced at the time of presentation (33).

While this discussion has focused on sporadic colorectal neoplasia and its increasing appearance in young adults, additional insight is provided by patients with known inherited predispositions to CRC, including familial adenomatous polyposis or hereditary nonpolyposis colon cancer (HNPCC), bearing, respectively, mutations in the gatekeeper genes, adenomatous polyposis coli, or mismatch repair genes (MLH1, MSH2, MSG6, PMS2, EPCAM) (48). These patients commonly develop CRA or CRC at younger ages, when CRC risk has been shown to be increased in association with excess weight (49,50). In a study of 937 HNPCC carriers followed at 14 institutions, with median age enrollment 44.9 years (36-53 years), obesity was associated with a 2.4-fold greater risk for CRC compared with normal and underweight reference groups. Interestingly, there was no increase in risk in HNPCC patients with obesity randomly assigned to aspirin (ASA), 600 mg daily, suggesting that obesity-promoted CRC in HNPCC patients may be reduced by regular ASA use (51).

Female breast cancer is the most common US malignancy included on the IARC obesity-associated cancer list, with peak incidence at 62 years and usual age range 55 to 84 years (20). Breast cancer in postmenopausal women is usually estrogen receptor positive and is associated with increased risk in association with obesity (4). Of the tumors listed in Table 1, breast cancer is unique in that a major variety, premenopausal breast cancer, characterized by estrogen receptor negative status, has been noted to occur at a relatively constant rate of 40% by age 40 years (52), and obesity is associated with an overall decreased risk of premenopausal breast cancer (53). Thus, because of the already significant occurrence of premenopausal breast cancer in patients under 40 years of age, it is difficult to determine if there is an obesity-associated shift to younger age. However, premenopausal women at high risk for breast cancer, including prior history of lobular carcinoma in situ, generally considered a multifocal premalignant precursor, have shown significantly increased risk of developing breast cancer in association with obesity (54). These high-risk premenopausal women fit well into the latent process depicted in Figure 1 that is accelerated by obesity. Moreover, unique insight is provided by patients with triple negative breast cancer (TNBC), tumors that lack expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (55). These tumors, commonly identified in premenopausal women, are refractory to hormonal and cytotoxic chemotherapy (55). In a retrospective review of invasive breast cancer among 1,064 patients from Walter Reed National Military Medical Center, 160 patients had TNBC, of whom 89 were below age 50 years and 148 either had overweight or obesity. Thus, TNBC in all patients, including those younger than age 50, is highly associated with obesity (56).

Further insight is provided by patients with hereditary breast and ovarian cancer wherein 80% of cases have been attributed to mutations in BRCA1/BRCA2 (57). In a series of 176 multigenerational kindreds, earlier appearance of breast cancer was noted in successive generations, with age at diagnosis shifting from 51.8 years in grandparents to 48.7 years in parents, followed by 41.9 years in probands and 34.7 in children (58). The shift to earlier age in successive generations was attributed to lifestyle factors including obesity. In other studies, weight gain and number of pregnancies have been shown to significantly increase risk of breast cancer, whereas weight loss between age 18 and 30 years has been associated with decreased risk of breast cancer (59).

Ductal carcinoma in situ (DCIS), with peak incidence during age 60 to 74 years, is a noninvasive breast cancer precursor and is unusual before age 35. DCIS has recently undergone a marked increase in detection and occurrence from an incidence of 1.8 per 100,000 in 1973-1975 to 32.5 in 2004. The increase has mainly occurred in patients older than 50; however, DCIS has been noted to be increased in all age groups in association with obesity (60). Thus, the breast cancer precursor DCIS, familial breast cancer associated with BRCA mutation, and TNBC are all increasing in incidence in young adults, and the increases are associated with obesity (54-60).

Renal cell carcinoma (RCC), the third most common IARC obesity-associated malignancy (6), has a peak incidence for diagnosis at age 64 (20); however, several retrospective series have reported RCC patients younger than 45 (61). Although up to 60% of patients with RCC have been reported as having overweight/obesity (62), the specific percentage of patients under 45 with excess weight has not been reported. However, in a case control study of 1,214 patients and 1,234 controls, early adult obesity was associated with a 60% increase in risk for RCC (63).

Endometrial cancer in the United States is the most common malignancy of the female genital tract. It is most frequently diagnosed in postmenopausal women aged 45 to 74 years (19,20). However, 2% to 14% of endometrial cancers have been reported to occur in women aged 40 years and younger (64). Sporadic endometrial

cancers in the young are usually associated with high BMI and with obesity rates reported to range from 37% to 60% (64). Many of these patients have polycystic ovary syndrome characterized by obesity, menstrual irregularity, infertility, and enlarged ovaries with multiple ovarian cysts (64).

Thyroid cancer commonly occurs in young adults, with 28% of new diagnoses in the 20- to 40-year-old age group. Its incidence in patients under 65 is increasing (65), extending down to the 45- to 49-year-old age group in some South American countries (66). In contrast with many tumors discussed in this article, thyroid cancer in young adults is usually curable because it most frequently is detected early as an asymptomatic neck mass. Cases in young adults show a female preponderance; appear to be differentiated, with papillary histology being more frequent than follicular; and frequently harbor a mutation in the RAS-RAF-MEK-ERK, mitogen activated protein kinase pathway (65).

Pancreatic cancer, with peak incidence in 70- to 80-year-old individuals, is uncommonly observed below age 45 and is increasing in frequency (19,20). Individuals with overweight or obesity between ages 20 and 39 years had 2 to 6 years' earlier onset of pancreatic cancer compared with normal-weight controls (67). A UK survey conducted between 1998 and 2006 showed no change in incidence rate for males under 50 years; however, a slight increased incidence was reported in females in the 20- to 39-year-old group (68). In a retrospective review covering 1993 to 2008, 33 patients (5.7%) were identified in the 50 years or younger age range. Only 3 (9%) had obesity compared with 4 (12%) of the matched controls (69).

Hepatocellular cancer (HCC), with overall incidence in Western countries peaking at 60 to 70 years of age, is one of the most common cancers on a worldwide basis (70). Incidence rates in the United States have increased by 2.5- to 3-fold over the past 35 years (71). On a global basis, HCC is associated with liver injury from different etiologies including viral infections with hepatitis B and hepatitis C, hepatotoxins including aflatoxin, chronic alcohol abuse, and metabolic alterations that occur with obesity, the latter leading to metabolic syndrome, consisting of obesity, diabetes, insulin resistance, and dyslipidemia (72). The common pathway by which each of these insults leads to HCC includes liver damage, followed by inflammation, usually leading to cirrhosis and then HCC, thereby providing an extended latent period for obesity-promoted carcinogenesis.

Obesity-mediated liver damage progresses through nonalcoholic fatty liver disease (NAFLD) showing steatosis and lipid deposition in liver cells without inflammation, proceeding to nonalcoholic steatohepatitis characterized by further fatty acid deposition and ballooning degeneration of hepatocytes with inflammation, leading to fibrosis, cirrhosis, and HCC (72). In Western countries, where overweight and obesity are common, NAFLD is present in 20% to 40% of the general population (72). The increase in obesity and its comorbidities, including diabetes and metabolic syndrome in Western countries, is projected to promote the incidence of HCC, especially in Hispanic men and African American women (73).

Because NAFLD is a predisposing risk factor for HCC, it is noteworthy that NAFLD is increasing in young adults in association with the increased incidence of obesity and metabolic syndrome (74). Thus, NAFLD in young adults aged 18 to 30 years old has increased 2.5-fold over the past three decades and is reported to be present in more than half (57.4%) of young adults with morbid obesity (74).

In a recent US study, the Liver Cancer Pooling Project, composed of 14 separate cohorts and containing 2,087 cases with prospectively measured BMI and waist circumference, showed that excess weight at the time of enrollment was associated with liver cancer in a dose response manner (71). Although most of the cohorts enrolled "older" Americans, 85 patients were younger than 50 years at the time of enrollment, and 18 of these were diagnosed with liver cancer before age 50, 3 of whom had obesity at the time of enrollment (P. Campbell and C. Newton, personal communication). This observation that 18 of 85 patients developed liver cancer before age 50, along with the high incidence of NAFLD in young adults with obesity, indicates the importance of careful surveillance of HCC as another malignancy likely to increase in young adults.

MM, characterized by malignant proliferation of plasma cells, anemia, elevated levels of a circulating monoclonal immunoglobulin, destructive bone lesions, and renal failure, is the second most common hematologic malignancy in the United States and the only primary malignancy of blood cells included by the IARC as related to obesity (6). MM is diagnosed with a peak incidence of approximately 69 years and has maintained a constant incidence for at least the past three decades (75). However, in three series reported since 1992, MM has been reported in patients younger than 45 with incidences of 2.2%, 9.6%, and 15% (76,77). None of these series reported BMI. In a pooled analysis of 242 MM cases in patients younger than 50 compared with 1,758 age-matched controls, patients showed a significant positive association of elevated BMI with risk of MM and a greater than twofold increase in MM risk for patients with severe obesity (78). Moreover, the incidence of MM has been noted to be increased in patients who reported heavy compared to lean body shapes during childhood and adolescence (75), providing support for a potentially long latent period for the impact of obesity on malignancy development. Interestingly, chromosomal abnormalities characteristic of MM have been shown to be no different in patients above or below age 45 (79).

MGUS, characterized by restricted proliferation of a predominant clone of plasma cells, not exceeding 10% of marrow cells and absence of diagnostic criteria for MM (80), is considered a universal premalignant precursor of MM with variable rates of progression (81). MGUS, identified in large population screenings by detection of circulating monoclonal immunoglobulins, is most common in the 80- to 96-year-old age group; however, it has significantly been reported in patients younger than age 50. In some of these cases, it has been projected to have been present for latent periods in excess of 20 years (82). Obesity has been shown to be associated with increased risk for MGUS in women (83). Interestingly, although it is considered a premalignant condition, MGUS shares many of the genetic and cytogenetic changes noted in MM, including activation of c-myc, del(17p), t(4:14), and 1q gains (81). In a retrospective study of 7,878 MGUS patients identified through the US Veterans Health Administration database, 39.8% had overweight and 33% had obesity. Moreover, risk of transformation of MGUS to MM was increased with obesity and black race (29,83).

Esophageal adenocarcinoma (EAC) and gastric cardia adenocarcinoma, both malignancies of glandular epithelium originating near the gastroesophageal junction, have undergone a rapid increase in

incidence over the past two to three decades (84,85). They are consistently associated with overweight/obesity, and 10% of patients presenting with EAC are noted to have morbid obesity (86). Although EAC has a peak incidence in the 80-year-old age group (87), in a retrospective study of 374 patients treated for EAC between 2000 and 2007, 63 (16.8%) were under age 50 (86). EAC may be preceded by a premalignant precursor, Barrett's esophagus (BE), commonly seen in younger patients, where it is associated with chronic inflammation, gastroesophageal reflux disease, and obesity (87).

In addition to their association with obesity at time of diagnosis, occurrence of both EAC and gastric cardia adenocarcinoma in later years is increased following elevated BMI during early adulthood (age 20) and with progressive weight gain between ages 30 and 50 years (73). In addition to the overall increase in occurrence of BE in young adults (87) there has been a notable increase in EAC in patients under age 40, and more than 10% of patients undergoing surgery for EAC are reported to be $\leq$50 years old (88).

Meningioma constitutes 20% to 30% of all intracranial neoplasms, with peak incidence in men in the 60- to 69-year-old age group and in the 70- to 79-year-old age group in women (89,90). Cranial irradiation and obesity are risk factors (89), sometimes with long latent periods of more than 20 years between radiation and diagnosis of meningioma. Meningioma in the pediatric age group is seen as part of hereditary syndromes such as neurofibromatosis with inherited NF2 mutations. Somatic mutations of the NF2 gene are frequently identified in sporadic cases of meningioma (90). In a report of 35 patients from a single institution and meta-analysis of more than 450 patients, meningioma occurring during first three decades of life, with an average age at diagnosis of 25 years, had a female predominance but no notation of occurrence of obesity (90).

In contrast to many of the other cancers discussed in this article, epithelial ovarian cancers are not uncommon among young women, in whom they are thought to coincide with activity of the female reproductive cycle (19,20). Increased BMI is a risk factor for epithelial ovarian cancers, and elevated levels of IGF-1, which frequently accompany obesity, are thought to contribute (91). Mutation of mismatch repair genes including germ line mutations occur in a small percentage of patients under 40.

Gallbladder cancer, a rare malignancy in the United States with peak incidence in the 80-year-old group, is rarely seen or reported in patients under 50 years of age (92). Risk factors for gallbladder cancer include obesity and chronic inflammation associated with gallstone disease. Chronic inflammation of 15 or more years has been estimated to result in gallbladder cancer in genetically predisposed individuals. Treatment of cholelithiasis and cholecystitis by surgical removal of the gallbladder has significantly decreased the incidence of gallbladder cancer (93).

Association Between Obesity-Linked and Young Adult Cancers

Of the 13 cancers identified by the IARC as being associated with increased body fat (6), most have their highest incidence rates in older adults. However, 5 of the 13 obesity-associated cancers, including breast, thyroid, uterus, ovary, and stomach cancer, have been identified by US SEER data as occurring in the top 20 cancers in 20- to 39-year-old females and 5 of the 13, including colorectal, thyroid, kidney, stomach and liver cancer, have been identified in the top 20 in 20- to 39-year-old males (18). Of the 13 IARC obesity-associated malignancies, all but gallbladder cancer have been well documented to occur in significant numbers in patients under 50 years of age, and 4 of these malignancies—colorectal, breast, thyroid, and possibly pancreatic cancer—have been reported to be increasing in the female young adult population. Moreover, five premalignant precursors, including CRA for CRC, BE for EAC, NAFLD for HCC, DCIS for breast cancer, and MGUS for MM, have been reported to be increasing in the young adult population in association with obesity. In addition, excess body weight and/or weight gain has been noted to precede presentation of these malignancies by long latent periods, in some cases by multiple decades.

In summary, many of the malignancies noted to occur with increasing frequency in young adults are among the 13 obesity-associated cancers. With the expanding worldwide incidence of overweight and obesity in children and young adults, the long latency period associated with many sporadic cancers, the demonstration in humans and animal models that obesity accelerates the development of cancer, and the probability that even obesity at young age has a long-term effect on tumor progression, it is highly likely if not imminent that obesity will lower the age of occurrence across the age spectrum, shifting multiple malignancies to younger age groups in general and to the young adult population in particular.

Overall, this assessment is limited because many of the reports of malignancies in young adults do not provide anthropomorphic measurements. Moreover, evaluation of these data often underestimates obesity because patients with advanced malignancy frequently present for evaluation after significant weight loss. Further evidence of an association of cancer and obesity in young adults will require more consistent reporting of anthropomorphic data at time of diagnosis as well as premorbid data when available. Because body mass may be considerably reduced at time of cancer diagnosis, it is important that these data be monitored in a prospective fashion among the healthy pediatric and young adult population and made available for analysis if and when malignancy is diagnosed. However, documenting and reporting this information are critically important to more firmly establish the relation of obesity to young adult cancers.

Disrupting the Linkage Between Obesity and Young Adult Cancers

The data cited in this article portend an imminent threat of the impact of the obesity pandemic on an age shift in occurrence of obesity-associated malignancies, including their appearance in young adults. This occurrence will require increased cooperation between adult, adolescent, and pediatric oncologists, endocrinologists, and weight management professionals for effectively evaluating and dealing with the looming crisis. The most effective way to curtail development of this problem is to prevent expansion of the obesity pandemic in both children and adults. This is a critical challenge since there are already 110 million children and adolescents and 640 million adults with obesity worldwide who constitute the at-risk

pool for development of obesity-accelerated malignancies (94,95). Moreover, this at-risk population is even further expanded by the demonstration that the effects of overweight/obesity may have a long latent period and, in some cases, precede the diagnosis of malignancy by decades. Thus, an important goal for medical professionals and supporting agencies is to encourage obesity prevention, weight loss, and increased physical activity in both children and young adults (3). In some cases, extreme measures such as bariatric surgery are being considered in children and young adults with obesity because of the potential consequences of disorders such as polycystic ovary syndrome and fatty liver disease. Special attention needs to be focused on detecting, monitoring, and reversing metabolic syndrome in all patients, especially young adults.

Interestingly, the demonstration that ASA reduced incidence of CRC in young HNPCC patients with obesity (51) indicates the importance of further research to improve cancer prevention strategies in patients with hereditary cancer syndromes and more broadly in young adults with overweight or obesity (51). Like ASA, potential chemopreventive agents for young adults with obesity must be relatively nontoxic and safe for long-term administration. Metformin, extensively used for treatment of type 2 diabetes mellitus, has already been shown, at low dose, to be safe and reduce recurrence of colorectal adenoma in nondiabetic patients following initial polyp resection (96). Because obesity-promoted cancers have been shown in some cases to involve epigenetic changes (97), other potential opportunities for chemoprevention in young adults include the use of epigenetic targeted therapies (98).

In terms of screening the young adult population for early signs of malignancy, what is clearly needed is a series of easily administered, minimally invasive, and cost-effective screening tools. These might include training and encouraging young women with obesity to perform breast self-examination, regular thyroid palpation by medical and dental practitioners, stool DNA testing for both upper and lower gastrointestinal pathologies (99), and further development of screening blood tests for circulating DNA, circulating tumor cells, and other potential biomarkers (100). Because overweight and obesity are lifestyle consequences, it is possible that they can be sufficiently altered by lifestyle modifications to avert the impending expansion of young adult cancers. O

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Immunotherapy for Prostate Cancer: Where Do We Go From Here?—PART 1: Prostate Cancer Vaccines:

http://www.cancernetwork.com/prostate-cancer/immunotherapy-prostate-cancer-where-do-we-go-herepart-1-prostate-cancer-vaccines/page/0/1

Abstract / Synopsis:

Immunotherapies have emerged as a revolutionary modality for cancer treatment, and a variety of immune-based approaches are currently being investigated in the field of prostate cancer. Despite the 2010 approval of sipuleucel-T, subsequent progress in prostate cancer immunotherapy development has been limited by disappointing results with novel vaccination approaches and by prostate cancer's general resistance to immune checkpoint blockade. Nevertheless, there remains strong preclinical and clinical evidence to suggest that prostate cancer is a susceptible target for immune therapies. Innovative strategies for vaccine development, adoptive cell transfer, alleviation of immunosuppression in the tumor microenvironment, and combinatorial approaches using existing drugs and novel immune agents hold great promise for improving the treatment of prostate cancer. The first article in this two-part series will provide an overview of both past and present therapeutic vaccination strategies for the promotion of antitumor immunity against prostate cancer. Later, in Part 2, we will discuss novel areas of clinical development and identify the trends that may define the future of prostate cancer immunotherapy.

Introduction

Prostate cancer is the most commonly diagnosed malignant tumor in American men and the second leading cause of cancer-related mortality.[1] Even with recent advances in multimodality therapy for localized disease, relapse occurs in 30% of patients,[2]while men with metastatic disease ultimately develop therapeutic resistance despite the advent of novel cytotoxic drugs, anti-androgen therapies, and radiopharmaceuticals. Immunologic approaches have long been of

interest in prostate cancer because the disease has several characteristics that theoretically make it a suitable immunotherapy target.[3] The prostate is a nonessential organ whose tissues produce multiple tumor-associated antigens (TAAs) for which specific T-cell populations targeting them have been identified. These T cells can potentially serve as the central effectors of adaptive antitumor immunity. Additionally, the relatively slow growth kinetics of prostate cancer may provide a longer window for the development of effective immune responses. Despite these potential advantages, prostate cancer is generally thought to be a “cold” tumor, with limited T-cell infiltration and minimal responses to date to single-agent immune checkpoint therapies. Prostate cancer has a relatively low tumor mutation burden,[4,5] which has frequently been considered an indicator of a tumor’s poor inherent responsiveness to checkpoint inhibition; in addition, emerging data are identifying the presence of specific genetic phenotypes that are associated with the development of less immunogenic intratumoral landscapes.[6] Furthermore, prostate cancer tumors have been known to downregulate human leukocyte antigen (HLA) class I expression, induce T-cell apoptosis, increase immunosuppressive cytokines, and increase suppressive regulatory T cell (Treg) populations in order to evade immune surveillance.[7,8] Consequently, there is a significant need to develop approaches that can circumvent the inherent immunosuppression of the prostate cancer tumor microenvironment. Clinical applications of immunotherapeutic approaches in prostate cancer have yielded mixed results, but spurred by the success of sipuleucel-T, the first therapeutic vaccine approved for use in human cancer, numerous novel vaccination approaches that enhance antitumor immunity are now being investigated (Table).

Sipuleucel-T

Sipuleucel-T consists of autologous peripheral blood–derived mononuclear cells cultured with a prostatic acid phosphatase (PAP) and granulocyte-macrophage colony-stimulating factor (GM-CSF) fusion protein. Sipuleucel-T was approved for use in the setting of asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer on the basis of three trials whose results demonstrated

clinical efficacy. An integrated analysis of two of the trials, D9901 and D9902A, demonstrated an improved median survival in those treated with sipuleucel-T of 23.2 months vs 18.9 months for placebo, which was equivalent to a 33% reduction in the risk of death (hazard ratio [HR], 1.5; 95% CI, 1.1–2.05; *P* = .011).[9] It should be noted, however, that overall survival (OS) was a secondary endpoint in these studies, and that the primary endpoint of improved progression-free survival (PFS) was not met. Concerns have also been raised over the pooling of data from two independent studies and over possible inequivalence of baseline disease characteristics among the compared subgroups.[10] The subsequent IMPACT trial randomized 512 patients with metastatic castration-resistant prostate cancer in a 2:1 ratio to sipuleucel-T or the control treatment and found a significant 4.1-month increase in OS for the therapy group, although with no difference in time to progression; there were no major differences in adverse effects between the two arms.[11]

Despite these results and the subsequent US Food and Drug Administration approval of sipuleucel-T, its widespread adoption has been hampered by the involved manufacturing process, concerns about detrimental effects of the leukapheresis procedures, the limited therapeutic window and magnitude of clinical benefit, and questions raised by the discordance between the PFS and OS outcomes. Of particular importance is the recognition of this phenomenon of improved survival without changes in PFS as a recurrent theme in immunotherapy trials. This has been noted in several other clinical contexts, including in pre-approval trials of checkpoint inhibitors in metastatic melanoma and renal cell carcinoma,[12,13] and it raises the important question of what are the most appropriate parameters for measuring efficacy in the age of novel immunotherapies.

Although the exact mechanism of action of sipuleucel-T is not known, correlative studies provide insight into clinical predictors of response and immunologic effects of the therapy. Retrospective analyses have suggested increased benefit in patients with more favorable prognostic features, such as lower baseline prostate-specific antigen (PSA) and lactate dehydrogenase (LDH) levels and better performance status.[14] Increased tumor burden is generally believed to correspond to greater

systemic immunosuppression, and the suggestion of a later onset of action of sipuleucel-T based on the delayed separation of Kaplan-Meier survival curves has led to recent recommendations to consider sipuleucel-T vaccination early in the treatment of metastatic castration-resistant prostate cancer.[15]

Mechanistically, sipuleucel-T has demonstrated robust activation of antigen-presenting cells (APCs), antigen-specific T-cell responses, and increases in cytokines associated with T-cell activation. The number of APCs and their activation, as measured by CD54 upregulation, have positively correlated with improved OS.[16] Interestingly, sipuleucel-T has also resulted in humoral antigen spread to a variety of targets beyond PAP, with immunoglobulin (Ig) G responses to PSA and LGALS3 that have correlated with improved OS.[17] A neoadjuvant trial of sipuleucel-T prior to radical prostatectomy found that treatment could increase the frequency of activated CD4+ and CD8+ T cells in the tumor microenvironment, particularly at the interface with adjacent benign tissue.[18] The broad stimulation of systemic immunity, along with the recruitment of possible effector T cells to tumor by sipuleucel-T, provides a further rationale for combining vaccination approaches with other activators of T-cell function. This robust immunologic response also suggests the need to consider further studies evaluating vaccination in the neoadjuvant and adjuvant settings for localized disease, when vaccination may enable the development of sustained antitumor immune surveillance.

Other Vaccine Approaches

Cell-based vaccines

Despite the approval of sipuleucel-T, a variety of alternate vaccine approaches have had much less success in the management of prostate cancer. GVAX is a cellular vaccine consisting of irradiated cells from PC-3 and LNCaP prostate cancer cell lines that are modified to constitutively express GM-CSF.[19,20] The theoretical advantages of this approach include the opportunity to induce immunologic responses to multiple TAAs and the possibility of mass-producing vaccines that can be

administered without the need for HLA matching.[21] Ultimately, two phase III trials to test the therapeutic efficacy of GVAX were undertaken. The VITAL-1 trial comparing GVAX to docetaxel plus prednisone in asymptomatic castration-resistant prostate cancer was terminated after a futility analysis demonstrated a less than 30% chance of meeting the improved survival endpoint. VITAL-2, which compared the combination of GVAX and docetaxel to docetaxel and prednisone was also stopped after an interim analysis showed an increased risk of death in the GVAX arm.[22] Clinical development of GVAX was ultimately halted.

Virus-based vaccines

PROSTVAC (PSA-TRICOM) is a cancer vaccine composed of a series of poxviral vectors (vaccinia during the initial priming vaccine and fowlpox for all boosts) engineered to express PSA and a triad of human T-cell costimulatory molecules (B7.1, intercellular adhesion molecule 1, and lymphocyte function-associated antigen 3).[23,24] A phase II study of 125 patients with minimally symptomatic metastatic castration-resistant prostate cancer randomized to placebo or PROSTVAC with adjuvant GM-CSF did not demonstrate improvement in the primary PFS endpoint but showed an increased median OS of 25.1 vs 16.6 months (HR, 0.56; P = .0061).[25] A similar trial by the National Cancer Institute that allowed for enrollment of patients with symptomatic or visceral metastatic castration-resistant prostate cancer demonstrated a median OS of 26.6 months, with 12/32 patients demonstrating PSA decline. Patients with lower-risk disease as defined by a Halabi model–predicted survival of > 18 months at the time of treatment had a particularly notable duration of survival (median OS, 37.3 months), suggesting the possibility that vaccination provides the greatest benefit for patients with lower tumor burden or a less aggressive phenotype.[23] In the setting of biochemical recurrence after definitive local therapy, 63% of patients treated with PROSTVAC in conjunction with GM-CSF were progression-free at 6 months, and there was a notable reduction in PSA doubling time following treatment.[26]

Based on the promising phase II data in patients with metastatic castration-resistant prostate cancer, PROSPECT (ClinicalTrials.gov

identifier: NCT01322490), a global phase III trial enrolling 1,297 patients with metastatic castration-resistant prostate cancer, was undertaken to evaluate the efficacy of PROSTVAC-VF ± GM-CSF. Unfortunately, this trial was stopped in September 2017 when a preplanned interim analysis found the therapy to be unlikely to meet its OS endpoint.

Despite the disappointing results of the PROSPECT trial, there are compelling data to demonstrate the immunogenicity of PSA-encoding poxviral vaccines. An aggregate evaluation of blood T-cell responses across seven early poxviral vaccine trials showed 57% of patients (59/104) with a twofold or greater increase in PSA-specific T cells following the vaccine. Interestingly, a majority of these patients also demonstrated the phenomenon of antigen-spreading, with documented T-cell response to non-PSA antigen targets.[27] A similar vaccination strategy incorporating only a single costimulatory molecule (B7.1) was administered in conjunction with radiotherapy for localized prostate cancer and was found to produce a significant increase in PSA-specific T cells compared with radiotherapy alone.[28] Consequently, PROSTVAC has been administered in combination with escalating doses of ipilimumab in a phase I trial in metastatic castration-resistant prostate cancer. This trial demonstrated no significant increase in adverse events with the combination compared with ipilimumab alone,[29] with 14/24 chemotherapy-naive patients (58%) experiencing PSA decline, and with 6/24 having declines of > 50%; moreover, the median OS in this trial was a robust 31.6 months.[30] Currently, the optimal clinical contexts and combination strategies for PROSTVAC remain questions of interest, with ongoing trials being conducted in the localized (NCT02326805, NCT03315871, NCT00096551), neoadjuvant (NCT02506114, NCT02153918), adjuvant (NCT02772562), biochemical recurrence (NCT00450463, NCT01875250), metastatic castration-sensitive prostate cancer (NCT02649855), and castration-resistant prostate cancer (NCT01867333, NCT02933255) settings.

DNA vaccines

DNA vaccines consist of closed circular DNA plasmids designed to encode an antigen of interest under a strong mammalian promoter. The first trial to evaluate a DNA vaccine encoding prostate-specific membrane antigen (PSMA) in combination with adjuvant GM-CSF was initiated nearly 2 decades ago and demonstrated the safety and feasibility of generating immune responses to self-antigens in prostate cancer patients.[31] A variation on this theme involved the use of PSMA fused to tetanus toxin in patients who had exhibited biochemical recurrence of prostate cancer. Here, all patients demonstrated an increase in CD4+ T cells targeting tetanus toxin fragment C and CD8+ T cells specific for the PSMA epitope; an increase in PSA doubling time was also seen.[32]

pTVG-HP is a DNA vaccine encoding human PAP (hPAP) that produced persistent hPAP-specific T-cell responses that correlated with favorable changes in PSA doubling time. These responses frequently occurred later in the course of DNA immunization, and the vaccine was able to augment responses when given as a booster, sustaining a persistent type 1 T helper cell–based T-cell response with an extended dosing schedule.[33]

KEY POINTS

- **To date, sipuleucel-T remains the only vaccination strategy approved for use in prostate cancer based on improvements in overall survival—although lack of PSA modulation, challenges of administration, and cost have limited its widespread utilization.**
- **Various vaccination approaches have so far failed to show significant clinical benefit in late-stage trials, but the consistent demonstration of antigen-specific immune responses and improvements in surrogate endpoints such as PSA doubling time with many vaccination strategies is reason for optimism about the future.**
- **Growing evidence suggests that implementation of vaccination strategies earlier in disease and/or in combination strategies may enhance clinical benefit. Future studies will have to investigate vaccination use in localized and low-tumor-burden states, in addition to use of vaccines in synergistic combinations with other immunostimulatory agents.**

Several prostate cancer DNA vaccine trials are now active, most notably trials utilizing pTVG-HP in combination with GM-CSF (NCT01341652), as a prime boost to sipuleucel-T therapy (NCT01706458), and with

programmed death 1 (PD-1) blockade via pembrolizumab (NCT02499835). The last of these strategies is based on preclinical models demonstrating upregulation of PD-1 in the T cells of mice treated with pTVG-HP DNA vaccinations, with preliminary data showing encouraging antitumor activity in a setting where single-agent PD-1 blockade has largely been ineffective.[34] Several novel vaccination strategies currently in early development include a DNA vaccine that encodes the androgen receptor ligand binding domain (NCT02411786), dual targeting with simultaneous use of partially humanized PSA and PSMA coding constructs (NCT02514213), and novel combinations with immunomodulatory agents and checkpoint inhibitors (NCT02616185).

Adenoviral vaccines

Another method for directly inducing immunogenic cell death of prostate tumor cells involves the use of a replication-deficient adenoviral vector expressing the herpes simplex virus thymidine-kinase gene (*HSV-TK*) delivered directly to localized prostate cancer (AdV-tk). Administration of the antiherpetic prodrug induces local cytotoxicity, and when combined with inflammation from standard debulking surgery or radiation, this therapy may theoretically activate both innate and adaptive antitumor immune responses. The HSV-TK protein also acts as a super-antigen–like molecule in this setting.[35] Following demonstration of safety in a phase I trial,[36,37] the combination of intraprostatic AdV-tk, androgen deprivation therapy, and radiation for high-risk localized disease achieved lower recurrence rates compared with historical controls.[38] There has also been suggestion of a prolonged time to recurrence when AdV-tk is utilized in the neoadjuvant setting.[39] These findings have led to a placebo-controlled phase III trial in patients with localized disease who are candidates for curative external beam radiation therapy (NCT01436968).

Dendritic cell vaccines

Dendritic cells (DCs), which capture, process, and present antigens to T cells,[40] have received considerable interest as a basis for cellular vaccines that can be manipulated to induce responses against TAAs.

Three main approaches have been evaluated in DC vaccination: ex vivo antigenic peptide loading followed by autologous infusion of the conditioned DCs, gene modification of DCs in vivo through the use of recombinant viruses, and ex vivo genetic engineering for antigen presentation with or without enhanced cosignaling.

DCVAC/PCa is a promising vaccination strategy that is now being evaluated in a global phase III clinical trial (VIABLE; NCT02111577). It is an autologous DC-based vaccine composed of Poly (I:C)–activated DCs pulsed with killed prostate cancer cells from the LNCaP cell line. Phase I and II trials showed that a regimen of DCVAC and metronomic cyclophosphamide co-administered with docetaxel increased OS by 7.2 months over historical controls with metastatic castration-resistant prostate cancer.[41] This regimen was well tolerated overall, with no serious anaphylactic reactions or adverse events attributed to immunotherapy. NCT02107430 is another active trial testing the efficacy of DCVAC/PCa in the adjuvant setting following definitive radiation for high-risk localized disease.

Different formulations of DC vaccines utilizing alternative sources of TAAs and other adjuvants are in early stages of development as well. These include DCs pulsed with recombinant human PSMA and recombinant survivin peptide,[42] with prostate cell line lysates,[43] with PSMA and inducible CD40,[44] and with the T-cell receptor γ chain alternate reading frame protein (TARP).[45] It remains to be seen how these vaccination approaches can be optimized and sequenced to enhance antitumor immunity and what modes of therapeutic utilization, likely in combinatorial approaches, will prove most effective.

Bacteria-based vaccines

Listeria monocytogenes is an intracellular pathogen that is actively phagocytosed by APCs and is able to subsequently replicate in the cytosol via escape from the phagosome. These pathogenic features enable the generation of both CD4 and CD8 responses, since Listeria antigens are processed through both the major histocompatibility complex (MHC) I and MHC II pathways.[46] The use of an attenuated

form of Listeria engineered to express TAAs leverages these immunogenic features to induce an antitumor immune response; vaccines with an attenuated Listeria vector are being investigated in a variety of disease contexts. Preclinical data have demonstrated the ability of Listeria vector vaccines to generate an antigen-specific tumor response and to induce tumor regression in murine prostate cancer models, both as a single agent[47] and when administered with radiation therapy.[48] Two commercial Listeria platforms are currently being evaluated in phase I clinical trials, for safety alone (ADU-741/JNJ-64041809; in NCT02625857) and in combination with PD-1 checkpoint blockade (ADXS31-142; in NCT02325557).

Peptide vaccines

Another approach to the stimulation of antitumor immune activity involves the use of personalized peptide vaccines (PPVs). These consist of multiple exogenously administered cancer-associated peptides that can be presented on HLA class I molecules for recognition by T cells. Adjuvants such as toll-like receptor ligands, Montanide ISA-51, or agonists of stimulator of interferon genes (STING) are used to stimulate polarized type 1 T helper cell or CD8+ T-cell responses.[49] Aided by rapid improvements in next-generation sequencing and the development of algorithms for epitope prediction, these peptides seek to induce robust and rapid cytotoxic T-lymphocyte activation without the costs and cell availability limitations of cell-based approaches.[50] The first randomized phase II trial of PPVs in prostate cancer, reported in 2010, was an open-label, multicenter, crossover study comparing a four-peptide vaccine plus a low dose of estramustine phosphate (EMP; 280 mg/d) with a standard EMP dose (560 mg/d) in patients with metastatic or nonmetastatic castration-resistant prostate cancer.[51] Median PFS was 8.5 months in the PPV-plus-EMP group and 2.8 months in the EMP-only group (P = .0012); the HR for OS was 0.3 (95% CI, 0.1–0.91) in favor of the PPV-plus-EMP group (log-rank P = .0328). The combination was tolerated without major adverse effects. Another study, which assessed PSA kinetics and immune responses associated with a PPV, found that peptide-specific IgG and T-cell responses strongly correlated with PSA doubling time, which in turn showed a correlation with OS.[52] These

markers may be important surrogates to monitor in light of the observation that PFS and OS often do not track together in the setting of prostate cancer immunotherapies. A recent trial of 72 patients with early-stage castration-resistant prostate cancer found that those treated with PPV plus low-dose dexamethasone vs dexamethasone alone showed marked improvements in PFS (22.0 vs 7.0 months; *P* = .0076) and OS (73.9 vs 34.9 months; *P* = .00084), a significant finding that needs to be validated in a phase III setting.[53] Combining PPV with low-dose cyclophosphamide in an attempt to abrogate immunosuppressive Treg populations did not affect clinical response; however, while Tregs were decreased with combination therapy, it is possible this immunostimulatory effect was compensated for by the increase in levels of myeloid-derived suppressor cells. Of note, a subset analysis revealed that patients who exhibited a humoral immune response to the peptide in the vaccine or increased peptide-specific cytotoxic T-lymphocyte activity in peripheral blood showed significantly longer survival.[54]

A novel vaccine approach involves use of peptides of the reverse transcriptase subunit of telomerase (hTERT), which is often overexpressed in cancer cells and which plays an important role in tumor proliferation. Earlier studies demonstrated extensive epitope spreading within hTERT following vaccination with a 16–amino acid hTERT peptide fragment,[55] and based on these data, a therapeutic hTERT vaccine consisting of the three highest-frequency hTERT peptides was tested in patients with prostate cancer. In this phase I study, hTERT vaccine and GM-CSF were administered to patients with metastatic hormone-naive prostate cancer who were beginning androgen deprivation therapy. Out of 22 patients, 21 also received radiotherapy to the prostate or adjacent bony lesions during the vaccination period. As expected, a majority of the patients experienced significant reduction in PSA levels, but in addition, 86% demonstrated an immune response to the administered peptides. Of note, 2 patients in the highest-dose (0.7 mg) peptide group experienced anaphylactic reactions; ultimately, intermediate peptide dosing at 0.3 mg was deemed safe and most immunogenic.[56] GX301 is a vaccine consisting of four telomerase peptides and the adjuvants Montanide ISA-51 and imiquimod; it was found to be safe and immunogenic in an early trial,[57] and a phase II

trial in patients with metastatic castration-resistant prostate cancer who have already been treated with docetaxel is now active (NCT02293707).

Conclusion

While later-phase trials of single-agent vaccination therapies beyond sipuleucel-T have not yielded significant clinical benefit to date, these studies have provided a valuable foundation that can guide the development of subsequent strategies for prostate cancer immunotherapy. It is clear from correlative clinical trial experiments that numerous vaccination approaches are able to induce immunologic responses to putative TAAs. Furthermore, patient subset analyses of clinical trials suggest that certain populations, particularly those with a lower tumor burden and those earlier in the course of disease progression, may ultimately be more likely to benefit from vaccination strategies. This argues for the need to carefully evaluate the patient populations being treated in vaccination trials and to consider utilization of vaccines in localized and oligometastatic settings.

Future vaccination approaches will undoubtedly seek to utilize vaccines in conjunction with the many agents now being developed to stimulate both the innate and adaptive immune system. Promising strategies may also look to incorporate vaccines in conjunction with existing modalities of treatment, such as radiation therapy, that are known to have immunomodulatory properties. We will discuss many of the alternative immunotherapeutic approaches currently under investigation in Part 2 of this series. As we continue to gain a deeper understanding of the immunogenic properties of prostate cancer vaccines and identify new ways to augment antitumor immunity, the full therapeutic promise of prostate cancer vaccination may yet be fulfilled.

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Effects of Cryotherapy on Objective and Subjective Symptoms of Paclitaxel-Induced Neuropathy

Prospective Self-Controlled Trial

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Abstract and Introduction

Abstract

Background Chemotherapy-induced peripheral neuropathy (CIPN) is a dose-limiting and disabling side effect of taxane anticancer agents. We prospectively evaluated the efficacy of cryotherapy for CIPN prevention.

Methods Breast cancer patients treated weekly with paclitaxel (80 mg/m^2 for one hour) wore frozen gloves and socks on the dominant side for 90 minutes, including the entire duration of drug infusion. Symptoms on the treated sides were compared with those on the untreated (nondominant) sides. The primary end point was CIPN incidence assessed by changes in tactile sensitivity from pretreatment baseline in a monofilament test at a cumulative dose of 960 mg/m^2. We also assessed thermosensory deficits, subjective symptoms (Patient Neuropathy Questionnaire [PNQ]), manipulative dexterity, and the time to events and hazard ratio by PNQ. All statistical tests were two-sided.

Results Among the 40 patients, four did not reach the cumulative dose (due to the occurrence of pneumonia, severe fatigue, severe liver dysfunction, and macular edema), leaving 36 patients for analysis. None dropped out due to cold intolerance. The incidence of objective and subjective CIPN signs was clinically and statistically significantly lower on the intervention side than on the control (hand: tactile sensitivity = 27.8% vs 80.6%, odds ratio [OR] = 20.00, 95% confidence interval [CI] = 3.20 to 828.96, $P < .001$; foot: tacile sensitivity = 25.0% vs 63.9%, OR = infinite, 95% CI = 3.32 to infinite, $P < .001$; hand: warm sense = 8.8% vs 32.4%, OR = 9.00, 95% CI = 1.25 to 394.48, $P = .02$; foot: warm sense: 33.4% vs 57.6%, OR = 5.00, 95% CI = 1.07 to 46.93, $P = .04$; hand: PNQ = 2.8% vs 41.7%, OR = infinite, 95% CI = 3.32 to infinite, $P < .001$; foot: PNQ = 2.8% vs 36.1%, OR = infinite, 95% CI = 2.78 to infinite, $P < .001$; hand: hazard ratio [HR] = 0.13, 95% CI = 0.05 to 0.34; foot: HR = 0.13, 95% CI = 0.04 to 0.38, dexterity mean delay = −2.5 seconds, SD = 12.0 seconds, vs + 8.6 seconds, SD = 25.8 seconds, $P = .005$).

Conclusions Cryotherapy is useful for preventing both the objective and subjective symptoms of CIPN and resultant dysfunction.

Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a frequent and disabling side effect of cancer treatment, primarily taxanes and platinum agents.[1] CIPN reduces health-related quality of life[2] and often results in dose delay, dose reduction, or treatment discontinuation.[3] A patient-reported outcome study found that CIPN numbness persisted in 67%–80% of patients for one year following the completion of paclitaxel therapy.[4] Duloxetine was recommended for CIPN; however, it has limited efficacy for the amelioration of chemotherapy-induced pain, and none for numbness or functional disability.[4,5] Furthermore, no established strategy exists for CIPN prevention.[4]

Therapeutic regional hypothermia (cryotherapy) can reduce chemotherapy-induced complications by decreasing regional perfusion with acceptable tolerability.[6] Frozen gloves and socks prevented docetaxel-induced nail and skin toxicity in prospective, self-controlled trials that compared the protected side with the nonprotected side.[7,8] A retrospective study indicated that the occurrences of docetaxel-induced peripheral neuropathy was lower in the patients who used frozen gloves and socks compared to the patients who did not wear them (35% vs. 57%).[9]

Because CIPN symptoms are largely subjective and many clinicians underestimate their severity using the Common Terminology Criteria for Adverse Events v. 4.0 (CTCAE),[10,11] prospective trials with patient-reported outcomes may be superior for evaluating preventative efficacy; however, additional end points, including objective and functional assessments, are also needed to control for the placebo response bias of patient-reported outcomes. A self-controlled design can mitigate the effects of other confounders, including individual differences in sensory detection. Therefore, we investigated the effectiveness of cryotherapy against paclitaxel-induced peripheral neuropathy in a prospective self-controlled trial with multiple end points (e.g., objective, subjective, and functional assessments).

Methods

Study Design

This self-controlled clinical trial evaluated the preventive effects of cryotherapy for CIPN. As in previous cryotherapy studies,[7,8] each patient wore frozen flexible gloves and socks (Elasto-Gel, 84400 APT Cedex, Akromed, France) on the dominant hand and foot from 15 minutes before paclitaxel administration to 15 minutes after the infusion was complete (90 minutes in total). Frozen gloves were replaced after the first 45 minutes. The nondominant side acted as the untreated control. Symptoms of CIPN were assessed before chemotherapy (baseline) and before every cycle of paclitaxel administration during outpatient care. We analyzed the time to events (the cumulative doses to subjective CIPN events [PNQ ≥ D]) and CIPN symptoms at the cumulative dose of 960 mg/m^2, which is the recommended dose for neo-adjuvant and adjuvant weekly paclitaxel therapy.[12]

To explore the risk factors for CIPN, we assessed the pharmacokinetics during the first administration of paclitaxel. Breast cancer patients were recruited from the Kyoto University Hospital (Kyoto, Japan) between May 2014 and August 2015 according to the following inclusion criteria: planned administration of weekly paclitaxel (80 mg/m^2 for one hour) for at least 12 cycles (cumulative dose of 960 mg/m^2), an Eastern Cooperative Oncology Group Performance Status of 0 or 1, and a provision of signed informed consent. The exclusion criteria were as follows: peripheral sensory/motor neuropathy (CTCAE grade ≥ 2); neuralgia or edema (CTCAE grade ≥ 2); tumor metastasis in bone, soft tissue, or skin of the hands or feet; the absence of one or more fingers or toes; Raynaud's symptoms; peripheral arterial ischemia; hand-foot syndrome; and any other reasons based on the primary physician's judgment.

This trial was approved by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine (G638) in accordance with Helsinki guidelines and was registered with the University Hospital Medical Information Network in February 2014 (UMIN000013398).

Outcome Measures

Primary End Point Assessment: Tactile Disturbance. The primary end point was the incidence of CIPN (any grade), defined as a decline in tactile sensation from the pretreatment baseline as assessed by the Semmes-Weinstein monofilament test (NIHON MEDIX, Chiba, Japan), which is a validated measure of peripheral neuropathy.[13] Patients were blinded and stimulated by 20 grades of nylon filaments. We set the Semmes-Weinstein monofilament test as the primary end point for two reasons: 1) it is a robust and patient-blinded assessment, which will decrease the effects of patient expectancy in this nonblinded intervention trial; and 2) patients suffer who undergo paclitaxel therapy from tactile problems more than other types of sensory or motor problems..[14] Patients experiencing at least a

diminished sensation in response to light touch after a cumulative dose of 960 mg/m^2 were counted as events in the primary end point assessment.

Thermosensory Disturbance (Objective Symptoms). Thermosensory disturbance was assessed using a thermal stimulator (Yufu Itonaga, Tokyo, Japan) with 3 °C and 48 °C outputs. We stimulated the patients' (with their eyes closed) hands or feet with hot or cold stimulation and assessed the sensation following the thermal stimulation (normal, delayed, or diminished compared with baseline). The delayed and diminished sensations at 12 cycles (cumulative dose, 960 mg/m^2) compared with the pretreatment baseline were classified as events.

Vibration Perception (Objective Symptoms). Vibration perception at the wrist and ankle was assessed by a C 128-Hz tuning fork (NITI-ON CO, LTD, Chiba, Japan). Patients no longer feeling vibrations within 10 seconds of application after 12 paclitaxel cycles were considered events. Patients who exhibited abnormal scores at baseline were excluded from the analysis.

Performance Speed (Objective Symptoms). Manipulative dexterity was assessed using the grooved pegboard test (Lafayette Instrument Company, Lafayette IN), a validated sensory motor speed test.[15] The pegboard has 25 holes, with randomly positioned slots for pegs and keys along one side. Each peg must be properly rotated to match the hole before it can be inserted. We measured the time (seconds) required for the insertion of 25 pegs by each hand (dominant vs nondominant) and calculated the mean difference from the baseline score.

Patient-Reported Assessment (Subjective Symptoms). Subjective symptoms were assessed using the Japanese version of the PNQ, a validated patient-reported questionnaire on neuropathy and activities of daily living (ADL) that correlates with quality of life.[11] The patient subjectively responded to each item, grading each as A (no neuropathy), B (mild neuropathy), C (moderate neuropathy that does not interfere with ADL), D (moderate neuropathy that interferes with ADL), or E (severe neuropathy that interferes with ADL).[11] We assessed the grades of CIPN, and patients having grades D or E, as severe as CTCAE grades 2 and 3,[11] were counted as severe CIPN events.

Cryotherapy Tolerability. Adherence to cryotherapy, pain, abnormality of sensation, and other discomforts due to cryotherapy were checked during every intervention.

Electrophysiological Signs. We measured the conduction velocity and action potential amplitude of the median nerve using Neuropack X1 (Nihon Kohden Corporation, Tokyo, Japan). The current perception thresholds on the hands and feet were also assessed using a Neurometer CPT (Neurotron, Towson, MD). Patients exhibiting abnormal values[16,17] were counted as events.

Pharmacokinetics. The pharmacokinetics of paclitaxel was assessed by plotting the area under the plasma concentration-time curve (AUC) for 24 hours, commencing immediately before administration ($AUC_{0–24}$). Blood samples were obtained prior to infusion and immediately before and one, three, five, and 23 hours after the end of the infusion. The plasma drug concentration was measured by high-performance liquid chromatography with tandem mass spectrometry.[18] Pharmacokinetic parameters were calculated according to the two-compartment model using the nonlinear least squares method in WinNonlin 6.4 (Pharsight, Inc., Mountain View, CA). Furthermore, we calculated the dose intensity (mg/m^2/wk) of paclitaxel as the cumulative dose (mg/m^2) divided by the administration period.

Statistical Analysis

The sample size was determined by referring to the sizes recorded in similar previous cryotherapy studies.[8] CIPN symptoms are presented as the incidence rate, odds ratio (OR), and 95% confidence interval (CI) of the hands and feet. The time to subjective CIPN was analyzed using the Kaplan-Meier method and Cox regression analysis. Manipulative dexterity was presented as the mean time difference (SD). McNemar's test (tactile, thermal, vibration perception, and subjective CIPN), log-rank test (time to CIPN events), and two-sided paired *t* test between intervention and control sides (manipulative dexterity) were used for statistical comparison. A *P* value of less than .05 was considered statistically

significant. All analyses were performed using SAS v. 9.3 (SAS Institute Inc., Cary, USA) and R (R Foundation for Statistical Computing, Vienna, Austria), supervised by a statistician. Data quality was ensured by an independent data center (Medical Research Support Co., Ltd., Osaka, Japan). All statistical tests were two-sided.

Results

Patient Recruitment and Characteristics

Among the 44 patients registered, four did not undergo any intervention. An additional four did not reach a cumulative paclitaxel dose of 960 mg/m^2, leaving 36 patients for the analysis (Figure 1;). A total of 25 patients completed paclitaxel therapy at a cumulative dose of 960 mg/m^2, and 11 underwent chemotherapy at a cumulative dose higher than 960 mg/m^2 (maximum = 4080 mg/m^2).

Table 1. Patient characteristics (n = 40)

Characteristic	
Mean age (SD), y	56.0 (13.8)
Mean weight (SD), kg	55.6 (7.5)
Mean body mass index (SD), kg/m^2	22.4 (4.3)
Mean area under the curve (SD), μg·h/mL	7.5 (1.4)
Smoker, No. (%)	3 (7.5)
Diabetes, No. (%)	3 (7.5)
Left-handed, No. (%)	3 (7.5)
Breast cancer, No. (%)	
Left	19 (47.5)
Right	18 (45.0)
Left and right	3 (7.5)
Treatment, No. (%)	
Neo-adjuvant	22 (27.5)
Adjuvant	11 (55.0)
Palliative	7 (17.5)

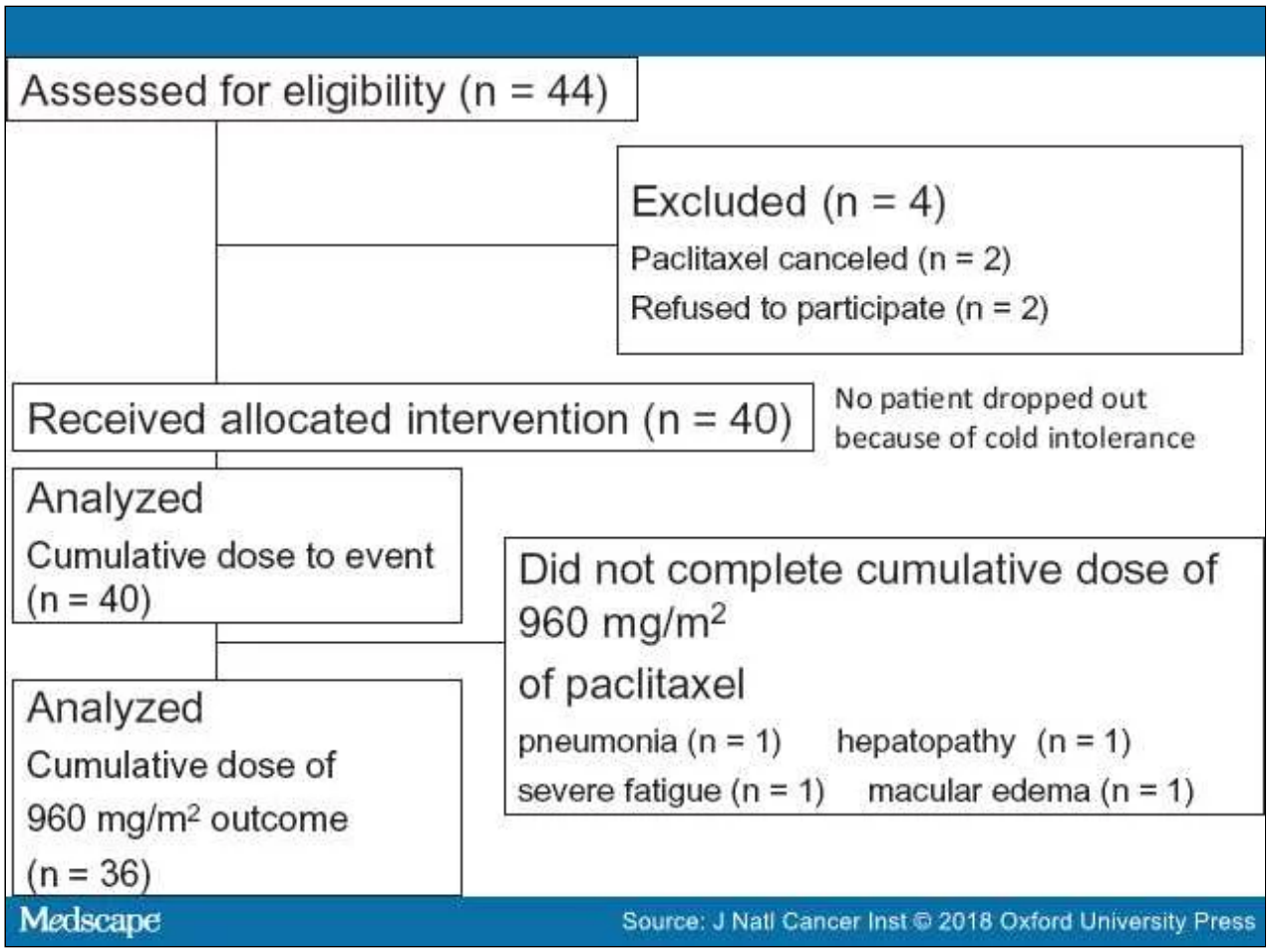


Figure 1.

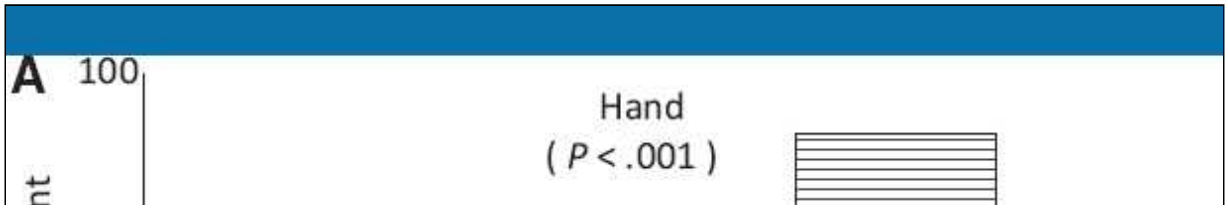
Study flow diagram. We included 40 patients who received the allocated intervention and analyzed the cumulative dose for subjective chemotherapy-induced peripheral neuropathy (CIPN) events. All CIPN signs were analyzed in the 36 patients who reached a cumulative dose of 960 mg/m^2. We compared the hands and feet of the intervention side to those of the control side.

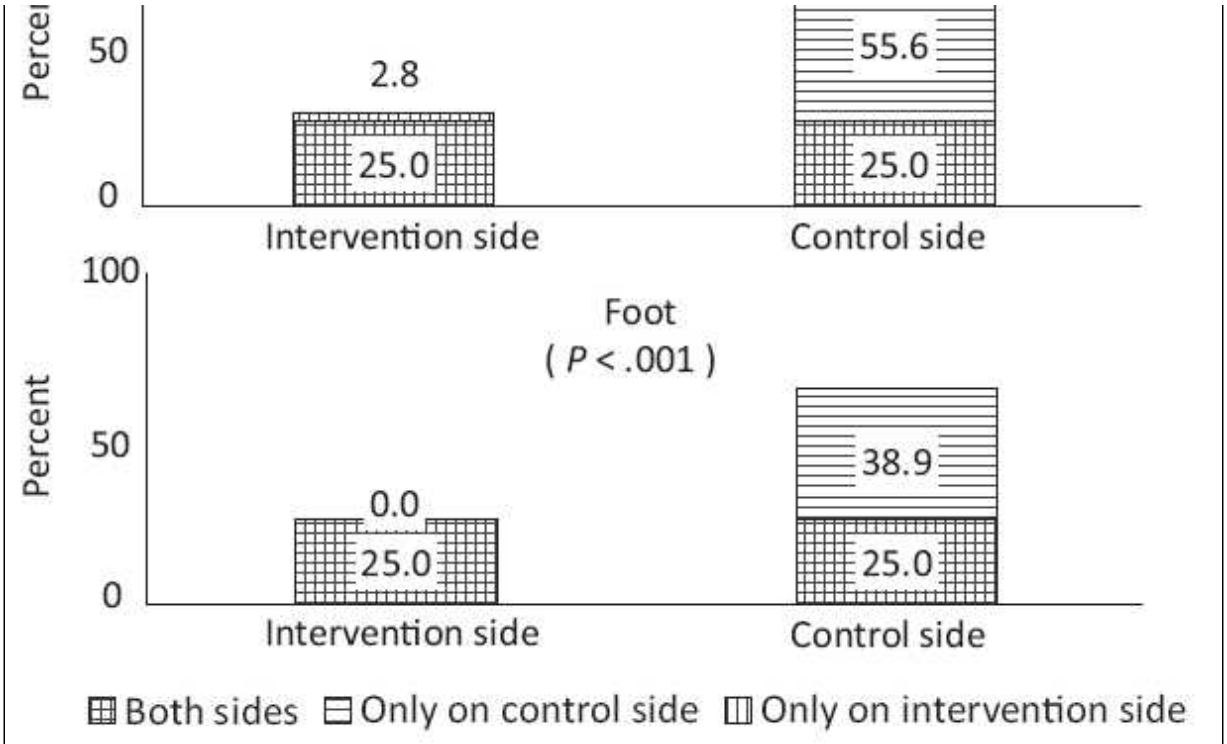
Cryotherapy Tolerability

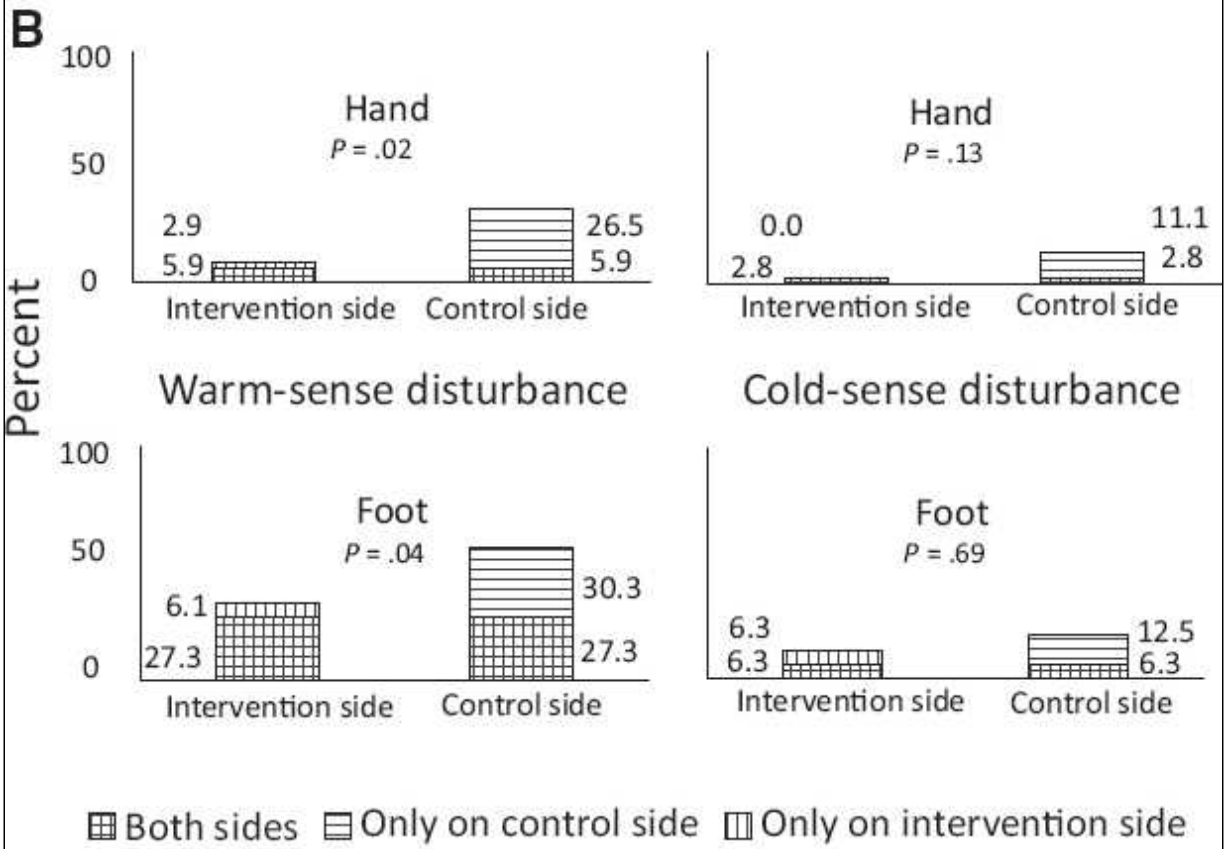
No patients dropped out due to cold intolerance. The most frequently reported adverse events (events/(person*cycle)) were pain (8.2%), sensory abnormalities (0.4%), and feeling cold (4.2%). The adverse events diminished immediately during or after cryotherapy intervention.

Primary End Point

The proportion of hands and feet exhibiting tactile deterioration were clinically and statistically significantly lower for the intervention side than the control side (hand: 27.8% vs 80.6%, OR = 20.00, 95% CI = 3.20 to 828.96, $P < .001$; foot: 25.0% vs 63.9%, OR = infinite, 95% CI = 3.32 to infinite, $P < .001$). The proportions include the patients who experienced CIPN from both the intervention and control sides of the hand and foot (Figure 2A).







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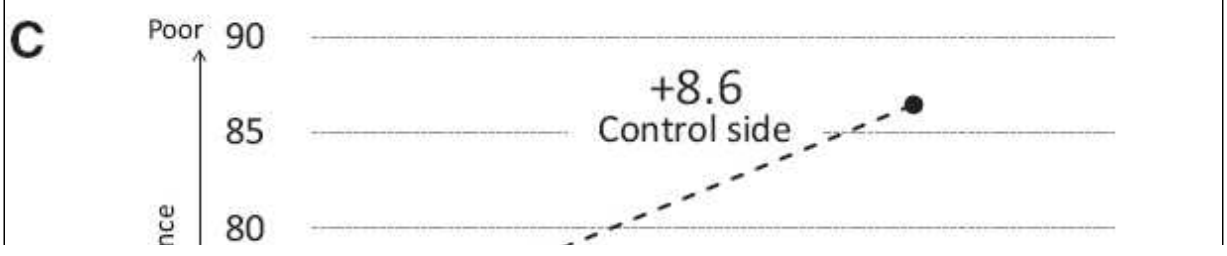


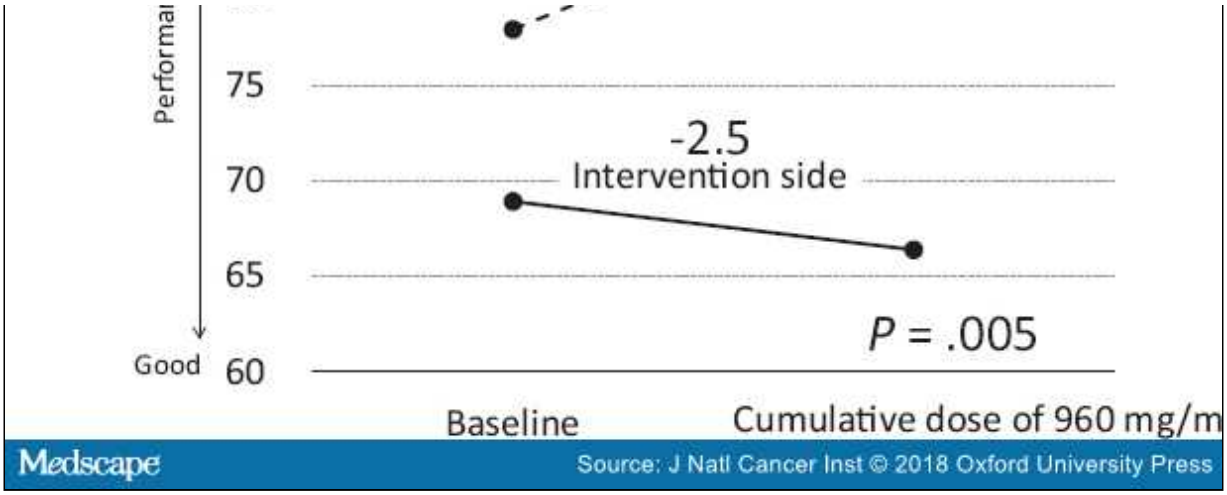
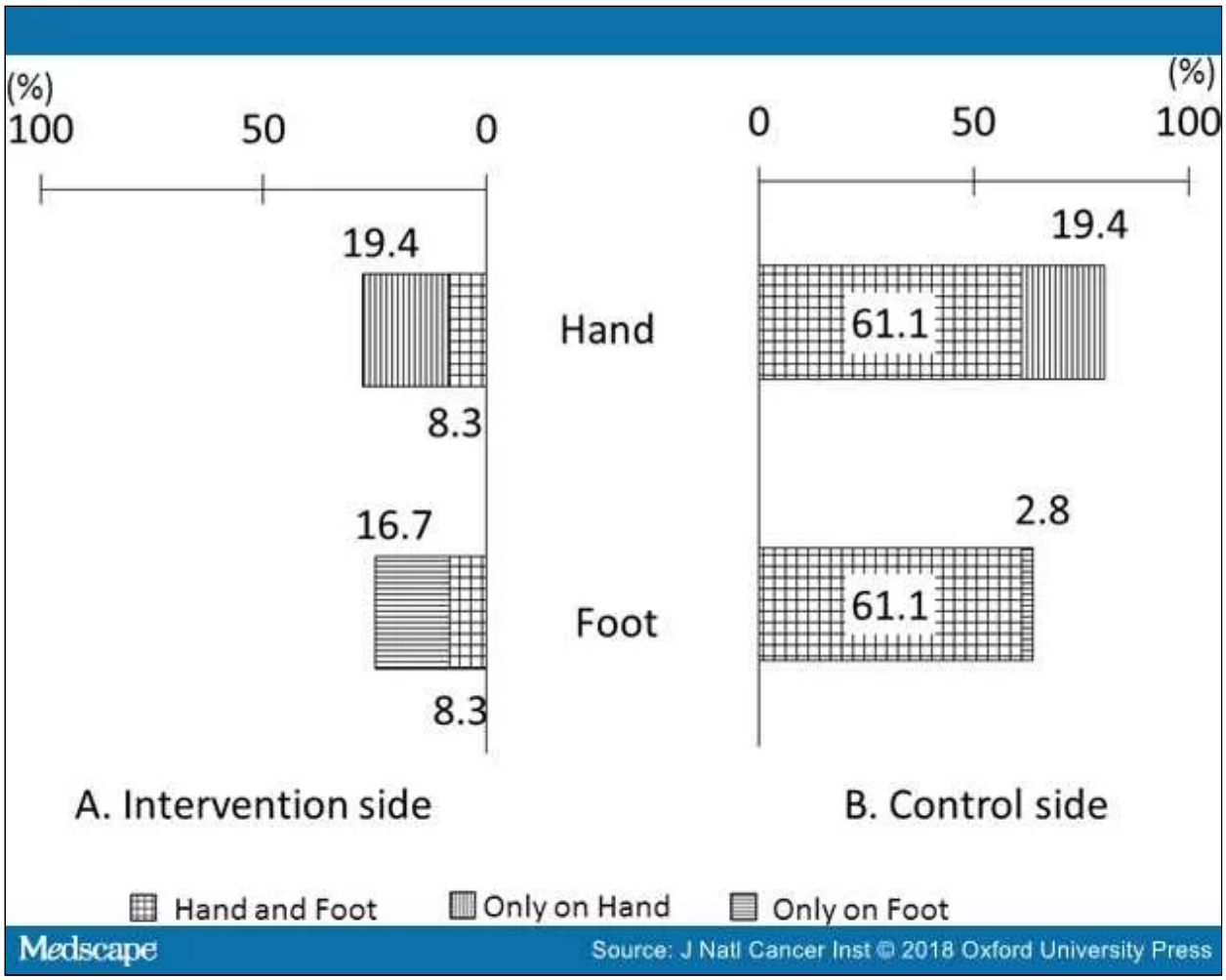


Figure 2.

Objective chemotherapy-induced peripheral neuropathy (CIPN) events at a cumulative dose of 960 mg/m^2. **A)** The efficacy of cryotherapy for reducing the primary end point, incidence of CIPN, was assessed by tactile-sensory deficits on the monofilament test. Any tactile deterioration from the pretreatment baseline in an intervention or control side hand or foot at a cumulative dose of 960 mg/m^2 was considered a CIPN event. The differential incidence between the intervention and control sides was evaluated using a two-sided McNemar's test (n = 36). **B)** Treatment with cryotherapy reduced thermosensory dysfunction. Only patients who exhibited a normal sensory threshold at baseline were included (hand: warm, n = 34, cold, n = 36; foot: warm, n = 33, cold, n = 32). Any response delay, response reduction, or thermal analgesia at a cumulative dose of 960 mg/m^2 was considered a CIPN. **C)** This figure shows manipulative dexterity deficits. *P* values were determined by a two-sided paired *t* test between the intervention and control side. The **solid line** denotes the intervention side, and the **dotted line** indicates the control side (N = 36).

Secondary End Points

Objective End Points. Figure 2B presents the incidence of thermosensory deficits. Patients who exhibited an abnormal thermal sense at baseline (hands warmth, n = 2; feet warmth, n = 3; and feet cold, n = 4) were excluded from the analysis. The incidence of a reduced perception of warmth was clinically and statistically significantly lower on the intervention side (hand: 8.8% vs 32.4%, OR = 9.00, 95% CI = 1.25 to 394.48, *P* = .02; foot: 33.4% vs 57.6%, OR = 5.00, 95% CI = 1.07 to 46.93, *P* = .04). The proportions include the patients who experienced CIPN from both the intervention and control sides of the hands and feet). In contrast, cold-sense deficits also tended to be numerically lower on the intervention side but showed no statistical significance (hand: 2.8% vs 13.9%, OR = inifinite, 95% CI = 0.66 to infinite, *P* = .13; foot: 12.6% vs 18.8%, OR = 2.00, 95% CI = 0.29 to 22.11, *P* = .69). The proportions include the patients who experienced CIPN from both the intervention and control sides of the hands and feet). For the incidence of vibration perception deficits, patients exhibiting an abnormal sense at the pretreatment baseline (hand, n = 5; foot, n = 7) were excluded from the analysis; however, the incidences tended to be numerically lower on the intervention side but showed no statistically significant differences between the intervention and control sides (hand: 9.7% vs 12.9%, OR = inifinite, 95% CI = 0.03 to infinite, *P* = 1.00; foot: 13.8% vs 24.1%, OR = inifinite, 95% CI = 0.41 to infinite, *P* = .25). The proportions include the patients who experienced CIPN from both the intervention and control sides of the hands and feet). The performance speed compared with the baseline level exhibited a greater delay on the control side (−2.5-second delay, SD = 12.0 seconds, on the intervention side vs +8.6-second delay, SD = 25.8 seconds, on the control side, *P* = .005) (Figure 2C). Some patients showed abnormal scores, and there were no statistically significant differences in nerve degeneration in the electrophysiological signs. Supplementary Figure 1 (available online) shows the incidence overlaps of CIPN in the hands and feet.



Supplementary Figure 1.

Incidence overlap of chemotherapy induced peripheral neuropathy (CIPN) in the hands and feet. CIPN was assessed by a Semmes-Weinstein monofilament test. Results for the (A) intervention and (B) control side are shown.

Subjective End Point. For sensory dysfunction, Figure 3 shows the subjective severity grades of CIPN at a cumulative dose of 960 mg/m^2 (PNQ grades A–E). The occurrences of CIPN (PNQ grades C–E) were prevented by cryotherapy (severe CIPN with grades D or E; hand: 2.8% vs 41.7%, OR = infinite, 95% CI = 3.32 to infinite, $P < .001$; foot: 2.8% vs 36.1%, OR = infinite, 95% CI = 2.78 to infinite, $P < .001$). A log-rank analysis of Kaplan-Meier curves (Figure 4) revealed that CIPN also occurred faster on the control side than on the intervention side (hand: HR = 0.13, 95% CI = 0.05 to 0.34, $P < .001$; foot: HR = 0.13, 95% CI = 0.04 to 0.38, $P = .007$). Only two patients reported motor dysfunction, which lasted less than one week.

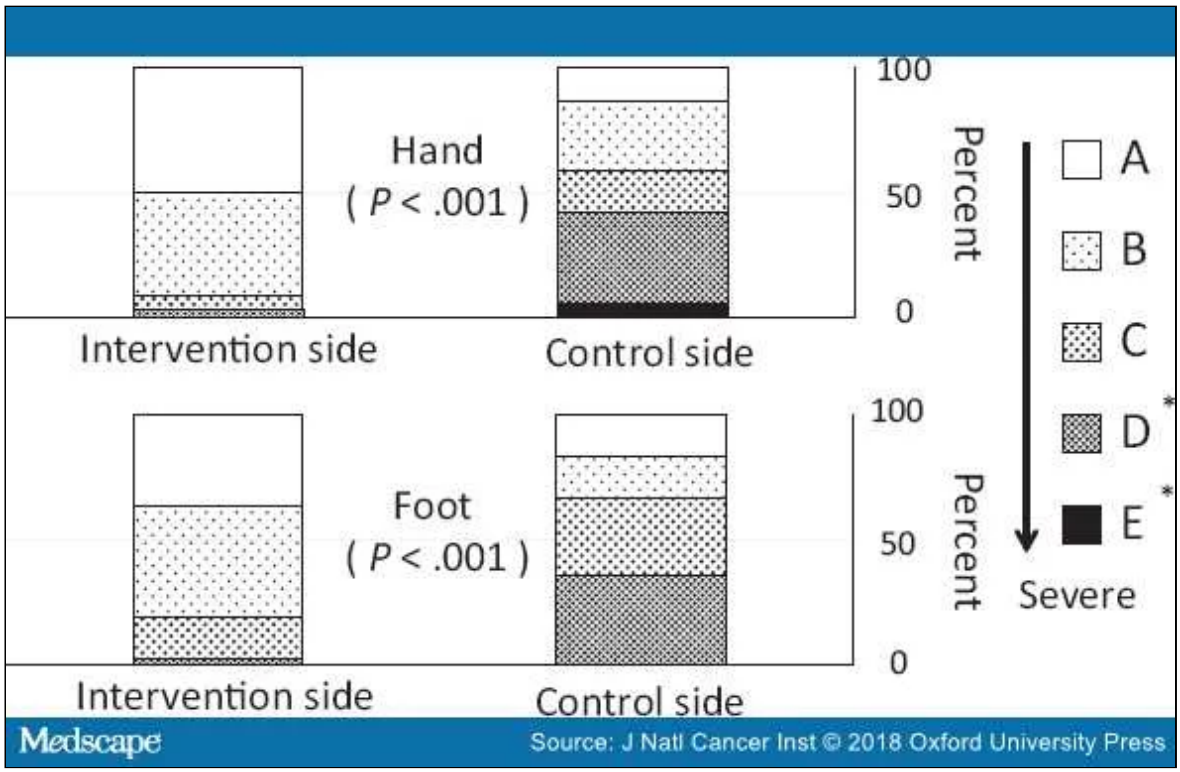


Figure 3.

Severity of subjective symptoms (at a cumulative dose of 960 mg/m^2). The administration of cryotherapy also reduced the subjective symptoms based on the Patient Neuropathy Questionnaire responses (the secondary end point, subjective) at a cumulative dose of 960 mg/m^2 (*P* values determined by the McNemar's test, n = 36). The subjective responses to each item were graded from A (no neuropathy) to E (severe neuropathy) by the patient. A rank of D or E indicates impaired activities of daily living. *Activities of daily living were interfered with.

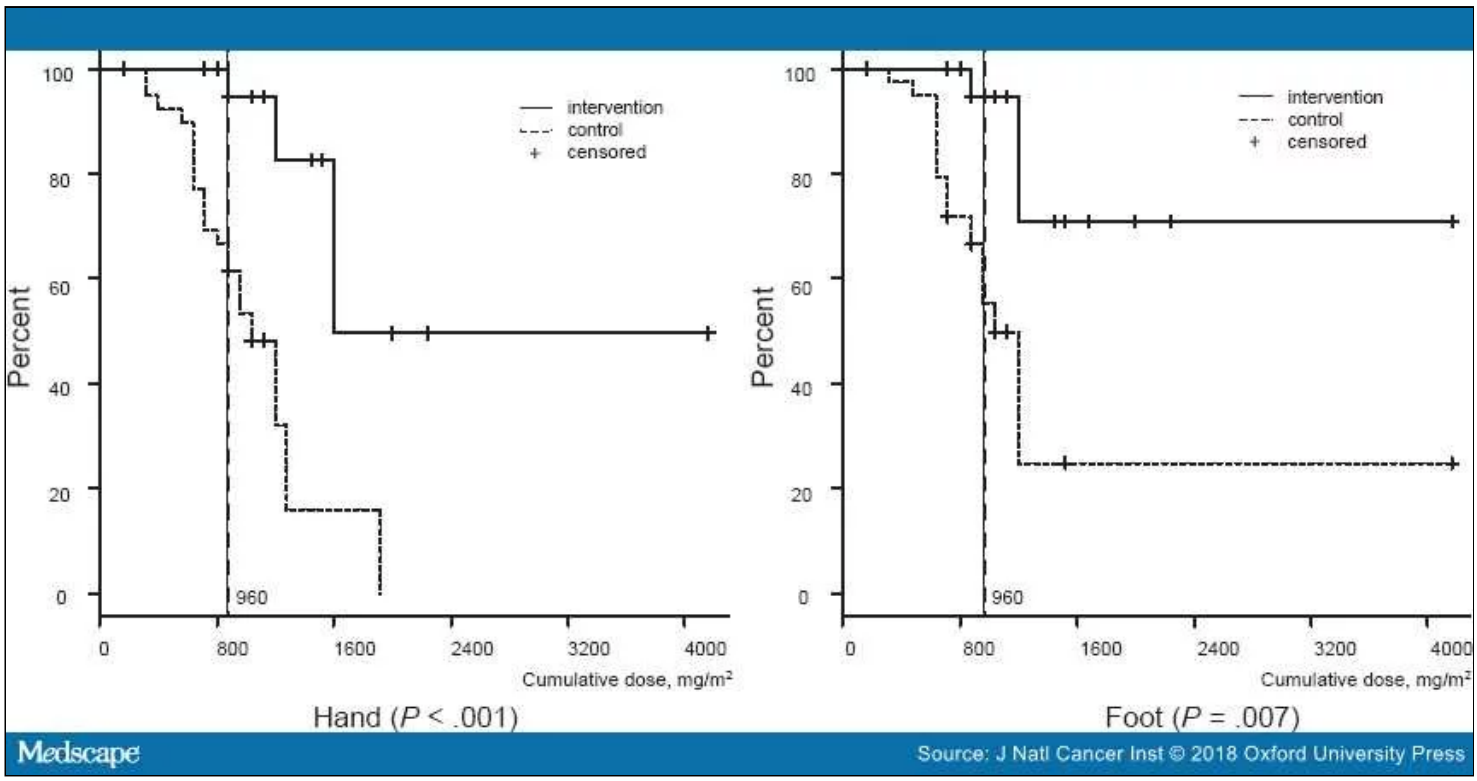


Figure 4.

The appearance of severe subjective neuropathy symptoms with cumulative dose. Severe subjective neuropathy symptoms (Patient Neuropathy Questionnaire ≥ D; moderate to severe tingling, pain, or numbness that interferes with activities of daily living) with cumulative dose were compared between the intervention and control sides using a log-rank test (n = 40). The **solid line** denotes the intervention side, and the **dotted line** indicates the control side. The **dotted vertical line** represents a cumulative dose of 960 mg/m^2. We included four censored patients who did not complete cumulative dose of 960 mg/m^2 paclitaxel due to pneumonia (n = 1), severe fatigue (n = 1), severe liver dysfunction (n = 1), and macular edema (n = 1). A two-sided log-rank test was used to calculate the *P* values.

Risk Factors of CIPN. We analyzed the effect of clinical factors on the time to subjective CIPN events (PNQ ≥ D) on the control side. No statistically significant differences were found for the time to events between the low AUC_{0-24} group, with the low AUC means below the median value (group mean = 6.6 μg·h/mL, SD = 0.5 μg·h/mL; total cohort median = 7.2 μg·h/mL) and the high AUC_{0-24} group (mean = 8.3 μg·h/mL, SD = 1.4 μg·h/mL) (hand: *P* = .54, foot: *P* = .56) (Figure 5A). The dose intensity varied because of the results of temporary delays due to chemotherapy-induced neutropenia. Symptoms occurred statistically earlier in the high–dose intensity group (mean = 75.0 mg/m^2/wk, SD = 4.8 mg/m^2/wk) above the median (68.6 mg/m^2/wk) than in the low–dose intensity group (mean = 56.6 mg/m^2/wk, SD = 7.0 mg/m^2/wk; hand: *P* = .001, foot: *P* = .003) (Figure 5B). We also examined the effects of other factors; however, none were statistically significant risk factors for CIPN (, availale online).

Supplementary Table 1.. Risk factors

Variable	Hand			Foot		
	CIPN* (*N* = 21)	no-CIPN* (*N* = 15)	*P* †	CIPN* (*N* = 21)	no-CIPN* (*N* = 15)	*P* †
Mean age, y (SD)	54.9 (14.5)	57.0 (13.4)	0.68	54.9 (14.5)	57.0 (13.4)	0.67
Mean body mass index, kg/m^2 (SD)	24.0 (3.3)	23.1 (2.5)	0.39	24.0 (3.3)	23.1 (2.5)	0.39
Smoking history, No. (%)	0 (0.0)	3 (20.0)	0.06	0 (0.0)	3 (20.0)	0.06
Diabetes, No. (%)	2 (9.5)	1 (6.6)	1.00	2 (9.5)	1 (6.6)	1.00
Mean peak serum concentration (SD)	3814.8 (760.4)	3759.7 (484.2)	0.81	3814.8 (760.4)	3759.7 (484.2)	0.81
Mean baseline tactile sensory (SD)	3.1 (0.5)	3.2 (0.4)	0.43	3.7 (0.49	3.7 (0.3)	0.67
Mean baseline vibration sensory (SD)	19.3 (6.4)	20.3 (10.1)	0.71	14.2 (7.1)	17.5 (9.1)	0.24
Mean baseline pegboard score (SD)	78.7 (16.0)	77.1 (26.1)	0.83	--	--	-

*Patients neurotoxicity questionnaire grade > C; moderate to severe tingling, pain or numbness on the control side. CIPN = Chemotherapy induced peripheral neuropathy. †P values were determined by a two-sided t-test (age, body mass index, peak serum concentration, baseline tactile thresholds, baseline vibration thresholds, and baseline pegboard score) or a two-sided Fisher's exact test (smoking history and diabetes) between CIPN and no-CIPN

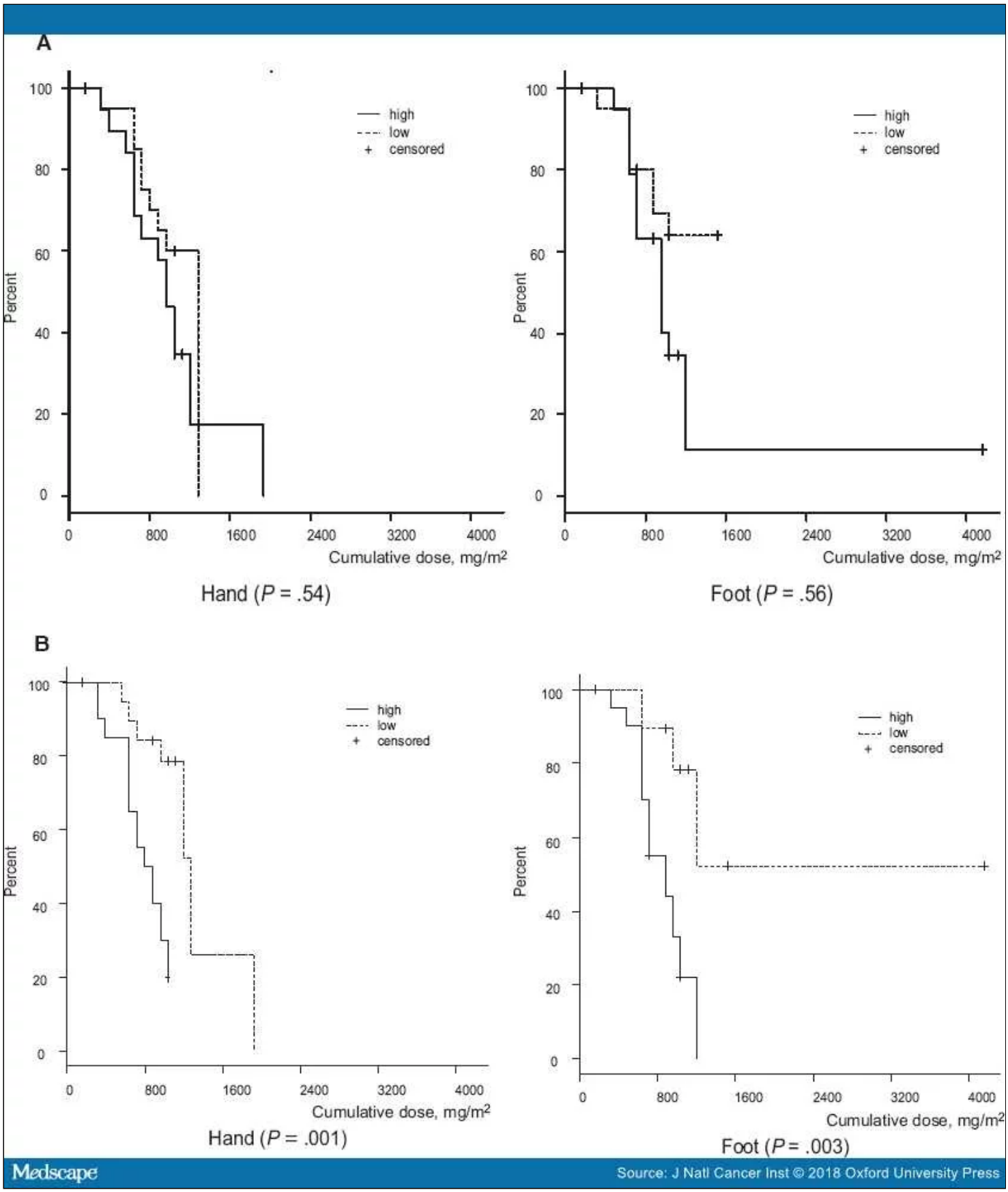

A
100
80
60
40
20
0
Percent
high
low
censored
0
800
1600
2400
3200
4000
Cumulative dose, mg/m2
Hand (P = .54)
Foot (P = .56)
B
Hand (P = .001)
Foot (P = .003)
Medscape
Source: J Natl Cancer Inst © 2018 Oxford University Press

Figure 5.

Cumulative dose to subjective symptoms (Patient Neuropathy Questionnaire rank ≥ D; moderate to severe tingling, pain, or numbness that interferes with activities of daily living [ADL]) on the control side to identify chemotherapy-induced peripheral neuropathy (CIPN) risk factors (n = 40). **A)** This figure presents the high/low area under the curve (AUC; μg·h/mL). The low-AUC group (mean = 6.6 μg·h/mL, SD = 0.5μg·h/mL) and high-AUC group (mean = 8.3 μg·h/mL, SD = 1.4 μg·h/mL) were divided by the median AUC (7.2 μg·h/mL). The **solid line** denotes the high-AUC group, and the **dotted line** indicates the low-AUC group. **B)** This figure presents high/low dose intensity (mg/m^2/wk). The low–dose intensity group (mean = 56.6 mg/m^2/wk, SD = 6.7 mg/m^2/wk) and high–dose intensity group (mean = 75.0 mg/m^2/wk, SD = 4.8 mg/m^2/wk) were divided by the median dose intensity (68.6 mg/m^2/wk). The **solid line** represents the high–dose intensity group, and the **dotted line** denotes the low–dose intensity group. A two-sided log-rank test was used to calculate the *P* values.

Electrophysiological Signs. For the incidence of electrophysiological signs, patients exhibiting a normal sense at the pretreatment baseline were included in the analysis (median nerve conduction velocity, n = 18; median nerve action potential amplitude, n = 14; current perception thresholds, hands, n = 11, feet, n = 8); they showed no statistically significant differences between intervention and control sides (nerve conduction velocity: 5.5% vs 5.5%, OR = 1.00, 95% CI = 0.01 to 78.50, *P* = 1.00; action potential: 28.5% vs 28.5%, OR = 1.00, 95% CI = 0.01 to 78.50, *P* = 1.00; current perception threshold; hand: 18.1% vs 27.2%, OR = inifinite, 95% CI = 0.03 to inifinite, *P* = 1.00, foot: 25.0% vs 25.0%, OR = 1.00, 95% CI = 0.01 to 78.50, *P* = 1.00) . All *P* values were analyzed using McNemar's test.

Discussion

Our findings support the efficacy of cryotherapy for CIPN prevention, as evidenced by a clinically and statistically significant reduction in patient-reported subjective symptoms, diminished objective signs (tactile and thermosensory), and prevention of manipulative dexterity. The development of subjective CIPN symptoms was clinically and statistically significantly delayed during the course of the paclitaxel treatment, the occurrence of subjective CIPN at a cumulative dose of 960 mg/m^2 was almost completely prevented, and the CIPN incidence as assessed by other objective modalities tended to be lower on the intervention side. Because the self-controlled design can reduce the effects of unknown potential confounders to levels lower than expected in randomized clinical trials, data consistency among the multiple assessments and large effect size, as exampled by a small hazard ratio, support the robustness of our conclusions despite the limited sample size.[19] Furthermore, no patients dropped out due to cold intolerance in response to cryotherapy.

Our study had several limitations. First, placebo effects are inevitable. To minimize differences in expectancy between the intervention and control sides, we supported a subjective symptom evaluation with objective measures. One potential confounder is that the control side may exhibit higher skin temperatures concomitant with a homeostatic whole-body temperature increase due to cooling on the intervention side; however, this influence was likely minimal because the incidence of CIPN symptoms on the control sides did not deviate substantially from that reported in previous studies.[1,2] A comparison between patients with and without intervention would control for this physiological response. Second, the nondominant hand and foot always acted as the control, as in previous studies of cryotherapy.[7,8] To the best of our knowledge, there have been no reports on bilateral differences in CIPN symptoms (either subjective or objective). Impairments in ADL are likely less severe when CIPN occurs in the nondominant hand due to easier compensation using the dominant hand. Third, we did not plan to follow the patients after the completion of paclitaxel treatment because postpaclitaxel therapy could impact the sensory status. In this study, patients underwent surgery (n = 10), radiotherapy (n = 8), hormonal therapy, and/or additional chemotherapy (n = 18) following paclitaxel therapy. The 30 patients who returned to our clinic within a median of 6.1 weeks (2 to 126 weeks) stated that there was no worsening of CIPN symptoms after the cessation of paclitaxel treatment. While previous studies have suggested that the development of additional CIPN signs or coasting is relatively rare after cessation of chemotherapy,[20,21] long-term follow-up would reveal the effects of cryotherapy on the natural course of CIPN signs and symptoms.

Compression therapy using surgical gloves modestly prevents CTCAE grade 2 or higher sensory and motor peripheral neuropathy with four cycles of triweekly nanoparticle albumin-bound paclitaxel.[22] Compression therapy and cryotherapy share an analogous mechanism of reduced drug exposure due to vasoconstriction during paclitaxel infusion. The low temperature associated with cryotherapy may also decrease the uptake of paclitaxel and damage of neurons or mechanotransductions, which might be related to decreased CIPN.[20,23]

Total drug exposure may also enhance the risk of CIPN. In a previous study, the CIPN incidence increased with AUC and time above the paclitaxel concentration threshold.[24] Although we found clinically and statistically significant differences in the cumulative dose to events between the high- and low-dose intensity groups, no differences were found between high- and low-AUC groups with a uniform dosage and relatively small variability in pharmacokinetics. Any other risk factor analyses have low power, and we could not identify any correlation between CIPN occurrence and the baseline assessments.

We conclude that cyrotherapy is a simple, safe, and effective strategy for the prevention of CIPN in patients with cancer undergoing paclitaxel treatment. Cyrotherapy could support the delivery of optimal chemotherapy by preventing a dose delay or reduction, as well as inhibiting the deterioration of quality of life in cancer patients during and after treatment.

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REVIEW

Could Intermittent Energy Restriction and Intermittent Fasting Reduce Rates of Cancer in Obese, Overweight, and Normal-Weight Subjects? A Summary of Evidence[1,2]

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ABSTRACT

Animal studies and human observational data link energy restriction (ER) to reduced rates of carcinogenesis. Most of these studies have involved continuous energy restriction (CER), but there is increasing public and scientific interest in the potential health and anticancer effects of intermittent energy restriction (IER) or intermittent fasting (IF), which comprise periods of marked ER or total fasting interspersed with periods of normal eating. This review summarizes animal studies that assessed tumor rates with IER and IF compared with CER or ad libitum feed consumption. The relevance of these animal data to human cancer is also considered by summarizing available human studies of the effects of IER or IF compared with CER on cancer biomarkers in obese, overweight, and normal-weight subjects. IER regimens that include periods of ER alternating with ad libitum feed consumption for 1, 2, or 3 wk have been reported to be superior to CER in reducing tumor rates in most spontaneous mice tumor models. Limited human data from short-term studies (≤6 mo) in overweight and obese subjects have shown that IER can lead to greater improvements in insulin sensitivity (homeostasis model assessment) than can CER, with comparable reductions in adipokines and inflammatory markers and minor changes in the insulin-like growth factor axis. There are currently no data comparing IER or IF with CER in normal-weight subjects. The benefits of IER in these short-term trials are of interest, but not sufficient evidence to recommend the use of IER above CER. Longer-term human studies of adherence to and efficacy and safety of IER are required in obese and overweight subjects, as well as normal-weight subjects. *Adv Nutr* 2016;7:690–705.

Keywords: intermittent energy restriction, intermittent fasting, cancer, obese, normal weight

Introduction

Excess adiposity and overnutrition are important causes of cancer. An increase in BMI (in kg/m^2) of 5 is associated with a 20–52% greater risk of 13 cancers, including endometrial, gall bladder, renal, rectal, postmenopausal breast, pancreatic, thyroid, colon, and esophageal cancers; leukemia; multiple myeloma; non-Hodgkin lymphoma; and malignant melanoma (1). Biomarker-calibrated energy intake is positively associated with total cancer, as well as with breast, colon, endometrial, and kidney cancer in postmenopausal women (2). Observational evidence indicates that weight reduction with energy restriction (ER)[3] reduces the risk of breast cancer (3, 4), whereas weight reduction with bariatric surgery reduces the risk of cancer, mainly in women (5).

Reduced tumor development with ER was first identified in a study by Rous (6), which demonstrated that ER delayed the development of recurrence and the growth of mammary tumors in mice. One hundred years of subsequent laboratory research has confirmed that ER prevents tumor development in rodents, and many studies indicate that ER prolongs the life-span (7). The comparator groups in these studies were mainly overfed, sedentary laboratory animals (8). Thus, these studies indicate that ER can reduce the cancer-promoting effects of obesity and overnutrition, but

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[3] Abbreviations used: ADER, alternate-day energy restriction; ADF, alternate-day fasting; CER, continuous energy restriction; CRP, C-reactive protein; CVD, cardiovascular disease; ER, energy restriction; IER, intermittent energy restriction; IF, intermittent fasting; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; MMTV, mouse mammary tumor virus; *mTOR*, mammalian target of rapamycin; NIA, National Institute on Aging; *p53*, tumor protein 53; ROS, reactive oxygen species; *sirt*, sirtuin.

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these findings may not apply to normal-weight animals or human subjects (BMI < 25), for whom ER may be ineffective or possibly detrimental.

The importance of the type of control in randomized studies of ER was demonstrated in 2 ongoing long-term primate studies. The Wisconsin National Primate Research Center study (9) indicated that a 20–25% daily or continuous energy restriction (CER) reduced diabetes and cardiovascular disease (CVD) compared with control animals consuming feed ad libitum (25% ER, $n = 38$; 2 CVD, 0 diabetes compared with controls, $n = 38$; 4 CVD, 16 diabetes). However, these diseases were not reduced in 20–25% CER-fed monkeys in a National Institute on Aging (NIA) study (10) compared with relatively lighter controls that received regulated rather than ad libitum portions of food (25% ER, $n = 40$; 3 CVD, 2 diabetes compared with controls, $n = 46$; 0 CVD, 5 diabetes). Thus, the data in the Wisconsin study suggested that ER reduced the risk of diabetes and CVD when it overcame the adverse effects of overnutrition and excess adiposity, but the data in the NIA study suggested that ER did not have these effects in lighter rhesus monkeys. Interestingly, the 20–25% CER led to comparable reductions in cancer rates in both studies. Cancer rates for ER and control in the Wisconsin National Primate Research Center study were 4 and 8, respectively; in the NIA study, rates were 0 and 6, respectively. Thus, a 25% CER had anticancer effects in lighter as well as heavier rhesus monkeys.

Most ER research has involved CER. Alternatives include intermittent energy restriction (IER) or intermittent fasting (IF), which comprises periods of marked ER or total fasting interspersed with periods of normal eating. These approaches recently have received a great deal of scientific and public interest (11). This increasingly popular dietary approach is the subject of many self-help books that claim that this pattern of eating is optimal for weight loss, reducing ill health, and promoting longevity. The attraction of IER above standard CER approaches is the assertion that IER can exert beneficial health effects when weight and total energy intake are maintained. These beneficial effects are claimed for normal-weight as well as overweight individuals. However, these claims for human health benefits are extrapolations of data from animal studies in which IER regimens often produced an overall ER, and reduced weight and adiposity compared with overweight controls who consumed food ad libitum.

The heightened scientific and public interest in IER and its adoption by numerous overweight and normal-weight subjects worldwide means existing data need to be summarized. Tannenbaum and Silverstone (12), early IER researchers, warned of the dangers "that research findings may be coupled with suggestions and guesses to build up concepts which by pyramided repetition become accepted."

This review article will summarize animal studies of tumor development with IER or IF compared with CER and their relative effects on key markers of tumorigenesis. The relevance of these animal data to human cancer is considered by summarizing available human studies of the effects of IER or IF compared with CER on cancer risk biomarkers in obese, overweight, and normal-weight subjects.

Current Status of Knowledge

Carlson and Hoetzel (13) first reported that IF in Wistar rats (no food 1 in 4 d, 1 in 3 d, or on alternate days, interspersed with days of normal eating) increased longevity by 15–20% and reduced mammary tumor growth by 65–90% compared with those consuming feed ad libitum. Reductions were proportional to the number of days of fasting per week and the amount of weight reduction. Several experimental intermittent feeding protocols in animals have been studied since then that included periods of IF (most commonly alternate days of total food deprivation) or IER (1–3 wk of 50–75% ER). The most-studied regimens in humans have been alternate-day fasting (ADF) or IER, with either 2 consecutive days/wk of ~65% ER, or alternate-day energy restriction (ADER), typically 75%. The term "intermittent fasting" is used in the literature to describe periods of either no intake (i.e., IF) or reduced intake (i.e., IER). However, there are potential different metabolic and biological responses between IF and IER. For example, there may be greater metabolic fluctuations during fasting periods and hyperphagia during nonrestricted periods with IF than with IER. We defined IF as periods of no intake and a complete ER, and IER as intermittent periods of reduced food intake and a partial ER. We will summarize data for IF and IER separately.

The review will address the following 4 key questions and highlight areas for further research: *1*) Do IER and IF bring about reductions in tumor rates when they achieve an overall ER or in the absence of an overall ER, and how does this compare with CER? *2*) Do IER and IF have beneficial effects on cancer risk biomarkers in humans when they achieve an overall ER or in the absence of an overall ER, and how does this compare with CER? *3*) Do IER and IF have cancer-protective effects in normal-weight as well as obese/overweight subjects? *4*) Are IER and IF safe, or could they have potential adverse effects in obese/overweight and normal-weight subjects?

The Effects of IF and IER on Tumors in Rodent Models

Spontaneous tumor models. A variety of IF regimens have been tested, ranging from alternate days of fasting to occasional periods of 5 d of fasting (**Table 1**). IF regimens reduced mammary tumor rates by 40–80% compared with ad libitum consumption (13, 15, 16). The antitumor effect of IF in these studies is proportional to the degree of overall ER and reduced body weight compared with the group consuming ad libitum. IF did not have antitumor effects on mammary (14) or prostate (21, 22) tumors when mice were allowed to overfeed on unrestricted days and their overall energy intake matched the energy intake of the group consuming feed ad libitum. The IF mice in one of these prostate tumor studies had higher serum insulin-like growth factor (IGF) I concentrations than did the mice consuming

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TABLE 1 Intermittent fasting and spontaneous tumor development in laboratory rodents[1]

Reference	Animal model, age at start of restriction, length of study, number of rodents	Study design and IF and AL feeding regimens	Final weight of IF rodents, % of weight of AL rodents	Overall ER with IF vs. AL, %	Tumor incidence or mortality, % of rodents	Outcomes
Mammary tumors						
Carlson and Hoetzel, 1946 (13)	Female Wistar Institute rats (OR), 6 wk old, over life span (98–104 wk), $n = 77$	3 different IF regimes vs. daily AL—IF: 1 in 4 d, 1 in 3 d, 1 in 2 d; AL: on nonrestricted days (AL diet: 46% fat, 26% carbohydrate, 27% protein)	IF: 1 in 4 d = 89, 1 in 3 d = 88, 1 in 2 d = 85	ND	AL: 37; IF: 1 in 4 d, 29; 1 in 3 d, 36; 1 in 2 d, 7 Rate of tumor growth (g/100 d)—AL: 134; IF: 1 in 4 d, 48; 1 in 3 d, 42; 1 in 2 d, 13	IF (1 in 2 d): 30% fewer tumors than AL ($P < 0.05$)
Tannenbaum and Silverstone, 1950 (14)	Female DBA inbred-strain mice (50% OR), mature (34 wk old), 110 wk, $n = 104$	IF vs. daily AL—IF: 2 separate days of food deprivation/wk (Monday and Thursday); AL: on nonrestricted days (AL diet: 6% fat, 75% carbohydrate, 19% protein)	93	4 (all nutrients)	AL: 80; IF, 89 Mean + SD age of onset—AL: 74.3 ± 3.1 wk; IF: 77.7 ± 2.4 wk	No difference between AL and IF
Shankaraiah et al., 1984 (15)	Female CH3/HE mice (OI), 5 wk old, over life span (median survival: 11–12 wk), $n = 96$	ADF vs. daily AL—IF: 1 in 2 d; AL: on nonrestricted days (AL diet: 6% fat, 75% carbohydrate, 19% protein)	93	ND	AL: 83; IF: 53	IF: 30% fewer tumors than AL ($P < 0.05$)
Chen et al., 1990 (16)	MMTV-induced tumors in female virgin CH3/0U mice (OI), 6–8 wk old, 84 wk, $n = 112$	IF vs. daily AL with high-fat, low-carbohydrate diet—IF: 2 periods of fasting/wk (1 × 2-d food deprivation, 2 d AL, 1 × 3-d food deprivation); AL: high-fat diet (68% fat, 0% carbohydrate, 32% protein)	Reported reduced weight, but percentage not specified	40 (all nutrients)	AL: 100 by 44 wk; IF: 12 by 88 wk; IF delayed tumors from 44 to 88 wk	IF (1 × 2 d, 1 × 3 d): 88% fewer tumors than high-fat, low-carbohydrate AL ($P < 0.05$)
Chen et al., 1990 (16)	MMTV-induced tumors in female virgin CH3/0U mice (OI), 6–8 wk old, 84 wk, $n = 112$	IF vs. daily AL with high-carbohydrate, low-fat diet—IF: 2 periods of fasting/wk (1 × 2-d food deprivation, 2 d AL, 1 × 3-d food deprivation); AL: low-fat diet (5% fat, 63% carbohydrate, 32% protein)	Reported reduced weight, but percentage not specified	32 (all nutrients)	AL: 100 by 44 wk; IF: 0 by 88 wk	IF (1 × 2 d, 1 × 3 d): 100% fewer tumors than high-carbohydrate, low-fat AL ($P < 0.05$)
Shao et al., 1990 (17)	Female CH3/BI mice, 16–20 wk old, 70 wk, $n = 60$	IF vs. daily AL with high-fat, low-carbohydrate diet—IF: 2 periods of fasting/wk (1 × 2-d food deprivation, 2 d AL, 1 × 3-d food deprivation); AL: high-fat diet (68% fat, 0% carbohydrate, 32% protein)	88	40 (all nutrients)	By 74 wk—AL: 69; IF: 17	IF (1 × 2 d, 1 × 3 d): 52% fewer tumors than high-fat, low-carbohydrate AL ($P < 0.05$)
Shao et al., 1990 (17)	Female CH3/BI mice, 16–20 wk old, 70 wk, $n = 60$	IF vs. daily AL with low-fat, high-carbohydrate diet—IF: 2 periods of fasting/wk (1 × 2-d food deprivation, 2 d AL, 1 × 3-d food deprivation); AL: low-fat diet (60% fat, 4.5% carbohydrate, 35.5% protein)	90	40 (all nutrients)	AL: 72; IF: 22	IF (1 × 2 d, 1 × 3 d): 50% fewer tumors than low-fat, high-carbohydrate AL ($P < 0.05$)

(Continued)

TABLE 1 (*Continued*)

Reference	Animal model, age at start of restriction, length of study, number of rodents	Study design and IF and AL feeding regimens	Final weight of IF rodents, % of weight of AL rodents	Overall ER with IF vs. AL, %	Tumor incidence or mortality, % of rodents	Outcomes
Siegel et al., 1988 (18)	Female Fischer rats inoculated with Mat 13762 rat mammary adenocarcinoma cells, 12–16 wk old, 11 d, $n = 30$	IF: 1 in 2 d vs. daily AL; initiated 7 d before inoculation	ND	ND	Mortality after 10 d—IF: 50; AL: 12 (host survival was 1–2 d longer with IF than AL, which represents a 50–75% inhibition of tumor growth)	IF (1 in 2 d): 38% fewer deaths than AL ($P < 0.025$)
Other tumors						
Berrigan et al., 2002 (19)	Male p53-deficient mice prone to lymphomas and sarcoma (model of Li Frameni syndrome), 28–40 wk old, 96 wk, $n = 94$	AL vs. IF 1 d/wk vs. 40% CER; AL diet: 11% fat, 68% carbohydrate, 21% protein	IF: 76; CER: 50	IF: 14 (all nutrients); CER: 40 [with reduced carbohydrate (50% restriction) = protein = fat]	Multiple tumor burden—AL: 40; IF: 26; CER: 25 (IF and CER not significantly lower) Longevity: AL = 313 d, IF = 357 d, CER = 383 d	IF and CER both delayed the onset of tumors and had 14% and 24% increased longevity, respectively, compared with AL ($P < 0.001$)
Chen et al., 2012 (20)	Female athymic BALB/c and beige-nude mice inoculated with human lung (A549), hepatic (HepG-2), and ovarian (SKOV-3) carcinoma cells, 6–8 wk old, 16 wk, $n = 48$	8 wk AL vs. 2 IF regimens (4 wk: 1 d food deprivation + 6 d AL; 4 wk: AL) (4 wk: 2 d food deprivation + 5 d AL; 4 wk AL); AL diet: 9% fat, 77% carbohydrate, 14% protein; diets initiated 4 wk after inoculation	IF: 1 or 2 d/wk = 100	IF: 1 d/wk, 103; 2 d/wk, 102	Mortality over 16 wk Lung—AL: 63; IF for 1 d: 31; IF for 2 d: 0 Hepatic—AL: 69; IF for 1 d: 38; IF for 2 d: 0 Ovarian—AL: 69; IF for 1 d: 38; IF for 2 d, 0	All tumors—IF (2 d): 63–69% decreased mortality at 16 wk vs. AL and 31–38% decreased mortality vs. IF 1 d ($P < 0.05$); reduced tumor growth via reduced IGF-I megakaryocyte growth and platelets and increased NK cell activity
Buschmeyer et al., 2010 (21)	Male SCID immunodeficient mice inoculated with LAPC-4 (androgen-sensitive human prostate cancer cells), 6 wk old, 4-wk diet intervention, $n = 105$	IF initiated once all tumors were larger than 200 mm^3; AL; IF [1 d food deprivation + 6 AL, 1 d food deprivation + 6 d PF (equal to AL group), 2 d food deprivation + 5 d AL, 2 d food deprivation + 5 d PF (equal to AL group)]; 28% CER; AL diet: 40% fat, 54% carbohydrate, 16% protein	IF (2 d food deprivation + 5 d PF): 92; CER: 83; other groups = 100	Intakes of all IF groups were the same or greater than intake of AL groups	Tumor growth >1500 mm^3 IF[2]: 1 d food deprivation + 6 d PF = 0.65 ($P = 0.26$); 2 d food deprivation + 5 d AL = 0.60 ($P = 0.18$); 2 d food deprivation + 5 d PF = 0.59 ($P = 0.16$) CER[2]: 7 d = 0.59 ($P = 0.17$)	Nonsignificant trend for reduced tumor growth with IF and CER vs. AL
Thomas et al., 2010 (22)	Male CB-17 immunodeficient mice inoculated with LAPC-4 (androgen-sensitive human prostate cancer cells), 8 wk old, 5-wk diet intervention, $n = 100$	IF initiated once all tumors were larger than 200 mm^3; AL; IF for 2 nonconsecutive days and 5 d AL; AL diet: 40% fat, 44% carbohydrate, 16% protein	IF: 100	IF intake was the same as AL intake	IF: tumor volume equal to AL ($P > 0.10$); survival equal to AL ($P = 0.37$)	IF had tumor rates equal to AL

[1] ADF, alternate-day fasting; AL, ad libitum feeding/fed; CER, continuous energy restriction; DBA, dilute brown nonagouti; ER, energy restriction; IF, intermittent fasting; IGF-I, insulin-like growth factor I; MMTV, mouse mammary tumor virus; ND, no data; OI, ovarian-independent animal model; OR, ovarian-responsive animal model; PF, pair-fed; p53, tumor protein 53; ref, reference; SCID, severe combined immunodeficiency.
[2] Values are HRs compared with AL.

feed ad libitum, but they did not have increased downstream protein kinase B signaling (22)

Berrigan et al. (19) reported that *p53*-deficient mice undertaking 1 d of food deprivation/wk (14% ER, 25% weight reduction) had reduced rates of neoplasms (mainly sarcoma) and an intermediate survival (355 d) that was less than those on a daily ER (40% ER, 50% weight reduction, 383 d survival) and greater than the group that consumed feed ad libitum (no change in weight, 313 d survival). Chen et al. (20) reported that 2 d of IF/wk and ad libitum eating for 5 d with no overall ER reduced the progression of lung, ovarian, and hepatic human xenografts in an immunocompromised mouse model (6- to 8-wk-old female athymic BALB/c and beige-nude mice). These reductions were associated with reduced IGF-I, megakaryocyte growth and platelet production, and increased natural killer activity. The relevance of this finding to human cancers is not known.

IER has been studied mainly in mouse models by Cleary et al. (23–28) at the University of Minnesota (**Table 2**). Mammary tumor studies tested cycles of 3 wk of 50% ER (mainly carbohydrate restriction) and 3 wk ad libitum consumption. Four studies in estrogen-responsive mouse mammary tumor virus (MMTV)–TGF-α mice all found IER to be superior to ad libitum consumption. IER was superior to isoenergetic CER in 3 of these studies (23–25), and equivalent in 1 study (26). Two additional studies were conducted in a MMTV human epidermal growth factor receptor 2 (HER2/neu) estrogen–unresponsive tumor model. One study found IER to be equivalent to CER, and both diets reduced tumor rates compared with an ad libitum diet (27). However, the second study, which used the same model, did not find significant differences in tumor rates between IER, CER, and ad libitum consumption (28).

Thus, an IER with 3 wk of alternate ER and ad libitum consumption may be equivalent or superior to an equivalent CER for overcoming the tumor-promoting effects of overnutrition in mice prone to developing estrogen receptor–positive MMTV-induced mammary tumors. The greater effects of IER compared with CER suggests that IER is exerting additional cancer-protective effects in addition to the effects of reduced weight. In contrast, the estrogen receptor–negative HER2/neu-positive tumor model appears less responsive to ER, with equivalent and modest effects of IER and CER. Ovarian cycling hormones were not assessed in these studies. Other investigators reported that both 25% CER and periods of 7 d of 50% ER could interrupt menstrual cycling in mice, resulting in significant reductions in estrogen (31, 32), which potentially accounts for the benefits of IER and CER in the estrogen-responsive mouse models.

The University of Minnesota group (29) also studied the effects of IER on the development of prostate cancer in a transgenic adenocarcinoma mouse prostate model. An IER regimen that involved 2 wk of 50% ER (mainly carbohydrate restriction) and 2 wk of controlled ad libitum consumption (an overall 25% ER) did not influence prostate cancer rates. However, IER increased time-to-tumor detection and survival compared with ad libitum consumption and an isoenergetic CER, along with associated greater reductions in serum IGF-I and leptin and higher serum adiponectin. A similar study of IER (1 wk of 50% ER and 1 wk of controlled ad libitum consumption) in LSL-KrasG12D/+; Pdx-1/Cre pancreatic cancer–prone mice reported fewer pancreatic lesions with IER than with isoenergetic CER and ad libitum feed consumption (30). The mechanism of this effect is not known, but it appears to be independent of IGF-I and the mammalian target of rapamycin (mTOR) pathway activity, which decreased in the CER but not in the IER group.

Carcinogen-induced tumor models. CER reduced tumor rates in a number of carcinogen-induced tumor models. In contrast, IER and IF appeared to be detrimental, and could increase tumor rates if they were commenced within 4 wk of carcinogen exposure, i.e., during the critical cancer-promotion stage (**Table 3**). IER did not have the cancer-protective effects of CER with carcinogen-induced mammary, hepatic, and colorectal tumors in rats (33, 37). Tagliaferro et al. (34) reported a 12% increased rate of mammary tumors in rats with IER compared with ad libitum feed consumption, despite an overall 14% ER compared with the group that consumed feed ad libitum. Likewise, IF increased tumor rates in rats compared with ad libitum feed consumption in carcinogen-induced models of colon (40) and liver (38) tumors. In contrast, introduction of IER and IF 4–8 wk after carcinogen exposure in rats reduced mammary carcinomas by 50% (35) and the development of preneoplastic liver lesions by 65% (39) compared with ad libitum feed consumption.

Summary of IF and IER in animal models. IF has been compared with ad libitum feeding in rodent models. IF reduced tumor rates and tumor growth mainly when there was an overall ER and reduced bodyweight. IF did not overcome the cancer-promoting effects of overnutrition in the majority of animal models when weight and overall energy intake were maintained.

IER regimens that included alternating periods of ER and ad libitum feed consumption for 1, 2 or 3 wk have been reported to be superior to CER in overcoming the tumor-promoting effects of overnutrition in some but not all animal tumor models. The greater cancer-protective effects of IER compared with CER suggest that IER exerted additional effects on these reduced-weight animals; hence, there are potential benefits for IER in normal-weight animals and subjects. IER and IF initiated at the time of carcinogen administration was not effective, whereas it was effective if given ≥4 wk after administration of the carcinogen. The relevance of carcinogen-induced tumors to the human situation is not clear, but it indicates that IER and IF regimens may not offer cancer protection in all situations.

Mechanistic Animal Studies of IF and IER

Cell proliferation. Reduced proliferation in epithelial cells could reduce cancer initiation and the subsequent promotion

TABLE 2 IER and spontaneous tumor development in laboratory rodents[1]

Study (ref)	Animal model, age at restriction, length of study, number of rodents	Study design and IER regimen	Final weight of IER rodents, % of weight of AL rodents	Final fat pad weight vs. rodents fed AL diet, %	Overall ER vs. AL group, %	Tumor incidence, % of rodents
Cleary et al., 2002 (23)	MMTV–TGF-α heterozygous Lep+lep^{ob} mice (OR), 10 wk old, 80 wk, n = 93	AL vs. IER vs. PF CER; IER: 3 wk 50% restriction, 3 wk AL	IER: 93 (assessed 1 wk into refeeding phase); CER: 92	IER: 93; CER: 90	IER: 20; CER: 20	AL: 77; IER: 3; CER: 44 (IER group had 74% fewer tumors than AL group and 41% fewer than CER group, $P < 0.05$)
Cleary et al., 2007 (24)	MMTV–TGF-α heterozygous Lep+lep^{ob} mice (OR), 10 wk old, 79–80 wk, n = 100	AL vs. IER vs. PF CER; IER: 3 wk 50% restriction, 3 wk AL	IER: 80 (restricted phase), 104 (refed phase); CER: 84	IER: 76 (restricted phase), 127 (refed phase); CER: 88	IER: 12; CER: 15	AL: 84; IER: 15; CER: 27 (IER group had 69% fewer tumors than AL group, $P < 0.05$)
Rogozina et al., 2009 (25)	MMTV–TGF-α heterozygous Lep+lep^{ob} mice (OR), 10 wk old, 79–82 wk, n = 225	AL vs. IER vs. PF CER; IER: 3 wk 50% restriction, 3 wk AL	IER: 71 (restricted phase), 81 (refed phase); CER: 80	IER: 20 (restricted phase); 45 (refed phase); CER: 40	IER: 25; CER: 27	AL: 71; IER: 9; CER: 35 (IER group had 62% fewer tumors than AL group, $P < 0.05$)
Dogan et al., 2010 (26)	MMTV–TGF-α Lep+lep^{ob} mice (OR); 10 wk old; mice killed at 13, 25, 37, 55, and 73 wk; n = 135	AL vs. IER vs. 25% CER; IER: 3 wk 50% restriction, 3 wk AL	At 73 wk—IER: 63 (restricted phase), 74 (refed phase); CER: 70	IER: 29 (restricted phase), 50 (refed phase); CER: 32	ND	AL: 45; IER: 11.5; CER: 20 (IER group had 33% fewer tumors than AL group, $P < 0.05$, and did not differ from CER group, $P > 0.05$)
Pape-Ansorge et al., 2002 (28)	MMTV–TGF-neu overexpress heterozygous HER2/neu estrogen-negative tumors (OI), 9 wk old, 80 wk, n = 96	AL vs. IER vs. PF CER; IER: 3 wk 50% restriction, 3 wk AL	IER: 89 (assessed 2 wk into refeeding phase); CER: 80	IER: 55 (assessed 2 wk into refeeding phase); CER: 47	IER: 10; CER: 16	AL: 37.5; IER: 22.5; CER: 33 (no statistical difference between groups)
Mizuno et al., 2013 (27)	MMTV-Her2/neu mice (OI), 8 wk old, 60 wk, n = 95	AL vs. IER vs. 25% CER; IER: 3 wk 50% restriction, 3 wk AL	IER: 70 (assessed at end of refeeding phase); CER: 73	ND	IER: 25; CER: 25	AL: 87; IER: 51; CER: 47 (IER and CER groups had fewer tumors than AL group, $P < 0.05$) Tumor-free survival—AL: 46.1 wk; IER: 49.1 wk; CER: 52.4 wk (CER group had delayed tumors vs. AL and IER groups, $P < 0.05$)
Bonordon et al., 2009 (29)	Prostate TRAMP, 5 wk old, 48 wk, n = 220	AL vs. IER vs. 25% CER; IER: 2 wk 50% restriction, 2 wk controlled refeeding	IER: 85; CER: 85	ND	IER: 25; CER: 25	AL: 95; IER: 91; CER: 95 (no difference between groups) Median survival—AL: 41 wk; IER: 46 wk; CER: 40 wk (IER group survived longer than AL group, $P > 0.05$) Time to tumor detection—AL: 33 wk; IER: 38 wk; CER: 35 wk (IER group had longer time to tumor detection than AL group, $P > 0.05$)
Lanza-Jacoby et al., 2014 (30)	Pancreatic LSL-KrasG12D/+ mice; Pdx-1/Cre, 6 wk old, 44 wk, n = 93	AL vs. IER vs. 25% CER; IER: 1 wk 50% restriction, 1 wk controlled refeeding	IER: 73: CER: 70	ND	IER: 25; CER: 25	PanIN-2 or greater lesion—AL: 70; IER: 27; CER: 40 (IER group had 43% fewer lesions than AL group and 13% fewer than CER group; IER < AL and CER, $P < 0.05$)

[1] Diet on nonrestricted days consisted of an ad libitum AIN-93M diet with 9% energy as fat, 77% carbohydrate, and 14% protein. Diet on restricted days consisted of a 50% ER diet achieved by reducing carbohydrate intake with equivalent fat and protein intakes in the ad libitum diet. Restricted days had a 65% reduction in carbohydrate compared with the ad libitum diet. AL, ad libitum feeding/fed; CER, continuous energy restriction; ER, energy restriction; IER, intermittent energy restriction; Lep, leptin; MMTV, mouse mammary tumor virus; ND, no data; Ob, obese; OI, ovarian-independent animal model; OR, ovarian-responsive animal model; PanIN, pancreatic intraepithelial neoplasia; PF, pair-fed; ref, reference; TRAMP, transgenic adenocarcinoma mouse prostate.

TABLE 3 IER, IF, and carcinogen-induced tumor development[1]

Study (ref)	Rat model, carcinogen, age of rats, length of study, number of rats	Study design and timing of IER/IF after initiation	IER/IF regimen	Diet composition on nonrestricted days	Diet composition on restricted days	Final body weight vs. rats fed AL diet, %	Final body fat stores vs. rats fed AL diet, %	Overall ER vs. AL group, %	Tumor incidence[2]
Mammary tumors									
Mehta et al., 1993 (33)	Virgin Sprague Dawley rats (OR), DMBA, 8 wk old, 10 wk post-DMBA, $n = 90$	AL vs. IER vs. CER, 40%; 1 wk post-DMBA	IER: 2 d AL, 2 d 40% ER	AL: 33% fat, 47% carbohydrate, 20% protein	Restrictions: 40% ER, 56% carbohydrate, 26% fat, 20% protein	IER: 85; CER: 72	ND	IER: 20; CER: 40	AL: 63; IER: 57; CER: 27 (CER rats had 40% fewer tumors than AL rats and 30% fewer than IER rats, $P < 0.05$)
Tagliaferro et al., 1996 (34)	Virgin Sprague Dawley rats (OR), MNU, 8 wk old, 18 wk post-MNU, $n = 159$	AL vs. IER; 11 d post-MNU	IER: 1 wk 33% restriction, 3 wk 107% AL	AL: 46% fat, 20% protein, 34% carbohydrate	33% restriction in energy, carbohydrate, fat, protein	90	73	14	AL: 54; IER: 66 (IER rats had 12% more tumors than AL rats, $P < 0.0001$)
Buison et al., 2005 (35)	Wistar Institute rats (OR), DMBA, 8 wk old, 50 wk, $n = 90$	AL vs. IER; 8 wk post-DMBA	4 IER cycles: 50% intake for 4–8 wk to lose 20% of weight; AL for 3 wk to regain weight	AL: 60% fat, 15% protein, 24% carbohydrate	IER: 50% ER, 50% fat restriction, 75% carbohydrate restriction	87	75	ND	AL: 17.6; IER: 8.8 Tumor burden (g/rat)—AL: 0.38 ± 0.88; IER: 0.08 ± 0.29 (no difference between groups)
Tessitore, 1998 (36)	Female Sprague Dawley rats, MNU, 7 wk old, 42 wk, $n = 30$	IF vs. AL; 1 wk post-MNU	IF: 3 cycles of 3 d food deprivation and 10 d AL	ND	NA	100	ND	ND	Number of malignant mammary tumors per rat—IF: 29; AL: 15
Hepatic tumors									
Hikita et al., 1997 (37)	Female Sprague Dawley rats, DENA, 7 wk old, 20 wk, $n = 30$	IF vs. AL; days 16–21 and 23–28 post-DENA	IF: 2 × 5 d food deprivation	13% fat, 29% protein, 56% carbohydrate	NA	100	ND	ND	Number of AHF—AL: 22,162 ± 6253; IF: 30,108 ± 14,512 (no significant difference between groups) AHF comprised a greater % of liver volume in IF (2.5 ± 1.2) vs. AL (1.2 ± 0.36) ($P = 0.05$)
Tomasi et al., 1999 (38)	Male Fischer 344 rats, DENA, 8 wk old, 52 wk, $n = 22$	IF vs. AL; 1 wk post-DENA	IF: 3 cycles of 3 d food deprivation and 11 d AL	13% fat, 29% protein, 56% carbohydrate	NA	99	ND	91	IF: 72; AL: 36 (IF group had 2-fold higher rates than rats fed the AL diet)
Rocha et al., 2002 (39)	Adult male Wistar rats, DENA, 10 wk old, 52 wk, $n = 36$	IF vs. AL; 4 wk post-DENA	IF: 48 h food deprivation/wk and 5 d AL for 4 wk for 48 wk	ND	NA	75	ND	ND	Glutathione *S*-transferase–positive liver foci area (mm^2/cm^2)—IF: 0.36 ± 0.51; AL: 0.95 ± 0.49 (IF rats had fewer liver foci than AL rats, $P < 0.05$)

(Continued)

TABLE 3 (*Continued*)

Study (ref)	Rat model, carcinogen, age of rats, length of study, number of rats	Study design and timing of IER/IF after initiation	IER/IF regimen	Diet composition on nonrestricted days	Diet composition on restricted days	Final body weight vs. rats fed AL diet, %	Final body fat stores vs. rats fed AL diet, %	Overall ER vs. AL group, %	Tumor incidence[2]
Colorectal tumors									
Caderni et al., 2002 (40)	Male Fischer 344 rats, AOM, 8 wk old, 13 wk, $n = 44$	IF vs. AL; 10 d post-AOM	IF: 5 cycles of 4 d food deprivation and 7–10 d AL	11% fat, 15% protein, 74% carbohydrate	NA	90	ND	0	Aberrant crypt foci/rat—IF: 4.3 ± 1.3; AL: 2.4 ± 0.4 (IF rats had more aberrant crypt foci than AL rats, $P < 0.005$)

[1] AHF, altered hepatic foci; AL, ad libitum feeding/fed; AOM, azoxymethane; CER, continuous energy restriction; DENA, diethylnitrosamine; DMBA, 7,12-dimethylbenz[a]anthracene; ER, energy restriction; IER, intermittent energy restriction; IF, intermittent fasting; MNU, methylnitrosourea; NA, not applicable; ND, no data; OR, ovarian-responsive animal model; ref, reference.
[2] Values are % of rats unless otherwise indicated.

of initiated tumor cells. A continuous ER of 25–30% has elicited marked reductions in mammary epithelial cell proliferation in mice (−70% to −90%), which is not seen with smaller daily restrictions, e.g., a 5% CER (31, 41). Mammary and prostate cell proliferation has been reduced with ADF or a sufficiently restricted ADER regimen (>85% restriction on restricted days). Mammary cell proliferation was reduced with both an 85% ADER (−60%) and an ADF (−65%) compared with ad libitum consumption, but not with a 75% ADER. Interestingly, reductions in proliferation with these regimens were comparable with reductions achieved with a 25% CER (−70%), but they were achieved without imposing an overall ER and without reducing body weight (31). Similarly, reductions in prostate cell proliferation were reported with an 85% ADER (−47%) (42) or ADF (−57%), but were not seen with a 50% ADER (43).

The reduced cell proliferation rates in these studies were reported on the morning immediately after the hyperphagic ad libitum day of ADER or ADF. This suggests that IER has a sustained effect on proliferation during both restricted and ad libitum days, provided that there is a sufficiently severe restriction on restricted days. However, IER and IF animals consume their daily energy intake within a few hours on feasting days, creating a greater self-imposed period of no food intake before the measurements, which may account for some of the reductions in proliferation observed.

CER decreases mammary cell proliferation in rodents, largely by loss of estrous cycle, reductions in reproductive hormone concentrations, and reduced IGF-I concentrations. Estrous cycles were unaffected in mice undergoing ADER or ADF (41). Reduced mammary and prostate cell proliferation in these studies has occurred alongside reduced serum IGF-I concentrations. The relevance of these data to the human situation is not known, because the effects of IER and IF on human IGF-I activity are not well characterized (see IGF-I, insulin, and insulin sensitivity section). Currently, to our knowledge, there are no human data on the effects of IER, IF, and CER on cell proliferation.

Stress resistance. ER is thought to reduce the risk of cancers and other diseases in part through hormesis, whereby ER acts as a low-intensity stressor that elicits cytoprotective effects via adaptive upregulation of cellular stress resistance pathways (44). These pathways include upregulation of kinases and deacetylases, including sirtuins, protein chaperones that coordinate protein synthesis, folding, disaggregation, and degradation (45); antioxidants; enzymes; and autophagy (44). In rats, ADF has been shown to be an effective form of ER in reducing tissue damage in the brain and heart compared with ad libitum consumption (46), and has been found to be superior to CER in protecting hippocampal neurons against excitotoxic injury (47).

Autophagy. Autophagy is reported to be transiently upregulated during the first 24 h of fasting in rodent liver, muscle, kidney, and heart, partly in response to increased ketones

(48). The effects of fasting on autophagy within the wider range of target tissues in humans affected by cancer have not been studied. Furthermore, the role of autophagy in the development of human cancers in different tissues is complex and not well defined (49).

Oxidative stress and antioxidant activity. Oxidative stress is linked to the development of cancer and accelerated aging, with the prevailing hypothesis being that reactive oxygen species (ROS) production should be limited to reduce cellular damage. A recent paradigm shift has highlighted the fact that ROS production may be required to evoke an obligatory mild cellular stress response, which in turn upregulates antioxidant pathways and lowers overall long-term oxidative stress (50). Thus, changes in antioxidant enzyme activity with IER or IF, especially when animals have adapted to increase their enzyme activity, may provide a more relevant marker of their impact on disease risk than ROS production per se.

In male Sprague Dawley rats, 4 wk of ADF with alternating 24 h of total food deprivation and 24 h of hyperphagia (150% ad libitum intake) and 14% overall ER did not affect antioxidant enzyme activity (glutathione peroxidase, glutathione reductase, or catalase) in the heart or liver after hyperphagic feed days, but led to decreased activity of catalase in the brain and glutathione peroxidase in muscle compared with rats that consumed an ad libitum diet (51). These rats experienced increased concentrations of some (carbonyls) but not all (malondialdehyde and protein nitration) oxidative damage markers in the brain and liver (51). An earlier study from this group, however, reported significant increases in antioxidant enzyme activity in muscle and adipose tissue after a longer-term 32-wk exposure to IF (measured after feed days) compared with isoenergetic ad libitum feed consumption (52). Descamps et al. (53) reported that 16 wk of ADF in mice increased superoxide dismutase activity in the brain, spleen, and mitochondria, but reduced superoxide dismutase activity in the liver compared with isoenergetic ad libitum feed consumption. Thus, IF appeared to have variable effects on antioxidant capacity in different tissues. Enzyme activity may increase with longer exposure to IF as a long term adaptation in response to the initial increase in oxidative stress with IF.

The effect of changes in antioxidant enzyme activities on the actual development of cancer is unclear. Increased antioxidant enzyme activity, along with reduced ROS production, in IF mice compared with those consuming ad libitum feed translated to reduced lymphoma incidence (0% for IF compared with 33% for controls that consumed ad libitum feed) (53). However, Uhley et al. (54) reported that 28 wk of a 20% CER in rats reduced mammary gland oxidative DNA damage (5-hydroxymethyl-2′-deoxyuridine) by 25% compared with ad libitum consumption, whereas an IER that was isoenergetic to the CER group (5 cycles of 6 wk of 50% IER and 2 wk of catch-up hyperphagia at 150% ad libitum intake) increased DNA damage by 30%. Thus, there is a potential for adverse effects with IER. Weight-loss trials of IER compared with CER in overweight/obese premenopausal women have shown inconsistent effects on advanced oxidative protein products. One study reported comparable 20% reductions with both IER and CER (55). A second study reported no change in advanced oxidative protein products with either approach (56).

Problems investigating IER in animal models and their relevance to human cancers. The most compelling data to support specific reductions in tumors with IER are rodent studies, which have reported reduced tumor rates compared with rates in continuously fed animals, despite apparently comparable body weights and energy intake (23, 29, 30). However, comparable-weight, IER, IF, and continuously fed animals could have different amounts and distribution of body fat, which are not often measured. Many animal studies are likely to be underpowered to assess modest differences in energy intake that may exist between IER and CER groups.

The adverse effects of IER and IF in some animal models may be the result of hyperphagia on nonrestricted days. Alternatively, periods of fasting with IF or energy or carbohydrate restriction with IER evoke surges in lipolysis and fat oxidation and increases in circulating FFAs and ketone bodies, which could be detrimental. Increased FFAs (57) and ketones (58) have been linked to the growth of certain cancers. Fasting for 1–7 d increased circulating FFAs 5- to 7-fold and ketone bodies 20-fold, which was associated with the growth of Walker carcinoma 256 and Jensen sarcoma in rats (59).

These potential adverse effects of fasting and ER in animal models are important to consider, but may not be an issue for humans. In contrast with animal studies, compensatory overfeeding is not seen in human studies. IER (2 consecutive d/wk) led to a 20–30% ER and not hyperphagia on unrestricted days in studies of overweight and obese humans (56). Likewise, ADER was associated with a 5% ER on unrestricted days in obese subjects (60).

The high fluxes in circulating FFAs and ketone bodies linked to reduced growth hormone production seen with fasting and ER in rodents are not seen in humans, particularly not in obese subjects who have reduced growth hormone production compared with lean subjects. (61). A 36-h total fast in obese and lean subjects increased circulating FFAs by 1.7- and 2.4-fold, respectively, and ketone bodies by 6- and 18-fold, respectively. Fasting induces more rapid rises in FFAs and ketones in women than in men (62). IER is likely to evoke a much smaller flux in FFAs and ketones than is IF (63). In our own studies, IER (2 d of 75% ER) led to a small (20%) increase in serum ketones and a 10–300% increase in concentrations of individual FFAs on the morning after the 2 restricted days (55, 56).

Studies of IER and IF in Obese and Overweight Humans

There are no data, to our knowledge, on the effects of IER and IF on cancer rates in humans. Here, we summarize available human data comparing the effects of IER and

IF with CER on cancer risk biomarkers that are thought to mediate the links between adiposity and energy intake and the development and growth of cancers, including insulin, IGF-I, leptin, adiponectin, cytokines, and inflammation-related molecules (64). Because many biomarkers are likely to have marked acute changes during restricted and feeding days of the IER, we have only reported this data when the day of measurement (feeding or fasting) has been specified, thus providing an accurate description of the overall metabolic effects of the IER and IF regimens. Findings are reported separately for obese and overweight subjects and for normal-weight subjects.

The effect of IER and IF on metabolic cancer risk markers

IGF-I, insulin, and insulin sensitivity. Marked reductions in serum IGF-I are thought to mediate the cancer-protective effects of CER, IER, and IF in rodent studies. In contrast, circulating concentrations of total IGF-I and bioactive IGF-I [determined by insulin-like growth factor binding protein (IGFBP) 1, IGFBP-2, and IGFBP-3] appear to be poor markers of the effects of ER and weight loss in humans. Serum IGF-I often increases alongside weight loss, ER, and exercise (65), and is inversely linked to general adiposity and hepatic fat (66). Serum IGF-I concentrations do not relate well to IGF-I bioactivity within tissues, which is notoriously difficult to assess in humans (67).

For completeness, we present data on the relative effects of IER, IF, and CER on circulating total and bioavailable IGF-I. We reported no change in circulating total IGF-I concentrations along with weight loss with IER or CER in either of our studies (55, 56). IER and CER both increased IGFBP-1 (26% and 28%, respectively) and IGFBP-2 (22% and 36%, respectively), but did not change serum bioavailable IGF-I (ultrafiltered) when measured after feed days. There was a further acute 17% increase in IGFBP-2 on the morning after the 2 restricted days of a 70% ER, but no measurable changes in total or serum bioavailable IGF-I (ultrafiltered) (55). Rasmussen et al. (68) previously reported that 4 d of 80% ER brought about acute reductions in serum free IGF-I (−48% assessed with a noncompetitive immunoradiometric assay) mainly via increases in IGFBP-2, as well as increases in the acid labile subunit. The overall effect of IER or IF on IGF-I bioactivity across feed and fast days has not been assessed.

Reduced insulin receptor activity is considered to be as important as or more important than IGF-I receptor activity in preventing cancers in humans (69). Continuous ER and weight loss are well known to reduce serum insulin and improve insulin sensitivity (70). A key question is whether IER may lead to greater improvements in insulin sensitivity than CER for an equivalent weight loss or overall ER. The greater nadir of ER possible with periods of IER, typically 50–75% compared with 25% with CER, specifically may reduce hepatic and visceral fat stores (70) and fat cell size (71), alter insulin receptor affinity (72), and elicit hormetic effects (44) or greater metabolic flexibility (73).

Our initial randomized trial compared IER (2 consecutive days, 70% ER/wk) to an isoenergetic CER (n = 105; 25% daily ER Mediterranean-type diet: 6.8 MJ/d) in overweight and obese healthy women. IER led to comparable reductions in body fat compared with CER over 6 mo [mean (SD) IER, −6.4 ± 1.5 kg; CER, 5.6 ± 1.3 kg; P = 0.34] (49). However, IER led to greater reductions in insulin resistance (HOMA-IR) than did CER [difference −23% (95% CI: −38.1%, −8.6%); P = 0.001] when measured during feed days. Our follow-up study reported that both an intermittent energy and carbohydrate restriction (IECR: 60% ER, 40 g carbohydrate, 3.39 MJ/d) and a less-restrictive intermittent low-carbohydrate diet allowing ad libitum protein and MUFAs [IECR with ad libitum protein and fat (IECR+PF): 4.78 MJ, 40 g carbohydrate/d] led to equivalent fat loss (−3.7 kg), both of which were 1.8-fold greater than that with CER. Reductions in insulin and insulin resistance occurred in both IER groups when measured after a feed day [IECR, −22% (95% CI: −35%, −11%); IECR+PF, −14% (95% CI: −27%, −5%) compared with CER, −4% (95% CI: −16%, 9%)]. The IER groups experienced a further 25% reduction in insulin resistance when measured immediately after restricted days.

Adiponectin and leptin. Leptin and adiponectin are produced by adipose tissue. Increasing adiposity increases leptin and lowers adiponectin. The resulting adiposity-related imbalance of leptin and adiponectin may have a role in cancer development and progression via the effects on insulin sensitivity and inflammation, and the direct effects on cell proliferation and apoptosis (64).

In overweight humans, CER only increases adiponectin with large reductions in weight (>10%) (74). Our IER group had a nonsignificant increase in adiponectin (10%, after feeding days) in association with a 7% weight loss, but there was no change with CER despite a comparable weight loss (P = 0.08) (55). Our follow-up IER study reported no change in adiponectin with IER (7% weight loss) and CER (4% weight loss) (56). Ten weeks of ADER (alternate days of 75% ER and ad libitum Mediterranean diet) led to a 30% increase in plasma adiponectin in obese subjects when measured after both restricted and feeding days, along with a 4% weight loss (75). Both of our IER studies reported large comparable reductions in leptin (40%) and the leptin-to-adiponectin ratio with IER and CER (55, 56).

Inflammatory markers. Weight loss with CER reduces circulating concentrations of C-reactive protein (CRP) by 2–3% for every 1% weight loss, whereas TNF-α and IL-6 are reduced by ~1–2% per 1% weight loss (65). Reductions in inflammatory markers with IER align with this and appear to be comparable with CER for a given weight loss (55, 56). Twelve weeks of ADER (alternate days of 75% ER and an ad libitum Mediterranean diet) reduced weight by 4%, but did not reduce CRP in obese subjects (75). Eight weeks of a

similar regimen tested in 10 obese subjects with asthma did not reduce CRP, but reduced TNF-α by 70% during both restricted and feeding days after 8% weight loss (76).

Summary for weight and biomarkers in overweight and obese subjects

The limited biomarker data show that IER and CER lead to comparable reductions in adipokines and inflammatory markers, and minor changes in the IGF axis. The greater reported improvements in insulin sensitivity with IER compared with CER have been based on HOMA-IR which suggests greater improvements in hepatic insulin sensitivity. These findings need to be verified with the use of robust methodologies, e.g., insulin clamp or other techniques.

Studies of IER, IF, and CER in cohorts of normal-weight and overweight humans

There are few data, to our knowledge, on the effects of IER and IF in a truly normal-weight population (i.e., BMI < 25 kg/m^2) (77). A number of studies (77–79, 84, 86, 87) have assessed the effects of IER, IF in cohorts that include both overweight and normal-weight subjects with variable results on markers of metabolism and cancer risk, but, to our knowledge, none of these studies have reported direct comparisons between IER or IF and CER.

Some IF and IER studies have imposed hyperphagia during ad libitum days to provide proof of principle of the effects of IF or IER without an overall ER (77–79, 84). Three short-term IF studies (2–3 wk) have assessed the effects of alternate days of a total 20–36 h fast interspersed with periods of hyperphagia (175–200% normal intake) (77–79). These studies have reported variable effects on insulin sensitivity after feasting days of the regimen, which was improved when measured by Halberg et al. (78) in normal-weight and overweight men, but was not replicated by Soeters et al. (77) in a population of leaner normal-weight men. Heilbronn et al. (79) reported impaired glucose uptake on the morning after fasting days in women but not men. This indicates some peripheral insulin resistance in women (80), most likely secondary to greater fluxes of FFAs after fasting days in women than in men (81). This is likely to be a benign observation and a normal adaptation to fasting that preserves lean body mass (82).

A potential beneficial effect observed in these studies includes increased *sirtuin (sirt) 1* gene expression in muscle (measured after a feasting day) (79). This promotes resistance to oxidative stress in animal models, although the role in human cancer is not resolved (83). An adverse effect was the tendency to reduce the number of mitochondria per cell in skeletal muscle when measured after feasting days of IF (79).

Wegman et al. (84) recently reported the effects of 3 wk of an IER with alternate days of 75% ER interspersed with days of 175% of normal intake in normal-weight and overweight subjects with and without an antioxidant supplement. Assessments immediately after fasting days (18 h after the last meal) showed reduced plasma insulin (–1.01 μU/mL). Gene expression changes in peripheral blood mononuclear cells in this study showed a tendency for increased expression of *sirt 3* (P = 0.08), but no changes in the expression of oxidative stress genes (84). Interestingly, the beneficial effects of IER reported in this study were abrogated when IER included an antioxidant supplement, which suggests that ROS production may be important in improving insulin resistance in association with IER. Similarly, antioxidants have been shown to blunt the insulin-sensitizing effects of exercise in normal-weight humans (85).

Other studies have tested the effects of IER in free-living normal-weight and overweight individuals without stipulating hyperphagia on feed days, thus achieving an overall reduction in energy intake. Varady et al. (86) tested a 12-wk ADER (75% ER on restricted days; n = 15) compared with no intervention controls (n = 15) in men and women. This IER had an overall 30% ER, which led to reductions in weight (−6%), body fat (−14%), leptin (−40%), and CRP (−50%), and increased adiponectin (+6%). Brandhorst et al. (87) recently reported the 3-mo pilot data of an IER that involved 5 d/mo of a low-protein ER (46–66% ER providing ~0.25 g protein/kg weight during restricted days) interspersed with normal intake for the remaining 25 d of the month. The diet was tested in 23 normal-weight and overweight subjects (BMI > 18.5 kg). Assessments at 3 mo, taken after 5 d of normal eating in 19 subjects who completed the study (82% of cohort) showed modest reductions in body weight (−2%), trunk fat (−3% by DXA), serum IGF-I (−15%), and glucose (−5.9%). These preliminary data show a potential for different formats for intermittent diets, although there are insufficient details of uptake to the study, adherence to IER, and intake on the nonrestricted days to inform the likely successful application of this eating pattern in the wider population.

Thus, short-term studies have demonstrated some potential, albeit not consistent benefits of IF and IER in groups of normal-weight and overweight subjects, some in the absence of an overall ER. One study conducted in a truly normal-weight group (77), however, did not find statistical differences in insulin sensitivity, and reported reduced resting energy expenditure and lowered skeletal muscle mTOR phosphorylation, which could reflect decreased skeletal muscle protein synthesis. Thus, ADF has the potential to reduce lean body mass and lead to unwanted gains in body fat and the associated detrimental effects in normal-weight subjects.

Is there an optimal pattern of restriction and macronutrient composition for IER and IF regimens?

The optimum duration, frequency, and severity of ER needs to strike a pragmatic balance between being achievable and delivering beneficial physiologic effects. There are numerous potential permutations of IER and IF that could be studied. IER is likely to be preferable to IF regimens in humans. Aside from a presumed greater compliance, IER regimens that provide 2496 kJ and 50 g protein on restricted days will help maintain nitrogen balance and muscle mass, which may not be achieved with periods of total fasting (88). IER will evoke a smaller flux in FFAs and ketones than IF (63), which has been linked to short-term impaired glucose tolerance with the resumption of normal feeding. The

longer-term implications of short-term impairments in glucose tolerance with repeated IF each week is not known.

An important question is whether the reported reduced tumor rates with IER are linked to periods of ER regardless of macronutrient intake, or whether they are specifically linked to intermittent reductions in carbohydrate, protein, or fat intake. Most animal studies of IF have reduced overall energy intake with equal reductions in all macronutrients. In contrast, the IER studies have maintained protein and fat content and reduced energy intake through lowering carbohydrate. Thus, the reduced rates of mammary (89), prostate (29, 89), and pancreatic (30) tumors and lymphomas (35) with IER have occurred with intermittent periods of 50% ER and a 75% restriction in carbohydrate. IER-fed animals in these studies have had an overall 10–25% ER and 35% reduction in carbohydrates compared with animals consuming an ad libitum diet.

Dietary protein has variable effects on tumor development within different animal models. Many rodent mammary tumor studies have reported reduced tumor rates with ER that has been achieved with reduced carbohydrate or fat alongside maintained or increased protein intakes (91–93). However, Fontana et al. (94) reported a 56–70% inhibition in tumor growth with a 7% protein diet compared with an isocaloric 21% protein diet in a WHIM16 breast- and castrate-resistant LuCaP23.1 prostate cancer model linked to reduced IGF/protein kinase B/mTOR pathway activity and altered epigenetic effects. The optimal protein intake to prevent cancer and optimize health in humans needs careful consideration. On a pragmatic note, compliance with the energy-restricted days of IER is likely to be increased with adequate protein, which prevents hyperphagia (95). Minimum protein requirements for health and to maintain adequate lean body mass from the overall diet are estimated to be 0.8 g good quality protein · kg body weight^{-1} · d^{-1} for normal-weight adults, with higher recommended amounts of ~1.2 g protein/kg body weight for older subjects, subjects with sarcopenia, and weight-losing subjects (96, 97).

IER studies have recommended healthy eating and not feasting on nonrestricted days. Typically, IER regimens tested in overweight and obese subjects result in an overall 30% ER. Feasting on nonrestricted days may offset some beneficial health effects of weight loss with IER. For example, a high-fat ADER (45% fat on feast days) produced weight loss that was equivalent to that of a low-fat ADER

TABLE 4 Comparative effects of IER or IF and CER on cancer-protective mechanisms in mouse and human studies[1]

	IER/IF and CER regimen effects		
		Human studies	
Cancer-protective mechanism	Study focus and murine model	Obese/overweight women [BMI (in kg/m^2) ≥ 25]: 6 mo IER, 2 d 70% ER, and 5 d normal diet; 25% overall ER (55)	Normal-weight M and F (BMI <25)
Reduced cell proliferation	Mammary epithelial cell proliferation in C57BL/6J female mice—IER (alternate days of 85% ER and AL; no overall ER): IER-fed mice showed reductions in proliferation on feeding days comparable to 25% CER–fed mice (31)	NCD	NCD
Reduced oxidative stress	Oxidative DNA damage in mammary epithelial cells in Wistar female rats—IER (6 wk 50% ER and 2 wk refeeding with AL; 30% overall ER): IER-fed rats showed increased oxidative DNA damage vs. 20% CER–fed group and group consuming food ad libitum (54)	Serum advanced oxidative protein products: the IER group showed reductions comparable with a 25% CER group on both restricted and AL days	NCD
Reduced IGF-I activity	Serum IGF-I: MMTV–TGF-α mice—IER (3 wk 50% ER and 3 wk AL; overall 12% ER): IER-fed mice showed reduced serum IGF-I on restricted days vs. a 15% CER group (101)	Serum total IGF-I and bioavailable IGF-I (ultrafiltered): the IER group showed concentrations comparable with a 25% CER group on both restricted and AL days; serum IGFBP-1 and IGFBP-2: IER showed higher concentrations on restricted days than 25% CER	NCD
Increased insulin sensitivity	NCD	HOMA-IR insulin sensitivity: IER, measured after an AL day, was 23% lower than that for 25% CER, with a further 25% reduction after the restricted days	NCD
Improved adipokine profile	Plasma adiponectin:leptin ratio: MMTV–TGF-α mice—IER (3 wk 50% ER and 3 wk AL; overall 12% ER): IER-fed mice showed a lower adiponectin:leptin ratio on both restricted and feeding days vs. a 25% CER–fed group (26)	Plasma adiponectin:leptin ratio: the IER group showed a ratio comparable to a 25% CER group on both restricted and feeding days	NCD
Reduced inflammation	NCD	Serum CRP and IL-6: the IER group had concentrations comparable to a 25% CER group on both restricted and feeding days	NCD

[1] AL, ad libitum feeding/fed; CER, continuous energy restriction; CRP, C-reactive protein; ER, energy restriction; F, female; IER, intermittent energy restriction; IF, intermittent fasting; IGF-I, insulin-like growth factor I; IGFBP, insulin-like growth factor binding protein; M, male; MMTV, mouse mammary tumor virus; NCD, no comparison data between IER and CER.

(25% fat on feast days; 5.4 ± 1.5 kg compared with −4.2 ± 0.6 kg) (98), but, despite weight loss, led to a harmful decrease in brachial artery flow–mediated dilation, which could increase the risk of atherosclerosis and hypertension (99).

Variable responses and adaptations to CER or repeated cycles of IER

A persistent observation is the large variability of response to IER within animal studies in genetically identical rodents under standardized conditions. For example, Berrigan et al. (18) reported that survival in *p53*-deficient mice varied between 161 and 462 d in the group consuming feed ad libitum and between 49 and 609 d in the ADF group. This biological variation may be linked in part to different epigenetic effects between animals, which are also likely to produce variable responses in humans.

Tachyphylaxis, a decrease in response, could occur with either prolonged stimulus with CER or repeated stimulus of IER or IF. Rogozina et al. (25) found that reductions in IGF-I during the ER period of IER were attenuated with repeated cycles of IER. Similarly, Thomas et al. (22) reported a metabolic adaption to twice weekly 24-h fasts, with greater glucose uptake and reductions in ketone production by week 7 of IF. Conversely, in lean individuals, Lim et al. (100) reported decreasing oxidative stress in response to repeated periods of hyperphagia and a presumed upregulation of antioxidant enzymes. Longer-term studies of IER and IF would allow this issue to be examined.

Conclusion

There are few data, to our knowledge, that inform about whether IER and IF have greater anticancer effects than an isoenergetic CER regimen or in the absence of an overall ER. The comparative effects of IER and CER on mechanisms linked to cancer risk within animal and human studies are summarized in **Table 4**, as well as the many gaps in these data.

Human studies of IER and IF mainly have been short-term, and involved small groups of selected subjects. These studies do not inform about any potential longer-term adaptations and effects on disease risk with longer-term IER or IF that may occur. Longer-term studies (>6 mo) of adherence to and efficacy and safety of IER and IF are required in obese, overweight, and normal-weight subjects.

The limited data on IER and IF show some, but by no means consistent, beneficial effects, and are currently insufficient to support claims about the anticancer effects of IER and IF. However, the popularity of intermittent dieting and some positive findings with IER compared with CER mean IER deserves further study. We need to heed the warning of Tannenbaum and Silverstone (11), who advised 70 y ago that "research findings (with IER and IF) get coupled with suggestions and guesses to build up concepts which by pyramided repetition become accepted." High-quality research comparing IER and IF with CER are required to ascertain any true health benefits and anticancer effects.

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CIMAvax-EGF: A New Therapeutic Vaccine for Advanced Non-Small Cell Lung Cancer Patients

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Lung cancer is the common fatal illness with the highest incidence and mortality globally. Epidermal growth factor receptor overexpression by tumor cells is associated with uncontrolled proliferation, angiogenesis, anti-apoptotic signals, metastization, and invasiveness. CIMAvax-EGF vaccine consists of a chemical conjugate of the EGF with the P64 protein derived from the Meningitis B bacteria and Montanide ISA 51, as adjuvant. The vaccine is projected to induce antibodies against EGF that results in EGF withdrawal. CIMAvax-EGF demonstrated to be safe and immunogenic in advanced non-small cell lung cancer (NSCLC) patients. The efficacy study was an open-label, multicentric Phase III clinical trial, which enrolled 405 advanced NSCLC patients. Patients with proven stage IIIB/IV NSCLC, who had completed four to six cycles of chemotherapy (CTP) were randomized to receive CIMAvax-EGF or best supportive care. CIMAvax-EGF resulted in a significantly larger overall survival in patients receiving at least four doses. High EGF concentration at baseline was a good predictive biomarker of the vaccine activity and a poor prognostic biomarker for the non-treated population. The proportion of CD8+CD28− cells, CD4 cells, and the CD4/CD8 ratio after first-line CTP was also associated with CIMAvax-EGF clinical benefit. After completing the Phase III, a Phase IV trial was done where the vaccine was administered in primary care units. Administering the vaccine at primary care institutions granted better access and treatment compliance. Safety was confirmed. Several clinical trials are currently ongoing to validate EGF as a predictive biomarker of CIMAvax-EGF efficacy.

Keywords: non-small cell lung cancer, cancer vaccine, clinical trial, CIMAvax-EGF, immunotherapy

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THE ROLE OF CHECKPOINT INHIBITORS (CPIs) IN THE CONTROL OF NON-SMALL CELL LUNG CANCER (NSCLC)

The strategy of triggering the immune system to control tumor progression is not new in cancer research but has been characterized by alternating trends of excitement or frustration. BCG, interferon, and interleukin-2 provided clinical evidences of antitumor activity, but their role in the oncology practice remained limited to few tumor localizations (1, 2). With the advent of immune "check-points" inhibitors, cancer immunotherapy has proven to radically increase the survival of patients bearing advanced melanoma, lymphoma, renal, lung, urothelial, and head and neck tumors (3, 4). Immunotherapy represents an "unconventional" way of treating cancer by targeting the immune system, not the tumor itself (5). The hypothesis is that hindering the "switch-off" receptors like CTLA-4 and PD1 in the lymphocytes, would set the immune system free to destroy cancer.

Antibodies against CTLA-4, progressive disease (PD)-1 (programmed death), and PD-1 ligands (PD1-L) represent a major step forward and are the first examples of broadly effective and durable cancer immunotherapies (5, 6).

Lung cancer is the common fatal illness with the highest incidence and mortality globally. NSCLC is the most common histological type of lung cancer (7). Albeit NSCLC is not a classical "immune-sensitive" cancer like melanoma or renal cell carcinoma, two anti-PD1 antibodies and one anti-PD1L antibody have been approved for the treatment for patients with advanced disease.

Nivolumab, a PD-1 CPI, was evaluated in a Phase III study in patients with non-squamous NSCLC that progressed during or after platinum-based doublet chemotherapy (CTP). Overall survival was longer with nivolumab than with docetaxel, a taxane derivative that inhibits the polymerization of microtubules. The median overall survival was 12.2 months in the nivolumab group and 9.4 months in the docetaxel group (8). As well, patients with advanced squamous cell NSCLC who have PD after first-line CTP were randomized to receive nivolumab or docetaxel. The median overall survival was 9.2 months with nivolumab vs. 6.0 months with docetaxel (9).

On the other hand, patients with previously treated NSCLC and PD-L1 expression on at least 1% of tumor cells were randomized to receive pembrolizumab (a different anti-PD1 antibody) at two-dose levels. Overall survival was significantly larger for pembrolizumab, at the two evaluated doses. Median overall survival was 10.4 months with pembrolizumab at 2 mg/kg, 12.7 months after pembrolizumab at 10 mg/kg and 8.5 months after receiving docetaxel. In patients with at least 50% of cells expressing PD-L1, median survival time (MST) was better with pembrolizumab (10).

Moreover, in patients with newly diagnosed stage IIIB/IV NSCLC and PD-L1 expression on 50% of cancer cells, pembrolizumab was associated with significantly longer progression-free and overall survival as compared to platinum-based CTP. A total of 305 patients were randomly allocated to platinum CTP or pembrolizumab. Patients in the pembrolizumab group had a median PFS of 10.3 months, compared to 6.0 months for the CTP group. The 6 months overall survival was 80.2% in the pembrolizumab arm vs. 72.4% in the CTP arm (11).

Finally, FDA has lately accepted atezolizumab (an anti-PD1L antibody) for treating CTP-refractory, metastatic NSCLC patients. The approval followed the findings from the randomized Phase III OAK and Phase II POPLAR clinical trials, indicating a median 4.2 months survival advantage over docetaxel CTP (MST in OAK trial: 13.8 vs. 9.6 months). OAK study participants included patients with varying PD-L1 status and both squamous and non-squamous tumors (12, 13).

In summary, three immunomodulatory drugs, two anti-PD1 antibodies (nivolumab and pembrolizumab), and one anti-PD1 ligand antibody (atezolizumab), have shown to improve the survival of advanced NSCLC, still considered an unmet medical need. **Table 1** summarizes the most important results of the three CPIs approved so far for second- or first-line therapy of advanced NSCLC patients.

EGF/EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) SYSTEM AND CIMAvax-EGF MECHANISM OF ACTION

Oncogenic mutations have arisen as key therapeutic targets for molecular treatments in several cancers (14). EGFR, a well-validated oncogene, is a 170-kDa membrane glycoprotein. The intracellular domain is associated with protein tyrosine kinase activity, and its overexpression by tumor cells alters the regulation of the cell cycle, blocks apoptosis, promotes angiogenesis, and increases the motility and invasiveness of the tumor cells (15).

Therefore, EGFR as well as its downstream mediators have been identified as important therapeutic targets. The approved small-tyrosine kinase inhibitors (TKIs) of EGFR, gefitinib (Iressa™), erlotinib (Tarceva™), and afatinib (tykerb™), are effective in a group of NSCLC patients whose tumors carry stimulating mutations within the kinase domain of EGFR (16–19). EGFR–TKIs are the best option as front-line therapy in EGFR mutant NSCLC patients. In pretreated NSCLC, EGFR–TKIs are more effective than conventional cytotoxic therapy, in existence of EGFR mutations (16–19). EGFR has seven known ligands, among which, EGF is one of the most critical (20, 21).

The strategy of "sequestering" EGF reproduces the "hormonal castration" therapy, known to be effective in hormone-dependent tumors such as breast and prostate, thus extending this concept to other types of malignant tumors.

The mechanism of action of CIMAvax-EGF consists on the formation of antibodies against EGF, breaking the tolerance to a self-protein. This is possible because the vaccine consists on a chemical conjugate of the recombinant EGF with the P64k protein derived from the *Neisseria meningitidis* (conjugate EGF-P64K) (**Figure 1**) and the adjuvant Montanide ISA 51 (22). CIMAvax-EGF is administered by the intramuscular route, at four injection sites (22, 23).

CIMAvax-EGF vaccine exerts its anti-cancer activity by targeting the immune system, inducing anti-EGF antibodies that result in the decline of the circulating EGF in sera (23, 24). This, in turn, significantly decreases the probability that the remaining EGF binds to its receptor (EGFR) on the surface of cancer cells. EGF withdrawal results in the loss of a key pro-proliferation and pro-survival signal for the neoplastic cells (23, 24). The vaccine has demonstrated to be safe and immunogenic in more than 5,000 advanced NSCLC patients (23, 24).

CIMAvax-EGF was approved as a maintenance treatment for patients with stage IIIB/IV NSCLC, after front-line CTP.

Two randomized studies have been completed so far. The Phase II clinical trial included 80 advanced NSCLC patients: 40 vaccinated and 40 treated with supportive care. Patients joined the trial after finalizing first-line CTP, regardless their objective response. CIMAvax-EGF was non-toxic and induced anti-EGF antibodies. Vaccinated subjects showed a trend toward better survival, which was not statistically significant at this sample size (25).

The efficacy study consisted in an open-label, multicentric Phase III clinical trial, which enrolled 405 advanced NSCLC patients, at 21 research sites. Patients with proven stage IIIB/IV NSCLC, who received four to six cycles of platinum-based

TABLE 1 | CPIs in the treatment of patients with advanced NSCLC.

Patient population	CPI arm	Control arm	MST	
			CPI arm (months)	Control arm (months)
Non-squamous NSCLC patients that progressed during or after platinum-based doublet chemotherapy (CTP)	Nivolumab	Docetaxel	12.2	9.4
Squamous NSCLC patients that progressed during or after platinum-based doublet CTP	Nivolumab	Docetaxel	9.2	6
Previously treated NSCLC with progressive disease (PD)-L1 expression on at least 1% of tumor cells	Pembrolizumab (2 mg/kg)	Docetaxel	10.4	8.5
Previously treated NSCLC with PD-L1 expression on at least 1% of tumor cells	Pembrolizumab (10 mg/kg)	Docetaxel	12.7	8.5
CTP-refractory, metastatic NSCLC	Atezolizumab	Docetaxel	13.8	9.6
Previously untreated advanced NSCLC with PD-L1 expression on at least 50% of tumor cells	Pembrolizumab (200 mg)	Carboplatin plus pemetrexed, cisplatin plus pemetrexed, carboplatin plus gemcitabine, cisplatin plus gemcitabine, carboplatin plus paclitaxel	6 months SV rate: 80.2%	6 months SV rate: 72.4%

NSCLC, non-small cell lung cancer; CPI, checkpoint inhibitor; MST, median survival time.

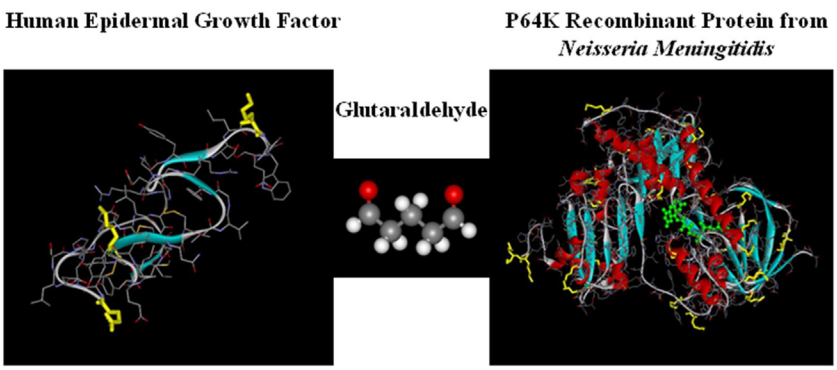

FIGURE 1 | CIMAvax-EGF composition. CIMAvax-EGF therapeutic vaccine consist on a chemical conjugate of the EGF with the P64K protein derived from *Neisseria meningitidis*.

CTP were randomized to vaccine arm [CIMAvax-EGF plus best supportive care (BSC)] or to control arm (BSC alone). Primary endpoint was overall survival while secondary endpoints were the assessment of serum EGF concentration, immunogenicity, and safety. All lung cancer patients completed front-line CTP achieving stable disease, partial, or complete response of the target lesions. Most subjects had cisplatin/carboplatin in combination with vinblastine, etoposide, or paclitaxel. Randomization (EGF cancer vaccine vs. BSC) was unbalanced (2:1), given the preliminary evidence of survival advantage shown in the Phase II study. Vaccine schedule consisted in four biweekly doses (induction phase) followed by monthly reimmunizations (maintenance). Cyclophosphamide was administered before vaccination at a low, immunomodulatory dose (200 mg/m^2). Vaccination was maintained until severe patient condition worsening (PS = 3) or unmanageable toxicity (26).

This study was registered in the National Public Registry of Clinical Trials; a WHO-validated public registry (http://www.who.int/ictrp/network/rpcec/en, RPCEC00000161). In total, 270 vaccinated and 135 controls were enrolled in the Phase III study. Both groups were well balanced according to the most important prognostic variables. The majority of the patients were men, current, or past smokers, with an ECOG performance status of 1. The most prevalent histology was squamous cell carcinoma, and they had stable disease or partial response after first-line platinum doublet. Vaccination was safe, and the most common adverse reactions were mild or moderate injection site events, fever, headache, chills, vomiting, and general malaise. CIMAvax-EGF significantly augmented overall survival when the Harrington–Fleming test was applied (26). The Harrington–Fleming is a weighted log-rank test that can be used once the non-proportionality of the hazard ratio is confirmed (27, 28). This waited log-rank is the ideal test when there is a deferred split of the time to event curve (27, 28). This is the case of therapeutic cancer vaccines or immune-modulatory drugs, which effect may manifest several months after the intervention. In this scenario, the projected hazard ratio does not apply from the beginning but at the separation of both curves. MST was 10.83 months for vaccinated vs. 8.86 months for non-vaccinated. In the Phase III trial, the 5-year survival rate was 14.4% for vaccinated subjects

TABLE 2 | CIMAvax-EGF in the treatment of patients with advanced NSCLC (Phase III clinical trial).

Patient population	CIMAvax-EGF arm	Control arm	MST	
			CIMAvax arm (months)	Control arm (months)
Stage IIIB/IV NSCLC patients, with at least stable disease after CTP (ITT)	CIMAvax-EGF	BSC	10.83	8.86
Stage IIIB/IV NSCLC patients, with at least stable disease after CTP (PP)	CIMAvax-EGF	BSC	12.43	9.43
Stage IIIB/IV NSCLC patients, with at least stable disease after CTP. Patients with (EGF) > 870 pg/ml	CIMAvax-EGF	BSC	14.66	8.63

NSCLC, non-small cell lung cancer; MST, median survival time; PD, progressive disease; CTP, chemotherapy; BSC, best supportive care.

vs. 7.9% for controls. The advantage was larger in those patients that completed vaccination induction consisting in four doses ("per protocol" scenario). The "per protocol" scenario is very relevant for CIMAvax-EGF given that several doses are required to break the tolerance and induce a protective response. MST was 12.43 months for vaccinated subjects completing induction vs. 9.43 months, for control patients (**Table 2**). Those controls that did not survived for at least 42 days (vaccine induction time) were excluded from the analysis. The 5-year survival rate was 16.62% for vaccinated patients vs. 6.2% for controls. A subgroup analysis considering demographic or tumor variables was done, and the larger gain was seen in smoker patients bearing squamous cell carcinomas with an ECOG 1 (26).

CIMAvax-EGF IMMUNOGENITY AND PREDICTIVE BIOMARKERS OF EFFICACY

Immune response was characterized in patients treated with CIMAvax-EGF (24). Anti-EGF antibodies induced by CIMAvax-EGF inhibited EGF–EGFR binding and abrogated EGFR activation (**Figure 2**). After immunization, there was a decrease in the circulating EGF which was inversely correlated with the antibody response. Antibody response also correlated with survival benefit since those patients displaying higher antibody titers exhibited better survival (24).

In the Phase III trial, a large proportion of patients (78.8%) met the good antibody response (GAR) condition (anti-EGF antibody titers $\geq$ 1:4,000 sera dilution). GAR condition was associated with longer survival in the preceding exploratory and Phase II trials. The geometric mean of the maximum antibody titers was 1:12,646 sera dilution, while the maximum anti-EGF titer was 1:1,024,000. Patients developing a GAR as soon as day 32 had a significant survival benefit (MST = 27.28 months) as compared to controls (26).

The functionality of anti-EGF antibodies was also evaluated. Sera from vaccinated patients inhibited the binding between EGF and its receptor. Median binding inhibition capacity was 20 and 40% after 5 and 12 months from vaccination, respectively (24). Furthermore, post-immune sera abrogated EGFR phosphorylation. Median phosphorylation inhibition was 65 and 85% after 5 and 12 months, respectively (24).

To discern the immune dominance of the antibody response induced by vaccination, several peptides mimicking the main EGF epitopes were synthesized. Sera from vaccinated patients were then tested for binding to the peptides in an enzyme-linked immunosorbed assay. In the Phase III study, 46% of the patients showed an immune-dominant response against the loop B of the EGF molecule (26).

The immune response of 19 long-term (more than 2 years) NSCLC survivors, regularly treated with CIMAvax-EG, was assessed (29). Previous studies showed that the anti-EGF antibody titers increased in vaccinated patients after repeated immunizations, until a plateau is reached (24–26). In long-term vaccinated patients, the anti-EGF antibody response remained high, reaching a plateau at 1:10,000 sera dilution. Although a deferred decrease in antibody titers was found in one third of the uninterrupted vaccinated patients, for the majority (two-thirds), there was no evidence of clonal exhaustion after 2 years of monthly vaccination. The immunodominance of the antibody response induced by CIMAvax-EGF was tested in long-term vaccinated subjects. The predominant response was against the loop B, which is the main binding site of EGF to EGFR. Long-lasting vaccination resulted in a reduction of serum EGF level. EGF concentration decreased to undetectable values in all continued vaccinated patients (29).

In summary, prolonged vaccination with CIMAvax-EGF induced high anti-EGF antibodies, capable to maintain serum EGF in undetectable levels. Toxicity was not exacerbated with lengthy vaccination. Long-term "EGF deficiency" did not result in deleterious effect for normal tissues. Previously, it was published that the lack of EGF produces delayed development of fetal tissue but no injury on healthy adult tissues (30).

During the last decade, the scientific community has been working hard on the development and evaluation of biomarkers for cancer drug development (31).

Several attempts have been done to find predictive biomarkers of clinical benefit of CIMAvax-EGF. Vaccinated patients with serum EGF concentration >870 pg/ml showed larger survival as compared with controls with the same EGF serum level. MST in this patient population was 14.66 months, as large as the survival of patients receiving other drugs as continuation or switch maintenance (26). MST was 8.63 months for those control patients with EGF concentration greater than 870 pg/ml (**Table 2**). Five-year survival rate for patients with high (EGF) was 23% for vaccinated patients, while no controls were alive at the referred time interval. The association between EGF levels and prognosis remained significant when the prognostic variables (gender, smoking history, performance status, and staging) were included in the multivariate analysis (26).

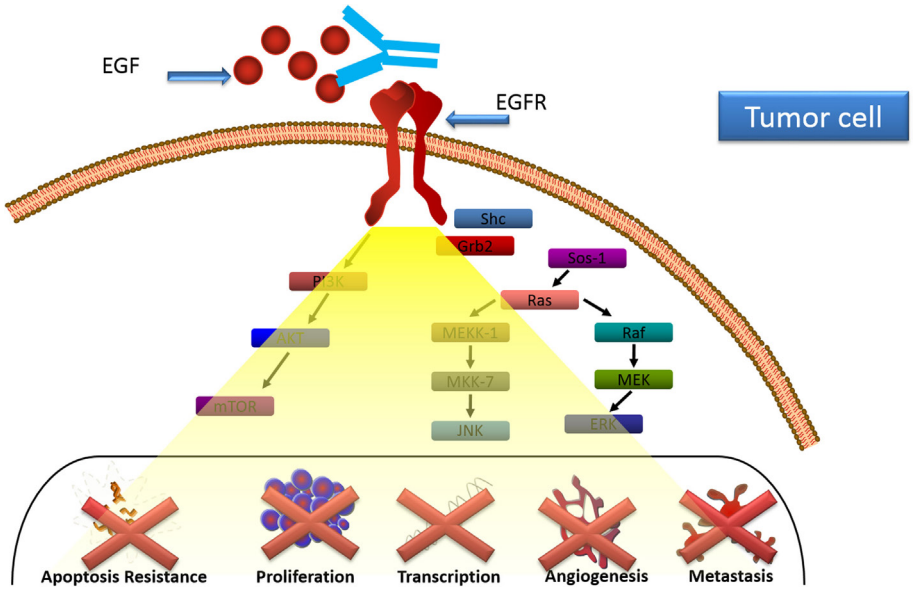

FIGURE 2 | CIMAvax-EGF mechanism of action. Anti-EGF antibodies induced by CIMAvax-EGF inhibit EGF–epidermal growth factor receptor (EGFR) binding and abrogate EGFR activation.

On the other hand, control patients with a high (EGF) had a significantly shorter survival (8.63 months) as compared with non-treated subjects with low (EGF) at baseline (15.06 months). In summary, the Phase III trial demonstrated that the EGF level in patients' sera could be simultaneously a biomarker of poor prognosis and a predictive factor of CIMAvax-EGF benefit. This result confirms the role of the EGF in the biology of the tumor but also provides a biomarker for selecting patients who benefit largely from vaccination with CIMAvax-EGF (26).

The impairment of immune system of cancer patients induced by the tumor together with the previous oncological therapies is largely proven. The evaluation of immunocompetence would provide evidences of which patients are going to benefit from immunotherapy (32). A deficit in the number of B cells, a reduced CD4/CD8 ratio and an increase in late-stage differentiated cells such as CD8+CD28− T cells distinguish the "immune-compromised" profile (33). In that logic, besides EGF concentration, the proportion of CD8+CD28− T cells, CD4 T cells, and the CD4/CD8 ratio after CTP was correlated with the clinical benefit of CIMAvax-EGF (33).

Vaccinated patients with CD4+ T cells counts greater than 40%, CD8+CD28− T cells counts lower than 24% and a CD4/CD8 ratio >2 after first-line platinum-based CTP, achieved a significantly large median survival, as compared to controls with the same phenotype. MST was 46.4 months for vaccinated patients with CD4+ counts >40% vs. 12.3 months for the matched controls, 37.2 vs. 14.3 months for vaccinated and controls with CD8+CD28− T cells counts <24% and 50.4 vs. 14.3 months for treated vs. non-treated patients with CD4/CD8 ratio >2 (33).

These findings highlight the potential value of T cell subpopulations and EGF serum levels, measured after front-line CTP, as predictive biomarkers of CIMAvax-EGF efficacy.

COMBINING CTP AND CIMAvax-EGF

CIMAvax-EGF is commonly administered after patients have finished first-line CTP. However, it would be important to start vaccination earlier in the course of the disease, given that the vaccine requires time to elicit a neutralizing response. In that sense, CIMAvax-EGF was administered concurrently with platinum doublets or even, before CTP (34). In addition, CTP and cancer vaccines could be additive through different mechanisms: by decreasing immunosuppressive cells such as T-regulatory and myeloid-derived suppressor cells, by stimulating massive antigen release leading to effective cross-priming, by modifying the tumor microenvironment and by augmenting the T-cells traffic of into the tumors (35). Oxaliplatin and cisplatin can stimulate antitumor responses, through the induction of immunogenic cell death (35). The release of new antigens can activate dendritic cells, which in turn, that activate cytotoxic lymphocytes (35, 36).

Dose-dense platinum CTP did not affect CIMAvax-EGF capacity to induce a potent antibody response. Immunogenicity in terms of percentage of good responders or immunodominance against loop B was better after vaccinating concurrently or before CTP, as compared to the standard sequential platinum doublets and vaccination (34). Increased immunogenicity could be explained by the earlier unset of vaccination or by the potentiating effect of the cytotoxic drugs.

CIMAvax-EGF IN PRIMARY CARE UNITS AND FUTURE PERSPECTIVES

After completing the Phase III, a Phase IV trial was launched where the family medicine physicians administered CIMAvax-EGF in primary health care units (policlinics). In total, 45 primary

level units together with 24 secondary level units (hospitals) participated in the study that enrolled more than 1,000 patients in 3 years. This study was registered in the National Public Registry of Clinical Trials (http://www.who.int/ictrp/network/rpcec/en, trial number RPCEC00000181). Administering the vaccine at primary care institutions granted better access and treatment compliance. Safety was confirmed; the most frequently reported adverse events were pain at the site of injection followed by fever, headache, chills, nausea, and dyspnea (22).

Overall survival of those patients that received at least one vaccine dose was 13.9 months (mean) and 7.0 months (median). Survival rate at 12 and 24 months was 34.8 of 18.1%, respectively. On the other hand, the overall survival of patients receiving at least the induction doses was 16.93 months (mean) and 9.9 months (median). The 12 and 24 months survival rate was of 44.1 and 23.3%, respectively.

In summary, CIMAvax-EGF was safe in patients with NSCLC at advanced stages treated in primary care facilities. The safety profile coincided with the previously described in controlled studies. CIMAvax-EGF also showed benefit in terms of survival, mainly in those subjects that completed four vaccine doses. Treatment with CIMAvax-EGF resulted in preliminary evidences of improvement in the quality of life, which was significant for the emotional functioning and the fatigue symptom. The use of medications to control pain was stable during vaccination (22).

Several clinical trials are currently ongoing. A new Phase III trial (WHO-validated public registry; http://www.who.int/ictrp/network/rpcec/en, trial number RPCEC00000208) is open for enrollment, where CIMAvax-EGF is used as switch maintenance in patients completing front-line CTP that has EGF concentration higher than 870 pg/ml (enrichment design). The main goal of the trial is to prospectively validate EGF as a predictive biomarker. In this scenario, the randomization is unbalanced (3:1) given the previous evidences of the clinical benefit of the vaccine. In addition, a new Phase IV (WHO-validated public registry; http://www.who.int/ictrp/network/rpcec/en, trial number PCEC00000205) was launched in 178 policlinics (at least one investigation site per state municipality) and 25 hospitals. Patients will be recruited by the oncologists in the specialized oncology services, but will be treated in their neighborhood, at the primary health care facilities. The aim is to grant vaccine access and to improve treatment compliance. In this trial, EGF concentration will be measured but not as an inclusion criterion. Instead, EGF at baseline will be retrospectively correlated with the clinical efficacy. An EGF quantification system was developed in the country by the National Center for Immunoassay, to accompany the vaccine prescription (37). Both studies will permit the consolidation of the scientific evidence of the EGF as a biomarker. Other translational studies are planned to gather more information on the relevance of the lymphocyte subpopulation as well as the individual tumor biology (mainly associated with EGFR mutations) for the CIMAvax-EGF efficacy.

AUTHOR CONTRIBUTIONS

DS was involved in the evaluation of immunogenicity and predictive biomarkers of CIMAvax-EGF efficacy (EGF concentration and immunophenotyping). TC was involved in trials' design and implementation. Both authors participated in the analysis, writing, and revision of the manuscript.

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