Cancer Treatment and Research
Series Editor: Steven T. Rosen

Vincenzo Zappia · Salvatore Panico
Gian Luigi Russo · Alfredo Budillon
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Advances in Nutrition and Cancer

Indexed in PubMed/Medline

Springer

Cancer Treatment and Research

Volume 159

Series Editor

Steven T. Rosen, Chicago, IL, USA

For further volumes:
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Advances in Nutrition and Cancer

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ISSN 0927-3042
ISBN 978-3-642-38006-8 ISBN 978-3-642-38007-5 (eBook)
DOI 10.1007/978-3-642-38007-5
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2013947358

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

This volume includes 26 contributions presented at the Third International Conference on *Advances in Nutrition and Cancer* held in Naples, Italy, in May 2012 at the National Institute for Cancer Research.

The major aim of the meeting was to illustrate the most recent and innovative projects in this area and to propose novel strategies for chemoprevention as well as molecular epidemiology and dietary intervention programs. During the conference a group of experts from different areas discussed pivotal and current topics on the key issues related to the interactions between human nutrition and malignancies.

Comparing the themes reported here with those discussed in the two previous meetings (1992, 1998), the major scientific advancements certainly derive from the extensive use of molecular biology, molecular epidemiology, and a variety of epigenetic approaches in nutrition research.

Today, cancer affects about 24 million people worldwide and is responsible for over six million deaths each year. Although early diagnosis has made some progress, in most cases tumors are still treated at advanced stages, with limited therapeutic success. Prevention is therefore a fundamental approach for fighting this severe pandemic. In this context the fundamental conclusion of Doll and Peto (1981), suggesting that at least 30 % of all cancers might be prevented by dietary regimens, is still relevant and has been confirmed by a large variety of results: the association among diet, nutrition, and cancer risk is not in question.

The first part of the volume focuses on general aspects relating life style, diet, and cancer. The molecular mechanisms underlying the connections among obesity, energy balance, physical activity, and cancer risk/progression are extensively covered as well as the presumptive association of salt intake and alcoholic and carbonated soft drinks with digestive tract cancers.

A large variety of phytochemicals and natural antioxidants have been characterized and proposed as potential chemopreventive/chemotherapeutic agents. The effects and mechanisms of the action of resveratrol, quercetin, and sulforaphane, as well as the conflicting results on selenium and selenoproteins, are reported in the second part.

It is well known that, in several tissues, diet can modulate the methylation status of the cells and epigenetic factors influenced by diet are receiving major attention in cancer prevention and as therapeutic targets. The third part is devoted to these

fundamental and most promising issues. An interesting new area deals with the adverse intrauterine environments induced by maternal diet, which influence DNA methylation and predispose to diseases in adulthood.

The fourth part deals with the beneficial effects of a functional food, olive oil, in cancer prevention. The evidence of the chemopreventive effects of extra-virgin olive oil, the major source of fat in the Mediterranean diet, associated with low incidence of cardiovascular diseases and of several tumors, such as breast cancer, is analyzed. Special emphasis has been placed on the effects of antioxidants on human hepatoma cells and on their chemical interactions with other food ingredients affecting nutritional and sensory quality.

The last two parts deal with fundamental results such as the epidemiologic evidence that lifestyle (including diet regimen) effectively prevents cancer recurrence.

Space has also been given to anti-angiogenesis: numerous preclinical, chemical, and epidemiological data have demonstrated that angiogenesis inhibition can be applied in cancer prevention and that several diet-derived chemopreventive components have angiogenesis as a common target. The relationship of the human gut microbiome with gastrointestinal malignancies has also been discussed; the understanding of the dynamic interplay between the gut microbiome, the immune system, and dietary exposure may contribute to future cancer prevention strategies.

As demonstrated in this volume, the inter-disciplinary approach has already yielded a rich harvest of basic knowledge concerning cancer development and will provide the seeds for future breakthroughs in clinical progress. The text is intended to furnish the reader with a general view of the state of the art in this very central and solid area of research. We will be satisfied if the multidisciplinary nature of these proceedings informs and stimulates the readers as much as it did the participants in the conference. It is our hope that the volume will encourage further research and understanding of all aspects of this intriguing and complex field.

Vincenzo Zappia

Acknowledgments

The Third International Conference on *Advances in Nutrition and Cancer*, held in May 2012, was sponsored by the National Institute for Cancer Research "Fondazione G. Pascale," Naples, the non-profit Association Arfacid onlus, Naples, the Oncology Research Center of Mercogliano, the Departments of Biochemistry and of General Pathology of the Second University of Naples, the Departments of Clinical Medicine and Molecular Oncology of the University of Naples "Federico II," the Institute of Food Science of the National Research Council, Avellino, and the Italian Institute for Philosophical Studies of Naples.

The meeting received the authoritative patronage of the Accademia Nazionale dei Lincei, Consiglio Nazionale delle Ricerche, Regione Campania, Comune di Napoli, Provincia di Napoli, Seconda Università degli Studi di Napoli, Università degli Studi di Napoli "Federico II," Lega Italiana per la Lotta contro i Tumori—Sezione di Napoli, Associazione Italiana di Oncologia Medica, Società Italiana di Biochimica, Società Italiana di Cancerologia, and the Ordine dei Medici di Napoli.

Contributors to the conference included: Banca di Credito Popolare, Roche, Acen, Biorad, DBA Italia, Delchimica, Diasorin, Euroclone, Farmacia Morrica, Istituto Varelli, Life Technologies, Microtech, Strategies Consulting, Vinci Biochem, and Carl Zeiss.

The editorial coordination between the editors, authors, and Springer Verlag was handled by Janet Gates and the organizing secretariat by Paola Colonna and Pina Carfora.

The editors acknowledge Dr. Giuseppe Iacomino for his valuable assistance in the Conference organization. They also express their gratitude to the authors of the articles and to Springer Verlag for having made possible the publication of this volume.

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Part I
Life Style, Diet and Cancer

The Role of Metabolic Carcinogenesis in Cancer Causation and Prevention: Evidence from the European Prospective Investigation into Cancer and Nutrition

Elio Riboli

Abstract

The theory that nutrition might be involved in the causation and prevention of cancer arose over 100 years ago from laboratory studies of the effect of diet on tumour growth. During the mid-20th century, the major focus of cancer epidemiology was on the role of tobacco and alcohol. It was not until the early 1980s, following a seminal report from Doll and Peto on cancer causes, that major research programmes on nutrition and cancer were instigated. The European Prospective Investigation into Cancer and Nutrition (EPIC) was established at IARC-WHO as a large prospective cohort study designed specifically to investigate the relationship of diet, nutritional factors, anthropometry and physical activity with cancer risk. Since the early 1990s, EPIC has made a major contribution to understanding the effect of these factors on population risk of cancer. This chapter summarises the development of the field of nutritional cancer epidemiology, and describes how the EPIC study was designed to investigate cancer and nutrition. Key findings from EPIC in the role of nutrition and metabolic factors and cancer are highlighted.

Keywords

Nutrition · Diet · Metabolic factors · Anthropometry · Steroid hormones · Cancer · Epidemiology · Large prospective cohort studies · European Prospective Investigation into Cancer and Nutrition

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_1,

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1 Historical Background

The theory that nutrition might be involved in the causation or prevention of cancer developed at the beginning of the 1900s with the first laboratory studies on the effect of different diets on the development and growth of tumours. This early research found that tumours, either spontaneous or transplanted, would grow in well-nourished rodents while calorie-restricted diets inhibited tumour growth. The calorie restriction required to inhibit tumour growth was substantial compared to ad libitum. Further studies showed that calorie restriction also prevented recurrence of spontaneous tumours transplanted after excision [30, 36, 42].

In the 1930s, Albert Tannenbaum and his collaborators, [44, 46, 47] commenced three decades of laboratory research on the effect of hypercaloric, hyperlipidic or hyperproteic diets on cancer causation using rodent models. They found that some tumours were particularly influenced by either a hypercaloric or hyperlipidic diet, or both, particularly tumours of the mammary gland, liver and lung. This was complemented by the work of Baumann who demonstrated that at least part of the tumour-promoting effect of increased dietary fat was due to corresponding increased caloric intake [29].

The first known epidemiological study on diet and cancer in humans was published in 1933. This study of 462 cancer patients and 435 controls found that people who consumed more vegetables had a reduced risk of cancer, and those who drank more beer had a higher risk of developing cancers of the upper digestive tract [41].

Tannenbaum led what was probably the first retrospective cohort study to investigate the effect of obesity on mortality from cancer and other diseases. The study used data from North American life insurance companies and found that there was a 30–50 % increase in mortality from cancer among people who were obese at the time they had first subscribed to the health insurance policy [45].

These results did not substantially modify mainstream approaches to cancer research; the prevailing hypothesis at the time was that cancer was caused by chemical or physical compounds that could be found in the living environment

including the air, drinking water, food (as contaminants or chemical additives) or in occupational settings. Therefore, the majority of studies on cancer were essentially designed to identify new carcinogens and understand the mechanism of chemical and physical carcinogenesis.

The two major factors being investigated as potential lifestyle-related carcinogens at the time were tobacco and alcohol. The role of tobacco was clearly identified in the 1950s by the studies led in England by Richard Doll and Bradford Hill [5–8], and in the US by Ernst Wynder [51]. These studies demonstrated that the carcinogenic effect of being a lifelong smoker was exceptionally strong. For example, starting smoking at around age 18–20 and smoking 20–30 cigarettes per day multiplied the risk of developing lung cancer by 30- to 40-fold. The identification of a number of chemical compounds present in cigarette smoke and their testing in laboratory carcinogenesis models rapidly provided strong mechanistic support to the epidemiological studies.

The paradigm for the accrual of scientific evidence linking alcohol to cancer was much less straightforward for a number of reasons, one being the strength of the association between alcohol consumption and cancer risk. In fact, the relative increase in cancer risk due to alcohol consumption is substantial but not as strong as with cigarettes and lung cancer. Typically, elevated alcohol consumption—for example in the order of 70–80 g of alcohol per day (equivalent to one bottle of wine or four pints of beer)—is associated with up to 5- or 6-fold increase in risk of developing cancers of the upper aerodigestive tract (mouth, pharynx, larynx and oesophagus) with a multiplicative effect for drinkers who also smoke. However, the relative risk increase for most other cancers (e.g. cancer of the colorectum, liver and breast) is much more modest, in the order of 1.5-fold.

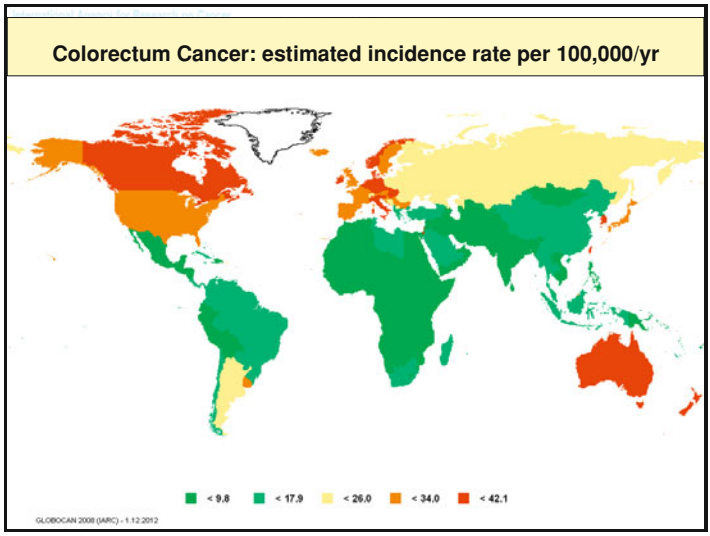
Evidence from experimental animal models to demonstrate that alcohol can cause cancer also took much longer to accrue than for chemicals in cigarette smoke. The main mechanisms that have been identified to date for the influence of alcohol on cancer risk [19] include DNA damage by acetaldehyde that may be particularly relevant for upper GI tract, liver; increased secretion and bioavailability of oestrogens, in relation to mammary tumours; production of reactive oxygen and nitrogen species and changes in folate metabolism.

As for diet, it was not until the 1960s and 1970s that the first case–control studies emerged that were designed with modern epidemiological methods to investigate the possible role of diet in the risk of developing cancer. The first studies investigated mainly cancers of the digestive tract (oesophagus, stomach, colon, rectum), respiratory tract (larynx, lung) and breast [31]. This renewed interest in nutrition was stimulated by the publication of data on cancer incidence in different global populations [9, 10].

Population-based cancer registry data indicated that the global incidence of many cancers varied enormously, with up to 15–20-fold differences in the incidence of some cancers between different populations around the world (Fig. 1).

For some cancers, such as lung or liver, the most likely explanation for these variations was found in the different history and prevalence of tobacco smoking or, as lately demonstrated, the incidence and prevalence of hepatitis B and C, exposure

(a)



(b)

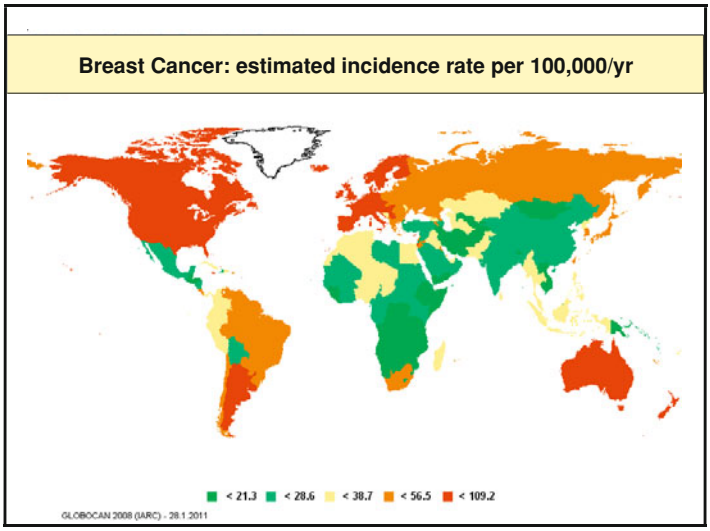


Fig. 1 GLOBOCAN 2008 estimated cancer incidence rate worldwide per 100,000/yr for (**a**) colorectum and (**b**) breast cancer. Reproduced from [14]

to aflatoxins from mould-contaminated food and alcoholic beverage consumption. For many other cancers, such as breast, colon and rectum, prostate and stomach, the explanations of the causes underlying these huge variations were unknown, but stimulated the hypothesis that diet could have a role in their aetiology.

Early dietary epidemiological studies investigated the association between cancer risk and particular foods. Gradually, some evidence emerged that consumption of certain foods might be associated with reduced cancer risk, particularly fruits, vegetables and fibre-rich cereals. Conversely, it was found that consumption of salt-preserved food (including pickled vegetables and salt-preserved meat), red meat and animal fat (as opposed to vegetable fat) could increase cancer risk.

Although this work enhanced the concept of a link between diet and cancer risk, the research focussed on the type of food, not on precise measurement of the total amount of food consumed, the corresponding nutrient intake or the total energy from food. There was also no notion of the potential importance of investigating the effects of weight and physical activity on cancer risk. At the time, it was considered that fat intake was most likely to increase cancer risk only via its chemical and nutritional characteristics, rather than by contributing to excessive total energy intake; obesity was not then considered a major risk factor and none of the studies had clearly separated the effect of caloric intake from the effects of fat intake.

In 1981, Richard Doll and Richard Peto published a milestone report on cancer causes in the US in which they estimated that 35 % of cancer could be due to nutrition, but with a very wide confidence interval of 10 or 70 % [11]. The publication of this report in combination with a report on Diet and Cancer from the US National Academy of Science (National Academy of Science 1982) provided significant impetus for IARC-WHO to initiate a new research programme on nutrition and cancer within the Unit of Analytical Epidemiology directed by Dr Rodolfo Saracci.

The establishment of large-scale prospective cohort studies specifically designed to investigate the relationship of diet, nutritional factors, anthropometry and physical activity to cancer risk contributed to a new era in cancer epidemiology. The first such studies were started in North America. Particularly, Walter Willett at Harvard University did seminal work in developing new diet questionnaires specifically tailored for use in very large cohort studies and in formalising the importance and the methods to account and adjust for the intake of macronutrients and the energy they provide.

On the European side, at IARC collaboration with the University of Lund, Sweden, commenced to design a prospective cohort study in Malmö, while in parallel the Danish Cancer Society started planning a similar project in Copenhagen and Aarhus.

Other researchers had started considering the design of prospective studies on nutrition in the Netherlands, the United Kingdom (Cambridge), Italy (Milan) and France (Paris).

At IARC, this new interest was built on through the development of a research programme on nutrition and cancer which formed the basis for initiating the planning of a multicentre European prospective cohort study that eventually became the European Prospective Investigation into Cancer and Nutrition (EPIC).

2 The Design and Establishment of the EPIC Project

The EPIC project was initiated in 1989 with a series of methodological studies aimed at testing the relative validity and reproducibility of diet measurement methods specifically designed to be used in large, multicentre and multilanguage cohort studies.

The basic design of these methodological studies consisted of two repeat measurements obtained with the newly designed diet assessment questionnaires, at the start and end of a one-year period. Diet questionnaire results were compared to a reference method consisting of the average diet intake estimated with 12 repeat 24-h dietary recalls (24 HDR) administered monthly throughout the study period.

Biomarkers measured in four repeat blood samples and in four repeat 24-h urine collections were used to compare with estimated diet intake of specific nutrients and foods [43].

These studies also pilot-tested the feasibility of collecting detailed lifestyle and medical history data and biological samples from a large number of study participants in a cost-effective manner. Successful completion of this methodological and pilot phase paved the way to the funding, by the Europe against Cancer programme of the European Commission and by a number of national institutions, of the full-scale project.

Enrolment for EPIC began in 1992 in 17 research centres in 7 core EPIC countries (France, Germany, Greece, Italy, The Netherlands, Spain and the UK). Over the next 2–3 years, additional research centres that were conducting similar prospective studies in Denmark, Norway and Sweden also joined the EPIC consortium.

Recruitment took place mostly from 1993–1999 and largely invited study participants from the general population in certain geographical areas with an age range of 35–70 years. Some exceptions existed, for example, the Utrecht cohort, which was based on women who underwent breast screening, the Oxford cohort that targeted both the general population and vegetarians and vegans and the French cohort composed of members of the national health insurance of school employees.

By the end of the study participant recruitment phase in 1999, EPIC became the largest prospective cohort study with a baseline biobank specifically designed to investigate the relationship between nutrition and cancer, with 521,330 participants from 23 study centres across 10 European countries (Fig. 2).

The prospective cohort approach included the collection of baseline questionnaires on diet and lifestyle factors, as well as the measurement according to standardised protocols of anthropometric characteristics (weight, height, sitting height, waist and hip circumferences), blood pressure and pulse rate. EPIC was also the first study to collect and store blood from a very large number of participants: 388,467 blood samples of 30 ml volume were collected and aliquoted into 28 plastic straws of citrated plasma and serum, stored in liquid nitrogen, with aliquots divided for security reasons between the central biorepository at IARC and in each national centre.

European Prospective Investigation into Cancer and Nutrition (EPIC): Key Investigators and Collaborating Centres

Imperial College London (ICL)
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International Agency for Research on Cancer/ World Health Organization (IARC, Lyon)
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France
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• Paolo Vineis, University of Turin and HuGeF Foundation.

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• Göran Hallmans, Mattias Johannson, Malin Sund, Umeå University.

United Kingdom
• Nicholas E Day, Kay-Tee Khaw, Sheila Bingham†, Nicolas Wareham, John Danesh, University of Cambridge.
• Timothy J Key, Ruth C Travis, University of Oxford.

Fig. 2 European centres participating in the EPIC consortium with key collaborators

A key feature of EPIC is its wide geographical coverage, which means that the study comprises populations with varying dietary and lifestyle habits and different underlying cancer incidence rates. This study design including heterogeneity in both the exposure of interest, and the disease outcome was conceived to increase the overall statistical power of identifying diet–diseases relationships.

On the other hand, this diversity in diet and language might introduce systematic over or underestimation of the intake of specific foods and nutrients across study centres. To address these methodological challenges, we developed a research programme aimed at improving diet measurement via the introduction of an in-built calibration sub-study. A detailed 24-h dietary recall assessment was made in 7 % of the cohort (38,000 individuals) across each of the EPIC countries;

this was used to calibrate results from each centre to correct for possible systematic between-centre over/underestimations in the baseline dietary assessments.

Since the initial baseline collection, cohort participants have been followed up to obtain information on changes in major dietary and lifestyle factors including a series of questions on whether the subjects had suffered from a number of common diseases and medical conditions.

Follow-up aimed at identifying cancer cases occurring among the EPIC cohort is mostly based on record linkage with population cancer registries data. In some EPIC centres (i.e. in France, Germany and Greece) where the area of residence of the participants does not coincide with that of comprehensive cancer registries, cancer incidence data are gathered via a combination of other methods including active follow-up of study subjects and their next-of-kin, health insurance records and clinical and pathology registries.

Data have since been stored in a central secured database located at IARC-WHO and are made available to EPIC collaborators for specific studies. So far, over 60,000 newly incident cancer cases have been reported, including around 14,000 breast, 3,600 lung, 5,200 colorectal, 1,180 pancreatic and 1,400 endometrial cancer cases, giving scope for well-powered studies on cancer causation.

3 Research Highlights on Nutrition and Metabolic Factors and Cancer

EPIC represents a large and mature resource for epidemiological studies of cancer aetiology. Data can be integrated from studies on diet, physical activity, anthropometry and biomarker measurements to build a broad picture of the role of nutrition and metabolic factors in cancer causation. Here, some highlights of this research within EPIC are summarised.

3.1 Foods and Nutrients

Specific research projects conducted within EPIC on the relationship between consumption of specific foods and nutrients and cancer risk have either identified or clarified a number of associations with the risk of developing specific cancers; these are briefly outlined below.

Fibre and particularly cereal fibre has been consistently found to be strongly associated with reduced risk of developing colorectal cancer [2, 3]. Red meat and processed meat were associated with increased risk of digestive tract cancers, including stomach, colon and rectum [17, 32].

The blood levels of a number of nutrients were found to be significantly associated with reduced cancer risk. Notable associations suggesting a protective effect include B vitamins and cancers of the colorectum [12] and lung [23], ascorbic acid and some carotenoids and cancer of the stomach [20], Vitamin D and cancer of the colorectum [22].

However, the focus of this paper is on the major findings concerning the association of anthropometric and metabolic factors with cancer risk.

3.2 Obesity

The relationship between body mass index (BMI) or other anthropometric measures and cancer risk has been investigated for a number of cancer types in the EPIC population. Body weight and BMI were found to be positively associated with risk of colon cancer in men, whereas weak or no associations exist in women. However, when waist circumference and waist–hip ratio (WHR), indicators of abdominal obesity, were analysed in the EPIC study, they were found to be predictive of subsequent risk of developing colon cancer in both men and women, with relative risk for the highest versus the lowest quintile of WHR of 1.51 for men (95 % CI = 1.06–2.15); and 1.52 for women (95 % CI = 1.12–2.05) [33].

The association of abdominal obesity with colon cancer risk may also vary depending on hormone replacement therapy (HRT) use in postmenopausal women as it appears more strongly in women who never took HRT; further studies will be needed to investigate this [33].

The relative risk for endometrial cancer for women with a waist circumference of $\geq$88 cm versus <80 cm was 1.76 (95 % CI = 1.42–2.19); weight, waist and hip circumferences and WHR were also strongly associated with increased risk of endometrial cancer [16].

In EPIC, overweight (expressed as BMI) was a significant predictor of breast cancer risk in postmenopausal women not using HRT. When restricting the analyses to women who were taking HRT at baseline or had taken HRT in the few years preceding baseline, an apparent weakening of the association of BMI with breast cancer risk was observed [27]. Similar results were reported by other cohort studies (for example [18]). However, this interpretation of the results does not take into account the real-time sequence of the two risk factors. In fact, these women first gained weight during their life and subsequently started taking (or not taking) HRT. Therefore, the interpretation should be that HRT increased breast cancer risk more markedly in lean women and progressively less markedly in overweight and obese women (Fig. 3).

Among premenopausal women, weight and BMI were slightly inversely associated with breast cancer. Although this effect was non-significant in EPIC, it was in agreement with a number of other studies, thus stimulating an unexpected research question. There are no known mechanisms that would satisfactorily explain why overweight and obese women would be at slightly reduced breast cancer risk before age 45–50.

This unexpected association was corroborated by the EPIC finding that increasing C-peptide levels as a marker of insulin resistance are associated in an opposite direction with breast cancer risk, predicting an increase in risk after menopause but a slight decrease in risk before menopause [49].

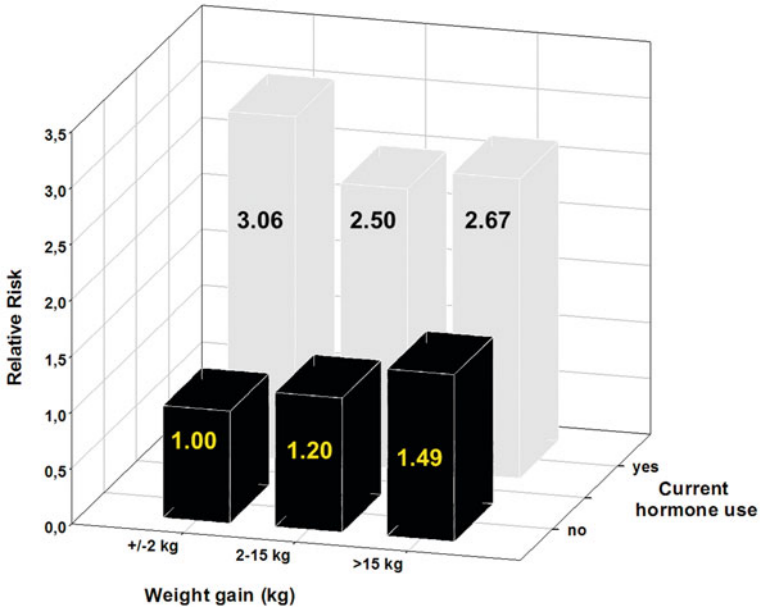

Fig. 3 Multivariate-adjusted relative risk of breast cancer by weight gain category and current hormone use among postmenopausal women in the EPIC study

Given the range of effects observed in different segments of the population, it will be important to understand the mechanisms by which obesity affects cancer risk. There is substantial evidence supporting the theory that hormonal mechanisms play a central role in breast cancer aetiology.

Moderate and high levels of physical activity, combining recreational and household activities, were associated with a reduced risk of breast cancer, independently of the level of overweight. Heterogeneous associations were observed by breast cancer hormone receptor status indicating hormone-related mechanisms [28, 40].

Similarly, a reduced colorectal cancer risk was observed in relation to increased physical activity. The risk was halved in subjects who were physically active and slim compared to those being overweight and sedentary [15].

No association with physical activity has been found for a number of other cancer types including lymphoid neoplasms [48].

3.3 Height

Consistent with the theory that early, rapid growth could predispose to increased cancer risk later in life, height was also shown to be associated with increased risk of a number of cancers and with overall cancer incidence and mortality [50].

The association is particularly evident for colorectal and breast cancer risk in EPIC. For colorectal cancer, tall men (≥180.5 cm) and women (≥167.5 cm) have a RR of 1.40 (95 % CI = 0.99–1.98) and 1.79 (95 % CI = 1.30–2.46), respectively, compared to short men (<168 cm) and short women (<156 cm) with a linear relationship over the full scale of height [33].

The risk of breast cancer for being tall compared to being short among pre- and postmenopausal women was 1.33 (95 % CI 0.96–1.84) and 1.40 (95 % CI 1.16–1.69), also with a linear relationship predicting an increased risk for each additional 5 cm of height of 1.05 (95 % CI 1.00–1.16) in premenopausal and 1.10 (95 % CI 1.05–1.16) in postmenopausal women [27].

The mechanism by which height affects cancer risk is unclear but is likely to be due to a combination of genetic, environmental, hormonal and nutritional factors. What is intriguing is that metabolic factors must operate in early age before full height is attained, to leave an "imprint" for risk of disease in later life. Understanding the factors that influence both growth and subsequent risk of different non-communicable diseases, including cancer and cardiovascular and respiratory diseases, should be an important subject for future research as it may pave the way to yet unknown disease mechanisms.

3.4 Insulin Resistance

Insulin resistance is a complex metabolic condition in which the uptake of glucose at the cellular level requires higher than normal levels of insulin. When the condition is mild, the pancreas is able to increase insulin synthesis and compensate for the higher demand. When the condition becomes more serious, the pancreas may not be able to produce enough insulin and therefore high levels of insulin coexist with high glycemia. This condition may precede the clinical appearance of type 2 diabetes for years or may persist at a mild level without ever leading to overt diabetes during a person's life.

The two most widely used serum markers of insulin resistance are C-peptide and glycated haemoglobin (HbA1c). Elevated C-peptide level is an earlier marker of insulin resistance than HbA1c. Glycated haemoglobin reaches above normal levels when high insulin secretion is not sufficient to compensate peripheral insulin resistance and control glycaemia while C-peptide, being a marker of insulin secretion, may start being elevated when glycaemia and HbA1c are still within the normal range. For this reason, we used C-peptide in a number of investigations in EPIC into the association of insulin resistance with subsequent cancer risk.

We first reported a strongly positive association between serum C-peptide levels and relative risk of colon and rectal cancer in the New York University Women's Health Study, with an odds ratio of 2.92 (95 % CI 1.26–6.75) for the highest versus lowest quintiles [24]. This was replicated for colon and rectum cancer within EPIC, with a much larger number of cancer cases, though the association was less strong [21].

A positive association has also been observed for cancer of the endometrium at all ages [4]. For cancer of the breast, an opposite effect was found in premenopausal versus postmenopausal women (as described above, [49]). Breast cancer risk was significantly reduced in subjects who were less than 50 years of age at cancer diagnosis and had elevated C-peptide levels at baseline [49].

3.5 Sex Steroid Hormones

An underlying hormonal mechanism is indicated for many of the associations of anthropometric traits with cancer as described above. This has led to detailed studies on individual hormonal factors and cancer risk, as exemplified by work on breast cancer in EPIC.

In postmenopausal women (where BMI is positively associated with breast cancer risk), BMI was positively correlated with serum levels of estrone, free estradiol and free testosterone [25]. Sex hormone–binding globulin (SHBG) levels showed an inverse relationship with BMI; this glycoprotein sequesters androgens and estrogens such that the bound form is unavailable to bind its cellular receptor. In addition to these observations, elevated levels of a panel of hormones was found to be a strong predictor of increased breast cancer risk, with SHBG the only factor to be associated with reduced breast cancer risk (Fig. 4) [25].

In contrast, in premenopausal women (where there is an inverse association of BMI and breast cancer risk), these associations were not apparent for estrogens or SHBG. However, elevated androgens were associated with increased risk of breast cancer [26].

4 Conclusions

It is clear that a number of chemical, physical and biological carcinogens have a major role in cancer causation and in some cases account for most of the variations in cancer incidence across the world. However, they cannot explain the global variations for a large number of cancers that represent a significant proportion of the world's total cancer burden.

Research conducted during past decades, and particularly those based on large prospective studies including EPIC, has found that diet, nutrition, physical activity and anthropometry have major influences on population risk of cancer.

From a scientific point of view, the unravelling of the mechanistic effects underlying the increased cancer risk associated with some components of current Western diet and lifestyle is challenging. The effect may be due to fine modulation or modest dysregulation of normal physiological processes rather than gross disruption, as may be the case for some strong exogenous carcinogens.

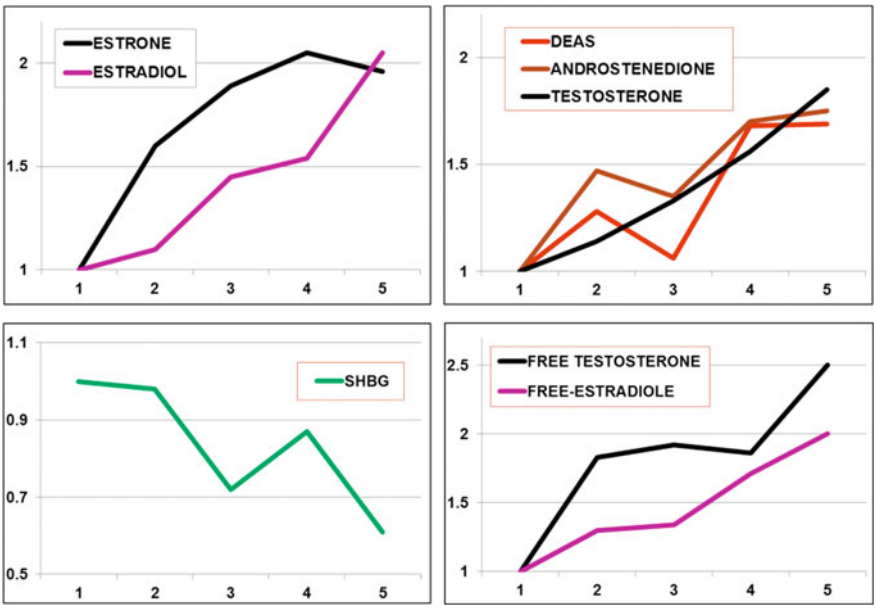

Fig. 4 Relative risk of breast cancer by quintiles of serum sex steroid levels among 300,000 postmenopausal women in the EPIC study with average follow-up of 8 years. Odds ratio estimated by conditional logistic regression with study centre, age at blood donation, time of day of blood donation and fasting status at blood donation as matching factors for breast cancer cases and control subjects. x axis: quintiles of hormone concentration from low [45] to high [10]; y axis: relative risk

From a public health point of view, these metabolic lifestyle factors are of great interest as they largely overlap with the major known risk factors of cardiovascular diseases and diabetes, therefore offering the opportunity of targeting the prevention of a number of non-communicable diseases with the same panel of public health interventions [13].

Acknowledgments The author would like to acknowledge Claire Westwood for assistance with preparation of the manuscript, and the following for financial support of the EPIC study: European Commission (DG-SANCO); International Agency for Research on Cancer; Danish Cancer Society (Denmark); Ligue Contre le Cancer, Institut Gustave Roussy, Mutuelle Générale de l'Education Nationale, Institut National de la Santé et de la Recherche Médicale (INSERM) (France); Deutsche Krebshilfe, Deutsches Krebsforschungszentrum and Federal Ministry of Education and Research (Germany); Ministry of Health and Social Solidarity, Stavros Niarchos Foundation and Hellenic Health Foundation (Greece); Italian Association for Research on Cancer (AIRC) and National Research Council (Italy); Dutch Ministry of Public Health, Welfare and Sports (VWS), Netherlands Cancer Registry (NKR), LK Research Funds, Dutch Prevention Funds, Dutch ZON (Zorg Onderzoek Nederland), World Cancer Research Fund (WCRF), Statistics Netherlands (The Netherlands); ERC-2009-AdG 232997 and Nordforsk, Nordic Centre of Excellence programme on Food, Nutrition and Health. (Norway); Health Research Fund (FIS), Regional Governments of Andalucía, Asturias, Basque Country, Murcia and Navarra, ISCIII RETIC (RD06/0020) (Spain); Swedish Cancer Society, Swedish Scientific Council and Regional

Government of Skåne and Västerbotten (Sweden); Cancer Research UK, Medical Research Council, Stroke Association, British Heart Foundation, Department of Health, Food Standards Agency, and Wellcome Trust (United Kingdom).

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Obesity, Energy Balance, and Cancer: A Mechanistic Perspective

Stephen D. Hursting

Abstract

Nearly 36 % of adults and 20 % of children in the USA are obese, defined as a body mass index (BMI) $\geq$30 kg/m^2. Obesity, which is accompanied by metabolic dysregulation often manifesting in the metabolic syndrome, is an established risk factor for many cancers. Within the growth-promoting, proinflammatory environment of the obese state, cross talk between macrophages, adipocytes, and epithelial cells occurs via obesity-associated hormones, cytokines, and other mediators that may enhance cancer risk and/or progression. This chapter synthesizes the evidence on key biological mechanisms underlying the obesity–cancer link, with particular emphasis on obesity-associated enhancements in growth factor signaling, inflammation, and vascular integrity processes, as well as obesity-dependent microenvironmental perturbations, including the epithelial-to-mesenchymal transition. These interrelated pathways represent possible mechanistic targets for disrupting the obesity–cancer link.

Keywords

Obesity · Inflammation · Growth factor signaling · Vascular perturbations · Insulin resistance

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_2,

Abbreviations

AMPK	AMP-activated kinase
BMI	Body mass index
CR	Caloric restriction
COX-2	Cyclooxygenase-2
DIO	Diet-induced obesity
EMT	Epithelial-to-mesenchymal transition
FGF-2	Fibroblast growth factor-2
IGF-1	Insulin-like growth factor-1
JAK	Janus kinase
mTOR	Mammalian target of rapamycin
MCP-1	Monocyte chemoattractant protein-1
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAI-1	Plasminogen activator inhibitor-1
PI3K	Phosphatidylinositol 3-kinase
STAT	Signal transducer activator of transcription
TNF-α	Tumor necrosis factor-alpha
tPA	Tissue-type plasminogen activators
uPA	Urokinase-type plasminogen activators
VEGF	Vascular endothelial growth factor
ZEB1	Zinc finger E-box-binding homeobox 1

Contents

1 Introduction

The prevalence of obesity, defined as a body mass index (BMI, body weight [in kilograms] divided by height [in meters] squared) $\geq$30 kg/m^2, has increased dramatically in recent decades in the United States, and nearly 36 % of adults and 20 % of children are now obese [1]. Among obese adults, approximately 60 % meet the criteria for the metabolic syndrome, a state of metabolic dysregulation characterized by insulin resistance, hyperglycemia, dyslipidemias (particularly hypertriglyceridemia), and hypertension [2]. In obesity and/or metabolic syndrome, alterations also occur in circulating levels of insulin, bioavailable insulin-like growth factor (IGF)-1, adipokines (e.g., leptin and adiponectin), inflammatory factors (e.g., cytokines), and vascular integrity–related factors (e.g., vascular endothelial growth factor [VEGF] and plasminogen activator inhibitor [PAI]-1) [3, 4]. Through these mediators, obesity and metabolic syndrome are linked to various chronic diseases [3, 5] including cardiovascular disease, type 2 diabetes, and the focus of this review, cancer.

Evidence-based cancer prevention guidelines urge avoiding obesity [6]. Overall, 14 % of all cancer deaths in men and 20 % of all cancer deaths in women are attributable to overweight and obesity [7]. Obesity is associated with increased mortality from cancer of the prostate and stomach in men; breast (postmenopausal), endometrium, cervix, uterus, and ovaries in women; and kidney (renal cell), colon, esophagus (adenocarcinoma), pancreas, gallbladder, and liver in both genders [7]. While the relationships between metabolic syndrome and specific cancers are less well established, first reports from the Metabolic Syndrome and Cancer Project, a European cohort study of approximately 580,000 adults, confirm associations between obesity (or BMI) in metabolic syndrome and risks of colorectal, thyroid, and cervical cancer [8]. With the increasing prevalence of obesity and metabolic syndrome, strategies to break the links between these conditions and cancer are urgently needed [3].

Herein, we discuss possible mechanisms underlying the links between obesity, metabolic syndrome, and cancer, with emphasis on obesity-associated enhancements in growth signaling, inflammation, and angiogenic processes and on the cross talk between macrophages, adipocytes, endothelial cells, and epithelial cells in many cancers. Specifically, we describe the dysregulation of growth signals (including insulin, IGF-1, downstream signaling pathways, and adipokines), cytokines and cellular cross talk, and vascular integrity factors in the obese state that may contribute to multifactorial enhancement of cancer processes. Components of these interrelated pathways offer possible mechanism-based targets for the prevention and control of cancers related to, or caused by, excess body weight and the metabolic syndrome. However, as we also discuss, key unanswered questions remain regarding the links between obesity, metabolic syndrome, and cancer and putative strategies to break them.

2 Dysregulated Growth Signals

2.1 Insulin and IGF-1

Insulin is a peptide hormone produced by pancreatic beta cells and released in response to elevated blood glucose. Hyperglycemia, a hallmark of metabolic syndrome, is associated with insulin resistance, aberrant glucose metabolism, chronic inflammation, and the production of other metabolic hormones such as IGF-1, leptin, and adiponectin [9]. Sharing ~50 % sequence homology with insulin, IGF-1 is a peptide growth factor produced primarily by the liver following stimulation by growth hormone. IGF-1 regulates growth and development of many tissues, particularly prenatally [10]. IGF-1 in circulation is typically bound to IGF-binding proteins (IGFBPs) that regulate the amount of free IGF-1 bioavailable to bind to the IGF-1 receptor (IGF-1R) and elicit growth or survival signaling [10]. In metabolic syndrome, the amount of bioavailable IGF-1 increases, possibly via hyperglycemia-induced suppression of IGFBP synthesis and/or hyperinsulinemia-induced promotion of hepatic growth hormone receptor expression and IGF-1 synthesis [9] Elevated circulating IGF-1 is an established risk factor for many cancer types [10, 11].

2.2 Signaling Pathways Downstream of the Insulin Receptor and IGF-1R

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway, downstream of the insulin receptor and IGF-1R, is one of the most commonly altered pathways in epithelial cancers [12]. This pathway integrates intracellular and environmental cues, such as growth factor concentrations and nutrient availability, to regulate cellular survival, proliferation, protein translation, and metabolism. Activation of receptor tyrosine kinases, such as the insulin receptor or IGF-1R, stimulates PI3K to produce lipid messengers that facilitate activation of the Akt cascade. Akt regulates the mammalian target of rapamycin (mTOR) [13], which regulates cell growth, cell proliferation and survival through downstream mediators. mTOR activation is inhibited by increased AMP-activated kinase (AMPK) under low nutrient conditions [14]. Increased activation of mTOR is common in tumors and many normal tissues from obese and/or diabetic mice [15], and specific mTOR inhibitors block the tumor-enhancing effects of obesity in mouse models [16, 17].

2.3 Leptin, Adiponectin and Their Ratio

Leptin is a peptide hormone produced by adipocytes, is positively correlated with adipose stores and nutritional status, and functions as an energy sensor to signal the brain to reduce appetite. In the obese state, adipose tissue overproduces leptin,

and the brain no longer responds to the signal. Insulin, glucocorticoids, tumor necrosis factor-alpha (TNF-α), and estrogens all stimulate leptin release [18]. Leptin has direct effects on peripheral tissues, indirect effects on hypothalamic pathways and modulates immune function, cytokine production, angiogenesis, carcinogenesis, and other biological processes [18]. The leptin receptor has similar homology to class I cytokines that signal through the Janus kinase and signal transducer activator of transcription (JAK/STAT) pathway that is often dysregulated in cancer [19].

Adiponectin is a hormone mainly secreted from visceral adipose tissue. Levels of adiponectin, in contrast to leptin, negatively correlate with adiposity. Adiponectin functions to counter the metabolic program associated with obesity and hyperleptinemia by modulating glucose metabolism, increasing fatty acid oxidation and insulin sensitivity, and decreasing production of inflammatory cytokines [20]. The possible mechanisms through which adiponectin exerts anticancer effects may include increasing insulin sensitivity, and decreasing insulin/IGF-1 and mTOR signaling via activation of AMPK. Adiponectin also reduces proinflammatory cytokine expression via inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [21–23].

In vitro, animal and epidemiologic evidence linking leptin [23–25] or adiponectin [21, 26–28] individually to cancer risk is mixed. Associations between the adiponectin-to-leptin ratio and the metabolic syndrome [29–31] and some cancers [32–34] are reported. Further characterization of these links is needed.

3 Chronic Inflammation

3.1 Cytokines and Cross Talk Between Adipocytes, Macrophages, and Epithelial Cells

Obesity and metabolic syndrome are associated with a low-grade, chronic state of inflammation characterized by increased circulating free fatty acids and chemoattraction of immune cells (such as macrophages that also produce inflammatory mediators) into the local milieu [35–37]. These effects are further amplified by the release of inflammatory cytokines such as interleukin (IL)-1β, IL-6, TNF-α, and monocyte chemoattractant protein (MCP)-1. Adipocytes can enlarge past the point of effective oxygen diffusion, which results in hypoxia and eventually necrosis. Free fatty acids escape the engorged/necrotic adipocytes and deposit in other tissues, which in turn promotes insulin resistance and diabetes (through down-regulation of insulin receptors and glucose transporters), hypertension, and fatty liver disease and also activates signaling molecules involved in epithelial carcinogenesis, such as NF-kB [38].

NF-κB is a transcription factor that is activated in response to bacterial and viral stimuli, growth factors, and inflammatory molecules (e.g., TNF-α, IL-6, and IL-1β) and is responsible for inducing gene expression associated with cell

proliferation, apoptosis, inflammation, metastasis, and angiogenesis. Activation of NF-κB is a common characteristic of many tumors and is associated with insulin resistance and elevated circulating levels of leptin, insulin, and/or IGF-1 [39, 40].

3.2 Inflammation and Cancer

The link between chronic inflammation and cancer development was first noticed nearly 150 years ago by Rudolph Virchow when he observed an abundance of leukocytes in neoplastic tissue [41]. Now, inflammation is a recognized hallmark of cancer, and growing evidence continues to indicate that chronic inflammation is associated with increased cancer risk [42–44]. Several tissue-specific inflammatory lesions are established neoplastic precursors for invasive cancer, including gastritis for gastric cancer, inflammatory bowel disease for colon cancer, and pancreatitis for pancreatic cancer [45, 46].

Tumor microenvironments are composed of multiple cell types including epithelial cells, fibroblasts, mast cells, and cells of the innate and adaptive immune system [46, 47]. As discussed previously, macrophages, which are activated in the obese state, infiltrate tumors and amplify the inflammatory tumor microenvironment, often through NF-kB-dependent production of cytokines and angiogenic factors [46]. Another important cancer-related inflammatory mediator is cyclooxygenase (COX)-2, an enzyme that is upregulated in most tumors and catalyzes the synthesis of the potent inflammatory lipid metabolite, prostaglandin E_2. COX-2 overexpression is an indicator of poor prognosis in multiple cancer types [48].

In some cancers, inflammatory conditions precede malignant changes (as previously mentioned), whereas, in other cancer types, genetic alterations and premalignant changes precede the inflammatory microenvironment and neoplasia [44]. Malignancies may thus be initiated or exacerbated by inflammation, and increased levels of inflammation markers may be a cause and/or consequence of cancer [43, 44]. In either scenario, the inflammatory microenvironment exerts tumor-promoting effects, with dysregulated inflammation pathways implicated in genetic instability and also cell proliferation, survival, angiogenesis, and metastasis associated with cancer [42, 43, 49].

4 Microenvironmental Factors

4.1 Vascular Perturbations

VEGF, a heparin-binding glycoprotein produced by adipocytes and tumor cells, has angiogenic, mitogenic, and vascular permeability–enhancing activities specific for endothelial cells [50]. Circulating levels of VEGF are increased in obese, relative to lean, humans and animals, and increased tumoral expression of VEGF is associated with poor prognosis in several obesity-related cancers [51]. The need

for nutrients and oxygen triggers tumor cells to produce VEGF, which leads to the formation of new blood vessels to nourish the rapidly growing tumor and may facilitate the metastatic spread of tumors cells [50].

Adipocytes communicate with endothelial cells by producing a variety of proangiogenic and vascular permeability–enhancing factors. These include VEGF, IGF-1, PAI-1, leptin, hepatocyte growth factor, and fibroblast growth factor-2 [52]. In the obese, nontumor setting, these factors stimulate neovascularization in support of the expanding fat mass. These adipose-derived factors may also contribute to obesity-associated enhancement of tumor angiogenesis. Bevacizumab-based therapy (i.e., anti-VEGF therapy), in combination with conventional chemotherapy, is considered a first-line treatment option for patients with advanced colorectal cancer; however, decreased efficacy in obese patients is reported and speculated to be associated with increased levels of VEGF (and/or other proangiogenic factors) produced by visceral white adipose tissue [53, 54]. The relative contributions of tumor-derived versus adipocyte-derived proangiogenic factors in tumor development, progression, and metastasis remain unclear.

PAI-1 is a serine protease inhibitor produced by endothelial cells, stromal cells, and adipocytes in visceral white adipose tissue [55]. Increased circulating PAI-1 levels, frequently found in obese subjects, are associated with increased risk of atherogenesis and cardiovascular disease, diabetes and several cancers [4, 55]. PAI-1, through its inhibition of urokinase-type and tissue-type plasminogen activators, regulates fibrinolysis and integrity of the extracellular matrix. PAI-1 is also involved in angiogenesis and thus may contribute to obesity-driven tumor cell growth, invasion, and metastasis [4]. Although PAI-1 levels in obese individuals may be reduced via weight loss or TNF-α blockade [56, 57], the role of PAI-1 in tumorigenesis remains controversial [55].

4.2 Epithelial-to-Mesenchymal Transition

Mouse model studies of cancer typically involve xenotransplantation of human tumor cell lines into immunodeficient mice [58, 59]. However, xenograft models are extremely limited due to their lack of normal tumor microenvironment. Immunodeficient mice have aberrant mammary gland development, lack normal immune/inflammatory responses, and resist developing DIO and CR phenotypes, which prevents elucidation of the link between energy balance and cancer development/progression in this system. One approach to overcome these limitations is the development of syngeneic transplant models in immunologically intact animals for studying the energy balance–cancer link. For example, a recent publication from our laboratory describes the development and characterization of a transplant model of claudin-low and basal-like mammary cancers, which overcomes many existing limitations of xenograft models by using (a) cells derived from a spontaneous MMTV-Wnt-1 mouse mammary tumor that, like basal-like breast cancers in women, are responsive to CR and obesity, and (b) a wild-type,

syngeneic host with normal immune function, mammary gland development and metabolic responses to energy balance modulation [60].

Our findings in this model indicate a mechanistic link between energy balance, the epithelial-to-mesenchymal transition (EMT), and tumor initiating cells (TICs) in breast cancer progression [60]. We speculate that DIO prepares fertile soil (tumor microenvironment), including changes in EMT, intratumoral adipocytes, and local and systemic hormones, growth factors, and cytokines, for enhanced tumor progression, and that determinants of growth in this fertile soil include the plant variety (intrinsic breast cancer subtype) and/or the seed density (extent of TIC enrichment). In contrast, CR may discourage tumor progression by acting on the soil antithetically to DIO, including promoting epithelial differentiation, discouraging EMT, preventing intratumoral adipocytes infiltration, and decreasing systemic hormones, growth factors, and cytokines. Future studies are warranted to determine whether the tumor-enhancing effects of DIO depend on the extent of TIC enrichment in different subtypes of breast cancer, as well as other epithelial cancers, and whether CR targets different (and perhaps TIC-independent) pathways than DIO to impact breast cancer progression.

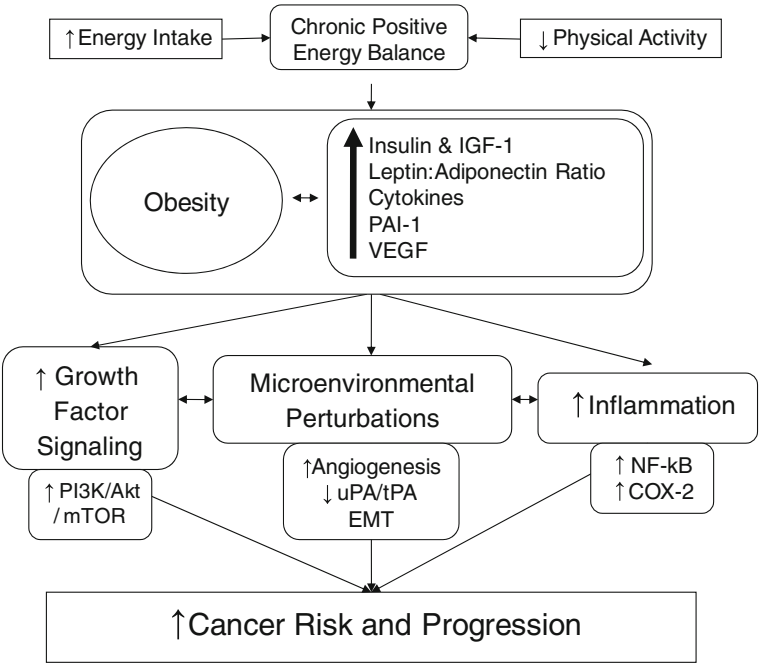

Fig. 1 Obesity and cancer: overview of mechanisms. An arrow preceding text denotes a directional effect (e.g., activity or concentration). Abbreviations: *IGF-1* insulin-like growth factor-1; *PAI-1* plasminogen activator inhibitor-1; *tPA* tissue-type plasminogen activator; *uPA* urokinase-type plasminogen activator; *VEGF* vascular endothelial growth factor; *EMT* epithelial-to-mesenchymal transition

Components of EMT may thus represent novel targets for preventing and/or controlling cancer and novel biomarkers of response to energy balance modulation or other interventions, particularly in obese women. It is plausible that energy-responsive growth signaling pathways regulate tumor cell differentiation and TIC proliferation through upregulation of obesity-associated serum hormones and cytokines, including those already described in this review. For example, increased circulating IGF-1 is associated with increased risk for pre- and postmenopausal breast cancer [61, 62] and increased tumor growth in animal models [16, 17, 63]. IGF-1 also plays a major role in stimulating expansion of normal breast stem cell populations during mammary gland development [64]. Additionally, IGF-1 receptor signaling potentiates SLUG-mediated EMT in hepatocytes [65] and increases ZEB1 mediated EMT in prostate cancer cells [66], although the relationship between IGF-1 receptor and EMT in breast cancer has not previously been reported. Metformin, a standard drug for diabetes that increases insulin sensitivity and lowers circulating insulin levels, effectively inhibits proliferation and survival of TICs [67, 68]. Obesity-associated increases in serum leptin levels have been previously shown to correlate with breast cancer risk and prognosis [69]. Leptin activates Hedgehog signaling and alters gene expression programs that may contribute to cell fate and EMT in hepatic stellate cells [70].

5 Summary and Conclusions

Obesity often results from chronic positive energy balance due to excessive energy intake and/or decreased energy expenditure (Fig. 1). Metabolic consequences include increased circulating levels of insulin and bioavailable IGF-1, and altered levels of adipokines, cytokines, and proangiogenic/vascular integrity factors. Activation of the pathways downstream of these critical systemic regulators leads to enhanced growth factor signaling, vascular perturbations, inflammation, as well as cancer-associated changes in the microenvironment, such as EMT, and thereby may increase cancer development and/or progression. **Components of these interrelated pathways represent promising mechanism-based targets for lifestyle or pharmacologic interventions to prevent or control cancer in obese or otherwise metabolically dysregulated individuals.** To accelerate the pace to identify new mechanism-based intervention targets to prevent or control obesity-related cancers, additional study is required to establish the causal relationships between specific components of obesity-responsive growth signaling, inflammation, and microenvironmental regulating pathways and cancer.

Acknowledgments Dr. S. Hursting is funded, in part, by grants from the National Cancer Institute (R01CA129409 and R01CA135306), the Breast Cancer Research Foundation (UTA09-001068), and the National Institute of Environmental Health Sciences (P30ES007784).

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Fruits and Vegetables: Updating the Epidemiologic Evidence for the WCRF/AICR Lifestyle Recommendations for Cancer Prevention

Teresa Norat, Dagfinn Aune, Doris Chan and Dora Romaguera

Abstract

The World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) current dietary recommendations for cancer prevention include "eating at least five portions/servings of a variety of non-starchy vegetables and or fruits every day". The most recent report coordinated by WCRF/AICR (2007) concluded that the evidence of a protective effect of fruits and vegetables on cancer was either "probable"—mouth, pharynx and larynx, oesophagus stomach, lung- or "limited suggestive"—nasopharynx, lung, colorectum, ovary, endometrium, pancreas, liver-. In a previous report published by WCRF/AICR in 1997, the evidence of the association of fruits and vegetables with cancer risk was considered convincing. This judgement was based mainly on the results of case-control studies. The association of fruit and vegetable intake and the risk of colorectal, breast and pancreatic cancer was re-examined in the Continuous Update Project (CUP) and the results were quantitatively summarised in meta-analyses. The CUP, with more data available, has confirmed the conclusion of the WCRF/AICR second expert report that there is no convincing evidence that fruits and vegetables play a role on cancer aetiology. On the other hand, evidence that is more consistent has been collected in the CUP about the role of dietary fibre and colorectal cancer. The evidence on the role of dietary fibre in colorectal cancer aetiology has been recently upgraded by the CUP expert panel from probable to convincing.

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_3,

Keywords
Lifestyle recommendation · Cancer prevention · Cancer risk

Abbreviations

WCRF/AICR	World Cancer Research Fund/American Institute for Cancer Research
CUP	Continuous Update Project
SLR	Systematic Literature Review
RR	Relative Risk
EPIC	European Investigation into Cancer and Nutrition

Contents

1 Introduction

In 2007, the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) launched the Second Expert Report "Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective" [24]. The Second Expert Report includes ten recommendations for cancer prevention, based on a comprehensive review of the scientific evidence in this area. The report was followed by a second publication entitled "Policy and Action for Cancer Prevention: Food, Nutrition, and Physical Activity—a Global Perspective" [25], which includes recommendations for policies and actions to politicians, policymakers, health professionals and others. The recommendations are based on the principle that patterns of food consumption and physical activity are determined by social, cultural, political and economic factors.

The Second Expert Report represents the most authoritative review of the topic ever produced. The process of the Second Expert Report was initiated with the elaboration by a group of experts (Task Force) of a detailed methodology for conducting systematic reviews of observational epidemiological studies and intervention studies on the aetiology of cancer in terms of food, nutrition and physical activity [23, http://www.dietandcancerreport.org/cancer_resource_center/downloads/SLR_Manual.pdf]. The feasibility, utility and reproducibility of the specification manual were tested and compared between two independent academic institutes, using endometrial cancer as a test case [22]. The epidemiological evidence of the relationship of food, nutrition and physical activity, and the risk of seventeen cancer sites, cancer survival and the determinants of obesity was systematically reviewed in nine academic or research institutions from Europe and the United States.

The systematic reviews of epidemiologic, experimental and laboratory studies constituted the basis upon which an international panel of experts commissioned by WCRF/AICR judged and graded the evidence on the role of foods, nutrition, physical activity and body fatness on cancer aetiology and established personal recommendations and public health goals for cancer prevention. National and international reports that had made recommendations for the prevention of other diseases were also taken into consideration, so that the recommendations for cancer prevention could be set in a broad public health context. Before any evidence was presented, the panel agreed a set of minimum criteria for each grade. The grades were *convincing; probable; limited; and substantial effect on risk unlikely*. The limited category was further divided into *limited suggestive* where it was suggestive of a causal relation and *limited no conclusion* where no conclusion could be drawn. In addition, to provide flexibility, certain characteristics of the evidence were used to upgrade or downgrade conclusions. The conclusions provided the basis for making recommendations, and the recommendations were based only on the conclusions for which the likely causality was judged probable or convincing.

The second expert report features eight general and two special recommendations (Table 1). The recommendations have a broad-based approach for public health purposes; they are formulated as advice on foods and diets for the prevention of cancer as a whole rather than on specific nutrients and particular cancer sites.

Nevertheless, the scientific evidence is continually accumulating, and new studies need to be considered in the context of the existing evidence. To ensure that the evidence is regularly updated in the future, WCRF/AICR is undertaking the Continuous Update Project (CUP) in collaboration with researchers from Imperial College London.

Table 1 2007 World Cancer Research Fund/American Institute for Cancer Research Personal Recommendations for Cancer Prevention

Recommendations
BODY FATNESS
Be as lean as possible within the normal range of body weight
Ensure that body weight throughout childhood and adolescent growth projects towards the lower end of the normal BMI range at age 21
Maintain body weight within the normal range from age 21
Avoid weight gain and increases in waist circumference throughout adulthood
PHYSICAL ACTIVITY
Be physically active as part of everyday life
Be moderately physically active, equivalent to brisk walking, for at least 30 min every day
As fitness improves, aim for $\geq$ 60 min of moderate or for $\geq$ 30 min of vigorous physical activity every day
Limit sedentary habits such as watching television
FOODS AND DRINKS THAT PROMOTE WEIGHT GAIN
Limit consumption of energy-dense foods. Avoid sugary drinks
Consume energy-dense foods sparingly
Avoid sugary drinks
Consume 'fast foods' sparingly, if at all
PLANT FOODS
Eat mostly foods of plant origin
Eat at least five portions/servings (at least 400 g or 14 oz) of a variety of non-starchy vegetables and of fruits every day
Eat relatively unprocessed cereals (grains) and/or pulses (legumes) with every meal
Limit refined starchy foods
People who consume starchy roots or tubers as staples also to ensure intake of sufficient non-starchy vegetables, fruits and pulses (legumes)
ANIMAL FOODS
Limit intake of red meat and avoid processed meat
People who eat red meat to consume less than 500 g (18 oz) a week, very little if any to be processed
ALCOHOLIC FOODS
Limit alcoholic drinks
If alcoholic drinks are consumed, limit consumption to no more than two drinks a day for men and one drink a day for women
PRESERVATION, PROCESSING, PREPARATION

(continued)

Table 1 (continued)

Recommendations
Limit consumption of salt
Avoid mouldy cereals (grains) or pulses (legumes)
Avoid salt-preserved, salted or salty foods; preserve foods without using salt
Limit consumption of processed foods with added salt to ensure an intake of less than 6 g (2.4 g sodium) a day
Do not eat mouldy cereals (grains) or pulses (legumes)
DIETARY SUPPLEMENTS
Aim to meet nutritional needs through diet alone
Dietary supplements are not recommended for cancer prevention
BREASTFEEDING
Mothers to breastfeed; children to be breastfed
Aim to breastfeed infants exclusively up to six months and continue with complementary feeding thereafter
CANCER SURVIVORS
Follow the recommendations for cancer prevention
All cancer survivors to receive nutritional care from an appropriately trained professional
If able to do so, and unless otherwise advised, aim to follow the recommendations for diet, healthy weight and physical activity

From [24]

2 The Continuous Update Project

The CUP is an ongoing systematic literature review on food, nutrition, physical activity and body fatness and cancer risk [http://www.wcrf.org/cancer_research/cup/index.php]. In order to ensure a consistent approach to reviewing the evidence, the CUP follows a scientific process of systematic literature reviews (SLRs) of the published evidence, similar to the process used to inform the WCRF/AICR Second Expert Report. Also, as part of the CUP process, WCRF/AICR has convened an independent panel of experts (the CUP panel), consisting of leading scientists in the field of diet, physical activity, obesity and cancer who will consider the evidence produced by the CUP SLRs together with the scientific evidence from mechanistic studies and draw conclusions before making recommendations. A separation between those who collect and summarise the evidence and those who judge it has as its objective that the CUP provides an impartial analysis and interpretation of the data as a basis for reviewing and where necessary revising the 2007 WCRF/AICR's cancer prevention recommendations.

Most of the recommendations of the second expert report were based on the results of prospective studies and randomised controlled trials, the two study designs on top of the hierarchy of evidence [15]. There is also extensive evidence that case-control studies and cohort studies in nutrition and cancer do not provide concordant results [20]. For that reason, the CUP will systematically review the evidence from cohort studies and randomised controlled trials. Case-control studies, cross-sectional studies and ecological studies will not be included in the reviews.

A feature of the CUP is the continuous update of a large database containing the results of all published cohort and randomised controlled trials on food, nutrition, physical activity and body fatness in relation to cancer risk (Fig. 1). The first step in the continuous update of the CUP database was to combine into one unique database the databases produced by the cancer-site systematic reviews and to standardise all the collected information to a common format. The CUP database constitutes the largest existing database of data on epidemiological data on nutrition and cancer. The database is the data source for the CUP meta-analyses that inform the expert panel.

3 Epidemiologic Evidence of the Relationship of Fruits and Vegetables and Cancer Risk

In 2003, a large systematic review on fruit and vegetable intake and the risk of cancer concluded that the overall data provided by case-control studies supported the view that fruit and vegetable intake was associated with a reduced risk of cancers of the oesophagus, lung, stomach and colorectal; breast cancer risk was inversely associated with the intake of vegetables but not with fruits; bladder cancer was associated with fruit but not with vegetable intake. However, the data from cohort studies although suggestive of a protective effect of both fruits and vegetables for most of the cancer sites considered were not as convincing as that from case-control studies. The only significant associations from cohort studies were for lung and bladder cancer and fruit intake [20]. The discrepancy of the overall results of case–control and cohort studies may be due to recall and selection biases. On the other hand, it was also possible that some associations may have been underestimated in prospective studies as a consequence of the combined effects of imprecise dietary measurements and limited variability in dietary intakes within each cohort.

In 2007, with more available evidence from cohort studies, the WCRF/AICR second expert report concluded that the evidence that fruit and vegetables intake modifies the risk of cancer was not convincing (Table 2). Fruit and vegetables intake was judged as probably related to the risk of cancers of the mouth, pharynx, larynx, oesophagus and stomach; fruit intake as probably related to the risk of lung cancer; foods containing carotene probably related to prostate cancer and foods

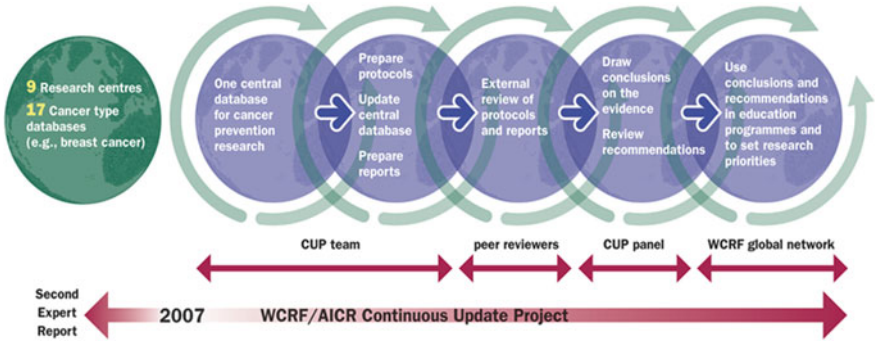

Fig. 1 Process of the Continuous Update Project

containing folate probably related to pancreatic cancer. Most of these judgements were based on the results of case–control studies.

In an integrated approach which considers that most diets related to a decreased risk of cancer are mainly made up of plant foods [24], the expert panel elaborated a recommendation on plant foods, which included consuming a variety of non-starchy vegetables and fruits every day and unprocessed cereals and pulses and limiting the consumption of refined starchy foods.

4 Updated Evidence from the Continuous Update Project on Plant Foods and Cancers of the Breast, Colorectum and Pancreas

The CUP SLRs on breast, colorectum and pancreatic cancers have been conducted by reviewers at Imperial College London. During the systematic literature review for the WCRF/AICR second expert report (until June 2006), the search and data extraction of published articles were conducted by reviewers of the Istituto Nazionale Tumori, Milan, Italy (for breast cancer), Wageningen University, the Netherlands (colorectal cancer), and the University of Leeds, UK (pancreatic cancer). The information collected in these centres has been included in the CUP-updated reviews.

The methods used for the CUP meta-analyses are described in detail elsewhere [11]. Brief, random effects models were used to calculate summary RRs and 95 % CIs for the highest versus the lowest level of intake and for the dose–response analysis [13]. The average of the natural logarithm of the RRs was estimated, and the RR from each study was weighted by the inverse of its variance. The method described by Greenland and Longnecker [14] was used in dose–response analysis to compute linear trends and 95 % CIs from the natural logarithms of the RRs and the CIs across categories of intake. When some data were missing, a number of approaches implemented in the search literature review for the WCRF/AICR Second Expert Report were used to derive the information required to estimate the

Table 2 Judgement of the panel of the WCRF/AICR second expert report on the grade of evidence of the association of fruits and vegetables and the risk of cancer

Grade of evidence	Decreases risk	
	Exposure	Cancer site
Convincing	There is no convincing evidence that fruits and vegetables modify the risk of cancer	
Probable	Non-starchy vegetables	Mouth, pharynx, larynx,
		Oesophagus
		Stomach
	Allium vegetables	Stomach
	Garlic	Colorectum
	Fruits	Mouth, pharynx, larynx,
		Oesophagus
		Lung
		Stomach
	Foods containing folate	Pancreas
Limited suggestive	Non-starchy vegetables	Nasopharynx
		Lung
		Colorectum
		Ovary
		Endometrium
	Carrots	Cervix
	Fruits	Nasopharynx
		Pancreas
		Liver
		Colorectum

Adapted from [24]

dose–response slope and its confidence interval from the available data where possible [8]. Eighty grams was used as a serving size for recalculation of the intakes to a common scale [grams per day (g/day)] in studies that reported intakes as frequency, as used in the previous search literature reviews of fruit and vegetable intake and cancer risk conducted for the second expert report [7]. Heterogeneity between studies was assessed using Q and I^2 statistics [17] Stata software was used for the statistical analyses.

- Continuous update on breast cancer, fruits and vegetables

In the 2007 WCRF/AICR report, the evidence for an association between fruit and vegetable intake and breast cancer risk was judged too limited or inconsistent for a conclusion to be made.

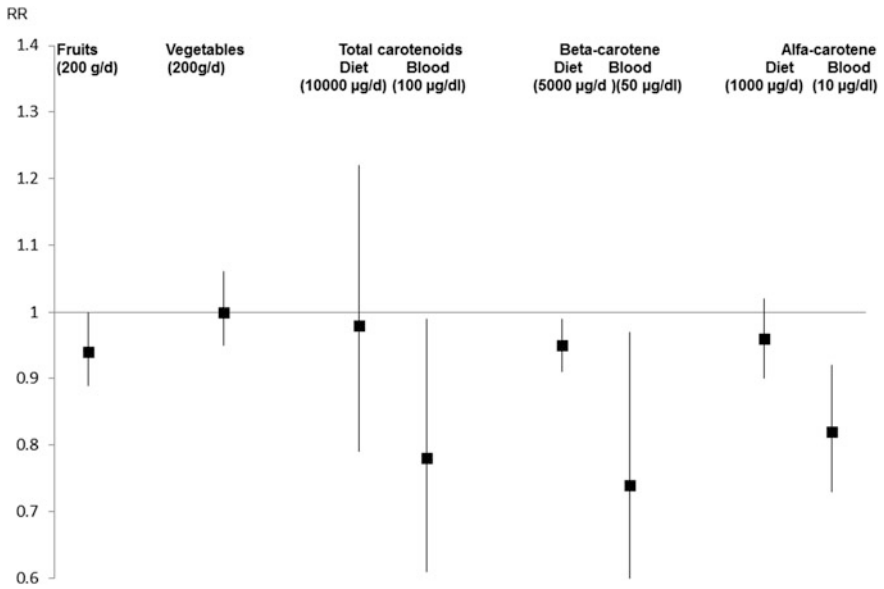

Fig. 2 Summary results of linear dose–response meta-analyses of breast and colorectal cancers and dietary intake of total fibre and by food source. Relative risks and confidence intervals per 10 g/day increase (figures based on data from the CUP [1, 2])

The updated CUP review of fifteen prospective studies published until April 2011 does not provide significant evidence that fruit and vegetable consumption may prevent breast cancer risk [3, 4]. A weak inverse association with fruit intake was observed when comparing the highest category of intake versus the lowest (relative risk [RR] = 0.92; 95 % CI = 0.86–0.98) with no evidence of heterogeneity. In linear dose–response models, a modest 6 % decrease in breast cancer risk for an increase of 200 g of fruit intake that was borderline statistically significant was estimated (RR = 0.94; 95 % CI = 0.89–1.00). No association was shown with vegetable intake in dose–response models (RR = 1.00; 95 % CI = 0.95–1.06 for an increase of 200 g of vegetable intake) or when comparing the highest versus the lowest category of consumption (RR = 1.00; 95 % CI = 0.95–1.06) [4].

Several observational studies have reported stronger associations between diet and disease endpoints when using biomarkers of diet, compared to the associations estimated using questionnaire data on diet [9, 16, 18]. These results suggest that some diet–disease associations might have been underestimated because of diet measurement error [12]. The meta-analyses of dietary carotenoids and blood carotenoids in the CUP are consistent with this observation (Fig. 2). In the CUP meta-analyses, breast cancer was not associated with dietary carotenoids (RR for 10,000 µg/d = 0.98; 95 % *CI* = 0.79–1.22), whereas a significant association with blood carotenoid levels was observed (RR = 0.78; 95 %CI = 0.61–0.99 for

100 μg/dl) [3]. Inverse significant associations were also observed for blood alpha- and beta-carotene, and these were stronger than the associations observed with the intake of the same macronutrients estimated with questionnaires. Carotenoids have been widely measured as dietary biomarkers of fruit and vegetable intake because their blood concentrations appear to be moderately correlated with diet (correlation ranges from more 0.2 to over 0.5). Carotenoids are lipid soluble, and their bioavailability may be modulated by the lipid content of the diet and other host factors and, presumably, genetic variability. Circulating blood levels of carotenoids probably provides a good estimation of their bioavailability, or overall level of body exposure, in different populations (*reviewed in* [19]).

It is also possible that the association of fruits and vegetables varies depending on the characteristics of the breast tumours. In the CUP, there were not enough published studies to conduct meta-analyses stratified by breast cancer tumour characteristics. The Pooling Project of Cohort Studies including 33,380 incident cases of breast cancer from 18 prospective cohort studies reported that intakes of alpha-carotene, beta-carotene and lutein/zeaxanthin were inversely associated with the risk of oestrogen-receptor-negative but not with the risk of oestrogen-receptor-positive breast cancers. In the studies participating in the project, the intake of carotenoids was estimated using data from questionnaires [26].

Fruits and vegetables, together with cereals, are the main sources of dietary fibre in many populations. In the CUP-updated review on breast cancer, breast cancer risk was inversely associated with dietary fibre (RR per 10 g/day = 0.95; CI = 95 % CI = 0.91–0.98). Inverse although non-significant associations were observed for fibre from fruits (RR = 0.88; 95 % CI = 0.75–1.03) and fibre from cereals (RR = 0.91; 95 % CI = 0.79–1.04), whereas no association was observed with fibre from vegetables (RR = 0.97; 95 % CI = 0.55–1.12) [1].

- Continuous update on colorectal cancer, fruits and vegetables

In the 2007 WCRF/AICR report, the panel judged that there was limited evidence that fruits and vegetables protect against colorectal cancer. There were a substantial number of studies on fruits, vegetables and colorectal cancer, but their results were not entirely consistent.

The updated CUP review on colorectal cancer identified 19 prospective studies published up to 31 December 2010. The overall results of cohort studies showed a weak association of colorectal cancer with fruit intake when comparing the highest versus the lowest levels of intake reported in the articles (RR = 0.90; 95 % CI = 0.83–0.98), but there was no dose–response relationship (RR per 100 g/day = 0.98; 95 % CI = 0.94–1.01). An inverse dose–response association with vegetable intake was observed (RR per 100 g/day = 0.98; 95 % CI = 0.97–0.99) as well as when comparing the highest versus the lowest category of exposure (RR = 0.91; 95 % CI = 0.86–0.96). The association of fruits and vegetables and colorectal cancer risk was also explored using fractional polynomial models [6]. The nonlinear models showed that the dose–response associations were more evident at very low levels of intake, suggesting that individuals with very low intake of fruits and vegetables might be at a modest increased risk of colorectal

Table 3 Convincing and probable conclusions from the Continuous Update Project report on colorectal cancer

Food, nutrition, physical activity and cancers of the colon and rectum		
	Decreases risk	Increases risk
Convincing	Physical activity	Red meat
	Foods containing dietary fibre	Processed meat
		Alcoholic drinks (men)
		Body fatness
		Abdominal fatness
		Adult-attained height
Probable	Garlic	Alcoholic drinks (women)
	Milk	
	Calcium	

From WCRF/AICR Continuous Update Project 2011, http://www.wcrf.org/cancer_research/cup/colorectal_cancer.php

cancer and that little benefit with respect to colorectal cancer would be observed by further increasing fruits and vegetables above 100 g/day [5].

With respect to dietary fibre, in the WCRF/AICR second expert report, a high intake of foods containing fibre was judged as probably protective against colorectal cancer. Because the studies on fibre intake and colorectal cancer had provided inconsistent results, and fibre intake tends to be related to healthy lifestyles, the panel acknowledged the possibility that the observed associations could at least be partially explained by residual cofounding.

The updated CUP meta-analysis of 16 prospective studies confirmed that the risk of colorectal cancer is inversely related to the intake of dietary fibre (RR per 10 g/day = 0.90; 95 % CI = 0.86–0.94) [2]. In CUP meta-analyses by food source of fibre, the inverse association was restricted to fibre from cereals (RR per 10 g/day = 0.90; 95 % CI = 0.83–0.97); an inverse association was observed with fibre from fruits, but it was not statistically significant (RR per 10 g/day = 0.93; 95 % CI = 0.82–1.05). No association with fibre from vegetables was observed (RR per 10 g/day = 0.98; 95 % CI = 0.91–1.06) (Fig. 2). The available data on dietary fibre and colorectal cancer were overall consistent; no significant heterogeneity across study results was detected ($I^2 = 0$ %). In view of the CUP results, in 2011, the CUP panel of experts modified the judgement of the 2007 WCRF/AICR second expert report panel. The evidence that foods containing dietary fibre protect against colorectal cancer was upgraded from probable to convincing (Table 3).

- Continuous update on pancreatic cancer, fruits and vegetables

The CUP systematic review on pancreatic cancer was updated with results of studies published up to 31 December 2011. During the CUP, the number of publications from prospective studies on pancreatic cancer increased substantially compared to the number of studies available for the reviews of the second expert report published in 2007. Ten cohort studies on vegetables and twelve on fruits and pancreatic cancer incidence and mortality were identified, from which eight studies were published after the second expert report.

The CUP meta-analysis studies do not provide evidence that fruit or vegetable intake is related to pancreatic cancer. The relative risks for an increase of 100 g were 1.00 (95 % $CI = 0.96–1.03$) for vegetables and 1.00 (95 % $CI = 0.95–1.05$) for fruits (unpublished data).

5 Does Adherence to the WCRF/AICR Recommendations Decrease the Risk of Cancer in a Population?

The study type that could provide direct evidence of the causal relationship underlying the assumption that adherence to the WCRF recommendations may modify cancer risk in a population is randomised controlled trials. However, such trials are not feasible, not only because of the time and resources needed to obtain a sufficient number of cases in the study population, but also because of the methodological difficulties in applying and maintaining the intervention, measuring its compliance, controlling for other related factors that may be modified during the intervention and other issues in measuring the effect in an unbiased manner. Instead, it is possible to obtain indirect evidence from observational prospective studies on whether individuals with lifestyles in concordance with the WCRF/AICR recommendations are at a lower risk of cancer. This has been explored in the European Prospective Investigation into Nutrition and Cancer (EPIC), a prospective study in 521,330 men and women, aged 25–70 years, who were recruited between 1992 and 2000 in ten European countries: Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom. At recruitment, participants completed extensive medical, dietary and lifestyle questionnaires, including questions on alcohol use, smoking status, physical activity, education, reproductive history, breastfeeding, exogenous hormone use and previous illnesses. Body weight and height were measured in most centres, and usual food intakes were measured by using country-specific validated dietary questionnaires. The details of the EPIC study have been described in detail elsewhere [10].

In order to assess the concordance with the WCRF/AICR recommendations, a score of concordance with the recommendations was developed and operationalised in 386,355 EPIC participants using the data collected at participants' recruitment [21]. The relationship of the score at baseline and subsequent cancer risk was investigated. After a median follow-up time of 11 years, 36,994 cancer cases were identified in EPIC. The results suggest that following the WCRF/AICR recommendations on diet, nutrition, physical activity and weight management for

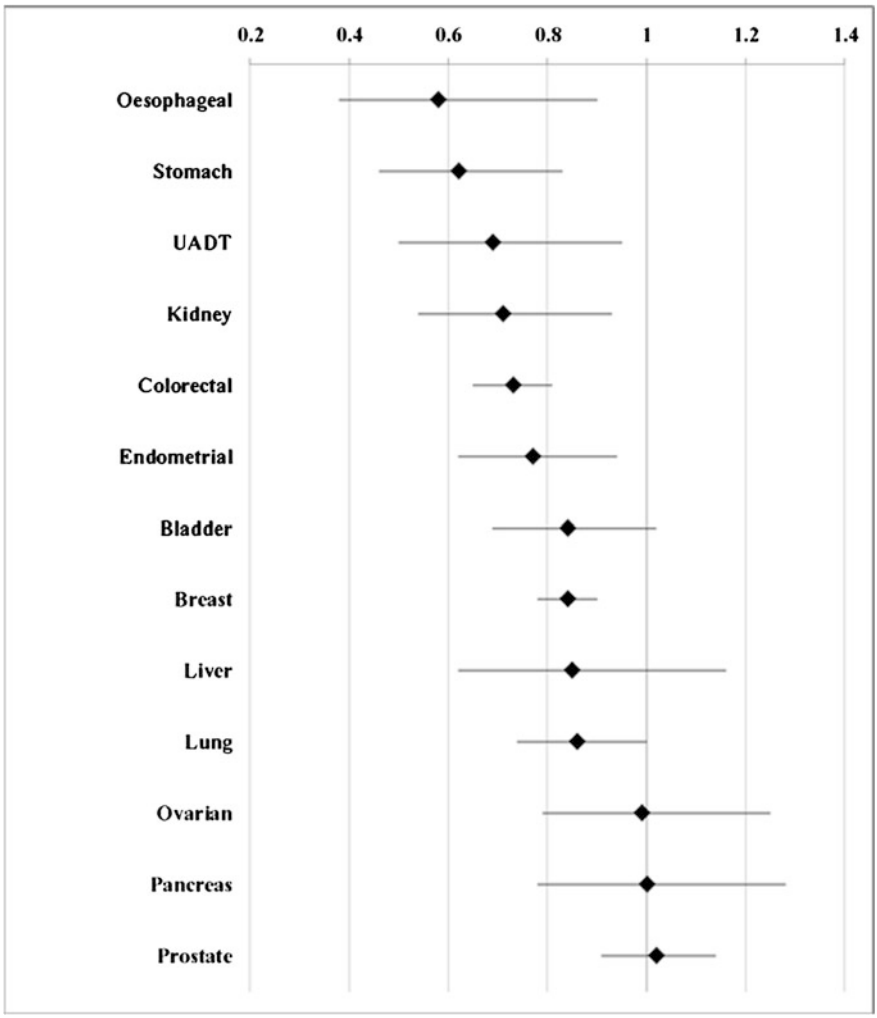

Fig. 3 Hazard ratios (95 % CIs) for specific cancer types associated with the highest versus lowest score of concordance with the WCRF–AICR recommendations in the EPIC population (based on data from [21])

cancer prevention are associated with a lower risk of developing most types of cancer. Participants within the highest category of the WCRF/AICR score ($\geq$5 points in men and $\geq$6 points in women) were 18 % less likely to develop any cancer compared with those in the first category of the score ($\leq$2 points in men and $\leq$ 3 points in women). The concordance with the WCRF/AICR recommendations assessed by the score was predictive of the risk of cancers of the upper aerodigestive tract, oesophagus, stomach, colorectum and endometrium and of breast cancer (Fig. 3). The WCRF/AICR score did not predict the risk of cancers

of the prostate, ovary, pancreas and bladder. These null associations are consistent with the judgements in the second expert report.

Amongst the score components, the concordance with the recommendations on plant foods was the strongest predictor of cancer risk. Participants within the highest category of the plant food component of the score ($\geq$5 points in men and $\geq$6 points in women) were 11 % (95 % CI = 6–16 %) less likely to develop any cancer compared with those in the first category of the score ($\leq$2 points in men and $\leq$3 points in women) [21]. The relative risk reductions estimated for other components ranged between 4 and 6 %. The recommendations on food that promote weight gain and breastfeeding were not related to overall cancer risk after adjustment for other components of the score. The analyses were adjusted for smoking, educational attainment and, in women, for ever use of oral contraceptives, hormone replacement therapy, age at first menarche, age at first pregnancy and menopausal status.

6 Final Considerations

The 2007 WCRF/AICR Second Expert Report concluded that there was no convincing evidence to support the view that fruits and vegetables may protect against cancer. The CUP, with more data, has confirmed the conclusions of the WCRF/AICR Second Expert Report.

However, the existing evidence collected before and during the CUP indicates that higher blood carotenoids are related to a decreased risk of breast cancer, but this association is not observed in most studies on dietary carotenoids. This discrepancy may be due to measurement errors of diet and points to the need to identify more biomarkers of diet and to improve methods for dietary assessment. On the other hand, the results of the CUP support that individuals with a low intake of fruits and vegetables might be at an increased risk of colorectal cancer. The CUP collected and summarised convincing data, showing that fibre intake plays a role in colorectal cancer prevention.

The operationalisation of a score based on the WCRF/AICR recommendations in the population participating in EPIC, a large prospective study on European populations, showed that adhering to the WCRF/AICR recommendations may contribute to prevent the risk of cancer in the population and that the score component with the highest predictive value was based on the recommendation on plant foods.

By design, randomised controlled trials are the type of studies that should be conducted to explore the cause–effect relationship required to demonstrate the preventability of cancer by dietary modifications. However, methodological issues make randomised controlled trials often unfeasible and inadequate for this purpose. Trials on specific nutrients may be important to investigate biological pathways and mechanisms, for which the identification of biomarkers of nutrient availability and of intermediate endpoints will be required.

Prospective studies investigating diet and cancer also have limitations, such as the measurement error of the dietary questionnaires, the limited number of validated biomarkers of dietary intake and the lack of repeated dietary assessment during follow-up. However, as shown in the extensive literature reviews conducted in the CUP and previous reports, prospective studies are currently the most important source of evidence on the relationship of cancer risk with food, nutrition, physical activity and body fatness.

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The Diet as a Cause of Human Prostate Cancer

William G. Nelson, Angelo M. DeMarzo and Srinivasan Yegnasubramanian

Abstract

Asymptomatic prostate inflammation and prostate cancer have reached epidemic proportions among men in the developed world. Animal model studies implicate dietary carcinogens, such as the heterocyclic amines from over-cooked meats and sex steroid hormones, particularly estrogens, as candidate etiologies for prostate cancer. Each acts by causing epithelial cell damage, triggering an inflammatory response that can evolve into a chronic or recurrent condition. This milieu appears to spawn proliferative inflammatory atrophy (PIA) lesions, a type of focal atrophy that represents the earliest of prostate cancer precursor lesions. Rare PIA lesions contain cells which exhibit high c-Myc expression, shortened telomere segments, and epigenetic silencing of genes such as *GSTP1*, encoding the π-class glutathione S-transferase, all characteristic of prostatic intraepithelial neoplasia (PIN) and prostate cancer. Subsequent genetic changes, such as the gene translocations/deletions that generate fusion transcripts between androgen-regulated genes (such as *TMPRSS2*) and genes encoding ETS family transcription factors (such as *ERG1*), arise in PIN lesions and may promote invasiveness characteristic of prostatic adenocarcinoma cells. Lethal prostate cancers contain markedly corrupted genomes and epigenomes. Epigenetic silencing, which seems to arise in response to the inflamed microenvironment generated by dietary carcinogens and/or estrogens as part of an epigenetic "catastrophe" affecting hundreds of genes, persists to drive clonal evolution through metastatic dissemination. The

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_4,

cause of the initial epigenetic "catastrophe" has not been determined but likely involves defective chromatin structure maintenance by over-exuberant DNA methylation or histone modification. With dietary carcinogens and estrogens driving pro-carcinogenic inflammation in the developed world, it is tempting to speculate that dietary components associated with decreased prostate cancer risk, such as intake of fruits and vegetables, especially tomatoes and crucifers, might act to attenuate the ravages of the chronic or recurrent inflammatory processes. Specifically, nutritional agents might prevent PIA lesions or reduce the propensity of PIA lesions to suffer "catastrophic" epigenome corruption.

Keywords

Prostate · Proliferative inflammatory atrophy · Heterocyclic amines · Epigenetics · DNA methylation

Abbreviations

PSA	Prostate-specific antigen
BPH	Benign prostatic hyperplasia
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PhIP	Phenylimidazopyridine
GST	Glutathione *S*-transferase
TLRs	Toll-like receptors
COX	Cyclooxygenase
NSAIDs	Non-steroidal anti-inflammatory drugs

Contents

Prostate cancer is the most common cancer diagnosed in men in the United States (US) and in Western Europe. Once rare in the rest of the world, the disease appears also to be on the rise throughout Asia and in many other developing nations. Diet

and lifestyle, along with risk factors such as age, family history, and sex steroid hormones, have long been thought to contribute to prostatic carcinogenesis [57]. However, more recently, molecular pathology insights have indicted chronic or recurrent epithelial cell injury, accompanied by innate and adaptive inflammatory responses, in the early steps of prostate cancer development [18]. As a consequence, dietary components capable of inducing such injury, such as the heterocyclic amines created by over-cooking meats, loom large as candidate prostate carcinogens, while dietary components able to limit cell and genome damage and/or attenuate prostate inflammation, may protect against prostate cancer development. The mechanism(s) by which dietary components, inherited susceptibility, and sex steroid hormones cause epithelial damage and/or drive inflammatory processes that lead to cancer as men age, if better understood, could provide new opportunities for prostate cancer prevention, improved prostate cancer screening strategies, and perhaps even better prostate cancer treatment outcomes.

1 Proliferative Inflammatory Atrophy: A Lesion that Links Epithelial Injury to Prostate Cancer

In regions of the world with high prostate cancer incidence, prostate inflammation is essentially ubiquitous [18]. Though mostly asymptomatic, particularly if affecting the peripheral zone of the prostate where cancers arise, prostatitis has long been known to drive prostate cancer diagnoses independent of its propensity to cause the disease, because it tends to elevate serum prostate-specific antigen (PSA) levels. In the inflamed prostate, damage to the barrier function of the prostate epithelium stereotypically causes backflow of prostate secretions, including secreted proteins like PSA, into the prostate parenchyma and ultimately into the bloodstream. Because the detection of PSA in serum serves as the primary trigger of prostate biopsy for prostate cancer detection and diagnosis, prostate inflammation is responsible for a significant fraction of more than 30 million PSA tests, leading to more than a million prostate biopsies looking for cancer, performed in the United States each year [36]. Nonetheless, serum PSA elevations as early as age 40 years are associated with an increased risk of prostate cancer later in life [23, 24].

This tendency for prostate inflammation to elevate serum PSA has greatly undermined attempts to test causal associations between prostatitis and prostate cancer in population studies. Restricting cancer association studies to symptomatic prostatitis has not been very helpful either. Symptomatic prostatitis typically reflects inflammation of the transition zone near the urethra where benign prostatic hyperplasia (BPH) arises, leading to irritative voiding symptoms which prompt as many as 2 million physician visits in the United States each year, each accompanied by serum PSA tests [50]. As a consequence, apparent correlations between symptomatic prostatitis and prostate cancer in epidemiologic studies, which are numerous, have been frequently attributed to the bias that such men might be more

likely subjected to prostate biopsy [19]. In addition, since symptomatic prostatitis does not reflect inflammation in the prostate peripheral zone where cancers arise, the condition presumably serves as a poor surrogate for pro-carcinogenic inflammatory processes. To circumvent these limitations and more directly test whether peripheral zone inflammation might be correlated with prostate cancer, an analysis of end-of-study prostate biopsies collected from placebo-treated subjects participating in the Prostate Cancer Prevention Trial of finasteride for asymptomatic men with serum PSA values <3 ng/mL was undertaken [83]. The results of this study, expected soon, should yield definitive insight into whether the presence of inflammation is directly associated with cancer within prostate glands.

Most of the inflammation in the prostate is a consequence of damage to the prostate epithelium, which can be caused by dietary carcinogens, estrogens, and inflammatory oxidants [18]. Microscopic examination of prostate tissues, particularly tissues from glands which contain prostate cancers, yields evidence of longstanding chronic or repeated tissue insults and innate immune responses. As an example, large numbers of corpora amylacea, microscopic laminated bodies containing calprotectin, myeloperoxidase, and α-defensins, from neutrophil granules, are often spread out through such prostate glands [71]. These bodies form as a consequence of neutrophil discharge during acute inflammation, and remain, like spent ammunition casings, even after the acute inflammatory process has subsided. Of interest, prostate tissues from rats prone to prostate inflammation and to prostate cancer also contain abundant corpora amylacea [32]. Characteristically, when the prostate epithelium suffers injury, focal atrophy lesions with dilated glandular lumens and immature epithelial cells appear [16, 17]. The epithelial cells act as if the normal secretory cell differentiation pathway has been abandoned in favor of a stress response with induced expression of α- and π-class glutathione *S*-transferases, cyclooxygenase-2, and other mediators of genome damage defense and cell survival [60, 86, 94]. Focal atrophy lesions, surrounded by corpora amylacea, mark prostate tissues that have suffered cell and tissue damage, and define a pro-carcinogenic milieu.

Proliferative inflammatory atrophy (PIA) lesions (Fig. 1), which comprise a subset of focal atrophy-type injury responses in the prostate, exhibit very high epithelial proliferation rates and infiltration by inflammatory cells [67]. This type of epithelial damage response is reminiscent of a number of other conditions in other organ sites, such as atrophic gastritis, hepatitis, and cirrhosis, that are known to lead to cancer development. Not surprisingly, PIA lesions also appear to be cancer precursor lesions, giving rise to prostatic intraepithelial neoplasia (PIN) or to prostate cancer directly [64]. The evidence that epithelial damage, leading to PIA, may initiate prostate cancer development, has accumulated over the past decade or so to include: (1) the detection of somatic genome and epigenome defects in PIA lesions that are identical to those seen in PIN and prostate cancer, (2) the visualization of direct morphological transitions between PIA and PIN and between PIA and prostate cancer, (3) the frequent appearance of somatic genetic and epigenetic alterations characteristic of PIN and cancer in PIA lesions, often to an intermediate degree between normal and neoplasia, (4) the greater prevalence

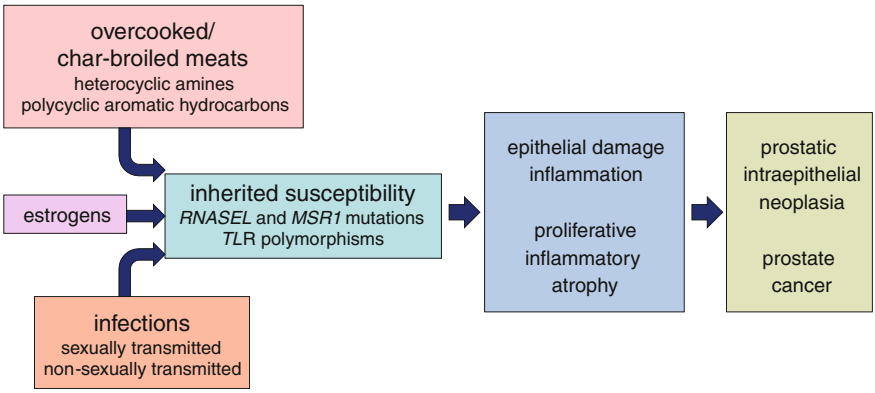

Fig. 1 Etiological factors for prostate cancer. Prostatic carcinogenesis follows damage to the prostate epithelium, regenerative proliferation, and chronic/recurrent inflammation, leading to proliferative inflammatory atrophy lesions. These lesions sprout cells with c-Myc activation, telomere shortening, and epigenetic gene inactivation, which give rise to prostatic intraepithelial neoplasia and prostate cancer, carrying targeted gene rearrangements, mutations, and an extensively corrupted epigenome

of PIA lesions in prostate peripheral zone regions in areas of the world with high prostate cancer risk, and (5) the propensity for preclinical models of prostatic carcinogenesis, including those using dietary carcinogens, to first show PIA lesions before cancers arise [18].

Prostate damage, followed by a maladaptive innate immune response and tissue injury response, may be the pathway that connects environmental exposures, including the diet, to prostate cancer development. Certainly, both in population studies and in molecular pathology analyses, the association between prostate inflammation, PIA, and prostate cancer is clear. Yet, the mechanisms by which dietary habits, or other exposures, lead to prostate damage have not been fully elucidated.

2 The Diet: A Source of Carcinogens that can Damage the Prostate Epithelium and Cause PIA

Epidemiology studies of prostate cancer have strongly implicated the diet as a major modulator of prostate cancer risk. Prostate cancer incidence and mortality varies among different geographic regions, with high prostate cancer risk in the United States and in Europe and low prostate cancer risk in Asia, yet immigrants from low-risk regions to high-risk regions typically adopt higher prostate cancer risks, particularly with cultural assimilation [27, 73]. This likely reflects dietary differences: either dietary habits in high-risk regions promote prostate cancer, dietary habits in low-risk regions prevent prostate cancer, or both. When examined in greater detail, the most consistent dietary association for prostate cancer appears

to be intake of red meats and/or animal fats [26, 43]. For red meats, cooking at high temperatures or char-broiling creates both heterocyclic aromatic amine and polycyclic aromatic hydrocarbon carcinogens [39, 44]. These cooking practices are also associated with an increased prostate cancer risk and may partially explain an increased propensity for prostate cancer development among African–American men versus Caucasian men in the United States [37, 82].

The best studied of these carcinogens for prostate cancer is 2-amino-1-methyl-6-phenylimidazopyridine (PhIP), the most abundant of the >20 heterocyclic amines that can appear in over-cooked meats [8, 85, 87]. In rat models, PhIP ingestion causes PIA and prostate cancer [74]. By itself, PhIP is non-toxic and non-mutagenic, but after activation, first to *N*–OH-PhIP by CYP1A1 or CYP1A2 in the liver or elsewhere and then to more reactive species in prostate epithelial cells by sulfotransferases or by a kinase/phosphatase, PhIP can cause marked prostate epithelial cell damage, elicit inflammatory responses, and form pro-mutagenic adducts with DNA [51]. Epithelial injury, accompanied by an inflammatory response, is a critical feature of PhIP prostate carcinogenesis in rats. Rats fed PhIP show genome mutations in ventral, dorsolateral, and anterior prostate lobes, but exhibit epithelial cell damage, inflammation, and PIA in only the ventral lobes [51]. For epidemiology studies, precise estimates of PhIP exposure from food frequency questionnaires, even with added questions about food preparation preferences, have proven difficult. This is likely because cooking practices can generate a wide variation of heterocyclic amine levels, though frequent intake of meat by men who prefer the meats to be well-done, pan-fried, or grilled, appears to be accompanied by an increased risk for prostate cancer [15]. More recent studies have suggested that germ line variants in PhIP-metabolizing enzymes, including cytochromes p450 enzymes, sulfotransferases, and UDP-glucuronide transferases, might affect prostate damage upon over-cooked meat consumption [40].

The influence of meat consumption may not be restricted to the initiation step of prostate cancer development. The natural history of human prostate cancer encompasses many years, with small prostate cancers appearing as early as age 20–30 years in the United States in autopsy studies, diagnoses of localized cancers seen at age 60–70 years, and death from prostate cancer occurring at age 70 years and older. This provides a large window of opportunity for chronic PhIP intake to influence the pathogenesis of prostate cancer. The rat models of PhIP prostate cancer development feature carcinogen exposure for a limited period of time after puberty, followed by observation for many months, isolating the action of PhIP on the initiation and early promotion steps of carcinogenesis [74]. However, during the pathogenesis of human prostate cancer, the most common somatic gene defect, epigenetic silencing of *GSTP1*, results in loss of π-class glutathione *S*-transferase (GST) expression, a phenotype that may be remarkably sensitive to PhIP-mediated cell and genome damage [56]. This change first appears in PIA lesions and persists in prostate carcinoma cells throughout metastatic dissemination [52, 91]. Thus, not only might PhIP cause the epithelial injury that leads to the formation of PIA lesions, but PhIP might also continue to inflict cell and genome damage for decades, driving ongoing malignant prostate cancer progression.

3 Non-Dietary Exposures that Cause Prostate Epithelial Damage, Prostate Inflammation, and PIA

Sex steroid hormones, infections, and inheritance have all been thought to influence prostate cancer development. Androgenic hormones, such as testosterone and dihydrotestosterone, along with a functioning androgen receptor, are needed for normal growth and development of all sex accessory glands, including the prostate and seminal vesicles, but there is little evidence that androgens cause prostate cancer *per se*. Androgen levels decline steadily throughout life in adult men, reaching a peak around age 21 years and falling thereafter, as prostate cancers begin to arise [66, 68]. In the United States, African-American men suffer more prostate cancers than Caucasians, despite similar age-adjusted androgen levels [66]. Also, in the prostates of adult men, androgen signaling is required for terminal differentiation to the columnar secretory epithelial cell phenotype, promoting the transcription and translation of genes like *PSA* and *TMPRSS2* and driving the production of secretions for the ejaculate. By acting in this way, androgens tend to suppress epithelial cell proliferation.

Of course, androgen signaling does play a significant role in the progression of established prostate cancers. The mechanism for this appears to involve the acquisition of somatic genome translocations and deletions which create fusion transcripts formed from androgen-regulated differentiation genes, such as *TMPRSS2* and others, and oncogenes, such as *ERG* and *ETV1* of the ETS family of transcription factors [84]. With these somatic genome defects, prostate cancer cells co-opt androgen signaling for the maintenance of a neoplastic phenotype. This may be the mechanistic basis for the frequent responses of advanced prostate cancers to androgen deprivation or to antiandrogens: interference with androgen signaling results in a reduction in the levels of *TMPRSS2-ERG* or other fusion transcripts in prostate cancer cells, attenuating cell growth and limiting cell survival. Remarkably, while androgens may act more to drive prostate cancer progression than to trigger prostate cancer initiation, new data have suggested that the initiation of androgen-target gene transcription might involve induction of DNA double-strand breaks by androgen receptor-associated TOP2B, a topoisomerase capable of resolving tangles in DNA, directed to sites in *TMPRSS2* most often involved in translocations and deletions [28]. These breaks, which lead to *TMPRSS2-ERG* fusions, likely occur after the emergence of PIA lesions, perhaps driving PIN cells to become more invasive carcinoma cells. Topoisomerases prevent DNA tangling by catalyzing DNA breakage/rejoining reactions to permit strand passage. The enzymes are well-known to be sensitive to many compounds which can disrupt rejoining reactions and create recombinogenic DNA double-strand breaks. However, whether dietary components are responsible for any TOP2B-mediated DNA double-strand breaks during the pathogenesis of prostate cancer has not been reported.

Unlike androgens, estrogens may act to damage the prostate epithelium and promote the early steps of prostatic carcinogenesis. Breast cancers and prostate cancers are generally coincident throughout the world, leading to the hypothesis that estrogens might cause both diseases [14]. This contention is largely supported by rodent models, where estrogen exposures lead both to prostate inflammation and to prostatic cancer [59]. As an example, exposure of adult male Wistar rats to 17ß-estradiol results in prostate inflammation whether or not dihydrotestosterone is also given [55]. Male rodents given perinatal or neonatal estrogen exposures manifest prostatitis in adulthood [55, 75, 76]. Estrogens likely trigger inflammation in rodent prostates via induction of autoimmunity, as the condition can be induced in non-estrogen-treated rats via adoptive transfer of T-cells from adult male rats given 17ß-estradiol [69]. The mechanisms by which estrogens cause autoimmune prostate inflammation have not been fully elucidated, but may involve pituitary prolactin secretion, differential action of estrogenic hormones on estrogen receptor isoforms in the prostate, and/or reactive oxygen species generation by estrogen redox cycling. Also, the influence of diet habits on estrogen levels in men, largely produced in fatty tissues by aromatase action on androgens, has not been determined. Of note, however, in the United States, African-Americans, with higher prostate cancer risks, tend to have higher estrogen levels than Caucasians [66].

Infectious causes of prostate cancer have been more difficult to pin down. Many infectious agents have been detected in prostate tissue specimens or prostate secretions, but which of these actually cause prostate damage or trigger prostate inflammatory responses has not been systematically determined [70]. The best studied have been sexually transmitted infections, where gonorrhea and chlamydia have been reported to elevate serum PSA levels, an indicator of damage to the epithelial barrier function of the prostate, in at least 32 % of cases [78]. Remarkably, despite effective antibiotic treatment, PSA values can remain elevated after such infections for many months, providing evidence for chronic epithelial damage and dysfunction. This is consistent with population studies finding an increased prostate cancer risk with mild increases in serum PSA at early ages and/or with a history of sexually transmitted infections [23, 24, 79]. This complicates the search for infectious etiologies for prostate cancer in population cohort studies. If a prostate infection can damage the prostate epithelium in a young man, leading to PIA and chronic prostate inflammation even without persistent colonization as the man ages, then definitively establishing which infectious pathogen was responsible for this "hit-and-run" phenomenon will be very difficult. Nonetheless, direct inoculation of rodent prostates with bacteria or viruses triggers marked inflammatory responses and subsequent prostate cancer precursor lesions [21, 22].

Finally, some of the inherited susceptibility to prostate cancer development may be explained by genes encoding participants driving the activation and intensity of innate inflammatory responses. Two such genes, *RNASEL* and *MSR1*, appear responsible for some familial clusters of prostate cancer [11, 90]. *RNASEL* encodes a ribonuclease that participates in an interferon-inducible RNA destruction pathway activated in response to viral infection or other cellular damaging stress; *MSR1* encodes subunits of a macrophage scavenger receptor that binds bacterial

lipopolysaccharide and lipoteichoic acid. Diminished function of either protein in mice reduces the ability to fully clear various infections [80, 96]. In population studies, fairly consistent associations have been seen between prostate cancer and polymorphic variants of genes encoding toll-like receptors (TLRs), such as *TLR4* and the cluster *TLR1-TLR6-TLR10* [77, 95]. TLRs can bind a broad range of pathogens and/or damaged cell components, acting via NF-κB signaling to promote vigorous innate immune responses [12].

Even though estrogens and infections seem to be able to cause prostate epithelial damage which might lead to PIA and prostate cancer in the absence of dietary influences, each of these processes could be impacted by dietary habits common in high-risk prostate cancer regions of the world. Estrogen levels tend to be higher in men with increased fatty tissues. The microbiome, a source of infections or of colonization resistance to infections, varies widely with dietary practices. By influencing the propensity for estrogens and infections to inflict prostate damage, the diet can indirectly act to promote prostate cancer. Similarly, the degree of dietary heterocyclic amine-mediated prostate damage is likely to be subject to the same host genetic factors that regulate the intensity of host responses to prostate infections. RNASEL can degrade human RNA as well as viral RNA, leading to apoptosis [89]. MSR1 helps clear circulating oxidized serum low-density lipoproteins [41]. TLRs are activated by damaged human cell components [12]. Thus, the diet likely exerts direct and indirect effects on human prostate cancer development.

4 Inflammation, PIA, and the Molecular Pathogenesis of Prostate Cancer

Life-threatening human prostate cancer cells contain 3,866 mutations (20 non-silent coding mutations), 108 rearrangements, 5,408 regions with DNA hypermethylation, shortened telomere sequences, and activated c-Myc protein [7, 58, 92]. Notably, the somatic mutations do not seem to have singled out any common "driver" of prostatic carcinogenesis, nor hinted at any base change signature more consistent with one type of carcinogen versus another. Instead, mutations seem to accumulate over time in individual cancers, influenced by whether acquired mismatch repair gene abnormalities have appeared and/or whether pro-mutagenic treatments have been used. The more consistent somatic genetic defect is the translocations described above, particularly those involving gene targets of androgen signaling fused to cancer genes, such as *TMPRSS2-ERG*, which may be attributed to errors in initiation of transcription in response to androgen action leading to TOP2B-associated DNA double-strand breaks [28]. This somatic genome defect appears to occur in PIN lesions and likely underlies the invasiveness characteristic of carcinoma. Of all of the somatic changes in prostate cancer cells, the most consistent and earliest seem to involve epigenetic gene silencing, telomere shortening, and c-Myc induction [58].

Gene silencing, resulting from increased DNA methylation at gene regulatory sequences, is a candidate-initiating event for human prostate cancer. As an example, *GSTP1*, encoding the π-class GST, an oxidant and carcinogen detoxifying enzyme, has been found to be epigenetically silenced in some 5–10 % of PIA lesions, >70 % of PIN lesions, and almost all prostate cancers [9, 52, 58]. *GSTP1* silencing has been attributed to *de novo* DNA hypermethylation at the 5′ regulatory region of the gene [45]. Of note, since such DNA methylation changes are potentially reversible, *that is*, the DNA sequence remains intact; persistent maintenance of *GSTP1* inactivation throughout prostate cancer progression has served as a priori evidence for a selective growth or survival advantage during prostatic carcinogenesis. The mechanism for such a selective advantage has not been elucidated. Certainly, *GSTP1* serves a caretaker gene function during carcinogenesis generally, as human prostate cancer cells devoid of GSTP1 better activate PhIP to cell and genome damaging species that cells with the enzyme, and mice carrying disrupted *Gstp1/2* genes develop more skin tumors in response to topical carcinogens, and more intestinal tumors in the setting of relentless inflammation, than wild-type mice [30, 65]. A clue to a selected phenotype may be in mice carrying *Pb-c-Myc* transgenes that develop prostate cancer [33]. Preliminary data hint that loss of π-class GST function in these mice triggers accelerated prostatic carcinogenesis.

GSTP1 is likely but one of many genes epigenetically silenced early during prostatic carcinogenesis, by some process tied to prostate inflammation and PIA [58]. Unfortunately, as of yet, the mechanism by which epigenetic silencing of any such genes occurs in the inflamed microenvironment of the prostate, or in other organs prone to cancer development, has remained elusive. Nonetheless, inflammation and epigenetic gene silencing appear to be major contributors to the earliest steps of epithelial carcinogenesis generally, with clear examples in inflammatory bowel disease, hepatitis, and gastritis in addition to PIA lesions in the prostate [81]. Presumably, the inflamed microenvironment promotes epigenetic gene silencing and aberrant DNA methylation by acting to influence the regulation of chromatin architecture, by interfering with fidelity of DNA methylation pattern preservation in such way as to promote over-methylation at certain gene sites, or by corrupting both chromatin structure and DNA methylation maintenance. In one mouse model study of intestinal tumorigenesis, new DNA methylation changes emerged in inflamed tissues at the loci of 250 genes, with 70 % of the genes known to be targeted by polycomb complexes for repression [29]. This observation nominates polycomb complex repression as a participant in the establishment of *de novo* DNA methylation changes in the setting of pro-neoplastic inflammation. In this type of mechanism, DNA methylation reinforces polycomb complex repression in some way to maintain epigenetic gene silencing. In support of a complementary type of mechanism, the inflammatory cytokine interleukin 1β was found to trigger gene silencing in certain cells by promoting nitric oxide generation, leading to an over-activation of DNA methyltransferases [31]. Which type of mechanism drives the epigenetic catastrophe in PIA lesions in the prostate is not clear. *GSTP1*, along with other stress-response genes, tends to be induced to high level expression in PIA cells, with rare PIA cells suffering loss of GSTP1

expression and *de novo* methylation [52]. Thus, epigenetic silencing, and DNA methylation, must occur despite transcriptional *trans*-activation.

In addition to DNA methylation, telomere shortening and over-expression of c-Myc protein consistently accompany human prostatic carcinogenesis [33, 49]. The telomeres of chromosomes, specialized structures containing ~2,000 repeats of the sequence 5′-TTAGGG-3′ maintained by the enzyme telomerase, act to prevent loss of DNA sequences which might otherwise occur with lagging-strand DNA synthesis during replication and to reduce illegitimate recombination [48]. Critically, short telomeres, reflecting replication in the absence of the enzyme telomerase or some sort of telomere sequence damage, are characteristic of PIN lesions and prostate cancer cells [49]. Of note, short telomere sequences have also been seen associated with hepatitis and inflammatory bowel disease [2, 38]. Perhaps, oxidants elaborated at sites of inflammation can damage and shorten telomere sequences in the prostate as well. c-Myc expression, also ubiquitous in human prostate cancer cells, can drive prostate tumorigenesis in mice: forced c-Myc expression in the mouse prostate leads to the appearance of neoplastic cells with increased nuclear and nucleolar size, with blunted differentiation, and with diminished expression of Nkx3.1, a prostate-specific homeodomain transcription factor and tumor suppressor [33]. Like telomere shortening, c-Myc over-expression may be influenced by an inflammatory milieu. Mice carrying defective *Apc* genes prone to intestinal tumorigenesis show c-Myc phosphorylation and stabilization in response to exposure to TLR ligands from the intestinal microflora [42].

PIA lesions, which are generated in response to cell and tissue damage accompanied by an induced inflammatory response, link exposures, like dietary carcinogens and estrogens, to prostate cancer. The earliest stereotypical molecular events, epigenetic gene silencing, telomere shortening, and c-Myc activation, arise in PIA lesions. However, the precise mechanisms by which these molecular accidents occur have not been elaborated. Each may have its origins, or at least be influenced, by either the damaging exposure, *for example,* a dietary carcinogen, or by the inflammatory response. In this way, the prostatic carcinogenesis may resemble exposure-driven cancer development in many organ sites.

5 Rational Interventions to Prevent Prostate Cancer

If the epidemic of prostate cancer in the developed world can be explained by exposures to dietary carcinogens and/or estrogens that lead to chronic prostate inflammation, rational prostate cancer prevention approaches should involve: (1) an avoidance of exposures, (2) an attenuation of prostate cell and tissue damage inflicted by carcinogens, and/or (3) a reduction in intensity or duration of inflammation in the prostate. Not surprisingly, epidemiologic and clinical trial evidence has emerged in support of each approach.

A reduction in dietary heterocyclic amine exposure could be accomplished not only by educating individuals to avoid eating over-cooked meats, but also by attempting to modify cooking practices at a population scale. Steaming,

microwaving, and marinating are all known to produce less heterocyclic amines than pan-frying, char-broiling, and retaining pan-dripping [85]. Measuring heterocyclic amine content in marketed cooked foods, along with appropriate incentives, might promote safer cooking practices by restaurants and by grocers. Until this occurs, carcinogen-inflicted prostate damage may be able to be lessened even despite continued consumption of over-cooked meats. Because heterocyclic amine carcinogens, like PhIP, need to be activated by metabolism to trigger cell and genome damage, interference with bioactivation might present an attractive strategy to limit carcinogenesis. Diets rich in inducers of phase 2 metabolic enzyme expression, which activate the Keap1-Nrf2 pathway, both reduce carcinogen damage generally in animal models and lower prostate cancer risk in human epidemiology studies [1, 13, 20]. The foods with the highest levels of phase 2 enzyme inducers, such as the isothiocyanate sulforaphane, are the cruciferous vegetables, like broccoli, Brussels sprouts, cauliflower, and others. In a study of normal human volunteers, intake of cruciferous vegetables reduced PhIP adduction to DNA in response to a cooked meat meal [88]. A reduction in estrogen exposures may be more difficult to achieve. Estrogens are produced via aromatase action and androgens in fatty tissues, a worrisome accompaniment to the obesity epidemic arising in the United States and other developed countries. Also, African-American men have higher estrogen levels than Caucasian men, even though androgen levels are similar and have higher prostate cancer risk [66]. Perhaps, if obesity can be controlled, prostate cancer risk might fall.

Anti-inflammatory approaches to prostate cancer prevention have yielded mixed results. Chronic or recurrent inflammation can generate reactive oxygen and nitrogen species that can cause cell and tissue damage [3, 34]. For this reason, dietary antioxidants or anti-inflammatory agents should act to protect prostate cells from the ravages of ongoing injury even if PIA lesions have already emerged. Certainly, some of the dietary components consumed in more commonly or abundantly in Asia, such as soy, may have anti-inflammatory properties relevant to the prostate; in rat models, soy-rich diets have been shown to reduce prostate inflammation [72]. In addition, prostate cancer epidemiology focused on cohorts in the developed world strongly suggests that inadequate intake of any number of antioxidant micronutrients, including vitamin E, selenium, and lycopenes from tomatoes, results in increased prostate cancer risk [10, 25, 93]. However, a recent large clinical trial, the selenium and vitamin E cancer prevention trial (SELECT), revealed that supplementation with selenium alone (hazard ratio (HR) of 1.04 with 99 % confidence interval of 0.87–1.24), with vitamin E alone (HR of 1.13 with 99 % confidence interval of 0.95–1.35), or with the combination (HR of 1.05 with 99 % confidence interval of 0.88–1.25) did not reduce prostate cancer incidence [46]. Whether this trial truly targeted men with inadequate antioxidant intake, rather than over-supplementing men with adequate intake, has not yet been reported.

Dietary and anti-inflammatory drugs might provide another strategy for lowering prostate cancer risk [6]. Cyclooxygenase (COX) inhibitors, including aspirin and non-steroidal anti-inflammatory drugs (NSAIDs), have been associated with

reduced incidence and mortality of many human cancers [5]. Unfortunately, these drugs also cause gastrointestinal bleeding and cardiovascular events, which even though rare, have limited widespread use for preventing cancer. The use of such drugs for prostate cancer prevention has proven even more problematic. The selective COX-2 inhibitors, including celecoxib and rofecoxib, seemed promising when used in rodent prostate cancer models, but have thus far not been found to be particularly effective in human trials [4, 53, 54]. One difference between the rodents and humans may be that *PTGS2*, which encodes human COX-2, is epigenetically silenced in almost all human prostate cancers, but not in any rodent prostate cancers [91, 94]. Despite this poor track record of success of COX inhibitors for prostate cancer risk reduction, epidemiology studies have detected a consistent inverse correlation between regular aspirin use and prostate cancer [35, 47, 61]. Whether this potential benefit can be attributed to non-selective COX inhibition or to some other anti-inflammatory property of the aspirin has not been resolved. The finding that statin drugs, which may also exhibit anti-inflammatory activity, are associated with lowered prostate cancer risks has also not been fully explained [62, 63].

6 Summary and Conclusions

In response to dietary carcinogens, excess estrogens, or both, chronic or recurrent prostate inflammation, induced by damage to the prostate epithelium, drives prostate carcinogenesis to epidemic rates in the developed world. This pathogenesis mechanism is revealed in the emergence of PIA, precursor lesions for PIN and prostate cancer. Cells in PIA lesions, attempting to regenerate the prostate epithelium despite ongoing inflammatory stresses, appear prone to suffer epigenome corruption, heralded by *GSTP1* silencing and de novo DNA methylation, telomere shortening, and c-Myc activation. Activation of androgen signaling in such cells risks targeted translocations, caused by androgen recruitment of TOP2B to binding sites at the loci of prostate cell differentiation genes, which lead to fusion gene transcripts, such as *TMPRSS2-ERG*, that drive malignant prostate cancer progression. Careful attention, at a population scale, to dietary habits, with changing of cooking practices to limit carcinogen production, with increased intake of cruciferous vegetables to reduce carcinogen bioactivation, and with increased intake of anti-inflammatory and antioxidant micronutrients to attenuate prostate inflammation, might contribute to lowering the societal burden of prostate cancer.<!Query ID="Q1" Text="No Query" –>

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Mediterranean Dietary Pattern and Chronic Diseases

Salvatore Panico, Amalia Mattiello, Camilla Panico and Paolo Chiodini

Abstract

The study of the relationship between the Mediterranean way of eating and the occurrence of diseases typical of the economically developed countries has been considered the starting point of nutritional epidemiology. From the Seven Countries Study in the 1950s to the recent European EPIC collaboration, the evaluation of the components of diet-affecting chronic diseases such as cardiovascular disease and cancer has been crucially based on the analysis of foods and nutrients characterizing the Mediterranean dietary habits. This long research history has been marked by a consistency of data over time when either single nutrients/food groups or more complex dietary patterns have been analyzed: The Mediterranean way of eating is a protective tool from cardiovascular diseases and many cancers. Italy has been a natural point of observation, starting from cardiovascular disease in the mid-1950s and continuing with major cancers. In spite of unfavorable lifestyle changes in the Italian population mostly due to globalization of unhealthy habits (richer diet and lower levels of physical activity), those individuals still close to the Mediterranean style are significantly protected. The very recent Italian data derived from the observation of about 50,000 individuals, participating in the

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_5,

Italian cohorts of the EPIC study, confirm these findings and are consistent with results from other European populations and in some cases also from North American populations. Moreover, several dietary trials suggest that such a way of eating improves both the metabolic risk condition for chronic disease and the occurrence of those diseases. In conclusion, a way of eating inspired by a Mediterranean dietary pattern is not only based on evidence but is also a palatable style that has contributed to protection from the epidemic of chronic diseases.

Keywords
Mediterranean diet · Chronic disease · Nutritional epidemiology

Abbreviation

EPIC European Investigation into Cancer and Nutrition

Contents

Food culture is the marker of any civilization; it is a historical identity derived from the daily lives of diverse populations. Within the context of Euro-Asian human development in the Mediterranean basin, the three-flag foods (wine, olive oil, and bread) were the basic features of that culture and became the heritage until contaminated by the Central–Northern European food culture (meat, lard, and butter) [40]. In spite of continuous cultural food exchange throughout the centuries, differences across Europe have remained, and even in globalized Europe, there are specific food markers for the Mediterranean culture, which identify individuals whose dietary habits adhere to the typical Mediterranean diet.

Table 1 The most frequent dietary components in 4 countries participating in EPIC

Germany	Butter, processed meats, fruit juices
United Kingdom	Tea, cakes, soft drinks, milk, butter
Greece	Vegetable oils, legumes and vegetables, fish
Italy	Vegetable oils, cereals and derived products, fruit

1 Dietary Habits in Europe in the Last Few Decades

We know from statistics available all over Europe in the twentieth century that Greek people, particularly in Crete, had the longest life expectancy in the world in the 1950s and 1960s, followed by Southern Italy, Spain, and France [61]. Since the 1950s, Ancel Keys and other colleagues studied the diets of people in the Mediterranean basin in relation to the observation that coronary heart disease was much less frequent in that region compared with Central–Northern Europe and North America. The characteristics of the Mediterranean diet, as determined at that time, were high intakes of cereals, grains, vegetables, dried beans, olive oil, garlic, fresh herbs, seafood, and fruit, with wine taken with food in moderation. Meat and poultry were also eaten in moderation, with poultry more frequently served than red meat. Animal fats in the form of butter, cream, and lard were not included in the diet [10]. In spite of important changes in dietary habits occurring all over Europe that have impressively reduced the differences in food consumption between Mediterranean and non-Mediterranean regions, some specific characteristics have recently been detected by the EPIC study (Table 1), indicating that it is still possible to identify well-known areas where food consumption is typical of some European populations [53].

EPIC is the major ongoing European investigation into nutrition and chronic diseases and is a unique scientific resource for information on cancer and, more recently, cardiovascular disease. The EPIC cohort consists of about 370,000 women and 150,000 men, mainly aged 35–69 years, recruited from 1992 to 1998 in 23 research centers in 10 European countries (Denmark, France, Germany, Greece, Italy, Norway, Spain, Sweden, the Netherlands, and the United Kingdom). Baseline questionnaires included detailed questions about current habitual diet and a supplementary dietary investigation on a sample of the entire cohort to minimize measurement errors [47, 48].

2 Keys' Diet–Heart Hypothesis

Ancel Keys, the American physiologist who observed a visible difference between the occurrence of myocardial infarction in the USA and occurrence of myocardial infarction in the Italian and Greek Mediterranean area where he was serving the

US Army during World War II, took the initiative to evaluate in depth the concept that at the population level, a "rich" diet (typical of non-Mediterranean countries) might be linked to higher plasma cholesterol and to an insidious tendency to coronary heart disease: It was a real breakthrough. He organized a meeting in Naples (Italy) in 1954 to discuss this research undertaking with prominent investigators from several countries [34] whose aim was to organize an investigation to test the diet–heart concept by careful population comparisons. This was the start of the "Seven Countries Study," a longitudinal survey of population samples from Finland, Greece, Italy, Japan, the Netherlands, the USA, and Yugoslavia, with the intention to follow the occurrence of disease in the coming years. After more than 50 years of analyses, the data are still valuable. The diet–heart concept was clearly confirmed by the results of the Seven Countries Study [32, 33]. In cross-population analyses, 10-year coronary mortality rate was significantly related to sample average saturated fatty acid intake in the habitual diet. Similar findings were obtained for serum cholesterol: The higher the sample average cholesterol level, the higher the coronary mortality rate. Finally, dietary saturated fatty acids were also closely related to serum cholesterol. The basic concept of a 3-way relationship was strongly reinforced by these observations: The evidence found is indicative of the fact that dietary lipid is an important determinant of serum cholesterol, and serum cholesterol is an important determinant of coronary heart disease. Several landmark metabolic-ward-controlled feeding trials carried out by Keys and his colleagues produced the data underlying the critically important equation they derived, on the relationship under isocaloric conditions of change in dietary lipids to change in serum total cholesterol: $ATC = 1.35(2\Delta SFA - \Delta PFA) + 1.5\ \Delta CHOL\ 1/2$, where ATC is change in serum cholesterol, ΔSFA and ΔPFA are change in percentage of total dietary calories from saturated and polyunsaturated fatty acids, respectively, and ΔCHOL is change in dietary cholesterol in mg/1,000 kcal. This equation—in continuous use in nutrition investigations—is referred to by all researchers as "Keys equation" [20, 31]. Other historical metabolic ward studies by Keys' group had also demonstrated that calorie balance on a "Western" diet influences blood cholesterol; weight gain increases serum cholesterol, and in obese individuals, weight loss produces a decrease; and that water-soluble fiber in the diet lowers serum cholesterol [7, 30]. All these contributions, produced before 1970, were reproduced by other investigators over time and worldwide. They were crucial to define the concept of major coronary risk factors as the basis for the population-wide prevention of epidemic cardiovascular disease [25]. This paramount work contributed to the Mediterranean diet becoming the "protective standard diet" for cardiovascular disease.

3 Simply an Issue of Dietary Fat and Lipids?

The conspicuous evidence of differences in dietary lipids and blood cholesterol between populations with high frequency and those with low frequency of coronary heart disease had a really profound impact on the history of cardiovascular disease research in etiology: Attention to the totality of dietary components was somehow neglected for years.

In a population-based investigation conducted in the 1980s in Italy (in random samples of the Italian population selected from all over the country), initial results on the relationship between diet and cardiovascular metabolic risk factors highlighted the complexity of interpreting the protective effect of the Mediterranean-style diet. An association was detected between the use of more "atherogenic" food with serum cholesterol and blood pressure, but also with higher levels of blood glucose [55], and an association was also detected with blood glucose when comparing individuals who consumed prevalently olive oil with those consuming prevalently butter [56]. In a cross-cultural trial including individuals living in a Mediterranean region compared with individuals living in Northern Europe and Australia, substituting dietary saturated for monounsaturated fat was found to impair insulin sensitivity [60].

The results of these studies showed the importance of other metabolic pathways associated with dietary habits and cardiovascular risk; the indication was that dietary components affect cardiovascular disease not only through the well-known involvement of lipid metabolism but also through carbohydrates. Numerous metabolic studies have confirmed these observations for cardiovascular disease etiology, as well as several recent investigations which have shown the importance of the carbohydrate metabolic pathway in the etiology and pathogenesis of many cancers, particularly linked to the proliferative effect of growth factors regulated by the action of insulin. These observations suggest the need for a more complex approach to understand the protective effect of diet on chronic diseases, mostly cardiovascular disease and cancer.

4 Dietary Indicators: Food, Nutrients and Patterns in the Study of the Etiology of Chronic Diseases

Traditional analyses in nutritional epidemiology typically examine diseases in relation to a single or a few nutrients or foods. In spite of the important amount of evidence produced by this approach, some conceptual and methodological limitations have to be considered. People do not eat isolated nutrients, but meals including several foods producing complex combinations of nutrients that may be interactive or synergistic [41]. Therefore, the single-nutrient approach may be inadequate. However, analyses based on a large number of nutrients or food items may produce statistically significant associations simply by chance [15]. We also know that some nutrients are highly correlated and that this makes it difficult to

examine their separate effects; this is due to reduction in the degree of independent variation in the nutrients when they are entered into a model simultaneously [36].

It is likely that the cumulative effects of multiple nutrients included in a dietary pattern may be sufficiently large to be detectable, whereas the effect of a single nutrient may be too small to detect [50]. If we evaluate the literature on some important dietary intervention trials, we can observe that altering dietary patterns has appeared to be more effective than single-nutrient supplementation as in the case of blood pressure–lowering trials [8].

Finally, because nutrient intakes are commonly associated with certain dietary patterns [27, 46], single-nutrient analysis may potentially be confounded by the effect of dietary patterns. If low dietary fat is associated with higher intakes of vegetables, fruits, fiber, folate, and whole grains, it may be that because intakes of vegetables, fruits, fiber, folate, and whole grains (as a food pattern) are independently associated with a reduced risk of coronary heart disease, these dietary components are potential confounders when the relationship between dietary fat and coronary disease is taken into consideration [59]. Interaction between dietary components is an obstacle to removing confounding efficiently so that any attempt to control this by statistical analysis may be unsuccessful. One solution might be the use of overall dietary pattern designed to consider how foods and nutrients are consumed in combinations [2, 13, 21, 23, 24, 26, 28, 57]. In analyses with dietary patterns, the colinearity of nutrients and foods is used advantageously because patterns are characterized on the basis of eating behavior [22].

5 Mediterranean Scores and Chronic Disease

As pointed out in the previous paragraph, the use of a complex index to identify dietary patterns is the way to group individuals into food-eating categories, which better characterizes their dietary habits. Scientific literature provides several examples of dietary pattern indicators; in this paper, we will focus our report on Mediterranean pattern indicators and chronic diseases.

The investigators of the Seven Countries Study developed the mediterranean adequacy index (MAI) to define the healthiness of a diet as assessed in 1960 in Nicotera, a Southern Italian rural community, where a pilot study of the Seven Countries Study was conducted [4, 5, 17].

In the Seven Countries Study, a strong inverse relationship between MAI and coronary heart disease mortality in 25 years was found [18]. In the two Italian rural cohorts of the Seven Countries Study, MAI showed the protective effect of a healthy Mediterranean dietary pattern versus the occurrence of fatal CHD events at 20 and 40 years [39].

Analyzing the longevity of an elderly Greek cohort, the investigators who developed the MEDSCORE (an index of Mediterranean adherence based on the components of the Greek diet) found that the greater the adherence to Mediterranean–Greek diet, the higher the survival [57]. A modified version of the Greek

index (m-MEDSCORE) has been used in several analyses in the EPIC studies. The modification was made to take into account the dietary features of Central and Northern European populations, yet maintaining the overall capability to distinguish between more and less adherence to the Mediterranean-type diet. High values of m-MEDSCORE were found to be more important in positively influencing longevity than the single nutrients in European countries [58]. The use of the score in the Spanish EPIC cohort has suggested that there is an inverse association between adherence to the Mediterranean diet and the incidence of fatal and non-fatal CVD in initially healthy middle-aged adults [37].

The EPIC study has also provided an important piece of evidence on the relationship between adherence to the Mediterranean diet measured through m-MEDSCORE and all cancers: The higher the score, the lower the risk of cancer [11]. Applying the m-MEDSCORE to breast cancer incidence data in a cohort of Asian-American women, a protective effect of high adherence to the Mediterranean diet was detected [63]. Moreover, recent evidence has been provided on diet and cognitive decline using the m-MEDSCORE: High levels of the index have been found in association with reduced risk of white matter detected through magnetic resonance scanning of the brain [19].

In the European cohorts of EPIC, several analyses were carried out to evaluate the relationship between m-MEDSCORE and some metabolic features, including metabolic syndrome, a condition known to indicate high-normal level of insulin resistance. High levels of m-MEDSCORE have been found to be associated with a lower increment of waist circumference over time [49], indicating that the weight gain of the Mediterranean populations detected in recent years [16, 42] is due to high energy intake (quantity and not quality of the food) and to some Westernization of their dietary habits [35], which have attenuated the "Mediterranean advantage" [45]. Moreover, a lower prevalence of metabolic syndrome was found to be associated with reduced endometrial [12], colorectal [6] and breast cancer [1]. This may indirectly confirm a role for the Mediterranean diet on the risk of those cancers. Convincing evidence is provided by recent meta-analyses, indicating that the components of metabolic syndrome are significantly reduced by a Mediterranean diet in comparison with a low-fat diet [43] and that metabolic syndrome is less frequent in individuals eating according to a Mediterranean pattern [29].

6 Mediterranean Dietary Patterns and Chronic Diseases in Recent Population Studies in Italy

The ongoing largest prospective investigation on the etiology of chronic diseases (mainly cancer and cardiovascular disease) in Italy is the Italian EPIC cohort. From 1993 to 1998, 47,749 volunteers (15,171 men and 32,578 women) were recruited to EPIC Italy from five centers: two in Northern Italy (Varese, $n = 12{,}083$ and Turin, $n = 10{,}604$), one in Central Italy (Florence, $n = 13{,}597$),

Table 2 The Italian Mediterranean Index: components producing scores

Positive score if highly consumed	Pasta
Positive score if highly consumed	Typical Mediterranean vegetables (i.e., raw and cooked leafy vegetables, raw tomatoes, onion and garlic, and all the other vegetables excluding cabbages and root vegetables)
	Fruit
	Legumes
	Olive oil
	Fish
Positive score if lowly consumed	Soft drinks
	Butter
	Red meat
	Potatoes
Positive score if moderately consumed	Alcohol

and two in Southern Italy (Ragusa, $n = 6403$ and Naples, $n = 5062$). All exposure variables and the procedures to detect cancer incidence and mortality were performed as in the European investigation [48]. It is worth remembering that diet was assessed using semi-quantitative food frequency questionnaires designed to capture local dietary habits [44]. EPICOR is the cardiovascular diseases section of EPIC Italy; specific validated procedures to detect the incidence of coronary and cerebrovascular events were designed and applied [2, 9, 14, 51].

In order to evaluate the role of dietary patterns on chronic diseases, an Italian Mediterranean Index was developed. Through the analysis of this index, a score is produced, which is based on intake of 11 items: high intakes of 6 typical Mediterranean foods (pasta; typical Mediterranean vegetables, that is, raw and cooked leafy vegetables, raw tomatoes, onion and garlic, and all the other vegetables excluding cabbages and root vegetables; fruit; legumes; olive oil; and fish); low intakes of 4 non-Mediterranean foods (soft drinks, butter, red meat, and potatoes); and also alcohol. If consumption of typical Mediterranean foods was in the third tertile of the distribution, the person received 1 point; all other intakes received 0 points. If consumption of non-Mediterranean foods was in the first tertile of the distribution, the person received 1 point. Ethanol received 1 point for intake up to 12 $g \cdot d^{-1}$; abstainers and persons who consumed >12 $g \cdot d^{-1}$ received a 0 (Table 2).

The Italian index is somewhat different from the Greek MEDSCORE. A major difference is the presence, among the components, of pasta, the typical Italian food [2]. Anthropology is binding: The food culture of Italians is markedly characterized by pasta; this is true especially for the Mediterranean part of the country. At the time of the reunification of Italy (1860–1861), the Prime Minister of the Kingdom of Savoia (Camillo Benso di Cavour, living in Turin in Northern Italy)

waiting for the conquest of Southern Italy by Giuseppe Garibaldi wrote to his Ambassador in Paris these codified words: "in a short time we will all eat macaroni" [40]. The recognized high quality of dietary information in EPIC is matched by high-quality information on other relevant variables in lifestyle, which are important considerations in the analysis. Taking into account several of these variables, the results are quite impressive as to the potentially protective role of Mediterranean dietary pattern on cardiovascular disease and cancer. Analyses performed using the Italian Mediterranean Index are coherent with those of other groups of dietary components typical of the Mediterranean diet.

For coronary heart disease in women, a protective effect for high versus low consumption of leafy vegetables and olive oil has been found [9]. Preliminary data presented at a scientific meeting suggest that the Italian Mediterranean index is inversely related to coronary heart disease incidence (fatal and non-fatal), close to statistical significance in women. Moreover, an indicator of a diet rich in high-glycemic index food (high-glycemic load), which is not typical of the isocaloric Mediterranean diet, has been found to be inversely related to coronary heart disease incidence, with more than 50 % significant excess risk in those consuming carbohydrates belonging to the high-glycemic index food [51].

For stroke, a reduced risk has been found in individuals scoring high in the Italian Mediterranean Index in comparison with those scoring low: significant relative risk: 0.47 for all types of stroke and 0.37 for ischemic stroke [2]. As an indirect confirmation, total antioxidant capacity determined in foods has been found to be protective: significant relative risk 0.57 in the comparison between upper and lower tertiles [14].

For colorectal cancer, a reduced significant risk has been detected for those scoring high in the Italian Mediterranean Index compared with those scoring low: relative risk 0.54 in men and 0.46 in women [3].

For breast cancer, a number of coherent results have been produced. In part of the cohort (the Milano cohort), data presented at a scientific meeting indicated that the Italian Mediterranean Index had been found to be significantly related to a reduced risk (relative risk 0.64 between upper and lower tertiles). As for coronary heart disease, the glycemic load of the diet has been found to be significantly inversely related to risk (1.46 for high- versus low-glycemic load) [52]. Following the same direction, the analysis of vegetable consumption has provided evidence for a significant protection for all types of vegetables (0.65 for all vegetables; 0.70 for leafy vegetables) [38].

7 Final Comments

The long history of the relationship between the Mediterranean diet and cardiovascular disease etiology is corroborated by recent data, and this is particularly true in Italy. In spite of unfavorable overall dietary changes in the Italian population, those individuals still close to the Mediterranean style are significantly

protected. The good news is that the same pattern is also protective for frequent cancers (i.e., breast and colorectal cancers). The Italian data are consistent with data from other European populations and in some cases also from Northern American populations: A comprehensive meta-analysis corroborates these findings [54]. As a result of this evidence, WHO strongly supports dietary modification in the direction of a Mediterranean style to prevent chronic diseases [62].

In conclusion, a way of eating inspired by a Mediterranean dietary pattern is not only based on evidence but is also a palatable style that has contributed to protection from the epidemic of chronic diseases.

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Dietary Salt Intake and Risk of Gastric Cancer

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Abstract

Humans began to use large amounts of salt for the main purpose of food preservation approximately 5,000 years ago and, although since then advanced technologies have been developed allowing drastic reduction in the use of salt for food storage, excess dietary salt intake remains very common. Gastric cancer is a common neoplasia, and dietary factors, including salt consumption, are considered relevant to its causation. A number of experimental studies supported the cocarcinogenic effect of salt through synergic action with Helicobacter pylori infection, in addition to some independent effects such as increase in the rate of cell proliferation and of endogenous mutations. Many epidemiological studies analyzed the relationship between excess salt intake and risk of gastric cancer. Both cross-sectional and prospective studies indicated a possibly dose-dependent positive association. In particular, a comprehensive meta-analysis of longitudinal studies detected a strong adverse effect of total salt intake and salt-rich foods on the risk of gastric cancer in the general population. Altogether, the epidemiological, clinical, and experimental evidence supports the possibility of a substantial reduction in the rates of gastric cancer through progressive reduction in population salt intake.

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_6,

Keywords
Salt intake · Gastric cancer · Risk · Food storage · Helicobacter pylori · Risk factor

Abbreviations

HP	*Helicobacter pylori*
COX-2	Cyclooxygenase-2
iNOS	Inducible nitric oxide synthases
CagA	Cytotoxin-associated gene A
MNNG	Methylnitronitrosoguanidine
MNU	N-nitroso-N-methylurea

Contents

1 Introduction

Sodium is the most abundant electrolyte in extracellular fluid and as such has a major role in the regulation of body fluid volume, plasma osmolality, and cell membrane potential. In the course of evolution, the human body has developed powerful mechanisms to preserve its sodium and chloride content and to maintain the sodium concentration in the extracellular fluid within a relatively narrow range [1]. Up to approximately 5,000 years ago, the sodium content in the human diet was extremely low since the salt content of natural foods is quite modest [2]. Switching to a much higher salt intake occurred as a result of the discovery that the addition of salt to many foods was useful to preserve them from rapid deterioration [3]; this high salt intake has been sustained since then despite the widespread availability of modern technologies for food preservation. The current unjustifiably large excess in dietary salt intake has meanwhile been recognized as a major worldwide hazard to community health being causally associated with epidemic disorders such as hypertension [4, 5], cardiovascular accidents [6], renal dysfunction [7], nephrolithiasis [8], and osteoporosis [9].

Gastric cancer is a common neoplasia and is the third leading cause of death from cancer worldwide [10, 11, 12]. Although there are large geographic and ethnic differences in its incidence and its overall rate has been slowly decreasing over recent decades, this disease is still an important public health burden and, at least partly, a preventable public health problem throughout the world [10, 11, 12]. Evidence has been provided for more or less strong associations between gastric cancer and a few dietary factors, among which is the dietary salt content and the habitual consumption of salt-rich foods. In this regard, many case-control studies detected an adverse effect of high salt consumption on the risk of gastric cancer WCRF [13]. Also, a number of prospective studies have been performed to investigate the relationship between salt or salted food consumption and risk of stomach cancer. A recent meta-analysis of these studies has shown that dietary salt intake is directly associated with the risk of gastric cancer, with progressively increasing risk across increasing levels of habitual consumption [14]. This epidemiological evidence is supported by the results of clinical and experimental studies which found that high salt intake may alter the viscosity of the gastric protective mucous barrier [15] and increase the colonization by *Helicobacter pylori* (HP), a recognized risk factor for gastric cancer [16]. High intra-gastric sodium concentrations were shown to cause mucosal damage and inflammation, which in turn has been reported to increase cell proliferation and endogenous mutations [17, 18].

This chapter will focus mainly on recent epidemiological advances concerning the association between excess salt intake and risk of gastric cancer and will also touch on the possible mechanisms whereby excess salt may promote the development of gastric cancer.

2 Salt Intake and Gastric Cancer: Epidemiological Evidence

Incidence rates of gastric cancer are different among geographic regions, and this variability may be due to lifestyle and/or environmental factors in addition to genetic susceptibility. Migration studies have focused on the association between salt and gastric cancer. Analyses of Japanese migrants showed a higher rate of decrease in gastric cancer events in Japanese individuals resident in Hawaii than in those who migrated to Brazil, whose rate in turn was similar to the rate of those who stayed in Japan [19]. These differences might indeed be explained by changes occurring in the habitually high dietary salt intake that migrant populations continued or not to maintain in host countries.

Earlier ecological investigations had also shown an adverse effect of high salt consumption on the risk of gastric cancer: The INTERSALT study, including 24 countries, showed a significant direct association between 24 h urinary sodium excretion (a valid proxy for dietary salt intake) and mortality from gastric cancer [20]. Also, the results of the analysis of 65 rural Chinese counties indicated a positive relationship between consumption of salt-preserved vegetables and

mortality from stomach cancer [21]. Similar results were obtained in five Japanese areas, where the authors found a positive association between 24 h urinary sodium excretion and mortality from gastric cancer [22, 23].

While there are remarkable differences in the prevalence of gastric cancer in different countries, a large number of case-control studies suggested an adverse effect of high dietary salt intake or habitual consumption of salted foods on the risk of gastric cancer not only in Asian countries [24, 25], but also in American [26, 27] and European populations [28, 29, 30]. In particular, a most recent European case-control investigation supported the conclusion that high salt consumption is an important risk factor for gastric cancer, with no differences according to HP infection and virulence, tumor site, and histological type [31]. A consensus document of the World Cancer Research Fund International in 2007 supported a relationship between salt consumption and risk of gastric cancer and reported the results of a meta-analysis of the case-control studies available at that time WCRF [13]. In particular, this analysis showed that habitual salted food consumption was positively associated with gastric cancer, with a fivefold increase in risk for each single serving per day.

Finally, as previously mentioned, a number of prospective studies estimated the predictive role of dietary salt intake on the risk of gastric cancer. The majority of these studies were carried out in Japan. Here, a large cohort including 39,065 male and female participants followed for an average of 11 years showed a significant association between total salt intake, assessed by questionnaire, and rate of gastric cancer in men (RR = 2.23) and a positive but not significantly similar trend in women (RR = 1.32) [32]. In addition, another two Japanese investigations detected a similar relationship between high salt intake and rate of gastric cancer, over 11 and 14 years of follow-up, respectively [33, 34]. At variance with these results, the analysis of a cohort of 12,000 male and female participants followed for 15 years in Hawaii demonstrated a positive trend only in women [35]. A large population study carried out in the Netherlands was also not able to detect a significant association between total reported salt intake and risk of gastric cancer (RR = 1.18) [36].

Prospective studies focusing on the consumption of particular salt-rich foods in general demonstrated an adverse effect of elevated consumption on the risk of stomach cancer. Thus, a strong positive association between processed meat consumption and gastric cancer was found in a very large European cohort, including 520,000 male and female participants, in which greater intake was associated with 68 % greater risk compared with lower consumption [37]. A similarly adverse effect of greater processed meat consumption was found in both men and women by the prospective investigation of a large American population sample including 970,000 individuals followed for an average of 14 years [38].

Similar results were found with regard to the consumption of salted fish. Thus, in a sample of 17,600 American men, greater consumption of salted fish correlated with higher mortality for gastric cancer (RR = 1.90) [39]. In a Japanese cohort, greater salted fish consumption was significantly associated with higher gastric cancer incidence in men (RR = 1.77), while in women there was only a

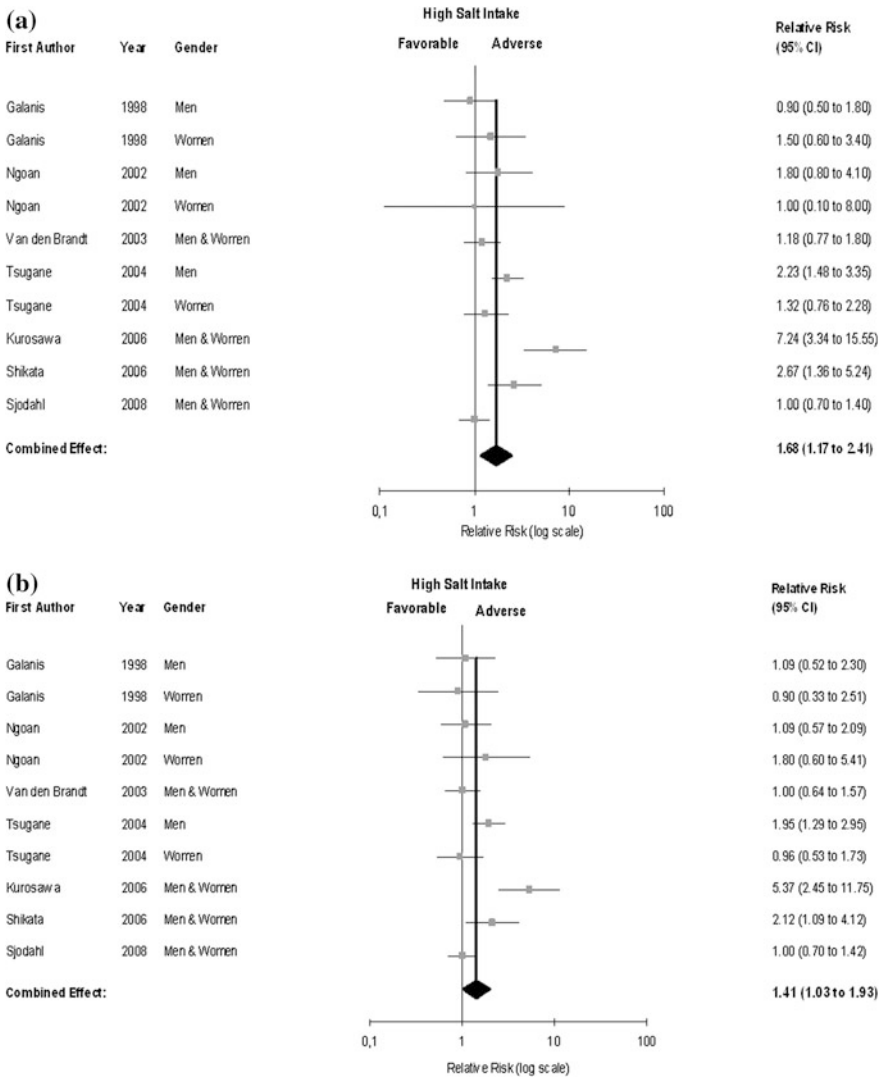

Fig. 1 *Salt intake and risk of gastric cancer*. **a** "*High*" *salt intake*: Forest plot of the risk of gastric cancer associated with "high" salt intake compared to "low" salt intake in 10 population cohorts from 7 published prospective studies. **b** "*Moderately high*" *salt intake*: forest plot of the risk of gastric cancer associated with "moderately high" salt intake compared to "low" salt intake in 10 population cohorts from 7 published prospective studies. Total population, $n = 268{,}718$; events, $n = 1{,}474$. Results are expressed as relative risk (RR) and 95 % confidence intervals (95 % C.I.). (Adapted from Ref. [14])

nonsignificant trend (RR = 1.17) [32]. However, the authors also reported that higher pickled food intake was associated with greater risk of gastric cancer accidents both in male and in female participants [32]. In another Japanese cohort

composed of over 55,000 atomic bomb survivors, there was also a positive trend between higher intake of pickled foods and risk of gastric cancer after 20 years of follow-up, which, however, did not attain statistical significance (RR = 1.11) [40].

In addition, studies on another common Asian dietary salt-rich food, that is, miso-soup, showed a nonsignificant positive trend between elevated habitual consumption of this type of food and gastric cancer events both in a small American cohort [35] and in a large Japanese population [41].

A recent systematic review of all the prospective studies available has shown unequivocally a direct and significant association between higher salt intake and risk of gastric cancer [14]. This meta-analysis was based on the results of seven studies published between 1998 and 2008 and included 270,000 people and 1,474 events. Pooled estimates based on a follow-up period ranging from 6 to 15 years indicated that there was a graded positive association between habitual salt consumption and incidence of gastric cancer. "High" salt intake and "moderately high" salt intake were associated with 68 and 41 % greater risk of gastric cancer, respectively, compared with "low" salt consumption (Fig. 1). The relationship was not significantly different in men and women, and likewise was not affected by the length of follow-up, the year of publication, and the participants' age at enrollment. Salt consumption was a particularly strong predictor of gastric cancer in the studies carried out in Japanese populations. Separate analyses indicated that the risk of gastric cancer was greater in individuals who reported to be habitual eaters of salt-rich foods: In particular, elevated consumption of pickled foods, salted fish, and processed meat were significantly associated with 27, 24, and 24 % greater risk of gastric cancer, respectively, whereas high miso-soup consumption showed a similarly nonsignificant trend (RR = 1.05) (Fig. 2).

Finally, Dias-Neto et al. [42] analyzed the association between salted food consumption, preference for salted foods (or the use of table salt), and gastric intestinal metaplasia. The study, that included cohort, case-control and cross-sectional investigations, showed a trend toward an adverse effect of high salt consumption that, however, did not reach statistical significance.

3 Salt Intake and *Helicobacter Pylori* Infection

Helicobacter pylori (HP), infection is one of the main predisposing factors for gastric cancer development. High salt intake increases the colonization by HP [16] and induces mucosal damage on persistent HP infection [16]. A few studies suggested a possible causal link between excess salt intake, HP infection, and carcinogenesis [34, 43, 44].

A cross-sectional study of 634 Japanese men (aged 40–49 years) reported that habitual consumption of salt-rich foods, in particular miso-soup and pickled vegetables, was associated with high prevalence of HP infection [45]. This association was also reported by EUROGAST, an international study that related the rate of national HP infection to the 24 h urinary sodium excretion levels assessed

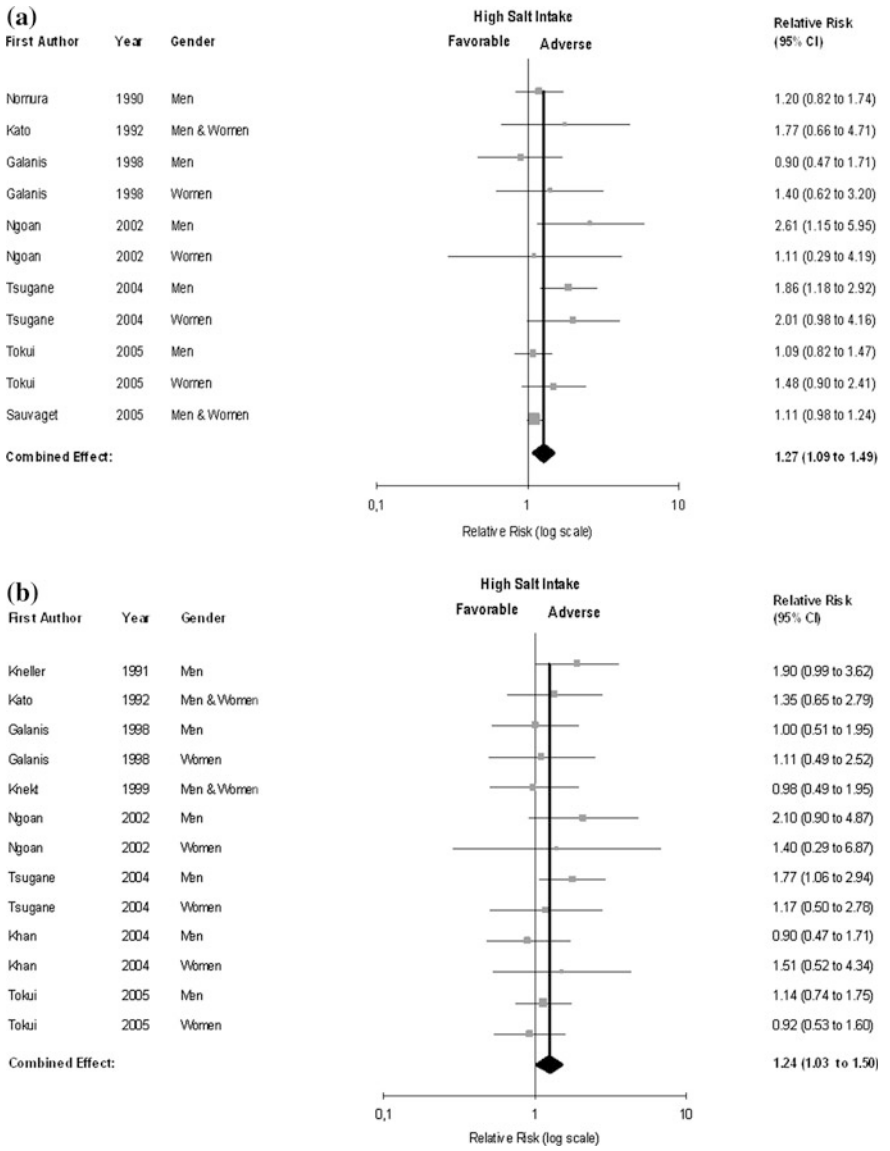

Fig. 2 *Salt-rich foods intake and risk of gastric cancer*. **a** *Pickled foods*: forest plot of the risk of gastric cancer associated with "high" pickled foods intake compared to "low" intake in 11 population cohorts from 7 published prospective studies. Total population, $n = 242{,}568$; events, $n = 2{,}858$. **b** *Salted fish*: forest plot of the risk of gastric cancer associated with "high" salted fish intake compared to "low" intake in 13 population cohorts from 8 published prospective studies. Total population, $n = 209{,}704$; events, $n = 1{,}447$. **c** *Processed meat*: Forest plot of the risk of gastric cancer associated with "high" processed meat intake compared to "low" intake in 7 population cohorts from 5 published prospective studies. Total population, $n = 1{,}578{,}092$; events, $n = 2{,}002$. **d** *Miso-soup*: Forest plot of the risk of gastric cancer associated with "high" miso-soup intake compared to "low" intake in 12 population cohorts from 8 published prospective studies. Total population, $n = 249{,}931$; events, $n = 3{,}022$. Results are expressed as relative risk (RR) and 95 % confidence intervals (95 % C.I.). (Adapted from Ref. [14])

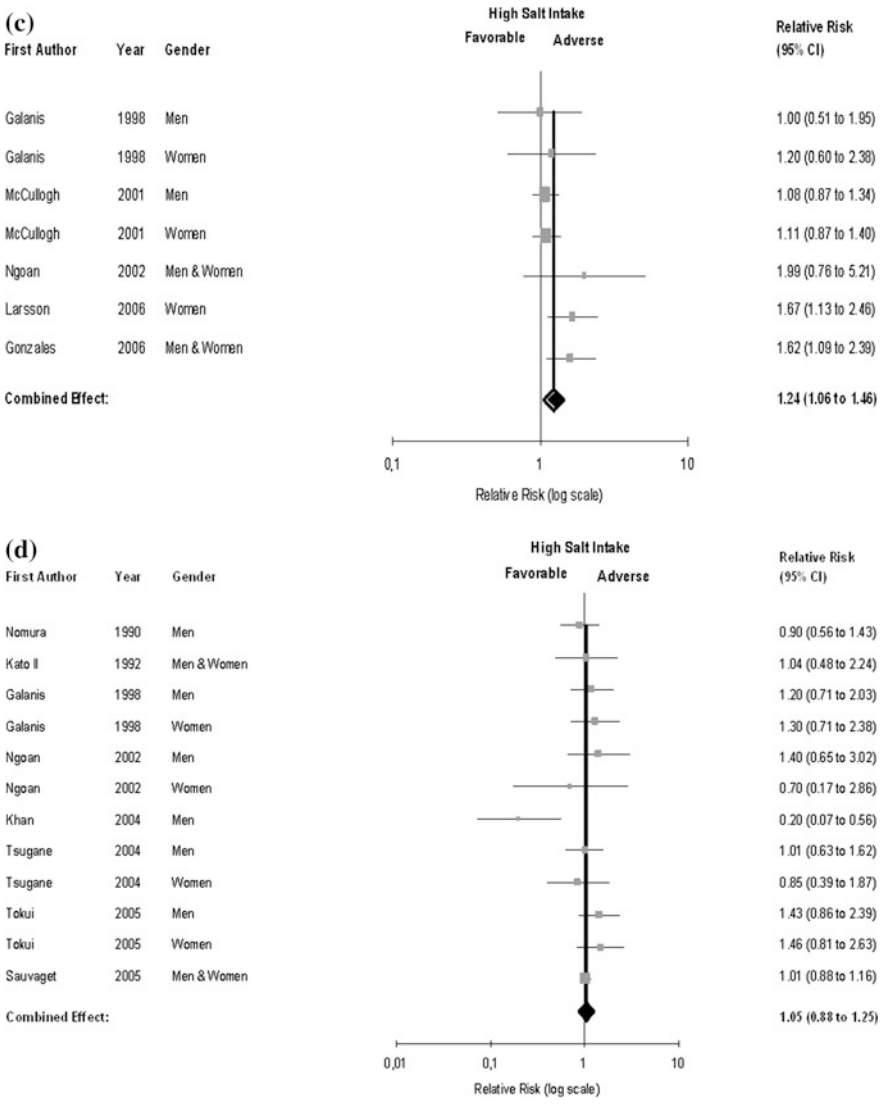

Fig. 2 Continued

by the INTERSALT project. A positive relationship between HP infection rates and urinary sodium excretion was detected in almost of all age and gender categories [46].

Two small case-control studies assessed *Helicobacter pylori* (HP), infection and salt intake in Japanese [44] and Korean [34] samples, after the development of gastric cancer. The authors found that the combination of high salt intake and HP infection was associated with gastric cancer compared to the combination of low

salt diet and no HP infection. In addition, the combination of high salt and HP infection was better associated with the risk of gastric cancer than the combination of *Helicobacter pylori* (HP), infection and low salt intake in a Japanese population sample [44].

The potential association between salted food consumption and HP infection in humans was also evaluated in a Japanese prospective study, which showed a positive association between high salt intake and gastric cancer only in the HP infection-positive group [34]. Moreover, the results indicated a strong effect of high salt intake on gastric carcinogenesis in individuals with both atrophic gastritis and HP infection.

Finally, the synergistic effects of dietary high salt intake and HP infection on gastric carcinogenesis were also found in animal studies. *Helicobacter pylori* (HP), infection exacerbated gastric mucosal damage on a salty diet in mice [47]. Moreover, in a study of Mongolian gerbils with HP infection, the cocarcinogenic effect of a high salt diet was confirmed, and high salt consumption was associated with elevated titers of anti-HP antibodies, hypergastrinemia, and inflammatory cell infiltration [43].

4 Other Biological Mechanisms and Experimental Evidence

A number of experimental studies addressed the question of the possible mechanisms of the adverse effect of excess salt intake toward susceptibility to gastric cancer. As previously mentioned, a powerful interaction has been detected between excess salt intake and HP infection, with high salt intake increasing the rate of colonization of the gastric mucosa by HP [16], enhancing surface mucous cells, and reducing gland mucous cell mucin [43]. A study in rats showed that high dietary salt intake reduced cell yield and produced an increase in the number of S phase cells, susceptible to mutagenesis [17]. In the same species, salt administration induced dose-dependent damage of the surface mucous cell layer and an increase in replicative DNA synthesis [18]. Moreover, in gerbils with HP infection, high dietary salt up-regulated the expression of COX-2 and iNOS [48], potentiated the effects of HP infection, and caused gastric cancer progression [49]. High salt intake was found to potentiate CagA expression (HP gene), increase the capacity of this gene to translocate into gastric epithelial cells, and improve the capacity of HP to alter the function of epithelial cells [50].

In addition, both hypergastrinemia induced by high salt intake in the presence of HP infection [43] and the synergic effect of this chronic hypergastrinemia and HP infection may contribute to parietal cell loss and gastric cancer progression [51].

A number of studies suggested that elevated salt intake may promote and/or enhance the effect of food-derived carcinogens, for example N-nitroso compounds [1, 15, 52], potent carcinogen that may induce tumors in several sites [53], by

affecting the viscosity of the protective mucous barrier [43] and damaging the gastric epithelium.

Finally, some experimental investigations on animal models showed a synergistic effect of high salt intake and chemical carcinogens (MNNG and MNU) in the development of gastric cancer [54, 55].

5 Perspectives

Gastric cancer remains one of the most common forms of cancer worldwide with approximately 870,000 new cases [56, 57] accounting for about 9.9 % of new cancers [58]. The mortality from this form of cancer remains high with more than 628,000 deaths (12.1 %) worldwide, equally represented in developed and developing countries [59]. While the worldwide incidence of gastric cancer has declined rapidly throughout recent decades, in particular in Western countries, in China, and other countries in East Asia, the decline has been less substantial than for other countries. In fact, an increased incidence has been observed in the oldest and youngest age groups [60]. Moreover, the age at onset of gastric cancer in the Chinese population is lower than in the West, and this may signal the effect of new environmental factors.

Most adult populations around the world have average daily salt intakes higher than 6 g (MRC [1, 61, 62], and for many in eastern Europe and Asia higher than 12 g [63], while WHO recommendations suggest that average population salt intake should be less than 5 g per day [64]. A population reduction in salt intake is recognized as a global priority for a highly cost-effective prevention of the epidemic of cardiovascular disease in both developed and developing countries [1, 65, 66, 67, 68, 69]. There is ample evidence suggesting the potential for further benefit by this policy in addition to its effects on cardiovascular disease [70].

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Alcoholic Beverages and Carbonated Soft Drinks: Consumption and Gastrointestinal Cancer Risks

Rosario Cuomo, Paolo Andreozzi and Francesco Paolo Zito

Abstract

Alcoholic beverages (ABs) and carbonated soft drinks (CSDs) are widely consumed worldwide. Given the high consumption of these beverages, the scientific community has increased its focus on their health impact. There is epidemiological evidence of a causal association between AB intake and digestive cancer, but the role of alcohol in determining cancer is not fully defined. Experimental studies have so far identified multiple mechanisms involved in carcinogenesis; ethanol itself is not carcinogenic but available data suggest that acetaldehyde (AA) and reactive oxygen species—both products of ethanol metabolism—have a genotoxic effect promoting carcinogenesis. Other carcinogenetic mechanisms include nutritional deficits, changes in DNA methylation, and impaired immune surveillance. As CSDs are often suspected to cause certain gastrointestinal disorders, consequently, some researchers have hypothesized their involvement in gastrointestinal cancers. Of all the ingredients, carbon dioxide is prevalently involved in the alteration of gastrointestinal physiology by a direct mucosal effect and indirect effects mediated by the mechanical pressure determined by gas. The role of sugar or artificial sweeteners is also debated as factors involved in the carcinogenic processes. However, several surveys have failed to show any associations between CSDs and esophageal, gastric, or colon cancers. On the other hand, a slight correlation between risk of pancreatic cancer and CSD consumption has been found.

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_7,

Keywords
Ethanol · Carbonated soft drink · Gastrointestinal cancer

Abbreviations

AA	Acetaldehyde
AB	Alcoholic beverages
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
CI	Confidence intervals
CSD	Carbonated soft drink
CYP2E1	Cytochrome P450 2E1
EGJAC	Adenocarcinoma of the esophagogastric junction
GCA	Gastric cardia adenocarcinoma
GI	Gastrointestinal
HCC	Hepatocellular carcinoma
MNNG	N-methyl-N′-nitro-N-nitrosoguanidine
NCGA	Noncardia gastric adenocarcinoma
NIH–AARP	National Institutes of Health–American Association of Retired Persons
RR	Relative risk
SAMe	S-adenosyl methionine

Contents

1 Introduction

Alcoholic beverages (ABs) and carbonated soft drinks (CSDs) are widely consumed worldwide. The last report of the World Health Organization estimated an average of 6.13 L of ethanol per adult per year in 2005, with higher levels of alcohol consumption in western regions and in countries with growing economies. Ethanol consumption has remained stable since 1990, although an increase has been observed among young people [26]. CSDs have rapidly become widespread in the last few decades, and they are consumed more than ABs. In fact, today they are the most popular type of beverage worldwide and most people drink them daily, making them one of the major components of energy intake [67]. In 2006, CSD consumption was equal to 30.2 L per capita; their consumption is increasing in developing countries, while it is stable or decreasing in North America and Europe [74]. Several studies have evaluated the effects of ABs and CSDs on human health, but they have provided diverging data. The health burden attributable to alcohol is very high: It contributes to important chronic diseases and represents the third cause of death in the world (3–8 % of all global deaths). ABs are linked to neuropsychiatric, cardiovascular, gastrointestinal and liver diseases, but also to suicide, violence, and injuries [58]. In addition, several types of cancers, mostly those of the gastrointestinal (GI) tract, are associated with ethanol consumption, as confirmed by a large number of human studies on the global or specific risk of cancer related to alcohol consumption.

As to CSDs, the assessment of their impact on human health is very difficult due to their complex constituents. There are both high and low calorie CSDs, containing, respectively, sugar and sweeteners. Several types of sweeteners are used in CSDs: aspartame, saccharin, sucralose, sorbitol, mannitol, and many others. The international scientific community is on the alert for the metabolic effects of these beverages. The principal concern regards the consumption of high calorie CSDs in children and adolescents, because several studies have shown that CSDs often replace more nutritive beverages such as milk or fruit juice [39]. An association between CSDs and obesity or the metabolic syndrome has been hypothesized, but also dental, pulmonary, or cardiovascular diseases have been related to their consumption. Direct or indirect carcinogenic effect of some ingredients in CSDs has been supposed but an association between their consumption and several types of cancer is still unclear. In this report, our aim is to address the relationship between ethanol ingestion or CSD consumption and neoplastic diseases, mainly focusing on GI cancers. To correctly evaluate this relationship, it is necessary to discuss ABs and CSDs individually. Our intention is to focus mainly on the epidemiological data regarding the risk of cancer as related to the consumption of these beverages and on the putative carcinogenic mechanisms. Regarding CSDs, given the large number of compounds contained in these beverages, we will consider primarily the effects of carbon dioxide and of other ingredients such as sugar, sweeteners, and additives on human health.

2 Alcoholic Beverages and Gastrointestinal Cancer

The growing interest in the relationship between alcohol and cancer is due to the large consumption of these beverages all over the world. A wealth of evidence has shown that ABs are a risk factor for several types of cancer. In particular, alcohol ingestion, even in moderate amounts, is associated with cancer of the digestive tract, but also with the oral cavity, larynx, and breast cancers [10]. This correlation is supported by a large number of epidemiological studies and animal experiments that have identified ethanol (or its metabolites) as a promoting factor of human carcinogenesis.

A recent report has estimated that 3.6 % of all cases of cancer are attributable to alcohol drinking, with high variability among the geographic regions of the world, related to the differences in the amounts consumed [10]. These data confirm the high health burden related to alcohol consumption. The growing evidence supporting the carcinogenic effects of alcohol has led the International Agency for Cancer Research to classify ethanol and acetaldehyde (AA) associated with ABs as definitely carcinogenic for humans [79]. Nevertheless, the exact pathways of alcohol carcinogenesis are not clearly identified, and probably, the mechanisms involved are manifold and differ in several target organs [10].

Table 1 Relative risks of gastrointestinal cancer associated with AB consumption

Organ	Alcohol consumption	RR	CI	Synergism
Esophagus				
Squamous cell carcinoma	210–420 g/week	1.93	1.08–3.45	Smoke
Pandeya et al. [71]	>420 g/week	4.67	2.28–9.55	
Adenocarcinoma	210–420 g/week	0.67	0.37–1.21	
Pandeya et al. [71]	>420 g/week	0.89	0.42–1.89	
Stomach	>25 g/day	1.07	1.04–1.10	Smoke
Bagnardi et al. [3]	>100 g/day	1.32	1.18–1.49	
	1–4 times/14 days	1.30	0.78–2.16	
Sjodahl et al. [81]	>4 times/14 days	1.49	0.78–2.83	
Colon and rectum	30–45 g/day	1.16	0.99–1.36	Folate deficiencies
Cho et al. [14]	>45 g/day	1.41	1.16–1.72	
Liver	>0 g/day	2.4	1.3–4.4	Viral hepatitis
Hassan et al. [40]	>80 g/day	4.5	1.4–14.8	
Pancreas	>60 g/day	1.38	0.86–2.23	Smoke–alcohol–female
Michaud et al. [62]				

2.1 Human Studies

Several epidemiological studies have investigated the correlation between cancer and alcohol consumption. A Canadian population-based case–control study has shown an increased relative risk (RR) of several cancers (esophagus, stomach, colon, liver, pancreas, lung, prostate) among alcohol consumers compared with abstainers and occasional drinkers [7].

However, the available data are contrasting, particularly for some types of cancer (stomach, pancreas, and colon). This disagreement is partially due to the lack of a recognized safe threshold of ethanol drinking [97], which does not allow us to discriminate the subjects as exposed or not to cancer risk, and by the difficulty of estimating an individual's alcohol intake. On the other hand, many studies do not consider the various types of beverages (wine, spirits, beer, etc.) or other risk factors, such as smoking, which may somehow contribute to cancer development. Furthermore, the different number of subjects recruited in the studies could also explain the contrasting results on cancer risk related to alcohol intake.

In this report, we will focus only on the epidemiological studies that have investigated the association between alcohol beverages and GI tract cancers (see Table 1).

2.1.1 Esophageal Cancer

A causal relationship between chronic alcohol consumption and esophageal squamous cell carcinoma (ESCC) has been demonstrated by various epidemiological studies; this relationship is characterized by both a dose-dependent risk and a synergistic effect with smoking. A recent large case–control study assessed the association between esophageal cancer and alcohol intake; the data show that the RR of ESCC is increased by a factor of 1.93 (CI 1.08–3.45) for a consumption of 210–419.9 g/day and 4.67 (CI 2.28–9.55) for a consumption of more 420 g/week. Current smokers who consumed more than 420 g alcohol/week had a RR of 21.87 (CI 3.90–122.49) [71]. Several studies confirm this association: Boffetta et al. [9] found an increased risk for ESCC already at a dose of 12 g alcohol daily ($RR = 1.37$), rising to an RR of 5.8 following an intake of 72 g alcohol daily.

With regard to esophageal adenocarcinoma (EA), earlier studies reported a significant increase in the risk of this cancer in relation to alcohol consumption [98]. However, recent population-based studies show a lack of association of alcohol consumption with either esophageal Barrett's esophagus or adenocarcinoma [2, 47, 71].

2.1.2 Gastric Cancer

There is no consistent evidence that alcohol intake affects the risk of stomach cancer. Data from epidemiologic studies do not definitively support a harmful effect of alcohol on the development of gastric cancer.

A meta-analysis of case–control and cohort studies reported a modest increase in RR of gastric cancer of 1.07 (CI 1.04–1.10) for a consumption of 25 g/day and

1.32 (CI 1.18–1.49) for a consumption of 100 g/day [3]. A subsequent large prospective cohort study failed to observe a statistically significantly association between light beer, wine, and hard liquor with gastric cancer, whereas the consumption of medium-strong/strong beer had a RR of 2.09 (CI 1.11–3.93) [50]. The authors suggest that constituents of beer other than alcohol may play a role in the increased risk of gastric cancer.

In addition, data from a recent cohort study in Norway suggest that alcohol consumption per se has no significant association with cancer risk, whereas the combined high exposure to cigarettes (>20/day) and alcohol (>5 occasions/14 days) increases the risk for gastric cancer (RR = 4.38 CI 1.72–11.17) in comparison with subjects who do not smoke or drink [81].

2.1.3 Colorectal Cancer

The carcinogenic effect of alcohol on the colon and rectum is debated. The low levels of folate observed in chronic alcoholics, consequent to an impaired metabolism and low intake of this vitamin, might increase the risk of colorectal cancer. In 1974, Breslow and Enstrom were the first to evaluate the association between beer consumption and the occurrence of rectal cancer [13]. Some subsequent individual studies and meta-analyses have supported this hypothesis, showing a modest effect of alcoholic consumption on colorectal cancer [17, 27]. A recent pooled analysis of cohort studies has confirmed the association between alcohol and colorectal cancer, showing that the risks were 1.16 (CI 0.99–1.36) and 1.41 (CI 1.16–1.72) for persons who consumed 30–45 g/day and more than 45 g/day of ethanol, respectively, compared with nondrinkers. No differences among different ABs regarding risk of colon versus rectal cancer were observed [14].

2.1.4 Hepatocellular Carcinoma

The association between alcohol and liver cancer, in particular hepatocellular carcinoma (HCC), is well established [72]. Alcohol consumption is considered the most important risk factor for HCC in populations with low prevalence of hepatitis B or C virus infection such as the USA and northern Europe. Probably, the main carcinogenic mechanism of alcohol-related HCC is through the development of liver cirrhosis, but DNA damage induced by reactive oxygen species or AA and the increased hepatic metabolism of carcinogens might also have a role. Furthermore, the concomitant presence of other factors such as tobacco and hepatitis B or C virus might increase HCC risk. There is evidence of an RR of 2.4 (CI 1.3–4.4) for global alcohol consumption and 4.5 (CI 1.4–14.8) for heavy alcohol intake (>80 g/day), with an increased RR of 53.9 (CI 7.0–415.7) in case of concomitant presence of chronic viral hepatitis and heavy alcohol consumption [40].

2.1.5 Pancreatic Cancer

The development of chronic pancreatitis as a result of alcohol drinking has suggested a possible role of ABs in pancreatic carcinogenesis. Some individual

studies have identified alcohol intake as a possible risk factor for pancreatic adenocarcinoma. However, the relevance of this association remains controversial.

A recent meta-analysis of 14 cohort studies has estimated an increase in pancreatic cancer risk for alcohol consumption >30 g/day for women (RR 1.41, CI 1.07–1.85) but not men compared with nondrinkers, and no effect of the specific types of alcoholic beverage on cancer risk was observed. These data were adjusted for year of exposure, age, smoking, body mass index, and presence of diabetes [32]. In a second meta-analysis, the data adjusted for the main confounding factors have not shown a significant association between alcohol consumption and cancer (RR 1.38, CI 0.86–2.23), although an increased risk was recorded in men who were heavy liquor consumers (>45 g/d) (RR 2.23, CI 1.02–4.87) [62].

More studies are needed to definitively clarify the possible causal association between pancreatic cancer and alcohol consumption.

2.2 Alcohol Damage: Animal Experiments

Experiment animal studies have been able to highlight some mechanisms underlying alcohol carcinogenesis. The carcinogenic effect of alcohol on animals depends on the experimental method. When alcohol is applied locally, the occurrence of a tumor is related to its irritant effect. On the other hand, a systematic administration has a stimulating effect on chemically induced carcinogenesis [72]. There is long-standing evidence that alcohol is not a carcinogen per se: It is not able to induce DNA mutations and chronic alcohol intake does not stimulate tumor growth or the metastatic spread of a transplanted tumor in mice [46]. As it is not directly involved in cancer development but acts by enhancing the effect of direct carcinogens, alcohol has to be considered a cocarcinogen. Moreover, experimental data also support the view that the carcinogenic effects of alcohol are related to the production of AA, rather than to alcohol itself [80].

It has been shown that the administration of AA to rats is able to induce hyperplastic and hyperproliferative changes in the epithelia of the upper GI tract [42]. Considering that several bacteria are able to metabolize alcohol into AA, some authors have hypothesized the role of oral bacteria in alcohol-related carcinogenesis of upper GI tract cancer [80].

In animal studies, the data on alcohol-related gastric cancer are contrasting. To explore the ability of alcohol to enhance the effect of known gastric carcinogens, some researchers have explored the synergic effect in coadministration experiment. However, in rodents treated with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), no differences were observed in the occurrence of gastric tumors induced by a chronic administration of 10 % ethanol in drinking water, compared with a control group [94].

Also data on liver carcinogenesis in experimental animals show that alcohol alone is unable to induce cancer. As to hepatocarcinogenesis, in animal models administered nitrosamines as tumor inducing compound, ethanol intake alone was

able to inhibit hepatocarcinogenesis [72]. Only the combination of alcohol and other carcinogenic factors, such as hepatectomy [86] or a low methyl donor diet [92], was related to the development of liver tumors in animal models.

2.3 Alcoholic Beverages and Gastrointestinal Cancers:Putative Mechanisms

The mechanisms underlying alcohol carcinogenesis are not fully understood and probably differ by target organs, as do other carcinogens that act at many sites. Evidence suggests that ethanol itself is not a carcinogen [46], although it might have a local effect by increasing the exposure of the mucosa to carcinogens. However, ethanol metabolism (AA production or induction of cytochrome P450 2E1), nutritional deficiencies, and impaired gene expression or immune surveillance seem to be the main mechanisms involved in alcohol-related carcinogenesis [72] (see Table 2).

In addition, as variants in genes for alcohol and folate metabolisms affect the occurrence of cancer for alcohol drinkers, there is evidence that alcohol-related cancer could be considered as a result of the interaction between individual susceptibility and the cellular toxic effects determined by ethanol exposure [22].

2.3.1 Local Effects

Strong evidence suggests that the acute administration of ethanol can increase the permeability of biological membranes and facilitate the entry of carcinogenic

Table 2 Main putative mechanisms involved in AB-related carcinogenesis

	Ethanol effects	Carcinogenic mechanism
Local effects	Solvent action of ethanol	Facilitation of carcinogen entrance into epithelial cells
	Morpho-functional changes of salivary glands	
	Production of reactive oxygen species and nitrogen species	Oxidative stress, lipid peroxidation, and DNA damage
Ethanol metabolism	Production of acetaldehyde	Production of stable adducts with DNA
	Induction of cytochrome CYP2E1	Impaired pro-carcinogen metabolism
Changes of gene expression	Folate deficiencies, inhibition of methionine synthetase and DNA methylase	Decreased methylation of tumor promoter genes
Immune surveillance	Inhibition of number and activity of NK cells	Facilitation of cancer growth
Nutritional deficiencies	Folate, vitamin A and beta-carotene deficiencies	Impaired DNA methylation and increase in oxidative stress

molecules into epithelial cells, acting as a solvent [96]. This effect could explain the synergic effect of alcohol and smoking in the carcinogenesis of head and neck, but not in nonsmokers. On the other hand, chronic alcohol exposure of the oral cavity results in a series of morphological and functional changes of aspecific defensive mechanisms: Alcohol-induced atrophy of parotid and submaxillary glands reduces the saliva flow and increases its viscosity, leading to a reduction in the oral clearance of carcinogens. Consequently, epithelial cells are exposed to higher concentration of carcinogens [55].

2.3.2 Ethanol Metabolism

Ethanol metabolism plays a critical role in alcohol carcinogenesis: In liver cells, ethanol is primarily oxidized via the enzyme alcohol dehydrogenase (ADH) or cytochrome P450 2E1 (CYP2E1) to AA, which is the main metabolite of ethanol metabolism. Subsequently, AA is converted into acetate by aldehyde dehydrogenase (ALDH). AA and reactive oxygen species (ROS) are able to induce DNA damage and promote carcinogenesis.

AA is the main metabolite of ethanol and a large number of experiments have shown its direct mutagenic and carcinogenic effects. In particular, AA is able to bind to DNA and make stable adducts by which it could trigger replication errors and mutations in oncogenes or oncosuppressors [24]. AA production depends on the activity of ADH and ALDH, which are encoded by multiple genes. Because these genes exist in several variants, some polymorphisms may result in elevated AA levels and predispose to alcohol-related cancers. An increasing number of studies suggest an interaction between genetic susceptibility and alcohol drinking on cancer risk in human beings. Most available data support the role of polymorphisms ADH1B, ALDH2, and, less strongly, CYP2E1 in alcohol carcinogenesis [22].

In addition, the production of reactive oxygen species and nitrogen species is a possible mechanism of alcohol-related carcinogenesis: Ethanol metabolism increases oxidative stress and induces lipid peroxidation. Lipid peroxidation products are known to interact with DNA in order to form exocyclic DNA adducts. This mechanism is particularly relevant for liver carcinogenesis [23, 41, 64].

Finally, the chronic consumption of ABs induces cytochrome CYP2E1, which is involved in the metabolism of various xenobiotics, including pro-carcinogens such as nitrosamines, aflatoxin, vinyl chloride, which occur already at low levels of alcohol intake (40 g alcohol per day) [70]. The interaction between ethanol and pro-carcinogen metabolism is complex and may depend, among others, on the degree of CYP2E1 induction, on the chemical structure of the pro-carcinogen, and on the presence or absence of ethanol in the body during pro-carcinogen metabolism [80].

2.3.3 Nutritional Deficiencies

Alcoholism is one of the main causes of malnutrition worldwide. Various vitamin and micronutrient deficiencies are associated with chronic consumption and may

contribute to cancer development, although their role is still unclear. Heavy alcohol consumption seems to affect folate metabolism as a consequence of its low intake and destruction by AA. Folate deficiency, by affecting the inhibition of transmethylation, seems to change gene expression with a potential role in carcinogenesis [85]. In addition, the impaired intake and metabolism of vitamin B12 and vitamin B6 observed in chronic alcoholics could contribute to an impaired DNA methylation [10]. Various studies have also pointed out a vitamin A and beta-carotene deficiency in alcohol carcinogenesis [93]. It has been shown that prolonged use of alcohol determines a low intake of retinoids and carotenoids, but also a breakdown of retinol through ROS production [53].

2.3.4 Changes in Gene Expression

DNA methylation hinders gene expression, whereas a reduced methylation enhances it. A decreased methylation of tumor promoter genes has been hypothesized as a mechanism for alcohol-related cancer development, and this mechanism seems to play a critical role in liver cancer. Some evidence has confirmed that chronic alcohol consumption induces important changes in the degree of DNA methylation [15].

Experimental studies have shown that alcohol ingestion may affect DNA methylation in different ways: folate deficiency, reduced activity of methionine synthetase—the enzyme that remethylates homocysteine to methionine, and the inhibition of DNA methylase—which transfers methyl groups to DNA [72]. In chronic intake, there is evidence of reduced S-adenosyl methionine (SAMe)—the methyl donor for DNA methylation reactions, consequent to a reduced activity of methyladenosyltransferase, with increased levels of homocysteine related to the inhibition of the DNA methyltransferase enzymes. SAMe deficiency induces DNA hypomethylation and increases DNA instability, associated with increased risk for cancer [65].

2.3.5 Alcohol and Immune Surveillance

It is well known that alcohol abuse is related to an immunodeficient state and that chronic alcoholics are more susceptible to infections. Evidence shows that alcohol drinking can reduce both innate and specific immune response and facilitate cancer cell growth [16]. In particular, as shown in animal studies, alcohol itself may contribute to an impaired immune surveillance by inhibiting number and lytic activity of NK cells following a single equivalent dose of ethanol [99]. Other factors such as malnutrition or vitamin deficiency may also affect immune surveillance and induce cancer development in chronic alcohol consumption.

3 Carbonated Soft Drink and Gastrointestinal Cancer

CSDs are the most popular beverages worldwide. Among all CSDs, regular cola is the most common drink in the world. This beverage was invented in 1886 in Atlanta, Georgia, by a pharmacist who, by accident or design, mixed carbonated water with sugar syrup, phosphoric acid, caffeine, and other natural flavors to

Table 3 Relative risks of gastrointestinal cancer associated with CSD consumption

Organ	CSD consumption	RR	CI
Esophagus			
Squamous cell carcinoma	1 can/week	0.70	0.41–1.18
	2–6 cans/week	0.66	0.39–1.11
Ren et al. [75]	>6 cans/week	0.85	0.46–1.56
	1–6 drinks/week	0.68	0.45–1.03
	7–20 drinks/week	0.67	0.45–1.01
Ibiebele et al. [44]	> 20 drinks/week	0.40	0.20–0.78
Adenocarcinoma	1 can/week	1.52	0.97–2.38
	2–6 cans/week	1.36	0.88–2.12
Ren et al. [75]	>6 cans/week	1.11	0.66–1.85
	1–6 drinks/week	1.12	0.74–1.70
	7–20 drinks/week	1.01	0.65–1.58
Ibiebele et al. [44]	> 20 drinks/week	0.94	0.53–1.66
Stomach			
Cardia adenocarcinoma	12–103 drinks/year total carbonated soft drinks	0.86	0.52–0.40
	104–364 drinks/year total carbonated soft drinks	0.97	0.62–1.51
Mayne et al. [59]	> 365 drinks/year total carbonated soft drinks	0.74	0.46–1.16
Noncardia adenocarcinoma	12–103 drinks/year total carbonated soft drinks	0.79	0.52–1.21
	104–364 drinks/year total carbonated soft drinks	0.78	0.52–1.16
Mayne et al. [59]	> 365 drinks/year total carbonated soft drinks	0.65	0.43–0.98
Colon and rectum	0–250 g/day	0.96	0.90–1.02
	250–550 g/day	1.08	0.87–1.34
Zhang et al. [100]	>550 g/day	0.94	0.66–1.32
Pancreas	≤3/week	1.05	0.84–1.31
Schernhammer et al. [77]	>3/week	1.13	0.81–1.58
	≤2/week	0.73	0.40–1.33
Mueller et al. [66][a]	>2/week	1.87	1.10–3.15

[a] Chinese population

create what is known as "the world's favorite soft drink." The ingredients of CSD are various, and the analysis of their health impact is difficult. Therefore, most studies do not describe the health effects of single ingredients but the general impact of the whole beverage on risk of disease.

Different opinions are reported about the interaction of carbonated beverages and the GI system, some suggesting a negative influence and others describing its benefits when consumed in a variety of conditions. This trend appears to be paralleled by the increase in their commercial diffusion. CSDs have been hypothesized to increase the risk of gastric reflux and EA, although several case–control studies have reported inverse or null associations with laryngeal or esophageal cancers [44, 48, 59, 101].

Soft drinks are the leading sources of added sugar in the US diet [37] and greatly contribute to hyperglycemia and hyperinsulinemia [12]. Some studies found that individuals who consume large quantities of soft drinks were at increased risk for obesity [33, 56, 78] and type 2 diabetes mellitus [20, 21], conditions that may be on the causal pathway to pancreatic cancer [43, 51, 97] (see Table 3).

3.1 Carbonated Soft Drink and Gastrointestinal Cancer: Human Studies

3.1.1 Esophageal Cancer

CSDs have been associated with gastroesophageal reflux, an established risk factor for EA. In a multicenter, population-based case–control study conducted in three geographic areas of the USA, and the associations between CSD intake and risk factor for esophageal carcinoma were examined [59]. In this study, subjects were asked about their usual frequency of consumption of "diet soft drinks or soda" and "regular soft drinks or soda, not diet" (per day, week, month, or year) during the 3–5-year period before diagnosis in patients, or the interview in control subjects. Frequencies for these items were combined to estimate total consumption and divided into quartiles. This analysis included 687 control subjects, 282 case patients with EA, 255 with gastric cardia adenocarcinoma (GCA), 206 with ESCC, and 352 with noncardia gastric adenocarcinoma (NCGA). Contrarily to the proposed hypothesis, this study showed that CSDs consumption was inversely associated with EA risk, primarily due to the intake of low calorie CSDs. High CSDs consumption did not increase the risk of any esophageal cancer subtype in men or women. These findings indicate that the consumption of these beverages (especially low calorie CSDs) is inversely associated with risk of EA, and thus it is not likely to have contributed to the rising incidence rates.

A comment on the study by Mayne et al. [57] emphasized that in the population studied, control subjects were younger than case patients with EA. Moreover, in their comment, the authors also stated that the control subjects had statistically significantly higher income than case patients with adenocarcinoma of the esophagus. The continuing survey of food intakes by individuals reported large differences in CSD consumption in terms of income [89]. Another criticism raised of Mayne's study was the lack of duration of CSD consumption. Indeed, CSDs consumption was ascertained for 3–5 years before the diagnosis of cancer, and it

was hypothesized that CSDs contribute to the development of adenocarcinoma of the esophagus by inducing gastroesophageal reflux for several decades.

Utilizing the data from the National Institutes of Health–American Association of Retired Persons (NIH–AARP) Diet and Health Study, some researchers investigated the relationship among hot tea, iced tea, coffee, and carbonated soft drink consumption and risk of incident of upper GI tract cancers [75]. Information on diet and health-related behaviors was mailed to AARP members aged 50–71 years who resided in eight US states (California, Florida, Louisiana, New Jersey, North Carolina, Pennsylvania, Georgia, and Michigan). The data retrieved from the database allowed the authors to verify the risk related to soft drink consumption and cancer development for 428 esophageal cancers (ESCC and EA). The study was performed considering categories of consumption based on number of cans (none, $\leq$1 can/week, 2–6 can/week, $\geq$1 can/day). A borderline nonsignificant association between CSDs intake and EA was found for those who drank $\leq$1 can/week, with a RR of 1.52 (CI 0.97–2.38;. however, for those with the greatest intake, with $\geq$ 1 can/day, the RR was 1.11 (CI 0.66–1.85).

Another study investigated the association between CSDs and risk of EA, adenocarcinoma of the esophagogastric junction (EGJAC), and ESCC in Australia, a nation with a rapidly increasing incidence of EA [44]. In this study, a food frequency questionnaire was used to collect data on carbonated soft drink consumption; a self-administered questionnaire was used to collect information on demographic, socioeconomic, and lifestyle-related factors from 1,484 control subjects, 294 cases with EA, 325 cases with EGJAC, and 238 cases with ESCC. The results of this study showed that a high intake of soft drinks was not associated with risk of EA (RR 0.94, CI 0.53–1.66, p for trend = 0.85) or EGJAC (RR 1.07, CI 0.67–1.73, p for trend = 0.89), but it was inversely associated with ESCC of the esophagus (RR 0.40, CI 0.20–0.78, p for trend = 0.04). Hence, in the Australian population studied, the authors observed that esophageal ESCC cases as well as other esophageal cancer cases were less likely to drink soft drinks than controls. ESCC patients were also older, less likely to be obese, and less likely to report reflux. Although the authors adjusted for these factors in all of their analyses, some degree of residual confounder cannot be ruled out to account for their finding. In any case, they showed that none of the esophageal cancers were related to CSD consumption.

Additional information on the inverse relationship between ESCC risk and CSD consumption come from Gallus et al. [29] who analyzed data from a hospital-based case-control study conducted in Northern Italy between 1992 and 1997. Case patients were 304 individuals with incident, histologically confirmed esophageal ESCC. Control subjects were 743 patients admitted to the same hospitals as case patients for a wide spectrum of acute, nonneoplastic conditions, not related to smoking, alcohol consumption, or long-term dietary modification. Control subjects were frequency matched with case patients by age, sex, year of interview, and area of residence. This study as well suggests that CSD consumption does not increase the risk of esophageal cancer and that a moderate inverse association between CSD intake and esophageal ESCC risk exists.

Data from a Swedish nationwide, population-based, case–control study were analyzed to determine whether there is an association between carbonated drink intake and EA or GCA [48]. During the data collection in 1995–1997, 189 patients with EA, 262 patients with GCA, and 820 control subjects underwent a personal interview. All cancers were histologically classified. Also in this other study, the frequency of intake of CSDs was not associated with risk of EA; high consumers (intake more than six times weekly) had a statistically nonsignificant lower risk compared with never users (RR 0.89, CI 0.49–1.64; $p = 0.77$). Moreover, this article stated that consumption of carbonated low-alcohol beer and combined intake of carbonated drinks were also not associated with risk of EA.

While these consistent findings [29, 44, 48, 75] across diverse populations exclude a risk for EA related to CSD consumption, data on ESCC lend credence to the observation that the risk of this cancer is inversely related to CSD consumption although a causal mechanism for this association remains elusive.

3.1.2 Gastric Cancer

An analysis of the relationship between gastric cancer (GC) and CSD consumption was performed utilizing the data from the National Institutes of Health–American Association of Retired Persons (NIH–AARP) Diet and Health Study [75]. The research cohort included 481,563 participants, 286,402 men and 195,161 women. The authors observed no evidence, in 455 gastric cancer patients (231 gastric cardia; 224 gastric noncardia), of a dose–response association between soft drink consumption and GC risk.

Mayne et al. [59] interviewed 255 patients with gastric cardia and 352 with gastric noncardia adenocarcinoma. The researchers asked patients about the usual frequency of consumption of diet or regular CSDs during the period 3–5 years before the diagnosis (case patients) or the interview (control subjects). Also in this study, no risk of gastric cancer related to CSDs consumption was found.

Similar data are reported in the article by Lagergren et al. [48] who analyzed 262 patients with gastric cardia adenocarcinoma. They failed to find any significant association between high consumption of CSDs (>6 times per week) and risk of this type of gastric cancer (RR 1.09, CI 0.64–1.85; $p = 0.64$).

3.1.3 Pancreatic Cancer

Sugar-sweetened soft drinks are a prevalent source of readily absorbable sugars and can be associated with an increased risk of obesity and diabetes. Diabetes mellitus and a diet high in glycemic load are both potential risk factors for pancreatic cancer. Some studies have explored the relationship between CSD consumption and pancreatic cancer.

Analyzing the database of the Nurses' Health Study and the Health Professionals Follow-Up Study performed in the USA, 392 pancreatic cancer were selected among 88,794 women and 49,364 men without cancer at baseline [77]. Soft drink consumption was first assessed at baseline and then updated periodically from 1980 until 1998. Patients who consumed more than three sugar-sweetened

soft drinks weekly experienced a nonsignificant RR of pancreatic cancer. Women in the highest category of sugar-sweetened soft drink intake (>3 drink/week) did experience a significant increase in risk (RR 1.57; CI 1.02–2.41; p for trend = 0.05), whereas there was no association between sugar-sweetened soft drink intake and pancreatic cancer among men. Among women, a borderline risk associated with higher sugar-sweetened soft drink intake was limited to those with elevated body mass index (>25 kg/m2; RR 1.89, CI 0.96–3.72; $p = 0.10$) or low physical activity (RR 2.02, CI 1.06–3.85; $p = 0.05$). In contrast, consumption of diet soft drinks was not associated with an elevated pancreatic cancer risk in either cohort.

Another survey showed a borderline positive association between soft drink consumption and the risk of pancreatic cancer [49]. Indeed, in 131 incident cases of pancreatic cancer the multivariate RR for the highest compared with the lowest consumption categories of soft drinks was 1.69 (CI 0.97–2.36, p for trend = 0.05).

A Chinese prospective cohort study examined the risk of pancreatic cancer in 60,524 individuals up to 14 years of follow-up. Pancreatic cancer cases were ascertained by record linkage of the cohort database with records of the population-based Singapore Cancer Registry and the Singapore Registry of Births and Deaths [66]. The analysis identified 142 incident cases of pancreatic cancer. Subjects consuming ≥2 soft drinks/wk experienced a statistically significant increased risk of pancreatic cancer (RR 1.87, CI 1.10–3.15; $p = 0.02$) compared with those who did not consume soft drinks.

Moreover, a recent analysis studied the association between CSD consumption and risk of pancreatic cancer. This is a pooled analysis of the data from 14 cohort studies conducted in the Pooling Project of Prospective Studies of Diet and Cancer—a large international consortium of several countries in the world [31]. In this study, 2,185 incident pancreatic cancer cases were identified among 853,894 individuals during a follow-up varying from 7 to 20 years. No statistically significant associations were observed between pancreatic cancer risk and CSD intake (RR 1.19, CI 0.98–1.46 comparing ≥250–0 g/d of CSD). However, when CSD intake was modeled as a continuous variable, a positive association with pancreatic cancer risk was evident (RR 1.06, CI 1.02–1.12).

The association between CSD consumption and pancreatic cancer risk was also studied in an Italian case–control study conducted in 1991–2008 on 326 pancreatic cancer cases and 652 matched controls [30]. This study concluded that CSD consumption is not materially related to pancreatic cancer risk.

Another two studies failed to find any association between soft drinks and pancreatic cancer risk [4, 68].

3.1.4 Colon Cancer

So far, only one study has verified the association between sugar-sweetened soft drink intake and the risk of developing colon cancer [100]. This study was conducted within the Pooling Project of Prospective Studies of Diet and Cancer (Pooling Project), an international consortium of cohort studies. In the 13 studies

of this pooled analysis, 5,604 incident invasive colon cancer case patients (1,858 men and 3,746 women) were identified among 239,193 men and 492,248 women followed for up to 6–20 years. Sugar-sweetened carbonated soft drink consumption was not associated with colon cancer risk (pooled multivariable RR 0.94, CI 0.66–1.32, comparing >550 g/d versus nondrinkers). When the authors examined diet CSDs, the pooled multivariable RR for a 375 g/d increment was 1.01 (CI = 0.94–1.08; measured in four studies, $n = 1{,}928$ case patients). The categorization of sugar-sweetened CSDs into cola and noncola beverages did not shown any association with risk of colon cancer.

3.1.5 Other GI Cancers

There are no references in the literature showing any relationship between carbonation of beverages and any GI cancer other than the ones previous cited.

3.1.6 Aspartame and GI Cancer

The growing obesity epidemic in industrialized countries, due in part to fast food and soft drink consumption, has lead to an increased demand for low calorie foods. Given the increasing market for these so-called diet or light products, additional new generation sweeteners have emerged, including aspartame. Epidemiologic data on the role of aspartame in humans are scanty [95]. The warning about the potential carcinogenicity of aspartame came from a study suggesting that an increase in brain cancer incidence was related to the introduction of this artificial sweetener on the market [69]. This study was later criticized because of uncertainties on brain cancer trends [90]. Moreover, a few subsequent case–control studies on brain cancer found no consistent evidence of an excess risk in relation to aspartame [36].

Regarding GI cancers, a study based on an integrated network of case–control studies conducted in Italy found no association between saccharin and other sweeteners on the risk of cancers of the oral cavity and pharynx, esophagus, colorectal, and cancers involving other sites [28]. In this study, an integrated network of case–control studies was conducted between 1991 and 2004 in Italy. Cancer cases involving the upper digestive tract comprised 598 patients with incident, histologically confirmed cancers of the oral cavity and pharynx, 304 of the esophagus, 1,225 of the colon, and 728 of the rectum, while controls were 7,028 patients (3,301 men and 3,727 women) admitted to the same hospitals for acute, nonneoplastic disorders. The RR for consumption of aspartame was 0.77 (CI 0.39–1.53) for cancers of the oral cavity and pharynx, 0.77 (CI 0.34–1.75) for esophageal, 0.90 (CI 0.70–1.16) for colon and 0.71 (CI 0.50–1.02) for rectal cancer. Therefore, this work indicated a lack of association between aspartame and risk of several common GI neoplasms.

An update of this study was published two years later [11], and it described 230 patients with incident, histologically confirmed cancers of the stomach and 547 corresponding controls, 326 of the pancreas, and 652 controls. All controls were patients admitted to the same hospitals as cases for acute, nonneoplastic disorders.

RR for ever users of sweeteners versus nonusers were 0.80 (CI 0.45–1.43) for gastric cancer and 0.62 (CI 0.37–1.04) for pancreatic cancer.

3.2 Carbonated Soft Drinks and GI Cancer: Animal Experiments

Belpoggi et al. [6] studied the long-term effects of Coca Cola, the best known CSD, administered as a substitute for drinking water on male and female Sprague–Dawley rats. The objective of the project was to study whether and how long-term consumption of Coca Cola affects the basic tumorigram of test animals. The bioassays were performed on rats at different ages, namely on males and females exposed to the drink since embryonic life or from 7 weeks of age and on males and females exposed from 30, 39, or 55 weeks of age. Animals were kept under observation until spontaneous death and then underwent complete necropsy. The results indicated (a) an increase in body weight in all animals treated; (b) a statistically significant higher incidence in females, both breeders and offspring, of malignant mammary tumors; (c) a statistically significant higher incidence of exocrine adenomas of the pancreas in both male and female breeders and offspring; and (d) no other tumors of the all body regions including the GI system. Humans do not consume this beverage under the same conditions as in this experimental design, where the consumption was very paradoxical; however, the results confirm that an exaggerated ingestion of high caloric beverages, such as regular soft drinks, can lead to a marked increase in body weight which in turn presents an increased risk for developing cancer.

Animal studies have failed to show a carcinogenic activity of aspartame and other sweeteners [35, 95]. Only three studies on animals (Sprague–Dawley rats and Swiss mice) treated with variable doses of aspartame and followed until natural death found an excess of malignant neoplasms, mainly lymphomas and leukemias in female rats, and hepatocellular carcinomas in male mice [82–84]. Such apparent excess can, however, be explained by the longer life of the animals treated with aspartame, as well as by the higher rates of infections in the study animals [87]. No studies in animals have shown an increased risk of other GI cancers [54]

3.3 Putative Mechanisms

In examining the properties of carbonated beverages, it is necessary to consider three major points: (a) the carbon dioxide with which these beverages are charged; (b) the sugar or sweetener contained; (c) the effect of other substances used by industries for their preparation. Of these, the most interesting and probably the most valuable one is carbon dioxide. Several mechanisms have been suggested as a possible cause of damage to the GI system due to CSDs and, consequently, a potential carcinogenicity [18] (see Table 4). Most of the information on the

Table 4 Putative mechanisms involved in CSD-related carcinogenesis

	Soft drink effects	Putative carcinogenic mechanism
CO_2 content	Increase in gastric pressure	Facilitation of esophageal acid reflux and induction of intestinal metaplasia (Barrett's esophagus)
	Increase in HCl secretion	
Sugar	High insulin levels increase insulin-like growth factor	Proliferation of pancreatic cancer cells
Aspartame	Production of direct carcinogenic such as formaldehyde	DNA damage (generation of formaldehyde adducts) and increase in chromosomal aberrations

relationship between carbonated beverage consumption and GI damage is related to the problem of gastroesophageal reflux disease (GERD). The main symptom of this disease is heartburn, which frequently occurs when the esophagus is exposed to gastric content. Recommendations generally made by physicians are to lose weight and, if necessary, modify lifestyle behaviors, body posture, and dietary habits, and generally to limit the consumption of carbonated beverages. Very few studies have postulated that carbonated beverages cause GERD [25, 38, 61]. Fass et al. [25] found that carbonated soft drink consumption was a predictor of GERD symptoms in a multivariate analysis. Another small study on healthy individuals, performed by manometric evaluation, found a lower esophageal sphincter pressure immediately after, and also later on, following the ingestion of carbonated water compared to noneffervescent water [38]. However, a more recent research, performed on healthy subjects with the aim to verify the effect on gastroesophageal reflux, of sweetened (sucrose 10 %) water added with increasing concentrations of carbon dioxide, showed no difference between carbonated and still water [19].

Carbonated beverages seem to influence stomach function by both mechanical and chemical effects. Data from various studies emphasize that the mechanical effect depends on the pressure exerted by gas and fluid volume on the gastric wall [19, 73]. These studies showed that symptoms related to gastric distress appear only with intakes of carbonated fluid greater than 300 ml [1]. On the other hand, a chemical effect related to carbon dioxide determines a slight increase in hydrochloric acid, which could influence the digestive process or worsen an acid-related disease [60]. No studies showing a direct involvement of carbon dioxide by both mechanical and chemical effects in any carcinogenicity process have been published.

Factors that raise insulin and glucose levels and promote obesity and diabetes, such as sugar-sweetened CSDs [78], may be positively associated with pancreatic cancer risk. High insulin concentrations may increase free insulin-like growth factor (IGF) levels by reducing levels of IGF-binding proteins [34]. Based on available evidence, exposure to IGF causes proliferation in pancreatic cancer cell lines [8]. Elevated insulin concentrations seem to activate IGF receptors, which may lead to cancer cell proliferation [52]. Elevated post-load and fasting plasma glucose [5, 45] and nonfasting plasma C-peptide [63] have been associated with an

increased risk of pancreatic cancer. This mechanism supports the hypothesis that sugar-sweetened beverages with a high glycemic load may increase the risk of pancreatic cancer [66].

The last topic to be addressed is that of sweeteners, like aspartame, whose use in recent years has increased in CSDs. However, there are some warnings regarding their safety; in fact, in recent years, three articles have been published by the same authors who reported the effects of aspartame on neoplastic induction in animals [82–84]. The interest of researchers regarding the potential carcinogenicity of aspartame stems from some in vitro tests of genotoxicity. Aspartame has been evaluated in vitro in a chromosomal aberration test where it showed a statistically significant increase (2.5–4.2-fold, compared to control values) in the percentage of aberrant cells or in the number of chromosomal aberrations per cell in all the doses tested but the substance was not mutagenic [76]. As to the data regarding liver carcinogenicity in Swiss mice [83], the authors hypothesized that since aspartame is completely metabolized in the GI tract to phenylalanine, aspartic acid, and methanol, the observed carcinogenic effects were not caused by aspartame itself but rather by its metabolites. In particular, the conversion of methanol into formaldehyde in the liver may result in the generation of formaldehyde adducts [91], which could explain the plausibility of the hepatocarcinogenic effects in male mice. On the other hand, the European Food Safety Authority states that there is a general consensus in the scientific community backed up by a considerable body of evidence that in mice, hepatic tumors induced by nongenotoxic compounds can be considered as irrelevant for human risk assessment [88].

4 Conclusions

The high level of consumption of ABs and CSDs has aroused much attention in the last few years for its impact on human health and GI cancers. However, the majority of the studies in this field are limited in terms of clinical, epidemiological, and experimental settings. Available data show a clear positive correlation between alcohol consumption and some types of GI cancer. This association is characterized by a dose-dependent effect and a synergic action of cigarette smoking. In line with this, the control of heavy alcohol consumption and cigarette smoking represents the main target for primary prevention of alcohol-related cancer.

On the other hand, given the heterogeneous composition of CSDs, it is difficult to study their effects on GI carcinogenesis. Several studies have shown no correlation between esophageal or gastric cancer and CSD consumption, whereas only a few studies have highlighted a positive relationship between caloric CSDs and pancreatic cancer. These findings are probably related to calorie intake and impairment of glucose metabolism control rather than to the consumption of CSDs per se. However, further studies should be performed to definitely rule out the involvement of CSDs in GI carcinogenesis.

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Part II
Natural Dietary Molecules

Anti-Inflammatory and Anticancer Drugs from Nature

Barbora Orlikova, Noémie Legrand, Jana Panning, Mario Dicato and Marc Diederich

Abstract

Over the centuries, plant extracts have been used to treat various diseases. Until now, natural products have played an important role in anticancer therapy as there are more than 500 compounds from terrestrial and marine plants or microorganisms, which have antioxidant, antiproliferative, or antiangiogenic properties and are therefore able to reduce tumor growth. The recent discovery of new natural products has been accelerated by novel technologies (high throughput screening of natural products in plants, animals, marine organisms, and microorganisms). Vincristine, irinotecan, etoposide, and paclitaxel are examples of compounds derived from plants that are used in cancer treatment. Similarly, actinomycin D, mitomycin C, bleomycin, doxorubicin, and L-asparaginase are drugs derived from microorganisms. In this review, we describe the molecular mechanisms of natural compounds with anti-inflammatory and anticancer activities.

Keywords

Natural compounds · Cancer · Inflammation · Cyclooxygenase · Nuclear factor kappa B · Chemotherapy · Chemoprevention

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_8,

Abbreviations

VEGF	Vascular endothelial growth factor
ROS	Reactive oxygen species
NF-κB	Transcription factor nuclear factor κB
N-pyrazinecarbonyl-L-phenyl-alanine-L-leucine boronic acid	Bortezomib
BAFF	B-cell activating factor
NIK	NF-κB-inducing kinase
STAT	Signal transducer and activator of transcription
COX-2	Cyclooxygenase-2
CDK	Cyclin-dependent kinase
EEF2	Eukaryotic elongation factor 2
PKC	Protein kinase C
PI_3K	Phosphoinositide 3-kinase
MAPK	Mitogen-activated protein kinase
MLCK	Myosin light-chain kinase
ATM	Ataxia telangiectasia mutated
PAL	Phenylalanine ammonia-lyase
EGCG	Epigallocatechin gallate
IKK	Inhibits IκB kinase
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ETA	11,14,17-eicosatrienoic acid

Contents

1 Introduction

The definition of a natural compound is very complex. Traditionally, a natural product is a chemical compound produced by living organisms and possessing biological or pharmacological activity. Moreover, a natural product can also be synthesized and thus be chemically identical to its natural counterpart. In order to elucidate the contribution of natural products in chemotherapeutic drug discovery and development, Newman and Cragg generated a drug classification after evaluation of all approved anticancer drugs between 1940 and 2010. In their analysis, 206 approved anticancer agents were classified into clearly defined groups [1].

While compounds belonging to the first 8 categories were classified as naturally derived or inspired compounds, the last category lacks any natural product inspiration and is therefore considered as the only truly synthetic class of compounds. Accordingly, 78.6 % of all approved anticancer agents are either natural products or based thereon, or agents that mimic them in one form or another (Fig. 1), and only 21.4 % were fully synthetic with no prototype or conception from nature. If we consider these data, it becomes clear that Mother Nature plays a predominant role in modern therapy. Moreover, this classification emphasizes the importance of the development of drugs from natural origins.

However, the use of natural products has limitations since living organisms sometimes synthesize only trace quantities of otherwise interesting bioactive compounds and natural products have limited bioactivity. Here, the polarity of the molecule often complicates its cellular uptake, thus leading to a reduced activity.

Nevertheless, natural products can be optimized, on the one hand, by removal, introduction or modification of functional groups of active natural product scaffolds in order to improve their bioactivity and, on the other hand, by natural product-inspired combinatorial synthesis thereby providing large libraries of compounds in a short time. These are the promising strategies of the future to obtain powerful drug leads.

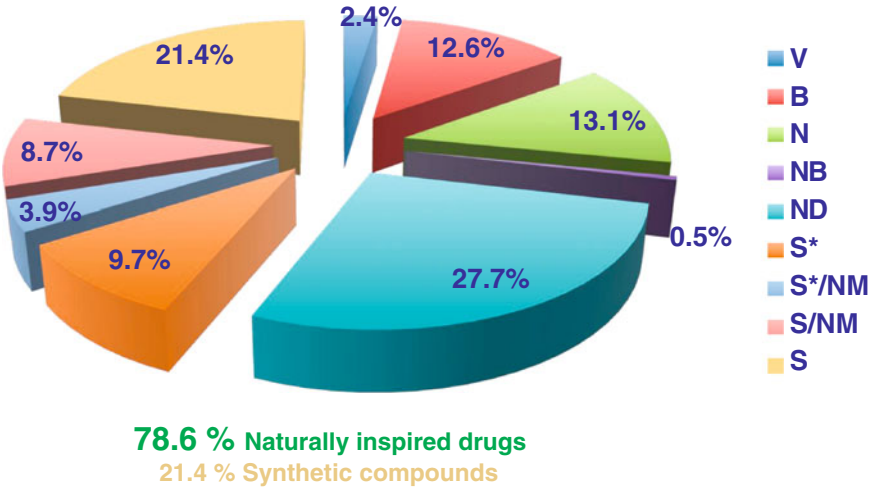

Fig. 1 Classification of cancer drugs according to Newman and Cragg [1]. According to this classification, nine categories can be identified. *N* unmodified natural products, *ND* derived from a natural product—usually after semisynthetic modification of the natural product, *NB* a natural botanical-defined mixture approved as such by the Food and Drug Administration (FDA) or similar organization, *V* vaccines, *B* biological entities, usually a large peptide or protein, *S** synthetic compounds but with a pharmacophore inspired from a natural product, *S*/NM* synthetic compound with natural product pharmacophore, showing competitive inhibition of the natural product substrate (mimicking a natural product), *S/NM* synthetic compounds showing competitive inhibition of the natural product substrate, and *S* synthetic compounds without any relationship to a natural product

2 Cancer and Leukemia

2.1 Introduction

About 2.46 million people developed cancer in Europe in 2008. The mean morbidity age is 69 years for both men and women. The most frequent cancer in women is breast cancer whereas prostate cancer is most frequent in men. Known factors for cancer are smoking, obesity, diet, and physical inactivity. Furthermore, UV light and viral infections are considered harmful. Importantly, cancer is an age-dependent disease and is characterized by uncontrolled growth and spread of cells. Today it is considered as a genetic disease with its development being a complicated, multi-step process. There are several main causes leading to the development of cancer. First, cancer can be caused by activation of oncogenes. DNA mutations caused by carcinogens from cigarette smoke, ionizing radiation, or UV light may lead to the activation of oncogenes, encoding for promoters of abnormal cell growth, such as growth factors or secondary messengers. Activation of oncogenes can also occur through chromosomal translocations. In this case, one

end of one gene is translocated to the beginning of another gene leading to the development of a fusion gene, which can be an oncogene. Finally, viral RNA can be transcribed in cDNA by reverse transcriptase that can then be integrated into the human cellular DNA. If this integration occurs within an oncogene, this oncogene might be activated. Moreover, cancer can be initiated by deactivation of tumor suppressor genes that control cell cycle, cell differentiation, and apoptosis. Thus, their inactivation leads to uncontrolled growth. Often the inhibition of the tumor suppressor gene function occurs by deactivation of the function of the transcribed protein. The binding of, for example, viral proteins to the tumor suppressor proteins can activate this inhibition.

2.2 The Hallmarks of Cancer

Disabled regulatory circuits characterize cancer cells. In opposition to normal cells, cancer cell proliferation and homeostasis are no longer under control [2]. Hanahan and Weinberg recently updated their seminal paper about the hallmarks of cancer and suggest that ten molecular alterations, including eight hallmarks and two enabling characteristics, contribute to malignant cell growth (Fig. 2) [3].

Self-sufficiency in growth signals Cancer cells are able to maintain chronic proliferation. On the one hand, cancer cells synthesize growth factors on their own, leading to autocrine stimulation. On the other hand, they stimulate normal cells in their environment to supply growth factors. Another possibility is an increased

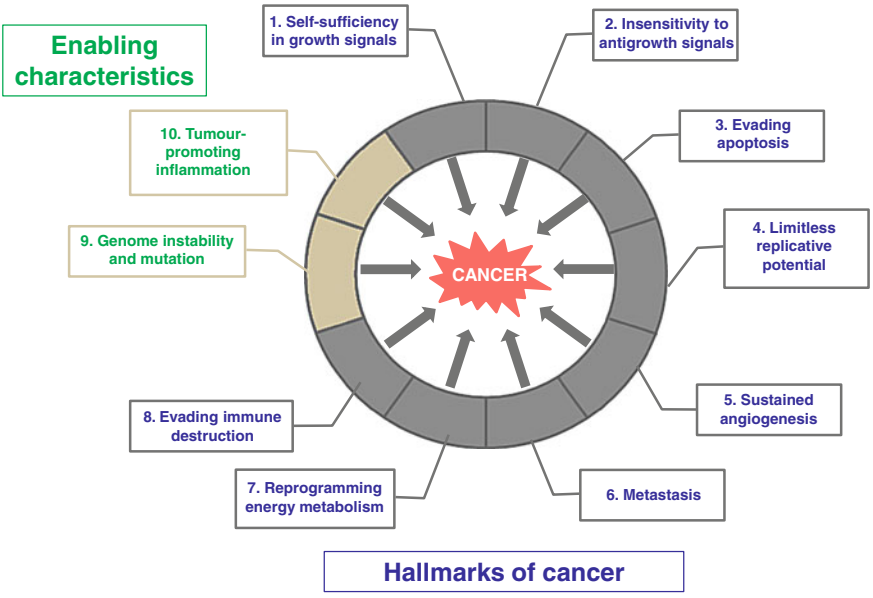

Fig. 2 Carcinogenesis depends on ten molecular alterations; modified from [3]

expression or a mutation of receptors on the cell surface, making the cancer cells hyper-responsive to the signals of growth factors.

Insensitivity to growth-inhibitory signals Tumor suppressor genes ensure limited cell growth and proliferation. As described above, tumor suppressor genes are often deactivated in cancer cells, leading to persistent cell proliferation.

Evasion of apoptosis Apoptosis is a natural barrier to cancer development. However, it can be inhibited or evaded by cancer cells. This happens due to mutations of tumor suppressor genes, overexpression of antiapoptotic genes including Bcl-2 or Bcl-xL or downregulation of pro-apoptotic factors like Bax.

Unlimited replicative potential Telomeres, tandem hexanucleotide repeats at the end of the chromosomes, ensure a limited number of cell cycles. With advanced proliferation, telomeres shorten until the ends of chromosomes cannot be protected any longer. End-to-end-fusion of chromosomes and thus unstable chromosomes is the consequence. The cell enters apoptosis. Through the expression of telomerase, a specialized DNA polymerase that adds telomere repeat segments to the ends of telomeric DNA, cancer cells are able to overcome this limitation and gain immortalization.

Sustained angiogenesis By overexpression of pro-angiogenic factors like vascular endothelial growth factor (VEGF) or downregulation of inhibitors like thrombospondin-1, the tumor is enabled to improve the accumulation of nutrients and oxygen and the elimination of metabolic waste and carbon dioxide.

Metastasis This dangerous hallmark is made possible by loss of cell-to-cell adhesion molecules, like E-cadherin. Metastasis proceeds as an array of several steps, called the invasion–metastasis cascade. The first step is the invasion followed by intravasation of cells into blood vessels. After that a transit of the cells through the lymphatic and hematogenous systems takes place. During the next step, extravasation, the cancer cells migrate into the parenchyma and form micrometastases. Finally, the micrometastases grow to macroscopic tumors, a process called colonization.

Recently, two new emerging hallmarks were described:

Reprogramming energy metabolism To allow uncontrolled cell proliferation, cancer cells have to adjust their metabolism in order to gain sufficient energy. It was found that under the influence of oncogenes and hypoxia, the cell metabolism changes to anaerobic glycolysis which in some cases leads to accumulation of glycolytic intermediates. Because of the increased need of glucose for this energy pathway, cancer cells often overexpress glucose transporters and thus increase glucose import into the cell.

Evading immune destruction The immune system is a natural barrier to tumor development. However, cancer cells can evade detection and destruction by immune cells by obstructing them, for example, through the secretion of immunosuppressive factors or the enticement of immunosuppressive inflammatory cells.

According to Hanahan and Weinberg, two enabling characteristics facilitate the acquisition of these hallmarks (Fig. 2).

Genomic instability and mutation In cancer cells, mutability is increased by a higher sensitivity to mutagenic compounds as well as a breakdown of the natural

genomic maintenance system and suppression of the surveillance systems controlling the integrity of the genome. The development of the hallmarks explained above depends strongly on changes in the genome [3].

Chronic inflammation By supplying the tumor with bioactive molecules, such as growth factors, survival factors, and pro-angiogenic factors, as well as reactive oxygen species (ROS), inflammation contributes to the development of the hallmarks of cancer [3].

These hallmarks and enabling characteristics can be considered as targets for modern anticancer therapy. Natural compounds are in fact excellent tools to investigate the importance of these molecular targets and they can also provide novel therapeutic and chemopreventive compounds to be used in biomedical applications [4–8].

3 Enabling Characteristics: Inflammation

3.1 Introduction

From the hallmarks of cancer and its enabling characteristics, inflammation and the related cell signaling mechanisms have retained much attention in recent years. It was already well documented by Mantovani et al. that an inflammatory environment is causative for cancer development (Fig. 3) [9]. Cells, enabled to proliferate due to DNA damage, continue to proliferate, their growth being supported by secreted cytokines and chemokines as well as growth factors produced by altered inflammatory cells [9, 10]. Natural compounds can efficiently inhibit this hallmark [11].

3.2 Nuclear Factor Kappa B Cell Signaling Pathways

A central molecular mechanism involved in inflammatory and innate immune response is the transcription factor nuclear factor κB (NF-κB) [12]. It has been shown that NF-κB transactivates more than 550 genes controlling most if not all of the ten alterations described above [13, 14]. Thus, NF-κB inhibitors such as bortezomib (N-pyrazinecarbonyl-L-phenyl-alanine-L-leucine boronic acid), which inhibits the proteasome, are considered a promising new approach in cancer treatment [14, 15].

The NF-κB family comprises five members: relA (p65), relB, cRel, NF-κB1 (p50), and NF-κB2 (p52) [14]. These proteins form homodimers and heterodimers [10, 14].

There are four pathways leading to the activation of NF-κB (Fig. 4).

1. The first one is called canonical NF-κB activation pathway. Under non-stimulated conditions, RelA-p50 dimers are associated with an inhibitory protein called Inhibitor of κB (Iκ B) and so remain in an inactive form in the cytoplasm.

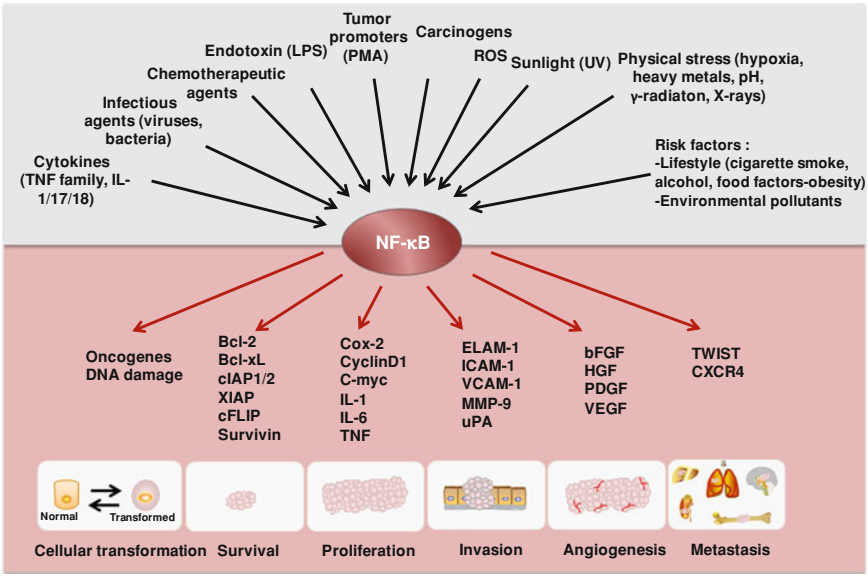

Fig. 3 Link between inflammation, NF-Kb, and cancer; modified from [10]

After stimulation of cell surface receptors with pro-inflammatory cytokines, for example, TNF-α, stress signals, or pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides, IκB kinase (IKK) is activated and it phosphorylates IκB. As a consequence, IκB is ubiquitinated and subsequently degraded by the proteasome. The released NF-κB transcription factor then translocates to the nucleus where it activates the transcription of many target genes involved in cancer initiation, promotion, and progression [14, 16, 17].

2. The second pathway is called non-canonical or alternative NF-κB signaling pathway and activates the p100-RelB complex. In this case, members of the TNF-superfamily like the B-cell activating factor (BAFF) or the CD40 ligand activate the NF-κB-inducing kinase (NIK) by binding to their receptors. NIK in turn activates IKKα. IKKα phosphorylates two serine residues of an IκB-like domain of p100. As a consequence, p100 is partially degraded by the proteasome and the p52-RelB complex is liberated [10, 14, 17].
3. The third NF-κB activation pathway—the atypical pathway—is independent from IκB degradation. The stimuli regulating this activation are still unclear. After a stimulation, p105 is present in complex with p50 and is phosphorylated by IKKs at serine residues of an IκB-like domain. Then, p105 is ubiquitinated and partly degraded by the proteasome, leading to formation of p50. Subsequently, Bcl-3 binds to the p50 homodimer forming a trimeric complex. Afterward, the complex translocates to the nucleus where Bcl-3 serves as a transcription activator by interaction with CBP/p300 [17, 18].

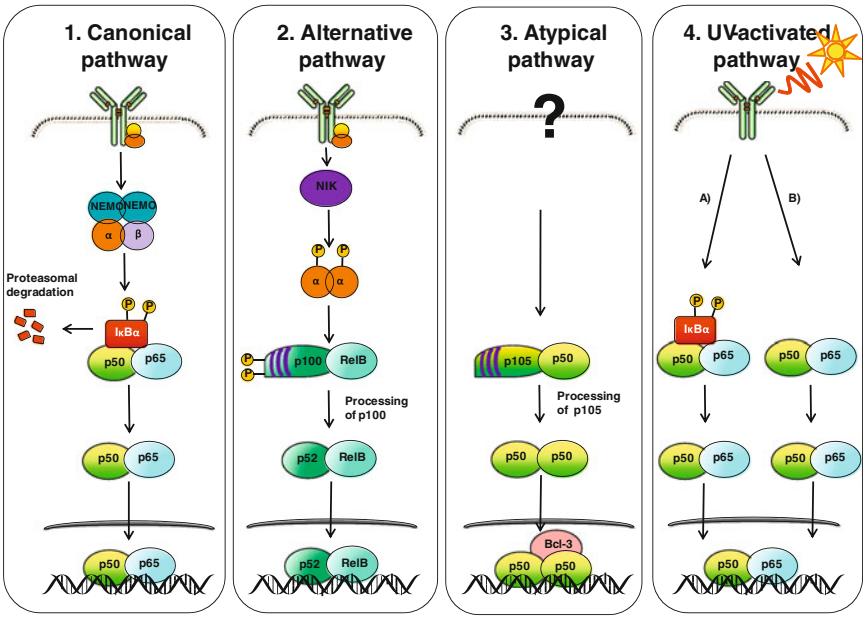

Fig. 4 The four pathways of NF-κB activation. **a** The classical or canonical NF-κB activation pathway activated by Toll-like, TNF, or T-cell receptor. **b** The alternative or non-canonical NF-κB signaling pathway activated by BAFF receptor or CD40. **c** The atypical pathway. **d** The UV-induced pathway with *A* the early-phase pathway and *B* the late-phase pathway (adapted from [17])

4. The fourth pathway is the UV-induced NF-κB activation pathway. UV light activates NF-κB by two different mechanisms: (a) the early-phase IκB-dependent pathway, leading to IκBα phosphorylation and proteasomal degradation and (b) the late-phase IκB-independent pathway during which NF-κB is released by an unknown mechanism [19, 20].

In healthy cells, the activation of NF-κB is a regulated process as transcription of IκB, the natural inhibitor of NF-κB, is under the control of this transcription factor [10]. By this auto-regulatory loop, NF-κB activation is limited in time and intensity [15]. In tumor cells, molecular alterations lead to a pathological and constitutive upregulation of NF-κB activation, resulting in the expression of genes being involved in the development of cancer [10, 13]. This activation of NF-κB has been described in many tumors [10].

However, natural compounds of various origins are able to efficiently counteract NF-κB activation and related cell signaling mechanisms. Recently, we provided evidence that nature provides excellent inhibitors of NF-κB: we showed that curcumin [21–23]; β-lapachone and emodin, sanguinarine, and capsaicin [24]; kawains [25]; naphtopyrones [26]; cardenolides [27, 28]; heteronemin [29]; polyketide derivatives [30]; chalcones [31, 32]; and neem leaf extract [33] efficiently inhibit NF-κB cell signaling pathways. Recent reports demonstrate that this

is a very active field of research worldwide [6, 22, 34–39]. It is noteworthy to mention that NF-κB is most often coregulated with the signal transducer and activator of transcription (STAT) signaling cascades and that natural compounds efficiently inhibit these regulatory pathways [40, 41].

3.3 Cyclooxygenase-2: A Key Mediator of Inflammation

Besides the NF-κB pathway, cyclooxygenase-2 (COX-2), the inducible form of the family of cyclooxygenases is an important mediator of inflammation, which has been found constitutively expressed in many forms of cancer including breast, colon, or prostate [42]. A number of studies showed that COX-2 is stably expressed since the early pre-neoplastic stages. This encourages many to consider COX-2 as a potential target in chemoprevention as well as in the treatment of cancer. Synthetic inhibitors of COX-2, which target enzymatic activity, are the only clinical strategy to counteract COX-2. However, these compounds present severe side effects, a factor that limits their prolonged intake, which is required for chemoprevention or during anticancer treatment. Accordingly, the discovery of natural COX-2 inhibitors might prevent the observed side effects and this has recently become an active field of research [43].

4 Natural Compounds as Anticancer Agents

4.1 Anticancer Compounds from Terrestrial Plants

To survive and defend themselves, plants have adopted multiple mechanisms based on compounds such as alkaloids, which are able to inhibit the growth of other plants. As an example, vincristine is a *Vinca* alkaloid isolated from *Catharanthus roseus*, the Madagascar periwinkle. It causes a metaphase arrest by binding to free tubulin dimers and thus prevents microtubule polymerization. Another well-known compound, etoposide is a natural podophyllotoxin of the May apple (*Podophyllum peltatum*) and is used in the treatment of most types of cancer (lung cancer, acute leukemia, testicular cancer, and breast cancer). Etoposide prevents the entry of tumor cells in mitosis by inhibiting the activity of topoisomerase II leading to double-strand DNA breaks. Moreover, paclitaxel and docetaxel are molecules of the taxane family with antitumor activities against breast or ovarian cancer. Paclitaxel is derived from the bark of the yew tree whereas docetaxel is isolated from the needles of yew trees of the Pacific Ocean (*Taxus brevifolia*). They stimulate the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization. This stability inhibits the normal dynamic reorganization of the microtubular network and leads to a mitotic arrest. Flavopiridol is the first cyclin-dependent kinase (CDK) inhibitor that was discovered [44]. This is a semisynthetic flavonoid. It interferes with the

phosphorylation of CDKs, inhibits cell division by preventing progression in G1 and G2 phase, and induces apoptosis of tumor cells. In addition, flavopiridol decreases expression of cyclin D1 [45], which is overexpressed in breast carcinoma cells. This compound also inhibits the activity of the complex cdk7/cyclin H, involved in the phosphorylation of other CDKs. Clinical trials of this compound are currently underway as well as combinatorial testing with other natural compounds such as paclitaxel and cisplatin [46, 47].

4.2 Compounds from Marine Organisms

There is a large chemical diversity in marine plants. The products isolated from marine sources possess cytotoxic activities against many cancers [5, 29, 34, 35, 38, 48–51].

In 1981, didemnin B was the first marine compound isolated from the Caribbean tunicate *Tridemnum solidum*. It prevents the translocation of the eukaryotic elongation factor 2 (EEF2) and thus inhibits protein synthesis [52]. Didemnin B activates caspases and thereby leads to apoptosis of cells [53].

Dolastatines are peptides derived from the Indian Ocean molluscs *Dollabella auricularia*. They possess antineoplastic properties. Dolastatin 15, for example, inhibits mitosis by interfering with microtubule formation, causing a blockage of cell division in metaphase [54].

Bryostatins were isolated from a species of bryozoan *Bugula neritina*. This species lives in symbiosis with a bacterium that secretes an active biomolecule: bryostatin. This compound recovers the larvae of bryozoans and acts as a repellent for fish predators. Bryostatins are macrolides and comprise a family of 15 derivatives. Bryostatin 1 is best known as an anticancer agent. This compound is able to induce ubiquitination and proteasome degradation of Bcl-2 in lymphoblastic leukemia and permits the growth of progenitor cells from bone marrow [55]. Bryostatins are potent activators of protein kinase C (PKC) and regulate the activation, growth, and differentiation of cells [46, 47].

4.3 Compounds from Microorganisms

Microorganisms have developed sophisticated mechanisms to survive in hostile environments and in tissues/organisms that have highly evolved immune systems. Thus, during the invasion of tissue, they implement different strategies. For example, they release toxins that can destroy the cells of the immune system and they can “camouflage” to avoid being recognized by the defense systems. Considering their multiple properties, the substances of microorganisms are of interest for cancer therapy.

Rapamycin is a macrolide isolated from *Streptomyces hygroscopicus* and has antifungal and antiproliferative properties. Rapamycin inhibits the mTOR pathway

that controls growth and cell proliferation [56]. Activation of mTOR allows the entry of cells into the cell cycle. Rapamycin prevents allograft rejection after transplantation and exerts its antiproliferative effect in vitro and in vivo on a large number of tumors [56]. It blocks cell cycle progression in G1 in T and B cells, osteosarcoma, and other tumor cells.

Wortmannin was isolated from *Penicillium fumiculosum* and is an inhibitor of phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and myosin light-chain kinase (MLCK), a serine-threonine kinase that phosphorylates myosin light chains involved in the contraction of smooth muscle [57]. Wortmannin blocks the kinase ataxia telangiectasia mutated (ATM) induced by DNA damage and inhibits the antiapoptotic effects of cytokines as well as the stabilization of p53 [46, 47].

4.4 Dietary Compounds

Cinnamic acid is a phenolic acid discovered and purified by Péligot and Dumas in 1834. It was isolated from the plant *Cinnamomum cassia* and is known for its antiseptic and antifungal properties. Cinnamic acid is an intermediate in the biosynthetic pathway of shikimic acid and of all phenylpropanoids. It is produced from phenylalanine by an enzyme, the phenylalanine ammonia-lyase (PAL) and it is then converted into paracoumaric acid by cinnamate 4-hydroxylase. Cinnamic acid can be converted into other compounds including hydroxycinnamic acids (caffeic acid, ferulic acid, and sinapic acid), coumarins (p-coumaric acid) by internal cyclization, flavonoids (quercetin), and lignins (coniferyl alcohol). Cinnamic acid is also a precursor of benzoic acid, salicylic acid, and gallic acid. It has been shown that cinnamic acid exhibited antitumor effects against melanoma, glioblastoma, and adenocarcinoma of the lung as well as the prostate [58]. Moreover, in colorectal cancer cells (Caco-2), cinnamic acid possesses an antiproliferative effect and dose-dependently inhibits DNA synthesis [59].

4.5 Chalcones

Polyphenols are important dietary compounds [60] with more than one phenol unit per molecule. The family comprises many thousands of molecules present in plants, fruits, and vegetables, involved in plant defense against ultraviolet radiation and pathogen aggression. They protect cells against tissue damage caused by free radicals and related pathologies such as cancer, inflammation, and cardiovascular diseases [61]. Chalcones represent an important polyphenolic subgroup with antioxidative, antibacterial, anti-inflammatory, anticancer, cytotoxic, and immunosuppressive potential. Chalcones are especially abundant in fruits like citruses or apples and vegetables that were traditionally used in herbal medicine [62]. Despite their apparent lack of specificity, chalcones were described to target distinct

regulatory proteins that modulate downstream signaling pathways. Specific structural requirements play an essential role and can be correlated with observed bioactivity [63–67]. Identification and characterization of natural chalcones as nutraceuticals or as novel therapeutic agents is a promising approach to fight inflammation and cancer [31, 32, 39].

5 Modulation of Cyclooxygenase-2 by Natural Products

There are a wide variety of natural products that can also modulate the expression of COX-2 at the transcriptional level, posttranscriptional or posttranslational level (Fig. 5) [43].

5.1 Regulation at the Transcriptional Level

Polyphenols represent a large source of compounds used in chemotherapy and chemoprevention. These compounds possess interesting anti-inflammatory and anticancer properties. One target of these compounds is transcription factor NF-κB. Many studies have investigated the ability of these compounds to decrease the function of COX-2 in regulating its expression at the transcriptional level (Fig. 5).

Many compounds modulate the expression of COX-2 by interfering with MAPK signaling pathways. Indeed, phosphorylation of p38 and ERK can be inhibited by curcumin, extracted from the root of *Curcuma longa*, which has anti-inflammatory and anticancer effects [68], by resveratrol, a compound mainly found in red wine and grapes [69] and by epigallocatechin gallate (EGCG) [70], a polyphenol from green tea.

Activation of JNK activates the transcription factor AP-1, which is inhibited by diallyl polysulfides from garlic and onion [71]. In addition, the PI3K/Akt pathway is inhibited by psoralidin, a compound isolated from the seeds of *Psoralea corylifolia* [72].

The inhibition of the activation of MAPKs prevents activation and nuclear translocation of transcription factors that bind to specific sites of the promoter of COX-2 and leads to inhibition of COX- 2 expression.

Natural products can also act directly on the activation of transcription factors. For example, apigenin, a flavone found in chamomile, inhibits IκB kinase (IKK), which phosphorylates and inhibits IκBα. IκBα sequesters NF-κB and prevents its transport into the nucleus and the interaction of its subunits (p65, p50) with the promoter.

Other transcription factors are also targets of natural products. AP-1 composed of c-jun and c-Fos subunits is inhibited by curcumin in carcinoma of the endometrium. This factor is also inhibited by resveratrol in mammary epithelial cells stimulated with non-carcinogenic LDCs [73], by diallyl trisulfides [71] and by flavonoid quercetin [74]. The activation of AP-1 leads to inflammation, cell

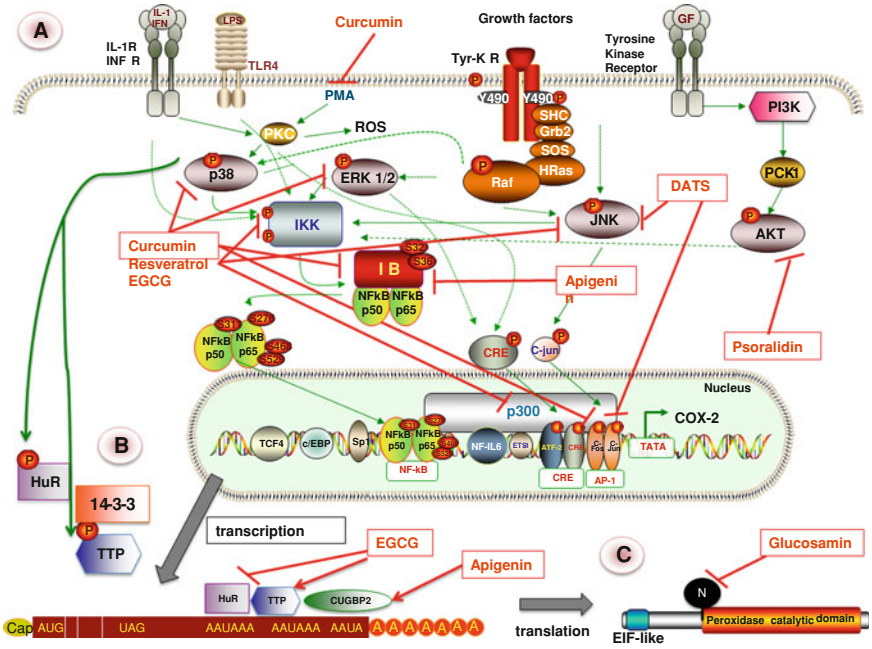

Fig. 5 Modulation of COX-2 expression by natural compounds. **a** Transcriptional regulation: Curcumin, resveratrol, and EGCG prevent p38, ERK, and JNK activation as well as IκBα degradation and IKK activation. Curcumin inhibits the effect of PMA and ETS-1 nuclear translocation. Diallyl trisulfides prevent JNK activation and c-Fos and c-Jun nuclear translocation. Apigenin prevents IκBα degradation. Psoralidin inhibits AKT activation. **b** Posttranscriptional regulation: EGCG is able to inhibit HuR binding to AREs and promotes TTP expression. Apigenin allows CUGBP2 binding to AREs as well as its association with HuR. **c** Posttranslational regulation: Glucosamine prevents N-glycosylation of the COX-2 protein

transformation, and immune response. AP-1 allows the epithelial-mesenchymal transition, which represents an early step in metastasis formation [75]. The transcription factor Ets-1 regulates the expression of factors involved in angiogenesis such as VEGF. Its expression can be inhibited by curcumin in human carcinomas of the endometrium [68]. PMA is an inducer of COX-2 expression. It binds to the site of diacylglycerol (DAG) of PKC and activates it. PKC promotes the formation of ROS. Curcumin has antioxidant properties and blocks the action of PMA [75].

5.2 Regulation at the Posttranscriptional Level

Modulation of mRNA stability and translation efficiency is crucial for COX-2 stability in immune diseases and cancer. In colorectal cancer, COX-2 mRNA is very stable. Few studies have investigated the effect of natural products on COX-2 expression at the posttranscriptional level (Fig. 5).

Several proteins including HuR and TTP modulate the stability of COX-2. Moreover, microRNAs such as miR-199 and miR-16 that bind AUUUUA-rich elements (ARE) in the 3′ untranslated region (UTR) regulate the fate of the COX-2 encoding mRNA. Some natural products regulate these proteins in order to stabilize or destabilize mRNA. EGCG, for example, inhibits HuR protein involved in mRNA stabilization and induces TTP protein that destabilizes mRNA COX-2 [70]. Curcumin is able to downregulate and upregulate expression of several miRNAs. Indeed, miR-199 expression is downregulated and miR-22 is upregulated by curcumin [76].

P38 MAPK is important in the regulation of mRNA stability. Indeed, p38 is able to phosphorylate HuR and TTP. Activating phosphorylation of HuR allows its transport into the cytoplasm and its attachment at the AREs sequences [77]. In contrast, phosphorylation of TTP is recognized by the 14-3-3 complex that binds to the protein and prevents its binding to AREs [78]. Curcumin [79] and resveratrol [69] prevent activation of p38.

Other compounds play a role in translation efficiency of mRNA of COX-2 by modulating the binding of certain proteins such as TIA-1 and TIAR on AREs. Apigenin, for example, stabilizes the transcript of COX-2, but prevents its translation into protein due to the interaction between HuR and CUGBP2 that inhibits translation [80]. It has been shown that cinnamon, glucocorticoids, and green tea promote the transcription of TTP [81].

Activation of p38 is important for regulating the expression of COX-2 at the transcriptional and posttranscriptional levels. At the transcriptional level, it enables the transcription of COX-2 by activating transcription factors such as NF-κB or AP-1. At the posttranscriptional level, it promotes the phosphorylation of HuR and allows its nucleocytoplasmic transport to stabilize COX-2 mRNA.

Selected compounds affect COX-2 only at the posttranscriptional level. This is the case of mangiferin, a glucosylxanthone extract from mango trees (*Mangifera indica*), which reduces mRNA stability of COX-2 without affecting the efficiency of transcription of COX-2 in microglial cells stimulated with lipopolysaccharides [82].

5.3 Posttranslational Regulation

The investigation of posttranslational modifications of COX-2 is a recent field of research. Initial results suggest a role as chemopreventive agents on the expression of COX-2 at this level (Fig. 5).

Polyunsaturated fatty acids such as omega-3 from fish or flax seeds are considered as preventive agents of cardiovascular diseases. The two main omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), exert antiproliferative effects against colon cancer [83], prostate cancer [84], and hepatocellular carcinoma [85]. An inverse correlation between the likelihood of developing aggressive forms of prostate cancer and regular consumption of

omega-3 fatty acids has been demonstrated [84]. In addition, administration of DHA, EPA or 11,14,17-eicosatrienoic acid (ETA), another omega-3 fatty acid, inhibits the expression of pro-inflammatory mediators in in vivo models of skin cancer [86]. Omega 3 reduces COX-2 expression and inhibits its activity [87]. Glucosamine is also an omega-3 fatty acid naturally present in the skeleton of crustaceans and is mainly used for the treatment of osteoarthritis. Recent data show that glucosamine can reduce the N-glycosylation of COX-2 in lung cancer cells [88] and hence decreases the activity of COX-2. The ability of this molecule to block the activity and stability of COX-2 reveals interesting anti-inflammatory properties in the treatment of cancer.

6 Conclusions

Nature has provided the largest library of medicinal tools and has become a foundation for sophisticated traditional pharmacopeia all over the world. Egyptian medicine, the Chinese *Materia Medica*, the Indian Ayurvedic system as well as in the ancient Western world–known Greek and Roman medicine have been based mostly on drugs of plant origin. Nowadays, natural products maintain their importance in modern cancer drug discovery. Secondary metabolites produced by organisms often include active compounds serving predominantly for defense, predation, or communication. Hence, it is not surprising that those biologically highly active compounds have become an inherent acquisition to modern medicine. The activity of these compounds against molecular mechanisms involved in inflammation and cancer is nowadays an active field of research. Compounds from natural sources can therefore be considered as valuable agents in both prevention and therapy.

Acknowledgments BO and NL are recipients of doctoral Télévie-Luxembourg grants. Research at the Laboratoire de Biologie Moléculaire et Cellulaire du Cancer is supported by "Recherche Cancer et Sang" foundation, "Recherches Scientifiques Luxembourg" association, "Een Haerz fir kriibskrank Kanner" association, the Action Lions "Vaincre le Cancer" association and Télévie Luxembourg. Further support was received from the European Union (ITN "RedCat" 215009, Interreg IVa project "Corena").

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Selenium and Cancer: A Story that Should not be Forgotten-Insights from Genomics

Catherine Méplan and John Hesketh

Abstract

Selenium (Se) is an essential micronutrient that is incorporated into selenoproteins. Although epidemiological studies suggest that low Se intake is associated with increased risk of various cancers, the results of supplementation trials have been confusing. These conflicting results may be due to different baseline Se status and/or genetic factors. In addition, mechanistic links between Se intake, selenoproteins and carcinogenesis are not clear. In this article, we discuss the functional significance of single-nucleotide polymorphisms (SNP) in selenoprotein genes and the evidence as to whether or not they influence risk of colorectal, prostate, lung or breast cancers. Both *in vitro* and *in vivo* studies have shown that a small number of SNPs in genes encoding glutathione peroxidases 1 and 4, selenoprotein P, selenoprotein S and 15-kDa selenoprotein have functional consequences. Data from case–control studies suggest that a variant at codon 198 in glutathione peroxidase 1 influences the effect of Se status on prostate cancer and risk, and it has also been associated with breast cancer and lung cancer risk, whereas variants in glutathione peroxidase 4, selenoprotein P and selenoprotein S may influence the risk of colorectal cancer. In addition, the results of gene microarray (transcriptomic) studies have identified novel selenoprotein biomarkers of Se status and novel downstream Se-targeted pathways. The work highlights the need to take baseline Se status and genetic factors into account in the design of future intervention trials.

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_9,

Keywords
SNP · Transcriptomics · Selenoproteins · Micronutrient · Colorectal Cancer · Prostate Cancer

Abbreviations

SNP	Single-nucleotide polymorphisms
GPX	Glutathione peroxidase
TR	Thioredoxin reductase
Sec	Selenocysteine
SECIS	Selenocysteine insertion sequence
Sel	Selenoprotein
SePP	Selenoprotein P
CRC	Colorectal cancer
GWAS	Genome-wide association studies
mTOR	Mammalian target of rapamycin

Contents

1 Introduction

Selenium (Se) is an essential micronutrient required for animal and human health [1, 2]. Dietary Se intake is highly dependent on the concentration of Se in the soil on which the crops were grown or on which the fodder for animals was produced-as a result, dietary Se intake, and resulting Se status, varies greatly throughout the

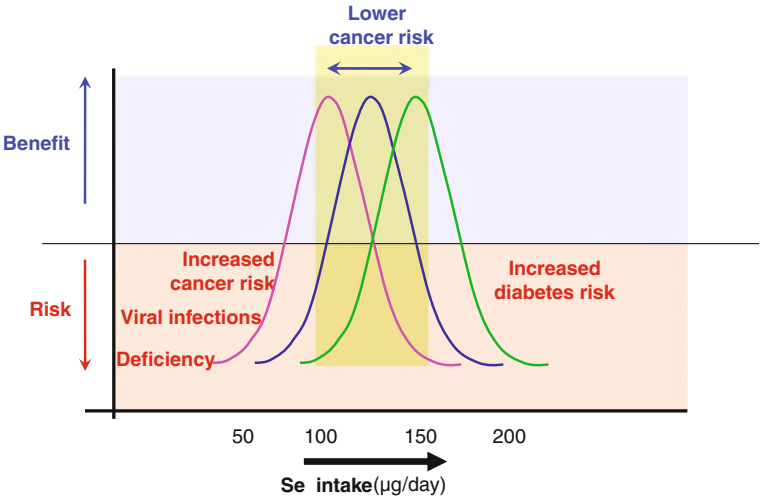

Fig. 1 Individualisation of benefits and risks associated with selenium intake. The graph suggests a possible relationship between Se intake and disease risk and benefit. Note the highlighted window of intake where there have been suggestions of lower cancer risk. The potential variation in the Se intake/cancer risk is illustrated by the three curves. This risk is affected by genetic factors, age and risk factors (such as environmental exposure to stress including alcohol intake)

world. Se deficiency can occur in populations eating locally sourced food from areas where the soil Se content is very low. For example in parts of China, very low Se intake (<10 µg/day) is a key factor contributing to a fatal viral myocarditis called Keshan disease. However, such severe deficiency is rare. In contrast, sub-optimal Se intake is more commonly observed, for example, in parts of Europe, Se intake is estimated at about 30–50 µg/day, compared with the present reference nutrient intake of 75 and 60 µg/day for men and women, respectively. Regions of New Zealand and Australia have comparable sub-optimal intakes. Thus, although in most populations, Se intake is not low enough to cause deficiency-associated disease and it may be inadequate for optimal health [1–3].

It is some 40 years since the first epidemiological study suggested that Se status is inversely related to risk of various cancers and that cancer mortality was lower in areas of the USA where crop Se was high compared to regions where it was lower [4]. Although similar findings were subsequently reported across China and worldwide and low serum Se was reported to be a pre-diagnostic indicator of higher cancer risk, particularly for gastrointestinal and prostate cancers (see [3]), subsequent studies have provided contradictory and inconsistent findings as regards the relationship of Se status and cancer risk (see [2, 3] for reviews). Despite these inconsistencies, there has been considerable interest in whether such low, sub-optimal Se intake is associated with increased susceptibility to cancers. Several supplementation trials have been carried out in relation to Se and cancer

risk, and initial results provided considerable impetus for Se and cancer susceptibility research. A Se supplementation trial carried out in the USA found that supplementation with 200 μg/day Se as selenised yeast led to reduced incidence of prostate and colorectal cancers in the lowest tertile of the population in terms of plasma Se status prior to supplementation [5]. A subsequent trial in China with a daily 50 μg/day supplement of selenised yeast was found to lower oesophageal cancer mortality [6]. However, this apparent positive benefit of increased Se intake on cancer risk has had to be balanced with apparent differing relationships of Se status and cancer risk in a range of case–control studies [3], the failure of Se supplementation to lower prostate cancer risk in the recent SELECT trial [7, 8] and possible increased risk of diabetes at relatively high Se intakes [9]. It now appears that as illustrated in Fig. 1, there is a narrow window of benefit between a plasma Se level which can be beneficial and either lower or higher levels where there is increased disease risk. Thus, as a result, there is debate over whether increased Se intake may be of benefit in lowering cancer risk.

Underlying the debate over the anti-cancer effects of increased Se intake and the conflicting results from supplementation trials are three factors. First, results from different trials can be complicated by the baseline levels of Se intake; notable in this respect is the difference between the two trials in the United States where baseline in the SELECT trial is above that in the lowest tertile of the population in the earlier trial where Se was observed to reduce cancer risk [5, 7, 8]. Thus, it is possible that in the SELECT trial, but not in the NPC trail, the baseline Se was already close to optimal in terms of prostate cancer prevention. Second, increased knowledge of human genetic variation is leading to a realisation that by causing interindividual variation in how individuals metabolise nutrients, genetic variation impinges on diet–disease risk interactions in a number of situations and this can lead to altered disease susceptibility; there are SNP in genes related to Se metabolism [10, 11], and as illustrated schematically in Fig. 1, such variation could potentially affect responses to dietary Se and the relative levels at which benefits of increased Se intake occur. Third, there is a lack of mechanistic understanding of the roles of several selenoproteins in target tissues such as the prostate and colon and therefore poor knowledge of the physiological consequences of low dietary Se. Figure 1 highlights the importance of defining individualised risk–benefit windows [12] integrating Se intake, genetic factors altering Se metabolism and selenoprotein function/expression, disease risk level (for example, reflecting environmental exposure to carcinogens) and changes in Se requirements as observed in the ageing population [13] before defining the risk–benefit window for Se for a population.

The advance of genomic sciences provides methods and approaches to address the two latter issues: genotyping techniques allow assessment of the effects of genetic influences on Se metabolism and the relationship with disease; microarray and proteomic approaches allow detailed studies of selenoprotein function. In this article, we will discuss how such genomic approaches are contributing to a description of the relationships between Se, selenoproteins and cancer risk.

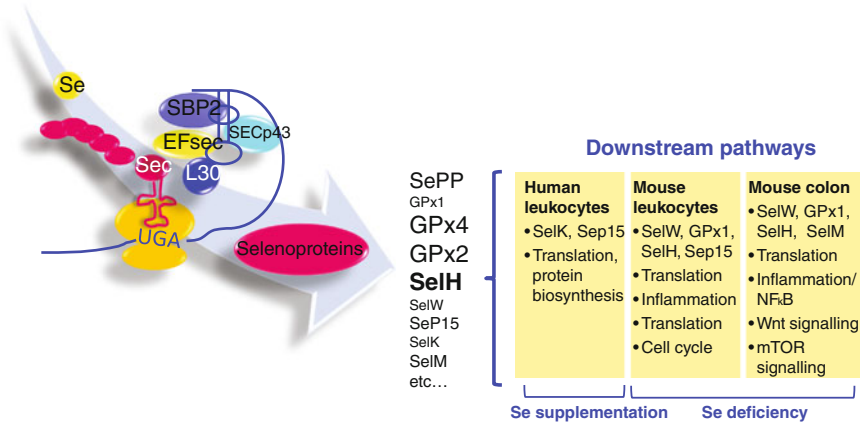

Fig. 2 Schematic diagram illustrating synthesis of selenoproteins, the selenoprotein hierarchy and effects of altered Se intake on downstream pathways. The scheme illustrates how Se is incorporated into a number of selenoproteins by a mechanism involving specific proteins that bind to a structure in the 3′ untranslated region of selenoprotein mRNAs. The size and font of the selenoproteins listed illustrate their position in the hierarchy of selenoprotein synthesis (bigger font, bold—higher in hierarchy). Changes in selenoprotein activity affect downstream pathways, and those found to be affected experimentally are shown in the *shaded boxes*

2 Selenoproteins

In organisms from bacteria to humans, Se in the food is incorporated into a number of Se-containing proteins, the selenoproteins. The physiological functions of Se are thought to be brought about by these selenoproteins [1, 14, 15]. In humans, Se is found in 25 selenoproteins in all of which it is present as the amino acid selenocysteine (Sec) [14]. Sec is incorporated into these proteins at UGA codons during translation of the appropriate mRNAs. This involves recoding of UGA codons for incorporation of Sec rather their usual function as a STOP codon signalling the termination of translation. A unique biochemical mechanism, illustrated schematically in Fig. 2, allows this recoding to occur: it involves a specific selenocysteyl-tRNA (tRNA-Sec), a stem-loop structure (Sec insertion sequence: SECIS) within the 3′ untranslated region (3′ UTR) and specific proteins that bind to the SECIS structure and so allow the UGA codon to recognise the tRNA-Sec. The tRNA-Sec is synthesised from selenide, ATP and seryl-tRNA.

Sec has a pKa of 5.47 (lower than that for cysteine), and in most selenoproteins, it is found at the active site where it appears in most cases to contribute to oxidoreductase activity. Many selenoproteins have antioxidant protection or redox functions. Of the glutathione peroxidases (GPx), GPx1 and GPx2 have been shown to protect cells from reactive oxygen species and reactive nitrogen species, GPx3 has been proposed to have an extracellular antioxidant role, and GPx4 has a complex set of functions including protecting cells from lipid hydroperoxides. The

three thioredoxin reductases (TR1-3) have oxidoreductase functions, but in this case, their function is not only related to maintenance of redox state but also related to the conversion of ribonucleotides to deoxyribonucleotides. The iodothyronine deiodinases carry out oxidoreductase reactions in thyroid hormone metabolism.

The oxidoreductase nature of the activity of less well-characterised selenoproteins has emerged recently. For example, structural studies indicate that selenoproteins H, L, T and W (SelH, SelL, SelT and SelW) and the 15-kDa selenoprotein all contain a thioredoxin-like redox fold [16, 17]. In addition, SelS, SelN, 15-kDa selenoprotein, SelK and SelM are components of the endoplasmic reticulum that appear to be involved in redox balance and the unfolded protein response [13, 16–18]. Despite these clues to the functions of these selenoproteins, details as to their biochemical roles are still limited.

Selenoprotein P (SePP) is unique in containing multiple Sec, 10 in humans. It is a secreted protein that makes up over 50 % of the total plasma Se content and is detected as multiple isoforms of which two are approximately 50 and 60 kDa in molecular weight [19, 20]. In the mouse, SePP has been shown to be essential for the maintenance of the Se content of extrahepatic tissues such as brain and testis [21, 22] and this has led to the proposal that the major function of SePP is to deliver delivery of Se from liver to other tissues. However, in addition, SePP has been shown to have antioxidant functions and this may be related to the findings that although expressed highly in the liver, SePP is also expressed in a range of other cell types including the colon [19, 23].

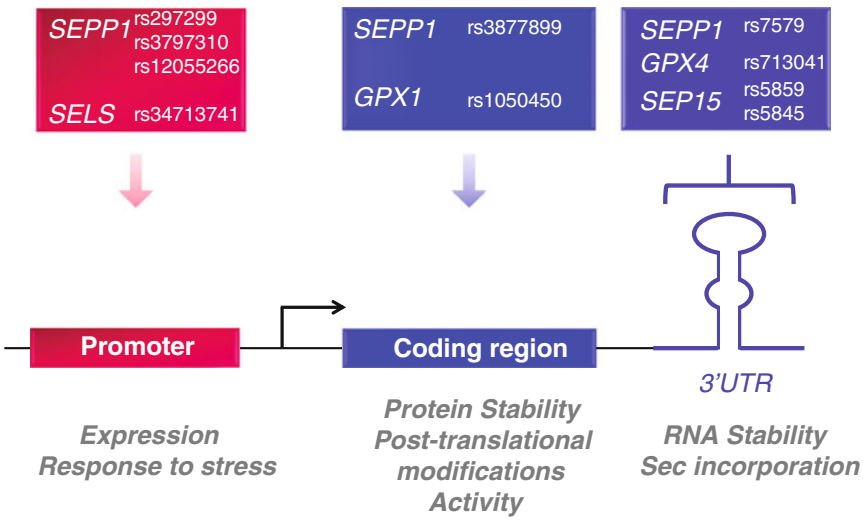

Fig. 3 Functional SNPs in selenoprotein genes. Functional single-nucleotide polymorphisms have been found in several selenoprotein genes. In the figure, they are identified by their rs numbers and gene name. The position of the SNPs in the different functional regions of the corresponding mRNA and their potential effects on selenoprotein expression is shown

2.1 Selenoprotein Hierarchy and its Implications

Dietary Se intake is essential for selenoprotein biosynthesis, and it regulates their synthesis to a different extent depending on the selenoprotein. Indeed, there is very strong evidence that the synthesis of different selenoproteins is affected differentially by low Se intake [14, 24, 25]. This has led to the concept of the "selenoprotein hierarchy", which proposes that changes in Se intake affect the synthesis of some selenoproteins more than others: for example, GPx1 protein and mRNA levels are more sensitive to Se deficiency than GPx4 in many tissues. Furthermore, the relative position of selenoproteins in this hierarchy differs between different tissues, and this causes the pattern of response of selenoproteins to Se supply to vary between different tissues. For example, in the rat, GPx4 is more sensitive to Se depletion in the liver than the heart and deiodinase activity is less sensitive to Se deficiency in the thyroid than in the liver [24]. On the basis of mRNA levels, GPx1, SelW, SelH and SelM appear to be most sensitive to Se supply in colonic cells and GPx1 and SelK in lymphocytes [26, 27]. There is evidence that interactions between the proteins of the Se incorporation machinery and the different selenoprotein 3′ UTRs are critical in the hierarchy (e.g. [28, 29]). As a consequence of the tissue-specific selenoprotein hierarchy, the effects of low Se supply on the pattern of selenoprotein expression differ between tissues, and in addition, this may have implications for involvement of different selenoproteins in tumorigenic processes or protective mechanisms in different tissues.

A change in activity of each selenoprotein would be expected to cause downstream metabolic effects that are specific for each protein. As illustrated in Fig. 2, since low Se intake affects synthesis of several selenoproteins, one would expect Se depletion to affect a range of downstream targets. The advent of genomic technologies, especially microarrays, is allowing integration of consequences of changes in Se intake in the context of the pattern of these downstream molecular pathways. This is particularly relevant to Se because of the tissue-specific selenoprotein hierarchy-changes in Se supply alter the pattern of selenoprotein synthesis (consequently affecting net activity of the corresponding protein) in a tissue-specific manner and thus also change the downstream metabolic effects differentially between tissues.

3 Selenoprotein Gene Variants

SNP can have functional effects on gene expression and ultimately protein activity either by causing amino acid changes in the protein or by influencing regulation of expression. The latter is the case for SNPs in gene promoter regions. In selenoprotein genes, gene variants in regions corresponding to the 3′ UTR have the potential to influence expression because of the key role of the SECIS and 3′ UTR–protein interactions in Sec incorporation. Indeed, in selenoprotein genes, SNPs in the gene regions corresponding to the 3'UTR, promoter and protein

coding regions have been reported to influence Se metabolism [10, 13]. This is illustrated schematically in Fig. 3.

On the basis of effects on levels of inflammatory markers such as TNF-alpha and interleukins, an SNP located at position −105 in the promoter of the *SELS* gene (rs34713741) has been shown to have functional consequences [30]. In addition, activity of the plasma glutathione peroxidase (GPX3) has been reported to be influenced by a number of variants [31]. A polymorphism (not a SNP) has been identified in the promoter of *SEPP1* gene corresponding to a complex repeated sequence $(TC)_n-T_{17}$ with one variant containing 3 TC repeats and the other one 5 [23]. This motif could alter a putative binding site for the transcription factor, nuclear factor of activated T cells (NFAT). However, this observation has not yet been confirmed and not been associated with disease risk.

Variants in the protein coding region of selenoprotein genes have been found to lead to amino acid changes and potentially changes in protein function. For example, a single-nucleotide change that leads to a proline-to-leucine change at codon 198 in the *GPX1* gene (rs1050450) has been found to reduce enzyme activity in the Leu variant protein [32]. This variant has been reported to influence the relationship between plasma Se and erythrocyte GPx1 activity [33] and also to be associated with white blood cell GPx1 activity [34]. This SNP is found widely in different ethnic groups, but the leucine variant is relatively rare (7–15 %). In addition, a G/A SNP (rs3877899) leads to an alanine-to-threonine amino acid change at codon 234, and this has been reported to influence the response of various blood cell and plasma selenoprotein activities to Se supplementation [35]. In addition, it affects the proportion of SePP isoforms in plasma and their relationship with lymphocyte GPx4 levels [20].

Several functionally significant SNPs have been identified in selenoprotein gene regions corresponding to the 3′ UTR. In GPX4, rs713041 has C/T allelic variants and both variants are relatively frequent in a range of ethnic groups [29, 36, 37]. In the first study of this SNP, it was observed that individuals carrying the two variants were reported to differ in lymphocyte leukotriene levels [36]. Subsequently, a strong body of evidence has been accumulated from both *in vitro* and *in vivo* experimental approaches to show that this SNP has functional effects. For example, in RNA–protein binding assays using Caco-2 cell extracts, the C variant shows stronger binding than the T variant [29]. In addition, when the two-variant 3'UTR sequences were linked to a reporter gene and transfected into Caco-2 cells, the T- and C-variant sequences showed differences in the extent to which they directed the expression of the reporter [37]. Furthermore, when clones of these transfected cells were selected so that expression levels of the T- and C-variant transgenes were similar, over-expression of the two variant 3′ UTRs was found to affect the expression of other selenoproteins such as GPx1 and SelH [38]. In human volunteers, the SNP was shown to affect the response of blood selenoprotein concentrations to Se supplementation with, in particular, T and C carriers showing differences in lymphocyte GPx1 protein levels and the ratio of GPx1:GPx4 protein concentrations during the washout period of the trial [29]. Overall, the data suggest that rs713041 affects the hierarchy of selenoprotein

synthesis with the C-variant 3′ UTR from GPX4 competing more strongly than the T variant for one or more proteins involved in Se incorporation. Computer prediction using Mfold indicates that the SNP influences the secondary structure of the 3'UTR around the SECIS, and therefore, our hypothesis is that rs713041 alters the binding of Sec incorporation machinery to the 3′ UTR of the GPX4 3′ UTR and so affects the pattern of selenoprotein synthesis [29, 37].

In addition, a SNP in the 3′ UTR of the *SEPP1* gene, rs7579, has been shown to be functionally significant. This is a G/A variant, and it has been found to influence the responses of human lymphocyte GPx4 and GPx1, plasma GPx3 and thioredoxin reductase 1 (TR1) and erythrocyte TR1 to supplementation [35]. The SNP affects response of plasma SePP protein levels and isoform pattern to supplementation [20, 35]. Additionally, in *SEP15*, there are two variants in the gene region that encodes the 3'UTR, a C/T substitution at position 811 (rs5845) and a G/A at position 1125 (rs5859), and based on reporter gene experiments these SNPs are thought to be functional [39].

4 Genetic Epidemiology of Selenoproteins and Cancer

Several lines of evidence, including reduced activity/expression of some selenoproteins in breast, prostate, colorectal and lung cancers and frequent loss of heterozygosity (LOH) at some selenoprotein loci, suggest a role for selenoproteins in the aetiology of cancer initiation and progression (e.g. [40–43]. These studies emphasise that factors which lower selenoprotein activity/concentration could contribute to cancer development and progression. As described above, functional polymorphisms have been shown to modulate expression levels, stability and activity of selenoproteins and several associations between these polymorphisms and cancers have been observed.

4.1 Colon Cancer

Several relatively small-scale genotyping studies have been carried out in relation to selected selenoprotein SNPs and colorectal cancer (CRC) risk. In a study of a Scottish population (∼300 cases, 189 controls), carriage of the C variant of rs 713041 in the GPX4 gene was found to be associated with CRC but not with increased risk of having adenomatous polyps [37]. Recently, however, this association was not replicated in a Korean population [44], and, in contrast, in a Czech population (832 cases, 705 controls), the T variant was found to be associated with increased CRC risk [45]. Measures of Se status were not available in the analyses of Czech and Korean subjects, so it is not possible to assess the influence of Se status on the impact of this SNP on disease risk. However, in this regard, it is interesting that in the UK study [37], preliminary data suggested that in CRC patients, Se status, as assessed by plasma Se levels and erythrocyte GPx1 activity,

was significantly lower in individuals with CC genotype than in individuals with CT or TT genotype.

In addition, genotyping for known functional SNPs in selenoprotein genes in the Czech and Korean (827 cases, 727 controls) studies mentioned above has shown that other genetic variants may influence CRC risk [44, 45]. Critically, in both populations, SNPs in the promoter region of *SELS* were found to modulate disease risk. The T allele of rs34713741 was associated with greater CRC risk (odds ratio of 1.68) in the Czech population, while a second variant in close proximity led to increased risk in females in the Korean population (odds ratio 2.25). The replication of the association in these two distinct populations strongly suggests that regardless of other genetic, lifestyle and dietary factors, SNPs in *SELS* promoter influence CRC risk. Interestingly, rs34713741 has also been linked to gastric cancer risk [46]. Additionally, in the Czech population, significant genetic interactions were observed between rs4880 (*SOD2*), rs713041 (*GPX4*) and rs960531 (*TXNRD2*), and between *SEPP1* and either *SEP15* or *GPX4* [45], suggesting that function of multiple selenoproteins in the colon affects CRC risk. Biologically these interactions reflect the combined metabolic interactions of GPx4, TR2 and MnSOD in response to oxidative stress and SePP in the delivery of Se for the synthesis of selenoproteins. The potential importance of variants in *SEPP1* is highlighted by results from a genotyping study of subjects from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial [47] in whom 44 tagged SNPs in *GPX1, GPX2*, *GPX3* and *GPX4*, *SEPP1* and thioredoxin reductase 1 (*TXNRD1*) genes were studied and 4 variants in *SEPP1* and one in the *TXNRD1* were found to be significantly associated with risk of advanced distal colorectal adenoma: one C/G variant in the 5′ gene region of *SEPP1* at −4166; three in the 3′ gene region of *SEPP1*; and one in *TXNRD1* at position −181. In addition, in this study there was a significant overall association with adenoma risk of all the variants of *SEPP1* and *TXNRD1*.

Using a tagged SNP approach, the association of variants in *GPX1-4* with colorectal cancer risk was assessed recently in a combination of three US-based case–control studies of colorectal adenoma rectal or colon cancer [48]. After adjustment for age and sex, the data show that three SNPs in *GPX3* (rs8177447, rs3828599 and rs736775) and one in *GPX2* (rs4902347) were significantly associated with rectal cancer but not with colon cancer or adenoma risk. Using a similar approach, SNPs and tagged SNPs covering variation in *GPX1-4* and *SEPP1* were analysed in 804 cases and 805 matched controls from the Women's Health Initiative Study [49]. This analysis found that only one SNP (rs8178974 in *GPX4*) was associated with colorectal cancer risk, but overall genetic variation in *GPX4* showed no association. However, the mean Se status of these individuals was high (135.6 μg/l serum Se), and this could have obscured any effects of genotype on disease risk. Indeed, selenoprotein synthesis is highly dependent on dietary Se intake, and so it is to be expected that the influence of the SNPs described here could be modified by Se intake. For that reason, it will be vital in future work to combine genotyping for selenoprotein SNPs with measures of Se status and carry out such studies in populations where Se status is relatively low.

4.2 Prostate Cancer

Recently, studies examining genotype effects in relation to prostate cancer risk have suggested that functional SNPs in *SEPP1 GPX1* and *SEP15* to modify susceptibility to prostate cancer [50–52]. In an European population, rs7579 in *SEPP1* was found to affect prostate cancer risk and rs1050450 found to affect the relationship between serum Se concentration and disease risk [50]. In contrast, a study of a second European population reported prostate cancer risk to be modulated by low Se status together with genotype for rs4880 in the *SOD2* gene and for rs3877899 in the *SEPP1* [52]. A study in the United States has suggested that both prostate cancer risk and survival are modified by a combination of genetic variation in *SEP15* gene and low Se status, although there was no affect on disease risk [51]. Recently, we have carried out a pathway-wide analysis of SNPs in all selenoprotein genes in a nested case–control cohort of prostate cancer patients and controls, and after an initial screen a second phase of genotyping indicated risk of advanced cancer is significantly modified by Se status and SNPs in *SELK*, *TR1* and *TR2* [53].

4.3 Breast Cancer

Although epidemiological studies have provided conflicting conclusions and little evidence to link Se intake/status with risk of breast cancer, a number of genotyping studies have been carried out to examine the relationship between genotype for selected SNPs in selenoprotein genes and disease risk. LOH at the *GPX1* locus was observed in about 36 % breast tumours analysed [41], and in addition, this study observed an association between rs1050450 in *GPX1* and breast cancer risk. Subsequently, an association of this SNP with breast cancer risk was confirmed in a Danish cohort, with the T allele being associated with increased risk [54]. On the contrary, a lack of association was observed in other studies carried out in the US and Canadian populations [55–57] and a meta-analysis suggests an overall lack of association despite an increased risk among African women [58]. However, the apparent lack of association could reflect the complex interaction between Se status and genotype for rs1050450. It appears that there may be an association between breast cancer risk and genotype for rs1050450 in populations with low Se intake such as European (e.g., Danish) populations but not in women with high Se intake (such as US and Canadian populations).

Another polymorphism corresponding to a variable number GCG repeats in the *GPX1* gene results in a protein with a variable number of alanines, and interestingly, one study has reported genotype for this variant to be associated with a significant increase in breast cancer risk in pre-menopausal women [57].

Hu et al. [39] observed an association between rs5859 in *SEP15* and breast cancer in African Americans but not in Caucasians. In addition, they observed LOH at the *SEP15* locus in tumour tissue, and a second study has also implicated

SEP15 allelic loss with the development of breast cancer among African American women [59]. The observed LOH at both *GPX1* and *SEP15* loci suggests a potential tumour suppressor role of the two selenoproteins in breast cancer aetiology.

4.4 Lung Cancer

rs1050450 in *GPX1* has also been associated with lung cancer risk [60–65]. In addition, as described above for breast cancer, LOH at the GPX1 locus was observed in lung tumours [66]. An interesting complex interaction between risk of lung cancer, genotype for rs5859 and Se status was observed in a Polish population [33] in whom it appears that, depending on Se status, the risk of lung cancer among smokers differs between genotype for rs5859. For individuals of AA genotype, an increasing plasma Se concentration was associated with a reduced lung cancer risk, whereas GG and GA genotypes were associated with decreased risk of lung cancer only among individuals with lower Se status, suggesting that Se supplementation would only benefit AA but could increase lung cancer in risk in GG and GA individuals.

4.5 Implications

It is evident from the preceding sections that although several studies of selenoprotein SNPs and cancer risk are relatively small and observations have mostly not been replicated, there are three themes emerging. First, there is replication that SNPs in the promoter region of *SELS* affect risk of colorectal or rectal cancer in two dissimilar cohorts [67] and repeated observations that rs1050450 in *GPX1* affects lung cancer risk (see Sect. 4.4).

Second, several studies have found that selenoprotein SNPs can affect cancer risk in combination with another genetic factor (a second SNP) or a lifestyle factor. Thus, the relation between prostate cancer risk and serum Se has been reported to be modified by variants in SEP15 [51], rs1050450 in *GPX1* or rs7579 in *SEPP1* [50]. In a separate prostate cancer study, a combination of variants in *SEPP1* (rs3877899) and *SOD2* (rs4880) affects disease risk [52]. Similarly, in addition to the effect of *SELS* promoter SNPS on CRC risk in a Czech cohort, SNP–SNP interactions between rs713041 (*GPX4*) and rs4880 (*SOD2*) or rs7579 in *SEPP1* were found to affect CRC risk [45]. A further example, the combination of rs1050450 in *GPX1* and rs4880 in *SOD2*, has been observed to modulate breast cancer risk.

Third, there is a trend towards SNPs in different selenoprotein genes affecting risk of different cancers. For example, as discussed above in Sects. 4.1–4.4, there is evidence that rs1050450 in *GPX1* affects risk of breast, bladder, lung and prostate cancer but no report of it affecting CRC risk. Rs713041 in *GPX4* has been linked to colorectal cancer risk, although different studies have given confusing results,

and there is one report of it affecting breast cancer recurrence but no link to prostate cancer risk. In addition, SNPs in the promoter region of *SELS* affect risk of cancers of the gastrointestinal tract, but there is no evidence for these variants affecting risks of other cancers. Surprisingly, in contrast, although both SEP15 and SelS are thought to function in the ER stress pathway, SNPs in *SEP15* have been associated with risk of a range of cancers—in prostate, breast, lung and rectal. SePP has such a central role in Se metabolism that it is not surprising that SNPs in *SEPP1* have been associated with risks of prostate and CRC cancer [45], and breast cancer (Méplan, Hesketh, Vogel unpublished data). However, the effects of the *SEPP1* SNPs are modulated by interactions with other variants, and these have been reported to be different between prostate and colorectal cancers (compare [44, 49, 52]), again suggesting a mechanistic difference in the tumorigenesis.

The observation that SNPs in different selenoprotein genes appear to affect cancer risk in different tissues could reflect either key roles for specific selenoproteins in the different tissues or their responses to different types of carcinogen or the tissue-specific hierarchy of selenoproteins. In addition, the modification of cancer risk by genetic interactions between selenoprotein SNPs probably reflects two aspects of Se metabolism—first, its incorporation into a limited number of selenoproteins by a common mechanism and second, the selenoprotein hierarchy, a result of which a change in expression of one selenoprotein may alter expression of another. An example of this is illustrated in the case/control colorectal cancer study in which we observed genetic interactions between SNPs in different selenoproteins overlapping with the known function of the corresponding proteins [44]. In particular, SNPs in *SEPP1*, which modulate response to Se, SePP plasma isoforms and potentially Se delivery [19, 34] interact with two SNPs in the 3′ UTR of *SEP15* and *GPX4*. These genetic interactions could mean that in an individual with sub-optimal Se intake and in whom there are the combined genotypes for a variant "poor" in Se delivery (in SEPP) and a variant "poor" for Secys insertion, synthesis of GPx4 or *SEP15* could be reduced, rendering colonic cells more sensitive to oxidative damage and the individuals more prone to colorectal cancer. Thus, the picture is complex when Se intake and possible factors affecting Se metabolism and its downstream pathways are also considered (as illustrated in Fig. 4). Thus, in future genetic epidemiological studies, it will be necessary to assess functional SNPs across the whole Se pathway in combination so as to model the contribution of variants throughout the pathway and in addition combine this with measures of Se status. The importance of gene–diet interactions, as well as potential involvement in lifestyle and environmental factors such as smoking, and the complex interplay between SNPs are likely reasons why to date genome-wide association studies (GWAS) have failed to identify selenoprotein SNPs in cancer risk.

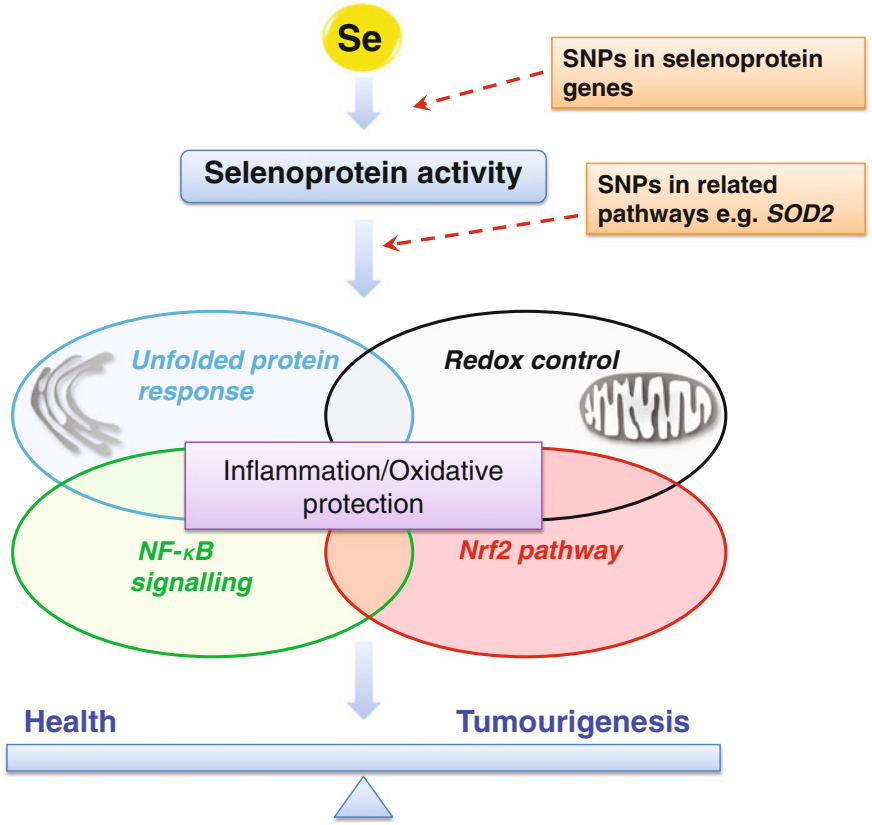

Fig. 4 Se intake and SNPs interact to affect multiple integrated metabolic responses. The scheme illustrates that both Se intake and SNPs affect selenoprotein activity and that in turn the pattern of selenoprotein activity affects multiple downstream pathways that are metabolically interlinked. Potentially it is the integrated response of these pathways that is likely to underlie the role of Se in tumorigenesis

5 Genomic Studies

5.1 Effects of Dietary Se Intake on the Transcriptome

Vital to the Se–cancer debate is increased knowledge of the biochemical functions of selenoproteins and how cellular biochemical function changes in response to altered Se intake. As mentioned in Sect. 2.1, Se availability does not alter the level and activity of all the selenoproteins to the same extent. On top of the overall pattern of selenoprotein expression, there is a further layer of complexity since changes in activity of individual selenoproteins in turn affects activity of downstream targets. With the advent of genomic techniques, it has become possible to

begin to unravel not only changes in the pattern of selenoprotein mRNA expression with microarrays but also the complex interaction of this pattern with downstream targets and pathways. Thus, an understanding of cell and tissue responses to altered Se intake and the biochemical functions of Se and selenoproteins can now be addressed from an integrated systems viewpoint. Using gene microarrays, we have done this by studying how changes in Se supply change the expression pattern of the global transcriptome in the mouse colon and in both human and mouse leucocytes; the major findings are illustrated schematically in Fig. 2.

Gene microarray analysis carried out on human lymphocyte RNA isolated from individuals before and after a 6-week supplementation with 100 μg/day sodium selenite [68] showed that expression of *SEP15* and *SELK* mRNAs was significantly affected by the supplementation, suggesting that these selenoproteins are most sensitive to Se supply in human lymphocytes. Subsequently, in a study where mice were fed a diet sub-optimal and marginally deficient in Se, a transcriptomic analysis of the colon showed that marginal Se deficiency led to lower expression of GPx1, SelH, SelW and SelM, showing that at least at the mRNA level, expression of these selenoproteins in the mouse colon is sensitive to dietary Se depletion [27]. This sensitivity of GPx1 and SelW to Se depletion is compatible with earlier observations on the selenoprotein hierarchy in a gut epithelial cell line [68], but the sensitivity of SelH and SelM in the colon had not previously been described. Interestingly, a study of splenic leucocyte RNA from the same mice showed SelW, GPx1, SelH and *SEP15* were sensitive to Se depletion [69], suggesting that expression of SelH and SelW may be particularly sensitive to Se depletion in a range of tissues. Comparison of the data from the human and mouse studies suggests that the changes in lymphocytes may be species specific and it will be important to define the relationship between Se status and selenoprotein expression in our ongoing experiments on RNA isolated from human colon biopsies.

In order to assess the nature of downstream targets sensitive to Se intake, pathway analyses of the microarray data obtained in these studies were carried out. In the study of the human lymphocyte expression data after Se supplementation, the protein biosynthetic pathways were the most sensitive to Se supplementation [68]. A comparable analysis of the mouse colon data showed that protein translation was top of the list of pathways altered by marginal Se deficiency. In addition, pathways related to protein synthesis (including "regulation of eiF4e and p70S6 kinase", "ribosomal proteins" and "mTOR signalling pathway") inflammatory signalling (TNF-α-NF-κB), cell cycle regulation (Wnt signalling) and proteasome degradation were also found to be modulated by Se intake. Experiments in Caco-2 gut epithelial cells transfected with a luciferase reporter driven by three NF-κB-binding sites showed that Se supply modulated the NF-κB response to TNF-α [70], providing further support for the hypothesis that Se modifies inflammatory pathways in the colonic epithelium. Interestingly, in mouse splenic leucocytes, pathways associated with inflammation were also found to be affected by Se depletion and real-time PCR analysis showed that more than 30 NF-κB

targets were down-regulated in moderate Se deficiency, confirming that Se depletion affects NF-κB signalling [69].

Overall, these transcriptomic analyses suggest that in the colon, and possibly other tissues, Se supply modulates protein synthesis, unfolded protein response, Wnt, Nrf2 and inflammatory pathways [27, 68, 69]. Many of these pathways are involved in regulating cellular responses to external or internal challenges: for example, Nrf2 to a xenobiotic and antioxidant responses, unfolded response pathways to viral and inflammatory challenges. In addition, several selenoproteins themselves, such as glutathione peroxidases and thioredoxin reductases, are key to antioxidant protective mechanisms. Thus, our hypothesis is that the selenoproteins play central roles in the ability of cells to respond to, and cope with, a range of challenges which affect downstream pathways involved in these processes.

5.2 Selenoprotein Function in the Colon

As far as the colon is concerned, the microarray experiments and genotyping experiments highlight several selenoproteins as being of particular importance; SelH, SelM and SelW are particularly sensitive to Se intake, and genetic variation in SelS appears to affect both gut health and inflammatory responses.

Present knowledge suggests that (1) these selenoproteins have functions in endoplasmic reticulum (ER) unfolded protein response, antioxidant protection and inflammatory signalling and that (2) there is biochemical crosstalk between endoplasmic reticulum stress, oxidative stress, Nrf2 signalling, mammalian target of rapamycin (mTOR) signalling and inflammatory signalling. For example, mTOR signalling regulates pathways controlling cell growth and protein synthesis; it modulates several factors involved in translation, including key elongation and initiation factors and their kinases. In addition, transcriptional regulation of *c-myc* (a target gene of the Wnt pathway) and mTOR nutrient sensing are linked through mTOR regulation of translation initiation and elongation. We speculate that the observed effects of Se on protein synthesis and translation pathways are linked to alterations in Wnt and/or mTOR signalling and that this represents one aspect of the interlinked biochemical network altered by Se intake and SNPs.

A second nodal pathway in underlying selenoprotein function is the ER stress pathway by which challenges such as nutrient starvation and oxidative stress compromise the protein folding capacity of the ER, so leading to the accumulation of misfolded proteins. As described in Sect. 2.1, the selenoproteins SEP15, SelM and SelS are found in the ER and have been postulated to function in the removal of misfolded proteins and inflammatory signalling. Furthermore, expression of SelS itself is modulated by a range of metabolic events such as ER stress [71] and inflammatory pathways [72]. Since these pathways are dysregulated during colorectal carcinogenesis, alteration of expression/activity of SelH, SelS or SelM by Se intake and/or genetic factors could modulate ER stress [13], with implications for a range of interacting biochemical pathways and ultimately tumorigenesis. Overall,

our hypothesis (see Fig. 4) is that together dietary Se intake and SNPs in selenoprotein genes modulate a number of interacting pathways implicated in responses of cells to oxidative, endoplasmic reticulum and inflammatory stresses [11]. On the basis of the known and suggested roles of selenoproteins in cell protection mechanisms and the downstream-targeted metabolic pathways affected by changes in Se status, it is possible that altered colonic selenoprotein expression modulates the ability of gut epithelial cells to cope with damaging metabolic challenges, and in turn, this may alter the risk of an individual to develop cancer. Defining the role of these selenoproteins in the colon should be a priority.

Use of siRNA to knock down gene expression is proving useful in investigating selenoprotein function. *GPX4* knock-down in a colonic epithelial cell line followed by transcriptomic analysis, real-time PCR and western blotting showed a major effect on genes encoding components of mitochondrial respiratory complexes I, IV and V [73]. *GPX4* knock-down also increased levels of mitochondrial reactive oxygen species and oxidised lipid and altered expression of apoptosis-inducing factor (AIF), consistent with earlier studies, indicating that low *GPX4* expression increases lipoxygenase-derived lipid hydroperoxides and AIF-induced apoptosis [74]. Since *GPX4* expression is increased in differentiated enterocytes [75], it has been suggested that the AIF-related protective role of GPx4 may be particularly important in differentiated enterocytes and that changes in mitochondrial oxidative function and apoptosis as a result of altered GPx4 activity may influence colonic cell proliferation [72]. The relationship between these effects and CRC risk is unknown, but it is tempting to speculate that mitochondrial function plays a central role in the biochemical networks that determine cell responses to low Se intake and selenoprotein genetic variation. In this regard, it is interesting both that knock-down and over-expression experiments have suggested that effects of GPx1 on mitochondrial function are central to its control of redox-regulated pathways [76] and that ER stress is linked with mitochondrial redox reactions and inflammatory pathways [77, 78].

6 Conclusions and Future Work

Se is present in 25 selenoproteins in humans, and changes in Se intake affect selenoprotein synthesis to a different extent depending on the protein and the tissue. The roles of the various selenoproteins in different tissues are not fully understood, especially in the case of the less well-characterised proteins. To better understand the relationship between Se intake and cancer, future work should focus on defining the roles of the various selenoproteins in tissues where cancers develop. However, it is only by adopting an integrated approach, taking into account the effect of Se supply on multiple selenoproteins within the context of downstream-targeted pathways and environmental stress that we will understand how Se affects overall cell responses and its role in preventing the carcinogenic process (illustrated schematically in Fig. 4). The data which have emerged from

genetic epidemiological studies indicate that it appears likely that different variants may affect risks of cancers in different tissues. This presumably reflects differing roles of selenoproteins and emphasises the importance of mechanistic studies of Se function in these tissues. Functional genomic approaches are essential to understand the role of Se in target tissues and how Se and selenoproteins may play a role in control of cell growth.

There is now strong evidence that that a number of SNPs in selenoprotein genes have functional consequences with regard to the expression of the proteins. However, it remains to be demonstrated conclusively whether these variants, or other selenoprotein SNPs, influence cancer risk. Despite this caveat, disease association studies have provided preliminary data, suggesting that there are links between SNPs in selenoprotein genes and cancer risk. For example, the available data suggest that risk of lung cancer may be linked to genotype for rs1050450 in *GPX1* and CRC risk may be linked to genotype for a functional variant in *SELS* and possibly also *GPX4* and *SEPP1* variants, but not rs1050450 in *GPX1*. In contrast, there is evidence that risk of breast cancer is affected by rs1050450 in *GPX1* in conjunction with lifestyle factors such as smoking. In addition, SNPs in *GPX1* and *SEPP1* have also been reported to affect prostate risk. Importantly, several studies indicate that cancer risk may be modulated by interactions between SNPs and/or between SNPs and Se status. Overall, the selenoprotein genotyping data in relation to cancer risk are tantalising and provocative but require confirmation and replication with genotyping for as many functional SNPs in the selenoprotein pathway as possible and in combination with measures of Se status. The goal should be to describe quantitatively the combined, and individual, effects of different SNPs throughout the selenoprotein pathway and their interactions and Se status on cancer risk.

Both genotyping and mechanistic studies suggest that Se and selenoproteins are relevant to our understanding of cancer biology. It is important that research into "Se and cancer" continues despite the negative result from the SELECT supplementation trial. Indeed, the results of the SELECT study underline the importance of taking a Se–gene interaction view of potential cancer risk. There is a need for a Se supplementation trial to be carried out in a population of relatively low baseline Se status (such as Europe), preferably also incorporating genotyping for selenoprotein SNPs so as to avoid issues of confounding factors.

Acknowledgements CM is supported by the Biotechnology and Biological Sciences Research Council (grant BB/H011471/1). Work in JEH's laboratory has been supported by Biotechnology and Biological Sciences Research Council, Food Standards Agency, Wellcome Trust, European Union, World Cancer Research Fund and Newcastle Healthcare Charity.

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Resveratrol: From Basic Studies to Bedside

Adriana Borriello, Debora Bencivenga, Ilaria Caldarelli, Annunziata Tramontano, Alessia Borgia, Vincenzo Zappia and Fulvio Della Ragione

Abstract

Plants produce a remarkable amount of low molecular mass natural products endowed with a large array of pivotal biological activities. Among these molecules, resveratrol (3,5,4'-trihydroxystilbene) has been identified as an important modulator of cell phenotype with a complex and pleiotropic mode of action. Extensive literature regarding its activity, mainly employing cellular models, suggests that this polyphenol controls cell proliferation, induces differentiation, and activates apoptosis and autophagy. The compound also modulates angiogenesis and inflammation. Similarly, studies on implanted cancers and chemical-induced tumors confirm the potential chemotherapeutical interest of the compound. Likewise, several reports clearly demonstrated, in animal models, that the compound might positively affect the development and evolution of chronic diseases including type 2 diabetes, obesity, coronary heart disease, metabolic syndrome, and neurogenerative pathologies. Finally, a number of investigations stated that the toxicity of the molecule is scarce. Despite these promising observations, few clinical trials have yet been performed to evaluate the effectiveness of the molecule both in prevention and treatment of human chronic disease. Preliminary findings therefore suggest the need for more extensive clinical investigations.

Keywords

Resveratrol · AMPK · PGC-1α · Sirtuin · Cancer therapy

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*, Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_10,

Abbreviations

AKT	cellular homolog of the transforming v-Akt protein
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
BSA	Body surface area
CD95	Cluster of differentiation 95
cdc2 kinase	Cell division control protein 2 kinase
Egr-1	Early growth response protein 1
FOXO	Forkhead box class O
HIF	Hypoxia-inducible factor
IGF-1	Insulin-like growth factor 1
MAP kinase	Mitogen-activated protein kinase
NAD	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
$p21^{Cip1}$	21 kDa protein cyclin-dependent kinase inhibitor protein 1
PDE	Phosphodiesterase
PGC-1α	Peroxisome proliferator–activated receptor gamma coactivator 1-alpha
PI3K	Phosphatidylinositol 3-kinases
PKC	Protein kinase C
Sirt-1	Sirtuin 1
TRAIL	TNF-related apoptosis-inducing ligand

Contents

1 Introduction

Resveratrol (Fig. 1) was mentioned for the first time in 1939 by Takaoka, who isolated it from "*Veratrum album*" [89]. The name of the polyphenol presumably comes from its occurrence in the resin of a *Veratrum* species. In 1997, Pezzuto and

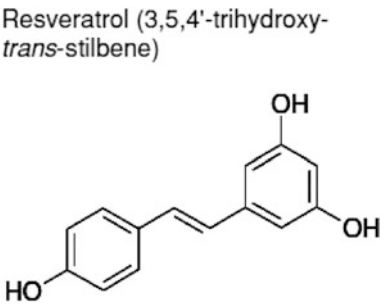

Fig. 1 Chemical structures of trans-resveratrol. Resveratrol (3,5,4′-trihydroxystilbene) is a derivate of stilbene (stilbenoid) and exists as two diastereomers: cis- (Z) and trans- (E). The trans-form is the preferred steric form in nature and is relatively stable. It can undergo isomerization to the cis- form when exposed to ultraviolet irradiation

colleagues published a study reporting that extracts of the non-edible Peruvian legume "*Cassia quinquangulata Rich*" (Leguminosae) showed a potent cycloxygenase 2 inhibitory activity [45]. They also found that resveratrol was the active principle of the extract [45].

In Pezzuto's paper, as harbinger of things to come, a large number of resveratrol anticancer activities were reported, affecting all the steps of cancerogenesis, namely initiation, promotion, and progression. Thereafter, an exponential number of reports on resveratrol accumulated and, so far, more than 5000 studies have been published (Fig. 2). In 1998, the effect of resveratrol on the growth and

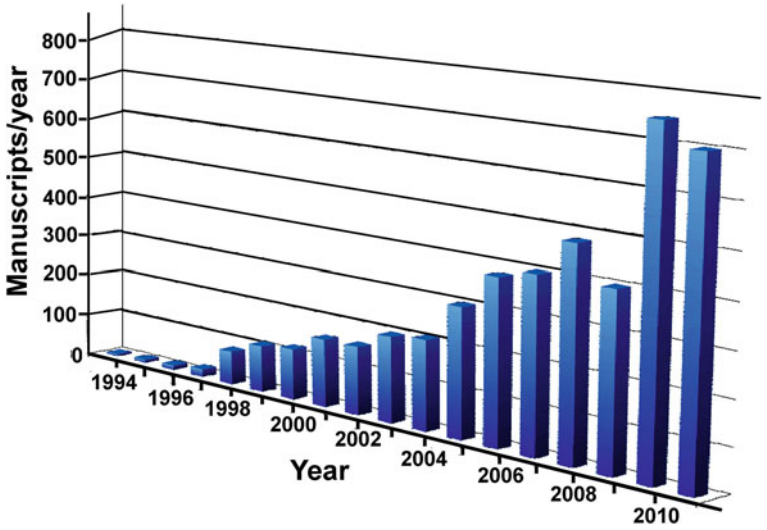

Fig. 2 Number of papers concerning resveratrol per year. The graph reports the number of published investigations during the period 1994–2011. The values were derived from Pub.Med.gov

differentiation of HL-60 cell line, a human promyelocytic cell line, was reported. In this study it was demonstrated, for the first time, that the molecule induces the myeloid commitment of the cells by hampering a specific cell cycle transition, that is, from G2 to M [29]. Importantly, biochemical analysis demonstrated definite changes in the cell division cycle engine, that is, the accumulation of cyclin A and the hyperphosphorylation of cdc2 kinase [29].

Although resveratrol is ubiquitous in nature, it is found in a limited number of edible substances, most notably in grapes. In turn, due to the peculiar processing methodology, resveratrol is found predominantly in red wines. Thus, resveratrol received intense and immediate attention. The notion that red wine prevents cancer and other diseases was very appealing and was strictly correlated to the so-called French Paradox. The correlation is now strongly questioned and, most intriguing, even whether or not a "French Paradox" exists is a matter of debate.

In brief, since 1997, resveratrol has been suggested to promote health in relation to various diseases or sufferings, covering a broad range of pathologies including cancer, heart disease, neurodegenerative pathologies, aging, inflammation, obesity, and diabetes. These diseases are clearly strictly interconnected and, for example, positive effects on obesity, inflammation, and aging might be important in the prevention of malignant transformation. Here, we will discuss the relationship between resveratrol and cancer, also taking into account the possibility of its use in therapy.

2 Resveratrol Effects on Established Cell Lines

The activities of resveratrol on cells have been extensively investigated and several hundred studies have been published on this topic. The majority of cellular models employed have been established from malignant tissues. Thus, these investigations suffer not only from the artificial in vitro growth conditions, but also from the strong intrinsic phenotypic variability due to the genetic and molecular alterations specific to the cancer of origin. Conversely, few analyses have been performed on cells derived from normal tissues.

The major phenotypic effects include the arrest of growth (at different phases of the cell cycle) [12, 13, 23, 29, 33, 52, 69]; the induction of differentiation [5, 14, 27, 29, 46, 47, 53, 104]; the activation of apoptosis, necrosis, and autophagy [1, 35, 55–57, 61, 63, 72, 73, 76, 92, 102]; anti-inflammatory activity [54, 107]; and interference with tumor angiogenesis [2, 16, 19, 85] among others. These activities appear particularly interesting in the field of human cancer treatment since they affect the major aspects of human malignant transformation [40]. In some studies, resveratrol has been associated with other compounds (frequently chemotherapeutics) that increase or hamper its effects. Several excellent reviews have critically appraised the ample literature on the ex vivo resveratrol activities, and this is not the aim of the present review [96 and references therein]. Interestingly, some studies report that the effects of the molecule are different at distinct

concentrations, inducing proliferation at low level and showing an anticancer function at higher concentration [18].

Some general conclusions might be drawn from the studies on cell lines. First, the polyphenol is endowed with a very large variety of promising biological activities that, in the main, are not related to resveratrol antioxidant capability. Second, the efficacious concentrations generally range between 10 and 50 μM. Third, the effects of the molecule frequently vary in relation to the concentration employed. Thus, resveratrol might be considered a hormetic compound in that the amount of molecule used is of critical importance for its activity [18]. This variability must be taken into consideration when translating in vitro experiments into clinical settings.

3 The Molecular Bases of Resveratrol Activity

The evaluation of the efficacious doses of an anticancer agent requires robust knowledge of its mechanisms of action. Over the past two decades, the molecular activities of resveratrol have been the subject of a vast number of investigations.

A multitude of data implicates resveratrol in an intricate web of pathways confirming the pleiotropic nature of the compound.

The molecule modulates various transduction pathways, including those correlated to MAP kinase [8, 12, 31, 105]; JNK [73, 79, 100], NF-κB [13, 14, 33, 42, 48, 55], AKT/PI3K [38, 39, 69, 98], PKC [7, 60, 75, 80, 86], CD95/TRAIL [26, 35, 56, 77, 78], and FOXO [23, 85].

Resveratrol controls apoptosis by altering the level of p53 [36, 90, 108]; caspases [3, 21, 64]; survivin [6, 41]; Bax, Bcl-2, and Bcl-xL [68, 70, 92]. The compound inhibits cyclooxygenase [87, 88, 110] and cytochrome P450 [22]; induces phase II drug metabolizing enzymes [24, 25, 51]; up-regulates antioxidant enzymes such as glutathione peroxidase [84], catalase [34], and quinone reductase [74]; and inhibits ornithine decarboxylase [95]. Intriguingly, resveratrol has been shown to regulate cathepsin D [94] and to inhibit HIF-α (hypoxia-inducible factor α) function [20, 101]. The effect on HIF-α factors (HIF-1α and HIF-2α) is particularly important since these proteins modulate the metabolism of glucose by enhancing its internalization and glycolitic metabolism [81].

During our studies on resveratrol, we demonstrated that, in K562 cells (an erythroleukemic cell line), the molecule up-regulated the cellular content of Egr-1 (early growth response) transcription factor by activating MAP kinase pathway [30]. In turn, Egr-1 increased the gene transcription of $p21^{Cip1}$, an inhibitor of cyclin-dependent kinases. $p21^{Cip1}$ accumulation was responsible for the resveratrol antiproliferative effect and, at least in part, for the induction of erythroid differentiation [30]. Resveratroinduced $p21^{Cip1}$ accumulation has also been observed by us and other research groups in different cell line models. This finding demonstrates, in general, that resveratrol affects specifically gene expression and the cell division cycle engine.

An additional mechanism of resveratrol action requires, however, particular attention and discussion, that is, its effect on sirtuin. Sirtuins are a family of enzymes that deacetylate proteins at the expense of NAD, thus possessing either protein deacetylase or mono-ribosyltransferase activity [37, 109]. Sirtuins have been implicated in the promotion of life extension in several species and in the modulation of gene transcription, apoptosis, and stress resistance, as well as energy expenditure control under low-calorie conditions [58, 103, 109].

In 2003, Howitz and colleagues reported that resveratrol is a powerful naturally occurring activator of yeast Sir2, the homolog of mammalian Sirt-1, and is also able to extend the life length of *Saccaromyces cerevisiae* [44]. Subsequently, the capability of resveratrol to elongate the duration of life was also confirmed in a worm (*Caenorhabditis elegans*) and in the fruit fly *Drosophila melanogaster* [9].

Subsequently, some of these results have been questioned, and now the anti-aging effect of resveratrol would appear to be unlikely [59, 67]. Similarly, whether the effect of resveratrol on Sirt-1 exists in vivo or it is only an in vitro activity has been the object of debate [11, 28]. On this aspect, however, no definite conclusion is available.

In the context of the effects of an altered nutrition in human physiology and pathology, it has been reported that resveratrol strongly ameliorates the performances of mice fed with a high-fat diet. The positive effect was correlated to Sirt1-dependent deacetylation and activation of PGC-1α, a master gene that activates oxidative metabolism by increasing the respiratory chain components and the mitochondria number and activity [50, 67]. Very recently, further studies on this topic have been reported. First, it has been demonstrated that the primary molecular effect of resveratrol is the inhibition of phosphodiesterase (PDE) that results in a cyclic AMP increase [65]. The up-regulation of the cyclic mononucleotide triggers a series of reactions resulting in the activation of AMP kinase (AMPK), a pivotal player in the control of caloric restriction. Then, AMPK regulates Sirt1 and PGC-1α (Fig. 3a). A different study reports that initial targets of resveratrol are Sirt1 or AMPK, alternatively, depending on the amount of resveratrol employed [71]. In both cases, the final result is the activation of PGC-1α (Fig. 3b).

Thus, although the two reports propose different primary resveratrol targets, final effectors are both AMPK and PGC-1α [65, 71]. Studies with knock-out mice might help in clarifying whether the differences in the observed mechanisms may depend or not on the resveratrol concentration employed. In both cases, the two investigations definitely demonstrate that resveratrol affects “in vivo” energy metabolism.

Up to the end of 2011, more than 50 studies analyzed the effect of resveratrol as an anticancer compound in animal models of different cancers, including skin cancer (non-melanoma skin cancer and melanoma); breast, gastric, colorectal, esophageal, prostate, and pancreatic cancers; hepatoma, neuroblastoma, fibrosarcoma, and leukemia (reviewed in [96]). In general, these preclinical studies suggest a positive activity of the molecule in lowering the progression of cancer, reducing its dimension, and decreasing the number of metastases.

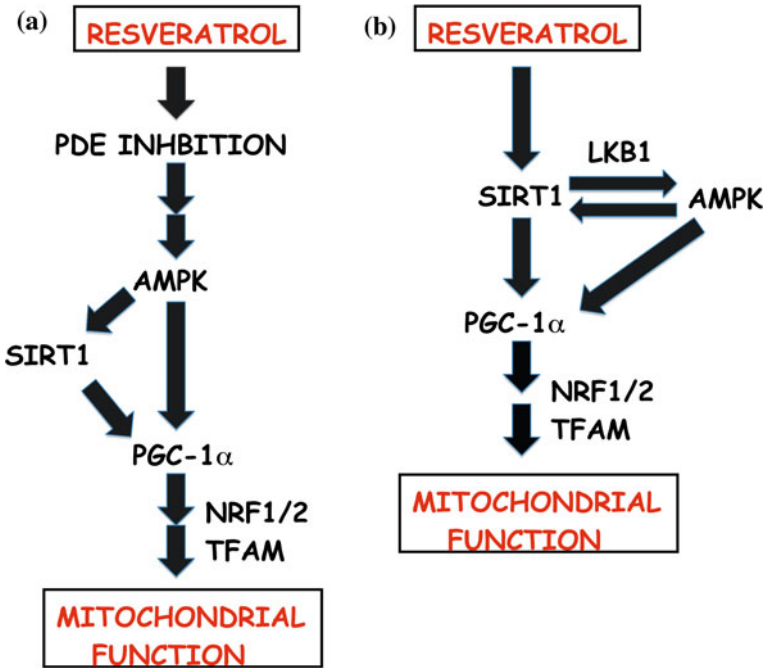

Fig. 3 Proposed molecular mechanisms of resveratrol affecting energy metabolism *Panel* a. The primary target of resveratrol is the phosphodiesterase activity that results in an increase in cyclic AMP. The up-regulation of cAMP stimulates the activity of AMPK. This kinase activates SIRT-1 and PGC-1α independently. Furthermore, SIRT-1 activates PGC-1α. Finally, PGC-1a along with additional transcription factors (NRF1/2 and TFAM) positively modulates the mitochondrial activity. *Panel* b. In this mechanism, the primary target of resveratrol is SIRT-1. The up-regulation of this enzyme results into the activation of both AMPK and PGC-1α. Moreover, AMPK also activates PGC-1α. Finally, PGC-1α and the two factors, NRF1/2 and TFAM, increase mitochondrial activity

These findings prompted studies to evaluate the possibility of translating the anticancer activities observed in preclinical studies into the use of resveratrol for cancer treatment. It is, however, necessary to emphasize that a large number of naturally occurring compounds show an anticancer activity in animal models but, when evaluated in clinical trials, the results obtained are very frequently unsatisfactory in terms of efficacy and toxicity.

4 Resveratrol Pharmacokinetics

It is indisputable that resveratrol modulates in vivo and in vitro a large array of intracellular molecular mechanisms as well as complex biological events and that the natural polyphenol has a positive effect on numerous experimental models of cancers. However, the doses of resveratrol required for reaching serum levels

comparable with those found efficacious in vitro have cast severe doubts on the potential usefulness of the molecule, particularly in dietary prevention strategies.

Resveratrol is usually well tolerated at least in the short-term or acute exposure experiments performed in humans. When eight healthy subjects were exposed for eight days to 2 grams of resveratrol twice/day, six of eight subjects had mild episodic diarrhea/loose stool. The symptoms typically appeared at the beginning of the treatment period, and one of the subjects developed a temporary rash and headache [49].

In a double-blinded, randomized, placebo-controlled study, up to 975 mg/day was given to healthy volunteers. Two adult subjects (male and female) of each group were treated with 25, 50, 100, or 150 mg, six times/day, for two days in total. Adverse effects were mild in severity and similar between all groups [4]. In a different study, 270 mg resveratrol was given to 19 volunteers for one week without causing any discomfort [99]. In a further report, healthy volunteers tolerated resveratrol well in a seven-day exposure study, but experimental details were not given, thus making the assessment of results challenging [32]. The same article describes a trial that included daily exposure to 2.5 g or 5 g resveratrol for 28 days. The authors reported that the adverse events were generally mild in nature and reversible, but the experimental details are scarce [32].

On the other hand, the prevention and/or treatment of malignancies (and other chronic diseases) might require therapies extended for several months/years and, thus, data on long-term resveratrol toxicity are of crucial importance. Unfortunately, this information is so far not available. Therefore, while resveratrol might be considered a food supplement and a relatively safe natural medication, further investigations are absolutely necessary to determine its long-term effects.

A central issue that needs to be clarified is resveratrol bioavailability compared with its therapeutic efficacy. This complex issue might be approached in different ways. As reported in a previous section, resveratrol in vitro effects (i.e., on cell systems) are mainly observed at concentrations ranging from 10 to 50 μM. However, these values do not consider that the polyphenol interacts with components of the culture medium (e.g., proteins, lipoproteins and others), and thus, the actual free resveratrol effective concentration might be significantly lower. In humans, when resveratrol was administered in a single dose of approximately 25 mg [4, 82, 83, 97], the plasma concentration of the free molecule ranged from 1 to 5 ng/ml (4-20 nM); administration of a higher dose (5 g) led to a value of serum free resveratrol of about 2.3 μM [4, 82, 83, 97]. The maximum peak plasma concentration was reached in the first 30–90 min after intake. Under these conditions, the corresponding concentration of the three main resveratrol metabolites (resveratrol-3-*O*-sulfate, resveratrol-3-*O*-glucuronide and resveratrol-4-*O*-glucuronide) exceeded that of the free compound by approximately 20-fold [4, 82, 83, 97]. Plasma half-lives of resveratrol and of its three major conjugates were similar (between 2.9 and 11.5 h). In urine, within 24-h postdose, excretion rates were highest during the initial 4-h collection period, while traces of resveratrol metabolites were detected in feces, consistent with an enterohepatic recirculation [4, 82, 83, 97]. Thus, bioavailability studies showed that, even after a high dose of

resveratrol administration, only a small amount of the free form is present in plasma and that treatments with high resveratrol amounts are required to reach serum levels corresponding to those necessary for the in vitro biological activities.

This methodological approach, however, is intrinsically poor, as it does not directly correlate the strategy of treatment and the serum dosage with the biological effects.

An interesting alternative methodology is to consider the resveratrol dosage employed in studies where clear in vivo effects were observed. In this respect, the study of Baur and colleagues might be useful [10]. The authors employed, in mouse treatment, a dosage of about 22.4 ± 0.4 mg kg^{-1} day^{-1} and observed significant phenotypic effects after approximately 110 weeks. The value corresponds to about 1568 mg for a man of 70 kg. As Baur and Sinclair [9] reported a concentration of 5 mg resveratrol per liter of some red wines, the above value would correspond to about 300 l of wine to be consumed every day.

This estimation, however, does not consider the so-called body surface area (BSA), a parameter that is necessary for a correct dose translation from mice to humans. Employing this parameter, 22.4 mg kg^{-1} (in mice) corresponds to 1.82 mg kg^{-1} (in humans), and, in turn, 1568 mg to about 128 mg. However, protective effects have been observed at a lower resveratrol dose, that is, 5.9 mg kg^{-1} day^{-1} in mice [50], equivalent to 33 mg for a man of 70 kg (using BSA correction). Since 33 mg resveratrol is contained in 6 l of wine, this still suggests that the importance of the dietary phytoalexin is questionable.

However, two more aspects should be taken into account which might allow us to suggest that resveratrol effects occur (at least in part) even in the presence of a normal diet (about 0.4 l of wine day^{-1}, around 1.5 mg day^{-1}).

First, the efficacy of dietary resveratrol might be higher than that showed by the compound taken in pill form as a purified preparation. Indeed, it has been suggested (but not demonstrated) that the presence in foods of other compounds (for example, other polyphenols) interfering with resveratrol removal might diminish the catabolism of the phytoalexin and increase its serum level [50]. Second, bioavailability data suggest that prolonged resveratrol treatment leads to an increase in serum resveratrol content as well as to its accumulation in specific cellular compartments (i.e., cellular membranes) or tissues, due to the molecule lipophilicity.

5 Clinical Studies on Resveratrol

Although few studies on the clinical effect of resveratrol treatments in humans are available, the existing data seem promising enough to warrant further investigation.

A very recent report, based on a cohort of one thousand people, showed a direct correlation between resveratrol dietary consumption and improvement of several cardiac risk parameters. The intake of resveratrol was evaluated by determining

levels of resveratrol itself and of its metabolites in urine [106]. A further investigation reported a trial where 75 subjects were divided into 3 groups and treated with placebo, grape extract, and grape extract enriched with a low amount of resveratrol. The authors claimed that after treatment for 1 year, a number of cardiac risk factors (C-reactive protein, tumor necrosis factor-α, plasminogen activator inhibitor type 1, interleukin-6/interleukin-10 ratio) were significantly decreased only in the group of subjects treated with resveratrol [93]. Finally, a pivotal study, published in Cell Metabolism, showed that in 11 obese patients, treatment with 150 mg/day of resveratrol for 30 days, strongly and positively influenced several parameters (i.e., increase in intramyocellular lipid levels and decrease in intrahepatic lipid content, circulating glucose, triglycerides, alanine-aminotransferase, and inflammation markers) and, biochemically, induced the up-regulation of muscle AMPK, SIRT-1, and PGC-1alpha activities, which are similar to the observations reported in the animal models [91]. The authors emphasized that the treatment was, however, performed employing resveratrol at a concentration 400-fold lower than that used in mice.

These three studies [91, 93, 106], suggest, but clearly do not definitely prove, that resveratrol affects metabolic parameters and risk factors which are also important for cancerogenesis.

Two other interesting studies were published in Cancer Research in 2010. These investigations evaluated the toxicity and metabolism of the polyphenol and its accumulation in both normal and malignant colon tissue [17, 66]. In these cases, the treatment was at high doses for a short period. The results of one study demonstrated that resveratrol shows very low toxicity and might reach concentrations negatively affecting IGF-1 level [17]. Lowering IGF-1 is considered to be one important parameter in anticancer activity. In the second investigation, patients affected by colon carcinomas were treated with resveratrol for 7 days before tumor removal. The results showed a clear, although limited, decrease in proliferation of malignant cells. Moreover, resveratrol accumulated in colon tissues ranging in concentration from 20 to 200 μM [66].

In addition to the studies reported above, several clinical trials of either dietary or supplemented resveratrol are currently at different stages of completion. Particularly, a search in www.clinicaltrials.gov by using the key term “resveratrol” revealed 53 studies at different stages (retrieved October 4, 2012). More specifically, 7 studies were active but not yet recruiting, 18 studies were active and recruiting, 21 studies were completed, 2 studies terminated, 4 studies with unknown status (i.e., information has not been updated recently), and 1 investigation was withdrawn. The majority of these trials investigate the effect of the molecule on type-2 diabetes, obesity, and cardiovascular diseases.

Eight studies directly evaluated the effect of resveratrol on cancer development. Six trials were completed and the results of four of these have been published [15, 43, 62, 66]. One trial is still recruiting the patients while the status of the last one is unknown.

The majority of studies (four) were devoted to investigating the effect of the polyphenol treatment on colon cancer while one analyzed the activity of the molecule on the development of cancers. The remaining three studies focused on resveratrol effects on gastrointestinal cancer, follicular lymphoma and multiple myeloma (using an association between resveratrol and bortezomib), but no conclusions are yet available from these investigations. In general, the major inference that can be deduced from the published studies [15, 43, 62, 66] is that the positive cancer chemopreventive properties showed by resveratrol warrant further investigation. The study designs for these trials (e.g., dosages/formulations of resveratrol, length of trial) vary greatly, with doses as high as 5 g in healthy adults.

A main limitation and criticism of the clinical resveratrol research already available is a lack of trials examining the long-term health effects of resveratrol. Importantly, it has been recently announced that the Danish Council for Strategic Research has granted $3.4 million for a four-year study to investigate resveratrol on the management of metabolic syndrome, osteoporosis, and chronic inflammation. The main purpose of this landmark study is to demonstratively prove that supplementary intake of resveratrol can neutralize the detrimental effects of excess body weight, specifically obesity. The effects to be measured include low-grade inflammation that is often associated with type 2 diabetes, non-alcoholic fatty liver disease, osteoporosis, and cancer.

In summary, further controlled clinical trials are clearly required to prove the preventive and therapeutic efficacy of either dietary or supplemented resveratrol.

6 Conclusion and Perspectives

The emerging data from human clinical trials on resveratrol suggest that the positive effects obtained in vitro and in animal models must be taken into serious consideration in view of a possible protective or therapeutic use of the molecule. As a matter of fact, a trivial comparison of the serum level reached by the molecule after treatment with the amount necessary in the cellular growth medium for obtaining phenotypic effects appears quite simplistic.

Indeed, recent findings indicate that even a very low dose of resveratrol treatment (8 mg/die) prolonged for one year significantly reduces a number of cardiac risk factors [93]. Probably, 8 mg resveratrol is still a value too high to be reached only by drinking wine (it corresponds to 1–3 liters, depending on the wine), but it is not extremely elevated considering that resveratrol occurs in various foods. On the other hand, the scarce toxicity of the molecule suggests that the use of the compound in the prevention or treatment of chronic diseases might warrant serious consideration.

On the other hand, it is not clear whether long-term resveratrol supplementation will preserve the benefits to ultimately impact the development of chronic disease, and the small number of clinical trials remains dwarfed compared to the thousands of basic science experiments.

Finally, to further evaluate resveratrol's potential for widespread use in human medicine, continued research exploring a gamut of responses in humans is obviously necessary. Future studies should aim to:

1. Investigate different dosages and/or formulations of resveratrol, in terms of both bioavailability and efficacy.
2. Evaluate the efficacy of resveratrol as a putative alternative for a given outcome/treatment. In some chronic diseases (type 2 diabetes, obesity, metabolic syndrome, cardiovascular diseases, and neuro-degenerative pathologies), resveratrol may be considered a serious alternative option in their prevention and treatment. In this regards, only the results of numerous and independent trials must be considered.
3. Study the effects of long-term resveratrol supplementation.
4. Determine the activity of resveratrol's metabolites.
5. Establish if resveratrol can have either additive or synergistic effects in combination with other therapies.
6. Determine whether genetic factors might explain differences in bioavailability and physiological responses to resveratrol between individuals.
7. Develop new strategies for resveratrol supplementation.

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Quercetin: A Pleiotropic Kinase Inhibitor Against Cancer

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Abstract

Increased consumption of fruits and vegetables can represent an easy strategy to significantly reduce the incidence of cancer. From this observation, derived mostly from epidemiological data, the new field of chemoprevention has emerged in the primary and secondary prevention of cancer. Chemoprevention is defined as the use of natural or synthetic compounds able to stop, reverse, or delay the process of tumorigenesis in its early stages. A large number of phytochemicals are potentially capable of simultaneously inhibiting and modulating several key factors regulating cell proliferation in cancer cells. Quercetin is a flavonoid possessing potential chemopreventive properties. It is a functionally pleiotropic molecule, possessing multiple intracellular targets, affecting different cell signaling processes usually altered in cancer cells, with limited toxicity on normal cells. Simultaneously targeting multiple pathways may help to kill malignant cells and slow down the onset of drug resistance. Among the different substrates triggered by quercetin, we have reviewed the ability of the molecule to inhibit protein kinases involved in deregulated cell growth in cancer cells.

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_11,

Keywords
Quercetin · Kinase inhibitor · Antioxidant · Chemoprevention · Flavonoids · Polyphenols

Abbreviations

Akt/PKB	v-akt murine thymoma viral oncogene homolog 1/protein kinase B
PI_3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
Hck	Haematopoietic cell kinase
GSK-3β	Glycogen synthase kinase-3β
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B cells
HO-1	Heme oxygenase-1
ROS	Reactive oxygen species
CK2	Casein kinase 2
Raf-1	v-raf-1 murine leukemia viral oncogene homolog 1
ERK1/2	Extracellular signal-regulated kinase 1/2
MEK-1 or MAP2K1	Mitogen-activated protein kinase kinase 1
PTEN	Phosphatase and tensin homolog detected on chromosome 10
IKKα/β	Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase α/β
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, α/β
S6K1	Ribosomal protein S6 kinase
$p90^{RSK}$	90kDa polypeptide
AMPK	AMP-activated protein kinase

Contents

1 Introduction

Hundreds of scientific studies demonstrate that non-nutritional compounds present in the human diet are able to prevent degenerative diseases such as cancer [82, 98, 100]. This heterogeneous class of molecules, generally known as phytochemicals, includes more than 10,000 compounds, and among them, more than 6,000 are polyphenols included in the class of flavonoids divided into the subclasses of flavones, chalcones, flavonols, flavanones, flavan-3-ols, isoflavones, anthocynidins, flavanonols [38]. They are present in foods of vegetal origin such as fruits and vegetables and beverages such as tea, wine, beer, and juices and in many dietary supplements or herbal remedies. Due to the variety of their physiological roles in plant tissues in regulating enzymes involved in cell metabolism and in mechanisms of defense against foreign agents (radiations, viruses, parasites), phytochemicals have been associated with pleiotropic effects in animal cells.

The increasing interest of scientists in phytochemicals has arisen following the demonstration that their biological targets in mammalian cells were the same as those involved in inflammatory processes and oncogenic transformation, such as alterations in cell cycle control, apoptosis evasion, angiogenesis, and metastases. These conclusions are derived from preclinical studies supported by a large quantity of epidemiological evidence, suggesting that a daily intake of phytochemicals can reduce the incidence of several types of cancers [18, 86, 98, 100]. However, despite this positive association, the field is still the subject of debate and criticism, since explanations on how a diet rich in fruits and vegetables can protect against degenerative diseases remain elusive and require further biochemical and genetic studies on cellular and animal models in order to depict the mechanism(s) of action of phytochemicals [5, 16].

In the last decade, we have focused our attention on the activity of quercetin (3,3′,4′,5,7-pentahydroxyflavone), one of the major dietary flavonoids, found in a broad range of fruits, vegetables, and beverages such as tea and wine, with a daily uptake between 10 and 100 mg, depending on eating habits [4, 43]. This molecule has been employed in different studies aimed to demonstrate its antioxidant, anti-inflammatory, anti-angiogenic, anti-proliferative, and pro-apoptotic effects [55, 85]. Here, we will focus our attention on the ability of the molecule to inhibit protein kinases involved in deregulated cell growth in cancer cells.

2 Antioxidant Properties of Quercetin

Among polyphenols, quercetin is one of the most potent antioxidants, as demonstrated in different in vitro [10] and in vivo studies [79]. In general, flavonoids are good antioxidants since they are able to stabilize radicals formed after scavenging several reactive oxygen species (ROS) molecules owing to extensive electron delocalization on multiple mesomeric structures existing for the aroxyl radical species of flavonoids [10]. Quercetin and kaempferol are highly efficient

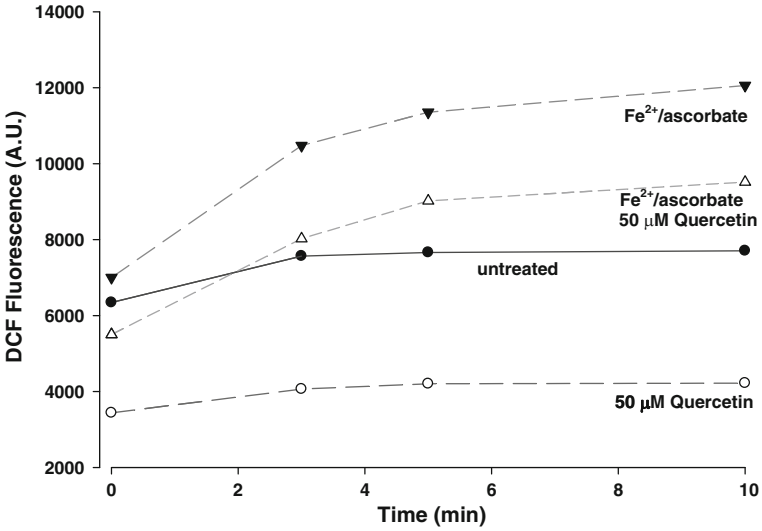

Fig. 1 Antioxidant effect of quercetin in U2OS cell line. Time course of basal and Fe^{2+}/ascorbate induced ROS production in U2OS cells treated with DMSO (0.1 %, vehicle) or quercetin 50 μM in DMEM medium with 10 % FBS. ROS were measured from 0 to 10 min as DCF fluorescence using the oxidation-sensitive probe, 5,6-carboxy-2′,7′-dichlorofluorescein diacetate, DCHF-DA (10 μM) [88]

radical scavengers, but only the quercetin aroxyl radical decays slowly enough to make this flavonol a potent antioxidant [10]. In fact, in several different cell lines, quercetin maintains its ability to efficiently scavenge intracellular ROS. An example is reported in Fig. 1, where in the presence or absence of an oxidative insult, quercetin maintains its ability to scavenge ROS, without affecting cell viability (not shown). However, the biological properties of the molecule do not always coincide with its antioxidant capacity, as discussed elsewhere [82]. For this reason, we suggest caution in interpreting the anticancer and pro-apoptotic functions of quercetin based only on its antioxidant ability. A good indication of the existence of a functional link between antioxidant capacity and biological effects may derive from the parallel use of compounds structurally and functionally similar to quercetin [e.g., myricetin and/or (+)catechin] which, like quercetin, maintains the ability to reduce ROS, but may not possess the same anticancer effects [82, 83].

The antioxidant potential of quercetin generates a paradox common to other polyphenols: The scavenging activity of the molecule against ROS chemically converts it into oxidative products which display a high reactivity toward thiols and can lead to the loss of protein function. Therefore, paradoxically, the net result between protection offered by quercetin and damage caused by its toxic products may weigh in favor of the latter. In lung cells, quercetin efficiently protects against H_2O_2-induced DNA damage, but this positive effect is counteracted by the

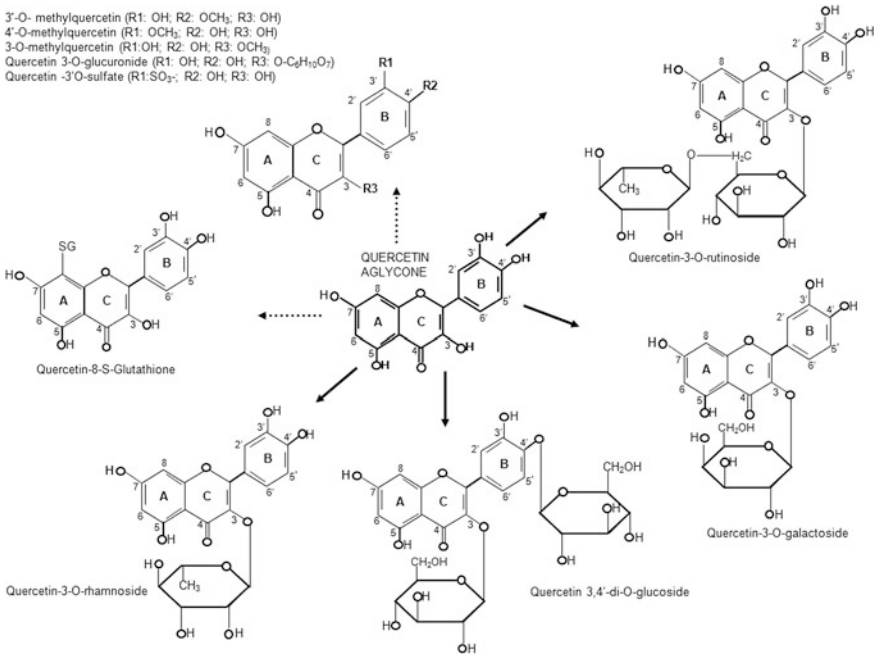

Fig. 2 Structure of quercetin and most representative quercetin glycosides and metabolites in humans

reduction in GSH level, an increase in LDH leakage and cytosolic-free calcium concentration [9]. It is interesting to note that quercetin was used at a concentration of 100 μM which usually results toxic in many cell lines and cannot practically be obtained in vivo. The same group also described the formation of a quercetin–quinone (QQ) species when the molecule is employed as an antioxidant. QQ, like other semiquinone radicals and quinones, is toxic because of its ability to arylate protein thiols. Protection against QQ may arise from GSH which, when present at the right concentration, quickly traps QQ [8]. However, potential toxic effects of the QQ species have not yet been proven in vivo.

3 Quercetin Bioavailability and Metabolism

The in vivo antioxidant properties of quercetin are influenced by its absorption, metabolism, and bioavailability. In food, quercetin is present in differently glycosylated forms, not as aglycone (i.e., without sugar groups; Fig. 2). Therefore, its bioavailability depends on the type of glycosides present in different food sources. After absorption, quercetin is metabolized in different organs, such as the small intestine, colon, liver, and kidney. Here, the molecule is conjugated to methyl and sulfate groups and glucuronic acid to generate its major conjugates in humans: 3-

O-methylquercetin (isorhamnetin), quercetin-3-O-glucuronide, 3-O-methylquercetin-3-O-glucuronide, quercetin-3′-O-sulfate, and quercetin glutathione conjugate [42, 57, 104] (Fig. 2). It is worth noting that the presence of sugar molecules in the different forms of quercetin glycosides can strongly reduce its antioxidant activities. Therefore, the aglycone, almost absent in food, is usually of higher antioxidant potency than the glycoside form, depending on where the sugar molecule is attached [68]. However, at least in rats, plasma obtained after quercetin administration was more resistant against copper sulfate-induced lipid peroxidation, strongly suggesting that some conjugated metabolites of quercetin act as effective antioxidants when plasma is subject to metal ion-induced lipid peroxidation [19]. Antioxidant activity of conjugated metabolites has also been observed in vitro [79, 92], and some of them, to a lesser extent, possess pro-apoptotic functions similar to the whole aglycone (reviewed in [85]). The absorption of quercetin is also influenced by gut microflora, which in humans metabolizes probably half of quercetin-3-rutinoside to phenyl-C2 acids [75]. As an example, quercetin-3-O-rhamnoglucoside and quercetin-3-O-rhamnoside are not recognized by human enzymes and are hydrolyzed to quercetin by several strains of *Bacteroides* [6, 90]. It is estimated that the number of bacteria able to metabolize quercetin-3-O-glucoside ranges between 10 and 109/g dry mass [91].

The net result of dietary quercetin absorption and metabolism is a total plasma concentration in the nanomolar range (<100 nM) that can be increased to micromolar concentrations after supplementation. As an example, supplementation with 1 g/day of quercetin for 28 days increased plasma concentrations to 1.5 μM [15, 65]. However, there were no effects on serum total LDL, thrombogenic risk factors, including platelet aggregation, platelet thromboxane B2 production, blood pressure, or resting heart rate [15]. A similar conclusion was reached by a more recent interventional study on healthy volunteers [26]. These findings are in contrast to results obtained from in vitro experiments and animal studies where strong antioxidant effects of quercetin were measured [24, 29, 30, 64]. This suggests that no protective effect of foods containing quercetin was mediated by factors other than those attributed to the molecule, or, as mentioned above, that quercetin may exert a protective effect independently from its antioxidant properties.

To summarize the results of the interventional studies performed on human subjects supplemented with quercetin, its glycosides, or quercetin-enriched food as reported in two excellent reviews [65, 90], the maximal concentration in plasma ranged between 0.14 and 7 μM, resulting from an ingestion of quercetin or quercetin equivalents of 0.008–4 gr (Table 1). In a simplistic way, one might speculate that if supplementation with fried onions (quercetin glucosides equivalent to 64 mg of aglycone) generates a peak plasma level of 0.8 μM [46, 47], then a 1,500 mg daily dose might reach a value not far from 10 μM, which represents the concentration necessary to justify the biological effects of the molecule observed in vitro [55]. Unfortunately, from data reported in Table 1, this does not seem to be the case. Probably, interindividual variability may account for the broad range of determinations published after quercetin supplementation in

Table 1 Bioavailability of quercetin, quercetin glycosides and quercetin-containing foods in humans

Flavonol	Source	Dose	Plasma concentration (μM)	Reference
Quercetin	Pure compound	4 g	<0.33	[36]
Quercetin	Pure compound	0.14 mg/kg body weight	0.15–0.42	[32]
Quercetin	Pure compound	8–20–50 mg	0.14–0.22–0.29	[27]
Quercetin	Pure compound	50–100–150 mg/die	0.189–0.295–0.431	[26]
Quercetin	Complete meal	87 mg quercetin eq	0.37	[64]
Quercetin	Onions	186 mg quercetin eq	2.18	[3]
Quercetin	Onions	50 mg quercetin eq	0.83	[66]
Quercetin	Onions	64 mg quercetin eq	0.65	[48]
Quercetin	Onions	68 mg quercetin eq	0.74	[46, 47]
Quercetin	Onions	100 mg quercetin eq	7.6	[33]
Quercetin	Apples	107 mg quercetin eq	0.3	[46, 47]
Quercetin	Apple cider	1.6 mg quercetin eq	0.14	[25]
Quercetin	Mixed black currant and apple juice	6.4 mg	1.1	[108]
Quercetin	Buckwheat tea	200 mg quercetin eq	2.1	[33]
Quercetin	Onions, apples, mixed	100 mg quercetin eq	0.8–0.18–0.4	[57]
Quercetin 3′glucoside	Pure compound	156 mg	5	[76]
Quercetin 4′glucoside	Pure compound	150 mg	3.5	[45]
Quercetin 4′glucoside	Pure compound	160 mg	4.5	[76]
Quercetin 4′glucoside	Pure compound	100 mg quercetin eq	7.0	[33]
Rutin	Pure compound	100 mg quercetin eq	0.3	[46, 47]
Rutin	Pure compound	190 mg	0.18	[45]
Rutin	Pure compound	500 mg	0.13–0.73	[11]
Rutin	Pure compound	8–20–50 mg quercetin eq	6.5–7.4–7.5	[27]
Rutin	Pure compound	200 mg quercetin eq	1.1	[33]

Modified from [65, 90]

healthy volunteers. These variations may be due to the different bioavailability of quercetin glycosides present in different foods and the polymorphism of intestinal enzymes in humans and animal models [65, 90].

To ameliorate quercetin bioavailability, novel strategies have been identified to increase its uptake in cellular and animal models taking advantage of the improvement in the field of nanoparticles. Nanocarriers modify modern drugs by increasing their efficacy, stability, and solubility; decreasing their toxicity; and sustaining their release. Drug delivery systems using nanotechnology based on liposomes and biodegradable polymers have increasingly attracted the attention of scientists in recent years. In the case of quercetin, gastrointestinal absorption increased by 5.71 times when administered in the form of solid lipid nanoparticles in rats, compared to controls [61]. The different approaches employed to package quercetin as nanoparticles and their efficacy in terms of "nanochemoprevention" and "nanochemotherapy" have been recently reviewed [59, 70, 85].

In conclusion, a dose as low as 150 mg/day significantly increases plasma quercetin concentrations. The most common dosage in studies has been 1,000 mg/day, generally divided into two doses [53, 104].

4 Quercetin Safety

A few excellent reviews have been published in recent years on the safety of quercetin [42, 74]. They agree that evidence of a possible in vivo toxicity of the molecule is lacking or elusive. Warnings on the safety use of quercetin have been raised since the original observation that the molecule positively reacted to the Ames test of in vitro mutagenesis on several bacterial strains. However, methylation at the different hydroxyl groups significantly reduced or abolished the mutagenic activity (reviewed in [42]). In vivo genotoxicity studies failed to confirm the mutagenicity shown in vitro. In fact, oral administration to mice and rats did not induce any significant changes in terms of micronuclei formation, chromosomal aberrations, sister chromatid exchange, or DNA damage (reviewed in [42]). In acute toxicity tests, no symptoms of toxicity were reported in rabbits receiving a single intravenous dose of 100–150 mg/kg body weight [2]. The results of subchronic and chronic toxicity studies on quercetin are more debatable. In 1999, the International Agency for Research on Cancer (IARC) concluded that quercetin is not classifiable as carcinogenic to humans [74]. During a 2-year study conducted by the National Toxicology Program (NTP), male F344/N rats fed 2 g/kg body weight/day of quercetin (corresponding to a dose of 140 g for a 70-kg individual for 728 days) showed severe chronic nephropathy, hyperplasia, and neoplasia of the renal tubular epithelium [73]. These data have been subjected to methodological criticisms, making these findings of unclear significance [44, 51] and parallel studies performed using the same rat model failed to confirm the renal histopathological effects of quercetin (Table 2 in [42]). However, a later study confirmed the original conclusions of the NTP study [41]. At lower doses, from 50 to 500 mg/kg/day, no significant adverse effects were reported.

Several 2-stage (initiation–promotion) carcinogenicity studies have been performed to determine the potential effect of quercetin on chemically induced carcinogenesis. The evidence resulting from the extensive review published by Harwood et al. [42] is that "the promotional activity of quercetin on chemically-induced organ-specific carcinogenesis is somewhat inconsistent." In a few cases, quercetin showed a tumor-enhancing activity, but the majority of the studies supported the anti-carcinogenic and chemoprotective effects of the molecule.

The absence of any relevant toxicity of quercetin on mammalian germ cells excluding negative effects of the molecule on reproduction and development has been confirmed by several studies [42]. A recent debate involved the potential use of the molecule as an antioxidant in the context of male reproduction and fertility. In fact, for the reasons discussed above, quercetin is useful in scavenging free radicals in the testicular compartment that generates oxidative stress leading to chromosome aberrations, but, on the other hand, during the process, quercetin becomes oxidized with the consequent consumption of glutathione, an increase in cytosolic calcium concentration and lactate dehydrogenase (LDH) leakage, leading to a reduction in sperm motility. A recent review suggested caution in the administration of quercetin in subjects affected by reproductive dysfunction coupled with elevated oxidative stress until the cellular toxicity of quercetin metabolites has been better investigated in adequate cellular and animal models [80].

In humans, the unique phase I clinical trial of quercetin so far completed recommended a dose of 1,400 mg/m^2, which corresponds to about 2.5 g for a 70-kg individual, administered via intravenous infusion at 3-week or weekly intervals [28]. At higher doses, up to 50 mg/kg (about 3.5 g/70 kg), renal toxicity was detected without signs of nephritis or obstructive uropathy. Overall, the absence of revealed cases of adverse effects on human health encourages the addition of food-grade quercetin to foods at levels that guarantee its safety and coincide with the amount consumed in diets rich in fruits and vegetables (200–500 mg/day).

5 Quercetin and Cancer

Surfing PubMed (http://www.ncbi.nlm.nih.gov/pubmed), it is easy to retrieve a significant number of recent reviews supporting the view that quercetin can be efficient at treating cancer by inducing cell death or cell cycle arrest preferentially in cancer cells versus their normal counterparts through a process involving the down-regulation of selective oncogenes or the up-regulation of tumor suppressor genes, which, in turn, enhance selective pathways leading to the elimination of cancer cells. In a recent work, we reviewed how the different biological activities of quercetin (antioxidant, anti-inflammatory, anti-proliferative, pro-apoptotic, and anti-angiogenic) span through all the stages of carcinogenesis from initiation to invasion and metastasis. The ability of the molecule to interfere with different targets identified by the pivotal studies by Hanahan and Weinberg as "hallmarks of cancer" [39, 40] supports the view of a pleiotropic inhibitor of cell growth with

synergistic effects against cancer cells. The literature on quercetin regarding the anti-proliferative and growth-suppressing effects, the induction of senescence and telomerase inhibition, the induction of cell death and autophagy, the anti-angiogenic activity, the activation of immune destruction, and the effects on other hallmarks of cancers has been reviewed elsewhere [85]. Here, we will focus on the ability of the molecule to bind and inhibit several kinases involved in growth control and whose regulation and expression are often altered in cancer cells.

5.1 Quercetin as a Protein Kinase Inhibitor

Since the demonstration in the early 1980s that quercetin was able to inhibit both tyrosine [17, 31] and serine–threonine [14] kinases, the molecule has been labeled as a non-specific protein kinase inhibitor. Evidence accumulating over time clearly indicated that many of its inhibited targets were kinases involved in cell growth; therefore, the concept emerged that quercetin may act on multiple targets exerting a pleiotropic activity in inhibiting cell proliferation in cancer cells. However, the direct binding between quercetin and the inhibited kinases has only been reported in a few cases and linked to a cause–effect mechanism responsible for growth suppression. As an example, quercetin interferes with the Akt/PKB-dependent pathway by inhibiting proliferation and inducing apoptosis on several cancer cell lines, without directly targeting Akt/PKB [34, 37, 54, 96, 97, 99]. The PI_3K-Akt/PKB pathway plays a critical role in mammalian cell survival signaling and has been shown to be activated in various cancers [1, 21]. In this context, the molecular target of quercetin is located upstream of Akt/PKB. In fact, the X-ray crystallographic structure of $PI_3K\gamma$ bound to quercetin indicates that the molecule fits into the ATP-binding site with a Kd value of 0.28 μM, slightly higher than that of myricetin (0.17 μM) [49, 105]. This structural finding is confirmed by biological data, indicating that the direct binding of PI_3K by quercetin inhibited the entire PI_3K-Akt pathway of signaling including inhibition of AP-1 and NF-κB activation [37, 49, 50]. Interestingly, quercetin is also a direct inhibitor of the protein kinase CK2. This kinase is a highly conserved serine/threonine kinase ubiquitously distributed in all investigated eukaryotes and involved in several intracellular pathways which control, among others, cell cycle, proliferation, apoptosis, and transformation [67, 87]. Recent evidence has indicated that the anti-apoptotic effect of CK2 is partially mediated by up-regulation of the Akt/PKB pathway by direct binding of CK2 subunits to Akt/PKB [35] and/or its capacity to hyperphosphorylate Ser129 of Akt/PKB [22, 78]. Moreover, CK2 indirectly activates Akt/PKB through phosphorylation and inactivation of tumor suppressor PTEN in a cluster of Ser/Thr residues located at the PTEN C terminus [103]. Native and recombinant CK2 is inhibited by quercetin with an IC_{50} ranging between 0.50 and 1 μM [60, 61, 63, 89]. It is worth noting that two independent kinases, namely CK2 and PI_3K, both converging on a proliferative signaling mediated by Akt/PKB are inhibited by quercetin, supporting the view of a pleiotropic activity of the molecule in growth control.

Structural studies indicate that quercetin can directly bind with Raf-1 and MEK-1 ex vivo and in vitro, with stronger inhibition of MEK-1 kinase activity than Raf-1 [56, 58]. In this case, quercetin seems to bind in a pocket separate from but adjacent to the ATP-binding site of MEK-1 [49]. This interaction leads to inhibition of TPA-induced phosphorylation of ERK and $p90^{RSK}$, and the activation of AP-1 and NF-κB. It is interesting that quercetin exerted stronger inhibitory effects than PD098059, a well-known pharmacologic inhibitor of MEK-1, while resveratrol did not affect either MEK-1 or Raf-1 kinase activity [49, 56, 58].

Quercetin has been used to obtain high-quality diffraction spectra of the Hck tyrosine kinase which belongs to the large family of the Src tyrosine kinases, a group of non-receptor tyrosine kinases that play a role in tumor cell proliferation, survival, and metastasis [109]. The Src family counts nine members which differ in cellular expression, localization, and function. Hck binds to B-cell receptor in unstimulated B cells, and its interaction with BCR-ABL in preclinical experiments in a myeloid leukemia cell line was essential for transformation signaling [62]. Both BCR-ABL and Src family kinases are targeted by dasatinib, the only agent approved for the treatment of patients with imatinib-resistant or imatinib-intolerant chronic myeloid leukemia [56, 58]. Quercetin binds to the Hck active site [93], but the biological consequences are unknown.

IKKα and IKKβ are two kinases involved in NF-κB activation. Following proliferative stimuli, they phosphorylate IκB proteins (IκBα and IκBβ) at serine residues in the N-terminal region. Phosphorylated IκBα and IκBβ are subsequently degraded by ubiquitination with the consequent release of NF-κB which then translocates to the nucleus where it up-regulates the transcription of target genes [23]. NF-κB is involved in cancer development; therefore, inhibition of its positive regulators, such as IKKα and IKKβ kinases, may result in anticancer activity. It was reported that quercetin suppressed TNF-induced NF-κB activation; however, its molecular target in the signaling cascade was not identified [71]. Later, it was shown that quercetin inhibited both IKKα and IKKβ with an apparent IC_{50} value of 11 and 4 μM, respectively [77]. Certainly quercetin behaves as a competitive inhibitor of ATP, but for both IKKα and IKKβ, the molecule significantly reduced the apparent V_{max} and increased the apparent K_m, indicating a mixed-type inhibition mechanism. In fact, the inhibition of IKKα and IKKβ by quercetin was protected by increased amounts of substrate IκBα, suggesting that the binding site of quercetin may overlap with both the ATP- and IκBα-binding sites [77].

More recently, it has been reported that quercetin, as well as the flavonoids luteolin and apigenin, inhibits GSK-3β. This kinase is a constitutively acting multi-functional serine–threonine kinase involved in diverse physiological pathways ranging from metabolism, cell cycle, gene expression, development, and oncogenesis to neuroprotection [81]. GSK-3β is overexpressed in the nucleus of pancreatic cancer cells, where it stimulates NF-κB activity and activates the inflammatory response cascade. An in vitro assay using the recombinant enzyme indicates an IC_{50} for quercetin of about 2 μM, and molecular dockings predict that the molecule fits within the ATP-binding site of GSK-3β [52].

A recent and interesting screening confirmed that quercetin displays specific inhibitory effects in various groups of kinases [7]. In this work, the authors employed a panel of 256 different recombinant kinases with a broad coverage of the human kinome to test the ability of quercetin in in vitro assays to directly inhibit their enzymatic activity. Quercetin decreased the activity of about 100 kinases by greater than 95 % at 30 μM, very close to the mean IC_{50} calculated for many cell lines [7, 55]. At 2 μM, quercetin decreased the activity of ~15 kinases by greater than 95 % and that of a remaining set of ~50 kinases by 80–95 %, suggesting that these kinases may represent specific targets for quercetin. Those with barely detectable activity after treatment with 2 μM quercetin were Cdc2-like kinase 1 (CLK1), insulin receptors (INSR-R), and muscle-specific kinase (MUSK) [7]. It is worth noting that the kinases in the human dendrogram-related kinome that were targeted by quercetin included tyrosine kinases (TK), tyrosine kinase-like kinases (TKL), serine/threonine protein kinases (STE), casein kinases (CK1), cAMP-dependent protein kinases (AGC), calcium/calmodulin protein kinase II kinases (CAMK) and the CMGC subclass, which includes cyclin-dependent kinase (CDK), mitogen-activated protein kinase (MAPK), glycogen synthase kinase (GSK), and CDK-like groups of kinases [7]. Despite the interesting results, the present work leaves important questions open: (1) It is unlikely that within the 15 protein kinases inhibited by a low concentration of quercetin (2 μM), PI_3K, Hck, CK2, MEK-1 are not present, although, apparently, they were included in the panel of tested kinases; (2) the inhibitory effect measured must be confirmed in cellular models on native kinases and associated with deregulation of cell growth or other biological effects.

A similar approach was followed earlier by Cohen's group in 2000 [20]. In this work, using a panel of 34 kinases obtained after tissue purification or expressed in prokaryotic or eukaryotic systems, the authors demonstrated in in vitro assays that at 20 μM concentration, quercetin was able to decrease by less than 30 % the enzymatic activity of the following kinases: MAPKAP-K1b (also known as $p90^{RSK}$; <20 %); S6K1 (<25 %); GSK-3β (<30 %); AMPK (<16 %); CK2 (<19 %); and PI_3K (<18 %). The inhibitory activity of quercetin against GSK-3β, CK2 and PI_3K shown in Cohen's group was later confirmed by independent studies cited above.

6 Conclusions: Pleiotropy Versus Synergy

The adjectives "pleiotropic" and "synergistic" when referred to biologically active molecules may lead to contradictory interpretations. Here, we reviewed evidence according to which, in cancer cells, quercetin at optimal intracellular concentrations can trigger multiple kinases known for their positive role in promoting cell growth in cancer cells if hyper-activated following transcriptional and/or post-translational events. Limiting the investigation to the intracellular effects of the molecule, that is, after quercetin has reached the target cells, pleiotropy refers

Table 2 Cellular kinases directly targeted by quercetin

Targets	Binding site	Concentration	Cellular effects	Reference
MEK-1	Activation loop	1–2 μM	Apoptosis	[49, 56, 58]
			Cell cycle	
			Growth arrest	
$PI_3K\gamma$	ATP-binding site	3.8 μM	Apoptosis	[105]
			Cell cycle	
			Growth arrest	
IKK α/β	ATP- and IκBα-binding sites	IC_{50} 11 μM (α)	Apoptosis	[77]
		IC_{50} 4 μM (β)	Inflammation	
Hck (Src tyr kinase family)	ATP-binding site	2 μM	Apoptosis	[93]
			Cell cycle	
			Growth arrest	
CK2	ATP-binding site	$IC_{50} = 0.92$ μM	Apoptosis	[89]
	Competitive inhibitor	$K_i = 1.18$ μM		
GSK-3β	ATP-binding site	$IC_{50} = 2$ μM	Apoptosis	[52]

Adapted from [85]

to its ability to bind and interfere with the activity of several effectors that insist on one or more pathways, converging on the same cellular process, for example apoptosis, cell cycle arrest, autophagy. A good example is the interaction of quercetin with the PI_3K-Akt/PKB-NF-κB signal transduction pathway. The parallel and contemporary inhibition of CK2 and PI_3K, both converging on Akt/PKB, may result in the switch-off of the pathway since the hyper activation of Akt/PKB by CK2/PI_3K phosphorylation is inhibited. Simultaneously, the inhibition of IKKα/IKKβ kinases by quercetin potentiates the inhibition of the pathway, since NF-κB nuclear translocation and activation are blocked. This example represents a case where pleiotropic and synergistic effects match and enhance the anti-proliferative efficacy of quercetin. However, several conditions must be verified to establish this positive behavior, such as (1) the intracellular concentration of quercetin must be high enough to inhibit all the kinases involved in the target pathway(s), since, as discussed above and reported in Table 2, the K_i and IC_{50} for the different enzymes are significantly different; (2) the target pathway(s) must be hyper-activated in cancer cells, but not in their normal counterparts; (3) the target kinase should not regulate antagonist processes, such as cell proliferation and cell death. To satisfy the first condition, it is necessary to reach micromolar intracellular concentrations of quercetin aglycone. This is almost impossible referring to a dietary uptake of the flavonoid for two main reasons: limited bioavailability and absence of the aglycone in the serum; in fact, the "total" quercetin measured in the blood after oral supplementation includes almost exclusively its metabolites [57,

65, 90]. However, it is interesting to note that according to a study on fibroblasts, O-methylated conjugates of quercetin can be converted to free aglycone in the cell [96, 97, 102]. Therefore, the obstacle of low bioavailability can apparently be overcome by increasing dosage, as demonstrated in animal models where oral consumption of 45–47 mg/day for 2 weeks in rats resulted in a plasma concentration of quercetin and its metabolites of 60 μM [94]. However, attention must be paid when comparing quercetin supplementation in animal models to humans, since due to differential tissue expression and genetic polymorphisms, the activity of metabolizing enzymes can differ highly among mammalian species [90]. Alternatively, the circulating concentration of free quercetin can be increased by intravenous administration, considering that individuals affected by cancer can well tolerate acute serum levels of 200–400 μM [28]. Obviously, this possibility implies a therapeutic use of the molecule, far from any chemopreventive application.

The second important condition that must be verified to justify the use of quercetin as a pleiotropic inhibitor of cancer cell growth is specificity for the malignant phenotype compared to normal cells (point B listed above). In this case, the position of several authors is interesting, who, evoking the hormetic nature of quercetin, describe the molecule as a double-edged sword, which acts as an antioxidant and protects cells from ROS when present at low concentrations in normal cells/tissues and becomes a pro-oxidant at concentrations higher than 30–50 μM, thereby generating ROS that kill cancer cells by activating apoptosis and other defensive processes [9, 104]. Although attractive, this hypothesis deserves extensive experimental validation and it is prone to several criticisms. One obvious disadvantage of increasing intracellular concentrations of quercetin is to favor the pro-oxidant reaction not only in cancer cells, but also in normal tissues, generating side effects that must be carefully monitored and, eventually, confined to therapeutic protocols where benefits and toxic side effects are weighted differently from those in chemopreventive applications [98]. Regarding the *hormetic* behavior of the molecule, we suggest caution in the use of a term taken from toxicology and which assumes different meanings depending on the scientific context in which it is adopted [12, 13, 101]. We recently published a hormetic classical U-shaped curve of quercetin in HeLa cells, where a significant increase in cell growth was observed when the molecule was applied at low concentrations (0.5 μM), comparable to those achievable in vivo after a meal abundant in quercetin-enriched foods [95]. For these reasons, it is necessary to carefully consider its use as a supplement or functional food, since potential benefits may be converted into damage, if precancerous cells are sensitive to the hormetic effect of the molecule. In addition, as we and others reported (Fig. 1 and [83, 84]), micromolar concentrations of quercetin continue to exert antioxidant activity by reducing ROS without interfering with cell viability, which seems to contradict the hypothesis of the specific pro-oxidant effects of the molecule in cancer cells.

Finally, we mentioned that the kinase(s) targeted by quercetin should not regulate antagonist processes in order to support its synergistic effects (point 3 listed above). A good example is represented by the MAP kinase. The RAS-RAF-

MEK-MAPK pathway is frequently deregulated in human cancers as a result of genetic alterations in their components or upstream activation of cell surface receptors, making the components of these signaling cascades interesting targets for therapeutic intervention [1, 21]. As reported above, quercetin interacts with MEK-1 and this binding results in a dose-dependent suppression of JB6 P+ cell transformation induced by epidermal growth factor or H-Ras, both of which are involved in the activation of MEK/ERK signaling [56, 58]. We also demonstrated that in leukemic cell lines and B cells isolated from chronic lymphocytic leukemia, quercetin is able to down-regulate the anti-apoptotic factor Mcl-1 through the inhibition of MEK/MAPK signaling pathways, leading to Mcl-1 instability.[1] These data suggest that, as expected, the apoptotic contribution of quercetin requires inactivation of the MEK/MAPK pathway. Other researchers have reported the opposite effect: (1) the activation of MEK/MAPK was required for quercetin-induced apoptosis in A549 lung carcinoma cells [72]; (2) quercetin decreased cell viability and induced DNA fragmentation in heat-shocked rat hepatoma H4 cells markedly stimulating MAPK [69]; (3) hepatoprotection of quercetin against oxidative stress by induction of metallothionein expression occurred by activating MAPK and enhancing Nrf2 DNA-binding activity [106]; and (4) protection of human hepatocytes from ethanol-derived oxidative stress by inducing HO-1 occurred via the MAPK/Nrf2 pathways [107]. In summary, the emerging paradox is that quercetin may or may not activate the MEK/MAPK pathway; if these events take place in the *wrong* cell line or in an *inappropriate* space-to-time window of the cell physiology, the consequences observed can be opposite to those expected. As an example, increasing MAPK activity by quercetin in a precancerous cell to protect it from stress damage can in turn result in an enhanced proliferative stimulus. Another piece of this complex puzzle regards the effects of quercetin on specific isoforms of cancer kinases when the molecule is added alone or in association with other drugs or pro-apoptotic inducers, such as death ligands (DL; anti-CD95; recombinant TRAIL). In fact, we demonstrated that quercetin slightly decreased PKCα activity, but when it was coadministrated with anti-CD95, PKCα activity increased by 12-fold compared to quercetin monotreatment [83]. This example demonstrates that quercetin can behave (directly and/or indirectly) as an inhibitor or activator of the same kinase and in the same cell line, depending on the stimulus applied and/or the physiological state of the malignant cell.

Here, we have analyzed the advantages and disadvantages of the multiple properties of quercetin in ameliorating pathological conditions associated with degenerative diseases such as cancer. Many of the paradoxes discussed in the present chapter and connected to quercetin can be easily extended to a large number of naturally occurring compounds possessing potential anticancer activity. Whether quercetin acts as an antioxidant or kinase inhibitor, the "first hits" of the molecule in vivo remain largely unknown. This may not be a problem if we accept the possibility that quercetin (and other phytochemicals) can be functionally

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pleiotropic, but this possibility must correlate with clinical efficacy. Unfortunately, no clinical trials have yet been published on cancer patients using quercetin in monotherapy or in combination with other chemotherapeutic drugs (Table 3 in [85]). The lack of such information represents the bottleneck common to many bioactive phytochemicals in view of their potential clinical use. Future studies on the possible use of quercetin in adjuvant cancer therapy are therefore urgently required.

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Sulforaphane as a Promising Molecule for Fighting Cancer

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Abstract

Cancer is a complex disease characterized by multiple genetic and molecular alterations involving transformation, deregulation of apoptosis, proliferation, invasion, angiogenesis, and metastasis. To grow, invade, and metastasize, tumors need host components and primary dysfunction in the tumor microenvironment, in addition to cell dysfunction, can be crucial for carcinogenesis. A great variety of phytochemicals have been shown to be potentially capable of inhibiting and modulating several relevant targets simultaneously and is therefore non-specific. Because of the enormous biological diversity of cancer, this pleiotropism might constitute an advantage. Phytochemicals, in particular diet-derived compounds, have therefore been proposed and applied in clinical trials as cancer chemopreventive/chemotherapeutic agents. Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables. SFN has proved to be an effective chemoprotective agent in cell culture, in carcinogen-induced and genetic animal cancer models, as well as in xenograft models of cancer. It promoted potent cytostatic and cytotoxic effects orchestrated by the modulation of different molecular targets. Cell vulnerability to SFN-mediated apoptosis was subject to regulation by cell-cycle-dependent mechanisms but was independent of a mutated p53 *status*. Moreover, combination of SFN with cytotoxic therapy potentiated the cytotoxic effect mediated by chemotherapy

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_12,

in vitro, thus suggesting its potential therapeutic benefit in clinical settings. Overall, SFN appears to be an effective and safe chemopreventive molecule and a promising tool to fight cancer.

Keywords

Sulforaphane · Isothiocyanates · Phase I and II enzymes · Angiogenesis · Metastatic process

Abbreviations

SFN	Sulforaphane
ITCs	Isothiocyanates
CYP	Cytochrome P450
GST	Glutathione-S-transferase
UGT	UDP-glucuronosyltransferase
NQO1	NAD(P)H:quinone oxidoreductase 1
Keap 1	Kelch-like ECH-associated protein 1
GSH	Glutathione
HCA	Heterocyclic amines
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
CDK	Cyclin-dependent kinase
HDAC	Histone deacetylase
VEGF	Vascular endothelial growth factor
MMP	Metalloprotease
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
Hif-1 α	Hypoxia-inducible factor-1 α
HO-1	Heme oxygenase-1
ARE	Antioxidant response element

Contents

1 Introduction

Cancer, traditionally thought of as a disease affecting mainly Western countries, is nowadays also spreading among populations living in developing countries. The diet and lifestyle typical of wealthier societies living in the West, often characterized by a greater exposure to carcinogens and by a widespread and historically rooted smoking habit, lie at the basis of the higher incidence of cancer in such countries. However, increasing industrialization and wealth has led many developing countries to experience rapid changes in their social habits, causing cancer incidence rates to reach the same level as in more industrialized countries and therefore turning cancer into a worldwide problem [72].

Cancer is a cellular disease that originates from multiple causes, genetic and/or epigenetic, and induces the alteration in cellular homeostasis with the loss of control of cell growth and proliferation.

The genesis of a tumor is an extremely complex process, in which initially normal cells accumulate mutations in critical genes. It involves many events and is experimentally described as a multistep process, in which it is possible to recognize at least three phases: initiation, promotion, and progression.

The initiation phase represents an acute event, characterized by damage to the cellular genome, which, if not properly repaired, is fixed in a stable and irreversible mutation after cell replication. The initiated cell acquires a potential capacity for individual growth, dissociated from the organism's control systems.

The promotion phase is characterized by an intense proliferative stimulus of cells started through epigenetic mechanisms. It is assumed that the promotion activity of chemicals is exerted by the induction of oxidative stress, the stimulation of cell proliferation, the promotion of pre-neoplastic lesions, and the inhibition of spontaneous cell death. The clone of cells proliferating in this stage acquires an appearance of a benign tumor, and the cell mass remains physically grouped and compact.

The progression is the third stage recognized and associated with the acquisition of a malignant phenotype. In this stage, some cells may detach from the tumor mass, invade nearby tissue, infiltrate the bloodstream or lymphatic vessels, and lead to the development of secondary cancers distant from the site of origin [87].

The challenge against cancer can be won only by fighting it at all levels. Thus, a good chemopreventive agent should be able to modulate all different stages of carcinogenesis, including initiation, promotion, and progression. In this context, natural substances, such as the isothiocyanates (ITCs) from cruciferous vegetables (Brussels sprouts, broccoli, cauliflower, and cabbage), are receiving great attention, due to their ability to modulate multiple targets involved in the carcinogenetic process. The ITCs generate through normal food processing and chewing from glucosinolates, a family of plant secondary metabolites that do not exert any biological activity. Among ITCs, SFN, which is obtained by hydrolysis of glucoraphanin, abundantly present in broccoli, was given special attention, being able to interfere at various levels of the carcinogenetic process [62].

2 Modulation of the Carcinogenetic Process

The now-proven chemopreventive activity of SFN is due to its ability to inhibit, reverse, or delay the development of all the different stages of carcinogenesis: initiation, promotion, and progression (Fig. 1). In fact, SFN can modulate the initiation phase both by preventing the metabolic activation of pro-carcinogens to carcinogens, through the inhibition of phase I metabolism, and by promoting the detoxification of carcinogens through the induction of phase II metabolism, and by protecting DNA from the insults of mutagenic compounds. SFN can also modulate the promotion phase by inhibiting the clonal expansion of transformed cells, through cytostatic and cytotoxic effects, such as cell cycle delay and/or arrest and apoptosis.

Finally, SFN can modulate the progression phase by inhibiting angiogenesis, the conversion from benign to malignant tumors and the metastatic process.

2.1 Modulation of the Initiation Phase

2.1.1 Inhibition of Phase I Enzymes

A fundamental step in blocking chemically induced carcinogenesis is the inhibition of phase I enzymes. These enzymes are involved in chemical reactions (oxidation, reduction, and hydrolysis) that generally lead to detoxification but are also involved in bioactivation of carcinogens. Almost all dietary and environmental carcinogens are really pro-carcinogens that are bioactivated by drug-metabolizing enzymes into highly reactive intermediates that can bind macromolecules (DNA, RNA, and protein). These events are primarily catalyzed by cytochrome P450 (CYP) enzymes. Thus, compounds able to modulate their expression and function may be important in the prevention of the carcinogenetic process. Phase I metabolism can be modulated by SFN. Many studies demonstrate that it can regulate the activity of CYP either by direct interaction or through the regulation of mRNA levels. For example, in rat hepatocytes, SFN inhibited in a dose-dependent manner the activities of CYPs 1A1 and 2B1/2, and in human hepatocytes, it markedly decreased CYP3A4 activity by reducing CYP3A4 mRNA level [59]. SFN is also a competitive inhibitor of CYP2E1 in microsomes from livers of acetone-treated rats. SFN does not have effect against CYP1A2, an enzyme involved in the metabolic activation of heterocyclic amines [4, 19].

Overall, SFN appears to be an agent able to inhibit several CYPs and, as a consequence, able to reduce the activation of pro-carcinogens.

2.1.2 Induction of Phase II Enzymes

A great body of literature has demonstrated, both in vitro and in vivo, that SFN is one of the most potent inducers of phase II enzymes such as epoxide hydrolase, ferritin, glutathione peroxidase, glutathione reductase, glutamate-cysteine synthetase [94], glutathione-S-transferase (GST) [93], heme oxygenase-1 (HO-1) [18],

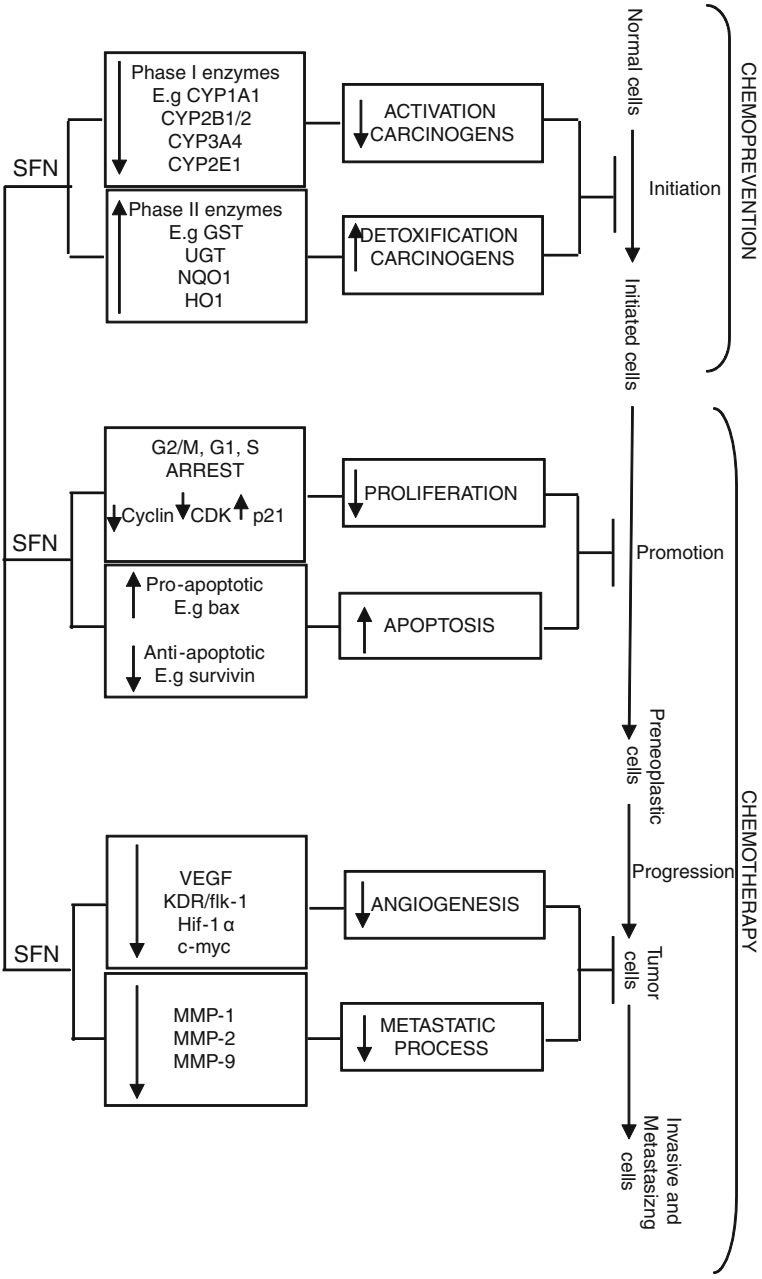

Fig. 1 SFN affects the three different phases of the carcinogenetic process acting as both chemopreventive and chemotherapeutic agent by modulating various molecular targets

thioredoxin reductase [3], and UDP-glucuronosyltransferase (UGT) 1A [6]. For example, in HepG2 human hepatoma cells, SFN increased the activity of NAD(P)H:quinone oxidoreductase 1 (NQO1) [46], as well as bilirubin glucuronidation [6]. SFN also promoted GST and NQO1 activities in murine Hepa1c1c7 [46, 60] and up-regulated GSTA1/2 and GSTP1 mRNA levels in primary rat hepatocytes, while in human primary hepatocytes, SFN induced mRNA levels of GSTA1/2 and GSTM1 [59]. Moreover, SFN exerted its ability as promoter of phase II metabolism in colon and prostate cancer cell lines. In particular, it increased in a dose- and time-dependent manner the activity of GSTA1 and UGT1A1 both in differentiated HT29 [6] and in undifferentiated CaCo-2 cells [88]. SFN increased GSTA1 and NQO1 mRNA levels, as well as the GST and NQO1 activity in human prostate cells [9]. In HepG2 human hepatoma cells and in human hepatocytes, SFN significantly reduced the level of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-DNA adducts in a dose-dependent manner, possibly due to the induction of UGT1A1 and GSTA1 mRNA expressions [2].

In vivo studies confirmed the activity of SFN as a potent inducer of phase II enzymes. GST and NQO1 activities were increased in tissues of rats orally treated with SFN. In SFN-treated rats, NQO1 and GST activities were increased in the stomach, duodenum, and bladder [64] and in the liver, colon, and pancreas [60].

SFN caused a significant increase in the enzymatic activities of NQO1 and total GST in prostatic tissues, in the liver, in the kidney and, most significantly, in the bladder [47]. An induction of phase II enzymes has recently been demonstrated in pre- and post-initiation phase studies of experimentally induced lung carcinogenesis in female Swiss albino mice treated with SFN [48].

SFN seems to exert the induction of phase II enzymes through the stimulation of transcription of their genes, which contain a specific DNA sequence, called antioxidant responsive element (ARE) [20, 63]. Genes that contain this enhancer sequence are regulated by the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which is located in the cytoplasm bound to the cytoskeletal protein Kelch-like ECH-associated protein 1 (Keap 1). In response to alteration in the cytosolic redox state, it breaks the bond between Keap 1 and Nrf2, the latter is thus activated, and free from the bond with the protein, Nrf2 can translocate to the nucleus where it induces the transcription of ARE containing genes. When Nrf2 is inactive, no type of gene expression induction by SFN is observed [21, 37, 38, 51, 68, 92].

Many studies show that this pathway is involved in cellular protection from oxidative stress and can be an effective therapeutic target in chronic disorders, such as cancer and neurodegenerative diseases [55]. SFN is considered as an indirect antioxidant, because it is able to protect cells from the excessive production of free radicals, not by acting as donors or electron acceptors, but by modulating the expression of phase II enzymes and by modulating glutathione (GSH) intracellular levels. SFN, in fact, has also been proved to be capable of increasing the synthesis of the light chain, but not of the heavy chain, of glutamylcysteine synthetase, an enzyme that catalyzes a limiting step of the synthesis of GSH [94].

All together, this evidence demonstrates the ability of SFN to promote carcinogen detoxification and cellular defenses against potential mutagenic events.

2.1.3 Protection of DNA from Insults of Mutagenic Compounds

SFN is able to counteract the genotoxicity of many carcinogenic compounds through different mechanisms of action. For example, exposure to heterocyclic amines (HCA) is associated with the development of some cancers such as breast, colon, and prostate cancers, and in vitro studies suggested that SFN is a potent inhibitor of mutagenesis induced by HCA [80].

In HepG2 human hepatoma cells, the co-treatment with SFN and PhIP significantly reduced the level of PhIP-DNA adducts. However, when PhIP-treated cells were post-treated with SFN, no decrease in the levels of PhIP-DNA adducts was detected. This finding suggests that SFN exerts a preventive action rather than induction of DNA repair enzymes [46].

In MCF-10F human mammary epithelial cells, SFN inhibited the formation of DNA adducts after exposure to benzo(a)pyrene and 1.6-dinitropyrene [84]. In human colorectal cells, SFN protected DNA from the single-stranded breaks induced by benzo(a)pyrene [8], while in human liver cells, which express CYP2E1 and CYP1A2, SFN contrasted the double-strand breaks induced by N-nitrosodimethylamine and 2-amino-3-methylimidazo [4,5-f] quinoline [5].

A study on human lymphocytes showed that SFN contrasts the genotoxicity of four different compounds: alkylating ethyl methanesulfonate, aneugen vincristine, oxidizing H_2O_2, and alkylating and oxidizing mitomycin C. In the case of ethyl methanesulfonate and mitomycin C, the antigenotoxic activity is due to the induction of apoptosis. On the contrary, the addition of SFN to cultures treated with vincristine and H_2O_2 does not result in any increase in the fraction of apoptotic cells, suggesting a different mechanism for the protective action of SFN, such as the inhibition of cell proliferation or the induction of specific enzymes [26].

Results from in vivo studies are not clear. Gills et al. [34] reported that when SFN is administered topically before and after 7,12-dimethylbenz(a)anthracene, in a classic two-stage carcinogenesis protocol (where effects on initiation are separated from effects on promotion), SFN does not decrease the percentage of tumor-bearing mice. On the other hand, Kuroiwa et al. [54] reported that SFN decreases the incidence of atypical hyperplasia in pancreatic ducts and the incidence of adenocarcinomas in a hamster model of pancreatic carcinogenesis.

These results suggest that SFN possesses an interesting antigenotoxic potential, but this activity has still not been completely elucidated.

2.2 Modulation of the Promotion Phase

2.2.1 Cytostatic Effects

SFN is able to modulate the cell cycle progression in several cellular models (prostate, colon, breast, bladder, and T cells) and to arrest cells in G1 phase [14, 77, 91], in S phase [89], or in G2/M phase [40, 73, 74], depending on the cell line

under study, the dose of treatment, and the time of exposure. These actions are associated with the modulation of cyclins and cyclin-dependent kinase (CDK) and CDK inhibitors.

In particular, the cell cycle arrest in G2/M phase has been observed in PC-3 and DU-145 human prostate cancer cells, in HCT-116, HT29, and Caco-2 human colon cancer cells [82], in F311 murine breast cancer cells [39], in MCF-7 human breast cancer cells [40], in Jurkat human T leukemia cells [23, 25], in U2-OS human osteosarcoma cells [53], and in a panel of human myeloma cell lines, as well as in primary myeloma tumor cells [43].

The ability of SFN to induce G2/M phase arrest is due to several mechanisms. In PC-3 prostate cancer cells, it has been associated with a significant reduction in the protein levels of cyclin B1, Cdc25B, and Cdc25C and with an increase in Tyr-15-phosphorylated CDK1. These events result in an activation of ChK2 that induces a rapid and sustained phosphorylation of Cdc25C at SER-16. The same Chk2-dependent G2/M arrest was seen in HCT-116 human colon cancer cells [82]. Moreover, in MCF-7 breast cancer cells, SFN caused an arrest in the mitotic phase, due to inhibition of tubulin polymerization [40], while in F311 breast cancer cells, the arrest was due to an aberrant and absent mitotic microtubule formation [39].

Although G2/M phase arrest is the main type of cell cycle arrest induced by SFN, arrest in an other phase occurs in prostate and colon cancer cells. In LnCap and in DU-145 human prostate cancer cells, SFN treatment caused a total arrest in G1 phase through inhibition of CDK4 activity, increased expression of p21, and decreased expression of cyclin D1 [91]. Similarly, in HT29 colon cancer cells, G1 phase arrest occurred after an increase in p21 levels and a decrease in cyclin D1, cyclin A, and c-myc [79].

Finally, S phase arrest, as a result of SFN treatment, has been reported in UM-UC human bladder cells [89].

An in vivo study clearly demonstrated that SFN inhibits the promotion phase through the inhibition of cell proliferation. SFN topically administrated significantly inhibited 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol 13-acetate-induced mouse skin tumorigenesis [34].

On the basis of this evidence, SFN has been shown to be an effective cytostatic agent able to modulate the cell cycle through several molecular mechanisms.

2.2.2 Cytotoxic Effects

The pro-apoptotic effect of SFN is not cell specific, and it has been described in different cell lines.

The first evidence was reported in colon cancer cells [32, 33, 42], subsequently, in prostate cancer cells [14, 15, 81], medulloblastoma [35], mammary [39, 75], ovary [12], pancreas [74], blood [23, 24], bladder [77, 89], skin [61] cells, and a panel of human myeloma cell lines, as well as in primary myeloma tumor cells [43] and in B16F-10 highly metastatic melanoma cells [36].

On these in vitro models, the ability of SFN to induce events characteristic of apoptosis, such as chromatin condensation, translocation of phosphatidylserine

across the plasma membrane, and DNA fragmentation has been demonstrated. Moreover, SFN is able to activate multiple targets of both intrinsic (mitochondria-mediated) and extrinsic (death receptor-mediated) pathways. Interestingly, glioblastoma cells, one of the most common and still difficult to treat brain tumors, responded to the treatment with SFN with an increase in apoptotic cell fraction induced by a caspase-dependent and caspase-independent mechanism [52].

At the basis of SFN pro-apoptotic action, numerous molecular mechanisms have been proposed such as maintenance of Cdc2 kinase in an active form [39, 73], activation of pro-apoptotic genes such as caspase [74], blocking of tubulin polymerization [39], increased oxidative stress [83], decreased levels of intracellular antioxidants [74], and nuclear factor signalling pathways [45]. Other studies on leukemia cells associate SFN-induced apoptosis to an increase in bax levels [23, 25]. In PC-3 prostate cancer cells, SFN promoted apoptosis by PARP cleavage and this result has been confirmed in vivo in PC-3 xenograft nude mice, where the apoptosis was associated with an increase in the expression of pro-apoptotic proteins, such as bax and bid [81].

In HT29 colon cancer cells, SFN increased bax expression, the release of cytochrome c, and PARP cleavage [33]. In myeloma cells, SFN induced apoptosis by the depletion of mitochondrial membrane potential, by PARP cleavage, and by caspases 3 and 9 increase, as well as by the down-regulation of antiapoptotic proteins including Mcl-1, X-IAP, c-IAP, and survivin [43].

In HeLa human cervical carcinoma cells and in HepG2 hepatocarcinoma cells, SFN induced apoptosis through the activation of caspase 3 [71], and in T98G and U87MG glioblastoma cells, SFN promoted apoptosis by an increase in $[Ca^{2+}]_I$, by an induction of calpain, and by the cytosolic up-regulation of Smac/Diablo [52]. A recent study investigated the anticancer effects of SFN on colorectal cancer using primary cancer cell lines and demonstrated an activation of caspase 3, a loss of mitochondrial membrane potential, the cleavage of pro-caspase 3, and an increase in caspase 2, 3, 8, and 9 activity [13].

Moreover, ERK and JNK signalling pathways are involved in the regulation of activator protein 1 that induces SFN-mediated apoptosis in PC-3 cells [92]. Studies performed on Jurkat T leukemia cells demonstrated that these cells were most sensitive to SFN-induced apoptosis in the G1 phase, less sensitive in G2/M phase, and least sensitive in S phase [29]. SFN is also able to cause cytodifferentiation and a significant increase in the apoptotic cell fraction of HL-60 human promyelocytic cells toward both granulocytic and macrophagic lineages, mediated by the involvement of phosphatidylinositol 3-kinase/protein kinase C [31]. Finally, studies on HEK293 embryonic kidney cells, HCT-116 human colon cancer cells [65], BPH-1, LnCaP, and PC-3 prostate epithelial cells also identified the ability of SFN to inhibit the activity of histone deacetylase (HDAC), as a new mechanism underlying its pro-apoptotic effect [16, 67]. These findings were also confirmed in mice orally treated with SFN, where significant inhibition of HDAC activity was detected contemporaneously with an increase in acetylated histones H3 and H4 [66].

Interestingly, SFN-induced apoptosis seems to be independent of p53, one of the most important tumor suppressor genes, often mutated in cancer cells. SFN treatment of HT29 colon cancer cells does not change p53 levels [33]. Studies performed on three types of fibroblasts characterized by a different p53 status (wild-type, knock-out, and mutated) demonstrated that a mutated p53 does not prevent SFN-induced apoptosis and suggest a p53-independent pathway [27, 28]. In HCT-116 colon cancer cells, SFN promoted apoptosis by an increase in caspase 7 and caspase 9 levels, both in p53 wild-type and in knock-out cells [70, 76].

On the whole, SFN has been shown to be an effective cytotoxic agent able to promote apoptosis of cancer cells through the modulation of different pathways.

2.3 Modulation of the Progression Phase

2.3.1 Inhibition of Angiogenesis

More recent studies demonstrated the ability of SFN to interfere with angiogenesis. Angiogenesis is the physiological process of formation of new blood vessels, essential for normal tissue growth, but it is also the mechanism that allows the tumor to nourish itself, to expand, and to form metastases [10]. Without this new formation of vessels able to create and develop a network of capillaries that allow the intratumoral delivery of oxygen and nutrient supply, the growth of a tumor is limited and does not reach the critical size of 1–2 mm^2.

The progression of angiogenesis requires the presence of certain pro-angiogenic molecules released by tumor cells, such as vascular endothelial growth factor (VEGF).

In HMC-1 human microvascular endothelial cells, SFN reduced the in vitro formation of microcapillaries, capillary-like tube, and cell migration by the inhibition of the expression of VEGF and its receptor KDR/flk-1 at the transcriptional level, the hypoxia-inducible factor-1 α (Hif-1 α), and c-Myc [7]. Using HUVEC human umbilical vein endothelial cells as a model of angiogenesis, it was demonstrated that SFN also inhibits the formation of blood vessels and induces a dose-dependent decrease in the proliferation of endothelial cells [1]. The inhibition of angiogenesis by the suppression of proliferation was also observed in bovine aortic endothelial cells [41].

Taken together these findings suggest that SFN is able to modulate all the essential steps of angiogenesis.

2.3.2 Inhibition of Conversion from Benign to Malignant Tumors

Several animal models have been used to evaluate in vivo antitumor effects of SFN. SFN demonstrated the ability to significantly inhibit the development of tumors, induced by several carcinogenic compounds in various organs, including the lung [17], pancreas [54], skin [34, 92], and stomach [21], especially through the modulation of the early stages of tumor development.

Moreover, other studies demonstrated the ability of SFN to inhibit the malignant progression of lung adenoma induced by tobacco carcinogens. In fact, in A/J mice treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and benzo(a)-pyrene, histopathological examination has shown a significant reduction in the malignant progression of lung cancer. The inhibitory activity of SFN has been associated with decreased cell proliferation and increased apoptosis, probably by modulation of caspase 3 [17].

2.3.3 Inhibition of the Metastatic Process

The ability to generate metastasis is one of the characteristics of malignant tumors. The metastatic process consists of a series of sequential events that lead cancer cells to spread, through the blood and lymphatic system, from the place of development to another organ.

It is a complex biological event linked to individual characteristics of the subject (general conditions and ability of immune response) and specific features of tumor cells (location, size, and histological characteristics) [22, 57, 69].

The spread of tumor cells begins locally through the release of enzymes able to digest the surrounding tissues, such as adipose tissue and cartilage. When tumor cells encounter blood or lymphatic vessels, they can destroy the wall and penetrate into the blood stream or lymphatic system and spread throughout the body [56, 58].

SFN demonstrated its inhibitory effect on the metastatic process in the appearance of lung metastases induced by B16F-10 highly metastatic melanoma cells in C57BL/6 mice, where it also caused a significant reduction in pulmonary fibrosis markers and in cellular proliferation markers, in addition to an increase in the survival of animals bearing metastases [90]. The mechanisms underlying these effects are due to the inhibition of the activation of metalloprotease (MMP) 2 and 9, a family of endoproteinase able to destroy most of the components of the extracellular matrix, allowing cell invasion and metastasis formation [11, 85]. Metalloproteases are also able to modulate certain neoplastic progression promoting factors such as cytokines and growth factors [95]. SFN inhibited oral carcinoma cell migration and invasion by the down-regulation of MMP-1 and MMP-2 [44], and it was demonstrated to reduce axillary lymph node metastasis of KPL-1 human breast cancer cells in female athymic mice [50].

3 Conclusions

Cancer is an extremely complex disease, characterized by numerous genetic and molecular alterations that represent potential targets for the development of new pharmacological strategy. To take into account the enormous biological complexity that characterizes a tumor, a promising strategy is based on the use of non-specific agents, able to inhibit or modulate many critical targets simultaneously. In this context, vegetable products represent an interesting source of multitarget

compounds. Since SFN was demonstrated to be an important inducer of phase II enzymes in 1992 and a promising chemopreventive agent, extensive studies of this ITC have followed which reveal that SFN also possesses important abilities that represent fundamental chemotherapeutic approaches. In fact, it is able to inhibit proliferation and to induce apoptosis and cytodifferentiation in several models in vitro and in vivo. Most tumor cells exhibit alterations in the ability to develop into non-proliferating adult cells. The SFN ability to induce terminal differentiation generates cells with no or limited replicative capacity, which must then undergo apoptosis [86]. Thus, differentiation represents an alternative approach to more traditional anticancer agents. Moreover, SFN is a promising antiangiogenic molecule and is able to inhibit the metastasis of cancer cells. There are no agents currently available to inhibit the metastasis of cancer cells [90]. This is due to the fact that the cancer cells in different metastases and even in single metastasis may respond differently to chemotherapy.

Emerging highlights indicates that SFN is able to induce apoptosis in cells with p53 mutated or knocked-out. Considering that most of the conventional chemotherapeutic agents require the presence of intact p53, this is an interesting point with important clinical implications. Accordingly, it is important to underline the ability of SFN to overcome resistance and enhance the efficacy of other chemotherapeutic agents [28, 30, 49].

Effectiveness is, however, not the only requirement to be pursued in the development of new molecules. Although larger-scale clinical trials are necessary, SFN seems to possess a favorable toxicological profile, considering the absence of genotoxicity [26] and its tolerability and safety in humans [78].

As previously discussed, the recent literature has clearly demonstrated that SFN is an effective and safe chemopreventive molecule and a promising tool for fighting cancer.<!Query ID="Q1" Text="No Query" –>

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Part III
Genetics and Epigenetics

Functions, Aberrations, and Advances for Chromatin Modulation in Cancer

Mariarosaria Conte and Lucia Altucci

Abstract

Cancer initiation and progression is causally connected to genome and epigenome deregulations. Epigenetic deregulations (such as DNA methylation, histone modifications, and miRNA-based modulation) have been increasingly reported in tumorigenesis and different chromatin-modulating enzymes have been discovered and classified and their aberrations connected to cancer. A better insight into alterations occurring on chromatin enzymes and their impact in cancer thus represents a crucial step in exploiting epigenetic targeting in cancer prevention and treatment.

Keywords

Cancer · Epi-modifications · Writers · Readers · Erasers · Therapy

Abbreviations

AML	Acute myeloid leukemia
AR	Androgen receptor
CARM1	Coactivator-associated arginine methyltransferase1
CBP	CREB-binding protein
CLL	Chronic lymphocytic leukemia

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_13,

DNMT	DNA methyltransferase
DZNep	Deazaneplanocin A
EHMT2	Histone-lysine N-methyltransferase
ER	Estrogen receptor
Eu-HMTase1	Euchromatic Histone Lysine N-Methyltransferase 1
FBXL11	F-box and leucine-rich repeat protein 1
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMT	Histone methyltransferase
JHDM1B	JmjC-domain-containing histone demethylase 1B
JMJD5	Jumonji domain containing 5
KDM	Lysine (K) demethylase
KDM2B	Histone demethylase 2B
KDM5	Histone demethylase 5
KDM8	Histone demethylase 8
KMT	Lysine methyltransferase
LSD1	Lysine-specific demethylase 1
MLL	Mixed lineage leukemia
MOZ	Monocytic leukemia zinc-finger protein
PCAF	P300/CBP-associated factor
PRC2	Polycomb-repressive complex 2
PRMT	Protein arginine methyltransferases
RBP2	Retinol-binding protein 2
SIRT	Sirtuin

Contents

1 Background

The study of epi-modifications and their implications is constantly being informed as our depth of knowledge increases. Understanding the histone code and its critical role in biology led to the study of the enzymes that "write" these modifications, the *writers*, those that "read" modified chromatin, the *readers,* and the *erasers*, able to eliminate a specific modification [1]. Therefore, epigenetic marks and their repercussions in cancer represent a crucial step toward new therapeutic strategies [2]. Epigenetic and genetic alterations accumulating in a given cell lead to the deregulation of pathways involved in proliferation, migration, growth, differentiation, transcription, and death signals. All these alterations cause the onset of cancer, which in turn results in changes in the surrounding stroma and disorders of the immune system.

2 Epi-enzymes in Cancer

Chromatin structure can be remodeled by specific mechanisms involving covalent modifications of histones and DNA. DNA triggers methylation of cytosine within CpG islands, while a multitude of modifications involve histones in both their N-terminal and C-terminal tails, or even in their globular domains. Histone modifications dynamically change chromatin structure (so-called "epigenetic plasticity") leading to relevant biological consequences in the cell. Moreover, the fact that modulation can also include non-histone substrates adds a level of complication [3]. Thus, attention has been focused on chromatin-modifying enzymes to define their deregulation in cancer and their potential as therapeutic targets.

2.1 HATs

Two main categories of HATs exist. A-type HATs acetylate histones within chromatin in the nucleus, whereas B-type HATs play a broader role in the cell. A-type HATs are classified into four families with sequence homology of the HAT domain and include Gcn5/PCAF [4], MYST [5], p300/CBP [6], and Rtt109, the latter reported to be mycotic-specific [7]. In prostate cancer, p300 and CBP are overexpressed, altering androgen receptor (AR)-responsive gene modulation. CBP is also translocated in t(8;16) in which MOZ, localized on 8p11, is fused to CBP [8]. Mutations in p300 (often C-terminal truncations) have also been found in many tumors [9–11]. Downregulation of PCAF has been connected to gastric cancer progression and linked to poor clinical outcome. Mutation in Tip60 is associated with prostate cancer through DNA repair deregulation and resistance to apoptosis. PCAF and Gcn5 are overexpressed in the central nervous system diseases in pediatric patients and Wilms tumors [12].

2.2 (De)Acetylation in Cancer and HDACs

The equilibrium between HDAC and HAT activities plays a key role in regulating gene expression. Future studies will clarify whether the modulation of acetylation is causally linked to cancer or whether it is caused by complex epi-deregulations. Ultimately, whether cancers characterized by overall alterations of the acetylation state may benefit (and in which sense) from an epi-therapeutic approach is an issue that still needs to be addressed.

HDACs play a key role in gene regulation and therefore in human cancers such as leukemia [13]. HDACs are divided into four classes: class I HDACs (HDAC1, HDAC2, HDAC3, and HDAC8), homologous to *Saccharomyces cerevisae* Rpd3; class II (further divided into class IIa and class IIb) HDACs (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10), homologous to yeast HDA1; class III HDACs (sirtuins) include enzymes homologous to yeast Sir2; class IV stands only for HDAC11 (for a scheme see Fig. 1). In particular, HDAC1 is highly expressed in many cancers including gastric, colon-rectal, hepatic, breast, and pancreatic cancer [14–16]. HDAC2 has been found mutated in colon cancer and is overexpressed in esophageal, prostate, and gastrointestinal carcinomas [17–19]. Many other cancers associated with poor prognosis, such as prostate, gastric, colorectal cancer, and chronic lymphocytic leukemia (CLL) [16, 18, 20, 21], have been reported to display HDAC3 overexpression. HDAC4 is expressed in a tissue-specific manner and promotes the growth of colon cancer cells through repression

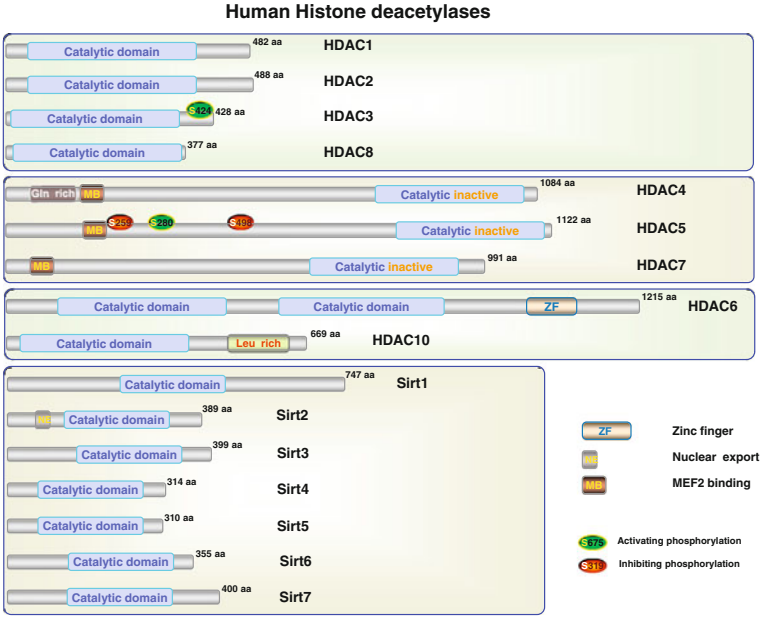

Fig. 1 Schematic representation of the human histone deacetylases

of the cell cycle regulator p21 [22]. HDAC5 downregulation has been reported in lung cancer [23], while its overexpression has been found in colon cancer [24]. Downregulated expression levels of HDAC6 have been observed in lymphoma [25], but higher expression levels are associated with oral squamous cell cancer [26]. The role of HDAC8 has been investigated in CLL in children [27]. Of the class III HDACs, SIRT1 is involved in carcinogenesis often in age-related neoplasms (for a scheme of SIRT1 targets in cancer and other diseases see Fig. 2). In particular, sirtuins are correlated to aging, cancer, and stress response. SIRT1 overexpression has been found in prostate, colon, skin cancers, and acute myeloid leukemia (AML) [28]. In addition to SIRT1, other sirtuins (such as SIRT4 and 7) are linked to cancer development [29]. Conversely, low levels of SIRT2 have been observed in gastric carcinoma and in gliomas. For some sirtuins, such as SIRT3, the scenario is complicated by the fact that both upregulation and downregulation have been reported in different forms of breast cancer. SIRT4 loss may contribute to diabetes, a major risk factor for cancer. SIRT6, which also displays ADP-ribosyltransferase activity, is widely overexpressed in brain and skeletal muscle. Finally, SIRT7 promotes active transcription of rRNA genes and lower levels of this enzyme have been found in non-proliferating tissues such as heart, brain, and skeletal muscle.

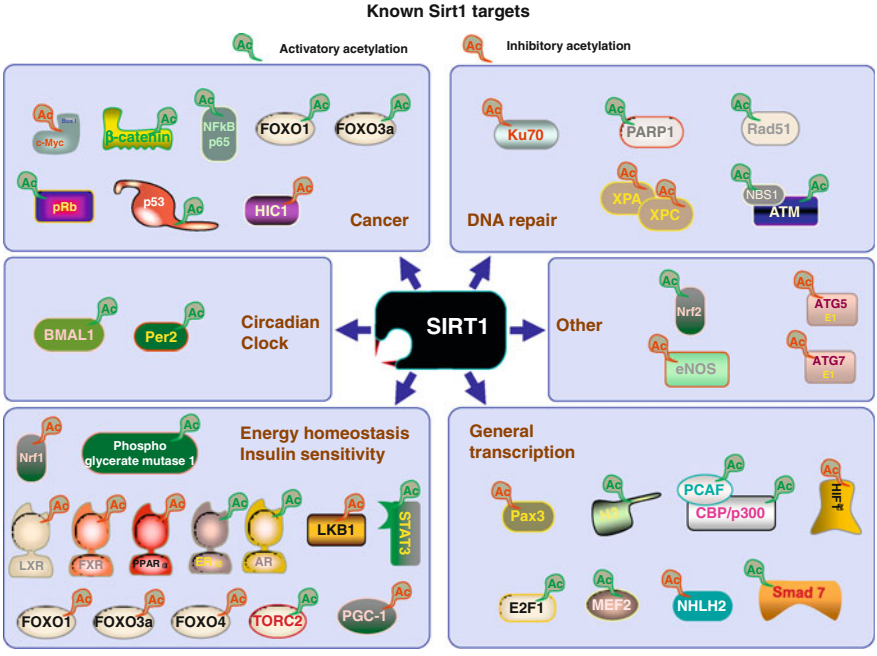

Fig. 2 Schematic representation of the SIRT1 targets

2.3 HMTs and KDMs

Histones are methylated by enzymes (HMT) such as arginine methyltransferases (PRMTs) and lysine methyltransferases (KMTs). PRMT1 is an important component of mixed lineage leukemia (MLL) oncogenic fusion proteins [30]. PRMT6 is responsible for H3R2 methylation; this methylation may counteract H3K4me3 activation. Elevated levels of PRMT1 have been detected in breast and colon cancers [31]. PRMT2 can interact with the estrogen receptor (ER) and acts as a strong coactivator of the AR. PRMT3 is involved in the regulation of protein synthesis, while PRMT4, also known as CARM1 (coactivator-associated arginine methyltransferase1), is known to control the arginine-regulated mechanism of transcription. CARM1 methylates histone H3 and the mutation decreases methyltransferase and p160 coactivator regulation [32]. CARM1 is overexpressed in breast tumors [33]. PRMT5 is strongly expressed in lymphoma and leukemia cells as well as in gastric carcinoma [34]. EZH2, an H3K27 KMT, is involved in the polycomb-repressive complex 2 (PRC2) and is highly expressed in several solid tumors such as prostate cancer [35] and in pro-B cells [36]. Overexpression of SUV39H1/2 (KMT1A/B) has been reported in dietary-induced tumors [37], while increased mRNA levels have been found in colon cancer cells. The lysine methyltransferase G9a contributes to H3K9 dimethylation, regulated in suppressor-gene silencing. G9a is overexpressed in various cancers such as leukemia, prostate, and lung cancer as well as hepatocellular carcinoma [38]. KMT1D (or Eu-HMTase1) is overexpressed in gland tumors [39], while SETDB1/ESET cooperates with DNA methyltransferase (DNMT) in the silencing of promoter regions in tumors via the trimethylation of histone H3 on K9. Numerous mutations and rearrangements of MLL1 (also called KMT2A) have been observed in leukemogenesis. MLL4 (also called KMT2D) is involved in liver oncogenesis. In addition, alteration of histone demethylases (KDM) also causes/contributes to cancer. In breast carcinoma, the downregulation of KDM1 (LSD1) is correlated to metastasis. The aberrant regulation of jumonji domain demethylases has been found in various cancer cell lines. KDM2B (JHDM1B/FBXL11) abolishes the dimethylation state of H3K36me2 or H3K4me3 by causing the downregulation of several proteins involved in the cell cycle such as p14, p15, and p16 in T-cell lymphomas. The KDM5 family, including RBP2 (KDM5A), PLU1 (KDM5B), and SMCX (KDM5C), are often overexpressed in a wide number of cancers such as gastric, cervical, lung, breast, prostate, and kidney cancer as well as leukemias. Another family of lysine demethylases, KDM8 or JMJD5, targets H3K36me2. This enzyme is overexpressed in breast, thyroid, adrenal, bladder, and liver cancers [40] and plays a key role in modulating cell proliferation.

2.4 DNMTs

DNA methylation has frequently been described as a silencing epigenetic event. Methylation is catalyzed by enzymes belonging to the DNMT family. Several cancers are often associated with DNA methylation states and DNA in particular is

more frequently hypomethylated than hypermethylated. Genomic DNA is the subject of intense ongoing studies aimed at better understanding alterations involved in transforming normal cells into pro-cancerous cells [41, 42].

3 Epi-Cancer Therapy

3.1 HDAC and HAT Modulators

HDACi are considered a potential strategy to reverse aberrations associated with cancer and many have been tested in clinical trials [43]. For an in-depth description of the role of HDACi in cancer (for the representation of some known HDACi see Fig. 3), we suggest the reader refers to [44–46] and references contained therein. Whether selective HDACi might offer better benefits (or lower side effects) over broadly acting HDACi (pan HDACi) remains as one of the questions to be addressed.

Unlike HDACi, few and poorly validated molecules modulating HATs are now available (see Fig. 4). In the last decade, two substrates analogous to peptide-CoA conjugates, Lys-CoA and H3-CoA-20, have been proposed as powerful p300 and PCAF inhibitors. However, their metabolic instability precludes the use as drugs. Other PCAF inhibitors comprise isothiazolones and analogs acting as acetylation inhibitors in a dose- and time-dependent manner. Garcinol is a B-type PBD active against viruses, bacteria, gastric ulcers and cancer, such as colon adenocarcinoma [47]. Garcinol derivatives (LTK-13, LTK-14, and the disulfonyl-substituted derivate LTK-19) have also been synthesized. Another promising HATi is anacardic acid (AA), the main component of cashew nut shell liquid. This compound reduces

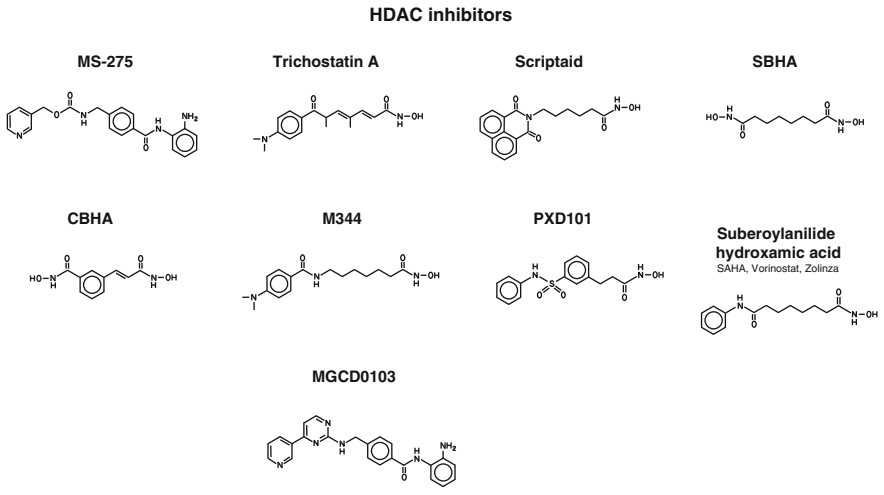

Fig. 3 Structure of some known HDAC inhibitors

breast cancer cell propagation by inhibiting ER-alpha DNA binding [48]. Gcn5-inhibitors, Y-butyrolactones, have also been synthesized and proposed as beneficial [49]. Curcumin, an inhibitor of p300, has been reported to be active in the prevention and treatment of several cancers. The development of HATi is less advanced than that of HDACi. This is likely related to the fact that HATs are more often mutated than overexpressed compared to HDACs. Although this has possibly delayed the development of inhibitors, selective HATi for mutated HATs in cancer may represent a valid alternative strategy with predictable features of specificity.

3.2 PRMT, KMT, and KDM Inhibitors

PRMT alterations correlate with many cancers. Hence, molecules able to modulate methyltransferases are desirable for treatment. Chaetocin, a micotoxin, is a SUV39H1 inhibitor that exerts antimyeloma activity in myeloma cells, and in vivo [50]. BIX01294, the first G9a and GLP inhibitor, inhibits the histone methyltransferase EHMT2, which acts as a corepressor for specific transcription factors and is strongly overexpressed in bladder carcinomas [51]. Moreover, acyl derivatives of p-aminosulfonamides and dapsone have also been identified. In particular, dapsone has been suggested for the treatment of glio-blastoma [52]. Tranylcypromine (trans-2-phenylcyclopropylamine) and its analogs are the best-known LSD1 inhibitors. Tranylcypromine has been proposed for the treatment of sarcomas, peripheral nerve sheath tumors and promyelocytic leukemia [53, 54]. Deazaneplanocin A (DZNep) interferes with the PRC2 and induces apoptosis in cancer cells. Inhibition of EZH2 by DZNep reduces proliferation in breast cancer cells [55]. In AML, cotreatment with DNZep and Panobinostat, an HDACi, exerts an apoptotic effect on primary leukemia cells [56].

3.3 DNA Methyltransferase Inhibitors

The Food and Drug Administration (FDA) has approved the DNMT-inhibitors 5-azacytidine (azacytidine) and 5-aza-2-deoxycytidine (decitabine) for

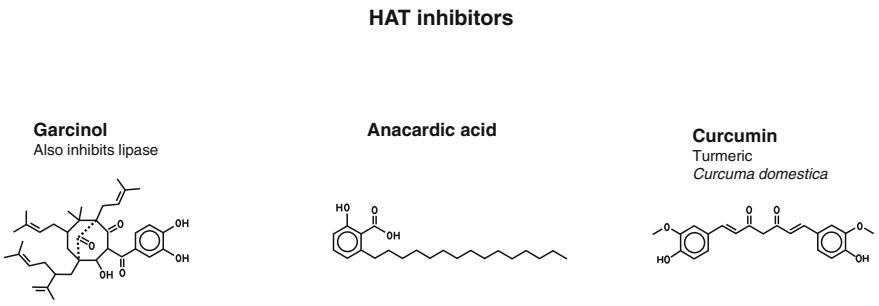

Fig. 4 Structure of some known HAT inhibitors

myelodysplastic syndromes (MDS) [57]. Decitabine has also been used for the re-expression of silenced ER in triple negative breast cancer. Another important DNA Methyltransferase Inhibitors (DNMTi) is zebularine, which is selectively incorporated into malignant cells and not into normal cells. Cancers such as ovarian and cervical carcinomas can be treated with the DNMTi hydralazine [58]. Non-nucleoside-targeted molecules have also been proposed for direct inhibition of DNMTs. The phosphorothioate antisense oligonucleotide MG98, in phase I study, has been tested in patients with myelodysplasia and AML [59]. RG108 shows demethylating action comparable to zebularine in lymphoid, myeloid, and colorectal cancers. The SGI-1027 inhibits DNMT1, DNMT3a, and DNMT3b and has been tested against hepatocellular, cervical, prostate, and breast cancer [60].

4 Conclusions

Disorders in DNA methylation and histone modifications can induce cancer promotion and progression. Chromatin changes may be regulated by *writers* and *erasers* and "mined" by *readers.* This is a complex epi-regulated scenario, where single modifications (and single enzymes) and the connection with other histone marks/enzymatic complexes need to be taken into account to understand epigenetic deregulation in malignancies. The fact that chromatin modifications are possibly mutually exclusive or additive and can influence each others' deposition makes chromatin modulation of gene expression a complex issue. A large number of high-throughput screening studies in cancer have emphasized the importance of some histone marks and epi-enzymes in cancer. Deciphering our present knowledge from bench to bedside will involve characterizing a new "targeted" therapy, where vast chromatin areas in cancer might be deregulated by the induced modification of their epigenetic status. Thus, a re-evaluation of the therapeutic schemes of the existing epi-cancer treatments together with a cautious patient stratification might be needed. Whether the anticancer activity of epi-modulators might be due to "clean" epi-effects alone, and/or to which degree non-epigenetic action needs to be evaluated, remains unclear. The clear modulation of non-histone targets by epi-molecules and the fact that few epi-treatments (mainly HDACi and DNA demethylating agents) have really entered the clinic represent a further complexity. The use of HDACi in cancer treatment suggests, for example, that histone acetylation can only be considered as information about the effects of treatment and not, as originally proposed, as response to treatment. Therefore, one choice might be the unbalanced presence of *writers/readers/erasers* (overexpression or silencing) or the mutation of these enzymes. Clearly, epi-enzymes mutated in cancer offer the stimulating option to synthesize selective molecules to modulate only the mutated enzyme, thus acquiring features of tumor-selective action. Interestingly, HDACs are more often quantitatively altered in cancer (excepted for HDAC2 mutation), whereas HATs are more frequently mutated. The implications of this apparent difference still need to be investigated. Moreover, discoveries progressively being

made in this field with newly identified chromatin marks and efforts for their mining may rapidly change our present view. Finally, whether selective or broader acting chromatin modulators should be chosen for epi-treatment in cancer is still under discussion and possibly may have more than one option. Of course, a broader modulator might have advantages in the case of multiple alterations of different modifiers and marks. Multiple epi-modulators (targeting more than one class of enzymes) may thus represent a promising approach in cancer. This last approach, which still needs to be fully validated, might use chromatin "re-modulation" in a more targeted manner.

Acknowledgments This work was supported by the EU: Blueprint (contract no 282510); Italian IHEC (Flag Project: EPIGEN); the Italian Association for Cancer Research (AIRC no 11812); Italian Ministry of University and Research (PRIN_2009PX2T2E_004); PON002782; PON0101227. We thank C. Fisher for editing the manuscript.

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Epigenetics and Epidemiology: Models of Study and Examples

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Abstract

Epidemiological studies have successfully identified several environmental causes of disease, but often these studies are limited by methodological problems (e.g. lack of sensitivity and specificity in exposure assessment; confounding). Proposed approaches to improve observational studies of environmental associations are Mendelian randomization and the meet-in-the-middle (MITM) approach. The latter uses signals from the growing field of -omics as putative intermediate biomarkers in the pathogenetic process that links exposure with disease. The first part of this approach consists in the association between exposure and disease. The next step consists in the study of the relationship between (biomarkers of) exposure and intermediate -omic biomarkers of early effect; thirdly, the relation between the disease outcome and intermediate -omic biomarkers is assessed. We propose that when an association is found in all three steps it is possible that there is a casual association. One of the associations that have been investigated extensively in the recent years but is not completely understood is that between environmental endocrine disruptors and breast cancer. Here we present an example of how the "meet-in-the-middle" approach can be used to address the role of endocrine disruptors, by reviewing the relevant literature.

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_14,

Keywords
Epigenetics · Epidemiology · Breast cancer · Endocrine disruptors · -Omic biomarkers · Meet-in-the-middle approach

Contents

1 Introduction

The evolution of living organisms is the expression of different and sometimes opposing forces. The main tension is between the need for stability and the need for change. The first is expressed, for example, by the great structural stability of DNA and of its coding system, conserved almost intact across species. However, without the ability to change organisms would not be able to adapt to changing environments and their continuous threats. Transposons (transposable elements), crossing-over at meiosis and epigenetic changes are some of the mechanisms that ensure the variety of genetic configurations that allow variation, adaptation and evolution.

As a very general rule, environmental stresses tend to increase (epi)genomic instability, and this is both a mechanism to enhance diversity and respond to threats and also a potentially harmful mechanism leading to disease.

Over the last two decades, much research has gone into the role of epigenetic changes in the development of cancer [10, 38]. The most broadly researched and studied epigenetic mechanism is DNA methylation [6]. First observed in the early 1980s in studies of carcinogenesis [10], DNA methylation mainly occurs at CpG dinucleotides, which are found grouped together in promoter regions of around 50 % of all human genes; the latter are also labelled CpG islands. DNA methylation involves the addition of a methyl group to a CpG dinucleotide [6]. Methylation of CpG islands can cause gene silencing. If this affects tumour suppressor genes, there may be the induction of cancer. Cancer cells have been seen, in fact, to have unusual patterns of methylation, including both hypermethylation of tumour suppressor genes and hypomethylation of proto-oncogenes or transposable elements [42].

Another epigenetic mechanism is dysregulation through histone modifications due to the phosphorylation, methylation or acetylation of histone tails [10], which are predominantly in charge of maintaining nucleosome structures [28, 36]. Disruption of normal histone modification mechanisms can cause changes in gene expression and has been observed in the development of some cancers [20, 28, 36]. For example, in vitro studies conducted on human cells by Jensen et al. [18], described alterations of histone H3 acetylation and changes in gene expression in genes linked to histones, due to exposure to the arsenicals AsIII and MMA. Likewise, in 2009 [55] found that arsenite exposure in human lung carcinoma cells led to increased methylation of H3K4 and caused repression of cellular transcription (we refer to arsenic as this is one of the environmental exposures best studied from the epigenetic perspective) [36].

A third main mechanism in which epigenetic changes occur is through micro-(mi)RNA expression, described as small non-coding RNA molecules of approximately 22 nucleotides. miRNAs are involved in genomic and cellular regulation and are thought to generally suppress gene activation. There are over 800 miRNAs in humans, and it is believed up to 30 % of genes in mammals are influenced by miRNAs [36]. Dysregulation of miRNA expression has been suggested to play a role in various diseases [1] such as arsenic-related kidney and bladder cancers [13].

It is believed that exposure to environmental carcinogens and the associated epigenetic changes such as the ones described above can occur in utero; epigenetic programming may play a major role in embryonic development and the health of an individual in the long term [6]. A study conducted by Martínez et al. in 2011 in rats showed that arsenic exposure during gestation resulted in altered patterns of DNA methylation of brain cells affecting memory, thus suggesting that as exposure in utero has long-standing effects on growth and development [31].

2 Breast Cancer, Endocrine Disruptors and -Omic Biomarkers: An Application of the *Meet-in-the-Middle* Approach

The identification of environmental causes of disease is limited by several methodological problems including, in particular, serious limitations in exposure assessment. One approach that has been proposed and extensively applied to improve observational studies of environmental associations is Mendelian randomization [7] in which the random assortment of genes from one generation to the following is used to address potential confounding. If a gene variant is associated with both exposure and disease, this lends credibility to the causal nature of the exposure–disease association, because in principle gene variants are inherited independently of environmental exposures, including confounders. In the meantime, recent years have seen the burgeoning of a number of high-throughput, powerful technologies for the investigation of biological molecules in body fluids

Table 1 -Omics technologies

-Omics	Example of platform	Characteristics
Transcriptomics	Human 4x44k DNA microarrays	Study of expression levels of mRNA molecules in a population of cells (gene expression profiling). Transcriptome is highly variable over time, between cell types and will change in response to environmental change
Proteomics [11]	Luminex multianalyte profiling system	Analysis of the total protein output encoded by the genome, mainly to identify proteins within the complex proteomic profile that discriminate between normal, benign, or disease status [8, 46]
Epigenomics [31]	High-density DNA microarrays	Study of epigenetic processes (mainly DNA methylation, histone modification but also RNA interference of gene expression by non-coding RNAs such as micro-RNA and siRNA) on a genome-wide scale
Metabolomics [13]	NMR spectroscopy or LC-mass spectrometry	Study of a complete set of low molecular weight metabolites that are present in a cell or organism at any given time in order to define individual metabolic profiles (and responses) that can be used to predict the onset of common diseases

or tissues, collectively called -omics. One way to assess the causal nature of environmental associations is what we call here the "*meet-in-the-middle*" approach, that is, to exploit the nature of -omic signals as putative intermediate markers in the pathogenetic process that links exposure with disease.

2.1 Biomarkers and -Omics Technology

New intermediate biomarkers can be identified on the basis of "-omics" technologies, such as transcriptomics or gene expression profiling, proteomics, epigenomics and metabolomics (Table 1). These intermediate biomarkers directly or indirectly report on events that lie on the continuum between exposure and disease.

Intermediate biomarkers can provide important mechanistic insight into the pathogenesis of environmental diseases. In contrast to traditional molecular biology, "-omics" technologies allow the use of the same methodology for the detection of cellular responses to different categories of chemicals and types of toxicity and provide mechanistic information on an exceptionally large scale. Previous results support the hypothesis that use of omics-based biomarkers in the context of the suggested "meet-in-the-middle" approach may facilitate the establishment of mechanistic links between environmental exposures and disease initiation and progression.

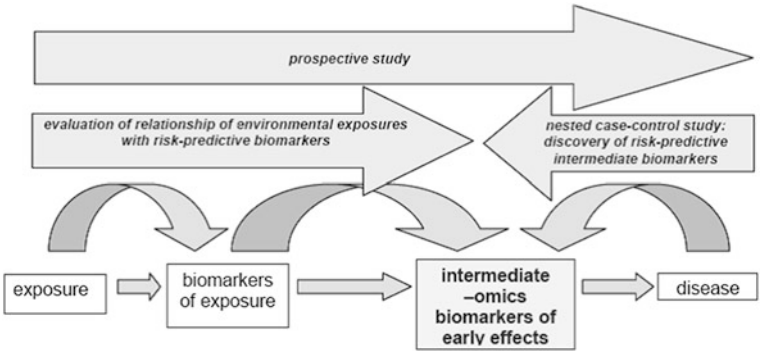

Fig. 1 "Meet-in-the-middle" approach

2.2 "Meet-in-the-Middle" Approach

We have recently described an innovative approach to tackle the challenge of identifying causal relationships which is known as the "*meet-in-the-middle*" (MITM) approach [13, 15]. This approach is based on a combination, within a prospective study, of (a) a prospective search for intermediate biomarkers which are elevated in subjects who eventually go on to develop disease and (b) a retrospective search for links of such biomarkers to past environmental exposures (Fig. 1).

The first part of this approach consists in the association between exposure and disease. The next step consists in the study of the relationship between (biomarkers of) exposure and intermediate -omic biomarkers of early effects; thirdly, the relationship between the disease outcome and intermediate -omic biomarkers is assessed. We propose that when an association is found in all three steps, it is possible that there is a causal association (in addition, of course, to the application of the well-known Hill's guidelines for causal assessment). Despite the similarity between Mendelian randomization and the MITM approach, an important difference is the lack of genetic instruments in the MITM approach which means that confounding is still a possibility.

One of the associations that have been investigated extensively in recent years but is still not completely understood is that between environmental endocrine disruptors and breast cancer. Here, we present an example of how the "meet-in-the-middle" approach can be used to address the case of breast cancer and endocrine disruptors, by reviewing the relevant literature.

2.3 Step 1: Endocrine Disrupting Chemicals and Breast Cancer

Many studies have focused on the association between exposure to Endocrine Disrupting Chemicals (EDCs) and breast cancer risk. EDCs are synthetic and

natural compounds in the environment that interfere with (i.e. mimic and/or antagonize) the actions of endogenous hormones by altering hormone synthesis, secretion, transport, binding, action or elimination and thereby disrupt the functions of the endocrine system. Exposure to EDCs can occur in many different ways. At times, it is intentional, for example in the case of medicinal use, but mostly it is accidental. Many EDCs are found in food, which can be the main source of exposure, for example in the case of BPA.

2.4 Step 2: Endocrine Disrupting Chemicals and Epigenomics

In this example, the -omics technology we have focused on was epigenomics. We searched PubMed for literature published up to August 2012, using the following search terms: "Endocrine Disruptors" OR "Bisphenol-A" OR "Diethylstilbestrol (DES)" OR "Cadmium" AND "epigenomics" OR "epigenetics" OR "DNA methylation" OR "histone modification" OR "micro-RNA".

We included 26 papers in our short review, together reporting 1,798 genes of which 11 genes were found in more than one paper. Below follows a description of the findings of these papers (overview in Table 2). Because of the relatively small number of reported miRNAs, there were none that were found by more than one study.

2.4.1 BPA

Exposure to bisphenol-A has been studied in animal models and has been associated with epigenetic changes which can pose health threats. Exposing mice perinatally to both high and low doses of BPA led to hypomethylation in the offspring [8, 52]; another study has shown an increased risk of prostate cancer in rats due to epigenetic alterations after BPA exposure [12, 34]. More recently, studies in human cell lines have been performed examining the association between bisphenol-A exposure and DNA methylation. Weng et al. compared three microarray datasets and found 170 genes that were identified in all three sets as differentially expressed after BPA exposure (57 up-regulated and 113 down-regulated). Moreover, hierarchical clustering indicated that the up-regulated genes were mainly found in ERα-negative breast cancer cell lines, while the down-regulated genes were mostly detected in ERα-positive breast cancer cell lines. Furthermore, DNA methylation analysis on the candidate gene *lysosomal-associated membrane protein 3* (*LAMP3*) showed increased DNA methylation levels in breast cancer cell lines compared to controls, primarily in tumours with a positive ERα-status [50]. Another study exposed human mammary epithelial cells (HMEC) to BPA and concluded that there was an increase in DNA methylation of 6 genes [35].

Avissar-Whiting et al. studied the expression of micro-RNAs in three placental cell lines after 6 days of exposure to BPA. After performing unsupervised hierarchical clustering, they reported different expression patterns for the different cell

Table 2 Genes found to be differentially methylated after EDC exposure and in relation to breast cancer

Exposure	Study, year	Number of significant associations found (n)	DYNC1LI2	ERLIN2	FGFR1	HN1L	IFNGR1	MOCS1	P16	PIM1	RUNX1T1	TAP2	VPS29
Exposure versus Epigenetics													
BPA	Dolinoy, 2007	1											
	Prins, 2008	8											
	Ho, 2008	8											
	Yaoi, 2008	13											
	Weng, 2010	170	X	X	X	X	X	X		X	X	X	
	Qin, 2012	6							X				
DES	Li, 1997, 2001, 2003	2											
	Sato, 2006	3											X
	Tang, 2008	14											X
	Sato, 2009	1											
	Bromer, 2009	1											
Cadmium	Benbrahim-Tallaa, 2007	2							X				
	Wang, 2011	1,574	X	X	X	X	X	X		X	X	X	
	Hossain, 2012	2											
Epigenetics versus Breast Cancer													

(continued)

Table 2 (continued)

Exposure	Study, year	Number of significant associations found (n)	DYNC1LI2	ERLIN2	FGFR1	HN1L	IFNGR1	MOCS1	P16	PIM1	RUNX1T1	TAP2	VPS29
	Lo, 2008	40							X				
	Jovanovic, 2010	43							X				
	Huang, 2010	186							X				
	Wang, 2010	14							X				
	Suijkerbuijk, 2010	19											

The top part of this table displays genes found to be differentially methylated after EDC (BPA, DES, cadmium) exposure. The lower part shows genes that have been reported to be associated with breast cancer in epigenetic studies. The goal of this table is a comparison of genes mentioned in both steps 2 and 3 of the MITM approaches

lines and a clear discrimination between cases and controls in two of the cell lines. There was an overlap of 21 mi-RNAs which were differentially expressed in both cell lines. Out of 4 of these annotated mi-RNAs only 1, miR-146a (up-regulated in both cell lines), was validated using RT-PCR [2]. Interestingly, Marsit et al. [30] reported up-regulation in the same mi-RNA after folate deficiency.

2.4.2 Diethylstilbestrol

Another well-known and extensively studied EDC is diethylstilbestrol (DES). The transgenerational effect after DES exposure that has been shown in epidemiological studies strongly indicates the involvement of epigenetic factors. Already in 1997, Li et al. showed a decrease in methylation of CpG sites in the lactoferrin gene after neonatal exposure of mice to DES and this hypomethylation was also detected in uterine tumours [26]. A few years later they reported hypomethylation of the exon-4 region of the c-fos gene in mice, after neonatal exposure to DES, but they could not show the same for the HOXA-10 and HOXA-11 gene [24, 25]. All the genes they studied had previously been reported to be differentially expressed in mice after DES exposure and are involved in the development of the reproductive organs [4, 29, 33, 51]. Bromer et al. [5] also studied the HOXA-10 gene in female offspring of DES exposed mice and reported an increase in DNA methylation after exposure in utero, but not in vitro or in vivo. Tang et al. [44] reported 14 genes (all but one of these genes were not reported in previous studies) with altered methylation levels after exposing mice to DES. Differential genome-wide DNA methylation [investigated by restriction landmark genomic scanning (RLGS)] was reported by Sato et al. in the uterus and epididymis of mice, after postnatal exposure to DES [39, 40].

Besides these DNA methylation studies, there are also a few studies which focused on mi-RNAs. Hsu et al. treated MDEC cells (mammosphere-derived epithelial cells) with DES and found 82 micro-RNAs with an altered expression in exposed cells versus control cells (of which 37 up-regulated and 45 down-regulated loci). After studying one of these (miR-9-3) in more detail, they found promoter hypermethylation, which might be associated with an increased risk of breast cancer [15].

MCF-7 cells were exposed to DES by Lee et al., and they reported a decreased expression of miR-34b [23]. Reduced expression of this micro-RNA has previously been associated with increased DNA methylation [45].

Finally, Warita et al. investigated the relationship between DES exposure and histone modification. They used chromatin immunoprecipitation (ChiP) and PCR to assess this association in TTE1 Leydig cells and reported reduced acetylation of histone H3 in the promoter of the *P450scc* gene [49].

2.4.3 Cadmium

Cadmium is the focus of many other studies investigating the role of EDCs in relation to epigenetic changes. Several animal studies concerning this exposure have been performed as well as studies using animal cell lines. Takiguchi et al.

reported hypomethylation after exposing TRL1215 cells (rat liver) to a dose of 2.5 μM of cadmium for 1 week, but hypermethylation after exposure for 10 weeks (after which the cells are probably transformed) [43]. Another study showed that both a low (140 mg/kg $CdCl_2$) and high (210 mg/kg $CdCl_2$) dose of cadmium for 60 days increased global methylation in both the liver and kidney of hens [53]. Zhu et al. used COBRA to assess LINE-1 methylation in the liver and testis of rats but found no difference between exposed and non-exposed rats after 8 days of post-natal exposure, nor after 70 days. Also, no difference was detected in the methylation level of the p53 promoter in the testis after cadmium exposure. However, cadmium exposure did seem to slow down the naturally occurring hypomethylation of part of the c-fos gene in the testis [56].

An epigenome-wide study was performed by Wang et al. in rat liver, assessing the methylation level of 385,000 probes after low dose, chronic cadmium exposure (compared to no exposure), using the MeDIP-Chip assay. They reported hypermethylation in the promoter region of 675 genes and hypomethylation in the promoter region of 899 genes. Looking at gene-specific methylation of genes regulating the apoptotic process, they found hypermethylation of CASP8 (resulting in decreased expression) and hypomethylation of TNF (also resulting in decreased expression) [47].

Studies involving human cells or cell lines include the study by Benbrahim-Tallaa et al. who reported global DNA hypermethylation after exposing human prostate epithelial cells to cadmium. When they investigated methylation levels of CpG islands specifically in the promoter region of 2 tumour suppressor genes (RASSF1A and p16), they found hypermethylation compared to non-exposed cells, which led to reduced expression [3]. Global hypermethylation was found by a study in which human embryo lung fibroblast (HLF) cells were long term exposed to cadmium. They plotted a dose-response relationship between increasing exposure and increasing methylation but only concentrations of 1.2 and 1.5 μmol/L led to a statistically significant increase compared to non-exposed cells [19]. Huang et al. [16] described a reduction in global DNA methylation of K562 cells (chronic myelogenous leukaemia cell line) after exposure to 2.0 μM of cadmium during 24 and 48 h.

The only study that investigated epigenetic effects of dietary cadmium exposure in humans was performed by Hossain et al. Cadmium levels were measured in blood (reflecting short-term exposure) and urine (reflecting long-term exposure) samples and peripheral blood was drawn to assess LINE-1 methylation levels, as well as methylation of the promoters of *p16* and *MLH1*. Higher levels of cadmium in urine were associated with hypomethylation of LINE-1 but no association was found between cadmium levels in blood and LINE-1 methylation, nor between cadmium concentrations in urine and blood and *p16* or *MLH1* methylation [14].

Changes in micro-RNA expression after 10 μM cadmium exposure were measured in human hepatoblastoma cells (HepG2) cells by Fabbri et al. They listed 12 miRNAs of which several are believed to play a role in tumour suppression [9].

2.4.4 Replicated Associations

When comparing the genes found in the studies described above, 11 genes were reported in two independent studies. These genes will be compared to the genes found in studies investigating the association between epigenomics and breast cancer, described in the next section, in an attempt to apply the "meet-in-the-middle" approach.

2.5 Step 3: Epigenomics and Breast Cancer

The final step in the "meet-in-the-middle" approach describes the association between -omics technology and breast cancer. It is of vital importance that new biomarkers are found, not only for the detection of breast cancer, but also for typing and treatment. Breast cancer has been among the earliest and most intensely studied diseases using epigenetic as well as other -omics technologies. Many papers have been published on these techniques in relation to breast cancer, and therefore, we have restricted this report to recent reviews on epigenetics.

In 2008, Lo et al. performed a literature search for studies reporting epigenetic changes, including DNA methylation, in breast cancer [27]. They described 40 genes to be hypermethylated in subjects with this disease, of which one was also found to be differentially methylated after exposure to EDCs. Both Jovanovic et al. [21] and Huang et al. [17] have recently written extensive reviews of papers studying the epigenetics of breast cancer. They have both come up with a list of hypermethylated and hypomethylated genes in human breast cancer cells. Jovanovic et al. [21] listed a selection of the 43 most frequently differentially methylated genes in breast cancer: 6 hypomethylated and 37 hypermethylated genes. One was also mentioned by the exposure and -omic studies above. Huang et al. reports many more genes commonly hyper- or hypomethylated in breast cancer, 186 in total. Of this long list, also one gene was reported by at least two studies as being differentially expressed after EDC exposure [17]. Finally, Wang et al. as well as Suijkerbuijk et al. reviewed the possibility of using the methylation status of certain genes as a biomarker for breast cancer. They reported 14 and 19 genes, respectively, of which 1 and 0 genes are also described in 2 studies as genes that undergo epigenetic changes after exposure to EDCs [41, 48]. Table 2 gives an overview of this. After comparing the genes found in epigenomics versus breast cancer studies to the 11 "validated" genes reported by two independent exposure versus -omics studies, one gene survives and its function will be shortly described below.

2.5.1 P16

P16 is the prototype of the gene that is called cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4). It has several different aliases, of which p16INK4a and CDKN2A are the most commonly used. Its location on the human genome is 9p21 and, besides other functions, it plays an important role in the cell cycle arrest and apoptosis. The gene is known as a tumour suppressor gene because

it is often mutated and deleted in several types of cancer. It has been shown that the promoter region is often hypermethylation in cancer, leading to reduced expression and silencing of this gene [37].

3 Discussion

The aim of much current research on breast cancer and environmental exposures is to identify intermediate biomarkers based on -omic technologies. Identification of these biomarkers is attempted by studying three possible associations: (1) the association between exposure and disease, (2) the association between exposure and intermediate -omic biomarkers of early effects, and (3) the association between the disease outcome and intermediate -omic biomarkers. When an association is found in all three steps, it is possible that there is a causal association and a new biomarker can be identified. It is also still possible, however, that there is no true causal relationship.

A major aim of biomarker validation is to characterize biomarker variability. The main components of biomarker variability that affect the design and interpretation of epidemiologic studies are as follows: (1) biological variability related to the subject (including inter-subject and intra-subject variability), (2) variability due to measurement error (including inter-laboratory and intra-laboratory variability), and (3) random error. Therefore, in epidemiologic studies using biomarkers, it is important to collect, whenever possible, (1) repeat samples (day-to-day, month-to-month, or year-to-year variation may be relevant depending on the marker), (2) information on potential confounders (factors influencing inter-subject variation), (3) information on conditions under which samples have been collected and laboratory analyses have been conducted (batch, assay, and specific procedures) [46].

In the present literature review, one gene (p16) has been reported to be differentially methylated after exposure to EDC and it has also been found to be differentially methylated in breast cancer cells and tissue. Exposure to EDC largely occurs through diet. One problem, however, is that this gene is reported in different studies and none of these studies have assessed all three steps of the MITM approach. Therefore, it is difficult to characterize biomarker variability. Nevertheless, the fact that this gene has been found in all three steps increases the plausibility of a causal link between EDC exposure and breast cancer risk.

One of the main challenges with (potential) intermediate biomarkers is to understand whether they belong to the causal pathway between exposure and disease, whether they are simply a side effect of exposure or disease, or whether their measurement is confounded by some other exposure. A way to establish that levels of EDCs in the blood can contribute to breast cancer risk independently of confounding by other risk factors would be to show that the levels of EDCs are associated with different genotypes or polymorphisms and that such genotypes or polymorphisms also predict disease (concept of *Mendelian randomization*). For

example, three studies found higher breast cancer risk associated with higher PCB exposures among postmenopausal white women with a polymorphism in the *CYP1A1* gene [22, 32, 54]). Because the different alleles of the *CYP1A1* gene are independent of confounding factors, since they are assorted randomly from one generation to the next, the finding that a polymorphism in the *CYP1A1* gene was associated with breast cancer would provide indirect proof of a genuine involvement of PCBs in the aetiology of the disease. However, many other chemicals are metabolized by CYP1A1.

It becomes clear that both the concept of Mendelian randomization as well as the "meet-in-the-middle" approach have great potential in identifying (intermediate) predictive biomarkers of disease but several challenges remain with the practical implications of these models.

Acknowledgments This work has been made possible by a grant from Compagnia di San Paolo to PV and KVV.

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Dietary Epigenetics in Cancer and Aging

Trygve O. Tollefsbol

Abstract

Although epigenetic aberrations frequently occur in aging and cancer and form a core component of these conditions, perhaps the most useful aspect of epigenetic processes is that they are readily reversible. Unlike genetic effects that also play a role in cancer and aging, epigenetic aberrations can be relatively easily corrected. One of the most widespread approaches to the epigenetic alterations in cancer and aging is dietary control. This can be achieved not only through the quality of the diet, but also through the quantity of calories that are consumed. Many phytochemicals such as sulforaphane from cruciferous vegetables and green tea have anticancer epigenetic effects and are also efficacious for preventing or treating the epigenetic aberrations of other age-associated diseases besides cancer. Likewise, the quantity of calories that are consumed has proven to be advantageous in preventing cancer and extending the lifespan through control of epigenetic mediators. The purpose of this chapter is to review some of the most recent advances in the epigenetics of cancer and aging and to provide insights into advances being made with respect to dietary intervention into these biological processes that have vast health implications and high translational potential.

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_15,

Keywords
Nutrition · Cancer · Epigenetic · Dietary · Aging

Abbreviations

CR	Caloric restriction
DNMT	DNA methyltransferases
EGCG	(-)-epigallocatechin-3-gallate
HAT	Histone methyltransferases
HDAC	Histone deacetylase
hTERT	Human telomerase reverse transcriptase
miRNA	microRNA
SAM	S-adenosylmethionine
SFN	Sulforaphane
siRNA	Short-interfering RNA
SIRT1	Sirtuin 1

Contents

1 Introduction

Although there are many different variations of the definition of epigenetics, perhaps the most widespread is that epigenetic processes involve changes that are heritable but are not encoded with the DNA sequence itself. There are numerous types of epigenetic mechanisms, and the three most important in mammals include changes in DNA methylation, histone modifications, and non-coding RNAs.

DNA methylation is the most studied of the epigenetic processes and is based on the addition of a methyl moiety (CH_3) donated enzymatically from S-adenosylmethionine (SAM) to the 5-position of cytosine, primarily occurring in CpG dinucleotides. This is carried out by three major methyltransferases (DNMT1, DNMT3A, and DNMT3B) in mammalian systems. DNMT1 is responsible for

maintaining the methylation pattern that is largely preserved with each mitotic division, and DNMT3A and DNMT3B are more involved with *de novo* methylation, the creation of new methylated cytosines (5-methylcytosines) at cytosine that were not previously methylated. In general, the more methylated a gene regulatory region becomes, the less transcription will occur from the promoter although there are notable exceptions to this dogma as occurs with the gene that encodes human telomerase reverse transcriptase (*hTERT*), the key regulatory gene of telomerase [11, 44].

Epigenetic changes are also mediated by histone modifications. Although histone acetylation and methylation are the most studied of these modifications, others also occur such as histone phosphorylation, ubiquitination, biotinylation, sumoylation, and ADP-ribosylation. The number of enzymes that carry out histone modifications is large relative to those that mediate DNA methylation and the two that often attract interest, especially with regard to cancer and aging, are the histone acetyltransferases (HATs) and the histone deactylases (HDACs) [26]. In general, the more acetylated the histone amino tails become, the more likely it is that the gene promoter region that contains those histones will have increased transcriptional activity [7].

Non-coding RNA, the third major type of epigenetic control in mammalian systems, is also important in gene expression. For example, microRNA (miRNA) consists of single-stranded non-coding RNAs that are usually about 21–23 nucleotides in length. These sequences suppress gene expression by altering the stability of gene transcripts and also by targeting the transcripts for degradation although miRNA may also lead to an increase in gene transcription [36]. Many miRNAs have now been identified and may regulate a large percentage of genes in mammals [14].

2 Cancer Epigenetics and Dietary Intervention

Environmental factors such as the diet are well known to influence gene expression and to contribute to cancer through epigenetic mechanisms [20]. To illustrate the importance of this, it has been reported that over half of the gene defects that occur in cancer are through epigenetic alterations when compared to genetic mutations [23]. General DNA hypomethylation not uncommonly occurs in cancer cells, while gene-specific hypermethylation often leads to inactivation of key genes such as the tumor suppressors [3]. It is thought that these changes in DNA methylation play an early role in cancer genesis and lead to aberrations in cellular proliferation as well as immortalization of previously normal cells. Changes in histone modifications are also prevalent in cancer cells. For example, increased activity of the HDACs is a very common feature of cancer cells and can lead to tumorigenesis through effects on epigenetic gene expression [13]. Alterations in miRNA are also common in cancer, and this occurs in many cases through defects in the expression of genes that play a role in cancer initiation or progression [14]. Perhaps most

importantly, a growing interest has been the collective interactions of epigenetic processes in the control of gene regulation aberrations in cancer. For instance, it is not uncommon for epigenetic mechanisms to act in concert which lead to alterations in gene expression in cancer cells [15].

2.1 The Epigenetics Diet in Cancer Prevention and Treatment

Many natural dietary agents which consist of bioactive compounds have been shown to be effective in cancer prevention and treatment, and these nutraceuticals often mediate favorable epigenetic changes [38, 50]. In fact, we have suggested an "epigenetics diet" that impacts the epigenome and that may be used in conjunction with other cancer prevention and chemotherapeutic strategies [18].

One of the foremost compounds that have been shown in a vast number of studies to have anticancer properties is (-)-epigallocatechin-3-gallate (EGCG) of green tea. Many studies have shown a positive connection between green tea (EGCG) consumption and the inhibition of numerous cancers [6, 25, 48]. Although EGCG has varied effects on cancer cells such as antioxidant properties, it has also been shown to have important epigenetic effects in that it can inhibit DNMT

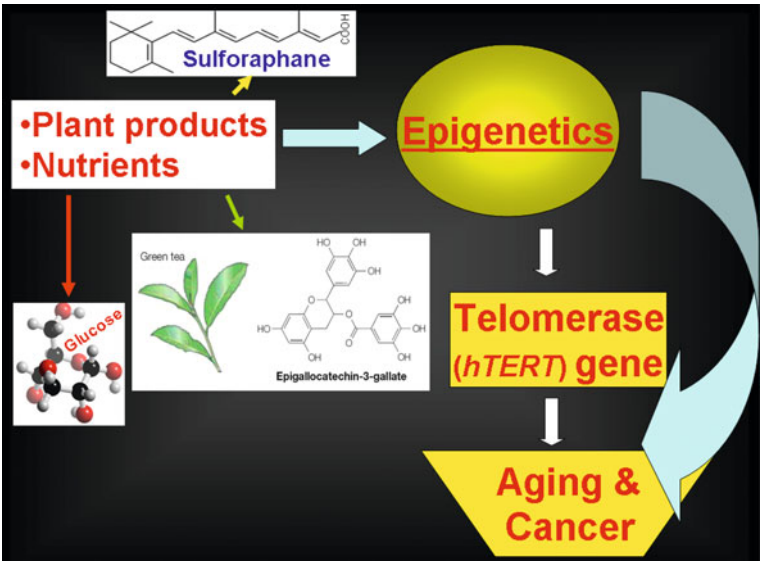

Fig. 1 Effects of components of the epigenetics diet on epigenetics, telomerase (hTERT), and aging and cancer. Plant products such as sulforaphane from cruciferous vegetables and (-)-epigallocatechin-3-gallate (EGCG) (structures shown) modify epigenetic processes that can have a direct impact on aging and cancer. They also lead to the down-regulation of *hTERT* which is central to both aging and cancer. The mechanisms for epigenetic modifications of the phytochemicals can vary depending on the particular compound. Glucose restriction also can impact epigenetic processes and affect aging and cancer

activity by directly interacting with the DNMTs [16]. This can effectively lead to the reversal of tumor suppressor epigenetic silencing of cancer cells and induce apoptosis of these cells. Further, we have found that the inhibition of DNMTs by EGCG can lead to the suppression of telomerase in cancer cells by down-regulating *hTERT* [4, 41]. *hTERT* activity in cancer cells is associated with increased DNA methylation of its gene regulatory region due to repressors binding to its promoter [4]. Since telomerase is central to tumor progression, this EGCG-mediated down-regulation of telomerase activity may have major implications in cancer prevention and treatment (Fig. 1).

Another key dietary component that has epigenetic effects is sulforaphane (SFN) of cruciferous vegetables such as broccoli, cauliflower, Brussels sprouts, cabbage, and kale. However, although we have shown that SFN can inhibit DNMT1 and DNMT3A [39], its most powerful effects are through inhibition of HDAC activity [12, 42, 43, 47]. As aforementioned, HDACs are often increased in cancer cells and inhibition of HDAC activity by SFN may have considerable potential in preventing HDAC increases in cancer cells. Since *hTERT* is also controlled though histone acetylation/deacetylation [11], we tested whether SFN may have an impact on *hTERT* gene regulation in breast cancer cells. We found that SFN treatment of MCF-7 and MDA-MB-231 cells leads to a dose- and time-dependent down-regulation of *hTERT* (Fig. 1). The SFN-induced hyperacetylation facilitated the binding of many hTERT repressor proteins such as MAD1 and CTCF to the *hTERT* gene regulatory region [39]. We also found that depletion of CTCF using siRNA attenuated the SFN-induced *hTERT* down-regulation in breast cancer cells [39].

Although dietary compounds such as EGCG in green tea and SFN in cruciferous vegetables have many other effects, it is clear that these compounds can affect the epigenetic control of key genes and greatly influence the initiation and progression of cancer [38]. In addition to EGCG and SFN, a number of other dietary compounds are also quite effective in cancer prevention. For instance, curcumin (tumeric), resveratrol (grapes and red wine), and genistein (soybeans) as well as many other phytochemicals have created considerable excitement for their epigenetic potential [38]. We feel that a diet consisting of epigenetic-modifying foods and beverages could have a major impact on the incidence of cancer worldwide and have therefore encouraged the epigenetic diet as a means to not only prevent cancers, but also perhaps treat many early stage cancers [18]. It is also highly feasible that a combination of these phytochemicals in the diet may show synergistic effects to further reduce the incidence of cancer [40].

3 Aging Epigenetics: The Impact of Dietary Factors

The single most important risk factor for developing cancer is age, and therefore, many investigations have explored the role of epigenetics in both cancer and aging with the intention that there may be links between these two important biological

processes. In fact, as with cancer, aging is associated with general genomic hypomethylation and regional or gene-specific hypermethylation [22, 37] which may be due to changes in the expression of the DNMTs [5, 34]. These modulations in DNA methylation during aging likely contribute to a number of changes in the regulation of epigenetically controlled genes such as *hTERT* to contribute to the aging process [33]. Moreover, histone modifications appear to play a major role in aging. In fact, sirtuin 1 (SIRT1) is a class III NAD^+-dependent HDACs that has shown remarkable effects on aging through increasing the lifespan of a diverse range of animal models [8, 24]. The SIRT1 enzyme appears to be an important nutrient sensor linked to metabolic rate. The redox potential, simplified as the NAD/NADH ratio, may be important in regulating SIRT1 activity which is a key indicator for oxygen consumption and the respiratory chain. Although many other epigenetic changes occur during the aging process, the regulation of DNA methylation and histone modifications as well as epigenetic control of *hTERT* and the role of SIRT1 in modulating aging biological processes have captured considerable interest.

3.1 Nutrient Quantities, Epigenetics and Aging

As aforementioned, not only is the quality of the diet an important factor, but the quantity of nutrients consumed is also a major player in cancer and aging. Caloric restriction (CR) is the most effective intervention into the aging process and maximum lifespan, and it is mediated in part by epigenetic mechanisms [49, 53]. The restriction of total calories by 25–60 % relative to normally fed controls while providing essential nutrients can lead to a 50 % increase in lifespan [9, 10, 21, 31, 45, 52]. DNA methylation may be altered in response to CR through its effects on specific gene loci leading to increased longevity [19]. Moreover, SIRT1, an important HDAC in the aging process, is strongly linked to CR. For example, many studies have shown that its activity is affected by CR both in vitro and in vivo [8, 17, 27, 32]. The longevity–extension effects of sirtuin were originally discovered in yeast [17], and activation of SIRT1 is often observed in various tissues of animals subjected to CR, while inactivation of SIRT1 may lead to ablation of the lifespan-extending effects of CR. It is therefore apparent that epigenetic processes are not only central to the aging process, but also that they are involved with key mediators of aging such as DNA methylation and SIRT1.

3.2 The Epigenetics Diet and Aging

Resveratrol, a dietary polyphenol phytochemical, is an important mediator of CR and acts as a SIRT1 mimic that leads to increased longevity both in vitro and in vivo [1, 2, 51, 54]. Besides resveratrol, many other polyphenols such as EGCG have been shown to have beneficial effects on the aging process [46]. Other

epigenetic diet components such as cruciferous vegetables and soybeans may also have advantageous effects on longevity through their cancer preventive properties [28, 31, 38]. For instance, consumption of components of the epigenetics diet over a period of time may lead to a decrease in age-associated diseases such as cancer and cardiovascular disease [35].

3.3 Glucose Restriction and Extension of the Hayflick Limit

The Hayflick limit refers to the cellular senescence process that is considered to be fundamental to biological aging. Since studies of CR have been confined to analysis of the organism longevity (including single-cell yeast), we sought to test whether CR could affect the Hayflick limit in mammalian systems. Moreover, the effects of CR on human aging have not yet been resolved largely because of the impracticality of CR studies using relatively long-lived human populations. Since cellular senescence is considered a fundamental basis of aging, we restricted glucose in cultures of human fibroblast cells and monitored the Hayflick limit of the cells [29]. The human cells that received only about 15 mg/L of glucose had a significantly reduced lifespan when compared to control human cells that received the normal level of 4.5 g/L of glucose in culture. We further noted that epigenetic alterations occurred in these cells in response to glucose restriction that led to a relatively modest increase (compared to cancer cells) in *hTERT* and a decrease in *p16*, a gene which slows cellular replication. We found that the epigenetic changes in these cells in response to CR were at least in part due to changes in DNA methylation and histone modifications in these cells (Fig. 2). Further, precancerous cells were found to have the opposite effects on *hTERT* and *p16* in response to glucose restriction and to lead to epigenetic-induced apoptosis of the cells [29] (Fig. 2).

In additional investigations, our studies have indicated that SIRT1 becomes elevated in the glucose-restricted human cells that lead to lifespan extension through epigenetic effects of SIRT1 on *p16* as well as genetic effects of SIRT1 on *p16* through the Akt/p70S6K1 pathway [30]. Therefore, CR is effective in extending the lifespan of not only animals, but also their individual cells. Since the Hayflick limit is a basic aspect of aging and there were no metabolic systemic factors involved in these studies, this suggests that CR works primarily at the cellular senescence level and that an important mediator of cellular senescence and aging is changes in epigenetic mechanisms in response to CR.

4 Conclusions

Epigenetic mechanisms are central to many aspects of both aging and cancer, and dietary factors are important means of alleviating many of the adverse effects of these biological processes. Both the quality and the quantity of the diet are crucial

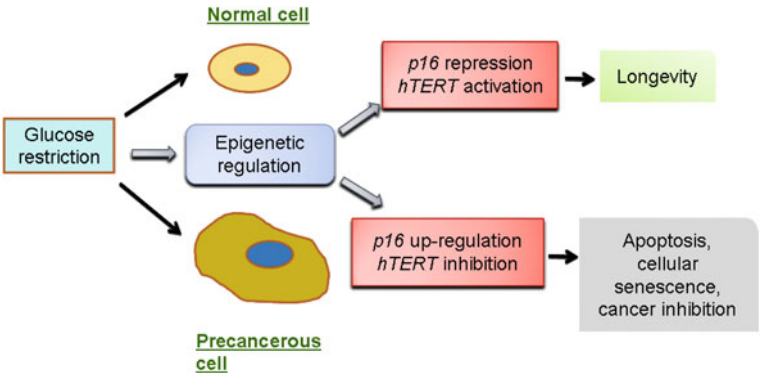

Fig. 2 Effects of glucose restriction on longevity and cancer inhibition through epigenetic regulation. Glucose restriction can impact epigenetic regulation in both normal and cancer cells. In normal cells, it leads to *p16* repression and *hTERT* activation to extend the Hayflick limit. In precancerous cells, the opposite effects on *p16* and *hTERT* lead to apoptosis, cellular senescence, and cancer inhibition of the glucose-restricted cells

in healthy aging and also significantly impact cancer. The quality of the diet is illustrated though the epigenetics diet consisting of consumption of phytochemicals that modulate epigenetic processes such as DNA methylation, histone modifications, and non-coding RNA. Substantial data that have been accumulated worldwide clearly show that the epigenetics diet has considerable potential in not only preventing cancer, but also reducing the incidence of age-related diseases. The quantity of the diet also has epigenetic effects in that reduction in calories impacts many epigenetic mechanisms such as the activity of SIRT1 which is an epigenetic mediator of a number of cellular processes. We have shown that CR at the cellular level can extend the lifespan of human cells and is likely a fundamental basis of the life-extending process of the epigenetic effects of CR. Many questions remain regarding the role of epigenetics in cancer and aging, but it is now clear that epigenetic mechanisms are basic aberrations in both cancer and aging and that the epigenetics diet is moving to the forefront of cancer and aging research as a safe and efficacious means to reduce the morbidity and mortality of these biological processes that claim so many human lives.

Acknowledgments This work was supported in part by grants from the National Cancer Institute (RO1 CA129415), the American Institute for Cancer Research, and the Norma Livingston Foundation.

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Breast Cancer and the Importance of Early Life Nutrition

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Abstract

Epigenetic processes play a central role in regulating the tissue-specific expression of genes. Alterations in these processes can lead to profound changes in phenotype and have been implicated in the pathogenesis of many human diseases including human cancer. There is growing evidence that the environment, particularly variations in diet, during specific developmental periods can induce changes in the epigenome, which are then stably maintained throughout life influencing susceptibility to cancer in later life. This chapter will review the evidence that alterations in early life nutritional exposure can affect breast cancer risk through the altered epigenetic regulation of genes and discuss how detection of such altered epigenetic marks in early life may provide biomarkers to detect individuals at increased risk of disease.

Keywords

Epigenetics · Nutrition · Transcription · Early life

Abbreviations

A^{vy}	Agouti viable yellow
AOX	Acetyl-CoA carboxylase

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_16,

ATM	Ataxia telangiectasia mutated
BMI	Body mass index
BRCA1	Breast cancer 1, early onset
BW	Birth weight
CVD	Cardiovascular disease
CpG	Cytosine and guanine nucleotides linked by phosphate
DES	Diethylstilbesterol
DHA	Docosahexaenoic acid
DNMT	DNA methyltransferase
ER	Estrogen receptor
EWAS	Epigenome-wide association studies
GR	Glucocorticoid receptor
HDAC	Histone deacetylase
HMT	Histone methyltransferase
IAP	Intracisternal A-particle
IGF1	Insulin growth factor 1
MeCP	Methyl CpG binding protein
MeDIP	Methylated DNA immunoprecipitation
miRNA	MicroRNA
MUFA	Monounsaturated fatty acid
ncRNA	Non-coding RNA
NCD	Non-communicable diseases
NMU	N-nitroso-N-methylurea
POMC	Pro-opiomelanocortin
PR	Protein restricted
PPAR	Peroxisomal proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
RXRα	Retinoid X receptor-α
TEB	Terminal end buds

Contents

1 Introduction

Traditionally, it has been widely accepted that cancer is caused by genetic alterations, such as mutations or deletions to our DNA. However, there is increasing evidence that some cancers including breast cancer may originate in part in utero. Human epidemiological studies have shown that early life environment plays a key role in the development of breast cancer. These findings have been replicated in a variety of animal models where both under- and overnutrition have been shown to influence the risk of cancer susceptibility in the offspring. The mechanism by which early life environment can induce distinct, stable phenotypes is beginning to be understood and may involve epigenetic processes. Recent studies have shown that a number of environmental factors in early life can induce changes in the epigenome, which are then stably maintained throughout life, suggesting that early life nutrition may modulate breast cancer risk by inducing persistent epigenetic changes that alter mammary gland development or structure decades before the onset of disease. This article will review the evidence that alterations in early life nutritional exposure can affect breast cancer risk through the altered epigenetic regulation of genes and discuss how detection of such altered epigenetic marks in early life may provide biomarkers to detect individuals at increased risk of disease.

1.1 Early Life Environment and Future Disease Risk

The association between the quality of the early life environment and subsequent risk of chronic disease in later life was first described by David Barker and colleagues who found a strong geographical relationship in the UK between the rates of infant mortality and risk of CVD 50–60 years later [1]. Numerous retrospective studies have subsequently shown that low birth weight is associated with an increased risk of a range of non-communicable diseases (NCDs) including CVD, type 2 diabetes, obesity, and hypertension in later life [2]. These findings have now been replicated in a number of animal models where in general pregnant rats or mice have been fed either, a globally restricted, low-protein or high-fat diet during pregnancy and/or lactation. Interestingly, the offspring born to such dams develop with very similar features, developing dyslipidemia, obesity, hypertension, hyperinsulinemia, and hyperleptinemia in later life [3]. The induction of different phenotypes by perturbations in early life nutrition is thought to be part of a normal adaptive mechanism whereby the organism acting through the process of developmental plasticity can adjust its developmental program in response to

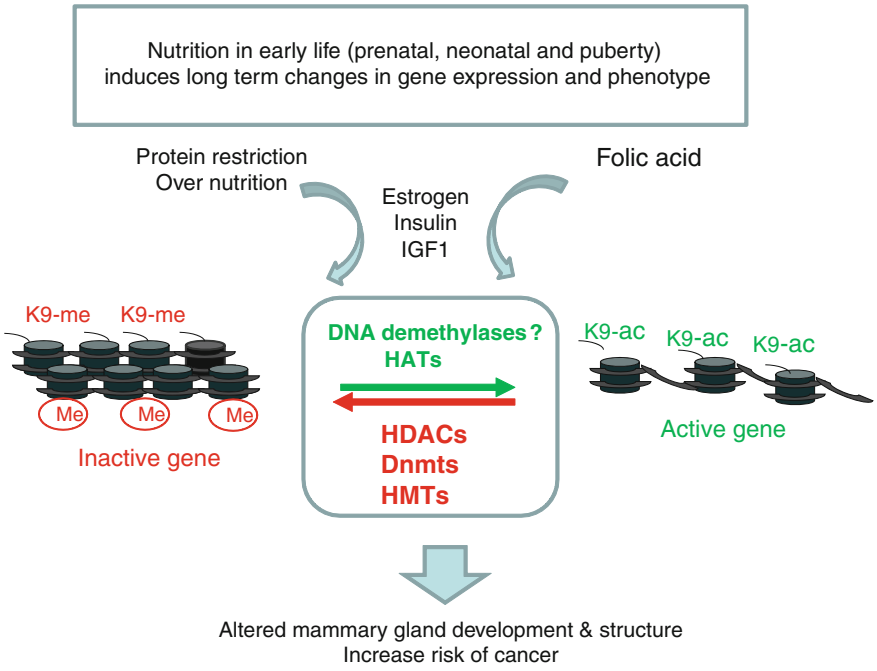

Fig. 1 Effect of early life nutrition on the epigenome. Nutrition in early life determines the balance between gene methylation and demethylation. Histone deacetylases (HDACs), histone methyltransferases (HMTs), and DNA methyltransferases (DNMTs) promote histone deacetylation, the methylation of K9 on histone H3, and DNA methylation resulting in a closed chromatin configuration and gene silencing. In contrast, histone acetyl transferases (HATs) promote the acetylation of lysine residues within the histone tails including acetylation of H3K9, resulting in an open chromatin configuration and gene transcription

environmental cues to aid fitness/survival in later life [4]. There is now evidence that the mechanism by which an organism can produce different phenotypes from a single genome in response to the environment is through the altered epigenetic regulation of genes.

2 Epigenetics

Epigenetic processes are integral in determining when and where specific genes are expressed. Alterations in the epigenetic regulation of genes can lead therefore to profound changes in phenotype [5, 6]. The major epigenetic processes are DNA methylation, histone modification, and non-coding RNAs (Fig. 1).

2.1 DNA Methylation

Methylation at the 5 position of cytosine in DNA within a CpG dinucleotide (the p denotes the intervening phosphate group) is a common modification in mammalian genomes and constitutes a stable epigenetic mark that is transmitted through DNA replication and cell division [7]. CpG dinucleotides are not randomly distributed throughout the genome but are clustered at the 5′ ends of genes/promoters in regions known as CpG islands. Hypermethylation of these CpG islands is associated with transcriptional repression, while hypomethylation of CpG islands is associated with transcriptional activation [8, 9].

DNA methylation is important for asymmetrical silencing of imprinted genes [10], X chromosome inactivation [11, 12], and for cell specification and tissue-specific gene expression [7]. Methylation of CpGs is largely established during embryogenesis or in early postnatal life. Following fertilization, maternal and paternal genomes undergo extensive demethylation followed by global methylation *de novo* just prior to blastocyst implantation [13, 14] during which the majority of CpGs are methylated, mainly in repressive heterochromatin regions and in repetitive sequences such as retrotransposable elements [15]. Lineage-specific methylation of tissue-specific genes also occurs throughout prenatal development and early postnatal life and determines the developmental fates of differentiating cells. The methylation of CpG dinucleotides *de novo* is catalyzed by DNA methyltransferases (DNMT) 3a and 3b, and is maintained through mitosis by gene-specific methylation of hemi-methylated DNA by DNMT1 [16].

2.2 Histone Modification

The DNA in our cells is packaged as chromatin. The basic unit of chromatin is a nucleosome which comprises 147 bp of DNA wrapped around a core of histone proteins (two copies of histone H2A, H2B, H3, and H4). Histone proteins contain two domains: a globular domain and an N-terminal tail domain. The N-terminal tails of the histones are subject to modifications including acetylation, methylation, ubiquitination, sumoylation, and phosphorylation [17]. Histone modification leads to the recruitment of effector proteins which in turn bring about specific cellular processes. The establishment of these marks on the histone tails is often referred to as the histone code. Histone acetylation is exclusively associated with active chromatin states, while the methylation of lysine can be either an active or repressive mark depending on the specific lysine involved [17]. Many families of histone-modifying enzymes have been identified, the so-called writers of the code include the histone acetyl transferases and methyltransferases, while the erasers include the deacetylases and demethylases [18, 19].

Cross talk between DNA methylation and histone modification clearly occurs. Methylated DNA is bound by methyl CpG binding protein-2 (MeCP2) which can recruit both histone deacetylases (HDACs), which remove acetyl groups from the

histones, a signal of transcriptionally active chromatin and histone methyltransferases (HMTs) such as SUV39H1 [20] which methylates lysine 9 on H3, resulting in a closed chromatin structure and transcriptional silencing. Recent studies have, however, shown that DNMT1 is recruited by a number of histone-modifying enzymes, such as HDAC1 and HDAC2, and the histone methyltransferases SUV39H1 and EZH2 [21, 22], suggesting that chromatin structure may also determine DNA methylation status and that there is a reciprocal relationship between these two processes.

2.3 Non-Coding RNAs

Non-coding RNAs (ncRNAs) have also been implicated in the epigenetic regulation of gene expression. ncRNAs can act either in cis or in trans. The *cis*-acting ncRNAs are the long/macro-ncRNAs (up to 100,000 nt), while the trans-acting ncRNAs include the microRNAs (miRNAs), which mostly target the 3′ untranslated region of mRNAs for degradation [23]. However, more recent studies have shown that the human miRNAs can also induce chromatin remodeling [24, 25], suggesting that DNA methylation, histone modification, and miRNAs may work in concert with each other to regulate gene expression.

2.4 Epigenetics, Aging, and Cancer

Epigenetic marks induced during development largely persist into adulthood. However, aging is associated with tissue-specific epigenetic drift, decreased activity of DNMT1, and global hypomethylation of the genome which leads to the activation of proto-oncogenes such as c-Myc and cN-ras [26]. However, hypermethylation of tumor suppressor gene promoters also occurs in aging [27]. Altered epigenetic regulation has been linked to the development of a number of cancers including breast, lung, prostate, and hematopoietic tissue [28–32]. For instance in sporadic breast cancer, Oct-4 and Sox2, which are normally exclusively expressed in stem cells where they play an essential role in maintaining pluripotency, are hypomethylated and highly expressed [33]. In contrast, the tumor suppressor gene BRCA1, which plays a pivotal role in DNA repair and which is mutated in inherited forms of breast cancer, is frequently hypermethylated and this is associated with poor prognosis and estrogen receptor (ER) negative tumors [34, 35]. These findings show that aging is not simply associated with a progressive decline in capacity to maintain DNA methylation but also involves selective dysregulation of epigenetic processes. The mechanism underlying such targeted hypermethylation is unclear. Spreading of methylation from heterochromatin to transcriptionally active regions of the genome has been suggested, although this does not explain why only certain DNA sequences are affected. However, one implication is that the level of methylation induced in early life sets the epigenetic background upon

which changes associated with aging operate and so variation in the epigenome induced during development may influence susceptibility to disease in later life [36].

2.5 Early Life Nutrition and the Epigenome

DNA methylation is the most stable epigenetic mark. However, there is growing evidence that the epigenome is susceptible to a number of environmental factors during specific periods of the life course, namely the prenatal, neonatal, and pubertal periods. One of the best examples of how nutrition in early life can alter phenotype is seen in studies from the honeybee. Female larvae fed different diets develop into either sterile worker bees or fertile queen bees, even though they are genetically identical [37]. However, the proportion of larvae developing into queen bees as opposed to sterile workers was significantly increased when the expression of DNA methyltransferase 3 (DNMT3) was silenced, suggesting that nutrition can profoundly affect phenotype and it does so through the altered methylation of DNA [37].

In mice, maternal diet has been shown to alter DNA methylation in the offspring. Differences in the intake of methyl donors and the cofactors for one-carbon metabolism during pregnancy in the agouti mouse induce differences in the coat color of the offspring. The murine A^{vy} mutation results from the insertion of an intracisternal A-particle (IAP) retrotransposon upstream of the agouti gene, which regulates the production of yellow-pigmented fur. Supplementation of pregnant mice with betaine, choline, folic acid, and vitamin B_{12} led to increased methylation of the agouti gene and shifted the distribution of coat color of the offspring from yellow (agouti) to brown (pseudo-agouti) [38].

There is also evidence in a number of animal models of nutritional programming that perturbations in early life nutrition alter the epigenetic control of a number of key regulators of lipid and glucose metabolism. For instance, feeding pregnant rats a protein-restricted (PR) diet induced hypomethylation of the GR and PPARα promoters in the livers of juvenile and adult offspring, which was associated with increased mRNA expression of these genes [39, 40]. Increased expression was also associated with an increase in acetylation of histones H3 and H4 and methylation of histone H3 at lysine K4 [41]. Sequencing analysis of the PPARα promoter showed that four specific CpGs were hypomethylated, and that two CpGs which were located within transcription factor response elements predicted the level of the transcription [42]. Thus, the effects of the maternal PR diet on the offspring are targeted to specific CpGs. Neonatal overfeeding induced by raising rat pups in small litters has also been shown to induce the hypermethylation of two CpG dinucleotides within the pro-opiomelanocortin (POMC) promoter, which are essential for POMC induction by leptin and insulin [43]. This suggests that overfeeding during early postnatal life, when the appetite circuitry within the hypothalamus is still developing, can alter the methylation of genes critical for

body weight regulation. Together these findings show that early life nutrition can induce long-term changes in the methylation of key genes involved in metabolism and appetite control, suggesting that such changes may underpin the developmental origins of metabolic disease.

3 Prenatal Environment and Cancer Risk

In contrast to the work on early life origins of metabolic disease, there have been far fewer studies on the effect of the prenatal environment on later risk of cancer. Studies have shown that prenatal exposure to the endocrine disrupting agent diethylstilbesterol (DES) increases the risk of cancer of the vagina and cervix in the daughters of DES-exposed mothers as well as increasing breast cancer risk [44], while exposure to ionizing radiation in utero or in early childhood increases cancers of the digestive system and reproductive tract in later life [45]. But for the most part studies to date have focussed on the relationship between birth weight (BW) and later cancer risk. The majority of these studies have shown a positive association between BW and risk of cancer; however, negative associations were found between BW and risk of hepatoblastoma and endometrial cancer [46].

Many of the studies examining the relationship between BW and later breast cancer risk have differed significantly in their design, particularly whether pre- and postmenopausal cancer was analyzed together or separately and the extent to which other variables were included in the analysis, making comparisons and conclusions challenging. However, a recent meta-analysis combining data from 26 published studies showed that that the risk of pre- but not postmenopausal breast cancer was consistently increased in individuals with higher BW [47]. The relative risk estimate of premenopausal breast cancer comparing women with high BW to women with low BW was 1.20 (CI 0.91–1.19) for cohort studies and 1.36 (CI 0.66–1.64) for case control studies. Interestingly, a number of these studies [48–50] reported a J- or U-shaped association between BW and premenopausal breast cancer risk, with babies born below 2,500 g and above 4,000 g both being associated with an increased risk of breast cancer compared to babies born within the normal range. However, BW in all of these studies should be viewed only as a very crude indicator of the intrauterine environment, which may have been compromised through a variety of maternal, environmental, or placental factors [51].

One important determinant of later cancer risk is maternal diet. In Norway, a decrease in calorie intake during the Second World War due to food rationing was associated with a decrease in breast cancer risk in women who were born during this period of famine or who were peripubertal at that time [52, 53]. In contrast, in studies of the Dutch Hunger Winter, a famine that occurred in the Netherlands in 1944 showed that women born to mothers exposed to famine during pregnancy had an increase in breast cancer risk [54, 55]. An increase in breast cancer risk was also observed for women who had been exposed to famine during childhood. This difference in outcome between the studies in Norway and the Netherlands may

reflect the severity of the famine in the Netherlands and/or the fact that the famine in the Netherlands ended abruptly [54] which may have resulted in a period of rapid catch-up growth which has been associated with raised levels of growth factors and hormones which may potentiate cancer risk.

It is not only prenatal nutrition that may alter breast cancer risk; studies have also shown that breast-feeding can reduce an infant's risk of developing breast cancer before menopause [56]. DHA, the long-chain PUFA present in breast milk, is known to be important for neuronal development, but the presence of DHA at this early stage of life may also affect the development of the mammary gland and subsequently modify future cancer risk. Interestingly, some studies in animals have shown that n-3 PUFAs have a protective effect and reduce cancer risk [57]. The rate of growth during early life is also an important determinant of subsequent breast cancer risk. Rapid growth in the peripubertal period between 8 and 14 years is associated with an increased risk of breast cancer, but BMI at 8, 10, 12, and 14 years of age is inversely associated with the risk of breast cancer [58].

3.1 Animal Models of Nutritional Programming of Cancer Risk

Animal studies also support the hypothesis that nutrition in early life can influence later breast cancer risk. Feeding a PR diet through pregnancy and lactation, which produces low BW offspring, doubled the incidence of early onset N-nitroso-N-methylurea (NMU) induced mammary tumors in the PR offspring compared to offspring from control fed dams [59]. PR offspring also displayed reduced post-natal ductal branching and epithelial invasion at 3 weeks, this was followed by a period of rapid compensatory mammary growth, and an increase in the expression of the insulin receptor, ER, and IGF1 in the PR offspring compared to controls.

There have also been studies which have shown that overnutrition in early life is associated with an increased cancer risk. De Assis et al., showed that offspring from dams fed a high-fat (a mixture of saturated, PUFA and MUFA) diet during gestation, had a higher BW and developed, in response to 7,12-dimethyl-benz[a]anthracene (DMBA) treatment, mammary tumors significantly earlier than in the DMBA treated offspring from control fed dams [60]. The mammary glands of the offspring from the high-fat fed dams also contained more terminal end buds (TEB), the structure that gives rise to mammary tumors [61] and increased epithelial density. The mammary glands of the high-fat offspring also had a higher number of proliferating cells, higher levels of the pro-survival factor AKT but lower levels of ER-a. The type of fat and timing of exposure may also be important. Feeding pregnant rats a diet high in n-6 PUFA leads to a significantly higher incidence of mammary tumors in the female offspring [62, 63]. The increase in tumor incidence was again associated with changes in mammary gland structure. A higher number of TEB was observed; these TEBs also persisted longer and exhibited a reduced level of differentiation to alveolar buds. Feeding high-n-6 PUFA diets during the peripubertal period in rats also increases mammary tumor

incidence, when compared with rats that were only exposed to high-n-6 PUFA diets postpuberty [64, 57]. In contrast, a low-fat n-3 PUFA diet during the peripubertal period resulted in a protective effect against mammary carcinogenesis in rats [57]. The low-n-3 PUFA diet reduced mammary cell proliferation and increased apoptosis, particularly in the TEB. Serum levels of 8-hydroxy-2′deoxyguanosine, a marker of DNA damage, were significantly reduced in these low-n-3 PUFA fed rats. Moreover, when high-fat n-6 PUFA diets were co-supplemented with n-3 PUFA via fish oil during pregnancy in rats, a decrease in breast cancer risk in the offspring was observed [65].

High BW, in early life, has been shown to be associated with high intrauterine exposure to estrogens and to increased maternal and/or cord blood concentrations of insulin, insulin-like growth factor 1 (IGF1), leptin, and adiponectin [66–69]. Compensatory catch-up growth observed in low BW individuals and in models of undernutrition is also associated with increased levels of such hormones [70]. High levels of such hormones and growth factors in early life when the epigenome is most susceptible to environmental factors is likely to have long-term effects on the development of the mammary gland potentially altering the rates of cell proliferation or apoptosis, stem cell number and differentiation, influencing the risk of breast cancer in later life. To date, a number of epigenetic changes have been reported in the mammary glands of offspring fed a PR or high-fat diet during pregnancy. Zheng et al. reported that the expression of the cell cycle inhibitors p16 and p21 was persistently decreased in the mammary gland of the PR offspring compared to controls [71, 72]. The decrease in p16 and p21 expression was accompanied by a decrease in histone acetylation and demethylation of K4 on histone H3. For p21, the effect of maternal diet on DNA methylation was also examined using bisulfite sequencing but no change in methylation was observed [72]. High-fat feeding during pregnancy has been shown to induce epigenetic changes in p16. Zheng et al. reported a decrease in histone H4 acetylation, decreased HDAC3 binding, but no difference again in DNA methylation levels [73] although here a MeDIP approach was used which measures methylation across a region rather than the methylation of individual CpGs.

3.2 Micronutrient Intake

A substantial body of human epidemiological and animal studies have shown that nutrients involved in one-carbon metabolism such as folate, vitamin B6, vitamin B2, vitamin B12, and choline are protective against a number of cancers, although the relationship between folate intake in adulthood and breast cancer risk is complex. The majority of studies, but not all, have shown an inverse relationship [74] between dietary folate intake and breast cancer risk. However, there are also studies which suggest that folic acid supplementation leads to increased breast cancer risk if given at doses $\geq$400 μg/d [75]. Folic acid intake has increased dramatically in some countries over the past 10 years due to fortification of food

with folic acid, consumption of folic acid supplements, and periconceptional folic acid supplementation taken for the prevention of neural tube defects [76–78]. A number of studies have explored the effect of supplementation with folic acid and other micronutrients during early life on later cancer risk. Folic acid supplementation for 3 weeks prior to mating and throughout pregnancy and lactation has been reported to lead to a significantly lower number of TEB in the offspring compared to offspring from the dams fed the control diet ($p = 0.014$) [79]. As TEBs are the structures that give rise to mammary tumors, fewer TEBs might suggest a decrease in tumor susceptibility although this was not tested in this study. In contrast, Ly et al. [80] have reported that both maternal and postweaning folic acid supplementation significantly increased the risk of mammary adenocarcinomas in the offspring (OR ¼ 2.1, 95 % CI 1.2–3.8, P ¼ 0.008 and OR ¼ 1.9, 95 % CI 1.1–3.3, P ¼ 0.03, respectively) after DMBA treatment [80]. Maternal folic acid supplementation in this study also significantly accelerated the rate of mammary adenocarcinoma appearance (P ¼ 0.002) and increased the multiplicity of mammary adenocarcinomas (P ¼ 0.008) in the offspring. This difference in response to folic acid supplementation in these two studies may reflect the difference between the effect of folic acid in an animal model with long latency periods where there is no exposure to a carcinogenic agent compared to models where mammary tumorigenesis is induced through exposure to an agent such as DMBA. Micronutrients may have an impact on carcinogenesis through their role in providing one-carbon moieties for the synthesis of nucleotides and/or for the synthesis of S-adenosyl methionine (SAM), the universal donor for nearly all methylation reactions, including that of DNA. Ly et al. have reported that maternal, but not postweaning, folic acid supplementation significantly reduced global DNA methylation ($p = 0.03$), whereas postweaning, but not maternal, folic acid supplementation significantly decreased DNA methyltransferase activity ($p = 0.05$) in non-neoplastic mammary glands of the offspring [81, 80], suggesting that epigenetic processes may be important in determining later cancer risk.

3.3 Identification of Epigenetic Biomarkers

If cancer risk reflects events across the life course, then epigenetic changes associated with cell transformation may be present years before the onset of clinical disease [82]. It may therefore be possible to detect epigenetic changes before overt signs of disease and use these to identify individuals at increased risk. However, in humans, the only tissues readily accessible for testing are blood, buccal, cord, or placenta at birth. Tissue-specific differences in gene methylation have clearly been well documented. However, Talens et al. have reported recently that DNA methylation levels measured in blood were equivalent in buccal cells for half of the candidate loci examined, despite the fact that these cell types originate from different germ layers (mesoderm and ectoderm, respectively) [83]. Interestingly, Godfrey et al. has recently reported in two independent cohorts that the

methylation state of a single CpG site in the promoter region of the transcription factor RXRA in the umbilical cord was strongly related to childhood adiposity in both boys and girls [84]; RXRA promoter methylation explained over a fifth of the variance in childhood fat mass, suggesting that epigenetic alterations may contribute a far greater proportion of an individual's risk to NCD than had previously been thought. This suggests the possibility that methylation levels in cord or other readily available tissues may provide useful proxy markers of methylation in more metabolically relevant tissues. Although this may be dependent on when the environmental challenge occurred, such a constraint during very early development is likely to affect all germ layers and so an imprint of this altered epigenetic mark may be detectable in all tissues, while exposures occurring later on in gestation may only induce tissue-specific effects.

Brennan et al. have reported recently in prediagnostic blood samples that methylation of an intragenic region of the ATM gene in peripheral blood DNA was associated with an increased risk of breast cancer [85]. There was no association between the time from blood collection to diagnosis which ranged from 1 month to 11 years and the level of ATM methylation, suggesting that this association is not explained by the presence of preclinical disease. ATM methylation at this site was also found to be stable over time, suggesting that ATM hypermethylation in peripheral blood represents a stable marker of predisposition. Wong et al. have also reported that peripheral blood methylation of BRCA1 is associated with a 3.5-fold (95 % CI, 1.4–10.5) increased risk of early onset breast cancer with a BRCA1 mutation-associated pathology [86]. Detectable BRCA1 methylation in peripheral blood was also associated with high levels of BRCA1 promoter methylation within the tumor suggesting that constitutional BRCA1 methylation may increase susceptibility to the development of BRCA1 hypermethylated tumors [86]. However, whether alterations in the methylation of ATM or BRCA1 were induced during early life when the epigenome is most susceptible to change remains to be determined. Nevertheless, these findings suggest that epigenetic marks in peripheral blood may provide useful predictive biomarkers of later disease risk. Moreover, with the development of new high-throughput genome-wide methylation analyses, it will now be possible to carry out epigenome-wide association studies (EWAS) to identify epigenetic marks in blood associated with increased breast cancer susceptibility. Many questions remain as to the mechanisms underlying such changes. For example, what is the range of nutritional factors that can induce such changes and what are the periods of susceptibility or the stability of such induced changes? Understanding these processes would allow the development of both effective predictive biomarkers of disease risk and targets for intervention strategies to reduce breast cancer risk.

4 Conclusion

Traditionally, it has been widely accepted that genetic alterations cause cancer. However, there is now a considerable body of evidence to suggest that variations in the quality of the early life environment affect future cancer risk through the altered epigenetic regulation of genes. The demonstration of a role for altered epigenetic regulation of genes in the developmental induction of breast cancer in early life together with the identification of potential biomarkers suggests the possibility of nutritional or perhaps pharmacological interventions which could modify long-term breast cancer risk.

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Part IV
Olive Oil in Cancer Prevention

Olive Oil and Other Dietary Lipids in Breast Cancer

Eduard Escrich, Montserrat Solanas and Raquel Moral

Abstract

Breast cancer is the most frequent malignant neoplasia among women worldwide. In addition to genetic and endocrine factors, the environment, and specifically nutritional factors, plays a key role in its aetiology. Epidemiological and in particular experimental studies have shown the link between dietary fat and breast cancer. Abundant data have attributed a potentially chemopreventive effect for extra-virgin olive oil (EVOO), the main source of fat in the Mediterranean diet, which is associated with low incidence and mortality rates from chronic diseases such as breast cancer. We have demonstrated the differential modulatory effect of dietary lipids on mammary carcinogenesis, mainly in studies developed in an experimental model. Thus, diets high in n-6 polyunsaturated fatty acids (PUFA) have a clear stimulating influence, whereas EVOO diets mainly have a negative modulatory effect on breast cancer development. The specific mechanisms involved are not fully understood, but nowadays, it is widely accepted that they are numerous and complex. Our group has contributed to improving the knowledge of these mechanisms by demonstrating the influence of dietary lipids on the structure and function of cell membranes, the modulation of cell-signalling transduction pathways, the regulation of gene expression and growth and sexual maturity.

Keywords

Breast cancer · Dietary lipids · EVOO · Experimental mammary cancer · n-6 PUFA · Olive oil · Corn oil

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_17,

Abbreviations

PUFA	Polyunsaturated fatty acids
MUFA	Monounsaturated fatty acids
OA	Oleic acid
EVOO	Extra-virgin olive oil
DMBA	Dimethyl-benz(α)anthracene
HCO	High corn oil
LA	Linoleic acid
FFA	Free fatty acids
PCNA	Proliferating cell nuclear antigen
CPT I	Carnitine palmitoyltransferase I
PPAR	Peroxisome proliferator receptor
VDUP1	Vitamin D3–upregulated protein 1
IGF	Insulin-like growth factor

Contents

1 Introduction

Breast cancer is by far the most frequent cancer among women worldwide (22.9 % of the total), followed by colorectal cancer (9.4 %) and cancer of the cervix of the uterus (8.8 %). It is also the most frequent cause of cancer death in women in both developing and developed regions (Fig. 1a) [1]. The aetiology of breast cancer is multi-factorial, with genetic, epigenetic and endocrine factors being the main factors involved [2, 3]. However, incidence rates of this neoplasia show a different geographical distribution, with the highest rates in developed regions of the world

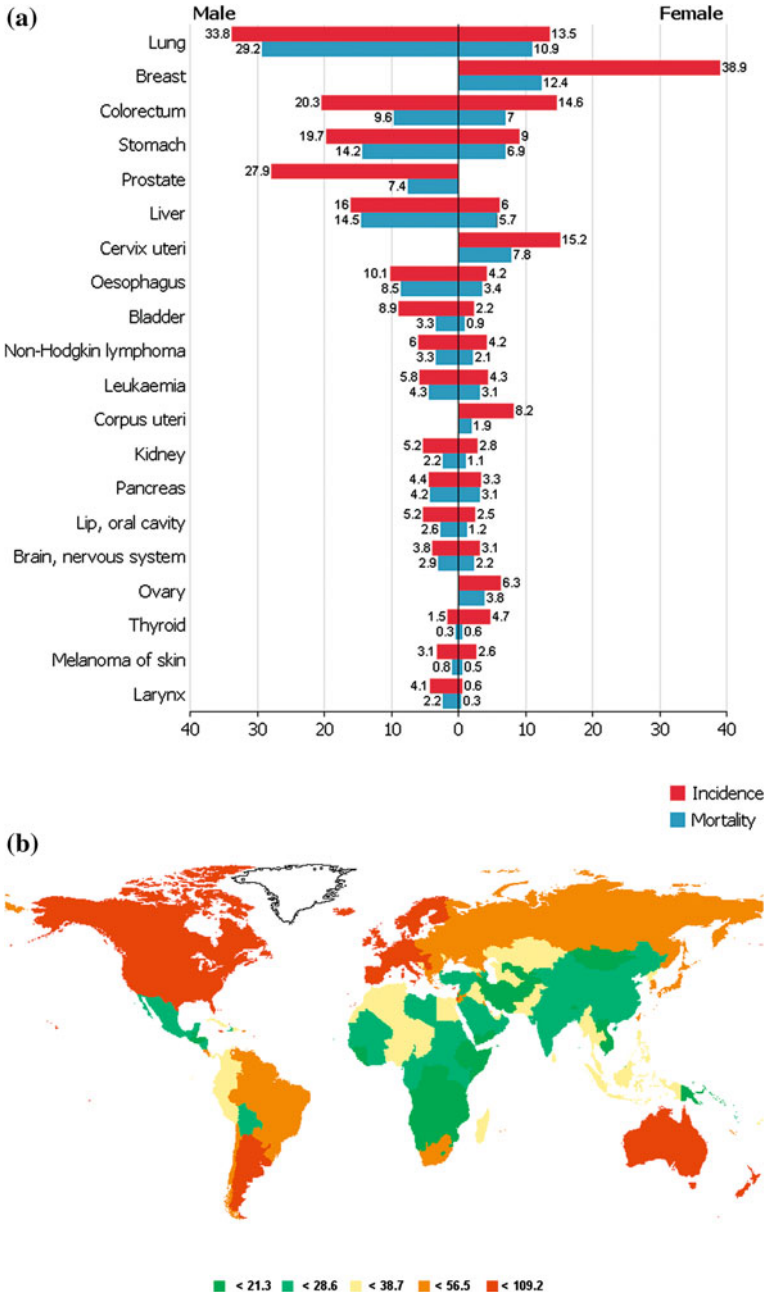

Fig. 1 **a** Estimated age-standardised incidence and mortality rates (per 100,000) by sex and cancer site worldwide. **b** Estimated age-standardised incidence rates of breast cancer per 100,000 (World) [4]

(except Japan) and with the lowest ones being found in most of the developing regions (Fig. 1b) [1, 4]. This indicates that environmental factors also play a role in the development of breast cancer. Among them, nutritional factors are the most remarkable due to firstly, the continuous exposition or deficiency to which a population may be submitted because of dietary habits and lifestyle and secondly, to the opportunity to adopt preventive measures based on such factors [5].

Among the numerous dietary components that have been related to breast cancer, lipids have been revealed as being significant ones. Epidemiological and especially experimental studies have found a relationship between dietary lipids and certain cancers, such as breast, colorectum, and prostate cancer [5–8]. The main epidemiological support for the association between dietary fat and breast cancer risk comes from ecological studies (correlational and those of migrant populations), while, in contrast, analytical epidemiology (case-control and prospective studies) has generated some inconsistent data and not all of the studies support a significant association. While a positive association between total and/or saturated fat intake and the risk of breast cancer appears to be well established, the relationship with specific subtypes of fat is less consistent [9–13]. Experimental studies, including those from our group, have provided important scientific evidence for the relationship between dietary lipids and breast cancer. Thus, beyond the stimulatory effect of fat-enriched diets, distinct types of lipids have a differential modulatory effect on breast cancer: stimulatory for saturated fat, mainly of animal source, and n-6 polyunsaturated fatty acids (PUFA) of vegetable origin mainly found in seed oils; and inhibitory for n-3 PUFA, of vegetable and especially marine origin. Concerning monounsaturated fatty acids (MUFA), mainly oleic acid (OA), present in high quantities in olive oil, although there is growing evidence for a chemopreventive effect, there are conflicting results too, with reports ranging from non-stimulating, weak stimulating to stimulating effects on tumour growth [7, 14–17].

Olive oil, the major energy source of the Mediterranean diet, has received much attention because of the abundant data showing that Mediterranean countries have lower rates of incidence and/or mortality from several chronic diseases such as cardiovascular diseases and some types of cancer, including that of the breast cancer, in comparison with other Western countries. The Mediterranean diet includes a variety of food patterns from different regions of the Mediterranean, and it is identified by the consumption of an abundance of plant foods (fruit, vegetables, cereals, grains and nuts), dairy products, fish, and olive oil [18] (Fig. 2). Extra-virgin olive oil (EVOO), the first-pressed olive oil, is characterised not only by its richness in OA (72–84 %), but also by the presence of minor bioactive compounds such as squalene, phenolic antioxidants (hydroxytyrosol, tyrosol and oleuropein), secoiridoids (oleuropein and its aglycon), flavonoids and lignans [19–21]. At present, it is well established that the potential healthy effects of EVOO can be attributed both to its particular fatty acid composition (specifically the high OA content, a suitable quantity of essential PUFA and a relatively low n-6 PUFA/n-3 PUFA ratio) and these minor bioactive compounds [22–25].

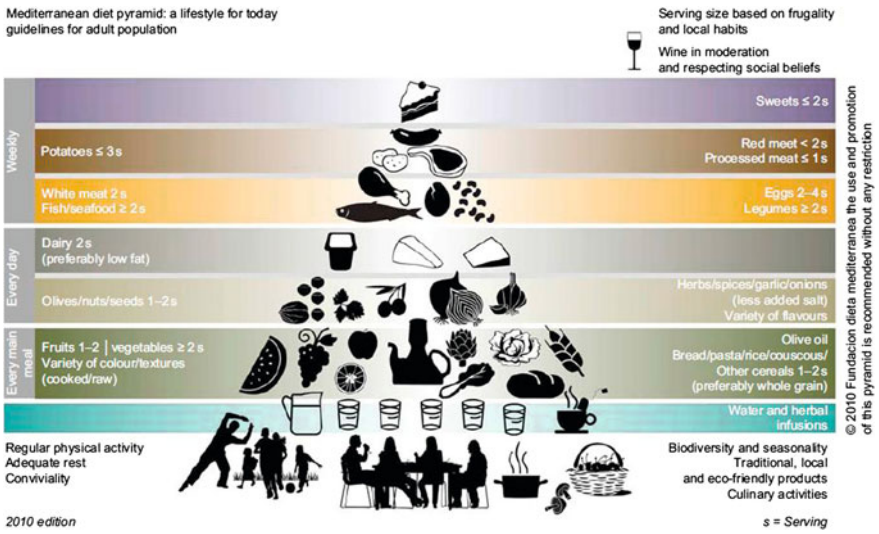

Fig. 2 The updated Mediterranean Diet pyramid (from the Mediterranean Diet Foundation) [89]

Our group has been investigating the effects of dietary lipids on breast cancer for more than 25 years in two main lines: the first one is in a chemically induced breast cancer model, using two types of fat: a seed oil, the corn oil rich in n-6 PUFA, and EVOO; and the second line is in human breast tumours. The aim of this chapter is to show the principal results obtained in the experimental model, where we focused our research on clinical carcinogenesis, tumour morphology and especially the molecular mechanisms by which these two types of fat may exert their differential modulatory effects on mammary carcinogenesis. Even though caution must be applied when extrapolating experimental data to a human population owing to the difficulty of obtaining data with controlled variables in humans, this type of study is essential to gain insight into the influence of nutritional factors on health.

2 Experimental Evidence for the Effects of Dietary Lipids on Mammary Carcinogenesis

The experimental studies were developed in a breast cancer model where cancer was induced with a single dose of dimethyl-benz(α)anthracene (DMBA), a polycyclic aromatic hydrocarbon, in the female Sprague–Dawley rat [26]. The suitability of this model for the possible application of the results in human breast cancer has previously been validated, and it is used extensively in breast cancer studies [27, 28]. For the investigation of the different effects that olive oil and n-6 PUFA may have on the initiation and promotion of mammary adenocarcinomas, diets were designed and validated as follows: a control low-fat diet (with 3 % corn

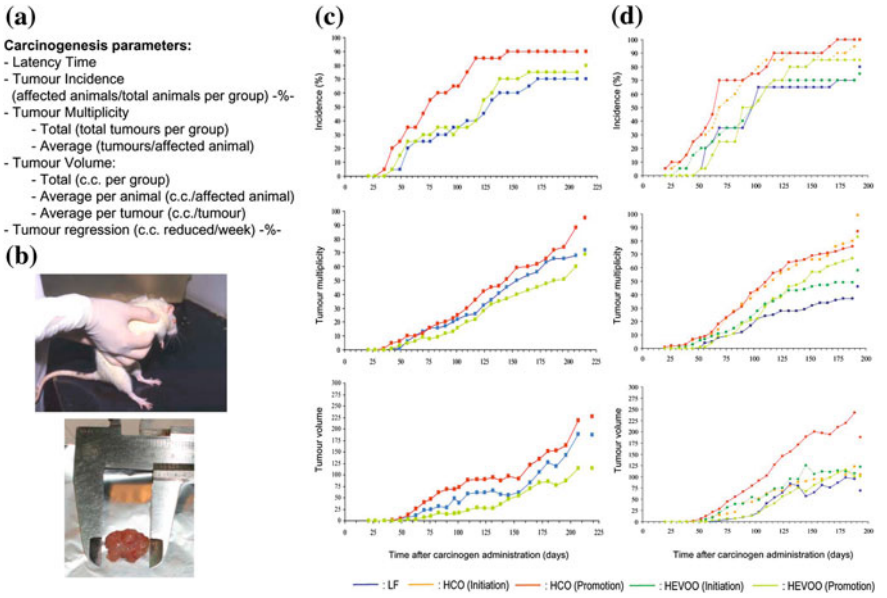

Fig. 3 Effects of high-seed oil and high-EVOO diets on clinical parameters of the rat DMBA-induced mammary carcinogenesis. Depending on the experimental design, animals were fed the high-corn oil (HCO) diet or the high-EVOO (HEVOO) diet from weaning onwards (initiation groups) or from carcinogen administration onwards (promotion groups). A low-fat diet (LF) group fed this diet all through the study was always included as a control group. Animals were examined and weighed weekly throughout the study and monitored for mammary tumour appearance weekly from 21 days after carcinogen administration (at 53 days of age) onwards. **a** Eight different clinical parameters in addition to suitable statistical analysis were defined. **b** Representative images of the animal exploration and of an experimental mammary adenocarcinoma. **c** Three representative parameters from an experimental series aiming to study the promotion of the carcinogenesis are shown. The HCO diet exerted a clear stimulatory effect in such a way that tumours appeared earlier, there were more affected animals, more tumours, and they were greater in comparison to the control group. In contrast, the high-EVOO diet mainly showed a protective effect, with latency time, incidence, and tumour content and volume similar to or under the control. **d** The same three representative parameters from another experimental series, where the dietary lipid effects on both initiation and promotion were studied, are shown. The HCO diet consistently exerted the enhancing-tumour effect whereas a weak stimulating influence was observed due to the HEVOO diet, although this effect was much lower than that of the HCO diet, even though it was also a high-fat diet

oil), a high-fat n-6 PUFA diet (with 20 % corn oil) and a high-fat n-9 MUFA diet (with 17 % EVOO and 3 % corn oil) [29–31].

To study the effects of these dietary lipids on experimental breast cancer, several clinical parameters were defined (latency time, tumour incidence, multiplicity and volume, and tumour regression) (Fig. 3a, b), in addition to suitable statistical analysis [32–35]. In the sixteen experimental series developed in our laboratory, we consistently demonstrated that diets rich in seed oils exert a clear stimulatory effect on mammary carcinogenesis in such a way that tumours appear

earlier, there are more affected animals, more tumours, and they are greater in comparison to the control group. In contrast, diets rich in EVOO, with the same total content in fat as the high-corn oil (HCO) diet, mainly showed a protective effect, with latency times, incidence, and tumour content and volume similar to or under that of the control group. Importantly, the study of tumour regression showed a slow tumour progression due to this kind of diet rather than a real, partial or total regression of the established tumours. These results suggest that the effects of EVOO diets would not be as potent as for using them as therapeutic agents, at least by themselves (Fig. 3c, d) [6–8, 31, 36–38]. Moreover, a weak stimulating influence was observed in some of the experimental series, although this effect was always much lower than that of the diet rich in seed oil, even though it was also a high-fat diet (Fig. 3d) [39]. These differences can be related to the timing of dietary intervention as well as to distinct varieties of olive oil used, as it was reported that the influence of olive oil on carcinogenesis depends on the unsaturated/saturated fatty acids and OA/linoleic acid (LA) ratios, and the relative composition of minor compounds of the oil [40]. Such findings are of interest considering the high percentage of olive oil used in the experimental diet, since all high-fat diets may have an unspecific stimulatory effect on carcinogenesis [41]. In this sense, a positive association between dietary energy supply and cancer mortality rates has been shown, and energy restriction has an indiscriminate inhibitory effect of carcinogenesis [42].

Therefore, these results indicate that high-n-6 PUFA diets confer a clinical behaviour of greater biological aggressiveness in mammary tumours, whereas EVOO diets show characteristics of lower malignancy in the tumours. It should be emphasised that this modulatory action of dietary lipids on mammary cancer has mainly been observed in the promotion stage of carcinogenesis, although an influence on the initiation stage cannot be ruled out [6, 8].

3 Influence of Dietary Lipids on the Morphological Malignancy of Mammary Adenocarcinomas

The differential effect of dietary lipids on mammary cancer has also been characterised morphologically by our group, for the first time ever, by means of a comprehensive histopathological analysis of mammary adenocarcinomas. This analysis was based on the study of eleven parameters, some of them being already widely used in human breast pathology and other characteristics of the experimental tumours, and a new histological grading system adapted to rat mammary carcinomas by our group. First, these studies showed a correspondence between the histological pattern and the clinical characteristics of each tumour, finding that tumours with a higher degree of biological aggressiveness displayed higher histopathological degrees, desmoplastic reaction, limfoplasmocitic infiltration, tumour necrosis and prevalent cribiform architectural patterns [43, 44]. Secondly, the analysis of the effect of different diets showed that diets rich in seed oil

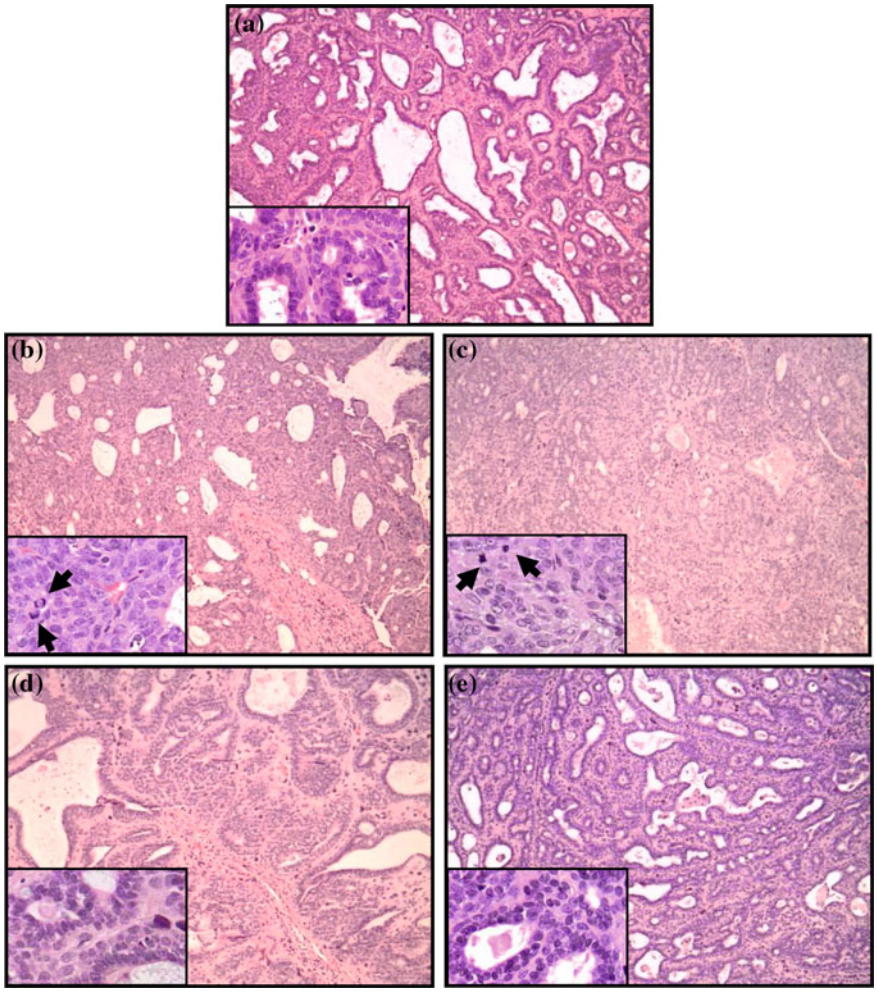

Fig. 4 Histopathological features of rat DMBA-induced mammary adenocarcinomas from the different dietary groups. The histopathological analysis of haematoxylin and eosin-stained sections of tumours from the different experimental groups revealed that diets rich in seed oil promoted adenocarcinomas with a higher degree of malignancy (**b** and **c**), in comparison to the tumours from the control (**a**) and EVOO groups (**d** and **e**). On the contrary, tumours from animals fed high-EVOO diets exhibited histopathological features compatible with a lower degree of malignancy than those from the animals fed the HCO diet, being more similar to control tumours. Low-power pictures at x100 original magnification, high-power pictures (insets) at x400 original magnification. Mitotic figures are pointed with arrows (**b** and **c**) [45]

promoted adenocarcinomas with a higher degree of malignancy, in comparison to the control and EVOO diets, shown by a high histological degree, stromal invasion, more prominent tumour necrosis and frequent cribiform pattern. On the contrary, tumours from animals fed with high-EVOO diets exhibited

histopathological features compatible with a lower degree of malignancy than those from the animals fed with the HCO diet, being more similar to control tumours. Thus, they displayed a low histopathological degree, few invasive and necrotic areas and extensive papillary areas (Fig. 4). Therefore, the different modulating effect of dietary lipids corresponds not only to different tumour clinical behaviour but also to different histopathological features [45].

4 Mechanisms of Action of Dietary Lipids on Breast Cancer

The specific mechanisms by which dietary fat in general and EVOO diets in particular may exert their modulatory effects on breast cancer have not been well elucidated. However, at present, there is increasing understanding that dietary lipids may act through diverse and complex mechanisms, probably in an integrated, simultaneous and/or sequential way. Our group has contributed to improving knowledge of these mechanisms by investigating possible alteration in hormonal status, the structure and function of cell membranes, cell-signalling transduction pathways, gene expression, and growth and sexual maturation, among others (Table 1) [46–48].

4.1 Alteration of Hormonal Status

Since breast cancer is a hormone-dependent neoplasia, firstly, we investigated whether dietary lipids modify the status of the main hormones regulating the growth and development of the normal mammary gland and tumours. Results showed that serum concentrations of luteinizing hormone, follicle-stimulating hormone, estradiol, progesterone, prolactin, insulin and corticosterone did not significantly change due to the effect of high-n-6 PUFA or high-EVOO diets [49], (E. Escrich, unpublished results). The data reported in the literature about the effect of dietary lipids on circulating hormone levels are inconclusive [50]. Thus, pregnant rats fed a high-n-6 PUFA diet have shown to either increase [51] or not modify [52] serum estradiol levels. Other authors have found lower concentrations of estradiol in lactating dams fed a 7% olive oil diet in comparison to those fed a 7% corn oil diet [53]. The discrepancy among results may be related to the cyclical nature of hormones, as their serum concentrations are highly dependent on the circadian rhythms and the phase of the oestrus cycle [54]. We also analysed the expression levels of sex hormone receptors in the mammary gland and in the experimental tumours, finding little difference due to the effect of dietary lipids. Thus, no significant differences were found in the expression of oestrogen receptors (ERα, ERβ1 and ERβ2). We observed higher levels of progesterone receptors in the mammary gland of rats fed the EVOO diet in relation to the animals fed the HCO diet at the age around puberty, which may be related to the development of lobulo-alveolar structures [39]. These results suggest that the

Table 1 Effects and some of the main molecular mechanisms of action of high-EVOO and high-seed oil diets on experimental mammary cancer[a]

		EVOO diets	Seed oil diets
Clinical behaviour		Indolent tumour growth	Increased tumour growth rate
		Protective or weak promoting effect	Stimulatory effect
Histopathological features		Low degree of histological malignancy	High degree of histological malignancy
Mechanisms of action	Proliferation	Decreased or similar	Increased
		↔ *mitotic activity*	←↑ *mitotic activity*
		↔ *PCNA levels*	↔ *PCNA levels*
		↔ *Cyclin D1 expression*	↔ *Cyclin D1 expression*
		↓ *p21Ras activity*	↔ *p21Ras activity*
		↑ *non-activated p21Ras levels*	↔ *non-activated p21Ras levels*
		↑ *MAPK activity (with↓ Akt activity)*	↑ *MAPK activity (without ↓ Akt activity)*
	Survival	Decreased	Increased
		↓ *Akt activity (↓ phospho-Akt levels)*	↑ *phospho-Akt/total Akt ratio*
	Apoptosis	Increased	No changes
		↓ *Akt activity and ↑ MAPK activity*	↑ *Akt activity and ↑ MAPK activity*
		↑ *active Caspase-3 levels*	↔ *active Caspase-3 levels*
		↑ *apoptotic cells*	↔ *apoptotic cells*
	Tumour regression	No	No
	DNA damage	Probably decreased	Probably increased
		↓ *Ubi-PCNA levels*	↑ *Ubi-PCNA levels*
		↔ *8-oxo-dG levels*	↔ *8-oxo-dG levels*
	Differentiation	Similar	Decreased
		↔ *PCPH expression*	↓ *PCPH expression*
		↔ *H19 and VDUP1 expression*	↓ *H19 and VDUP1 expression*

[a] The effects are compared with those of a control low-fat diet. ↑: increase; ↓: decrease; ↔: no changes

modulatory effects of dietary lipids on breast cancer do not seem to be exerted by altering hormonal status, which is such an important parameter of the normal and tumorous breast. Likewise, we also analysed several plasmatic biochemical parameters and found only a decrease in total cholesterol levels due to both high-n-6 PUFA and high-EVOO diets (E. Escrich, unpublished results).

4.2 Modifications of Cell-Membrane Structure and Function

Membrane lipid composition influences membrane function and is highly regulated by cells. Moreover, it may vary depending on the lipids usually ingested, mainly due to n-3 PUFA and the n-3 PUFA/n-6 PUFA ratio. Diet-induced modifications in membrane lipid composition have been associated with changes in membrane fluidity, lipid-mediated signalling pathways and lipid peroxidation levels in cell membranes [55]. Therefore, the influence of dietary lipids on tumour fatty acid composition was studied in our laboratory, through the analysis of fourteen fatty acids in six lipidic fractions. Mammary adenocarcinomas from rats fed a high-seed oil diet, more aggressive clinically and histopathologically, in comparison to control diet tumours, displayed a significant increase in the LA relative content and a decrease in that of the OA, significantly in phosphatidylcholine, phosphatidylethanolamine and free fatty acids (FFA). Having taken into account that the hydrolysis products of these phospholipids and FFA have been linked to tumour cell proliferation, these results are in agreement with experimental and epidemiologic studies showing the detrimental effect of diets rich in LA and the beneficial one of the EVOO diets in mammary carcinogenesis [36]. There is also evidence that adherence to the Mediterranean diet may affect the structural properties of the erythrocyte cell membrane of hypertensive patients, according to the beneficial effect of this diet on hypertension [56].

4.3 Influence on Signal Transduction Pathways

Next, our research focused on the study of the possible alterations in gene function and activity proteins involved in cell-signalling pathways. Specifically, we explored the Ras-mediated signalling pathway, which has been demonstrated to have an important role in mammary development and human breast cancer [57]. The results showed that a diet rich in EVOO significantly decreases p21Ras activity but increases protein levels, thus suggesting an increase in the relative levels of the non-activated protein due to this diet. The decrease in p21Ras activity was not the result of a lower *ras* mutation rate nor a reduced expression of gene-codifying proteins involved in the post-translational modification of Ras, such as HMG-CoA reductase and squalene synthase [58]. Other experimental studies have suggested that an olive oil diet may have a protective effect on colon cancer through the effect of its minor compound squalene, inhibiting the HMG-CoA

reductase. However, such effects have been observed at very high concentrations of squalene (1% of diet) [59].

Unexpectedly, the lower p21Ras activity was not associated with changes in the expression or activity in the ErbB1, ErbB2 and ErbB3 tyrosine kinase membrane receptors, which have a key role in human breast cancer [60]. On the contrary, we observed significantly decreased levels of the 80-kDa ErbB4-cleaved protein due to the high-EVOO diet. Although the biological significance of the ErbB4-truncated form in breast cancer is poorly known, it has been related to ErbB4 activation [61]. The EVOO diet also downregulated the pro-survival Ras/PI3 K/Akt pathway and upregulated the Raf/Erk pathway as compared with the control. In contrast, the high-n-6 PUFA diet neither modified Ras activity nor ErbB receptors, but rather enhanced the Raf/Erk pathway, suggesting an unspecific effect of both high-fat diets upregulating the ERK1/2 pathway. Moreover, the high-n-6 PUFA diet displayed the highest phospho-Akt:total Akt ratio, consistent with the stimulating effect of this diet on tumour growth. Neither of the two diets altered the RalA/B-mediated pathway.

The results obtained in the analysis of Ras and its main effectors led us to hypothesise a pro-apoptotic scenario due to the EVOO diet. Therefore, we investigated the apoptotic state in the mammary tumours from the different experimental groups. The relative levels of Caspase-3, which is the main executioner of the caspase cascade involved in both extrinsic and intrinsic apoptosis [62], were significantly increased in tumours from the animals fed the olive oil diet, which was concordant with the proposed increased apoptosis owing to this diet. This result was confirmed by TUNEL assays [58]. These results are in line with some studies that have reported that olive oil, oleic acid and some minor compounds of olive oil can modulate apoptosis [53, 63–65].

Our results also showed that the high-EVOO diet did not exert any significant effect on tumour cell proliferation, as the analysis of the mitotic activity and the proliferating cell nuclear antigen (PCNA) expression levels indicated. Thus, the high-EVOO diet may exert its modulatory effect on breast cancer by altering the proliferation/apoptosis balance, shifting it in favour of apoptosis, which might contribute to the ability of this diet to negatively regulate tumour growth. The high-seed oil diet, however, increased tumour mitotic activity, consistent with its effect in transducing survival/proliferation signals and, therefore, with its tumour-enhancing effect [58]. The effect that olive oil may have on tumour proliferation is still not well elucidated. *In vitro* experiments have found evidence that some minor compounds of olive oil, such as hydroxytyrosol and oleuropein, inhibit proliferation of breast cancer cells [63], while oleate has been reported to have a stimulatory effect on cell proliferation [64].

Interestingly, mono-ubiquitinated PCNA levels were also significantly decreased due to the EVOO diet and non-significantly increased due to the high-corn oil one. Since PCNA ubiquitination specifically occurs when DNA is damaged or replication forks stalled, these results suggest less DNA damage owing to the EVOO diet and higher genotoxic stress due to the high-seed oil diet. However, no changes were found in the levels of the pre-mutagenic 8-oxo-dG base, probably

because of the effects of dietary lipids on other DNA lesions. The role of lipid peroxidation in the modulation of mammary tumour development is not fully understood, and it remains controversial. Although it is accepted as a central mechanism of cancer initiation [14], the cancer-inhibiting effect of long-chain n-3 PUFA has been attributed, at least partially, to the formation of peroxidation products [66–68]. Moreover, accumulating evidence suggests that oxidative stress-induced apoptosis plays an important role in the anticarcinogenic effect of several chemopreventive agents, including some polyphenols [69, 70]. The effect of high-EVOO diets on oxidative stress is currently being addressed by our group.

4.4 Effects on Gene Expression

It is well-known that different dietary components can modulate the expression of specific genes. The best-characterised effect of fatty acids on gene expression is the modulation of genes involved in lipid, carbohydrate and protein metabolism [71]. Moreover, the effects of dietary lipids may be modified by the disease, as we have observed changes in the normal regulation of the expression of carnitine palmitoyltransferase I (CPT I) and mitochondrial HMG-CoA synthase and peroxisome proliferator receptor α (PPARα) by high-n-6 PUFA diets in the liver of tumour-bearing rats [72]. Although data regarding the effect of dietary lipids in cancer-related genes are scarcer, there is a growing body of evidence about their role in the regulation of genes involved in cell growth, survival, apoptosis and differentiation [71]. We found in experimental mammary tumours that the high-EVOO diet and the HCO diet had different influences on the modulation of the expression of the ErbB family of membrane receptors, especially c-erbB1. Thus, the HCO diet increased the ratio between the 9.5-kb mRNA of c-erbB1/EGFR (coding the functional full-length receptor) and the 2.7-kb mRNA (coding an inactive truncated receptor), whereas the high-EVOO diet decreased this ratio in mammary adenocarcinomas. In addition, this diet tended to decrease c-erbB2/neu mRNA and p185ErbB2/Neu protein levels, whereas the HCO diet did not modify the expression of this receptor [37]. On the other hand, the expression levels of c-Ha-ras1, a key transductor of ErbB proliferative signalling, were not modified by these diets [73, 74]. High-fat diets did not modify either the expression levels of HMG-CoA reductase or squalene synthase [58], which codify enzymes of the mevalonate pathway, the source of the prenyl groups needed for the post-translational modification of p21Ras and its consequent activation [75].

Since cell dedifferentiation is a process linked to cancer, our group also studied the dietary lipid effects on cell differentiation-related genes in DMBA-induced mammary tumours. We analysed the expression of known mammary differentiation markers, α- and β-casein and transferrin, in addition to β-actin, which is also related to tumour invasion capability. The results showed that the expression levels of caseins were not related to the degree of tumour morphological differentiation nor to the clinical behaviour, which suggested that these genes were not good

biomarkers of the modifications that dietary lipids conferred to the mammary adenocarcinomas. On the contrary, transferrin expression tended to be increased in the high-olive oil diet groups, what is in accordance with the less aggressive phenotype of the tumours as a result of this diet. Moreover, the high-seed oil diet, but not the high-EVOO diet, increased the β-actin mRNA expression, though no changes in the protein levels were observed. This last result, in addition to the increase observed in ZBP1, the transporter of the β-actin transcript from the nucleus to the cell periphery, suggested a deregulated transport and translation of this mRNA associated with tumours of a higher degree of malignancy, those of the groups fed the high-seed oil diet [76]. We also analysed the influence of dietary lipids on PCPH expression, a gene whose product acts synergistically with Ras. The results revealed a significant increase in PCPH levels with the mammary gland differentiation degree. In adenocarcinomas, PCPH expression not only decreased, in comparison with the normal tissue, but was also deregulated. Moreover, the most malignant tumours, those from the high-seed oil diet groups, exhibited the lowest PCPH expression levels [77].

New high-throughput techniques for studying differential gene expression allowed a different experimental approach in order to study the effects of dietary lipids on cancer. By means of cDNA microarray analyses, we identified 4 genes significantly downregulated due to the effect of the high-seed oil diet but not modified by the high-EVOO diet. These genes were α2u-globulin, Vitamin D3-upregulated protein 1 (VDUP1), the imprinted gene H19 and an unknown gene. As these genes, specifically H19 and VDUP1, are potentially related to cell differentiation and proliferation, their downregulation by the high-seed oil diet suggests that they may be involved in the tumour-stimulating effect of this diet on breast cancer [78]. Moreover, the insulin-like growth factor (IGF) II gene, related to proliferation and reciprocally imprinted with H19, was upregulated by the HCO diet and downregulated by the high-EVOO diet. Furthermore, both high-fat diets also had an opposite effect on the activity of thioredoxin, an oxidoreductase inhibited by VDUP1 which promotes cell growth and has an antiapoptotic action [79]. This result was in accordance with the opposing effect of the HCO and EVOO diets on mammary carcinogenesis.

4.5 Effects on Growth and Sexual Maturation

We have also focused our attention on how dietary lipids may act on cancer initiation, by modulating the susceptibility or resistance of the mammary gland to neoplastic transformation. There is striking evidence showing the importance of early-life events, including food and nutrition, in modifying the risk of breast cancer in later life [80]. Some of the mechanisms that we are currently investigating are growth and sexual maturation, including mammary gland differentiation, xenobiotic detoxification and oxidative stress.

The mammary gland has the particularity that, unlike other organs, after birth it remains highly undifferentiated until the onset of puberty. Reproductive events

increasing the number of menstrual cycles and therefore lifetime exposure to oestrogens, such as early menarche, are recognised as factors increasing breast cancer risk [5]. Thus, modifiable nutritional factors influencing the timing of puberty may modify susceptibility to mammary transformation, advancing, retarding or expanding the windows of this susceptibility. Our results indicated an advance in growth and sexual maturation due to the effect of the high-fat diets, such an effect being stronger in the case of the high-seed oil diet than in the high-EVOO diet [39]. This situation has been demonstrated clinically, morphologically and molecularly in the rat DMBA-induced breast cancer model. Clinically, after determining vaginal changes accompanying sexual maturity, scoring them from State 0 to State 3, we concluded that puberty onset was significantly advanced in the high-fat diet groups, especially in the HCO diet group. Thus, for example, rats fed the high-seed oil diet arrived at State 3, the fully mature state, significantly earlier than did the control group. However, when comparing the days spanning between states (1–2, 2–3, and 1–3), no differences were found among groups. Therefore, irrespective of the day of puberty onset, the evolution of the maturity process did not change due to dietary lipids. Moreover, no differences were found due to the diet in the time between the onset of puberty and the first oestrous or in the cycle of the rats. Furthermore, for a specific state, there were no differences in body weight among the groups. However, as already mentioned, the high-seed oil diet group arrived earlier at a specific state than did the low-fat diet group. On the other hand, for a specific day, the high-seed oil diet group had a higher estimated body weight than did the low-fat diet group. The lack of differences in the body weight, when arriving at each stage, is in agreement with the fact that sexual maturity is related to the acquisition of a threshold level of body mass, as has already been reported in humans [81]. The advance in puberty onset due to the effect of the HCO diet was confirmed morphologically. Thus, the histological analysis of ovaries showed a higher number of corpora lutea, indicative of ovule release, in the high-seed oil diet group than in the control at post-puberty (post-natal day 51), suggesting a greater fertility of these animals. Finally, the effect on sexual maturity was also characterised molecularly by means of the analysis of the hypothalamic expression of the marker of puberty kisspeptin-1 (KiSS1) [82]. At post-natal day 36, the high-seed oil diet group tended to have higher levels of KiSS1 than did the low-fat diet group, in accordance with advanced puberty onset in that group. No differences were found at other ages tested since KiSS1 is highly dependent on the oestral phase.

Given that the development of the mammary gland is another target of sexual maturity, we conducted morphological analysis of the mammary glands by identification of the normal epithelial structures (terminal end buds (TEB), terminal ducts (TD), alveolar ducts (AB); and lobules Type 1 (Lob1), lobules Type 2 (Lob2), and lobules Type 3 (Lob3)) at different days of development. However, results showed few differences due to the effect of the high-fat diets. They induced subtle modifications at post-puberty, such as a higher number of differentiated Lob2 s at 51 days, without decreasing the number of undifferentiated structures which are the target of malignant transformation (TEB and TD) [39]. The few

modifications observed in the morphology of the glands at the time of the highest susceptibility to transformation suggest that dietary lipids may modify mammary cancer risk inducing other changes at a molecular level. At the end of the study, the highest mammary gland density was found in the high-seed oil group, followed by the low-fat and the high-olive oil diet groups, but differences were not significant [39].

Definitively, these results suggest that one of the mechanisms by which dietary lipids may differentially modulate breast carcinogenesis at the level of the initiation stage would be their impact on the reproductive state of the individuals. One interesting possibility is that dietary lipids may act by influencing differentially the level of the body-fat stores. Consequently, we began to evaluate this possibility. Firstly, we undertook it by means of the analysis of body-weight evolution and different body mass indexes. The results showed an increased weight throughout the study in the rats fed the high-seed oil diet, in comparison with the ones fed the control diet, whereas the high-EVOO diet, with the same percentage of fat as the HCO diet, did not change body weight or mass, when compared to the control. At the end of the study, body weights and mass indexes were also higher due to the seed oil diet, as compared to the low-fat diet [39]. Several studies have also reported an effect of high-n-6 PUFA diets on body weight, but there is little experimental data regarding the effects of olive oil diets. In this sense, a 30 % EVOO diet has also been shown to produce lower body-weight gain in rats when compared to a 30 % corn oil diet [83]. On the other hand, there are human epidemiological data suggesting that the Mediterranean diet may have a protective effect on obesity [84–86]. The mechanisms by which n-6 PUFA and EVOO diets may differentially affect the body energy balance and, thus, sexual maturity and breast cancer risk, are currently being explored in our laboratory and the results will be part of an independent publication.

5 Conclusions

There is increasing epidemiological and experimental evidence supporting the beneficial effects of olive oil, especially EVOO, which is the major energy source in the Mediterranean diet, in relation to breast cancer and other diseases [87, 88]. The regular intake of EVOO provides a high supply of the MUFA oleic acid, as well as a variety of biologically active compounds such as phenolic antioxidants. These two classes of components, by means of different specific mechanisms, seem to be responsible for the preventive effects observed of olive oil against breast cancer.

In an experimental breast cancer model, we have shown that dietary lipids differentially modulate the clinical behaviour and the histopathological features of the experimental mammary adenocarcinomas. While diets rich in n-6 PUFA have a clear stimulatory effect, which is translated into a tumour phenotype of greater malignancy, high-EVOO diets exert a negative modulatory effect to a weak

promoting effect, conferring to the tumours, in any case, characteristics compatible with lower aggressiveness. Therefore, beyond its energy content, the type of dietary lipid consumed is essential in its final effect on the development and progression of breast cancer. The mechanisms by which EVOO and other dietary lipids may exert their effects are diverse, such as changes in the composition of cell membranes, the modulation of cell-signalling pathways involved in cell proliferation and differentiation, cell survival and apoptosis, and the influence on growth and sexual maturity. Some of the parameters that we have studied, in addition to experimental data published in the literature using lower percentages of olive oil in the diet, suggest that EVOO may have a beneficial effect on breast cancer risk if its consumption is moderated. In fact, the potential chemopreventive effect of EVOO should be considered within the context of the Mediterranean food pattern and lifestyle as a healthy and well-balanced choice from childhood and throughout life.

Acknowledgments The present study was supported by grants from the "Plan Nacional de I + D+I (AGL2006-0769 and AGL2011-24778)"; "Fundación Patrimonio Comunal Olivarero 2008-2012" (FPCO2008-165.396); "Agencia para el Aceite de Oliva del Ministerio de Medio Ambiente y de Medio Rural y Marino 2008-2012" (AAO2008-165.471); "Organización Interprofesional del Aceite de Oliva Español 2009-2013" (OIP2009-165.646); and the "Departaments d'Agricultura, Alimentació i Acció Rural, i de Salut de la Generalitat de Catalunya" (GC2010-165.000). The sponsors had no role in study designs, data collection and analyses, interpretation of results, preparation of the manuscript, the decision to submit the manuscript for publication or the writing of the manuscript. The authors thank Irmgard Costa, M. Carmen Ruiz de Villa and Raquel Escrich for their collaboration in these studies.

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A Holistic Approach to Study the Effects of Natural Antioxidants on Inflammation and Liver Cancer

Susan Costantini, Giovanni Colonna and Giuseppe Castello

Abstract

The limited effectiveness of chemotherapy and the high recurrence rate of cancers highlight the urgent need to identify new molecular targets and to develop new treatments. Numerous epidemiological studies have recently highlighted the existence of an inverse association between fruit and vegetable consumption, natural antioxidants, and cancer risk; in fact, antioxidant intake through diet or supplements of plant origin is strongly recommended for cancer prevention and cure. In general, antioxidants are substances of vegetable, mineral, or animal origin that neutralize free radicals and protect the body from their negative actions on the plasma membrane, proteins, and DNA. Hence, cancer can be prevented by the stimulation of the immune system to destroy cancer cells or to block their proliferation. Since living organisms may be studied as a whole complex system by the "omics sciences" which tend toward understanding and describing the global information of genes, mRNA, proteins, and metabolites, our aim is to use bioinformatics and systems biology to study cytokinome, which plays an important role in the evolution of inflammatory processes and is also a key component in the evolution of cancer, a disease recognized as depending on chronic inflammation and also with the concomitant presence of type 2 diabetes and obesity. On the whole, we define

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_18,

cytokinome as the totality of these proteins and their interactions in and around biological cells. Understanding the complex interaction network of cytokines in patients affected by cancers should be very useful both to follow the evolution of cancer from its early stages and to define innovative therapeutic strategies by using systems biology approaches. In this paper, we review some results of our group in the light of the "omics" logic, and in particular (1) the need for a global approach to study complex systems such as multifactorial cancer and, in particular, hepatocellular carcinoma, (2) the correlation between natural antioxidants, inflammation, and liver cancer, (3) the challenge and significance of the cytokinome profile, (4) the evaluation of the cytokinome profile of patients with type 2 diabetes and/or chronic hepatitis C infection, and (5) adipokine interactome.

Keywords

Systems biology · Complex systems · Cytokinome · Inflammation

Abbreviations

ACE	Angiotensin-converting enzyme
ADIPOQ	Adiponectin
AGT	Angiotensin II
CHC	Chronic hepatitis C
CHD	CHC and type 2 diabetes
CXCL1	Chemokine (CXC motif) ligand 1
CXCL9	Chemokine (CXC motif) ligand 9
GIP	Glucose-dependent insulinotropic peptide
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
IL-1	Interleukin-1
JAK	Janus kinase
LC	CHC-related cirrhosis
LCD	CHC-related cirrhosis and type 2 diabetes
MIF	Macrophage migration inhibitory factor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NR3C1	Nuclear receptor subfamily 3 group C, member 1
PAI-1	Plasminogen activator inhibitor 1
D (POLR2D)	Polymerase (RNA) II (DNA directed) polypeptide
RBP4	Retinol binding protein 4
RELA	V-rel reticuloendotheliosis viral oncogene homolog A
ROS	Reactive oxygen species
SFR5	Secreted frizzled-related protein 5
STAT3	signal transducer and activator of transcription

T2D	Type 2 diabetes
TNF	Tumor necrosis factor
USF1	Upstream stimulatory factor 1

Contents

1 The New Logic of Biology in the "Omics" era

Until the last century, the approach of biological science was to break down the biological object under study into its elementary parts and to analyze all the individual units in order to explain life processes. This was a typical analytical and reductionist procedure, which contributed to the understanding of almost all properties of molecular parts of living organisms, such as genes, proteins, and metabolites. It focused on the study of each single component of the system under consideration but was not able to predict the behavior of the system as a whole. A system can be defined as a number of interacting elements existing within a boundary that is surrounded by an environment. Its properties are dependent on the nature of its component elements. The system is also able to interact with and to respond to external inputs. When the number of interacting components becomes vast, we speak of a "complex system." A striking property of a complex system is that it generates its overall and unpredictable properties (also called emerging properties) from the interactions among its components. In effect, we can say that the system behaves in a complex way when the dynamically interacting parties induce emerging properties such as adaptability, self-organization, and the ability to respond under disturbance. In this way, these nonlinear interactions allow a number of possible states with new emergent behaviors which are not predictable from the simple sum of the component parts. These principles have been applied to study stock markets, ecosystems, and flocks of birds but only recently to biology.

In biology, we have a limited knowledge of the living organism as a whole, and the laws regarding the organizational forces of biological systems are indeed essential to solve and to understand the collective phenomena and the framework

for the functionality of the systems. The "omics sciences" are an initial effort to study the behaviors of the "biology actors" in an environment as a whole, aimed at understanding and describing the comprehensive information of genes (by genomics), mRNA (by transcriptomics), proteins (by proteomics), and metabolites (by metabolomics). Nevertheless, a method able to describe the relationships between different "omics" levels has not yet been developed. The growing quantity of data produced by the biological sciences and by the new techniques of cellular and molecular analysis must be analyzed not only by considering their single components but also by studying the relationships between them. Only in this way it will be possible to avoid those typical, analytical, and reductionist procedures which have already been unable to explain natural phenomena, such as protein folding or some major diseases.

It is now widely accepted that dividing complex systems into smaller components and simpler units is inadequate to understand the organization and onset of an emerging property. It is necessary to focus on the interactions and on the networks between the components in order to reproduce the characteristics of their complexity.

In this context, it is necessary to consider the enormous number of possible connections between the units, on which all possible states of the systems depend. Mathematical tools, such as graphs and neural nodes, are commonly used to understand their functional networks because only in this way we can be obtained a comprehensive view of the metabolic behavior at the basis of different pathological processes such as cancer. Another major concern is that the perturbations deriving from the environment are also able to induce changes in the entire system.

2 A Holistic Approach to Study the Effects of Natural Antioxidants on Inflammation and Liver Cancer

Much has been written about the presence of antioxidants in human nutrition and the ability of these compounds to interact with reactive oxygen species (ROS) produced by oxidative metabolism [1]. Certainly, an antioxidant must interact with cellular metabolic processes to exert its action as well as to be metabolized and eliminated [2]. Enough is known about the specific molecular properties belonging to antioxidants but little is known of their actual physiological benefits and metabolic activity [2]. In fact, many articles deal with the action that antioxidants show locally, in specific cellular areas, often with contrasting results but little is known about their activities and global effects exerted in the wider metabolic network. Generally, we try to understand their actions with epidemiological studies which, by their nature, reveal macroscopic effects on populations of individuals for limited periods of time. What often appears is that these effects are characterized by U-shaped activity curves, with a broad minimum corresponding to the concentration which should be physiologically active, and with the arms of the ascending curve revealing concentrations with no physiological or even toxic

effects [3, 4]. The issue becomes even more complex when combining a diet containing these antioxidants in cancer patients with the intent to prevent or limit the progressive effects of the disease. Since cancer is a biologically multi-etiological disease with different cellular and metabolic specificities which suggest that each cancer is a different disease [5], it complicates our ability to relate the overall effects of dietary antioxidant compounds in patients with cancer.

In fact, the scientific rationale used in the vast majority of studies still heavily reflects the reductionist logic widely used until the early second millennium, which provided us with important information about the properties of individual biological molecules and/or a specific cellular area, but with poorly integrated and overall vision. In the early years of this millennium, thanks to the Human Genome Project [6], biologists developed a holistic logical view of biology, which was adequately supported by the "omics" technologies and the ability to conduct complex statistical analysis of massive amounts of data, under the name of systems biology [7]. In recent years, our research group has been applying this comprehensive approach to hepatocellular carcinoma, a lethal form of cancer with virtually little chance of survival [8], which is widespread throughout the world as well as in the Campania region in southern Italy, where it represents one of the most dangerous cancers.

Our main objective is to study the chronic inflammatory process that leads to liver cancer in the presence or absence of type 2 diabetes and obesity by experimental and computational data [9]. These studies, conducted through a comprehensive approach, take into account not only data derived from various "omics" sciences (genomics, transcriptomics, proteomics, and metabolomics) but also the possible relationships between them in order to obtain and define the complete metabolic network regulating the development and progression of this cancer [7]. In this way, it will be possible to obtain a global comprehensive view and not a reductionist one. By integrating the various "omics" levels related to the hepatocyte under physiological and/or cancerous conditions, it will be possible to have a differential view of the metabolic networks for helping us to identify the stages and alterations due to the progression of the disease, identifying those fundamental relational metabolic nodes that control important physiological functions. Only under these conditions, we can have some hope of using this information for predictive medicine which would allow targeted metabolic or pharmacological approaches by identifying pathological changes in nodes at early stages of the disease. To obtain this result, we have to populate the various levels with specific data for the layer. Figure 1 shows a scheme of our logical approach that we have recently applied to a much simpler biological model. The data that populate the various levels must be integrated in a coherent way with respect to the problem to be solved. The environment (represented mainly from the food components of diet) is seen to exert a perturbation of the cellular system at different levels. A key role is to clearly define the info signaling system of the metabolic network. In fact, it is important to know what types of information are differentially exchanged by normal and pathological cells to understand the progression of a disease and whether food supplements, proposed to counteract oxidative stress, are successful.

For this reason, we use a modern technique defined as multiplex immunoassay, which evaluates the serum levels of about 50 cytokines, chemokines, and growth factors and reveals the dynamics of cytokinome signaling in patients. This is an important approach if hinged on the "omics" logic. The cells recognize the cytokine signals through appropriate receptors placed on their membranes. However, the study of these receptors have certain limitations because (1) only the more abundant receptors were studied, even if very sensitive assays by antibodies and fluorescence (ELISA) were used; (2) receptors showed pleiotropy, that is, they have good affinity for various cytokines, therefore, similar messages can be brought by different cytokines; (3) the meaning of a biological message is known only for some receptors. In particular, we do not know the meaning of all those messages carried out by the underrepresented cytokines (the less concentrated ones at plasma level). We do not also understand whether messages are recognized by the receptor as only redundant or with diverse informative content [10, 11]. Protein chips of considerable and improved sensitivity are now available. They allow the simultaneous determination of several cytokines using a new assay based on a fluorescence/laser/antibody technology (multiplex technology). This assay evaluates very small samples of serum, plasma, or cellular supernatants and is the most accurate, sensitive, and reproducible cytokine assay available. A very interesting aspect of this technology is also to demonstrate quantitatively the presence of the underrepresented cytokines [12, 13]. These cytokine patterns, being part of the new holistic logic, can be indicated as "cytokinome" [14]. Cytokines are involved in cell information signaling as an informative network similar to the Internet, where capillaries connect all the cellular systems of an organism. A comprehensive description of this network is important for understanding the pathogenetic evolution of many chronic inflammatory diseases. However, many questions remain unanswered. In the case of chronic inflammatory diseases, which cytokines develop in the time of the whole cytokine pattern? Does their evolution in time begin in the same way and are they common for all diseases or is pathology correlated and addressed by different types or classes of cytokines? Which cytokinome develops during the disease? Knowing these answers would help to evaluate the dynamics of cytokinomes during the progression of chronic inflammatory diseases but, above all, might predict the prognosis of the disease in advance. Another element of complexity in the cytokine network is also introduced by the fact that some genes encoding cytokines can give rise to variant forms of cytokines (isoforms) by means of alternative splicing, yielding molecules with slight structural differences but biologically significant changes of activity. This explains why it is always useful to analyze the gene expression profile correlated with the cytokines. In fact, previous studies have identified important mutations in some cancers, but they were primarily focused on a limited set of genes and, thus, provided a restricted view of the mutational spectrum. Although correct and comprehensive understanding of cytokine functions can be obtained from simultaneous and coherent measurements of the serum concentrations of cytokines, another advantage in the use of the cytokinome approach is by the technology of multiplexing. The inherent difficulty of independent measurements

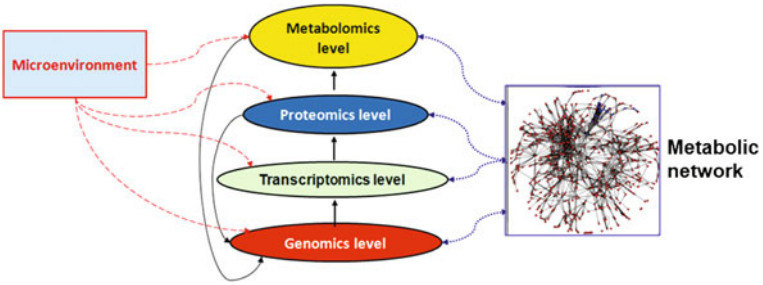

Fig. 1 Scheme of the relationships between the different omics levels

of cytokine concentration to obtain correct internal ratios among the various molecules present in the same biological fluid arises because of the difference in concentrations which span several magnitudes of orders. However, it is now possible to effectively characterize the serum levels of cytokines and reliable internal concentration ratios by using a broad-spectrum bead-based multiplex immunoassay. Therefore, all the data related to cytokine evaluations can be analyzed and modeled computationally by using graphs or networks connecting the various data groups (related to gene and protein expression obtained by microarrays and by multiplex biometric ELISA-based immunoassay) in terms of dynamic probabilistic maps of metabolic and/or physiological activities and/or pathogenetic pathways. Hence, the definition and evaluation of a human cytokinome may become an important tool in the future to analyze the interaction network of cytokines both in healthy individuals and in patients affected by cancer. Using these computational models, it will be easier to understand and investigate how the regression of a chronic inflammation process, by acting on the cellular populations of cytokines, can block the progression of the cancer and how this knowledge can be a useful prognostic and diagnostic tool for clinicians.

3 The Cytokinome Profile of Patients with Type 2 Diabetes and/or Chronic Hepatitis C Infection

The complexity of the metabolic events that lead the liver to progress to hepatocellular carcinoma is still a serious obstacle to assess the molecular mechanisms which drive the evolution of various diseases (hepatitis viruses, diabetes, obesity), which in turn often drive, acting independently or not, the progression to cancer. An initial distinction between the different info-signals that drive the progression of the pathology is therefore important in order to correctly identify their origin and metabolic significance in order to assess their real importance in the progression of the disease.

However, if one does not proceed on this basis, it is virtually impossible to evaluate the metabolic significance of any nutritional supplement. It is evident that the macroscopic or phenotypic effects of these supplements may always be demonstrated but we will never know how they are exerted at the level of the metabolic network. Therefore, our present work is to identify the origin of many pathogenic cytokines, which can be shown by using the panels of multiplexing technology.

In general, both type 2 diabetes (T2D) and chronic hepatitis C (CHC) infection are associated with increased risk of developing hepatocellular carcinoma (HCC) [15, 16]. Cytokines are known to play an important role not only in the mechanisms of insulin resistance and glucose disposal defects but also in the pathological processes occurring in the liver during viral infection [12]. Therefore, serum levels of a panel of numerous cytokines, chemokines, adipokines, and growth factors were evaluated at the same time by BioPlex assay in patients with chronic hepatitis C (CHC), CHC-related cirrhosis (LC), type 2 diabetes (T2D), CHC and type 2 diabetes (CHD), CHC-related cirrhosis and type 2 diabetes (LCD) and in healthy controls, to identify those molecules that might be useful for discriminating the various stages of these diseases [9].

The results obtained showed that expressions of β-NGF, CXCL1, CXCL9, C-peptide, GIP, and adiponectin were up-regulated in all the patient groups except in those with T2D, suggesting that they may be associated with CHC infection leading to fibrotic, cirrhotic, and cancer progression, also in the presence of type 2 diabetes (CHD and LCD). Moreover, since type 2 diabetes has been recognized as a cofactor that can modify the course of CHC infection and can be used as an independent predictor of HCC [16], the significant molecules in patients with T2D and CHC were compared. In particular, it has been shown that (1) ghrelin and leptin levels are lower in CHC patients than in those with T2D, (2) IL-1α, insulin, and PAI-1 levels are higher in CHC patients than in those with T2D, (3) CXCL1, CXCL9, β-NGF, C-peptide, GIP, and adiponectin are higher only in CHC patients, and (4) HGF and glucagon are higher only in T2D patients.

All these molecules were analyzed by Ingenuity Pathway Analysis 7.1 (Ingenuity Systems, Inc., Redwood City, CA, USA), which created a network on the basis of associated functions and data mining from experimental studies reported in the literature (Fig. 2). This graph presents five hub genes such as signal transducer and activator of transcription 3 (STAT3), NR3C1 (nuclear receptor subfamily 3, group C, member 1), NF-κB (called also RELA), TP53 (tumor p53), and upstream stimulatory factor 1 (USF1). In particular, STAT3 is induced from the leptin [17] and has a role in suppressing IFN induction of STAT1-dependent inflammatory genes, such as CXCL9 [18]. Therefore, it was possible to hypothesize that in T2D patients, the higher level of leptin induced the STAT3 increased expression and the related CXCL9 suppression. On the other hand, NR3C1 is the receptor to which cortisol and other glucocorticoids bind and interact with STAT3 and RELA [19]. The network reported in Fig. 2 shows that RELA modulates the expression of TP53, MIF, CXCL9, and IL-1α, whereas TP53 regulated that of CXCL1, IL-2R, IL-1α, MIF, PAI-1 (called also SERPINE1), and IL-18. Therefore,

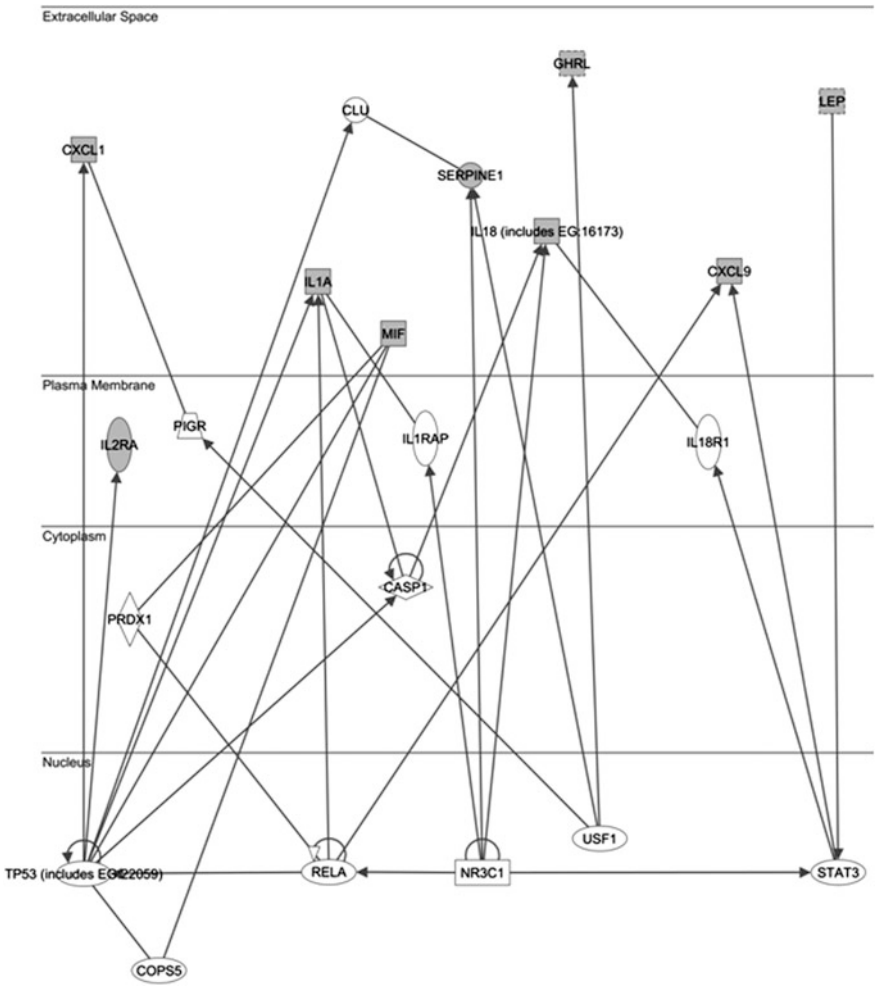

Fig. 2 From [9]. The graph shows the closely associated network for significant cytokines in T2D and CHC patients (evidenced with *gray symbols*) and other molecules (evidenced with *white symbols*) obtained by ingenuity pathway analysis software

this can explain why CXCL1, IL-1α, IL-18, and PAI-1 as well as CXCL9 are higher in CHC patients. As regards USF1, it is involved in the regulation of numerous genes of glucose and lipid metabolism as well as hepatic metabolism. Expression of USF1 was down-regulated during the progression of liver fibrosis in CHC patients [20] and this explains the lower ghrelin expression in CHC patients with respect to those with T2D. Moreover, it is worth noting that HGF has been found at normal values only in CHC patients. This growth factor stimulates mitogenic, motogenic (including metastogenesis), and morphogenic effects on epithelial and endothelial cells via its receptor, proto-oncogene Met. The

activation of Met by HGF binding is linked to cell growth and survival, through activation of both the PI3-kinase/PDK/Akt and the Ras/Raf/MEK/ERK pathways, and to cell mobility and cytoskeletal organization via activation of the Rho-GTPases, Rho, Rac, and CDC42. HGF and Met have been associated with progression, invasiveness, and metastasis in a number of cancer types, and for these reasons, their signaling is a major target for the development of cancer therapeutics [21]. Hence, HGF increased levels in all patient groups except in CHC suggested that this protein can be used as an index for predicting, in patients with T2D, the progression of chronic inflammation to cancer [13]. Overall, this study has demonstrated that the simultaneous quantitative determination of a large panel of cytokines, is able to report the correct ratios and dynamics between highly and poorly represented molecules, and represents an accurate, simple, specific, non-invasive, reproducible, and inexpensive method that, in future, could be included in routine clinical practice to monitor the association of type 2 diabetes and/or CHC to liver cirrhosis and, possibly, to cancer, and to improve the prognosis of these diseases.

4 Adipokine Interactome

Adipose tissue is now known to express and secrete a variety of adipokines, which act at both the local (autocrine/paracrine) and systemic (endocrine) levels. In addition to these efferent signals, adipose tissue expresses numerous receptors that allow it to respond to afferent signals from traditional hormone systems. Besides the biological repertoire necessary for storing and releasing energy, adipose tissue contains the metabolic machinery to permit communication with distant organs. Through this interactive network, adipose tissue is integrally involved in coordinating a variety of biological processes including energy metabolism, neuroendocrine function, and immune function [22]. Recently in our group, we started to study the complex interaction network of the adipokines that represents a possible link between type 2 diabetes, obesity, and cancer [23]. This interconnected network has been extracted from the whole human proteome collected by public databases such as Biogrid, HPRD, MINT, and Pathway Interaction Database and manually curated and updated by the Center for BioMedical Computing (CBMC) at the University of Verona (the earlier version contains 11,120 nodes) [24]. The network of the adipokines is composed of 729 nodes (proteins) with 21,289 edges (interactions) with two isolated components such as ADIPOQ and SFRP-5 direct interactants. The connected component of this network comprises 724 nodes and 21,286 interactions with an average clustering coefficient of 0.586. In particular, the evaluation of the average clustering coefficient distribution identifies a modular organization of metabolic networks and the value related to the network heterogeneity (variance of the connectivity) reflects the tendency of a network to contain hub nodes.

In a network, the bottlenecked proteins represent key connectors and, hence, important and essential nodes of the network [25], whereas the betweenness centrality is appreciated as the amount of traffic that a vertex or edge has to handle in a network [26] and the stress centrality indicates a high number of shortest paths [27]. Therefore, the degrees of bottleneckness, betweenness, and stress were calculated to characterize the important and essential nodes in the network. In particular, only angiotensin II (AGT) among all the adipokines showed a high degree of these values. Concerning AGT first-order interactome, we found that this adipokine interacts with 19 proteins and 27 interactions and regulates numerous metabolic processes related to the regulation of arterial blood pressure. Moreover, angiotensin-converting enzyme 2 (ACE2) can be considered a weak link between AGT and ghrelin that in turn interacts with leptin and IL-6. Furthermore, in the IL-6 network, there are some components, such as JAK1, JAK2, STAT1, and STAT3, that are involved in the regulation of phosphorylation of other adipokines, for example TNF and omentin. Moreover, the detailed analysis of the adipokine network showed that POLR2D (namely RBP4) was another highly clustered component of the network with an average coefficient of 0.840. It interacts with 257 proteins by 10,920 interactions, some of which are related to different cellular activities such as RNA splicing, metabolic paths, gene expression, and DNA repair. In particular, many adipokines such as IL-6, IL-8, adipsin, TNF, and resistin interact by second-order degree with RBP4. All the data demonstrated that the adipokines interact with each other, create a complex network, and demonstrate that they work together to promote obesity and cancers [23].

5 Conclusions

Numerous epidemiological studies have highlighted the existence of an inverse association between consumption of fruit and vegetables rich in natural antioxidants and the risk of cancer. In fact, the intake of antioxidants through diet or taking supplements of plant origin has been strongly recommended for cancer prevention and treatment (Storner and Mukhtar 1995). In general, antioxidants are substances of vegetable origin and, in some cases, mineral or animal origin that can neutralize free radicals and protect the body from their negative actions on the plasma membrane, proteins, and DNA [28, 29].

However, we have often noted that studies on antioxidant supplements which focused only on detailed metabolic effects with a very limited metabolic view are poorly connected with the broad metabolic network which is present in the whole organism and its physiological meaning, if it exists at all, is wasted. For this reason, we believe that a systems biology approach is the best way to overcome this limitation, even if it takes more time, is much more complex because of the numerous skills needed, and, above all, requires rigorous computational analysis.

Our studies have focused on the understanding of the metabolic network that supports hepatocarcinoma progression and, because there has been a resurgence of interest in the connection between inflammation, type 2 diabetes, obesity, and cancer over the past few years, we believe that cytokines are among the molecules that play an important role in the evolution of these processes. In fact, they are proteins that are expressed before and during the inflammatory process and play a key signaling role at various levels of disease, so that they can be considered as specific markers of cancer and of its specific evolutionary stages [12, 13]. Recently, the serum levels of many cytokines, chemokines, adipokines, and growth factors in patients with type 2 diabetes, CHC, CHC-related cirrhosis, CHC and type 2 diabetes, and CHC-related cirrhosis and type 2 diabetes have been evaluated by a broad-spectrum bead-based multiplex immunoassay. The data obtained has shown that the serum levels of some proteins are significantly up-regulated in all the patients or in those with only one disease and are often higher, even if in different amounts, when both diseases are associated. Moreover, some molecules have shown significant correlations with clinical/biochemical data, suggesting the possibility of defining mini-panels that can be used as specific markers for the different stages of the disease. However, all these observations have demonstrated that an integrated approach is much more powerful than isolated measurements to evaluate specific stages of these two complex pathologies (type 2 diabetes and CHC) alone or when they are concomitant in a patient [9].

Therefore, we emphasize that all the data related to cytokine evaluation should be modeled computationally by using graphs or networks connecting the various data groups in terms of dynamic probabilistic maps of metabolic and/or physiological activities and/or pathogenetic pathways. In fact, only in this way, it is possible to define the human cytokinome that may be a useful tool to analyze the interaction network of cytokines both in healthy individuals and in patients affected by different diseases [14].

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Extra Virgin Olive Oil: From Composition to "Molecular Gastronomy"

Raffaele Sacchi, Antonello Paduano, Maria Savarese, Paola Vitaglione and Vincenzo Fogliano

Abstract

The aim of this chapter is to provide a brief overview of the recent results of studies on extra virgin olive oil (EVOO) and its interactions with other food ingredients during cooking, to highlight basic molecular aspects of the "magic" of EVOO and its role in Mediterranean gastronomy. The use of raw EVOO added to foods after cooking (or as a salad oil) is the best way to express the original flavour and to maximize the intake of natural antioxidants and compounds related to positive effects on human health (hypotensive, anti-inflammatory, and anti-cancerogenic, among others). EVOO, however, also exhibits its protective properties during/after cooking. Different chemical interactions between biophenolic compounds and other food ingredients (water, milk proteins, carotenoids of tomato, omega-3 polyunsaturated fatty acids in canned-in-oil fish and meat or fish proteins) occur. Even during cooking, EVOO exhibits strong antioxidant properties and influences the overall flavour of cooked foods. The physical (partitioning, emulsion) and chemical (hydrolysis, covalent binding, antioxidant properties) phenomena occurring during cooking of EVOO are discussed with emphasis on the changes in the sensory (bitterness and fruity flavour) and nutritional qualities of some traditional Mediterranean foods. In particular, tomato–oil interactions during cooking, fish canning in EVOO, meat marinated in EVOO before cooking and roasting and frying in EVOO are examined. The interactions between EVOO antioxidants and flavours with milk proteins are also briefly discussed.

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_19,

Keywords
Extra virgin olive oil · Molecular gastronomy · Mediterranean diet

Abbreviations

EFSA	European food safety authority
EVOO	Extra virgin olive oil
HA	Heterocyclic Amine
HPLC	High-performance liquid chromatography
HPTA	Hydroxy pentacyclic triterpene acids
LC–MS	Liquid chromatography–Mass spectrometry
NMR	Nuclear magnetic resonance

Contents

1 Olive Oil Composition and Healthy Effects

In the last few years, several papers have been published correlating the in vitro and in vivo positive actions on human health of extra virgin olive oil (EVOO) with its chemical composition [16, 31]. EVOO health properties have been attributed to both the high level of oleic acid in the triacylglycerol profile, and the many different minor compounds (squalene, tocopherols, phenolic compounds such as phenyl alcohols, secoiridoids, and lignans) present in the unsaponifiable fraction of EVOO, and these properties in turn have been related to preventive effects on human health [16]. Antimicrobial, antioxidant, and anti-inflammatory activities have principally been associated with EVOO (for a review see [9]). As regards EVOO anti-inflammatory benefits, particular attention has been paid to the phenolic compound named oleocanthal (for a review see [18]). It was demonstrated to have ibuprofen-like activity, as both compounds inhibit the same cyclooxygenase enzymes in the prostaglandin-biosynthesis pathway [5]. However, a scientific

demonstration that oleocanthal, in the amount contained in EVOO, is the only compound responsible for the anti-inflammatory effect of VOO is still lacking [14]. In fact, mounting evidence in food science and health research indicates that it is the complex mixture of polyphenols in foods, more than individual compounds, that can synergistically act towards a final health effect. In the case of EVOO, this concept has been demonstrated for pinoresinol and colon cancer [12]. The authors reported that pinoresinol-rich EVOO extracts have potent chemopreventive properties in colon cancer cells and that this result was achieved at substantially lower concentrations in EVOO than with purified pinoresinol [12].

Recently the European Food Safety Authority (EFSA) Panel on Dietetic Products, nutrition and allergies (NDA) provided the scientific opinion on: *scientific substantiation of health claims in relation to polyphenols in olive and protection of LDL particles from oxidative damage, maintenance of normal blood HDL-cholesterol concentrations, maintenance of normal blood pressure, anti-inflammatory properties, contributes to the upper respiratory tract health, can help to maintain a normal function of gastrointestinal tract, and contributes to body defences against external agents. The food constituent, which is the subject of the health claims, is polyphenols in olive (olive fruit, olive mill waste waters or olive oil, Olea europaea L. extract and leaf). On the basis of the data presented, the Panel concludes that a cause and effect relationship has been established between the consumption of olive oil polyphenols (standardised by the content of hydroxytyrosol and its derivatives) and protection of LDL particles from oxidative damage. The Panel considers that in order to bear the claim, 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) in olive oil should be consumed daily* [11].

Together with chemical stability towards oxidation processes (high oxidation stability) due to antioxidant capacity, phenolic compounds in EVOO also contribute to its bitter and pungent taste. The phenol compounds, the natural volatile compounds with the fruity aroma, make EVOO both a "functional food" and a "food flavour". EVOOs, however, show a great variability in composition and sensory properties due to the olive variety, environment, growth, degree of ripening, extraction and storage technology [31]. There is a large variation in the EVOOs on the market in terms of their chemical and sensory qualities, in the range of their nutritional properties and of their price. In particular, food chemists and panels of experts agree that the more bitter EVOOs have a higher quality, so EVOO taste may be a good driver for consumers to choose products with healthy characteristics [36].

Changes in phenolic and volatile compounds also arise during EVOO cooking, as well as many interactions with other ingredients used in Mediterranean food preparations. While there are several studies on the effects of agronomic and technological parameters on the quality of olive oil, only a few papers have analysed its culinary aspects. The aim of this paper is to make a brief overview of the recent results on this subject and to highlight key molecular characteristics to explain the "magic" of EVOO and its role in Mediterranean gastronomy.

2 EVOO in Mediterranean "Molecular Gastronomy"

The use of raw EVOO added to foods after cooking (or as a salad oil) is the best way to express the original flavour and to maximize the intake of natural antioxidants and EVOO compounds associated with positive effects on human health (hypotensive, anti-inflammatory, and anti-cancerogenic, among others). EVOO, however, also exhibits its protective properties when used in cooking. Chemical interactions between biophenolic compounds and other food ingredients (water, milk proteins, carotenoids of tomato, omega-3 polyunsaturated fatty acids in canned-in-oil fish and meat, or fish proteins) have been investigated in many studies. Even during cooking, in fact, EVOO exhibits strong antioxidant properties and influences the overall flavour of cooked foods. The physical (partitioning, emulsion) and chemical (hydrolysis, covalent binding, antioxidant properties) phenomena occurring when EVOO is cooked with other food ingredients involves changes in the sensory (bitterness and fruity flavour) and nutritional qualities of some traditional Mediterranean foods. Molecular interactions also occur during cooking between phenolic compounds of EVOO and other compounds in different cooked food systems, thus increasing the healthy and protective effect of some cooked foods. They will be discussed in detail in the following paragraphs.

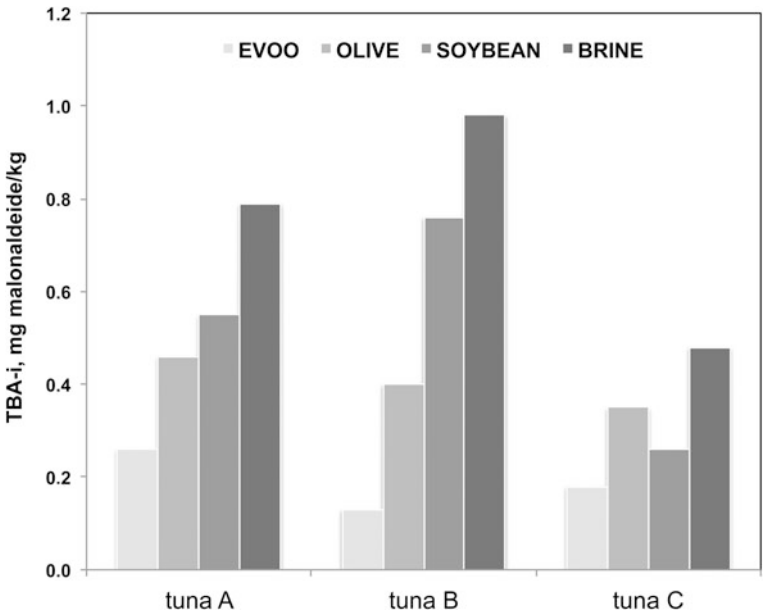


Fig. 1 Oxidation in three different canned tuna sterilized with different filling media (TBA-i, mg malonaldeyde/kg)

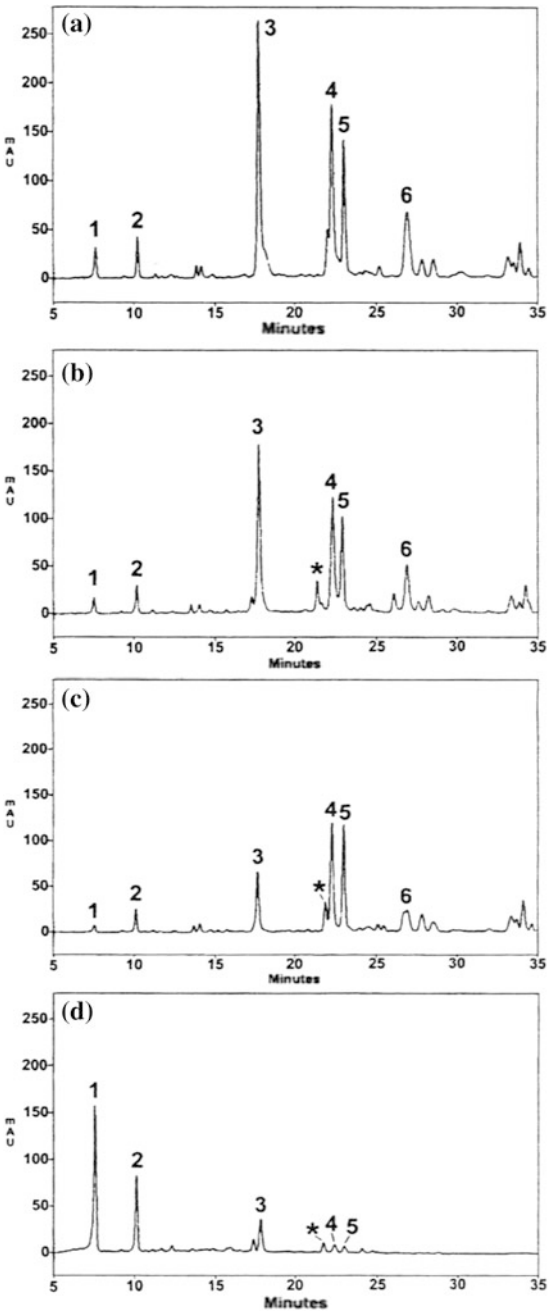


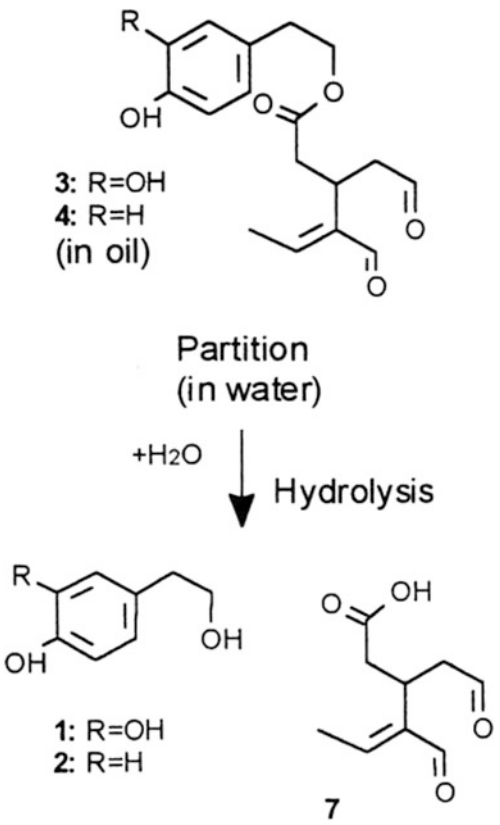
Fig. 2 Behaviour of phenolic antioxidant compounds during sterilization: (**a**) initial EVOO; (**b**) sterilized EVOO; (**c**) oily phase after sterilization; (**d**) water phase after sterilization. Peaks identification: hydroxytyrosol (*1*), tyrosol (*2*), dialdehydic form of decarboxymethyl oleuropein (*3*) and ligstroside (*4*) aglycons, pinoresinol (*5*), aldehydic form of oleuropein (*6*), * unknown compound

2.1 EVOO in Fish Canning

The interactions between EVOO used as filling oil and canned tuna muscles have been studied in several of our experiments, with the aim of preserving the intake of native n-3 PUFAs present in fresh fish muscle before tuna canning.

The level of n-3 PUFAs measured by proton NMR spectroscopy was significantly higher in tuna canned in EVOO compared to those recovered in tuna muscles canned and sterilized in soybean or refined olive oil, as well as in brine [21, 22]. The level of oxidation found in lipids extracted from canned tuna muscles was also lower in EVOO (Fig. 1).

The protective effect of EVOO during/after the thermal treatment of the cans can be attributed to the properties of natural antioxidants of EVOO which are not present in other filling oils or brine [13, 23]. In particular, by monitoring the level of phenol compounds in filling oils before and after the sterilization of the cans, a clear partitioning from the oil phase towards the water phase (muscle) was observed, combined with an hydrolysis of secoiridoid aglycons (Fig. 2 and Scheme 1) [7, 32].



Scheme 1 Formation of hydroxytyrosol (*1*), tyrosol (*2*), and dialdehydic form of decarboxymethyl elenolic acid (*7*) in brine from the dialdehydic form of decarboxymethyl oleuropein (*3*) and ligstroside (*4*) aglycons during the sterilization of virgin olive oil–brine mixtures

The combination of these two phenomena (partitioning and hydrolysis) leads to the accumulation of hydrophilic phenolic antioxidants (hydroxytyrosol and tyrosol) on the muscle surface, protecting the n-3 PUFA from thermal oxidation.

2.2 Tomato–EVOO Interactions

Fresh tomato and tomato products are characterized by a healthy value and anti-tumour activity, especially towards prostate cancer [28]. The intake of carotenoids (lycopene) has been related to anti-carcinogenic activity and anti-atherogenic effects both in vitro and in vivo [27]. Several epidemiological studies have suggested that a high consumption of tomatoes and tomato products containing lycopene may protect against CVD and reduce risk of several types of cancer, most notably those of the prostate, breast, lung, and digestive tract. Serum and tissue levels of lycopene have also been inversely related to a risk of chronic disease [27]. In the human diet, both fresh and transformed tomatoes are consumed around the world but the typical (Italian) Mediterranean gastronomy is characterized by a substantial use of tomato and olive oil in combination with pasta and pizza.

In recent years, we have studied the behaviour of different model systems simulating the cooking of tomato sauces in combination with small quantities of EVOO. In particular, a traditional tomato sauce preparation (i.e. the *Neapolitan*

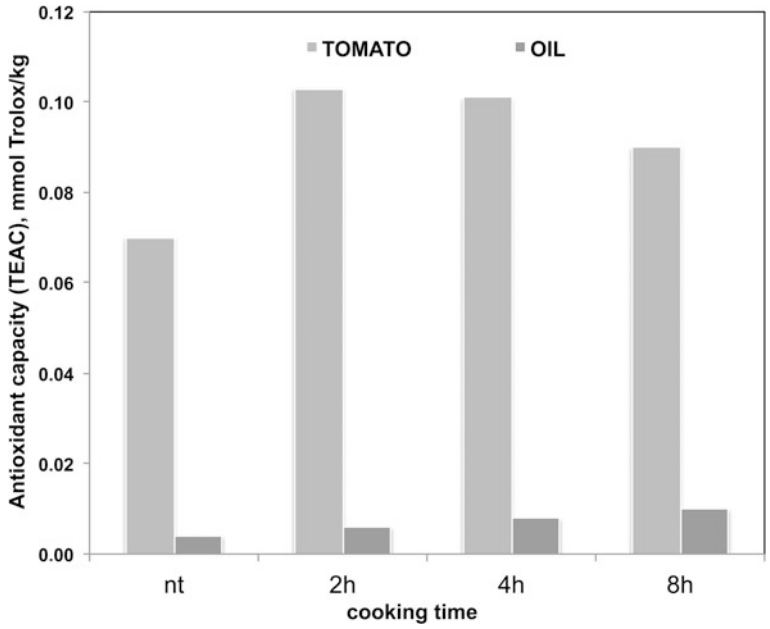


Fig. 3 Antioxidant capacity (TEAC, mmol Trolox equivalents/kg) of tomato and oil phases during cooking of a tomato–EVOO sauce submitted to heating test

Ragù) requires lengthy cooking (6–10 h) at medium–low temperatures (70–80 °C) in traditional earthenware pots. What happens when a tomato–oil system is heated in these conditions? A loss in the antioxidant content can be expected at the end of cooking, and this has been verified in tomato sauce submitted to a heating test [29]. On the contary, we observed a significant increase in the antioxidant properties during cooking with EVOO with a protective action of secoiridoids present in virgin olive oil on tomato carotenoids (Fig. 3).

The partitioning of carotenoids towards the oil phase (Fig. 4), as well as that of some flavonoids such as naringenin, was also observed during cooking and this was also related to the carotenoid bioavailability in these food preparations.

These observations and data can be of interest in relation to cancer prevention properties of certain *traditional functional foods* such as *Ragù*. These traditional preparations can be considered very healthy as they allow the intake of molecules having both antioxidant and protective roles.

2.3 Meat Marinating in EVOO Before Roasting

Another traditional process in the Mediterranean style of cooking is marinating meat and fish in oil, wine, and herbs (oregano, rosemary) before roasting and the use of an oil–lemon juice or oil–red wine emulsion during the cooking to wet the

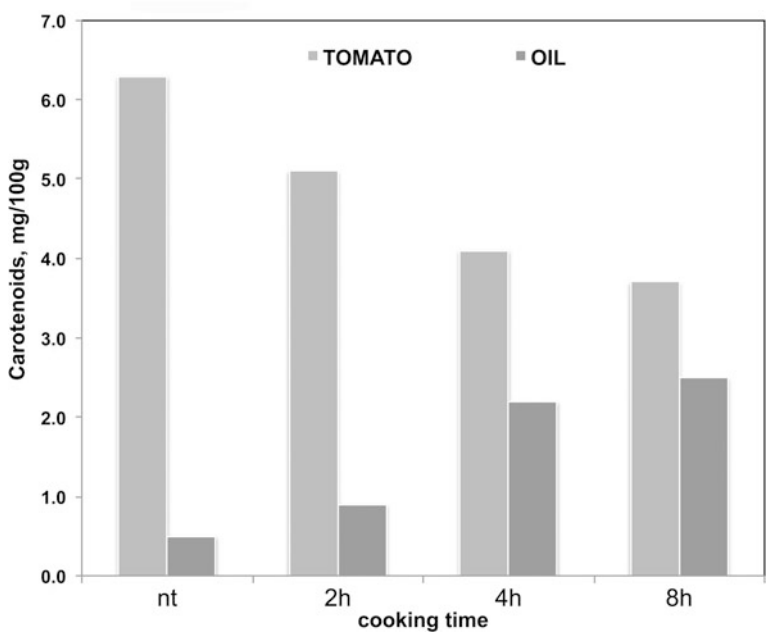


Fig. 4 Carotenoid content (mg/100 g) of a tomato–EVOO sauce submitted to heating test

meat/fish surface. This tradition, in part lost in the home and in restaurant practice, has been demonstrated to be related to a protective effect on protein degradation during the thermal treatment of cooking [25, 30, 35].

When phenol compounds or EVOOs are added to model systems simulating cooking, they both show a relevant inhibition in heterocyclic amine (HAs) formation. Mutagenic HAs are formed at low levels during cooking of meat and fish, and some of them are considered to be possible human carcinogens. The formation of HAs may be affected by the presence of synthetic or naturally occurring antioxidants. Monti et al. [25] studied the effect of EVOO phenolic compounds, identified and quantified by LC–MS, on the formation of HAs in a model system. An aqueous solution of creatinine, glucose, and glycine was heated in the presence of two samples of EVOO differing only in the composition of phenolic compounds. The addition of EVOO to the model system inhibited the formation of 2-amino-3-methylimidazo[4,5-f]quinoxaline, of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline and of 2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline by between 30 and 50 % compared with the control. Fresh-made olive oil, which contained large quantities of dihydroxyphenylethanol derivatives, inhibited HA formation more than a 1-year-old oil did. The inhibition of HA formation was also verified using phenolic compounds extracted from VOO, demonstrating that EVOO can play an interesting functional role during the cooking/roasting of meat and fish by preventing the formation of potential carcinogenic molecules.

Interestingly, the presence of EVOO phenolic compounds is able to reduce the formation of acrylamide even in low moisture systems such as biscuits [4].

2.4 Frying in EVOO

Frying in EVOO can also minimize the production of potentially toxic compounds (such as acrylamide and hydroxy-alkenals), as well as the uptake of healthy biophenolic antioxidants in the crust of fried-in-EVOO foods. The phenolic compounds of EVOO are quite stable during frying and have been found even after several hours of frying [1, 10], and they can interact with the food matrix inhibiting the formation of dangerous compounds.

The relationship between EVOO phenol compounds and the formation of acrylamide in potato crisps was first investigated by Napolitano et al. [26]. The phenolic composition of 20 EVOO samples was screened by LC–MS, and four oils, characterized by different phenol compound patterns, were selected for frying experiments. Slices of potatoes were fried at 180 °C for 5, 10, and 15 min in EVOO, and the acrylamide content was determined by LC–MS. EVOO phenolic compounds are not degraded during frying and the colour of the crisps was not significantly different among the four EVOOs. Acrylamide concentration in crisps increased during frying time, but the formation was faster in the oil having the lowest concentration of phenolic compounds. Moreover, the EVOO having the highest concentration of ortho-diphenolic compounds was able to efficiently

inhibit acrylamide formation in crisps cooked in mild to moderate frying conditions. The use of ortho-diphenolic-rich EVOOs was proposed as a reliable mitigation strategy to reduce acrylamide formation in domestic deep-frying.

Hydroxy-alkenals are other potentially toxic and carcinogenic compounds arising from the thermal decomposition of PUFAs during frying [15]. We applied high-resolution proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy (400–600 MHz) to the quantitative analysis of hydroperoxide decomposition products in thermally oxidized oils [33]. Different oils (EVOO, sunflower, and soybean) were heated in a thermostatic bath fryer (180 °C for 360 min). Proton NMR quantitative aldehyde analysis (*n*-alkanals, *trans*-2-alkenals, 4-hydroxy-*trans*-2-alkenals, alka-2,4-dienals) of fifteen oil samples (0, 60, 120, 240, and 360 min heating) was compared with that of total polar compounds through silica column chromatography and of short-chain fatty acids determined by gas chromatography. The formation of the different aldehydes during 6 h of heating was followed in the three oils. Alka-2,4-dienals and *trans*-2-alkenals were the major products detected by NMR in all heated oils. The production of *trans*-2-alkenals was almost the same in EVOO and seeds oils, while *n*-alkanals were produced at the end of the heating in a larger amount in EVOO.

The 4-hydroxy-2-alkenals, in particular, were not detected (threshold 0.1 mM/L) in EVOO after 6 h of heating. The formation of these compounds is, in fact, related to the decomposition of conjugated hydroperoxydienes arising from the oxidation of polyunsaturated fatty acids [15]. In olive oil, the low amount of linoleic (5–10 %) and linolenic (less than 1 %) acids explains these findings. For the same reason, alka-2,4-dienals were formed in small amounts (1.1 mM/L oil after 6 h heating) with respect to polyunsaturated seed oils.

Other studies focused on the uptake of phenol compounds in fried-in-EVOO foods. The crust of French fries, when EVOO is used as frying oil in continuous frying, was demonstrated to absorb a significant amount of phenol compounds that can be extracted and quantified by LC–MS from the fried potatoes [34]. Kalogeropoulos et al. [19] studied the behaviour of finfish, representing the most popular fish species in Greece, during pan-frying in virgin olive oil. Analyses for polyphenols, hydroxy pentacyclic triterpene acids (HPTA), and α-tocopherol were performed in the fresh and fried oils and fish. Nine polyphenols were determined in the frying oil samples; six of them were also found in fried fish. The terpenic acids, oleanolic, maslinic, and ursolic, were also determined in frying oils and fried fish. Besides water loss and oil absorption, pan-frying caused the partial loss of all the antioxidants studied in the fried oils, as well as their enrichment in the fried fish. The polarity of the antioxidants studied, affected to some extent their partition between the frying oil and the water-containing fish.

The same authors [20] studied potatoes, green peppers, courgettes, and eggplants shallow fried in EVOO according to Mediterranean traditional culinary practice. Courgettes and eggplants were also blanketed with wheat flour or batter prior to frying. Among 12 polyphenols determined, tyrosol predominated in frying oils and courgette samples, while chlorogenic acid was the major phenolic species in the other vegetable samples. Besides water loss and oil absorption, shallow

frying resulted in partial loss of all the antioxidants studied in frying oils and enrichment of fried vegetables with olive oil antioxidants, which was to some extent affected by the type of vegetable fried and the culinary practice followed. The overall retention of the antioxidants in oil and food ranged from 32 to 64 % for α-tocopherol, 25–70 % for polyphenols, and 35–83 % for HPTA. It appears that vegetables fried in EVOO provide an additional intake of α-tocopherol, terpenic acids, and polyphenols such as tyrosol and chlorogenic acid.

The performance of virgin olive oil and a commercial vegetable shortening was also investigated by Andrikopoulos et al. [2] during 10 successive pan-fryings of potatoes at 180 °C for a total period of 60 min and during 10 successive deep-fryings at 170 °C for a total period of 120 min. For both the oils tested, the effect of pan-frying was worse than the effect of deep-frying. The same was true for visible spectrum and total phenols in virgin olive oil. Both oils performed similarly during pan-frying, while virgin olive oil performed better during deep-frying. A very strong correlation between octanoic acid formation and total polar artefacts in the whole data set was observed.

Andrikopoulos et al. [3] also submitted virgin olive oil, sunflower oil, and a vegetable shortening to deep-frying and pan-frying of potatoes, for eight successive sessions, under the usual domestic practice. The frying oil absorption by the potatoes was quantified within 6.1–12.8 %, depending on the oil type and the frying process. The retention of total phenolics ranged from 70 to 80 % (first frying) to 20–30 % (eighth frying). Tannic acid, oleuropein, and hydroxytyrosol-elenolic acid dialdeydic form showed remarkable resistance in all frying sessions in both frying methods, while hydroxytyrosol and hydroxytyrosol-elenolic acid were eliminated faster. The deterioration of the other phenolic species accounts for 40–50 and 20–30 % for deep-frying and pan-frying, respectively, after three to four frying sessions, which are the most usual in the household kitchen.

The migration of health promoting micro-constituents from frying vegetable oils to French fries was also studied by Chiou et al. [8], who analysed the behaviour of vitamin E in this thermal process.

2.5 EVOO and Milk Proteins

Interactions between olive oil compounds and milk proteins can also affect the perception and nutritional quality of the food matrix. Meynier et al. [24] demonstrated that hexanal and t-2-hexenal, volatile compounds found in high amounts in EVOO, form covalent bonds with whey proteins and sodium caseinate, causing changes of amino acid composition of proteins in the presence of oxidized lipids. A similar behaviour can be observed from the interaction between EVOO phenolic compounds and milk protein, with a decrease in the perceived bitterness and pungency in these food systems. Another important issue is the perception of volatile and non-volatile (biophenols) compounds in relation to oil-in-water emulsions. In the case of emulsions, hydrophobic flavour components can be

perceived at lower concentrations in water than in oil, and many of the lipid oxidation products show high solubility in the oil phase [6].

In food systems in which EVOO and milk proteins are present, interactions cause strong modifications of the sensory properties of EVOO, with an evident loss of bitterness and pungency.

3 Conclusions

The scientific evidence briefly discussed here has demonstrated an active functional role for EVOO, not only as a source of antioxidants as raw ingredients in the Mediterranean diet but also in protecting other food components during cooking.

Several modifications and positive interactions with other components (carotenoids, omega-3 PUFAs, and proteins) occur, and foods cooked with EVOO can improve their nutritional quality and anti-carcinogenic effects, which has been well demonstrated for cooked tomato–EVOO mixtures [17].

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Part V
Life Style Prevention of Cancer

Life Style Prevention of Cancer Recurrence: The Yin and the Yang

Franco Berrino

Abstract

There is increasing evidence that lifestyle after the diagnosis of cancer may affect prognosis. Several studies have shown that a Western dietary pattern, obesity, weight gain, a sedentary lifestyle, metabolic syndrome, high serum levels of insulin, growth factors, and inflammatory cytokines after the diagnosis of cancer are associated with an increased incidence of recurrences. Most studies have been on breast and colon cancer. However, in the clinical management of cancer, little attention is presently paid to improving lifestyle and controlling body weight. Lifestyle intervention trials are needed to corroborate or confute the observational results on cancer recurrences, but, even now, there is no contraindication to promoting moderate physical exercise, moderate calorie restriction (CR), and a Mediterranean dietary pattern. In fact, the AICR/WCRF 2007 systematic literature review recommends cancer patients to adopt the lifestyle recommended for the prevention of cancer. Interestingly, the evidence-based AICR/WCRF recommendations coincide with traditional rules, based on far Eastern philosophy, of avoiding extremely *yin* food, such as sugared beverages and calorie-dense foods, and extremely *yang* food, such as processed meat, and relying on the equilibrium of slightly *yang* food, such as whole-grain unprocessed cereals, eaten with slightly *yin* food, such as legumes and vegetables.

Keywords

Macrobiotic diet · Lifestyle · Mediterranean dietary pattern · Breast cancer

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_20,

Abbreviations

CMF Cyclophosphamide/methotrexate/5-fluorouracil
CR Calorie restriction
MS Metabolic syndrome

Contents

1 Introduction

Worldwide, we are entering a period of unprecedented high prevalence of age-related chronic non-communicable diseases, whose related disabilities are projected to inflate into an unprecedented economic and social challenge. Human chronic diseases are complex non-linear processes that depend on a large number of interconnected genetic and metabolic pathways which should be tackled with a many faceted preventive strategy. In animals, calorie restriction (CR) is the most potent dietary intervention for preventing cancer and other age-related chronic diseases, and for prolonging life. In humans, calorie-dense diet and sedentary lifestyle are responsible for the growing prevalence of metabolic syndrome (MS), which, together with tobacco, is the major preventable cause of the most prevalent chronic diseases, mediated by the increased availability of insulin, growth factors, and inflammatory cytokines. There is increasing evidence that MS is also a major determinant of cancer recurrence [6, 44, 46, 49, 55, 59, 60]. We and others have shown that a sustainable CR, associated with decreased prevalence of MS, can be obtained through a comprehensive change in dietary habits, reducing animal food and refined carbohydrates, and increasing whole-grain cereal products, legumes, and vegetables [2, 13]. Chronic diseases are largely due to modifiable Western lifestyle factors. To date, however, investment in promoting potentially preventive and sustainable lifestyle modification is disproportionately low relative to its potential return.

Progress in cancer and other chronic disease biology has been extremely fast in the last few decades, emphasizing the relevance of complexity as opposed to the previously dominant reductionist view of nature, which has been very productive

for the prevention of cardiovascular diseases (based on treatment of specific risk factors) but largely ineffective for cancer. The cancer chemoprevention experiments carried out so far in humans do not seem to have paid much attention to this evolving view of complexity. Most were based on the supplementation or the avoidance of a single or a few nutrients, and most failed or ended with dubious results. Multi-faceted intervention is probably needed to favourably affect the complex biological systems involved in the development of cancer. A single agent or factor acting on a single or a few pathways might actually fail because of the existence of redundant vicarious pathways, or even be dangerous because of its interference with potentially preventive pathways.

2 The "Yin" Food and the "Yang" Food

All foods derive from living organisms, either animal or plant, which are extremely complex and have evolved, together with human ancestors, over millions of years. The availability of food has influenced human evolution, and vice versa. Poisonous foods have been progressively recognized and eliminated from the diet, and natural selection has favoured the adoption of food patterns permitting healthy reproductive life. In the last 10–30,000 years, cereals have become the staple food of almost all human populations, and humans had experienced the advantage of mixing cereals and legumes in the same dish thousands of years before acquiring the knowledge of their complementary aminoacidic composition. Two recent large cohort studies on the intake of dietary fibres and mortality, the EPIC study in Europe [8] and the NCI-ARP study in the US [45], consistently showed that a high intake of cereal fibres is associated with lower mortality from cancer, cardiovascular, pulmonary, gastrointestinal, and infectious diseases. In addition, vegetable fibres were associated with some protection, but not fruit fibres. Interestingly, the isolated administration of whole-grain cereal components—fibres, vitamins, or minerals—did not seem to elicit any protective effect [26], suggesting more than an additive influence of cereal constituents on health, the so-called "food synergy" [23–25].

Whole-grain cereals (mainly brown rice, millet, barley, oats) and occasionally buckwheat and wheat pasta are the basic components of a macrobiotic diet, which also includes 20–30 % locally grown vegetables, 5–10 % beans, including traditional soy products, sea vegetables and occasionally fruits, nuts, and fish [33]. Interestingly enough, the 2007 WCRF/AICR recommendations for the prevention of cancer[1] (Table 1) broadly coincide with the macrobiotic recommendations of avoiding the habitual intake of extremely "yin" food, such as sugar and sugared beverages, alcoholic beverages, and refined flours, as well as of extremely "yang" food, such as processed meat, salty food, and red meat, while the central recommendation is to "Eat mostly food of plant origin, with a variety of non-starchy

[1] www.dietandcancerreport.org

Table 1 WCRF recommendations

• Be as lean as possible within the normal range of body weight
• Be physically active as part of everyday life
• Limit consumption of energy-dense food and avoid sugary drinks
• Eat mostly food of plant origin, with "a variety of non-starchy vegetables and of fruit every day" and "relatively unprocessed cereals (grains) and/or pulses (legumes) with every meal"
• Limit intake of red meat and avoid processed meat
• Limit alcoholic drinks
• Limit consumption of salt and avoid mouldy cereals or pulses
• Aim to meet nutritional needs through diet alone
• Mothers to breastfeed; children to be breastfed
• Cancer survivors to follow the recommendations for cancer prevention

Fig. 1 Tao pictogram representing the concept of yin and yang

vegetables and of fruit every day and with unprocessed cereals and/or pulses with every meal", which is also the basic characteristic of the Mediterranean diet, as well as the staple food of most populations before the industrial revolution: pasta with beans in Italy, cuscus with chickpeas in north Africa, rice with soy products in Asia, maize with black beans in central America, and millet with peanuts in Black Africa.

Table 2 Nutritional WCRF recommendations ordered by yin–yang criteria

Avoid sugary drinks	▼▼▼
Limit alcoholic drinks	▼▼
Limit energy-dense food	▼
Eat mostly food of plant origin with relatively unprocessed cereals, pulses, and vegetables	
Limit intake of red meat	▲
Limit consumption of salt	▲▲
Avoid processed meat	▲▲▲

In Chinese philosophy, the concept of *yin–yang* is used to describe how polar opposites or seemingly contrary forces are interconnected and interdependent in the natural world, and how they cause everything to happen. Yin and yang are not opposing forces, but complementary forces. Everything has both yin and yang aspects. The concept of yin and yang is present in the Tao pictogram (Fig. 1), symbolically representing a walking man sustaining on his shoulder a bamboo cane with two baskets, one carrying the yin energy and the other the yang energy. Only if there is a perfect equilibrium between the two energies can the man make his way towards the Tao. Classically, "yang" refers to the "sunny side", and "yin" to the "shadowy side". While "yin" is dark, passive, feminine, cold, wet, diffuse, weak and is associated with the earth and the night, "yang" is bright, active, masculine, hot, dry, focused, strong and associated with the sky and the sun. Animal food is yang, especially red and salted meat, and vegetable food is yin, the most yin being refined foods such as oils, alcoholic beverages, and sugar.

Table 2 shows the WCRF/AICR recommendations ordered according to the macrobiotic classification of food in terms of yin and yang. Yang is symbolized by a triangle soundly resting on its base, while yin is symbolized by a triangle in difficult equilibrium on its vertex. In synthesis, the WCRF/AICR recommendations are to avoid extremely yin and yang food, to limit unbalanced yin and yang food, and to rest on slightly yang food, such as whole-grain unprocessed cereals, equilibrated by slightly yin food, such as legumes and vegetables.

In fact, observing the gastronomic traditions of all populations living in temperate climates, one usually notices attempts to equilibrate yang and yin food: fish is usually served with a boiled potato, turkey is filled with chestnuts, red meat is served with some salad and a glass of wine; in Italy, salted ham is served with melon; in Sicily, citrus fruits are eaten with salt; and hamburger, very yang because made of red meat and cooked at a high temperature, is usually eaten with very yin sugared beverages on ice. Only in very cold (yin) climates, one may have two very yang food at breakfast, such as egg and bacon. In hot climates one would prefer yin fruits and sweets. A key principle of the Taoist philosophy is to emphasize *wu-wei* (literally non-action), action through non-action, basically meaning respecting nature, harmonizing with nature, "naturalness", and, in nutrition, choosing natural, simple, harmonizing foods, instead of extremely processed foods.

3 Synergy in Food

The advantage of choosing simple food profiting from the synergy between different food components is increasingly recognized [25]. The bioavailability of omega-3 fatty acids is several folds higher if they are taken from fish rather than from supplements. Supplementing a liposoluble vitamin may decrease the absorption of other vitamins that use the same transporter mechanism. The results of most randomized controlled trials of nutrient supplements (mainly antioxidant vitamins and minerals and B vitamins) to prevent cancer or cardiovascular diseases

were either null or showed adverse effects. On the contrary, observational studies on food patterns showed a lower incidence of chronic disease, in persons characterized by a high score of prudent or Mediterranean food pattern. A significantly lower risk was reported for diabetes [51], cardiovascular diseases [18, 30], stroke [1], Alzheimer [53], and several cancers, including breast [9, 57, 61], stomach [3], colon [15], and pancreatic cancer [27].

Constituents of natural foods are coordinated, and their concentration varies in different environments. Vegetables growing in the mountains, for instance, are richer in polyunsaturated fatty acids, which protect from low temperatures, and foods containing high quantities of polyunsaturated fatty acids are usually richer in antioxidant phytochemicals. There is increasing evidence that phytochemicals affect gene expression, favouring or inhibiting DNA methylation, histone acetylation, or micro-RNA. Specific DNA methylations and histone modifications, usually associated with pro-inflammatory micro-environments, have been implicated in several cancers. In principle, as epigenetic changes can be transmitted over consecutive mitotic divisions, those occurring before conception or during embryonic development will have a much greater impact on the overall epigenetic status of the organism. Nevertheless, at any age, environmental factors, such as diet and physical activity, may modify DNA methylation and histone acetylation [14, 36]. Global hypomethylation and site-specific hypermethylation are common features of human tumours. Diet can profoundly alter epigenetic patterns, but the causal link between diet and epigenetics in the development of human disease is still poorly understood. A challenging research field is developing to determine which adverse epigenomic marks are reversible by specific drugs, nutrients, or lifestyle changes. Over 50 bioactive phytochemicals have been demonstrated to be active on DNA methyltransferase, or histone acetylase/deacetylase. Experimental studies on animals or cultured human cell lines support their role in the prevention of cancer, but have often been conducted at concentrations far beyond those documented in humans. It is very difficult to predict from these results the effects of these substances on disease prevention in humans. There is evidence, however, that continuous exposure at physiological concentrations can remodel the epigenome in a cumulative fashion [52]. Moreover, in these experiments, whole food extracts usually have significantly greater physiological effects than isolated specific constituents: “Mother Nature knew what she was doing when she created plant foods: vegetables, fruit, whole grains, nuts, and legumes are great examples of foods that are rich in a combination of important vitamins, minerals, fiber, protein, antioxidants, and more” (cited from [24]). As there is still much we do not know or do not understand about the interaction of biologically active plant food components, it is better to eat food as close to its natural form as possible: let us harmonize with nature.

4 Cancer and Lifestyle

Several studies have shown that a Western dietary pattern, obesity, weight gain, a sedentary lifestyle, metabolic syndrome, high serum levels of insulin, and growth factors after the diagnosis of cancer are associated with an increased incidence of recurrences and with lower cancer-specific survival. Most studies were on breast and colon cancer. However, in the clinical management of cancer, little attention is presently paid to improving lifestyle and controlling body weight.

Lifestyle intervention trials are needed to corroborate or confute the observational results on cancer recurrences, but, even now, there is no contraindication to promoting moderate physical exercise, moderate CR, and a Mediterranean dietary pattern. Several studies showed that obese people have lower overall and disease-free survival when diagnosed with breast cancer [10, 37, 50, 54], colorectal cancer [11, 20], and prostate cancer [17, 38], independently from the stage at diagnosis and other biological characteristics of the disease. Recently, a clinical study suggested that obesity is an unfavourable prognostic factor also for non-Hodgkin lymphoma [16]. A meta-analysis of 43 studies that followed up breast cancer patients showed poorer survival among obese compared with non-obese women, for both overall [HR 1.33; 95 % confidence interval (CI) 1.21, 1.47] and breast cancer–specific survival (HR 1.33; 95 % CI 1.19, 1.50). Similar results were obtained using waist–hip ratio as the measure of obesity (HR 1.31; 1.08, 1.58). The effect was present in both premenopausal and postmenopausal patients, and in treatment and observational cohorts [50].

There is evidence, moreover, that weight gain after the diagnosis of breast cancer is associated with an increased risk of total and breast cancer mortality. In a study [43], each 5-kg gain was associated with a 13 % increase in breast cancer–specific mortality; weight loss after diagnosis was associated with increased mortality from all causes, and mortality from breast cancer was (non-significantly) lower if compared to stable weight. Several studies also reported that the weight gain usually observed during CMF-based adjuvant chemotherapy was associated with a worse prognosis [5, 31]. We showed that weight gain during adjuvant chemotherapy can be prevented [59]. Little attention, however, is presently paid to weight control in the clinical management of breast cancer.

A randomized controlled trial carried out on 2,500 breast cancer patients to test the efficacy of reducing dietary fat intake showed a borderline significant 24 % reduction in new breast cancer events (local and distance recurrences and contralateral breast cancer) [7]. In this study, women in the intervention arm lost, on average, 2.1 kg over 5-year follow-up. Another trial carried out on over 3,000 patients to test the effect of reducing fat intake and increasing the consumption of fruit and vegetables (mainly through fruit and vegetable juices) did not find any protection; in this case, however, the design was isocaloric, and the women randomized in the intervention group actually gained some weight [48]. The likely reason for such a discrepancy is that a moderate calorie restriction may protect from breast cancer recurrence.

Observational studies on thousands of patients operated for breast cancer [21, 22, 47] or colorectal cancer [20, 40] showed that those who practise moderate physical exercise after diagnosis experience a lower risk of recurrence and death. In colon cancer patients, there is no evidence of any effect in stage I or stage IV, but there is a marked effect in stage II and III. In breast cancer patients, the effect of physical activity is apparently confined to oestrogen receptor–positive tumours, with a reduction in cancer-specific mortality of the order of 30–50 % for women practising at least 30 min per day of a physical exercise whose intensity corresponds to brisk walking. The mechanism by which physical exercise is protective is likely to involve its effect on insulin sensitivity.

An observational study of the dietary pattern of colon cancer patients participating in a randomized study of adjuvant chemotherapy showed that a high score in Western diet was associated with a significantly increased risk of recurrences. Foods with higher factor load included cheese, fat condiments, margarines, sweets, red meat, and processed meat [39]. Similar studies on breast cancer patients, on the contrary, found that a Western dietary pattern was associated with lower all-cause mortality but not breast cancer–specific mortality [32, 35]. A diet rich in dairy products and poor in vegetables has also been found to be associated with worse survival in two studies on ovarian cancer patients [12, 41]. These studies, however, relied only on the cases' recall of prediagnosis dietary intake. Observational studies suggest that specific vegetable food, such as soy food [4, 19, 29, 56] and cruciferous vegetables, may help to prevent breast cancer recurrences, both in patients under hormonal treatment and not. Also marine fatty acids from food are likely to be associated with a reduced risk of breast cancer recurrence (and all-cause mortality): a study that assessed dietary intake with repeated 24-h recalls over 6 years found that breast cancer patients whose consumption of long-chain omega-3 fatty acids was in the upper two tertiles had 25 % reduced risk of additional breast cancer events [27, 58]. The consumption of omega-3 supplements did not show any protective effect. Studies on multivitamin use and breast cancer outcome have given intriguing and contradictory results [34, 42].

The DIANA (DIet and ANdrogen) trials of dietary intervention for the prevention of breast cancer showed that a few months of Mediterranean and macrobiotic diet are sufficient to favourably modify the metabolic and endocrine characteristics of women at high risk of breast cancer, namely to reduce insulin and the bioavailability of growth factors and sex hormones [2, 28]. The ongoing DIANA-5 project [60] is a randomized controlled trial of diet and physical activity on 2,000 breast cancer patients at high risk of recurrence because of MS and/or high serum insulin or testosterone level. Its aim is to test the hypothesis that it would be possible to significantly reduce breast cancer recurrences through a comprehensive lifestyle modification, which includes moderate physical exercise, moderate calorie restriction, and a diet mostly based on food of plant origin, with an ample variety of unrefined grains, legumes, and seasonal vegetables and fruit, according to the 2007 World Cancer Research Fund recommendations.[2]

[2] www.dietandcancerreport.org

If this and other studies fulfil the promise of reducing cancer-related morbidity and improving quality of life, dietary advice and weight control support for breast cancer survivors shall become a new standard of care.

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Part VI
Emerging Breakthroughs

New Perspective for an Old Antidiabetic Drug: Metformin as Anticancer Agent

Alessandra Leone, Elena Di Gennaro, Francesca Bruzzese, Antonio Avallone and Alfredo Budillon

Abstract

Metformin, an inexpensive, well-tolerated oral agent that is commonly used in the first-line treatment for type 2 diabetes, has become the focus of intense research as a potential anticancer agent. This research reflects a convergence of epidemiologic, clinical, and preclinical evidence, suggesting that metformin may lower cancer risk in diabetics and improve outcomes of many common cancers. Notably, metformin mediates an approximately 30 % reduction in the lifetime risk of cancer in diabetic patients. There is growing recognition that metformin may act (1) directly on cancer cells, primarily by impacting mitochondrial respiration leading to the activation of the AMP-activated protein kinase (AMPK), which controls energy homeostasis in cells, but also through other mechanisms or (2) indirectly on the host metabolism, largely through AMPK-mediated reduction in hepatic gluconeogenesis, leading to reduced circulating insulin levels and decreased insulin/IGF-1 receptor-mediated activation of the PI_3K pathway. Support for this comes from the observation that metformin inhibits cancer cell growth in vitro and delays the onset of tobacco carcinogen-induced lung cancer in mice and that metformin and its

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_21,

analog phenformin delay spontaneous tumor development cancer-prone transgenic mice. The potential for both direct antitumor effects and indirect host-mediated effects has sparked enormous interest, but has led to added challenges in translating preclinical findings to the clinical setting. Nonetheless, the accumulation of evidence has been sufficient to justify initiation of clinical trials of metformin as an anticancer agent in the clinical setting, including a large-scale adjuvant study in breast cancer, with additional studies planned.

Keywords

Metformin · Phenformin · Diabetes mellitus · AMP-activated protein kinase · PI_3K pathway

Abbreviations

ACC	Acetyl-CoA carboxylase
ACF	Aberrant crypt foci
ACL	ATP citrate lyase
AKT/PKB	Protein kinase B
CaMKK2	Ca^{2+}/calmodulin-activated kinase kinase
cAMP	Cyclic adenosine monophosphate
CI	Confidence interval
CRTCS	cAMP-responsive element binding protein (CREB)-regulated transcription coactivator
CSC	Cancer stem cell
DM	Diabetes mellitus
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FAS	Fatty acid synthase
FOXO	Forkhead box O
GLUT	Glucose transporter
GPCR	G-protein-coupled receptor
HCC	Hepatocellular carcinoma
HIF-1α	Hypoxia-inducible factor 1α
HOMA	Homeostasis model assessment
IGF	Insulin growth factor
IGFBP	Insulin growth factor binding protein
IGF-R	Insulin growth factor receptor
IR	Insulin receptor
IRS	Insulin receptor substrates
LKB1	Liver kinase B1
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
mTOR	Mammalian target of rapamycin

mTORC1	mTOR complex 1
NAFLD	Non-alcoholic fatty liver disease
Nampt	Nicotinamide phosphoribosyltransferase
OCT	Organic cation transporter
PDK1-3	Pyruvate dehydrogenase kinase 1 and 3
PET	Positron emission tomography
PI_3K	Phosphatidylinositol 3 kinase
rag	Recombination activating gene
RR	Relative risk
SREBP-1	Sterol regulator element binding protein 1
TSC	Tuberous sclerosis
UMIN	University Hospital Medical Information Network
VEGF	Vascular endothelial growth factor

Contents

1 Introduction

Diabetes mellitus (DM) is a metabolic disorder that is characterized by chronic hyperglycemia and aberrant carbohydrate, fat, and protein metabolism that result from defects in insulin secretion, insulin action, or both. It represents a major global health problem that has been recognized and treated for centuries. Since the Middle Ages, Europeans have treated "thirst and frequent urination", two symptoms of DM patients, using extracts of *Galega officinalis,* an herbaceous plant that was later found to contain active components such as galegine, a guanidine derivate. In the 1920s, galegine was found to exhibit the ability to decrease blood sugar and insulin resistance. In the 1950s, biguanide derivates such as phenformin and metformin were introduced into the clinic and represented an important milestone in the development of oral antidiabetic pharmacotherapy. Phenformin has been withdrawn from usage in the United States and in most other countries

since 1978 due to rare fatal cases of lactic acidosis, whereas metformin still represents a preferred front-line agent for antidiabetic therapy alongside diet and exercise because of its transient GI toxicity and very rare severe lactic acidosis toxicity [80].

Recently, a series of studies and meta-analyses have shown an increased risk of cancer in DM patients. In particular, meta-analyses have revealed a strong association between diabetes and cancers of the pancreas or liver, the main organs implicated during the deregulation of the metabolic equilibrium that is typical in DM [80]. Altered metabolic pathologies, such as hyperglycemia and hyperinsulinemia, as well as other DM-associated factors, such as obesity and high saturated fat diets, are also independent risk factors for cancer, illustrating a close correlation between the two diseases [39].

In contrast, several retrospective epidemiological reports have highlighted that the use of metformin in diabetic patients correlated with a reduced lifetime incidence of cancer. Moreover, metabolic reprogramming is now considered an emerging critical hallmark of cancer and represents a target for anticancer therapy [73]. In this regard, among the 24 provocative questions posed last year by NCI director Harold Varmus on a dedicated Web site,[1] which aim to identify perplexing problems to guide progress in cancer research, a critical one is how we determine the mechanism by which some drugs, commonly and chronically used for other indications (such as metformin and others), can protect against cancer incidence and mortality [9]. Thus, is metformin a diabetic drug for cancer or it is a cancer drug for diabetics? [53]. In this chapter, we will review all of these aspects in an effort to navigate through this highly debated field and to answer some of these questions. Thus, it is not surprising to observe that in the last 3 years, an increasing number of studies about metformin and cancer have been published on PubMed (Fig. 1).

2 Cancer and Metabolism

The increased glucose uptake of cancer cells is a phenomenon that was first described by the Nobel Prize winner Otto Warburg in the 1920s. The so-called Warburg effect is the observation that the metabolism of most tumor cells is characterized by increased glycolysis that is maintained even during conditions of high oxygen tension (i.e., aerobic glycolysis), followed by elevated lactate production levels. This metabolic status contrasts with the low glycolytic rates associated with the oxidation of mitochondrial pyruvate that are exhibited by most normal cells [12]. Moreover, Warburg suggested that this altered metabolism is a fundamental cause of cancer. At present, we know that cancer cells use the elevated amounts of glucose as a carbon source for anabolic reactions to drive glycolytic intermediates into various biosynthetic pathways that are essential for

[1] http://provocativequestions.nci.nih.gov

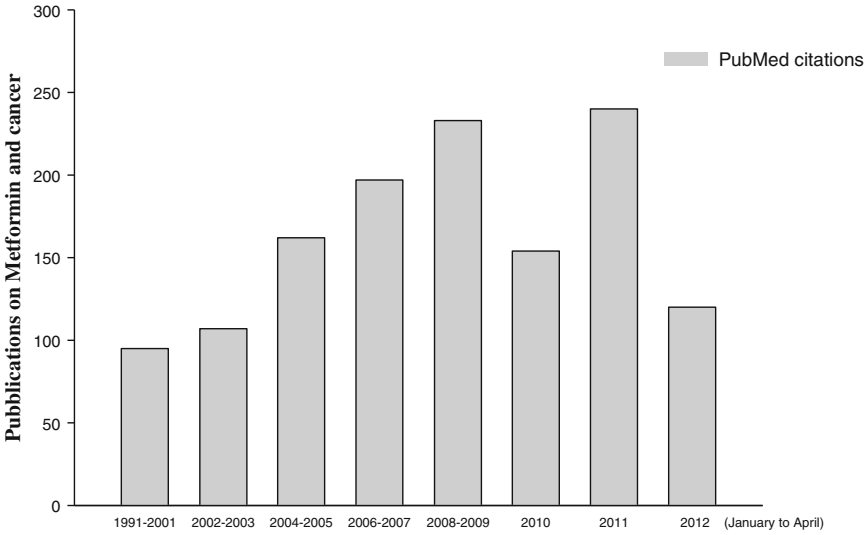

Fig. 1 The *graph* shows the increasing number of citations regarding the relationship between metformin and cancer, recorded in PubMed in the last 20 years (adjusted at May 2012)

cancer cell growth [47]. Notably, as the alteration in glucose metabolism occurs early during carcinogenesis, the Warburg effect represents the basis of the current usage of positron emission tomography (PET) for the diagnosis of cancer using radiolabeled deoxyglucose, which exhibits increased uptake by both primary and metastatic cancer cells [25, 79].

The preferential use of aerobic glycolysis facilitates the survival of the highly proliferative cells during conditions of continuously changing oxygen tension, which is otherwise lethal for the normal cells that depend on oxidative phosphorylation for ATP production [68]. Moreover, cancer cells, by producing lactic acid, affect cellular pH, which can promote tumor invasion and suppression of anticancer immune effectors. Finally, tumors can metabolize glucose via the pentose phosphate system to generate NADPH, which can contribute to fatty acid synthesis and defend against chemotherapeutic agents [25, 73].

Although the Warburg effect has been known for many years, the precise molecular mechanisms underlying this phenomenon remain unknown. Consequently, it is also difficult to identify a selective target for anticancer approaches. Some reports have suggested that hexokinase, which is a metabolic enzyme that catalyzes the conversion of glucose to glucose-6-phosphate and which is the rate-limiting first step in the glycolytic pathway, might represent an anticancer target [87]. In addition, nicotinamide phosphoribosyltransferase (Nampt) and its product, nicotinamide adenine dinucleotide (NAD), which play crucial roles in the regulation of several metabolic reactions that are implicated in the glycolytic pathway as well as in the regulation of factors that affect both tumor progression and the inflammatory response (e.g., sirtuins), might represent therapeutic targets [24].

The increased de novo biosynthesis of fatty acids has also been considered a crucial metabolic alteration of cancer cells and is associated with the hyperactivity of lipogenic enzymes such as ATP citrate lyase (ACL), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS) [48]. FAS is overexpressed in many cancers [81], and chemical inhibitors of FAS have been shown to decrease proliferation and increase apoptosis in cancer cells [48]. Recent studies have suggested that in cancer cells, the Warburg effect might depend on mitochondrial uncoupling—the abrogation of ATP synthesis in response to mitochondrial membrane potential in cancer cells—leading to a decreased pyruvate flux through the Krebs cycle and a shift in the oxidation of non-glucose carbon sources (e.g., fatty acids) to maintain mitochondrial integrity and function [71]. However, other reports have suggested that the Warburg effect does not necessarily involve in permanent mitochondrial dysfunction [87]. Interestingly, both uncoupled mitochondria, which render cells more resistant to cytotoxic insults, and increased fatty acid oxidation, which has been linked to chemoresistance, might represent additional therapeutic targets for cancer treatment.

Another relevant target for the metabolic reprogramming of cancer cells is the hypoxia-inducible factor 1α (HIF-1α), a transcription factor that mediates the hypoxia-induced gene expression changes that are thought to be adaptive for cells upon exposure to a reduced oxygen environment. Such genes encompass those involved in glycolysis and include the glucose transporters (GLUT1 and GLUT3). Moreover, recent reports have demonstrated that HIF-1α induces pyruvate dehydrogenase kinase 1 and 3 (PDK1-3) expression, which facilitates the phosphorylation and inhibition of pyruvate dehydrogenase and, consequently, mitochondrial respiration in cancer cells [44, 52, 65].

In summary, the switching of cellular metabolism from mitochondrial respiration to glycolysis drives cancer transformation and progression as well as chemoresistance and should therefore be considered a crucial target for anticancer therapy.

3 Diabetes and Cancer

DM comprises a group of metabolic disorders, which include two predominant subtypes—types 1 and 2—that are characterized by different metabolic activities. Type 1 diabetes (5–10 % of all diabetics) is associated with the complete absence of endogenous insulin that is attributed to the autoimmune destruction of insulin-secreting β-pancreatic cells and requires the exogenous administration of insulin. In contrast, type 2 diabetes (90 % of all diabetics) is characterized by the long-term presence of hyperglycemia and hyperinsulinemia associated with insulin resistance in the peripheral tissues. In the latter subtype of diabetes, treatment with exogenous insulin is required only when the β-pancreatic cells become non-functional [39, 80]. Currently, a large variety of drugs are available for the treatment for DM, including insulin, insulin analogs, and insulin secretagogues

that function by compensating for the lack of insulin production by the patient's β-pancreatic cells. Another class of drugs includes insulin sensitizers, such as the oral antidiabetic metformin or the thiazolidinediones, which can overcome insulin resistance. Lastly, glucosidase inhibitors, such as acarbose, function therapeutically by acting on carbohydrate digestion to prevent the development of postprandial hyperglycemia. Generally, all of the classes of diabetic medications are coupled with recommended lifestyle changes including diet and exercise, which might decrease or prevent the obesity that is often associated with DM [62].

DM is associated with several complications, such as retinopathy, nephropathy, cardiovascular diseases, and, as reported in several studies, with an increased risk of cancer.

Several meta-analyses have evaluated the relative risk (RR) of cancer in both case-control and cohort studies of diabetics, demonstrating a mild increase in cancer (and in cancer mortality). Although this increase applies to both solid and hematological malignancies, it is more evident for certain site-specific cancers. Elevated relative risk (RR) has been shown for pancreatic (RR 2.50, 95 % CI 1.8–3.5) and liver (RR 1.94, 95 % CI 1.53–2.46) cancers, which are the main organs involved in the development of DM [22, 37].

The precise relationship between diabetes and pancreatic cancer is difficult to delineate because previous meta-analyses did not distinguish whether diabetes was a preexisting condition that promoted the development of exocrine pancreatic cancer or if it was a consequence of cancer.

Recently, the diagnosis of new-onset diabetes has been used as a potential early diagnostic indicator for pancreatic cancer in screening programs enrolling middle-aged patients. In elderly patients, it represents a high probability indicator of pancreatic cancer, with a 3 year risk that is eight times higher in diabetic than in non-diabetic patients of similar age and sex. Additional studies have demonstrated that new-onset diabetes is an early event that is attributed to cytokine production by pancreatic tumors rather than to alterations in normal pancreatic tissues [15, 64]. Interestingly, the RR remained higher for new-onset diabetic patients when the meta-analyses were adjusted for age, race, and cigarette smoking as well as for post-load glucose levels. These findings indicated that hyperglycemia and prediabetic status might represent risk factors for pancreatic cancer and that insulin plays a prominent role in promoting cancer progression. However, insulin alone is insufficient to promote cancer progression because the pancreatic cells in type 1 diabetic patients are not exposed to increased insulin levels compared to other tissues [64, 80].

The increased incidence of liver cancer and, in particular, of hepatocellular carcinoma (HCC) in diabetic patients is mainly due to the elevated insulin levels that the liver cells are exposed to via portal circulation. This condition is evident in both physiological and pathological situations, particularly in type 2 DM, which is characterized by exacerbated states of hyperinsulinemia and insulin resistance. Significantly, in type 1 diabetic patients treated with exogenous insulin, the insulin levels in the liver cells are the same as those in other organs. As has been previously described for pancreatic cancer, the elevated insulin levels in liver cancer

are insufficient to explain the correlation between cancer and diabetes. Therefore, several epidemiological studies have analyzed other factors. For instance, hepatic steatosis and cirrhosis, as well as hepatitis B and C infections, have been implicated as connecting factors between the two diseases. Similarly, non-alcoholic fatty liver disease (NAFLD) might represent a main cancer risk factor in obese diabetic patients and in 80 % of type 2 diabetic patients [16, 18].

In conclusion, the relationship between diabetes and cancer remains unclear and requires re-evaluation because DM is not a singular disease. Rather, DM is a group of metabolic disorders in which each disorder is characterized by its own metabolic and hormonal abnormalities that affect patients differently. Thus, it is difficult to consider diabetic patients as a homogeneous cohort, and further studies are required to understand the complex relationship between cancer and diabetes.

Moreover, a group of confounding factors exist which are based on lifestyle, such as lack of physical activity, obesity, smoking, sex, ethnicity, comorbidity, duration of treatment, quality of metabolic control, and number of antidiabetic drugs changed during the treatments. Such factors might influence the meta-analysis reports. In this regard, a recent study has demonstrated that whereas insulin levels might represent a physiological indicator for early follow-up (first 5 years after diagnosis), the obesity-related factors, such as leptin, might represent an important marker over time for long-term follow-up of breast cancer [27].

At the molecular level, several mechanisms might account for the tumor growth observed in diabetic patients. Diabetes can promote carcinogenesis through the action of insulin and its complex downstream signaling network, which induces not only a modulation of metabolic pathways but also a modulation of mitogenic signaling via the following two distinct mechanisms: (a) systemic mechanisms attributed to specific alterations including hyperglycemia and hyperinsulinemia and (b) site-specific mechanisms that affect specific organs [80]. Interestingly, hyperinsulinemia can promote tumorigenesis directly by activating the insulin receptor (IR) in epithelial tissues or indirectly by influencing the levels of other modulators such as insulin growth factors (IGFs), sex hormones, and inflammatory mediators. For instance, when insulin binds to the A isoform of IR, which is predominantly expressed in cancer cells, it can trigger mitogenic signaling pathways that act through adaptor proteins such as IR substrates (IRS1-4). This signaling results in the activation of the mitogen-activated protein kinase (MAPK) pathway—activation that is preserved in the presence of insulin resistance—and in the induction of survival signaling that is mediated by PI3K, by AKT, and by the mammalian target of rapamycin (mTOR) [39, 55]. As mentioned above, insulin can also act indirectly via IGFs and their cognate receptors (IGF-Rs). Insulin resistance and elevated levels of insulin can displace IGFs from insulin growth factor binding proteins (IGFBPs), resulting in increased levels of free IGFs, which can constitutively promote tumor growth and cancer progression. Breast cancer cell lines have been observed to exhibit an interaction between insulin with the IR-A homodimer, the isoform of the IR that is widely expressed in this tumor, and insulin with the IR-A/IGF1R heterodimer to stimulate tumorigenesis and to promote survival pathways [6, 56, 66]. Moreover, the polymorphic form of IGF1R has

been demonstrated to be associated with non-small-cell lung cancer, whereas a polymorphism of IGF2R is correlated with gastric cancer risk [36, 86]. Furthermore, the activation of IGF1R/IR has been detected at elevated levels in breast cancers, independently of the specific tumor subtype (e.g., luminal, triple negative, or Her2+) and is associated with poor survival and resistance to targeted therapies, including those targeting the estrogen receptor (ER) or the Her family members. Moreover, the authors suggested that a specific IGF-IR tyrosine kinase inhibitor, BMS-536924, can be used to overcome such resistance and to promote improved survival, independently of breast cancer subtype [50].

Ultimately, elevated insulin levels are associated with insulin resistance and can promote tumorigenesis by increasing the free estrogen levels produced in the ovaries, by blocking sex-hormone-binding globulin, and by increasing the conversion of androgen to estrogen in adipose tissue [39, 80].

4 Metformin and Cancer

4.1 Epidemiological Studies

Metformin (1, 1-dimethylbiguanide hydrochloride) is a biguanide derivative and belongs to a class of oral hypoglycemic agents. Metformin acts principally on hepatocytes, myocytes, adipocytes, and β cells in the pancreas (Fig. 2). In the liver, metformin inhibits hepatic glucose production while increasing insulin sensitivity in the peripheral tissues, leading to increased glucose uptake and usage by the skeletal muscle and adipose tissues. The primary effects are a reduction in plasma insulin and glucose levels, followed by an enhancement of blood glucose control and a decreased incidence of complications that are correlated with diabetes [8, 20]. At the molecular level, the principal metabolic mediator of the glucose-lowering effects of metformin is the activation of AMPK, a serine–threonine kinase that is involved in the regulation of cellular energy metabolism [45, 67] (see in Sect. 5). Metformin is one of the most widely prescribed front-line drugs for the treatment for type 2 diabetes because of its relatively low cost and reputation as a safe drug, as well as its effects in cardiovascular disease prevention [7]. The mild-to-moderate toxicity of metformin treatment in terms of gastrointestinal disturbances, such as nausea, vomiting, and diarrhea, might be prevented by dose reduction. Although the incidence is rare, lactic acidosis is the most severe adverse event, occurring predominantly in elderly patients who present with hepatic, cardiac, or renal comorbidities [20].

In the past decade, epidemiological observations have suggested that the use of metformin in DM patients is correlated with a reduction in cancer incidence. A case-control study conducted by Evans et al. on 923 cases of cancer in 11,876 newly diagnosed type 2 diabetic patients revealed for the first time that the overall cancer incidence was lower in diabetic patients treated with metformin compared to patients treated with other drugs. Moreover, the duration of treatment and

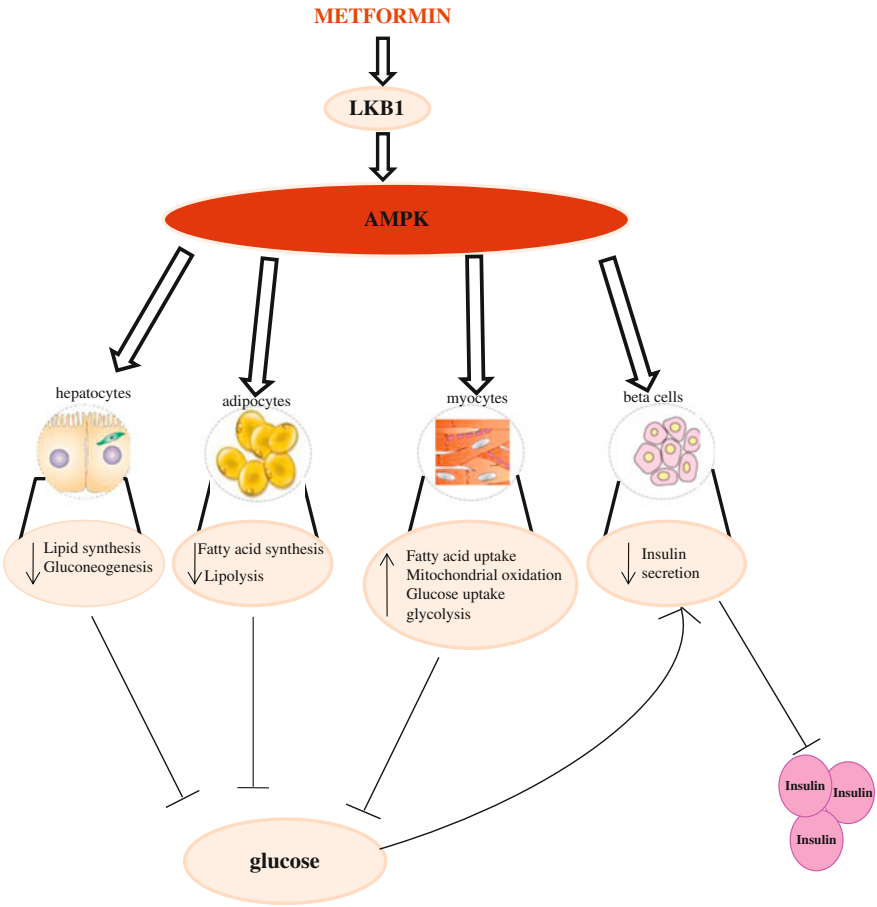

Fig. 2 Effects of metformin on various tissues in patients with diabetes mellitus type 2, leading to downregulation of ATP-consuming pathways and increase in ATP-generating pathways

number of prescriptions further influenced the cancer incidence: The increased duration of treatment and number of prescriptions correlated with a lower incidence of cancer [23].

Since this study, an increasing number of retrospective analyses have been performed. In 2006, Bowker et al. performed a population study on administrative data derived from 5. 4 year follow-ups of a cohort of 10,309 diabetic patients who received different treatments (metformin, sulfonylureas and/or insulin). This study also demonstrated reduced cancer deaths in patients who were treated with metformin alone without insulin compared to those who were treated with sulfonylureas regardless of insulin treatment [11, 62]. Unfortunately, an untreated control group was not included in this analysis, so whether the cancer risk was reduced by metformin or increased by insulin cannot be definitively asserted.

Other observational studies reported similar trends [3, 60], and interestingly, the association of metformin with reduced incidence/mortality of cancer was also maintained in the presence of insulin or after the adjustment of insulin doses, suggesting that metformin exerts an insulin-independent indirect mechanism of action [60].

Recently, several meta-analyses have been performed to understand the role of metformin in cancer incidence/mortality. Decensi et al. examined 11 epidemiological studies (5 comprehensive studies of all cancers and 6 studies of single cancer sites) that were extrapolated from a comprehensive literature search, which was not subjected to language or time restriction. The data derived from the analyses demonstrated that metformin treatment is associated with a 31 % reduction in cancer incidence and mortality and that this reduction exhibits a dose-response trend. Interestingly, a significant correlation between cancer risk and metformin treatment was observed in hepatocellular and pancreatic cancer patients, whereas no significant correlation has been reported in patients presenting with colon, breast, or prostate cancers, suggesting that metformin elicits cancer site-specific effects [19]. Recently, another study of 1,353 type 2 diabetic patients confirmed that after a median follow-up of 9.6 years, metformin exerted anticancer protective effects in a dose-dependent manner [1, 49].

An additional meta-analysis, which was performed on large population-based data from different countries, confirmed that metformin reduced the incidence and mortality of cancer at any site by approximately 33 %, with the variability in reduction depending on the site. Metformin treatment decreased the risk of colon, lung, and liver cancers but did not affect the development of hormone-dependent prostate and breast cancers or of pancreatic or gastric cancers [61].

Retrospective analyses have also been performed to understand the role of metformin on the development of metastasis, as well as whether this drug could improve the efficacy of chemotherapy. In particular, an analysis of diabetic patients with lung cancer revealed that the use of metformin reduced the occurrence of metastatic disease [57, 58] and improved chemotherapeutic outcomes [78]. Similarly, Jiralerspong observed that metformin increased the pathological complete response in breast cancer patients receiving neoadjuvant chemotherapy. Unfortunately, metformin did not improve the estimated 3 year relapse-free survival rate [1, 42]. In contrast, recently, metformin has been shown not to induce any beneficial effect on overall survival, on disease-free survival, or on the development of distal metastasis in triple-negative breast cancer cells [4].

In conclusion, several studies have suggested the use of metformin as an anticancer drug, but some limitations need to be considered. The majority of studies were retrospective, and the data were not obtained from population-based registries but rather from clinical and hospital data. Moreover, diabetic patients received a variety of treatments and were not randomized either for the administration of metformin or for objective criteria (for example, some patients with a history of cancer were included in cancer risk studies). Furthermore, an imbalance in important cancer risks and prognostic factors was evident. Nonetheless, such

observational studies have suggested the plausible antitumor effects of metformin and have provided the basis for the initiation of prospective clinical trials.

4.2 Mechanisms of Action and Preclinical Studies

Several mechanisms of action have been proposed to explain the antitumor effects of metformin, although at the molecular level, the main effect is the activation of AMPK [21, 63]. AMPK acts as a cellular sensor of metabolism and stresses, such as hypoxia, oxidative stress, ischemia, and others, which lead to an increased ratio of AMP:ATP and the consequential increase in AMPK activation [20]. AMPK could be activated by metformin via the following three independent mechanisms: (1) by LKB1 (liver kinase B1), which induces phosphorylation of Thr 172 in the α catalytic subunit of AMPK [51]; (2) indirectly through the inhibition of complex I of the respiratory chain; and (3) by the activation of other inhibitors of mitochondrial ATP synthesis, such as oligomycin. The blockade of complex I of the respiratory chain results in low oxygen consumption, followed by modulation of NAD^+/NADH ratios and an increase in the AMP:ATP ratio, resulting in increased AMPK activation [1]. In mammals, another important kinase is Ca^{2+}/calmodulin-activated kinase kinase (CaMKK2), which activates an AMPK alternative pathway in response to increases in intracellular Ca^{2+} without altering the AMP:ATP ratio [31]. During physiological conditions, activated AMPK exerts its hypoglycemic action on the liver, β-pancreatic cells, and muscle and adipose tissue, where it enhances glucose uptake by inducing elevated levels of Glut1. AMPK also induces ATP-generating pathways, such as glycolysis, while inhibiting the ATP-consuming pathways such as gluconeogenesis, glycogen synthesis, and cholesterol synthesis [39, 45, 67]. The activation of AMPK is also crucial for the induction of the oxidative catabolism of glucose and fatty acids as well as for the regulation of mitochondrial biogenesis. Furthermore, the activation of AMPK also results in the inhibition of protein synthesis by blocking the mTOR pathway [31]. Overall, the targeting of AMPK elicits both a hypoglycemic effect and a direct effect on several pathways involved in tumor development.

Based on these findings, AMPK might be considered as a tumor suppressor. Notably, the upstream kinase LKB1 has been demonstrated to be a tumor suppressor gene that is frequently mutated in solid tumors. The LKB1 gene exhibits loss of function mutations in approximately 30 % of non-small-cell lung cancers and in 20 % of cervical cancers [40, 83]. Mutations in LKB1 have been demonstrated to influence cell growth and cell cycle progression, as well as cell polarity [30, 82]. Moreover, LKB1 is often comutated with kRAS in non-small-cell lung cancers, inducing an increase in tumor incidence and metastasis [30]. The inhibition of AMPK results in B-Raf V600E mutation-harboring melanomas, in which LKB1 is phosphorylated at two C-terminal sites [88] and is unable to activate AMPK. Moreover, the hyperactivation of AKT is frequently found in many tumors and induces the phosphorylation of AMPK at Ser485, which inhibits the

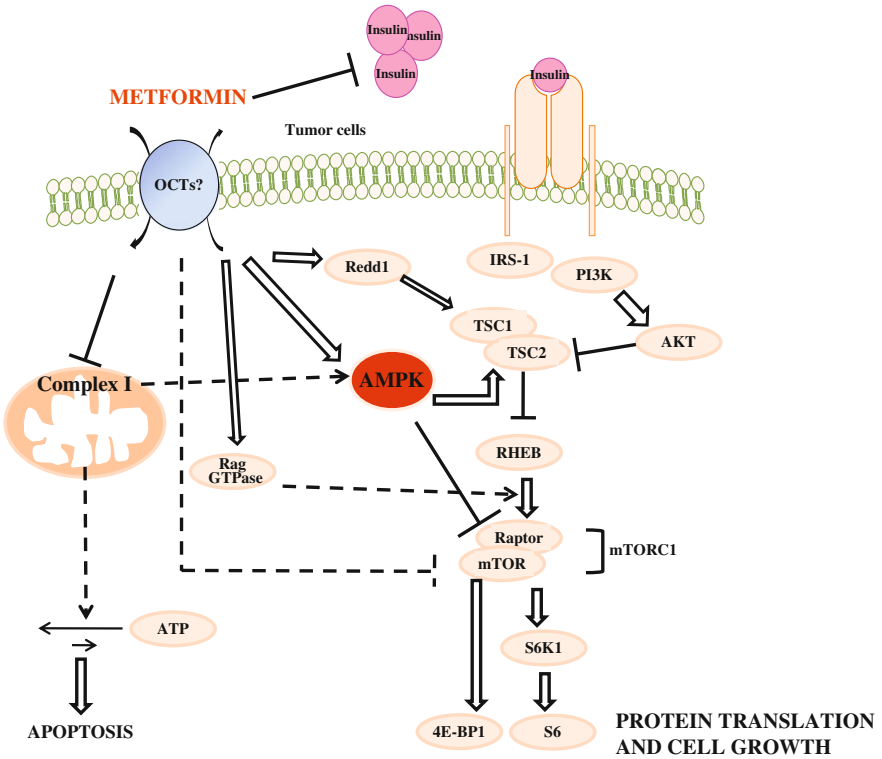

Fig. 3 Metformin exerts its antitumor effects by insulin-dependent and insulin-independent mechanisms. Metformin can suppress cell growth by inactivation of AKT–mTOR pathway and/or may act on both AMPK and mitochondrial complex I, leading, respectively, to increase in apoptosis and downregulation of protein translation and inhibition of cell growth

phosphorylation and activation of AMPK by LKB1 [34]. The relevance of AMPK as a favorable prognostic marker has been shown by another study, in which the concomitant increases in pAMPK and pMAPK3/1 were implicated as prognostic markers of favorable outcome for the treatment of colorectal cancer patients, suggesting a possible interaction of these two pathways and new therapeutic strategies [2].

The anticancer effects of metformin have been proposed to be exerted via the following two distinct mechanisms: an indirect insulin-dependent effect and a tumor-direct insulin-independent effect (Fig. 3). The indirect effect of metformin is exerted on the liver, where metformin inhibits hepatic gluconeogenesis by inducing the activation of AMPK, followed by a reduction in circulating levels of glucose and insulin. In vitro and in vivo studies have reported that metformin, similar to other biguanides, induces the hyperactivation of 4E-binding protein in glucose-deprived cells, leading to inhibition of the unfolded protein response and strong inhibition of the mTOR pathway [56]. Moreover, Buzzai and colleagues

demonstrated that in colorectal cell lines, glucose deprivation induced p53-dependent autophagy by the activation of AMPK in response to metformin [14]. In contrast, p53 status does not appear to be relevant for the metformin sensitivity of breast cancer cells. In response to the deprivation of glucose, metformin can induce apoptosis by both caspase-dependent and caspase-independent mechanisms concomitantly with changes in mitochondrial morphology and membrane permeability that depend on the cellular energy collapse that is related to the depletion of NAD^+ levels [90]. Furthermore, pancreatic cancer cells have recently been shown to exhibit crosstalk between insulin/IGF1 receptors and G-protein-coupled receptors (GPCRs), which leads to increased cell growth and survival signaling induced by mTORC1, ERK, and PI3 K pathways. Because ERK and PI3 K are well-defined effectors of KRAS, which is mutated in 90 % of pancreatic cancers, these crosstalk results are reinforced by that mutation. Interestingly, metformin has been observed to disrupt the crosstalk between IR/IGF1R and GPCRs, reducing cellular proliferation in both cell lines [46] and xenograft models [70].

The indirect effect of metformin has been confirmed in a study by Memmott et al., who observed in vivo that metformin prevented tobacco carcinogen-induced lung tumorigenesis. In particular, metformin has been shown to reduce tumor burden and simultaneously to markedly downregulate mTOR in tumors. Interestingly, metformin inhibits mTOR by activating AMPK in the liver, but not in the lung, where it indirectly downregulates IR/IGF1R and decreases AKT [59].

The direct effects of metformin on cancer growth are independent of insulin levels and might involve in the activation of AMPK, which inhibits several pathways, such as lipogenic pathways, via the suppression of SREBP-1 (sterol regulator element binding protein 1), via the regulation of the cell cycle by the phosphorylation of p53 and FOXO3a [84], or via the modulation of the estrogen-dependent pathway by the phosphorylation of CRTCS, a cAMP-responsive element binding protein (CREB)-regulated transcription coactivator [13]. The direct, AMPK-dependent effect also involves the direct action of AMPK on TSC2 and, as a consequence of its downstream signaling, reduces both protein synthesis and cell cycle progression. Interestingly, during the loss of TSC2, AMPK has been proposed to act directly on mTOR by phosphorylating the mTOR-associated protein Raptor [29]. Furthermore, a correlation between AMPK activation and adiponectin, a protein involved in metabolic signaling, has recently been demonstrated. Physiological concentrations of adiponectin were found to inhibit prostate and colon cancer growth by activating AMPK and inhibiting S6 K in patients presenting with normal weight. This finding has not been evident in obese patients who present with low levels of adiponectin, leading to reduced activation of AMPK and increased tumor growth. Consequently, the authors have suggested that metformin, by acting as an endogenous AMPK activator, can overcome resistance to adiponectin, thereby inhibiting cell growth [85].

Metformin has recently been observed to exhibit a direct action on mTOR, independently of TSC2 and AMPK [43]. That study found that in MEFs, metformin exerted an antitumor effect by inhibiting Rag, a GTPase protein involved in a direct translocation of mTORC1 to a cellular compartment that contains Rheb, a

protein that induces mTOR activity [43]. To confirm this finding, a recent study demonstrated, using pancreatic cells, that metformin directly influenced the mTOR pathway in a p53-dependent manner via an AMPK-independent mechanism to increase REDD1, a negative regulator of mTOR. Moreover, the induction of REDD1 decreased cyclin D1 independently of AMPK1, which resulted in cell cycle arrest [8]. Lastly, an additional AMPK-independent tumor-direct effect of metformin could be related to the modulation of complex I of the respiratory chain and to the consequent regulation of the ATP levels that can subsequently affect apoptosis.

Based on the elevated heterogeneity of tumors, several recent studies have suggested that metformin acts selectively on a subset of cancer cells and, in particular, on cancer stem cells (CSCs) [5, 33]. Hirsch et al. [33] have demonstrated this hypothesis for the first time, observing that metformin selectively targeted the CSCs in triple-negative breast cancer cells and elicited synergistic antitumor activity in combination with doxorubicin. This finding has been confirmed recently in mouse xenografts, in which the simultaneous administration of metformin in combination with several chemotherapeutic drugs reduced tumor growth and prevented relapse in several cancer cell models, presumably by inhibiting the highly tumorigenic CSC-like cells. Moreover, the synergistic interaction of metformin with chemotherapeutic drugs has also been confirmed [38]. Interestingly, combinatorial treatment with metformin and chemotherapeutic drugs has been performed in another study, in which the combinatorial administration of metformin and paclitaxel converged on AMPK activation to reduce cell growth [69]. Recently, metformin has been demonstrated to increase the sensitivity of cancer cells to radiotherapy and to exert cytotoxicity on CSCs, overcoming their radioresistance via the activation of AMPK and the suppression of mTOR [75].

Metformin, similar to its analog biguanide phenformin, is a cation, and both the direct and indirect selective antitumor effects of metformin depend on the expression of organic cation transporters (OCT1, 2 and 3). In diabetic patients, metformin enters hepatic cells via OCT1 transporters during high levels of exposure to the drug via the hepatic portal vein. This cationic transporter is highly expressed in hepatocytes; in mice exhibiting a global knockout of OCT1, as well as polymorphisms of OCT1, the hepatic uptake of metformin is severely impaired, resulting in reduced hypoglycemic effects [41, 59, 74, 89]. Little is known about the expression of OCT1 in neoplastic cells. However, recently, OCT1 expression has been demonstrated to be highly variable in both epithelial ovarian cancer cell lines and primary human tumors. Interestingly, the knockdown of OCT1 in epithelial ovarian cancer cell lines, as well as the application of the OCT1 inhibitor quinidine, reduced the antitumor effect of metformin but did not affect the antineoplastic activity of phenformin [31, “personal communication”, 72].

4.3 Clinical Studies

Prospective and ongoing clinical trials are aimed at investigating the safety and the efficacy of metformin in cancer patients, independent of diabetic status, to analyze its role as a chemopreventive agent and to identify its biological effects (Fig. 3). More than fifty studies on the effects of metformin in cancer patients are currently registered at NCI's cancer.gov. The majority of these trials are phase II breast cancer studies, which include biomarker analysis and the administration of metformin either as a single agent or in combination with other treatment modalities.

Limited clinical trials have been published so far regarding metformin. Hadad et al. conducted a preoperative, "window of opportunity" randomized trial, in which they showed the possible biological effects of metformin on tumor tissues. Metformin was administered to non-diabetic breast cancer patients before surgery, and the antitumor effects were compared with those of the untreated control group. Interestingly, the patients did not exhibit any quantifiable change in tumor size after 2–3 weeks of metformin treatment. However, an analysis of the tumor-derived biopsies revealed decreased insulin levels and a decrease in Ki67 staining, a marker of proliferation, indicating possible biological effects on tumor tissues [30]. In contrast, a recent study demonstrated that, overall, presurgery treatment with metformin did not affect Ki67 levels compared to treatment with the placebo arm in non-diabetic breast cancer patients. Interestingly, in a planned subgroup analysis, the effects of metformin on Ki67 could be stratified on the basis of insulin resistance according to the HOMA index (homeostasis model assessment). No correlation was observed between the insulin levels and metformin treatment in patients with HOMA indices greater than 2.8, whereas changes in Ki67 levels were noted in patients with HOMA indices less than 2.8, suggesting that metformin exerts its effects indirectly, depending on the grade of insulin resistance. Intriguingly, a similar stratification of Ki67 by HOMA index was noted in women who were overweight or obese, had abdominal obesity, or partook in moderate alcohol consumption [10].

According to observational studies in which elevated levels of peptide-C have been associated with poor outcome in non-diabetic breast cancer patients [17], Goodwin et al. [27, 28] demonstrated that metformin, when administered at a standard dose (1,500 mg/day), reduced insulin levels in non-diabetic breast cancer survivors by 22 % without relapse. Recently, the same group proposed a neoadjuvant "window of opportunity" study, in which metformin would be administered three times daily for 2–4 weeks prior to surgery. They identified the following potential predictors of metformin benefit: elevated BMI, physical inactivity, high fasting insulin as markers of host influence and tumor immunopositivity for Ki67 and TUNEL staining, and the presence of OCT1 and LKB1 as markers of tumor influence. Interestingly, a clear increase in TUNEL staining and a decrease in Ki67 have been observed to correlate with metabolic changes, following the administration of metformin [27, "personal communication", 76].

A chemopreventive role for metformin has been demonstrated by a short-term clinical trial performed on rectal aberrant crypt foci (ACF), which is considered as an endoscopic surrogate marker for colorectal cancer. In non-diabetic patients with ACF, treatment with metformin significantly decreased the number of ACF after 1 month of therapy compared to the control group. Moreover, the authors observed a downregulation of colonic epithelial proliferative activity in the same group, as evaluated using the proliferating cell nuclear antigen labeling index. In contrast, no significant apoptotic modulation was detected [35]. As the biological significance of ACF as a surrogate marker for colorectal cancer remains controversial, the same group has recently registered a prospective randomized controlled trial in the University Hospital Medical Information Network (UMIN) Clinical Trials Registry as UMIN000006254, in which the chemopreventive effects of metformin will be evaluated in metachronous colorectal polyps and in non-diabetic post-polypectomy patients [32].

5 Conclusions

Here, we have reported on several lines of evidence supporting a role for the biguanide metformin as an antitumor agent. Nonetheless, some issues remain unresolved, such as the principal mechanism of action of metformin (direct or indirect/host effect), the characteristics of the patient and/or tumor that can influence responses to metformin, what types of cancer respond to treatment with biguanides, and which therapeutic setting could enhance the benefits of metformin (chemoprevention, neoadjuvant, adjuvant, combined, or single-agent administration).

Several reports have suggested that the antitumor activity of metformin appears to be elicited by both direct and indirect mechanisms, through lowering insulin levels and by directly affecting the tumor tissues via both AMPK-dependent and AMPK-independent mechanisms. Interestingly, a principal mediator of metformin activity, AMPK, is defective in many tumor cells. Several mechanisms can cooperate to induce the loss of AMPK activation and consequently might affect the antitumor effects of metformin. Paradoxically, metformin might be more effective for the treatment for tumors in which AMPK activation has been lost because it can cause greater decreases in ATP levels and more apoptosis.

The mechanism of action of metformin has remained obscure due to the limitations of the preclinical models, in which higher levels of metformin, glucose, and insulin were used, compared to more physiological/clinical conditions. For example, the dose range of metformin in clinical/epidemiological studies is 250–2,250 mg/day, whereas the preclinical doses range from 45-fold increased dosages for in vivo studies to 10,000-fold increased dosages for in vitro studies, in which mM concentrations of metformin have been used. Moreover, tissue culture media contain up to 3- to 5-fold excess of glucose and up to a 40-fold excess of insulin. However, low doses of metformin (approximately 10 μM) have recently

been demonstrated to be sufficient to induce moderate activation of AMPK and the consequential activation of downstream pathways [77].

Recently, a phase III randomized trial was registered to address these issues, in which the effects of metformin that was administered in combination with chemotherapy were compared to those of placebo in early breast cancer patients. In this study, the patients were stratified for hormone receptor status, for HER2 expression, and for the chemotherapeutic drugs used, such as paclitaxel, docetaxel, doxorubicin, and cyclophosphamide [26]. The primary outcome was the rate of cancer-free survival, whereas the secondary outcomes were overall survival, disease-free survival and adverse events, and factors that were associated with correlative analysis, such as weight, fasting insulin levels, and tumor tissue. The primary hypothesis of this study was that elevated fasting insulin at baseline that exhibited a significant reduction at 6 months would predict metformin benefit ([27], "personal communication", [28]).

Which type of biguanide is best for clinical application remains unclear. For example, if direct action on the tumor is required, then the OCT1 receptor expression levels become crucial, and it might therefore be better to reconsider the use of phenformin, which does not require the presence of OCT1 receptors to penetrate the cells.

Lastly, a recent, controversial study reported that the mutant V600E Braf gene, which is present in 50 % of melanomas, conferred in vitro resistance to metformin treatment by activating RSK to prevent AMPK activation. Moreover, metformin treatment accelerated tumor growth and induced VEGF-A expression in vivo. Interestingly, combined anti-VEGF treatment synergistically reduced the tumor growth of the Braf mutant but not of the wild-type cells [54].

In conclusion, numerous studies have clearly demonstrated a new application of the "old" antidiabetic drug metformin as an anticancer drug. However, further in-depth knowledge of its mechanism of action is necessary to identify the optimal therapeutic and clinical context in which to use it as an antitumor drug.

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Gut Microbes, Diet, and Cancer

Meredith A. J. Hullar, Andrea N. Burnett-Hartman and Johanna W. Lampe

Abstract

An expanding body of evidence supports a role for gut microbes in the etiology of cancer. Previously, the focus was on identifying individual bacterial species that directly initiate or promote gastrointestinal malignancies; however, the capacity of gut microbes to influence systemic inflammation and other downstream pathways suggests that the gut microbial community may also affect risk of cancer in tissues outside of the gastrointestinal tract. Functional contributions of the gut microbiota that may influence cancer susceptibility in the broad sense include (1) harvesting otherwise inaccessible nutrients and/or sources of energy from the diet (i.e., fermentation of dietary fibers and resistant starch); (2) metabolism of xenobiotics, both potentially beneficial or detrimental (i.e., dietary constituents, drugs, carcinogens, etc.); (3) renewal of gut epithelial cells and maintenance of mucosal integrity; and (4) affecting immune system development and activity. Understanding the complex and dynamic interplay between the gut microbiome, host immune system, and dietary exposures may help elucidate mechanisms for carcinogenesis and guide future cancer prevention and treatment strategies.

Supported by US NIH grants U01 CA162077, R01 DK084157 and Fred Hutchinson Cancer Research Center.

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_22,

Keywords
Microbiota · Gut microbiome · Gut microbial community · Cancer prevention

Abbreviations

PRR	Pattern recognition receptors
ETFB	Enterogenic *Bacteroides fragilis*
LPS	Lipopolysaccharide
TLR-4	Toll-like receptor -4
LBP	LPS-binding protein
MSI	Microsatellite unstable
CIN	Chromosomal unstable
SRB	Sulfate-reducing bacteria
UC	Ulcerative colitis
NOC	N-nitroso compounds
ODC	Ornithine decarboxylase
DFMO	Difluoromethylornithine
ODMA	O-desmethylangolensin
ITC	Isothiocyanates
EHC	Enterohepatic circulation
SBA	Secondary bile acids
CA	Cholic acid
DCA	Deoxycholic acid
LCA	Lithocholic acid
E_2	Estradiol
E_1	Estrone
E_1S	Estrone-3-sulfate
E_3	Estriol
DHEA	Dehydroepiandrosterone
16α-OHE1	16α-hydroxyestrone
TRFLP	Terminal restriction length polymorphism
SHBG	Sex-hormone-binding globulin

Contents

1 Introduction

Representing a vast ecosystem, the indigenous bacteria in the human gut have various physiological effects and carry out multiple metabolic functions that can influence the health of the host. Bacteria are hypothesized to benefit the host in many ways. These favorable effects include (1) facilitating the metabolic conversion and uptake of beneficial dietary components; (2) producing beneficial fermentation end products that affect intestinal pH and interact with gut mucosa epithelial cells; (3) excluding pathogens by competing for attachment sites within the gut mucosa; (4) interacting with the intestinal immune system and contributing to the regulation of immune function; (5) transforming or excreting toxic substances; and (6) generating fecal bulk that decreases transit time and dilutes toxic substances [127]. Laboratory studies of the phenotypic differences between germfree and conventional animals illustrate the importance of the normal microbiota for overall host health [128, 142]; germfree animals tended to have a lower body temperature, smaller lymph nodes, lack of deconjugation of bilirubin and bile acids, an absence of urease and β-glucuronidase activities, and lower organ weights [20, 139].

Carcinogenesis has been associated with the microbiome through direct and indirect routes (Fig. 1). Direct pathways include colonization of epithelia by pathogens or direct interaction with the innate immune system via bacterial antigenic particles with pattern recognition receptors (PRR, e.g. toll-like receptor). Indirect pathways include bacterial production of carcinogens and chemoprotective factors from exogenous sources, such as diet, or from endogenous sources, such as compounds resulting from human metabolism (e.g., bile acids and steroid hormones). We present below epidemiologic and experimental evidence for associations of the gut microbiome, diet, and cancer.

2 Molecular Characterization of the Gut Microbial Community

Bacteria colonize throughout the gastrointestinal tract, and to a great extent, bacteria in fecal samples reflect the bacterial composition in the lumen of the large intestine [49, 129]. The adult human intestine is host to a diverse community of

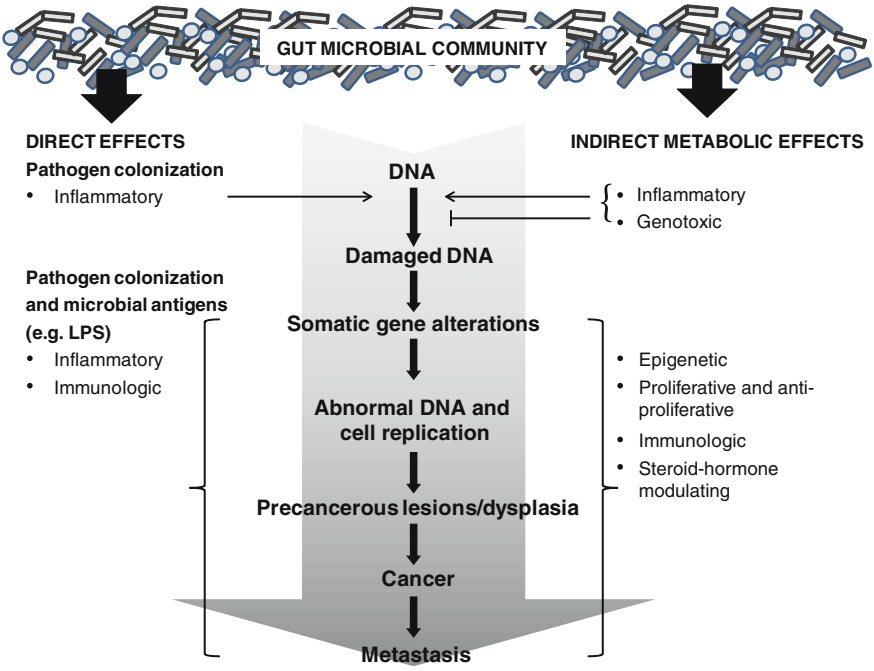

Fig. 1 Direct and indirect mechanisms by which the gut microbial community may influence cancer risk. Direct colonization of gut epithelium by pathogens, as well as effects of microbial antigens (e.g., lipopolysaccharide—LPS), contribute to inflammation and altered immune function. Indirectly, microbial metabolites of exogenous substrates (i.e., dietary constituents) and endogenous host compounds (i.e., steroid hormones, bile acids, etc.) can affect the carcinogenesis continuum within the colon, as well as in other tissues via systemic effects

microorganisms, including more than 800 species of bacteria [119]. However, the gut microbial community is distributed predominantly among two bacterial divisions, the Bacteroidetes and the Firmicutes, and one Archaeal species, *Methanobacter brevii* [49, 62, 119, 143]. A metagenomic analysis of the gut community also suggests that there is a core microbiome that individuals share; however, at the bacterial species level, large variation in gut microbial composition between individuals is observed [49, 62, 119, 143]. Therefore, the task of identifying particular bacteria associated with a specific phenotype in humans can be difficult. Conventional culture techniques for isolating and identifying active bacteria are arduous and time consuming. Furthermore, quantifying bacteria with these techniques is limited because it is estimated that approximately 40–60 % of mammalian bacterial species from the intestine cannot be cultured with conventional techniques [20, 140].

Because of the problems inherent in conventional culture techniques, studies of gut microbial communities have turned to molecular sequence-based approaches to identify intestinal bacterial species [5, 53, 65, 92, 150]. Bacterial DNA and

RNA can be identified regardless of whether the bacterium itself can be cultured. For phylogenetic-based approaches, the 16S ribosomal RNA (rRNA) gene is ideal, because it contains regions of the DNA that are conserved across bacterial species, as well as sequences that are unique to a specific bacterial species. Furthermore, the relationship between rRNA content and growth rate in enteric bacteria is well established, and rRNA content per cell varies with growth rate under different nutrient conditions; thus, 16S rRNA content can be used as an estimate of microbial biomass [112, 124, 131] and the physiologically active bacteria. These molecular assays can be used to focus at the domain level (i.e., Eubacteria and Archae), the phylum level (i.e., Bacteroidetes and Firmicutes), the functional group level (i.e., sulfate-reducing bacteria), or the species level (i.e., Clostridia sp.).

Comparative omics technologies provide an opportunity to link microbial community structure and function to human health and disease. In a recent study using a metagenomic approach to catalog the genes in the microbiomes from 124 individuals, Qin et al. [119] identified 3.3 million bacterial sequences. This approach was used to putatively categorize humans into three classes, or enterotypes, based on the composition and functional potential of their gut microbiome [9]. This is intriguing because it suggests that the underlying physiology of the gut microbiome and thus, how the human host is influenced by the microbiome, varies in a potentially predictable way. However, the presence of a gene does not necessarily imply that it is actively being expressed and shaping microbiome–host interactions. Functional metagenomic approaches need to be integrated with other approaches to assess which of these genes are actively expressed (metatranscriptomics) and translated to functioning proteins (metaproteomics). The integration of these 'omics technologies can also assess the presence of the metabolic pathways, such as sulfate reduction, nitrate reduction, secondary bile acid formation, and others that interact with diet to influence human health. They can also be used to measure the direct effects of pathogens that may promote carcinogenesis in epithelial cells. Evidence for the influence of the gut microbiome as a direct or indirect agent of carcinogenesis has been evident in the epidemiologic literature (see below). Coupled with studies of in vitro systems, mouse models, and controlled human interventions, we can start to understand the mechanisms associated with the gut microbiome which influence human health and risk of disease. However, until we can sample the gut microbiome in a prospective fashion, it will be hard to understand truly the causal effect of the gut microbiome on disease outcomes [86].

3 Direct Effects of the Gut Microbiome in Cancer Development

3.1 Gut Microbes as Infectious Agents

It is now clear that infectious agents are important to the development of specific cancer types; cervical, anal, penile, oropharyngeal, liver, and stomach cancers, along with certain types of lymphomas, have well-established infectious etiologies [23, 42]. Approximately 20 % of the total worldwide cancer burden is attributable to known infectious agents, and this proportion is expected to increase over time [23, 153]. The majority of known infection-associated cancers are caused by viral agents, such as the link between cervical cancer and oncogenic human papillomavirus alpha types, or between liver cancer and hepatitis C and B viruses [116]. However, bacteria, and in particular microbes found in the gut, have also been implicated as carcinogenic agents [96].

Helicobacter pylori, considered a class I carcinogen by the International Agency for Research on Cancer (IARC), is an established cause of gastric cancer and MALT lymphoma and accounts for approximately 5.5 % of cancers worldwide [72, 116]. Chronic carriage of *Salmonella typhi*, the causative agent of typhoid fever, is hypothesized to be linked with gallbladder cancer [90]. Additionally, several species of bacteria have been identified as potential candidates associated with colorectal cancer; these include *Streptococcus bovis* (also known as *S. gallolyticus*), *Fusobacterium nucleatum, H. pylori, Coriobacterialies,* and enterotoxigenic *Bacteroides fragilis* (ETFB) [27, 31, 63, 100].

Most of the studies linking potential infectious bacterial agents to carcinomas of the gut have used a case–control design. Although case–control studies are efficient for studying rare diseases, they are unable to establish the temporal relationship between infection and cancer [99]. This methodological concern is important to the interpretation of case–control study results. For cancer, the microenvironment around the tumor becomes more anaerobic, and possibly, more susceptible to infection as the cancer progresses [68]. Therefore, it is unclear if the malignant tumor creates an ideal environment for infection with specific bacterial agents, or if bacterial infection precedes the carcinoma and acts to drive carcinogenesis via inflammation or other pathways. Furthermore, some bacterial species may be important in early carcinogenesis but may not be able to tolerate the new tumor environment as the cancer develops; these potentially important agents would be missed in case–control studies of cancer in the gut. Thus, prospective studies and studies of precursors to cancer, such as colorectal adenomas, are important to determining causality in the relationship between bacterial agents and cancer.

In addition to determining the temporal relationship between bacterial agents and cancer, mechanisms by which infectious agents may promote carcinogenesis should be established. Each of the bacterial species discussed briefly above are hypothesized to follow a more traditional model of microbial carcinogenesis by

promoting cancer directly at the site of infection. In this model, the microbe would infect the gut mucosa, resulting in a chronic, local inflammatory response which triggers cellular proliferation, cytokine production, and oxidative DNA damage due to an increase in reactive oxygen species [37]. Over time, DNA damage would accumulate in the infected cells, as well as adjacent cells, with mutations in tumor suppressor genes and oncogenes driving morphologic changes that eventually progress to a malignant tumor. Although this model is certainly important for *H. pylori* and the development of gastric cancer [51], increasing evidence points toward the potential for gut microbes to affect carcinogenesis at anatomic sites beyond the gastrointestinal tract and to have complex interactions with diet [36, 123]. For example, some researchers hypothesize that *H. pylori* and high salt intake may act synergistically to promote gastric cancer [147]. Further, recent studies have shown that polyamine catabolism contributes to ETFB-induced colon tumorigenesis in mice [63] (see dietary polyamine section below). Diet may also serve as a potential source of infection for possible carcinogenic agents. For example, high red meat intake is associated with an increased risk of colorectal cancer [114], and it is possible that this relationship is mediated by potentially carcinogenic bacterial contaminants of red meat products, such as *S. bovis* [84].

3.2 Gut Microbial Antigenic Particles Associated with Inflammation

Lipopolysaccharide (LPS, also known as endotoxin) is a bacterial cell wall component in gram-negative bacteria that is associated with low-grade, chronic inflammation in obesity [29, 30, 38] and colorectal cancer [28, 56]. LPS acts through toll-like receptor-4 (TLR-4), a PRR associated with innate immunity, which triggers TGF-β-mediated pathways [1, 97]. This leads to the expression of various genes that promote neoplasia, including those of growth factors and inflammatory mediators. Serum LPS-binding protein (LBP), a protein that binds LPS upon activation of TLR-4, is correlated with circulating concentrations of LPS [117], and a recent prospective study showed that polymorphisms in the LBP gene were associated with increased colorectal cancer risk [33]. To date, no studies have evaluated the association of these mutations to the distribution of gram-negative bacteria in the gut microbiome and cancer risk.

4 Indirect Effects of the Gut Microbiome in Cancer Development and Prevention

Host diet influences the amount and types of bacteria present in the gut, and gut microbial metabolism of dietary compounds affects the production of both protective and harmful metabolites. Therefore, the interaction between dietary intake and the commensal gut bacteria may ultimately influence cancer risk in humans.

Table 1 Summary of the potential impact on cancer risk for gut microbial metabolism of specific exogenous and endogenous compounds

Bacterial metabolism	Substrate sources	Bacterial species	Potential impact on cancer
Exogenous substrates			
Sulfate reduction to produce hydrogen sulfide (H_2S)	Dietary protein, especially sulfur-containing amino acids, and inorganic sulfur sources (SO_4 in water)	Sulfate-reducing bacteria (SRB)	H_2S has cytotoxic and genotoxic effects
Nitrate reduction to nitrite resulting in N-nitroso compounds (NOC)	Meat, particularly red meat	Multiple gut bacterial species	NOC can form DNA adducts
Polyamine production	Ornithine	Multiple gut bacterial species	Polyamines associated with increased inflammatory microenvironments
Flavonoid metabolism: daidzein to equol	Soybeans	*Enterococcus faecium* strain EPI1, *Lactobacillus mucosae* strain EPI2, *Finegoldia magna* strain EPI3, and *Veillonella* sp.	Equol production is associated with lower risks of breast and prostate cancer in high-soy-consuming populations
Metabolism of glucosinolates to isothiocyanates (ITC)	Cruciferous vegetables, such as broccoli, cabbage, kale, and brussels sprouts	*Escherichia coli, Bacteroides thetaiotaomicron, Enterococcus faecalis, Enterococcus faecium, Peptostreptococcus sp.*, and *Bifidobacterium sp.*	ITC have anti-carcinogenic properties, including causing cell cycle arrest and inducing apoptosis
Endogenous substrates			
Production of secondary bile acids (SBA) deoxycholic acid and lithocholic acid	Primary bile acids: cholic acid and chenodeoxycholic acid	Multiple *Clostridium sp.*	SBA may have tumor-promoting activity, and some studies associate high fecal SBA levels with increased risk of colorectal cancer
Metabolism of endogenous estrogens	Estradiol (E_2), estrone 16α-hydroxyestrone, 16-oxoestradiol, 15α-hydroxyestrone	Numerous reactions carried out by a wide variety of bacteria including *Clostridium paraputrificum*, *Bacteroides* sp., *Eubacterium lentum*	Higher circulating concentrations of E_2 associated with breast cancer risk

Cancers arise as a consequence of genomic and epigenomic instability. This instability permits the accumulation of genetic and epigenetic alterations that transform normal, healthy cells into cancer cells. Microbial metabolites may influence the development of microsatellite unstable (MSI) or chromosomal unstable (CIN) tumors through direct genotoxic effects on DNA, as well as by modulating DNA repair systems and by modulating epigenetic mechanisms through histone acetylation or CpG Island methylation [98, 137]. Groups of bacteria with unique metabolism—such as chemolithoheterotrophs that use inorganic compounds as an electron acceptor and organic carbon sources for growth and organoheterotrophs that use organic carbon for both respiration and growth—have been associated with cancer. We describe below several examples of metabolism unique to bacteria that support mechanisms by which the gut microbiome can 1) produce metabolites from exogenous sources (i.e., diet) that may influence tumorigenesis or 2) alter exposure to circulating levels of endogenous compounds, such as steroid hormones or bile acids, that influence tumorigenesis (Table 1).

4.1 Gut Microbial Metabolism of Exogenous Substrates Associated with Carcinogenesis

4.1.1 Sulfate Reduction

Hydrogen sulfide (H_2S) is produced by sulfate-reducing bacteria (SRB) and has been shown to have both cytotoxic and genotoxic effects in cell culture studies [13, 44, 75]. For example, using a modified comet assay, Attene-Ramos et al. [13] showed that H_2S resulted in genomic DNA damage. Sulfide has also been shown to prevent the oxidation of butyrate by colonic epithelial cells, thereby reducing ATP formation and energy harvest [34]. This lowers the absorption of ions, mucus formation, and cellular detoxification. Roediger et al. [121, 122] reported decreased fatty acid oxidation in colonocytes exposed to H_2S, and there is evidence that sulfide alters cellular redox potential which, in turn, alters cell proliferation [44].

The role of SRB in inflammatory bowel disease and colorectal cancer has been evaluated in several epidemiologic and clinical studies [15, 60, 80, 118, 121, 122, 130]. Genomic instability associated with sporadic colon cancer and ulcerative colitis (UC), a risk factor for colon cancer, is hypothesized to result in part from H_2S exposure [14]. In a population-based study ($n = 55$), the distribution of SRB varied between different ethnic groups and the prevalence of SRB was associated with the diets of groups having higher rates of colon cancer [115]. UC has been linked to increased inflammation possibly associated with H_2S generated by SRB [52]. Furthermore, when analyzed by culture methods, fecal samples from patients with UC showed higher concentrations of SRB than feces from patients without UC [61].

SRB are often members of the normal gut microbiota, and diet can influence their distribution and activity. Dietary protein, especially sulfur-containing amino

acids, and inorganic sulfur sources (SO_4 in water) contribute to H_2S production [46]. In a controlled feeding study, Magee et al. [95] showed that H_2S was significantly related to the amount of meat protein consumed. In mice, inorganic sulfate consumption enhanced sulfate reduction [46], and sulfonated proteins (e.g., mucins) enhanced sulfate reduction and inhibited methanogenesis in a continuous culture inoculated with fecal slurry [59]. These studies show that SRB are required for sulfate reduction to occur and that diet can alter their abundance. Most SRB form a fairly phylogenetically discreet group found in the delta subdivision of the delta Proteobacteria. One exception is Desulfotomaculum, which is found in the Clostridium subdivision of the gram-positive bacteria [71]. The functional genes, dissimilatory (bi) sulfite reductase (*dsrA*) [154] and the adenosine-5'-phosphosulfate reductase (*apsA*), are key enzymes in the sulfate reduction pathway [45, 55].

4.1.2 Nitrate Reduction

Epidemiologic studies have suggested an increased risk of colon cancer associated with red and processed meat consumption [114]. Numerous constituents in red and processed meats may contribute to this increased risk [17], including protein and other nitrogenous residues which allow for increased gut bacterial production of N-nitroso compounds (NOC) [22]. Nitrate can be reduced endogenously to nitrite via nitrate reductase produced by the gut bacteria, and nitrite can interact with organic compounds to form NOC. Many classes of NOC have been identified in feces, including nitrosamines, nitrosamides, and nitrosoguanidine [25]. NOC can form DNA adducts which induce mutations. For example, it has been shown that some NOC are alkylating agents that induce GC to AT transitions at the second base of codon 12 or 13 of the K-*ras* gene—this is a common mutation found in colorectal tumors with K-*ras* mutations [25]. More recently, transcriptomic analysis of colon biopsies was compared in inflammatory bowel disease patients diagnosed with UC and irritable bowel syndrome patients without inflammation [66]. The investigators associated gene expression levels with fecal NOC in all study participants (cases and controls) and, using network analysis, found chromatin modification linked to altered regulation of 11 histone genes. This suggested that epigenetic mechanisms may be relevant to NOC-induced carcinogenesis.

Diet can influence NOC concentrations. Meat consumption increases the amount of nitrogenous residues in the colon [136], and in a controlled feeding study in eight men, there was a dose–response between intake of meat and fecal concentrations of NOC [73]. Fecal water genotoxicity correlated with colonic gene expression changes in pro-carcinogenic pathways, including DNA damage repair, cell cycle, and apoptosis pathways in a 7-day dietary intervention with red meat [67]. Additional controlled feeding studies in men showed that, while heme iron increased fecal NOC, protein sources low in heme (i.e., white meat and protein from vegetable sources) did not increase fecal NOC [22, 39]. The independent effect of heme on NOC suggested that chemical catalysis, in addition to bacterial N-nitrosation, may be responsible for the dose-dependent effect of red meat on

increasing endogenous intestinal N-nitrosation [39]. The addition of broccoli, brussels sprouts, or green peas to a high red meat diet had no effect on mean levels of fecal NOC, but the addition of soy statistically significantly suppressed fecal NOC [74].

The importance of gut bacteria in N-nitrosation has been demonstrated by the fact that N-nitrosation does not occur in germfree rats given nitrate as a nitrosating agent, but it does occur in rats harboring a conventional gut microbiota [103]. A number of facultative and anaerobic bacteria are able to catalyze the formation of NOC via nitrate reductase, and dissimilatory nitrate reduction is carried out by a number of bacteria distributed across bacterial groups. The *nar*G gene is responsible for the reduction of nitrate to nitrite. Interestingly, recent studies in humans observed an increased risk of colorectal cancer in people ingesting more than three servings of red meat per week and who had a polymorphism in the nucleotide excision repair pathway [79]. However, the combined effect of degree of gut microbial formation of NOC and variation in host DNA repair mechanisms on colorectal cancer risk has not been investigated.

4.1.3 Polyamine Production

Polyamine exposure has been linked to inflammation in colonic mucosa and subsequent colon cancer risk [58]. Ornithine is converted to the polyamine putrescine, which is a precursor of spermidine and spermine. While this pathway is important to normal growth, the polyamines can also be oxidized to produce reactive oxygen species contributing to a chronic inflammatory microenvironment. An increased flux of polyamines into epithelium up-regulates eukaryotic ornithine decarboxylase (ODC), a key regulatory enzyme involved in polyamine synthesis and up-regulated in colon cancer. This may favor colon cancers that have up-regulated the MAPK signaling pathway downstream from K-*ras* mutations found in CIN tumors [76, 89, 105].

Although polyamines are produced endogenously, both polyamines from diet, as well as those generated by microbial metabolism of dietary precursors, influence levels to which gut epithelial cells are exposed. Gerner et al. [57] showed that the efficacy of chemopreventive treatments can be influenced by modulation of dietary putrescine; however, modulation of the gut microbiome is a chemoprevention avenue that has not received attention. A common chemoprevention treatment in familial adenomatous polyposis is to block ODC with difluoromethylornithine (DFMO) and nonsteroidal anti-inflammatory drugs [58]. DFMO acts on eukaryotic production of polyamines; however, it may be less effective in altering the supply of bacterially produced putrescine. Given the phylogenetic and structural diversity of bacterial amino acid decarboxylases [91], there are multiple, diverse pathways by which bacteria produce polyamines and influence lumenal polyamine concentrations. Thus, while DFMO may influence eukaryotic production of polyamines, bacterial production may not be influenced. For example, DFMO has been shown to be effective in altering growth rates in *H. pylori*, but not those of other enterics, such as *E. coli* and *C. rodentium* [16]. Identifying new

approaches for reducing polyamine production by gut microbes or altering the gut microbial community may be another chemoprevention strategy in high-risk patient populations.

4.1.4 Flavonoid Metabolism

Epidemiologic studies have shown that the consumption of foods of plant origin is associated with lower risk of several cancers [152]. Flavonoids are polyphenolic compounds and are the most abundant phytonutrients in the human diet. Categorized into six major subgroups, they have various cancer-impeding activities, such as reducing DNA damage via antioxidant properties or interacting with inflammation pathways (reviewed in [138]). High inter-individual variation in excretion and circulating concentrations and the extent of metabolism is probably a reflection of variation in the gut microbiome.

Probably the most extensively studied flavonoid with regard to bacterial metabolism is the soy isoflavone daidzein. Studies have shown that approximately 30–50 and 80–90 % of the population are able to metabolize daidzein to equol [54, 88] and O-desmethylangolensin (ODMA) [6, 81], respectively. Several in vitro studies suggest that equol is more biologically active than its precursor daidzein. For example, equol has been shown to be more estrogenic [101], is a more potent antioxidant than daidzein [7, 120, 144, 145], and has a higher effective free fraction in serum than both genistein and 17β-estradiol [110]. This has led to increased interest in equol producers as potential "responders" to soy consumption; over 10 years ago, Setchell et al. [133] hypothesized that the failure to "bacteriotype" individuals for their ability to produce equol in previous intervention studies of soy or isoflavone supplements could explain the variable results seen in such studies. Some, although not all, studies have shown a lower risk of breast cancer and prostate cancers associated with equol production (reviewed in [87]) and favorable associations, in terms of breast cancer risk, between equol production and circulating concentrations of steroid hormones, urinary estrogen metabolites, and mammographic breast density [10, 48, 54, 113].

Human intestinal bacteria are responsible for the production of equol and ODMA [11, 32]. Certain bacteria have been identified that are capable of carrying out discrete steps in the pathway to equol production [135], but other work also suggests that a consortium of bacteria consisting of *Enterococcus faecium* strain EPI1, *Lactobacillus mucosae* strain EPI2, *Finegoldia magna* strain EPI3, and an as yet undescribed species related to *Veillonella sp*. may be involved in equol production [43].

4.1.5 Metabolism of Glucosinolates from Brassica

Consumption of cruciferous or Brassica vegetables has been shown to be inversely associated with risk of some cancers [85]. Isothiocyanates (ITC), the hydrolysis products of glucosinolates, have been shown to have anti-carcinogenic properties both in vitro and in vivo (reviewed in [111]). The biologic effects of ITC are diverse, including interaction with multiple signaling pathways important to

carcinogenesis as well as cross talk between pathways. The inhibitory activity of ITC against tumorigenesis is inferred by its ability to modulate Phase 1 and 2 biotransformation enzyme activities, thereby affecting several processes related to chemical carcinogenesis, such as the metabolism and DNA binding of carcinogens [70]. In vitro studies have also indicated that ITC cause cell cycle arrest and induce apoptosis [109].

Glucosinolates are converted into ITC by either the plant myrosinases or bacterially produced thioglucosidases. Cooking cruciferous vegetables deactivates the plant myrosinases, and given that most cruciferous vegetables consumed by humans are cooked, gut bacteria play a critical role in converting glucosinolates to ITC. Previous studies have shown that certain species of bacteria, such as *Escherichia coli, Bacteroides thetaiotaomicron, Enterococcus faecalis, E. faecium, Peptostreptococcus sp.*, and *Bifidobacterium sp.,* isolated from the human gut or feces can convert glucosinolates into ITC and other derivatives [26, 50, 69]. Controlled feeding studies in humans have shown significant inter-individual differences in urinary ITC excretion after participants consumed the same amount of cruciferous vegetables that had been either heated or microwaved prior to consumption to remove the plant myrosinase activity [125, 134, 146]. Similar effects have been found in controlled feeding studies with rats [35, 126]. This suggests inter-individual differences in the activity or composition of the intestinal bacteria involved in ITC formation. In support of this hypothesis, we showed recently that the fecal bacteria from individuals who excrete higher amounts of ITC in their urine after a standard meal of cooked broccoli metabolize more glucoraphanin [93].

4.2 Gut Microbial Metabolism of Endogenous Substrates Associated with Carcinogenesis

4.2.1 Production of Secondary Bile Acids

The secondary bile acid (SBA), deoxycholic acid (DCA), a colonic bacterial transformation product, has been implicated in gallstone formation [141] and colorectal carcinogenesis [21, 106]. The primary bile acids, cholic acid (CA) and chenodeoxycholic acid, are synthesized in the liver from cholesterol, are conjugated with either glycine or taurine, and undergo enterohepatic circulation (EHC). Although EHC of bile acids between the liver, gallbladder, and intestines is approximately 95 % efficient, up to 5 % of bile acids escape EHC and are transformed by anaerobic bacteria in the colon to SBA, DCA and lithocholic acid (LCA). Diet can affect the amount of bile acids entering the colon, and studies suggest that high-fat diets may result in higher fecal SBA concentrations [40, 104]. SBA have been shown to have tumor-promoting actions in animal studies, and some studies in humans have shown increased risks of colorectal cancer associated with high fecal bile acid concentrations (reviewed in [104]). However, not all studies have shown such associations [107]. Associations between fecal bile acid

concentrations and colon cancer are complex given that factors such as gut transit time and pH may also influence fecal SBA concentrations, and it has been suggested that fecal bile acid concentrations may overestimate the amount of DCA in the bile acid pool given that nearly all bile acids that escape EHC are converted to SBA before excretion [104]. Nonetheless, serum DCA levels, which may reflect the bile acid pool more accurately, also have been shown to be higher in patients with colon cancer than in healthy individuals [18, 19]. A potential mechanism for associations between DCA and colon cancer is that DCA may change the balance between apoptosis, proliferation, and differentiation in the intestinal epithelium [64], acting through interference of tumor suppression and enhancing stimulation of growth via cell signaling pathways.

The bacteria responsible for DCA formation have been identified and belong to the genus *Clostridium* [47, 83, 148, 149]. These bacteria are classified into two classes with either high or low 7α-hydroxylating activity which may explain some of the inter-individual variation in DCA concentrations. In addition, the bile acid inducible (*bai*) operon, involved in the bacterial 7α-dehydroxylation of CA to DCA, has been characterized [148].

4.2.2 Metabolism of Endogenous Estrogens

Breast cancer risk is associated with higher levels of circulating estrogens, such as estradiol (E_2), estrone (E_1), E_1 sulfate (E_1S), estriol (E_3) and dehydroepiandrosterone (DHEA) [82], and 16α-hydroxyestrone (16α-OHE_1) in urine [10]. Both genetic and environmental factors that alter circulating estrogen levels may influence the risk of breast cancer.

Estrogens circulate in the bloodstream either free or bound to protein, conjugated or unconjugated, and either interact with target tissue or are excreted. Estrogens are sulfated, glucuronidated, or methylated in the liver, and about 50 % are excreted in urine and the other half are excreted in bile and undergo EHC. In the colon, some of the compounds are excreted in feces and some are metabolized by the gut microbiome (reviewed in [108]). The gut microbiome can (1) increase the exposure to circulating hormones via deconjugation and (2) influence the composition (or types) of hormones in circulation via hydroxylation/dehydroxylation and methylation/demethylation. β-Glucosidases, β-glucuronidases, and sulfatases from a wide variety of gut bacteria hydrolyze hormone conjugates [41]. These unconjugated estrogens are reabsorbed into the bloodstream and excreted in urine or can undergo EHC again [132]. Adlercreutz and others showed over 30 years ago that when patients were given antibiotics, urinary estrogen excretion decreased suggesting that gut bacteria are important in regulating EHC of estrogens and therefore exposure [3, 4, 102]. Diet may also influence serum hormone concentrations either directly by binding of estrogens by dietary fiber [8] or indirectly by influencing gut microbial community metabolism of estrogens [2].

Many gut bacteria are capable of performing the initial step of hydrolyzing conjugated steroids [151], which enables further metabolism by intestinal bacteria to occur. Microbial metabolism of estrogens includes reduction, oxidation, and the

Table 2 Associations between serum hormone concentrations (outcome)[a], the gut microbial community (exposure), and diet (exposure) in premenopausal women

	β-Coefficient	p value
Estrone sulfate		
Archaea[b]	−0.11	0.028
Total fiber	−0.01	0.019
Alu I Axis 1	−0.18	0.017
Total fiber	−0.01	0.094
Total fat	−0.005	0.045
Free estradiol: all estradiol		
Alu I Axis 2	0.21	0.013
Total fiber	−0.01	0.099
Total estradiol		
Rsa I Axis 2	0.17	0.028
Total fiber	−0.011	0.053

[a] Using stepwise GLM that considered inclusion at 0.10 level for hormones, percent body fat, and potentially confounding demographic factors
[b] Adjustment for adiposity

generation of E_2 from E_1, as well as from E_2-3-glucuronide, E_1 from E_2, and from estrone-3-sulfate (E_1S), and E_3 from 16α-OHE_1. In addition, in vitro incubation showed conversion of E_1, E_2, and 16α-dehydroxyestrone to E_3, 16-oxoestradiol to 16-epiestriol, and 15α-hydroxyestrone to 15α-hydroxyestradiol [77, 78, 94]. Ring-A reduction is catalyzed by enzymes from a wide variety of bacteria including *Clostridium paraputrificum* and *Bacteroides* sp., and reductive dehydroxylation of ring-D and oxidative reactions, such as dehydrogenation, is carried out by bacteria such as *Eubacterium lentum* [24].

We recently evaluated associations between gut microbial community composition and circulating steroid hormone concentrations in a cross-sectional study of 115 healthy premenopausal women, age 40–45 years [12]. The gut microbial community from fecal samples was measured by terminal restriction length polymorphism (TRFLP) analysis with two restriction enzymes, Alu I (predominantly Bacteroidetes) and Rsa I (predominantly Firmicutes), and quantitative PCR (qPCR) of the 16S rRNA genes of Bacteria, Bacteroides, Clostridia Cluster XIVa, and Archaea. Regression models were fit to assess associations between hormones and the gut microbial community structure. The outcomes measured were serum concentrations of E_1, E_1S, total and free E_2, sex-hormone-binding globulin (SHBG), free E_2:total E_2 ratio, and exposures were gut microbial community multivariate axes from TRFLP analysis and qPCR. The final solution for NMS analysis of the Alu I TRFLP patterns had stable stress values of 16.65, after 400 iterations. The three axes cumulatively explained 79.7 % of the variation in both subsets of TRFLP data. Axes 1, 2, and 3 explained 26.2, 31.7, and 21.7 %, respectively. The final

solution for NMS analysis of the Rsa I TRFLP patterns had stable stress values of 16.87, after 400 iterations. NMS analysis for Rsa I TRFLP explained a total of 82 % of the variation in the data for axis 1 (23 %), axis 2 (29 %), and axis 3 (29 %). E_1 ($p < 0.004$), E_1S ($p < 0.017$), E_2, and free E_2:total E_2 ratio were significantly associated with the gut microbial community using ALU I ($p < 0.05$). E_1S, E_2, and free E_2:total E_2 ratio were significantly associated with the gut microbial community described by Rsa I ($p < 0.05$). E_2 free E_2:total E_2 ratio, and SHBG were associated with Bacteroides. E_1 and E_1S were associated with Archaea ($p < 0.05$). When adjusted for dietary factors and demographics, there was a significant association between E_1S and either Archaea or Alu Axis I, free E_2:total E_2 and Alu Axis 2, and E_2 and Rsa Axis I, suggesting that the composition of the gut microbiome may be a factor in determining concentrations and types of circulating steroid hormones. This study suggests that it is important to consider an exposure such as diet within the context of gut microbial community (Table 2).

5 Conclusions

Aspects of human health are influenced by the interaction of the gut microbiome, diet, and host physiology. We presented examples of microbially mediated pathways associated with cancer. These pathways involve both 1) direct contact of the pathogen with human host and 2) indirect effects of microbial metabolism of exogenous and endogenous substrates. These pathways alter inflammation, modify DNA leading to mutations, or influence epigenetics and gene silencing. Recent metagenomic studies of the gut microbiome have revealed the varied anaerobic metabolisms, both chemoheterotrophic and organoheterotrophic, involved in fermentation and the production of metabolites that are either beneficial or harmful to the host [9, 143]. In addition, these approaches have characterized differences in the composition of the microbial community associated with tumor and nontumor regions in the colon [100]. Inter-individual variation in cancer risk may therefore be associated with microbial biomass, composition, and function, and the interaction with host factors such as diet. Future studies need to consider the gut microbiome as a contributing functional unit in relation to host exposures in order to better understand both its impact and those of the exposure on cancer risk and to design appropriate prevention strategies.

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Inflammatory Angiogenesis and the Tumor Microenvironment as Targets for Cancer Therapy and Prevention

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Abstract

In addition to aberrant transformed cells, tumors are tissues that contain host components, including stromal cells, vascular cells (ECs) and their precursors, and immune cells. All these constituents interact with each other at the cellular and molecular levels, resulting in the production of an intricate and heterogeneous complex of cells and matrix defined as the tumor microenvironment. Several pathways involved in these interactions have been investigated both in pathological and physiological scenarios, and diverse molecules are currently targets of chemotherapeutic and preventive drugs. Many phytochemicals and their derivatives show the ability to inhibit tumor progression, angiogenesis, and metastasis, exerting effects on the tumor microenvironment. In this review, we will outline the principal players and mechanisms involved in the tumor microenvironment network and we will discuss some interesting compounds aimed at interrupting these interactions and blocking tumor insurgence and progression. The considerations provided will be crucial for the design of new preventive approaches to the reduction in

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_23,

cancer risk that need to be applied to large populations composed of apparently healthy individuals.

Keywords

Tumour microenvironment · Angiogenesis · Inflammation · Anti-angiogenic therapy · Chemoprevention

Abbreviation

IGF1-R	Insulin-like growth factor 1-receptor
IκB	Inhibitor kinase B
IKK	Inhibitor kinase kinase
IL-13	Interleukin-13
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-12	Interleukin-12
IL1Rα	Interleukin-1 receptor-alpha
IL1β	Interleukin-1 beta
JAK	Activating Janus kinase
MAPK	Mitogen-activated phospho kinase
MDM2	Murine double minute 2
MDM4	Mouse double minute 4
MDSC	myeloid-derived suppressor cell
MMP-1	Matrix metallo protease-1
MMP-2	Matrix metallo protease-2
MMP-7	Matrix metallo protease-7
MMP-9	Matrix metallo protease-9
mTOR	Mammalian target of rapamycin
N1	N1-polarized neutrophils
N2	N2-polarized neutrophils
NAC	N-acetylcysteine
NF-κB	Nuclear factor-kappa B
NSAIDs	Non-steroidal anti-inflammatory drugs
NSCLC	Non-small cell lung cancer
PDGF	Platelet-derived growth factor
PDGFR-β	Platelet-derived growth factor receptor-beta
PDGFR	Platelet-derived growth factor receptor

PI3K	Phosphoinositide 3-kinase
PIG3	p53-inducible gene 3
PlGF	Placental growth factor
PMNs	Polymorphonuclear neutrophils
Raf	Root abundant factor
RARs	Retinoid acid receptors
RARβ	Retinoid acid receptor-beta
RCC	Renal cell carcinoma
RelA	NFkB subunit-A
RelB	v-rel reticuloendotheliosis viral oncogene homolog B
RET	Rearranged during transfection
RHD	Rel homology domain
RITA	Reactivation of p53 and induction of tumor cell apoptosis
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
STAT	Signal transducer and activator of transcription
STAT3	Signal transducer and activator of transcription 3
SV40	Simian virus 40
TAM	Tumor-associated macrophage
TEM	Tie2-expressing macrophage
TGFα	Transforming growth factor-alpha
TGFβ	Transforming growth factor-beta
Th	T helper
Th2	T helper type-2 polarization
TKIs	Tyrosine kinase inhibitors
TNF	Tumor necrosis factor
TNFα	Tumor necrosis factor-alpha
TP53TG1	TP53 target 1
TSP-1	Thrombospondin-1
VEGF-A	Vascular endothelial growth factor A isoform
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vSMC	Vascular smooth muscle cell
WIP1	Wound-induced protein 1

Contents

1 Tumor Microenvironment

Despite the awareness that the host plays a major role in carcinogenesis and tumor progression, dating back to the observation of inflammatory cells in cancer made by Virchow in 1863, and the essentially contemporary "seed and soil" hypothesis postulated by Paget in 1889, these concepts were essentially ignored for nearly a century [14]. Currently, the idea of cancer as a tissue and developing not simply due to mutations in a single cell but rather as a complex process creating a permissive environment within the host has now taken hold [44]. Furthermore, the conception of the tumor microenvironment as being both a target of cancer therapy, for example, targeting angiogenesis [46] in a growing array of tumor types and perhaps even more importantly, as a target for cancer prevention [8, 10] is becoming a reality.

Inflammation is now recognized as a driving force in carcinogenesis [31, 114]. Many environmental causes of cancer and risk factors are associated with some form of chronic inflammation. Up to 20 % of cancers are linked to chronic infections, 30 % can be attributed to tobacco smoking and inhaled pollutants (such as silica and asbestos), and 35 % to dietary factors (20 % of cancer burden is linked to obesity) [77].

The effect of inflammation on tumor progression is related to its ability to drive the angiogenic switch [7, 89, 106]. Cancer cells themselves produce a series of proangiogenic factors, such as vascular endothelial growth factor (VEGF), that interact with specific receptors inducing endothelial cell recruitment and activation. Activated endothelial cells give rise to a tumor-associated vasculature, a process termed tumor angiogenesis, which is quite different from the normal tissue vasculature, presenting tortuous, disorganized and fenestrated vessels, high macromolecule diffusion coefficients, and an insufficient or absent functional lymphatic network leading to high interstitial pressure. This tumor vascularization is essential for cancer progression, as it allows tumor expansion, local invasion, and metastatic dissemination, indicating that tumor angiogenesis is a critical early event in carcinogenesis [22, 47, 54, 59].

1.1 Cellular Players

1.1.1 Inflammatory Cells

Many studies demonstrate the tight correlation between cancer and inflammation (Table 1), and recently, cancer-related inflammation (CRI) has been defined as the seventh hallmark of cancer, due to the pivotal role of these components in the tumor microenvironment [28, 60]. Inflammatory components of the microenvironment are several cell types that participate and sustain CRI. The major populations of inflammatory cells infiltrating tumors are tumor-associated macrophages (TAMs), but also Tie2-expressing monocytes (TEM), myeloid-derived suppressor cells (MDSCs), and neutrophils are involved in this process. The role of tumor-infiltrating macrophages has been well established. Macrophages, in fact, are able to assume different phenotypes depending on microenvironmental stimuli, such as chemokines, fostering tumor progression or development of the specific immune response [31, 106, 121]. The most important population of infiltrating tumor inflammatory cells are tumor-associated macrophages (TAMs) that produce a series of growth factors, cytokines, and extracellular matrix-modeling molecules inducing tumor cell proliferation and angiogenesis. TAMs originate from monocyte precursors that, attracted by tumor cells mainly through CCL2 and CSF-1, extravasate from the bloodstream. TAMs themselves produce CCL2, establishing an autocrine positive loop of regulation [141]. Several studies show that TAMs localize in necrotic and hypoxic areas, which produce HIF1-dependent molecules (VEGF, CXCL12, and CXCR4), which in turn regulate TAM migration [104]. Activated TAMs produce a series of proangiogenic factors that sustain and promote tumor growth; among others, they release VEGF, PDGF, TGFβ, and members of the FGF family. Further, TAMs produce numerous angiogenesis-modulating enzymes such as matrix metalloproteinases MMP-7, MMP-2, MMP-9, MMP-1, as well as cyclooxygenase (COX)-2 [106].

Several studies have shown a correlation between the presence of infiltrating TAM and the poor prognosis of cancers such as lymphomas [76, 165] and breast cancer [20]. Recently, a monoclonal antibody against CCL2, developed and tested in murine models of glioma, was able to significantly reduce the percentage of TAMs and increases survival of tumor-bearing mice [182]. TEMs are a subset of Tie2-expressing TAMs that are closely associated with endothelial cells and tumor angiogenesis [27].

Polymorphonuclear neutrophils (PMN) are the most abundant subpopulation of leukocytes in the blood and mainly involved in innate immune response against microorganisms. However, their flexibility and regulatory capacity is becoming increasingly appreciated [96, 149]. Upon stimulation, neutrophils are rapidly recruited into affected tissues, where they start to degranulate releasing proteases and generating reactive oxygen species (ROS). These factors can produce tissue damage and cell lysis, as well as potential DNA damage acting as both an initiator and promoter [96]. Increased levels of neutrophils have been observed in several cancers type such as breast and colon cancer and in inflammatory lesion and

Table 1 Mechanisms of action of some chemoprevention agents

Compound	Functional targets		Molecular targets			
	↓Inflammation	Angioprevention	↓NF-κB	↓Akt	↓STAT3	↑AMPK
NAC	+	+	+	+*	+*	+*
Fenretinide	–	+	?	+	–	–
Aspirin	+	+	+	+	+	+**
Metformin	+	+	+	+	+	+
EGCG	+	+	+	+	+	+
Xanthohumol	+	+	+	+	–	–
Deguelin	+	+	+	+	+	+
Hyperforin	+	+	+	+	–	–
Curcumin	+	+	+	+	+	+
Resveratrol	+	+	+	+	+	+
CDDO-Me	+	+	+	+	+	+
CDDO-Im	+	+	+	+	+	–
Tamoxifen	–	+	+	–	+***	–

Most of these cancer prevention agents have been shown to inhibit (↓) inflammation and angiogenesis (angioprevention) as well as the NF-κB and Akt pathways. Several have also been shown to block the STAT3 pathway and activate (↑) AMPK (which in turn inhibits Akt downstream). + Activity present, – No evidence for activity in the literature. * Activity based on contrasting oxidative stress. ** Activity due to salicylate, a metabolite in vivo. *** Only one report using raloxifene. For references, see the following reviews: [2, 8, 10, 138, 139]

ulcerated areas [104]. PMNs have a direct role in the angiogenesis induced by CXCR2 binding chemokines [16], which induce VEGF production by PMN [131]. We proposed the hypothesis that PMN within tumors could show a similar phenotypic polarization to that of tumor macrophages [106] to anti-tumor "PMN1" (or N1) and protumor "PMN2" (or N2) subsets, based on several lines of evidence [73, 107, 142]. Recent studies in experimental models have provided extensive support for the existence of pro-angiogenic ("N2") or anti-tumor (N1) neutrophil phenotypes [49]. The major player in the switch between the two phenotypes appears to be TGFβ, as increased levels of TGFβ stimulated polarization toward N2 neutrophils [48, 49]. Neutrophils can produce a plethora of cytokines, including tumor necrosis factor α (TNFα), interleukin (IL)1β, IL12, IL1Rα, and VEGF; chemokines (CXCL1, CXCL8, CXCL9, CCL3, and CCL4) and proteases such as MMP9 [106].

1.1.2 Fibroblasts and Myofibroblasts

Fibroblasts form the structural scaffold the stroma, of tissues and they play a pivotal role in the tumor microenvironment. In physiological conditions, they are almost quiescent, while upon tissue injury, fibroblasts are activated to increase

production of extracellular matrix components such as collagen and fibronectin [125]. Furthermore, they participate in the formation of basement membrane by secreting laminin and type IV collagen. In initial phases of the carcinogenesis process, fibroblasts inhibit tumor progression through gap junctions between activated fibroblasts. Later, during tumor progression, cancer-associated fibroblasts (CAFs) favor tumor growth and progression modifying the tumor stroma [116]. Mechanical tensions drive differentiation of activated fibroblasts toward myofibroblasts, and recent studies have suggested that fibrosis itself is a major driving force for carcinogenesis [75]. Matrix rigidity is known to regulate several correlates of carcinogenesis and epithelial-mesenchymal transition (EMT), the major route toward invasive tumor growth and dissemination [74]. CAFs release into the stroma surrounding the tumor several cytokines and growth factors such as hepatocyte growth factor (HGF), fibroblast growth factor (FGF), CXCL2, IL6, and TGFβ [60]. In addition, CAFs produce CXCL12, also known as stromal-derived factor-1 (SDF-1), that attracts endothelial cell precursors (EPCs), thus promoting angiogenesis [110, 172]. Recently, it has been demonstrated that in a cohort of patients affected by mobile tongue squamous cell carcinoma, CAF density was a reliable marker for predicting prognosis [15].

1.1.3 Endothelial Cells and Pericytes

The tumor vessel architecture is quite different from that of normal vessels, mainly due to altered endothelial-endothelial and endothelial-pericyte cell interactions producing poorly developed, tortuous vessels. These vessels show structural variations such as fenestrated walls and a paucity of pericytes, resulting in functional alterations. Angiogenesis, the formation of new blood vessels starting from preexisting vessels, is a complex process constituted by different steps. Initially, there is an increase in vascular permeability due to extracellular matrix breakdown driven by proteinases such as metalloproteinases and the action of major angiogenesis factors that are also permeability agents, in particular VEGF [71]. Subsequently endothelial cells migrate toward the source of the angiogenic stimulus, proliferate and then differentiate. Mural cells stabilize the endothelial cells sprouting to make new vessels. The process requires a fine regulation of the balance between stimulatory and inhibitory signals mediated by angiopoietins, integrins, chemokines, oxygen sensors, and others [21]. In normal vessels, pericytes are an integral part of the capillary bed, coordinating intracellular signaling between endothelial cells and other components of vessel to prevent leakage. The vascular tone and contraction of blood vessels are maintained by vascular smooth muscle cells (vSMCs) that form single or multiple layers around vessels [128].

In contrast, tumor vessels are immature due to dysregulation of levels of pro-angiogenic and anti-angiogenic factors, resulting in an imbalanced expression of angiogenic cytokines and inhibitors. During tumorigenesis, the tumor microenvironment provides attracting stimuli that recruit endothelial precursor cells (EPCs) from the bone marrow and drives differentiation toward mature endothelial or mural cells [66]. One of the major molecular mediators of this process is VEGF-A,

produced by numerous neoplastic cells. VEGF-A, upregulated by hypoxia, systemically can bind receptors on EPCs, inducing their mobilization. VEGF-A and VEGF-A receptors are already targeted by several compounds such as monoclonal antibodies and small molecule inhibitors that exert anti-tumor anti-angiogenic activity. Targeting angiogenesis is coming into focus also as a way of preventing cancer (Table 1) as well as a therapeutic modality [8, 9, 153].

Recently, the role of pericytes in tumor vasculature has been revaluated. Pericytes are fibroblastic/smooth muscle-like cells that are in close contact with endothelial cells. Their recruitment, differentiation, function, and homeostasis are finely regulated by a range of signaling pathways, such as platelet-derived growth factor (PDGF), TGFβ, and angiopoietins [117]. Pericytes can protect endothelial cells from anti-VEGF therapy, and tumor vasculatures without sufficient pericyte coverage are more sensitive to anti-angiogenic therapies [117]. For these reasons, several groups have evaluated the feasibility to target both endothelial cells, through anti-VEGF therapy, and pericytes by anti-PDGF therapy. These dual-targeted therapies have an increased efficacy in a variety of mouse models [17, 42], but recent studies have shown that there are still some issues in clinical trials [92]. Interestingly, two small molecule tyrosine kinase inhibitors (TKIs) approved anti-angiogeneic agents that are active in renal cell carcinoma and other cancers (Sorafenib and Sunitinib) target both VEGFRs and PDGFRs. However, the recent experimental evidence suggesting that inhibition of angiogenesis may promote metastatic dissemination [43, 111] has been more recently linked to inhibition of pericyte coverage and subsequent facilitation of tumor cell access to the vasculature [26, 170].

1.2 Molecular Pathways

Molecular pathway deregulation within the microenvironment occurs in all the phases of carcinogenesis as a consequence of extensive stimulation of cytokines, chemokines, and other factors, determining a type of cellular "Hobson's choice" between life and death. In this dynamic imbalance, one could use chemicals targeting these signaling pathways at different stages, according to the cellular context. Here, we briefly depict the main pathways modulated by a series of natural compounds we subsequently describe below. Although they may seem simply parallel or diverging roads, they cross each other in such a tangled manner that the final outcome, or destination, is quite unpredictable and circumstantial [152, 166].

1.2.1 p53

p53 is a key regulator of cell fate activated in response to cellular stress such as that induced by deregulation of mitogenic oncogenes, DNA damage, nutrient deprivation, hypoxia, and loss of stromal support [161]. Moll and Schramm [101] defined it as "an acrobat in tumorigenesis" to describe the multiple interactions the

p53 protein can engage in with other molecular partners that confer it the "good cop/bad cop" role in cell survival [136]. p53 was discovered in 1979 as a 55-kDa protein produced by the host that co-immunoprecipitated with the large T antigen in SV40-transformed cells [85]. In normal tissue, the level of p53 was not detectable, but it was found over-expressed in a large number of tumors [32]. This along with other experimental evidence [97, 100] could account for a role in positive regulation of cell proliferation. But in the late 1980s, p53 was found mutated with consequent loss of function in almost half of all human cancers [61, 62]. The large T antigen forms a complex with Rb and p53 that inactivates both of these molecules, cooperating in the potent cell transformation capacity of the SV40 virus. In addition, injection of wild-type p53 in rat fibroblasts prevented transformation by other oncogenes [88]. These findings gained wide acceptance, and p53 was defined as a tumor suppressor gene. Originally it was thought to be the only product encoded by the TP53 gene, currently the p53 family includes at least nine isoforms originated by alternative splicing, alternative promoter usage, or translation initiation. p53 is a transcription factor that binds specific DNA response element (RE) sequences, activating genes involved in cell cycle control (p21, GADD45, WIP1, mdm-2, EGFR, PCNA, Cyclin D1, Cyclin G, TGFa, and 14-3-3 s), DNA repair (GADD45, PCNA, and p21), apoptosis (BAX, Bcl-L, FAS1, FASL, IGF-BP3, PAG608 and DR5/KILLER and GML), angiogenesis (TSP-1, BAI1), and cellular stress response (TP53TG1, CSR, and PIG3) [122]. More interesting, recent findings suggest that not just cell cycle arrest or apoptosis, but induction of senescence with consequent occurrence of immune responses by the organism, can be involved in tumor progression triggered by p53 [173]. A new complexity in its role as a tumor suppressor is given by the recent discovery that p53 can regulate microRNA expression [63] and that it posses non-transcriptional activity in the cytoplasm triggering autophagy or apoptosis, interacting directly with Bcl-2 family members [56]. p53 activity is tightly regulated by its gatekeeper MDM2. MDM2 binds the transactivation domain of p53 blocking its transcriptional activity [102] and inducing p53 ubiquitinylation leading to degradation in the proteasome [83]. Since MDM2 is a direct transcriptional target of p53 [169], the system is tightly regulated. Like MDM2, MDM4 binds p53 cooperating in its degradation [90]. Tuning the MDM2–p53 loop is a promising target for anticancer therapy.

Oncogene signaling appears to activate an angiogenic program within tumor cells [79, 123, 124], often through stabilization of HIF1α [103, 133] increasing VEGF and other proangiogenic factors. However, oncosuppressors are also involved in regulation of the angiogenic program in normal and tumor cells [50, 150]. In particular, angiogenesis is modulated by the master regulator and critical tumor suppressor p53, one of the key molecules affected by, and an effector of, many chemotherapeutics [50, 150]. p53 represents a multivalent tumor suppressor whose importance in preventing cancer is demonstrated by frequent inactivation of its pathway in tumors [109].

Many functions have been attributed to p53 that includes, for example, direct roles in repair and recombination, association with proteins involved in genome

stability, and chromatin modification. However, its broadest cellular impact is that of transcription factor (TF). In its role as a master regulator, the large network of genes subjects to p53 control comprise a diverse group of biological activities that include apoptosis, cell cycle regulation, senescence, energy metabolism, immune response, motility and migration, and cell-to-cell communication [162].

A growing body of evidence is accumulating indicating that p53 can also suppress angiogenesis through several different mechanisms, which include the following: (1) enhanced production of anti-angiogenic molecules in the extracellular matrix, derived from collagen by the activity of collagen prolyl-4-hydroxylase [151], a transcriptional target of p53; (2) repression of transcription of proangiogenic molecules such as VEGF and bFGF [150]; (3) stimulation of HIF1α degradation by direct binding [126]; (4) modulation of microRNA such as miR107 targeting HIF1β [174]; and (5) inhibition of the angiogenic switch in tumors in concert with p19ARF [155]. Moreover, nutlin-3, a non-genotoxic activator of the p53 pathway, has been shown to inhibit endothelial cell migration and tube formation, while showing minor effects on endothelial cell cycle and apoptosis [132]. Nutilins and RITA, two drugs that disrupt MDM2-p53 interaction, show good anticancer activity in preclinical studies [70, 157] which will hopefully be tested in the clinic.

1.2.2 NF-κB

NF-κB is a transcription factor that binds a specific DNA sequence [134]. It is involved in the control of a vast number of normal cellular processes (immune and inflammatory responses, development, cellular growth, and apoptosis) and, when deregulated, NF-κB plays a role in several diseases, including chronic inflammation, heart disease, and cancer [11, 84].

NF-κB consists of hetero- and homo-dimers of five different proteins (p50, p52, p65 RelA, RelB, and c-Rel) sharing a conserved N-terminal region, called the Rel homology domain (RHD) which can bind DNA. There is also a family of inhibitory partners, IκB [13]. In most cells, NF-κB is trapped in the cytoplasm due to direct binding to one of the seven members of the IκB family. The upstream regulator of this interaction is the IκB kinase (IKK), consisting of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ). Upon activation by extracellular signals, IKK phosphorylates IκB, which subsequently enters into the polyubiquitination degradation pathway, releasing NF-κB from cytoplasmatic exile. Once in the nucleus, NF-κB can regulate a plethora of genes coding for cytokines, chemokines, immunoreceptors, cell adhesion molecules, cell surface receptors, and pro- and anti-apoptotic proteins [53]. Many different stimuli can activate NF-κB through three different pathways. The canonical one is triggered by proinflammatory cytokines and viral/microbial infection and, via IKK activation—IκB degradation—p50:RelA nuclear localization, leads to the expression of genes involved in innate immunity and inhibition of apoptosis [77, 135]. In the non-canonical pathway, upon stimulation by TNF cytokine family members, p52:RelB is generated by processing of the precursor complex p100:RelB. This

leads to modulation of genes involved in adaptive immune response and secondary lymphoid organ development [171]. The third pathway, the atypical one, does not require IKK activation. DNA damage (UV irradiation), certain chemotherapeutic drugs, or ARF activation results in repression, rather than activation, of anti-apoptotic genes by p50:RelA functioning as tumor suppressor [112]. Excessive production of activating cytokines (TNFα, IL-1β) [51], oncogenes such as ras and myc, or genetic alterations in the gene coding NF-κB [30, 127] determines constitutive expression of NF-κB in many malignances. As a cancer-promoting factor, NF-κB represses apoptosis [140] and promotes angiogenesis [24], tumor metastasis [68], and cell cycle progression [67]. Its inhibitory effect on apoptosis accounts for NF-κB mediated chemoresistance, although in a few cases (paclitaxel, doxorubicin), NF-κB is required for drug-induced cell death [18, 68]. Due to its intricate involvement in cancer and inflammation, researchers continue to place substantial effort in elaborating new strategies to specifically target the NF-κB pathway and its regulatory partners (Table 1), often adopting natural compounds (curcumin, resveratrol), synthetic drugs (thalidomide), or biomolecular tools (cell permeable peptides, microRNA, and decoy oligodeoxynucleotides) [12, 78, 87].

1.2.3 Signal Transducer and Activator of Transcription

Cytokines and growth factors secreted by tumor cells trigger constitutive NF-κB activation in cancer cells and other components of the tumor microenvironment [57, 91, 131]. Specific factors, such as IL-6 and FGF, are activators of signal transducer and activator of transcription 3 (STAT3) [41, 181]. In mammals, there are seven STAT proteins each encoded in a distinct gene and for some of them different alternative splicing isoforms exist. Originally identified through the study of IFN-induced responses [35], STAT's share structurally and functionally conserved domains except for the carboxy-terminal transcriptional activation domain that is quite divergent and contributes to STAT specificity [178]. The most highly conserved domain is the SH2 domain accountable not only for the capability of STAT's to homo-heterodimerize [137], but also to bind to specific phosphotyrosine motifs. Indeed, this latter SH2 feature is essential for STAT signaling, enabling STAT recruitment to the cytokine receptor and association with the activating Janus kinase (JAK) [58]. Of the seven STAT proteins known, constitutive activation of STAT3, STAT5, and STAT6 has been implicated in human cancers such as leukemias, multiple myeloma, and several solid tumors. This finding, together with their role in NF-κB signaling pathway, makes STATs suitable targets for cancer therapy. STAT3 was first identified as the acute-phase response factor (APRF) for its role in activating transcription of IL-6 responsive genes [5]. Subsequent studies have shown that constitutive activation of STAT3 occurs in cells transformed by oncogenic tyrosine kinases such as Src [177, 178]. The mechanism of constitutive STAT3 activation is mainly due to autocrine and paracrine production of IL-6 from the tumor inflammatory environment leading to STAT3 phosphorylation. Interestingly, IL-6 induces STAT3, which in turn induces IL-6 in a feed-forward activation loop. Activation of STAT3 protects cells from

apoptosis and promotes cell cycle progression and metastasis [23]. Several studies have demonstrated links between the STAT3 and NF-κB pathways: STAT3 is activated by IL-6 [105, 181] and COX-2 [34], transcriptional targets of NF-κB. On the other hand, in normal immune cells, STAT3 controls NF-κB activation by inhibiting IKK [166]. Unphosphorylated STAT3 binds unphosphorylated NF-κB and accumulates in the nucleus, where they activate a subset of NF-κB-dependent genes [175]. Furthermore, recently, Lee and colleagues [86] revealed how STAT3 directly interacts with RelA and facilities its acetylation by p300, leading to prolonged NF-κB nuclear retention in both tumor and tumor-associated immune cells. STAT6 was first shown to be activated by IL-4 [65] and subsequently by IL-13 that shares a receptor chain with IL-4. Interleukin-4 plays an important role in modulating the balance of T helper (Th)-cell subsets, favoring expansion of the Th2 lineage relative to Th1. Indeed, STAT6 knockout mice are unable to develop Th2 cells [148].

2 Cancer Chemoprevention

2.1 N-acetylcysteine

N-acetylcysteine (NAC) appeared to be one of the more promising agents [39] in the protection of microenvironment (Table 1). It is a synthetic precursor of cysteine and glutathione, the body's most relevant antioxidant. Increasing glutathione levels, NAC itself is considered an antioxidant drug and plays a key role in buffering the oxidative stress generated by ROS in arthritis, cancer, cardiovascular and respiratory disease [179]. In the 1960s, NAC was first used as mucolytic drug [119] and to prevent chronic obstructive pulmonary disease exacerbation. Later in the 1980s, several experiments in animal models unveiled its cancer preventive potential showing that NAC can prevent carcinogen-mediated oxidative DNA damage and suppress or delay the development of tumors or preneoplastic lesions in rodents [37, 38]. Moreover, NAC can prevent cellular damage following HIV1-mediated oxidative stress due to low levels of cysteine and glutathione [118].

Recently, this antioxidant agent has been proposed as an eligible drug for breast cancer prevention, due to the reported protection from DNA adduct formation in a mouse epithelial cell line [160]. The molecular mechanisms by which NAC accomplishes its protective role have been extensively investigated. It inhibits the NF-κB, AP1, and MAPK pathways, counteracting proinflammatory and growth factor signaling and reducing cell damage following chemotherapy or radiotherapy [147]. Reducing AKT phosphorylation, NAC also targets the microenvironment compartment, supporting its anticancer effect by preventing angiogenesis and tumor metastasis in experimental models [6, 94]. Despite all the makings of a good chemopreventive drug, NAC was very disappointing in clinical trials: in the BRONCUS study, it did not show any benefit in pulmonary disease [40]; in the EUROSCAN study, patients with head and neck or lung cancer did not benefit

from NAC administration [156]. In addition, there is no strong clinical evidence of a benefit in cardiovascular diseases, nor in liver or in kidney diseases [3]. Furthermore, experimental studies disclose, as for other antioxidant agents, a possible carcinogenic effect for NAC by DNA damage induction in the presence of CuII [108].

2.2 Fenretinide

Fenretinide, also known as N-(4-hydroxyphenyl)retinamide (4HPR), is the retinoid acid analogue most used in cancer chemoprevention. Although it is known that the intracellular receptor of retinoic acid belongs to the steroid/thyroid hormone superfamily, its analogue exerts its activity affecting several pathways inside tumor cells. In hepatocellular carcinoma, lung, head and neck cancer, and several ovarian cell lines, 4HPR induces apoptosis, binding RARβ and thereby activating caspase 3 [19, 129]. RARβ is one of the three retinoid acid receptors (RARs) involved in normal cell growth and regulation. Fenretinide is able to induce apoptosis also in a caspase-independent manner, impairing redox balance, triggering ROS production and cytocrome c release. Moreover, it activates ceramide/sphingomyelin-mediated pathways [159]. These mechanisms of action are active in myeloid leukemia cells [72], prostate cancer cells [55], human pancreatic cells [98], neuroblastoma [33], retinoblastoma cells [154, 159], and liver cancer cells [176].

Furthermore, fenretinide affects the angiogenic component of the tumor microenvironment (Table 1). It directly impairs endothelial cell functions, interfering with the production, release, and activation of several growth factors and cytokines. Fifteen years ago Pienta and co-workers demonstrated that 4HPR inhibits endothelial cell motility, the ability to form capillary-like structures, and neovascularization in a prostate cancer model [115]. We later demonstrated that in retinoblastoma and Kaposi's sarcoma models, 4HPR reduces tumor angiogenesis [45, 154]. In addition, fenretinide affects cell viability targeting other pathways including IGF1/IGF1 receptor, TGFβ, and NF-κB [138].

Several clinical trials have assessed the efficacy of fenretinide both in chemopreventive and in chemotherapeutic settings. The largest phase III clinical trial was the multicenter, randomized chemoprevention trial coordinated by the Istituto Nazionale dei Tumori of Milan, started in 1987 to evaluate the effects of fenretinide on tumor incidence in patients with stage I breast cancer. The results of this study show that fenretinide has a chemopreventive activity in premenopausal women, although it appeared to have a paradoxical opposite effect in postmenopausal women. Currently, there are clinical trials in a chemopreventive setting for cervical cancer, bladder cancer, and ovarian cancer. Other trials aim to assess the efficacy of 4HPR as a chemotherapeutic drug involving patients affected by head and neck cancer, lymphoma, neuroblastoma, kidney cancer, glioma, small cell lung cancer, and prostate cancer.

3 Therapies Targeting the Tumor Microenvironment

It is clear that targeting the tumor microenvironment is a promising alternative to, or in addition to, conventional cancer therapy targeting the tumor cell itself. This theory is supported by findings suggesting that the tumor microenvironment could be the sole causative mechanism for cancer [163, 164]. In addition, the tumor microenvironment appears to be readily and effectively targeted by several promising cancer chemoprevention agents, preventing or delaying the insurgence of a clinically relevant tumor.

Angiogenesis is essential for growth of a solid tumor beyond a clinically indolent small cluster of cells and, later, to metastasize, the cause of death for the majority of oncology patients. This explains the substantial efforts by researchers and industry in designing effective anti-angiogenic therapies. In 1971, Folkman foresaw the role of angiogenesis in carcinogenesis [46]. Following genetic or environmental alterations, tumor cells secrete proangiogenic growth factors (such as VEGF) determining the "angiogenic switch" leading to the formation of tumor vasculature. All the pathways and molecular players involved in the acquisition of this angiogenic phenotype are good candidates for anti-angiogenic therapy. Most studies have been focused on the VEGF/VEGFR pathway. Bevacizumab is a humanized monoclonal antibody that inhibits the isoforms of VEGF (VEGF-A) from binding its receptors. It was the first anti-angiogenic drug approved by FDA, for treatment of colorectal cancer given in combination with a chemotherapy regimen. Bevacizumab is almost always effective only when combined with chemotherapy, probably due to the "normalization" effect on tumor vessels. This "renovation" of the erratic and irregular tumor vasculature reduces leakiness and reduces interstitial pressure, allowing for better drug delivery and efficacy [71, 80, 99].

Aflibercept (VEGF-Trap) is a molecule obtained from fusion of the extracellular domains of VEGFR-1 and VEGFR-2 with the Fc fragment of IgG. This fusion protein inhibits the binding of VEGF or PlGF to their cellular receptors, blocking angiogenesis and tumor growth in preclinical studies. It is currently being evaluated in clinical trials for the treatment of acute myeloid leukemia, glioblastoma, carcinoma, metastatic pancreatic cancer, and small cell lung cancers among others [64].

Another class of compounds extensively studied is the TKIs targeting receptor tyrosine kinases, also known as RTK inhibitors. Since some receptors are expressed by both endothelial and cancer cells, these molecules may be effective on both types of cells. The first compound belonging to this class was semaxanib, first tested in combination with 5-fluorouracil, but it was discontinued due to disappointing results and high toxicity [130]. Sunitinib is a small oral molecule targeting the VEGFR, PDGFR, c-kit, RET, colony-stimulating factor (CSF)-1R that showed promising results in clinical trial in patients with metastatic renal cell carcinoma (RCC) and achieved FDA approval for therapy of metastatic RCC and gastrointestinal stromal tumor (GIST) malignancies [28]. Belonging to the same

class of compounds, sorafenib inhibits VEGFR, PDGFR-β, and c-kit. In several tumor cell lines, this molecule also targets Raf kinases and the ERK1/2-MAPK signaling. It is approved for treatment of metastatic RCC and unresectable hepatocellular carcinoma [167].

As inhibitor of the EGFR/HER1, erlotinib is thought to exert its anticancer activity by repressing expression of growth factors including VEGF. It is approved for the treatment of pancreatic cancer in combination with gemcitabine and for non-small cell lung cancer (NSCLC) [120]. Imatinib is an inhibitor of PDGFR and of Bcr/Abl used for therapy of chronic myeloid leukemia (CML). It also has anti-angiogenic effects in vitro, affecting endothelial cells and pericytes due to its interaction with PDGFR. Targeting pericytes is a valid approach to compromise vasculature, although the drawback is that the leaking vasculature may promote metastasis. Thus, well-designed therapy is required to avoid potentially adverse effects [144].

Beside its beneficial application, long-term anti-VEGF therapy has side effects, and among these are cardiovascular complications and acquired resistance. The former is due to the broad action of these compounds on both tumor and non-tumor-associated endothelial cells. The latter can be the result of angiogenesis signal redundancy and compensatory effects that will require a multiple target approach to overcome these drawbacks.

Due to the complexity of the entangled network behind inter- and intra-cellular communications, many other compounds have been designed to target signaling pathways also affecting angiogenesis. Cetuximab is a monoclonal antibody targeting EGFR, it represses proangiogenic cytokine expression in metastatic colorectal cancer [82].

LY294002 and wortmannin are two inhibitors of the PI3K family members that did not move to clinical application due to high toxicity [145]. The AKT/mTOR pathway is targeted by perifosine (an alkylphospholipid), rapamycin and its analogues (temsirolimus and everolimus); these compounds are effective treatment for renal cell carcinoma [69]. Another nodal hub in the signaling network is hypoxia inducible factor, HIF. HIF is targeted at multiple levels by compounds inhibiting its transcriptional activity [180], preventing DNA binding (Echinomycin) [81], or interaction with cofactors such as p300 (Chetomin) [143] or HSP90 (Geldanamycin) [95]. Other approaches targeting HIF are preventing its expression and accumulation (2-Methoxyestradiol) by inhibition of thioredoxins (PX-12). Geldanamycin, 2-Methoxyestradiol, and PX-12 are in clinical trials and showing some preliminary effects [29].

Several epidemiologic studies have shown a lower incidence of colon, breast, and gastric cancers in long-term-treated patients with anti-inflammatory drug (NSAIDs) or other COX-2 inhibitors [10]. COX-2 correlates with poor prognosis in patients affected by different cancer types, such as adenocarcinoma, ovary, and colon cancer. The COX-2 inhibitor celecoxib (Celebrex®) is now on clinical trial for several cancers, including prostate, head and neck, and non-small cell lung cancers [52].

One of the most important proinflammatory molecules is TNFα. TNFα activates the NF-κB pathway and tissue remodeling necessary for tumor growth and spread. To counteract TNFα activity, several approaches have been employed, such as a soluble TNF receptor, etanercept, a chimeric monoclonal antibody against TNF, infliximab, and a pegylated humanized anti-TNFα fragment, CDP870 [1]. These inhibitors are currently clinically used for several chronic inflammatory diseases, the most common being rheumatoid arthritis. Currently, there are several ongoing clinical trials evaluating TNFα inhibitors as anti-neoplastic drugs, but to date, there is no clear evidence demonstrating efficacy (http://clinicaltrials.gov/).

In addition to the molecular pathways described, the inflammatory cells composing tumor microenvironment may constitute suitable targets for treating cancer. As previously mentioned, TAMs play a pivotal role in cancer-related inflammation; they are mainly recruited by CSF-1 and CCL2. One possible way to inhibit TAM infiltration is to target the CSF-1/CFS-1 receptor (CSF-1R) or CCL2/CCL2 receptor (CCR2) axis. The RTK inhibitor, sunitinib, is able to block CSF-1R in vitro experiments [25]. CCR2 inhibitors are now under investigation by several pharmaceuticals companies. Recently, Merck has developed MK0812, currently in phase II clinical trial for relapsing-remitting multiple sclerosis and rheumatoid arthritis [168] (http://clinicaltrials.gov/).

Upon recruitment, TAMs produce a plethora of proangiogenic and protumorigenic factors that foster carcinogenesis; for these reasons, other strategies aim to target activated TAMs. Recent results show that zoledronic acid, known to reduce skeletal problems associated with bone metastasis, also acts by impairing myeloid differentiation toward TAMs and inhibiting TAMs ability to produce MMP9 [158]. Furthermore, CAFs are also a recent target for therapy. CAFs produce SDF-1, the ligand for CXCR4, which attracts endothelial cell precursors (EPCs), promoting angiogenesis. An inhibitor of CXCR4, AMD3100, has been developed and is currently in clinical trials for the treatment of acute myeloid leukemia, lymphoma, relapsed/refractory multiple myeloma, and others malignancies [36].

Targeting the tumor microenvironment has, at least in theory, the advantage that the non-tumor cell populations are genetically stable and thus less prone to accumulate mutations that lead to acquired drug resistance. However, this assumption is not entirely correct. For example, recent experiments have suggested that endothelial cells can originate from tumor cells and genetic alterations in tumor-associated endothelial cells have been found in some cases [4, 93, 113, 146].

4 Conclusions

In conclusion, an efficient tumor therapy has to target more than the tumor cell itself, but has to hit all the cellular players: endothelial cells, pericytes, immune cells, and fibroblasts, which take part in this multifaceted process. Numerous steps forward have been made to achieve this goal but more must be done to more

effectively target cancer and the carcinogenesis process. Given the vast amount of experimental data and clinical and preclinical observations, it is clear that to eradicate cancer, a therapy targeting only tumor cells is not enough. In several therapeutic protocols, anti-angiogenic drugs such as bevacizumab, sunitinib, and sorafenib are included, and anti-inflammatory adjuvant therapies that are tailored to the tumor microenvironment exist. Despite these multitarget approaches, often these therapies fail, in part due to the late stage of clinical detection and therapy administration. Thus, an interesting alternative strategy is to attack the tumor in its initial phases, when it is not yet clinically detectable, through cancer chemoprevention. One of the key elements in this approach is the tumor microenvironment, or better the prevention of the establishment of the tumor microenvironment thus blocking tumor growth and progression.

We have described some of the scientific rationale underlying this approach and the biological scenario in which the interaction between the tumor microenvironment and drugs takes place. All the steps of carcinogenesis, genetic mutations in preneoplastic cells, interaction, and molecular signaling with the surrounding microenvironment and tumor homeostasis are putative targets for drugs. Here, we have reported recent updates in some of the most promising compounds currently under investigation for cancer chemoprevention both in preclinical and in clinical studies. The encouraging results, although sometimes controversial, collected in the studies reported, clearly highlight the importance of the tumor microenvironment and show how chemopreventive approaches could be a valid strategy to prevent and control cancer progression.

Acknowledgments These studies were supported by grants from the AIRC (Associazione Italiana per la Ricerca sul Cancro), the Ministero della Salute, the and ISS (Istituto Superiore della Sanità). AB is a FIRC (Fondazione Italiana per la Ricerca sul Cancro) fellow. AP is the recipient of the "Caterina Forni" AIRC fellowship. We thank Dr Paola Corradino (Multimedica IRCCS) for data management.

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Nutriomes and Personalised Nutrition for DNA Damage Prevention, Telomere Integrity Maintenance and Cancer Growth Control

Michael F. Fenech

Abstract

DNA damage at the base sequence and chromosome level is a fundamental cause of developmental and degenerative diseases. Multiple micronutrients and their interactions with the inherited and/or acquired genome determine DNA damage and genomic instability rates. The challenge is to identify for each individual the combination of micronutrients and their doses (i.e. the nutriome) that optimises genome stability, including telomere integrity and functionality and DNA repair. Using nutrient array systems with high-content analysis diagnostics of DNA damage, cell death and cell growth, it is possible to define, on an individual basis, the optimal nutriome for DNA damage prevention and cancer growth control. This knowledge can also be used to improve culture systems for cells used in therapeutics such as stem cells to ensure that they are not genetically aberrant when returned to the body. Furthermore, this information could be used to design dietary patterns that deliver the micronutrient combinations and concentrations required for preventing DNA damage by micronutrient deficiency or excess. Using this approach, new knowledge could be obtained to identify the dietary restrictions and/or supplementations required to control specific cancers, which is particularly important given that reliable validated advice is not yet available for those diagnosed with cancer.

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_24,

Keywords
Nutriomes · DNA damage · Telomere · Genome stability · Personalised nutrition

Abbreviations

MRI	Magnetic resonance imaging
TRF1; TRF2	Telomeric-repeat binding factors 1 and 2
MTHFR	Methylenetetrahydrofolate reductase
ADH1	Alcohol dehydrogenase
ALDH2	Aldehyde dehydrogenase
CBMN Cyt	Cytokinesis-blocked micronucleus cytome
MTAP	Methylthioadenosine phosphorylase

Contents

1 Background and Current Status

DNA damage at the base sequence and chromosome level is the most fundamental cause of developmental and degenerative diseases (including accelerated ageing) and is predictive prospectively of these conditions [1–3] Hundreds of genes are involved in maintenance of genome integrity, and there is great variation amongst individuals with respect to common polymorphisms that impact on the activity of these enzymes [4–8]. The proteins encoded by those genes required for DNA replication, DNA repair or detoxification of potential genotoxins depend on essential cofactors that are obtained from the diet for optimal function [9–24]. Dietary profile differs between individuals to varying extents depending on their acquired or inherited dietary preferences and food availability; furthermore, uptake of micronutrients from the digestive system and transport into cells of the body also vary depending on genetics and altered expression of transporters that occurs with age [25–27]. Nutritional factors are required for genome maintenance not only in vivo but also in vitro which varies greatly depending on the culture

medium used [28, 29]. Maintenance of genome integrity in vitro is critical particularly in long-term culture of cells (e.g. stem cells) which may be taken out of the body for expansion and then returned to the original donor or other recipients for medical therapy reasons because DNA damage accumulated in vitro may result in oncogenic events in stem cells [30, 31]. Currently, dietary reference values (e.g. recommended daily intakes, upper safety limits) and culture medium recipes and conditions do not take into consideration impact on genome integrity and yet harm to the DNA sequence and/or the epigenome is the most fundamental and critical pathology underlying cellular and organism health and disease.

2 Nutriomes, DNA Damage and Telomere Maintenance in Vivo

Using food frequency questionnaire data and the cytokinesis-blocked micronucleus assay, one of the best validated biomarkers of DNA damage [1], we were successful in identifying nine micronutrients associated with this biomarker of chromosome breakage or loss. Increased dietary intake of vitamin E, calcium, folate, retinol and nicotinic acid was associated with reduced DNA damage, whilst increased intake of riboflavin, pantothenic acid and biotin was associated with more DNA damage and beta-carotene showed a U-shaped relationship, such that moderate intake was beneficial but excess intake was not [32]. The results also showed interactive effects amongst these factors.

Using the results of these studies, it is possible to imagine classifying preferred foods for DNA damage prevention based on their content of the nutriome consisting of vitamin E, calcium, folate, retinol, beta-carotene and nicotinic acid as shown in Fig. 1. It is evident from this that beef and bananas are a poor source of this genome-protective nutriome compared to almonds, wheat bran, cheese, broccoli and tuna. The approach of studying nutriomes may prove more efficacious in identifying those foods and dietary patterns that can best protect against fundamental pathologies at the genome level. Recently, the use of principal component analysis was reported for the first time to determine the plasma nutriomes associated with improved cognitive function and MRI measures of the brain [33]. Similar approaches could be used in relation to identifying nutrient biomarker patterns associated with DNA damage and cancer prevention.

This more detailed knowledge may help to refine dietary recommendations for optimal health. For example, the most recent WHO/FAO report recommends a minimum of 400 g of fruit and vegetables per day for the prevention of chronic diseases such as heart disease, cancer, diabetes and obesity, as well as for the prevention and alleviation of several micronutrient deficiencies, especially in less developed countries [34]. This recommendation could be misleading as shown in Table 1, using folate as an example of a key micronutrient required for genome integrity and normal foetal development [35, 36]. There are essentially four different types of vegetables some of which are actually fruits. Which vegetables one

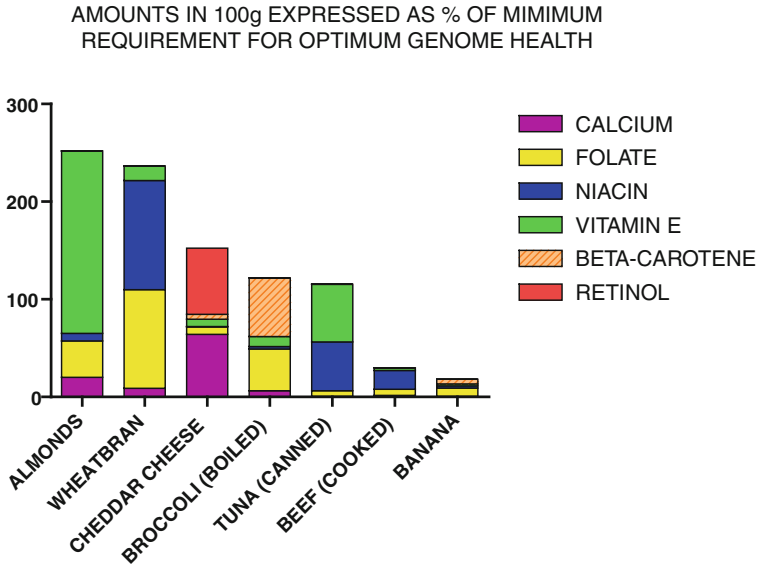

Fig. 1 Content of micronutrients associated with reduced DNA damage in selected common foods. The height of each bar for each micronutrient within the separate foods corresponds to the amount of the micronutrient expressed as the percentage of the minimum daily intake associated with a reduced micronucleus frequency index in lymphocytes as determined in the study of Fenech et al. [32]. The relative contribution of each of the micronutrients (if present) is indicated by the height of each specifically coloured bar. The nutrient content of the foods was determined using published food content tables such as the USDA Nutrient Database (http://www.nal.usda.gov/fnic/foodcomp/search/) and McCance and Widdowson's Composition of Foods Sixth Summary Edition

chooses or prefers can make a great difference to their folate intake or the amount consumed to achieve the daily requirement of folate. For high folate vegetables (i.e. pulses and/or leafy or cruciferous vegetables), it is sufficient to consume 400 g per day to meet the recommended dietary allowance of 400 μg folate per day, but if one prefers root/tuber vegetables or "fruit vegetables", then it is necessary to consume 2.5 kg per day which is impractical and could be prohibitively expensive. Furthermore, the folate level in fruit vegetables is even less than half that of roots and tubers. This example alone indicates the evident inadequacy of current recommendations with respect to maximising the efficiency of obtaining the required intakes of important micronutrients such as folate. We urgently need more precise recommendations based on nutrient dense foods such as pulses and grains and particularly those foods and combinations that deliver the nutrient combinations for genome integrity maintenance.

A critical region of the genome that is now attracting more attention by nutritional genomics scientists is the telomere. Telomeres are a TTAGGG tandem repeat sequence that caps the ends of chromosomes and have the unique function, together with the shelterin proteins associated with them (e.g. TRF1 and TRF2), of

preventing fusion of chromosome ends which would cause chromosomal instability by the formation of dicentric chromosomes, anaphase bridges and cycles of chromosome breakage and further fusions due to the generation of uncapped chromosome ends. This is the so-called breakage–fusion–bridge cycle fuelled by excessive telomere shortening and/or dysfunction of the telomere/telosome complex [37, 38]. It is likely that nutrition may play a protective role because (1) oxidation of guanine in the telomere sequence can prevent TRF1 and TRF2 binding required for telomere stability and function, and oxidation of guanine could be prevented by adequate dietary antioxidant intake; (2) folate is required to prevent accumulation of uracil in the telomere sequence that could lead to breaks within the telomere or subtelomere leading to telomere deletions and loss, whilst subtelomere hypomethylation can lead to loss of telomere length control leading to telomere dysfunction and (3) niacin is required to provide NAD for tankyrase activity which is essential for accessibility of telomerase for telomere maintenance [39, 40].

In vivo studies have shown that folate, vitamin D, omega-3 fatty acids, multivitamin use, weight loss by caloric restriction and cereal fibre intake tend to be associated with longer telomeres whilst oxidative stress, high plasma homocysteine, intake of processed meat and linoleic acid, psychological stress and obesity tend to be associated with shorter telomeres [41–47]. The significance of these results remains unclear as they are largely data from single studies and the association of telomere shortening with unhealthy ageing has recently been questioned given that both excessively short and long telomeres have been associated prospectively with cancer risk [48].

Table 1 Folate content of vegetables (DFE in μg per 100 g)[a]

High folate (HF) vegetables		Low folate (LF) vegetables	
Pulses	Leafy or cruciferous vegetables	Roots or tubers	"Fruit" vegetables
Red kidney beans (130)	Broccoli (93)	Onions (16)	Tomato (15)
Mung beans (60)	Brussel sprouts (60)	Potato (22)	Pumpkin (9)
Chickpeas (171)	Cabbage (43)	Turnip (9)	Cucumber (6)
Lentils (180)	Endive (142)	Parsnip (57)	Capsicum (11)
Peas (59)	Spinach (146)	Swede (21)	Eggplant (14)
Lima beans (50)	Lettuce (73)	Carrot (14)	Olives (0)
Mean (108)	Mean (93)	Mean (23)	Mean (10)
Mean (100)		Mean (16)	

[a] Data from USDA Nutrient Database (http://www.nal.usda.gov/fnic/foodcomp/search/)
DFE dietary folate equivalent, DFE values are shown in brackets

3 Nutritional Needs and Knowledge Gaps in Tissue Culture Systems

A critical issue in tissue culture is the evident lack of physiological conditions in terms of both composition of culture medium and oxygen tension, both of which have profound impacts on the rate of growth of cells and their level of chromosomal instability. For example, recipes of culture media can vary enormously between each other with respect to minerals and vitamins and often the concentration is supra-physiological relative to human serum or deficient depending on the micronutrient. RPMI 1640 culture medium, one of the most commonly used for culturing human cells, is supra-physiological for folate, methionine and riboflavin and deficient for iron, copper, zinc, calcium, magnesium and sulphur relative to human serum (Table 2). Whilst some of the deficiencies in culture medium may be addressed by the addition of foetal bovine serum this is only added at 5–10 % which would still render culture medium deficient if the micronutrient is absent or deficient in the recipe. It is evident that current culture media are not physiological relative to human plasma, and therefore, data obtained from in vitro experiments need to be treated with caution if attempts are made to extrapolate to in vivo predictions. The latter can only become feasible once physiological culture media are developed that are equivalent in composition to human plasma and other body fluids (e.g. cerebro-spinal fluid, interstitial fluid) and if the oxygen tension used is similar to that experienced by tissues in the body. Physiological oxygen tension is at least 2–4 times lower than that of atmospheric oxygen typically used in cell culture incubators. It was shown that cells grown under physiological oxygen conditions experience less oxidative stress and paradoxically grow more slowly compared to cells in atmospheric oxygen incubators [49, 50]. Faster growth does not necessarily result in better genome stability because the former could be due to permissiveness of cell cycle checkpoints causing a reduction in cell cycle time and/or reduced apoptosis of cells with DNA damage. We and others have shown that DNA damage, cell death and cell growth in cultured cells are strongly affected by concentration of essential micronutrients, such that both deficiency or excess within the physiological range can profoundly harm the genome and alter cell growth and survival kinetics [23, 28, 29, 51–54]. The use of excessively high concentrations of methyl donors (e.g. folate, methionine, choline, vitamin B12) in culture medium theoretically may lead to an adverse DNA methylation pattern that may inappropriately silence important house-keeping genes, although strong evidence for this hypothesis is currently lacking [55]. It is evident that, given the wide spectrum of micronutrients required for genome maintenance and repair, the development of physiological culture medium composition is an important prerequisite to enable the determination of optimal culture conditions for growth of human cells in a genomically stable state and to explore the impact of various micronutrient combinations (i.e. nutriomes) and dosages against different genetic backgrounds. These developments are also critical if we are to use in vitro data reliably to predict in vivo nutritional effects on an individual basis. In this regard, it

Table 2 Comparison of concentration of some micronutrients between a single sample of human serum and normal complete RPMI 1640 culture medium (data not previously published)

Micronutrient	Concentration	Human serum	RPMI 1640 culture medium
Folate	μmol/L	0.028	2.3
Methionine	μmol/L	30	100
Riboflavin	μmol/L	0.05	0.53
Iron	mg/L	0.84	0.19
Copper	mg/L	1.4	<0.1
Zinc	mg/L	0.94	0.17
Calcium	mg/L	98	26
Magnesium	mg/L	20	11
Sodium	mg/L	3,400	3,200
Potassium	mg/L	154	200
Phosphorous	mg/L	121	174
Sulphur	mg/L	1,110	64

is important to note that concentrations of micronutrients achievable in vitro might not be possible in vivo due to excretion and redistribution within tissues. Furthermore, with respect to body fluids, we only have good knowledge on possible micronutrient concentrations in blood plasma and our knowledge about interstitial fluids surrounding organs (e.g. cerebrospinal fluid) or within tissues is at this stage rudimentary. We need to consider optima both within the physiological and supra-physiological range but only use "physiological dose ranges" achievable in vivo for in vivo predictions using in vitro models.

With respect to optimising in vitro and in vivo cellular health, it is becoming increasingly recognised that parameters of genome and epigenome damage are exquisitely sensitive to changes in micronutrient concentration even within the "normal" physiological range [23, 28, 29, 52, 55, 56]. It is therefore practical, feasible and desirable to start re-examining dietary reference values so that recommended intakes coincide with the attainment of tissue concentrations that are consistent with minimised DNA damage. For a detailed recent review on the status of validation of DNA damage, biomarkers for measuring the genomic impact of malnutrition and a proposed roadmap for determining nutrient and nutriome requirements for optimal genome maintenance refer to Fenech [1, 18].

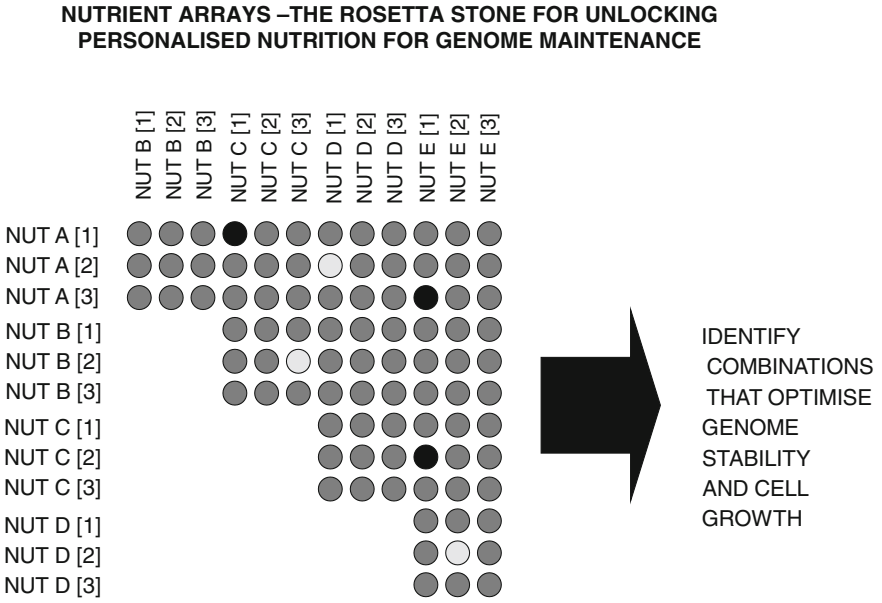

Fig. 2 Theoretical example of a simple nutrient array microculture system. NUT = single-nutrient or multiple nutrient combination; A–E = different types of nutrients or nutrient combinations; 1–3 = increasing dose levels. The different grey level colouring is simply an indication of the potential variability in cell growth, viability and genome stability that may be observed depending on the combinations used. The challenge is to identify the best combination or combinations for each individual

4 Testing Nutriomes in Nutrient Arrays

The biggest challenge in nutritional genomics is to make the quantum leap from a reductionist single-nutrient–single-gene interaction approach to studying the interaction of a complete nutrient combination (i.e. the nutriome) with the whole genome on an individual by individual basis. The ultimate goal is effectively to find for each individual the nutriome that best matches their genome so that cellular function and genome and epigenome maintenance are optimised. The "Rosetta Stone" (mechanism or code) to unravel this puzzle lies in developing nutrient arrays in microculture systems, such that multiple nutriomes can be simultaneously tested whilst taking into consideration impact of dosage in the assessment (Fig. 2). The microwell that produces cells than can proliferate adequately and viably whilst maintaining optimal genome and epigenome stability is likely to represent the best nutriome match for that individual's cells. The development of high-content automated analyses of DNA damage has already become feasible using quantitative image cytometry [57–60], such that multiple measures can be captured simultaneously in interphase cells including the number

of cells and their nuclear DNA content, multiple measures of genome stability such as telomere length and aneuploidy by FISH, oxidised guanine and DNA methylation by immunohistochemistry, chromosome damage and telomere end fusions by micronucleus cytome assays in cytokinesis-blocked binucleated cells and so on.

Such a system would also identify the deficiency and safe upper limit range for that individual for multiple micronutrients within a single scan and identify any unexpected combinations that could prove counter-intuitively cytotoxic or genotoxic. The plausibility of such a possibility is supported by our observation that genome instability increased under low folate conditions (20 nM) if riboflavin concentration was increased to replete status [29] possibly because the latter, which is the precursor of the FAD cofactor for MTHFR, increases MTHFR activity which catalyses the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate making the former folate species less bioavailable for dTMP synthesis from dUMP and thus increasing uracil in DNA. Excessive uracil in DNA causes abasic sites and DNA strand breaks when uracil glycosylases attempt to repair this highly mutagenic lesion [1, 18, 55]. Therefore, it is important to develop a nutrient array system that can efficiently interrogate multiple micronutrient combinations at different dosages. This type of approach has the added advantage that it becomes possible to identify an individual's nutriome for genome health maintenance without needing to know the person's genetic background. Furthermore, such systems could also be used to compare the response of different genotypes under the same nutriome conditions and estimate the percentage of the variance of the biomarkers measured that is explained by different genotype and different nutrients in the nutriomes tested including their interactions.

Prototypes of this approach have been designed by our group and others to investigate the following interactive effects on DNA damage, cell death and cell growth:

1. different ratios of sulphur- and seleno-methionine at constant physiological methionine concentration [54].
2. folate concentration with alcohol [61].
3. alcohol/acetaldehyde concentration and ADH1 or ALDH2 genotype [62, 63].
4. folate concentration with BRCA1 or BRCA2 genotype [64, 65].
5. folate concentration with riboflavin concentration with MTHFR C677T genotype [29].

In these studies, the CBMN Cyt assay was used to obtain multiple measures of chromosomal instability, cell death and cell division [66, 67]. The results of this approach are very promising because not only can they readily define the percentage variation in genotoxicity, cytotoxicity, metabolite and cell growth biomarkers which is attributable to a specific micronutrient, genotype and interactions between these parameters but they can also define the shape of the nutrient/DNA damage dose-response curve for genetically defined cell types. The use of the CBMN Cyt assay is particularly relevant for this purpose because the relative incidence of DNA damage, cell death events and cytostasis varies as

micronutrients and their concentrations within a nutriome are increased or decreased in multiple combinations. The relevant nutriomes within a single metabolic pathway may involve more than just two micronutrients; for example, the folate-methionine cycle requires folate in various forms as a substrate and betaine, vitamin B12, vitamin B6 and vitamin B2 as cofactors. Therefore, the nutrient array should also be designed to interrogate combinations of multiple micronutrients simultaneously in a dose-related manner and at different or contrasting dosage levels for each micronutrient relative to the others.

The in vitro nutrient array system would also be an ideal mechanism to test whether the predictions of emerging nutrigenomic mathematical models in specific key metabolic pathways [68, 69] actually hold true because this system is less likely than in vivo human models to be affected by problems relating to compliance to dietary intervention and unexpected lifestyle and exposure variables such as stress and recreational drug consumption as well as environmental genotoxicants which can impact on the genome damage indices measured. Furthermore, it is financially prohibitive to test multiple micronutrient combinations in vivo.

5 Nutrient Restriction for Cancer Growth Control

One of the greatest challenges in ageing populations is the need to prevent the proliferation of cancers which accumulate with age. Currently, there is no validated advice on the appropriate diet to adopt once a person is diagnosed with cancer because our knowledge on nutrient-gene interaction with respect to cancers is rudimentary. Furthermore, there is concern that supplementation with certain nutrients that are required for genome maintenance and cell growth (e.g. folate, methionine) may stimulate the cancer growth. Is it possible to identify the nutriome that prevents the growth of each cancer?

Ideally, nutriomes in nutrient array systems will be not only able to interrogate the optimal nutritional requirements for growth and genome maintenance of normal cells from an individual but also to verify that such a nutriome does not stimulate growth of cancer cells that the individual might have. Cancer cells are likely to have a markedly different genotype to that of the host's normal cells and could respond differently to the same nutriome environment. For example, some cancer cells amplify the high affinity folic acid receptor [68] giving them a distinct potential advantage over normal cells, when folate is limiting, in accessing folate from the surrounding fluid. The ideal nutriome for an ageing or cancer-prone individual would be the combination that not only sustains the replenishment of normal cells in a genetically integral manner but also inhibits the growth of cancer cells. It is conceivable that both normal cells and cancer cells from an individual could be simultaneously tested within a single-nutrient array system.

A nutrigenetic dietary restriction strategy for cancer growth control may be feasible. The strategy I propose is based on the following steps:

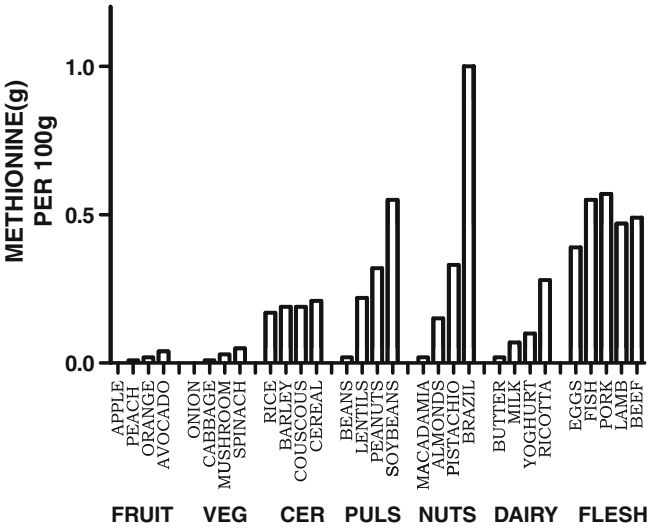

Fig. 3 The methionine content of typical fruits, vegetables (VEG), cereals (CER), pulses (PULS), nuts, dairy and flesh foods in grams per 100 grams. The methionine content was derived from tables such as the USDA Nutrient Database (http://www.nal.usda.gov/fnic/foodcomp/search/) and McCance and Widdowson's Composition of Foods Sixth Summary Edition

1. Identify the genetic defect that causes the cancer to be specifically and strongly dependent for a micronutrient required for DNA repair or growth.
2. Test susceptibility to micronutrient restriction in vitro using a nutriome-nutrient array system.
3. (1) Design personalised dietary restriction treatment to improve therapeutic ratio of therapy with genotoxic drugs or (2) design personalised dietary restriction therapy to inhibit growth or induce cell death of residual cancers post-therapy.

Recently, considerable interest has emerged regarding the use of methionine restriction to prolong healthy lifespan and to control growth of cancers (recently reviewed by Cavuoto and Fenech [70]). The reason for this possibility is that methionine dependency phenotype is a common feature of cancers and appears to be caused by mutations in methionine metabolism genes in either the salvage or de novo pathways. The best characterised and most common of these mutations is the deletion of the MTAP gene in the salvage pathway which often occurs together with the common CDKN2A (p16INK4) deletion found in 8–60 % of cancers depending on the cancer site. The coincidence of these two important genetic events occurs because these two genes are in very close proximity on the p arm of chromosome 9. A high level of methionine tends to fuel the growth of cells because it is required for polyamine synthesis. When MTAP is deleted in cancer cells, they are unable to regenerate methionine and thus are completely dependent

on dietary supply. Knowing that a cancer is methionine dependent therefore brings forward the possibility to give targeted nutritional methionine restriction advice to control a person's cancer should it have this critical mutation. The feasibility of this approach is currently being tested in our laboratory. Should methionine restriction prove to be a viable modality for personalised nutrition for cancer growth control, particularly in those individuals with cancers with the MTAP deletion, it will be necessary to give greater attention to the dietary sources of methionine.

A close inspection of Fig. 3 shows that the richest sources of methionine tend to be flesh foods, eggs, certain dairy foods (e.g. cheese), certain nuts (e.g. Brazil nuts) and certain legumes (e.g. soya beans). The poorest sources are fruits and vegetables. It is evident that a carefully constructed vegan diet rich in fruits and vegetables would be required to achieve a strong restriction of methionine intake.

6 Conclusions

In conclusion, the investigation of nutriomes and the use of nutrient array systems to interrogate genomic responses to multiple nutrient doses and combinations are in principle feasible and hold great promise to define the complete nutritional requirements, including supplementations and restrictions, of any cell type to either optimally sustain its growth and reproduction in a genetically stable manner in the case of normal differentiated progenitor cells and stem cells or suppress its growth and cause its death in the case of cancer cells.

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Novel Approaches in Melanoma Prevention and Therapy

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Abstract

The incidence of cutaneous melanoma has risen at a rate significantly higher than that for other malignancies. This increase persists despite efforts to educate the public about the dangers of excess exposure to UV radiation from both the sun and tanning beds. Melanoma affects a relatively younger population and is notorious for its propensity to metastasize and for its poor response to current therapeutic regimens. These factors make prevention an integral component to the goal of decreasing melanoma-related mortality. Transformation of melanocytes into malignant melanoma involves the interplay between genetic factors, UV exposure, and the tumor microenvironment. The roles of UV radiation in the etiology of melanoma are mediated by both direct damage of DNA through formation of photoproducts and production of reactive oxygen species (ROS). Many of the promising antioxidant agents under development for the prevention of melanoma are derived from foodstuffs. B-Raf is a member of the Raf kinase family of serine/threonine-specific protein kinases that plays a role in regulating the MAP kinase/ERKs signaling pathway. About 50 % of melanomas harbor activating BRAF mutations. BRAF mutations are found in 59 % of the melanomas arising in skin with intermittent sun exposure, such as trunk and arms, as compared with only 23 % of the acral melanomas, 11 % of

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_25,

mucosal melanomas, and 0 % of uveal melanomas. Two new agents, ipilimumab and vemurafenib, have been shown to improve outcome of advanced melanoma as presented at the plenary session of the 2011 annual meeting of the American Society of Clinical Oncology. Vemurafenib is the first personalized compound which demonstrated an improvement in progression-free survival (PFS) and overall survival (OS) in metastatic melanoma harboring the BRAFV600 mutation and represents the first drug of a class that exerts its anti-proliferative activity through inhibition of a highly specific molecular target. GSK2118436 (dabrafenib), the second BRAF inhibitor, in phase I and II trial obtained similar results to vemurafenib. A phase III trial is now ongoing. Taken together, the early clinical development of vemurafenib and dabrafenib clearly confirms that BRAF inhibitors can halt or reverse disease in patients with melanomas carrying this mutation, improving survival times compared with historically standard treatments (chemotherapy and interleukin-2). The clinical development of other new BRAF inhibitors such as RAF265 and LGX818 is now ongoing. Combination strategies of BRAF inhibitors with ipilimumab, an anti-CTLA-4 antibody, and/or MEK inhibitors or metformin are now under investigation in clinical trials.

Keywords
Melanoma · BRAF · Ipilimumab · Vemurafenib · Dabrafenib

Abbreviations

GTE	Green tea extracts
EGCG	epigallocatechin-3-gallate
CSD	chronic sun damage
CTLA-4	cytotoxic T lymphocyte–associated antigen 4
cSCC	Cutaneous squamous cell carcinoma
VEGFR-2	vascular endothelial growth factor receptor 2

Contents

1 Introduction

The incidence of cutaneous melanoma has risen at a rate significantly higher than that for other malignancies, with a yearly increase among the white population of approximately 3 % for the past 30 years. This increase persists despite efforts to educate the public about the dangers of excess exposure to UV radiation from both the sun and tanning beds [1]. Other risk factors for melanoma include fair skin and inability to tan, red hair, light eye color, numerous common or atypical nevi, age >50 years, male gender, and a personal or family history of the disease [2]. When detected at the earliest stages, more than 90 % of patients diagnosed with cutaneous melanoma are cured by surgical excision of the lesion; however, 5 year survival rates fall to 60 % for those with regional metastases and to less than 16 % for those with distant metastases [3]. Unlike many other cancers, melanoma affects a relatively younger population and is notorious for its propensity to metastasize and for its poor response to current therapeutic regimens. These factors make prevention an integral component to the goal of decreasing melanoma-related morbidity and mortality.

Epidemiologic data indicate that UV radiation is the major environmental carcinogen for melanoma. UV exposure in the B (UV-B; 260–320 nm) and A (UV-A; 320–400 nm) wave length ranges is the most biologically relevant environmental risk factor for sporadic melanoma. The exact mechanism, however, whereby UV irradiation induces melanoma has not been determined. It is now appreciated that transformation of melanocytes into malignant melanoma involves the interplay between genetic factors, UV exposure, and the tumor microenvironment Gruji [4].

Twenty researchers from nine different nations have performed an analysis of over 20 epidemiological studies on the incidence of skin cancers among users of tanning beds. The data reviewed show that the risk of melanoma increases by 75 % in individuals who begin tanning salon use prior to age 30. The full report of this meta-analysis was published in "The Lancet Oncology" [5] and supports the recommendation of the world health organization (WHO) to avoid sunlamps and excessive exposure to sunlight. The International Agency for Research on Cancer (IARC) has upgraded tanning equipment that emits UV rays from the category of "probable carcinogens" to "carcinogenic to humans" (WHO IARC working Group [6]). With this new classification, tanning beds and sunlamps join a class of cancer risk factors that includes asbestos, radon, smoking, alcohol, and hepatitis.

2 Prevention Strategies Against Melanoma

Melanoma prevention includes primary, secondary, and tertiary strategies. Primary prevention takes place at the earliest stage of carcinogenesis, that is, at the initiation stage, prior to the time of the "first hit." Primary prevention strategies, such as sunscreen, reduce initial mutations suffered by a "normal" cell. Prevention

regimens that use screening protocols to detect premalignant lesions early, or treat with agents designed to prevent premalignant lesions from progressing to invasive cancer, constitute secondary prevention. Tertiary prevention entails efforts to decrease recurrences and increase survival in patients who have already undergone treatment for cancer.

Exposure to UV radiation causes DNA damage directly through formation of photoproducts and indirectly through oxidative damage from reactive oxygen species (ROS), both of which contribute to UV-induced carcinogenesis and photoaging [7]. Many of the promising antioxidant agents under development for the prevention of melanoma act to reduce oxidative DNA damage and are derived from foodstuffs.

2.1 Diet and Melanoma

2.1.1 Lycopene

Lycopene, a red-colored compound found primarily in tomatoes, is the most efficient singlet oxygen quencher (and antioxidant) of all of the carotenoids. A new report from the United Kingdom showed a significant reduction in UV-induced photo damage in the skin of 20 healthy women who consumed 55 g of tomato paste (16 mg of lycopene) in olive oil, versus olive oil alone, daily for 12 weeks [8]. However, the utility of lycopene as a primary melanoma prevention agent still requires preclinical assessment in a mouse model of melanoma.

2.1.2 Sulforaphane

Sulforaphane (SF) is a small-molecule antioxidant first isolated from broccoli sprouts, in a study designed to identify the chemical entity(s) that might help explain the observation that consumption of large quantities of cruciferous vegetables decreases cancer risk. SF, unlike the carotenoids which are able to quench ROS directly, derives its antioxidant capacity from the ability to up-regulate natural antioxidant enzymes in tissues. Topical application of SF was shown to induce the expression of antioxidant enzymes and increase the MED for UV radiation in human skin [9]. A second study demonstrated that oral delivery via broccoli sprout supplement in the feed of mice was able to reduce the number of UV-induced squamous cell carcinomas in supplemented mice [10]. However, the utility of SF for primary prevention of melanoma remains untested in a relevant mouse model.

2.1.3 Green tea Extracts (GTE)

The anticancer properties of green tea are attributed to polyphenols, principally (-) epigallocatechin-3-gallate (EGCG). The chemical properties of EGCG indicate that it can act both as a sunscreen and as a quencher of free radicals, although it is doubtful that these properties alone account for its biological activity [11]. Neither GTE nor EGCG has been tested for the prevention of UV-induced melanoma in a

mouse model, but utility in a secondary prevention modality is suggested by the finding that EGCG inhibits the formation of lung metastases after tail vein injection of B16 melanoma cells in a syngeneic mouse model of melanoma [12]. Topical EGCG inhibits erythema, oxidative stress, and infiltration of inflammatory leukocytes and enhances pyrimidine dimer repair in DNA, in UV-irradiated human skin. A number of skin-care products, including sunscreens, contain GTE, although in many cases their polyphenol content is not standardized. Further preclinical and clinical studies of GTE and EGCG for the prevention of skin cancers including melanoma are certainly warranted.

2.2 The Role of BRAF Protein in Melanoma

B-Raf is a member of the Raf kinase family of serine/threonine-specific protein kinases. This protein plays a role in regulating the MAP kinase/ERK signaling pathway, affecting cell division, differentiation, and secretion [13]. Mutations in this gene have been found in different cancers, including melanoma, papillary thyroid carcinoma, colorectal cancer, non-Hodgkin lymphoma, non-small-cell lung carcinoma, and adenocarcinoma of the lung [14].

More than 30 mutations of the *BRAF* gene associated with human cancers have been identified. The frequency of BRAF mutations varies widely in human cancers. In fact, about 50 % of melanomas harbor activating BRAF mutations; among the BRAF mutations observed in melanoma, over 90 % are at codon 600 (e.g., V600E mutation). The second most common mutation is BRAFV600 K [15]. The high prevalence of BRAF mutations in cutaneous melanoma and the known epidemiological link between UV and melanoma have prompted speculation that the BRAFV600E mutation is induced by UV damage. Specifically, BRAF mutations are common (59 %) in melanomas arising in skin with intermittent sun exposure, such as trunk and arms, but are found in only 23 % of the acral melanomas and 11 % of mucosal melanomas [16] and are absent in uveal melanoma [17, 18]. A clear understanding of the UV–BRAF link is complicated by the observation that melanomas from chronically sun-exposed areas (defined by histopathologic evidence of solar elastosis) possess only an 11 % frequency of BRAFV600E mutation [19]. Together, these contrasting observations highlight the uncertainties surrounding the molecular factors driving BRAF mutation, in particular, the role of sun exposure. BRAFV600E has been implicated in different mechanisms of melanoma progression, including the activation of the downstream MEK/ERK pathway, evasion of senescence and apoptosis, unchecked replicative potential, angiogenesis (through MEK-dependent activation of HIF-1α and VEGF), tissue invasion, and metastasis (via up-regulation of several proteins involved in migration, integrin signaling, cell contractility, tumor- and microenvironment-derived interleukin-8), as well as the evasion of immune response [20]. In primary melanomas, clinicopathologic features consistently reported to be associated with mutant *BRAF* include younger age, fewer markers of chronic sun damage (CSD) in

surrounding skin, higher total body nevus counts, and specific histopathologic findings (large epithelioid cytomorphology, heavy melanization, and prominent upward epidermal scatter of melanocytes).

An Australian group [21] reported results from a study about the frequency and type of oncogenic *BRAF* mutations in metastatic melanoma and correlation of *BRAF* status with clinicopathologic features and outcome. 197 patients with AJCC stage IIIC unresectable melanoma or stage IV melanoma were enrolled in the study. Mutated *BRAF* was found in tumors from 95 patients (48 %). Patients with mutant *BRAF* melanoma were younger than patients with wild-type *BRAF* melanoma at diagnosis of distant metastases (median age, 55.8 versus 63.1 years, respectively) and at diagnosis of primary melanoma (median age, 51.9 versus 60.6 years, respectively). Other features of patients with metastatic melanoma, including the disease-free interval, were not significantly different between patients with mutant and wild-type *BRAF*. This study showed that the overall prognosis of patients with *BRAF*-mutant metastatic melanoma is no better in terms of disease-free survival than those with *BRAF* wild-type metastatic melanoma. Despite this, two-thirds of *BRAF*-mutant patients who were newly diagnosed during the study period did not receive a *BRAF* inhibitor and demonstrated a consistent trend to poorer outcome in terms of overall survival compared with *BRAF* wild-type patients. Correlates of mutant *BRAF* in the antecedent primary melanoma were site (trunk), earlier age of onset, lack of CSD, nodular and superficial spreading histopathologic subtypes, and the number of mitoses. These findings support the concept that mutant *BRAF* melanoma is biologically heterogeneous and acts in concert with other genetic alterations to produce a particular phenotype. The presence of an activating mutation in the *BRAF* oncogene had no impact on time to distant or unresectable metastasis but was associated with a worse outcome thereafter. The survival analysis after diagnosis of distant metastases was confounded by the subset of *BRAF*-mutant patients who received the *BRAF* inhibitor for two reasons: first, treatment with BRAF inhibitor extends overall survival; second, patients selected to participate in clinical trials may have better prognostic factors, including better performance status, absence of symptomatic brain metastases, and other non-measurable factors. In patients with metastatic melanoma, the presence of mutant *BRAF* had no impact on the interval from diagnosis of first-ever melanoma to first distant metastasis (including first unresectable locoregional recurrence). The use of *BRAF* inhibitor in the subgroup of BRAF-mutated patients confounded the survival analysis, particularly because this subgroup seems to have prolonged survival compared with the remaining patients. It seems that the presence of a *BRAF* mutation is associated with poorer survival in patients with metastatic melanoma, but this warrants confirmation in a historical cohort of patients from a time when *BRAF* inhibitors were not available.

2.3 The Treatment for Advanced Melanoma

2.3.1 The Treatment for Advanced Melanoma Before 2011

In phase III studies, dacarbazine, the only chemotherapeutic agent approved by the US Food and Drug Administration (FDA) for the treatment for metastatic melanoma prior to 2011, was associated with a response rate of 7–12 % and a median overall survival of 5.6–7.8 months after the initiation of treatment [22].

2.3.2 The Treatment for Advanced Melanoma After 2011

Two extraordinary advances were recently recognized when positive results from two separate studies of new therapies, ipilimumab and vemurafenib, for the treatment for advanced melanoma were published. Ipilimumab is a fully human antibody that binds to CTLA-4 (cytotoxic T lymphocyte–associated antigen 4), a molecule on cytotoxic T lymphocytes that is believed to play a critical role in regulating natural immune responses [23]. The absence or presence of CTLA-4 can augment or suppress the immune system's T-cell response in fighting disease. Ipilimumab is designed to block the activity of CTLA-4, thereby sustaining an active immune response in its attack on melanoma cells. The effect of ipilimumab to reverse immuno tolerance to the melanoma is associated with a long-term response in a subpopulation of patients as well as induction of autoimmune side effects [24].

The selective and potent inhibitor of oncogenic mutant BRAF [25], vemurafenib (PLX4032/RG7204/RO5185426), showed very positive results in clinical studies. The phase II trial involving patients previously treated for melanoma harboring BRAFV600E mutation studied activity of vemurafenib with respect to overall response rate (primary endpoint, defined as percentage of treated patients with a tumor response), duration of response, and overall survival. The trial enrolled 132 patients who were treated with vemurafenib at the dose of 960 mg orally twice daily (until the development of unacceptable toxic effects or disease progression) and showed a confirmed response rate of 53 %, with a median duration of response of 6.7 months and a median overall survival of 15.9 months, an unprecedented outcome in melanoma patients [26].

During the follow-up period (median was 12.9 months, range 0.6–20.1), 24 % of patients received ipilimumab after their disease progressed while receiving vemurafenib. In an unplanned post hoc analysis, median overall survival remained at 15.9 months (95 % CI 11.6–18.3 %) [27].

2.3.3 The BRIM-3 Results

A phase III trial, the 2-arm randomized BRIM-3 (BRAF inhibitor in melanoma-3) study, compared vemurafenib, 960 mg orally twice daily, to dacarbazine chemotherapy, 1,000 mg/m^2 administered every 3 weeks, as first-line therapy in metastatic melanoma patients. Progression-free survival (PFS) and overall survival (OS) were both primary endpoints in this trial. 675 patients with unresectable, previously untreated stage IIIC or stage IV metastatic melanoma harboring

BRAFV600E mutation (BRAFV600E mut) were enrolled. The results obtained showed a relative reduction of 63 % in the risk of death and 74 % in the risk of tumor progression in BRAFV600E mut patients. After a longer follow-up, the median OS for vemurafenib arm is of 13.2 months compared to 9.9 months in the dacarbazine arm. Notably, about 38 % of patients required dose reduction in the vemurafenib arm [28]. The most common adverse events of vemurafenib were cutaneous events, arthralgia, and fatigue. Photosensitivity skin reactions of grade 2 or 3 in BRIM-3 trial were seen in 12 % of the patients, with grade 3 reactions characterized by blistering that often could be prevented with sunblock. Adverse events in BRIM-3 trial led to dose modification or interruption in 129 of 336 patients (38 %) in the vemurafenib group. Cutaneous squamous cell carcinoma (cSCC), keratoacanthoma, or both developed in 61 patients (18 %). All lesions were treated by simple excision. Vemurafenib was well tolerated in all the clinical trials so far completed. In the BRIM-3 trial, the incidence of grade 1–2 and grade 3–4 adverse events was similar to those from prior studies.

Vemurafenib was the first personalized compound which demonstrated an improvement in PFS and OS in metastatic melanoma harboring the BRAFV600 mutation. The BRIM-3 trial results represent the most dramatic advance ever achieved in the treatment for metastatic melanoma. On August 17, 2011, the US Food and Drug Administration (FDA) approved vemurafenib for first- and second-line therapy of unresectable or metastatic melanoma with the BRAFV600E mutation as detected by a concurrently FDA-approved test. On December 15, 2011, the European Medicine Agency (EMA)'s Committee for Human Medicinal Products (CHMP) recommended the granting of a marketing authorization for vemurafenib to treat patients with metastatic or unresectable melanoma harboring BRAFV600 mutation. European Commission approval for marketing in 27 EU countries was finally granted on February 17, 2012 (Patient W.A.I.T. Indicator EFPIA 2010).

2.3.4 Future of BRAF Inhibitor Treatment

Clinical trials of GSK2118436 (dabrafenib), a second BRAF inhibitor, in phase I and phase II trials [29] obtained results similar to those of vemurafenib. Dabrafenib is a highly specific inhibitor of mutant V600EBRAF (mt BRAF). In fact, in melanoma cell lines, it inhibits mt BRAF about four times more potently than vemurafenib while maintaining a comparable selectivity for mt BRAF over wild-type BRAF (wt BRAF). Phase I results appear to be at least as impressive as the phase I results for vemurafenib, both in terms of safety and in terms of efficacy. In fact, Dabrafenib was evaluated in a phase I clinical trial of similar design to the vemurafenib study, and the results were even more striking. A total of 93 patients were enrolled during the dose-escalation portion of the trial, 85 of whom had metastatic melanoma, and of those, 76 had activating mutations in BRAF. Among the 16 patients treated at the two highest dose levels, there were 10 measurable responses observed (63 % response rate). A phase III trial is now ongoing. In addition, responses have been seen in melanoma patients with brain metastases.

Taken together, the early clinical development of vemurafenib and dabrafenib clearly confirms that BRAF inhibitors can (at least temporarily) halt or reverse disease in patients with melanomas carrying this mutation, apparently improving survival times compared with historically standard treatments (chemotherapy and interleukin-2).

RAF265 is another orally bioavailable, selective inhibitor of RAF, including BRAF and CRAF and mutant BRAF. RAF265 also has anti-angiogenic activity through inhibition of vascular endothelial growth factor receptor 2 (VEGFR-2). In a BRAF-mutant xenograft mouse model, RAF-265 produced dose-dependent tumor regression ([30], Stuart et al. 2011). RAF265 is currently being investigated in phase I clinical trials in patients with advanced malignant melanoma.

LGX818 [31] is a potent and selective RAF kinase inhibitor with unique biochemical properties that contribute to an excellent pharmacological profile. LGX818 has selective anti-proliferative and apoptotic activity in cells expressing BRAFV600E. In the A375 (BRAFV600E) human melanoma cell line, LGX818 suppresses phospho-ERK ($EC_{50} = 3$ nM) leading to potent inhibition of proliferation. LGX818 induced tumor regression in multiple BRAF-mutant human tumor xenograft models grown in immune-compromised mice and rats at doses as low as 1 mg/kg. A phase I clinical trial in patients with BRAF-mutant tumors is ongoing (http://cancertrialaustraliaa.com/Clinical-Trials-Register.aspx. Cancer Trials Australia).[32]

2.3.5 Combination Strategies

A clinical phase I/II trial has been designed to evaluate the safety, tolerability, and efficacy of vemurafenib in combination with ipilimumab. [33] This therapy combines targeted and immunotherapy approaches. The two drugs have different mechanisms of action, with ipilimumab (first- and second-line treatment in USA, second-line treatment in Europe at the dose of 3 mg/kg to treat patients with late-stage metastatic unresectable melanoma) sustaining an active immune response. Vemurafenib and ipilimumab also show a different pattern of action: While vemurafenib has been demonstrated to have quick action, rapid metabolic shutdown, but disease progression after a median of 6–8 months, ipilimumab works more slowly with the ability to make the disease chronic.

Another combination is being tested in the BRIM-7 trial [34]. This phase Ib, dose-escalation study aims at evaluating the safety, tolerability, and pharmacokinetics of vemurafenib in combination with GDC-0973 in patients with BRAFV600E-positive metastatic melanoma who have progressed after treatment with vemurafenib alone. GDC-0973 is a potent and highly selective inhibitor of MEK1/2, downstream targets of BRAF. The rationale underlying this combined therapy is two-fold: The first is the expectation of additive or possibly synergistic effects upon PFS; the second concerns the possibility to avoid the toxicities that may accompany the paradoxical stimulation of the MEK pathway when BRAF inhibitors are used as single agents. This may prove to be a basis for the cSCCs and keratoacanthomas reported in most trials of BRAF inhibitors as single agents.

Recent phase I and phase II trials showed that this approach is safe and able to lower the toxicity of either agent alone. 43 BRAF-mutated melanoma patients (all BRAF inhibitor naïve) were enrolled in the phase I study. Grade 3 adverse effects included generalized rash (n = 2, 4 %) and neutropenia (n = 2, 4 %). Skin toxicity ≥ grade 2 occurred in nine (20 %) pts; of these, there were grade 2 rash (n = 4, 8 %) and grade macular rash (n = 1). No cSCCs or hyperproliferative skin lesions have occurred at any dose level. Of 16 evaluable pts, 13 pts had partial response (PR) and 3 stable disease (SD) for an ORR of 81 % (95 % CI 54.4–96.0 %) and all but 2 patients remain on study. In another combination trial of 10 evaluable patients who received 150 mg BID dabrafenib + ≥1 mg QD trametinib, 9 pts had PR and 1 SD. Trametinib at 2 mg QD combines safely with dabrafenib 150 mg BID, with no SCC thus far and decreased frequency of rash compared to previous trials of single-agent dabrafenib and trametinib, respectively [35]. The preliminary antitumor activity warrants further investigation; the randomized phase II trial (Part 3) is accruing.

Single-agent metformin inhibited proliferation in 12 out of 19 cell lines irrespective of the BRAF mutation status, but in one $NRAS^{Q61K}$ mutant cell line, it powerfully stimulated cell growth [36]. Synergistic anti-proliferative effects of the combination of metformin with vemurafenib were observed in 6 out of 11 BRAFV600E mutants, including highly synergistic effects in two BRAFV600E mutant melanoma cell lines. Antagonistic effects were noted in some cell lines, in particular in BRAFV600E mutant cell lines resistant to single-agent vemurafenib. Seven out of 8 BRAF wild-type cell lines showed marginally synergistic anti-proliferative effects with the combination, and one cell line had highly antagonistic effects with the combination. The differential effects were not only dependent on the sensitivity to each drug alone, but also dependent on effects on cell cycle or signaling pathways. The combination of vemurafenib and metformin tended to have stronger anti-proliferative effects on $BRAFV600E^{-}$mutant cell lines [36]. However, determinants of vemurafenib and metformin synergism or antagonism need to be understood with greater detail before any potential clinical utility of this combination can be tested.

3 Conclusions

UV radiation is the major environmental carcinogen for melanoma. The exact mechanism, however, whereby UV irradiation induces melanoma has not been determined, but it is now established that transformation of melanocytes into malignant melanoma involves the interplay between genetic factors, UV exposure, and the tumor microenvironment. The analysis of over 20 epidemiological studies on the incidence of skin cancers among users of tanning beds shows that the risk of melanoma increases by 75 % in individuals who begin tanning salon use prior to age 30 and has upgraded tanning equipment that emits UV rays from the category of "probable carcinogens" to "carcinogenic to humans." With this new

classification, tanning beds and sunlamps join a class of cancer risk factors that includes asbestos, radon, smoking, alcohol, and hepatitis. The roles of UV radiation in the etiology of melanoma are mediated by both direct damage of DNA through formation of photoproducts and production of reactive oxygen species (ROS), which contribute to UV-induced oxidative stress, inflammation, and photoaging. Many of the promising antioxidant agents under development for the prevention of melanoma are derived from foodstuffs. The development and use of primary, secondary, and tertiary prevention strategies have the potential to safely reduce cancer-causing mutations and the resultant transformation to malignancy. Successful prevention strategies have enormous potential to reduce both morbidity and mortality from melanoma.

Metastatic melanoma has historically had a poor prognosis because of lack of responsiveness to traditional chemotherapeutics (dacarbazine); about 50 % of all the melanoma harbors an activating mutation in BRAF. BRAF inhibitors represent an excellent model of anticancer-targeted therapy, showing both a very good clinical activity and a good safety profile. The categorization of metastatic melanoma patients into BRAF-mutated and BRAF wild-type subsets represents a paradigm shift in thinking about prognosis and therapy of melanoma. As with other types of cancer, the discovery of a molecular target has translated into the identification of a therapeutic target (e.g., HER2 for breast cancer, CD20 in NH lymphomas, EGFR in colorectal and lung cancer…). The same pattern has occurred with BRAF-mutant melanoma. The presence of this mutation is linked with an aggressive disease and with a younger age of onset, but through the development of specific inhibitors, the prognosis of this subset of melanoma patients has radically improved. Indeed, combinations of BRAF inhibitors with other active agents such as ipilimumab or with synergistic agents targeting the same pathway such as MEK inhibitors have the potential to improve outcome even further by increasing efficacy and reducing toxicity or side effects. So, based on the promising results of single-agent trials, it is now perhaps possible to design a different future for melanoma patients: combination strategies [37].

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Concluding Remarks

Rodolfo Saracci

Contents

An attempt at summarizing, however incompletely, the variety of topics and research perspectives as presented at the third conference of "Advances in Nutrition and Cancer" in Naples, May 2012 and herein reported in this volume would be at best repetitious, at worst confusing. Therefore, this summary will address a few selected issues that have emerged or are emerging since the previous conference "Advances in Nutrition and Cancer 2", held in Naples in October 1998.

First one thing is certain, the world, and in particular the world of nutrition as relevant to human health, has changed substantially since 1998. Humankind has increased in number, weight and height. As world population has grown from about six to seven billion, overweight and obesity are rampant not only in economically advanced countries but also in several less economically prosperous regions. There appears to be no country where average adult height has regressed, in fact, it has been reported to be either stable or increasing. Food has been adequate to constitute the key determinant of these major and still ongoing trends. Unless effective preventive measures, centred on nutrition, are implemented on a world scale, these trends will continue into the future, resulting in an increase in the absolute number of cancers and probable increases in the rates of occurrence of certain cancers at

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V. Zappia et al. (eds.), *Advances in Nutrition and Cancer*,
Cancer Treatment and Research 159, DOI: 10.1007/978-3-642-38007-5_26,

sites shown to be associated with overweight—which reflects lifetime caloric imbalance—or with height—which reflects nutrition in early life phases. A fertile stream of "life course" research has in fact developed investigating in particular the role of nutrition in intrauterine life, childhood and adolescence and its effects on adult long-term health and diseases, including cancers [1].

Second, major revolutionary changes have occurred in biology. The first complete sequencing of the human genome in 2003 seems almost a remote event in view of the subsequent bourgeoning evolution of genomics, immediately overlapped by that of epigenomics. Both are driven by the remarkable technological developments of the last two decades that now permit systematic and global approaches to different fields of biological research. One example is the investigation, not of selected micro-organisms of the intestinal flora but of the "universe" represented by the human gut microbiome, namely the collective genomes of the gut microbial community, capable of operating on dietary constituents and generating compounds that may have a variety of roles in carcinogenesis [2]. Similarly, all the metabolites (the "metabolome") of a variety of plant extracts can be measured by NMR techniques to identify compounds of potential interest for human pharmacology, including those in the field of oncology. Other projects of this type aim at characterizing in full the optimal combination of micronutrients that at the individual level (individual "nutriome") may optimize DNA protection against lesions [3] or influence DNA methylation patterns which are potential epigenetic pathways of carcinogenesis, as identifiable through genome-wide methylation assays [4]. In the next 10 years, it will be of the utmost interest to see how far these approaches may change our understanding of the basic mechanisms through which nutritional factors contribute to the occurrence of cancer. Innovative methods will certainly be needed to analyse the massive amount of data such approaches will generate.

Besides these new systemic approaches, genomics and epigenomics have greatly increased the breadth and depth of targeted investigations ranging from the study of epigenetic modifiers inducing cancer cell–selective death [5] to research on nutrients producing epigenetic effects on the telomerase gene that conditions the lifespan of normal and cancerous human cells [6].

In sharp contrast to these rapidly advancing frontiers, other fields of research have shown relatively slow progress. There are in fact issues for which in my recollection development was solicited 15 years ago and is still solicited today, a sign that advancement has been far less than was hoped for. These issues include in the first place the need for better, specific biomarkers of exposure to nutrients and foods, in the three categories of recovery, concentration and replacement biomarkers. For instance, in the category of recovery biomarkers, which is considered the gold standard, we remain essentially limited to the doubly labelled water method to assess energy intake and also to measurements of nitrogen, potassium and sodium in urine. Improved measurement of what and how people actually eat today, what and how they ate yesterday and further back in time is another seemingly permanent issue on the research agenda. On the same line of the above-mentioned systemic approaches, correlating extensive metabolic profiles

with patterns of food and nutrient intake is one of the new avenues being explored to examine whether such profiles may constitute reliable biomarkers of specific intakes. Controlled randomized trials to evaluate causal links between food and nutrient intake and biomarkers of intake or early and late health effects continue to be advocated. However, they are usually laborious, problematic in several practical respects and continue to be rather uncommon.

Between the very fast and the slow progressing frontiers of research stand the frontline closer to the study of nutrition as practically materialized by people's eating habits. On these frontlines, some new findings have been reported since 1998 while other previous results have received confirmation. It has been known for a long time that the multiple nutrients composing any diet may interplay reciprocally to affect absorption, metabolism and biological actions. Hence, the need to investigate the effects not only of nutrients but also of individual foods, classes of foods and food patterns. Fruits and vegetables are rich in antioxidants and have been credited with preventive effects on cancer at several sites, including colorectal cancer. Recent evidence from a meta-analysis of all prospective studies published up to the end of 2010 [7] only partially confirms this protective effect, which has been demonstrated when subjects with a high intake of fruit and vegetables are compared to subjects with a very low intake. However, for those with an adequate intake of fruit and vegetables, no further protection is gained by increasing the level above 100 g per day. The emerging evidence since the 1990s of "Mediterranean diet(s)" which are protective against not only cardiovascular diseases but also metabolic diseases and cancers has been confirmed in different contexts [8]. Within the fundamental and fine-tuned balance between caloric intake and caloric expenditure that prevents overweight (with the help of regular physical activity which promotes fitness as well), these diets share common components while maintaining some local variants, for instance, the consumption of pasta in Italy. The common denominators are as follows: a high intake of cereals, legumes, fruit, vegetables and fish, a low intake of red and processed meat and of alcohol, with olive oil as an important element of the mix and a high ratio of monounsaturated to saturated fats.

These qualitative and quantitative specifications fit nicely into the general recommendation: "Moderation in everything, including moderation". A recipe for not only good health but also, as Aristotle already knew, a "good life", which is at the same time balanced and dynamic.

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