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Commentary

Ins and outs of dietary phytochemicals in cancer chemoprevention

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ABSTRACT

A voluminous number of evidence suggests that an increased consumption of fruit and vegetables is a relatively easy and practical strategy to reduce significantly the incidence of chronic diseases, such as cancer, cardiovascular diseases and other aging-related pathologies. This review will critically discuss the applications of chemical and dietary chemoprevention, intending the protecting effects against cancer of chemically synthesized molecules, or phytochemicals present in the regular diet. The length of chemopreventive treatments requires the administration of low doses of chemopreventive agents, to avoid toxic side effects. This poses the question, here discussed, of the bioavailability of these compounds, usually very modest. Another key issue is whether purified phytochemicals have the same protective effects, as do the whole food or mixture of foods in which these compounds are present. These aspects will be analysed at the light of the ''antioxidant hypothesis'' in cancer prevention and the ''combination chemoprevention'', both referring to the pleiotropic and synergistic effects of compounds present in the diet. Single molecules may evolve in perfect chemopreventive agents, as in the case of tamoxifen, or generate ambiguity. Resveratrol and quercetin represent two paradoxes, discussed here.

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1. Introduction

According to the recent 29th report on the health status of the United States, the overall rate for leading causes of death for all ages between 1950 and 2002 substantially decreased [1]. This important result is primarily due to the significant reduction of deaths for stroke and heart diseases starting from the seventies (in 2002 the age-adjusted death rate for heart diseases was 59% lower than in 1950). On the opposite, the overall rates for cancer, the second leading cause of death throughout the same period, has not been so positive. Cancer mortality rose between 1960 and 1990 and then reversed direction very slowly through 2003 for all races and both sexes combined [1,2]. Female lung cancer incidence rate increased from 1975 to 2003, decelerating since 1991, while breast cancer stabilized from 2001 to 2003 [2]. According to other studies, several common forms of epithelial malignancies (e.g., lung, colorectal, prostate, pancreas, breast and ovary), for both sexes, showed a negative trends in the last 30 years [3]. These statistical and epidemiological data provide the rationale to implement cancer prevention programmes.

According to a seminal paper published in 1981 and based on epidemiological studies, an average of 35% of overall human death rate for cancer is associated to nutritional

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factors [4]. This hypothesis, re-evaluated at the light of compelling evidence on an inverse relationship between fruits and vegetables consumption and risk of cancer [5], has two rational explanations: (i) the presence in the diet of suspected carcinogens; (ii) the absence in the diet of compounds possessing cancer preventing (chemopreventive) properties. Starting from the early 1980s, in United States, several governmental agencies promoted programmes on cancer prevention that had considerably grown since then.[1] Dietary guidelines have been established in order to reduce the risk of cancer. Actually, several hundreds molecules are studied as potential ''chemopreventive agents'', and over 50 compounds are being tested in clinical trials [6]. Despite these enormous efforts, chemoprevention represents a highly controversial topic due to ethical, economic and scientific issues.

This review will focus on some key aspects of cancer chemoprevention, including bioavailability and pleiotropy of potential chemopreventive agents. Differences existing between dietary and pharmacological forms of chemoprevention will be discussed.

2. *Chemoprevention*: what does it mean? An historical perspective

When, Dr. Michael Sporn coined for the first time the term ''chemoprevention'', referring to the activity of natural forms of vitamin A in preventing the development and progression of epithelial cancer [7], he originated a novel field in cancer research. Accordingly to a more modern and complete definition, chemoprevention includes the use of natural or pharmacological agents to suppress, arrest or reverse carcinogenesis, at its *early* stages [3]. The term ''chemoprevention'', passed from 4000 to 10,000 citations in PubMed in the last quinquennium, is now broadly used to indicate the ability of a molecule not only to prevent, but also to cure cancer. In other cases, the attribute ''chemopreventive'' has been associated to a life style, such as a correct diet. As a consequence, ''chemopreventive'' effects are largely described in the current Literature and result from a plethora of different experimental approaches: epidemiological studies on human subjects; clinical trials on patients; studies on animal models where carcinogenesis was experimentally induced; *in vitro* tests on cell lines.

Following the indications of the National Cancer Institute (NCI), five classes of chemopreventive agents show promising results in clinic, and are considered of high priority: selective estrogen receptor modulator (SERMs); non-steroidal anti-inflammatory drug (NSAIDs); calcium compounds; glucocorticoids; retinoids.[1] Many of these compounds are chemically synthesized and have already been applied in pharmacological therapies to cure diseases different from cancer, before their use as chemopreventive drugs. In parallel, the NCI, based on numerous reports describing the anticancer activity of naturally occurring molecules [8,9], identified about 40 edible plants possessing potential chemopreventive compounds, globally known as phytochemicals. However, in many cases, the chemopreventive effects of these compounds are primarily based on cell culture and animal model studies, and only few of them are entering clinical trials [6] (Table 1). Therefore, from a functional, pharmacological and clinical point of view, it would be useful to distinguish between ''pharmacological'' and ''dietary'' chemoprevention.

For the purpose of this review, I will use the term ''phytochemicals'' to indicate the following classes of non-nutrient compounds present in fruit and vegetables: carotenoids, polyphenols, alkaloids, nitrogen-containing and organosulfur compounds. Together with carotenoids, polyphenols represent the most studied class of phytochemicals. They includes the following sub-groups: phenolic acid, tannins, stilbenes, coumarins and flavonoids (reviewed in [10]). Most of these compounds possess strong antioxidant properties that contributed to formulate the ''antioxidant hypothesis'' in cancer prevention.

3. ''Pharmacological'' chemoprevention

Actually, the concept of ''multistep carcinogenesis'' proposes that cancer generates over a period of time due to the accumulation of somatic mutations in a single cell, resulting in gradual phenotypic changes, from a normal to a pre-neoplastic cell, that progresses to neoplastic [9,11]. These different stages in carcinogenesis are generally described as: initiation (days), promotion (several years), and progression (1–5 years). Initiation is irreversible and includes the initial hit by chemical or physical carcinogenic agents directly at DNA level. Promotion, which involves epigenetic mechanisms, is usually a relatively slow and reversible process leading to accumulation of pre-malignant cells abnormally dividing. Progression is generally irreversible, and leads to the final stage of carcinogenesis with tumor growth and acquisition of invasiveness and metastatic potential [9,11]. Excellent animal models and transgenic mice have been established to study multistep carcinogenesis for breast, prostate, colon, lung cancer [11–13]. The passage from pre-malignant to malignant cell involves activation of proto-oncogenes and/or inactivation of tumor suppressor genes (reviewed in [11]). Both categories of genes, when mutated, cause alterations in key cellular processes linked to cell growth and proliferation. A good chemopreventive agent should be able to interfere with one or more phases of the multistep carcinogenesis process.

The efficacy of a novel chemopreventive agent is measured throughout the same procedures applied to a new drug. It must satisfy the following requirements: (1) primary prevention in high risk healthy individuals; (2) cancer prevention in individuals that already had developed pre-malignant lesions; (3) prevention of secondary forms of cancers in patients already treated for a primary cancer [14,15]. The final endpoint of all three aspects of chemoprevention is the attainment of clinical evidence for cancer reduction.

During the past two decades, NCI established a system for a scientific approach to developing chemopreventive agents starting from epidemiological and basic laboratory data, and ending with stepwise clinical trials. These procedures finally led the Food and Drug Administration (FDA) to apply chemoprevention to human subjects [14,15]. Preclinical

[1] http://www3.cancer.gov/prevention/cadrg.

Table 1 – Dietary chemopreventive compounds in active clinical trials[a]

Chemicals	Nutrients	Source	Clinical trial phase	Type of cancer
Vitamins	Vitamin D	Dairy products	II–III	Large bowel adenomas[b]
	Folic acid	Vegetables	II	Peripheral T-cell lymphoma[c]
			I	Colorectal cancer[d]
	Vitamin E	Vegetable oils	III	Chemotherapic patients[e]
	Ascorbic acid	Vegetables and Fruits	II	Non-APL acute myelogenous leukaemia[f]
			II	Multiple mieloma[g]
Minerals	Calcium	Dairy products, vegetables	II–III	Large bowel adenomas[b]
	Selenium	Vegetables, fruits, cereal grains, meat, fish	III	Non small cell lung cancer[h]
			III	Adenomatous colorectal polyps
Carotenoids	Lycopene	Tomatoes	II	Uremia-associated urothelial carcinoma
			Not specified	Healthy subjects[i]
Flavonoids	Genistein	Soybeans, soy products	II	Breast cancer[j]
			II	Healthy women[k]
			II	Pancreatic cancer[l]
	Proanthocyanidins	Vegetables, fruits, black tea	I	Postmenopausal women[k]
Phenolic acids	Resveratrol	Grapes, red wine	I–II	Colon cancer
			I	Healthy subjects
	Curcumin	Turmeric, curry, mustard	II	Colorectal cancer
			II	Pancreatic cancer
			II	Smokers[m]
			Not specified	Multiple mieloma[n]
	Epigallocatechin-3-gallate	Green tea	II	Oral leukoplakia
			II	Cervical cancer[o]
			II	Smokers[p]
			I	Esophageal cancer[q]

[a] Type of treatment: chemoprevention, chemoprotection, chemosensitazion/potentiation. Data from http://www.cancer.gov/clinicaltrials.
[b] And/or calcium.
[c] With pralatrexate and vitamin B12.
[d] Folate-depleted vs. folate-supplemented diet for the prevention of colorectal cancer in patients at high risk for colorectal neoplasia.
[e] Preventing chemotherapy-induced peripheral neuropathy in patients undergoing curative neurotoxic chemotherapy for cancer.
[f] With arsenic trioxide.
[g] With bortezomib and melphalan.
[h] Previously resected stage I.
[i] Preventing prostate cancer.
[j] With gemcitabine hydrochloride.
[k] High risk breast cancer.
[l] With aberrant crypt foci.
[m] With gemcitabine hydrochloride and erlotinib hydrochloride.
[n] With/without bioperine.
[o] In patients with human papillomavirus and low-grade cervical intraepithelial neoplasia.
[p] With chronic obstructive pulmonary diseases.
[q] With Barrett's esophagus.

evaluation and phase I clinical trials can be omitted, if the toxicity and pharmacokinetic of the potential chemopreventive agent is already known from animal studies. Phase I might be further accelerated for drugs already approved for other purposes in humans at doses comparable to those established for chemopreventive trials [14]. Phase II trials evaluate the efficacy of a compound on a relatively small population of subject (50–100 for 6–12 months) at high risk of a specific form of cancer potentially responsive to the putative chemopreventive molecule. Phase II chemopreventive trials are randomized, blinded, placebo-controlled studies, including intermediate surrogate biomarkers (e.g., biochemical, genetic, molecular, histological), able to predict the occurrence of cancer in a relatively short period. Finally, phase III clinical trials are randomized, controlled, large scale trials lasting several years (5–10 years or longer), since cancer incidence is usually taken as the primary endpoint. For their nature and length, these studies adsorb a significant amount of financial resources and, therefore, need a careful revision and selection [6].

A clear demonstration of chemopreventive activity associated to a single molecule is tamoxifen. Tamoxifen is currently the only agent approved by FDA in United States for the prevention of breast cancer in women at high-risk of oestrogen-receptor-positive tumours, based on a 49% reduction in invasive breast cancer in the National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1 trial [16]. The decision to test tamoxifen as potential chemopreventive agents derived from several *in vitro* studies indicating that tamoxifen acts as a selective oestrogen-receptor modulator (SERM), binds and inhibits oestrogen receptors in one organ (such as breast), and activates the same receptors in other

organs (bone and uterus) [3,16]. In addition, tamoxifen: (1) prevents breast cancer in rats; (2) shows an excellent safety profile when used in the adjuvant treatment of breast cancer; (3) possesses minimal toxic effects; (4) has beneficial effects on the maintain bone mineral density; (5) reduces the risk of developing a controlateral breast cancer in women with a history of breast cancer [16,17]. Actually, another SERM, raloxifene, is tested as breast cancer chemopreventive agent in an ongoing study of tamoxifen and raloxifene (STAR), based on its ability to prevent osteoporosis in postmenopausal women [17].

Nowadays, several drugs are under consideration by the FDA as potential chemopreventive compounds and are undergoing clinical trials [14,15]. Positive results have been already obtained for the prevention of: (i) colorectal adenoma and carcinoma with NSAIDs; (ii) bladder cancer with dietary vitamin C; (iii) prostate cancer with finasteride. A summary of these studies is reported on Tables 3–10 in reference [15].

Despite these encouraging results, many chemopreventive studies failed, ending with no results or an increased incidence of cancer. The most illustrative example in this sense is represented by the ATBC Cancer Prevention Study, where 29,133 male smokers, ages 50–69 years, were treated daily with β-carotene, vitamin E, both β-carotene and vitamin E, or placebo. The alarming results obtained after 5–8 years of follow-up, caused the immediate end of the trial, since β-carotene supplementation was associated with an increased risk of lung cancer development [18]. However, it must be mentioned that analysis of the secondary endpoints of the same study indicated that α-tocopherol supplementation reduced of 32 and 41% prostate cancer incidence and mortality, respectively [19]. The ATBC Cancer Prevention Study was realized on the basis of strong epidemiological data [18], but probably lacked the necessary support deriving from animal and cellular models [3].

The partial failure of this study indicates that a chemopreventive programme only based on epidemiological data linking dietary habits to risk of developing cancer is not sufficient to justify a large and costly clinical trial. As I will discuss later in this review, the road to develop and evaluate potential chemopreventive agents includes the collection of experimental data obtained from cell cultures and animal models.

4. *"Dietary"* chemoprevention

As stated above, many phytochemicals present in a diet rich in fruit and vegetables have been proposed as potential chemopreventive agents, and selected for ongoing phases I–III clinical trials (Table 1). These compounds can be divided into two main groups: cancer-blocking and cancer-suppressing agents. The former prevent carcinogens to hit their cellular targets (initiation) by several mechanisms: enhancing carcinogen detoxification, modifying carcinogen uptake and metabolism, scavenging reactive oxygen species (ROS) and other oxidative species, enhancing DNA repair. Cancer-suppressing agents inhibit cancer promotion and progression after the formation of pre-neoplastic cells by interfering with cell cycle regulation, signal transduction, transcriptional regulation, and apoptosis [6,9].

Several epidemiological studies suggest a positive association between consumption of a diet rich in fruits and vegetables and a lower incidence of stomach, esophagus, lung, oral cavity and pharynx, endometrium, pancreas, and colon cancers [5,20,21]. These studies support the view that more than 70% of all types of cancers do not dependent on the individual genetic background and can be prevented by changes in lifestyle, such as a correct diet [22]. Based on these scientific evidences, a number of important educational programmes started in USA and Europe to increase vegetable consumption in order to lower cancer incidence.[2] However, these conclusions have been recently questioned by an authoritative work, where the authors reported the results of two cohort studies reaching the conclusion that the association between fruit and vegetables intake and cancer incidence was null [23]. A modest reduction in risk was only observed for cardiovascular diseases. Although the work from Willett's group [23] raised many controversies and criticisms due to the statistical methods applied by the Authors [24], it posed a serious question on the real beneficial effects of a vegetables rich diet on cancer protection. In addition, it is paradoxical that the same diet may efficiently protect against diseases, which differ from a molecular and physiopathological point of view, such as cancer and cardiovascular diseases. It may be that a cause-effect relation exists between diet and specific form of cancers, as recently proved for non-Hodgkin lymphoma [25].

The rationale behind the protective effects of fruit and vegetables is the presence of antioxidant molecules, *i.e.* carotenoids and polyphenols, able to scavenge oxidant species efficiently. ROS include a variety of diverse chemical molecules extremely unstable, such as superoxide anions and hydroxyl radicals, whereas others, like hydrogen peroxide, are freely diffusible, relatively long-lived and able to cause DNA damage. Superoxide or hydroxyl radicals can either be generated exogenously, or produced intracellularly from cytosolic enzymes (NADPH oxidases) and mitochondria. The primary production of mitochondrial superoxide radicals occurs at the level of complex III [26]. The physiological homeostasis of ROS is maintained by a sophisticate system of intracellular antioxidant defences, including the enzymes SOD, catalase, glutathione peroxidase, peroxiredoxins and low molecular mass molecules, such as glutathione, present in millimolar concentrations within cells [26]. A physiological production of ROS is associated with important cellular processes in normal cells [27], while lowering ROS levels below the steady-state level may interrupt the physiological role of oxidants in cellular proliferation and host defence [26,28–30].

Increased ROS production has been described in cancer cells and linked to genomic instability and cancer initiation, progression and maintenance [30]. This theorem generates an obvious corollary: loading the pre-malignant cell with antioxidants (from food or supplements) by a chemopreventive intervention might lead to lowering ROS and protect cells from cancer. However two main questions rise from this paradigm:

[2] http://www.iarc.fr/epic/; http://www.5aday.gov.

(i) ''when'' the chemopreventive intervention must take place to show efficacy? (ii) What happens if the antioxidant treatment does not occur at the ''appropriate time''? In fact, a recent and provocative study suggests that increasing ROS generation over an established threshold by lowering antioxidant defences may contribute to kill cancer cells [28,29]. Using selected cell lines, the authors demonstrated an increased level of oxidative stress in oncogenic transformed cell lines, as expected. However, the administration of beta-phenylethyl isothiocyanate (PEITC) increased the oxidative stress by reducing the cellular capacity to counteract ROS increase. PEITC achieved this effect by depleting cellular levels of reduced GSH (gluthathione) and inhibiting GSH peroxidase [29]. As a result, PEITC administration induced ROS cytotoxicity similarly to other chemotherapeutic agents [31]. If the results of this study will be confirmed by further investigations, the consequence for the use of antioxidant in chemoprevention should be revised, since: (i) a treatment with dietary compounds able to scavenge ROS in malignant cells during cancer progression and/or propagation might lower the ROS threshold, ''saving'' the cell from drug-induced death; (ii) if the ROS-mediated mechanism of cancer cell death is correct, combined treatments using chemotherapeutic drugs and antioxidant compounds, need to be carefully re-evaluated. In fact, these protocols, by protecting cellular antioxidant defences, might abolish the ROS-induced cytotoxic effect of drugs due to increased oxidative stress. This scenario can explain the failure of several clinical trials where vitamin or other antioxidant micronutrients were given to cancer patients in association with traditional chemotherapeutic agents [3].

Antioxidant treatment could be more effective during the ''initiation'' phase, when a slight increase in ROS concentrations over the physiological threshold can cause genotoxic damage at DNA level. However, also in this case, choosing the right timing for the pharmacological treatment is essential. In fact, as briefly mentioned above, it is generally thought that the initial hit in cancer transformation is a stochastic event that takes place in a short time; therefore, only a ''daily'' chemopreventive therapy, primarily on healthy individuals with high-medium risk of cancer, may show efficacy. At the moment, this hypothetical strategy of intervention is not easily feasible for safety and ethical issues, as extensively discussed [3,19].

5. The "*Antioxidant hypothesis*" in chemoprevention

A part from the controversial aspects described above, the ''antioxidant hypothesis'' is strongly sustained in the Literature. It asserts that ''being the antioxidants able to prevent or reduce oxidative damage, their increased uptake from the diet will reduce the risk of chronic diseases'' [32]. It is worthwhile to note that a large part of studies supporting the antioxidant hypothesis against cancer are based on cell lines studies and on animal model where tumors were experimentally induced by high doses of carcinogens [3,8,9,33,34]. However, growing experimental evidence suggest that antioxidants present in food act as chemopreventive agents independent of their ability to scavenge ROS. Many of them interfere with signal transduction regulation at different levels: modulate hormones/growth factors activities, inhibit oncogenes and activate tumour suppressor genes, induce terminal differentiation, activate apoptosis, restore immuno response, inhibit angiogenesis, decrease inflammation. They counteract the activity of exogenous and endogenous potential carcinogens suppressing phase 1 reactions and/or activating phase 2 reactions, both catalyzed by metabolizing enzymes. These enzymes are involved in two major biotransformation reactions: phase 1 reactions that add or expose functional groups to xenobiotics such as –OH, –SH, NH_2 or –COOH, and phase 2 reactions that involve the conjugation of large water soluble biomolecules (glucuronidation, sulfation, acetylation, methylation, and conjugation with glutathione) to the xenobiotics. These two groups of enzymes are also known as activating enzymes and detoxifying enzymes, respectively, based on the biological consequences of enzymatic reactions. The cytochrome P450 family, a group of important monooxygenase enzymes, metabolises many xenobiotics, catalyzes many types of phase 1 reactions converting products in chemically more reactive and thereby can potentially interact with DNA and proteins causing aberrant mutations and/or alteration of signalling pathways. The other side of the coin shows the detoxifying phase 2 enzymes, generally categorized in four major families: UDP-glucuronosyltransferase; sulfotranferases; glutathione S-transferase (GST), *N*-acetyltransferases. These enzymes compete with the activating cytochrome P450 enzymes by eliminating reactive electrophiles via reduction, or conjugation with endogenous substrates. The dynamic equilibrium between carcinogen-activating enzymes and detoxifying enzymes can be fundamental to determine the cell fate after exposition to carcinogens [35].

Plant polyphenolic antioxidants represent one of the major class of dietary components characterized by a double function: antioxidant and regulators detoxifying enzymes (e.g., GST, NADPH: quinone oxidoreductase and heme oxygenase 1) [34]. The study of these enzymes led to the discovery of the antioxidant response element/electrophile responsive element (ARE/EpRE), an enhancer sequence, that regulates the cellular response to potential chemopreventive agents present in the diet (reviewed in [36]). More recently, it has been shown that ARE/EpRE regulates the transcription of several other genes, including cyclooxygenase 2 (*COX2*), and apolipoprotein A-I. Several different pathways are triggered by dietary phytochemicals, leading to ARE/EpRE activation. These include PKC, PI3K, MAPK, JNK pathways (reviewed in [9,37]), and are characterized by the ability to interfere with the interaction between *Nrf2,* the transcription factor, and its main negative regulator, *Keap 1*. As a consequence, *Nrf2*, sequestered by *Keap 1,* is blocked in an inactive form into the cytoplasm [38]. Although the real mechanism of action is still unclear, the *Nrf2-Keap 1* heterodimer works as an intracellular sensor against changes in electrophiles or ROS concentrations [38]. How phytochemicals interfere with this mechanism is still unclear. It can be evoked the capacity of several phase 2 gene inducers (including phytochemicals), to increase ROS, despite their antioxidant nature (discussed in [9]).

Other pathways involving phytochemicals activity in cancer prevention, such as MAP kinase pathways [39], NFκB

activation [9,37], TGF-β serine–threonine kinase signalling and β-catenin pathways are reviewed elsewhere [9,37,39].

6. Bioavailability of chemopreventive agents

In the voluminous Literature on the anticancer activity of dietary phytochemicals, the fundamental importance of their bioavailability and metabolism has been sometime neglected.

As clearly stated by the Chemoprevention Working Group [19], one of the main features of a potential chemopreventive agent is its ''safety'': it must be administered at doses much lower compared to a classical chemotherapeutic drug, since the receivers might be ''healthy'' subjects. However, from a Literature screening, appears clear that the concentrations generally used in the scientific papers are in the range of pharmacological doses or higher. As an example, flavonoids are the most abundant polyphenols in our diet and include different classes of compounds with a variable grade of antioxidant capacity (reviewed in [40]). Their uptake is primarily a consequence of their distribution in the original food, and depend on food processing (cocking, pressing, etc.). Therefore, it is extremely difficult to precisely calculate the daily intake of polyphenols, although dated reports refer to 1 g/die of total phenols [40]. Since the biological properties of polyphenols depend on their bioavailability, it is mandatory to study this issue before discussing their chemopreventive efficacy. In fact, the different chemical structures of polyphenols determine their selective gut absorption. Urine and plasma levels represent good markers to establish bioavailability of polyphenols and their metabolites. Generally, less than 10% of polyphenols, or their metabolites, ingested are found in urine and plasma, where concentrations barely reach 1 μM [40]. Polyphenols are usually present in the gut as glycosyl-derivatives and removal of the sugar moiety by glycosidases is required to pass the small intestine barrier. After hydrolysis to the free aglycone, polyphenols undergo modifications similar to common drugs: they are conjugated by methylation, sulfation, glucuronidation or a combination of them. This is a crucial point in terms of chemopreventive activity. In fact, conjugates can significantly change biological properties of the original compounds. As clearly stated by Scalbert and Williamson [40], when polyphenols are administered at pharmacological doses (hundreds of milligrams), or consumed in a polyphenol-rich diet (higher than 1 g/dose), they can readily saturate the conjugation pathways, leading to a detectable, unconjugated compounds in the plasma. Concentrations employed influence not only quality and quantity of circulating species, but also the tissues distribution of polyphenols and their relative metabolites. In fact, large doses are metabolized in the liver, while small doses are primarily metabolized by the intestinal mucosa [40,41]. Blood concentration of a single polyphenol, or its metabolites, does not significantly influence the antioxidant capacity of the plasma. Nevertheless, when the contribution of dietary polyphenols (0.5–1 g/die) is considered *in toto*, plasma antioxidant power increases to a value ranging between 50 and 75 μM equivalent of vitamin C [42]. These concentrations are in the same range of those used when a single polyphenol is added to cell lines or administered to animal models.

Based on this information, a functional link between presence in the diet of polyphenols with potential chemopreventive properties and ''antioxidant hypothesis'' in cancer prevention can be sustained. The general low bioavailability of single compounds, together with the complex transformation reactions they undergo, makes difficult a cause-effect analysis. On the contrary, when ingested in the whole food, ''combination'' of antioxidant phytochemicals (see below) might suggest a rationale for dietary chemoprevention.

7. Two paradoxes in chemoprevention: resveratrol and quercetin

7.1. *Resveratrol*

A number of excellent reviews have been recently published on resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), a phytoalexin firstly described as a component in the root of the weed *Polygonum cuspidatum*, whose extracts are very well known in Asian medicine [43,44], and also present in grape skin, wine and peanuts. Resveratrol raised his importance as a cancer preventive agent after a paper published in *Science* journal in 1997 [45]. However, what seems paradoxical is the discovery that resveratrol was initially present in the scientific Literature as cardiovascular protective agent, able to explain the ''French paradox''. In fact, the molecule inhibits platelet aggregation, prevents LDL oxidation by means of its antioxidant properties, exerts vasorelaxing effect on animal model (reviewed in [46]). After the Jang's paper [45], resveratrol became synonym of naturally occurred molecules possessing chemopreventive activities (reviewed in [43,44]. Finally, and more recently, resveratrol showed potential anti-ageing properties [47,48] (Fig. 1).

Several *in vivo* studies, recently reviewed [44], sustain resveratrol efficacy in inhibiting or retarding tumor growth and/or progression in animal models inoculated with malignant cell lines, or treated with tumorigenesis-induced drugs (benzo-[α]pyrene, DMBA, azoxymethane). However, lack of *in vivo* efficacy of resveratrol was reported by others [49]. *In vitro*, resveratrol and its analogs trigger numerous intracellular pathways leading to cell growth arrest. These include inhibition of ERK1/2-mediated signal transduction pathways, inhibition of PMA-dependent PKC activation, downregulation of β-catenin expression, block of cell cycle progression by inhibition of Cdk1 and Cdk4 kinase activities, activation of pRb, induction of apoptotic events, such as activation of caspases, p53 and Bax and inhibition of Bcl2 (reviewed in [43,44]).

Recently, Sinclair's group and others described the anti-ageing properties of resveratrol. The molecule is the most potent activation of sirtuins transcription and function over 20,000 compounds tested [48], resulting in extended life span in yeast, worm, fly, fish, and mice ([47] and references therein). Sirtuins (*Sir2* in yeast, *SIRT1–7* in mammals) are NAD-dependent deacetylases involved in gene silencing processes of ageing, blocking apoptosis and promoting cell survival [50]. The pro-survival properties of resveratrol have been confirmed by a recent paper showing its ability to increase aerobic capacity in mice by inducing genes for oxidative phosphor-

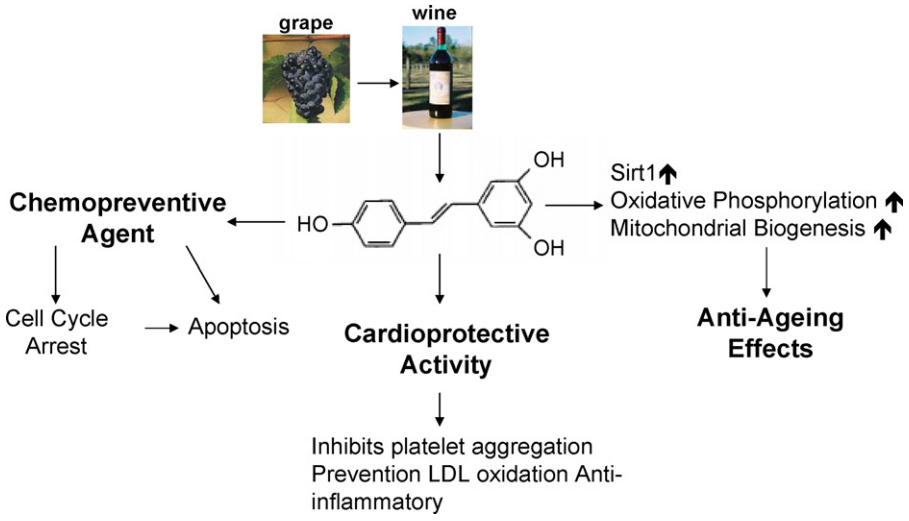


Fig. 1 – Pleiotropic activities of resveratrol against degenerative diseases (see text for details).

ylation and mitochondrial biogenesis in a *SIRT1*-dependent manner [51]. This appears as a potential contradiction respect to the ''antioxidant hypothesis'' mentioned above: as a chemopreventive agent and an antioxidant, resveratrol counteracts ROS production and inhibit cell growth; however, as an anti-ageing compound, the same molecule increases mitochondrial metabolism and, in turns, ROS production, enhancing cell survival. On the other hand, resveratrol seems to question the function of histone deacetylases in cancer therapy. In fact, while histone deacetylases ''inhibitors'' represent novel and potent chemotherapeutic drugs [52], resveratrol, apparently, functions in the same way ''activating'' the same enzymes.

Trying to find an explanation to these controversial data on resveratrol, I may suggest to take a careful look to the *in vivo* bioavailability of the molecule. As extensively reviewed ([53] and references therein), dietary resveratrol (up to 25 mg) is rapidly adsorbed and predominantly present in the plasma as glucoronide and sulphate conjugates. In addition, when administered in food, such as wine or grape juice, resveratrol metabolism is significantly inhibited by other polyphenols due to competitive reactions with metabolizing phase II enzymes [53], resulting in an increased concentration of the free form. Despite this, the free aglycone is almost undetectable in human plasma. Therefore, caution is suggested [53] when interpreting the voluminous Literature on anticancer activity of resveratrol, only based on *in vitro* studies on cell lines where the molecule is given at pharmacological concentrations (25–50 μM), as aglycone, a form almost absent in plasma and urine.

The explanation for the functional pleiotropy of resveratrol might be found in the values of its circulating concentrations. The recently discovered anti-aging properties of resveratrol seem to take the lead in the resveratrol saga. In fact, the molecule activates *in vivo* Sirt1 at very low concentration (nanomolar range) [48], a value potentially in agreement with resveratrol bioavailability. On the opposite, moving from physiological to pharmacological concentrations, as those employed in the *in vitro* experiments, predominates the anticancer properties of the molecule: inhibition of cell growth, activation of apoptotic events, arrest of cell cycle progression, depending on the cell line employed and the biomarkers analysed. However, at high doses, the term chemopreventive agent, referred to resveratrol, appears inappropriate.

7.2. *Quercetin*

Resveratrol represents an example of naturally occurring molecule possessing variegate and beneficial effects in terms of human health. On the opposite, for many other chemopreventive compounds, both protective and genotoxic activities have been described. Quercetin can be included in this category.

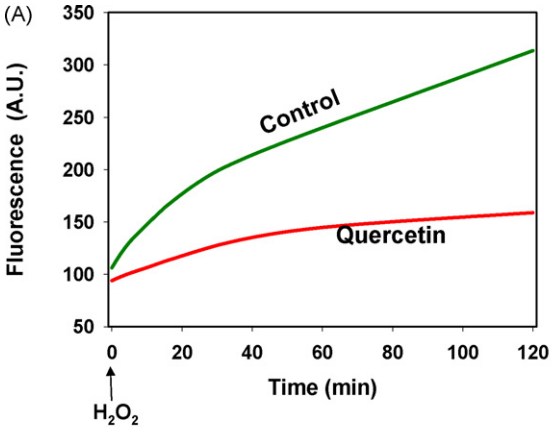
Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is one of the major dietary flavonoid, found in a broad range of fruit, vegetables and beverage such as tea and wine, with a daily intake in Western countries of 25–30 mg. The anti-oxidant, anti-inflammatory, anti-proliferative or apoptotic effects of the molecule have been largely analyzed in cell culture models (reviewed in [54]). Among polyphenols, quercetin is one of the most potent antioxidant, as demonstrated in different *in vitro* [55,56], and *in vivo* studies [57]. At molecular level, quercetin acts as anticancer agent by down-regulating the expression of oncogenes (*H-ras*, *c-myc* and *K-ras*) and anti-oncogenes (p53) [58], or up-regulating cell cycle control protein (p21WAF1 and p27KIP1) [59]. In addition, quercetin inhibits different tyrosine and serine–threonine kinases, whose activities are linked to survival pathways (MAPK, AKT/PKB) [60]. In animal models, quercetin inhibits cancer growth and induces apoptosis [61]. A phase I clinical trial indicated that the molecule can be safely administered and its plasma levels are sufficient to inhibit lymphocyte tyrosine kinase activity [62]. Consumption of quercetin in onions and apples was found to be inversely associated with lung cancer risk in Hawaii [63]. The effect of onions was particularly strong against squamous cell carcinoma. Increased plasma level of quercetin following a meal of onions was accompanied by increased resistance to strand breakage by lymphocyte DNA and decreased levels of some oxidative metabolites in the urine [64].

On the other side, the potential risk associated to quercetin consumption at pharmacological doses has been reported. In a recent review [65], the author drew an interesting paradox of quercetin biological activity, although limited to cell lines. At 50 μM concentration, quercetin was able to reduce by 35–40% the DNA damage caused by an oxidative insult; however, at the same concentration, quercetin increases *per se* the DNA breaks of 4-fold, indicating a clear genotoxic damage [65]. Similarly, the hazard in quercetin administration has been described on cell lines [66] and animal models [67]. These observations confirm the hypothesis formulated by Ames more than 20 years ago on the contemporary presence of mutagenic and protective activities in our regular diet [68].

Recently, our group demonstrated that, at micromolar concentration, quercetin was neither cytotoxic, nor apoptotic *per se*; however, when associated to death receptors inducers (e.g., the CD95 agonist anti-CD95, or recombinant TRAIL), synergistically increased apoptosis in different cell lines [55,69,70] and in *ex vivo* models [70]. As reported in Fig. 2, the enhancing effect of the molecule on apoptotic process was ''independent'' of its antioxidant activity. In fact, quercetin maintains its ability to efficiently scavenge ROS (Fig. 2A), acting as strong antioxidant among polyphenols [56]; however, this property is not required for its pro-apoptotic activity. In fact, as reported in Fig. 2B, other flavonoids, structurally and functionally similar to quercetin, maintain the ability to lower ROS, similarly to quercetin, but are ineffective in inducing apoptosis when associated to death receptor effectors.

These data support the hypothesis that naturally occurring bioactive compounds may act in synergy with drugs in pharmacological applications. In the case of quercetin, at relatively low, not toxic doses, the molecule synergized the effect of TRAIL on malignant cell lines normally resistant to the administration of the single drug. This combination therapy may allow the use of lower concentration of chemotherapeutic drugs with an increased efficacy. How quercetin exerts this activity at molecular level need further studies: its pleiotropic nature makes difficult the identification of the most sensitive cellular target.

Metaphorically, quercetin, when used at low concentrations, causes multiple wounds, without killing the malignant cell, which increases its vulnerability to the ''magic'' bullet represented by the chemotherapeutic drug. Other phytochemicals with comparable pleiotropic activity might work in the same way.



(B)

Molecule	↓ROS(%)	↑Apoptosis (%)
Quercetin 50 μM	60	>50
(+) Catechin 100 μM	40	0
Myricetin 50 μM	50	5
Quercetrin 50 μM	40	0

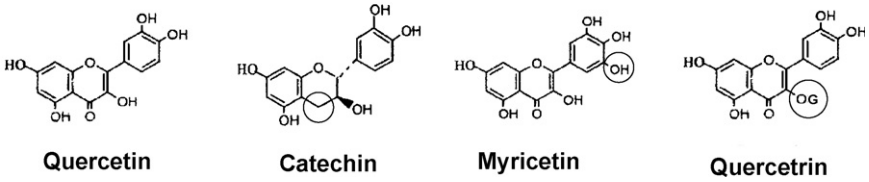


Fig. 2 – (A) Antioxidant effect of quercetin. The molecule (50 μM) lowered ROS in HPB-ALL cell lines stimulated with 10 μM H_2O_2 (for experimental details see [55]). (B) Antioxidant molecules structurally similar to quercetin (chemical formula reported below) lowered ROS, similarly to quercetin, but were unable to induce apoptosis, when administered in association with anti-CD95 (for experimental details see [9]). Circles indicates structural differences respect to quercetin. G, in quercetrin formula, indicates the glycosyl-group.

8. Conclusion: in *combination stat virtus*

Many reviews on cancer chemoprevention end with ''warnings'' on the low bioavailability of the active compounds, the difficulty in extrapolating conclusions only based on experimental data, the high cost of large scale clinical trials, the ethical problems correlated to the preventive treatment of healthy subjects, and so on. Each of these considerations is, *per se*, correct; therefore, putting together all of them, chemoprevention may result as a science since in its infancy, with many controversies which need be resolved. Certainly, more caution should be paid by the scientific community in elaborating conclusions from studies on cell lines. They suffer of several disadvantages: (1) use of very high concentration of the active compound; (2) absence of the ''right'' control experiments (*i.e.*, effects on non-malignant cells); (3) use of ''pure'' molecules, neglecting the potential effects of their metabolites; (4) generation of possible artefacts due to the interaction of the putative chemopreventive agents with cell culture components. This is a phenomenon diffuse and often underestimated, as reported by Halliwell's works [71]. However, the use of cell cultures to study naturally occurring chemopreventive agents has great advantages: it is simply, cheap, versatile, allows rapid screenings of multiple compounds and can orientate the scientist towards the molecular mechanism underlying the biological activities measured. Cell culture data may represent the green light to start a chemopreventive study, but should not go over.

In the classical way of studying cancer, research starts from the disease followed by gene identification and ending with specific cancer gene targeting and drug development. As recently reviewed [72], in chemoprevention, natural products, such as phytochemicals, take the top of the pyramid: based on epidemiological and experimental studies, these agents can be isolated from their natural sources, purified and assayed to test their ability in killing precancerous and cancerous cells (Fig. 3). *In vitro* studies should be limited to this phase. The procedure should continue with preclinical studies on animal models and phases I–III clinical trials described above. The result can lead to chemopreventive agents and/or anticancer drugs with pharmacological applications in chemotherapy (Fig. 3). The substantial difference between these two fates is the dose of treatment. This view might unify pharmacological and dietary chemoprevention and might help to overcome many criticisms in chemopreventive strategies.

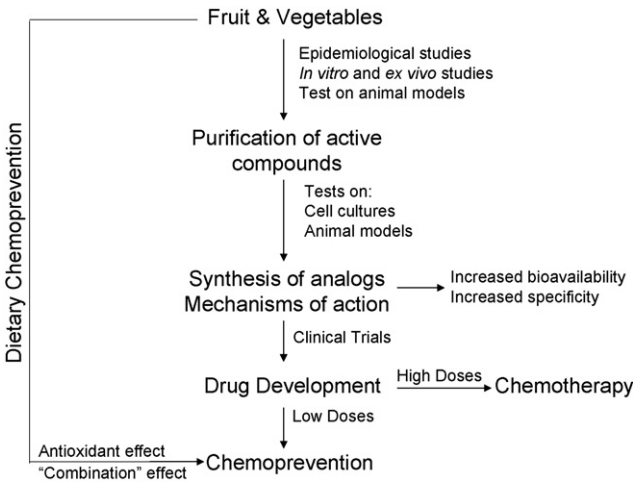


Fig. 3 – Approach to cancer prevention based on potential chemopreventive compounds present in the diet (see text for details).

Dietary chemoprevention deserves further analyses. A primary question remained unsolved regards how the same dietetic habit, *i.e.* fruit and vegetables, can protect against diseases with different genetic, molecular and physiopathological mechanisms, such as cardiovascular diseases and cancer. The ''antioxidant hypothesis'' in disease prevention might help, since a regular antioxidant diet may balance a small disequilibrium in ROS homeostasis occurring in ''normal'' cells, because of daily carcinogenic insults deriving from the lifestyle. The limited availability of dietary antioxidants, discussed above, that significantly reduces their presence in the target cells, might not represent a limitation. In fact, low concentration of antioxidants are, probably, sufficient to ''correct'' low increases in intracellular ROS, delaying, in long term, the occurrence of the pre-malignant event in cancer progression and in the development of atherosclerotic plaques. Perhaps, this speculative explanation has the merit to reconcile low bioavailability of dietary phytochemicals with disease prevention, and fit with the positive effects of some of them, such as resveratrol (discussed above) and quercetin [73], with prolonged life expectancy. The apparent contradiction described for resveratrol and quercetin referring to their opposite and/or different effects, can be explained paraphrasing Paracelsus: ''all substances are poisons ... the right dose differentiates a poison from a remedy''. One attribute common to the majority of phytochemicals is ''pleiotropy'', a word with a Greek etymology that means ''in more places''. In functional terms, the word possesses a negative meaning if associated to lack of specificity of the compound; it assumes a positive significance when indicates the capacity to trigger multiple cellular targets, increasing, in such a way, the efficacy of the molecule under investigation. Pleiotropy and hormesis might help to explain what happens in many experimental models. The passage from low to high concentrations of a single compound might generates a U-shape curve. In the case of resveratrol, low and high concentrations might correlate with the anti-aging and anti-cancer effects, respectively. Since diet provides low concentration of resveratrol, I will expect a more brilliant future of the molecule as anti-ageing, than chemopreventive agent. At high concentrations, resveratrol, or its synthetic analogs, might lead to pharmacological applications in cancer therapy, as suggested by the ongoing clinical trials.

As discussed in this review and in others, combination treatments might represent a new strategy that can play a major role in the future of cancer prevention, similarly to combination chemotherapy in the treatment of invasive cancers [3]. The association of several molecules (as naturally happens in some foods) might be more effective in cancer prevention, than single compounds. This view generated the development of ''combination chemoprevention'', intending that low doses of chemopreventive agents differing in the mode of action may increase efficacy and minimize toxicity, generating a synergistic effect [3,74]. Quercetin represents an example of this theory. Combination of two chemopreventive

agents are often seen in phases II and III clinical trials [14,15] (Table 1), with alternate results, as mentioned above. The benefit deriving from natural phytochemicals at low doses does not mean that the same molecules work ''better'' at higher doses, or when associated together. This is particularly true for dietary supplements, which often represent a jungle of not well characterized preparations, lacking a fundamental information direct to the consumers: data on the pharmacological dose versus the physiological, or recommended dietary allowance [19]. Paradoxically, the most effective combination chemopreventive preparation is diet. The several hundreds compounds present in food differ for molecular structure, stability, solubility, metabolism and cellular uptake; they are subjected to competitive and/or synergistic activity that cannot be simply reproduced in a pill.

The synergistic activity of a complex mixture made by known chemical compounds can be easily tested *in vitro*. My laboratory made the following simple experiment on cell lines. The most representative polyphenols present in a food with well-documented chemopreventive properties were characterized and quantitated by HPLC. Subsequently, the anti-proliferative activity of each single molecule was tested on cell lines at exactly the same concentration present in the original food extract (usually in the range of 0.1–5 μM). None of them showed any effect on cell growth and/or apoptosis. Mixing them in different combinations, keeping constant their final concentrations, reproduced the anti-proliferative and apoptogenic effects of the whole extract.[3]

In terms of public health, cancer eradication in Western Countries represents one of the scientific missions of the new millennium. The incredible acceleration subsequent to the introduction of the omics sciences might shorten this objective. However, the drug-on-demand strategy postulated by the novel field of pharmacogenomics is still in its infancy. Therefore, traditional pharmacological and surgical approaches remain, actually, the only way to cure efficiently cancer patients. Even when therapy and early diagnosis will reach their optimal efficacy, preventive strategies will remain mandatory. The controversies in chemoprevention that this and other reviews tried to analyse will be optimistically solved with the advance of scientific knowledge, as history of science taught. Therefore, science and politics should not hesitate to sustain and increment chemoprevention to fight cancer.

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