

Graduate School in Molecular Sciences and Plant, Food
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**PROTECTIVE ROLE OF DIETARY
BIOACTIVE COMPOUNDS:
MECHANISMS AND HYPOTHESIS**

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Abstract

Scientific evidences support the protective role of diets rich in fruit and vegetables against chronic diseases, like cancer and cardiovascular disease. In particular two classes of chemopreventive phytochemicals, i.e. **isothiocyanates** presence in Cruciferae and **anthocyanins** (ACNs) presence in berries and other colored fruits and vegetables, are considered in this thesis.

Cruciferae and especially *Brassica* genus contain high concentration of constituents with antioxidant properties (e.g. carotenoids, vitamin C, folate) as well as glucosinolate precursors of isothiocyanates (ITCs) and indoles that modulate xenobiotic biotransformation enzymes, such as Glutathione S-Transferase (GST). The protective effect of broccoli (*Brassica oleracea* L. var. *italica*) was tested through two different study protocols: a **regular-intake** intervention study with daily consumption of one serving of steamed broccoli for 10 days and a **single-meal** study (i.e. single consumption of broccoli, 250g). Analyzed broccoli resulted to be a good source of antioxidant compounds (carotenoids, vitamin C) and glucosinolates as ITCs. Subjects involved in the study were young male smokers recruited on the basis of anthropometric characteristics, food habits and their GSTM1 genotype.

Thirty subjects were enrolled for the **regular-intake study**. A single blind randomized cross-over experimental design was scheduled. Fasting blood samples were collected at the beginning and at the end of each treatment period (0, 10, 30, 40 days). Concentration of carotenoids, lutein, β -carotene and folate was assessed in plasma and serum samples. Lymphocytes were used for the determination of biomarkers of oxidative stress: cell resistance against oxidative stress, endogenous DNA damage (i.e. oxidized purines), *in vitro* DNA repair activity and mRNA expression of OGG1, NUDT1 and HO-1 levels. Cell protection against H₂O₂-induced DNA damage was higher after broccoli diet with respect to control diet in the whole group of subjects. Folate and lutein concentrations increased significantly after broccoli diet. Broccoli intake caused a more consistent and significant effect of protection against DNA damage in GSTM1 null (-27.6%) subjects compared to GSTM1 positive (-13.1%) individuals ($p < 0.05$). Oxidized purines decreased significantly ($p < 0.05$) after broccoli intake (-22.6%) while no effect of polymorphism was observed. DNA repair activity and OGG1, HO-1 and NUDT1 mRNA expression levels did not differ throughout the intervention study.

Twelve volunteers were selected for the **single-meal study**. Fasting blood samples were collected before broccoli consumption and after 3, 6, 8 and 24 h from broccoli intake in order to evaluate: cell resistance to oxidative DNA damage, bioavailability of bioactive compounds (folate, lutein, β -carotene and vitamin C) and total GST activity. In particular, serum folate, plasma vitamin C and β -carotene concentrations significantly increased. Furthermore, plasma GST activity increased significantly in individuals with GSTM1 positive genotype at 6 h with respect to 3 h and 24 h but not compared to baseline. Interestingly, *ex-vivo* induced DNA damage was significantly reduced after 24 h from broccoli consumption.

In conclusion, these two studies demonstrated that 10 days of consumption of steamed broccoli could improve defence against DNA damage, without affecting repair activity in young healthy smokers. Moreover, a single portion of steamed broccoli was able to decrease DNA damage and to modulate GST activity. Even if preliminary, our data suggest a “diet/genetic” interaction.

Delphinidin-3-glucoside (Dp-3-glc) and cyanidin-3-glucoside (Cy-3-glc) are two **ACNs** able to inhibit tumor cell proliferation.

An *in vitro* study was designed to investigate the effect of Dp-3-glc and Cy-3-glc on the angiogenic and Dp-3-glc on the procoagulant activities of human microvascular endothelial cells (HMEC-1).

HMEC-1 were incubated for up to 24h with culture media \pm ACNs (0.1, 1, 10, 100 μ M) alone or in combination with purified proangiogenic factor (VEGF), or bacterial endotoxin (LPS). Angiogenesis was evaluated by the capillary-like tube formation in Matrigel and the wound healing assay; while the pro-coagulant activity was tested by the thrombin generation (TG) assay and Tissue Factor (TF) expression as antigen, one-stage recalcification assay and mRNA quantitation.

Results showed that Dp-3-glc was able to inhibit angiogenesis in resting cells and in VEGF-stimulated conditions at 100 μ M; interestingly, the inhibition of the migratory VEGF-dependent stimulus started at the Dp-3-glc concentration of 1 μ M. In the TG assay Dp-3-glc significantly contrasted with the pro-thrombotic stimulus of LPS starting from 10 μ M on HMEC-1 intact monolayer and TF expression at 100 μ M concentration. Differently, Cy-3-glc did not show any effect on angiogenesis.

In conclusion, this study, in terms of anti-angiogenic and anti-coagulant properties of Dp-3-glc make this compound a potential cancer chemopreventive agent.

Together our results, from both *in vivo* studies on humans and *in vitro* cell culture models, support potential health benefits derived from the intake of fruit (coloured berries) and vegetable (broccoli).

Riassunto

Evidenze scientifiche dimostrano il ruolo protettivo frutta e verdura nei confronti delle malattie croniche, come cancro e patologie cardiovascolari. In particolare due classi di composti chemopreventivi sono stati presi in considerazione in questa tesi: **isotiocianati** (Cruciferae) ed **antocianine** (ACN) (bacche e vegetali colorati).

Le Cruciferae, specialmente il genere *Brassica* contengono un'elevata concentrazione di composti ad attività antiossidante (carotenoidi, vitamina C e folati) e glucosinolati, precursori di isotiocianati (ITCs) e indoli che modulano enzimi di biotrasformazione degli xenobiotici, come la Glutathione-S-Trasferasi (GST).

L'effetto protettivo dei broccoli (*Brassica oleracea* L. var. *italica*) è stato valutato tramite due protocolli: uno studio d'intervento con consumo regolare di una porzione di broccoli cotti a vapore al giorno per 10 giorni, e uno studio singola porzione (250g). I broccoli analizzati sono una buona fonte di composti ad attività antiossidante (carotenoidi, vitamina C) e glucosinolati come ITCs. I soggetti coinvolti nello studio erano giovani maschi fumatori reclutati sulla base di caratteristiche antropometriche, abitudini alimentari e genotipo GSTM1.

Trenta soggetti sono stati arruolati per lo studio sul **consumo regolare** (singolo cieco randomizzato cross-over), raccogliendo i campioni di sangue all'inizio e alla fine di ogni periodo di trattamento (0, 10, 30, 40 giorni). La concentrazione di carotenoidi, luteina, β -carotene e folati è stata determinata in plasma e siero. I linfociti sono stati usati per determinare biomarker di stress ossidativo: resistenza del DNA contro stress ossidativo, danno endogeno al DNA (purine ossidate), attività di riparazione del DNA ed espressione dell'mRNA di OGG1, NUDT1 e HO-1. La protezione contro danno al DNA indotto da H₂O₂ era più elevata dopo la dieta broccoli rispetto alla dieta controllo in tutto il gruppo di soggetti, con concomitante aumento delle concentrazioni di folati e luteina, e diminuzione delle purine ossidate (-22,6%). Si è visto un effetto più consistente di tutela contro i danni del DNA in individui con genotipo GSTM1 nulli (-27,6%) rispetto ai soggetti GSTM1 positivo (-13,1%) (p <0,05). L'attività di riparazione del DNA e i livelli di espressione di mRNA di OGG1, HO-1 e NUDT1 non differivano per tutto lo studio di intervento.

Dodici volontari sono stati selezionati per lo studio a **singolo pasto**, raccogliendo i campioni di sangue prima del consumo di broccoli e dopo 3, 6, 8 e 24 ore, valutando: resistenza del DNA contro stress ossidativo, biodisponibilità di folati, luteina, β -carotene e vitamina C e attività totale della GST. Il consumo del pasto di broccoli ha determinato un aumento dei valori plasmatici di folati, vitamina C e β -carotene e attività della GST, e una riduzione del danno al DNA indotto *ex-vivo* in modo maggiore nei soggetti con genotipo GSTM1 positivo.

In conclusione, questi due studi dimostrano che 10 giorni di consumo di broccoli cotti al vapore potrebbero migliorare la difesa contro i danni del DNA, ma non influenzano l'attività di riparazione del DNA in giovani fumatori sani. Inoltre, una singola porzione di broccoli al vapore è in grado di ridurre il danno al DNA e di modulare l'attività di GST. Anche se preliminari, i nostri dati suggeriscono una interazione "dieta/profilo genetico".

Delfinidina-3-glucoside (Dp-3-glc) e cianidina-3-glucoside (Cy-3-glc) sono due **ACN** in grado di inibire la proliferazione delle cellule tumorali.

Uno studio *in vitro* è stato progettato per studiare l'effetto di Dp-3-glc e Cy-3-glc sull'angiogenesi e Dp-3-glc sull'attività procoagulante di cellule endoteliali microvascolari (HMEC-1). Le HMEC-1 sono state incubate per un massimo di 24 ore con terreni di coltura \pm ACN (0,1, 1, 10, 100 μ M) da sole o in combinazione con il VEGF, o endotossina batterica (LPS). L'angiogenesi è stata valutata mediante i saggi di formazione di strutture capillaro-simili in Matrigel e "wound healing", mentre l'attività pro-coagulante è stata testata mediante il test di generazione di trombina (TG) e l'espressione del fattore tissutale (TF), come antigene, saggio di ricalcificazione e quantificazione dell'mRNA. I risultati mostrano che la Dp-3-glc ha inibito l'angiogenesi in condizioni basali e stimulate da VEGF a 100 μ M; l'inibizione dello stimolo migratorio del VEGF è iniziato alla concentrazione di 1 μ M. Nel test di TG, la Dp-3-glc ha significativamente contrastato lo stimolo pro-trombotico di LPS a partire dalla concentrazione 10 μ M su monostrato intatto di HMEC-1 e l'espressione di TF a 100 μ M. Diversamente, la Cy-3-glc non ha mostrato alcun effetto sulla angiogenesi. In conclusione, la Dp-3-glc ha dimostrato proprietà anti-angiogeniche e anti-coagulanti che rendono questo composto un potenziale agente chemiopreventivo del cancro.

Nel complesso i nostri risultati degli studi *in vivo* sull'uomo e in modelli *in vitro* in coltura cellulare sostengono benefici potenziali per la salute provenienti dal consumo di frutti (bacche colorate) e ortaggi (broccoli).

INDEX

0. PREFACE	1
0.1 References	2
1. STATE OF THE ART	3
1.1 References	31
2. AIMS OF THE STUDY	37
2.1 References	38
3. RESULTS AND DISCUSSION	40
3.1 TOPIC I: Role of broccoli intake, a glucosinolate-rich source, on biomarkers of oxidative stress in young smokers	40
3.2 Materials and methods	50
3.2.1 Characteristics and analyses of the product	50
3.2.2 Regular-intake dietary intervention study	52
3.2.3 Single-meal study	57
3.3 Results and discussion TOPIC I	59
3.3.1 Characteristics and analyses of the product	59
3.3.2 Regular-intake dietary intervention study	59
3.3.2.1 Discussion regular-intake study	62
3.3.3 Single-meal study	67
3.3.3.1 Discussion of single-meal study	69
3.4 Conclusions TOPIC I	73
3.5 References	74
3.6 TOPIC II: Effect of anthocyanins on angiogenic and pro-thrombotic properties of endothelial cells <i>in vitro</i>	84
3.7 Materials and methods	105
3.7.1 Angiogenesis study	106
3.7.2 HMEC-1 pro-coagulant properties	107
3.8 Results and discussion TOPIC II:	111
3.8.1 Angiogenesis study	111
3.8.2 HMEC-1 pro-coagulant properties	115
3.8.3 Discussion	118
3.9 Conclusions TOPIC II	121
3.10 References	122
4. GLOSSARY	130
APPENDIX 1- PUBLICATION AND TRAINING ACTIVITIES	136
APPENDIX 2- COPIES OF PAPER	138
APPENDIX 3- FOOD FREQUENCY QUESTIONNAIRES	148

0. PREFACE

Chronic diseases are one of the main causes of death world-wide. In particular, the increase of degenerative diseases, such as metabolic syndrome, cancer and cardiovascular disease, in our country seems to be related to a modification of lifestyle and dietary habits.

The rise in chronic diseases reflects a significant change in diet habits, physical activity levels, and tobacco consumption world-wide as a result of industrialization, urbanization, economic development and food market globalization. People are consuming a more energy-dense, nutrient-poor diet and are less physically active. These are no longer only diseases of the developed world: about 80% of all cardiovascular disease (CVD) deaths world-wide took place in developing, low and middle-income countries, while these countries also accounted for 86% of the global CVD disease burden. In developing countries people are being exposed to these risk factors for longer periods and a high proportion of disease takes place in people of working age. As reported by the World Health Organization (WHO, 2003) chronic conditions, including cardiovascular diseases (CVD), diabetes, obesity, cancers and respiratory diseases, account for 59% of the 57 million deaths annually and 46% of the global burden of disease. Five out of the 10 leading global disease burden risk factors identified by World Health Report 2002 - high blood pressure, high cholesterol, obesity, physical inactivity and insufficient consumption of fruits and vegetables - are strongly related to diet and physical activity. Together with alcohol and tobacco use, these preventable risks play a key role in the development of chronic diseases, which frequently involve overlapping risk factors and chronic conditions. In particular, low fruit and vegetable intake accounts for 2.7 million deaths.

There is good evidence that a change in **dietary habits**, physical activity and tobacco control can produce rapid changes in population risk factors and disease burden prevalence for these chronic diseases. Thus, the demonstration of functional effects of foods is crucial for the development of health promoting strategies.

The healthy effect of fruit and vegetable seems to be related to their content in vitamins, minerals and non-nutrient components, like fibre and bioactive compounds, called phytochemicals. This is a collective term for a variety of plant components that often perform important functions in the plant, such as providing colour, flavour, or protection, but are not essential in the human diet. All of these compounds have been shown either in humans or in laboratory experiments to have potential benefit for health wellness when they are included in diets. However, the bioavailability of these compounds is variable and their ultimate health effects uncertain.

The importance of diet and its relationship to disease prevention and maintenance of health has been the topic of numerous studies. Recently, the concept of the ability of some foods to carriers of bioactive compounds with possible effect on disease prevention, disease arrest or therapy has been under world-wide investigation.

Multidisciplinary approaches could allow demonstration of the importance of either whole diet or single food, starting from epidemiological relevance to the understanding of cellular and molecular mechanisms.

In particular, attention has been given to study and development of **functional foods**. Functional foods are any healthy food declared to have an health-promoting or disease-preventing properties beyond the basic function of supplying nutrients (i.e. fermented foods with live cultures with probiotic benefits; fruit and vegetable with effects on cardiovascular disease or anti-tumor properties, etc.).

Functional food products need to include health claims on their label describing their benefits. A nutrition claim states that a food has beneficial nutritional properties. An **health claim** is any statement on labels, advertising or other marketing products describing health benefits resulted from its consumption. Health claims must be supported by credible scientific evidence regarding a relationship between a substance (specific food or food component) and a disease or health-related condition. Both of these elements - a substance and a disease - must be present in the health claim. An example of an authorized health claim is: "Calcium may reduce the risk of osteoporosis". The European Commission has requested the European Food Safety Authority (EFSA) to provide relevant scientific advice for the setting of nutrient profiles (conditions concerning the nutrient content of foods) that foods or certain groups of foods must respect in order to bear nutrition and health claims.

The Nutrition EFSA Scientific Panel on dietetic products, nutrition and allergies (NDA) of Europe's food safety watchdog delivered in 2008 scientific advice to assist the European Commission and Member States in defining nutrient profile for foods bearing nutrition and health claims. The Panel has defined scientific criteria that could be utilised by EU policy makers in assessing which foods may carry nutrition and health claims. The Panel concluded that the main scientific consideration in establishing nutrient profiles is the potential of a food to adversely affect overall dietary balance, as defined by nutrient intake recommendations. The dietary role of different food groups must also be taken into account and the nutrient profiles should be consistent with food-based dietary guidelines established in EU Member States.

Since now, only few products obtained health claim, as most of results in literature failed in the demonstration of a cause-effect relationship between the intake of food and the benefit claimed.

Current research efforts are directed toward understanding the mechanisms and identifying both individual bioactive plant components and whole foods that may improve protection against chronic diseases.

0.1 References

World Health Organization (2003) Information sheet: Chronic disease - key risk factors include high cholesterol, high blood pressure, low fruit and vegetable intake

1. STATE OF THE ART

Vegetables and fruits intake: an overview

Nutrition science investigates the metabolic and physiological responses of the body to diet. With advances in the fields of molecular biology, biochemistry, and genetics, the study of nutrition is increasingly concerned with metabolism and metabolic pathways: the sequences of biochemical steps through which substances in living things change from one form to another.

Vegetables and fruits are generally low in energy density (with a few exceptions) and, when consumed in different assortment, are sources of many vitamins, minerals, and other bioactive compounds (phytochemicals). Many non-starchy vegetables, including salad vegetables and fruits, may be eaten raw and may also be cooked. Legumes are high in protein. Traditional diets all over the world combine cereals (grains) with pulses (legumes) and, in this way, ensure sufficient protein of adequate quality, usually with small amounts of animal foods. Nuts and seeds are concentrated sources of numerous micronutrients and of essential fatty acids. All these foods are sources of dietary fibre. Many herbs and spices have potent pharmacological as well as culinary properties. Consumption of vegetables and fruits is very variable: high around the Mediterranean littoral and some tropical countries; low in many low-income countries, including some in which fruits are abundant. Consumption of legumes is also very variable: beans and chickpeas and their products are basic foods in a number of Latin American, Middle Eastern, and Asian countries, but pulses are insignificant in typical North American and most European diets. Consumption of nuts, seeds, herbs, and spices also varies. Traditional Middle Eastern and Indian cuisines use a great variety of herbs and spices; garlic, usually classified as a herb, is consumed in remarkable quantities in some countries (WCRF/AICR 2007).

Vegetables and fruits (including berries), nuts and seeds, and herbs and spices, where they grow and can be cultivated, have always been part of human diets. Gatherer-hunters and pastoral peoples probably consumed more than relatively impoverished urban dwellers: for them, vegetables were the main sources of many vitamins, and fruits were a main source of energy, from sugar (also found in wild honey). They are consumed abundantly as part of many long-established traditional cuisines, around the Mediterranean littoral, the Middle East, in many Asian countries, and the Pacific islands, where substantial amounts of meat, dairy products, and other animal foods are traditionally consumed only occasionally. In contrast, monotonous 'poverty' diets include few of these foods. Globally, consumption of these foods is lower than now generally recommended. Vegetables and fruits are sometimes seen as relatively expensive. Well stocked supermarkets usually now display a variety of local and imported fresh vegetables and fruits, although supplies in smaller stores are more variable. Consumption of fresh vegetables and fruits in many tropical countries in Africa and Latin America is low: on average people in Brazil, for example, consume roughly the same as people in Britain. The explanation may be that in Africa, many rural communities are obliged to grow cash crops that displace gardens, and that in

Latin America knowledge of the value – and pleasure – of many indigenous vegetables and fruits has been lost. Many programmes in tropical countries are now dedicated to regaining this knowledge (Burkitt & Trowell, 1977).

Even before the discovery of vitamins as essential nutrients beginning in the early 20th century, vegetables and fruits have been recommended as ‘protective foods’. Early reports concerned with nutritional deficiencies paid less attention to pulses (legumes), nuts, and seeds, even though these plant foods contain protein, and nuts and seeds are nutrient- and also energy-dense, perhaps because they are not much consumed in the countries where most such reports were compiled. Instead, as already mentioned, priority was given to energy- and nutrient-dense foods of animal origin. By the 1980s, most reports concerned with prevention of chronic diseases recommended relatively high intakes of vegetables and fruits and sometimes also legumes, either because these foods were seen as nourishing substitutes for energy-dense fatty or sugary foods, or else because they were identified as positively protective against cardiovascular disease (Trowell & Burkitt, 1986). Evidence that vegetables and fruits might be protective against some cancers emerged in the 1990s (WHO 2003).

A common recommendation has been for at least five portions (or at least 400 g) of vegetables and fruits a day (WHO 1990).

Nutritional characteristics of vegetables and fruits

There are six major classes of nutrients: carbohydrates, fats, minerals, protein, vitamins, and water. These nutrient classes can be categorized as either macronutrients (needed in relatively large amounts) or micronutrients (needed in smaller quantities). The macronutrients include carbohydrates, fats, protein, and water. The micronutrients are minerals and vitamins.

The macronutrients (excluding water) provide structural material (amino acids from which proteins are built, and lipids from which cell membranes and some signaling molecules are built), and energy. Vitamins, minerals, fiber, and water do not provide energy, but are required for other reasons. A third class of dietary material, fiber (i.e., non-digestible material such as cellulose), is also required, for both mechanical and biochemical reasons, although the exact reasons remain unclear.

Other micronutrients include bioactive compounds, which are said to influence (or protect) some body systems. In particular plants contain a wide range of biologically active compounds, some of which are known as phytochemicals. There may be as many as 100,000 different compounds, which determine particular properties in plants, and in the fruits and vegetables they produce, such as flavour and colour. Phytochemicals are classified according to their chemical structure and functional characteristics, and include salicylates, phytosterols, saponins, glucosinolates, polyphenols, protease inhibitors, monoterpenes, phytoestrogens, sulphides, terpenes, and lectins.

It is widely believed that the health benefits of diets high in fruits and vegetables are likely to be due partly to the presence of phytochemicals. For instance, several act as antioxidants, preventing oxidative damage to cells, proteins, and DNA. It is likely that

other bioactive compounds have yet to be identified, and those that are known may have additional properties in the body that are not yet understood. But it is thought that nutrients, phytochemicals, and other, as yet unknown, bioactive components act together to influence physiological responses. Although many of these substances are bioactive, they are not essential in the diet and there is no daily requirement, so they are not classed as nutrients. Humans have developed tastes for some phytochemicals, such as the hot flavours of mustard oil, bitter alkaloids, and irritating capsaicins. There is genetically inherited variation in sensitivity to some tastes, for example, the bitter taste of isothiocyanates in cruciferous vegetables such as cabbage.

The composition of fruits and vegetables depends both on species and on subtype, as well as on the environmental, farming, production, and storage conditions. These include factors such as sun exposure, soil quality, agricultural practices, harvesting time, ripeness, length of time between harvest and consumption, and preservation and preparation methods.

Functions of different classes of bioactive compounds and their food sources

Vegetables, fruits, legumes, nuts, and seeds are sources of a wide variety of micronutrients and other bioactive compounds. Foods containing several of these constituents have been identified in the systematic literature reviews as being inversely associated with cancer risk. These are: carotenoids (including β -carotene and lycopene), folate, vitamin C, vitamin D, vitamin E, polyphenols (e.g. quercetin), pyridoxine, and selenium, explained below. However, it is not possible to ascribe the association between these foods and lower cancer risk to a causal effect of specific compounds with confidence, as each food contains a complex mixture of different constituents, all of which might also contribute to any observed effect.

Carotenoids are found in varying concentrations in all vegetables, particularly those that are red or orange. They are a family of more than 600 fat-soluble red/orange pigments that comprise xanthophylls (such as lutein) and carotenes (such as α - and β -carotene, and lycopene). Some carotenoids, most importantly β -carotene, can be converted by the body to retinol and are sometimes called pro-vitamin A carotenoids. These compounds tend to be the main dietary source of vitamin A in low-income countries. Only about half of the 50 or so carotenoids in human diets can be absorbed. They have antioxidant and other bioactivities. Sources of carotenoids include spinach, kale, butternut squash, pumpkin, red (bell) peppers, carrots, tomatoes, cantaloupe melon, and sweet potatoes. **β -carotene** is found in yellow, orange, and green fruits and green, leafy vegetables including carrots, spinach, lettuce, tomatoes, sweet potatoes, broccoli, cantaloupe melon, oranges, and winter squash (pumpkin). As a rule of thumb, the greater the intensity of the colour of the fruit or vegetable the more β -carotene it contains. The most concentrated source of **lycopene** is tomatoes, but it is also present in watermelon, red (bell) peppers, pink or red grapefruit, pink-fleshed guava, and persimmons (kaki).

The B-vitamin **folate** is a family of compounds essential for human health. Folic acid, the synthetic form, is used to fortify manufactured cereal products, spreads, and, in

some countries, flour or grains. Folates are involved in a number of metabolic pathways, especially in the synthesis of purines and pyrimidines, which are important for DNA synthesis and cell replication. Sources of dietary folate include liver, beans, spinach, broccoli, romaine lettuce, chicory, oranges, and papaya.

Vitamin C (ascorbic acid) is a water-soluble vitamin. Humans, like a small number of other animals, cannot synthesise vitamin C, so it is an essential part of diets. Vitamin C is essential for collagen synthesis and also has antioxidant activity. Severe deficiency causes scurvy. It is added to many foods, including bread and soft drinks, in small amounts as an antioxidant preservative. Natural dietary sources are vegetables, tubers, and fruits, including red/yellow (bell) peppers, kiwi fruits, broccoli, papaya, citrus fruits, strawberries, and potatoes, but it is destroyed by heat or contact with the air (for instance, when vegetables are chopped), or lost into cooking water.

Vitamin E is a fat-soluble vitamin and a potent antioxidant that occurs as eight different forms: α - and γ -tocopherol are the most common. The most important dietary sources of vitamin E are vegetable oils such as palm, sunflower, corn, soya bean, and olive oils. Nuts, sunflower seeds, and wheat-germ are also sources. Whole-grains, fish, peanut butter, green, leafy vegetables, and fortified breakfast cereals also contain this vitamin.

Pyridoxine is one of a group of water-soluble compounds collectively known as vitamin B6. This vitamin is involved in neurotransmitter synthesis, red blood cell formation and function, niacin (vitamin B3) formation, steroid hormone function, and nucleic acid synthesis (Leklem, 1999). Food sources include bananas, fish, poultry, liver, potatoes eaten with the skin, green, leafy vegetables, beans, legumes, nuts, wholegrains, and fortified breakfast cereals .

Selenium is a mineral element that occurs in different chemical forms. It is toxic in large amounts, but is essential in the diet at trace levels. It is present at varying concentrations in different soils; and since plants take up selenium from the soil, these levels determine the amount present in vegetables. Thus selenium deficiency is more prevalent in regions where the soil selenium content is low. Selenium is a component of the amino acids selenocysteine and selenomethionine, which are integrated into proteins to form selenoproteins. Selenoproteins include antioxidant enzymes such as glutathione peroxidases, thioredoxin reductase, which is important for DNA synthesis, and iodothyronine deiodinase, which is important for the synthesis of thyroid hormones (Geissler & Powers, 2005). Dietary sources of selenium include brazil nuts, fish, wholegrains, wheatgerm, and sunflower seeds.

The **polyphenols** is a group of chemical substances found in plants, characterized by the presence of more than one phenol unit or building block per molecule, they are not essential dietary components. Polyphenols are generally divided into hydrolyzable tannins (gallic acid esters of glucose and other sugars) and phenylpropanoids, such as lignins, flavonoids, and condensed tannins. They are derived from secondary plant metabolism of the shikimate pathway, where they exert different functions. They possess antioxidant activity and are widely studied for the mechanisms involved in their health benefit effects. One of the most studied is quercetin, a flavonoid. Many

studies in cultured cells and animals suggest that quercetin has antioxidant activity, which could give rise to a range of biological activities, including reducing inflammation. Quercetin is found in apples, green and black tea, onions, raspberries, red wine, red grapes, citrus fruits, leafy, green vegetables, cherries, elderberries, broccoli, blueberries, cranberries, and bilberries.

Different levels of scientific evidence

Three different and consequent levels of scientific evidence occur to demonstrate the (protective) effect of a food on human health. Through epidemiological evidence we can study the relationship between the consumption of a certain food and its protective effect; by means of experimental study it is possible to investigate molecular and cellular mechanisms and bioavailability of most interesting compounds by *in vitro* and *in vivo* models. Finally intervention trials on humans will be necessary to confirm the relationship between the intake of foods and their protective effect and to help in the study of the mechanisms involved.

A brief explanation of each one of these steps is given below.

1. Epidemiological evidence: relationship food intake-protective effect.

Epidemiological research describes and seeks to explain the distribution of health and disease within human populations. The methods used are based mainly on comparative observations made at the level of whole populations, special groups (such as migrants), or individuals within populations. This type of investigation is known as **observational**. By relating differences in circumstances and behavior to differences in the incidence of disease, associations are identified that may be causal. In epidemiological studies, an 'exposure' is a factor or condition that may increase or decrease the risk of disease.

Descriptive studies give information about statistics on disease incidence or mortality. Descriptive epidemiology informs cancer surveillance programmes, and is a basic tool for determining patterns of cancer, relative rates of cancer and other diseases, and changes in patterns and trends over time. Remarkable changes in the incidence of cancers provide first lines of evidence pointing to causation due to corresponding changes in environmental circumstances. Like all types of study, descriptive epidemiology has limitations. Apparent trends in cancer incidence and mortality may be due in part to changes and developments in screening, diagnosis, or treatment.

Ecological studies are designed to explore relationships between environmental factors and disease amongst populations rather than individuals. While ecological studies, like other observational studies, may suggest a relationship between a specific environmental factor (such as an aspect of food and nutrition) and disease, the actual causal relationship may be with a different 'confounding' factor, which may or may not be associated with the environmental factor being investigated. Ecological studies are often used to identify associations or trends that warrant further investigation. They have special strengths, particularly when conducted between populations, either internationally, or cross-culturally among different populations within a country.

Thus, the contrast in dietary intake between countries is often much larger than the contrast within countries. In addition, average national diets are likely to be more stable over time than the diets of communities, families, or individual people. For most countries, the changes in overall national dietary intakes over a decade or two are relatively small. **Migrant studies** compare cancer rates for migrants, and for their offspring, in their current country of residence, with rates in their country of origin. (Last, 2001). These studies show that populations migrating between areas with different cancer incidence rates acquire the rates characteristic of their new location for some cancers, often after only one or two generations. This shows that environmental, rather than inherited, factors are primarily responsible for the large differences in cancer rates in different regions and countries. Those diseases for which incidence shifts with migration, such as cancer, are diseases with evidently important environmental causes.

In **case-control studies**, individuals diagnosed with a specific type of cancer ('cases') are compared with otherwise similar individuals who have not been diagnosed with cancer ('controls'). The control group is a sample of the population from which the cases arose, and provides an estimate of how the exposures being studied are distributed in that population. Identifying and enrolling appropriate controls is a major challenge in case-control studies.

In prospective **cohort studies** (usually simply called cohort studies), the diets, body compositions, and/or physical activity levels of a large group (cohort) of people who are assumed to be healthy are assessed, and the group is followed over a period of time. During the follow-up period, some members of the cohort will develop and be diagnosed with cancer, while others will not, and comparisons are then made between these two groups. Because measurements are made before any cancer diagnosis, cohort studies are not subject to recall bias. A single cohort study allows examination of the effects of diet and physical activity on multiple types of cancer and other diseases. Also, in cohort studies, blood and tissue samples are often collected and stored for future analysis. Finally, cohort studies provide the opportunity to obtain repeated assessments of participants' diets at regular intervals, which may improve the dietary assessment. Cohort studies may need to be very large (up to tens or even hundreds of thousands of participants) to have sufficient statistical power to identify factors that may increase cancer risk by as little as 20 or 30 per cent. Also, meaningful comparisons between cases and non-cases can be made only for factors that vary sufficiently within the cohort. Cohort studies are expensive, so they have been conducted mostly in high-income countries.

Meta-analysis is a method used to combine the results of several studies addressing similar questions. Unless an epidemiological study is sufficiently large, modest but potentially important associations can be missed, simply because of the inadequate statistical power of the individual study. Meta-analysis is used to provide summaries of selected collections of studies. Study-level meta-analysis provides single estimates of effect using information from multiple studies of the same design. These summary

estimates can provide evidence regarding the presence or absence of an association, as well as examining possible dose-response relationships (WCRE/AICR 2007).

Epidemiological evidence and relationship cancer-lifestyle

The first evidence suggesting that cancer is a largely preventable disease has come from studies noting variations in cancer incidence across time and place. The most impressive initial evidence showing that patterns of cancer are altered by environmental factors, and are not mainly genetically determined, comes from studies describing changes in the rates of different cancers in genetically identical populations that migrate from their native countries to other countries. Such studies consistently show that changes in the rates of some of the most common cancers, including those of the stomach, colorectum, breast, and prostate, can be remarkable, even over one or two generations. Patterns of food and drink, of physical activity, and of body composition have changed remarkably throughout human history. With industrialization and urbanisation, food supplies usually become more secure, and more food is available for consumption. In general, diets become more energy dense, containing fewer starchy foods, more fats and oils, sugars, and additives, and often more alcoholic drinks. At the same time, patterns of physical activity change: populations become increasingly sedentary, their need for energy from food drops, and rates of overweight and obesity increase. These changes correlate with changes in the patterns of cancer throughout the world. Middle and low-income regions and countries within Africa, Asia, and Latin America have generally experienced comparatively high rates of cancers of the upper aerodigestive tract (of the mouth, pharynx, larynx, nasopharynx, and oesophagus), and of the stomach, liver (primary), and cervix. Rates of some cancers, especially stomach cancer, are now generally decreasing. In contrast, high-income countries, and urbanized and industrialised areas of middle- and low-income regions and countries, have higher rates of colorectal cancer and of hormone-related cancers (of the breast, ovary, endometrium, and prostate). Lung cancer is now the most common type in the world because of the increase in tobacco smoking and exposure to environmental tobacco smoke. Rates of these cancers, some of which may have been historically rare, are increasing. Globally, the number of people with cancer is projected to double by the year 2030, with most of this increase likely to occur in middle- and low-income countries. Such an increase would only partly be accounted for by the projected rise in the size and average age of the global population. This makes the task of cancer prevention all the more urgent and important.

2. Experimental evidence: studies to investigate mechanisms of action

Epidemiological studies all have strengths and limitations. So do laboratory and mechanistic studies; their main strength is control. The environment of these research studies is defined by chosen experimental conditions: precise manipulations can be made and relatively exact measures taken. Occasionally the test participant is a human volunteer, but usually these studies are conducted in animals (*in vivo*) or using human or animal cells grown in the laboratory (*in vitro*). Rodents (usually rats or mice) are the

most commonly used animals in laboratory experiments. Their relatively short lifespan provides comparatively fast results in cancer studies, and they offer a 'whole body system' suited to a wide variety of tests. Rodent studies can show how nutrients and other compounds might affect the cancer process. But it is known that some interventions that affect rodents do not affect humans, or do not affect them in the same ways or to the same degrees, and vice versa. Also, experiments on animals may be highly artificial, using special breeds of rodents initially given massive doses of carcinogenic agents, and then fed nutrients or other substances at levels far higher than humans would normally consume, or could ethically be given. Human or animal cells, sometimes derived from particular cancers, can be grown *in vitro* in the laboratory and used in experiments to help researchers understand mechanisms that may lead to the development of cancer. *In vitro* studies are conducted using cells or other test systems. Human cells, animal cells, mechanistic test systems, and bacterial systems can be used. Cell cultures can be primary, where tissue (such as a tumor biopsy) is taken directly from humans or animals and then cultured; or secondary, where the original cells are cultured a number of times. Such cell lines are commonly used in laboratory research, and can become immortal – cultured again and again. The cells or tissues are subjected to potential carcinogens, and then markers of damage are measured. Conducting studies *in vitro* has two main advantages. First, specific, well defined interventions can be tested; and second, intracellular mechanisms can be examined. However, these studies do not allow the study of integrated systems, such as how organs or the whole body responds to the interventions. Therefore extrapolation of results to humans is limited.

When we consume a food or drink, the nutrients contained are released from the matrix, absorbed into the bloodstream and transported to their respective target tissues (EUFIC, 2010). However, not all nutrients can be utilized to the same extent. In other words, they differ in their **bioavailability**. Several definitions exist for nutrient bioavailability, but broadly it refers to the proportion of a nutrient that is absorbed from the diet and used for normal body functions (Aggett, 2010). The following components describe the different steps of the metabolic pathway where changes in nutrient bioavailability may occur (Aggett, 2010):

- release of the nutrient from the physicochemical dietary matrix;
- effects of digestive enzymes in the intestine;
- binding and uptake by the intestinal mucosa;
- transfer across the gut wall (passing through the cells, in-between them or both) to the blood or lymphatic circulation;
- systemic distribution;
- systemic deposition (stores);
- metabolic and functional use;
- excretion (via urine or faeces).

As it is evident from this list, the bioavailability of a nutrient is governed by external and internal factors. The bioavailability of macronutrients – carbohydrates, proteins, fats – is usually very high at more than 90% of the amount ingested. On the other

hand, micronutrients, i.e. vitamins and minerals, and bioactive phytochemicals can vary widely in the extent they are absorbed and utilised. The first step in making a nutrient bioavailable is to liberate it from the food matrix and turn it into a chemical form that can bind to and enter the gut cells or pass between them. Collectively this is referred to as **bioaccessibility** (Holst & Williamson, 2008). Nutrients are rendered bioaccessible by the processes of chewing (mastication) and initial enzymatic digestion of the food in the mouth, mixing with acid and further enzymes in the gastric juice upon swallowing, and finally release into the small intestine, the major site of nutrient absorption. Here, yet more enzymes, supplied by the pancreatic juice, continue breaking down the food matrix. In addition to the bodily means of mastication and enzyme action, the digestibility of food matrices, especially of plant foods, is aided by cooking or pureeing the food. Minerals and other nutrients exist in different chemical forms in the food and this can influence their bioavailability. Nutrients can interact with one another or with other dietary components at the site of absorption, resulting in either a change in bioavailability or – if enhancers and inhibitors cancel each other out – a nil effect. Enhancers can act in different ways such as keeping a nutrient soluble or protecting it from interaction with inhibitors. For example, since carotenoids are fat-soluble, adding small quantities of fat or oil to the meal (3-5 g per meal) improves their bioavailability. Inhibitors may reduce nutrient bioavailability by: binding the nutrient in question in a form that is not recognized by the uptake systems on the surface of intestinal cells, rendering the nutrient insoluble and thus unavailable for absorption, or competing for the same uptake system. Systemic factors include deficiency of a certain nutrient or changes in physiologic state, e.g. pregnancy. In both cases, the body may respond by increasing the respective nutrient absorptive pathway or utilisation to meet the increased demand. Internal or host-related factors can be subdivided into gastrointestinal and systemic factors. Thus, the study of bioavailability of whole food or phytochemicals needs a scientific approach. Bioavailability studies can be assessed both on human and animals models. Compounds of interest or their metabolite are monitored during a certain time in blood, urine or faeces and target tissues (in particular for animal models), in order to obtain a quantification over the time of the presence of the constituent of interest within the body. Parameters of kinetics of a compound are: C_{max} (the maximal concentration achieved in blood); $T_{1/2}$ (halftime for reaching the half of C_{max} in blood); AUC (area under the curve) in a curve concentration (y assis) and time (x assis) (Figure 1.1).

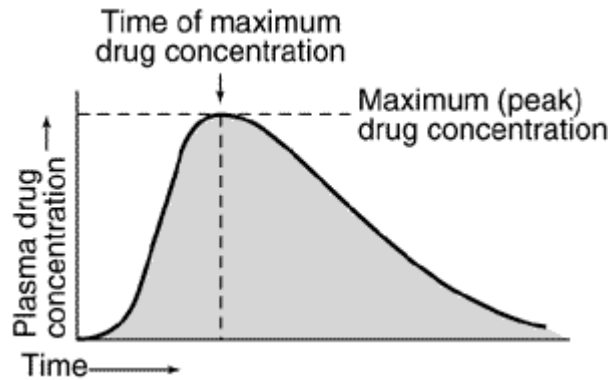


Figure 1.1 Bioavailability curve.

3. Human intervention studies: the most important *in vivo* evidence

A randomized controlled trial (RCT) is an experiment of design in which participants are randomly assigned to groups, often called intervention and control groups, to receive or not receive an experimental intervention. The main use of RCTs has generally been to test the efficacy of drugs and other medical treatments. In a 'double blind' RCT, neither the participants nor the investigators know to which group (intervention or control) the participant has been assigned. Blinding is used because the knowledge of group assignment might influence study results, but it is usually impossible to achieve with trials involving physical activity, or those investigating foods and drinks in their usual form. An effective use of RCTs is to test the effects of supplementation with specified doses of dietary micronutrients (as pills or by other means). However, pharmacological doses of supplements are often studied – doses much higher than can be derived from diets – and results may not be directly relevant to dietary intakes of that micronutrient. Such trials may yield powerful evidence of the effect of a specific dietary constituent. However, they are often conducted as a result of promising epidemiological studies that have shown protective effects of a particular group of foods, and there is always a possibility that the actual active agent or combination of agents in the foods has not been used in the trial. Dietary constituents that are or may be protective when contained within foods may have unexpected effects in isolation, especially at doses higher than those found in normal diets. RCTs are also used to test interventions designed to change behavior, including dietary intakes and physical activity. Such trials require a high level of commitment by participants, and learning how to conduct them well is a topic of active investigation. A unique and important strength of sufficiently large RCTs is that confounding variables, both known and unknown, will on average be distributed equally between the treatment and control groups, and will therefore not bias the study results.

The research approaches in the evaluation of the protective role of diet

Many scientists are studying dietary cancer prevention, yet often data are missing on how to translate much of the research generated into clear guidelines for the consumer.

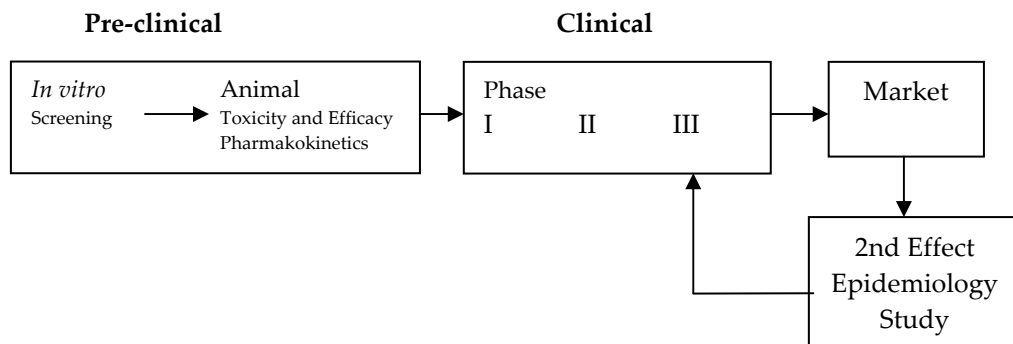
For example, “broccoli may decrease risk for prostate cancer” sounds clear, but leaves both the consumer and the clinician interested in designing a robust clinical trial unsure of dose, frequency of inclusion into the diet, or whether variety or preparation method is important for gaining the health benefit, or even if a sulforaphane (SF) supplement could replace whole broccoli. As a result of this lack of information on foods that have health benefits, neither the clinician nor the consumer has any knowledge of an effective dose. This is of particular concern when considering cancer prevention, since there is no easy, short-term endpoint/health outcome in order to judge effectiveness, such as plasma cholesterol levels for cardiovascular health. Clinical trials carried out prior to filling these knowledge gaps may not be optimized for these parameters and may provide confusing, disappointing, and maybe even harmful results. Substantial data gaps must be filled, to provide the detailed, evidence-based information necessary for the optimized design of these clinical trials: epidemiological data alone cannot provide the information necessary to design a robust clinical trial; *in vitro* data, even based on epidemiological studies, cannot provide the necessary detail or justification for designing a robust clinical trial; animal modeling of efficacy, bioavailability, and kinetics are essential for designing a robust clinical trial (Jeffery & Keck, 2008).

Whereas epidemiological studies are an excellent source of material for hypothesis generation, basing marketing, or lifestyle guidelines on epidemiological data alone may not always prove useful. A recent report even suggested that epidemiological studies are considerably less than 50% reproducible (Tuma, 2007). This low reproducibility might be due to our lack of knowledge of different aspects of the food under study, such as changes in the content of bioactive components with plant variety or cooking method. Mechanistic evidence for bioactivity is often derived from *in vitro* studies of purified components isolated from foods, and has gone far to persuade scientists of the potential benefit of plant foods, even though cell culture studies do not address disposition. The effects of bioactive components on cellular physiology may change with both dose and cell type. It is well known that all compounds are toxic and that a safe and tolerable upper level needs to be determined, particularly when bioactive components are isolated from whole foods and provided as dietary supplements. Potential interactions with drugs, dietary supplements and other bioactive food components, positive or negative, may also be of concern. Many foods contain more than one bioactive component, and yet scientists have a tendency to identify a major bioactive and then equate effects of that individual component with the effect of the whole food. Cell culture studies can be very informative about mechanism, but it is not enough to know that a component has bioactivity in cell culture. Cell culture studies may use doses that cannot be achieved physiologically, and they cannot provide information on bioavailability. Additionally, they may miss interactions with additional components in the whole food. For these reasons, *in vitro* studies do not extrapolate directly to dietary effects. The greatest gap in our understanding of health effects of bioactive food components may be details on disposition: bioavailability from different products, distribution, and metabolism, the

effective dose and the tolerable upper level. Animal studies can provide information on many of these questions to permit moving forward to small clinical studies. Animal studies can compare the purified component(s) used in cell culture with the complex foods in our diet, confirming (or refuting) the mechanisms identified in cell culture. Although animal studies do not always reflect *in vitro* findings, it is imperative to compare similar doses, forms, and extent of exposure before rejecting *in vitro* findings. Animal studies can highlight or dismiss concerns over bioavailability, efficacy, and kinetics. Frequently only a fraction of a dietary dose is absorbed. Animal studies can provide information about digestion, kinetics, and metabolism that may, for example, suggest specific processing methods to optimize bioavailability. Once a metabolic pathway is identified in animals, detecting and confirming it in a small clinical study can be relatively straightforward. Once efficacy has been established in animal models, translation to humans requires measurement of exposure and disposition, as well as measurement of efficacy. Whereas animal studies do not always appear to translate successfully to clinical findings, this is often because of differences in dose, exposure route, duration and frequency of exposure, or genetic diversity within the human population under study (Jeffery & Keck, 2008).

Availability of robust biomarkers of efficacy in healthy individuals is still extremely sparse. Whereas multiple biomarkers for effective maintenance of health have been developed in association with cardiac health, there remains a lack of biomarkers for determining successful prevention of cancers. As these are identified, it will be necessary to determine how new biomarkers can best be utilized to help the general public choose a diet that prevents cancer. Methods for the evaluation of safety and efficacy of drugs undergoing development typically start with *in vitro* screening assays, and all steps are well established, as outlined in Figure 1.2-A. For an optimal scientific approach to study the health benefits of foods it is essential to conduct detailed preclinical studies and small human studies before taking the step to fully randomized, double blind placebo-controlled human trials (Figure 1.2-B).

A: Drug development



B: An optimal scientific approach to the study of foods with health benefits

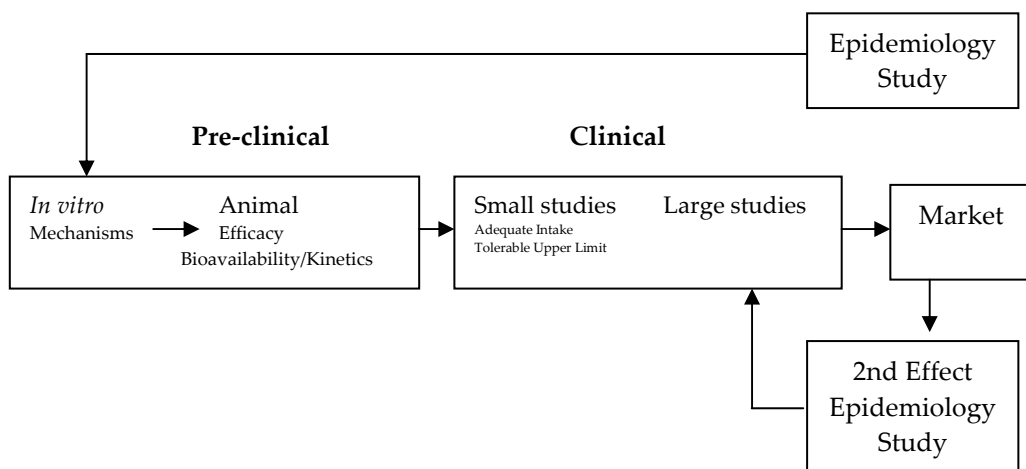


Figure 1.2 *A: A drug development; B: An optimal scientific approach to the study of foods with health benefits. A solid line represents common practice and dotted line represents less frequent occurrence (Jeffery & Keck, 2008).*

Biomarkers

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of normal and disease processes or pharmacological responses (Biomarkers Definitions Working Group (2001)).

A biomarker has been described as “a biological molecule that can be modified by an environmental or endogenous factor; the variation in the molecule can be quantified”. In case of oxidative processes that often occur in the organism as the result of a high production of reactive species (ROS and RNS), a biomarker can be considered as “a biological molecule that has arisen from attack by reactive oxygen, nitrogen or halide

species." Reactive species originate from a range of cellular processes, external factors and/or disease states (Ferguson *et al.*, 2006). Reactive species can cause damage to lipids, proteins and DNA. Therefore, biomarkers used in intervention studies regard lipids, proteins and DNA, as well as plasma antioxidant status as several molecules (such as phytochemicals) could act as antioxidants counteracting oxidant species.

The cancer process

Carcinogenesis is characterized by a complex process that involves a series of individual steps. Tumor development has been generally considered to consist in three distinct steps: initiation, promotion and progression (Figure 1.3).

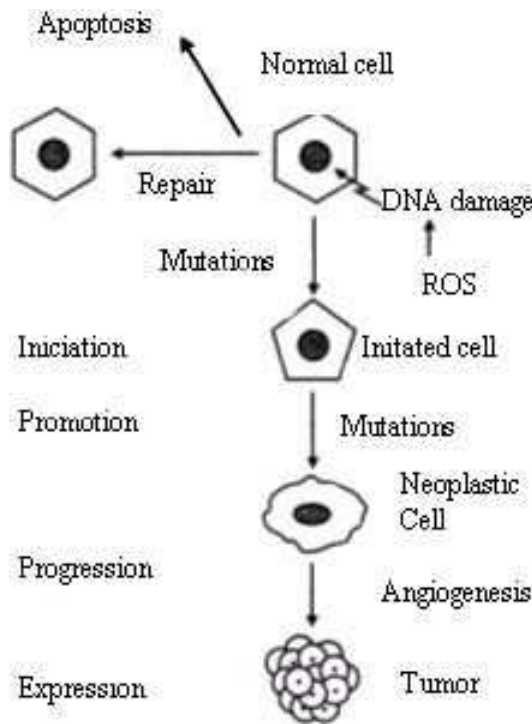


Figure 1.3 *Carcinogenic process. Multistage process, which simplified, comprises initiation (attack by ROS, carcinogen), accumulation of carcinogenic mutations, progresses through preneoplastic stages by the acquisition of more mutations, promotion by a tumor promoter, progression and development of angiogenic potential leading to expression of tumor (Trueba et al., 2004)*

Initiation is an irreversible event that begins when cells in normal tissues are exposed to a carcinogen and their genomic DNA undergoes damage that remains unrepaired or misrepaired. In case of chemically-induced carcinogenesis, initiation involves uptake of a given carcinogenic agent with subsequent distribution and transport to organs and tissues where metabolism occurs, the interaction of a reactive metabolite with cellular DNA with subsequent structural alterations in the DNA molecule, and final fixation of

the genotoxic damage to cause mutation. The resulting somatic mutation in a damaged cell can be reproduced during mitosis, which gives rise to a clone of mutated cells. **Promotion** is the expansion of the damaged cells to form an actively proliferating multi-cellular premalignant tumor cell population. **Progression** is the irreversible process which produces a new clone of tumor cells with increased proliferative capacity, invasiveness and metastatic potential (Fimognari *et al.*, 2008).

Food and nutrition modify the risk of cancers at a large number of sites. This means that some foods and drinks, dietary constituents (or their balance in diets), and methods of food production, processing, preservation, and preparation influence the development of some cancers. More recently, evidence has accumulated about the effects of physical activity and body composition on the risk of a number of cancers, suggesting that bioenergetics is another factor determining cancer risk and tumor behavior (WCRF/AICR 2007). Since the mid-1990s, great progress has been made in understanding the cancer process, and which internal and external factors modify cancer risk. Mapping of the human genome has enabled the establishment and development of new disciplines devoted to understanding biological processes at the most basic level, including those that prevent cancer, those that cause cancer, and those that modify its behavior. Evolution in living organisms depends on the accumulation of adaptations as a result of changes in the expression of the genetic information carried in DNA. Even with no changes in the DNA, alterations in how the message in the genetic code is translated can lead to functional changes. More importantly, the DNA itself is susceptible to mutation — changes in the genetic code itself — as a result of damage from external causes such as radiation or simply due to the process of metabolism. Such mutations are the essential basis for human evolution, by producing adaptations that are beneficial in particular environmental circumstances. At the same time, some mutations can contribute to the harmful changes in cells that eventually lead to cancer. The integrity of the genetic information is protected by many systems that prevent DNA damage, or remove or repair damaged DNA if it occurs. Imperfections in these systems limit the ability to block all damage and allow both helpful and harmful mutations to occur. Cancers result when sufficient mutations have accumulated, most presenting at an age that was rarely reached in the evolutionary past of human beings. The development of cancer may be seen as a corollary of the ability of humans to evolve and adapt. Ultimately it is both the genetic information (genotype) and its expression that control the characteristics (or phenotype) of an individual. Any exposure during the life course that affects the genotype or its expression may also have an effect on the phenotype. At any point in time, the phenotype is related not only to the genotype but also to a host of environmental factors, including nutritional exposures. This accumulated metabolic experience may begin during maternal and early life, and proceed throughout a person's lifetime (WCRF/AICR 2007).

In the introduction of each topic of this PhD thesis detailed information will be given regard the mechanisms studied in the different research models.

Cancer

Cancer is a group of more than 100 diseases characterized by uncontrolled cellular growth as a result of changes in the genetic information of cells. Cells and tissues are complex systems with critical stages and checkpoints to ensure normal growth, development, and function. Normally the division, differentiation, and death of cells are carefully regulated. All cancers start as a single cell that has lost control of its normal growth and replication processes. Human adults are made up of around 10^{13} (or 10 000 000 000 000) cells, which are renewed and replaced constantly. About 5–10 per cent of cancers result directly from inheriting genes associated with cancer, but the majority involve alterations or damage accumulated over time to the genetic material within cells. The causes of damage are both endogenous (internal) and exogenous (environmental). Food, nutrition, and physical activity are important environmental factors in the development of cancer. Each type of cancer has different characteristics, but one feature of all these diseases is unregulated cell growth and/or cell death. Apart from haematological cancers such as leukaemias, this results in a tumour or mass, and cancerous cells often invade the surrounding tissue. Spread of cancer cells from the primary site to other parts of the body is called metastasis. Benign tumours do not invade or metastasise. Malignant tumours do not remain localised but can invade and/or metastasise.

Genetic material

The genetic material of mammalian cells is composed of double-stranded DNA made from four organic bases – cytosine, guanine, adenine, and thymine – within a helical spine comprising deoxyribose (a sugar) and phosphate. The combination of a base with phosphate and deoxyribose is called a nucleotide. Humans have 3 billion base pairs in the DNA code that encode approximately 30 000 different genes. The nucleus of a cell contains DNA, and the information in the code is ‘read’ to generate proteins in the cytoplasm of the cell. This is achieved by transcribing the DNA into RNA, and then translating the information in RNA to synthesise protein. For transcription, the two DNA strands separate and an intermediary, complementary copy of the DNA is made from mRNA (which differs slightly in structure from DNA and is single stranded). For translation, the RNA leaves the nucleus and binds to an organelle in the cytoplasm called the ribosome. The RNA nucleotides encode for 21 different amino acids, with the ribosome moving along the RNA molecule and translating the genetic code into a sequence of amino acids that build into a protein. The normal metabolic processes in cells are controlled by proteins, each of which is a product of a single gene from the DNA in the nucleus. Although each cell in the body contains exactly the same genes, cells from different organs have different structures and functions because there is a process of regulation that determines which genes are expressed; that is, which genes are turned on and which are not. This differential gene expression varies not only from tissue to tissue but also from time to time over the course of a person’s life, from embryonic and fetal stages onwards.

Gene expression is regulated by promoter regions of genes in the DNA, as well as by epigenetic factors – those that alter gene expression without changing the nucleotide sequence. The availability of nutrients within the immediate environment influences these processes (Figure 1.4).

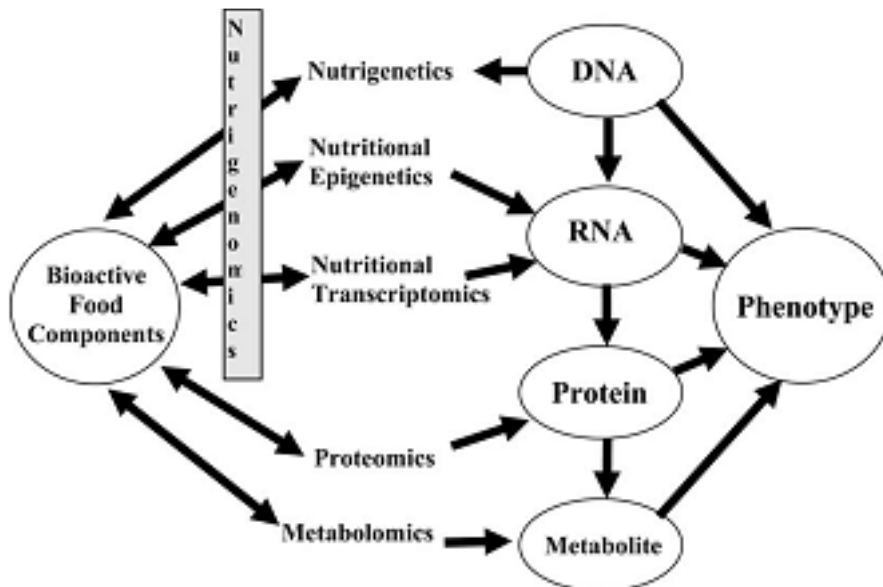


Figure 1.4 *The basis for the study of food, nutrition, bioactive food components and the cancer process. The genetic message in the DNA code is translated into RNA, and then into protein synthesis, and so determines the metabolic process. Research methods called “-omics” address these different stages.*

An integrated framework that simultaneously examines genetics and associated polymorphisms with diet-related diseases (nutrigenetics), nutrient induced changes in DNA methylation and chromatin alterations (nutritional epigenomics), nutrient induced changes in gene expression (nutritional transcriptomics), and altered formation and/or bioactivation of proteins (proteomics) will allow for a greater understanding of the interrelationships between diet and cancer risk and tumor behavior. Since the response to a bioactive food component may be subtle, careful attention will need to be given to characterizing how the quantity and timing of exposure influence small molecular weight cellular constituents (metabolomics).

Nutrigenomics and cancer

Unraveling links between diet and cancer is complex, as thousands of dietary components are consumed each day; a typical diet may provide more than 25000 bioactive food constituents (Craig, 1997). Assessing intakes of some constituents is difficult due to wide variations in the amounts of bioactive components within a particular food (McNaughton & Marks, 2003; Finley, 2006). Dietary constituents modify a multitude of processes in both normal and cancer cells (Milner, 2004; Finley, 2005). The response is further complicated since a single, bioactive food constituent can

modify multiple steps in the cancer process. Likewise, many of these processes can be influenced by several food components. Normal and cancer cells also differ in their responses to bioactive food components in terms of the dose (quantity), timing, and duration of exposure required to bring about effects. To unravel the contribution of nutrition to cancer, the biological processes underpinning cancer development need to be understood. Extensive evidence exists for nutritional factors in several processes related to cancer development (Figure 1.5).

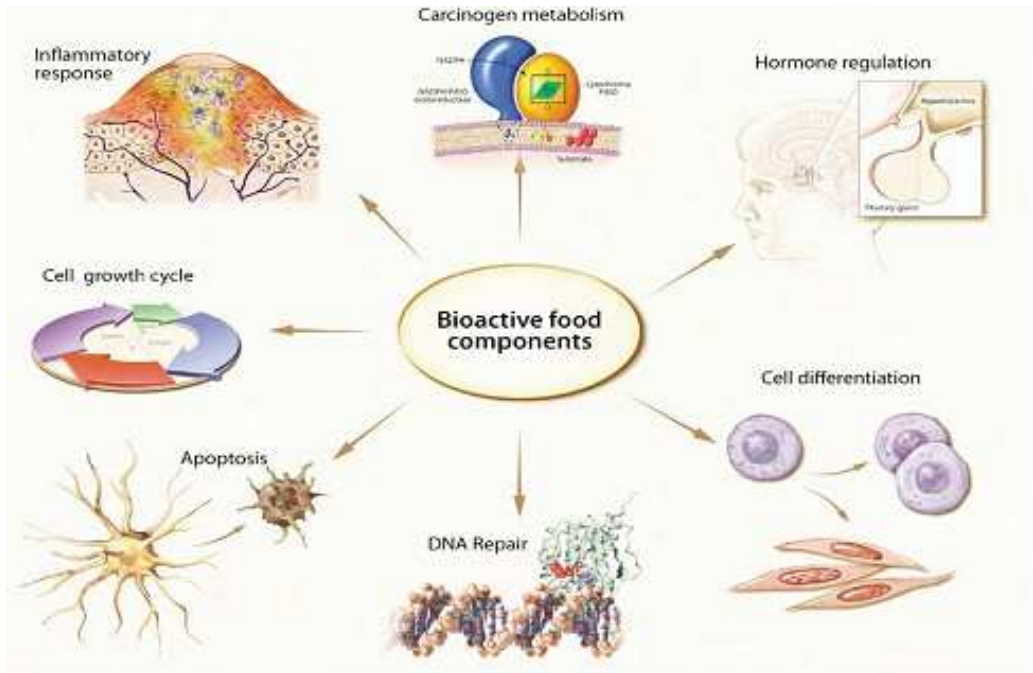


Figure 1.5 *Bioactive food components may modify simultaneously more than one cancer process including such diverse events as carcinogen metabolism, hormonal balance, cell signalling, cell-cycle control, apoptosis, and angiogenesis. (Trujillo et al., 2006)*

However, because of the complexity of the process, it is not possible to conclude that modifying any one, or more, of these processes influences cancer risk. The recent expansion of knowledge in molecular biology has allowed new techniques to be developed to explain these mechanisms. Nutrigenomics is a new field with profound implications in cancer prevention and therapy, since it seeks to clarify the impact of nutrition in the maintenance of genome stability, and to dissect out the influence of genotype in determining our response to diet. Nutrigenomics is the study of nutritional influences on the phenotypic variability of individuals based on genomic diversity (Figure 1.4). This determines the sequence and functions of genes, and studies single nucleotide polymorphisms (SNPs), and amplifications and deletions within the DNA sequence as modifiers of the response to foods and beverages and their constituents. Nutritional epigenomics is another key determinant of gene

expression patterns. It includes non-coding modification of genes (such as methylation, changes in histone homeostasis, miRNA, and DNA stability) in response to nutrition. Nutritional transcriptomics is the study of gene expression patterns at the RNA level, and it can identify common nutritional response elements in gene promoters that can be modulated by diet. Proteomics studies the proteins that can be expressed by a cell, many of which can be influenced by nutrition. Metabolomics studies the range of metabolic processes in a cell and metabolic regulation in cells or tissues, which again are heavily influenced by food, nutrition, and physical activity.

Cellular processes

The role of nutrition in cancer depends on how it impacts on fundamental cellular processes including the cell cycle. To understand cancer biology, it is important first to understand normal cellular processes. The integrity of tissues and organs depends on a regulated balance between cell proliferation and death, and appropriate cell differentiation. This regulation is controlled by several types of genes including oncogenes and tumor suppressor genes, and factors in the cellular environment that influence their expression. Maintenance of the DNA sequence and structure as cells divide is essential: several cellular mechanisms exist to ensure this is achieved.

Cell signalling

Cells detect and respond to external stimuli and send messages to other cells through a molecular mechanism known as cell signalling. Cells within a tissue normally communicate with each other through a network of locally produced chemicals called cytokines (including some growth factors). Cell proliferation is a tightly controlled and coordinated process, and is stimulated by growth factors. These soluble proteins can be produced locally, either from the same cell (autocrine), or from different cells (paracrine), or as hormones (endocrine) produced by a distant tissue and transported in the blood. Growth factors bind to specific receptors on the cell surface and transmit a signal into the cell, which is relayed to the nucleus. In the nucleus, genes are switched on to produce the proteins necessary for cell division. Getting the growth signal from the outside of the cell to the nucleus requires a series of steps. The shape of the receptor changes when the growth factor binds to it, which causes part of the receptor to become activated, usually by a process called phosphorylation. A regulated process of phosphorylation and dephosphorylation is necessary for the appropriate initiation, transmission, and cessation of signals.

Gene expression

Gene expression is the process by which the information within a gene is 'turned on' or 'turned off'. The information is used to create the associated proteins and modify the amounts produced. Also see Figure 1.3. Transcription factors are proteins involved in the regulation of gene expression and carry the signal from the cytoplasm to the nucleus. They bind to the promoter regions of genes and have the effect of either switching gene expression on or off. There are also nuclear receptors, such as retinoic acid receptors, that function as transcription factors by binding directly to specific DNA sequences. Some so-called 'housekeeping' genes are expressed by almost all cell types. These genes generally encode proteins that participate in basic cell functions

such as metabolic pathways and synthesis, and processing of DNA, RNA, or proteins. Other genes have more restricted expression, and are expressed only in specific cell types, and/or stages of development. Gene expression can also be influenced by changes outside the DNA of genes. DNA is closely organised and tightly packaged in the nucleus of cells. To achieve this, DNA is spooled around proteins called histones. Histone structure can be modified either, like DNA itself, by methylation, or more commonly by acetylation (addition of an acetyl group). Acetylation and deacetylation (removal) are mediated by the enzymes histone acetyl transferase (HAT) and histone deacetylase (HDAC), respectively. HATs relax the packaged DNA structure, which is associated with enhanced transcription, whereas HDACs stabilise the structure with higher levels of packaging, and so suppress transcription. Butyrate, produced in the colon by bacterial fermentation of non-starch polysaccharide (dietary fibre), diallyl disulphide from garlic and other allium vegetables, and sulphoraphane, a glucosinolate from cruciferous vegetables, can behave as histone deacetylase inhibitors (Dashwood et al., 2006), and act to maintain DNA stability or enhance transcription. Micro RNAs (miRNAs) are RNA molecules that do not encode proteins; instead they function as negative regulators of gene expression. Some mutations in miRNAs have been associated with human cancers, and they can function as oncogenes or tumour suppressor genes. miRNAs are short, single-stranded RNA molecules of approximately 22 nucleotides. For instance, to silence (turn off) genes, they may bind to complementary mRNA sequences and degrade them before they have been translated. Profiling miRNA signatures within cancer cells may aid the diagnosis, classification, and treatment of cancer. For example, a certain miRNA that is down-regulated in lung cancer is associated with decreased survival (Esquela-Kerscher & Slack, 2006). Research on the interactions between nutrition and non-coding RNA molecules is at an early stage but is potentially relevant to cancer.

DNA damage and repair

Each time a cell divides into two new daughter cells, there is potential for an error in replication of the DNA. These mutations result in non-functioning genes or in proteins with altered amino acid sequences that can change cell function. DNA is continuously exposed to damage from products of normal intracellular metabolism, including reactive oxygen species, hydroxyl radicals, and hydrogen peroxide; and also to damage from external factors such as ultra-violet (UV) light, as well as other environmental factors including food, nutrition, and physical activity. There are several mechanisms for DNA repair, a vital defense in maintaining cellular integrity and preventing a cell being transformed from normal to cancerous (see also chapter 3.1). Various studies suggest that nutritional status and/or certain food constituents may influence DNA repair. Data from observational studies suggest that severe malnutrition can impair DNA repair (Gonzalez et al., 2002). Although the processes of cell development, signalling, and DNA repair are tightly controlled, errors will occur during the trillions of cell divisions that occur over a lifetime. This may result in inappropriate proliferation or failure of damaged cells to die. These changes could provide the altered cell with a growth advantage over the normal cells in the tissue. If

additional alterations occur, this can result in a cell with the potential to become cancerous.

Causes of cancer

A number of different types of exogenous (environmental) factors are known causes of cancer. These include some aspects of food and nutrition that are established as carcinogenic by the International Agency for Research on Cancer, although it is difficult to estimate the proportion of cancers directly attributable to these. Known causes of cancer include: tobacco smoking and its use, infectious agents, medication, radiation, and industrial chemicals, and also carcinogenic agents in food and drink.

1. Endogenous causes

Inherited germ line mutations

As mentioned, only a minority (5–10 per cent) of cancers are linked to single inherited genes. Such inherited alterations are termed germ line mutations, and are passed on from egg or sperm DNA. Individuals with inherited germ line mutations will not definitely get cancer but have an increased risk of developing cancer compared with the general population. Often mutations in tumor suppressor genes increase the chance of developing cancer at a young age.

The other type of genetic mutation — somatic gene changes — develops during the life course. Such somatic mutations are not passed on to offspring. This DNA damage is caused by exposure to external factors such as radiation or carcinogens, or harmful products of normal aerobic metabolism.

Oxidative stress

Reactive oxygen species generated through normal oxidative metabolism have the potential to cause extensive DNA damage. The body has several mechanisms, which can scavenge reactive oxygen species to prevent such damage occurring, or block the effects. Reactive oxygen species cause oxidative damage to DNA. During repair, the damaged, oxidized bases are excreted in the urine. Levels of urinary 8-hydroxy-2'-deoxyguanosine, an oxidative DNA damage adduct, can be used as an indicator of oxidative DNA damage in humans and rodents. Antioxidants can scavenge reactive oxygen species. Vitamins C and E can donate electrons to free radicals and block their damaging activity. Dietary constituents such as ITCs and polyphenols can also activate the signaling pathways that lead to activation of the antioxidant response element, and up-regulation of the expression of detoxifying enzymes.

Inflammation

Inflammation is a physiological response to infection, foreign bodies, trauma, or chemical or other irritation, and in the acute phase can be helpful. However, chronic inflammation can result in DNA damage and cancer promotion. Chronically inflamed tissue is infiltrated with a variety of inflammatory cells that produce a wide variety of bioactive chemicals. These include cytokines, growth factors, reactive oxygen and nitrogen species, cyclooxygenase, and lipoxygenase products. A chronic inflammatory environment can increase proliferation and differentiation, inhibit apoptosis (programmed cell death), and induce angiogenesis (generation of new blood vessels).

Epidemiological and experimental evidence has demonstrated that long-term use of non-steroidal anti-inflammatory drugs can inhibit cancer development in a number of tissues including colon, oesophagus, and breast (Coussens & Werb, 2002; Hawkes, 2005). Cancer induced by inflammation may be susceptible to nutritional influences. Thus, dietary constituents could be involved in generation of reactive oxygen species, could influence antioxidant defenses, or could suppress the inflammatory process. For example, the glucocorticoid receptor pathway and the vitamin D receptor are capable of suppressing inflammation.

The immune system can be divided into innate and adaptive responses. Innate immunity provides initial defenses. Adaptive immunity develops later and involves activation of lymphocytes and their differentiation into effector and memory cells. The 'immune surveillance' hypothesis proposes that both the innate and adaptive immune systems constantly survey for and eliminate newly formed cancer cells, and that onset and progression of cancer are kept under control by the immune system (Smyth *et al.*, 2006). Immuno-surveillance requires that the immune system recognizes something different about cancer cells compared with normal cells within the same tissue – often different proteins (termed tumor antigens) that are expressed on the surface of a cancer cell. This recognition of 'altered self' allows the immune system to generate a response to these tumor antigens. They can be proteins that are only expressed by cancer cells, and newly expressed during cancer development; or proteins that have become mutated during the cancer process and so are different from the non-mutated protein; or proteins expressed due to differentiation of cancer cells (termed differentiation antigens); or proteins that are normally expressed by cells but that are expressed at much higher levels by cancer cells. Evasion of immune-surveillance is sometimes referred to as a further hallmark of cancer (Zitvogel *et al.*, 2006), although the evidence remains speculative. Specialized mucosal cells form the interface between the inside and outside of the body (Miura *et al.*, 1998). These are normally an efficient barrier against pathogens. The gut barrier consists of gut-associated lymphoid cells that can sense pathogens, and participate in innate and adaptive responses (Tlaskalova-Hogenova *et al.*, 2002). The function of these cells is dependent on nutrition. For example, n-3 PUFAs can enhance immunity, whereas high concentrations of n-6 unsaturated fatty acids can have a suppressive effect (Gleeson *et al.*, 2004). Various factors have been shown to modulate both inflammation and immunity, including vitamins A and E, copper, selenium, zinc, PUFAs, and epigallocatechin-3-gallate (EGCG) from green tea (Kubena & McMurray, 1996).

The cytokine IL-6 can act as a pro- or anti-inflammatory cytokine. In cancer, IL-6 can either stimulate proliferation or exert anti-tumor effects by enhancing both innate and adaptive immunity (Tripathi *et al.*, 2003). Dietary phytoestrogens, such as soy isoflavones, downregulate IL-6 gene expression and thus potentially influence the development of hormone-related cancers (Dijsselbloem *et al.*, 2004).

2. Exogenous causes

Tobacco use

Tobacco causes an estimated 20 per cent of all cancer deaths, and an estimated total of 1.2 million in 2002 World Health Organization and International Agency for Research on Cancer (World Cancer Report 2003). Smokers have increased risk of a number of different cancers. Worldwide, around 80 per cent of lung cancer cases in men and 50 per cent in women are caused by tobacco smoking (Mackay *et al.*, 2006). In 2002, out of all new cases of cancer in low-income countries, over 1 in 5 in men and almost 4 per cent in women were attributable to tobacco. In high-income countries, one third of all new cancer cases in men and just over 1 in 8 in women were attributed to tobacco smoking. Cigarette smoke contains at least 80 known mutagenic carcinogens, including arsenic, cadmium, ammonia, formaldehyde, and benzopyrene. Each will have a separate mechanism for causing cancer. For example, following metabolic activation, the activated derivative of benzopyrene, benzo(a)pyrenediol epoxide, can form DNA adducts in lung epithelial cells (Nishikawa *et al.*, 2004). Cigarette smoke is a powerful carcinogen and also a source of oxidative stress. Compared with non-smokers, active smokers have lower circulating concentrations of several antioxidant micronutrients including α -carotene, β -carotene, cryptoxanthin, and ascorbic acid (Alberg, 2002).

Carcinogenic agents in food

Food may be contaminated with natural or man-made carcinogenic toxicants. Moulds and the toxins produced by some moulds cause DNA adducts and are carcinogenic. Aflatoxin B, a product of the *Aspergillus* fungus and a common contaminant of cereals (grains) and peanuts, is an established cause of liver cancer. Fumonisin B, a toxin produced by the fungus *Fusarium verticillioides*, may be found on maize and may be carcinogenic, although epidemiological studies are lacking. Some carcinogenic compounds are formed during food preparation. Heterocyclic amines are formed by cooking meat at high temperatures, and polycyclic aromatic hydrocarbons can be produced in meat and fish that has been grilled (broiled) or barbecued (charbroiled) over a direct flame. High environmental concentrations of polycyclic aromatic hydrocarbons, which also come from pollution caused by traffic and industry, can contaminate other foods such as cereals, vegetables, and fruits. Some *N*-nitroso compounds are carcinogens, and are formed in foods containing added nitrates or nitrites; examples include fish and meat preserved with salting or preservatives, and smoking or drying. These carcinogens can also be generated from ingested foods containing nitrate or nitrite. *N*-nitroso compounds are also produced endogenously in the stomach and colon of people who eat large amounts of red meat or take nitrite supplements.

Nutrition and cancer

The majority of cancers are not inherited. Cancer is, however, a disease of altered gene expression that originates in changes to DNA, the carrier of genetic information. For a

cell to be transformed from normal to cancerous, it has to acquire different phenotypic characteristics that result from alterations to the genotype. Most cancers develop to the stage of being clinically identifiable only years or decades after the initial DNA damage. Cancer development, or carcinogenesis, requires a series of cellular changes. No single gene causes cancer. It is a multistep process caused by accumulated errors in the genes that control cellular processes. One genetic mutation may allow a single trait (such as increased survival) to be acquired by a lineage of cells, and descendants of these cells may then acquire additional genetic mutations. However, cancer only develops when several genes are altered that confer growth and survival advantages over neighbouring normal cells. The capacity of a cell to achieve effective cancer prevention or repair is dependent on the extracellular microenvironment, including the availability of energy and the presence of appropriate macro- and micronutrients. Tumors are not simply masses of cancer cells. Rather, they are heterogeneous collections of cancer cells with many other cell types — so-called stromal cells; cancer cells communicate with stromal cells within the tumor. The tumor microenvironment comprises many cell types including infiltrating immune cells such as lymphocytes and macrophages, endothelial cells, nerve cells, and fibroblasts. All these cell types can produce growth factors, inflammatory mediators, and cytokines, which can support malignant transformation and tumor growth, and attenuate host responses. In addition, factors produced by the cancer cells themselves modulate the activity and behavior of the tumor stroma. Initiation is the exposure of a cell or tissue to an agent that results in the first genetic mutation. This can be an inherited mutation or an exogenous or endogenous (produced through oxidative metabolism) factor. Even without external oxidative stress, hundreds of sites within DNA are damaged each day but are normally repaired or eliminated. Exposure to the carcinogen initiates DNA damage, usually via the formation of DNA adducts. If left uncorrected, these adducts can be transferred to daughter cells during division and confer the potential for neoplastic (new and abnormal) growth. Initiation alone is insufficient for cancer to develop. An initiated cell must go through a process of clonal expansion during promotion to become neoplastic; the larger the number of initiated cells, the greater the risk of progressing to cancer. Promotion involves exposure of the initiated cell to a promoting agent. This may allow alterations in the rate of proliferation or additional DNA damage to occur, leading to further mutations within the same cell, which alter gene expression and cellular proliferation. Finally, these initiated and promoted cells grow and expand to form a tumor mass. DNA damage continues at this stage and cancer cells often contain multiple copies of chromosomes. This clear, sequential process is typical of experimentally induced cancers but may be less clear in sporadic cancers in humans. At the end of the multistage process of carcinogenesis, the cell will bear some or all of the hallmarks of cancer (Hanahan & Weinberg, 2000). Several genes can contribute to each hallmark and one gene (for example p53) can contribute to several of the hallmarks. These hallmarks or traits are shared by most, if not all, cancer cells. The six hallmarks of cancer cells are (Hanahan & Weinberg, 2000):

- 1) self-sufficiency in growth signals;

- 2) insensitivity to antigrowth signals;
- 3) limitless replicative potential;
- 4) evasion of apoptosis;
- 5) sustained angiogenesis;
- 6) tissue evasion and metastasis.

Food, nutrition, and physical activity-related factors influence cellular processes and lead to cells accumulating these traits.

1) Growth signal autonomy

Unlike normal cells, cancer cells are not dependent on external growth factors to stimulate their division. Instead, they can generate their own signals or respond to lower concentrations of external signals. This frees cancer cells from the growth constraints of normal cells.

2) Insensitivity to antigrowth signals

Normal cells also receive growth inhibitory signals. Indeed, most cells of the body are quiescent and not actively dividing. Cells respond to negative environmental signals such as contact with other cells. Cancer cells have acquired mutations that interfere with these pathways and so do not respond to growth inhibitory signals.

3) Limitless replicative potential

Normal cells can divide a finite number of times. Once they have replicated 60 or 70 times they stop — a process termed senescence, which is thought to constitute a protective mechanism against unlimited proliferation. This preordained number of cell doublings is controlled by telomeres. Telomeres are segments of DNA on the ends of chromosomes, which are shortened during each round of DNA replication. Eventually when the telomeres are too short, the cell can no longer divide and it undergoes apoptosis. By contrast, cancer cells have acquired the ability to maintain the length of their telomeres, which means they can replicate endlessly. Recent work has suggested that senescence can be induced prematurely, particularly in premalignant cells, by activation of the normal, non-mutated forms of genes such as p53 and Rb (Hanahan & Weinberg, 2000). This senescence is a normal active process involving genetic and phenotypic changes that may protect against cancer development; for example, it may be one mechanism preventing benign moles from progressing to malignant melanoma. However, in malignant melanoma, cell markers of senescence are lost (Sharpless & DePinho, 2005). In experimental conditions, many constituents of food such as retinol, calcium, allyl sulphide, n-3 fatty acids, and genistein are known to influence progression of cells through the cell cycle. These studies, when conducted in cells in culture, need to be assessed cautiously because they may not always adequately reflect events *in vivo*. However, they can and do provide evidence additional to that gained from epidemiological studies.

Specific dietary components have effects on cell cycle progression and proliferation in experimental settings. Some known or hypothesized benefits of some dietary constituents are summarized here.

Vitamin A (in the form of retinol) can lead to cell cycle arrest (Bohnsack & Hirschi, 2004). Retinoids and carotenoids inhibit proliferation by binding retinoid receptors on

the cell surface. Reduced expression of retinoid receptors occurs during development of lung cancer (Lotan, 1999); retinoic acid receptor silencing is also common in other malignancies. Retinoic acid, a metabolite of vitamin A, has been used as a chemopreventive and therapeutic agent in cervical cancer (Palan *et al.*, 1998). Retinoids can inhibit proliferation of initiated cells by inducing apoptosis or inducing differentiation of abnormal cells back to normal (Butterworth *et al.*, 1982). Retinoids may also cause regression of precancerous lesions in the cervix (Abu *et al.*, 2005). **Butyrate and diallyl disulphide** can act as histone deacetylase inhibitors (Dashwood *et al.*, 2006) , and arrest the cell cycle. **Folate** is a necessary cofactor for DNA synthesis, and deficiency can reduce cell proliferation due to decreased DNA synthesis. **Phenolic compounds**, including genistein and EGCG, can inhibit some cyclins and cyclin-dependent kinases (Chen & Kong, 2005). Specifically, in people with oral leukoplakia, green tea (which contains EGCG) has been associated with significant decreases in the size of cancers and of micronuclei formation in exfoliated oral cells (Li *et al.*, 1999).

Phytoestrogens are found in high concentrations in soya beans, and have been shown *in vitro* to exhibit a plethora of different anti-cancer effects, including inhibiting proliferation (Strauss *et al.*, 1998; Adlercreutz, 2002). Only about one third of people have the types of microbial flora in their gut that are capable of metabolising the dietary isoflavone daidzein to equol. Compared with Western populations, Asian populations are more likely to produce equol, and this affects the expression of genes involved in cell signalling and differentiation, and cell division. Equol can also modulate oestrogenresponsive oestrogenresponsive genes (Niculescu *et al.*, 2007). **Glucosinolates** from cruciferous vegetables are converted in the liver to ITCs, which can arrest cell cycle progression, as well as induce phase II enzymes, which can promote carcinogen excretion. In a variety of animal studies, certain dietary components have shown reductions in experimentally induced cancers. **Allyl sulphides** in garlic inhibit experimentally induced colon tumor formation. Although this is not completely understood, experiments with diallyl disulphide suggest a block in the G2/M phase in the progression of the cell cycle, and induction of apoptosis (Knowles & Milner, 2001). Fish oil supplements decrease the number of tumors in experimental models of colorectal cancer (Rao *et al.*, 2001). **Long-chain n-3 PUFAs** in fish oils can limit tumor cell proliferation (Nkondjock *et al.*, 2003; Roynette *et al.*, 2004) by modifying signaling pathways, (Gupta *et al.*, 2000; Price *et al.*, 2000; Novak *et al.*, 2003) for example, by decreasing signaling of activated oncogenes (Collett *et al.*, 2001). Animals that receive a diet supplemented with n-3 fatty acids have fewer colonic tumors than those fed a diet supplemented with corn oil (Chapkin *et al.*, 1998), due to dietary fibre-altering, fatty acid-binding, protein expression in colonocytes during tumor development.

Various growth factors and hormones involved in normal cell processes can be used or produced by cancer cells to maintain or augment uncontrolled cell proliferation. The receptor for IGF-1 is over-expressed on many cancer cells. IGF-1 can enhance the growth of a variety of cancer cell lines (LeRoith *et al.*, 1995) by stimulating progression of the cell cycle from G1 to S phase (Calle & Kaaks, 2004). Insulin itself can also act as a

growth factor for tumor cell proliferation, both by binding to the insulin receptor on cancer cells and by stimulating increased IGF-1 production by the liver (Calle & Kaaks, 2004; Yakar *et al.*, 2005). Insulin resistance increases with body fatness, in particular abdominal fatness, and the pancreas compensates by increasing insulin production. This hyperinsulinaemia is associated with a risk of cancers of the colon and endometrium, and possibly of the pancreas and kidney (Calle & Kaaks, 2004). Leptin, a hormone produced by fat cells, can also stimulate proliferation of many premalignant and malignant cell types (Fenton *et al.*, 2005), as can a number of sex steroid hormones. Physical activity improves insulin sensitivity and decreases levels of insulin (Grimm, 1999). However, exercise has little or no long-term effects on circulating IGF-1 levels (Schmitz *et al.*, 2002; McTiernan *et al.*, 2005). IGF binding activity may increase with physical activity, and thus overall IGF-1 bioavailability and activity may decrease. Physical activity decreases serum estrogen and androgens in postmenopausal women. In premenopausal women, it decreases circulating estrogens, increases cycle length, and decreases ovulation, all of which provide a protective effect for breast and endometrial cancers.

In experimental animals, energy restriction leads to a reduction in cell proliferation (Hursting *et al.*, 2003). At the molecular level, dietary energy restriction affects levels of cell cycle control proteins (decreased cyclins, increased levels of cyclin-dependent kinase inhibitors, and decreased cyclin-dependent kinases), leading to reduced Rb phosphorylation and inhibited cell cycle progression (Thompson *et al.*, 2003). This, in turn, may directly inhibit tumor growth and/or indirectly reduce cancer development by reducing the number of cell divisions, thus reducing the chances for incorrect DNA replication or preventing damaged DNA from being replicated.

4) Evasion of apoptosis

Apoptosis is the tightly regulated process of cell death that controls cell numbers, removes damaged cells, and prevents damaged cells being replicated, thereby maintaining tissue integrity and protecting against cancer. Ultimately, cells break into small membrane-surrounded fragments (apoptotic bodies) that are phagocytosed without inducing inflammation. Triggers for apoptosis in normal cells include DNA damage, disruption of the cell cycle, hypoxia, reactive oxygen species, and physical or chemical insult. Two nonexclusive pathways, the intrinsic (mitochondrial) pathway or the extrinsic (death-receptor) pathway, can be activated. Both involve activation of caspases, a family of protease enzymes that cleave intracellular proteins (Fischer & Schulze-Osthoff, 2005). In apoptosis, p53 functions as a transcriptional activator of genes encoding apoptosis effectors. p53 can also exert a direct apoptotic effect by damaging mitochondria (Mihara *et al.*, 2003). Cancer cells have acquired mutations in genes regulating apoptosis and therefore can evade apoptotic signals. Defects in apoptosis are often observed in established cancers. In cancer cells, many signals that normally induce apoptosis, such as damaged DNA or expression of activated oncogenes, are present but apoptosis is not induced. This avoidance of apoptosis allows further opportunity for additional mutations to develop. In cancer cells with mutations in p53 or other members of this family, apoptosis may not occur.

Additionally, mutations in genes that would normally activate p53 or regulate its activity, or in genes that should be switched on as a result of p53 activation, can have the same effect. Cancer cells with upregulated expression of IGF-1R and increased responses to IGF-1 have decreased apoptosis (Yakar *et al.*, 2005). In experimental settings, energy restriction creates a proapoptotic environment, adjacent to premalignant and malignant breast pathologies (Thompson *et al.*, 2004). Long-chain n-3 PUFAs in fish oils limit tumor cell proliferation, increasing apoptotic potential along the crypt axis, promoting differentiation and limiting angiogenesis (Rao *et al.*, 2001; Nkondjock *et al.*, 2003; Roynette *et al.*, 2004). Reactive oxygen species can induce apoptosis, but it is also possible that scavenging of reactive oxygen species by dietary antioxidants can delay or inhibit apoptosis, and thus favor survival of premalignant cells. Indeed, this could explain why dietary antioxidant intervention trials have produced mixed results (Salganik *et al.*, 2000; Albright *et al.*, 2004).

Many dietary components have been shown to induce apoptosis in cultured cancer cells and in experimental models of cancer (Watson *et al.*, 2000). These include EGCG, curcumin, genistein, indole-3-carbinol, resveratrol, ITCs, lycopene, capsaicin, and organosulphur compounds (Khan *et al.*, 2007). In premalignant cells, retinoids, polyphenols, and vanilloids stimulate apoptosis (Sun *et al.*, 2004). Alpha-tocopherol (a form of vitamin E) has been shown both to induce (Gunawardena *et al.*, 2000) and to protect against apoptosis (Takahashi *et al.*, 1998).

5) Sustained angiogenesis

Angiogenesis, the formation of new blood vessels, is essential for the supply of nutrients and oxygen to any growing tissue, including tumors. Most cells within tissues reside within 100 μ m of a capillary blood vessel. The generation of blood vessels in adults is fairly constant and tightly controlled by a balance of angiogenesis inducers and inhibitors. For a cancer to progress to a larger size, it must acquire the ability to induce angiogenesis. Currently about 35 proteins have been identified as angiogenesis activators or inhibitors (Folkman, 2003; Folkman, 2006). In experimental settings, one of the first dietary components for which a beneficial anti-angiogenic effect was clearly demonstrated was EGCG from green tea (Cao & Cao, 1999). Now some 20 different compounds consisting mainly of flavonoids and isoflavones (including genistein) are documented as being able to modulate the angiogenic process. Diets high in n-6 fatty acids are associated with poor prognosis in breast cancer patients, whereas those high in n-3 fatty acids appear to suppress angiogenesis (Rose & Connolly, 2000). Curcumin, quercetin, and resveratrol have all been shown to inhibit the angiogenic factor, vascular endothelial growth factor (VEGF), in cultured cancer cells. Garlic extract may inhibit experimentally induced angiogenesis, as it can suppress endothelial cell motility, proliferation, and tube formation (Matsuura *et al.*, 2006). Phytoestrogens found in high concentrations in soya beans have also been shown to inhibit angiogenesis (Strauss *et al.*, 1998). Energy restriction reduces blood vessel density in premalignant and malignant breast pathologies (Thompson *et al.*, 2004). Exercise increases the levels of a circulating endogenous VEGF inhibitor in healthy people, which could decrease plasma levels of VEGF (Bailey *et al.*, 2006).

6) Tissue invasion and metastasis

Normal cells in solid tissues maintain their position in the body and generally do not migrate. As a cancer increases, it eventually reaches the membrane encapsulating the organ. Tumor cells secrete enzymes such as matrix metalloproteases (MMPs), which digest the membrane and allow the cancer to invade adjacent tissue. Once through the membrane, cancer cells can access other sites via the blood and lymphatic systems. This migration of cancer cells, or metastasis, is a common characteristic of most cancer deaths. There is limited evidence for dietary components to influence these late stages of cancer, although *in vitro*, EGCG, resveratrol, quercetin, curcumin, and genistein can inhibit one or more MMPs. Vitamin C can inhibit MMP production by a number of human cancer cell lines and prevent invasion of these lines *in vitro* (Roomi et al., 2006). Vitamin E can inhibit metastasis of pre-established tumors in mouse models of breast cancer (Hahn *et al.*, 2006).

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2. AIMS OF THE STUDY

The intake of several foods rich in bioactive constituents has been identified in the literature reviews as being associated with lower chronic-degenerative disease risk (Liu, 2004). The bioactivity of these compounds is in part related to their antioxidant properties which are important to decrease the risk of the onset development of many of the chronic degenerative diseases (Espin *et al.*, 2007).

Despite of this consideration, more studies are needed to demonstrate the protective role of specific food and the mechanisms of action of bioactive compounds.

This PhD research project will be divided in two parts.

1) The first part will concern to the evaluation of the impact of bioactive compounds in Cruciferae, on humans with different genetic polymorphisms of enzymes, involved in endogenous biotransformation systems. Particularly, two types of study will be designed analyzing: the bioavailability of protective compounds from broccoli and their role in the protection against biomarkers of oxidative stress, taking into account individual genetic susceptibility. The response of different biomarkers to both single meal and regular consumption of broccoli will be investigated. DNA damage will be evaluated as resistance to exogenous damage, endogenous DNA damage and OGG1 repair ability; while OGG1, HO-1 and NUDT1 mRNA levels will be considered as markers of repair and defence systems.

Recently, the inverse relationship found between cruciferous vegetable intake and cancer risk increased interest among scientists involved in cancer prevention studies. Dietary or environmental carcinogens must be activated to induce cancer. The ability of dietary constituents in cruciferous vegetables to inhibit phase I activation and to induce phase II enzymes are of primary importance to their cancer chemopreventive effects (Fimognari *et al.*, 2008). In particular, phase II enzymes decrease carcinogenicity by blocking carcinogen metabolic activation and enhancing carcinogen detoxification. Glutathione-S-Transferase (GST), is included in the phase II family enzymes activated by Cruciferae consumption (Jeffery & Keck, 2008). Moreover its genetic polymorphism is related to cancer risk (Qiu *et al.*, 2010; Wan *et al.*, 2010). Thus, we will evaluate the different susceptibility to risk of different GST polymorphisms in relation to broccoli consumption. Controversial data are found in literature, and human intervention studies have not clearly demonstrated the relationship between GST polymorphisms and protective effect of broccoli. It is hypothesized that GST genotypes associated with lower protection against carcinogens benefit most from cruciferous vegetable consumption.

The first part of this PhD research project will be carried out at the Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche (DiSTAM)-sezione Nutrizione (Università degli Studi di Milano), under the direction of Prof. Marisa Porrini and Dr. Patrizia Riso.

2) The second part will be focused on the study of mechanisms of action involved at cellular level induced by single compounds. Since most cancers are dependent on the growth of tumor blood vessels, angiogenesis may thus provide an

efficient strategy to delay or block tumor growth. Migration and proliferation of endothelial cells are critical steps in angiogenesis since these events allow cells to disseminate from pre-existing vessels to form new capillary structures (Folkman, 2006). Moreover, blood coagulation and cancer biology are strictly related (ten Cate & Falanga, 2008).

Capillary-like formation assay is a reliable approach able to test pro- or anti-angiogenic properties of a chemical compound. After a review of the literature two anthocyanins, i.e. Delphinidin-3-glucoside (Dp-3-glc) and Cyanidin-3-glucoside (Cy-3-glc), will be selected for this type of studies. These two compounds are able to inhibit tumor cell proliferation (Katsube *et al.*, 2003; Fimognari *et al.*, 2004; Olsson *et al.*, 2004), but it is not known whether they can also act on pro-angiogenic and the pro-thrombotic properties of endothelial cells. Models of angiogenesis on human microvascular endothelial cells will be used to test the role of Dp-3-glc and Cy-3-glc in capillary network formation and wound healing assay in resting and VEGF-stimulated conditions. The same cell line will be used to test the protective role of Dp-3-glc in pro-coagulant activity both in resting and LPS-stimulated conditions. The results will enhance our knowledge of the intricate roles and functions that ACNs act at both cellular and molecular level.

This part of research will be performed at the Hemostasis and Thrombosis Center, Ospedali Riuniti di Bergamo, Italy, supervised by Dr. Anna Falanga and Dr. Marina Marchetti.

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3. RESULTS AND DISCUSSION

3.1 TOPIC I: **Role of broccoli intake, a glucosinolate-rich source, on biomarkers of oxidative stress in young smokers**

Abstract

Scientific evidences support the protective role of diets rich in fruit and vegetables against chronic diseases, like cancer and cardiovascular disease (WCRF/AICR 2007). Fruits and vegetables compounds, able to reduce the risk of chronic diseases, include vitamins, minerals and numerous phytochemicals. The latter are bioactive compounds that have been shown to exert a wide range of biological activities. Since they are ingested regularly and in significant amounts as part of the diet, they may have a noticeable long-term beneficial effect.

Cruciferae and especially *Brassica* genus contain high concentration of constituents with antioxidant properties (e.g. carotenoids, vitamin C, folate) as well as glucosinolate precursors of isothiocyanates (ITCs) and indoles that modulate xenobiotic biotransformation enzymes, such as Glutathione S-Transferase (GST). Different GST polymorphisms seem to be related to altered protective effect against xenobiotics. The protective effect of broccoli (*Brassica oleracea* L. var. *italica*) was tested through two different study protocols performed at DiSTAM-sezione Nutrizione (Università degli Studi di Milano): a **regular-intake** intervention study with daily consumption of one serving of steamed broccoli for 10 days and a **single-meal** study (i.e. with one serving of broccoli). Broccoli were chemically characterized for the content in antioxidant compounds (carotenoids, vitamin C) and glucosinolates as isothiocyanates (ITCs). One portion of 250g of steamed broccoli provided: 2.75 mg of lutein, 1.50 mg of β -carotene, 172 mg of vitamin C and 160 μ mol of phenethyl- isothiocyanate (PEITC) equivalents of ITCs.

Subjects involved in the study were young male smokers recruited on the basis of anthropometric characteristics, food habits and their GSTM1 genotype.

Thirty subjects were enrolled for the **regular-intake study**. A single blind randomized cross-over experimental design was scheduled: 15 volunteers followed the sequence broccoli diet/wash-out/control diet; while the others followed the opposite sequence. Broccoli and control diet were 10 days long and they were spaced out from 20 days of free diet. One serving consisted of 250 g of steamed broccoli. Fasting blood samples were collected at the beginning and at the end of each treatment period (0, 10, 30, 40 days). Plasma, serum and lymphocytes were separated and stored for specific analyses. Concentration of carotenoids, lutein, β -carotene and folate was assessed in plasma and serum samples. Lymphocytes were separated and used immediately for the determination of cell resistance against oxidative stress (H_2O_2 -induced DNA strands breaks) through the COMET assay. Additionally, lymphocytes were isolated and appropriately stored at $-80^\circ C$ for the subsequent determination of biomarkers of oxidative stress: endogenous DNA damage (i.e. oxidized purines), *in vitro* DNA repair activity and mRNA expression of OGG1, NUDT1 and HO-1 levels. Twenty-seven male

smokers of the 30 enrolled completed the regular-intake study (age 22.1±2.5 y; BMI 23.0±2.7 kg/m²). Cell protection against H₂O₂-induced DNA damage was higher after broccoli diet with respect to control diet in the whole group of subjects. Folate and lutein concentrations increased significantly after broccoli diet. By analyzing data considering genotype as independent factor, broccoli intake caused a more consistent and significant effect of protection against DNA damage in GSTM1 null (-27.6%) compared to GSTM1 positive (-13.1%) subjects (p<0.05). Oxidatively damaged purine bases decreased significantly (p<0.05) after broccoli intake (-22.6%), while no effect of polymorphism was observed. DNA repair activity and OGG1, HO-1 and NUDT1 mRNA expression levels did not differ throughout the intervention study.

Twelve volunteers were selected for the **single-meal study**. One serving of 250 g of steamed broccoli with 70 g of pasta was consumed as first meal. Fasting blood samples were collected before broccoli consumption and at 3, 6, 8 and 24 h after broccoli intake. Plasma, serum and lymphocytes were separated and stored for specific analyses.

Cell resistance to oxidative stress (H₂O₂-induced DNA strands breaks) was determined through the COMET assay. Additionally lymphocytes were isolated and appropriately stored at -80°C for the subsequent determination of concentration of bioactive compounds (folate, lutein, β-carotene and vitamin C) and total GST activity. Total GST activity in plasma and lymphocytes was calculated by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione, and normalized by the amount of proteins found in each sample. All the 12 selected volunteers (age 20.8±1.6 y; BMI 22.5±2.1 kg/m²) completed the single meal study. Serum folate, plasma vitamin C and β-carotene concentrations increased significantly after 3h for the first two, and at 6h for β-carotene; while antioxidant content in lymphocytes did not show any increase after broccoli meal. In general, individuals with positive GSTM1 genotype showed a greater plasma and lymphocyte GST activity compared to individuals with GSTM1 null genotype. After broccoli consumption, plasma GST activity increased significantly (p<0.05) in individuals with GSTM1 positive genotype at 6 h with respect to 3 h and 24 h but not compared to baseline. No significant difference was observed in lymphocyte GST activity. A significant reduction of *ex-vivo* induced DNA damage (p<0.05) after 24 h from broccoli consumption intake with respect to time 0, 3 and 6 h was observed. Broccoli under controlled dietary conditions modulated GST activity even if it seems to be more evident in individuals with GSTM1 positive genotype, who showed also a greater protection against DNA damage.

In conclusion, these two studies demonstrated that 10 days of consumption of steamed broccoli could improve defence against DNA damage but did not affect repair activity in young healthy smokers. Our data are in accordance with shown protective effects of cruciferous vegetables, including Brussel sprouts, broccoli and watercress, on biomarkers of oxidative damage to DNA in human intervention studies. Also a single portion of steamed broccoli was able to decrease DNA damage and to modulate GST activity. Even if preliminary, our data suggest a “diet/genetic” interaction. In particular we supported the hypothesis that GSTM1 null genotype subjects might have particular benefit from regular brassica vegetable intake.

Introduction

As reported in detail in the State of the Art (chapter 1) the onset of several chronic diseases is linked to oxidative stress. In fact, oxidative DNA damage formed during oxidative stress and DNA repair may mediate several cellular processes, like replication and transcription, mutagenesis and apoptosis and thus may be for the organism development as well as its pathogenesis, including cancer. Diet seems to play a significant role in these mechanisms and the aetiology of several cancers. The evidence of the protective role for diets rich in fruit and vegetable is convincing and, associated with an healthy lifestyle, provides low incidence of cancer (WCRF/AICR 2007). In this regard, a class of specific vegetable food with hypothesized health benefits is Cruciferae vegetable. Despite many *in vitro* and animal studies, only little evidence is present in dietary controlled human intervention studies.

Intake and health benefits of *Cruciferae*

Specific groups of vegetables seem to offer enhanced sources of protective phytochemicals, notably Cruciferae and especially *Brassica* genus (Moreno *et al.*, 2006), which contain many putative protective agents. Cruciferae include 20 species in the *Brassica* genus (Table 3.1.1).

Table 3.1.1 *Cruciferous vegetables (adapted from Thomson and Green 2003): species and common names of different plants*

Genus	Species	Plant
<i>Brassica</i>	<i>oleracea</i>	Broccoli, brussel sprouts, cabbage, savoy cabbage, red cabbage, cauliflower, collards, kale, Chinese kale, Italian broccoli, asparagus broccoli, sea kale, wild cabbage, kohlrabi
<i>Brassica</i>	<i>rapa</i>	Chinese cabbage, celery ,cabbage, spinaci mustard, turnip, turnip tops, turnip broccoli, toria
<i>Brassica</i>	<i>napus</i>	Rape, colza, Siberian kale, rutabaga
<i>Brassica</i>	<i>japonica</i>	wasabi
<i>Brassica</i>	<i>juncea</i>	Chinese mustards, mustard greens, curled mustard, pak choi, bok celery, brown mustard
<i>Brassica</i>	<i>alba</i>	White mustard
<i>Brassica</i>	<i>nigra</i>	Black mustard oil, black mustard seeds
<i>Sisymbrium</i>	<i>officinale</i>	Hedge mustard
<i>Nasturtium</i>	<i>amoracea</i>	Horseradish
<i>Nasturtium</i>	<i>officinale</i>	Watercress

Bioactive compounds contained in *Brassica* include glucosinolates (GLS), phenolic compounds and antioxidants such as carotenoids (lutein and β -carotene) and vitamins (C, K1 and folate). The single active compounds responsible for chemopreventive properties probably do not exist since the protective activity results from the synergistic action of many different compounds, but glucosinolates are indeed the

most distinguishing compounds of these vegetables, and more than 120 GLS have been characterized so far (Halkier & Gershenzon, 2006; Sonderby *et al.*, 2010).

Table 3.1.2 shows a list of epidemiological studies which pointed out an effect on cancer prevention, related to cruciferous vegetables consumption.

Table 3.1.2 *Epidemiological studies on cruciferous vegetables consumption*

Cruciferous vegetable	Study population	References
Mustard green and Kale	Singapore	(MacLennan <i>et al.</i> , 1977)
Cabbage, Cauliflower, Rutabaga	Norway	(Kvale <i>et al.</i> , 1983)
Broccoli	Massachusset	(Colditz <i>et al.</i> , 1985)
Cruciferous vegetables	Greece	(Katsouyanni <i>et al.</i> , 1986)
Broccoli	Texas	(Bond <i>et al.</i> , 1987)
Broccoli	Louisiana	(Fontham <i>et al.</i> , 1988)
Brassica vegetables	Hawaii	(Le Marchand <i>et al.</i> , 1989)
Broccoli	Hawaii	(Goodman <i>et al.</i> , 1992)
Brassica vegetables	Shanghai	(Zhang <i>et al.</i> , 1992)
Cabbage	Japan	(Gao <i>et al.</i> , 1993)
Cabbage and Cauliflower	India	(Sankaranarayanan <i>et al.</i> , 1994)
Brassica vegetables	The Netherlands	(Voorrips <i>et al.</i> , 2000)
Brassica vegetables	USA	(Slattery <i>et al.</i> , 2000)
Brassica vegetables	Sweden	(Terry <i>et al.</i> , 2001)

The chemical structure of glucosinolate consists of a β -D-glucopyranose residue linked via a sulfur atom to a *N*-hydroximiniosulfate ester, plus a variable R group derived from one of eight amino acids (Halkier & Gershenzon, 2006).

Biosynthesis of glucosinolates in vegetables can be conveniently divided into three separate phases (Figure 3.1.1):

1. certain aliphatic and aromatic amino acids (e.g. methionine and phenylalanine for aromatic glucosinolates and tryptophan for indoles) are elongated by inserting methylene groups into their side chains;
2. the amino acid moiety itself, whether elongated or not, is metabolically reconfigured to give the core structure of glucosinolates;
3. the initially formed glucosinolates are modified by various secondary transformations which consist in the de-saturation and hydroxylation of the chain (Halkier & Gershenzon, 2006).

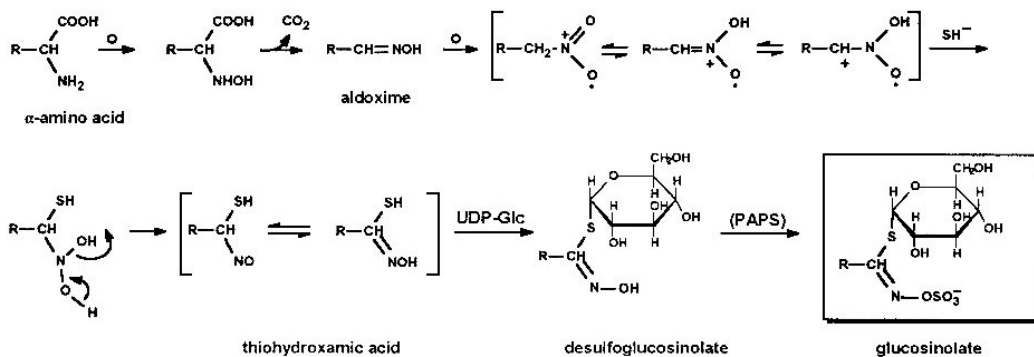


Figure 3.1.1 Biosynthesis of glucosinolates (Fahey *et al.*, 2001)

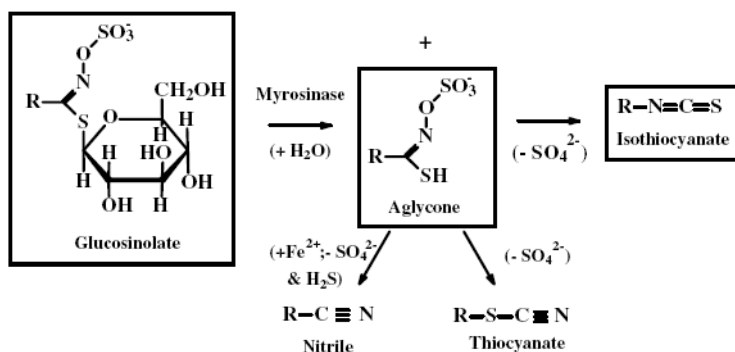


Figure 3.1.2 Formation of isothiocyanates, nitriles and thiocyanates from glucosinolates by myrosinase-catalyzed degradation (Thornalley, 2002)

Glucosinolates are sulfur-rich, anionic natural products that following hydrolysis by myrosinases (endogenous thioglucosidases) produce several different products (for example isothiocyanates, thiocyanates, and nitriles) (Figure 3.1.2).

Isothiocyanates are absorbed in the intestinal cells by passive diffusion and transported to liver by serum albumine. They are electrophilic and thus react with nucleophilic agents including thiols. The main reactive group in the cell is glutathione. The reaction between isothiocyanate and glutathione is spontaneous but can also be catalyzed by glutathione S-transferase (GST). The first product of this reaction is a dithiocarbamate, which can be further metabolized to the cysteine conjugate and then N-acetylated to form the corresponding mercapturic acid which is readily excreted in urine (Ye *et al.*, 2002).

Sulforaphane (1-isothiocyanato-4-methylsulfinyl butane) is the major isothiocyanate derived from the 4-methylsulfinylbutyl glucosinolate that is accumulated in broccoli florets.

Table 3.1.3 *Mechanisms of chemoprevention associated to isothiocyanates adapted from (Fimognari et al., 2008) and (Thomson and Green 2003)*

Chemo-preventive mechanism	Cruciferous/constituent	References
Apoptosis and regulation of cell cycle	Indole-3-carbinol	(Chung <i>et al.</i> , 2000)
	Sulforaphane	(Chiao <i>et al.</i> , 2000)
Inhibition of phase I enzymes	Dithiolthione	(Steinkellner <i>et al.</i> , 2001)
	Ascorbigen	(Hecht, 2000)
	Phenethylthiocyanate	
Induction of phase II enzymes	Dithiolthione	(Brooks <i>et al.</i> , 2001)
	Indole-3-carbinol	(Morimitsu <i>et al.</i> , 2000)
	Isothiocyanates	(Hecht, 2000)
	Sulforaphane	(Steinkellner <i>et al.</i> , 2001)
	Indole	
Inhibition of alpha hydroxylation of nitrosamine	Indoles	(Steinkellner <i>et al.</i> , 2001)
	Isothiocyanates	(Hecht, 2000)
Radical and electrophil scavenging	Indole-3-carbinol Ascorbigen	(Shertzter & Senft, 2000)

It is a potent inducer of phase II detoxification enzymes, inhibits phase I enzymes and can induce cell cycle arrest and apoptosis (Traka *et al.*, 2005). However, there are many other natural bioactive constituents which may be responsible of chemopreventive mechanisms: indole-3-carbinol, benzyl isothiocyanate and phenyl isothiocyanate (Visanji *et al.*, 2004). They have been extensively studied in experimental *in vitro* and *in vivo* carcinogenesis models. Numerous studies have consistently shown that chemoprotective agents influence carcinogenesis during the initiation and promotion phases of cancer development. In Table 3.1.3 the mechanisms for chemoprevention by constituents of cruciferous vegetables are reported.

Cancer protective mechanisms of sulforaphane have been widely studied with several *in vitro* studies as reported in Table 3.1.4.

In recent years, the inverse relationship found between cruciferous vegetable intake and cancer risk increased interest among scientists involved in cancer prevention studies.

Dietary or environmental carcinogens must be activated to induce cancer. The ability of dietary constituents in cruciferous vegetables to inhibit phase I enzymes activation are of primary importance to their cancer chemopreventive effects. The ability of cruciferous vegetables to activate phase II drug-metabolizing enzymes was demonstrated in 1979. Phase II enzymes decrease carcinogenicity by blocking carcinogen metabolic activation and enhancing carcinogen detoxification.

Table 3.1.4 Mechanisms of chemoprevention associated to sulforaphane adapted from (Fimognari *et al.*, 2008; Jeffery & Keck, 2008)

Endpoints	SF dose (μM)	Cancer cell type	References
Induction of phase II detoxification enzyme	0.4-0.8	Liver (mouse)	(Zhang <i>et al.</i> , 1992)
	2-5	Liver (human)	(Bacon <i>et al.</i> , 2007)
	5-10	Colon (human)	(Bacon <i>et al.</i> , 2007)
	0.5-5	Prostate (human)	(Brooks <i>et al.</i> , 2001)
	0.2	Liver (mouse)	(Posner <i>et al.</i> , 1994)
Inhibition of histone deacetylation	15	Prostate (human)	(Myzak <i>et al.</i> , 2006)
	5-25	Breast (human)	(Pledgie-Tracy <i>et al.</i> , 2007)
Cell cycle arrest	15	Colon (human)	(Gamet-Payraastre <i>et al.</i> , 2000)
	25-50	Colon (human)	(Jakubikova <i>et al.</i> , 2005)
	10-40	Prostate (human)	(Herman-Antosiewicz <i>et al.</i> , 2007)
	15	Breast (human)	(Jackson & Singletary, 2004)
	30	T-cells (human)	(Fimognari <i>et al.</i> , 2002)
Apoptosis	15	Colon (human)	(Gamet-Payraastre <i>et al.</i> , 2000)
	10-20	Prostate (human)	(Choi <i>et al.</i> , 2007; Herman-Antosiewicz <i>et al.</i> , 2007)
	30	T-cells (human)	(Fimognari <i>et al.</i> , 2002)
Inhibit cell growth	27	Bladder (human)	(Shan <i>et al.</i> , 2006)
	40	Ovarian (human)	(Chaudhuri <i>et al.</i> , 2007)
	8-10	Breast (human)	(Pledgie-Tracy <i>et al.</i> , 2007)
	15	Breast (human)	(Jackson & Singletary, 2004)
	10-30	T-cells (human)	(Fimognari <i>et al.</i> , 2002)
	12	Bladder (human)	(Tang <i>et al.</i> , 2006)

In **Phase I reactions**, a small polar group (containing both positive and negative charges) is either exposed on the toxicant or added to the toxicant. The three main Phase I reactions are oxidation, reduction, and hydrolysis. The most common enzymatic process is through the addition of oxygen catalyzed by cytochrome P450 enzymes. Some of the intermediates formed in this process may be electrophiles, which can react with nucleophilic sites in critical macromolecules such as DNA, RNA and protein and form a covalent binding product called adducts. DNA adducts that persist unrepaired can cause miscoding and thus produce mutations in critical genes.

A xenobiotic that has undergone a Phase I reaction is now a new intermediate metabolite that contains a reactive chemical group, e.g., hydroxyl (-OH), amino (-NH₂), and carboxyl (-COOH). Many of these intermediate metabolites do not possess sufficient hydrophilicity to permit elimination from the body. These metabolites must undergo additional biotransformation as a Phase II reaction.

Phase II reactions are conjugation reactions, that is, a molecule normally present in the body is added to the reactive site of the Phase I metabolite. The result is a conjugated metabolite that is more water-soluble than the original xenobiotic or Phase I metabolite. Usually the Phase II metabolite is quite hydrophilic and can be readily eliminated from the body. Some of the Phase II enzymes are quinone reductase, glutathione-S-transferase, UDP-glucuronosyl transferase and sulfotransferase.

Blocking carcinogen metabolic activation by Phase I enzymes and enhancing carcinogen detoxification by Phase II enzymes are two ways to decrease carcinogenicity (Fimognari *et al.*, 2008).

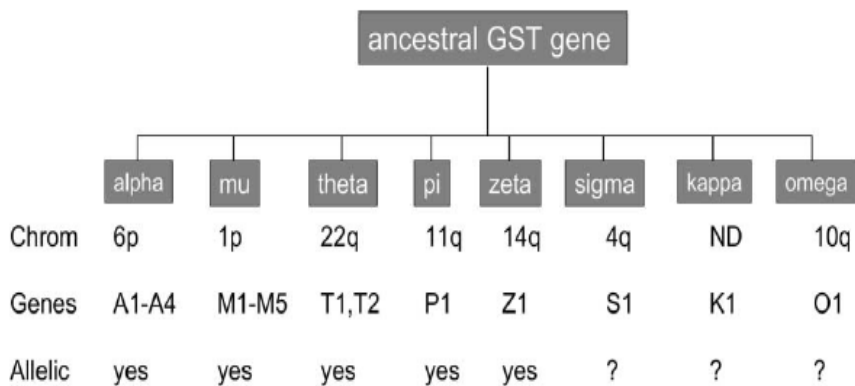


Figure 3.1.3 *The glutathione-S-transferase supergene family, (adapted from (Strange et al., 2001) On the basis of sequence homology and immunologic cross-reactivity, human cytosolic GSTs have been grouped into seven families, designated GST- α , - μ , π , σ , ω , θ , and ζ . The GST- μ family is encoded by a 100-kb gene cluster at 1p13.3 arranged as 5'-GSTM4-GSTM2-GSTM1-GSTM5-GSTM3-3'. Deletion of the GSTM1 gene, GSTM1*0, frequently affects both alleles, resulting in the so-called null genotype, GSTM1-/- (Roodi et al., 2004).*

In recent years, evidence has accumulated to support hypothesis that genetic **polymorphisms** in carcinogen-metabolizing enzymes may be of importance in determining individual susceptibility to cancer. The Glutathione S-transferases (GSTs), a family of multifunctional enzymes (Figure 3.1.3), metabolize a variety of xenobiotics with a large overlap in substrate specificity. Individuals who are homozygous for the null GSTM1 or null GSTT1 alleles lack the respective enzyme functions. The null GSTM1 genotype appears to be common in both Asians and Caucasians, whereas the null GSTT1 genotype exhibits population frequencies that depend on ethnicity. The GSTM1 and GSTT1 defects seem to be associated with increased risk of certain cancers; however, conflicting data have been observed (Chen *et al.*, 1996; Qiu *et al.*, 2010; Wan *et al.*, 2010). This may reflect the fact that certain GSTs may be more efficient in detoxifying certain carcinogens, but others may result in a higher risk for some types of exposure, which has been especially demonstrated with GSTT1 (Huang *et al.*, 2000; Agalliu *et al.*, 2006; Lampe, 2007).

DNA repair and defence systems

Mammals have five types of DNA repair system (Hoeijmakers, 2001; Larsen *et al.*, 2005):

1. Direct reversal (DR) corrects rather than removes damaged DNA bases;

2. Base-excision repair (BER) corrects DNA damage caused by reactive oxygen species, deamination, and hydroxylation arising from cellular metabolism and spontaneous depurination.
3. Nucleotide-excision repair (NER) removes lesions that distort the structure of the DNA helix, such as pyrimidine dimers and DNA adducts.
4. Recombinational repair (RER). Homologous recombination and non-homologous endjoining repair double-strand breaks. Homologous recombination is used when a second identical DNA copy is available, for example after replication; non-homologous end-joining re-links the 'broken' ends of a double-strand break.
5. DNA mismatch repair (MMR) detects and repairs copying errors made during replication.

One protective mechanism of action of *Brassica* vegetables is thought to involve reduction of free radical-related molecular damage and this could be particularly important in subjects with high exposure to oxidative stress. Cigarette smoke contains a large amount of reactive oxygen species (ROS) as well as other substances which generate ROS (Frei *et al.*, 1991). ROS can cause oxidative damage to DNA such as oxidized bases and strand breaks. These DNA lesions, including the promutagenic 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) could be implicated in early steps of cancer development (Evans *et al.*, 2004; Cooke *et al.*, 2006). Fortunately, mammalian cells have a large array of repair systems for the removal of oxidized DNA lesions; the most important pathway for oxidized bases is considered to be base excision repair (BER), whereas the nucleotide excision repair enzymes most likely functions as a back-up system. The repair of 8-oxodG by the BER system is initiated by the enzyme 8-oxoguanine DNA glycosylase (OGG1), which cleaves the N-glycosidic bond leaving behind a free base and an AP-site (apurinic/apyrimidinic site), as shown in Figure 3.1.4 (Moller *et al.*, 2003; Loft & Moller, 2006; Tudek *et al.*, 2006).

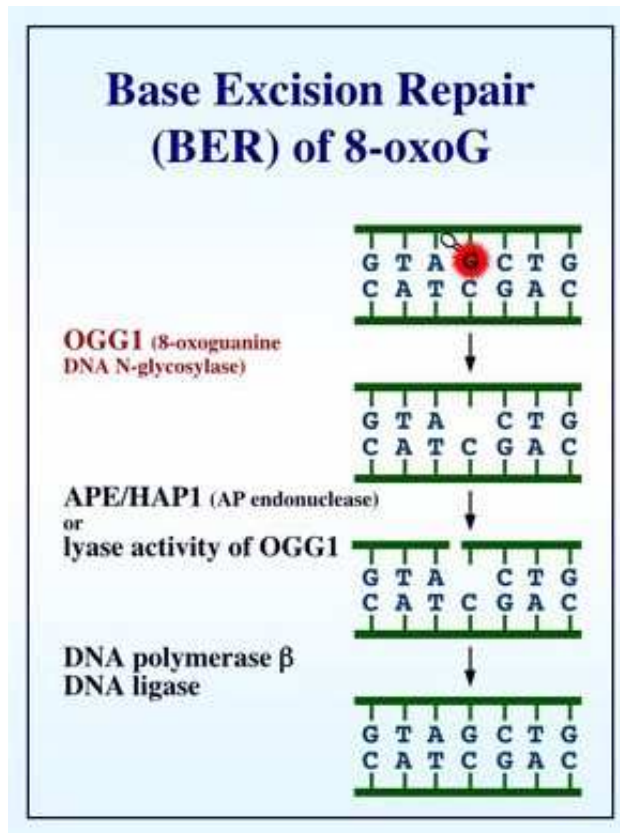


Figure 3.14 *OGG1 cleaves the glycosylic bond between 8-oxoG and the deoxyribose, thereby releasing the damaged base from DNA. The remaining abasic site is then cleaved 5' to the lesion by an AP endonuclease, APE, also called HAP1, or by the lyase activity of OGG1. The dRPase (deoxyribose phosphate lyase) activity of DNA polymerase β cleaves 3' to the lesion, and the missing nucleotide is inserted by DNA polymerase β , followed by ligation. Additional repair proteins are involved in this process.*

Nucleotide pool modification is an important source of nucleic acids damage. Intracellular free nucleotides play essential roles as precursors in the synthesis of DNA and RNA, and as molecules for energy storage, cofactors of metabolic pathways and regulators of signal transduction. Free nucleotides can, however, undergo various chemical modifications by endogenous and exogenous reactive molecules, some of which are inevitably produced in living cells (Iyama *et al.*, 2010).

In the nucleotide pool, dGTP can be oxidized to 8-oxo-2'-deoxyguanosine 5'-triphosphate (8-oxo-dGTP), which may give rise to 8-oxodG in DNA if the oxidized nucleotide is incorporated during replication or repair. 8-OxodGTP is removed from the nucleotide pool by the nucleoside diphosphate linked moiety X-type motif 1 (NUDT1) enzyme (Loft & Moller, 2006), as described in Figure 3.1.5. Misincorporation of oxidized nucleoside triphosphates into DNA/RNA during replication and transcription can cause mutations that may result in carcinogenesis or

neurodegeneration. The protein encoded by this gene is an enzyme that hydrolyzes oxidized purine nucleoside triphosphates, such as 8-oxo-dGTP, 8-oxo-dATP, 2-hydroxy-dATP, and 2-hydroxy rATP, to monophosphates, thereby preventing misincorporation. The encoded protein is localized mainly in the cytoplasm, with some in the mitochondria, suggesting that it is involved in the sanitization of nucleotide pools both for nuclear and mitochondrial genomes.

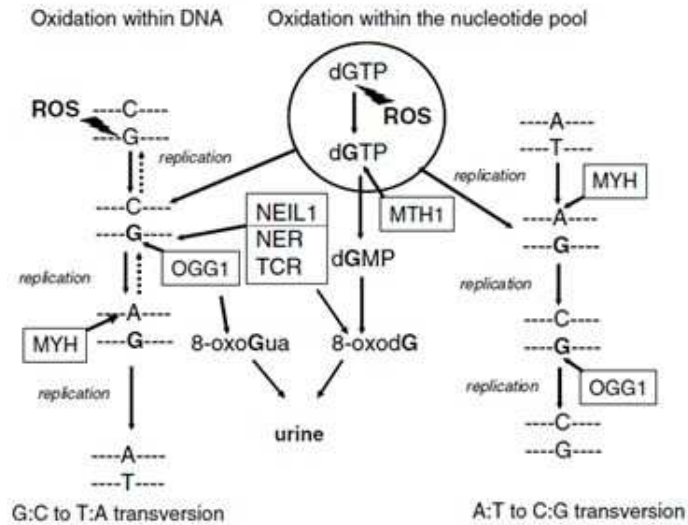


Figure 3.1.5 Oxidation of guanine in DNA and the nucleotide pool with consequences in terms of potential mutations as well as the involved repair pathways. NER, nucleotide excision repair; TCR, transcription coupled repair. Bold G indicates oxidized guanine (Loft & Moller, 2006).

Heme oxygenase-1 (**HO-1**) is responsible of the degradation of heme to biliverdin and is a well known stress response protein inducible by various agents able to generate ROS and thereby causing oxidative stress (e.g. U.V., X-ray radiation, oxidants such as H₂O₂, heavy metals, pro-inflammatory mediators). HO-1 is found over-expressed in various tissues affected by diseases such as atherosclerosis, hypertension, lung injury and cancer. Thus the level of HO-1 mRNA can be considered an important oxidative stress response and defence enzyme (Risom *et al.*, 2003).

3.2 Materials and methods

3.2.1 Characteristics and analyses of the product

"Marathon" broccoli (*Brassica oleracea* L. *Var italica*) was used for this intervention study. It was a kind gift from Di Stasi Company, Italy. The company guaranteed that the whole amount of vegetables used were from a single batch of production (same condition of growth). The vegetables arrived at the DiSTAM laboratory one day after the harvest and were immediately processed at the Experimental Institute of Agricultural Products and Technologies (IVTPA, Milan). Broccoli florets were handled

gently, excised from the main stem, immediately blanched (high temperature steaming of vegetables to deactivate enzymes) and, after cooling at room temperature for some minutes, frozen at -25°C. Aliquots of broccoli (raw and steamed) were lyophilized and finely ground before their analytical characterization.

Analysis of Carotenoids

Lyophilized broccoli (50 mg) were extracted at least four times (until colourless) in an ultrasonic bath with 5 mL tetrahydrofuran (THF), vortexed for 3 min and centrifuged at 1300 x g for 10 minutes.

The supernatants were combined, dried under nitrogen and stored at -80°C until HPLC analysis. The residue was dissolved in 100 µL eluent (MetOH:THF, 95:5) and put into dark vials for HPLC analysis. The HPLC system (Waters- Alliance and DAD) was equipped with a Vydac 201TP54 (250 x 4.6 mm) column. The elution was carried out by linear gradient using MetOH and THF as eluents, the gradient was: MetOH 100% for 5 min; MetOH:THF (95:5) for 15 min and MetOH:THF (85:15) for 10 min. The flow rate was 1.2 mL/min. Lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene were acquired at 445 nm wavelength. Lycopene was acquired at 472 nm. The standards were from SIGMA (Taufkirchen, Germany) and Roche (Basel, Switzerland).

Analysis of Vitamin C

About 5 g of freshly steamed broccoli ready to eat were gently chopped with a fork, and the vitamin quickly stabilized with metaphosphoric acid (MPA 10%) and vortexed for about 5 min. The final volume was 50 mL; the supernatant was centrifuged for 5 min at 2000 x g and immediately injected into HPLC system. Samples were compared with an external calibration curve (5-20 µg/mL; 14-114 µmol/L) of ascorbic acid (Sigma) freshly prepared with MPA 10%.

HPLC quantification was performed by injecting 50 µL into the HPLC system: pump Waters 510; detector UV-Vis Waters mod 486MS set at 245 nm; column C₁₈ Atlantis 5 µm 250 x 4.6 mm Waters; eluent 0.1% formic acid flow 1.4 mL/min.

Analysis of Isothiocyanates (ITCs) derived glucosinolates

Broccoli samples were analyzed for content in non-indolyl glucosinolates measuring related isothiocyanates through the cyclocondensation reaction according to (Conaway *et al.*, 2000). Indole glucosinolates were not measured as, with this method, their hydrolysis products decompose spontaneously (Shapiro *et al.*, 1998).

Samples of lyophilized powder (0.2 g) were weighed in 10 mL glass tubes and extracted with 3.5 mL MetOH:water (70:30, v/v). Samples were heated in a heating bath at 70°C for 10 min. The mixture was centrifuged (1500 x g; 10 min, 4°C), supernatant was removed and pellets were re-extracted twice: the first one with MetOH:water 70:30 and the last one with only water. All supernatants were combined and made up to a final volume of 10 mL. This extract underwent the cyclocondensation reaction and ITCs were quantified after myrosinase treatment. One mL of extract was incubated with 1 mL myrosinase (Sigma T4528), 0.35 U/mL in 0.1 mol/L potassium phosphate buffer pH 6.6 at 37°C for 2 h, 100 µL of mixture were used for the cyclocondensation reaction. The cyclocondensation reaction was performed in 5 mL tubes, 0.1 mL of supernatant was added to 0.4 mL distilled water, 0.5 mL of 100

mmol/L potassium phosphate buffer pH 8.5 and 1 mL of 20 nmol/L 1,2 benzenedithiole (SIGMA 27086) in acetonitrile; the mixture was incubated for 2 h at 65°C. Once derivatized, samples were immediately loaded into an auto-sampler and injected, after filtration with 0.45 µm PTFE filters. 1,3 benzenedithiole-2-thione peak was eluted isocratically with 80% MeOH and 20% water (flow rate 1.8 mL/min) on a Whatman Partisil 10 ODS-2 reverse-phase (4.6 x 250 mm) column equipped with Whatman guard cartridges RP. The HPLC system was an Alliance Waters 2996 coupled with DAD photodiode array detector 2996; the acquisition was performed at 365 nm wavelength. The peak of 1,3 benzenedithiole-2-thione was identified on the basis of retention time and UV spectra. Samples were compared with an external standard curve by using concentrations of 100 ng/mL (0.61 µmol/L), 250 ng/mL (1.53 µmol/L), 500 ng/mL (3.06 µmol/L), 1000 ng/mL (6.13 µmol/L) of PEITC (phenethyl isothiocyanate, Sigma 253731) in acetonitrile that was treated with cyclocondensation reaction as the samples. Results were expressed as µmol/L PEITC equivalents.

3.2.2 Regular-intake dietary intervention study

Subjects

Healthy males were recruited from the student population of the Università degli Studi di Milano and enrolled on the basis of their food habits, evaluated by means of a food frequency questionnaire (APPENDIX 3), and GSTM1 genotype. Subjects with high (>5 portions/day) or low (<2 portions/day) intake of fruit vegetables and those who followed macrobiotic or other alternative diets and who took supplements were excluded. Smoker males were included in the study if they smoked more than 10 cigarettes/day. The study was proved by the Local Ethical Committee and was in accordance with the Declaration of Helsinki. Informed consent was signed by each participant, who received a fee for the participation to the intervention study.

Polymorphisms analysis/GSTM1 genotyping

A PCR method was used to detect the presence or absence of the *GSTM1* gene in genomic DNA samples obtained from blood samples through DNeasy® Blood and Tissue kit (QIAGEN) as reported on handbook.

Then genomic DNA was amplified in a total reaction volume of 25 µl containing 4 µl of DNA, 1,5 mM dNTPs, 1.5 mM MgCl₂, 5 pmol of each oligonucleotide primer and 0.12 µl (5U/µl) TaqGOLD. DNA samples were amplified using the following primers: for *GSTM1* forward 5'-GGG CTCA AAT ATA CGG TGG A and reverse 5'- GGA GGA ACT CCC TGA AAA GC; for albumine forward 5'- AAA GCC AGA GCT GGA AGT CA and reverse 5'- CAG CTT TGG GAA ATC TCT GG; with a PCR product of 233 and 349 bp respectively.

PCR was performed with: TaqGOLD activation for 5 min at 95°C, the temperature profile repeated for the first two cycles was: denaturing 30 sec at 95°C, annealing 15 sec at 60°C and extension 30 sec at 72°C; for other 2 cycles: denaturing 30 sec at 95°C, annealing 15 sec at 58°C and extension 30 sec at 72°C; for other 2 cycles: denaturing 30 sec at 95°C, annealing 15 sec at 56°C and extension 30 sec at 72°C; and 29 cycles with

denaturing 30 sec at 95°C, annealing 15 sec at 54°C and extension 30 sec at 72°C; final extension for 5 min at 72°C and storage at 4°C. The amplified products were electrophoresed through 2% agarose gel and visualized by ethidium bromide staining.

Experimental design

A single blind randomized cross-over dietary intervention study was scheduled as represented in Figure 3.2.2.1. Thirty subjects were randomly divided into two groups of 15 subjects each: group 1 was assigned to the sequence broccoli diet/wash-out/control diet, whereas group 2 followed the sequence control diet/wash-out/broccoli diet. In this way each participant was his own control, which excluded confounding by factors that are stable within an individual over time but vary among subjects. Broccoli and control diet were 10 d long and they were spaced out from a 20 d wash-out period between dietary treatments. During the control diet subjects could consume their habitual diet avoiding the intake of cruciferous vegetables. A list of not allowed vegetables was given and a dietary daily diary was demanded during both the broccoli and control diet periods (APPENDIX 3).

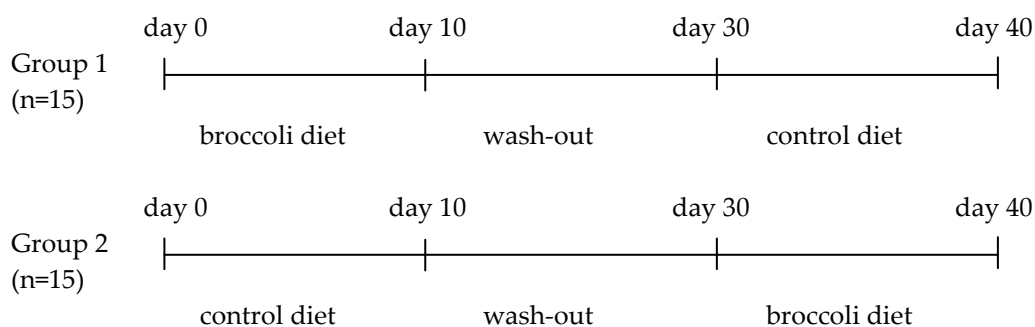


Figure 3.2.2.1 Scheme of cross-over dietary intervention trial

Diet

During the period of supplementation with broccoli, subjects received a daily portion (250 g) of “Marathon” broccoli (*Brassica oleracea* L. var. *italica*) frozen as previously described. During the trial, every day a defined amount of broccoli was steam-cooked for 15 min and portioned into appropriate food containers. On Friday subjects were given with two portions to eat during the weekend.

Blood samples

Blood samples were collected at the beginning and at the end of each treatment period (0, 10, 30, 40 d), early in the morning after overnight fasting. Samples were drawn into microtubes with heparin as the anticoagulant. Lymphocytes were separated by density gradient centrifugation of 100 μ L whole blood with Histopaque 1077 (Sigma Chemicals Co, St Louis, MO, USA). The lymphocyte layer was removed from the gradient, washed with Phosphate Buffer Saline (PBS, Sigma), centrifuged and used immediately for the determination of cell resistance to an oxidative stress (H_2O_2 -induced DNA

strand breaks). Additional lymphocytes were isolated, diluted into a solution containing 50% fetal bovine serum (FBS, Gibco, Gaithersburg, MD, U.S.A.), 40% culture medium (RPMI 1640, Gibco) and 10% dimethylsulfoxide (DMSO, Sigma) and stored at -80°C for the subsequent determination of: oxidized bases (i.e. formamidopyrimidine DNA glycosylase (FPG) sensitive sites), OGG1 DNA repair activity and mRNA expression levels of OGG1, HO-1 and NUDT1.

Detection of Biomarkers of Oxidative Stress

Hydrogen peroxide sensitivity estimation by the Comet assay

The resistance of lymphocytes to DNA oxidative stress was evaluated by means of the Comet assay, already applied in previous studies (Riso *et al.*, 1999; Riso *et al.*, 2004).

The measurement of H₂O₂-induced DNA strand breaks is considered to be a biomarker of the cellular resistance to oxidative stress because H₂O₂ generates DNA damage through oxidative attack.

Separated lymphocytes were fixed with agarose on fully frosted slides (Richardson Supply Co, London, U.K.). Two slides for each subject were prepared. One slide was subjected to a H₂O₂ treatment (submerging in a solution of H₂O₂ 500 µmol/l in PBS for 5 min in the dark) while the other one acted as a control (submerging in PBS for 5 min). Slides were put in lysis buffer (0.1 M Na₂EDTA; 10 mM TRIS; 2.5 M NaCl; 1 % sarcosine; pH 10 with NaOH; 1% triton X-100 and 1% DMSO added just before use) for 1 h at 4°C and then were left for 40 min in electrophoresis buffer alkaline solution (10 M NaOH, 0.2 M Na₂EDTA, pH>13) in a horizontal electrophoresis tank, prior to electrophoresis in the same solution at 25 V, 300 mA for 20 min in the dark. Slides were successively neutralized to pH 7, stained with ethidium bromide (2 µg/ml), washed in PBS, drained, and lastly covered with coverslips. One hundred cells for each slide were electronically captured using an epifluorescence microscope (Olympus CX 41, Olympus Italia) attached to a high-sensitivity CCD video-camera (CFW 1808M, Scion Corporation, Germany) and to a computer provided with an image analysis system (Cometa 1.5, Comet Program developed and improved on Image Pro Plus 5.1; Immagini e Computer, Bareggio, Milan, Italy). Levels of strand breaks were calculated as percentage DNA in tail. For each subject, the percentage DNA in tail of control cells (not treated with H₂O₂, i.e. background strand breaks, SBs in PBS) was subtracted from the percentage DNA in tail of treated cells.

FPG-sensitive sites estimation by the Comet assay

Detection of oxidative endogenous DNA base damage was performed by means of the enzyme FPG, which introduces breaks at sites of oxidized purines such as 8-oxo-2'-deoxyguanosine (8-oxodG, the major purine oxidation product), as well as other altered purines. The European Comet assay Validation Group demonstrated that the intra-assay variation of the comet assay is very low and we could detect dose-dependent increase in FPG sites in coded samples (Forchhammer *et al.*, 2010).

The concentration of FPG enzyme was selected after a dose-finding test through the Comet assay. Cryo-preserved lymphocytes were thawed and washed with fresh RPMI and cold PBS and then fixed with agarose on fully frosted slides to perform the Comet

assay as above described. After the lysis phase, slides were immersed in Endo Buffer (EB, 40 mM HEPES; 0.1 M KCl; 0.5 mM EDTA; 0.2 mg/ml bovine serum albumine (BSA); pH 8.0 with KOH, used also for the enzyme dilution) for 5 min for 3 times and then added with 50 μ L of a diluted solution of FPG (100 ng/mL) or with buffer alone (control) and incubated at 37°C for 45 minutes. The FPG enzyme was a gift from Prof Andrew Collins (University of Oslo, Norway). FPG added to the DNA in gel converts altered purines into DNA breaks (Collins *et al.*, 1996). Alkaline treatment and electrophoresis then followed. Net enzyme-sensitive sites, calculated by subtracting % DNA in the tail after incubation with buffer alone (EB, background SBs) from the % DNA in the tail with enzyme, indicated the extent of base oxidation.

DNA repair activity

The DNA repair activity was determined in cryo-preserved lymphocytes. Assessment of DNA repair incisions was analysed by a new version of the Comet assay as described by (Guarnieri *et al.*, 2008).

The repair activity was measured as the incision activity of substrate DNA treated with a photosensitiser/white light, which generates 8-oxoG (Collins *et al.*, 2001a; Collins & Horvathova, 2001; Collins, 2004; Guarnieri *et al.*, 2008). Oxidized bases were introduced into A549 lung epithelial cells substrate nuclei by irradiating cells with white light in PBS solution with 1 μ M Ro 19-8022 at 0°C (the photosensitiser was a gift from Hoffman-LaRoche, Basel, Switzerland). The cells were washed and re-suspended in freezing medium (50% FBS, 40% RPMI 1640 and 10 % DMSO) to a concentration of 3×10^6 cells/mL and frozen at -80°C.

For the preparation of human lymphocytes extracts, the cryo-preserved cells were centrifuged (300 g, 5 min, 4°C), and the pellet was re-suspended in buffer A (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, pH 7.8) at a volume of 20 μ L per 10^6 cells. The re-suspended cells were divided in aliquots of 50 μ L to which 12 μ L 1% Triton X-100 was added. The lysate was centrifuged (700 g, 5 min, 4°C) and the supernatant was mixed with 200 μ L buffer B (40 mM HEPES, 0.1 M KCl, 0.5 mM Na₂EDTA, 0.2 mM BSA, pH 8). Approximately 3×10^4 substrate cells were embedded in agarose and applied on 85×100 mm GelBond® films and lysed as described for the Comet assay. After 3 times washing (5 min each) in buffer B, repair incisions were detected by incubation of the agarose-embedded nuclei with 60 μ L lymphocytes extract or buffer B for 20 min at 37°C. The subsequent alkaline treatment and electrophoresis were identical to the conditions used to determine DNA damage using the Comet assay. An assay control (a sample for FPG sensitive-sites evaluation) was included in each electrophoresis run.

After neutralisation with 0.4 M Tris-HCl (pH 7.5) cells were placed in 96% ethanol for 1.5h or overnight. Nuclei were visualized as previously described for strand breaks and FPG-sensitive sites estimation after staining with 40 μ l ethidium bromide (4 μ g/ml) in PBS solution. The repair activity of the lymphocytes extract was determined as the difference in %DNA in the tail between parallel gels incubated with extract and control solution.

Quantification of OGG1, HO-1 and NUDT1 mRNA expression in lymphocytes by real time RT-PCR

Lymphocytes samples were stored in freezing medium as above described. Approximately 0.4 µg RNA was used for cDNA synthesis in a reaction volume of 20 µL using the TaqMan GeneAmp RT-PCR kit as recommended by Applied Biosystems (Nærum, Denmark). Quantative PCR reactions were carried out in ABI PRISM 7900HT (Applied Biosystems), using primers and cDNA-specific probes purchased from Applied Biosystems. We used as the reference gene 18S rRNA, which is commercially available as a probe and primer solution (Eukaryotic 18S rRNA Endogenous Control, 4352930E; Applied Biosystems). Below are probes and primers for the genes. Sequence accession ID numbers are from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>; accessed 12 February 2007): *hHO-1*: forward primer: 5'-CAT GAG GAA CTT TCA GAA GGG C-3'; reverse primer: GAT GTG GTA CAG GGA GGC CAT-3; TaqMan probe: 5'-6-FAM-TGA CCC GAG ACG GCT TCA AGC AGC TG-TAMRA-3' (NM_002133). *hOGG1*: forward primer: 5'-AAA TTC CAA GGT GTG CGA CTG-3'; reverse primer: 5'-GCG ATG TTG TTG TTG GAG GA-3'; TaqMan probe: 5'-6-FAM-CAA GAC CCC ATC GAA TGC CTT TTC TCT TTTAMRA- 3' (U96710). *hNUDT1*: forward primer: 5'-CAT CGAGGA TGG GGC TAG -3'; reverse primer: CAG AAG ACA TGC ACG TCC ATG A-3'; TaqMan probe: 5'-6-FAM-TCG CCC ACG AAC TCA AAC ACG ATC T-TAMRA-3' (D16581). We performed the PCR reactions in triplicate using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. For the PCR reaction the following protocol was used: activation of TAQ polymerase for 20 sec at 95°C, followed by a total of 45 temperature cycles for 0.01 sec at 95°C and 20 sec at 60°C. In each run a standard was included and verified on the efficiency plot, and the variation coefficients of the repeated measurements were 2.98% (Brauner *et al.*, 2007).

Determination of carotenoids (lutein and β-carotene) in plasma

Fasting blood samples were drawn into Vacuntainers™ containing heparine as anticoagulant. Plasma was obtained by centrifugation at 250 g for 15 min at 4°C. After centrifugation the samples were stored at -80°C in aliquots for no longer than 2 months. Carotenoids were extracted from plasma using the method developed by (Porrini *et al.*, 1998). Briefly, the compounds were extracted from 100 µL of plasma with hexane after a first step of deproteinization with absolute ethanol containing echineone as internal standard. After extraction, the solvent was evaporated under N₂ and samples stored at -80°C until HPLC analysis. HPLC conditions were the same as used for the determination of carotenoids in broccoli samples. Recoveries were between 80 and 100%; coefficients of variation were lower than 8% for both the carotenoids.

Determination of folate in serum

Fasting blood samples were drawn into Vacuntainers™ containing silicone. Samples were allowed to clot for 30 minutes before centrifugation for 15 min at 250 g at 4°C. Serum was immediately moved, aliquoted and stored at -80°C. Analysis of folate in

serum was performed using AIA-PACK FOLATE enzyme immunoassay kit (Tosoh Bioscience; Inc; Grova City U.S.A.).

Statistical analysis

An analysis of variance (ANOVA) with sequence (broccoli intake vs control diet or *vice versa*) as independent factor was used in order to evaluate the presence of a carry-over effect. As it was not detected (always $p > 0.05$), data from the two groups of subjects were considered independently from the sequence of treatment and analyzed together using ANOVA for repeated-measure design. This analysis with type of treatment and time as independent factors was used to investigate the effect of broccoli consumption on: cell resistance against induced DNA oxidative stress, levels of FPG-sensitive sites, OGG1 DNA repair activity, OGG1, HO-1 and NUDT1 mRNA expressions.

Data were also stratified by considering polymorphisms in GSTM1 gene as independent factor to evaluate the genetic susceptibility of these variables.

Post-hoc analyses of differences between mean values of treatments or genotypes were further assessed by Fisher Least Significant Difference (LSD) test. Differences were considered significant at $p < 0.05$. Regression analysis was applied to verify correlation between % changes in plasma carotenoids and serum folate concentrations registered in the four points of experimentation and the % changes in the end point of DNA damage considered. Statistical analyses were performed by means of STATISTICA 5.0 software (Statsoft Inc, Tulsa, OK).

3.2.3 Single-meal study

Experimental design

Twelve volunteers were selected for the “single-meal” study. Volunteers were divided into 4 groups of 3 subjects. Each group was analyzed the same day, as reported in Figure 3.2.1.1.

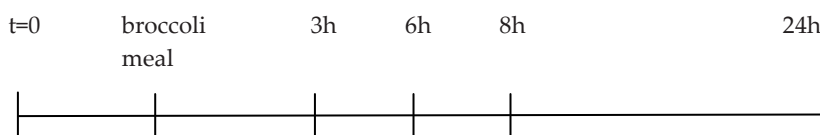


Figure 3.2.3.1 Scheme of single-meal intervention study for each group of 3 subjects.

Diet

For the experimentation “Marathon” broccoli (*Brassica oleracea* L. var. *italica*) were steamed under standardized condition (10 min), portioned and served to the volunteers, together with 70 g pasta (“Penne Rigate”, Barilla, Foggia, Italy). One serving was consumed as first meal at 9.30 in the morning.

Throughout the experimental period, subjects were instructed to observe some rules and to avoid smoking 30 min before blood collection.

Blood samples

Fasting blood samples were collected before broccoli and pasta consumption and then at 3, 6, 8 and 24h after broccoli intake in Vacuntainers™ containing heparine as anticoagulant. Lymphocytes were isolated as described in paragraph 3.2.2 for detection of Oxidative DNA damage with the Comet assay. Additional lymphocytes samples were stored at -80°C in PBS and used for GST activity quantification after three cycles of freezing/thawing. Plasma was obtained by centrifugation at 250 x g for 15 minutes at 4°C of whole blood samples. After centrifugation the samples were stored at -80°C in aliquots for no longer than 2 months.

Determination of carotenoids (lutein and β-carotene) in plasma and folate in serum

As reported for “regular-intake” intervention trial.

Determination of vitamin C in plasma and lymphocytes

One hundred microliter MPA (10%) solution were added to 100 µL plasma in duplicate; the samples were vortexed, centrifuged at 2200 x g for 2 minutes in order to separate the supernatant and injected. Lymphocyte pellets were suspended in 150 µL PBS. From each sample four aliquots were obtained. Two aliquots of 40 µL were added with 40 µL MPA (10%) solution and vortexed, while two aliquots of 35 µL were used for protein quantification. All the samples were kept at -80°C until HPLC analysis. Vitamin C concentrations in lymphocytes were expressed adjusting the results for protein concentrations. Then the prepared samples were injected as reported for the analysis of vitamin C in broccoli (paragraph 3.2.1).

Detection of Oxidative DNA damage in lymphocytes with the Comet assay

Hydrogen peroxide sensitivity estimation was performed on freshly lymphocytes as described in paragraph 3.2.2 for “regular-intake” study.

GST activity in plasma and lymphocytes

Plasma and lymphocytes were separated as previously described for the “regular-intake” study.

GST analysis was performed by TECAN spectrophotometer Infinite F200 a microplate reader and a 96-well plate (Greiner 96 wells, crystal clear, 500x346x176mm).

Total GST activity in plasma and lymphocyte was calculated by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. The reaction is accompanied by an increase of absorbance at 340nm. CDNB solution (20 mM CDNB in EtOH 95%) and GSH solution (20 mM GSH distilled water) were diluted 1:16 with buffer solution (0.1 M KH₂PO₄, 1mM EDTA in distilled water; pH 6.5). In each well 88 µL CDNB and 88 µL GSH solutions were gently mixed and added with 50 µL of plasma or lymphocytes samples or buffer solution. Reaction was monitored for 5 min at 340 nm. GST activity obtained was standardized to mg protein plasma or lymphocytes content.

Protein quantification

Proteins were analyzed by BCA™ Protein Assay Kit (Pierce, rockford, IL). The assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. This method combined the well-known reduction of Cu⁺² to Cu⁺¹ by protein in alkaline medium with the highly

sensitive and selective colorimetric detection of the cuprous cation (Cu⁺¹) using a reagent containing BCA.

Statistical analysis

An analysis of variance (ANOVA) was performed with time as independent factor was used to investigate the effect of broccoli consumption on: cell resistance against induced DNA oxidative stress, GST activity, antioxidant compound concentrations.

Data were also stratified by considering polymorphisms in GSTM1 gene as independent factor to evaluate the genetic susceptibility of these variables.

Post-hoc analyses of differences between mean values of treatments or genotypes were further assessed by Fisher Least Significant Difference (LSD) test. Differences were considered significant at p<0.05. Statistical analyses were performed by means of STATISTICA 5.0 software (Statsoft Inc, Tulsa, OK).

3.3 Results and discussion TOPIC I

3.3.1 Characteristic and analysis of the product

In table 3.3.1.1 the content of vitamins and protective compounds in steamed broccoli are summarized. We chose the portion of 250 g of broccoli after the quantification of glucosinolates as non-indolyl ITCs to provide 160 µmol of ITCs. One serving of steamed broccoli provided very low amount of carotenoids in particular lutein and beta-carotene, high amount of vitamin C (three time the italian recommended daily intake) and a discreet amount of isothiocyanates by considering data in literature (200 µmol).

Table 3.3.1.1 *Lutein, β-carotene, vitamin C and isothiocyanates content in one portion of steamed broccoli (mean ± SD). (*) Data are expressed as µmol PEITC (phenethyl isothiocyanate) equivalents.*

Steamed broccoli	mg/portion
Lutein	2.75±0.10
β-Carotene	1.50±0.03
Vitamin C	172.00±3.25
Isothiocyanates	160.00±0.05*

3.3.2 Regular-intake dietary intervention study

Characteristics of subjects

Twenty-seven male smokers of the 30 enrolled completed the “regular-intake” study (age 22.1±2.5 y; BMI 23.0±2.7 kg/m²); while 3 volunteers dropped out during the experimental period. Fourteen were GSTM1 positive and 13 were GSTM1 null genotype (Figure 3.3.2.1).

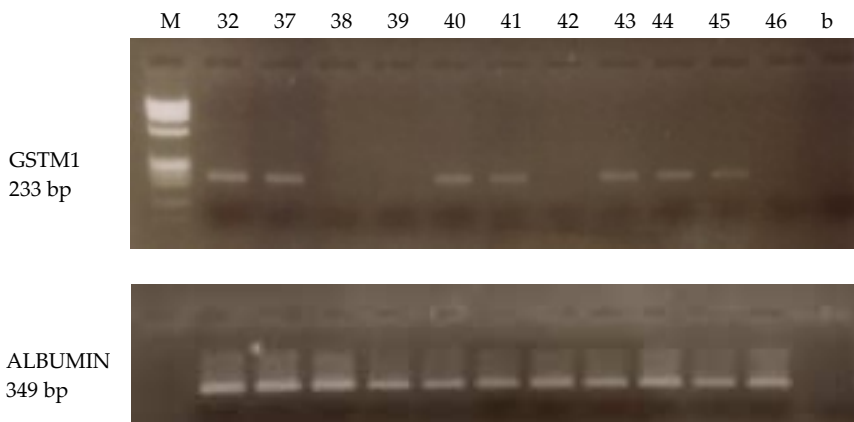


Figure 3.3.2.1 Example of image of gel electrophoresis of PCR for GSTM1 determination.
M: DNA molecular marker
Number of subjects
b: blank, no template sample

No side effects were observed in the whole group of subjects.

In table 3.3.2.2 characteristic of subjects at recruitment are summarized.

Table 3.3.2.2 Characteristics of subjects at recruitment (mean value \pm SD)

Characteristics	Number of subjects	Results
Age (years)	27	22.1 \pm 2.5
BMI (kg*m ⁻²)	27	23.0 \pm 2.7
H ₂ O ₂ - induced DNA strand breaks (% DNA in tail)	27	62.9 \pm 8.8
Oxidized purines (% DNA in tail)	19	14.0 \pm 6.3
OGG1 repair activity	11	1.9 \pm 2.3
NUDT1/18S mRNA (*10 ⁻⁶)	17	0.5 \pm 1.3
OGG1/18S mRNA (*10 ⁻⁶)	17	4.6 \pm 5.8
HO-1/18S mRNA (*10 ⁻⁵)	17	1.6 \pm 3.0

BMI: body mass index;

NUDT1: human nucleoside diphosphate linked moiety X-type motif 1;

OGG1: human 7,8-dihydro-8-oxoguanine-DNA glycosylase;

HO-1: human heme oxygenase 1;

18S: reference gene 18S rRNA.

Oxidative lymphocyte DNA damage

The level of H₂O₂-induced DNA strand breaks in lymphocytes decreased by 23% [95% CI: +13%, +34%] in the period of broccoli intake as compared to the control diet. This difference was driven by a reduction in the level of H₂O₂-induced DNA strand breaks after the broccoli intake period (-22%, [95% CI: -13%, -31%]), whereas no difference was detected after the control diet. (-0.4%, [95% CI: -10%, +9.4%]). The levels of FPG-sensitive sites and H₂O₂-induced strand breaks are reported in Table 3.2.4.3. The subjects had 41% [95% CI: +10%, +72%] lower level of FPG-sensitive sites after 10 days of broccoli consumption with respect to the control diet. The difference was driven by decreased level of FPG sites in the period of broccoli intake (-34%, [95% CI: -53%,

+15%]); whereas it was unaltered during the control diet period (+4%, [95% CI: -20%, +28%]). Analysis of effect-modification by the GSTM1 genotype revealed significant single-factor effects of the genotype ($p < 0.05$) and diet ($p < 0.01$) on H₂O₂-induced DNA strand breaks, whereas the interaction was not statistically significant ($p > 0.05$). The subjects with GSTM1 null genotype had higher baseline levels of H₂O₂-induced strand breaks (Table 3.3.2.3), however at the end of the 10 days broccoli diet there was no difference in the resistance to *ex vivo* generation of strand breaks depending on the genotype. Consequently, the effect on resistance towards *ex vivo* generation of strand breaks following broccoli intake (% changes) was most pronounced in GSTM1 null subjects (-27%, [95% CI: -37.9%, -17.4%]), whereas the subjects with GSTM1 positive genotype had lower effect on the level of resistance (-13%, [95% CI: -27.3%, +1.1%]).

Table 3.3.2.3 Results of “regular intake” study (Mean value±SD).

	Bbd	Abd	Bcd	Acid
Background SBs in PBS (% DNA in tail)				
All subjects (n=27)	7.5±2.6	7.7±2.3	7.4±2.8	7.4±2.6
H₂O₂-induced DNA SBs (% DNA in tail)				
All subjects (n=27)	61.4±11.1	47.9±11.4 ^c	55.4±13.3	55.2±9.6
GSTM1 positive (n=14)	57.6±11.8	48.8±12.1 ^a	52.4±12.7	54.0±9.4
GSTM1null (n=13)	65.4±9.1	46.9±11.1 ^c	58.7±13.7	56.5±10.1
Background SBs in EB (% DNA in tail)				
All subjects (n=19)	23.0±8.0	21.3±6.5	21.8±8.8	19.8±8.0
Oxidized purines (%DNA in tail)				
All subjects (n=19)	16.5±7.7	11.0±5.4 ^b	13.2±9.8	13.8±7.5
GSTM1 positive (n=9)	17.3±7.2	11.1±5.2 ^a	17.1±12.0	16.1±9.4
GSTM1 null (n=10)	15.9±8.4	10.9±5.9 ^a	9.8±6.0	11.6±4.8
DNA repair activity (%DNA in tail)				
All subjects (n=11)	3.7±3.0	3.6±2.5	3.0±3.0	4.4±3.1
OGG1/18S mRNA (*10 ⁻⁶)				
All subjects (n=17)	6.4±12.4	5.4±6.3	4.2±3.9	6.2±5.5
HO-1/18S mRNA (*10 ⁻⁵)				
All subjects (n=17)	3.3±9.0	2.2±4.5	1.5±2.7	1.8±1.6
NUDT1/18S mRNA (*10 ⁻⁶)				
All subjects (n=17)	1.6±5.5	0.8±2.0	0.6±1.3	0.5±0.7

Bbd: before broccoli diet, Abd: after broccoli diet, Bcd: before control diet, Acid: after control diet.

SBs: DNA strand breaks. NUDT1: human nucleoside diphosphate linked moiety X-type motif 1; OGG1: human 7,8-dihydro-8-oxoguanine-DNA glycosylase; HO-1: human heme oxygenase 1; 18S: reference gene 18S rRNA.

Significant difference between “before” and “after” each treatment: a: $p < 0.05$; b: $p < 0.005$; c: $p < 0.001$.

In contrast, the effect of broccoli consumption on the level of FPG sensitive sites was not statistically related to GSTM1 polymorphism. DNA repair activity was analyzed on lymphocyte samples of 11 subjects (with 7 GSTM1 positive and 4 GSTM1 null genotypes): it did not differ throughout the intervention study. OGG1, HO-1 and NUDT1 mRNA expression evaluated on 17 samples (with 8 GSTM1 positive and 9 GSTM1null genotypes) were not significantly modulated (Table 3.3.2.3).

Carotenoids and folate concentrations in blood

The circulating levels of folate and lutein significantly increased after broccoli consumption (+17.1% and +39.3% respectively), while no significant effect on β -carotene concentration was observed (Table 3.3.2.4).

Regression analysis showed an inverse and significant correlation between changes in serum folate concentrations and changes in H₂O₂-induced strand breaks ($R^2=0.23$; $p<0.001$).

Table 3.3.2.4 Concentrations of folate (in serum), lutein and β -carotene (in plasma) before and after broccoli and control diet. Data are expressed as mean value \pm SD.

	Bbd	Abd	Bcd	Acid
Folate (nmol/L) (n=27)	11.3 \pm 3.9	13.2 \pm 3.3**	12.0 \pm 5.9	12.1 \pm 4.7
Lutein (μ mol/L) (n=27)	0.31 \pm 0.13	0.43 \pm 0.21*	0.30 \pm 0.14	0.29 \pm 0.13
β-carotene (μ mol/L) (n=27)	0.28 \pm 0.25	0.31 \pm 0.22	0.28 \pm 0.24	0.29 \pm 0.29

Bbd: before broccoli diet, Abd: after broccoli diet, Bcd: before control diet, Acid: after control diet.

Significant difference between "before" and "after" each treatment: * $p < 0.001$; ** $p < 0.01$.

3.3.2.1 Discussion of regular-intake study

The present dietary intervention study showed that 10 days of consumption of steamed broccoli (200g/day) by young smokers decreased the level of endogenous oxidized DNA bases and H₂O₂-induced DNA strand breaks in lymphocytes. Whereas, OGG1 repair activity and mRNA expression levels of OGG1, NUDT1 and HO-1 in the same cells were unaltered, at least in the sub-sample of data analyzed. Moreover broccoli diet induced also an increase in folate and lutein concentrations in blood.

These data are in accordance with the fairly consistently shown protective effects of cruciferous vegetables, including Brussels sprouts, broccoli and watercress, on biomarkers of oxidative damage to DNA in human intervention studies (Verhagen *et al.*, 1995; Verhagen *et al.*, 1997; Gill *et al.*, 2004; Murashima *et al.*, 2004; Gill *et al.*, 2007; Hoelzl *et al.*, 2008; Riso *et al.*, 2009b).

Verhagen and colleagues investigated on the effect of consumption of 300g/day of Brussel sprouts in non-smokers healthy volunteers measuring 8-oxodG in 24h urine after 3 weeks in the first study and 1 week in the second one of intake. They found a decrease of 28% in urine 8-oxodG in the first study performed on male subjects (Verhagen *et al.*, 1995); and in the following trial the same effect was determined only in the male volunteers and not in females (Verhagen *et al.*, 1997). Gill *et al.* (2004) designed a dietary intervention trial where 18 subjects (male and female, non-smokers, subdivided into the control and treatment groups) consumed 113g of Cruciferous and Leguminous Sprouts (CLSs, composed of equal mix of sprouts of broccoli, radish, alphaaplha and clover) daily for 14 days. A significant anti-genotoxic effect against H₂O₂-induced DNA damage was shown in peripheral blood lymphocytes of the supplemented diet group respect to the control group; whereas no significant

induction in detoxifying enzymes (GST, GPX and SOD) was observed during the study, neither plasma antioxidant levels or activity were altered (Gill *et al.*, 2004).

Murashima and colleagues the same year investigated the effect of broccoli sprouts on the induction of various biochemical oxidative stress markers. Twelve healthy subjects (6 males and 6 females) consumed fresh broccoli sprouts (100 g/day) for 1 week. With treatment, total cholesterol and LDL cholesterol decreased, and HDL cholesterol increased significantly. Plasma cystine decreased significantly. All subjects showed reduced serum phosphatidylcholine hydroperoxide, urinary 8-isoprostane and 8-oxodG, and increased serum coenzyme CoQ(10)H(2)/CoQ(10) ratio (Murashima *et al.*, 2004). In a more recent intervention trial, Hoezl and colleagues (2008) found a protective effect of Brussel sprouts consumption (300g/day for 6 days) on peripheral human lymphocytes against 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and oxidative DNA-damage (measured as H(2)O(2)-induced DNA strand breaks and FPG and EndoIII sensitive sites) (Hoezl *et al.*, 2008).

Such effects may be particularly important in subjects exposed to high levels of ROS, e.g. from **cigarette smoke**. Moreover smoking is strongly associated with lung cancer risk and smokers tend to consume fewer vegetables and fruits than non-smokers (Subar *et al.*, 1990; McPhillips *et al.*, 1994; Cranganu & Camporeale, 2009). Tang and colleagues (2010) observed significant linear inverse associations between intake of fruits, total vegetables, and cruciferous vegetables and risk of lung cancer in a case-control study conducted in the US. Increasing cruciferous vegetable intake was significantly associated with reduction of lung cancer risk among smokers. The authors concluded that a diet high in cruciferous vegetables, particularly when consumed as raw, may reduce the risk of lung cancer among smokers (Tang *et al.*, 2010). Similar results were found recently by Lam and colleagues (2010) with a nested case-control study matched on cigarette smoking. The authors found an inverse association between cruciferous vegetables intake and lung cancer risk more significant for former and current smokers (Lam *et al.*, 2010), confirming results of a previous systematic review (Lam *et al.*, 2009).

Indeed, moving to dietary intervention trials, Gill *et al* (2007) showed that smokers had larger reduction in basal and H₂O₂-induced DNA strand breaks in lymphocytes than non-smokers following an intervention with 85 g of raw watercress daily for 8 weeks (30 smokers and 30 non-smokers) (Gill *et al.*, 2007). Similarly, Riso and colleagues (2009) found that FPG sites in lymphocytes decreased only in smokers, whereas the level of H₂O₂-induced DNA strand breaks decreased significantly both in smokers (n=10) and non-smokers (n=10) consuming 200 g of broccoli daily for 10 days (Riso *et al.*, 2009b).

A lowered level of **FPG-sensitive sites** in lymphocytes can in principle be caused by a decreased rate of guanine oxidation in DNA or in the nucleotide pool or an increased rate of repair (Loft & Moller, 2006). It has previously been observed that consumption of kiwi fruits increased the DNA repair activity and lowered the level of FPG sites in lymphocytes (Collins *et al.*, 2003). In addition, Guarneri *et al* (2008) investigated the repair activity towards oxidized DNA in human mononuclear blood cells in two

placebo-controlled antioxidant intervention studies, one with well-nourished subjects who consumed 600g/day of fruits and vegetables or tablets containing the same amount of vitamins and minerals for 24 days and another one with poorly nourished male smokers who consumed 500 mg/day vitamin C as slow- or plain- release formulations together with 182 mg/day vitamin E for 4 weeks (Guarnieri *et al.*, 2008). Only male smokers supplemented with slow-release vitamin C tablets had increased DNA repair activity by about 27%, while there was no significant change in subjects supplemented with tablets with plain-release formulation of vitamin C and in subjects who consumed fruits and vegetables. In the present study, with a reduced level of FPG sites after broccoli intake no change in repair activity towards oxidatively damaged guanine in DNA in extracts from the same pool of lymphocytes was found. Similarly, there was no change in the **mRNA expression of OGG1 and NUDT1 levels**, the protein of which is responsible for repair of oxidised guanine in DNA and the nucleotide pool, respectively. A possible limitation of the present study is the number of missing data (due to samples storage problems) in the analysis of DNA repair activity and the mRNA expression. Still, it should be emphasized that neither the OGG1 expression nor the repair activity pointed in the direction of increased level after broccoli intake, indicating that we would not find beneficial effects with even a substantially larger number of subjects in the study.

An increased resistance toward **H₂O₂-induced DNA strand breaks** was detected in lymphocytes. Thus, these data point mainly to increased resistance to oxidative stress in lymphocytes after a broccoli rich diet. This phenomena induced by cruciferous vegetables could be related to up-regulation of **antioxidant, phase II and other defence enzymes and/or increased levels of scavengers of ROS**. No change in the expression of **HO-1** was found; HO-1 is highly susceptible to oxidative stress and also a part of the *nrf2*- dependent gene battery of phase II enzymes frequently ascribed protective effects of cruciferous vegetables (AICR 2004). Moreover, in a previous study with a similar broccoli intervention no modulation of GST activity in plasma was detected (Riso *et al.*, 2009a). Similarly, in the watercress consumption study described above by Gill et al (2007) no change in mRNA levels of CAT, GPX1, GSTA4, GSTP1, SOD2 or UGT1A1 was found in lymphocytes despite considerable decreases in basal and oxidatively induced DNA damage (Hofmann *et al.*, 2009). In the same study the activity of SOD and GPX in red cells was not significantly changed overall, although there seemed to be an increase among subjects with the *GSTM1* null genotype (Hofmann *et al.*, 2009). In another study, the activity of SOD and GPX were unchanged after Brussels sprouts intake despite reduction of oxidatively induced DNA damage in lymphocytes as previously reported (Hoelzl *et al.*, 2008). Moreover, in a 3-phase crossover dietary intervention trial (standard broccoli, high glucosinolate broccoli, and water), the intake of standard broccoli changed the expression of only one gene tested in a very large battery of xenobiotic metabolism genes, through microarray and real-time RT-PCR analyses, in gastric mucosal tissue 6h after the consumption. On the contrary, high glucosinolate broccoli up-regulated the expression of more and different

genes, including thioredoxin reductase, aldoketoreductases, and glutamate cysteine ligase modifier subunit (Gasper *et al.*, 2007).

In the present study differences in the level of **background DNA strand breaks** after the broccoli supplementation were not detected. This is in keeping with the conclusion from a literature review showing that only very few publications have reported effect on background DNA strand breaks in leukocytes (or subsets of leukocytes such as lymphocytes or mononuclear blood cells), whereas there is a larger proportion of publications that have reported an effect of antioxidant supplementation in terms of FPG sensitive sites or 8-oxodG in leukocytes (Moller & Loft, 2006). The null effect in terms of background DNA strand breaks in this study is further strengthened by the fact that it was determined in both the analyses of H₂O₂-sensitivity (with PBS) and FPG sensitive sites (with Endo Buffer, EB). The level of background DNA strand breaks analyzed was higher in the estimation of oxidized purines than the analysis of H₂O₂-sensitivity. The difference in the level of DNA strand breaks between the two measurements may be due to methodological differences because the determination of H₂O₂-induced strand breaks was carried out on fresh lymphocytes separated from blood samples, whereas the level of FPG-sensitive sites was determined on cryopreserved samples. It should be noted that the higher level of strand breaks in the determination of FPG sensitive sites does not affect the validity of the analysis of oxidatively damaged DNA. In fact the level of FPG-sensitive sites measured is similar to the levels reported in other studies (Moller, 2006). However, **inter-individual and intra-individual variation** in the levels of oxidized purines and sensitivity to H₂O₂-induced DNA strand breaks were observed. In addition, there is large effect of broccoli intake in some subjects, whereas other subjects appear to have no benefit. We cannot determine whether this heterogeneity is because some subjects are non-responders, since this trait would only be revealed if the person participated in several independent intervention trials. Nevertheless, it should be emphasized that this cross-over study was not completely controlled in regard to habits of the subjects. Environmental and occupational exposures could affect the level of DNA damage and sensitivity to H₂O₂-induced DNA strand breaks, such as sunlight, air pollution, exhaustive exercise or therapeutics (Moller *et al.*, 2000). In addition, it is also possible that some subjects entering a trial on beneficial effects of dietary products change toward a healthier lifestyle. In the present study this might be displayed as decreased levels of FPG-sites and increased resistance toward H₂O₂-induced DNA strand breaks. Still, the reduction of DNA damage, as found in this study, may be due to **direct or indirect antioxidant scavenger functions** of compounds in cruciferous vegetables, including vitamin C, carotenoids, polyphenols, folates and/or some ICTs. Indeed, the previous intervention trial (Riso *et al.*, 2009a) showed that the broccoli consumption regime used in the present study increased the plasma concentrations of lutein and β -carotene in smokers respectively by about 45% and 33% as well as folate (about 16%) and non-indolyl ITCs (about 86%). The present study confirmed an increase of **folate** and **lutein** concentrations after broccoli diet, while, differently from the previous study, β -carotene levels were not significantly increased after the intervention

(probably because of the higher individual variability). A significant inverse correlation between the % changes in folate levels and those of H₂O₂-induced DNA damage was detected; this finding supported the contribution of the vitamin to the DNA protection against *ex-vivo* induced damage and/or repair. Despite the lack of correlation for single carotenoids a role of carotenoids in the DNA protection was not excluded because the effect of these antioxidants might depend on the synergy of multiple bioactive substances contained in whole food rather than single compounds.

The broccoli used in the present study had lower concentration of ITCs compared to the broccoli used the previous study (about 160 µmol vs. 200 µmol ITCs per portion provided), whereas there was the same level of protection against *ex vivo* generation of DNA strand breaks and endogenous levels of FPG-sites in lymphocytes from smokers (Riso *et al.*, 2009b). Thus the mechanisms for reduction of oxidative damage to DNA do not seem to involve specifically the ITCs with enzyme inducing properties. This further supports the notion that whole foods can exert a protective effect in virtue of the numerous compounds present able to act in synergy.

Data from epidemiological studies show that selected sub-groups of population have more evident protective effect by the consumption of *Brassica* gender vegetables. In particular **GST genetic polymorphisms** seem to play an important role at this regard. Some cross-sectional studies on air pollution exposure and diet do suggest that subjects with the GSTM1 null and positive genotype differ in respect to levels and response of FPG-sensitive sites and H₂O₂-induced strand breaks in leukocytes (Dusinska *et al.*, 2001; Avogbe *et al.*, 2005; Dusinska & Collins, 2008).

However, in intervention trials, the relationship between GST genetic polymorphisms and the protective role of *Brassica* gender vegetables is more difficult to evaluate.

A review by Steck & Herbert (2009), summarizes the results of feeding studies in humans that examine effects of polymorphisms in GSTs on ITC metabolite excretion, evaluates the evidence for modulation of Heterocyclic aromatic amines (HAA) mutagenicity by ITCs, and discusses the need for feeding studies examining potential interactions among polymorphic genes encoding phase I and phase II metabolizing enzymes, meat intake, and Brassica intake to elucidate their role in cancer etiology (Steck & Hebert, 2009).

We registered a higher baseline level of DNA SBs in GSTM1 null genotype subjects respect to the positive ones. The GSTM1 null genotype individuals may have a lower cell protection against oxidative stress and a lower ability to detoxify xenobiotics. Since GST is involved in the biotransformation of tobacco smoke carcinogens, such as polycyclic aromatic hydrocarbons, individuals that lack some of the enzyme isoforms and are subjected to smoke oxidative stress may have an increased risk of developing cancer. In this study broccoli consumption induced an increased resistance to H₂O₂-induced DNA strand breaks in GSTM1 null subjects as compared to the positive ones.

3.3.3 Single-meal study

Characteristics of subjects

All the 12 selected volunteers completed the study, in Table 3.3.3.1 the characteristics of subjects at recruitment were summarized. Six were GSTM1 positive and 6 were GSTM1 null genotype. Results were analyzed in 9 subjects (4 GSTM1 positive genotype and 5 GSTM1 null genotype) because data of 3 subjects were outliers possibly due to analytical problems.

Table 3.3.3.1 *Characteristics of subjects at recruitment (mean ± SD)*

Characteristics	Results
Age (years)	20.8±1.6
BMI (kg*m ⁻²)	22.5±2.1
H ₂ O ₂ -induced strand breaks (% DNA in tail)	53.3±9.3
GST activity in lymphocytes (nmol/min/mg*pr)	39.7±11.4
GST activity in plasma (pmol/min/mg*pr)	65.7±19.3

BMI: body max index

Carotenoids, folate and vitamin C levels

Plasma **carotenoid** concentrations measured at baseline and up to 24 h after consumption of broccoli (Table 3.3.3.2). Plasma lutein did not change along the time after broccoli consumption. Only slight modification for β-carotene was detected. In particular despite the low concentration provided by the portion of broccoli, β-carotene increased significantly ($p<0.05$) at 6h by about 0.04 μmol/L (+11%) respect to baseline, after its intake. Lymphocyte carotenoid concentrations measured from baseline and up to 24 h after consumption of broccoli meal, are represented in table 3.3.3.2: no significant difference was found at any time considered.

Plasma and lymphocyte **vitamin C** concentrations measured at baseline and up to 24 h after the consumption of broccoli, are represented in table 3.2.4.6. Plasma vitamin C concentrations increased significantly ($p<0.0001$) 3h after broccoli consumption by about 21.6 μmol/L (+35%). The increase was also present at 6 and 24 h with respect to baseline ($p<0.001$). The average increase corresponded to about 17.7 μmol/L (+28.6%) and 5.2 μmol/L (+8.5%) respectively. Vitamin C concentrations did not increase in lymphocytes following the broccoli intake.

Serum **folate** concentrations measured at baseline and up to 24 h after consumption of broccoli, are represented in table 3.2.4.6. Folate concentrations increased significantly ($p<0.0001$) 3 h after broccoli intake by about 8.3 nmol/L (+70%). The increase was also significantly different ($p<0.05$) after 6 h and 24h respect to baseline 4.8 nmol/L (+41%) and 1.1 nmol/L (+9.6%) respectively.

Table 3.3.3.2 Carotenoids, vitamin C, folate levels in plasma, lymphocytes and serum samples (mean \pm SD). Data with different letters are significantly different ($p < 0.05$) on the same row.

Time	0	3 h	6 h	24 h
β -carotene in plasma ($\mu\text{mol/L}$)	0.38 \pm 0.20ab	0.39 \pm 0.22ac	0.41 \pm 0.28bc	0.40 \pm 0.22ac
Lutein in plasma ($\mu\text{mol/L}$)	0.40 \pm 0.16	0.39 \pm 0.13	0.38 \pm 0.13	0.41 \pm 0.11
β -carotene in lymphocytes (nmol*mg p)	0.08 \pm 0.05	0.06 \pm 0.04	0.07 \pm 0.04	0.07 \pm 0.04
Lutein in lymphocytes (nmol*mg p)	0.14 \pm 0.04	0.12 \pm 0.05	0.12 \pm 0.05	0.12 \pm 0.05
Vitamin C in plasma ($\mu\text{mol/L}$)	61.7 \pm 19.5a	83.3 \pm 20.6b	79.4 \pm 21.5c	66.9 \pm 18.1d
Vitamin C in lymphocytes (nmol*mg p)	17.5 \pm 2.2	15.2 \pm 2.9	16.6 \pm 3.8	17.5 \pm 3.8
Folate in serum (nmol/L)	11.8 \pm 3.6a	20.1 \pm 4.2b	16.7 \pm 4.2c	13.0 \pm 4.3d

Oxidative lymphocyte DNA damage

A significant reduction of *ex-vivo* induced DNA damage ($p < 0.05$) after 24 h from broccoli consumption intake with respect to time 0, 3 and 6 h was observed.

One-way ANOVA showed a significant reduction of *ex-vivo* induced DNA damage ($p < 0.05$) after 24 h from broccoli consumption intake with respect to 0 h, 3 h and 6 h.

A further analysis by considering the different GST polymorphism as independent factor, showed that individuals GSTM1 positive polymorphism had lower DNA damage ($p < 0.05$) at 24h with respect to 0, 3, and 6h; while no significant difference was registered in individuals with GSTM1 null genotype. Individuals with GSTM1 positive genotype showed a greater protection against DNA damage (Table 3.3.3.3).

Table 3.3.3.3 Results of H_2O_2 -induced DNA strand breaks single-dose study (Mean \pm SD). Data with different letters are significantly different ($p < 0.05$) on the same row.

Time	0	3 h	6 h	24 h
H_2O_2-induced DNA strand breaks (% DNA in tail)				
All subjects (n=9)	53.3 \pm 9.3	54.7 \pm 7.8	58.1 \pm 12.5	43.4 \pm 16.4 ^a
GSTM1 positive (n=4)	54.6 \pm 10.3	56.0 \pm 5.6	66.0 \pm 7.3	33.2 \pm 13.1 ^a
GSTM1 null (n=5)	52.2 \pm 9.5	53.6 \pm 9.8	51.8 \pm 12.7	51.5 \pm 15.0

GST activity in plasma and lymphocytes

GST activity in plasma and lymphocytes analyzed in 9 subjects is reported in Table 3.3.3.4. In general, individuals with positive GSTM1 genotype showed a greater plasma and lymphocyte GST activity compared to individuals with GSTM1 null genotype. However lymphocyte GST activity resulted significantly different ($p < 0.05$) between individuals with GSTM1 positive and GSTM1 null genotype. After broccoli consumption, plasma GST activity significantly increased ($p < 0.05$) in individuals with GSTM1 positive genotype at 6 h with respect to 3 h and 24 h but not with respect to baseline. No significant difference was observed in lymphocytes GST activity (Table 3.3.3.4).

Table 3.3.3.4 Results of GST activity in plasma and lymphocytes of single-dose study (Mean±SD). Data with different letters are significantly different ($p<0.05$) on the same row.

Time	0	3 h	6 h	24 h
GST activity (n=9)				
Plasma (pmol/min/mg*pr)				
All subjects	65.7±19.3	58.1±24.5	74.5±30.0	58.9±13.3
GSTM1 positive (n=5)	76.2±17.3 ^a	68.6±29.4 ^{ab}	87.5±35.2 ^b	64.5±15.3 ^a
GSTM1 null (n=4)	52.6±13.4	45.0±5.9	58.1±10.8	51.9±6.3
Lymphocytes(nmol/min/mg*pr)				
All subjects	39.7±11.4	36.6±21.3	35.1±27.3	35.8±12.9
GSTM1 positive (n=5)	45.9±10.8	49.2±20.9	50.4±24.6	41.5±13.7
GSTM1 null (n=4)	31.8±6.9	21.0±6.8	16.1±17.8	28.7±8.2

3.3.3.1 Discussion of single-meal study

A single portion of steamed broccoli was able to decrease DNA damage and to modulate plasma GST activity. Broccoli protective effect has been related to multiple substances such as carotenoids, vitamin C, isothiocyanates and polyphenols. They display complementary and overlapping mechanisms of action, including the induction of detoxification enzymes and antioxidant effects.

In the present study, we have monitored the plasma concentrations of carotenoids, vitamin C and folate along the time after the intake of one portion of broccoli. The portion of broccoli (250 g) provided about 2.8 mg lutein, 1.5 mg β -carotene, 172 mg vitamin C and 160 μ mol ITCs.

The consumption of broccoli significantly increased some of the compounds such as vitamin C, folate and β -carotene in plasma. A significant increase of **vitamin C plasma** concentration was found 3 h after the consumption of broccoli and the levels were maintained significantly high until 24 h with respect to baseline. Few studies have shown similar effect after the consumption of vegetables and fruit. In fact in a recent study, in which seven healthy, non-smoking female volunteers assumed a single portion (300 ml) of orange juice providing 150 mg vitamin C, the concentration of vitamin C increased significantly 1 h after the drink reaching a peak at 2-4 h, however at 24 h plasma concentrations were comparable to baseline values (Guarnieri *et al.*, 2007). One possible reason to explain the differences between studies could be related to differences among food matrices. Orange juice is a simple food matrix, thus vitamin C could be more quickly absorbed with respect to broccoli. Also in a recent intervention trial, where smokers and no smokers consumed broccoli for 10 days, no differences in vitamin C concentration in plasma after intake was found (Riso *et al.*, 2009a), even if the amount of vitamin C provided was high (from 60 to 150 mg per portion).

Concerning **carotenoids**, no significant differences were observed for their concentration in plasma, except for β -carotene. The levels of β -carotene increased significantly following consumption of broccoli (+ 13% respect to baseline). However, limited studies have shown similar increase. In the study of Riso and colleagues (2009a), with 200 g of broccoli for 10 days, plasma lutein and β -carotene increased by

about 45% and 33% respectively. Similar results were found after 4 days of broccoli intake with an increase of 133% and about 28% for lutein and β -carotene plasma levels respectively (van het Hof *et al.*, 1999). Also Bohn and co-authors (2010), in a 8-week randomized controlled clinical trial of male smokers, found a significant increase in carotenoid concentrations in plasma in the two intervention groups (diet with various antioxidant-rich foods and kiwifruit diet) compared to the control group; in particular lutein and β -carotene increased by about 37% and 50% respectively after the antioxidant-rich diet; while after kiwifruit diet the increase of lutein plasma concentration was of 37% (Bohn *et al.*, 2010). Brown and colleagues (1989) did not find significant differences in plasma β -carotene concentrations after one portion of broccoli (600 g providing 6 mg β -carotene) (Brown *et al.*, 1989). Similarly, the intake for one week of 200 g of broccoli induced a significant increase in lutein and not in β -carotene concentrations in plasma (Granado *et al.*, 2006).

We have found similar results for lutein concentration, in fact no significant difference was observed after broccoli intake. Three possible reasons can be hypothesized to explain the lack of increase of lutein. The first one could be related to the low amount present in broccoli. The second one could be due to the fact that lutein peak in plasma could have been occurred between 8 and 24 h after broccoli intake. In fact, in accordance with data reported in literature, each carotenoid shows its own pattern of absorption, clearance and metabolism. In a human study in which single equimolar doses (0.5 μ mol/kg body weight) of lutein and β -carotene in oil solution were given to eight adults subjects, the mean serum concentration of lutein had a single maximum at 16 h, β -carotene had a first peak at 6 h and then a second at 32 h (Brown *et al.*, 1989). The third hypothesis could be related to an effect of competition between β -carotene and lutein for absorption. Kostic and colleagues showed that simultaneous ingestion of purified lutein and β -carotene decreased the bioavailability of lutein and consequently of the absorption (Kostic *et al.*, 1995).

From our results no significant difference in the concentration of the carotenoids and vitamin C in lymphocytes was observed. Probably, one portion of broccoli was not sufficient to increase the antioxidant content of such cells.

Broccoli is also a good source of **folate** (generally the concentration vary from 90-100 μ g per 100 g of broccoli). Folate play an important role in nucleotide biosynthesis, DNA methylation and methionine biosynthesis (Blakley & Cocco, 1984). A sub-optimal status of folate is often associated to increased risk of cardiovascular disease (Boushey *et al.*, 1995) and cancer (Kim, 1999). A high intake of folate, on the contrary, may hide a vitamin B12 deficiency, thus fortification may be at risk for some population groups (e.g. elderly people), while the intake of naturally rich vegetables should be advised.

In a short intervention study, no significant increase was found after 4 day consumption of 300 g broccoli providing 260 μ g folate (van het Hof *et al.*, 1999). In the study by Riso *et al.* (2009a), a significant increase of serum folate (about 15%) was registered after 10 days of broccoli consumption (200g), afterwards values decreased to basal levels after cruciferous free diet (Riso *et al.*, 2009a). Even if in the present study it was not possible to determine folate in broccoli, a significant increase in serum

concentration was shown. In particular, the concentration of folate increased by 70% after 3 h the broccoli intake. The levels were maintained significantly high until 24 h with respect to baseline.

Apart from antioxidants and vitamins, broccoli are a very important source of glucosinolates whose bioactive derivate are **isothiocyanates** (ITCs). Studies in literature demonstrated that ITCs are rapidly absorbed, cleared from blood and eliminated almost exclusively in urine (Shapiro *et al.*, 1998). In fact after absorption, ITCs react with glutathione; the reaction is spontaneous but can also be catalyzed by glutathione-S-transferase (GST), a family of iso-enzyme involved in the detoxification of carcinogens. In the present study we evaluated whether one portion of broccoli could modulate **GST activity**; moreover we further try to verify whether differences between subjects with different GST polymorphisms could be revealed. After broccoli intake, plasma GST activity significantly increased at 6 h. This increment was partly due a fluctuation at 3 h but it may be also due to an effect of the absorption of ITCs. An increase of ITCs at 6 h was in fact demonstrated by Conaway after 200 g steamed broccoli intake and was found in a pilot study performed in our laboratory (Conaway *et al.*, 2000). It was recently demonstrated that a regular intake of broccoli can increase the basal circulating levels of ITCs, in fact after 10 days of broccoli consumption (200g), ITCs levels doubled (Riso *et al.*, 2009a).

In general, individuals with positive GSTM1 genotype showed a greater plasma and lymphocyte GST activity with respect to individuals with GSTM1 null genotype (Lampe & Peterson, 2002). This suggests that individuals with GSTM1 null genotype may had, in the whole, a low GST activity and consequently a lower ability to excrete ITCs with respect to GSTM1 positive subjects. In lymphocytes of GSTM1 null genotype subjects we found GST activity significantly lower than GSTM1 positive individuals. After broccoli intake the increase of GST activity at 6 h was present only in individuals with GSTM1 positive genotype. This suggests that GSTM1 could be involved in the metabolism of ITCs, which seem able to increase the activity in individuals with GSTM1 positive genotype. However considering the small number of individuals and the heterogeneous results obtained, more studies are necessary to support the role of ITCs in the modulation of GST activity. Data in literature illustrated that cruciferous vegetable diets could modulate GST iso-enzymes level or activity, and the modulation effects were differently affected by GSTM1 genotype. Individuals with GSTM1 null genotype showed a reduction of lymphocyte activity between response to basal and vegetable-supplemented diets (Lampe *et al.*, 2000); instead Pool-Zobel showed that the induction of GSTM1 activity was more pronounced in individuals with positive genotype (Pool-Zobel *et al.*, 2005). Gasper *et al.* (2005) suggest that GSTM1 null subjects can excrete essentially all SFN metabolites via the mercapturic acid pathway after broccoli consumption; on the contrary only 70% of the ingested SFN could be excreted by GSTM1 positive subjects consuming the same amount of broccoli. The authors speculated that, in contrast to GSTM1 null genotype subjects, in GSTM1 positive ones a proportion of SFN could be retained within the body and this may mediate the anticarcinogenic activity of broccoli (Gasper *et al.*, 2005). This hypothesis was sustained

by recent results obtained by Steck et al (2007) (Steck *et al.*, 2007). However the regular consumption of broccoli (200g) for 10 days did not affect total GST activity, evaluated using CDNB as substrate (Riso *et al.*, 2009a). The same results were found in another study for GST(CDNB) activity, while GSTM1 null genotypes had an increased serum GST- α level and GST(NBD-CI) activity (Lampe & Peterson, 2002). Considering these findings, it may be that the effect on GST activity could be examined with other substrates than CDNB or with specific GST evaluation.

Another aim of the study was also to evaluate the effect of the portion of broccoli on lymphocyte protection from an *ex vivo* induced **DNA oxidative damage**. As a consequence of broccoli intake the cell resistance to DNA damage increased significantly at 24 h. A further analysis showed that only GSTM1 positive individuals increased significantly their protection ability at 24 h. This result, together with the observation that GST activity increased at 6 h from broccoli intake in GSTM1 positive individuals, could support the hypothesis of a lower disease risk of such subjects and a higher response to broccoli intake with respect to GSTM1 null individuals. As previously reported, Gasper *et al.*, (2005) found a lower concentration of sulforaphane metabolites excreted in the urine of GSTM1-positive persons than in null ones (Gasper *et al.*, 2005). Researchers speculated that SFN could be stored from tissues or have different metabolic pathway. This could explain why GSTM1-positive genotype had more protection from a small amount of broccoli. However researchers suggest that GSTM1 null individuals may compensate their genotype increasing the frequency of broccoli consumption in order to obtain effects similar to that observed in GSTM1 positive subjects (Gasper *et al.*, 2005).

One of the hypothesis tested in the present study is that an increase of plasma and cell levels of antioxidants could determine a modulation of biomarkers such as lymphocytes resistance to oxidative stress and cell enzyme activity. For example studies showed an increase of the resistance to DNA oxidatively-induced damage, but no effect on endogenous DNA damage, in concomitance with an increase of plasma vitamin C concentration after the consumption of 0.5 L of a kiwifruit drink (Collins *et al.*, 2001b). Similar results were also shown by Panayotidis & Collins (1997) in a human study, in which a single large dose of antioxidants (vitamin C, vitamin E and β -carotene) was introduced (Panayotidis & Collins, 1997). Also Guarnieri and colleagues (2007) found a protective effect of a single dose of Blood Orange Juice with a decrease of DNA damage after 3h (18%), maintained constant until 24 h (16%) (Guarnieri *et al.*, 2007). However Moller *et al* (2003) did not find an effect on DNA damage biomarkers after the intake of a 600 g fruit and vegetable diet or a supplementation containing the same amount of minerals and vitamins for 24 days (Moller *et al.*, 2003). In a very recent dietary intervention trial 10 servings/day for 2 weeks of botanically defined fruit and vegetable did not affect endogenous DNA damage and repair measures in lymphocytes (Chang *et al.*, 2010). In a similar study, where two groups of individuals consumed antagonistic diets, a protective effect of the diet rich in organic products, integral grains, fruit and vegetable and poor in industrialized products was found against oxidative DNA damage and DNA damage induced by H₂O₂ (Prado *et al.*, 2010).

However in the present study we have demonstrated that the protection against DNA damage was not correlated with plasma concentration of vitamin C, folate or carotenoids at 24 h. This result supports some hypothesis reported in literature by which the antioxidant protection is not exerted by single compounds but by their synergy. This may require long time or, as in this case, be apparent already after 24 h of the intake of a food portion.

3.4 Conclusions TOPIC I

Many epidemiological studies suggest that a diet rich in fruits and vegetables, in particular vegetables of the Brassicaceae family, is associated with a decreased risk and incidence of many degenerative diseases such as cancer.

Up to now, “regular-intake” study is the first published work reporting the possible relationship between GSTM1 polymorphism and modulation of DNA damage following broccoli intake (Riso *et al.*, 2010). We found that individuals with GSTM1 null genotype could benefit more than subjects with GSTM1 positive genotype from *Brassica* vegetables intake for 10 days. Differently, in the “single-meal” study, one portion of broccoli was able to affect more GSTM1 positive genotype subjects respect to the GSTM1 null genotypes, compared to the protection against DNA damage and modulation of GST activity. Broccoli seems to be a good bioavailable source of vitamin C, folate and carotenoids in both studies.

Is the protective effect due to the action of ITCs? It seems that ITCs have a slower excretion in those individuals that lack GST enzyme. Moreover ITCs induce the expression of the same enzyme, increasing their own metabolism. So the amount of broccoli ingested, the accumulating exposure given by a regular consumption can make the difference. If these results will be confirmed in future studies where a larger group of subjects is recruited, it will be possible to verify whether higher amounts of broccoli or longer time of consumption could increase GST activity and protection against DNA damage also in null genotype subjects.

It has been demonstrated that altered metabolism leads to a higher bioavailability of sulphoraphane or total ITCs, as shown in studies with administration of a single broccoli meal, although differences were small and the interpretation is debated (Gasper *et al.*, 2005; Steck *et al.*, 2007; Lampe, 2009).

The impact of GSTM1 on possible cancer protective effects of cruciferous vegetables is also debated with effect mainly shown with the GSTM1 and GSTT1 double null genotype in Chinese and European populations (Kim & Park, 2009), whereas the GSTM1 positive genotype might convey beneficial effects in subjects from North America (Gasper *et al.*, 2005). Intake of cruciferous vegetables, including broccoli, is most popular in the latter region, whereas other *Brassica* vegetables in the former region as well as multiple other dietary, life style and genetic differences can play roles in these apparent discrepancies. Even within a single family of vegetables, such as the crucifers and a specific genus, *Brassica*, effects may differ between different vegetables, and there may be alternative routes of metabolism for the different ITC (namely sulforaphane in the broccoli studies). There is speculation that there may be

differential effects of different Brassica vegetables on cancer risk because of differences in their phytochemical constitution (Gasper *et al.*, 2005). Other factors that may explain the differential excretion of ITCs between individuals include the amount of chewing, which affects the release of myrosinase from the vegetable. In the case of cooked vegetables, where myrosinase has been inactivated, conversion of glucosinolates to ITCs still occurs via the gastrointestinal microflora, so differences in ITC excretion between individuals also may be related to differences in the gastrointestinal microflora. Finally, there it is supposed that the GSTs affect the rate of ITC excretion, rather than the absolute amount excreted; in which case, a single urinary measurement of ITC excretion would have little value within either a feeding study or epidemiologic study designed to account for this difference in rate (Steck & Hebert, 2009).

In conclusion, this PhD research revealed a protective effect exerted by both regular intake and a single portion of broccoli, as far as DNA damage is concerned, in young male smokers who are exposed to oxidative stress. This outcome may be due to improved antioxidant status and synergic action of all the bioactive compounds present in broccoli.

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3.6 TOPIC II: Effect of anthocyanins on angiogenic and pro-thrombotic properties of endothelial cells *in vitro*

Abstract

Anthocyanins (ACNs), compounds of the flavonoid class, are natural pigments that provide colours from dark blue to purple to fruits and vegetables, including edible berries. Studies that investigated the effects of ACNs on health showed that these flavonoids possess anti-inflammatory and anti-carcinogenic properties. Delphinidin-3-glucoside (Dp-3-glc) and cyanidin-3-glucoside (Cy-3-glc) are two ACNs contained in berries. These two compounds are able to inhibit tumor cell proliferation, but it is not known whether they can also act on pro-angiogenic and the pro-thrombotic properties of endothelial cells.

An *in vitro* study was designed to investigate the effect of Dp-3-glc on the angiogenic and the pro-coagulant activities of human microvascular endothelial cells (HMEC-1).

HMEC-1 were incubated for up to 24h with culture media \pm Dp-3-glc (0.1, 1, 10, 100 μ M) alone (i.e. resting conditions) or in combination with purified proangiogenic factor (VEGF, 50 ng/mL), or bacterial endotoxin (LPS, 10 μ g/mL), stimulated conditions. After incubation, angiogenesis was evaluated by the capillary-like tube formation in Matrigel and the wound healing assay, while procoagulant activity was tested by the thrombin generation assay (TG) and Tissue Factor (TF) expression by antigen, one-stage recalcification assay on normal plasma and mRNA quantitation.

HMEC-1 were also incubated for 24h with culture media \pm Cy-3-glc (0.1, 1, 10, 100 μ M) alone or in combination with purified proangiogenic factor (VEGF) to evaluate if Cy-3-glc was able to affect HMEC-1 angiogenesis, by the capillary-like tube formation in Matrigel and the wound healing assay.

The results show that Dp-3-glc inhibited capillary tube formation both in resting and in VEGF-stimulated conditions, reaching statistically significant reduction and inhibition of stimulus at 100 μ M ($p < 0.05$). At the same concentration, Dp-3-glc significantly reduced the migration of resting HMEC-1; interestingly, the inhibition of the migratory VEGF-dependent stimulus started at the Dp-3-glc concentration of 1 μ M. In the TG assay Dp-3-glc significantly counteracted the pro-thrombotic stimulus of LPS starting from 10 μ M on HMEC-1 intact monolayers; while the ACN inhibited LPS-induced TF expression at 100 μ M concentration. Differently, Cy-3-glc did not show any effect on capillary-like tube formation and migration of HMEC-1 both alone and in combination with VEGF.

In conclusion, the data show that Dp-3-glc can affect angiogenic, migratory and procoagulant properties of endothelial cells. These findings, together with the known capacity of Dp-3-glc to affect tumor cell proliferation, make this compound a potential cancer chemopreventive agent.

Introduction

Anthocyanins: chemical structure, sources and bioavailability

Berries such as cranberries, blueberries, strawberries, blackcurrant and raspberries contain significant amounts of non-nutritive phytochemicals including the

polyphenols and other antioxidants. Polyphenols are defined chemically as substances that have an aromatic ring with an hydroxyl substituents, including esters and glycosides. Because of the diverse range of their chemical structures, polyphenols are grouped into different classes, depending on the number of phenol rings they possess and the type and number of structural elements binding the phenolic rings together (Manach *et al.*, 2004). The classes include: simple phenolic acids (e.g. ferulic acid, gallic acid, Figure 3.6.1); stilbenes (e.g. resveratrol, Figure 3.6.1); more complex chalcones (e.g. phloridzin, Figure 3.6.1); flavonoids, split into seven subclasses that include flavonols (e.g. quercetin, Figure 3.6.1), flavanols (e.g. epigallocatechingallate, Figure 3.6.1), flavones, flavanones, flavanonols, isoflavones and anthocyanins (Nicholson *et al.*, 2008).

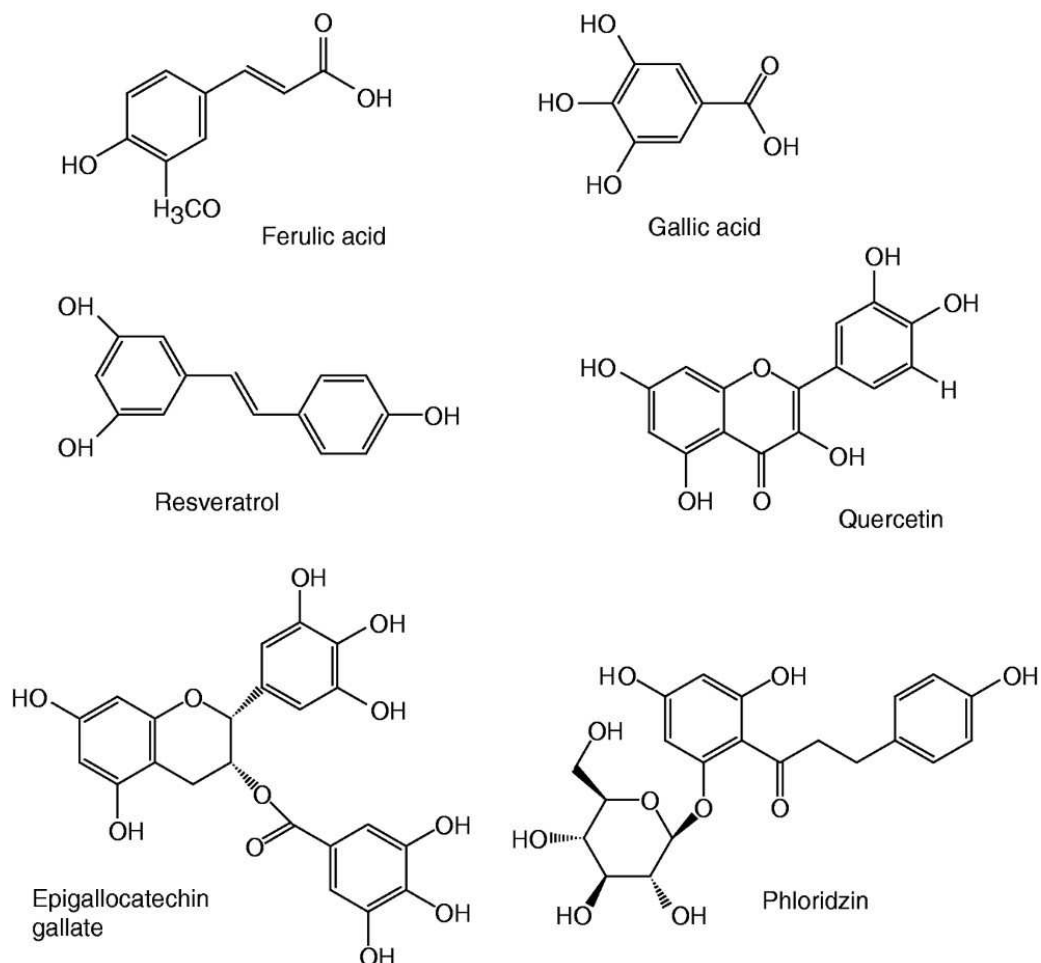


Figure 3.6.1 Chemical structures of selected polyphenols (Nicholson *et al.*, 2008)

Anthocyanins (ACNs) are flavonoids, which are the largest group of water-soluble pigments, occur ubiquitously in the plant kingdom and confer bright red or blue

coloration on many fruits (particularly berries), vegetables, grains, flowers and leaves. They can be found in nature in different structural arrangements (Figure 3.6.2), depending on the number of glycosylating sugars, their position in the aglycone (anthocyanidin) and the degree and nature of esterification of the sugar with aliphatic or aromatic acids (Giordano *et al.*, 2007).

Depending on the nutrition habits, ACNs daily intake has been estimated to be from 3-15 mg up to 200 mg in US (Wu *et al.*, 2006; Duthie, 2007; Crozier *et al.*, 2009; Fernandes *et al.*, 2010); the anthocyanidins intake in the US has been estimated to be 180-255 mg/day (Cooke *et al.*, 2005).

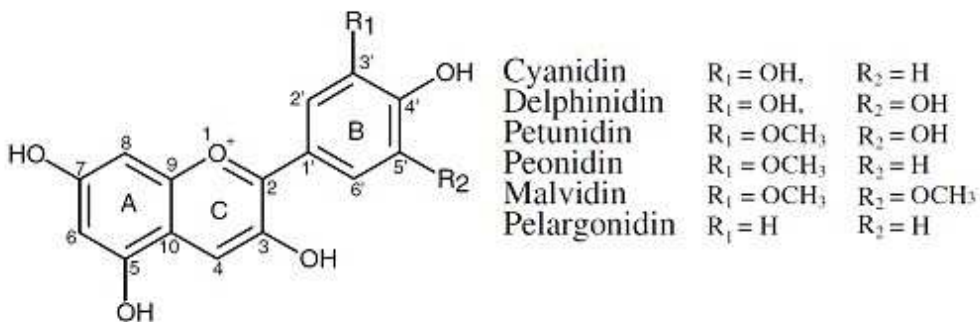


Figure 3.6.2 Chemical structures of selected anthocyanidins (Nicholson *et al.*, 2008)

Bioavailability studies are considered a fundamental issue, in order to understand the biological effects of anthocyanins. This implies the development of sensitive and validated analytical methods to quantify anthocyanins in biological fluids such as plasma and urine.

Following the ingestion of dietary flavonoids which exist in plants predominantly as glycoside conjugates, absorption of some but not all components into the circulatory system occurs in the small intestine (Silberberg *et al.*, 2006). Typically, this is associated with hydrolysis, releasing the aglycone, as a result of the action of lactase phloridzin hydrolase (LPH) in the brush-border of the small intestine epithelial cells. LPH exhibits broad substrate specificity for flavonoid-O-β-D-glucosides, and the released aglycone may then enter the epithelial cells by passive diffusion as a result of its increased lipophilicity and its proximity to the cellular membrane (Day *et al.*, 2000). An alternative site of hydrolysis is a cytosolic β-glucosidase (CBG) within the epithelial cells. In order for CBG-mediated hydrolysis to occur the polar glucosides must be transported into the epithelial cells, possibly with the involvement of the active sodium-dependent glucose transporter SGLT1 (Gee *et al.*, 2000). Thus, it has been accepted that there are two possible routes by which the glucoside conjugates are hydrolysed and the resultant aglycones appear in the epithelial cells, namely 'LPH/diffusion' and 'transport/CBG'. However, a recent investigation, in which SGLT1

was expressed in *Xenopus laevis* oocytes, indicated that SGLT1 does not transport flavonoids and that glycosylated flavonoids, and some aglycones, have the capability to inhibit the glucose transporter (Kottra & Daniel, 2007). Prior to passage into the blood stream the aglycones undergo metabolism, forming sulfate, glucuronide and/or methylated metabolites through the respective action of sulfotransferases (SULT), uridine-5'-diphosphate glucuronosyltransferases (UGTs) and catechol-O-methyltransferases (COMT). There is also efflux of at least some of the metabolites back into the lumen of the small intestine and this is thought to involve members of the adenosine triphosphate (ATP)-binding cassette (ABC) family of transporters including multidrug resistance protein (MRP) and P-glycoprotein (P-gp). Once in the bloodstream, metabolites can be subjected to phase II metabolism with further conversions occurring in the liver, where enterohepatic transport in the bile may result in some recycling back to the small intestine (Donovan *et al.*, 2006).

Flavonoids and their metabolites not absorbed in the small intestine can be absorbed in the large intestine but will be subjected to the action of the colonic microflora, which will cleave conjugating moieties and subject the resultant aglycones to ring fission, leading to the production of phenolic acids and hydroxycinnamates. These can be absorbed and ultimately excreted in urine in substantial quantities that, in most instances, are well in excess of the flavonoid metabolites that entered the circulatory system via the small intestine.

A detailed review on the bioavailability of polyphenols in humans was published in 2005 by Manach and colleagues (Manach *et al.*, 2005). Much of the research covered involved feeding volunteers a single supplement and monitoring the levels of flavonoids in plasma and urine over a 24 h period. As flavonoid metabolites were rarely available, analysis almost invariably involved treatment of samples with mollusc glucuronidase/sulfatase preparations and subsequent quantification of the released aglycones by HPLC using either absorbance, fluorescence or electrochemical detection. While at the time such studies provided valuable insights, it is important to appreciate their potential shortcomings. Namely, information yielded on the metabolites produced is very indirect, and quantitative estimates, although precise, are not necessarily accurate as there are few data on the efficiency with which the enzymes hydrolyse individual metabolites and release the aglycone (Mullen *et al.*, 2008a). Indeed, the only study on the subject to date reports that the use of enzyme hydrolysis results in an underestimation of isoflavone metabolites (Gu *et al.*, 2005). Anthocyanins, for people who eat berries and drink red wine on a routine basis, are major dietary components. Although there are exceptions, unlike other flavonoids that are absorbed and excreted, most anthocyanins do not appear to undergo extensive metabolism of the parent glycosides to glucurono, sulfo or methyl derivatives (McGhie *et al.*, 2003; Ichiyangi *et al.*, 2005). In feeding studies with animals and humans, typically ca. 0.1% of the quantities ingested, and sometimes much less, has been detected in urine. The available data imply that the determinants of absorption and excretion are influenced not only by the nature of the sugar moiety but also by the structure of the anthocyanidin aglycone (McGhie *et al.*, 2003; Wu *et al.*, 2005). The complex array of

information on anthocyanin bioavailability obtained with human and animal test systems has been reviewed by Prior and Wu (Prior & Wu, 2006). One of the reasons for the complicated picture that has emerged is that many feeds have involved berry or fruit supplements containing several structurally diverse anthocyanins. For instance, **black raspberries** contain five cyanidin-3-O-sugar conjugates ranging from mono to trisaccharides while **blueberries** contain a total of 12 anthocyanins, principally 3-O-glucosides, galactosides and arabinosides of cyanidin, delphinidin, petunidin and malvidin (McGhie *et al.*, 2003). This makes the complex anthocyanin content of plasma and urine exceedingly difficult, if not impossible, to assess in terms of absorption, excretion and potential phase I and phase II metabolism, especially when 3'-O-methylation can convert cyanidin to peonidin, and delphinidin to petunidin, and 5'-O-methylation converts petunidin to malvidin. Much simpler anthocyanin profiles are found in **strawberries** and **blackberries**, both of which contain one predominant anthocyanin, pelargonidin-3-O-glucoside in the former and cyanidin-3-O-glucoside in the latter (Wu *et al.*, 2006). As a consequence, data on anthocyanin bioavailability after ingestion of these berries by humans are potentially more straightforward to interpret. In a recent human study, 200 g of **strawberries** containing 222 μmol of pelargonidin-3-O-glucoside and trace quantities of pelargonidin-3-O-rutinoside (13 μmol) and cyanidin-3-O-glucoside (6 μmol) were consumed by six subjects, after which plasma and urine were collected over a 24 h period (Mullen *et al.*, 2008b). The plasma contained a pelargonidin-O-glucuronide in substantial quantities along with non-quantifiable amounts of three other pelargonidin-O-glucuronides and pelargonidin-3-O-glucoside, the latter perhaps derived from removal of the 6"-rhamnose moiety from pelargonidin-3-O-rutinoside. The main pelargonidin-O-glucuronide had a C_{max} of 274 ± 24 nmol/L, a T_{max} of 1.1 ± 0.4 h, in keeping with small intestine absorption, and $T_{1/2}$ of 2.1 ± 0.7 h. All the plasma anthocyanins also appeared in urine along with small quantities of pelargonidin aglycone and a pelargonidin-O-sulfate. The pelargonidin-O-glucuronide, that was the main metabolite in plasma, was by far the predominant component in urine, accounting over 0–24 h for 1498 nmol of a total of 1672 nmol of anthocyanins excreted. This corresponds to 0.75% of pelargonidin-3-O-glucoside intake. There is, therefore, no evidence of substantive post-absorption metabolism prior to excretion. In an earlier feeding study with **strawberries**, Felgines and co-workers (Felgines *et al.*, 2003) reported a urinary excretion equivalent to 1.8% of the 179 μmol of ingested pelargonidin-3-O-glucoside and this is also similar to values obtained in a 15–60 μmol dose study with strawberries (Carkeet *et al.*, 2008). These urinary recoveries are high for anthocyanins, and suggest that pelargonidin-3-O-glucoside is absorbed more readily than other anthocyanins. In a separate human feeding study with 200 g of **blackberries** containing 960 μmol of cyanidin-3-O-glucoside, 12 anthocyanins were excreted including unmetabolised cyanidin-3-O-glucoside, a cyanidin-O-glucuronide and a peonidin-O-glucuronide in quantities equivalent to 0.16% of intake (Felgines *et al.*, 2005). This suggests that pelargonidin-3-O-glucoside, while it is metabolized to fewer products, may be absorbed more readily than cyanidin-3-O-glucoside. However, the high cyanidin-3-O-glucoside content of the

blackberry supplement may have had an impact on absorption and/or excretion. In the circumstances, it would be of interest to carry out a feeding study and to determine not only the urine but also the plasma anthocyanin profile after ingestion of blackberries and strawberries containing similar quantities of anthocyanins. After a single dose of **black currant** (87.9 μmol , 58.8 mg of ACNs), the pharmacokinetics of delphinidin-3-glucoside (Dp-3-glc), delphinidin-3-rutinoside (Dp-3-rut), cyaniding-3-glucoside (Cy-3-glc) and cyaniding-3-rutinoside (Cy-3-rut) were analyzed in human plasma: T_{max} were equal for Dp-3-rut and Cy-3-rut (1.25h), which were greater than the values of Dp-3-glc and Cy-3-glc (0.75h). These suggest that absorption is influenced by the difference in the sugars moiety. The C_{max} and AUC values varied in the order: Dp-3-rut>Cy-3-glc>Dp-3-glc>Cy-3-glc, which was the same order as the quantity of the four components in the black currant ACNs ingested (Nakamura *et al.*, 2010). These data confirmed the previous study of the same authors (Matsumoto *et al.*, 2001). In another study with the ingestion of a single dose of black currant juice (330 mL, providing 98.2 mg of Dp-3-glc, 493.3 mg of Dp-3-rut, 60.2 mg of Cy-3-glc, 358.2 mg of Cy-3-rut and 29.3 mg of 4 unidentified ACNs) in the morning, blood samples were collected in the following 6h and urine until the following day at 4 pm. The four major native anthocyanidin glycosides of blackcurrant juice, delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside and cyanidin-3-rutinoside, were detected and identified in low amounts by HPLC and LC-MS in plasma and urine post-ingestion. Elimination of the anthocyanins was fast (maximum excretion after 1 h) and plasma levels (0–128.6 nmol/l) and total urinary excretion (0.07– 1.35 mg; 0.007–0.133% of the dose ingested) were low (Rechner *et al.*, 2002). The quantitative results derived in this study indicate that the majority of the ingested polyphenols from the blackcurrant juice are subjected to metabolism in the colon. The ingestion of high amounts of polyphenols have also been linked with increased urinary excretion of hippuric acids, such as hippuric acid, 3-hydroxyhippuric acid, and 4-hydroxyhippuric acid (Rechner *et al.*, 2002).

Another source of ACNs is **blood orange juice**, which contains 72 mg/L of ACNs (mainly represented by Cy-3-glc, 25.8 mg/L, Dp-3-glc, 4 mg/L and cyanidin-3-(6-malonylglucoside), Cy-M-glc, 17.9 mg/L). The analysis of samples obtained from human plasma and urine after the ingestion of a single dose of two-fold concentrated blood orange juice (500 mL), permitted to detect the three compounds in biological samples. Plasma levels after 1h of the ingestion were: Cy-3-glc 1.98 ± 0.55 ng/mL, Dp-3-glc 0.20 ± 0.04 ng/mL, Cy-M-glc 0.38 ± 0.06 ng/mL; urinary excretion within 4 h after the ingestion was respectively: 1.51 ± 0.55 μg , 14.89 ± 3.28 μg and 3.07 ± 0.50 μg for Dp-3-glc, Cy-3-glc and Cy-M-glc (Giordano *et al.*, 2007). A regular consumption of blood orange juice (600 mL/day for 21 days) provided 3.5 mg/100 mL of Cy-3-glc, and 1.2 mg/100 mL of cyanidin-3-glucoside-6''-malonyl (Cy-3-glc-6-M) in a dietary intervention study by Riso and colleagues (2005). The Cy-3-glc plasma concentration was very low and close to the detection limit of the instrument at baseline and after the washout period (about 0.6 nmol/L on average) and increased to 8 nmol/L after 3 weeks of blood orange juice

intake, i.e., an increase of more than 10-fold. Both the aglycon and the Cy-3-glc-6-M were not detected in plasma (Riso *et al.*, 2005).

Wild blueberry (WB, *Vaccinium augustifolium*) is composed of different anthocyanidins such as delphinidin, malvidin, petunidin, cyanidin and peonidin. Del Bo' and colleagues (2010) investigated ACNs absorption, metabolism, and distribution in the plasma, liver, brain, and their excretion in urine and feces in the Sprague-Dawley (SD) rats fed a wild blueberry-enriched diet (8%) over time (4 and 8 weeks). Anthocyanins were not detected in the plasma, liver, and brain samples after dietary treatments. Since they are rapidly metabolized and the animals were sacrificed 3-4 h after the last meal, it was not possible to detect their native forms in the plasma, liver, or brain tissues. ACNs content in the urine significantly increased at 8 weeks compared to that at 4 weeks (i.e., Cy-gal, Cy-glc, Pt-gal, Pn-gal, Pt-ara, Mv-gal, and Mv-glc-ac). ACNs that were not absorbed or excreted by bile, were detected in the feces. Pn-gal, Pt-ara, and Pn-glc-ac were the dominant excretory products after WB consumption (Del Bo *et al.*, 2010a). Phenolic acids and in particular hippuric acid were the principal metabolites detected in all the analyzed tissues. The amount of metabolites increased with time of exposure to the ACNs, suggesting a modulatory effect on metabolic pathways or an increased efficiency in absorption/excretion in the older animals (8 weeks vs 4 weeks) (Del Bo *et al.*, 2010a).

A point of note is that anthocyanins are readily distinguished from other flavonoids as they undergo re-arrangements in response to pH. The red flavylium cation predominates at pH 1–3 but as the pH increases to 4 and above the colourless carbinol pseudobase is the major component along with smaller amounts of the colourless chalcone pseudobase and the blue quinoidal base (Clifford *et al.*, 2000). Anthocyanins are traditionally extracted and analyzed in acidic medium as the red flavylium cation is the most stable form. However, it is not known what forms predominate *in vivo*. The limited available experimental evidence indicates that, in the acidic conditions that prevail in the stomach, anthocyanins are in the red flavylium form, but once they enter more basic conditions in the small intestine the carbinol pseudobase is likely to predominate. It could be that the colourless carbinol pseudobase is the main form in the small intestine where it undergoes limited absorption, possibly being metabolised to conjugates that are overlooked because they cannot be converted to red flavylium forms prior to the eventual analysis. It is also possible that significant amounts of the carbinol pseudobase might pass into the large intestine where degradation, to as-yet undetermined products, occurs due to the action of colonic bacteria. More subtle scenarios may exist, and detailed information is unlikely to be forthcoming until ring-labelled ¹⁴C-anthocyanins become available (Crozier *et al.*, 2009).

The percentage of intake that is absorbed varies with structure (for example being significantly modulated by which sugars are attached to a flavonoid aglycone), and the food matrix. The exact yields and proportions of metabolites from any substrate will vary not only with the individual's genetic profile but also with the composition and competence of that individual's intestinal microflora. Any biological effects produced by these metabolites will be a function of the concentration achieved at the relevant site

and the susceptibility of the organelle (receptor, enzyme, transporter, etc.) that again might vary with the individual's genetic profile. It is not possible to quantify the magnitude of the variation produced by these factors, but it would not be unreasonable to assume an order of magnitude overall (Crozier *et al.*, 2009).

There is now emerging evidence that some phytochemicals, at concentrations that might be achieved under normal dietary circumstances, can exert modulatory effects in cells through selective actions on multiple intracellular signalling cascades, which are vital for cellular functions such as growth, proliferation and death (apoptosis). The identification of these molecular targets is an important first step that must be attained before the molecular mode(s) of action can be elucidated and understood, and this understanding is essential in order to formulate dietary strategies that might manage or even prevent certain non-communicable diseases. Emphasis on the mode of action in subsequent sections will cover investigations that either deal with clinical and preclinical studies or *in vitro* investigations that use either phenolic compounds and/or their main *in vivo* metabolites at concentrations that, at least, approach what might be achieved through diet (Crozier *et al.*, 2009).

Biological activities of ACNs

Anthocyanins possess a variety of pharmacological properties rendering them interesting as potential chemo-preventive agents. Antioxidant activity (capacity to scavenge free-radicals) shown by ACNs *in vitro* studies has been thought to be the most important property, because it is involved in the onset development of many of the chronic degenerative diseases (LDL oxidation in atheroma plaque development, DNA oxidation and cancer, oxidation and ageing, inflammation, etc.) (Espin *et al.*, 2007). However many recent studies reported the effects of ACNs on cellular events related to tumor progression or promotion (Fimognari *et al.*, 2008). Promising research has shown that ACNs may serve an important role in promoting health by reducing the risk of atherosclerosis and cancer, and ameliorating inflammation (Neto, 2007).

Chemoprevention, which is referred to as the use of nontoxic natural or synthetic chemicals to intervene in multistage carcinogenesis, has emerged as promising approach to reduce the risk cancer and to control cancer, alternative therapy that has some limitation and drawbreaks in treating patients. Rational and successful implementation of chemopreventive strategies rely on the precise understanding of underlying molecular mechanisms. Alteration of carcinogen metabolism and subsequent DNA adduction may represent an important mechanism by which a given agent modulates chemically-induced carcinogenesis in the initiation stage. Modulation of carcinogen metabolism, cell-cycle regulation and/or induction if apoptosis are two of the main mechanisms considered for recent chemopreventive strategies (Fimognari *et al.*, 2008).

Antioxidant activity

The phenolic structure of anthocyanins conveys marked antioxidant activity in model systems via donation of electrons or transfer of hydrogen atoms from hydroxyl moieties to free radicals. Several studies confirmed antioxidant properties of ACNs.

Accumulation of oxidative DNA damage over the human lifespan may be a significant factor in cancer development (Halliwell, 2002). Reactive oxygen species generated endogenously through aerobic metabolism are potent genotoxins, causing mutations, DNA strand breakage and oxidative DNA base damage *in vitro* and *in vivo*. The anticarcinogenic value of a diet high in fruits and vegetables may be due to the relatively high abundance of antioxidants in these foods, which act to inhibit oxidative DNA damage through a variety of mechanisms including free radical scavenging and metal chelation. Berry extracts and individual berry components, including the anthocyanins, are strong antioxidants *in vitro* and exhibit a broad spectrum of antioxidant activity in chemical and cellular systems (Duthie, 2007). Flavonoids are strong metal chelators and are able to suppress peroxy and hydroxyl radical-induced supercoiled DNA strand scission and generation of reactive oxygen species from activated human granulocytes (Gasiorowski *et al.*, 1997; Duthie *et al.*, 2000). Quercetin, the anthocyanidin cyanidin and its glycoside cyanidin 3-glycoside are powerful inhibitors of oxidant-induced DNA damage in normal human colon mucosal cells *in vitro* (Duthie *et al.*, 2005), while 3-glucopyranosides of delphinidin, cyanidin, petunidin, peonidin and malvidin ameliorate the effects of vitamin E depletion on oxidative DNA damage in rat liver (Ramirez-Tortosa *et al.*, 2001). **Black raspberry** extract significantly inhibits O-8oxo-dG formation and tumorigenesis in animals treated with the carcinogen AZM (Harris *et al.*, 2001). Regular **blood orange juice** consumption (600 mL/day for 21 days) determined a significant increase in lymphocyte DNA resistance to oxidative stress in a human intervention trial (Riso *et al.*, 2005). The effect of the intake of a single portion of blood orange juice (BOJ, 300 ml, providing 150 mg vitamin C) on mononuclear blood cell (MNBC) DNA damage, compared with a drink supplemented with the same amount of vitamin C (C-drink) or sugars (S-drink). DNA damage significantly decreased 3 h after BOJ intake (about 18 %; $P < 0.01$) and remained constant at 24 h (about 16 %; $P < 0.01$). No effect of the C-drink and S-drink was observed (Guarnieri *et al.*, 2007). An *in vitro* study was assessed to determine if the organic **red oranges** have a higher phytochemical content (i. e., phenolics, anthocyanins and ascorbic acid), total antioxidant activity and *in vitro* bioactivity, in terms of protective effect against oxidative damage at cellular level, than nonorganic red oranges. Results demonstrated that organic oranges had significantly higher total phenolics, total anthocyanins and ascorbic acid levels than the corresponding non-organic oranges (all $p < 0.05$). Moreover, the organic orange extracts had a higher total antioxidant activity capacity of inhibiting the production of conjugated diene containing lipids and free radicals in rat cardiomyocytes and differentiated Caco-2 cells, respectively (Tarozzi *et al.*, 2006). In another *in vitro* study, HUVEC were treated with **ferulic acid, quercetin and resveratrol** (Nicholson *et al.*, 2008). Quantitative RT-PCR studies indicated that resveratrol (0.1 μM) significantly increased the expression of the gene encoding endothelial NO synthase (eNOS), which synthesises the vasodilator molecule NO, and both resveratrol and quercetin decreased expression of the potent vasoconstrictor, endothelin-1 (ET-1), while ferulic acid had no effect. The effects of resveratrol (0.1 μM) were also investigated when HUVEC were

under oxidative stress following treatment with H₂O₂ (0–50 μM), which dose-dependently increased expression of eNOS and ET-1. Resveratrol stimulated eNOS mRNA in the absence of H₂O₂ and still allowed the increase with H₂O₂, but the effects were not additive. In contrast, resveratrol blocked the stimulatory effect of H₂O₂ on ET-1 expression. Hence, resveratrol has potent effects at a physiological concentration (0.1 μM) that would be expected to result in vasodilation and therefore help reduce blood pressure and the risk of CVD (Nicholson *et al.*, 2008).

Anthocyanins, including cyanidin and several glycosides are antimutagens in both the Ames and sister chromatid exchange (SCE) genotoxicity tests (Gasiorowski *et al.*, 1997) and effectively inhibit oxidative DNA damage in rat smooth muscle cells (Lazze *et al.*, 2003). Anthocyanins are bioactive in different *in vivo* model systems. An effect on the resistance of H₂O₂-induced DNA damage was detected in the Sprague-Dawley (SD) rat fed a **wild blueberry**-enriched diet (8%) over time (8 weeks), but not after four weeks. No significant effect was detected in plasma antioxidant capacity at four and eight weeks (Del Bo *et al.*, 2010b). Hepatic O-8oxo-dG was significantly decreased in vitamin E-deficient rats fed a complex anthocyanin extract containing glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin (1 g/kg diet) for 2 wk (Ramirez-Tortosa *et al.*, 2001). However, in a study using only cyanidin-3-glycoside at a more nutritionally relevant concentration, no effect was seen on endogenous DNA strand breakage and oxidative base damage in isolated lymphocytes and O-8oxo-dG levels in liver and colon from vitamin E-deficient rats. Moreover, lymphocytes isolated from vitamin E-deficient rats fed cyanidin-3-glycoside were as susceptible to induced oxidative stress as rats fed control diet (Duthie *et al.*, 2005). These data suggest that certain polyphenols can modulate oxidative DNA damage *in vivo*, but others cannot. Moreover, in those studies where complex anthocyanin extracts or single anthocyanin aglycones or glycosides have been shown to be effective against oxidative damage, the phytochemicals have generally been fed at supra-nutritional or pharmacological doses.

Modulation of phase I and II drug metabolizing enzyme activities and inhibition of DNA damage

Berry phytochemicals may exert their anticarcinogenic effect by modulating the enzyme systems that metabolise carcinogens or procarcinogens to genotoxins. Activation of the procarcinogen may be inhibited or it may be converted to a less reactive compound before it is able to bind to DNA and initiate mutagenesis and carcinogenesis.

Berry extracts inhibit mutagenesis by metabolically activated carcinogens in the Ames test, but are not as effective against carcinogens acting directly on DNA (Ono *et al.*, 2003). Several freeze-dried berry extracts inhibit NMBA-induced esophageal cancer *in vivo*, with strawberry, black raspberry and blackberry preparations decreasing esophageal papilloma numbers by 24–56% relative to control animals. Inhibition of tumorigenesis was associated with a concomitant decrease in the level of the DNA adduct, O-6-methylguanine, suggesting that the berry extract was acting to inhibit formation of the mutagenic agent from the procarcinogen (Kresty *et al.*, 2001). Moreover, tumor burden was reduced up to 64% when berry extract was fed after

cancer initiation by NMBA indicating that berry extracts function as potent chemoprotectants at a number of sites along the cancer cascade. The cytochrome P450 superfamily of enzymes metabolise a large number of procarcinogens to reactive intermediates that covalently bind to DNA and induce malignant transformation. P450 activity can be induced or inhibited by flavonoids. Ellagic acid inhibits mutagenesis and carcinogenesis by acting on both P450 xenobiotic metabolism and several phase 2 detoxifying enzymes. Rats fed ellagic acid have significantly fewer carcinogen-induced hepatic and esophageal tumours and this reduction in tumour burden is associated with changes in the activity of several enzymes involved in carcinogen activation and detoxification. Liver P450 reductase activity was decreased significantly (25%) in rats fed ellagic acid for 23 days, while glutathione transferase (GST), NADPH quinone reductase and UDP glucuronyltransferase (UDPGT) activities increased. *In vitro* studies indicate that specific isozymes of rat liver P450s are inhibited by ellagic acid, with those isozymes inhibited the most, generally the most important in the bioactivation of procarcinogens in rats and humans (Ahn *et al.*, 1996). In addition to altering activation or binding of the chemical carcinogen with DNA, black raspberry extract also alters expression of genes associated with inflammation and carcinogenesis. COX-2 gene expression and subsequently prostaglandin production and nitric oxide synthase activity are inhibited in premalignant rat esophageal cells following feeding with berry extract (Stoner *et al.*, 2006). A study on human colorectal adenocarcinoma HT-29 cancer cells was carried out to evaluate if anthocyanin fractions from high bush blueberry cultivars affect the activity of the detoxifying enzymes GST and QR. The QR activity was lower in all cells treated with an anthocyanin fraction from Tifblue, Powderblue, Brightblue, and Brightwell cultivars than in control cells ($P < 0.05$). The activity decreased gradually when treated with increased concentrations of anthocyanin fractions (50-150 $\mu\text{g}/\text{mL}$) in the Tifblue and Powderblue cultivars. The GST activity was lower ($P < 0.05$) in cells treated with anthocyanin fractions from all of the cultivars and at all concentrations (Srivastava *et al.*, 2007)

Inhibition of cell growth and induction of apoptosis

Anthocyanidins, including delphinidin, malvidin, peonidin and petunidin inhibit proliferation of cancer cells derived from various tissues including colon, breast, blood and lung at high micromolar concentrations (Cooke *et al.*, 2005), as shown in Table 3.6.1. Similarly, cyanidin and a mixture of several of its glycosides dose-dependently inhibit HCT116 and HT29 colon cancer cell growth, with an IC_{50} of 63 and 780 μM , respectively (Serraino *et al.*, 2003). In general, potency reflects the chemical structure of the polyphenol with the anthocyanin glycoside less effective than the parent aglycone (Cooke *et al.*, 2005). Cyanidin and its glucoside cyanidin-3-glycoside, reduce oxidant-induced DNA strand breakage in normal human lymphocytes *ex vivo* and are as potent chemoprotectants as the flavonols quercetin and myricetin. Moreover, the anthocyanins were more effective than several other antioxidants including kaempferol, silymarin, vitamin C, vitamin E and its water-soluble analogue Trolox suppress cancer cell metastasis by inhibiting the motility, adhesiveness and invasiveness of the metastatic human lung cancer cell line A549 (Chen *et al.*, 2006).

Cultured lung cells pretreated with anthocyanin metabolites (0–100 μ M) for 24 h have a reduced capacity to migrate through a matrigel layer. Both cyaniding-3-glucoside and cyaniding-2-rutinoside inhibited cell invasion by approximately 40% at the highest concentration tested (Chen *et al.*, 2006). Cyanidin 3-glucoside was also a potent inhibitor of cell adhesion (Chen *et al.*, 2006). A study on human colorectal adenocarcinoma HT-29 cancer cells was carried out to evaluate if anthocyanin fractions from high bush blueberry cultivars increase apoptosis using two different methods: DNA fragmentation and caspase-3 activity. The results indicated that apoptosis was confirmed in HT-29 cells when treated with anthocyanins from blueberry cultivars at 50-150 μ g/mL concentrations (Srivastava *et al.*, 2007).

Table 3.6.1 Summary of *in vitro* growth inhibitory effects of anthocyanidins, anthocyanins and anthocyanin-rich extracts on different cell lines adapted from (Cooke *et al.*, 2005).

Compounds	References
Delphinidin	(Meiers <i>et al.</i> , 2001; Katsube <i>et al.</i> , 2003; Lazze <i>et al.</i> , 2004; Marko <i>et al.</i> , 2004; Zhang <i>et al.</i> , 2005)
Cyanidin	(Meiers <i>et al.</i> , 2001; Kang <i>et al.</i> , 2003; Katsube <i>et al.</i> , 2003; Hyun & Chung, 2004; Marko <i>et al.</i> , 2004; Zhang <i>et al.</i> , 2005)
Malvidin	(Meiers <i>et al.</i> , 2001; Hyun & Chung, 2004; Zhang <i>et al.</i> , 2005)
Peonidin	(Marko <i>et al.</i> , 2004)
Pelargonidin	(Marko <i>et al.</i> , 2004; Zhang <i>et al.</i> , 2005)
Petunidin	(Zhang <i>et al.</i> , 2005)
Delphinidin-3-galactoside	(Katsube <i>et al.</i> , 2003)
Delphinidin-3-glucoside	(Katsube <i>et al.</i>, 2003; Olsson <i>et al.</i>, 2004)
Cyanidin-3-galactoside	(Meiers <i>et al.</i> , 2001)
Cyanidin-3-glucoside	(Fimognari <i>et al.</i>, 2004; Olsson <i>et al.</i>, 2004)
Malvidin-3-glucoside	(Meiers <i>et al.</i> , 2001; Olsson <i>et al.</i> , 2004)
Chokeberry extract	(Malik <i>et al.</i> , 2003; Olsson <i>et al.</i> , 2004; Zhao <i>et al.</i> , 2004)
Grape extract	(Singletary <i>et al.</i> , 2003; Olsson <i>et al.</i> , 2004; Zhao <i>et al.</i> , 2004)
Cherry extract	(Kang <i>et al.</i> , 2003; Zhao <i>et al.</i> , 2004)
Bilberry extract	(Katsube <i>et al.</i> , 2003)
Black currant extract	(Olsson <i>et al.</i> , 2004)
Cranberry anthocianins	Seeram <i>et al.</i> , 2004)

Several studies report that anthocyanins inhibit tumor formation *in vivo*. Mammary tumor formation, multiplicity and tumor volume is decreased in DMBA-treated female rats fed 15 different anthocyanins from grape juice (Singletary *et al.*, 2003), while anthocyanins, given either as a mixture (estimated as 6.4 mg/day), a single pure cyanidin solution (estimated as 1.6 mg/day) or as freeze-dried tart cherries (600 mg/day) inhibit dysplasia in cancer-susceptible mice (Apc^{min}). Mice consuming anthocyanins, cyanidin or tart cherries had significantly fewer and smaller caecal adenomas than mice consuming the control diet. The authors calculated that the comparative human doses required to modulate tumor formation would be approximately 2.4 or 0.6 g of anthocyanins or cyanidin, respectively, which is obviously supraphysiological and unachievable by normal dietary intervention (Serraino *et al.*, 2003). Disturbingly, tart cherry consumption was associated with a significantly increased tumor volume in the small intestine. Conversely, cyanidin 3-

glucoside decreased the number of malignant and benign skin tumors in mice treated with DMBA as an initiator and TPA as promotor (Ding *et al.*, 2006)

Some anthocyanins suppress cancer cell growth *in vitro* by modifying cell signalling pathways. Benzo[a]pyrene diolepoxide (BPDE) treatment of mouse epidermal JB6 CI 41 cells induces activated protein 1 (AP-1) and nuclear factor kappaB (NFkB). Changes in expression of these proteins, which function normally to regulate cell proliferation and cell cycle control, are believed to be involved in human cancer development. Pretreatment of JB6 CI 41 mouse cells with methanol-extracted black raspberry fractions (1–100 µg/mL) dose-dependently inhibits carcinogen-induced AP-1 or NFkB protein expression in a luciferase reporter assay without affecting carcinogen/DNA binding (Huang *et al.*, 2002). Inhibition of the AP-1 pathway or NFkB activation was mediated by a decrease in the phosphorylation of members of the MAPK protein family and inhibition of Ikbα phosphorylation and degradation, respectively (Huang *et al.*, 2002). Berry extracts were inhibitory only when given prior to or during carcinogen exposure. Similarly, methanol-extracted blackberry extract dramatically inhibited BPDE-induced activation of AP-1 and NFkB and subsequent expression of vascular endothelial growth factor (VEGF) and COX-2, proteins believed to have a key role in tumor promotion and progression. Further studies indicated that the blackberry extract was altering the cell signalling pathways responsible for activating AP-1 and NFkB by inhibiting MAPKs activity and Ikbα phosphorylation (Lu *et al.*, 2006). Blackberry and strawberry extracts and cyaniding-3-glucoside produced similar effects in the AP-1 and MAPK pathways in JB6 mouse cell treated with UVB or TPA (Feng *et al.*, 2004; Wang *et al.*, 2005; Ding *et al.*, 2006). Whether down-regulation of this gene cascade alters tumorigenesis *in vivo* remains to be established. The process of metastasis, the spread of malignant cancer cells from their primary location to secondary sites in the body, is dependent on specific changes within the tumor. Cancer cells must attain increased motility, surface adhesion properties and increased extracellular protease activity to facilitate cell movement and invasiveness. Degradation and rupture of the extracellular matrix by proteases such as metalloproteinases (MMPs), serine proteinases and cathepsins, allow cancer cell migration and metastasis. Two glucosides of the anthocyanidin cyanidin (cyanidin 3-rutinoside and cyanidin 3-glucoside) reduce the invasive ability of the highly metastatic human lung cancer cell line A549 by differentially altering transcription of several proteases involved in carcinogenesis (Chen *et al.*, 2006). Expression of matrix metalloproteinase-2 (MMP-2) and urokinase-plasminogen activator (u-PA) was inhibited, while conversely, expression of tissue inhibitor of matrix metalloprotein-2 (TIMP-2) and plasminogen activator inhibitor (PAI), endogenous inhibitors of MMP-2 and u-PA, respectively, was increased (Chen *et al.*, 2006). Extracts from blueberry down-regulate MMP activity and increase TIMP-1 and TIMP-2 activity in human prostate cancer cells *in vitro* (Manach *et al.*, 2004). In a study of Katsube *et al.* (2003), among ethanol extracts of 10 edible berries, bilberry extract was found to be the most effective at inhibiting the growth of HL60 human leukemia cells and HCT116 human colon carcinoma cells *in vitro*. Bilberry extract induced apoptotic cell bodies and

nucleosomal DNA fragmentation in HL60 cells. The proportion of apoptotic cells induced by bilberry extract in HCT116 was much lower than that in HL60 cells, and DNA fragmentation was not induced in the former. Of the extracts tested, that from bilberry contained the largest amounts of phenolic compounds, including anthocyanins, and showed the greatest 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. Pure delphinidin and malvidin, like the glycosides isolated from the bilberry extract, induced apoptosis in HL60 cells. These results indicate that the bilberry extract and the anthocyanins, bearing delphinidin or malvidin as the aglycon, inhibit the growth of HL60 cells through the induction of apoptosis. Only pure delphinidin and the glycoside isolated from the bilberry extract, but not malvidin and the glycoside, inhibited the growth of HCT116 cells (Katsube *et al.*, 2003). Another study evaluated the bioactivities of phenolic compounds in rabbiteye blueberries and assessed their potential antiproliferation and apoptosis induction effects using two colon cancer cell lines, HT-29 and Caco-2. Polyphenols in three blueberry cultivars, Briteblue, Tifblue, and Powderblue, were extracted, freeze-dried and further separated into phenolic acids, tannins, flavonols, and anthocyanins. The dried extracts and fractions were added to the cell culture medium to test for antiproliferation activities and induction of apoptosis. Flavonol and tannin fractions resulted in 50% inhibition of cell proliferation at concentrations of 70-100 and 50-100 $\mu\text{g}/\text{mL}$ in HT-29 and Caco-2 cells, respectively. The phenolic acid fraction showed relatively lower bioactivities with 50% inhibition at ca. 1000 $\mu\text{g}/\text{mL}$. The greatest antiproliferation effect among all four fractions was from the anthocyanin fractions. Both HT-29 and Caco-2 cell growth was significantly inhibited by >50% by the anthocyanin fractions at concentrations of 15-50 $\mu\text{g}/\text{mL}$. Anthocyanin fractions also resulted in 2-7 times increases in DNA fragmentation, indicating the induction of apoptosis (Yi *et al.*, 2005). In the study of Shin *et al.* (2009), the authors investigated if the anthocyanins isolated from meoru (*Vitis coignetiae* Pulliat) exerted antiproliferative and anti-invasive and apoptotic effects on human hepatoma Hep3B cells. It was found that the anthocyanins could inhibit cell growth by 75% at the concentration of 400 $\mu\text{g}/\text{mL}$ for 48 h. Flow cytometric analysis showed that the anthocyanins increased the amount of DNA fragments (sub-G1 fraction) in a dose-dependent manner, which is closely related to mitochondrial dysfunction and reduction in antiapoptotic proteins (Bcl-2, χIAP , cIAP-1, and cIAP-2). The anthocyanins also significantly inhibited the migration and invasion of Hep3B cells through a matrigel-coated chamber (Shin *et al.*, 2009). Anthocyanins, cyanidin-3-glucoside (Cy-3-glc) and delphinidin-3-glucoside (Dp-3-glc) and their respective vinylpyranoanthocyanin-catechins (portisins) inhibited, in a dose-dependent manner, the growth of the breast cancer cell line (ER- α) MCF-7. The cytotoxicity effect was higher when cells were treated with Dp-3-glc and its respective portisin, however this did not correspond to modification on mRNA ER expression (Fernandes *et al.*, 2010).

Inhibition of angiogenesis

Angiogenesis is the process by which new blood vessels are formed from pre-existing vessels. Angiogenesis, a normal physiological process in growth and development and in wound healing, is also a fundamental step in the transition of tumors from a benign

to a malignant or invasive state. Tumors induce angiogenesis by secreting growth factors such as vascular endothelial growth factor (VEGF) to induce capillary growth into the tumor allowing tumor expansion and spread. VEGF is a chemical signal produced by cells that stimulates the growth of new blood vessels. It is part of the system that restores the oxygen supply to tissues when blood circulation is inadequate. When VEGF is over-expressed, it can contribute to disease. Solid cancers cannot grow beyond a limited size without an adequate blood supply; cancers that can express VEGF are able to grow and metastasize (Folkman, 2006).

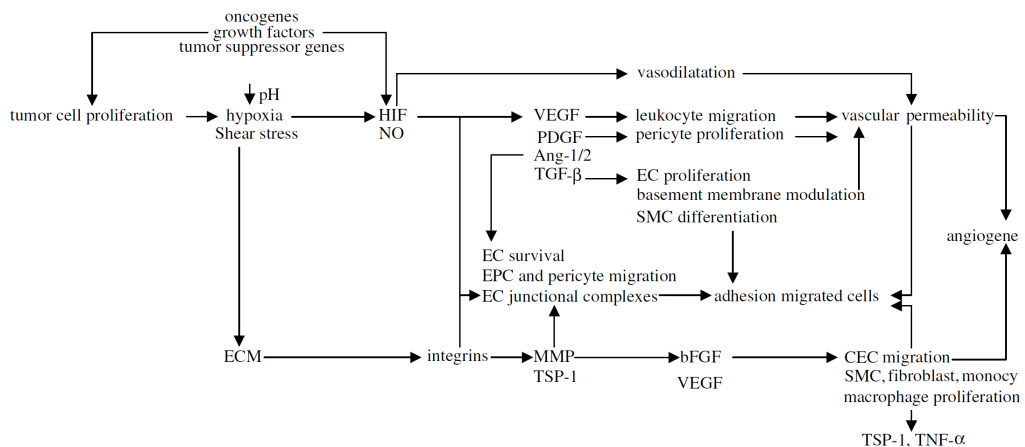


Figure 3.6.3 Interaction of key-players of the angiogenic process. *Ang 1/2*, angiopoietin 1 and 2; *bFGF*, basic fibroblast growth factor; *CEC*, circulating endothelial cell; *EC*, endothelial cell; *ECM*, extracellular matrix; *EPC*, endothelial progenitor cell; *HIF*, hypoxia-inducible factor; *MMP*, matrix metalloproteinase; *NO*, nitric oxide; *PDGF*, platelet-derived growth factor; *SMC*, smooth muscle cell; *TGF- β* , tumor growth factor- β ; *TNF- α* , tumour necrosis factor- α ; *TSP-1*, thrombospondin-1; and *VEGF*, vascular endothelial growth factor. Adapted from (de Vos et al., 2004).

Cancer cells can dissociate from the main tumor and be carried in the circulation to a site distant from the primary tumor where they implant and begin formation of a secondary tumor (Figure 3.6.3).

Several berry derivatives have potent anti-angiogenesis properties *in vitro*. In a series of elegant experiments, berry extracts (wild blueberry, bilberry, cranberry, elderberry, raspberry seed and strawberry) were tested for their ability to inhibit angiogenesis via altered VEGF expression and invasiveness. Human HaCaT keratinocytes, which are normally quiescent, retain the ability to initiate angiogenesis in response to certain stimuli. Each of the berry extracts inhibited hydrogen peroxide and $TNF-\alpha$ induced VEGF expression in these cells. Inducible VEGF expression was related to the flavonoid component of the berry extracts, independently of their antioxidant potential (Roy et al., 2002). Moreover, in the same study, berry extracts were able to inhibit angiogenesis in human dermal microvascular endothelial cells (Roy et al., 2002).

Similarly, crude whole black raspberry extract (at a concentration of 0.1% w/v) was antiangiogenic in a human placental tissue-based fibrin clot angiogenesis assay (Liu *et al.*, 2005). Fractionation of crude extract revealed a highly potent anti-angiogenic component that accounted for only 1% of the fresh weight of the whole berries. This highly active fraction completely inhibited angiogenic initiation and vessel sprouting. Several active compounds, including gallic acid, were subsequently identified, although none was as effective individually as the whole fraction, indicating that these compounds act synergistically (Liu *et al.*, 2005). Berry extracts can also inhibit angiogenesis in animals. Hemangiomas are abnormally dense collections of dilated capillaries that can occur in the skin or internal organs and have been used to model angiogenesis *in vivo*. Mouse endothelioma cells (EOMA) injected into compatible host animals proliferate to form blood vessel conduits that fuse with the systemic circulation, drawing blood into the hemangioma. EOMA cells pretreated in culture either with wild blueberry or a proprietary berry mix (OptiBerry) were unable to form hemangioma tumors as effectively as placebo-treated cells after injection into host mice. In the wild blueberry treatment group, less than 50% of the animals tested positive for the presence of a hemangioma and in those animals that tested positively, tumor mass was below 50% of that observed in the untreated group. Moreover, macrophage infiltration was significantly lower in these animals (Atalay *et al.*, 2003). Macrophages, recruited to sites of inflammation or infection, produce growth factors and cytokines that regulate angiogenesis. Berry extracts may work to inhibit angiogenesis by inhibiting inducible expression of NFkB and basal expression of monocyte chemoattractant protein 1 (MCP-1) (Atalay *et al.*, 2003).

Basal and VEGF-stimulated HUVEC migration and proliferation are potently inhibited by delphinidin (10-20 µg/mL). Flow cytometric analysis demonstrated that delphinidin inhibition of proliferation is correlated with the blockade of cell cycle in G(0)/G(1) phase. Western blot analysis showed that delphinidin reverses the VEGF-induced decrease in expression of cyclin-dependent kinase inhibitor p27^(kip1) and the VEGF-induced increase of cyclin D1 and cyclin A, both being necessary to achieve the G(1)-to-S transition. Furthermore, delphinidin inhibits neovascularisation *in vivo* in chorioallantoic membrane model (Favot *et al.*, 2003).

Among the tested anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin), Lamy and colleagues (2006) found that delphinidin was the most potent angiogenic inhibitor. *In vitro*, low concentrations of delphinidin (25 µM) inhibited VEGF-induced tyrosine phosphorylation of VEGF receptor (VEGFR)-2, leading to the inhibition of downstream signaling triggered by VEGFR-2. Inhibition of VEGFR-2 by delphinidin repressed the VEGF-induced activation of ERK-1/2 signaling and the chemotactic motility of HUVEC as well as their differentiation into capillary-like tubular structures in Matrigel and within fibrin gels. *In vivo*, delphinidin (300 µM) was able to suppress basic fibroblast growth factor-induced vessel formation in the mouse Matrigel plug assay (Lamy *et al.*, 2006).

Recently, tumor vascular targeting has expanded to include not only endothelial cells (EC) but also smooth muscle cells (SMC), which contribute to a mature and functional

vasculature. In a more recent study, Lamy showed that delphinidin (25 μM) also inhibits activation of the platelet-derived growth factor (PDGF)-BB receptor-beta [platelet-derived growth factor receptor-beta (PDGFR-beta)] in SMC and that this inhibition may contribute to its antitumor effect. The inhibitory effect of delphinidin on PDGFR-beta was very rapid and led to the inhibition of PDGF-BB-induced activation of extracellular signal-regulated kinase (ERK)-1/2 signaling, in a dose-dependent manner, with a complete inhibition at 20 μM , and of the chemotactic motility of SMC, as well as the differentiation and stabilization of EC and SMC into capillary-like tubular structures in a three-dimensional co-culture system. Using an anthocyan-rich extract of berries, the authors showed that berry extracts were able to suppress the synergistic induction of vessel formation by basic fibroblast growth factor-2 and PDGF-BB in the mouse Matrigel plug assay. Oral administration of the berry extract also significantly retarded tumor growth in a lung carcinoma xenograft model (Lamy *et al.*, 2008).

A very recent study (Matsunaga *et al.*, 2010) was designed to examine the antiangiogenic properties and antioxidant activities of the main anthocyanidins (delphinidin, cyanidin and malvidin) found as constituents in *Vaccinium myrtillus* (bilberry) anthocyanosides (VMA) and of N-acetyl-L-cysteine (NAC). Each of these anthocyanidins concentration-dependently inhibited VEGF-induced tube formation in a co-culture of human umbilical vein endothelial cells (HUVECs) and fibroblasts, the effect of each anthocyanidin being significant at 3 and/or 10 μM , while NAC significantly inhibited such tube formation at 1 μM (the only concentration tested). Moreover, each anthocyanidin (0.3-10 μM) and NAC (1-1000 μM) concentration-dependently scavenged the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The inhibitory effects against angiogenesis were similar among the anthocyanidins, as were those against the DPPH radical. Moreover, their radical-scavenging effects were induced by concentrations that were at or below those that induced their antiangiogenic effects. These findings indicate that the inhibitory effect of VMA on angiogenesis may depend on those of its main constituent anthocyanidins (delphinidin, cyanidin and malvidin), presumably via antioxidant effects (Matsunaga *et al.*, 2010).

Antinflammatory activity

Other effects of ACNs associated with protection from oxidative stress involve their role in reducing the inflammatory response as demonstrated by a decline of markers such IL-1- β , IL-6, TNF- α in rats (Tsuda *et al.*, 2002) and in humans (Bagchi *et al.*, 2004; Neto, 2007).

In a review on anti-inflammatory compounds of plant origin, Calixto and colleagues (2004) found that many phenolic compounds exert their effects through the modulation of the cytokine system. In particular the effects described for ACNs and phorogucinol derivatives seem to be dependent on the blockade of NF κ B activation, but might also include the inhibition of other pro-inflammatory pathways (Calixto *et al.*, 2004). Tsuda and colleagues (2002) reported that oral administration of Cy-3-glc suppressed the zymosan-induced response in rats. Treatments with Cy-3-glc also

reduced the elevation of NO_x, TNF- α , IL-1 β , IL-6 and CINC-1 concentrations. Furthermore, Cy-3-glc normalized several acute phase proteins in rats treated with zymosan. Kim and co-authors (2006) examined the inhibition of the expression of some inflammatory genes associated with ischemia-reperfusion (I/R) injury by anthocyanins isolated from black soybean seed coat in (TNF- α)-treated bovine aortic endothelial cells. In addition, its potential use on I/R-injury was investigated using rats subjected to 30-min occlusion of left descending coronary artery followed by 24-h reperfusion. Western blot analysis and luciferase activity assay showed that anthocyanins inhibited TNF- α -induced VCAM-1, ICAM-1, and COX-2 levels, which is through NF κ B-dependent pathway. Further, anthocyanins protected myocardial injury from I/R in rats (Kim *et al.*, 2006). CD40 is a member of TNF receptor superfamily that provides activation signals not only in antigen-presenting cells but also in a variety of non-immune cells, including vascular cells like endothelial cells (ECs). Intracellular tumor necrosis factor receptor-associated factors (TRAFs) translocation to lipid rafts is a key element in CD40-induced signaling. Treatment of ECs with anthocyanin (Cy-3-glc and Pn-3-glc, 100 μ M) prevented from CD40-induced proinflammatory status, measured by production of IL-6, IL-8, and monocyte chemoattractant protein-1 through inhibiting CD40-induced NF κ B activation. TRAF-2 played pivotal role in CD40-NF κ B pathway as TRAF-2 small interference RNA (siRNA) diminished CD40-induced NF κ B activation and inflammation. TRAF-2 overexpression increased CD40-mediated NF κ B activation. Moreover, TRAF-2 almost totally recruited to lipid rafts after stimulation by CD40 ligand and depletion of cholesterol diminished CD40-mediated NF κ B activation. Exposure to anthocyanin not only interrupted TRAF-2 recruitment to lipid rafts but also decreased cholesterol content in Triton X-100 insoluble lipid rafts. However, anthocyanin did not influence the interaction between CD40 ligand and CD40 receptor. So the authors conclude that their findings suggest that anthocyanin protects from CD40-induced proinflammatory signaling by preventing TRAF-2 translocation to lipid rafts through regulation of cholesterol distribution, which thereby may represent a mechanism that would explain the anti-inflammatory response of anthocyanin (Xia *et al.*, 2007).

Sustained microglial activation in the central nervous system (CNS) has been extensively investigated in age-related neurodegenerative diseases and has been postulated to lead to neuronal cell loss in these conditions. Lau and co-authors (2007) investigated the effect of blueberry extract on preventing inflammation-induced activation of microglia. Results indicated that treatments with blueberry extract inhibited the production of the inflammatory mediator nitric oxide (NO) as well as the cytokines IL-1 β and TNF α , in cell conditioned media from lipopolysaccharide (LPS)-activated BV2 microglia. Also, mRNA and protein levels of inducible nitric oxide synthase and COX-2 in LPS-activated BV2 cells were significantly reduced by treatments with blueberry extract (Lau *et al.*, 2007)

Relationship thrombosis-tumor: the role of endothelium

Endothelial cells are involved in many aspects of vascular biology, including: vasoconstriction and vasodilatation, and hence the control of blood pressure; blood

clotting (thrombosis & fibrinolysis); atherosclerosis; formation of new blood vessels (angiogenesis); inflammation and swelling (oedema) (Grandel & Grimminger, 2003). Endothelial cells also play an important role in the passage of various materials and of white blood cells from bloodstream to tissues and vice versa (Figure 3.6.4). In some organs, endothelial cells are highly differentiated to perform specialized 'filtering' functions. Examples of such unique endothelial structures include the renal glomerulus and the blood-brain barrier (Grandel & Grimminger, 2003).

Under physiological conditions, endothelial cells generate an active antithrombotic surface that inhibits clotting activation and reduce the adhesion of platelets and other circulating cells. When exposed to physical or biochemical stimuli, endothelium undertakes a series of phenotypic changes that leads to a shift towards a prothrombotic condition. In particular, bacterial products (e.g. lipopolysaccharide, LPS), as well as inflammatory cytokines, stimulate endothelial cells to express procoagulant tissue factor (TF) and cell adhesion molecules (Dauphinee & Karsan, 2006). It seems that LPS directly induce the prothrombotic state by upregulating the endothelial expression of TF through an NFkB-dependent mechanism (Parry & Mackman, 1995).

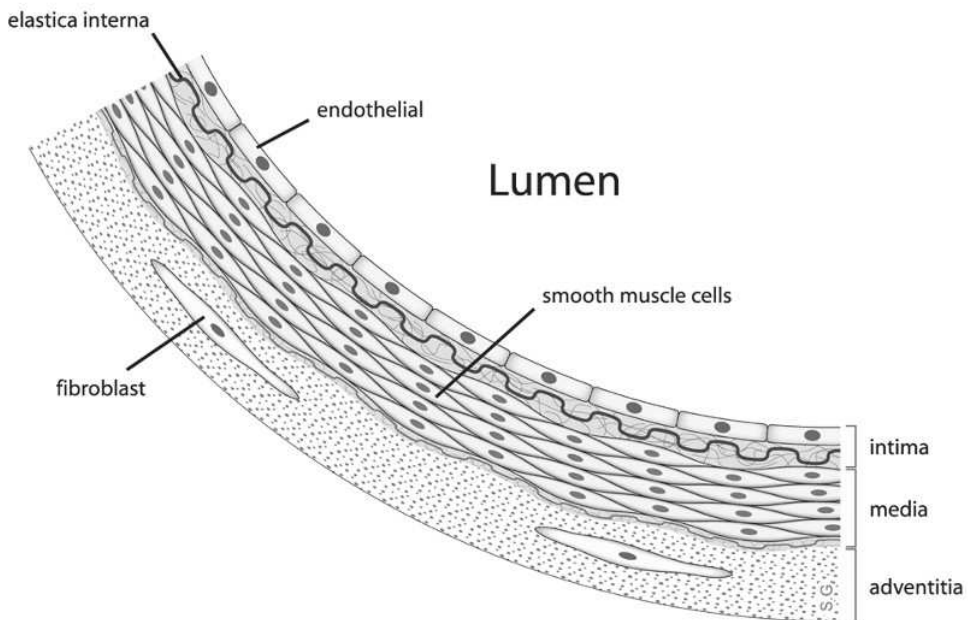


Figure 3.6.4 *Endothelium is the layer of thin specialized epithelium, comprised of a simple squamous layer of cells that line the interior surface of blood vessels, forming an interface between circulating blood in the lumen and the rest of the vessel wall (simple squamous epithelium). Endothelial cells line the entire circulatory system, from the heart to the smallest capillary (Jaffe, 1987).*

These modifications can lead to the **activation of the blood clotting cascade** (Figure 3.6.5), which culminates in fibrin generation, in addition to increasing cellular adhesion processes, which can further stimulate localized coagulation. These phenomena are also involved in the progression of chronic inflammatory diseases and are of particular relevance at microcirculation level, where the interactions between the vascular wall and circulating cells are enhanced. TF, also known as FIII, is a cell-bound transmembrane glycoprotein that is constitutively expressed by fibroblasts and smooth muscle cells of the vessel wall (Furie & Furie, 2008). It is also constitutively expressed in many tissues such as the brain, heart, lungs, kidneys, testes, and placenta, highlighting the importance of this glycoprotein (Semeraro & Colucci, 1997). Inflammatory stimuli (eg, LPS in sepsis), adhesion molecules (eg, P-selectin expressed on activated platelets and CD40 ligand expressed on activated white blood cells), inflammatory cytokines (eg, interleukin-6 and tumor necrosis factor), and oxidized low-density lipoprotein (LDL) can induce TF expression in monocytes and endothelial cells (Grignani & Maiolo, 2000). Circulating microparticles derived from monocytes and other cell types have also been shown to express TF on their surface (Panes *et al.*, 2007).

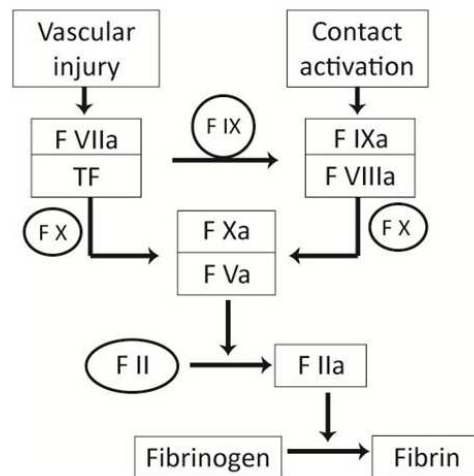


Figure 3.6.5 Overview of the coagulation cascade

TF not only plays an important role in coagulation but also mediates intracellular signaling events in inflammation, apoptosis, embryonic development, and cell migration (Rao & Pendurthi, 2005). In veins, endothelial cell activation results in the expression of adhesion molecules that then tether TF-bearing monocytes or microparticles onto their surface. It is thought that these tethered cells may trigger venous thrombosis (Furie & Furie, 2008). TF-bearing cells or microparticles provide the key cellular surface for the initiation of coagulation (Furie & Furie, 2008). Coagulation is initiated when TF binds to circulating FVIIa, forming a catalytic complex, TF-FVIIa, the so-called extrinsic tenase complex, on the phospholipid surface of the TF-bearing cell membrane (Hoffman & Monroe, 2007; Furie & Furie, 2008). See also Figure 3.6.5.

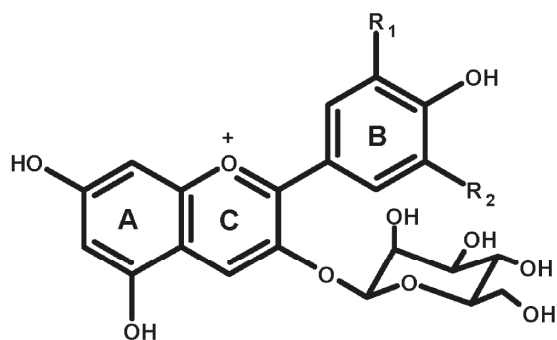
The extrinsic tenase complex then activates FIX and FX, and the resultant FXa converts a small amount of prothrombin (FII) to thrombin. This small amount of thrombin triggers the amplification phase by activating platelets as well as FV, FVIII, and FXI. Activated platelets provide a surface on which the coagulation factors assemble together. FXIa generates additional FIXa, which then binds to FVIIIa on the surface of activated platelets to form the intrinsic tenase complex. This complex propagates coagulation by efficiently activating FX. FXa binds to FVa on the surface of activated platelets to form the prothrombinase complex. A potent activator of prothrombin, the prothrombinase complex triggers a burst of thrombin generation (Hoffman & Monroe, 2007; Furie & Furie, 2008).

Blood coagulation and cancer biology are strictly related. In patients with cancer, blood coagulation is activated in the direction of a prothrombotic state. Moreover, a pro-coagulant environment may promote cancer progression in different ways. Blood coagulation proteins interact with cells in the vasculature to maintain hemostasis. However, many proteins that are involved in coagulation and anticoagulation, as well as fibrinolysis, are also found in extravascular tissues. In different organs, these proteins may be involved in cell-signaling mechanisms, through interaction with cell receptors like protease-activated receptors (PARs). Such interactions may drive inflammation, angiogenesis and cell proliferation. The potential procarcinogenic actions of proteases like thrombin may be counteracted by the anticoagulant and anti-inflammatory actions of the protein C-thrombomodulin mechanism (ten Cate & Falanga, 2008). Patients with hematologic malignancies are at high risk of thrombotic or hemorrhagic complications. The incidence of these events is greatly variable and is influenced by many factors, including the type of disease, the type of chemotherapy, and the use of a central venous device. As in solid tumors, a number of clinical risk factors have been identified and contribute to the increasing thrombotic rate in hematologic malignancies. Biologic properties of the tumor cells can influence the hypercoagulable state of patients with these malignancies by several mechanisms. Of interest, oncogenes responsible for neoplastic transformation in leukemia also may be involved in clotting activation (Falanga & Marchetti, 2009). In the blood of cancer patients, the balance is usually shifted towards a procoagulant direction. The resulting excess thrombin- and fibrin-forming activity promotes venous thrombosis and may in the extravascular compartment stimulate cancer progression. The activation of platelets and their interaction with leukocytes may propagate this process. In addition to the therapeutic modulation of the prothrombotic environment, the induction of specific anticoagulant proteins including thrombomodulin may have effects on tumor growth or dissemination, but the nature of these effects still remains hard to predict (ten Cate & Falanga, 2008). Recently, emphasis has been given to the potential risk of cancer therapy (both surgery and chemotherapy) in enhancing the risk for thromboembolic disease. Possible contributory causes for thromboembolic disease in cancer include the capacity of tumor cells and their products to interact with platelets, clotting and fibrinolytic systems, as well as their interactions with endothelial cells and tumor-associated macrophages. Cytokine release, acute phase reaction and

neovascularization may contribute, in cancer patient, to *in vivo* clotting activation, which is well documented by several plasmatic markers of an hypercoagulable state. Last but not least, a direct pathogenetic role of clotting activation in the progression of malignancy has been repeatedly proposed on the basis of pharmacological studies with anticoagulant/fibrinolytic drugs in experimental animals and selected clinical malignancies, as well as, lately, in genetically modified animal models (e.g. mice transgenic for PAI-1) (Donati, 1994).

Given this background and the important role of vegetables and fruits intake in the protection and prevention against tumor development, it is of interest to determine bioactive compounds action at the microvascular level. In particular little is known about the effects of phytochemicals on angiogenesis and procoagulant activity of endothelial cells.

We designed an *in vitro* study to investigate the effect of selected fruit bioactive compounds, delphinidin-3-glucoside (Dp-3-glc) and cyanidin-3-glucoside (Cy-3-glc) (Figure 3.6.6), on different endothelial cell functions involved in both angiogenesis and hemostasis.



$R_1 = \text{OH}; R_2 = \text{H}$: Cyanidin-3-glucoside

$R_1 = \text{OH}; R_2 = \text{OH}$: Delphinidin-3-glucoside

Figure 3.6.6 Chemical structures of Cyanidin-3-glucoside (Cy-3-glc) and Delphinidin-3-glucoside (Dp-3-glc)

3.7 Materials and methods

Endothelial cells

The immortalized human microvascular endothelial cell line-1 (HMEC-1) was kindly supplied by Dr. F.J. Candal (CDC, Centers for Disease Control and Prevention, Atlanta, GA, USA) (Ribeiro *et al.*, 1995). HMEC-1 were grown in RPMI 1640 medium (Gibco, Gaithersburg, MD, U.S.A.) supplemented with 5% fetal bovine serum (FBS,

Gibco), 2 mM L-glutamine (Gibco), 10 ng/ml epidermal growth factor (ICN, Costa Mesa, CA, U.S.A.) and 1 µg/ml hydrocortisone (ICN), 100 µg/ml streptomycin (Bristol-Myers Squibb, Princeton, NJ, USA), 5 µg/ml amphotericin B (Bristol-Myers Squibb). HMEC-1 were kept in a 5% CO₂/95% air atmosphere in a humidified incubator (Heraeus, Milan, Italy) at 37°C and serially passaged in T25 flasks (Falcon, Becton Dickinson, Mountain View, CA, USA) by trypsin/EDTA treatment (0.25% trypsin/EDTA solution, Life Technologies, Inc., Rockville, MD, U.S.A.) at 1:3 split ratio, twice a week. At the end of each experiment, endothelial cells were counted under microscopy and the Trypan Blue exclusion test was used to determine cell viability. More than 95% of cells resulted viable in all experiments.

Preparation of Pool of platelet-poor plasma (PPP)

Pool of platelet-poor plasma (PPP) was isolated from citrated blood of 40 healthy volunteers by double sequential centrifugation at 3,000 g for 15 min at room temperature, aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C until testing.

3.7.1 Angiogenesis study

Since most cancers are dependent on the growth of tumor blood vessels, angiogenesis may thus provide an efficient strategy to delay or block tumor growth. Capillary-like formation assay is a reliable approach able to test pro- or anti-angiogenic properties of a chemical compound (Folkman, 2003). The method is based on the differentiation of endothelial cells to form capillary-like structures on a basement membrane matrix, Matrigel. It is a matrix of a mouse basement membrane neoplasm. Matrigel is composed of a complex mixture of basement proteins, including laminin, type IV collagen, entactin/nitrogen and proteoheparan sulfate, and also contains growth factors. Migration and proliferation of endothelial cells are critical steps in angiogenesis since these events allow cells to disseminate from pre-existing vessels to form new capillary structures.

Capillary-like tube formation assay in Matrigel

A Matrigel-based capillary-genesis assay was performed on HMEC-1 to assess the ability of these cells to form an organized tubular network. Standard Delphinidin-3-O-glucoside (Dp-3-glc) and Cyanidin-3-glucoside (Cy-3-glc) were purchased from Polyphenols Laboratory (Sadnes, Norway), their chemical structures are represented in Figure 3.6.6. They were stored dried at -20°C until the execution of each experiment. Appropriate amounts of Dp-3-glc and Cy-3-glc were dissolved in RPMI 1640 medium at the appropriate concentrations just before each experiment. The day before the experiment confluent HMEC-1 were starved overnight with fresh RPMI 1640 medium supplemented with 1% FBS. Growth factor-reduced Matrigel® (Becton Dickinson) was thawed overnight on ice at +4°C. The day of the assay Matrigel was placed on the bottom of 96-well plate, and left at 37°C for 1h to gelificate. Starved HMEC-1 were collected by enzymatic detachment (0.25% trypsin-EDTA), counted and re-suspended

in RPMI 1640 medium supplemented with 0.5% FBS. Then, 10,000 HMEC-1 cells/well were seeded on Matrigel and incubated at 37°C in a 5% CO₂-humidified atmosphere with 50 ng/mL of purified recombinant human (rHu) VEGF¹⁶⁵ (R&D System, Minneapolis, MN), in absence or presence of increasing doses of Dp-3-glc and Cy-3-glc (0.1-1-10-100 μM). In resting condition experiments, the effect of ACN was evaluated in the absence of VEGF pro-angiogenic stimulus. After 24h of incubation tube formation was examined under inverted light microscopy (LEICA DM IRB, Germany). Digital images were acquired using a microcamera system (Optikam PRO 3 Digital Camera, Ponteranica, Italy) at 5X magnification. Three images per well were randomly taken in different areas of the well, selecting those areas that were distinct and distant enough to not overlap each other. Total tube length was determined by an image analysis software (Image J, National Institutes of Health, Bethesda, MD, U.S.A.). Photo capture and tube length quantification were performed by two observers blinded to treatment group. Results are expressed as mm tube length/cm² area.

Wound healing assay

A wound healing assay was set-up to determine the capacity of HMEC-1 to migrate/proliferate in both resting and rHu VEGF¹⁶⁵-stimulated condition. The same method was recently used in our laboratory to determine the ability of heparins to affect HMEC-1 migration (Vignoli et al., in press 2010).

Particularly, HMEC-1 were collected by enzymatic detachment (0.25% trypsin-EDTA solution), counted and re-suspended in RPMI 1640 medium with 5% FBS. Then 10,000 cell/well (100 μL) were seeded in a 96-well culture plate and incubated at 37°C in a 5% CO₂-humidified atmosphere until they reached the confluence (i.e. 3-4 days). In the middle of the well, a wound was created by aspiration of cells with vacuum system using a p10 μl tip. After 2 washes with RPMI 1640 medium, pre-treatment images were acquired using a microcamera system (5X objective), as explained for capillary-like tube formation assay. Then washing medium was removed and HMEC-1 were incubated at 37°C in the incubator with 50 ng/mL of purified VEGF¹⁶⁵, in absence or presence of increasing doses of Dp-3-glc and Cy-3-glc (0.1-1-10-100 μM). In resting condition experiments, the effect of ACN was evaluated in the absence of VEGF pro-angiogenic stimulus. After 18h incubation post-treatment images were acquired. For each well pre- and post-treatment images were compared, and the area occupied by migrating/proliferating cells ("re-growth area") was calculated using the ImageJ 1.42q software. Results are expressed as percentage of re-growth, assuming the area occupied by control cells as 100% of re-growth.

3.7.2 HMEC-1 pro-coagulant properties

Activation of the coagulation system and ensuing thrombin generation is dependent on expression of TF and the simultaneous down-regulation of endothelial-bound anticoagulant mechanisms and endogenous fibrinolysis. Thrombin generation (TG), converting fibrinogen to fibrin and leading to clot formation, is the endpoint of a

complex series of proteolytic reactions, as reported in detail in the introduction of this topic (Gailani & Broze, 1991; Hemker & Beguin, 1995).

In previous works conducted in the Hemostasis & Thrombosis Center in Bergamo, treatment of HMEC-1 with LPS induced a time-dependent increase in TF expression evaluated as activity, antigen and mRNA expression with a maximum effect at 4-6h and then a progressive decline towards basal values at 24-48h (Vignoli *et al.*, 2006). In order to evaluate the activity of Dp-3-glc on LPS-induced TF-expression, we chose the 4h time point, i.e. when the effect of the bacterial endotoxin stimulus was maximum.

Thrombin generation assay (TG) on HMEC-1 intact monolayer

HMEC-1 were collected by enzymatic detachment (0.25% trypsin-EDTA solution), counted and re-suspended in RPMI 1640 medium containing 5% FBS. Then 10,000 cell/well (100 μ L) were seeded in a 96-well plate and incubated at 37°C and 5% CO₂ in a humidified atmosphere. At confluence (i.e. 3-4 days), HMEC-1 were incubated for 4h in complete culture medium with increasing concentrations of Dp-3-glc (1, 10, 100 μ M) both in the absence or presence of 10 μ g/ml LPS (Lipopolysaccharide, bacterial endotoxin from *Escherichia coli*, Sigma) or complete culture medium (control cells, CTR). Additional experiments were performed with Dp-3-glc alone with 24h of incubation. At the end of incubation, HMEC-1 were washed 3 times with phosphate-buffered saline (PBS, pH 7.4), and immediately tested for their capacity to induce thrombin generation (TG) in normal pool plasma (PPP) by the Calibrated Automated Thrombogram (CAT) assay (Hemker *et al.*, 2006). This method uses a low-affinity fluorogenic substrate for thrombin to monitor the formation and inhibition of thrombin in a plasma sample triggered by TF, phospholipids, and CaCl₂. Briefly, 80 μ L PPP was added to each culture well plate together with 20 μ L of HEPES, or rHuTF (1 pM and 5 pM plus 4 μ M phospholipids) or calibration factor (CAL). After 10 minutes at 37°C, the reaction was started by the addition of 20 μ L of a mixture containing the fluorogenic substrate (Z-Gly-Gly-Arg-AMC; Bachem, Bubendorf, Switzerland) and CaCl₂. Fluorescence was read in a Fluoroskan Ascent reader (Thermo Labsystems, Helsinki, Finland), and thrombin generation curves (Figure 3.7.2.1) were calculated using Thrombinoscope software (Thrombinoscope, Maastricht, The Netherlands). TG curves were described in terms of lag-time, time to Peak (ttPeak), and area under the curve (endogenous thrombin potential, ETP).

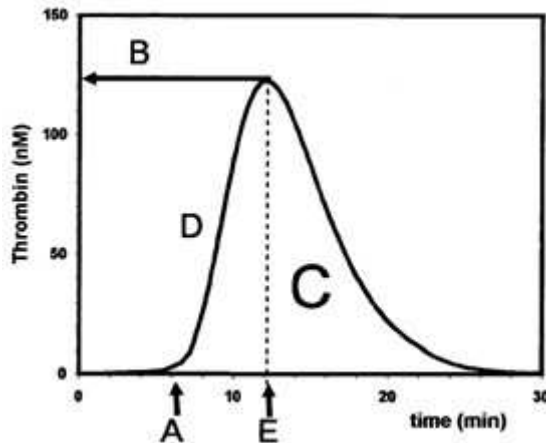


Figure 3.7.2.1 The parameters of the thrombogram. A) Lag-time (min), B) Peak height (nM), C) Endogenous thrombin potential (ETP) (= area under the curve)(nM x min), D) maximal rising slope (nM/min), E) time to peak (min), (Hemker & Beguin, 1995).

TG of HMEC-1 lysates

HMEC-1 were collected by enzymatic detachment (0.25% trypsin-EDTA solution), counted and re-suspended in RPMI 1640 medium containing 5% FBS. Then 10^6 cells were seeded in T25 flasks and incubated at 37°C and 5% CO₂ in a humidified atmosphere until they were confluent (3-4 days). LPS in the absence or presence of Dp-3-glc (1, 10, 100 μM) or culture medium (control cells, CTR) was then added. After 4h, cells were washed three times with saline, detached by scraping and re-suspended in saline for cell count.

TG was evaluated in HMEC-1 lysates (3×10^6 cells/mL PBS), obtained after three cycles of freezing/thawing, by the CAT assay in PPP (Hemker *et al.*, 2006). Briefly, 80 μl plasma was pipetted into the well of a microtiter plate together with 20 μl of HMEC-1 PBS lysate, or rHuTF (1 pM and 5 pM plus 4 μM phospholipids) or calibration factor (CAL). After 10 minutes at 37°C, the reaction was started by the addition of 20 μl of a mixture containing the fluorogenic substrate (Z-Gly-Gly-Arg-AMC) and CaCl₂. TG was quantified as described above.

HMEC-1 procoagulant activity

HMEC-1 lysates were prepared as for TG evaluation. PCA was evaluated in HMEC-1 lysates by the one-stage re-calcification assay of PPP. PCA was referred to a calibration curve constructed with different dilutions (up to 10^{-6}) of a standard rabbit brain thromboplastin (RBT; Sigma). Results are expressed as standard thromboplastin arbitrary units (URBT)/ 10^6 cells, 1 unit the activity of 1 mEquiv/ ml of RBT in the coagulation assay.

HMEC-1 TF antigen

HMEC-1 were collected by enzymatic detachment (0.25% trypsin-EDTA solution), counted and re-suspended in RPMI 1640 medium containing 5% FBS. Then 10^6 cells were seeded in T25 flasks and incubated at 37°C and 5% CO₂ in a humidified

atmosphere until they were confluent (3-4 days). LPS in the absence or presence of Dp-3-glc (1, 10, 100 μ M) or culture medium (CTR) was then added. After 4h, cells were washed three times with saline, detached by scraping and re-suspended in saline for cell count.

TF antigen levels were quantified in HMEC-1 extracts (0.5×10^6 cell/mL) in TRIS buffer (50 mmol/l Tris, 100 mmol/l NaCl and 1% Triton-X 100, pH 7.5) by IMUBIND® Tissue factor ELISA (American Diagnostica Inc., Stamford, CT, USA) according to the manufacturer's instructions. Results are expressed as pg/ 10^6 cells.

HMEC-1 TF mRNA expression

Evaluation of mRNA expression of TF in HMEC-1 was determined with Real Time RT-PCR. HMEC-1 were cultured and treated in T25 flasks as for the previous experiments. Then 1.5×10^6 washed cells were re-suspended in 350 μ L guanidinium thiocyanate and stored at -80°C until RNA extraction.

Total cellular RNA was extracted using RNeasy® Mini kit (Qiagen). Retro-transcription of cDNA was performed with RT-kit plus (Nanogen Advanced Diagnostics S.r.l., Italy) according to manufacturer's instructions. cDNA was used for amplification of Tissue Factor (TF) in Real Time PCR (Rotor Gene™ 6000, Explera a Qiagen Company, Italy). Simultaneous amplification of the invariant housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed. The following oligonucleotides were used : TF forward: 5'- AGG TGG GAA CAA AAG TGA ATG TG-3', and TF reverse exon 4-5: 5'-TTT TGG CTG TTT TCT TTC CTG AAC-3'; GAPDH forward: 5'-GAA GGT GAA GGT CGG AGT C-3' and GAPDH reverse: 5'-GAA GAT GGT GAT GGG ATT TC-3', (Laboratoires Eurobio, Les Ulis, France). Thermocycling consisted of: 2 min at 95°C for Go Taq® Hot Start Polymerase activation, 50 cycles of 15 sec at 95°C followed by of 60 sec at 56°C (acquiring step), and 60 sec at 72°C and stopped with a melting curve (ramp from 50°C to 99°C rising by 1°C each step, waiting for 90 sec of pre-melt conditioning on first step and 5 sec for each step afterwards).

PCR amplification was performed with Go Taq qPCR Master Mix (Promega Corporation, Madison, USA): 12.5 μ L of Go Taq qPCR Master Mix 2X, 0.5 μ L of each oligonucleotide (0.08 μ M), 2 μ L of cDNA (100 ng) and 9.5 μ L of nuclease-free water in a final volume of 25 μ L. Data analysis was performed with comparative quantitation of Ct sample values. The internal calibrator selected was CTR (not treated cells) with reported concentration of 1 fold. Then the ratio of TF on GAPDH values used as control was calculated for each sample.

Statistical analysis

Student's paired and unpaired t-test was used for the determination of significance levels between the different treatments. Differences were considered significant when $p < 0.05$.

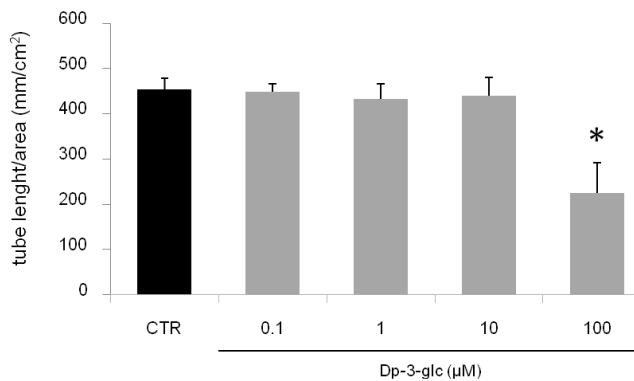
3.8 Results and discussion TOPIC II

3.8.1 Angiogenesis study

Capillary-like tube formation assay in Matrigel

To assess whether Dp-3-glc and Cy-3-glc might affect the formation of capillary-like tubes, HMEC-1 were seeded on Matrigel and treated for 24h with 0.1-1-10-100 μM Dp-3-glc or Cy-3-glc in the absence of exogenous pro-angiogenic stimuli (i.e. resting condition).

A



B

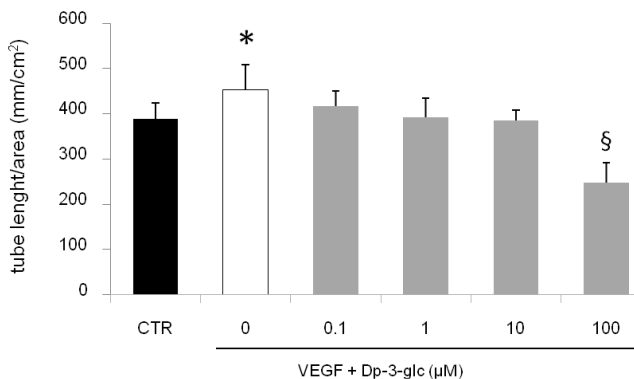
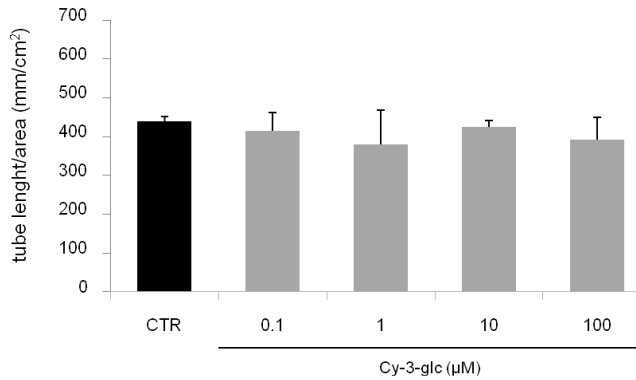


Figure 3.8.1.1 *Effect of Dp-3-glc on capillary-like tube formation. Dp-3-glc showed a significant inhibitory effect in tube formation at the 100 μM concentration in resting (panel A) and VEGF-stimulated (panel B) conditions. Data are expressed as the mean of three independent experiments \pm SD. * $p < 0.05$ vs CTR; § $p < 0.05$ vs VEGF.*

As shown in Figure 3.8.1.1-A, only at 100 μM concentration Dp-3-glc affected in a statistically significant manner ($p < 0.005$) the capacity of HMEC-1 to form the capillary-like tubes, with a reduction of 50% compared to control cells (CTR). Similar

experiments were performed with rHuVEGF¹⁶⁵-stimulated HMEC-1. Treatment with 50 ng/ml rHuVEGF¹⁶⁵ induced a statistically significant ($p < 0.005$) increase in capillary tube length compared to control cells. The addition of Dp-3-glc was able to dose-dependently counteract VEGF stimulus, reaching a complete inhibition at the concentration of 100 μM ($p < 0.05$) (Figure 3.8.1.1-B).

A



B

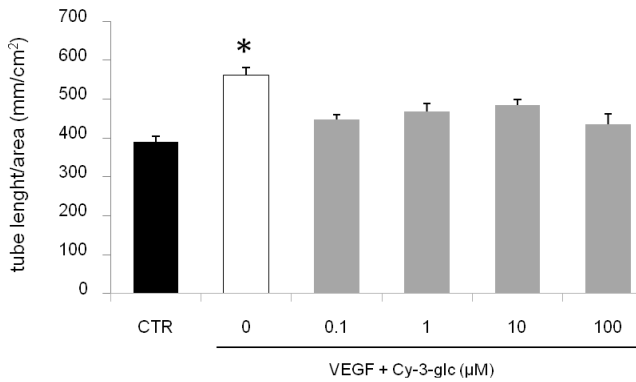


Figure 3.8.1.2 *Effect of Cy-3-glc on capillary-like tube formation. Cy-3-glc did not show a significant inhibitory effect in tube formation in resting (panel A) and VEGF-stimulated (panel B) conditions. Data are expressed as the mean of three independent experiments \pm SD. * $p < 0.05$ vs CTR*

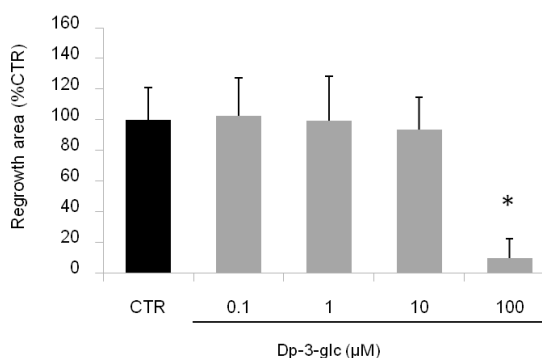
Differently, Cy-3-glc in the same experimental conditions did not show any statistically significant inhibitory effect on the formation of capillary structures, in either resting or VEGF-stimulated HMEC-1 (Figure 3.8.1.2).

Wound healing assay

The effect of Dp-3-glc and Cy-3-glc on HMEC-1 migration/proliferation was tested in both resting and VEGF-stimulated conditions by the wound healing assay.

In resting conditions (Figure 3.8.1.3-A), 100 μM Dp-3-glc significantly decreased by 90% the migration of HMEC-1 ($p < 0.0001$). At lower Dp-3-glc concentrations no statistically significant effects on HMEC-1 migration were observed. VEGF induced a significant ($p < 0.001$) increase in HMEC-1 re-growth: this effect that was significantly counteracted by Dp-3-glc starting from the concentration of 1 μM , reaching a complete (100%) inhibition at 100 μM ($p < 0.01$), (Figure 3.8.1.3-B).

A



B

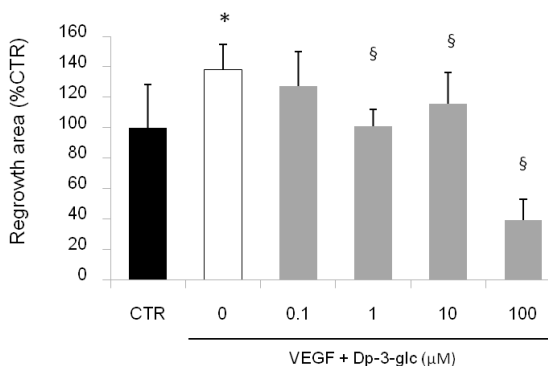
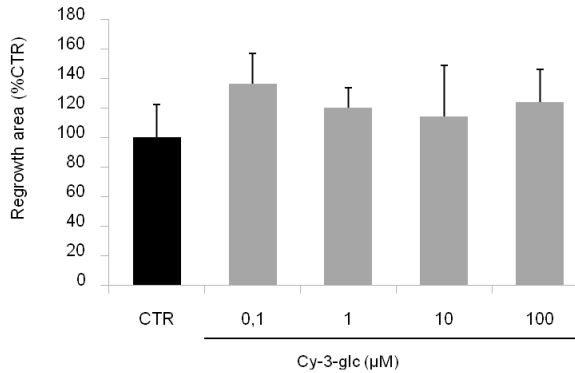


Figure 3.8.1.3 Effect of Dp-3-glc on migration in the wound healing assay. Dp-3-glc showed a significant inhibitory effect of re-growth area at the maximum concentration in resting conditions (panel A) and at 1 μM in combination with VEGF (panel B). Data are expressed as the mean of three independent experiments \pm SD. * $p < 0.05$ vs CTR; § $p < 0.05$ vs VEGF.

As observed with the capillary-like tube formation assay, Cy-3-glc did not show any statistically significant effect on HMEC-1 migration in both resting and VEGF-stimulated condition (Figure 3.8.1.4).

A



B

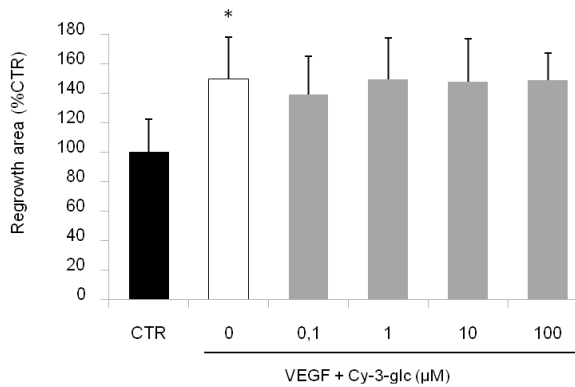


Figure 3.8.1.4 Effect of Cy-3-glc on migration in the wound healing assay. Cy-3-glc did not show a significant inhibitory effect in re-growth area in resting (A, on the left) and VEGF-stimulated (B, on the right) conditions. Data are expressed as the mean of three independent experiments \pm SD. * $p < 0.05$ vs CTR

3.8.2 HMEC-1 pro-coagulant properties

TG on HMEC-1 intact monolayer

The study of the effect of Dp-3-glc on endothelial cell-induced TG was performed in 2 different experimental conditions: resting and LPS-stimulated HMEC-1.

In the absence of any procoagulant stimulus, the treatment with Dp-3-glc (from 4h to 24h) did not significantly affect TG potential of HMEC-1 monolayer (data not shown). Differently, as shown in Figure 3.8.2.1, the pro-thrombotic stimulus of LPS induced an increase in TG potential of HMEC-1, expressed by a significant ($p < 0.05$) reduction in lag-time (1.9 vs 5.1 min) and ttPeak (6.8 vs 10.0 min), and an increase of ETP (1021 vs 749 nM*min) compared to control cells. Dp-3-glc significantly counteracted the prothrombotic stimulus of LPS starting from 10 μM : at this concentration, the values of lag-time, ttPeak and ETP were similar to that of unstimulated HMEC-1 cells.

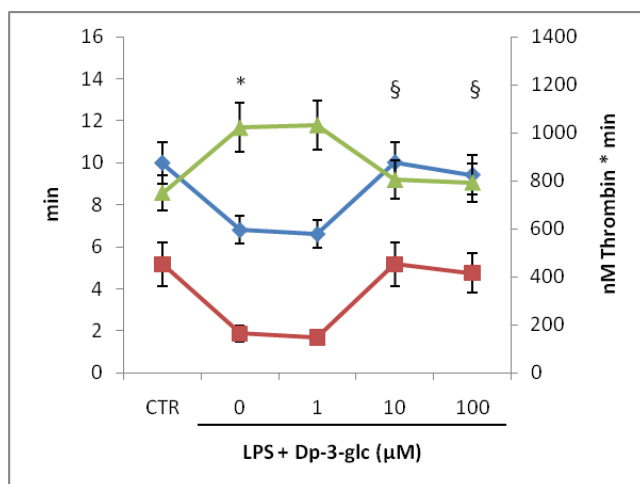


Figure 3.8.2.1 *Dp-3-glc anti-thrombotic effect on TG potential of LPS-stimulated HMEC-1 monolayer. Dp-3-glc showed a significant effect (* $p < 0.05$ vs CTR; § $p < 0.05$ vs LPS) on thrombogram parameters when added to LPS. Data are expressed as mean \pm SD of three independent experiments. Red square: Lag-time (min); green triangle: ETP (nM*min); blue diamond: ttPeak (min).*

TG of HMEC-1 lysates

TG of PBS lysates of HMEC-1 treated with LPS demonstrated a significant ($p < 0.05$) reduction in lag-time (4.1 vs 8.5 min) and ttPeak (10.3 vs 14.3 min), and an increase of ETP (1118 vs 975 nM*min) compared to control cells. The addition of Dp-3-glc did not significantly affect any TG parameter (Figure 3.8.2.2).

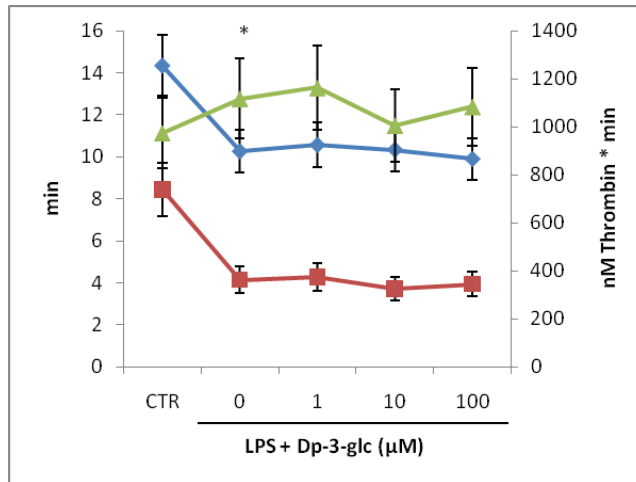


Figure 3.8.2.2 *Dp-3-glc effect on TG potential of HMEC-1 lysates. LPS induced a statistically significant effect on the three parameters considered (* $p < 0.05$ vs CTR): the addition of Dp-3-glc to LPS did not determine any statistically significant effect compared to LPS alone. Data are expressed as mean \pm SD of three independent experiments. Red square: Lag-time (min); green triangle: ETP (nM*min); blue diamond: ttPeak (min).*

HMEC-1 procoagulant activity

Figure 3.8.2.3 shows the results of PCA determination in cell lysates after 4 h incubation of HMEC-1 with LPS in absence or presence of increasing concentrations of Dp-3-glc. LPS induced a significant ($p < 0.05$) increase of TF-PCA; while Dp-3-glc at the concentration of 100 μ M produced a statistically relevant inhibition of 59% in URBT as compared to LPS-treated lysates ($p < 0.05$).

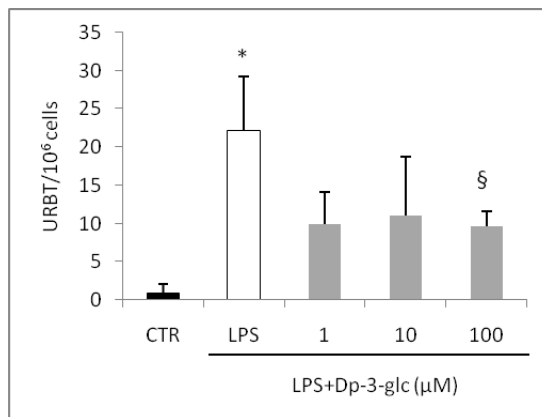


Figure 3.8.2.3 *Effect of Dp-3-glc on PCA of HMEC-1 stimulated with LPS. Dp-3-glc showed a significant effect (* $p < 0.05$ vs CTR, § $p < 0.05$ vs LPS) at the maximum concentration tested. Data are expressed as mean \pm SD of three independent experiments.*

HMEC-1 TF antigen

Figure 3.8.2.4 shows the results of TF antigen determination in extracts of HMEC-1 after 4 h incubation of these cells with LPS, in absence or presence of increasing concentrations of Dp-3-glc. In particular, TF antigen was dose-dependently decreased by Dp-3-glc, reaching a 25% inhibition at the concentration of 100 μ M ($p < 0.05$).

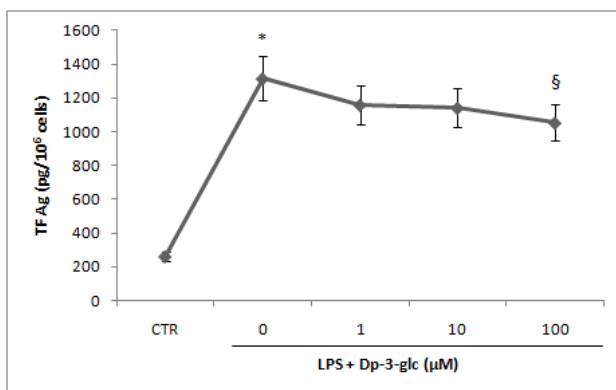


Figure 3.8.2.4 Effect of Dp-3-glc on TF antigen expression by HMEC-1 stimulated with LPS. Data are expressed as mean \pm SD of three independent experiments. * $p < 0.05$ vs CTR, § $p < 0.05$ vs LPS

HMEC-1 TF mRNA expression

The expression of TF was determined by quantitative Real Time RT-PCR on HMEC-1 treated with LPS in combination with Dp-3-glc for 4h. A significant increase (85%) of TF mRNA expression was induced by LPS treatment ($p < 0.05$). The combination with the anthocyanin Dp-3-glc determined a significant effect at the maximum concentration tested (100 μ M), with a inhibition of 28% versus LPS alone, as shown in Figure 3.8.2.5.

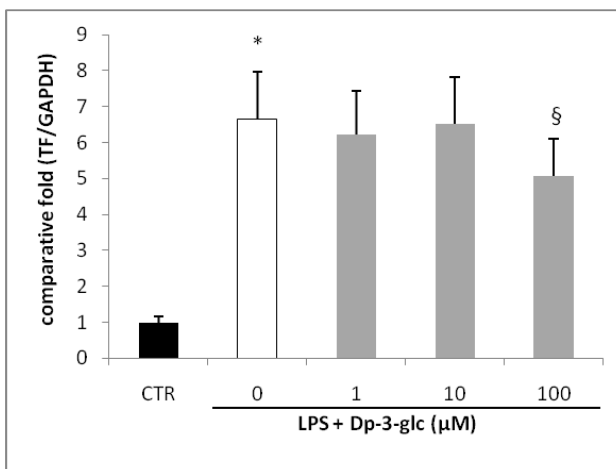


Figure 3.8.2.5 Dp-3-glc effect on TF mRNA expression of HMEC-1. Data are expressed as mean \pm SD of three independent experiments. * $p < 0.05$ vs CTR; § $p < 0.05$ vs LPS.

3.8.3 Discussion

In the present study we evaluated whether two anthocyanins (i.e. Dp-3-glc and Cy-3-glc, mainly represented in black currant, wild blueberry, blood orange, and other colored fruits and vegetables) can affect angiogenic properties of endothelium; moreover, we investigated on the capacity of Dp-3-glc to affect thrombotic properties of microvascular endothelial cells.

The **anti-proliferative activity** of these natural compounds against cancer cell lines have been already studied and available in the literature.

Katsube and colleagues (2003) demonstrated that, among ethanol extracts of 10 edible berries, bilberry extract was found to be the most effective at inhibiting the growth of HL60 human leukemia cells and HCT116 human colon carcinoma cells *in vitro*. In particular, the anthocyanin **Dp-3-glc** was able to inhibit the growth of HCT116 cells of 85% and 80% with the concentrations 863 μM and 431 μM respectively; while for HL60 cells of 75% with 216 μM (Katsube *et al.*, 2003).

Olsson and colleagues (2004) investigated on the effects of 10 different extracts of fruits and berries on cell proliferation of colon cancer cells HT29 and breast cancer cells MCF-7. The fruits and berries used were rosehips, blueberries, black currant, black chokeberries, apple, sea buckthorn, plum, lingonberries, cherries, and raspberries. The extracts decreased the proliferation of both colon cancer cells HT29 and breast cancer cells MCF-7, and the effect was concentration-dependent. In particular, standards **Dp-3-glc** and **Cy-3-glc** decreased significantly ($p < 0.001$) the proliferation of both cell lines to a high extent (87 and 82% respectively) at the concentration of 200 $\mu\text{g/mL}$ (431 μM) in the medium. At a lower concentration of the anthocyanin standards, i.e. 20 $\mu\text{g/mL}$, no effects was found on the proliferation of HT29 and MCF-7 cells (Olsson *et al.*, 2004).

Fimognari *et al.* (2004) investigated the *in vitro* capacity of the anthocyanin cyanidin-3-O-beta-glucopyranoside (**Cy-3-glc**) to induce apoptosis in Jurkat T-lymphoblastoid, as well as apoptosis and differentiation in HL-60 promyelocytic cells. Although Cy-3-glc induced apoptosis (as well as necrosis) in the two systems, HL-60 cells were much less sensitive than T-lymphoblastoid cells. In particular, IC₅₀ for Jurkat cells was 391 μM , while in HL-60 cells the growth inhibition of 37% was reached with the concentration of 446 μM . Moreover, treatment of HL-60 cells with Cy-3-glc caused differentiation into macrophage-like cells and granulocytes. The authors conclude that these interesting biological properties should encourage further investigation into the chemopreventive and/or chemotherapeutic potential of Cy-3-glc (Fimognari *et al.*, 2004).

Recently, Fernandes and colleagues (2010) published a work on the influence of anthocyanins and other polyphenols on breast cancer cell (MCF-7) proliferation. The antiproliferative effect was higher when cells were treated with **Dp-3-glc**, even if it was not followed by modification of ER expression. Differently, **Cy-3-glc** showed no significant antiproliferative effect on MCF-7 proliferation (Fernandes *et al.*, 2010).

However, no studies are available on the effects of anthocyanins on tumor-induced neoangiogenesis. Angiogenesis, the formation of new vessels, has a major role in

tumor growth, dissemination and metastasis in both solid and hematological tumors (Folkman, 2003). Angiogenesis is driven by microvascular endothelial cells, which upon activation by angiogenic growth factors degrade their basement membrane, migrate into the interstitial matrix, proliferate and form new capillary structures (Marchetti *et al.*, 2003). The angiogenic switch in cancer is a highly complex event and involves several tumor-derived factors. Inhibition of angiogenesis is one of the proposed strategies to control cancer.

The results of our **angiogenesis** study indicate that Dp-3-glc can interfere with both resting and activated endothelium, by a standard stimulus like VEGF. In fact, we obtained an increase in capillary tube formation and cell migration induced by VEGF respect to basal HMEC-1, while treatment Dp-3-glc was able to inhibit VEGF stimulus at 1 μM concentration. Differently, Cy-3-glc did not show the same effect. The different behaviour of the two anthocyanins could be due to their different chemical structure given that the only structural feature which differs in their structures is the hydroxylation pattern in ring B (Figure 3.6.6), as hypothesized also by Fernandes *et al* (2010). No *in vitro* studies are available on the effects of Dp-3-glc and Cy-3-glc on microvascular endothelial cells, in fact the attention has been more directed to the anti-angiogenic activity exerted by anthocyanidins. **Delphinidin** (Dp), aglycone form of Dp-3-glc, was recently demonstrated to inhibit proliferation migration and angiogenesis of human umbilical vein endothelial cells (HUVECs) (Favot *et al.*, 2003; Lamy *et al.*, 2006; Lamy *et al.*, 2008; Matsunaga *et al.*, 2010). Briefly, Matsunaga and colleagues (2010) examined the anti-angiogenic properties and antioxidant activities of the main anthocyanidins (**delphinidin, cyanidin and malvidin**) found as constituents in bilberry (*Vaccinium myrtillus*). Each of these anthocyanidins dose-dependently inhibited VEGF-induced tube formation in a co-culture of HUVECs and fibroblasts, with the effect of each anthocyanidin being significant at 3 and/or 10 μM . Moreover, their radical-scavenging effects were induced by concentrations that were at or below those that induced their anti-angiogenic effects (Matsunaga *et al.*, 2010). Lamy and colleagues in 2006 conducted a study where, among the anthocyanidins tested (cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin), **delphinidin** was the most potent angiogenic inhibitor. *In vitro*, low concentrations of Dp inhibited VEGF-induced tyrosine phosphorylation of VEGF receptor (VEGFR)-2, leading to the inhibition of downstream signaling triggered by VEGFR-2. Inhibition of VEGFR-2 by Dp reserved the VEGF-induced activation of ERK-1/2 signaling and the chemotactic motility of HUVECs as well as their differentiation into capillary-like tubular structures in Matrigel and within fibrin gels. *In vivo*, Dp was able to suppress basic fibroblast growth factor-induced vessel formation in the mouse Matrigel plug assay (Lamy *et al.*, 2006). In a subsequent study (2008), Lamy and colleagues showed that Dp also inhibits activation of the platelet-derived growth factor (PDGF)-BB receptor- β [platelet-derived growth factor receptor-beta (PDGFR- β)] in pulmonary aortic smooth muscle cells (PASMC) and that this inhibition may contribute to its antitumor effect. The inhibitory effect of delphinidin on PDGFR- β was very rapid and led to the inhibition of PDGF-BB-induced activation of extracellular signal-regulated kinase

(ERK)-1/2 signaling and of the chemotactic motility of PASM_C, as well as the differentiation and stabilization of HUVEC and PASM_C into capillary-like tubular structures in a three-dimensional co-culture system. Using an anthocyan-rich extract of berries, the authors showed that berry extracts were able to suppress the synergistic induction of vessel formation by basic fibroblast growth factor-2 and PDGF-BB in the mouse Matrigel plug assay. Oral administration of the berry extract also significantly retarded tumor growth in a lung carcinoma xenograft model. Taken together, these results provide new insight into the molecular mechanisms underlying the antiangiogenic activity of delphinidin that will be helpful for the development of dietary-based chemopreventive strategies (Lamy *et al.*, 2008). Favot and colleagues (2003) verified that VEGF-stimulated HUVEC cell migration and proliferation were potently inhibited by **Dp** in concentration-dependent manner (with maximum effect of 50% inhibition for migration and proliferation at 64 μ M). Flow cytometric analysis demonstrated that Dp inhibition of proliferation is correlated with the blockade of cell cycle in G(0)/G(1) phase. Western blot analysis showed that Dp reversed the VEGF-induced decrease in expression of cyclin-dependent kinase inhibitor p27(kip1) and the VEGF-induced increase of cyclin D1 and cyclin A, both being necessary to achieve the G(1)-to-S transition. Furthermore, Dp inhibited neovascularisation *in vivo* in chorioallantoic membrane model (Favot *et al.*, 2003). In a series of experiments, berry extracts were tested for their ability to inhibit angiogenesis via altered VEGF expression and invasiveness (Roy *et al.*, 2002). Anti-angiogenic properties were investigated in two cell lines: immortalized HaCaT human keratinocytes and human microvascular dermal endothelial (HMVE) cells. First the effect of selected berry samples on TNF α and H₂O₂-induced VEGF expression by HaCaT cells was investigated. The authors speculate that the flavonoid component of berry samples may be responsible for the observed effect on inducible VEGF expression and release. Next, an *in vitro* angiogenesis assay was performed with HMVE cells; two of the berry mix samples (50 μ g/mL) impaired *in vitro* angiogenesis (Roy *et al.*, 2002). Our study of **anti-coagulant effects** of Dp-3-glc was carried out on HMEC-1 stimulated by LPS, a known activator of endothelial pro-coagulant response. In these experiments, the dose of LPS was used according to a standard procedure established on the basis of recent studies (Falanga *et al.*, 2003). TF is the major activator of blood coagulation. The reduction in TF expression on microvascular endothelial cells may be important for maintaining physiological endothelial function and preventing thrombotic microangiopathies. The results of our study indicate that Dp-3-glc significantly counteract LPS-induced TF expression at microvascular site starting at 10 μ M concentration when tested with TG assay on intact monolayer of HMEC-1. Determination of TG on HMEC-1 lysates did not reveal any anti-coagulant effect, even at the highest dose tested. On the contrary, in the subsequent experiments performed, Dp-3-glc treatment (100 μ M) induced a significant reduction in total PCA on HMEC-1 lysates, determined with the one-stage recalcification assay. The further quantitation of TF, as antigen and as mRNA expression, showed a significant inhibition induced of LPS activation of HMEC-1 procoagulant conditions by 100 μ M Dp-3-glc.

To our knowledge, there are no other studies in the literature investigating the anti-thrombotic effect of anthocyanins on microvascular cell lines models.

Yamamoto and colleagues (2006) found that mulberry (*Morus alba*) varieties could be grouped into subclasses with either anti- or prothrombotic activities. A shear-induced *in vitro* platelet reactivity/thrombolysis test (Gorog Thrombosis Test) was used to screen for antiplatelet and thrombolytic activities. In case of effectiveness, it was followed by an *in vivo* test of laser-induced thrombosis in mice. Antioxidant activities and polyphenolic contents were also evaluated and they did not affect platelets but might enhance endogenous thrombolysis, thus causing an overall antithrombotic effect (Yamamoto et al., 2006).

Berry phytochemicals have been widely studied with *in vitro* and animal models and different protective effects were demonstrated, as reported in detail in the introduction of this topic (paragraph 3.7).

However, which single berry constituent(s) induce this protective response remains uncertain, and in the vast majority of studies the concentration of extract or phytochemical employed is substantially higher than could ever be achieved nutritionally (Duthie, 2007). We have also to consider that berry compounds are extensively metabolized and further converted by colonic microflora into related molecules. These compounds may persist *in vivo*, accumulate in target tissues and contribute significantly to the biological effects. Moreover, the levels of this type of phytochemicals may be underestimated due to limitations in laboratory extraction procedures because these compounds may bind to proteins, etc., causing their extraction for chemical analyses to be difficult (Seeram, 2008). However, the anthocyanins delphinidin-3-glucoside and cyanidin-3-glucoside, selected for this study, have been reported to be incorporated into human plasma with their structures maintained or converted from other structures (see also paragraph 3.6 for details).

3.9 Conclusions TOPIC II

Our results, together with the known capacity of Dp-3-glc to affect tumor cell proliferation, make this compound a potential cancer chemopreventive agent. Although considerable progress has been made in understanding the role that bioactive compounds, like anthocyanins, play in affecting human health and disease, there are still important gaps in our knowledge concerning the biology and chemistry of these compounds. Future studies should be designed to enhance our knowledge of the intricate roles and functions that ACNs can act at both cellular and molecular level. Moreover there is little evidence from human intervention studies, which provide the confirmation of the protective role of bioactive compounds. This *in vitro* study supports a protective effect on human endothelium. However, the concentration tested *in vitro* hardly could be achieved *in vivo*. Thus, the next step might be organization of a human feeding study where biomarkers of angiogenesis, such as VEGF, or marker of coagulation status, such as TF, TG and PCA could be easily analyzed in plasma samples.

In conclusion, our results support health promotion strategies that favour fruit and vegetable consumption for prevention and protection against chronic diseases.

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4. GLOSSARY

Angiogenesis: the physiological process involving the growth of new blood vessels from pre-existing vessels, essential for the supply of nutrients and oxygen to any growing tissue, including tumors.

Anthocyanidin: polyphenol, flavonoid, aglycone form of anthocyanidin.

Anthocyanin: polyphenol, flavonoid, which are the largest group of water-soluble pigments; they occur ubiquitously in the plant kingdom and confer bright red or blue coloration on many fruits (particularly berries), vegetables, grains, flowers and leaves. They can be found in different structural arrangements depending on the number of glycosylating sugars, their position in the aglycone (anthocyanidin) and the degree and nature of esterification of the sugar with aliphatic or aromatic acids.

Antioxidant: a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols.

Bioactive compound: a chemical compound or substance produced by a living organism, found in nature that usually has a pharmacological or biological activity.

Bioavailability: in pharmacology, used to describe the fraction of an administered dose of unchanged drug that reaches the systemic circulation, one of the principal pharmacokinetic properties of drugs. **Nutrient bioavailability** is the proportion of a nutrient that is absorbed from the diet and used for normal body functions.

Biomarker: a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

Biotransformation: the chemical modification (or modifications) made by an organism on a chemical compound. It means chemical alteration of chemicals such as (but not limited to) nutrients, amino acids, toxins, or drugs in the body. It is also needed to render nonpolar compounds polar so that they are not reabsorbed in renal tubules and are excreted.

Blood: a specialized bodily fluid that delivers necessary substances to the body's cells – such as nutrients and oxygen – and transports waste products away from those same cells.

BMI: body mass index, is a heuristic measure of body weight based on a person's weight and height (weight in kg/(height in m)²).

Brassica: a genus of plants in the mustard family (Brassicaceae or Cruciferae).

Broccoli: is a plant of the family Brassicaceae (formerly Cruciferae). It is classified in the *Italica* cultivar group of the species *Brassica oleracea*. Broccoli has large flower heads, usually green in color, arranged in a tree-like fashion on branches sprouting from a thick, edible stalk. The mass of flower heads is surrounded by leaves.

Cancer: a class of diseases in which a cell, or a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited, and do not invade or metastasize.

Carcinogen: any substance, radionuclide or radiation, that is an agent directly involved in causing cancer. This may be due to the ability to damage the genome or to the disruption of cellular metabolic processes. Several radioactive substances are considered carcinogens, but their carcinogenic activity is attributed to the radiation, for example gamma rays and alpha particles, which they emit. Common examples of carcinogens are inhaled asbestos, certain dioxins, and tobacco smoke.

Chronic disease: a disease that is long-lasting or recurrent. The term chronic describes the course of the disease, or its rate of onset and development. Examples of chronic diseases include: asthma, chronic osteoarticular diseases, chronic respiratory diseases, diabetes mellitus, cardiovascular diseases, osteoporosis, cancer.

Clotting: a complex process by which blood forms clots. It is an important part of hemostasis (the cessation of blood loss from a damaged vessel), wherein a damaged blood vessel wall is covered by a platelet and fibrin-containing clot to stop bleeding and begin repair of the damaged vessel. Disorders of coagulation can lead to an increased risk of bleeding (hemorrhage) or obstructive clotting (thrombosis).

COMET assay: Single Cell Gel Electrophoresis, a sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell. It involves the encapsulation of cells in a low-melting-point agarose suspension, lysis of the cells in neutral or alkaline (pH>13) conditions, and electrophoresis of the suspended lysed cells. This is followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage.

Cruciferae: an older name of Brassicaceae, meaning "cross-bearing", because the four petals of their flowers are reminiscent of a cross.

Diet: the sum of food consumed by a person or other organism.

DNA: DeoxyriboNucleic Acid, a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms.

DNA damage: it may be due to environmental factors and normal metabolic processes. Affects the primary structure of the double helix; that is, the bases themselves

are chemically modified. These modifications can in turn disrupt the molecules' regular helical structure by introducing non-native chemical bonds or bulky adducts that do not fit in the standard double helix. There are five main types of damage to DNA due to endogenous cellular processes. oxidation of bases [e.g. 8-oxo-7,8-dihydroguanine (8-oxoG)] and generation of DNA strand interruptions from reactive oxygen species. alkylation of bases (usually methylation), such as formation of 7-methylguanine, 1-methyladenine, 6-O-Methylguanine; hydrolysis of bases, such as deamination, depurination and depyrimidination; "bulky adduct formation" (i.e. benzo[a]pyrene diol epoxide-dG adduct); mismatch of bases, due to errors in DNA replication, in which the wrong DNA base is stitched into place in a newly forming DNA strand, or a DNA base is skipped over or mistakenly inserted.

Endothelium: the thin layer of cells that lines the interior surface of blood vessels, forming an interface between circulating blood in the lumen and the rest of the vessel wall. These cells are called endothelial cells. Endothelial cells line the entire circulatory system, from the heart to the smallest capillary. Endothelial tissue is a specialized type of epithelium tissue (one of the four types of biological tissue in animals). More specifically, it is simple squamous epithelium.

Epidemiology: the study of patterns of health and illness and associated factors at the population level. It is the cornerstone method of public health research, and helps inform evidence-based medicine for identifying risk factors for disease and determining optimal treatment approaches to clinical practice and for preventative medicine. In the study of communicable and non-communicable diseases, epidemiologists are involved in outbreak investigation to study design, data collection, statistical analysis, documentation of results and submission for publication.

Flavonoid: a class of plant secondary metabolites, belonging to the class of polyphenols.

Polyphenol: a chemical compound belonging to a group of chemical substances found in plants, characterized by the presence of more than one phenol unit or building block per molecule. Derived from secondary plant metabolism of the shikimate pathway.

Functional food: any healthy food claimed to have a health-promoting or disease-preventing property beyond the basic function of supplying nutrients.

Genotype: the genetic constitution of a cell, an organism, or an individual (i.e. the specific allele make-up of the individual) usually with reference to a specific character under consideration.

Glucosinolate: a class of organic compounds that contain sulfur and nitrogen and are derived from glucose and an amino acid. They occur as secondary metabolites of almost all plants of the order Brassicales (including the family Brassicaceae, Capparidaceae and Caricaceae), but also in the genus *Drypetes* (family Euphorbiaceae).

GST: Glutathione-S-Transferase family enzymes, which catalyse the conjugation of reduced glutathione — via a sulfhydryl group — to electrophilic centers on a wide variety of substrates. This activity detoxifies endogenous compounds such as peroxidised lipids, as well as breakdown of xenobiotics. GSTs may also bind toxins and function as transport proteins. The mammalian GST super-family consists of cytosolic dimeric isoenzymes of 45–55 kDa size that have been assigned to at least six classes: Alpha, Mu, Pi, Theta, Zeta and Omega.

Health claims: a statement usually put on food labels, they are claims by manufacturers of food products that their food has been scientifically demonstrated to reduce the risk of developing a disease or condition.

HMEC-1: Human microvascular endothelial cell line 1. They are an immortalized cell line that retains microvascular endothelial cell characteristics. HMEC-1 cells exhibit typical cobblestone morphology when grown in monolayer culture, rapidly form tubules when cultured on Matrigel. HMEC-1 cells express cell surface molecules typically associated with endothelial cells and cell adhesion molecules.

Human intervention study: used to test interventions designed to change behavior, including dietary intakes and physical activity. Such trials require a high level of commitment by participants.

Inflammation: a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process.

Isothiocyanate: the chemical group $-N=C=S$, formed by substituting sulfur for oxygen in the isocyanate group. Many natural isothiocyanates from plants are produced by enzymatic conversion of metabolites called glucosinolates.

LPS: lipopolysaccharides, large molecules consisting of a lipid and a polysaccharide joined by a covalent bond; they are found in the outer membrane of Gram-negative bacteria, act as endotoxins and elicit strong immune responses in animals.

Lymphocyte: a type of white blood cell in the vertebrate immune system.

Metabolic syndrome: a combination of medical disorders that increase the risk of developing cardiovascular disease and diabetes.

Metabolism: the set of chemical reactions that happen in living organisms to maintain life.

mRNA: messenger RiboNucleic Acid, it is a molecule of RNA encoding a chemical "blueprint" for a protein product. mRNA is transcribed from a DNA template, and carries coding information to the sites of protein synthesis: the ribosomes. Here, the nucleic acid polymer is translated into a polymer of amino acids: a protein. In mRNA as in DNA, genetic information is encoded in the sequence of nucleotides arranged into codons consisting of three bases each. Each codon encodes for a specific amino acid, except the stop codons that terminate protein synthesis. This process requires two other types of RNA: transfer RNA (tRNA) mediates recognition of the codon and provides the corresponding amino acid, while ribosomal RNA (rRNA) is the central component of the ribosome's protein manufacturing machinery.

Nutri-genomics: the study of the effects of foods and food constituents on gene expression. It is about how our DNA is transcribed into mRNA and then to proteins and provides a basis for understanding the biological activity of food components. Nutri-genomics has also been described by the influence of genetic variation on nutrition by correlating gene expression or single-nucleotide polymorphisms with a nutrient's absorption, metabolism, elimination or biological effects. By doing so, nutri-genomics aims to develop rational means to optimise nutrition, with respect to the subject's genotype.

Oxidative stress: an imbalance between the production and manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of tissues can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA.

Phytochemical: chemical compounds that occur naturally in plants. The term is generally used to refer to those chemicals that may affect health, but are not yet established as essential nutrients.

Plasma: the yellow liquid component of blood in which the blood cells in whole blood are normally suspended. It makes up about 55% of the total blood volume. It is the intravascular fluid part of extracellular fluid (all body fluid outside of cells). It is mostly water (90% by volume) and contains dissolved proteins, glucose, clotting factors, mineral ions, hormones and carbon dioxide (plasma being the main medium for excretory product transportation). Blood plasma is prepared by spinning a tube of fresh blood containing an anti-coagulant in a centrifuge until the blood cells fall to the bottom of the tube.

Polymorphism: genetic polymorphism is the simultaneous occurrence in the same locality of two or more discontinuous forms in such proportions that the rarest of them cannot be maintained just by recurrent mutation. Genetic polymorphism is actively and steadily maintained in populations by natural selection.

Prevention: preventive medicine or preventive care refers to measures taken to prevent diseases, (or injuries) rather than curing them or treating their symptoms.

Serum: the component that is neither a blood cell (serum does not contain white or red blood cells) nor a clotting factor; it is the blood plasma with the fibrinogens removed. Serum includes all proteins not used in blood clotting (coagulation) and all the electrolytes, antibodies, antigens, hormones, and any exogenous substances (e.g., drugs and microorganisms).

TF: Tissue Factor, also called platelet tissue factor, factor III, thrombokinase, or CD142. It is a protein present in subendothelial tissue, platelets, and leukocytes necessary for the initiation of thrombin formation from the zymogen prothrombin.

TG: Thrombin Generation, converting fibrinogen to fibrin and leading to clot formation. It is the endpoint of a complex series of proteolytic reactions of the clotting cascade.

Thrombosis: the formation of a blood clot (thrombus; Greek: θρόμβος) inside a blood vessel, obstructing the flow of blood through the circulatory system. When a blood vessel is injured, the body uses platelets and fibrin to form a blood clot to prevent blood loss. Alternatively, even when a blood vessel is not injured, blood clots may form in the body if the proper conditions present themselves.

VEGF: Vascular Endothelial Growth Factor, a signal protein produced by cells that stimulates the growth of new blood vessels. It is part of the system that restores the oxygen supply to tissues when blood circulation is inadequate. VEGF's normal function is to create new blood vessels during embryonic development, new blood vessels after injury, muscle following exercise, and new vessels (collateral circulation) to bypass blocked vessels. When VEGF is over-expressed, it can contribute to disease. Solid cancers cannot grow beyond a limited size without an adequate blood supply; cancers that can express VEGF are able to grow and metastasize.

Wound healing: an intricate process in which an organ repairs itself after injury. The classic model of wound healing is divided into three or four sequential, yet overlapping, phases: (1) hemostasis (not considered a phase by some authors), (2) inflammatory, (3) proliferative and (4) remodeling. The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction.

APPENDIX 1- PUBLICATION AND TRAINING ACTIVITIES

Picozzi C., Bonacina G., Vigentini I., Foschino R. (2010) Genetic diversity in Italian *Lactobacillus sanfranciscensis* strains assessed by Multilocus Sequence Typing and Pulsed Field Gel Electrophoresis analyses. *Microbiology*. 2010 Jul; 156(Pt 7):2035-45. Epub 2010 Apr 1

Riso P., Martini D., Moller P., Loft S., Bonacina G., Moro M., Porrini M. DNA damage and repair activity after broccoli intake in young healthy smokers. *Mutagenesis* 2010 Aug 16. [Epub ahead of print]

Vignoli A., Marchetti M., Russo L., Cantatalino E., Diani E., Bonacina G., Falanga A. "LMWH bemiparin and ULMWH RO-14 reduce the endothelial angiogenic features elicited by leukemia, lung cancer, or breast cancer cells", *Cancer Investigations*, in press.

Bonacina G., poster communication 1st year PhD "Protective role of vegetable food consumption: mechanisms and hypothesis"; 12th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Reggio Calabria, Reggio Calabria, September 12-14, 2007, pp 501-502

Bonacina G., poster communication 2nd year PhD "Protective role of vegetable food consumption: mechanisms and hypothesis"; 14th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Università degli Studi di Sassari, Oristano, September 16-18, 2009, pp 97-99

Bonacina G., oral communication 3rd year PhD "Protective role of bioactive dietary compounds: mechanisms and hypothesis"; 15th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Università degli Studi di Napoli Federico II, Portici, September 15-17 2010, pp 13-17

Marchetti M., Vignoli A., Cantalino E., Diani E., Bonacina G., Falanga A. Poster communication 3047 "Very low molecular weight heparins retain the capacity to inhibit endothelial cell migration and capillary-like tube formation induced by tumor cells" 51st American Society of Hematology (ASH) Annual Meeting and Exposition (December 5-8, 2009), New Orleans, LA, USA

Bonacina G., Marchetti M., Vignoli A., Diani E., Porrini M. and Falanga A.; poster selected for oral presentation: "Effects of an anthocyanin from wild blueberries (delphinidin-3-glucoside) on the proangiogenic and prothrombotic properties of endothelial cells" 5th International Conference on Thrombosis and Hemostasis Issues

in Cancer (ICTHIC) (April 23-25, 2010), Stresa, Italy, published on Thrombosis Research Volume 125, Supplement 2, (April 2010), page S189

Vignoli A., Marchetti M., Cantatalino E., Diani E., Bonacina G., Falanga A. Poster communication: "Very low molecular weight heparins (LMWH) retain the capacity to inhibit endothelial cell migration and capillary-like tube formation induced by tumor cells" 5th International Conference on Thrombosis and Hemostasis Issues in Cancer (ICTHIC) (April 23-25, 2010), Stresa, Italy, published on Thrombosis Research Volume 125, Supplement 2, (April 2010), page S191

Bonacina G., Marchetti M., Vignoli A., Diani E., Panova-Noeva M., Riso P., Porrini M. and Falanga A. Oral communication "Delphinidin-3-glucoside, an anthocyanin from wild blueberries, protects endothelial cells by proangiogenic and prothrombotic stimuli" XXI Congresso Nazionale Siset 2010 (October 28-31 2010, Bologna, Italy)

RESEARCH PROJECTS

Consumo di Brassicaceae e protezione dallo stress ossidativo: studio di intervento dietetico nell'uomo- Ministry of Education, University and Research (MIUR)-PRIN prot. 2005058197

Environmental Cancer Risk, Nutrition and Individual Susceptibility (ECNIS) Network of Excellence (operating in the context of the 6th EU Framework Programme for Research and Development) entitled the 'ECNIS Comet Assay Validation Group' (ECVAG) for the purpose of validation of the comet assay with respect to measures of DNA damage formation and its repair, with special focus on its application in biomonitoring studies.

Use of biosensors within a multidisciplinary approach for the study of degenerative disease prevention through diet

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Progetto di Ricerca Finalizzata Oncologica 2006 - **Caratterizzazione delle molecole coinvolte nella regolazione delle angiogenesi tumorali**

Fondazione Monzino Centro Cardiologico S.p.A., Milano & Hemostasis and Thrombosis Center, Ospedali Riuniti di Bergamo.

DNA damage and repair activity after broccoli intake in young healthy smokers

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Cruciferous vegetables contain compounds with antioxidant properties (e.g. carotenoids, vitamin C and folates) and can alter the activity of xenobiotic metabolism (i.e. isothiocyanates). These constituents may be particularly important for subjects who are exposed to free radicals and genotoxic compounds, including smokers. The aim of the study was to evaluate the effect of broccoli intake on biomarkers of DNA damage and repair. Twenty-seven young healthy smokers consumed a portion of steamed broccoli (250 g/day) or a control diet for 10 days each within a crossover design with a washout period. Blood was collected before and after each period. The level of oxidatively damaged DNA lesions (formamidopyrimidine DNA glycosylase-sensitive sites), resistance to *ex vivo* H₂O₂ treatment and repair of oxidised DNA lesions were measured in peripheral blood mononuclear cells (PBMCs). We also measured mRNA expression levels of repair and defence enzymes: 8-oxoguanine DNA glycosylase (OGG1), nucleoside diphosphate linked moiety X-type motif 1 (NUDT1) and heme oxygenase 1 (HO-1). After broccoli consumption, the level of oxidised DNA lesions decreased by 41% (95% confidence interval: 10%, 72%) and the resistance to H₂O₂-induced DNA strand breaks increased by 23% (95% CI: 13%, 34%). Following broccoli intake, a higher protection was observed in subjects with glutathione S-transferase (GST) M1-null genotype. The expression level and activity of repair enzymes was unaltered. In conclusion, broccoli intake was associated with increased protection against H₂O₂-induced DNA strand breaks and lower levels of oxidised DNA bases in PBMCs from smokers. This protective effect could be related to an overall improved antioxidant status.

Introduction

Diet, together with healthy lifestyle, plays a significant protective role against the development of several chronic diseases such as cancers (1). Specific groups of vegetables are particularly rich in potentially protective phytochemicals; the Cruciferae and especially *Brassica* gender contains high concentration of constituents with antioxidant properties (e.g. carotenoids, vitamin C and folates) as well as glucosinolate precursors of isothiocyanates (ITCs) and indoles that modulate the activity of xenobiotic biotransformation (2). Indeed, many studies point to specific cancer protective effects of cruciferous vegetables towards at least lung and stomach cancer (3,4).

One protective mechanism of action of *Brassica* vegetables is thought to involve reduction of free radical-related molecular damage and this could be particularly important in subjects with high exposure. Cigarette smoke contains a large amount of reactive oxygen species (ROS) as well as other substances that generate ROS (5). ROS can cause oxidative damage to DNA such as oxidised bases and strand breaks. These DNA lesions, including the promutagenic 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) could be implicated in early steps of cancer development (6,7). Fortunately, mammalian cells have a large array of repair systems for the removal of oxidised DNA lesions; the most important pathway for oxidised bases is considered to be base excision repair (BER), whereas the nucleotide excision repair enzymes most likely functions as a backup system. The repair of 8-oxodG by the BER system is initiated by 8-oxoguanine DNA glycosylase (OGG1), which cleaves the N-glycosidic bond leaving behind a free base and an AP-site (apurinic/pyrimidinic site) (8,9). In the nucleotide pool, dGTP can be oxidised to 8-oxo-2'-deoxyguanosine 5'-triphosphate (8-oxo-dGTP), which may give rise to 8-oxodG in DNA if the oxidised nucleotide is incorporated during replication or repair. 8-OxodGTP is removed from the nucleotide pool by the nucleoside diphosphate linked moiety X-type motif 1 (NUDT1) enzyme (10).

In a previous study (11), we observed that the intake of broccoli increased resistance to *ex vivo* H₂O₂-induced DNA strand breaks in peripheral blood mononuclear cells (PBMCs) of smokers and non-smokers, whereas oxidation of purines was reduced significantly only in smokers. It has been debated whether or not smokers *per se* have different levels of biomarkers measured by the comet assay; for instance, it has been suggested that publications from countries in southern part of Europe had a tendency to show a large effect of smoking because the brands of tobacco products were stronger than the brands in the Northern part of Europe (12). This is in keeping with a meta-analysis documenting that smokers have higher level of DNA damage measured by the comet assay, although there was large heterogeneity between publications (13).

Results obtained in our previous dietary intervention study (11) indicate that broccoli constituents may act either as free radical scavengers or may increase the endogenous defense against oxidative stress, and this could be particularly

important in subjects who smoke. For this reason, we developed a new intervention study to further investigate the effect of broccoli consumption on DNA damage (endogenous and oxidatively induced) and defense systems, including OGG1 repair activity as well as expression levels of *OGG1*, *NUDT1* and *heme oxygenase (decycling) 1 (HMOX1)* or commonly referred to as *HO-1* in PBMCs from smokers. HO-1 is the rate-limiting enzyme in the degradation of heme to equimolar biliverdin, carbon monoxide and ferrous iron and is considered an important inducible protective mechanism against oxidative stress (14). The transcription factors for expression of *HO-1* include the redox-sensitive *nrf2*, which is also involved in the induction of phase II enzymes through the antioxidant responsive element by ITCs (15). Thus, we may hypothesize that an enhanced expression of *HO-1* in lymphocytes can be considered as part of such enzyme induction and a response to oxidative stress, possibly modulated by nutritional interventions. Studies in cultured cells indicated that ITCs can enhance *HO-1* expression (16); to the best of our knowledge, such findings have not been reported or investigated in a human intervention trial.

The subjects enrolled in our study were also genotyped for the common polymorphism determining the presence of glutathione S-transferase (GST) M1, which is involved in the elimination of ITCs and may modulate the effects of cruciferous vegetables (3,4,17–19).

Materials and methods

Dietary intervention study

Thirty healthy smokers were recruited within the student population of the University of Milan and enrolled on the basis of their food habits, evaluated by means of a food frequency questionnaire, in order to have a homogeneous group for lifestyle and food intake and comparable with the characteristics of the group previously studied. The subjects were included in the study if they were males <30 years and if they smoked >10 cigarettes per day.

Exclusion criteria were high (more than five portions per day) or low (less than two portions per day) intake of fruit and vegetables, regular use of medications or dietary supplements, habitual alcohol consumption (less than three drinks per week were tolerated) and adherence to specific vegetarian diets (e.g. vegan or macrobiotic). The study was approved by the Local Ethical Committee and in accordance with the Declaration of Helsinki and informed consent was signed by each participant. Characteristics of subjects recruited for the study are reported in Table 1.

The investigation was designed as a crossover study. The volunteers were randomly divided into two groups of 15 subjects as follows: Group 1 was assigned to the sequence broccoli diet/washout/control diet, whereas Group 2 followed the sequence control diet/washout/broccoli diet. The period of broccoli or control diet was 10 days long and there was a 20-day washout period between dietary treatments. The study was conducted during the period of February to May 2007, which was carried out independently of our previous

intervention study on broccoli consumption (March to May 2006). Five of the subjects in our previous study were also recruited for the present study.

Broccoli and control diet

We used frozen 'Marathon' broccoli (*Brassica oleracea* L. var. *italica*) (Di Stasi Company, Basilicata, Italy), as in the previous study, however, this product, grown 1 year later, had a lower concentration of glucosinolates as evaluated by high-performance liquid chromatography analysis (108 versus 200 µmol ITCs per portion), thus the portion prepared for the volunteers was slightly increased. Apart from ITCs, each portion of broccoli provided 3.1 mg of lutein, 1.4 mg of β-carotene and 146 mg of vitamin C. We have used plasma concentrations of carotenoids as marker of broccoli intake and effectiveness of the intervention because these phytochemicals are not expected to be biotransformed by GST enzymes, plasma concentrations of ITCs were considered a priori to be suboptimal as marker because there could be effect-modification by GST polymorphisms. Moreover, we evaluated folate plasma concentrations, which were significantly increased in the previous broccoli intervention.

During the trial, every day broccoli was steam-cooked for 15 min and portioned (250 g) into appropriate food containers that were given to the subjects.

On Fridays, subjects were given two extra portions of broccoli to eat during the weekend. The subjects were instructed to keep their habitual diet during the entire trial. In addition, they were told to avoid the intake of cruciferous vegetables throughout the trial. We assessed the food intake during the study by a food diary, which the subjects were instructed to update daily.

Blood samples. Peripheral blood samples were collected in microtubes with heparin at the beginning and at the end of each treatment period (0, 10, 30 and 40 days). Samples were drawn early in the morning after overnight fasting.

PBMCs were isolated by density gradient centrifugation of 100 µl whole blood with Histopaque 1077 (Sigma Chemicals Co, St Louis, MO, USA). The PBMCs layer was removed from the gradient and the cells were washed with phosphate-buffered saline (PBS), centrifuged and used immediately for the determination of *in vivo* resistance to H₂O₂-induced DNA strand breaks. A different batch of isolated PBMCs was suspended in a solution containing 50% foetal bovine serum (FBS), 40% culture medium (RPMI) and 10% dimethyl sulphoxide (DMSO) and stored at -80°C for the determination of formamidopyrimidine DNA glycosylase (FPG)-sensitive sites, DNA repair activity and expression levels of *OGG1*, *HO-1* and *NUDT1*. Moreover, serum and plasma were isolated from blood in order to evaluate carotenoids and folate concentrations.

Polymorphisms analysis GSTM1. A polymerase chain reaction (PCR) method was used to detect the presence or absence of the *GSTM1* and *GSTT1* genes in genomic DNA samples obtained from blood samples through DNeasy® Blood and Tissue kit (QIAGEN) following the manufacturer's technical instruction.

DNA was amplified in a total reaction volume of 25 µl containing 4 µl of DNA, 1.5 mM dNTPs, 1.5 mM MgCl₂, 5 pmol of each oligonucleotide primer and 0.12 µl (5 U/µl) TaqGOLD. DNA samples were amplified using the following primers: for *GSTM1*, forward primer 5'-GGGCTCAAATA-TACGGTGGG and reverse primer 5'-GGAGGAAGTCCCTGAAAAGC; for *GSTT1*, forward primer 5'-GACCTTCCTTACTGGTCTC and reverse primer 5'-GAAGAGGTCCCTCCCAACT; for albumin, forward primer 5'-AAAGC-CAGAGCTGGAAGTCA and reverse primer 5'-CAGCTTTGG-GAAATCTCTGG.

PCR was performed with TaqGOLD activation for 5 min at 95°C, the temperature profile repeated for the first two cycles was denaturing 30 sec at 95°C, annealing 15 sec at 60°C and extension 30 sec at 72°C; for other two cycles, denaturing 30 sec at 95°C, annealing 15 sec at 60°C and extension 30 sec at 72°C; for other two cycles, denaturing 30 sec at 95°C, annealing 15 sec at 56°C and extension 30 sec at 72°C and 29 cycles with denaturing 30 sec at 95°C, annealing 15 sec at 54°C and extension 30 sec at 72°C and final extension for 5 min at 72°C and storage at 4°C. The amplified products were electrophoresed through 2% agarose gel and visualised by ethidium bromide staining.

Resistance to H₂O₂-induced DNA strand breaks in PBMCs. The resistance to H₂O₂-induced strand breaks in PBMCs was evaluated by means of the Comet assay as previously reported (20). The measurement of H₂O₂-induced DNA strand breaks is considered to be a marker of the cellular resistance to oxidative stress because H₂O₂ generates DNA damage by oxidative attack. Briefly, each measurement consisted of duplicate slides as follows: one slide was subjected to a H₂O₂ treatment (submerging it into a solution of H₂O₂ 500 µM in PBS for 5 min in the dark), whereas the other one acted as a control (submerging it in

Table 1. Characteristics of subjects before the intervention study (mean values ± SD)

Characteristics	Number of subjects	Results
Age (years)	27	22.1 ± 2.5
BMI (kg/m ²)	27	23.0 ± 2.7
H ₂ O ₂ -induced strand breaks (% DNA in tail)	27	62.9 ± 8.8
FPG-sensitive sites (%DNA in tail)	27	14.0 ± 6.3
OGG1 activity (%DNA in tail)	11	1.9 ± 2.3
mRNA expression of <i>NUDT1/18S</i> (× 10 ⁻³)	17	0.5 ± 1.3
mRNA expression of <i>OGG1/18S</i> (× 10 ⁻³)	17	4.6 ± 5.8
mRNA expression of <i>HO-1/18S</i> (× 10 ⁻³)	17	1.6 ± 3.0

BMI, body mass index.

PBS for 5 min). The slides were then immersed in lysis solution (0.1 M Na₂EDTA, 10 mM Tris, 2.5 M NaCl, 1% sarcosine, pH 10 with NaOH, 1% Triton X-100 and 1% DMSO added just before use). After lysis of the cells, slides were immersed in an electrophoresis solution (0.3 M NaOH, 1 mM Na₂EDTA; pH > 13) for 40 min, prior to an electric field being applied (25 V, 300 mA, 20 min). The slides were then neutralised to pH 7, stained with ethidium bromide (2 µg/ml) and analysed using an epifluorescence microscope (Olympus CX 41; Olympus Italia) attached to a high-sensitivity CCD video-camera (CFW 1808M; Scion Corporation, Germany) and to a computer provided with an image analysis system (Cometa 1.5; Immagini e Computer, Bareggio, Milan, Italy). DNA damage was expressed as percentage DNA in tail. For each subject, the percentage DNA in tail of control cells (not treated with H₂O₂) was subtracted from the percentage DNA in tail of treated cells.

FPG-sensitive sites. Detection of endogenous oxidised DNA base was performed by means of the enzyme FPG, in order to detect the major purine oxidation product (8-oxo-7,8-dihydroguanine) as well as other altered purines. We have participated in the European Comet Assay Validation Group, demonstrating that the intra-assay variation of the comet assay is very low and we could detect dose-dependent increase in FPG sites in coded samples from monocytic THP1 standardised cells (21,22). In the present study, cryopreserved PBMCs were used, thus a higher inter-individual variability is not excluded. Moreover, cold storage can partially affect the amount of strand breaks; however, the subtraction of endonuclease buffer (EB) data from FPG data is sufficient to correct for this effect.

In brief, cryo-preserved PBMCs were thawed and washed with fresh RPMI medium and cold PBS and then embedded in agarose on fully frosted slides to perform the Comet assay as above described. After the lysis phase, slides were immersed in 40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg/ml bovine serum albumin (BSA), pH 8.0 with KOH, used also for the enzyme dilution for three times 5 min each. Then, 50 µl of a diluted solution of FPG (100 ng/ml) or buffer alone (control) was added and the slides incubated at 37°C for 45 min. The FPG enzyme was a gift from Professor Andrew Collins, University of Oslo, Norway. FPG, added to the DNA in the gel, converts altered purines into DNA breaks (23).

Alkaline treatment and electrophoresis then followed. Net enzyme-sensitive sites were calculated by subtracting the % DNA in tail in the slides incubated with buffer from the % DNA in tail obtained by incubating the slides with FPG.

DNA repair activity. The DNA repair activity was determined in cryopreserved PBMCs. Assessment of DNA repair incisions was analysed by the Comet assay as previously described by Guarnieri *et al.* (24).

The repair activity was measured as the incision activity of substrate DNA treated with Ro19-8022/white light, which generates 8-oxo-7,8-dihydroguanine (24–26). Oxidised bases were introduced into A549 lung epithelial cells substrate nuclei by irradiating cells with white light in PBS with 1 µM Ro19-8022 (the photosensitizer was a gift from F. Hoffmann-La Roche, Basel, Switzerland) at 0°C. The cells were washed and resuspended in freezing medium (50% FBS, 40% RPMI and 10% DMSO) to a concentration of 3×10^6 cells/ml and frozen at -80°C.

For the preparation of human PBMCs extracts, the cryo-preserved cells were centrifuged (300 g, 5 min and 4°C), and the pellet was resuspended in buffer A (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 0.1 mM dithiothreitol and 10% glycerol; pH 7.8) at a volume of 20 µl per 10^6 cells. The resuspended cells were divided in aliquots of 50 µl to which 12 µl 1% Triton X-100 was added. The lysate was centrifuged (700 g, 5 min and 4°C) and the supernatant was mixed with 200 µl buffer B (40 mM HEPES, 0.1 M KCl, 0.5 mM Na₂EDTA and 0.2 mM BSA; pH 8). Approximately 3×10^7 substrate cells were embedded in agarose and applied on 85 × 100 mm GelBond® films and lysed as described for the Comet assay. After three times washing (5 min each) in buffer B, repair incisions were detected by incubation of the agarose-embedded nuclei with 60 µl PBMCs extract or buffer B for 20 min at 37°C. The subsequent alkaline treatment and electrophoresis were identical to the conditions used to determine DNA damage using the Comet assay. An assay control (a sample for FPG-sensitive sites evaluation) was included in each electrophoresis run.

After neutralisation with 0.4 M Tris-HCl (pH 7.5), cells were placed in 96% ethanol for 1.5 h or overnight. Nuclei were visualised as previously described for strand breaks and FPG-sensitive sites estimation after staining with 40 µl ethidium bromide (4 µg/ml) in PBS solution. The repair activity of the PBMCs extract was determined as the difference in % DNA in tail between parallel gels incubated with extract and control solution.

Quantification of OGG1, HO-1 and NUDT1 mRNA expression in PBMCs by real time reverse transcription-PCR. PBMCs samples were stored in freezing medium. Approximately 0.4 µg RNA was used for complementary DNA

(cDNA) synthesis in a reaction volume of 20 µl using the TaqMan GeneAmp RT-PCR Kit as recommended by Applied Biosystems (Naem, Denmark). Quantitative PCR reactions were carried out in ABI PRISM 7900HT (Applied Biosystems), using primers and cDNA-specific probes purchased from Applied Biosystems. We used as the reference gene 18S rRNA, which is commercially available as a probe and primer solution (Eukaryotic 18S rRNA Endogenous Control, 4352930E; Applied Biosystems). Below are probes and primers for the genes. Sequence accession ID numbers are from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>; accessed 12 February 2007): *HO-1*, forward primer 5'-CATGAGGAACATTCAGAAAGGGC-3' and reverse primer GATGTGGTACAGGGAGGGCCAT-3; TaqMan probe: 5'-6-FAM-TGACCC-GAGACGGCTTCAAGCAGCTG-TAMRA-3' (NM_002133); *OGG1*, forward primer 5'-AAATCCCAAGGTGTGGCACTG-3' and reverse primer 5'-GGGATGTTGTTGGAGGA-3'; TaqMan probe: 5'-6-FAM-CAA-GACCCCATCGAATGCCTTTCTCTTTTAMRA-3' (U96710); *NUDT1*, forward primer 5'-CATCGAGGATGGGGCTAG-3' and reverse primer CAGAAGACATGCACGTCCATGA-3'; TaqMan probe: 5'-6-FAM-TCCGCCCAAGCAACTCAAACACCATGATCT-TAMRA-3' (D16881). The PCRs were performed in triplicate using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. For the PCR, the following protocol was used as follows: activation of Taq polymerase for 20 sec at 95°C, followed by a total of 45 temperature cycles for 0.01 sec at 95°C and 20 sec at 60°C. In each run, a standard was included and verified on the efficiency plot, and the variation coefficients of the repeated measurements were 2.98% (27).

Determination of carotenoids and folate. Lutein, β-carotene and folate were analysed in blood as previously reported (26). These dietary markers were selected as we previously have demonstrated that the plasma concentrations was increased after 10 days of broccoli consumption, whereas no effect on vitamin C level was observed in the previous study (28).

Statistical analysis. The results were analysed by analysis of variance (ANOVA) with sequence (broccoli intake versus control diet or *vice versa*) as independent factor in order to assess carry-over effects. As no carry-over effects were detected (always $P > 0.05$) data were analysed by ANOVA for repeated measure design. Only results from subjects with a complete set of data available were included in the statistical analysis. This analysis with type of treatment and time as independent factors was used to investigate the effect of broccoli consumption on cell resistance against H₂O₂-induced strand breaks, levels of endogenous oxidatively damaged DNA lesions (FPG-sensitive sites) and DNA repair activity and *OGG1*, *HO-1* and *NUDT1* mRNA expression. Moreover, ANOVA with type of treatment as independent factor was used to evaluate percentage changes [i.e. (after treatment - before treatment)/before treatment × 100] in the different end point of DNA damage considered following broccoli diet with respect to control diet. Lastly, data were also analysed by adding GSTM1 genotype as independent factor.

Differences were considered significant at $P < 0.05$; post-hoc analysis of differences between treatments or genotypes were assessed by the Fisher Least Significant Difference test with $P < 0.05$ as level of statistical significance.

Regression analysis was applied in order to verify correlations between the % changes in plasma carotenoids and folate concentrations registered in the whole experimentation (broccoli and control diet treatments) and the % changes in the end points of DNA damage considered.

Statistical analyses were performed by means of STATISTICA 5.0 software (Statsoft Inc, Tulsa, OK, USA).

Results

Twenty-seven subjects completed the entire study, whereas three subjects dropped out during the experimental period. We ended up having fewer results on oxidatively damaged DNA, repair activity and mRNA expression because of problems during the storage of the samples and we have only included data where the complete set of the four samples were available. No side effects were observed in the whole group of subjects. Figure 1 and Figure 2 show the levels of H₂O₂-induced strand breaks and FPG-sensitive sites in PBMCs following the broccoli diet and control diet treatments, for each single subject.

Despite the inter-individual variation in the response to treatments (where biological variability cannot be excluded),

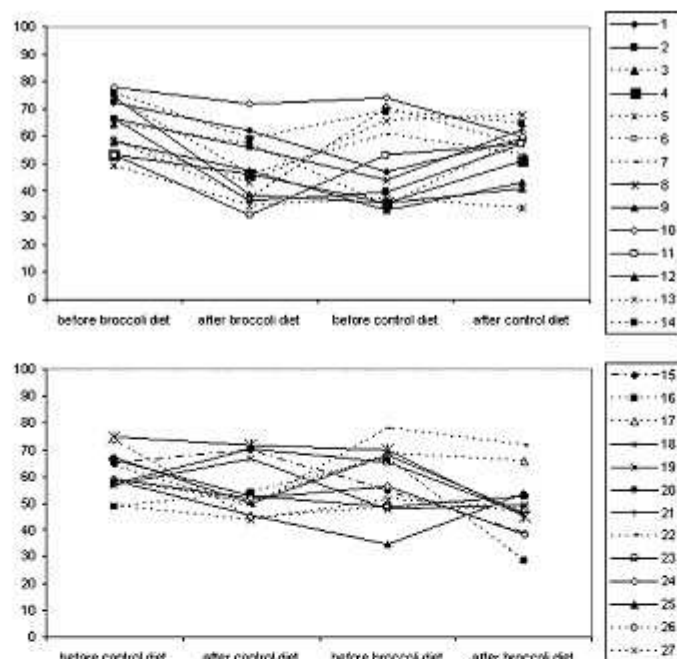


Fig. 1. Effect of broccoli diet and control diet on H_2O_2 -induced strand breaks evaluated by means of comet assay. (a) Individual values registered in Group 1 ($n = 14$) assigned to the sequence broccoli diet/washout/control diet; (b) Individual values registered in Group 2 ($n = 13$) assigned to the sequence control diet/washout/broccoli diet. The symbols (and identification numbers) represent different individuals.

there was a significant reduction in the level of DNA damage following the broccoli diet ($P < 0.01$).

The levels of H_2O_2 -induced strand breaks and FPG-sensitive sites before and after each period are reported in Table II. Background strand breaks (PBS and EB) are also reported. The mean level of H_2O_2 -induced strand breaks in PBMCs was decreased by 23% (95% CI: 13%, 34%) in the period of broccoli intake as compared to the period of control diet (Table II). This difference was driven by a reduction in the level of H_2O_2 -induced strand breaks after the broccoli intake period [-22% (95% CI: -13%, -31%)], whereas there was no difference in the resistance after the control diet [-0.4% (95% CI: -10%, +9.4%)].

The subjects had 41% (95% CI: 10%, 72%) lower level of FPG-sensitive sites in PBMCs after 10 days of broccoli consumption compared with the control diet. This difference was driven by decreased level of FPG sites in the period of broccoli intake [-34% (95% CI: -53%, 15%)], whereas it was unaltered during the control diet period [4% (95% CI: -20%, +28%)].

Analysis of effect-modification by the GSTM1 polymorphism revealed significant single-factor effects of the genotype ($P < 0.05$) and diet ($P < 0.01$) on H_2O_2 -induced strand breaks, whereas the interaction was not statistically significant ($P > 0.05$). The subjects with GSTM1-null genotype had higher baseline levels of H_2O_2 -induced DNA strand breaks (Table II); however, at the end of the 10 days period of broccoli

consumption, there was no difference in the resistance to *ex vivo* generation of strand breaks depending on the genotypes. Consequently, the effect on resistance towards *ex vivo* generation of strand breaks following broccoli intake (% changes) was most pronounced in subjects with the GSTM1-null genotype (-27.6%, 95% CI: -37.9%, -17.4%), whereas the subjects with GSTM1-positive genotype had lower level of resistance (-13.1, 95% CI: -27.3%, 1.1%) as showed in Figure 3.

In contrast, the effect of broccoli consumption on the level of FPG-sensitive sites was not significantly related to GSTM1 polymorphism (Table II).

The OGG1 repair incision activity, gene expression levels of *OGG1*, *HO-1* and *NUDT1* are reported in Table II; there was no significant effect of broccoli intake on these biomarkers in PBMCs.

The circulating levels of folate and lutein significantly increased after broccoli consumption (+17.1 and +39.3%, respectively), whereas no significant effect on β -carotene concentration was observed (Table III).

Regression analysis showed an inverse and significant correlation between changes in serum folate concentrations and changes in H_2O_2 -induced strand breaks ($R^2 = 0.23$; $P < 0.001$).

Discussion

The present study showed that 10 days of consumption of steamed broccoli by smokers decreased the level of

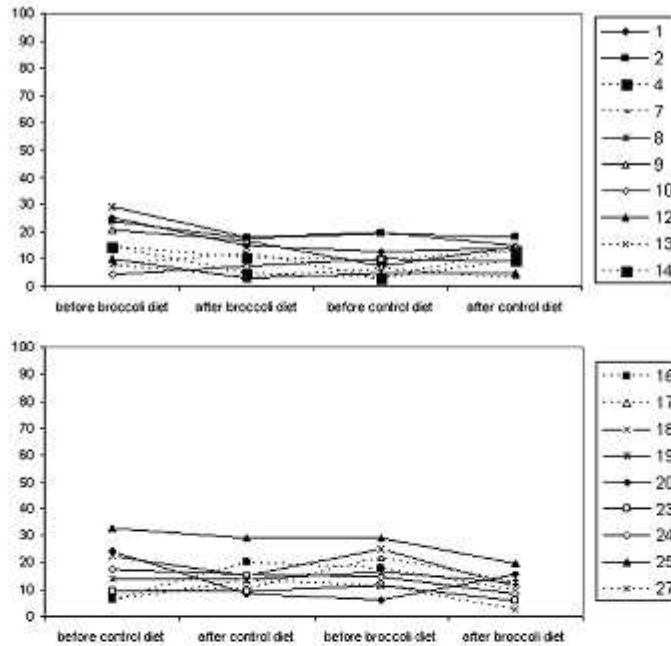


Fig. 2. Effect of broccoli diet and control diet on FPG-sensitive sites evaluated by means of comet assay. (a) Individual values registered in Group 1 ($n = 10$) assigned to the sequence broccoli diet/washout/control diet; (b) Individual values registered in Group 2 ($n = 9$) assigned to the sequence control diet/washout/broccoli diet. The symbols (and identification numbers) represent different individuals.

Table II. H_2O_2 -induced strand breaks, FPG-sensitive sites, DNA repair activity and mRNA expression of *OGG1*, *NUDT1* and *HO-1* before and after broccoli and control diet in smokers

	Before broccoli diet, mean (SD)	After broccoli diet, mean (SD)	Before control diet, mean (SD)	After control diet, mean (SD)
Background SBs (% DNA in tail, PBS)				
All subjects ($n = 27$)	7.5 (2.6)	7.7 (2.3)	7.4 (2.8)	7.4 (2.6)
H_2O_2 -induced SBs (% DNA in tail)				
All subjects ($n = 27$)	61.4 (11.1)	47.9 (11.4) [*]	55.4 (13.3)	55.2 (9.6)
GSTM1+ ($n = 14$)	57.6 (11.8)	48.8 (12.1) ^{***}	52.4 (12.7)	54.0 (9.4)
GSTM1- ($n = 13$)	65.4 (9.1)	46.9 (11.1) [*]	58.7 (13.7)	56.5 (10.1)
Background SBs (% DNA in tail, EB)				
All subjects ($n = 19$)	23.0 (8.0)	21.3 (6.5)	21.8 (8.8)	19.8 (8.0)
FPG-sensitive sites (% DNA in tail)				
All subjects ($n = 19$)	16.5 (7.7)	11.0 (5.4) ^{**}	13.2 (9.8)	13.8 (7.5)
GSTM1+ ($n = 9$)	17.3 (7.2)	11.1 (5.1) ^{***}	17.1 (12.0)	16.1 (9.4)
GSTM1- ($n = 10$)	15.9 (8.4)	10.9 (5.9) ^{***}	9.8 (6.0)	11.6 (4.8)
DNA repair activity (% DNA in tail)				
All subjects ($n = 11$)	3.7 (3.0)	3.6 (2.5)	3.0 (3.0)	4.4 (3.1)
OGG1 mRNA ($\times 10^{-3}$) ^a				
All subjects ($n = 17$)	6.4 (12.4)	5.4 (6.3)	4.2 (3.9)	6.2 (5.5)
HO-1 mRNA ($\times 10^{-3}$) ^a				
All subjects ($n = 17$)	3.3 (9.0)	2.2 (4.5)	1.5 (2.7)	1.8 (1.6)
NUDT1 mRNA ($\times 10^{-3}$) ^a				
All subjects ($n = 17$)	1.6 (3.5)	0.8 (2.0)	0.6 (1.3)	0.5 (0.7)

SB, strand break.

^aThe mRNA levels are reported as the fold compared to 18S.

^{*}Significant difference between 'before' and 'after' each treatment, $P < 0.001$.

^{**}Significant difference between before and after each treatment, $P < 0.005$.

^{***}Significant difference between before and after each treatment, $P < 0.05$.

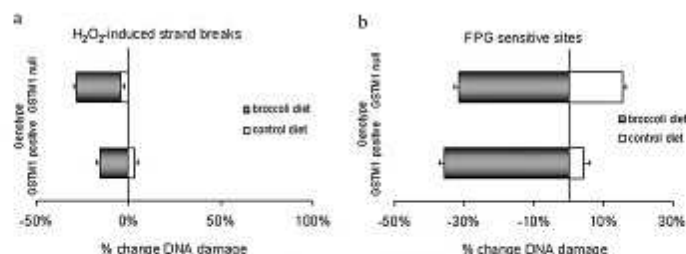


Fig. 3. Effect of broccoli diet and control diet on end points of DNA damage (FPG-sensitive sites and H_2O_2 -induced strand breaks) evaluated by means of comet assay. Data reported are mean % change DNA damage [(after treatment - before treatment)/before treatment \times 100] and standard error. (a) % change DNA damage (H_2O_2 -induced strand breaks) in GSTM1-null ($n = 13$) and GSTM1-positive ($n = 14$) subjects. (b) % change DNA damage (FPG-sensitive sites) in GSTM1-null ($n = 13$) and GSTM1-positive ($n = 14$) subjects.

Table III. Concentrations of folate (in serum), lutein and β -carotene (in plasma) before and after broccoli and control diet

	Before broccoli diet, mean (SD)	After broccoli diet, mean (SD)	Before control diet, mean (SD)	After control diet, mean (SD)
Folate (μ mol/l) ($n = 27$)	11.3 (3.9)	13.2 (3.3)**	12.0 (3.9)	12.1 (4.7)
Lutein (μ mol/l) ($n = 27$)	0.31 (0.13)	0.43 (0.21)	0.30 (0.14)	0.29 (0.13)
β -carotene (μ mol/l) ($n = 27$)	0.28 (0.25)	0.31 (0.22)*	0.28 (0.24)	0.29 (0.29)

*Significant difference between before and after each treatment. $P < 0.001$.

**Significant difference between before and after each treatment. $P < 0.01$.

endogenous oxidised DNA bases and H_2O_2 -induced DNA strand breaks. Whereas, OGG1 repair activity and gene expression levels of *OGG1*, *NUDT1* and *HO-1* were unaltered, at least in the sub-sample of data analysed.

Our data are in accordance with the rather consistently shown protective effects of cruciferous vegetables, including Brussels sprouts, broccoli and watercress, on biomarkers of oxidative damage to DNA in human intervention studies (11,29–34). Such effects may be particularly important in subjects exposed to high levels of ROS, e.g. from cigarette smoke. Indeed, Gill *et al.* (33) showed that smokers had larger reduction in basal and H_2O_2 -induced strand breaks in PBMCs than non-smokers following an intervention with 85 g of raw watercress daily for 8 weeks. Similarly, we have found that FPG sites in PBMCs decreased only in smokers, whereas the level of H_2O_2 -induced DNA strand breaks decreased significantly both in smokers ($n = 10$) and non-smokers ($n = 10$) consuming 200 g of broccoli daily for 10 days (11).

A lowered level of FPG sites in PBMCs can in principle be caused by a decreased rate of guanine oxidation in DNA or in the nucleotide pool or an increased rate of repair (10). It has previously been observed that consumption of kiwi fruits increased the DNA repair activity and lowered the level of FPG sites in lymphocytes (35). In addition, Guarnieri *et al.* (24) investigated the repair activity towards oxidised DNA in human mononuclear blood cells in two placebo-controlled antioxidant intervention studies, one with well-nourished subjects who consumed 600 g/day of fruits and vegetables or tablets containing the same amount of vitamins and minerals for 24 days and another one with poorly nourished male smokers who consumed 500 mg/day vitamin C as slow- or plain-release formulations together with 182 mg/day vitamin E for 4 weeks. Only male smokers supplemented with slow-release vitamin C tablets had increased DNA repair activity by $\sim 27\%$, whereas there was no significant change in

subjects supplemented with tablets with plain-release formulation of vitamin C and in subjects who consumed fruits and vegetables. In the present study, with a reduced level of FPG sites after broccoli intake, we found no change in repair activity towards oxidatively damaged guanine in DNA in extracts from the same pool of PBMCs. Similarly, there was no change in the gene expression of *OGG1* and *NUDT1*, the protein of which is responsible for repair of oxidised guanine in DNA and the nucleotide pool, respectively. A possible limitation of the present study is the number of missing data (due to samples storage problems) in the analysis of DNA repair activity and the mRNA expression. Still, it should be emphasised that neither the *OGG1* expression nor the repair activity pointed in the direction of increased level after broccoli intake, indicating that we would not find beneficial effects with even a substantially larger number of subjects in the study.

We found increased resistance towards H_2O_2 -induced strand breaks in PBMCs. Thus, our data point mainly to increased resistance to oxidative stress in PBMCs after a broccoli rich diet. Increased resistance to oxidative stress induced by cruciferous vegetables could be related to upregulation of antioxidant, phase II and other defence enzymes and/or increased levels of scavengers of ROS. We found no change in the expression of *HO-1*, which is highly susceptible to oxidative stress and also a part of the *nr2*-dependent gene battery of phase II enzymes frequently ascribed protective effects of cruciferous vegetables (2). Moreover, in our previous study with a similar broccoli intervention, we did not find modulation of GST activity in plasma (28). Similarly, in the watercress consumption study described above (36), no change in mRNA levels of catalase, glutathione peroxidase 1, glutathione S-transferase A4, glutathione S-transferase P1, superoxide dismutase 2 or UDP glucuronosyl transferase A1 was found in lymphocytes despite considerable decreases in basal and oxidatively induced DNA damage. In the same study,

the activity of SOD and GPX in red cells was not significantly changed overall, although there appeared to be an increase among subjects with the *GSTM1*-null genotype (36). In another study, the activity of SOD and GPX were unchanged after Brussels sprouts intake despite reduction of oxidatively induced DNA damage in lymphocytes (34). Moreover, the intake of regular broccoli changed the expression of only one gene tested in a very large battery of xenobiotic metabolism genes through microarray analysis. On the contrary, high sulphoraphane broccoli changed the expression of more and different genes in the gastric mucosa (37).

We did not detect differences in the level of DNA strand breaks after the broccoli supplementation. This is in keeping with the conclusion from a literature review showing that only very few publications have reported effect on DNA strand breaks in leukocytes (or subsets of leukocytes such as lymphocytes or mononuclear blood cells), whereas there is a larger proportion of publications that have reported an effect of antioxidant supplementation in terms of FPG-sensitive sites or 8-oxodG in leukocytes (38). The null effect in terms of DNA strand breaks in our study is further strengthened by the fact that it was determined in both the analysis of H₂O₂ sensitivity and FPG-sensitive sites. We detected a higher level of DNA strand breaks in the assessment of FPG-sensitive sites than the analysis of H₂O₂ sensitivity. We attribute the difference in the level of DNA strand breaks between the two measurements to methodological differences because the determination of H₂O₂-sensitive sites was carried out on fresh samples, whereas the level of FPG-sensitive sites was determined on cryopreserved samples. It should be noted that the higher level of strand breaks in the determination of FPG-sensitive sites does not affect the validity of the analysis of oxidatively damaged DNA. We have measured a level of FPG-sensitive sites that is similar to the levels reported in other studies (39). However, we did observe inter-individual and intra-individual variation in the level of FPG-sensitive sites and sensitivity to H₂O₂-induced DNA strand breaks. In addition, there is large effect of broccoli intake in some subjects, whereas other subjects appear to have no benefit. We cannot determine whether this heterogeneity is because some subjects are non-responders because this trait would only be revealed if the person participated in several independent intervention trials. However, it should be emphasized that our crossover study was not completely controlled in regard to habits of the subjects. Environmental and occupational exposures could affect the level of DNA damage and sensitivity to H₂O₂-induced DNA strand breaks such as sunlight, air pollution, exhaustive exercise or therapeutics (12). In addition, it is also possible that some subjects entering a trial on beneficial effects of dietary products change toward a healthier lifestyle. In our study this might be displayed as decreased levels of FPG sites and increased resistance toward H₂O₂-induced DNA strand breaks. Still, we believe that the reduction of DNA damage, as found in the present study, may be due to direct or indirect antioxidant scavenger functions of compounds in cruciferous vegetables, including vitamin C, carotenoids, polyphenols, folates and/or some ICTs. Indeed, we have previously shown that the broccoli consumption regime used in the present study increased the plasma concentrations of lutein and β -carotene in smokers, respectively, by ~45 and 33% as well as folate (~16%) and non-indolyl ICTs (~86%) (28). The present study confirms an increase of folate and lutein concentrations after broccoli

consumption, whereas, differently from the previous study, β -carotene levels were not significantly increased after the intervention (probably because of the higher individual variability). We also found a significant inverse correlation between the % changes in folate levels and those of H₂O₂-induced DNA damage possibly providing support for the contribution of the vitamin to the DNA protection against *ex vivo* induced damage and/or repair. Despite the lack of correlation for single carotenoids, we do not exclude a role of carotenoids in the DNA protection because the effect of these antioxidants might depend on the synergy of multiple bioactive substances rather than single compounds.

The broccoli used in the present study had lower concentration of ITCs compared to the broccoli used in our previous study (~110 versus 200 μ mol ITCs per portion provided), whereas there was the same level of protection against *ex vivo* generation of DNA strand breaks and endogenous levels of FPG sites in PMBCs from smokers (11). Thus the mechanisms for reduction of oxidative damage to DNA do not seem to involve specifically the ITCs with enzyme inducing properties. This further supports the notion that whole foods can exert a protective effect in virtue of the numerous compounds present, able to act in synergy.

To our knowledge, this study is the first reporting the possible effect-modification by *GSTM1* polymorphism in relation to modulation of DNA damage following broccoli consumption. In particular, we found an increased resistance to H₂O₂-induced strand breaks after broccoli intake in the group of subjects with the *GSTM1*-null genotype with respect to the positive genotype. Although large-scale systematic studies on the effect of the *GSTM1* polymorphism have not been published, some cross sectional studies on air pollution exposure and diet do suggest that subjects with the *GSTM1*-null and -positive genotype differ in respect to levels and response of FPG sites and H₂O₂-induced strand breaks in leukocytes (40–42). Individuals with *GSTM1*-null genotype could benefit more than subjects with *GSTM1*-positive genotype from *Brassica* vegetables intake because altered metabolism leads to a higher bioavailability of sulphoraphane or total ITCs as shown in studies with administration of a single broccoli meal, although differences were small and the interpretation is debated (17,19). The impact of *GSTM1* on possible cancer protective effects of cruciferous vegetables is also debated with effect mainly shown with the *GSTM1*- and *GSTT1*-double-null genotype in Chinese and European populations (4), whereas the *GSTM1*-positive genotype might convey beneficial effects in subjects from North America (17). Intake of cruciferous vegetables, including broccoli, is most popular in the latter region, whereas other *Brassica* vegetables in the former region as well as multiple other dietary, lifestyle and genetic differences can play roles in these apparent discrepancies.

In conclusion, in the present study, the intake of broccoli seems protective, as far as DNA damage is concerned, in smokers who are exposed to oxidative stress. This protective effect may be due to improved antioxidant status.

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Oral communication

Titolo: Delphinidin-3-glucoside, an anthocyanin from wild blueberries, protects endothelial cells by proangiogenic and prothrombotic stimuli

OC149

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Testo:

Anthocyanins (ACNs) are natural pigments that provide colors from dark blue to purple to fruit and vegetables, such as edible berries, and belong to the flavonoid class. Since these compounds are ingested regularly with the diet and their intake had been estimated to exceed 200 mg/day, they may have a relevant long-term effect. Studies investigating the health effects of ACNs have observed anti-inflammatory and anticarcinogenic properties. Delphinidin-3-glucoside (Dp-3-g) is one of the ACNs contained in wild blueberries (*Vaccinium angustifolium*) able to inhibit tumor cell proliferation. It is not known whether Dp-3-g can also act on proangiogenic and prothrombotic properties of endothelial cells.

We designed an in vitro study to investigate the effect of Dp-3-g on the angiogenic and the procoagulant activities of human microvascular endothelial cells (HMEC-1). HMEC-1 were incubated for up to 24h with culture media Dp-3-g (0.1, 1, 10, 100 microM) alone or in combination with purified proangiogenic factors (VEGF, FGF-2), or bacterial endotoxin (LPS, 10 microg/ml). After incubation, angiogenesis was evaluated by capillary-like tube formation Matrigel and wound healing assay, while procoagulant activity was tested by the thrombin generation assay (TG).

The results show that Dp-3-g inhibited capillary tube formation, both alone and in combination with proangiogenic factors, reaching significant reduction at 100 microM compared to control cells ($p < 0.05$). The same concentration of Dp-3-g significantly reduced the migration of HMEC-1. In the TG assay, Dp-3-g significantly counteracted the prothrombotic stimulus of LPS starting from 10 microM.

In conclusion, the data show that Dp-3-g can affect angiogenic, migratory and procoagulant properties of endothelial cells. These findings, together with the known capacity of Dp-3-g to affect tumor cell proliferation, make this compound a potential cancer chemopreventive agent. Our results support health promotion strategies that favour colored fruit and vegetables

5th International Conference on Thrombosis and Hemostasis Issues in Cancer (ICTHIC) (April 23-25, 2010)
 Poster selected for oral presentation.

CFPAC1 (Table). The clotting times (CT) mirrored TF expression levels with CFPAC1 giving the fastest time and MIAPaCa-2 the slowest. Blocking of cell-expressed TF with a saturating concentration of a polyclonal antibody (American Diagnostica) increased CT in all cases. The highest cell invasion was also observed with CFPAC1, followed by AsPC1 and MIAPaCa-2. Blocking of TF decreased cell invasion to approximately 50% in both CFPAC1 and AsPC1, but showed minimal effect on the already low levels observed with MIAPaCa-2.

Conclusions: Clotting time and cell invasion appear to be strongly correlated, with both processes being mediated, at least in part, via TF.

Table 1. Expression of Tissue Factor and growth factor receptors on pancreatic cell lines: correlation with TF activity and cell invasion^a

Pancreatic cell line	Mean clotting time (s)		Mean cell invasion/HPF		Growth factor receptor expression (% of positive cells/MFI)				
	Plasma + Cells	Anti-TF antibody added	Cells + serum	Anti-TF antibody added	TF	EGFR	VEGFR 1	VEGFR 2	VEGFR 3
MIAPaCa-2	120	>241	40	35	0/0	3/0	12/2	14/3	20/5
AsPC1	74	119	86	47	4/8	87/17	22/4	24/15	33/5
CFPAC1	28	95	243	134	85/135	90/91	0/0	11/3	0/0

^aNote: The MFI for the negative control samples have been subtracted from all values. Clotting time experiments were performed in triplicate while cell invasion experiments were performed in duplicate.

PO-85

Effects of an anthocyanin (delphinidin-3-glucoside) from wild blueberries on the proangiogenic and prothrombotic properties of endothelial cells

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Wild blueberries (*Vaccinium angustifolium*) are one of the main sources of anthocyanins (ACNs). They are glycosides of the flavonoid class, which are natural colorants of fruit and vegetables. It is known that ACNs can affect some cellular events 'in vitro' involved in tumor progression, including angiogenesis, proliferation, invasion and apoptosis. Since these compounds are ingested regularly with the diet, they may have a relevant long-term effect. Delphinidin-3-glucoside (Dp-3-g) is one of the ACNs contained in wild blueberries able to inhibit tumor cell proliferation. It is not known whether Dp-3-g can also act on proangiogenic and prothrombotic properties of endothelial cells.

We designed this study to investigate on the effect of Dp-3-g on the angiogenic and the procoagulant activities of human microvascular endothelial cells (HMEC-1) 'in vitro'. HMEC-1 were incubated for up to 24 h with culture media ± Dp-3-g (0.1, 1, 10, 25, 50, 100 μM), or purified proangiogenic factors (VEGF, FGF-2), or endotoxin (LPS, 10 μg/ml). After incubation, angiogenesis was evaluated by both capillary-like tube formation in Matrigel and by the wound healing assay, and procoagulant activity tested by thrombin generation assay (TG).

The results show that Dp-3-g inhibited capillary tube formation in a dose-dependent manner reaching about 90% reduction at 100 μM compared to control cells (p<0.05). The same concentrations of Dp-3-g had a similar effect on migration/proliferation of HMEC-1. A significant reduction of TG of unstimulated and LPS-stimulated HMEC-1 was also observed starting with 10 μM Dp-3-g.

In conclusion the data show that Dp-3-g affect angiogenic, migratory and procoagulant properties of endothelial cells. These findings, together with the capacity of Dp-3-g to affect tumor cell proliferation, make this compound a potential cancer chemopreventive agent. Further investigations are needed to better understand cellular mechanisms. Our results support health promotion strategies that favour coloured fruit and vegetables consumption.

PO-86

Platelet proteins modified by oxidative stress in myelodysplastic syndromes

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The myelodysplastic syndromes (MDSs) are heterogenous groups of clonal hematologic disorders characterized clinically and morphologically by ineffective hematopoiesis. The early stages of MDSs are characterized by dysplasia and increased apoptosis of bone marrow cells resulting in peripheral blood cytopenias. Ineffective erythropoiesis and multiple blood transfusions lead to iron overload. Excess of iron in cells and plasma catalyses the generation of reactive oxygen species. Oxidative imbalance with consequent activation of nitric oxide synthases leads to the increased production of nitric oxide (NO). NO rapidly reacts in the present of dissolved oxygen with free thiol groups of cystein and thereby modifies cellular as well as plasmatic proteins. S-nitrosylation is an important NO dependent postranslational modifications that regulates a large variety of cellular functions and signalling events. The aim of this study was to identify S-nitrosylated proteins detected in platelets of myelodysplastic patients and normals. Platelets were isolated by centrifugation from EDTA blood of patients with MDS diagnosis. Platelets of healthy donors were used as normals. S-nitrosylated proteins from washed platelets were detected by Biotin-switch technique. Biotinylated proteins were separated by 2D SDS-PAGE and identified by LC/MS. Biotin-switch technique is a specific method for detection of protein S-nitrosylation. We identified several platelet proteins, which were markedly S-nitrosylated in myelodysplastic patients as compared with platelets of normals. Patients with myelodysplastic syndromes have hemorrhagic tendency mainly attributed to thrombocytopenia. According to recent studies, impaired platelets has been characterized within MDS patients contributing to defect aggregation. S-nitrosylation of platelet proteins leads to significant functional changes. Therefore oxidative modification of platelet proteins may play an important role in hemostasis in myelodysplastic syndromes. Acknowledgements: This study was supported by Grants NS10633-3/2009 and MZ 02373601 from Ministry of Health, by Grant KAN200670701 from the Academy of Sciences, and by Baxter; Czech Republic.

PO-87

Prognostic value of vascular endothelial growth factor (VEGF) serum level and immunohistochemical expression in non-treated patient with chronic lymphocytic leukemia

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Introduction: Enhancement of angiogenesis in chronic lymphocytic leukemia (CLL) has been recognized more recently. Vascular endothelial growth factor (VEGF) is the major pro-angiogenic factor in humans and his transduction pathway may be very active in CLL cells contributing to their enhanced survival.

Aims: 1. To assess VEGF serum level (sVEGF) and VEGF immunohistochemical expression on malignant CLL cell; 2. To examine the possible association of sVEGF and VEGF immunohistochemical expression with clinical course, pattern of marrow infiltration, Rai stage, CD 38 positivity, LDH, beta-2 microglobulin (β2m) and cytogenetic abnormalities detected by FISH.

Materials and Methods: To predict the risk of disease progression, we analyzed serum sVEGF using ELISA technique and bone marrow samples using immunohistochemical staining in 33 Binet stage A de novo CLL patients and 30 controls.

Results: The sVEGF was not significantly elevated (p=0.31) in CLL patients (mean: 70.9 pg/ml; range: 16-483) compared with age- and sex-matched healthy controls (mean: 44.7 pg/ml; range: 22-69.2). sVEGF level positively correlated with elevated LDH level (p=0.008), but no correlation with other clinico-biological features was found. Bone marrow VEGF was significantly higher (p<0.0001) in CLL patients (mean: 78%±15.71) compared to controls (mean:15.4%±6.19) but no correlation with other clinico-biological features was found. In univariate analysis sVEGF (p=0.018), Rai substages (p=0.027), PBL (p=0.030), β2m level (p=0.008) and diffuse bone marrow infiltration (p=0.006) were significantly associated with increased risk of disease progression. But, in multivariate analysis only sVEGF (p=0.002) and β2m level (p=0.008) retained their prognostic significance.

Conclusions: Serum VEGF level may improve the assessment of individual prognosis of patients with early CLL while significance of bone marrow VEGF expression on malignant cells was not proved. Further assessment of real clinical significance of VEGF in CLL patients is required.

APPENDIX 3- FOOD FREQUENCY QUESTIONNAIRES

ABITUDINI ALIMENTARI

COLAZIONE

- Frequenza
 sempre mai a volte
- Tempo dedicato al consumo
 0 - 5 min 5 - 10 min > 10 min
- Luogo di consumo
 casa bar altro

• Da cosa è composta abitualmente?

PRANZO

- Frequenza
 sempre mai a volte
- Tempo dedicato al consumo
 < 30 min 30 - 60 min > 60 min
- Luogo di consumo
 casa mensa bar
 ristorante altro

• Generalmente, a fine pasto, si ritiene:

- soddisfatto insoddisfatto

• Composizione abituale

	sempre	mai	1-3 volte a sett.	4-6 volte a sett.
primo				
secondo				
contorno di verdura				
pane				
frutta				
toast, panini, pizzette				
caffè				
vino e/o birra				

CENA

- Frequenza
 sempre mai a volte
- Tempo dedicato al consumo
 < 30 min 30 - 60 min > 60 min
- Luogo di consumo
 casa mensa bar
 ristorante altro

• Generalmente, a fine pasto, si ritiene:

- soddisfatto insoddisfatto

• Composizione abituale

	sempre	mai	1-3 volte a sett.	4-6 volte a sett.
primo				
secondo				
contorno di verdura				
pane				
frutta				
toast, panini, pizzette				
caffè				
vino e/o birra				

SPUNTINI

- Frequenza
 sempre mai a volte
- Quante volte al giorno
 una due più di due
- Luogo di consumo
 casa bar altro

• Come sono composti abitualmente

Quando mangia, lei ritiene di essere:

- veloce normale lento

Generalmente, quando ha finito di mangiare, si ritiene:

- molto sazio sazio normale con fame

Dopo i pasti principali avverte sonnolenza?

- sì no

Cosa apprezza di più in un cibo? Ordini da 1 (più apprezzato) a 5 (meno apprezzato) in base al suo giudizio

- aspetto esteriore aspetto nutritivo profumo sapore
 nulla in particolare

Ritiene che le quantità di alimenti da lei abitualmente consumate siano:

- abbondanti normali scarse

Lei è allergico o non gradisce qualche cibo in particolare?

- no sì

Se sì, indichi quali:

È un fumatore?

- no sì Quante sigarette fuma al giorno?

Stai seguendo una dieta?

- no sì

Se sì, di che tipo? Per quale motivo?

Stai assumendo farmaci?

- no sì

Se sì, indichi quali (nome commerciale):

Stai assumendo integratori alimentari (vitamine, fibra, lievito di birra...)?

- no sì

Se sì, indichi quali:

Esprima il suo gradimento per i seguenti alimenti con un punteggio da 1 (meno gradito) a 9 (più gradito).

ORTAGGI E FRUTTA

1	Insalata
2	Pomodori da insalata
3	Carote
4	Patate
5	Patatine fritte
6	Spinaci
7	Melanzane
8	Broccoli
9	Cavolfiore
10	Cavolini di Bruxelles
11	Cavolo cappuccio
12	Zucchine
13	Zucca
14	Aglione
15	Cipolla
16	Arance
17	Banane
18	Mele
19	Pere
20	Mandarini
21	Uva
22	Pesche
23	Albicocche
24	Prugne
25	Kiwi
26	Melone
27	Frutta secca (noci, nocciole, mandorle...)

DOLCI, CEREALI E DERIVATI

28	Dolci in generale
29	Biscotti secchi

45	Gnocchi
46	Lasagne
47	Polenta
48	Ravioli ripieni di carne
49	Ravioli ripieni di verdura
50	Pizza

LEGUMI E DERIVATI

51	Fagioli
52	Piselli
53	Lenticchie
54	Soia
55	Tofu

CARNE, PESCE, UOVA

56	Carne in generale
57	Bistecca
58	Arrosto
59	Polpette
60	Pollo
61	Tacchino
62	Maiale
63	Carne in scatola
64	Mortadella
65	Prosciutto cotto
66	Prosciutto crudo
67	Salame
68	Pancetta
69	Bresaola
70	Fegato di vitello
71	Pesce fresco in generale (orata, trota...)
72	Pesce azzurro (alice, sgombro, sardine...)

30	Biscotti farciti
31	Biscotti al cacao
32	Merendine ripiene al cioccolato
33	Merendine ripiene marmellata
34	Merendine ripiene crema
35	Croissants
36	Crackers normali
37	Crackers integrali
38	Pane comune
39	Pane integrale
40	Panino burro e marmellata
41	Pasta
42	Pasta integrale
43	Riso
44	Riso integrale

BEVANDE E ALCOLICI

85	Acqua minerale naturale
86	Acqua minerale gassata
87	Birra
88	Vino bianco
89	Vino rosso
90	Superalcolici
91	Bibite analcoliche
92	The
93	Caffè
94	Infusi e tisane

73	Tonno in scatola
74	Gamberetti
75	Vongole
76	Uova in generale
77	Frittata

LATTE E DERIVATI

78	Latte intero
79	Latte scremato
80	Yogurt bianco
81	Yogurt alla frutta
82	Formaggi freschi (mozzarella, crescenza...)
83	Formaggi stagionati (parmigiano, fontina...)
84	Formaggi molli (certosino, asiago...)

PRODOTTI VARI

95	Burro
96	Olio d'oliva
97	Olio di semi
98	Aceto
99	Sugo a base di pomodoro e cipolla
100	Sugo a base di pomodoro e aglio
101	Ragù
102	Pesto alla genovese
103	Gelato alla frutta
104	Gelato alla crema
105	Cioccolato
106	Marmellata di frutta
107	Miele

	SEMPRE	N° VOLTE A SETTIMANA	N° VOLTE AL MESE	MAI	NOTE
PRIMI					
Pasta/riso al pomodoro					
Pasta/riso al ragù					
Pasta con verdure					
Minestrone, passato...					
Pasta/minestra di legumi					
SECONDI					
Spezzatino					
Carne alla pizzaiola					
CONTORNI					
Pomodori					
Peperoni					
Carote					
Insalata					
Spinaci, erbe					
Broccoli					
Cavolini di Bruxelles					
Cavolfiori					
Verze					
Cime di rapa					
Melanzane					
Zucca					
FRUTTA					
Arance					
Mandarini					
Pompelmi					
Kiwi					
ALTRO					
Pizza margherita					
Pizza con verdure					
Torte salate con verdure					

BEVANDE					
Tè					
Tè verde					
Vino bianco					
Vino rosso					
Caffè					
Succhi di frutta					
Spremuta d'arance					
CONDIMENTI					
Olio d'oliva					
Olio di semi					
Burro					

NOTE

DIARIO ALIMENTARE

NOME
COGNOME
DIETA STANDARD

DATA

COLAZIONE

alimento / piatto	quantità

SPUNTINO

PRANZO

SPUNTINO

CENA

SPUNTINO

DIARIO ALIMENTARE

NOME
COGNOME
DIETA BROCCOLI

DATA

COLAZIONE

alimento / piatto	quantità

SPUNTINO

PRANZO

SPUNTINO

CENA

SPUNTINO
