The Modulation of Lung Cancer Risk by Glutathione *S*-Transferase M1-Diet Interactions

Master thesis

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Abstract

Fruits and vegetables are considered to be protective against lung cancer, as is shown in epidemiological studies. They contain antioxidants which protect against oxidative DNA damage and induce detoxifying enzymes. Isothiocyanates, present in cruciferous vegetables, have chemo-preventive abilities by inhibition of phase I enzymes and induction of phase II enzymes. A lack of phase II glutathione *S*-transferases (GSTs) results in a higher lung cancer risk, since reactive carcinogen metabolites are no longer detoxified. These GST enzymes are also responsible for the elimination of isothiocyanates. A homozygous deletion in *GST* genes can therefore result in a higher concentration or longer exposure to the protective isothiocyanates, while antioxidants might compensate this loss by induction of other phase II enzymes. It has been suggested that people lacking certain glutathione *S*-transferase genes benefit more from the chemo-protective abilities of vegetables. We evaluated the modulating effects of the *GSTM1* genotype on the protective abilities of vegetable consumption against lung cancer risk by assessing epidemiological and molecular epidemiological studies. Nine out of thirteen epidemiological studies, report a lowered lung cancer risk for subjects with high vegetable intake and a homozygous deletion of the *GSTM1* gene, compared to subjects without this deletion. In the molecular epidemiological studies there is some discrepancy between results of studies measuring bulky lesions (e.g. DNA adducts) and oxidative parameters as biomarkers of lung cancer risk, possibly because of different mechanistic origins. Overall, eight of the thirteen molecular epidemiological studies report an inverse association between high antioxidant levels (present in vegetables) and molecular damage which is only seen in *GSTM1*-null subjects. From these results we can conclude that the *GSTM1*-null genotype in combination with certain constituents of vegetables have a probable protective effect against lung cancer.

Keywords: glutathione *S*-transferase, lung cancer, isothiocyanates, antioxidants, DNA damage.

Abbreviations: BaP, benzo(a)pyrene; CI, confidence interval; CYP, cytochrome P450; GST, glutathione *S*-transferase; GSTM1, glutathione *S*-transferase, mu class, isoenzyme 1; ITC, isothiocyanate; OR, odds ratio; PAH, polycyclic aromatic hydrocarbon; ROS, reactive oxygen species.

1. Introduction

For a long period of time, lung cancer has been the most common cancer worldwide. In 2008, the International Agency for Research on Cancer (IARC) reported 1.61 million new lung cancer cases, representing 12.7% of all new cancer cases (1). With 1.38 million annual deaths (18%), is lung cancer also the leading cause of death by cancer. The most important risk factor for lung cancer is smoking, as it accounts for 70% of all trachea, bronchus and lung cancer deaths (2). For smokers, the inhalation of nicotine is addictive because of the rewarding effects in the brain. Usually people start smoking at young age under peer pressure and maintain smoking predominantly because of a genetically determined dependence making quitting very difficult. (3). Nicotine itself has hardly carcinogenic activity, but with each puff of a cigarette a smoker inhales a mixture of more than 60 known carcinogens, along with co-carcinogens, toxicants, tumour promoters, oxidants, free radicals and inflammatory agents (4-6). More than 3800 chemicals have been identified in tobacco smoke, comprising at least 20 known lung carcinogens including tobacco-specific nitrosamines, aromatic amines and polycyclic aromatic hydrocarbons (PAHs) (7). Besides smoking, additional risk factors are environmental tobacco smoke (passive smoking), urban air pollution, diet and occupational exposure to asbestos and soot. The similarity between these risk factors can be found in the identity of the carcinogens involved: PAHs. PAHs are formed when organic substances (e.g. tobacco or meat) are burnt incompletely. Uptake of PAHs increases lung cancer risk, because several known PAH-metabolites have DNA damaging properties (8). Metabolism of carcinogens like PAHs is needed to facilitate detoxification and excretion, but some of the metabolites are reactive and bind to DNA. Carcinogen binding to the DNA can result in DNA adducts and subsequent somatic mutations. When these mutations occur in genes involved in cellular differentiation and proliferation, like oncogenes and tumour suppressor genes, this may lead to loss of normal cellular growth, genomic instability and cancer. This has been shown for the PAH benzo(a)pyrene (BaP) included in tobacco smoke; exposure leads to BaP-diol-epoxide DNA adducts that induce G to T transitions in the p53 tumour suppressor gene (9). With this mutation, BaP is directly involved in lung carcinogenesis.

On the other hand, several dietary factors have been found to be protective against lung cancer; many reports in the scientific literature focus on the protective effects of vegetables with special emphasis on antioxidants and isothiocyanates (ITCs), present in high concentrations in cruciferous vegetables (10-12). Furthermore, genetic variants have been found that seem to affect susceptibility to lung cancer. Most of them are polymorphisms in genes that are involved in the metabolism of PAHs and ITCs, like glutathione *S*-transferases (e.g. GSTM1) and cytochrome P450s (e.g. CYP1A1) (13). GSTM1 has a specific role in detoxification of PAHs and is therefore one of the most extensively investigated polymorphisms in relation to lung cancer. In this report, we evaluate the evidence for an interaction between *GSTM1* polymorphisms and dietary habits with regard to lung cancer risk in epidemiological and molecular epidemiological studies.

2. An epidemiological view on the protective ability of diet against lung cancer

Consumption of fruits and vegetables is considered healthy, because they are a source of many vitamins, minerals and antioxidants. Vegetables and fruits were labelled as 'protective foods', even before the discovery of vitamins as essential nutrients in the early 20th century (14). In the 1970s and 1980s, most reports focused on the protective ability of fruits and vegetables against cardiovascular disease (15-17). Since the 1990s, there has been emerging evidence that vegetables and fruits might

also be protective against some cancers (18-20). The potential protective agents include a variety of vitamins (e.g. vitamins A, C, E), their precursors (e.g. β-carotene) and minerals (e.g. selenium, calcium) (21). In 1996, Steinmetz and Potter published a review, which included 13 case-control studies conducted in the period of 1977-1994. All studies showed an inverse association for consumption of (green) vegetables or fruits and lung cancer (22). The World Cancer Research Fund/ American Institute for Cancer Research acknowledged this protection against lung cancer in their 2007 publication of Food, Nutrition, Physical Activity and the Prevention of Cancer: a Global Perspective (23). Here, they state that fruits probably protect against lung cancer and limited evidence suggests that (non-starchy) vegetables are protective as well. Furthermore, they mention the discrepancy between results from case-control studies and prospective cohort studies performed since the mid-1990s. The conflicting results obtained from cohort studies have made the overall evidence that vegetables or fruits protect against cancers somewhat less impressive. For instance, inverse associations between cruciferous vegetable intake and lung cancer risk have been reported by cohort studies of Voorrips *et al.* (2000) (24), Neuhouser *et al.* (2003) (25) and Feskanich *et al.* (2000; women) (26). In contrast, cohort studies from Feskanisch *et al.* (2000; men) (26) and Miller *et al.* (2004) (27) found no inverse association. In order to address these discrepancies and to evaluate the overall association between cruciferous vegetable intake and lung cancer risk, Lam *et al.* performed a meta-analysis using these cohort studies. The pooled odds ratio was 0.83 (95% CI, 0.63-1.08) (28). However, the same study reported a pooled odds ratio of 0.78 (95% CI, 0.70-0.88) for 12 case-control studies. Thus, the risk for lung cancer in subjects with the highest cruciferous vegetable intake was 22% lower in case-control studies and 17% lower in cohort studies compared with subjects with the lowest or no intake of cruciferous vegetables. Although these estimates are very close to each other, the data collected from the cohort studies were not statistically significant. A possible explanation for this discrepancy is the modulation of lung cancer risk by genetic variation in the various populations, which deserves further study. Specific (phase II) detoxifying enzymes might influence the ability to metabolise the protective ITCs and the carcinogenic compounds. Genetic polymorphisms in these metabolizing enzymes can modulate the effect of vegetables and therefore modify the risk of developing lung cancer.

3. The role of Glutathione *S*-transferase in the metabolism of ITCs and carcinogens

Isothiocyanates (ITCs) are present in relatively high concentrations in cruciferous vegetables (broccoli, Brussels sprouts, cabbage and watercress), predominately as glucosinolate precursors (29). The general structure of an isothiocyanate consists of –N=C=S. The ITC compounds are formed by hydrolysis catalyzed by the myrosinase enzyme. This enzyme is present in the plant itself, but can only come in contact with the glucosinolates after destruction of the plant cells by for instance chewing and preparation of the vegetables for consumption (10). The suggested mechanism of protection against cancer by ITCs is the modulation of biotransformation enzymes (12). The biotransformation pathway consists of several important steps in the activation and detoxification of a variety of xenobiotics, including drugs, toxins and carcinogens (30). Phase I enzymes, mainly cytochrome p450s (CYPs), are generally thought to be responsible for the metabolic activation of carcinogenic compounds. These enzymes catalyze reactions that increase the reactivity of (hydrophobic) compounds and prepare them for the next step. This second step (detoxification) is executed by phase II enzymes, which increase the solubility of the compound and promote its elimination (31). Phase II enzymes consist of glutathione *S*-transferases (GSTs), UDP-glucoronosyl transferases (UGTs) and NADPH quinone oxidoreductases (NQOs). The GSTs detoxify compounds by

conjugation with glutathione which increases water solubility for easier secretion. Furthermore, the processing of the reactive phase I products protects the DNA against damage by bulky lesions and oxidative stress by ROS (12).

ITCs have been found to act protective at three phases of carcinogenesis. Firstly, they can inhibit phase I enzymes and therefore prevent carcinogen activation (11). Next to inhibition of phase I enzymes, ITCs are also potent inducers of phase II enzymes (32). The induction of the enzymes promoting detoxification and elimination of the carcinogenic compound is reported as an important mechanism for protection against many carcinogens (33, 34). Mechanistic studies revealed a third protective ability of ITCs; activation of the protein kinase pathway by cellular stress may induce apoptosis (35). Another defence mechanism against oxidative stress-induced lung cancer is provided by antioxidants. Antioxidants (e.g. ascorbid acid, α-,γ-tocopherol, caretonoids) scavenge the ROS before they are able to interact with cellular components and cause oxidative damage (36). In addition, antioxidants induce expression of several genes involved in detoxification, including phase II enzymes like GSTs, mainly through the antioxidant-response element (ARE) (37, 38).

Next to their role in the metabolism of xenobiotics, GSTs are also important in the metabolism of food ingredients, including ITCs. The goal of metabolism of xenobiotics and ITCs by GSTs is similar, namely the elimination from the body. There are genetic polymorphisms known in humans which alter the activity of GST enzymes and therefore might influence the effect of ITCs on cancer risk (39). In humans, the *GST* gene superfamily is divided in four classes of isoenzymes; Alpha, Mu, Theta and Pi (40). The GSTM1 (Mu class, isoenzyme 1) enzyme is responsible for the detoxification of diol epoxides formed by polycyclic aromatic hydrocarbons (PAHs) in cigarette smoke (41). A common polymorphism of the *GSTM1* gene is a homozygous deletion, resulting in a so-called *GSTM1*-null phenotype. This homozygous deletion is present in approximately 50% of the Caucasian population, but might differ in other ethnicities (42). *GSTM1*-null subjects have no activity of the corresponding GST protein which results in a decreased ability of carcinogen detoxification. People who lack the GSTM1 enzyme are suggested to have a higher risk of developing lung cancer, possible because of the higher levels of DNA adducts found (43). A meta-analysis published by Carlsten *et al.* (44) showed that the *GSTM1*-null variant was indeed associated with an increased risk of lung cancer (OR 1.22; 95% CI, 1.14-1.30). The loss of GSTM1 activity might be replaced by other phase II enzymes, enhanced by antioxidant or isothiocyanate intake. In addition, individuals lacking this GSTM1 enzyme might have a slower elimination of ITCs. This may lead to a higher concentration or a prolonged exposure to the chemo-preventive ITCs after consumption of cruciferous vegetables. It has therefore been proposed that people with a *GSTM1*-null genotype benefit more of vegetable consumption, and have a higher protection against lung cancer by vegetable intake (29).

The modulating effects of the *GSTM1* genotype on the protective effects of vegetable consumption against lung cancer risk, can be studied by epidemiological studies with lung cancer as endpoint, but could also be assessed by molecular epidemiological studies, in which biomarkers of lung cancer risk were used as early end point. Results from both study types will be evaluated in this report.

4. Epidemiological data on *GSTM1*-diet interactions modulating lung cancer risk

To assess the effect of *GSTM1* genotypes on the interaction between vegetable intake and lung cancer risk, we have searched for epidemiological studies reporting lung cancer odds ratios (ORs) in Pubmed and Scopus, using the search terms GSTM1, lung cancer and one of the following dietary

factors: diet, vegetables, isothiocyanates, nutrition, micronutrients, antioxidants, vitamins or tea. Reports were excluded if a) there were no original data (review or meta-analysis); b) they were not conducted in humans and/or c) there was no association examined or reported regarding GSTM1, dietary intake and lung cancer. The search resulted in thirteen articles, with publication years ranging from 1999 to 2011 (table 1). Of these, eight were case control studies and five nested case-control studies. Of the thirteen studies, one reported α-tocopherol supplementation (45), six studies measured isothiocyanate as dietary factor, of which three based on intake by food frequency questionnaires (46-48) and three on urinary levels (49-51). Furthermore, there was one study which examined the effect of a 'healthy' or 'unhealthy' diet (52), one study focussed on green tea consumption (53) and four reported vegetable intake/consumption, of which three included cruciferous vegetables only (54-56) and one vegetables in general (57).

One out of thirteen studies reported no modulation of lung cancer risk by interactions between the *GSTM1* genotype and diet. Fowke *et al*. showed that the *GSTM1* genotype had no effect on the association between urinary ITC levels and lung cancer risk in Chinese non-smoking women (49). Within the group of women with detectable ITC levels, the odds ratios for lung cancer stratified by *GSTM1* genotype were 1.12 (95% CI 0.44-2.88) for *GSTM1*-null subjects and 0.97 (95% CI 0.29-3.23) for *GSTM1*-positive subjects. There were three studies which reported a protective role of high vegetable intake against lung cancer in *GSTM1*+ individuals. The study of Spitz *et al.* (46) was conducted in male and female subjects from the USA. A recalculation of the original data by Lam *et al.* revealed a protective effect for *GSTM1*-positive subjects with high ITC intake (OR 0.56, 95% CI 0.38-0.82) but not for *GSTM1*-null subjects (OR 0.81, 95% CI 0.55-1.19) (28). Another American study, performed by Wang *et al*., showed that individuals with high cruciferous vegetable intake were protected against lung cancer only when they were *GSTM1*-positive (55). The results of a Danish prospective study, by Sørensen *et al.,* showed an indication for an increased risk of lung cancer in *GSTM1*-null subjects with higher vegetable intake compared to the *GSTM1*-positive subjects (ORs were 1.14, 95% CI 1.00-1.30 and 0.91, 95% CI 0.78-1.06, respectively) (57).

On the other hand, there were nine studies which showed an (indicative) protective effect of high vegetable intake against lung cancer in *GSTM1*-null carriers. Woodson *et al.* investigated the protective ability against lung cancer of α-tocopherol and β-carotene in smokers (45). In the group of subjects with the longest smoking years, only *GSTM1*- subjects appear to benefit from α-tocopherol supplementation. A recalculation of the original data resulted in odds ratios of 1.41 (95% CI 0.68-2.93) for *GSTM1*+ subjects and 0.40 (95% CI 0.19-0.85) for *GSTM1*- subjects receiving α-tocopherol supplementation, compared to the subjects with no supplementation. For β-carotene, no effect on lung cancer risk was seen in both *GSTM1*+ and *GSTM1*- carriers. London *et al.* reported a protective effect of ITCs in a cohort of Chinese men (50). This protective effect was found exclusively in individuals with a homozygous deletion of the *GSTM1* gene (OR 0.36, 95% CI 0.20-0.63). Another study, by Zhao *et al*., revealed similar results in Chinese women (47). Here, only *GSTM1*-null subjects benefitted from high ITC intake and had a lowered lung cancer risk (OR 0.55, 95% CI 0.33-0.93). A study of Carpenter *et al.* (2009) revealed a reduced lung cancer risk in US citizens with higher ITC intake that was stronger among *GSTM1*-null subjects (OR 0.52, 95% CI 0.31-0.86 for *GSTM1*-null; OR 0.77, 95% CI 0.49-1.21 for *GSTM1*-positive) (48). An extensive European case control study by Brennan *et al*., containing 2141 cases and 2168 controls, confirmed these results. Cruciferous vegetable consumption protected against lung cancer in subjects with a *GSTM1*-null genotype (OR

Table 1. Evidence table of epidemiological studies reporting lung cancer risk in odds ratios based on the association between dietary constituent and *GSTM1* status.

Author, year	Cases/controls; population	Study type	Dietary constituent	Intake/exposure	Effect of GSTM1 on diet-lung cancer interaction	GSTM1 status	OR (95% CI)
Woodson, *et al.*, 1999 (45)	319/333; Caucasians	Nested case-control	α-tocopherol, β-carotene	α-tocopherol supplementation; Yes vs. no	yes	GSTM1+ GSTM1-	1.41 (0.68-2.93)* 0.40 (0.19-0.85)*
Spitz, *et al.*, 2000 (46)	503/465; Caucasians	Case-control	Isothiocyanate	Intake; > median vs. ≤ median	Yes	GSTM1+ GSTM1-	0.56 (0.38-0.82)† 0.81 (0.55-1.19)†
Zhao, *et al.*, 2001 (47)	233/187; Asians	Case-control	Isothiocyanate	Intake; > median vs. ≤ median	Yes	GSTM1+ GSTM1-	0.78 (0.39-1.59) 0.55 (0.33-0.93)
Carpenter, *et al.*, 2009 (48)	311/622; Caucasians & African Americans	Case-control	Isothiocyanates	Intake; ≥ median vs. < median	Yes	GSTM1+ GSTM1-	0.77 (0.49-1.21) 0.52 (0.31-0.86)
Fowke, *et al.*, 2011 (49)	209/787; Asians	Nested case-control	Isothiocyanates	Urinary level; detectable vs. undetectable	No	GSTM1+ GSTM1-	0.97 (0.29-3.23) 1.12 (0.44-2.88)
London, *et al.*, 2000 (50)	232/710; Asians	Nested case-control	Isothiocyanate	Urinary levels; detectable vs. undetectable	Yes	GSTM1+ GSTM1-	1.22 (0.67-2.24) 0.36 (0.20-0.63)
Chou, *et al.*, 2005 (51)	30/60; Asians	Nested case-control	Isothiocyanates	Urinary levels; detectable vs. undetectable	Yes, indication	GSTM1+ GSTM1-	2.00 (0.10-25.3) 0.30 (0.10-1.20)
Tsai, *et al.*, 2003 (52)	254/184; Caucasians	Case-control	Dietary pattern	'healthy' vs. 'unhealthy'	Yes, indication	GSTM1+ GSTM1-	1.22 (0.56-2.66) 0.46 (0.21-1.01)
Bonner, *et al.*, 2005 (53)	122/122; Asians	Case-control	Green tea	Consumption; daily vs. never	Yes, indication	GSTM1+ GSTM1-	1.67 (0.47-5.88) 0.36 (0.12-1.13)
Brennan, *et al.*, 2005 (54)	2141/2168; Caucasians	Case-control	Cruciferous vegetables	Consumption; high vs. low	Yes	GSTM1+ GSTM1-	0.89 (0.67-1.18) 0.67 (0.49-0.91)
Wang, *et al.*, 2004 (55)	716/939; Caucasians	Case-control	Cruciferous vegetables	Intake; highest vs. lowest	Yes	GSTM1+ GSTM1-	0.61 (0.39-0.95) 1.15 (0.78-1.68)
Lewis, *et al.*, 2002 (56)	122/123; Caucasians & Latin Americans	Case-control	Cruciferous vegetables	Consumption; high vs. low	Yes , indication	GSTM1+ GSTM1-	0.65 (0.16-2.66) 0.27 (0.06-1.33)
Sørensen, *et al.*, 2007 (57)	430/767; Caucasians	Nested case-control	Vegetables	Intake; per 50% increase	Yes, indication	GSTM1+ GSTM1-	0.79 (0.55-1.14) 1.14 (1.00-1.30)

* Recalculation of original data (not adjusted for age and daily cigarettes smoked) based on Bland & Altman (58)

† Recalculation of original data reported by Lam, *et al.* (28)

0.67, 95% CI 0.49-0.91), whereas no protection was found in people with a *GSTM1*-positive genotype (OR 0.89, 95% CI 0.67-1.18) (54). Four other studies showed an indicative protective effect of vegetables in *GSTM1*-null carriers on lung cancer risk. Chou *et al*. performed a nested case control study in China and reported odds ratios of 2.0 (95% CI 0.1-25.3) for *GSTM1*-positive subjects and 0.3 (95% CI 0.1-1.2) for *GSTM1*-null subjects with detectable ITC urinary levels (51). A study on cruciferous vegetable consumption, by Lewis *et al*., showed lowered odds ratios for *GSTM1*+ and *GSTM1*- subjects with high intake (56). The highest and most probable protection against lung cancer by vegetable intake was found in subjects with a homozygous deletion in *GSTM1* (OR 0.27, 95% CI 0.06-1.33; OR 0.65, 95% CI 0.16-2.66 for GSTM1+). People with a *GSTM1*-null genotype seemed to benefit more from a healthy diet than people that were *GSTM1*-positive. This was shown in an American case control study reported by Tsai *et al*.; odds ratios for *GSTM1*-null and *GSTM1*-positive were 0.46 (95% CI 0.21-1.01) and 1.22 (95% CI 0.56-2.66), respectively (52). Furthermore, Bonner *et al.* showed that green tea consumption seems to be protective against lung cancer in *GSTM1*-null subjects (OR 0.36, 95% CI 0.12-1.13), but not in *GSTM1*-positive subjects (OR 1.22, 95% CI 0.56-2.66) (53).

Overall, it can be concluded that the majority of the epidemiological studies indicate that high vegetable intake has a protective effect against lung cancer, especially in *GSTM1*-null individuals. However, not all of these studies report statistical significant results and the overall results are not consistent. This suggests that this conclusion should be considered with care and more epidemiological studies are needed for confirmation.

5. *GSTM1*-diet interactions on molecular damage as a biomarker of lung cancer risk; molecular epidemiological studies

Most traditional epidemiological studies measure mortality and morbidity (indicators) in relation to exposure to risk and protective factors. Several limiting factors are applicable to these approaches, like attenuation of underlying relationships by exposure misclassification (59). Therefore, molecular biological techniques have been implemented into epidemiological studies to improve better and earlier detection of toxic/carcinogenic exposures and subsequent risk. These molecular epidemiological approaches measure toxic substances, structures or processes in the body (called biomarkers) that influence or predict the incidence or outcome of harmful effects or disease (60). The use of biomarkers in the cancer epidemiology field was proposed in 1982 by Perera and Weinstein (61). Biomarkers are often classified in three categories: 1) markers of internal/effective dose, 2) markers of biologically early effect and 3) markers of susceptibility. Examples of internal/effective dose markers are urinary metabolites, DNA adducts, albumin adducts and haemoglobin adducts (62). Early biological effects can be measured with mutations or chromosomal aberrations/ translocations. Polymorphisms of metabolic genes and DNA repair are involved in disease susceptibility (63). Biomarkers for DNA damage are among the most extensively used intermediate endpoints, including DNA adducts, chromosomal aberrations, DNA oxidation and DNA strand breaks (64).

In molecular epidemiological studies, biomarkers are often used as a measure of carcinogen exposure and subsequent risk for lung cancer (65, 66). To assess the interaction of the *GSTM1* gene and diet on these biomarkers, we have searched in Pubmed and Scopus for studies with specific search terms; GSTM1 in combination with a dietary factor and a biomarker/damage parameter.

Table 2A. Molecular epidemiological studies reporting bulky lesions as DNA damage parameters and dietary constituents in relation to the *GSTM1* polymorphism.

Author, year	DNA damage parameter	Methodology	Dietary constituent	Investigated population	Effect of GSTM1 on diet-DNA damage interaction	Type of effect
Grinberg-Funes, *et al.*, 1994 (67)	PAH-DNA adducts in mononuclear cells	ELISA	Vitamin A,C,E, β-carotene	63 male smokers	Yes	Inverse association between vitamin E or C levels and DNA adducts, only in *GSTM1*-null subjects.
Mooney, *et al.*, 1997 (68)	PAH-DNA adducts in leukocytes	ELISA	Retinol, β- carotene, α-tocopherol	159 smokers	Yes	Inverse association between retinol or β-carotene levels and DNA adducts, significant in *GSTM1*-null subjects only.
Mooney, *et al.*, 2005 (69)	B(a)P-DNA adducts in leukocytes	HPLC	Vitamin C+E	309 healthy subjects	Yes	Inverse association between vitamin C+E intake and B(a)P-DNA adducts, only in (female) *GSTM1*-null subjects.
Wang, *et al.*, 1997 (70)	DNA adducts in lymphocytes	PPL	β-carotene, α-tocopherol	192 healthy male subjects	No	No significant association between β-carotene or α-tocopherol and DNA adducts in *GSTM1*-null and *GSTM1*-positive subjects.
Palli, *et al.*, 2003 (71)	DNA adducts in leukocytes	PPL	6 carotenoids, retinol, α-,γ-tocopherol	331 healthy subjects	Yes	Inverse association between levels of α- and β-carotene and retinol and DNA adducts, only in *GSTM1*-null subjects.
Palli, *et al.*, 2004 (72)	DNA adducts in leukocytes	PPL	Mediterranean diet; > 120 foods & drinks	634 healthy subjects	Yes	Inverse association between consumption/intake of leafy vegetables, white meat, vitamin C, vitamin E, β-carotene and DNA adducts, only in *GSTM1*-null subjects.
Chen, *et al.*, 2000 (73)	AFB_1-Albumin adducts in plasma	ELISA	Retinol, selenium, α-tocopherol, α-,β-carotene	304 healthy adults	Yes	Inverse association between selenium levels and AFB_1-Albumin adducts, significant in *GSTM1*-null subjects only.

Abbreviations: PAH, polycyclic aromatic hydrocarbon; B(a)P, benzo(a)pyrene; AFB1, aflatoxin B_1-albumin; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; PPL, ^{32}P-postlabelling.

Table 2B. Molecular epidemiological studies reporting oxidative stress related damage as DNA damage parameters and dietary constituents in relation to the *GSTM1* polymorphism.

Author, year	DNA damage parameter	Methodology	Dietary constituent	Investigated population	Effect of GSTM1 on diet-DNA damage interaction	Type of effect
Marotta, *et al.*, 2006 (74)	8-OHdG in leukocytes	HPLC-ECD	Papaya	54 elderly subjects	Yes	Inverse association between papaya intake and 8-OHdG concentrations, only in *GSTM1*-null subjects.
Hakim, *et al.*, 2004 (75)	8-OHdG concentration in urine	ELISA	Green and black tea	133 heavy smokers	Yes	Inverse association between green tea consumption and 8-OHdG levels, but only significant in *GSTM1*+ subjects.
Wilms, *et al.*, 2007 (76)	H_2O_2 induced strand breaks in lymphocytes	Comet assay	Ascorbic acid, quercetin	12 healthy subjects	No	Inverse association between quercetin and strand breaks in all subjects.
Riso, *et al.*, 2010 (77)	H_2O_2 induced strand breaks, oxidised DNA lesions in mononuclear cells	Comet assay	Broccoli	27 young healthy smokers	Yes/No	Inverse association between broccoli intake and H_2O_2 induced strand breaks in *GSTM1*-null subjects (significant difference with *GSTM1*+ subjects). Inverse association between broccoli intake and DNA lesions in all subjects.
Pool-Zobel, *et al.*, 1998 (78)	Oxidative DNA damage in lymphocytes	Comet assay	Carotenoid-containing vegetable juice	23 healthy male subjects	No	Inverse association between intake of carrot juice and oxidative DNA damage in all subjects.
Lee, *et al.*, 2010 (79)	Lipid peroxidation (conjugated dienes) in plasma	Comet assay	Licorice (antioxidant)	40 healthy male smokers	Yes	Inverse association between licorice intake and conjugated dienes, but only significant in *GSTM1*+ subjects.

Abbreviations: 8OHdG, 8-hydroxydeoxyguanosine; HPLC-ECD, high-performance liquid chromatography – electrochemical detection; ELISA, enzyme-linked immunosorbent assay.

Dietary factors were: diet, nutrition, micronutrients, antioxidant, chemo-preventive agents, vegetables and vitamins. The biomarkers/damage parameters were (DNA) adducts, DNA damage, chromosomal aberrations, sister chromatid exchange and lipid peroxidation. Here, the exclusion criteria were a) no original data available (review or meta-analysis), b) study not performed in humans, c) association between diet, GSTM1 and biomarker not studied and d) observational studies with calculated lung cancer risks (ORs). Thirteen scientific publications met these criteria. Of these thirteen, seven publications reported bulky lesions as biomarkers and were included in table 2A (67-73). Three measured PAH-DNA adducts by immuno-assay or HPLC (67-69), three aromatic DNA adducts by postlabelling (70-72), and one aflatoxin B_1-albumin adducts (73). Six out of thirteen studies measured oxidative stress related damage and were included in table 2B (74-79). Two of these used 8-OHdG concentration as a marker of oxidative DNA damage (74, 75). Furthermore, there were three reports with measurements of oxidised strand breaks/DNA damage (76-78) and one included lipid peroxidation (79).

Of the seven studies reporting bulky lesions, only one found no effect of *GSTM1* on the diet-DNA damage interaction. This Japanese study, by Wang *et al.,* measured DNA adducts in lymphocytes of healthy male subjects. There was no significant association between the DNA adduct levels and β-carotene or α-tocopherol plasma levels in *GSTM1*-null or *GSTM1*-positive subjects (70). All six other studies described protective effects of diet specifically in the *GSTM1*-null genotype against different types of DNA damages or other potential biomarkers of cancer risk. Grinberg-Funes reported that significant inverse associations between vitamin E or C levels and PAH-DNA adducts were limited to the null genotype of *GSTM1* (67). Mooney *et al.* measured PAH-DNA adducts as well, but in combination with β-carotene, α-tocopherol and retinol. Significant inverse association for β-carotene or retinol and DNA adducts were only found for subjects lacking the *GSTM1* gene (β=-0.3, p=0.05 and β=-1.089, p=0.04) (68). In 2005, Mooney *et al.* conducted a relatively large study, measuring B(a)P-DNA adduct levels as an intermediate cancer risk marker. This study described that supplementation of vitamin C and E resulted in a 43% decrease of adducts in female *GSTM1*-null subjects (95% CI: 0.35-0.94) compared to the non-significant decrease of 27% in female *GSTM1*-positive subjects (95% CI: 0.48-1.10) (69). A protective effect of vegetables in *GSTM1*-null subjects was also found by the two largest studies, including 331 and 634 subjects, performed by Palli *et al.* (71, 72). In 2003, Palli *et al.* reported the association between micronutrients and DNA adducts in leukocytes, taking *GST* polymorphisms into account (71, 72). An inverse association was found for α- and β-carotene in *GSTM1*-null subjects only (p for trend= 0.02 for both). Their other study (2004) focused on the modulation of the interaction between major food groups and DNA adducts by *GST* genes. Stratification by *GSTM1* resulted in significant inverse associations between DNA damage and increasing consumption of white meat (p=0.04), leafy vegetables (p=0.01) and intake of β-carotene (p=0.02), vitamin C (p=0.04) and vitamin E (p=0.05) (72). In contrast to the more abundant DNA adducts, Chen *et al.* measured aflatoxin B_1-albumin adducts (73). Their report described a predominant protective effect of dietary habits in the group of subjects carrying the *GSTM1*-null genotype. Inverse associations between plasma selenium and DNA adducts were found in both *GSTM1*-null and *GSTM1*-positive subjects, but this association was only significant in those without a functional *GSTM1* gene (β=-0.56, p=0.02; *GSTM1*+ β=-0.28, p=0.25).

There is less consistency between the studies reporting oxidative stress related damage. Three studies found no effect of *GSTM1* on the diet-DNA damage interaction. Pool-Zobel *et al.* measured oxidative DNA damage as a biomarker, in healthy male subjects. They found a reduction in DNA

damage after vegetable consumption, but this effect was equal for both *GSTM1*-null and *GSTM1*-positive subjects (78). A Dutch study, by Wilms *et al.,* described that the *GSTM1* genotype had no influence on the protective ability of quercetin and ascorbic acid supplementation against *ex vivo* H_2O_2-induced strand breaks (76). Quercetin gave a non significant but protective effect in all subjects, for ascorbic acid there was no protective effect observed. In addition, Riso *et al.* investigated the role of *GSTM1* polymorphisms on protection by broccoli against oxidatively damaged DNA lesions (77). They found an inverse association between broccoli intake and oxidised DNA lesions in all subjects. On the other hand, they reported that the *GSTM1* genotype did affect the protective ability of broccoli on H_2O_2-induced strand breaks. In subjects with the *GSTM1*-null genotype the strand breaks were reduced with 27.6% (95% CI -37.9, -17.4), whereas the broccoli-rich diet caused no significant protection in *GSTM1*-positive subjects (-13.1%, 95% CI: -27.3, 1.1). One other study reported a protective effect against lung cancer of the *GSTM1*-null genotype in combination with vegetable intake. Marotta *et al.* assessed 8-OHdG concentrations of elderly patients on a papaya-supplemented diet. Here, an inverse association was reported between papaya intake and 8-OHdG concentrations in *GSTM1*-null subjects only (74).

On the other hand, two studies reported that the *GSTM1*-positive genotype induced the protective effect by dietary factors against carcinogens. This was shown in two studies conducted in smokers. Hakim *et al.* tested the protective effect of green tea and measured urinary 8-OHdG levels. This resulted in an inverse association between green tea consumption and 8-OHdG levels which was only significant in *GSTM1*-positive subjects (75). In addition, Lee *et al*. studied licorice (antioxidant) supplementation and found a decrease of conjugated dienes, a measure of lipid peroxidation, in the *GSTM1*-positive group only (79).

Overall, there appears to be a distinction in results from molecular epidemiological studies reporting bulky lesions or oxidative parameters as biomarkers. Table 2A shows that six out of seven publications report an inverse association between high vegetable/antioxidant intake and PAH induced bulky lesions (adducts), but only in *GSTM1*-null subjects. This indicates a probable induced protective effect against lung cancer by *GSTM1*-null in combination with high vegetable intake. In contrast, results from table 2B are quite diverse and therefore hard to interpret. No clear effect was found of a *GSTM1*-diet interaction on lung cancer, by measuring oxidative stress related damage.

6. Discussion

The high prevalence of the *GSTM1*-null genotype and the indication that a homozygous deletion of this gene is beneficial in relation to vegetable intake and lung cancer risk marks the importance for human health. The interaction between diet and (lung) cancer has been intensively investigated over several decades. Since Seidegard *et al.* (80) stated in 1986 that a genetic polymorphism of a metabolic enzyme might be influencing lung cancer susceptibility, the interest aroused for a potential interaction between these enzymes and dietary intake on lung cancer risk. From the 1990s on, several studies reported interactions between *GSTM1* and dietary factors on lung cancer (28, 29, 81), but no review evaluated both epidemiological and molecular epidemiological results yet.

An overall evaluation of all studies results in a probable protection against lung cancer by vegetables/antioxidants in individuals carrying the *GSTM1*-null genotype. Nine out of thirteen observational epidemiological studies gave lower odds ratios for *GSTM1*-null subjects with high intake of (constituents of) vegetables. In addition, eight out of thirteen molecular epidemiological

studies showed inverse associations between vegetable constituents/antioxidants and biomarkers for lung cancer in *GSTM1*-null subjects only. This is in agreement with the hypothesis that *GSTM1*-null subjects are more protected against lung cancer because they cannot metabolize ITCs and therefore benefit longer of the chemo-preventive abilities of vegetables.

In observational epidemiological studies, vegetable or ITC intake is often measured with a dietary questionnaire. All observational studies included in table 1 used dietary questionnaires, either with or without a personal interviewer. However, the use of questionnaires for diet intake is considered a weakness for the study because of the possible measurement errors (28). In addition, for the retrospective case control studies there is a higher chance for dietary recall bias than for the prospective cohort studies which collect data on dietary intake before the subjects are diagnosed with cancer (30). Nevertheless, no distinction in results is seen between the case-control and nested case-control studies in table 1. Furthermore, dietary intake is measured in various ways for different studies and is often not comparable. Several publications use subjective measures as high and low intake or appoint a median of intake. Therefore, it might be hard to draw an overall conclusion from such different food intake measures. And, it is often not known, or taken into account, if the food is processed or not. For cruciferous vegetables it is known that cooking affects the bioavailability by inactivation of the myrosinase enzyme (30). Therefore, eating raw or cooked vegetables is influencing the biological potency and thereby also the degree of protection against cancer.

In all studies *GSTM1*-null genotypes were compared with *GSTM1*-positive genotypes. For *GSTM1*-null it is clear that all subjects with a homozygous deletion of this *GST*-gene are included in this group. For *GSTM1*-positive this is not so clear; this classification might include heterozygote and homozygote individuals ($GSTM1^{+}/_{-}$ or $GSTM1^{+}/_{+}$). In most studies, no distinction was made between these subgroups while it might be interesting to evaluate the risks for heterozygote and homozygote *GSTM1*+ subjects separately. Furthermore, the potential role for confounding by cigarette smoking should be evaluated in each study since smoking is the main cause of lung cancer and the diet of smokers is generally less healthy than for non-smokers (82). Of the eight epidemiological studies which investigated their total population (smokers and non-smokers), seven adjusted for different smoking characteristics. But although they performed statistical adjustments on smoking, there still exists the possibility of residual confounding by biases in assessment of smoking exposure (83). Besides the studies that included all subjects, three studies focused only on smokers (45, 46, 52) and two on non-smokers (49, 56). Making an overall risk evaluation might be influenced by these different study set-ups, especially when two out of three studies including smokers appear to have contradictory results compared to the majority of studies which show a protective effect of vegetables in *GSTM1*-null subjects. In addition, the study of Fowke *et al.* includes a non-smoking population and is the only epidemiological study reporting no effect of vegetables. The absence of smoking reduces the exposure to carcinogens tremendously and therefore as well the possibility of vegetables to protect against these carcinogens in both *GSTM1*+ and *GSTM1*- subjects. This might be an explanation for the absence of a modulatory effect on lung cancer risk by vegetable intake and *GSTM1* genotype.

Although characteristics like increased sensitivity are favouring the use of biomarkers in molecular epidemiological studies, there are some limitations as well. The type of biomarker used is important in terms of reliability as a detection of early effects or cancer risk. Some biomarkers are not really relevant in carcinogenesis since they represent tolerable molecular variation or reversible biological

changes (lipid peroxidation, sister chromatid exchange) (84). Other biomarkers are more relevant because of measurements of irreversible molecular alterations that eventually may lead to cancer. Several known biomarkers are good predictors of exposure, but not necessarily for health risk as well. PAH-DNA adducts were reported to be increased in smokers, and higher in lung cancer cases compared to controls, suggesting to be predictive of both exposure and risk (65). Chromosomal aberrations are considered as a good marker for both the early effect and cancer risk. For most toxic substances it is known that exposure leads to induction of chromosomal aberrations (84). In addition, a meta-analysis of Norppa *et al.* resulted in an increased cancer risk at high levels of chromosomal alterations, suggesting that chromosomal alterations were predictors of cancer (85). Despite the promising results of biomarker studies using chromosomal aberrations, none of the thirteen molecular epidemiological studies in tables 2a and 2b measured this predictor. Future studies on *GSTM1*-null and diet in relation to lung cancer should perhaps consider chromosomal aberrations more often as a biomarker of lung cancer risk.

Practical and ethical issues are limiting the use of carcinogen target organs since they are often not easily accessible by routine sampling (86). Therefore, biomarker studies often use easily obtainable surrogate tissues or cells, like leukocytes. However, these surrogates are not always the best option in terms of reliability, because they might not be actual targets of carcinogens or they are less sensitive (59). The different types of surrogate tissues or cells used also differ from each other by specificity. Mononuclear blood cells, a subgroup of the leukocytes, are reported to hold the highest levels of DNA adducts, suggesting that these might be more sensitive cell types and perhaps more reliable biomarkers than using the overall group of leukocytes (86). Furthermore, subsets of leukocytes might have variable life-spans, which makes an overall estimate of total damage uncertain (86). Immune irregularities easily affect the number and lifespan of the subtypes. Therefore, it might be necessary to separate different leukocyte types to obtain a more reliable estimate of DNA damage. On the other hand, it is possible that limiting to only one type of leukocyte might narrow the view too much. There were three molecular epidemiological studies which found no associations at all between the *GSTM1* genotype, diet and the biomarker of exposure (70, 76, 78). These studies used different biomarkers but all measured biomarkers in lymphocytes only. It is possible that the type of cell used explains the lack of associations in these studies.

In addition, an obvious difference in study size is present between the epidemiological and molecular epidemiological studies. In the epidemiological reports only one of all thirteen studies has a sample size smaller than 100 (51), while in molecular epidemiology this is six out of thirteen; four of these contain even less than 50 subjects (76-79). These four extreme small studies are all included in table 2b, which shows a clear distinction in study size of the bulky lesions and oxidative stress related studies as well. These small numbers in total study populations leads to even smaller subpopulations of subjects with characteristics of interest. A possible explanation is the laborious work of processing all the blood and urine samples for analysis and the limitation that is caused by simultaneous evaluation of all samples (87). But the fact remains that small sample sizes lower the statistical power of the study, and spurious effects may be seen. It is therefore notable to see that of the four small molecular epidemiological studies reporting oxidised DNA damage (sample size<50), three could not find a probable association between the *GSTM1* genotype, diet and oxidative DNA damage (76-78).

Furthermore, a striking difference in results is seen between the molecular epidemiological studies reporting oxidative stress related damage as a biomarker, and the studies reporting bulky lesions. All

but one study with (DNA) adducts report a protective effect against lung cancer by high vegetable (constituent) intake in *GSTM1*-null in subjects. On the contrary, the results of the studies measuring oxidative parameters are far less consistent. This might be explained by the different mechanisms that underlie the formation of DNA adducts and oxidative DNA damage. Figure 1 shows the suggested mechanisms of how cigarette smoke can cause lung cancer. Lung cancer can be caused by bulky DNA lesions (carcinogen-DNA binding), chronic oxidative stress (caused by ROS), or both. ROS can be formed by redox cycling with carcinogen metabolites. Next to enzymatic processes, including metabolism of xenobiotics, other ways of ROS formation are known. Inflammatory responses may induce ROS as well (88). In addition, transition metals (e.g. iron) can catalyze the transition of hydrogen peroxide to hydroxyl radicals in the Fenton reaction (89). Since air particles like particulate matter (PM) can cause inflammation-induced ROS, ROS are present in cigarette smoke and hydrogen peroxide is present in ambient air, oxidative damage can occur without the interference of the GST pathway (88, 90). Subjects lacking the *GSTM1* gene can overcome the loss of xenobiotic elimination by the chemo-protective abilities of vegetables. Vegetables can therefore protect against oxidative damage that arose from xenobiotic exposure. But since oxidative stress can be caused without involvement of GSTs, vegetable intake has no effect on this part of the ROS-induced oxidative damage in *GSTM1*-null subjects. The multiple possibilities of oxidative damage induction might be the explanation of the discrepancy in results. Furthermore, this shows that the *GSTM1* gene is far more involved in the protection against xenobiotics than against oxidative stress.

The role of isothiocyanates, antioxidants and GSTM1 in the mechanistic pathway leading to lung cancer

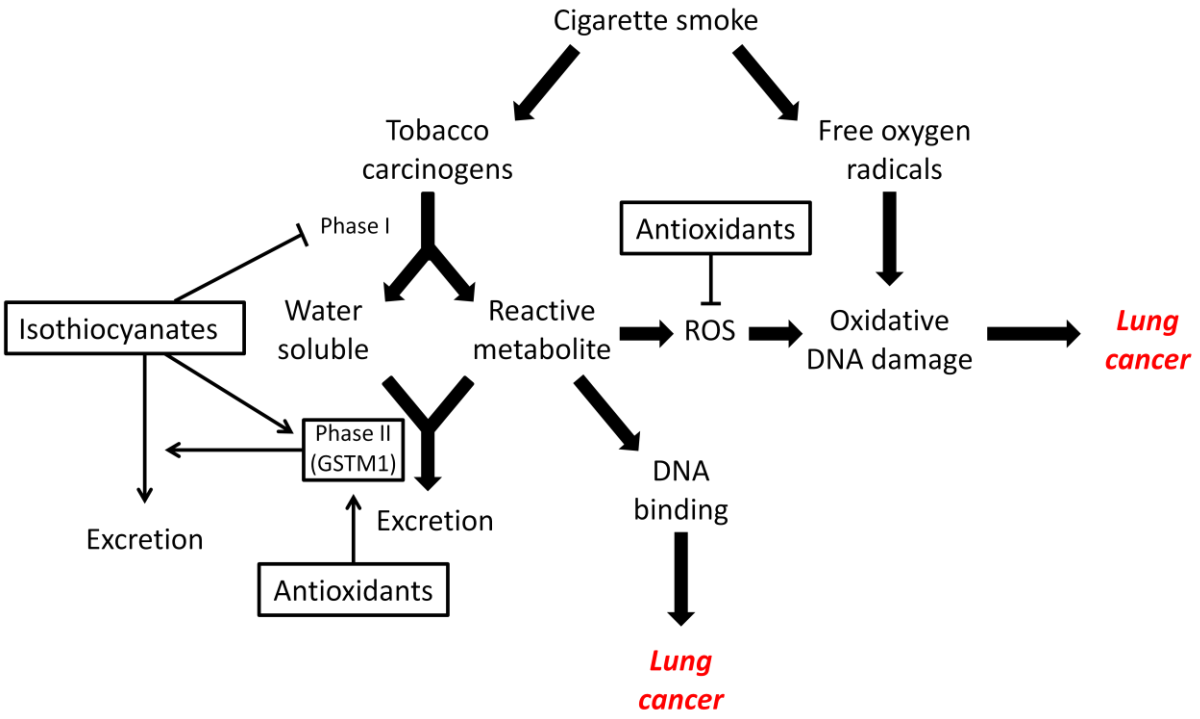

Figure 1. The possible mechanism of lung cancer development; caused by cigarette smoke. Lung cancer can be caused by reactive metabolites (that can bind to the DNA) and oxygen radicals (that can cause oxidative stress); either available in or caused by cigarette smoke. Antioxidants act as protection against oxidative damage by scavenging ROS, but also induce expression of genes encoding phase II enzymes, including GSTs. Isothiocyanates inhibit phase I enzymes and stimulate phase II enzymes, and thereby enhance carcinogen excretion. A homozygous deletion in the GSTM1 gene (GSTM1-null) prevents isothiocyanate excretion and might therefore be advantages in combination with high vegetable intake.

As is seen in tables 1 and 2, there is also a clear distinction in the type of dietary constituents measured in the epidemiological and molecular epidemiological studies. Epidemiological studies tend to focus only on (cruciferous) vegetables or the known chemo-preventive constituent; ITCs. On the other hand, in molecular epidemiology studies measurements are done on (namely) antioxidant intake/levels. The comparison of the outcomes from these different study types might be hard because of the different mechanisms that underlie isothiocyanate and antioxidant protection (figure 1). Isothiocyanates prevent DNA binding of carcinogens by inhibition of phase I enzymes and induction of phase II enzymes; this prevents oxidative DNA damage as well (indirect). Antioxidants are reported to scavenge ROS and thereby prevent oxidative DNA damage. In addition, antioxidants induce phase II enzyme activity (including GSTs) by enhancing expression of genes encoding detoxifying and defence proteins. It would therefore be useful if future studies include both vegetable/isothiocyanate and antioxidant intake when investigating the interaction with *GSTM1* polymorphisms in relation to lung cancer risk. Next to *GSTM1*, it would also be useful to include other single nucleotide polymorphisms (SNPs). This might result in a more complete view of the role of *GST* genes and diet in modulation of lung cancer risk.

Despite the lack of consistency, the majority of both the epidemiological and molecular epidemiological studies show an association between the *GSTM1* gene and diet in relation to lung cancer. However, there is a clear discrepancy in results of molecular epidemiological studies reporting bulky lesions and oxidative parameters. This might be attributable to some of the limiting factors of the studies or the difference in underlying mechanisms. The overall results show a possible interaction between the *GSTM1*-null polymorphism and diet on lung cancer risk. We can conclude that the *GSTM1*-null genotype in combination with certain constituents of vegetables have a probable protective effect against lung cancer.

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