Genotoxicity and carcinogenicity of acrylamide: a critical review

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Summary. In 2002, public health concerns were raised by Swedish studies showing that relatively high levels of acrylamide were formed during the frying, roasting, or baking of a variety of foods, including potatoes, cereal products and coffee at temperatures above 120 °C. Acrylamide possesses a range of hazardous properties, the key effects being carcinogenicity, genotoxicity, neurotoxicity and reproductive toxicity. Acrylamide is clearly carcinogenic in studies in animals, in which it causes increased tumour incidence at a variety of sites. Although the mechanisms for tumour induction in experimental animals have not yet fully elucidated, the *in vivo* genotoxicity at gene and chromosome level in somatic and germ cells in rodents cannot be discounted from contributing to it. At this time, there is no information to indicate any significant difference between rodents and humans in sensitivity to cancer formation from acrylamide. The present available epidemiological studies of human industrial and accidental exposures have to be considered not suitable for use in the cancer risk assessment of acrylamide in food, due to several limitations. In reviewing the genotoxicity and carcinogenicity of acrylamide, the author has taken into account also the evaluations made by the IARC in 1994, the FAO/WHO in 2002 by the European Commission Scientific Committee on Food (SCF) in 2002 and by the Joint FAO/WHO Expert Committee on Food Additive (JECFA) in 2005.

Key words: acrylamide, glycidamide, carcinogenicity, genotoxicity, risk assessment.

Riassunto *(Genotossicità e cancerogenicità dell'acrilamide: una rassegna critica)*. Nel 2002 le autorità nazionali ed internazionali furono allertate dalla scoperta, da parte di ricercatori svedesi, che livelli relativamente elevati di acrilamide si formavano, a temperature sopra i 120 °C, in prodotti di largo consumo, compresi patate, cereali e caffè. L'acrilamide possiede diverse proprietà tossiche, le principali delle quali sono la cancerogenicità, la genotossicità, la neurotossicità e la tossicità riproduttiva. L'acrilamide è una sostanza chiaramente cancerogena in animali da esperimento, topi e ratti, con formazione di tumori in diversi siti. Anche se i meccanismi di azione coinvolti nella formazione di tumori non sono stati ancora completamente chiariti, l'attività genotossica dell'acrilamide a livello genico e cromosomico *in vitro* e *in vivo,* quest'ultima in cellule somatiche e germinali in roditori, non permette di escludere un meccanismo di tipo genotossico nella induzione di tumori da parte dell'acrilamide. Gli studi epidemiologici disponibili, anche se negativi, non possono essere considerati adatti per la valutazione del rischio cancerogeno associato all'esposizione all'acrilamide attraverso la dieta, a causa di forti limitazioni. In questa rassegna dei dati di genotossicità e cancerogenicità dell'acrilamide, l'autore ha tenuto conto, tra l'altro, delle valutazioni fatte dalla IARC nel 1994, dalla FAO/WHO nel 2002, dal Comitato Scientifico dell'Alimentazione Umana (SCF) della Commissione Europea nel 2002 e dal Joint FAO/WHO Expert Committee on Food Additives (JECFA) nel 2005.

Parole chiave: acrilamide, glicidamide, carcinogenicità, genotossicità, valutazione del rischio.

INTRODUCTION

Acrylamide has been produced since the 1950 by hydration of acrylonitrile. It is used mainly to produce water-soluble polyacrylamides used as flocculents for clarifying drinking-water, for treating municipal and industrial waste waters and as flow control agents in oil-well operations. Other major uses are in soil stabilization, in grout for repairing sewers and manhole and in acrylamide gels used in biotechnology laboratories. The major routes of exposure at the workplace appear to be dermal absorption of acrylamide monomer from solution and inhalation of dry monomer or aerosols of acrylamide solution. Exposure occurs during acrylamide and polyacrylamide manufacture, during acrylamide grouting and during laboratory preparation of polyacrylamide gels. Other uses are as cosmetic additives (*e.g.*: creams, body lotions, shampoo) and direct and indirect food additives such as in paper and paperboard food packaging and coating. Acrylamide is also a component of tobacco smoke, which indicates that it can be formed by heating of biological material [1]. Acrylamide is readily taken up by all routes:

- inhalation, *e.g.*: manufacture, sewer line maintenance, cigarette smoking;

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- ingestion, *e.g.*: drinking water from flocculent-treated water, eating sugar (polyacrylamides used in refining process), eating certain foods cooked/processed at high T° (fried potatoes, cereals, etc.) (recent findings);
- skin absorption, *e.g.*: grout workers for sewer repair manufacture; cosmetics; polyacrylamide gels for electrophoresis in research laboratories.

For the general population, non-food exposures to acrylamide are to residual monomer in polyacrylamides. For the general (non-smoking) population exposure to low levels of residual acrylamide may occur through drinking water contaminated from the use of polyacrylamide flocculents in water treatment and from the use of polyacrylamides in cosmetics and toiletries. In April 2002 the Swedish National Food Administration announced that, on the basis of the findings of researches from Stockholm University guided by Margareta Törnqvist, acrylamide is formed in large consumer food products (*e.g.*, potato chips, french fries, processed cereals) when prepared/cooked at above 120 degrees Celsius. Short after, the Swedish findings were confirmed in several other European countries as well as in the USA. The levels found at that time were much higher than the levels recommended by WHO for drinking water (0.5 µg/L) corresponding to 1 µg/day for a person who assumes 2 l per day [2].

In light of the concern expressed by various European Union (EU) member states and of the public alarm, a consultation was convened by FAO/WHO in 2002 [3]. The consultation recognized the presence of acrylamide in food "as a major concern in humans based on the ability to induce cancer and heritable mutations in laboratory animals". Based on the data available at that time, food was estimated to make a significant contribution to total exposure of the general population to acrylamide. Average intakes were estimated to be in the range of 0.3 to 0.8 µg acrylamide/kg bw/day for developed countries, corresponding to about 21-26 µg/day for a 70 kg person. Within a population it was anticipated that children would generally have exposures 2-3 times higher and therefore they were at higher risk than adults.

A similar conclusion was reached by the European Commission (EC) Scientific Committee on Food (SCF) in 2002 [4].

A comprehensive review of human exposure and internal dose assessments of acrylamide in food is that published in 2005 by Dybing *et al.* [5].

In 2005 [6] the FAO/WHO Joint Expert Committee on Food Additives (JECFA) met in Rome from 9 to 17 February to evaluate the health risks of several food contaminants, including acrylamide. A summary report is available at www.who.int/ipcs/jecfa/summaries/en and www.fao.org/es/ESN/jecfa/whatisnew_en.stm.

To estimate the risk posed to humans, JECFA used a risk assessment approach known as the margin of exposure (MOE). The value of the MOE indicates the level of concern to assist risk managers in setting priorities for implementing measures to protect public health. The MOE is calculated by dividing the toxicity estimate from animal experiments by the estimated intake from food. Consequently, the lower the MOE the greater is the public health concern. JECFA considered the available human studies not suitable for use in the risk assessment of acrylamide in food. For the MOE calculation the most sensitive carcinogenicity estimate of 0.30 mg/kg body weight per day from the animal studies was selected. For the intake estimates, intake values of 0.001 and 0.004 mg acrylamide/kg body weight per day were selected to represent intakes by the general population and high consumers, respectively. The MOE were thus calculated to be 300 for the general population and 75 for high consumers (large amounts of foods that contain acrylamide). JECFA considered these margins to be low for a substance that causes cancer in animals. JECFA concluded that these MOE for acrylamide in food may indicate human health concern.

JECFA also noted that there is still considerably uncertainty in determining the precise risk level for human health, due to insufficient knowledge of the mechanisms of action and the limited data used for intake assessment.

REGULATIONS

In the EU acrylamide is listed in Annex 1 of Directive 67/548/EEC (Dangerous Substances Directive) [7] and is classified acrylamide as follows:

- Carcinogenesis: Category 2, with the risk phrase "May cause cancer".
- Mutagenesis: Category 2, with the risk phrase "May cause heritable genetic damage".
- Reproductive toxicity: Category 3, with the risk phrase "Possible risk of impaired fertility".

The European Commission (EC) Directive 88/379/EEC [8] states that any preparation containing greater than 0.1% w/w acrylamide would require classification and labeling as a Category 2 carcinogen. All polyacrylamides in the EU contain less than 0.1% w/w free acrylamide monomer. Certain countries (*e.g.*, UK, Netherlands) require the registration of polyacrylamide products for drinking water treatment, typical free acrylamide being less than 0.025% (w/w of polymer).

Polyacrylamides with no more than 0.1% free acrylamide in general have approval as direct and indirect food additives such as in paper and paperboard and coating under Germany, Netherlands and US-FDA regulations.

In 1996 WHO [2] considered acrylamide as genotoxic carcinogen and carried out a risk assessment using a non-threshold approach. On the basis of combined mammary, thyroid and uterine tumors in female rats in a drinking-water bio-assay [9] and using the linearized multistage model, guideline values associated with excess lifetime cancer risks of 10^{-4}, 10^{-5}, and 10^{-6} were estimated to be 5, 0.5 and 0.05 µg/l, respectively.

The Occupational Safety and Health Administration (OSHA) final rule permissible exposure limit (PEL) is 0.03 mg/m^3 for an 8 hour time-weighted average (TWA).

The US-EPA classifies acrylamide as "reasonably anticipated to be a human carcinogen" based on sufficient evidence in experimental animals and inadequate data in humans (from the Ninth Report on Carcinogens).

The overall evaluation made by the International Agency for Research on Cancer (IARC) in 1994 [1] was: "acrylamide is probably carcinogenic to humans" (Group 2A), based on inadequate evidence in humans and sufficient evidence in experimental animals.

At the European Commission level an important human health risk reduction strategy for acrylamide was adopted in 2003 [10]. The risk reduction strategy is based on a comprehensive Risk Assessment Report (RAR) (EC, 2000) [11] available on the Internet site of the European Chemical Bureau: http://ecb.jrc.it/existing-substances/ . Important information regarding the evaluation of the human health risks associated with acrylamide exposure for workers and consumers can be found in two other documents [12, 13].

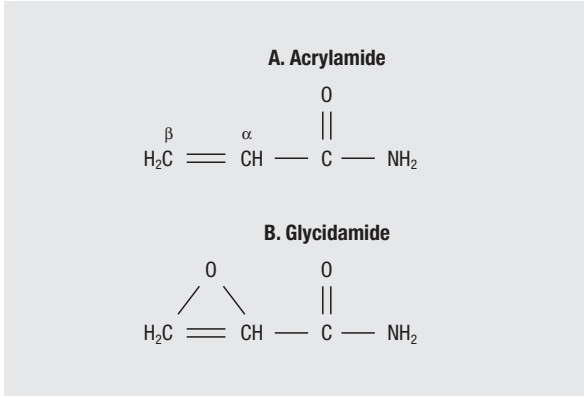

Fig. 1 | *Structure of acrylamide and glycidamide.*

METABOLISM AND REACTIVITY

Metabolism

Acrylamide is rapidly absorbed by mucosae and skin and, if inhaled, by the lungs and widely distributed in the body in all animal species so far investigated (rats, mice, dogs, minipigs). Acrylamide passes in the blood and for its hydrosolubility is diffused evenly in all the body. In mice it has been shown accumulation of acrylamide or its metabolites in the reproductive organ of males (testis) and rapid distribution to the developing fetus in pregnant females. Acrylamide was also present in the milk of treated lactating rats. Acrylamide is largely oxidized in mice, rats and humans to glycidamide. The more plausible candidate for its oxidation is the P450 cytochrome isoform 2E1, which oxidizes alcohols and is induced by ethanol [14]. Evidence was supported by comparison between wild type and CYP2E1 null mice strains for urinary metabolites of acrylamide. Whereas in the wild type about 50% of the administered dose of acrylamide was metabolized to glycidamide, in the CYP2E1 null strains no glycidamide was detected. Evidence for glycidamide formation in humans was shown in samples of haemoglobin taken from workers exposed to high levels of acrylamide [15]. Both acrylamide and glycidamide are equally distributed among the tissues and have half-lives of about 5 h in rats. The conversion of acrylamide to glycidamide is saturable, ranging from 50% of very low doses to 13% at 100 mg/kg bw in rats. Both compounds are detoxified by glutathione conjugation, and glycidamide is also detoxified by hydrolysis. The oral administration of 50 mg/kg bw to rodents, showed that in the rat the amount of the urinary glutathione derivative was 1.5-fold higher that in mice. Excretion of acrylamide and metabolites is rapid and occurs mostly via the urine where N-acetyl-S(2-carbamoylethyl)cysteine is found from the degradation of the conjugated glutathione.

Presently, one can say that in humans, at relatively low doses, glycidamide is formed at higher extent than in rats, very likely because of the higher levels of CYP2E1.

Reactivity

As shown in *Figure 1*, acrylamide is a fairly simple compound. It has an alpha,beta unsaturated double bond and this imparts much of its activity. A first important consequence is its metabolic conversion to glycidamide, an epoxide metabolite very reactive with electrophile (DNA). There is a second important reaction; it is called a Michael-type reaction in which the beta-carbon reacts with a nucleophile (*e.g.*, proteins). This is a kind of direct-acting alkylating reaction. These two reactions are important because they explain the primary targets of acrylamide (proteins) and of glycidamide (DNA).

Acrylamide reacts rapidly with SH groups. Extensive reaction occurs with proteins too, both on their SH groups and with amino groups, specially with amino terminals; the latter, having a low pk, are partly undissociated *in vivo*. In contrast, the *in vitro* reaction with DNA is modest and slow, and does not occur *in vivo*.

Acrylamide is highly water soluble, but is equally well soluble in some organic solvents like methanol, ethanol and acetone.

Adducts with haemoglobin

The most important reaction of acrylamide with proteins is the adduction to haemoglobin (Hb). Adducts are formed at the SH groups and on the amino groups of the N-terminal valines.

The reaction with Hb is extensive and only in the erythrocytes the concentration of Hb-A is higher that those determined by the even body distribution of acrylamide.

The acrylamide adducted on Hb is about the 12% of the administered dose. While Hb-adduct formation was a linear function of dose when the epoxide metabolite glycidamide was administered to experimental animals, Hb-adduct formation by either acrylamide or glycidamide was strongly dose-dependent in treated rats. At a low concentration, the ratio of the dose received of glycidamide to that of acrylamide was estimated to be 0.58 [16].

The formation of glycidamide and acrylamide adducts with Hb is directly proportional in man and rat. Comparison of free acrylamide in plasma, valine adducts on Hb and urinary S-(2-carboxyethyl)cysteine indicate that the rate of elimination of acrylamide is at least five times lower in man than in rats. Therefore, since the integrated concentration-time ratio for gly-

cidamide to acrylamide adducts in man (0.3) is about one-half of that of rats (0.58) at low doses, the tissue dose of glycidamide may be higher in man than in rats, on the basis of an equal uptake of acrylamide. Hb-adducts are used as a measure of human exposure to electrophilic compounds over the previous 4 months (the lifespan of erythrocytes), but are not an indicator of toxicity. Adducts formation at the N-terminal valine of Hb has been used as a marker of *in vivo* exposure to acrylamide, using an analytical procedure employing a modified Edman degradation [15]. A similar approach has been used for measurement of Hb-adducts to glycidamide. The detection of this adduct both in rodents and humans confirms the formation of glycidamide *in vivo* in humans. Measurement of Hb-adducts can give an integrated measure of the exposure of the last 3-4 months.

Adducts with cytoskeletal proteins

Binding of ^{14}C-acrylamide to neurofilament and microtubular proteins has been studied in rats [17]. Binding of microtubule-associated proteins 1 and 2 was about ten times greater that to tubulin. Other proteins that bound acrylamide significantly were that to tubulin, with possible spindle perturbance and chromosome malsegregation (*e.g.*, aneuploidy). Other proteins that bound acrylamide significantly were heavy- and medium-weight neurofilaments.

Adducts with protamines

In mice treated *i.p.* injection with ^{14}C-acrylamide the radioactivity associated with purified protamine closely paralleled the total radioactivity associated with sperm. Very little radioactivity (< 0.5%) was associated with sperm DNA. The period of maximal protamine alkylation corresponded to the period of maximal induction of dominant lethal mutations (about 8 days after dosing) [18].

DNA adduct formation

Adduct formation of acrylamide with DNA also occurs, although the reaction is very slow. Among the products *in vitro* are formamidoethyl or carboxyethyl adducts with exocyclic amino groups or ring nitrogens in DNA bases. The mutagenic significance and repair capabilities of these adducts are unknown.

The only adduct so far detected in mice and rats has been an adduct of glycidamide with guanine: N-7-(2carbamoyl-2-hydroxyethyl)guanine [19]. The levels of DNA adducts in mice were higher than in rats, as expected, due to the higher metabolic conversion of acrylamide to glycidamide in mice compared with rats. The DNA adduct on guanine was found in different organs, showing that its formation occurs ubiquitously.

The half-life of the DNA adduct was not determined, but by analogy with other small electrophilic molecules, can be estimated in a few days. Much longer is the persistence of Hb-adducts, as proteins do not have repair systems as DNA and the half-life is determined only by the physiological turnover of Hb which is around 90 days. At present, data on DNA adduct formation after acrylamide exposure in humans is lacking. Glycidamide is expected, because of its structure and reaction with DNA, to be of more significance than acrylamide to the genotoxic effects of acrylamide *in vivo*.

GENOTOXICITY

The genotoxicity of acrylamide, as well as of its reactive metabolite epoxide glycidamide, has been studied extensively.

In vitro

As reported by IARC [1] and FAO/WHO [3], acrylamide did not induce gene mutations in *Salmonella typhimurium* (Ames test), very likely because of the scarce presence or lack in the S9 mix of the specific isozyme (most plausible the P450 cytochrome 2E1) capable of metabolizing small hydrophilic molecules like acrylamide. Conversely, glycidamide induces gene mutations in *S. typhimurium* strains TA15 35 and TA100 with and without metabolic activation (S9 mix). Acrylamide showed equivocal, negative or weakly positive results in mammalian gene mutations assays, while it induced chromosomal aberrations, micronuclei (a mixed breakage(prevalent)-aneuploidy mechanism was shown), sister chromatid exchanges (SCE), polyploidy, aneuploidy and other mitotic disturbances (*e.g.* c-mitosis) in the absence of metabolic activation. Acrylamide, at micromolar concentrations, induced gene mutations in transgenic big blue mouse embryonic fibroblast cells, which might potentially be ascribed to its DNA-adduct-inducing property [20]. The mutational spectrum revealed an excess of G → C transversions and A → G transitions in the treated cells, compared with controls. Moreover, compared with the powerful mutagen and carcinogen benzo[a]-pyrene diol epoxide (BPDE), acrylamide was indeed a weak yet distinguishable mutagen in this test system.

Acrylamide did not induce unscheduled DNA synthesis (UDS) in rat hepatocytes, while glycidamide induced UDS in human mammary cells and rat hepatocytes.

In vitro cell transformation is not a reliable indicator of genotoxicity. Nevertheless, acrylamide induced cell transformation in three different cell lines (BALB/3T3, NIH/3T3 and C3H/10T½) in the presence as well as in the absence of exogenous metabolic activation.

The overall *in vitro* pattern is that acrylamide is not mutagenic in bacterial cells, differently from glycidamide; it is weakly, direct-acting mutagenic in mammalian cells. Acrylamide is clearly a direct-acting clastogen in mammalian cells in which it induces also, at lower extent, aneuploidy, polyploidy and other mitotic disturbances. There are inconsistent findings in the UDS studies carried out with acrylamide, differently from glycidamide which was clearly positive for this end-point.

In vivo

Drosophila. A mixture of positive and negative results were reported in published and unpublished studies investigating somatic mutations as well as sex-linked

recessive mutations. The overall significance was considered to be equivocal [1].

Somatic cells in rodents. Induction of structural chromosome aberrations, micronuclei or polyploidy was observed in various studies in mice treated *in vivo* (dose around 100 mg/kg bw) [1, 3].

The results of a series of low doses in the flow cytometer-based micronucleus assay in mice have been recently reported [21]. In one experiment, the effects of 22 doses *i.p.* injection (2 mice per dose) ranging from 2.5 to 100 mg/kg bw were studied. In the second experiment 7 doses (5 mice per dose) ranging from 1 to 30 mg/kg bw were used. A clear increase in the frequency of micronucleated polychromatic erythrocytes in the peripheral blood of the treated animals was observed even at the lowest doses used. The dose-response relationship was linear with a tendency to have a steeper rise at the lowest doses. Furthermore, the low DNA content measured in the micronuclei indicated an absence of whole chromosomes, *i.e.* no aneugenic effect of acrylamide, thus suggesting a clastogenic (chromosome breaking) mechanism. Although the doses used in this study are still much higher than those for acrylamide-exposed humans, the dose-response determined, with an absence of a threshold and with a tendency for a relatively stronger effect at the lowest doses, indicates that exact risk estimations for human exposure to low doses of acrylamide is a difficult task.

In another publication [22] it has been shown that glycidamide, the presumed genotoxic metabolite of acrylamide, induces micronuclei in bone marrow cells of mice and rats treated by *i.p.* injection. The mice were treated with three doses (0.18, 0.35 and 0.70 mmol/kg bw). For the rats two different doses (0.7 and 1.4 mmol/kg bw) were used. In mice, a linear-quadratic dose-dependent curve was observed, while in rats no positive dose-response relationship was obtained, probably due to toxic effects to the bone marrow. The most important aspect of this study is that after treatment with synthetic glycidamide the frequency of micronuclei per unit of the *in vivo* dose of glycidamide in the mouse is very similar to that obtained in a previous study, where mice were treated with acrylamide and glycidamide as a metabolite. This finding strongly supports the view that glycidamide is the predominant genotoxic factor in acrylamide exposure.

A weak, positive result was reported in the mouse spot test after single or 3 daily *i.p.* injections of 0, 50 or 75 mg/kg bw [1, 3]. As known, the mouse spot test can detect not only gene mutations but also chromosomal aberrations, chromosomal loss and somatic recombination.

Acrylamide did not cause unscheduled DNA synthesis (UDS) in the liver of rats received single or repeated (5x) *i.p.* injections of 0, 30 or 100 mg/kd bw [1, 3].

In a transgenic mouse model (MutaMouse) acrylamide induced a small increase in the mutation frequencies (three-fold and six-fold). These results were considered equivocal due to the high variation in the control mutation frequencies [1, 3].

Overall, based on the results of *in vivo* mammalian somatic cell assays, acrylamide appears to be clearly genotoxic at relatively high doses, producing positive results particularly in the micronucleus assay. The pattern of results indicate clastogenicity or, at lower extent, interference with spindle apparatus (polyploidy, aneuploidy) rather that gene mutation activity. Glycidamide is clearly positive in the micronucleus assay in mice and, with lower potency, in rats, with a predominant chromosome-breaking mechanism instead of chromosome loss.

GERM CELLS

Cytogenetics

Groups of male mice received single *i.p.* injections of 0 or 75 mg/kg bw acrylamide and mated with untreated females 7 days later or received 125 mg/kg bw with mating 7 or 28 days later, or 5 daily injections of 50 mg/kg with mating 7 days after [23]. In addition, flow cytometry was performed for cells taken from testicular preparations at 3 and 35 days after treatment with up to 150 mg/kg bw acrylamide. A statistically significant, dose-related increase in the frequency of aberrations (chromosome fragments, dicentrics and translocations being prominent) was reported from one-cell zygotes following mating at 7 days and to a lesser extent at 28 days. For repeated exposure 85% of zygotes contained chromosome aberrations. From testicular cell populations a marked decrease (about 74% of control values) in tetraploid cells was noted 3 days after single exposure. At 35 days a statistically significant dose-related decrease in the percentage of elongated spermatids (70% of control values at 150 mg/kg) and a statistically significant increase in elongated (elongating diploid spermatids) were noted suggesting impaired chromosome segregation during mitosis.

Groups of male mice received approximately 60 mg/kg/day by dietary administration for 1, 2 or 3 weeks or a single *i.p.* injection of 0 or 100 mg/kg [24]. An increased incidence of spermatogonia with aneuploidy, chromosome breaks and sister chromatid exchanges was seen in both types of exposure. A marked increase in sex-chromosome and autosomal univalents, fragments and rearrangements was also reported.

In a limited study (only one sampling time) carried out in male mice received single *i.p.* injections of 0, 50, 100 or 125 mg/kg acrylamide non increases in the incidences of chromosome/chromatid aberrations or hyperploidy were observed in spermatogonia and spermatocytes 24 h after treatment [25].

As part of a dominant lethal assay described below [26] a cytogenetic analysis was carried out on rat spermatocytes taken from males exposed to 0, 1.5, 3 or 6 mg/kg/day acrylamide in drinking water for 80 days and after a 12-week recovery period. No increase in structural aberrations was observed, with only a slight increase in reciprocal translocations. Due to the lack of further details regarding the experimental protocol, no firm conclusions can be derived from this poorly described study.

Germ cell micronucleus assay

Induction of micronuclei in spermatids was studied in rats received single *i.p.* injections of 0, 50 or 100 mg/kg acrylamide [27]. A statistically significant increase in the number of micronuclei was noted at 50 mg/kg.

Induction of micronuclei in spermatocytes was studied in rats received 0, 50 or 100 mg/kg acrylamide by single *i.p.* injection. Exposure or 4 daily injections of 50 mg/kg [28]. Micronuclei formation was analyzed on days 1, 3, 15, 18, 19 and 20 after treatment. The highest increases (day 18-20) correspond to spermatids initially exposed in the pre-leptotene stage; this result is consistent with other similar studies. Statistically significant increases in the incidence of micronuclei were noted 18-20 days after injections of 50 mg/kg:

- similar increase in micronuclei induction was re ported in another publication;
- in spermatids (post-meiotic stages) of mice exposed to acrylamide [29]. In the same study, a statistically significant increase of sister chromatid exchanges (SCE) in differentiating spermatogonia was also reported.

DNA synthesis and repair

UDS was studied by autoradiography in mice received single *i.p.* injections of 0, 8, 16, 31, 63, or 125 mg/kg acrylamide [30]. In addition, groups of male mice received a single *i.p.* injection of 0 or 125 mg/kg acrylamide with tritiated thymidine injected into the testes 6 h later. Also, groups of 4 male mice received *i.p.* injections of 46 mg/kg 14C-acrylamide; DNA was extracted from liver and testes samples 1-24 h after treatment and analyses for radioactivity. In the first experiment, a clear increase of UDS was noted with the maximum response occurring 6 h after tritiated thymidine injection. For the second experiment non significant increases of UDS were observed during the first 10 days after exposure to acrylamide, but an increase of UDS was noted from days 12-27. In the third experiment, DNA alkylation was observed, with the maximum levels 4-6 h post-administration in the testes and 1-2 h post-administration in the liver. The DNA alkylation levels in the testes were about 10-fold lower that in the liver.

UDS induction was studied in an *in vivo* test in F344 rats received single or repeated (5x) *i.p.* injections of 0, 30, or 100 mg/kg acrylamide [31]. A statistically significant increase of UDS, comparable to the positive controls MMS and cyclophosphamide, was noted only after repeated administration of 30 mg/kg. Glycidamide induced UDS in mouse spermatids *in vivo* [30].

Dominant lethal assays

All dominant lethal studies performed in mice and rats showed an effect from acrylamide exposure [32-37]. Induction of dominant lethal mutations was observed after all routes of administration (*i.p.* injection, gavage, dermal and drinking water). The lowest effective dose was observed following drinking water exposure (9.2 mg/kg bw for 20 weeks). The other positive results in the dominant lethal assay were in the range of 25-125 mg/kg bw. Consistently, the developmental stages sensitive to dominant lethal effects (late spermatids to early spermatozoa) correspond to increased DNA breakage and parallel the pattern of sperm alkylation and protamine alkylation.

Heritable translocation assays

Three studies showed increased frequencies of heritable translocations in mice. In the earlier study [38], male mice were exposed for 5 days at 40 or 50 mg acrylamide/kg bw/day. Two other studies were carried out, one with a 5 day exposure at 50 mg acrylamide/kg bw/day [39] and one with single doses of 50 and 100 mg acrylamide/kg bw/day [40].

Later on [41] data relative to the effects of acrylamide after dermal exposure in mouse experiments were published. The aim of the study was to determine a correction factor for heritable translocations induced by *i.p.* injection *vs*. dermal exposure in order to derive a more reliable quantification of genetic risk for humans. Total translocation frequency after dermal exposure was 8.6% as compared to 21.9% after *i.p.* injection exposures to 5 x 50 mg/kg A per day. For the calculated ratio of *i.p.* injection *vs*. dermal of 1: 0.39 there are, according to the authors, two caveats: 1) the exposed germ cell stages by *i.p.* injection and dermal were overlapping and not identical; 2) the dermal exposed males may have been exposed also orally, to an unknown extent, since they were not prevented by collars to lick some of the solutions applied to their backs.

Specific locus assays

Two studies showed positive results in the specific locus mutation assay in mice, which allows detection of both small and large gene lesions. In one study [42] male mice received 5 repeated *i.p.* injection doses of 50 mg/kg bw/day. Increased frequencies of specific locus mutations were observed for males mated with females on days 8-14 and 15-21 after treatment suggestive of specific locus mutations in the late stages of spermatogenesis (spermatids and spermatozoa).

In a second study [32] male mice received *i.p.* injections of 0, 100, or 125 mg/kg aqueous acrylamide. A high frequency of specific locus mutations was noted for males mated with females 5-8 days and 9-12 days after injection (6-14 mutations per locus per 10^5 gametes for males receiving 100 and 125 mg/kg *vs*. 1.3 per 10^5 gametes in the controls); this would indicate that specific locus mutations occurred in spermatids and spermatozoa. Cytogenetic analysis showed that most of the specific locus mutations were multi-locus (large) lesions.

ASSESSMENT OF GENETIC RISK

An assessment of the heritable genetic risk presented by acrylamide was carried out by a reference group of specialists in germ cell mutagenesis comprising Kerry Dearfield, George Douglas, Udo Ehling, Martha Moore, Gary Sega and David Brusick, on be-

half of the EU and the US-EPA and published in 1995 [43]. Using data from the specific locus mutation and heritable translocation assays, the modified direct and doubling dose approaches were used to quantitate genetic risk in humans for three exposure scenarios (ingestion, inhalation and dermal). The doubling dose approach is considered preferable to the direct (modified direct) approach, also because it does not require a specific estimate of the number of human loci which mutate to dominant disease alleles as does the direct approach. The doubling dose method requires an estimate of the overall spontaneous frequency in humans to dominant disease alleles, and these data are more easily available. The estimate of spontaneous mutation rate (1.5×10^{-3}) from the UNSCEAR (1986) [44] was used. For the spontaneous chromosomal aberration rate, an estimate made by Sankanarayanan (1982) [45] was used. These mutation frequencies in humans were used by applying the doubling dose approach (ICPEMC, 1983a, b; 1984) [46-48]. With these approaches and their underlying assumptions concerning extrapolation factors (including germ cell stage specificity, DNA repair variability, locus specificity), number of human loci associated with dominant disease alleles, and spontaneous mutation rates, an assessment of heritable genetic risk was calculated for the three scenarios. The number of new genetic chromosomal diseases caused by an assumed average intake of 0.013 µg acrylamide/kg bw/day via drinking water were up to 3 offspring potentially affected per 108 offspring with the model based on a doubling dose. One can note that the average intake via drinking water is about 20-60 times lower than the average intake of acrylamide from fried-processed foodstuffs in the general population estimated by FAO-WHO (2002) [3].

The calculations made by Dearfield *et al.* [43] for genic diseases give numbers about 500 times lower. Estimates for inhalation or dermal exposures suggest much higher risks for induced genetic disease in offspring from fathers occupationally exposed. For instance, a much greater risk (1.4×10^{-4}) of chromosomal disease is suggested for affected offspring from fathers dermally exposed at the upper range of acrylamide concentrations during grouting work (0.13 mg/kg bw/day). The late calculation only applies to the chromosomal damage occurring late in spermatogenesis, *i.e.* only during the lifetime of the exposed sperm, which means under the period of exposure or shortly after.

The calculations of genetic risk are based on a number of more or less uncertain estimations when extrapolating from mice to man. Another limitation is that only male mice were treated. The uncertainty in the figures presented is therefore considerable. However, one has to note that these calculations were made by the experts of germ cell mutagenesis and these experts concluded that the doubling dose method used is the most reliable.

In conclusion, acrylamide is a clear germ cell mutagen in experimental animals, with the potential to induce heritable genetic damage at gene and chromosomal level.

POSSIBLE MECHANISMS FOR ACRYLAMIDE GENOTOXIC EFFECTS

It has become increasingly clear that acrylamide has different reactivities that can contribute to its mechanisms and effects. The complex pattern of genotoxicity results indicate that not only acrylamide has activity via Michael-type reactions, but its metabolic product, the epoxide glycidamide, also has biological activity via direct nucleophilic substitution. *In vivo* conversion of acrylamide to glycidamide has been shown in rodents and humans. Both acrylamide and glycidamide appear to freely distribute systematically in the body, having also access to the germ cell components. While both compounds react with proteins, form Hb-adducts, they differ markedly in their reactivity with DNA. Acrylamide has high affinity to proteins producing adducts (*e.g.* with hemoglobin and protamines) and rather weak capacity to bind DNA. It is unable to induce gene mutations in bacteria; there is a need of long time to detect DNA binding after exposure; it induced genetic effects preferentially at chromosomal level, with the induction of structural chromosome aberrations and, at lower extent, numerical chromosome changes, including aneuploidy. The similar sensitive germ cell cycle stages as seen for dominant lethal mutations and heritable translocations suggest protamine binding. Acrylamide has effects on synaptoneural complex and on the spindle. Recent findings suggest that the induction of micronuclei *in vivo* by acrylamide exposure are essentially due to glycidamide by a chromosome-breaking mechanism and not by chromosome loss. Conversely, glycidamide has strong binding to DNA and relatively weak (compared to acrylamide) binding to proteins; predominant DNA-adduct *in vivo* was glycidamide-DNA adduct; major adduct, as expected for an epoxide, was at N-7 Guanine. DNA-adduct level *in vivo* was similar in several tissues; glycidamide induces directly gene mutations in bacteria; there is no delay in UDS induction after exposure; it induces micronuclei *in vivo*. Glycidamide-adducts may be more responsible for the gene mutations induction also in spermatogonia, while the acrylamide protein adducts as the protamine binding may explain some of the chromosomal effects seen in the germ cells. Therefore, exposure to acrylamide can have several results dependent upon the relative amounts of parent (acrylamide) *vs*. its epoxide metabolite (glycidamide) and their respective activities.

The practical relevance of these different mechanisms is substantial: the mechanism which involves cross-linking the chromosomes or the chromosomal-associated proteins (protamines) is a threshold-based, non linear event, for which there are safe levels. For the mutagenic effects induced by glycidamide and which involves DNA, perhaps as few as 1 adduct could be responsible for it. This is a non threshold-based, linear response for which there may be no safe dose. At germ cell level, the different mechanisms have undoubtedly implications for long-term *vs.* short-term outcomes and quality of sperm. Gene mutations in spermatogonia would indicate long-term risk, whereas protamine

alkylation in post-meiotic germ cell stages would indicate short-term risks. The real problem is that both mechanisms can occur.

CARCINOGENICITY OF ACRYLAMIDE

Animal experiments

Two long-term standard bioassay have been carried out, both in F344 rats given acrylamide via drinking water for up to 2 years.

In the first study [9] the animals (60 males and 80 females) received doses of acrylamide corresponding to 0, 0.01, 0.5 and 2.0 mg/kg bw/day. The maximum tolerated dose (HTD) appeared to have been reached at the high dose group as suggested by the reduced body weight increase.

In males, acrylamide induced a statistically significant increase of benign follicular cell adenomas of thyroid at the highest dose level (7/59 *vs*. 1/60). In males there was a statistically significant increase of malignant testicular mesotheliomas at 0.5 and 2 mg/kg/day (11/60 and 10/60 *vs*. 3/60 or 18% and 17% *vs*. 5%); the historical incidence was 3.1% (range: 2-6%). In males there was a non-significant increase in malignant astrocytomas in the spinal cord at the highest dose (3/60 *vs*. 1/60). There was also a non-significant increase of malignant astrocytomas in the brain of females at the highest dose (3/60 *vs*. 0/60), glial proliferation in the brain suggestive of an earlier tumor (3/60 *vs*. 0/60), and malignant astrocytomas in the spinal cord (3/61 *vs*. 1/60). Malignant astrocytomas were also observed in the brain (3/60, 0/60, 0/60, 2/60, 2/69) and glial proliferation (0/60, 0/60, 0/60, 1/60, 1/60). The effects in astrocytomas do not slow any clear dose-response and their toxicological significance is unclear. In females there was a non-significant increase of benign follicular cell adenomas of the thyroid at the highest dose (3/60 *vs.* 1/58) and a statistically significant increase of the adenocarcinomas in the uterus at the highest dose (5/60 *vs*. 1/60 or 8.3% *vs*. 1.7%) (historical control range: 0-2.3%). In the females, there was a statistically significant increase in the incidence of benign papillomas in the oral cavity at the highest dose (7/61 *vs*. 0/60) and a non-significant increase in focal hyperplasia (4/60 *vs*. 1/60). The incidence of malignant carcinomas did not show any clear dose-response (0/60, 0/60, 0/60, 2/60, 1/60). For males, the incidence of tumors in the oral cavity did not show any clear exposure relationship (carcinomas: 2/60, 0/60, 1/60, 0/60, 2/60; papillomas: 4/60, 7/60, 0/60, 5/60, 4/60) although there was a statistically significant increase in focal hyperplasia of the hard palate (0/60, 1/60, 1/60, 4/60, 5/60). In the females there were increases in benign and malignant tumors of mammary glands (10/60, 11/60, 9/60, 19/58, 23/61 and 2/60, 1/60, 1/60, 2/58 and 6/61, respectively), benign pituitary gland adenomas (25/59, 30/60, 32/60, 27/60, 32/60) and benign tumors of the clitoral gland (0/2, 1/3, 3/4, 2/4, 5/5). In males there were increased incidences of benign tumors of the adrenal glands (pheochromocytomas) (3/60, 7/59, 7/60, 5/60, 10/60). The increased incidences of mammary tumors, benign pituitary adenomas and adrenal pheochromocytomas are of doubtful toxicological significance due to the poor dose-response and high historical control incidence. For clitoridal adenomas the total number examined was too small to draw any conclusion.

A second standard 2-years bioassay was conducted in F344 rats via drinking water [49]. Groups of 75-204 males received 0, 0.1, 0.5 and 2 mg/kg bw/day, while groups of 50-100 females received 0, 1 and 3 mg/kg bw/day acrylamide. In the males, there were increased incidences of thyroid follicular adenomas (attaining statistical significance only at the highest dose) and a non-significant increase in carcinomas (3/204, 9/203, 5/101, 12/75 and 3/204, 3/204, 0/102, 3/75, respectively). Also in the females, there were increases in thyroid follicular adenomas and carcinomas (0/100, 7/100, 16/100 and 2/100, 3/100, 7/100, respectively). In the males, there was a statistically significant increase in malignant scrotal mesotheliomas at the highest dose (13/75 *vs.* 8/204). In the females, the combined incidence of mammary gland adenocarcinomas (2/96, 2/94 and 4/95) and fibroadenomas (9/96, 20/94 and 26/95) was significantly increased in both acrylamide dosed groups. In the brain, the following increased incidences of benign and malignant tumors were observed: astrocytomas (1/204, 0/98, 0/50, 2/75 or 0.5%, 0, 0, 3% in the males, and 0/100, 2/100 and 2/100 or 0, 2% and 2% in females), meningiomas (0/100, 2/100 and 3/100 in females; non-increase in males), malignant reticulosis (0/100, 2/100 and 3/100 in females; non-increase in males). These tumors may not be related to acrylamide exposure; combined historical control data from NTP studies indicate a range of glial cell tumors in the brain up to 4%. Individual occurrences of astrocytomas in the spinal cord were observed at very low incidence in both males and females without statistical significance. No significant increases were noted for neoplastic changes in the uterus, clitoral gland and oral cavity; however, sections did not appear to be taken from the oral cavity.

Overall, for acrylamide exposed rats there are clear increases in tumors in several organs. Some of the tumor types show a possible relationship with disturbed endocrine function (*e.g.*, thyroid, testicular mesotheliomas, adrenals) and raise the possibility of an indirect, hormonal mechanism. However, due to the clear *in vivo* genotoxicity of acrylamide, one can not exclude that also these types of tumors could have arisen after a direct damage to the hormone-producing organ. There is also a suggestion of tumors of the brain of the spinal cord, although the picture is not clear, due to the fact that these tissues represent possible targets for acrylamide activity. The lowest carcinogenic effective dose observed in these studies was 1-2 mg/kg bw/day.

As reported by the IARC (1994) [1], in a screening adenoma bioassay, acrylamide, given orally or by *i.p.* injection, increased the incidence and the multiplicity of lung tumors in strain A/J mice. The relevance of these results is doubtful, due to the high spontaneous incidence of lung tumors in this strain of mice.

In initiation-promotion bioassays [50, 51] acrylamide was also tested as "initiating agent" for skin carcinogenesis after oral, *i.p.* injection and topical administration to mice of one strain and after oral administration to mice of another strain, followed by topical application of the promoting agent 12-0-tetradecanoyl phorbol 13-acetate (TPA). Acrylamide induced a dose-related increase in the incidence of squamous-cell papillomas and carcinomas of the skin in all four experiments. However, there was not increase of tumors when mice were treated with acrylamide alone, not followed by application of TPA, suggesting that in this condition acrylamide was an "initiating agent".

Human data

Occupational epidemiology of acrylamide started about 20 years ago, while population based studies have been carried out only recently, after the discovery of acrylamide presence in fried/baked foods.

Occupational cancer epidemiology

A cohort of 371 white workers, six of which women, was analyzed in acrylamide and monomer production [52]. The results were substantially negative; however, the statistical power of this study was severely limited by the small number of the cohort components. The acrylamide monomer process began in 1995, while the polymer production started in 1965. Measurements of environmental concentrations showed a decrease over time: the TWA ranged from 0.1 to 1.0 mg/m^3 before 1957 and between 0.1 and 0.6 mg/m^3 in the period 1957-70; the values were below 0.1 mg/m^3 after 1970. Exposure to acrylonitrile was possible in the monomer production area. Exposure to acrylamide occurred in the polymer production by inhalation of dust, containing about 1% residual acrylamide. Dust concentrations were above 2 mg/m^3 (time-weighed average) in packaging and drying operations and lower in other jobs. Dermal absorption or ingestion of polyacrylamide dust could not be assessed. Workers were identified from census lists for the period 1955-1979. Only 19% had started work before 1960; 76% had worked for less than four years (14 workers had been also exposed to organic dyes for at least five years). Mortality was examined from the initial work to the end of December 1982. Standardized mortality ratios (SMRs) were estimated: 29 death *vs*. 38 expected (SMR 0.76). The number of deaths from all cancers was slightly higher than expected (11 observed; SMR, 1.39; 95% C.I., 0.70-2-49). The increase was related to cancer of the respiratory system (4 observed; SMR, 2.02; 95% C.I., 0.57-5.39). Excluding workers previously exposed to organic dyes no increase for respiratory tract cancer was observed and 2 cases of digestive tract cancer were observed against 1.6 expected.

A much larger study including 3 plants in the USA and 1 in the Netherlands, the cohort being of 8854 individuals, with potential exposure to acrylamide between 1925 and 1976 was carried out [53, 54]. 96% (8508: 7242 white and 1266 non-white) had worked in the USA. Follow-up from 1925 to 1983 was complete for 94% of the cohort. Death certificates were obtained for 95% in the USA and for 82% in the Netherlands. Exposure estimates were derived from each job in the four plants from ambient monotoring data from 1977 onwards. An individual cumulative exposure index was calculated by combining the estimated average daily exposure and the number of days spent in each job. Exposure to acrylamide was defined as cumulative exposure greater that 10.001 mg/m3/year; 2203 were exposed. Smoking habits were available for 35% of the cohort. Among the exposed workers there was a significant deficit for mortality from all causes (SMR, 0.81) with no trend in cancer mortality with increase of cumulative exposure. Weakly increased risks were observed for pancreas cancer (8 observed; SMR, 2.03, 95% C.I., 0.87-4.00) and for Hodgkin's disease (5 observed; SMR, 1.29, 95% C.I., 0.42-3.00). This study was updated in 1999 [55] This update was restricted to 8508 workers in the USA during 1984-94. The study showed the following results: SMR, 0.65 for brain/central nervous system; SMR, 2.11 for thyroid gland; SMR, 0.28 for testis and other male genital organs; SMR, 1.10 for respiratory tract cancers. None of these values was statistically significant or associated with exposure. A significant 2.26-fold risk (95% C.I., 1.03-4.29) was observed for pancreas cancers in workers with cumulative exposure to acrylamide higher than 0.30 mg/m3/years (9 deaths). It was stated that this study would have been able to detect a 25% increase in total cancer, 50% increase in respiratory trait cancers, and a 3-fold increase in brain and central nervous system cancers with a power of 80%.

In conclusion, at occupational level, no statistically significant dose-related increased incidence of cancer at any site was associated with exposure to acrylamide, with the only exception of a doubling of pancreatic cancer incidence in workers with the highest cumulative exposure.

The issue of background dietary exposure to acrylamide was not addressed in these studies.

Population based studies

One study [56] was an *a posteriori* study, utilizing six previous case-control studies on oro-pharyngeal, esophagus, laryngeal, colon, breast and ovary tumors, accompanied by detailed questionnaires on food consumptions, including reference to fried/baked potatoes. These data were used to evaluate the differences in acrylamide intake from food between the case and the control groups for each of the six tumor types. No determination of acrylamide in foods, nor molecular epidemiology (*e.g.*: use of biomarkers of exposure like Hb-adducts), was obviously carried out in this posthumous study. Moreover, all the tumor types indicated by the occupational epidemiology were absent among the tumor types investigated in this study. As expected, the results were substantially negative both for the weak, multi-site carcinogenicity of acrylamide and for the fact that controls were equally exposed to considerable amounts of acrylamide, thus diminishing further the low analytical power of the research.

In another study [57] the authors re-analyzed a population-based Swedish case-control study encompassing cases with cancer of the large bowel (n. = 591), bladder (n. = 263) and kidney (n. = 133), and 538 healthy controls; dietary acrylamide was assessed by linking extensive food frequency data with acrylamide levels in certain food items recorded by the Swedish National Food Administration. No excess risk, nor any trend of cancer of the bowel, bladder or kidney in high consumers of 14 different food items was shown.

The results of both these epidemiological population-based studies have to be considered "inconclusive", due to their low statistical power and other strong limitations (*e.g.*: poor exposure data, difficulty to find controls not exposed to acrylamide, limited number of tumor types investigated).

Summary of the carcinogenicity and mode of action

Acrylamide is carcinogenic in standard bioassays in rats via drinking water, producing increased incidences in a number of benign and malignant tumors in a variety of sites (*e.g.*: thyroid, adrenals, testis). The tumor types observed show a possible relationship with disturbed endocrine function and raise the possibility of a hormonal mechanism. However, the clear-cut genotoxicity of acrylamide *in vivo* in somatic and germ cells, and its metabolic conversion in the genotoxic epoxide glycidamide, able to form DNA-adducts *in vivo* in several organs, strongly suggest that a genotoxic mechanism for the carcinogenicity of acrylamide cannot be ruled out. Very likely, acrylamide is a weak, multi-site carcinogenic agent. The presently available data on mice present various limitations; further studies, preferably with B6C3F1 strain of mice used by the US-NTP, would be very valuable to compare mice and rats.

The human data from the occupational epidemiology as well as from the population-based studies so far carried out are in good agreement with the expectations. Their negative results, or better, their inconclusive results, are quite predictable, considering their low statistical power and the limited range of exposure doses, and also the weak carcinogenic potency of acrylamide as indicated by the risk assessment exercises. One has to remember that the results of the epidemiological studies can never prove that an agent is not carcinogenic; they can only provide an upper bound of the carcinogenic potential of acrylamide, which in any case is much higher than what is considered tolerable by some regulatory agencies, *i.e.*, one extra case during a lifetime per 10^4-10^5 individuals.

Quantitative cancer risk assessment

The results of quantitative cancer risk assessment can be given as the "unit risk", *i.e.* the theoretical risk associated with a certain unit dose, for example an average intake of 1 µg/kg bw/day, under a lifetime exposure (*e.g.*: 70 years). If exposures occur under shorter periods, the risk is thought to diminish proportionally. So far, several cancer risk estimates have been developed, all based on the results of the 2-years bioassays in rats administered acrylamide via drinking water. The outcome of these estimations vary by a factor of about 25, since they were based on different mathematical models. The mathematical models used are based on a number of assumptions (*e.g.*: non-threshold, linearity in the extrapolation from high to low doses) which are considered very conservative. Considering a lifetime (70 years) daily exposure to 1 µg acrylamide/kg bw, WHO (1996) [2] estimated a risk of 0.64×10^{-3} by applying a linearized multistage model without species conversion factor. The US-EPA (1996) [58], by using the same model as WHO but by applying a species conversion factor estimated a risk of 4.5×10^{-3}. Törnqvist *et al.* (1998) [59], by applying the linearized model (additive) estimated a risk of 7.4×10^{-3}. Törnqvist *et al.* (1998) [59] and Granath *et al.* (1999) [60], by applying a multiplicative model based on dosimetry estimated a risk of 16×10^{-3x}, with x denoting risk relative to the background cancer death rate; for comparison with the other figures that relate to cancer incidence, it can be multiplied by a factor of 1.4. The Norwegian Authority [61] has estimated a risk between 1.0 and 1.6×10^{-3}, by applying a different method based on the use of T25 [62].

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