Improved Basis for Cancer Risk Assessment of Acrylamide from Food

Determination of Glycidamide in Vivo Doses

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Abstract

Acrylamide is formed in heat processing of many common foods. According to animal cancer tests acrylamide is a carcinogen. To estimate the cancer risk from exposure via food, the response at high doses in the cancer tests with rats has to be extrapolated to the exposure levels in humans. Acrylamide is biotransformed to the epoxide glycidamide, which is assumed to be the cancer-risk increasing agent. Therefore in vivo doses of both acrylamide and glycidamide should be measured in rats and humans and related to the acrylamide intake. In vivo doses (area under the time-concentration curve, AUC) of reactive compounds can be determined from measured reaction products, adducts, to hemoglobin (Hb).

A study in mice showed that the food matrix does not have an influence on the absorbed amount of acrylamide from food. There was a linear dose-response of Hb-adduct levels from acrylamide and glycidamide.

For cancer risk assessment it is important to describe variations between individuals in intake and in AUC. Hb-adduct levels of acrylamide and glycidamide were studied in two large groups. In non-smokers the acrylamide and glycidamide-adduct levels varied with a factor of 5 and 8, respectively. The influence of other compounds in the diet on metabolic formation/elimination of glycidamide was demonstrated by associations between the ratio of glycidamide-to-acrylamide-adduct levels and alcohol intake. Furthermore, a non-linearity between glycidamide and acrylamide-adduct levels was shown at low exposure levels. AUCs from acrylamide and glycidamide in rats exposed as in the cancer tests were measured and compared with AUCs in humans exposed to acrylamide through food. The AUC of glycidamide per given dose of acrylamide were somewhat higher in humans than in the rats. Altogether the generated data could be used to improve the cancer risk estimate of acrylamide in food. The obtained data strengthen earlier preliminary cancer risk estimates of acrylamide.

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Abbreviations

AA	Acrylamide
AAMA	N-acetyl-S-(2-carbamoylethyl)-L-cysteine
AUC	Area under the curve
BW	Body weight
GC/MS	Gas chromatogaphy – mass spectrometry
CYP	Cytochrome P-450
DNA	Deoxyribonucleic acid
EH	Epoxide hydrolase
EU	European Union
FFQ	Food frequency questionnaire
GA	Glycidamide
GAMA	N-acetyl-S-(2-carbamoyl-2-hydroxy-ethyl)-L-cysteine
GSH	Glutathione
GST	Glutathione-S-transferase
Hb	Hemoglobin
LC/MS	Liquid chromatography – mass spectrometry
MN	Micro nucleous
MOA	Mode of action
MOE	Margin of exposure
MS	Mass spectrometry
NICI	Negative ion chemical ionization
NOAEL	No-observed-adversed-effect-level
PFPITC	Pentafluorophenyl isothiocyanate
PFPTH	Pentafluorophenylthiohydantoin
SPE	Solid phase extraction

List of papers

The thesis is based on the following papers:

I **Vikström A.C.,** Eriksson S,. Paulsson B., Karlsson P., Athanassiadis I., Törnqvist M. (2008), Internal doses of acrylamide and glycidamide in mice fed diets with low acrylamide contents, *Mol. Nutr. Food Res*. **52**, 974-980.

II Törnqvist M., Paulsson B., **Vikström A.C.,** Granath F. (2008), Approach for cancer risk estimation of acrylamide in food on the basis of animal cancer tests and in vivo dosimetry, *J. Agric. Food Chem*., **56**, 6004-6012.

III **Vikström A.C.,** Wilson K.M., Paulsson B.,Athanassiadis I., Grönberg H., Adami H-O., Adolfsson J., Mucci L.A., Bälter K., Törnqvist M. (2010), Alcohol influence on acrylamide to glycidamide metabolism assessed with hemoglobin-adducts and questionnaire data, *Food Chem. Tox*., **48**, 820-824.

IV **Vikström A.C.**, Paulsson B., Axmon A., Warholm M., Wirfält E., Törnqvist M. (2010) In vivo doses from acrylamide in food – variation between and within individuals, *manuscript*.

V **Vikström A.C**., Abramsson-Zetterberg L., Naruszewicz M., Athanassiadis I., Granath F., Törnqvist M. (2010) In vivo doses of acrylamide and glycidamide in humans after intake of acrylalmide-rich food, *manuscript.*

Permissions to reproduce the articles (I-III) in this thesis were kindly obtained from the publishers.

Following studies have also contributet to the contents of my thesis:

- Abramsson-Zetterberg, L., **Vikström, A.C**., Hellenäs, K.-E., and Törnqvist, M., (2008), Differences in the frequency of micronucleated erythrocytes in humans in relation to consumption of fried carbohydrate-rich food, *Mutat. Res.*, **653**, 50-56.

- Naruszewic, M., Zapolska-Downar, D., Kosmider, A., Nowicka, G., Kozlowska-Wojciechowska, M., **Vikström, A.C**., and Törnqvist, M., (2009), Chronic intake of potato chips in humans increases the production of reactive oxygen radicals by leukocytes and increases plasma C-reactive protein: a pilot study, *Am. J. Clin. Nutr.*, **89**, 773-777.

- Wilson, K.M., Bälter, K., Adami, H.-O., Grönberg, H., **Vikström, A.C**., Paulsson, B., Törnqvist, M., and Mucci, L.A., (2009), Acrylamide exposure and prostate cancer risk in the Cancer of the Prostate in Sweden Study: a validation and case-control analysis, *Int. J. Cancer*, **24**, 2384-2390.

1 Introduction

1.1 Background to this thesis

In 2002 the general public got aware of that acrylamide is formed in cooking/heating of many common foodstuffs [1,2]. Just a few years before, acrylamide had been recognized by the Swedish public as the toxic component in the leakage of the grouting material Rocha-Gil®, used in a tunnel construction work at Hallandsås in the south-west of Sweden [3]. Exposure of tunnel-construction workers, residents, and animals was clarified by biomarker studies in our research group [4,5]. Further studies on acrylamide then led to the finding that acrylamide is formed in food [1,2]. Acrylamide is classified as "probably carcinogenic to humans" (Group 2A) by the International Agency for Research on Cancer (IARC) since 1994 [6]. The possible cancer risk associated with the exposure to dietary acrylamide has been debated since 2002. The overall aim of this thesis is to contribute to an improved basis for the cancer risk assessment of acrylamide exposure via food.

1.2 Cancer

Cancer has become one of the primary causes of death in developed countries. Cancer was the second cause after cardiovascular diseases in Sweden 1984 [7] and in the U.S. 2005 [8]. In Sweden, the number of diagnosed cancer cases was approximately 52 000 in 2008 and approximately 20 000 people die every year due to a cancer disease [9]. Diagnosed cases have increased by an average of 2.7% per year during the two last decades [10]. The prognosis for cancer patients in Sweden has however improved the last four decades. For all cancer diseases the average five-year survival rate was approximately 35% for men and 48% for women in the 1970's. These figures are now approximately 70% for both genders [10]. Tumor development is generally a slow process of about 5-30 years, therefore the cancer incidence increases with age. In Sweden, the probability to develop cancer is approximately 30% before the age of 75 [10]. The fraction of individuals that survive cancer will continue to increase in developed countries due to better treatments. In contrast, low- and middle-resource countries will experience an increase of deaths due to cancer as socioeconomic factors impede development of health care systems [11].

The mechanisms for tumor development are complex, and research over the last 30 years has shown that hundreds of genes and proteins are involved. Cancer development is a multi-step process, where mutations, changes in the

genetic material in the cell nucleus, are initial steps. Promoting factors are then needed for the initiated cell to develop towards a tumor. Mutations can arise from exposure to chemicals or radiation (e.g. ionizing and UV) [12]. Most chemical substances that are known to be mutagenic are electrophilically reactive, or are activated via biotransformation into electrophiles [13].

1.3 Influence of lifestyle factors on cancer risk

Lifestyle factors influence the exposure to potentially carcinogenic, often mutagenic, compounds. For instance lung cancer is strongly associated with smoking. Dietary habits are considered as a lifestyle factor and diet is related to obesity. The importance of weight control, physical activity, and dietary patterns in reducing cancer risk is a general observation (e.g. [11,14]). Many carcinogenic compounds are formed in food treating processes; for instance heterocyclic amines and polycyclic aromatic carbons in the heat processing of red meat at high temperatures [12]. A wide array of substances in the human diet has been found to stimulate the development of tumors in animal experiments [15]. In contrast, naturally occurring compounds like for instance phytochemicals could have cancer preventive effects, though most mechanisms of action are still unknown [15].

1.4 Acrylamide formation

Acrylamide is formed in the reaction between reducing sugars and the amino acid asparagine [16,17]. Therefore high levels of acrylamide are found in cooked food items that are rich in carbohydrates, for instance bread and potato products [18,19]. Acrylamide formation also depends on parameters like temperature, time, pH and the water content in the matrix [20,21]. Acrylamide is only one of many compounds formed in the so called Maillard reaction in the cooking process; others give taste and color to foods.

1.5 Biotransformation of acrylamide to glycidamide

Acrylamide has been shown to be carcinogenic in cancer tests in rats [22,23] and mice [24,25]. In the body, acrylamide is biotransformed to the epoxide glycidamide (Figure 1). Glycidamide reacts with DNA [26,27], is genotoxic (reviewed in [28]), and is considered to be the cancer-initiating agent in acrylamide exposure.

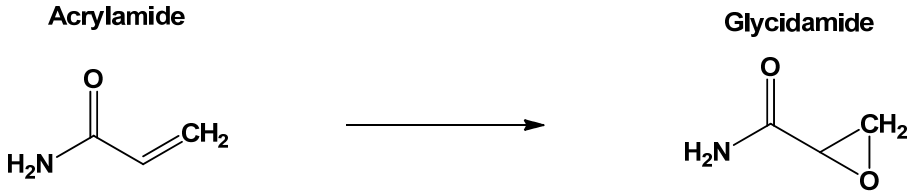

Figure 1. Acrylamide is biotransformed to glycidamide via phase 1 metabolism.

1.6 Hemoglobin adducts used as biomarkers for in vivo dose

Stable compounds could be analysed as such in for instance blood but, electrophilically reactive compounds/metabolites with short half-lives are difficult to measure. Therefore electrophiles are usually analyzed as stable reaction products, adducts, to for instance proteins. In the 1970's, adducts to hemoglobin (Hb) were suggested to be suitable for the measurement of aromatic amines [29]. Independent of these studies, methods for measurements of adducts to blood proteins, in particular to Hb, that further should be used for quantitation of in vivo doses (area under the concentration-time curve, AUC) were developed by Ehrenberg and co-workers [30-32]. The in vivo dose (AUC) of a compound hence describes the average concentration of a compound a person/animal has been exposed to during a certain time period. The AUC also reflects the net effect of all enzymatic, chemical and physiological factors on rates of formation and elimination of the reactive chemical. Therefore, the in vivo dose (AUC) can be used as a basis for interspecies and high-to-low dose extrapolation in risk assessment of genotoxic agents [33].

1.7 Risk assessment of acrylamide

In developed countries, all citizens seem to be exposed to acrylamide, as it is present in a broad range of staple foods [34,35]. There are no regulations by authorities in Sweden or in other countries concerning allowed levels of acrylamide in commercial foodstuffs. An exception is the maximum level in drinking water which in the European Union (EU) is 0.1 μg/L [36]. Average daily exposures to acrylamide have been estimated to approximately 0.5 μg/kg bw in Western countries (reviewed in ref. no. [35]).

Cancer risk assessment of acrylamide via food is challenging, as the whole population is exposed by low daily intakes. Further, one uncertainty is that risk assessments are made by extrapolation from high exposure levels in cancer tests of acrylamide in the rat [22,23] to low exposure levels for humans (Fig 2). For instance the U. S. Environmental Protection Agency (EPA) arrived at an estimate for life time risk at 4.5 cancer cases per 1000 individuals at an exposure level of 1 µg acrylamide/kg body weight (bw) and day [37].

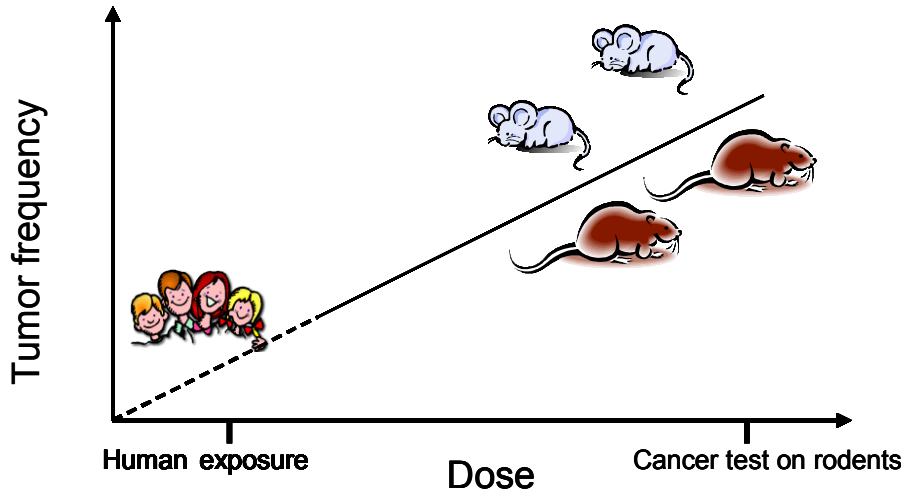

Figure 2. Risk assessment of acrylamide is done by extrapolation of tumor frequency data obtained in the cancer tests on rodents down to exposure levels that are relevant for humans. The acrylamide exposure doses in the cancer tests are 10^3 higher than estimated intakes of acrylamide via food for humans.

2 Aims of this thesis

The overall aim of this thesis is to improve the risk assessment of acrylamide exposure via food. The exposure of acrylamide should be related to the in vivo doses (AUC) of acrylamide and glycidamide. As glycidamide is considered the cancer-risk increasing agent in acrylamide exposure, the focus will be on glycidamide. Hb-adducts are used to measure the AUC.

The primary aims are to investigate:

- A possible influence of the food matrix on the absorbed fraction of acrylamide from food.
- The variation in AUC from glycidamide between individuals and in individuals over time. Inter-individual variation should be studied in a sufficiently large cohort with large variation in intake of acrylamide from food. Intra-individual variation should be studied in a group of individuals donating blood over a period of 1-2 years.
- The relationship between food frequency questionnaire data and Hb-adducts of acrylamide and glycidamide.
- Calculate AUCs of acrylamide and glycidamide in humans and relate these to exposure to acrylamide in food.
- Compare these AUCs in humans to AUCs in rodents exposed at the same high acrylamide levels as in cancer tests (see Figure 2).
- Dose-response curve for glycidamide formation at low doses of acrylamide exposure.

3 Background

3.1 Sources of acrylamide exposure to humans

There are other known sources of acrylamide (CAS nr 79-06-1) exposure than via food. Acrylamide is an industrial chemical, produced from acrylonitrile since the 1950s (cf. [58]). There are several studies of occupational exposure to acrylamide in different settings (e.g. [5,38-40]). In the past, cases of accidental contamination of the environment by acrylamide have been described, when the monomer was released from the usage of polyacrylamide [41], for instance the leakage of the grouting agent Rocha-Gil ® in the south of Sweden [5]. Another source of acrylamide exposure is smoking [42], as acrylamide has been found in cigarette smoke at levels of a few micrograms per cigarette [43]. High doses of acrylamide have been measured from occupational exposures (Table 1). These doses are far higher than the exposure measured from food which is the largest exposure source to the general public.

Table 1. Hb-adduct levels of acrylamide measured in individuals with background exposure due to food in non-smokers, smoking or occupational exposure.

Type of exposure	Acrylamide adduct levels (pmol/g globin)	References
Background exposure	20-110	[44-47]
Smoking	30-420	[42,47,48]
Occupational exposure	Up to 20 000 – 30 000	[5,38,42,49]

3.2 Acrylamide levels in food; data bases and the use in epidemiology

Acrylamide levels in food (Table 2) have been measured in several studies (e.g. [2,18]) and, the collected data is found in national and international data bases (e.g. [50,51]). These data bases have been used for estimation of the mean daily exposures of acrylamide; in adults estimated to approximately 0.5 µg/kg bw (reviewed in ref. no. [35]). For children the general intake seems to be slightly higher than for adults [52-54].

Mitigation strategies in order to reduce levels of acrylamide in food have been developed in collaborations between scientists and food agencies and these have further been shared with the industry [50,55]. Nevertheless, a report from the European Food Safety Authority (EFSA) reveals that acrylamide levels in common food-stuffs, in general did not decrease during 2003-2007 [50].

Table 2. Acrylamide levels measured in some common food products/groups in Sweden. Boiled products are included to exemplify that acrylamide is formed at negligible low levels by this cooking method even when the precursors for formation are present. The table is adjusted from Svensson et al. [18].

Food product / group	**Acrylamide concentration (µg/kg)**	
	Median	Min-Max
Potato crisps	980	330-2300
French fries	410	300-1100
Cookies/biscuits/wafers	410	< 30-640
Crisp breads	135	< 30-1900
Breakfast cereals	100	< 30-1400
Boiled potato, spaghetti, rice	< 30	

3.2.1 Epidemiological studies of acrylamide intake via food

In epidemiological studies on the association between acrylamide intake and cancer risk, the intake of acrylamide has generally been estimated from food frequency questionnaire (FFQ) data linked to the data bases on acrylamide content in foods. Most published epidemiological studies concerning acrylamide in food have shown negative associations between exposure and cancer (different sites) (reviewed in [56]). However, these studies have been criticized for too low statistical power [57], as for instance they included relatively few subjects.

3.3 Toxicological effects of acrylamide

Acrylamide is well studied with regard to its toxicological effects (e.g. [58]), briefly:

- Neurological symptoms were early observed in experimental animals (reviewed in ref. no. [59]) and in occupationally exposed humans [5,60].
- Developmental and reproductive effects have been observed in experimental animals (reviewed in ref. no. [61]).
- Acrylamide has been shown to be carcinogenic in cancer tests on rat [22,23] and mice [24,25]. Acrylamide provoked tumors in rodents of both sexes in multiple sites in the cancer tests.
- Genotoxicity of acrylamide and glycidamide is discussed below.

3.3.1 Genotoxicity of acrylamide and glycidamide

Acrylamide has clearly been shown to be genotoxic, both in vitro and in vivo. Acrylamide exposure induces both chromosomal aberrations and gene mutations in germ and somatic cells in mice and cell cultures (reviewed in ref. no. [6,28]).

Many studies have convincingly showed that the genotoxicity is due to the biotransformation of acrylamide to glycidamide. For instance DNA-adducts of glycidamide, and not of acrylamide, to guanine and to a lesser extent adenine have been analyzed in exposed rodents [26,27]. Studies in CYP2E1 knockout mice showed the importance of the conversion of acrylamide to glycidamide [62-64]. In the knockout mice only Hb adducts of acrylamide could be measured at significant levels. In the wildtype mice, linear dose-response of DNA adducts of glycidamide (liver, lung, and testes) and of Hb adducts of acrylamide and glycidamide were detected [62]. In the knockout mice genetic damage in somatic and germ cells was determined with the Comet assay and micronuclei measurement [62,63]. Other studies carried out on Big Blue mice and Big Blue rats, showed increased mutation frequencies after exposure to acrylamide or to glycidamide [65,66].

A lot of test systems have been developed to detect genotoxicity of compounds. One of the most common tests is the micronuclei test; both in vivo and in vitro systems are available. Micronucleus is an extra little nucleolus produced from the main nucleolus during the cytokinesis. Micronuclei are results of broken chromosomes and/or whole chromosomes. Such micronuclei can easily be detected and the frequency of micronuclei is a sensitive and commonly used endpoint for clastogenic effects. Linear dose-response has been shown for both acrylamide and glycidamide for micronucleated erythrocytes in rodents (different administration routes) [65,67,68] at exposure levels down to 2.5 mg acrylamide /kg bw in mice [69].

3.4 Biomarkers of acrylamide and glycidamide

Both acrylamide and glycidamide react with nucleophilic sites in proteins. Stable reaction products, adducts, are formed (reviewed in refs. no. [70,71]). DNA adducts of glycidamide have been measured in acrylamide dosed animals; N7-(2-carbamoyl-2-hydroxyethyl)guanine and N3-(2-carbamoyl-2-hydroxyethyl)adenine, respectively [26,27]. DNA-adducts describe the genotoxic dose to target tissues from exposure to acrylamide. However, there are no reports on analysis of DNA-adducts of glycidamide in humans, due to the insufficient sensitivity of the applied analytical methods. Urinary metabolites of acrylamide have been measured in both animals and humans,

and are often used in toxicokinetic studies. (Biomonitoring of acrylamide has been reviewed in ref. no. [72]).

3.4.1 In vivo dose (AUC)

That the life-span of erythrocytes is known, in adult humans approximately 120 days, makes calculations of in vivo dose (AUC) from Hb-adduct levels possible. During constant exposure to electrophilically reactive compounds, as for instance acrylamide exposure via food, the Hb-adduct level will reach a steady-state level. That is, an equilibrium between formation of adducts due to exposure and the disappearance of adducts due to elimination of erythrocytes (reviewed in ref. no. [70]). From the steady-state adduct level the AUC for a day or for a longer time period is calculated. In exposure experiments, the mean daily adduct increment can be used for the calculation of the daily AUC. The in vivo dose (AUC) (Figure 3) can be inferred from the Hb-adduct level when the reaction rate between the electrophile and the *N*-terminal valine is known (Figure 4) (see Paper II and V).

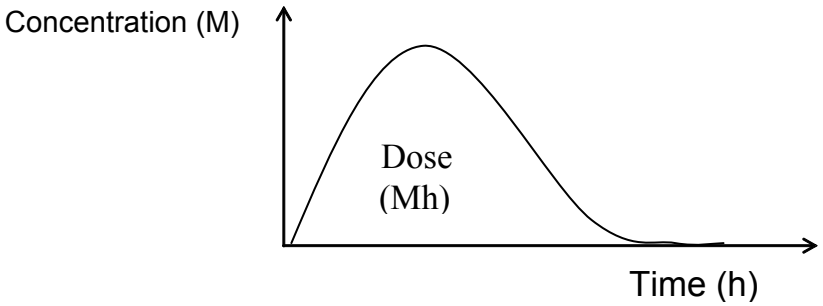

Figure 3. The dose curve after acute exposure to a compound. The dose is the integrated area under the concentration-time curve (AUC) with the unit [(mol x L^{-1}=M) x h].

3.4.2 The N-alkyl Edman method for analysis of Hb adducts

Electrophiles react at several sites of Hb, depending of the reactivity of the electrophile and on the strength of the nucleophilic site. The N-alkyl Edman method is based on analysis of adducts to the *N*-terminal valine, as valine is the N-terminal amino acid in all four protein chains of Hb and also a major reactive site for many electrophiles. Further, valine containing a substituted amino group cannot be used in protein synthesis, in contrast to other substituted amino acids. This makes adducts to the *N*-terminal valine suitable biomarkers as they can only be formed by reaction with electrophilic compounds [73].

The N-alkyl Edman method is based on that the detachment of N-alkylated *N*-terminal valine residues in Hb is favored in the reaction with an Edman

reagent (Figure 4) [74]. Stable derivatives are formed, suitable for gas chromatography (GC) mass spectrometric (MS) analysis [74,75]. The method was adjusted for analysis of Hb-adducts of acrylamide, for instance applied for monitoring of occupational exposure (e.g. [38]). Later adjustments made simultaneous analysis of acrylamide and glycidamide by GC-MS/MS possible [49].

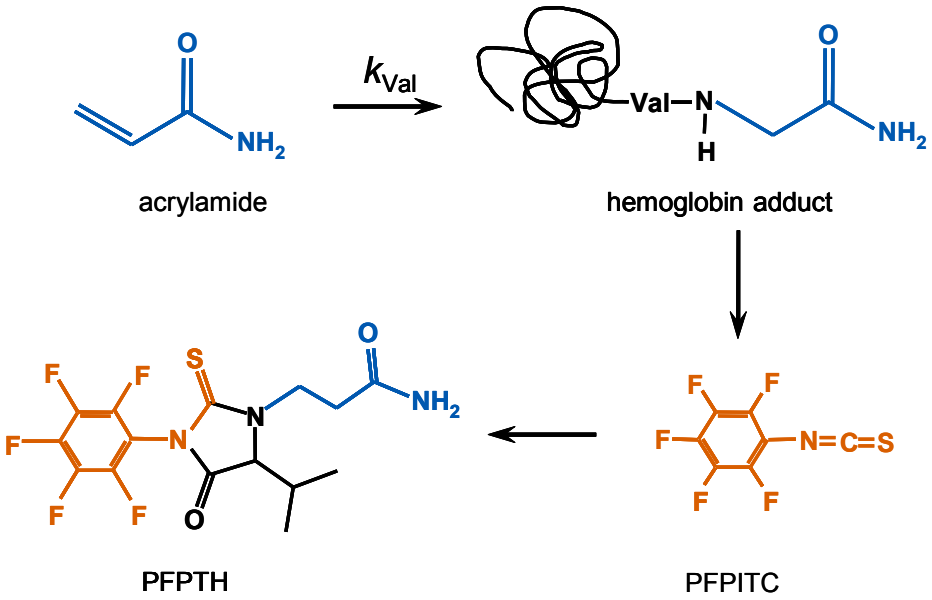

Figure 4. The Edman methodology is based on the reaction of electrophilic compounds to the N-terminal valine-N in hemoglobin (Hb). If the reaction-rate constant (k*val*) for the formation of the adduct is known, the in vivo dose (AUC) can be inferred from the adduct level. In this example the adduct formation is illustrated with acrylamide. For adduct analysis, hemoglobin is isolated and stable derivatives are formed (pentafluorophenylthiohydantoins, PFPTH) by coupling reaction with the Edman reagent pentafluorophenyl isothiocyanate (PFPITC) and detachment of the adduct as PFPTHs.

The method has been adopted by several research groups, mainly for monitoring of the acrylamide- and glycidamide-exposure to the general public (e.g. [44,46,47,76]). Adjustments have been made to achieve a faster sample preparation step by using solid phase extraction (SPE) techniques, instead of liquid/liquid isolation of the derivative, and to perform the analyses by liquid chromatography (LC)-MS/MS instead of GC-MS/MS (e.g. [46,77]).

3.5 Toxicokinetics of acrylamide

The absorption, distribution, metabolism and excretion of a compound is be described as toxicokinetics. Certain assumptions are made, for instance when the distribution in the body is described. For small, uncharged and polar compounds, like acrylamide and glycidamide, the high water-solubility facilitates the distribution [78]. Equilibrium between blood and tissues are reached rapidly after absorption. Both acrylamide and glycidamide could be

approximated by a one-compartment model and the disappearance of the compound in blood or in plasma is assumed to follow first order kinetics.

3.5.1 Studies of acrylamide in rodents

Toxicokinetics of acrylamide was initially studied in rodent models at high dose ranges (mg/kg bw). Studies in rats using oral administration at doses of 10-20 mg [^{14}C]-acrylamide/kg bw, showed that acrylamide was readily absorbed and distributed throughout the organism [79,80]. Determined elimination half-lives *(t½)* for acrylamide and glycidamide obtained in animal studies have depended on the exposure doses, exposure routes and species [79,81-84]. For instance, when mice were exposed to acrylamide via diet (0.1 mg acrylamide/kg bw) the half-lives were 1.2/3.0 h (males/females) and 2.6/3.7 h for acrylamide and glycidamide, respectively, assessed by measuring plasma concentrations over time (AUC) [82]. A study in rats at the same experimental conditions gave somewhat longer half-lives: 3.1/3.9 h (males/females) and 3.0/3.9 h for acrylamide and glycidamide, respectively [83]. The half-lives determined in the same study after intravenous (i.v.) injection were shorter [83].

In rats and mice the major detoxification pathway of acrylamide is via glutathione (GSH) conjugation, resulting in urinary excretion of the mercapturic acid derivative N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA) [85,86]. A second route for acrylamide metabolism is oxidation by cytochrome P450 2E1 (CYP2E1) to glycidamide [62,86]. Glycidamide reacts with GSH and the GSH-conjugate is excreted as two regioisomeric forms, N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA) and N-acetyl-S-(1-carbamoyl-2-hydroxyethyl)-L-cysteine (iso-GAMA) via urine [85,86]. Glycidamide can also be metabolized to glyceramide; (2,3-dihydroxypropionamide) [86] possibly catalyzed by epoxide hydrolase [87]. Small amounts of acrylamide and glycidamide as such, are excreted as well [86]. Figure 5 illustrates metabolic pathways for acrylamide.

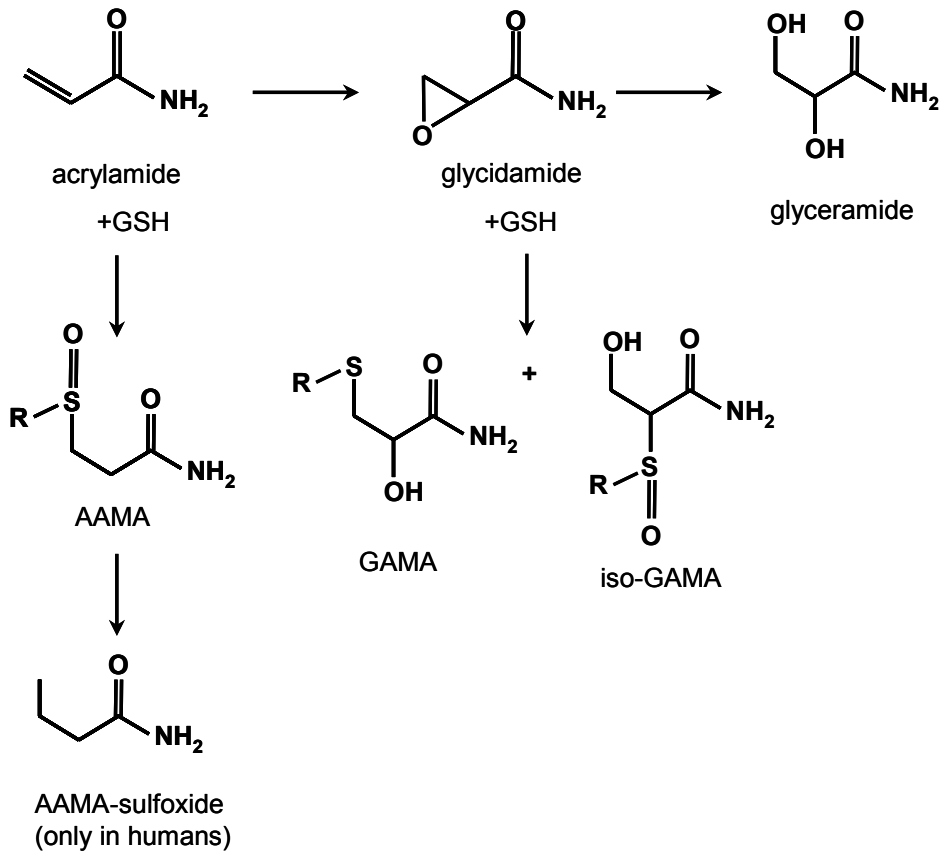

Figure 5. The biotransformation of acrylamide to glycidamide and metabolism to urinary metabolites are illustrated. AAMA-sulfoxide has been analyzed in humans but not in rodents.

3.5.2 Studies of acrylamide in humans

Some data from acrylamide exposure to humans exists, where acrylamide has been administered via food [88,89], in drinking water [90,91] or by dermal application [90,91]. In humans, acrylamide is readily absorbed and distributed [88-91], crosses the human placenta [92,93], and has also been detected in breast milk [52,89,93]. So far, the most exact data available for humans is an experiment with oral exposure to acrylamide at very high doses (single doses in drinking water; 0.5-3 mg ($^{13}C_3$)-acrylamide/kg bw) [90,91], that is 10^3 times the average exposure via food. From this study an elimination half-life *(t½)* for acrylamide of 3.1-3.5 h was calculated from measured urinary metabolites [91]. In another study six study persons were exposed to 1 mg acrylamide via a meal of potato chips, corresponding to 12 µg acrylamide/kg bw [88]. From measured urinary metabolites the half-life for acrylamide was estimated to 2.4 h [88]. The same design was used by Doroshyenko et al. [94] in a study that included 16 individuals exposed to 15 µg acrylamide/kg bw. The mean half-life of acrylamide was 5.0 h, the study showed a large individual variation though (range 2.3-25 h) [94].

Analyses of urinary metabolites [88,91] show that the metabolism in humans is similar to that in rodents. It is reasonable to assume that acrylamide is metabolized by CYP2E1 in humans as in mice (cf. [95]). Further, acrylamide is metabolized in vitro to glycidamide in human CYP2E1 supersomes, human liver microsomes as well as in genetically engineered V79 cells expressing human CYP2E1 [96]. CYP2E1 is mainly expressed in the liver, but also in lung, kidney, bone marrow and lymphocytes [97]. In contrast to rodents, fractions of AAMA further oxidized to AAMA-sulfoxide have been analyzed in humans [91].

3.6 Non-linear response in Hb-adducts to exposure of acrylamide in rodents at high exposure levels

The relationships between exposure dose and in vivo dose (AUC) of acrylamide and glycidamide were first studied by analysis of adducts to cysteine in hemoglobin of rats. A non-linear relationship was shown between exposure doses of 0.1-5 mg/kg bw (intraperitoneal injection, i.p.) and acrylamide-adduct levels [98]. Relatively higher levels of glycidamide adducts than of acrylamide-adducts to cysteine at the lower exposure doses of acrylamide (below 10 mg/kg bw), indicated saturation of the metabolism to glycidamide [81]. The deviation from linearity between exposure to acrylamide and Hb adducts of glycidamide, has been demonstrated in several studies in rodents exposed by various routes [67,79,99].

3.7 Cancer risk assessment of acrylamide

In most cancer risk assessment epidemiological data is not available or, data is not convincingly strong, to exclude or to support a carcinogenic effect. Therefore, risk assessments have to be based on data from animal studies. About 100 compounds are classified as human carcinogens based on sufficient human data (Group 1, IARC) [100]. This data is for instance achieved in studies on workers exposed at high levels during long time periods.

3.7.1 Epidemiological data on acrylamide exposure

The epidemiological studies that the last years have investigated associations between cancer and acrylamide exposure via food have with few exceptions [46,101-103] turned out negative (reviewed in ref. no. [56]). Three reports described positive associations of acrylamide intake assessed by FFQ data and hormone induced breast cancer and ovarian cancer or renal cell cancer [101-103]; one between Hb-adducts of acrylamide and hormone-induced breast cancer [46].

Epidemiological studies addressing the cancer risk associated with exposure of acrylamide intake via food have been criticized for too low statistical power. For instance, it is difficult to make correct individual intake estimates from food frequency questionnaire (FFQ) data as used FFQs are not designed to measure acrylamide exposure (see section 4.4). Furthermore, as there is exposures to everyone, the risk for misclassification is high [57]. Estimation of cancer risks based on epidemiological studies at low exposures is generally not possible, due to insufficient statistical power to detect relative risks lower than 10-20% [6]. It has been suggested that a case-control study of acrylamide would have to include hundreds of thousands of cases when intake is estimated from FFQs [57]. Epidemiological studies so far published on dietary acrylamide intake and cancer risk have generally comprised some hundreds or maximum some thousands of cases (e.g. refs. no. [102,103,105-112]).

A mortality study in 371 workers producing the acrylamide monomer showed no significant increase in cancer deaths cases due to acrylamide exposure [113]. Collins et al. [114] studied a larger cohort of 8854 workers of which 2293 were exposed to acrylamide. The study has been further evaluated at several occasions, though no consisting evidence on cancer risk increase has been reported [114,115]. However, the power to detect the expected small increased cancer risk was low, as the exposure levels to workers was by average 0.35 μg/kg bw, not much higher than to controls [115,116].

3.7.2 Human cancer risk assessment based on animal cancer tests

The following parameters could be considered important in cancer risk assessment of a genotoxic compound based on animal cancer tests:

- Exposure levels/doses in humans; mean values and variations (discussed in Background)
- In vivo doses (AUC) in humans (mean and variation)
- Dose-response relationship for carcinogenic effects
- Mechanism of action
- Model for extrapolation of the risk to humans

Dose-response relationship for toxic effects

It is generally accepted that for non-genotoxic compounds an exposure level exists below which no toxicological effects will be expressed. This threshold is often determined as a No-Observed-Adverse-Effect-Level (NOAEL) in a dose-response curve that could be based on animal or human data. The NOAEL would be the starting point in risk assessment where extrapolation to

adequate exposure levels for humans is performed by the use of default assumptions and uncertainty factors [117].

Genotoxic effects, for instance mutations, are considered stochastic; therefore a linear dose-response down to zero is considered the best assumption. Risk assessments are generally carried out by applying models on data obtained from animal studies, often long-term cancer tests on rodents [118].

Mode of action

Out of 14 risk assessments on acrylamide published between 1976 and 2002, nine hypothesized a genotoxic mode of action (MOA), and two documents discussed a combined genotoxic and hormonal mechanism (reviewed in ref. no. [119]). The EC report on acrylamide from 2002 suggested a hormonal mechanism due to some of the tumor types detected in the cancer tests on rat [58]. Other researchers have also suggested a hormonal MOA based on animal data but also due to the positive epidemiological studies (see section 3.7.1) (reviewed in ref. no. [120]). However, as shown earlier there is convincing evidence for that glycidamide is the genotoxic agent in acrylamide exposure (see section 3.3.1). Therefore glycidamide is assumed to be the cancer–initiating agent in the rodent cancer tests with acrylamide. Further, linear dose-response for biological effects has been shown in several studies at low exposure levels (see section 3.3.1). The National Toxicology Program has carried out new cancer tests on rodents exposed to acrylamide or glycidamide; data is still not available. These tests will give important information about the MOA as the comparison between acrylamide and glycidamide will support or exclude that the tumor formation is exclusively due to glycidamide genotoxicity.

Model for extrapolation of the risk to humans

The choice of data from the cancer tests that are used (tumor sites, sensitive sex, doses) and the used extrapolation model differ between organisations/researchers. Some cancer risk estimates for acrylamide expressed as the risk at a daily intake of 1 μg acrylamide/kg bw compiled in Table 3. As seen, the risk figures vary between 0.7 and 16 per 1000 individuals. This thesis work concerns the improvement of the basis for the risk estimate from 1998 by Törnqvist et al. [121].

Table 3. Some estimates of life time cancer risk due to acrylamide exposure of 1 µg/kg bw and day, and the corresponding extrapolation model for the risk assessment.

World Health Organization (WHO), 1996 [122]: **0.7 per 1000 individuals** Linear extrapolation, no consideration of metabolic differences between species.
U.S. Environmental Protective Agency (EPA), 1993 [37]: **4.5 per 1000 individuals** Extrapolation from rat to human via administered dose per m^2 body surface area. A transpecies factor of 7.05 was used, that is $3\sqrt{}$ the ratio of human-to-rat body weight.
Dybing and Sanner, 2003 [123]: **1.6 per 1000 individuals** Extrapolation from rat to human via administered dose per m^2 body surface area. A transpecies factor of 4.1 was used, that is $4\sqrt{}$ the ratio of human-to-rat body weight.
Törnqvist et al., 1998 [121] and Paper II: **16 per 1000 individuals** Relative risk model: extrapolation via in vivo dose (AUC) of glycidamide, the genotoxic metabolite.

3.7.3 The relative risk model

The model, a multiplicative risk model, is based on the dose (AUC) of the genotoxic compound in the target tissue [124]. This target dose can be approximated from the in vivo dose (AUC) in blood and hence determined with Hb-adduct measurements. The risk per AUC in animal cancer test is extrapolated to risk at AUC of human exposure levels without transpecies default assumptions. The relative risk model that in our research group has been evaluated for applicability to genotoxic chemicals was initially developed for risk assessment of ionizing radiation [125,126]. The applicability of the model to genotoxic chemicals has been evaluated using cancer test data for ethylene oxide [33] and butadiene [127].

A simplified description of the relative risk model

The model assumes that the cancer risk increment (ΔP) is linearly dependent on the target dose (AUC) (D) of the genotoxic compound and on the cancer/tumor background incidence (P^0) according to $\Delta P=\beta\ P^0\ D$. The interpretation is that the background promotive conditions determine the spectrum of spontaneous tumours as well as tumours induced by an exogenous agent. The risk increment is expressed with the relative risk coefficient (β) per unit of target dose, which also could be expressed as a doubling dose ($1/\beta$). In the evaluation of cancer-test data for ionizing radiation it has been indicated that β is approximately the same for different tumor sites, animal strains and species [33].

4 Results and discussion

4.1 Method for analysis of Hb-adducts of acrylamide and glycidamide

The original N-alkyl Edman method for analysis of adducts with the N-termini in hemogobin (Hb) [74] has been applied in this thesis. The first step in the procedure is globin precipitation from blood [128]. Globin samples are then dissolved in formamide, followed by addition of the Edman reagent pentafluorophenyl isothiocyanate (PFPITC). The coupling and detachment reaction of PFPITC and the N-termini in Hb forms stable reaction products; pentafluorophenylthiohydantoins (PFPTHs) (see Figure 4, section 3.4.2). Only N-terminals with an adduct is detached in the reaction as the required ring-closure is favored by the adduct (by a so called gem-dialkyl effect) [129]. This makes the method highly sensitive. The PFPTH derivatives are isolated and purified with liquid/liquid extraction. A further step, where samples are treated with acidic acetone, is needed to derivatize the polar glycidamide moiety before analysis on GC-MS analysis [49]. This is a time consuming procedure, but the method has been proven robust and the variation between analyses low.

An intent within this thesis work was to simplify the tedious and time-consuming procedures in this method, by using solid phase extraction (SPE) and adjust analysis to HPLC-MS/MS. Different SPE cartridges in combination with different solvents were tried out for the isolation step The same standards and calibration material was used as for analysis with GC-MS. As the overall variation in the examined methods based on SPE isolation and HPLC-MS analysis was higher that in the traditional method (RSD = 6% in Paper V) the method development work was discontinued and the work of this thesis was carried out with the traditional method. However, a parallel project within the research group with the aim to develop a high through-put method for analysis of Hb adducts on LC-MS/MS has been more successful. The so called FIRE procedure has shown good reproducibility for acrylamide- and glycidamide-adducts [130]. The most time-saving step in the FIRE procedure is the use of the fluorescein isothiocyanate (FITC) as the Edman reagent, which allows the coupling/detachment reaction directly in whole blood [130].

4.2 Factors with influence on acrylamide uptake from food (Paper I)

As discussed earlier, intake estimates are based on questionnaire data linked to data bases on information on acrylamide levels in different foods. However, for calculations of uptake, knowledge about the bioavailability of acrylamide is important, and whether the bioavailability differs between food matrixes. The later question was addressed in Paper 1.

4.2.1 Mice exposed to acrylamide at low doses (Paper I)

We wanted to investigate if the food matrix has an impact on the absorbed amount of acrylamide from food (Paper I). In this study on mice, we compared two different diets, one with a fibre-rich component and the other with as little fibres as possible. The two different diets contained heated soft whole kernel bread or a heated, commercially available, mashed-potato powder and white bread as acrylamide sources/matrix. Mice were fed the two different diets at two exposure levels of each and a control group was fed regular standard feed (totally 5 groups) during 28 days. No significant difference in the responses to the diets, measured with Hb-adducts of acrylamide and glycidamide, was observed (Figures 1B and 2 in Paper I). The results hence indicate that the absorbed fraction of acrylamide from food does not depend on the matrix. At these levels of exposure, 3-50 µg acrylamide /kg bw and day, both acrylamide-adduct and glycidamide-adduct levels showed linear dose-response.

A second aim of Paper I was to compare the influence of different extraction methods on acrylamide concentrations in food. It had been shown that water- versus alkaline-extraction, when used for acrylamide analysis in food, gave different results [131]. The commonly practiced neutral water extraction resulted in lower levels of acrylamide than the alkaline method, therefore it was important to establish which method reflected the, from food, absorbed fraction of acrylamide. According to Figure 1A and B in Paper I, the neutral water extraction related the exposure of acrylamide via the different diets to the measured Hb-adducts of acrylamide and to glycidamide. The water extraction method was recommended for analysis of acrylamide in foods, based on these results. If the alkaline method had showed a better association to “bioavailable” acrylamide than the water extraction, calculations on acrylamide intakes in humans could have been underestimated. (These results were first reported in 2005, S. Eriksson thesis [132]). It was later shown that the “extra” acrylamide extracted with the alcalic method was formed by another precursor at these conditions [133].

4.2.2 Bioavailability of acrylamide in food determined in rodent studies

Intents to determine the bioavailability of acrylamide administered to rodents, reveals differences in bioavailability between routes of administration (for calculation of bioavailability, *F*, see Eq. 1). In the mouse the bioavailability was determined after administration of the same dose of acrylamide (0.1 mg/kg bw) via gavage or fortified diet. The bioavailability was approximately 2-fold higher via gavage (32-52%) than the fortified diet (23%) [82]. The same conditions were used in a study in rats that showed similar results (F_{gavage}=60-98% and F_{diet}=32-44%) [83]. In another study mice were exposed to acrylamide via crisp bread and urinary metabolites of acrylamide was measured. These were then compared to the recovery of radioactivity in urine measured after subcutaneous injections of [^{14}C]acrylamide [134]. The authors suggested a close to 100% uptake as estimated by this method.

$$\text{Bioavailability, } F = \frac{AUC_{p.o.} \times Adm.dose_{i.v}}{AUC_{i.v.} \times Adm.dose_{p.o}} \qquad \text{Eq, 1}$$

Per oral=po; Intra venous=iv; Administered dose=Adm.dose

4.3 In vivo doses (AUC) of acrylamide and glycidamide in rats exposed as in cancer tests (Paper II)

A main aim of this thesis was to compare in vivo doses (AUC) of acrylamide and glycidamide in humans exposed to acrylamide via food to AUCs in rats exposed at the same levels as those used in cancer tests. A first step was to determine the AUCs in the exposed rats.

In Paper II, the AUC of acrylamide and glycidamide in Fisher 344 rats should be determined. Groups of males and females were exposed to acrylamide via water during one week at doses between 0.1-2.0 mg/kg bw. Female and male control animals were given tap water. Hb-adducts were measured in blood samples from all animals. A gender difference in adduct-level increment of acrylamide and of glycidamide per exposure dose of acrylamide was observed (see Figure I, Paper II); females showed higher adduct levels than males.

Second-order rate constants for the reaction of acrylamide or glycidamide to the N-terminal valine were determined in vitro. For calculation of AUC (Table 4) the adduct levels were adjusted considering the increase and decrease of hemoglobin Hb-adducts over time. The AUCs per administered dose of acrylamide (nMh/μg acrylamide per kg bw) were: 48 and 38 for acrylamide, and 24 and 18 for glycidamide, determined in females and males, respectively.

The relative risk mode showed a god fit to the tumor data in the evaluation with in vivo doses (AUC). A doubling dose of 13 mMh glycidamide was obtained (1/ β). The relative risk model is a crude, mechanism-based model for cancer risk asssessment of genotoxic agents, but manageable and straightforward if AUC data is available. However, there might be possible to further refine the model by, for instance, exclusion of overrepresented tumor sites in the rodent tests (c.f. [127]).

The AUCs determined in this study were comparable to the preliminary estimation by Törnqvist et al. 1998 [121] (from earlier data from intraperitoneal injections in Sprague-Dawley rats). The risk estimate determined by Törnqvist et al. (Table 3) was hence not changed by the result of Paper II. Compared to other risk estimates this figure is rather high (cf. Table 3).

4.4 Food frequency questionnaire data in relation to Hb-adducts

4.4.1 A study of the "Cancer of the Prostate in Sweden Cohort"

In collaboration with other research groups we used data from the population-based case-control study "Cancer of the Prostate in Sweden" (CAPS) to compare FFQ data to different stages of prostate cancer and measured Hb adducts from acrylamide to intake of acrylamide assessed by FFQs in non-smokers [135]. Dietary data was available for 1499 cases and 1118 controls that had answered a self-administered 261-item FFQ that assessed usual intake of foods over the previous 12 months. Acrylamide intake was calculated based on the consumption of 18 food products by multiplying the acrylamide content of a serving of the food by the frequency of consumption of that food (Figure 6). Hb adducts of acrylamide were measured in blood samples from a subset of 170 cases and 161 controls. The study showed low correlation between intake estimated by FFQ and Hb-adducts of acrylamide in non-smokers (p=0.25, 95% confidence interval: 0.14–0.35) [135]. No significant associations were found between acrylamide exposure and risk of prostate cancer (different endpoints) [135].

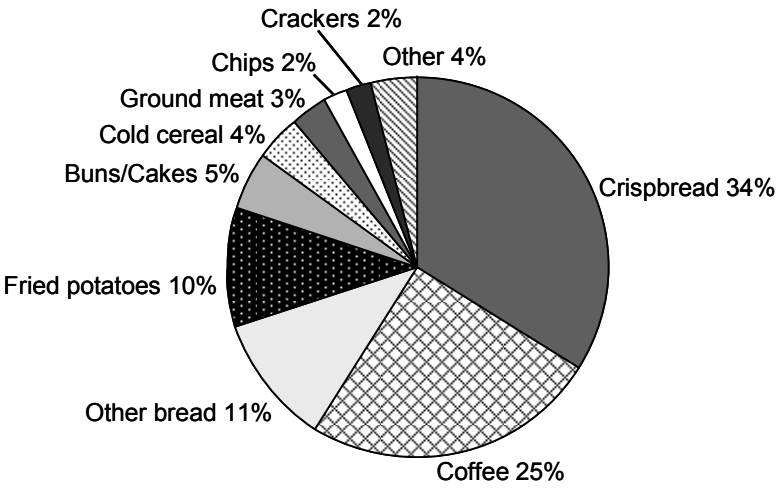

Figure 6. Contribution of foods to acrylamide intake for 331 Swedish men in the CAPS cohort (Figure modified from ref. no. [135]).

4.4.2 Discussion

Low to moderate associations between estimated acrylamide intakes by FFQ data and biomarkers have been shown in several studies [45,48,135,136]. This is for instance explained by that the used FFQs are not designed to specifically measure acrylamide intake and that acrylamide is present in a broad range of food items. Further, the concentration of acrylamide in food-stuffs varies between brands and even between batches [137].

It was shown in the Malmö Diet and Cancer (MDC) cohort that the variation between individuals in the internal dose of acrylamide assessed with Hb-adducts was approximately 10 times lower than by FFQ assessed intakes [48]. The most comprehensive study (n=828) has rejected questionnaire data as a suitable source for estimations of acrylamide intake from food when the FFQ was evaluated with Hb-adduct measurements [45]. However, as biomarkers do not describe the same time-window of exposure as FFQs do, this must be taken in account in the evaluation. Hb-adducts is a measure of the mean exposure for the last months, FFQ data, on the other hand, normally describes the mean intake over the last year.

Table 5. Exposure studies on hemoglobin (Hb) adduct-levels of AA and GA in humans.

Reference	***Exposure/exposed***	***Subjects***	***AA-adducts (pmol/g globin)***		***GA-adducts (pmol/g globin)***	
		(n)	*range*	*median*	*range*	*median*
Bergmark *et al.* 1997	smoker	10	27-148	mean=116	n.a.	
	non-smoker	8	24-49	mean=31	n.a.	
Schettgen *et al.* 2002	smoker+non-smoker	72	<11-294		n.a.	
Paulsson *et al.* 2003	non-smoker	5	27±6 (mean±SD)		26±6 (mean±SD)	
Schettgen *et al.* 2004	mother/	11	18-104	21	n.a.	
	child	11	6-43	10	n.a.	
Schettgen *et al.* 2004	smoker	16	25–199	44	22-119	44
	non-smoker	13	7–31	18	9-23	18
Hagmar *et al*. 2005 and	smoker	72	30-420	152	n.a.	
Wirfält *et al.* 2007	non-smoker	70	20-100	31	n.a.	
Kütting *et al.* 2005	smoker	1	104		n.a.	
	non-smoker	9	18-34	20	n.a.	
Sherer *et al.* 2007	smoker	264	84±42 (mean±SD)		n.a.	
	non-smoker	100	28±7 (mean±SD)		n.a.	
Urban *et al*. 2006	smoker	60	82±44 (mean±SD)		n.a.	
	non-smoker	60	28±7 (mean±SD)		n.a.	
Vesper *et al.* 2006	male, smoker+non-smoker	70	27-453	133	27-240	92
	female, smoker+non-smoker	26	31-325	84	36-239	128
Chevolleau *et al.* 2006	male, smoker	8	24-119	56	15-57	30
	male, non-smoker	21	10-70	31	13-45	21
	female, smoker	8	16-163	27	15-62	28
	female, non-smoker	31	9-30	22	12-47	23
Vesper *et al.* 2007	smoker+non-smoker	161	7-610		4-364	
Bjellaas *et al.* 2007[l)]	smoker	6	99-211	166	29-99	83
	non-smoker	44	18-66	37	7-46	18
Kütting *et al.* 2008	smoker	130	ca 8-325[b)]		n.a.	
	non-smoker	828	ca 2-103[b)]		n.a.	
Thonning Olesen *et al*. 2008	smoker	264	18-90[c)]	35[c)]	19-128[c)]	59[c)]
	non-smoker	484	35-273[c)]	123[c)]	8-49[c)]	21[c)]

Vesper *et al.* 2008	male, non-smoker	120	24.1–97.8[c)]	43[c)]	15.6–80.0[c)]	38.7[c)]
	female, non-smoker	135	24.2–85.5[c)]	41.7[c)]	21.0-86.7[c)]	40.7[c)]
	male, smoker	120	45.4–280[c)]	127[c)]	33.7-193[c)]	84.8[c)]
	female, smoker	135	43.7–294[c)]	117[c)]	35.3-219[c)]	100[c)]
Wilson *et al.* 2009a	male, non-smoker	331		32-56[e)]		
Wilson *et al.* 2009b	female, non-smoker	296		43.9		49.4
Vikström *et al.* 2010	male, non-smoker	161	16-179		5-122	
(Paper III)	male, smoker	19	65-275		25-200	
Vikström *et al.* 2010	male, non-smoker	33	39	16-97	39	21-160
(Paper IV)	female, non-smoker	35	38	16-89	38	19-158
	male, smoker	29	139	23-148	139	38-341
	female, smoker	39	164	36-427	164	28-375
Vesper *et al.* 2010	male, non-smoker	ca. 2650	48-51[c)]	GM=50	48-52[c)]	GM=50
	female, non-smoker	ca. 3100	48-53[c)]	GM=51	49-55[c)]	GM=52
	male, smoker	ca. 850	79-96[c)]	GM=108	79-96[c)]	GM=87
	female, smoker	ca. 500	95-115[c)]	GM=120	95-115[c)]	GM=104

n. a. = not analysed, GM = geometric mean
a) before and after controlled intake of potato chip
b) estimated from figure
c) between 5% and 95%
d) estimated from median values
e) between 25% and 75%

4.5 Variation of acrylamide- and glycidamide-adduct levels between and within individuals (Paper III and IV)

4.5.1 Hb-adduct levels of acrylamide and glycidamide in humans

The variation of acrylamide-adduct background levels in non-smokers in different studies is approximately between a factor of 5 [46,48] up to 20 [45], in the range of 5-100 pmol/g globin. Analysis of glycidamide-adducts have reported background levels generally below 100 pmol/g globin [46,47]. (For a summary of studies on Hb-adduct levels of acrylamide and glycidamide in smokers and non-smokers, see Table 5 (refs. included in table:. [42,44-48,54,76,93,136-146]).

When, Hb-adduct levels in different populations was compared, significant differences (p<0.5-0.001) between country groups (9 European countries) of non-smokers (n=255) was shown [76]. Also, differences between ethnic groups in the U.S. have recently been described [54]. More important was the overall increase of both acrylamide- and glycidamide-adduct levels with decreasing age groups in this study [54], supporting previous reports on higher acrylamide intakes in children than in adults [52-54].

4.5.2 Variation between individuals (Paper III and IV)

The acrylamide- and glycidamide-adduct levels measured in two large cohorts, CAPS and MDC are discussed in this thesis. The CAPS cohort of only men is described in section 4.4.1.

In the subsample from the MDC cohort both non-smoking and smoking men and women were included. Participants had been selected to obtain maximal variation in the acrylamide-exposure from food (based on FFQ data) [48]. The results from the MDC cohort showed no difference between males and females of Hb-adduct levels of acrylamide or glycidamide (Paper IV). As expected, smokers in both studies showed higher adduct-levels than non-smokers. The glycidamide-adduct to acrylamide-adduct-level ratios were higher at the lowest acrylamide adducts levels in both studies (Paper III and Paper IV) (Figure 7). The median value of acrylamide adducts was somewhat lower in the CAPS cohort than in the MDC cohort (31 and 39 pmol/globin, respectively). When non-smokers were divided in two groups by the median values of acrylamide adduct-levels in respective study, the groups with the lowest acrylamide adduct levels showed the highest ratios of glycidamide to acrylamide adducts.

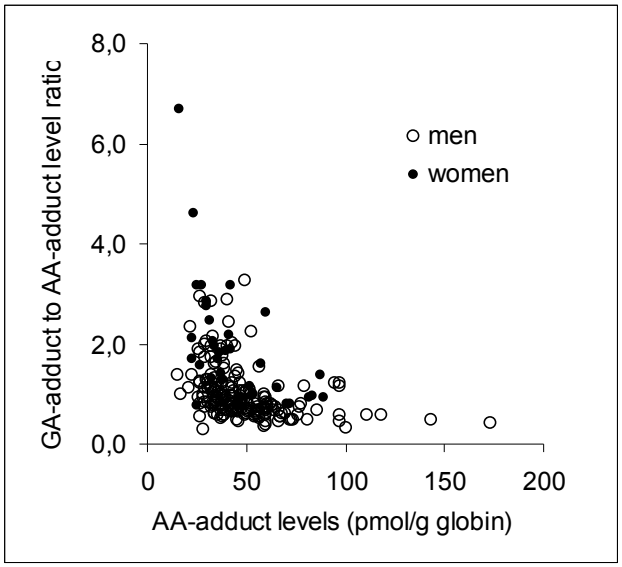

Figure 7. Acrylamide (AA)-adduct levels and corresponding glycidamide (GA)-adduct to AA-adduct-level ratio in all non-smoking subjects included in the MDC and the CAPS cohorts (194 men and 34 women).

4.5.3 Variation over time (Paper IV)

The variation over 16 months of acrylamide-adduct levels in 14 subjects, men and women, was determined from Hb-adduct analysis from three sampling occasions. The variations of adduct levels and of the ratio of glycidamide to acrylamide adduct-levels over time within individuals were as large as the variations between individuals (Table 2 in Paper IV). That adduct levels and the ratio varies over time, in some individuals with a factor of two between sampling occasions, might contribute to the explanation of poor associations of adduct measurements and questionnaire data. That the in vivo dose (AUC) of glycidamide, the genotoxic component in acrylamide exposure, varies over time and not in co-variation with acrylamide (see Figure 8), further contributes to the weak, if any, association between acrylamide intake and cancer incidence in epidemiological studies.

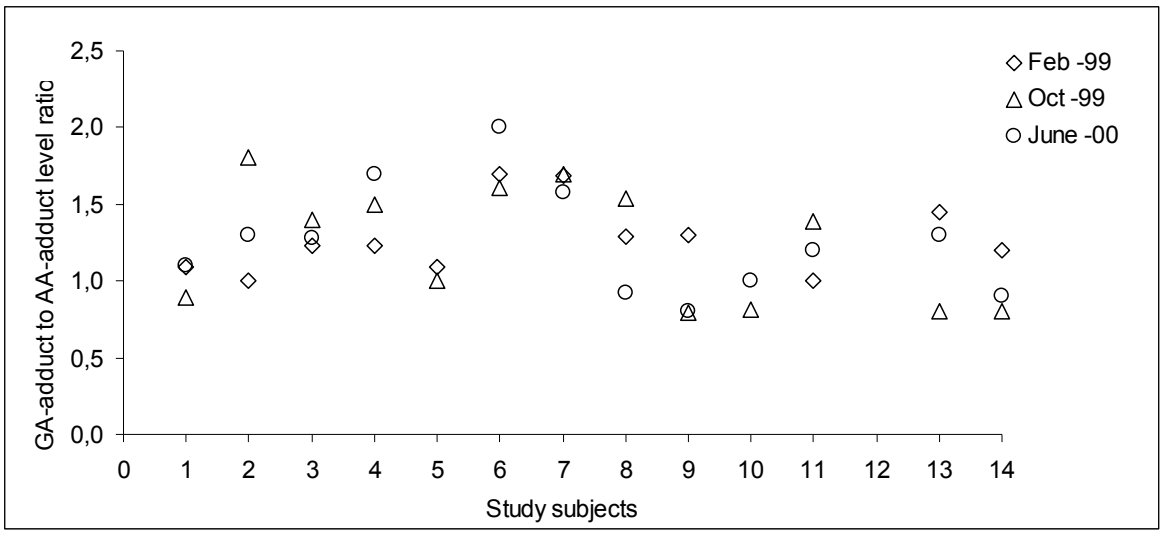

Figure 8. Variation over 16 months of the ratio of glycidamide (GA)-to-acrylamide (AA)-adduct levels in 13 subjects; two-three sampling occasions per subject with 8 months between.

4.6 Factors with an influence on the acrylamide biotransformation to glycidamide (Paper III)

4.6.1 Variation in the adduct-level ratio of glycidamide to acrylamide

Variations of acrylamide-adduct levels between individuals are mainly a result of different intake levels via food. The variation of glycidamide-adduct levels and in the glycidamide-adduct to acrylamide-adduct-level ratio are however due to differences in the formation/elimination of glycidamide. Glycidamide exposure via food could possibly have contributed to the variation, but only extremely low levels (≤1.5 μg/kg) of glycidamide has been suggested to be formed in food [147]. No other exposure sources are known, but it has been indicated that acrylamide might be produced endogenously, though at negligible low levels [148]. It has been discussed that environmental tobacco smoke would be a possible source of acrylamide exposure. In Paper IV, association between GA-adduct levels and passive smoking was shown in 72 non-smokers.

4.6.2 Polymorphism in genes coding for metabolic enzymes

An explanation to the differences in the metabolic rate of acrylamide to glycidamide could be polymorphisms in the genes coding for metabolic enzymes (for metabolism of acrylamide, see Figure 5), which lead to individual differences in the response to exposure of certain chemicals. Several of the enzymes suggested to be involved in the acrylamide metabolism are known to be polymorphic in humans due to common genetic variants as deletions of specific genes or differences in the coding sequences (e.g. [87,97,149]). From in vitro studies with human blood it has been

suggested that conjugations of acrylamide and of glycidamide with glutathione (GSH) are primarily non-catalyzed [150]. These results have been supported in a study on humans [94,97,149]. Nevertheless, a small study (n=49) have showed positive associations between glycidamide-adduct to acrylamide-adduct-level ratios and the GSTM1 null and GSTT1 null genotypes in non-smokers [151].

4.6.3 Influence on the acrylamide metabolism

If polymorphism is not the main explanation to the variations seen in the ratio between glycidamide and acrylamide-adduct levels, other factors must have an influence on the acrylamide metabolism. Factors suggested to influence the glycidamide- to acrylamide-adduct-level ratio are body mass index [76] and gender [76,104]. Other substances in food stuffs could also have an influence on acrylamide metabolism, for instance diallyl sulfide, an ingredient in garlic [152,153] and alcohol [76]. Natural remedies, medications and other compounds in food that are biotransformed via CYP2E1 have the possibility to affect the acrylamide metabolism.

4.6.4 Alcohol influence on acrylamide to glycidamide biotransformation (Paper III)

In Paper III, we examined the effect of alcohol on acrylamide metabolism. Hb adducts of acrylamide and the corresponding glycidamide-adduct to acrylamide-adduct-level ratio were compared to questionnaire data on alcohol intake. The ratio was used as a measure for individual differences in metabolism. It would have been of interest to study other substances than alcohol in relation to acrylamide biotransformation. Though, it is generally difficult to get detailed data on intakes of specific compounds from commonly used questionnaires. Further, for a study on influence on biotransformation, the mechanism of interaction needs to be known. The pitfalls of using FFQ data for acrylamide-intake calculations have previously been discussed, there are difficulties with estimation of intake of other substances by questionnaire data as well. For instance, it is generally considered that people tends to underestimate their alcohol intake. However, it seems as questionnaire data could be used to correctly classify alcohol consumption by groups (e.g. ref. no. [154]).

We used Hb-adduct data from the CAPS study, from which we had presented data on the correlation between Hb adducts of acrylamide and acrylamide intake assessed by FFQs in non-smokers (see section 4.4.1 and ref. no. [146]). As alcohol induces CYP2E1 in humans [155], we expected to se an increase in the ratio of glycidamide-to-acrylamide adduct levels with increased intake of alcohol. However, the study showed a possible competitive mechanism as, the ratio decreased with increased alcohol intake. An interesting observation

was that when the intake was stratified by acrylamide-adduct levels (cut at the median value) the trend only remained in men with the lowest levels (see Table 3, Paper III). That the impact of other substances on the acrylamide to glycidamide biotransformation is measurable at the lowest acrylamide-adduct levels, could explain that the variation in the ratio is highest at these low levels (see Figure 2, Paper III). The influence of alcohol on acrylamide metabolism was supported by the association between glycidamide-adduct levels and alcohol in non-smokers in Paper IV.

4.7 In vivo dose (AUCs) of acrylamide and glycidamide in humans exposed to acrylamide via food (Paper V)

One main aim of this thesis was to determine in vivo doses (AUC) of acrylamide and glycidamide in humans exposed to acrylamide via food (Paper V) and compare these to the approximated AUCs in cancer-test rats (see Paper I). In Paper V, non-smoking men and women were exposed to acrylamide via food ("Study 1") or via potato chips ("Study 2"). Blood samples were collected at the start and the end of exposure periods and Hb-adducts of acrylamide and glycidamide were analyzed and further used for AUC calculations. The participants in Study 1 were exposed to an acrylamide-rich diet during four days; mean daily intakes were approximately 11 μg/kg bw. In Study 2 participants increased there acrylamide intake by addition of 160 mg potato chips per day, corresponding to approximately 2.5 μg acrylamide/kg bw and day, to baseline diets during 28 days. Second-order reaction rate constants for formation of Hb-adducts of acrylamide and glycidamide were carefully determined (Figures 1 and 2 and Table 4 in Paper V). From adduct-level increments, adjusted for the increase/decrease of Hb and determined rate constants the AUC were inferred (Table 6). The differences of the calculated AUCs were of a factor of two between the studies, both for acrylamide and glycidamide. However, we judged the results from Study 1 to be more accurate as the intake of acrylamide was better controlled than in Study 2, for instance was the exposure period shorter in Study 1, and chemical analysis of all food products was included (cf. paper V, and ref. no. [156]).

The main result from these two studies was that the in vivo dose (AUC) of glycidamide per exposure dose of acrylamide was nearly 2-fold higher in humans exposed via food than in the rats exposed at 10^3 higher levels. In humans, the AUC of acrylamide and glycidamide per exposure dose of acrylamide were higher in Paper V than previously estimated by Törnqvist et al. in 1998 [121]. The results from these two studies included in this thesis indicate that the preliminary risk estimate from 1998 has been strengthened.

Table 6. The in vivo doses (AUC) of acrylamide (AA) and glycidamide (GA) per exposure dose acrylamide (nMh/µg AA per kg bw) in rats (Paper II) and humans (Paper V). Both studies in Paper V show higher AUC values than estimated by Törnqvist et al in 1998 [121]. The AUC for GA in humans (Paper V) is comparable to the AUC in rats, exposed at acrylamide levels as in the cancer tests.

	AUC per exposure dose (nMh/µg AA per kg bw)	
	AA	GA
Studies in the rat		
Paper II: ♀ / ♂	48/34	24/18
Mean	**41**	**21**
Törnqvist et al. 1998	28	16
Studies in humans		
Paper V: Study 1, High	212	49
Study , Medium	120	21
Mean	**166**	**35**
Törnqvist et al. 1998	94	28

5 Overall conclusions

5.1 Variations of Hb-adduct levels

Hb-adduct levels of acrylamide and glycidamide were studied in two relatively large groups in this thesis work. The main conclusion from the studies on variation between non-smoking individuals is that the acrylamide- and glycidamide-adduct levels vary with a factor of approximately 5 and 8, respectively. The data obtained in Paper V show that intakes of an acrylamide-rich meal at single occasions could result in 30% increase of the acrylamide-adduct level (at mean adduct levels of about 30 pmol/g). The variation over time of adduct levels studied in a small group (n=14) was shown to be approximately a factor of two. More conspicuous, was the observation that the ratio of glycidamide-to-acrylamide-adduct levels varies over time. The primary explanation for the variation in this ratio is influences on the formation/elimination of glycidamide. As an association between alcohol intake and adduct data was shown in Paper III, we draw the conclusion that other substances in food could have an influence on glycidamide formation/elimination.

5.2 Glycidamide adduct dose-response to acrylamide exposure

At a group level, in non-smoking individuals the glycidamide-to-acrylamide-adduct level ratio increases with decreasing acrylamide intakes. This non-linear response, showed at the lowest intakes, has intriged us. At high acrylamide intake levels the relation between acrylamide- and glycidamide-adduct levels is fairly stable, approximately around 0.7 (Paper V).

One of few animal studies with exposure levels relevant for human acrylamide intakes via food is the mice study described in Paper I. Mice were exposed to acrylamide via food at 3-50 μg/kg bw during one month. No obvious deviation from linearity of glycidamide–adduct levels from acrylamide exposure was shown in the mice. Evaluation of the data in Paper V made us suggest an alternative explanation the non-linear relationship in humans. There might be a contribution to the glycidamide-adduct level from another source than dietary acrylamide. An “extra background” level from glycidamide would give a relatively larger contribution at low adduct levels.

The observed non-liniarity complicates the risk assessment. At the present state of knowledge, the best approximation of the glycidamide-to-acrylamide AUC ratio was achieved in Paper V from the experiment with controlled intake.

5.3 Evaluation of the use of questionnaire data for intake calculation

Evaluation of the variations in adduct levels further contributes to the explanation on why Hb-adducts and FFQ data have shown low associations at an individual level.

To evalute the applicability of questionnare data and of Hb-adduct measurements we compared the acrylamide intake estimated from dietary history to intake calculated from adduct data in the MDC cohort (Paper IV). As expected, the correlation between the intake estimates from dietary history and the intake calculated from adduct data on an individual level was weak, as shown in Figure 10. However, when calculated at a group level, the figures for the two measures were comparable. The conclusion is, that questionnaire data or Hb-adduct data of acrylamide could be used to estimate mean intakes for a population. At an individual level, it would be possible to discriminate between individuals with high and low intakes with both methods, but difficult to assess small differences. Further, at an individual level both questionnaire data on acrylamide intake or Hb-adduct measurements from single blood samples are probably not sufficiently accurate to be used in studies were intake is compared to biological endpoints in cohort studies.

5.4 In vivo doses (AUCs) determined in humans and rats

The aim of this thesis was to contribute to improved quantitative data for cancer risk assessment of acrylamide exposure via food. Determination of in vivo doses (AUC) in rats exposed at the same acrylamide levels as rats in cancer tests was a first step (Paper I). The AUCs of acrylamide and glycidamide were further measured in humans after controlled acrylamide exposure via food (Paper V). To get as accurate values as possible, two studies with different exposure scenarios were used. The AUCs of acrylamide and glycidamide per given dose of acrylamide were somewhat higher in humans than in the rats exposed at 10^3 times higher levels (Table 6). By these studies the quantitative data for interspecies extrapolation was improved compared to the data from 1998 (Table 6). In vivo dose data generated within this thesis strengthen the previous risk estimate. The measured AUCs reflect the net effect of all enzymatic, chemical and physiological factors on rates of

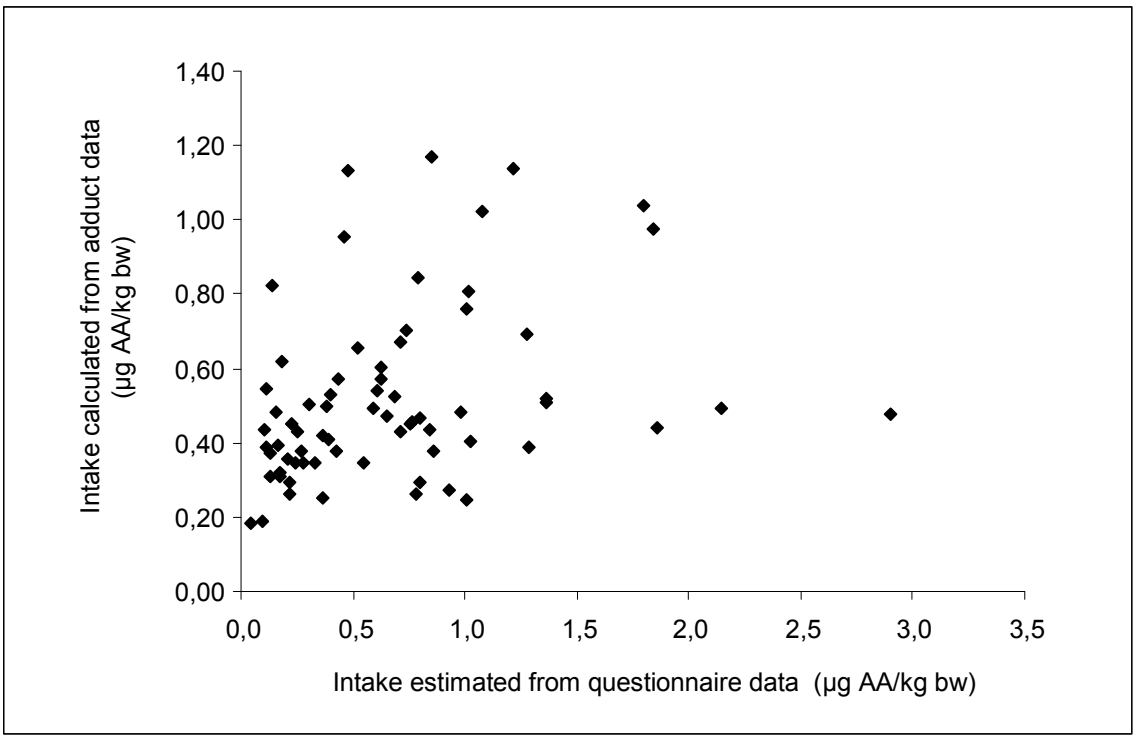

Figure 10. Intake of acrylamide (AA) estimated from questionnaire data (µg/kg bw) versus intake calculated from adduct data (pmol/per µg AA/kg bw). (Data from non-smokers in the MDC study in Paper IV.)

formation and elimination. Therefore no default assumptions have to be used in the inter-species extrapolation. Considering the rather high risk estimates (Table 3), there are a few precautions: the quantitative extrapolation from animal cancer tests to humans, using the relative risk model, so far is only demonstrated for ionizing radiation. Furthermore, we cannot exclude that there is some overestimation in the risk estimate due to overrepresentation of tumors in sensitive sites in the rat.

5.5 Perspectives on acrylamide exposure

Acrylamide is only one of many potentially caricinogenic compounds that we are exposued to via food. Others are naturally occurring substances, additives or are naturally formed during food processing. Besides acrylamide, many of the other compunds formed in the Maillard reaction are considered to be potentially toxic or potential carcinogens. Hundreds of chemicals have been estimated to be formed in the Maillard reaction or by lipid oxidation (see the Heatox project, ref. no. [34]).

One way to relate exposure of a compound to its associated risk, is the margin of exposure (MOE) approach. MOE is simply the ratio between the dose leading to tumor formation in experimental animals and the human intake, and hence reflect but do not define the risk [157]. The MOE value should be as large as possible, figures of MOEs of 10 000 or higher could be considered

as a low level of concern according to EFSA [157]. The MOE can be estimated by different methods, but the most conservative figures for acrylamide are 130-280 [158]. These values are for instance comparable to those of aflatoxin, but 1000 times lower than the figures for benzo(*a*)pyrene [158].

We are all exposed to acrylamide as it is present in such a broad range of food products. As heated food is a part of our daily diet it is impossible to totally exclude the acrylamide exposure. However, as showed in this thesis (paper V), there is linear dose-response between acrylamide intake and in vivo doses (AUC) of glycidamide in humans, therefore, reducing the highest intakes of acrylamide would be beneficial in a risk perspective. Further, when reducing acrylamide in diet probably levels of many other potential carcinogens formed in the Maillard reaction will decrease as well.

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