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## **Differences in hemoglobin adduct levels of acrylamide in the general population with respect to dietary intake, smoking habits and gender**

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**Key words:** Acrylamide; dietary intake; hemoglobin adducts

**Abstract**

The variation in dietary exposure to acrylamide (AA) has been studied through measurement of hemoglobin adduct levels from AA, as a measurement of internal dose, in a sample from the blood bank of the Malmö Diet and Cancer cohort (n=28,098). The blood donors are well characterised with regard to their food habits, and 142 individuals were selected to obtain highest possible variation in the adduct levels from AA (none, random or high intake of coffee, fried potato, crisp bread and snacks, food items estimated to have high levels of AA).

Among 70 non-smokers the AA-adduct levels varied by a factor of 5, and ranged between 0.02 and 0.1 nmol/g, with considerable overlap in AA-adduct levels between the different dietary groups. There was a significant difference between men with high dietary exposure to AA compared to men with low dietary exposure ( $P = 0.04$ ). No such difference was found for women. As expected a higher level (range: 0.03 – 0.43 nmol/g) of the AA adduct, due to AA in tobacco smoke, was found in smokers. Smoking women with high dietary exposure to AA had significantly higher AA-adduct levels compared to smoking women with low dietary exposure ( $P=0.01$ ). No such significant difference was found in smoking men. The median hemoglobin (Hb)- adduct level in the randomly selected group of non-smokers was compatible with earlier studies (0.031 nmol/g).

The variation in the average internal dose, measured as Hb adducts, was somewhat smaller than estimated for daily intake by food consumption questionnaires in other studies. Thus, the observed relatively narrow inter-individual variation in AA-adduct levels means that estimates of individual dietary AA intake have to be very precise if they should be useful in future cancer epidemiology.

## 1. Introduction

The finding that the industrial chemical substance acrylamide (AA) is formed in high amounts during cooking of food [1,2] led to concerns about exposure and health risk for the general population [3]. AA is a known neurotoxic and clastogenic substance, shown to be carcinogenic in animals and, therefore, classified as a probable human carcinogen [4,5].

The chemical reactivity of AA, an  $\alpha,\beta$ -unsaturated carbonyl compound, and its epoxy-metabolite glycidamide (GA) plays a crucial role on the toxic potency of AA. Both compounds are reactive towards biomacromolecules [6], however, AA being much less reactive than GA towards DNA [7-9]. This fact, together with the genotoxicity of GA, has led to the assumption that GA is the genotoxic agent and probably also the cancer-risk increasing factor in AA exposure (e.g. [10]).

Exposure to AA in work environments has been studied by measurements of stable reaction products (adducts) to hemoglobin (Hb), as a measure of internal dose [11-15]. In exposed workers dose-response relationships between Hb adducts and neurotoxic effects have been demonstrated [14,16]. Hb adducts accumulate over the life span of the erythrocytes and during chronic exposure the adduct level reflects the average exposure/internal dose during the last months. Other measures of exposure of AA, so far rarely used, are mercapturic acids in urine, or free AA in plasma and urine, reflecting the exposure during the last days [16,17].

Indeed, Hb adducts was the tool which led to the finding of AA formation in food. The observation of a regularly occurring background level (about 0.03 nmol per g globin) of adducts from AA to N-termini in Hb in non-smoking and occupationally unexposed control persons indicated the existence of another general exposure source [12,14]. The tracing of this unknown exposure source led to the demonstration of the formation of AA during cooking at elevated temperatures (cf. [18]).

A temperature-dependent formation of AA in human foodstuffs during cooking (at temperatures above 120° C) was demonstrated and carbohydrate-rich foods, particularly potatoes, showed very high AA levels (up to mg per kg) [2]. Follow-up studies verified the findings (e.g. [19]) and today data bases on levels of AA in food are available (e.g. [20]). It has been shown that formation of AA involves the Maillard reaction, and that asparagine in reactions with reducing sugar, particularly fructose, is the likely source of AA [21,22].

The dietary intake of AA has been estimated on the basis of AA levels measured in food and food consumption statistics. The average intake for adults in Sweden, the Netherlands and Norway has been estimated to approximately 0.5 µg/kg body weight (bw) per day (i.e. about 35 µg/day) [23-25]. A higher intake was estimated for children [24,25]. According to Svensson et al. the main source of the average AA intake in Sweden is coffee, contributing to about 40 % [23]. Potato products (potato crisps, French fries, fried potato products), contribute to about 35 % of the total intake; bread, crisp bread and biscuits to about 20 %, snacks (except potato crisps) to a few percent, and breakfast cereals contribute to a few percent. In the Dutch population potato crisps are estimated to give the largest contribution to the AA intake [24].

The cancer risk from AA has been estimated using data from cancer tests on rats, using different risk models. According to WHO, a life-time daily intake of 1 µg/kg bw AA will result in a life-time excess risk of approximately 0.7 cancer cases per 1000 individuals [26]. This is very similar to an estimate from the Scientific Committee of the Norwegian Food Control Authority, of around 1.3 cancer cases per 1000 (see [25]), while the model from US EPA results in an excess risk of 4.5 per 1000 [27]. A multiplicative model has been shown to be adaptable to the cancer test data with AA, and a preliminary risk estimation of AA according to this model, based on doses of GA in vivo, has indicated a somewhat higher risk than US EPA [28,29].

From the existing cancer risk estimates of AA, which still need to be improved, it could be inferred that dietary exposure to AA is not negligible from a population perspective. On the other hand the estimated risk to the individual is not very high. No associations have been observed between dietary AA exposure and cancer risk in the epidemiological studies so far carried out [30-33]. However, these studies have used indirect estimates of dietary intakes, and have been criticized for having too low statistical power to be of relevance for assessing the low risk enhancements that could be expected from animal-based risk models [34,35]. A cancer risk from an evenly distributed risk factor in the population could hardly be detected and assessed by standard epidemiological methods, even if it contributes to the overall cancer incidence. It is a prerequisite for performing conclusive epidemiological studies that there is enough exposure contrast within the population.

The data available up to now is insufficient for a picture of the distribution of the dietary AA exposure in the general population. The aim of the present study was therefore to estimate the highest possible variation in AA adduct levels in the general Swedish population by measurement of Hb-adduct levels in samples from a blood bank of donors, well characterized with regard to their food habits. In the selection of individuals for study, also considering smoking habits and gender, subjects were contrasted with regard to estimated AA intake. Subjects with extremely high intakes of food items (coffee, fried potato, crisp bread and snacks) estimated to give a large contribution to the total intake of AA, were compared with subjects with no consumption of these food items, and with randomly selected control subjects.

## **2. Subjects and methods**

### *2.1 Malmö Diet and Cancer Cohort*

The baseline examinations of the Malmö Diet and Cancer (MDC) cohort (n=28,098) were conducted from March 1991 until October 1996 [36]. Eligible participants were men in the

age-range 46 to 73 years, and women in the age-range 45 to 73 years, living in the City of Malmö (the third largest city of Sweden) and with Swedish reading and writing skills. Participants visited the study centre twice. During the first visit, the study procedures and questionnaires were explained, direct measurements made and blood samples collected. Two weeks later, the questionnaires completed at home were reviewed and the diet history interview conducted. The Ethical committee at Lund University has approved the study.

## *2.2 Dietary data*

Information on “usual” dietary habits was collected through a modified diet history method, combining a menu-book (recording of cooked meals during 7 days) and a diet history questionnaire [37]. In the menu book, participants recorded cooked meals, cold beverages (i.e., milk, juice, soft drinks, water and alcoholic beverages), drugs, natural remedies, and dietary supplements during seven consecutive days. In the diet history questionnaire the general meal pattern and the frequency and portion-size information of foods consumed regularly and with low day-to-day variation (i.e., hot beverages, sandwiches, edible fats, breakfast cereals, yoghurt, milk, fruits, cakes, candies and snacks) were recorded. The reference period of the questionnaire was the preceding year. The food group variables considered in this study were coffee, crisp bread (incl. both high and low fibre varieties), fried potato (incl. pan-fried and deep-fried potato), and snacks (incl. potato crisps and other types of salted snacks).

Mis-reporting of intake is an ongoing controversy in studies using self-report instruments to collect dietary information in epidemiological studies [38,39]. Goldberg et al. [38] have suggested that the ratio between energy intake (EI) and Basal Metabolic Rate (BMR) obtained from the equations recommended by World Health Organisation [40], may be used to identify individuals who report too low or too high habitual energy intakes. A ratio of 1.35 has been suggested as the lower cut-off for reasonable habitual energy intakes. This study excluded individuals below the lower cut-off (potential “under-reporters”) and those above the 99th

percentile of the EI-BMR ratio (potential “over-reporters”). Consequently, a total of 7,128 men and 10,176 women were considered for the present study.

### *2.3 Other variables*

Information on age and gender was obtained through the person-identification number. Smoking habits were in the MDC collected through a self-administrated questionnaire.

### *2.4 Selection of study sample*

Study participants were selected from four strata defined by gender and smoking status. Current smokers were contrasted against never smokers and former smokers that had not smoked for the last year. First, ten individuals from each of the four categories were selected at random. Secondly, individuals not reporting any consumption for either of the four food groups were identified, and five zero-consumers were selected at random from each sex-smoking status category. Thirdly, individuals of each category were ranked on their reported food group consumption. The five highest-ranking individuals of each food group variable were identified and included in the study sample. Because some difficulties occurred in finding individuals with high consumption of only one food group in the female smoking category, a decision was made to select six female smokers with high consumption of snacks. In addition, one female smoker that ranked high on both coffee and fried potato, was included in analysis. Thus a study sample of 142 individuals distributed over three broad dietary-exposure categories for AA was identified: random (n=40), low (n=20) and high (n=82). Seventy-two were smokers and 70 non-smokers.

### *2.5 MDC biobank*

Non-fasting blood samples had been collected, separated, frozen, and stored in different fractions at -80 °C and -140 °C [41,42]. The MDC biological bank is in good condition. The



quality control program has been evaluated, and did not reveal any quality problems of blood components [41,42].

### 2.6 Analysis of Hb adducts from acrylamide

The stored deep-frozen erythrocyte fractions for the selected subjects (n = 142) were prepared for analysis of Hb adducts according to the N-alkyl Edman method described earlier ([43] with modifications and materials for AA analysis according to [12]).

The thawed erythrocyte sample was diluted with purified water, added to acidic isopropanol and centrifuged. The pellet (the cell residues) was discarded and the globin was precipitated from the supernatant by addition of ethyl acetate [44]. The globin samples were derivatized with pentafluorophenyl isothiocyanate (PFPTC) leading to detachment of the AA adduct to the N-terminal valine in Hb as *N*-(2-carbamoylethyl)valine-PFPTH (AA-Val-PFPTH). A deuterated internal standard, AA-d<sub>7</sub>Val-PFPTH, was added (ca. 0.1 nmol/g globin) to the derivatized samples before the isolation by extraction and further purification of the formed AA-Val-PFPTH.

The analyses (one analysis per sample) were performed with a GC-MS/MS (Finnigan TSQ 700) in the negative ion/chemical ionization (NICI) mode. For quantification, single reaction monitoring (SRM) was used and the intensity ratio of selected product ions from AA-Val-PFPTH and AA-d<sub>7</sub>Val-PFPTH was compared with a linear calibration curve (prepared from in vitro alkylated Hb; range 0 – 1 nmol/g, duplicate samples of eight points,  $r^2 = 0.998$ ). The limit of detection was estimated to about 0.006 nmol/g globin (valid for 40 mg derivatized globin samples dissolved in 50  $\mu$ L toluene from which 1  $\mu$ L injected into the GC-MS/MS). The coefficient of variation (CV) for analysis of blood samples for AA-adduct levels up to 0.1 nmol/g globin, was less than 10%. These estimations are valid for samples prepared and analyzed on the same occasion and the use of internal standard from the same batch, that are the conditions applied in this study.

## 2.7 Statistics

Comparisons between the groups were made with Mann-Whitney's U-test, and  $P < 0.05$  was considered as statistically significant.

## 3. Results

Among non-smokers, the AA-adduct values approximately varied with a factor of 5, ranging between 0.02 and 0.10 nmol/g (Figure 1). Men with an estimated high dietary exposure to AA had significantly higher AA-adduct level compared with men with lower estimated dietary exposure (medians 0.043 and 0.030 nmol/g;  $P = 0.04$ ). However, no such difference was seen for women (medians 0.040 and 0.041 nmol/g;  $P > 0.5$ ).

Among smokers much higher AA-adduct levels were observed compared with non-smokers (Figure 2). Moreover, within the group of smokers the AA-adduct levels approximately varied with a factor of 10, ranging between 0.03 and 0.43 nmol/g. Smoking women with a high dietary exposure to AA, had significantly higher AA-adduct levels compared with smoking women with a low dietary exposure (medians 0.186 and 0.079 nmol/g;  $P = 0.01$ ). However, no such clear-cut difference was seen for smoking men (medians 0.146 and 0.093 nmol/g;  $P = 0.15$ ).

The median AA-adduct level among the non-smoking randomly selected subjects was 0.031 nmol/g, whereas the corresponding figure among smokers was 0.152.

## 4. Discussion

A variation by a factor of 5 in the level of the background Hb adducts to N-termini from AA was obtained in the present study of 70 non-smokers, where individuals with high or extreme intakes of food-stuffs with high levels of AA were included. There is no study published earlier

where detailed information on dietary habits of donors in a blood bank has been explored to obtain extreme groups with regard to dietary intake of AA, for studies of variations in internal doses of AA measured through Hb adducts.

As expected non-smoking men with a high estimated dietary exposure of AA had significantly higher AA-adduct levels than men with no such dietary exposure, while in contrast such a difference was not seen for women. We have presently no good explanation for this gender difference. A more global assessment of the participants' dietary habits might shed some light on this discrepancy. It should also be noted that there was a considerable overlap in AA adducts between the different dietary groups. This means that it was not possible to predict the AA-adduct level for a certain individual, based on dietary grouping. A more detailed dietary modelling might enhance this predictability.

All previous data on the background level of the Hb adduct from AA [12-15, 45-46], except for the study by Schettgen et al. [47], came from the same laboratory as in the present study, using the same instruments and standards for quantification etc. In these published studies of altogether about 50 non-smoking individuals the adduct level varies between 0.02 and 0.07 nmol/g Hb. The variation in the background level of the adduct from AA to N-termini in Hb (0.02 – 0.10 nmol/g Hb) obtained in the present study was somewhat larger than found in earlier studies. Schettgen et al. had, however, obtained adduct levels ranging with a factor of 5 (0.012 – 0.05 nmol/g Hb) in a study of 25 non-smokers [47].

AA is mainly detoxified by conjugation with glutathione (GSH), but is also transformed to GA by P450 (CYP2E1) [48,49]. In vitro studies indicate no influence of GSH transferase (GST) in the detoxification of AA, which then would mean that polymorphism in GST has no influence on the variation of the level of Hb adducts from AA, i.e. the relation between intake dose and internal dose of AA [50]. The relatively narrow variation in the AA- adduct level obtained in this study also supports this view.

The present study is not evaluated in detail with regard to tobacco use; however, the higher adduct levels observed in smokers (Figure 2) are in agreement with earlier studies. In the study by Bergmark an average incremental AA- adduct level of about 0.006 nmol/g Hb per smoked cigarette and day was obtained [12]. This would indicate that continuous smoking of 5 cig/day, contributes to a level of the AA-adduct to N-termini in Hb of 0.030 nmol/g, i.e. approximately the average background level in non-smokers [12].

The tracing of background exposure through biomarker measurement requires a sensitive analytical method, that allows identification through mass spectrometry. Furthermore, the possibility of establishing absolute quantitative relationships between levels of biomarker (internal dose) and intake considerably strengthens the usefulness of such analytical techniques. In this context measurement of adducts to N-terminal valines in Hb by the method used, applicable in laboratory animals and humans, has many advantages [51]. Measurement of the adduct from AA to cysteine in Hb, which also has been applied in animal studies [52,53], is less useful (reviewed in [51]). Furthermore, studies are rendered difficult if the adduct is formed as an artefact which has been shown to occur in the case of ethylene oxide, but not for AA, during storage of blood samples. Therefore measurement of AA adducts in blood bank samples as in the present study is reliable.

During continuous exposure the adduct level in Hb reflects a steady-state level, which could be used for calculation of average daily increment in adduct level and average daily dose (defined as concentration  $\times$  time [54]) during preceding months the average intake could then be estimated, provided that the rate of elimination is known. In the case of AA, Calleman [55] has estimated the rate of detoxification in humans from studies at relatively high exposure levels to approximately  $0.15 \text{ h}^{-1}$ . Using detoxification rate and the rate constant for the formation of adducts determined by Bergmark et al. [11], a background level of 0.030 nmol/g Hb of the AA adduct would correspond to an intake of  $1.2 \mu\text{g/kg bw and day}$ . This is higher than the estimated intake ( $0.5 \mu\text{g/kg bw and day}$ ) from food consumption statistics and

measured AA levels in food. Additional data is needed to permit reliable comparisons of these estimations. However, at the present stage there is no reason to believe that there exists any other dominating source of the Hb adduct, which is evenly distributed over the population and masks the adduct level increment from dietary AA.

With regard to cancer risk estimation of AA, it could be considered important to measure the average daily intake for an estimation of the total life-time dose, assuming that a genotoxic action without threshold is the mechanism for increased cancer risk at low doses ([56], cf. [57]). The assumption that GA is the genotoxic agent in AA exposure implies that it is important also to measure the ratio between internal doses of AA and GA. The data so far published indicate that the ratio between the adduct levels to N-terminal valine of GA and AA is about 1:1 [11,45], however with a lower value obtained by Licea-Perez et al. [13]. Additional data is now generated by several laboratories (see [58]). A ratio of 1:1 between the adduct levels of GA and AA would according to rate constants determined by Bergmark mean that the dose (i.e. concentration  $\times$  time) of GA is about 30 % of the dose of AA [11]. The reaction-kinetic parameters underlying these calculations, however, have to be further evaluated.

According to Svensson et al. [26] the estimated daily intake of AA in Sweden varied with a factor of ca. 7 between the 5<sup>th</sup> and 95<sup>th</sup> percentile of the adult population. However, in the present study, where we in contrast to the study by Svensson et al., use data from a population sample of middle-aged and older adults with extreme intakes of specific foods, we demonstrated that the variation in the average internal dose is smaller. Although from a sample with a more narrow age range, our results may indicate that the cancer risk factor AA is more evenly distributed in the population than what could be deduced from Svensson et al. [26].

The knowledge about the rather narrow variation in the average internal dose of AA may in part explain the results obtained in epidemiological studies where the association between dietary AA and cancer in different sites has been studied. Cancer of the large bowel, bladder

and kidney has been assessed in two Swedish case control studies, which were originally created for evaluating the carcinogenic effects of heterocyclic amines from diet [30,31]. Individual dietary AA intakes were crudely estimated from FFQs and reported levels of AA in frequently consumed food products. No associations were observed between dietary AA and cancer risk for any of these three types of cancer. Moreover, in case-control studies from Switzerland and Italy there was no association between intake of fried potatoes and coffee (assumed markers of AA intake) and a number of neoplasms [32,33]. The caveat with these studies was, however, that the statistical power was far too low to exclude the weak risk excesses that could be expected from the animal based models [34,35].

The observed relatively narrow inter-individual variation in AA adduct levels in this population based study means that estimates of individual dietary AA intake have to be very precise if they should be useful in future cancer epidemiology. A prerequisite for applying dietary estimates in cancer studies must be that they are preceded by validation studies e.g. against AA adducts. Cancer risk estimation of dietary exposure to AA for humans has still to be based on toxicological studies, possibly in combination with epidemiological studies using biomarkers of exposure.

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## LEGENDS

**Figure 1.** Hb adducts of acrylamide (nmol/g) among non-smoking subjects with an estimated low or high dietary exposure to acrylamide, and among subjects randomly selected from the study base. Filled circles represent men and unfilled circles represent women. The medians are shown by horizontal bars.

**Figure 2.** Hb adducts of acrylamide (nmol/g) among smokers with an estimated low or high dietary exposure to acrylamide, and among smokers randomly selected from the study base. Filled circles represent men and unfilled circles represent women. The medians are shown by horizontal bars.

Fig1

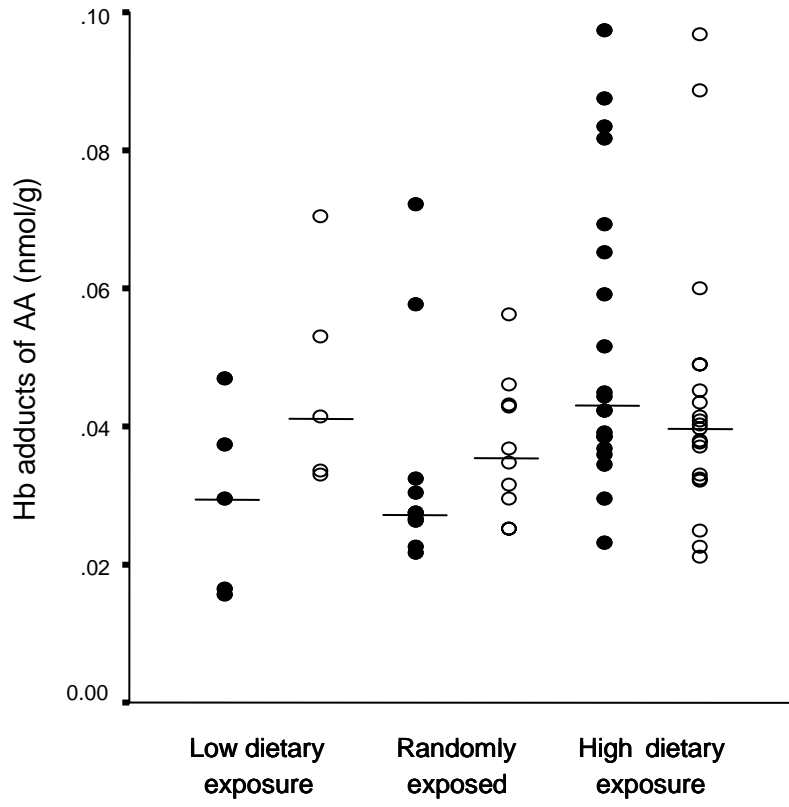


Fig. 2

