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Hemoglobin Adducts and Mercapturic Acid Excretion of Acrylamide and Glycidamide in One Study Population

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The aim of this study was to determine the relationship between the oxidative and reductive metabolic pathways of acrylamide (AA) in the nonsmoking general population. For the first time both the blood protein adducts and the urinary metabolites of AA and glycidamide (GA) were quantified in an especially designed study group with even distribution of age and gender. The hemoglobin adducts *N*-carbamoylethylvaline (AAVal) and *N*-(*R*,*S*)-2-hydroxy-2-carbamoylethylvaline (GAVal) were detected by GC-MS/MS in all blood samples with median levels of 30 and 34 pmol/g of globin, respectively. Concentrations ranged from 15 to 71 pmol/g of globin for AAVal and from 14 to 66 pmol/g of globin for GAVal. The ratio GAVal/AAVal was 0.4−2.7 (median = 1.1). The urinary metabolites were determined by LC-MS/MS. Of all urine samples examined 99% of *N*-acetyl-*S*-(2-carbamoylethyl)-L-cysteine (AAMA) levels and 73% of *N*-(*R*/*S*)-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA) levels were above the LOD (1.5 μg/L). Concentrations ranged from <LOD to 229 μg/L (median = 29 μg/L) for AAMA and from <LOD to 85 μg/L (median = 7 μg/L) for GAMA. The ratio of GAMA/AAMA varied from 0.004 to 1.4 (median = 0.3). Using hemoglobin adduct levels in blood and mercapturic acid excretion in urine for calculation of daily AA intake gave practically identical values. The median daily intakes were 0.43 (0.21−1.04) μg/kg of body weight(bw)/day using Hb adducts and 0.51 (<LOD−2.32) μg/kg of bw/day using mercapturic acids for calculations. Children take up approximately 1.3−1.5 times more AA per kilogram of body weight than adults. The ratio GAMA/AAMA is significantly higher in the group of young children (6−10 years) with a median level of 0.5. A gender-related difference in internal exposure and metabolism was not observed.

KEYWORDS: Acrylamide; glycidamide; human metabolism; toxicokinetics; human exposure; daily intake; mercapturic acids; hemoglobin adducts

INTRODUCTION

From studies on laboratory animals it has long been known that the administration of acrylamide (AA) results in the formation of tumors in various tissue (*1*). AA was categorized by the International Agency for Cancer Research into group 2A to be " probably carcinogenic to humans" (*2*–*4*). Epoxidation via cytochrome P-450 2E1 (CYP2E1) to glycidamide (GA) appears to be a prerequisite for the genotoxicity of AA in vitro and in experimental animals (*5, 6*). In 2002, Swedish researchers discovered that AA is formed during the frying and baking of potato and cereal products (*7*). Since then, many studies dealing with the formation, uptake, metabolism, and excretion of AA in humans at environmental dose levels have been conducted.

In **Figure 1** the metabolic pathways of AA are depicted. Phase I and phase II reactions are involved in the metabolism of AA. Cytochrome oxidase CYP2E1 was identified to be the involved enzyme in the oxidation of AA to the highly reactive epoxide GA (*8*). AA and GA react with glutathione and are excreted as mercapturic acids in the urine. *N*-Acetyl-*S*-(2-carbamoylethyl)-L-cysteine (AAMA) is the major metabolite resulting from the reductive metabolic pathway. Studies on toxicokinetics of AA in humans report that about half of the ingested AA dose is eliminated as AAMA. The mercapturic acid *N*-(*R*/*S*)-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA) is excreted with up to 5% of the applied dose (*9*–*11*). The mercapturic acids

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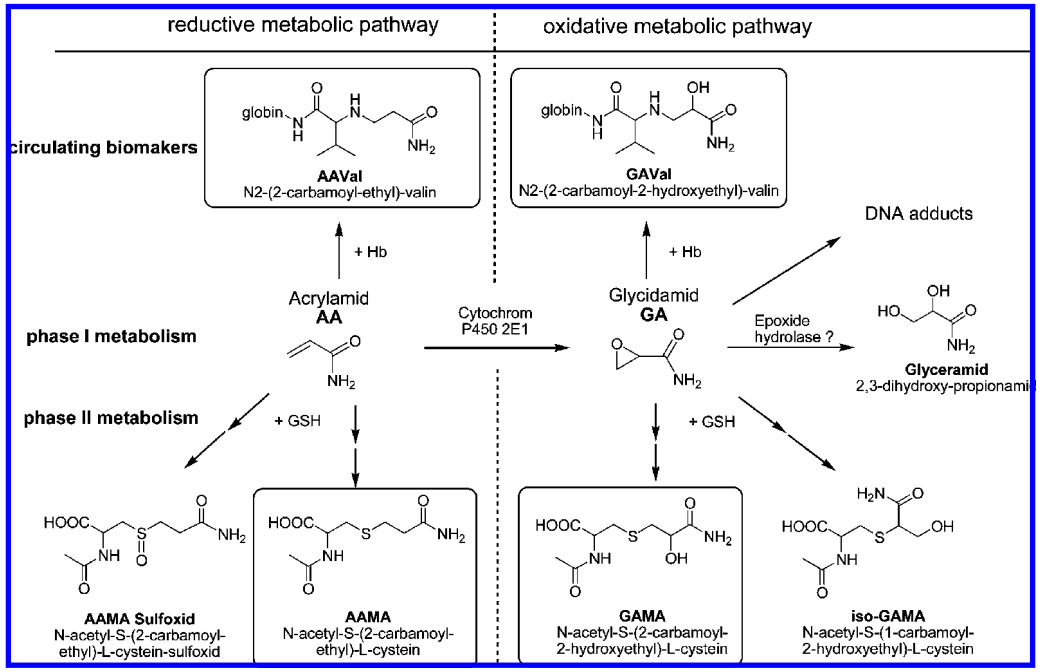

Figure 1. Metabolism of AA, status quo, partly adopted from refs *12* and *34*. Target analytes of this study are framed.

of AA and GA in human urine have proven to be valid biomarkers for AA exposure of the general population (*12–16*).

During the phase of distribution and metabolic activation, AA and GA react with nucleophilic sites throughout the body. The concentration of hemoglobin adducts reflects the internal effective doses of AA and GA. In laboratory animals it could be shown that the GA−hemoglobin adduct concentrations significantly correlate with liver−GA−DNA concentrations (*17*). Thus, hemoglobin adducts are biomarkers for the biochemical effect (reaction with nucleophiles) and are moreover regarded as surrogates for the determination of DNA adducts (*18*). The hemoglobin adducts, *N*2-(2-carbamoylethyl)valine (AAVal) and *N*2-(2-carbamoyl-2-hydroxyethyl)valine (GAVal) are approved parameters for the assessment of the internal burden and biochemical effect (*19–21*).

The balance between toxification and detoxification is crucial for the development of genotoxic action in vivo. This relationship between oxidative and reductive metabolic pathways, the excretion of urinary metabolites in combination with the presence of the circulating biomarkers, is the subject of this study. For this purpose, blood and urine samples were analyzed for hemoglobin adducts and urinary metabolites. With this study we link the two parameters for the biochemical effect (hemoglobin adducts) and internal exposure (hemoglobin adducts and mercapturic acids) to get more details about the metabolic fate of AA in humans. The relationship between the excreted urinary metabolites and the reaction products of AA and GA with hemoglobin are reported. With the acquired data on the exposure, we could estimate the actual amount of AA that is taken up in this study population.

MATERIALS AND METHODS

Subjects. Of an original collective of 1000 habitants in Bavaria (*22*), we randomly selected a subcollective. All test persons were nonsmokers, which was ascertained by the hemoglobin adduct of acrylonitrile in the blood of all participants according to the method of Schettgen et al. (*19*). The subcollective comprised 91 white Caucasian children, adolescents, and adults of the general population (45 males, 46 females), aged from 6 to 80 years (median = 36 years). An even distribution of age and gender was approached by selecting persons of different age groups: 5−10, 11−20, 21−30, 31−40, 41−50, 51−60, and >60 years. Each age group contained 11−15 individuals with about half male and half female subjects. In the blood samples and the respective urine samples, the hemoglobin adducts and mercapturic acids of AA and GA were analyzed.

All participants were informed in writing about the aims of our study and gave written consent about the donation of blood and urine. This study was approved by the committee on ethics in medical research of the University of Erlangen−Nuremberg.

Determination of Hemoglobin Adducts. The method applied for the determination of valine adducts of acrylamide (AAVal), glycidamide (GAVal), and acrylonitrile (CEVal) is based on the method of Paulsson et al. (*23*), which includes a modified Edman degradation of the alkylated N-terminal valine and a subsequent acetonization of the glycidamide−valine−pentafluorophenylthiohydantoin derivative. As the valine adducts of acrylonitrile and acrylamide remain unaffected in the derivatization, this procedure allows the simultaneous determination of hemoglobin adducts of acrylonitrile, acrylamide, and glycidamide in one analytical run.

Standards Used for Calibration. *N*-2-Carbamoylethylvaline−leucide−anilide, *N*-(*R*,*S*)-2-hydroxy-2-carbamoylehtylvaline−leucine−anilide, and *N*-cyanoethylvaline−leucine−anilide purchased from Bachem Biochemica (Heidelberg, Germany) with a chemical purity of >99%.

Internal Standards. Deuterium-labeled internal standards were synthesized by adding d_3-acrylamide or d_3-acrylonitrile to solutions of hemolyzed nonsmoker erythrocytes. After the reaction time of 4 h, the globin was isolated as described below (Sample Preparation). One hundred milligrams of this globin was dissolved in 3 mL of formamide and further diluted (1:100) to the final working solution. Nonlabeled AAVal and GAVal that result from the native origin of the used poolglobin were below the limit of detection (LOD).

For the quantification of GAVal, acetonized d_7-labeled pentafluorophenyl thiohydantoin derivate of the GA−valine adduct was used (gift of the Institute of Food Science in Kaiserslautern).

Calibration. For the calibration human globin was spiked with dipeptide standards in the concentration range of 0−1500 pmol/g of globin. Endogenous adduct concentrations were 27 pmol/g of globin for AAVal and 23 pmol/g of globin for GAVal; CEVal was <LOD. Linear calibration curves were obtained by plotting the quotients of the peak areas of AAVal, GAVal, and CEVal and the corresponding deuterium-labeled standards as a function of the concentrations used.

Table 1. Results of This Study, Urinary Metabolites, Hemoglobin Adducts, and the Ratios Analyzed in Blood and Urine Samples

			AAMA		GAMA		ratio,	AAVal,	GAVal,	ratio,
		creatinine, g/L	μg/L	μg/g of crea	μg/L	μg/g of crea	GAMA/AAMA	pmol/g of globin	pmol/g of globin	GAVal/AAVal
all	median	0.8	29	30	7	10	0.3	30	34	1.1
$n = 91$	95th perc	2.3	95	83	32	28	0.9	51	52	1.7
	range	0.1−3.1	<LOD−229	<LOD−138	<LOD−85	<LOD−38	0.004−1.4	15−71	14−66	0.4−2.7
gender										
female	median	0.7	22	30	5	10	0.3	30	34	1.1
$n = 46$	95th perc	2.2	67	73	32	27	1.0	54	54	1.6
	range	0.1−3.1	<LOD−95	<LOD−81	<LOD−85	<LOD−38	0.02−1.4	16−67	18−66	0.4−2.0
male	median	1.0	34	30	9	10	0.3	30	34	1.1
$n = 45$	95th perc	2.3	147	109	30	30	0.9	48	52	1.7
	range	0.3−2.8	4−229	9−138	<LOD−48	<LOD−35	0.004−1.3	15−71	14−56	0.5−2.7
age groups										
6−10 years	median	0.7	37	46	16	18	0.5	38	36	1.0
$n = 12$	range	0.3−1.9	3−83	8−98	3−43	9−34	0.2−1.3	26−59	22−52	0.6−1.4
11−18 years	median	1.4	44	30	15	11	0.3	34	38	1.0
$n = 11$	range	0.6−2.8	11−85	14−62	4−85	4−38	0.2−1.4	21−71	14−66	0.5−1.4
21−29 years	median	0.9	32	34	9	10	0.3	27	30	1.1
$n = 14$	range	0.2−3.1	4−197	14−138	<LOD−29	<LOD−26	0.02−1.0	18−50	20−41	0.4−1.6
31−39 years	median	0.5	11	28	<LOD	7	0.3	27	30	1.0
$n = 13$	range	0.1−2.7	<LOD−229	<LOD−85	<LOD−30	<LOD−14	0.004−1.0	21−47	18−47	0.8−1.9
41−49 years	median	0.8	26	28	5	8	0.2	27	34	1.2
$n = 15$	range	0.1−2.1	3−75	6−81	<LOD−25	<LOD−15	0.02−0.9	16−41	17−50	0.6−1.7
51−59 years	median	0.8	47	37	7	13	0.4	31	37	1.3
$n = 13$	range	0.2−2.3	3−95	12−132	<LOD−35	<LOD−28	0.01−0.6	22−46	23−52	0.8−2.0
61−80 years	median	0.9	21	23	7	8	0.4	30	31	1.1
$n = 13$	range	0.2−1.9	5−69	14−49	<LOD−14	<LOD−19	0.01−0.8	15−55	20−59	0.7−2.7

From the resulting graph (correlation coefficient; $r > 0,9$) the slope is used to calculate the hemoglobin adduct concentrations in the blood samples of the test persons.

Sample Preparation. Globin was isolated as described previously (*24, 25*).

Erythrocytes were isolated from 5 mL of whole blood by centrifugation, washed three times with 0.9% sodium chloride solution, and hemolyzed with bidistilled water and subsequent freezing at −18 °C. Globin was then isolated by precipitation with ethyl acetate and, after several wash steps, dried overnight.

Isolated globin was then dissolved in formamide, and labeled internal standards (d_3-AA-globin, d_3-ACN-globin, and acetonized d_7-GA-derivative) were added. The solution was then alkalized with NaOH, and the derivatizing reagent pentafluorophenylisothiocyanate (PFPITC) was added. The modified Edman degradation of the alkylated N-terminal valine occurred overnight. Then saturated aqueous NaCl solution was added to the formamide phase to improve the efficiency of extraction for the pentafluorophenyl thiohydantoine (PFPTH) derivative of the glycidamide−valine adduct. The pentafluorophenyl thiohydantoine derivatives of the N-terminal valine were extracted with diethyl ether and evaporated to dryness, and the residue was dissolved in toluene. Subsequently, the solution was washed with water and Na_2CO_3. After the evaporation of the toluene phase, acetonization of the glycidamide adduct was carried out by the addition of 1% H_2SO_4 in acetone (v/v) and kept overnight. The acidic solution was then neutralized, toluene was added, and the solution was washed and evaporated to dryness; the residues were redissolved in 50 μL of toluene. One microliter was then analyzed by GC-MS/MS in NCI mode.

The LOD was 4 pmol/g of globin (= 0.1 μg/L of blood) for CEVal, AAVal, and GAVal. The adducts were quantified by isotope dilution. For quality control purposes, one smoker's blood sample has been included in each analytical series. The between-series precisions for CEVal, AAVal, and GAVal were found to be 8% ($n = 6$; mean concentration = 267 pmol/g), 7% ($n = 6$; mean concentration = 183 pmol/g), and 6% ($n = 6$; mean concentration = 86 pmol/g), respectively.

Determination of Mercapturic Acids. The analytical method applied to quantify AAMA and GAMA in the urine samples is described elsewhere (*26*). The analytical standard *N*-(*R*/*S*)-acetyl-*S*-(2-carbamoylethyl)-L-cysteine was synthesised in our laboratory with a chemical purity of >95%. *N*-(*R*/*S*)-Acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine was custom synthesized (Institut für Dünnschichttechnologie and Mikrosensorik, Teltow, Germany; chemical purity > 90%). Briefly, to 4 mL of each urine sample were added isotope-labeled internal standard solutions (d_3-AAMA, d_3-GAMA). The analytes were extracted at an ENV+ (Isolute IST) SPE cartridge prior to ESI-RP-LC-MS/MS analysis (Sciex API 2000) in the negative ionization mode.

Each series included calibration standards and quality control material that were prepared by diluting aqueous working solutions of AAMA and GAMA with urine in the conentration range from 5 to 500 μg/L. Linear calibration curves were obtained by plotting the quotients of the peak areas of AAMA and GAMA and the corresponding d_3-labeled standards as a function of the concentrations used. The correlation coefficients were all higher than $r = 0.99$ for both AAMA and GAMA. Detection limits for both analytes were 1.5 μg/L urine. The analytival method is characterized by a between-day imprecision of 5% ($n = 6$, mean concentration = AAMA, 16 μg/L; GAMA, 20 μg/L) for both analytes.

The creatinine content of each urine sample was determined according to the method fo Larsen (*27*).

The statistical test were conducted using SPSS 14.0 software.

RESULTS AND DISCUSSION

Data of the effective doses, not only of AA but also of the actual genotoxic agent GA, play a key role in the discussion of the AA-based cancer risk of the general population. In this study, the protein adducts of AA and GA were analyzed as parameters for the biochemical effects, as well as the excretion of the urinary metabolites as a parameter of exposure in one study population. The concentrations of the hemoglobin adducts AAVal and GAVal and of the mercapturic acids AAMA and GAMA are summarized in **Table 1**.

The hemoglobin adduct of AA was detected and quantified in all blood samples of this study population in the range from 15 to 71 pmol/g of globin. The median of 30 pmol/g of globin is higher than the median levels we quantified in previous pilot studies at 21 pmol/g of globin (*28*) and 18 pmol/g of globin (*24*). However, the median level is comparable with another national study on 60 nonsmokers in which Urban et al. determined a median level of 28 pmol/g of globin (*15*). Additionally, the median AAVal level in this study is in

Table 2. National and International Comparison of Hemoglobin Adduct Levels in the Nonsmoking General Population, Median and Ranges[a]

	N	AAVal, pmol/g of globin, median (range)	GAVal pmol/g of globin, median (range)	GAVal/AAVal, median (range)
Paulsson et al. (2003)	5	27 (ns)	26 (ns)	1.0 (ns)
Schettgen et al. (2003)	25	21 (12−50)	nm	nm
Schettgen et al. (2004)	13	18 (7−31)	18 (9−23)	0.9 (0.5−1.7)
Hagmar et al. (2005)	70	31 (20−100)	nm	nm
Urban et al. (2006)	60	28 (18−51)	nm	nm
Chevolleau et al. (2007)	52	27 (9−70)	22 (12−47)	1.0 (0.2−2.1)
Bjellaas et al. (2007)	44	37 (18−66)	18 (7−46)	0.5 (0.1−1.1)
this study	91	30 (15−71)	34 (14−66)	1.1 (0.4−2.7)

[a] ns, not specified; nm, not measured.

excellent accordance with the median levels that were quatified in studies on nonsmokers of the general population in other European countries such as Sweden, which reported median levels of 27 and 31 pmol/g of globin (*23, 29*). Studies in Norway resulted in a median level of 37 pmol/g of globin (*21*) and in France in a median level of 26 pmol/g of globin for the nonsmoking general population (**Table 2**).

The hemoglobin adduct of GA was also quantified in 100% of the study subjects with a median level of 34 pmol/g of globin, in the range from 14 to 66 pmol/g of globin. In comparison with results of other studies on the nonsmoking general population (**Table 2**), the median level in our study is comparably high, but in the same order of magnitude. Overall, the median GAVal levels vary between 18 and 34 pmol/g of globin. For example, the median GAVal level of the nonsmoking general population in Sweden was 18 pmol/g of globin (*21*), and in France a median level of 22 pmol/g of globin (*30*) was reported.

Using the hemoglobin adduct levels in each blood sample a ratio is calculated that reflects the relationship of genotoxic to nongenotoxic dose, of GA and AA, in each test person.

The ratio between GAVal and AAVal detected in this study population varies considerably between individuals, in the range from 0.4 to 2.7. On an average basis, the adducts of AA and GA are formed in equal concentrations with a median ratio of 1.1. There is a highly significant correlation between the Hb adducts of GA and AA (**Figure 2**) ($r = 0.533$; $p < 0.001$).

The median ratios, reported on the international level (**Table 2**), vary between 0.5 and 1.1. This coefficient may be used to characterize interspecies differences in the carcinogenic potential of AA.

Several publications in the past dealt with the **interspecies differences** between humans, rats, and mice. Previously, Paulson et al. (*31*) reported that the ratio GAVal/AAVal in humans of 1.0 is more similar to the ratio in rats of 1.8 in comparison to the ratio in mice of 5.4. These adduct levels and the results were measured in control blood samples from laboratory animals and humans. The oxidation of AA to GA is much more pronounced in mice, which is confirmed also in more recent studies (*17*). The working group of T. R. Fennell compared levels of AAVal and GAVal after oral application of doses of 50 and 3 mg/kg (*32*) to rats with those found in humans after dosings of 0.5, 1.0, and 3 mg/kg AA (*33*). The ratios reported in the rats are for the lower dose (3 mg/kg) 0.8 and for the higher dose (50 mg/kg), 0.4. In humans, after the ingestion of 0.5, 1, and 3 mg/kg AA, the ratio was reported to be between 0.36 and 0.44 in a similar range. Although these doses are >1000 times higher than the environmental doses taken up with the diet and formation of GA may be favored at lower doses, the ratios we detect in the general population in the range of 0.4−2.7 are in the same order of magnitude.

We may conclude that the metabolisms of humans and rats are comparable with respect to the extent of oxidation and the formation of the ultimate carcinogen. This finding is relevant for the assessment of carcinogenic risk of AA to humans.

The reductive mercapturic acid, AAMA, was quantified in all urine samples except one, in the range from <LOD to 229 μg/L, with a median level of 29 μg/L (**Table 1**). This median level is in agreement with other studies of Boettcher et al. (*12*), Urban et al. (*15*), and Bjellaas et al. (*16*) that reported median levels of 29, 42, and 32 μg/L, respectively (**Table 3**).

The oxidative mercapturic acid, GAMA, was excreted in 75% of the test persons, in the range from <LOD to 85 μg/L, with a median level of 7 μg/L. The median level for the GAMA was also well comparable with the median levels (**Table 3**) detected in other studies that vary between 3 and 9 μg/L in the median (*12, 15, 16*).

There is a highly significant correlation between AAMA and GAMA ($r = 0.467$; $p < 0.001$). This shows that AA is the common source for both mercapturic acids. What needs to be highlighted in this context is that 30% of the adults excrete the reductive AAMA in considerable amounts without excreting GAMA, however. This might be due to the individual enzymatic status or can be a product of differences in the kinetics of the two mercapturic acids (*9*). We know that only about 4 h after AA application does the GAMA excretion set in, whereas the AAMA excretion occurs directly after application. Thus, the sampling time might have a decisive influence on the ratio (morning urine vs afternoon urine) (*16*).

The ratio GAMA/AAMA, reflecting the rate of oxidative metabolism, was determined with a median of 0.3. In comparison to other studies that analyzed median ratios of 0.1−0.2 (*12, 15, 16*), the median ratio of our study is high (**Table 3**), and there is a considerable variation in the range of 0.004−1.4. This is probably due to the special design of our study group, which is characterized by an even distribution of age. The comparably higher excretion of GAMA in the urine of the children and adolescents (see below), in comparison to adults might have a decisive influence on the described ratio.

This coefficient GAMA/AAMA may also be used to characterize **interspecies differences** in the carcinogenic potential of AA. After gavage of a AA dose of 0.1 mg/kg to male rats, 31% of the dose was excreted as AAMA and 28% was excreted as GAMA within 24 h, whereas male mice excreted 7% AAMA and 16% GAMA. From this results coefficients GAMA/AAMA of rats of 0.9 and of mice of 2.3 were calculated. These data show that in mice the oxidative metabolic pathway is pronounced (*34*).

Comparing the ratios of the two parameters, we observed that more reactive GA binds to nucleophilic sites throughout the body (median ratio GAVal/AAVal = 1.1) than we would expect by looking at the mercapturic acid excretion (median ratio GAMA/AAMA = 0.3). Bearing in mind that the direct comparison of results from animal feeding studies exhibits many flaws, we may conclude that the human metabolism seems to be quite similar to the rat metabolism and that the cancer risk extrapolation that is based on rodent studies is set on reasonable circumstances.

Relationship between Blood Adducts and Urine Metabolites. Between the parameters of reductive metabolic pathway AAVal and AAMA (Pearson, $p = 0.02$; $r = 0.25$) and oxidative pathway GAVal and GAMA (Pearson, $p = 0.03$; $r = 0.24$), no significant correlation was observed. This goes in line with the fact that the mercapturic acids show the immediate AA

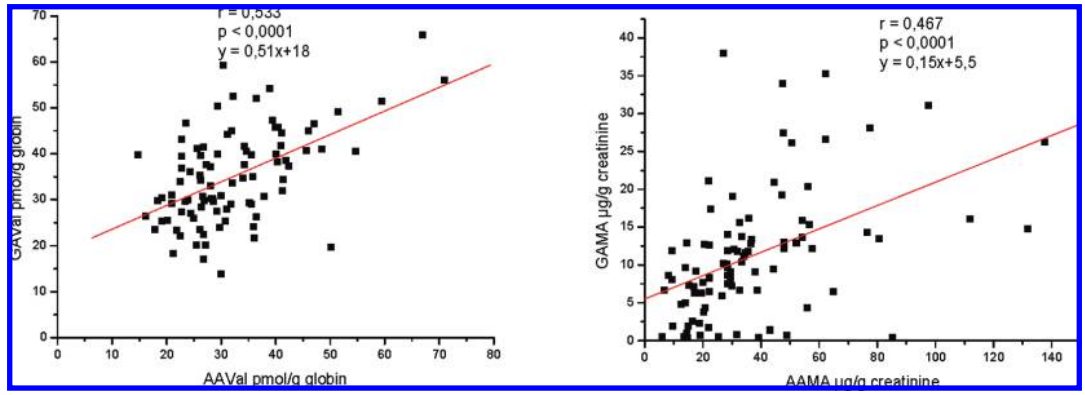

Figure 2. Correlation (Pearson) of the parameters GAVal to AAVal and GAMA to AAMA.

Table 3. National and International Comparison of Urinary Metabolite Concentrations in the Urine of the Nonsmoking General Population, Median and Ranges[a]

	N	AAMA, μg/L, median (range)	GAMA, μg/L, median (range)	GAMA/AAMA, median (range)
Boettcher et al. (2005)	16	29 (3−83)	5 (<LOD−14)	0.2 (0.1−0.5)
Urban et al. (2006)	60	42 (ns)	9 (ns)	0.2 (ns)
Bjellaas et al. (2007)[b]	47	32 (2−307)	3 (<LOD−17)	0.1 (0.01−0.2)
this study	91	29 (<LOD−229)	7 (<LOD−85)	0.3 (0.004−1.4)

[a] ns, not specified; nm, not measured. [b] Twenty-four hour urinary collection.

exposure, whereas the hemoglobin adducts reflect the exposure over the last 4 months. The average lifespan of the erythrocytes is 120 days.

Relationship between AA Exposure and Gender. The study group consisted of 46 female and 45 male test persons. The internal exposure toward AA and GA, measured as hemogobin adducts, is virtually the same for both sexes. The blood adduct levels, which can be used as long-term exposure markers, are in both sexes identical with median levels of 30 pmol/g of globin for AAVal and 34 pmol/g of globin for GAVal.

At the same time, there was a gender-related difference in the volume-based concentrations of excreted AAMA and GAMA. This indicates that men take up higher amounts of AA through their diet, but one should consider that when the creatinine-based concentrations are compared, this gender difference disappears (see **Table 1**). This is probably due to the clearly lower creatinine excretion in women (median in our collective = 0.7 g/L of urine) than in men (median in our collective = 1 g/L of urine).

The ratios GAMA/AAMA and GAVal/AAVal were the same in both females and males, with median levels of 0.3 and 1.1, respectively. This may be interpreted to indicate that both sexes are similar with respect to oxidative metabolism.

Relationship between AA Exposure and Age. The AAVal levels in the 6−10-year-old children (median = 38 pmol/g of globin) and also the group of children and adolescents from 11 to 18 years of age (median = 34 pmol/g of globin) are higher than in the group of adults (**Table 1**). The median levels in the adult age groups vary between 27 and 31 pmol/g of globin. For the analysis of the relationship between exposure and age the adult age groups were summarized (21−80 years, median AAVal = 29 pmol/g), and a significant difference was recorded ($p < 0.05$, Mann−Whitney *U*). This relationship between exposure and age is confirmed by the results of the whole group of >1000 persons (*22, 35*).

Using the mercapturic acids as parameters for the internal exposure, this age-related trend was not observed. The median AAMA levels of all age groups are in the range from 11 to 47 μg/L.

The interpretation of urinary metabolite concentrations in different age groups is rather complex, and various factors need to be considered. Comparison of the creatinine- and volume-based concentrations has flaws as both the daily water uptake and excreted urine volume, as well as the daily creatinine excretion, which is directly proportional to body weight and height, are stronly associated with age.

The high variation, moreover, is due to the fact that the urinary metabolites have short half-lives and thus reflect the AA exposure during the past 24 h. The mercapturic acid levels are dependent on whether the test person had a meal with high AA levels or not. They do not reflect the long-term dietary habits of the individual persons.

For this the hemoglobin adducts are the more suitable biomarker as they reflect the long-term exposure.

Relationship between AA Metabolism and Age. What needs to be highlighted when looking at the mercapturic acid excretion is that in the groups of 6−10 and 11−18 years, the GAMA could be detected in all urine samples. In contrast, there was no GAMA found in 30% of the urine samples in the older age groups (21−80 years), although they excreted considerable amounts of AAMA.

Accordingly, the median levels of GAMA are significantly higher in the younger age groups, with 16 μg/L in the young children from ages 6 to 10 and 15 μg/L in the group of older children and teenagers (11−18 years). The median GAMA levels in the group of adults are between <LOD and 9 μg/L. For the statistical analysis, the adult age groups were summarized (21−80 years, median GAMA = 8 μg/L), and a significant difference was recorded ($p < 0.05$; Mann−Whitney *U*).

Moreover, the ratio of the oxidative mercapturic acid to the reductive mercapturic acid, GAMA/AAMA, is clearly higher in the group of young children (ages 6−10 years) with a median level of 0.5. In the other age groups the ratios GAMA/AAMA are between 0.2 and 0.4. Between the young children and the summarized adult age groups (age 21−80 years, median ratio = 0.3) a highly significant difference in the GAMA/AAMA ratio was analyzed ($p < 0.05$; Mann−Whitney *U*).

In another study on the excretion of the mercapturic acids AAMA and GAMA in 100 children at the age of 7 years, this finding (ratio GAMA/AAMA = 0.5) was confirmed (*36*).

From these results on the excretion of urinary metabolites in the different age groups we may conclude that children and teenagers excrete significantly higher GAMA concentrations.

This indicates that in the younger age groups more genotoxic GA is generated and hence excreted with the urine. It needs to be highlighted that in the group of young children (6−10 years), the ratio of the oxidative mercapturic acid to the reductive mercapturic acid, GAMA/AAMA, is significantly higher with a median level of 0.5. That the higher oxidative excretion of GAMA is not proportional to a higher GAVal level in the respective blood samples indicates that besides the higher oxidative metabolism, also the detoxification via conjugation with glutathion is pronounced. This might be explained by age-related differences in the expression of phase I and II enzymes that are known to be tissue specific and can undergo developmental regulation (*37*). The role of the involved enzymes and the way they are influenced by diverse factors such as lifestyle or age need to be further investigated.

The higher dialy intake of AA by children (see below), in combination with the fact that tissue, undergoing proliferation and terminal differentiation, is particularly suscepible to carcinogenesis (*37*), should be of concern.

Daily Intake Estimation. By reason of the health of the general population and the possible carcinogenic risk due to AA in the daily diet, it is of utmost interest which doses are taken up. Calculations based on questionaire-based assessment of dietary habits have shown serious drawbacks (*38*). Biomarkers offer the possibility to calculate the AA doses actually taken up. This can be done using hemoglobin adduct levels (formula 1) as well as the mercapturic acid concentrations (formula 2).

On the basis of the AAVal concentrations in relation to the reaction rate constat ($k = 4.4 \times 10^{-6}$ L g of globin^{-1} h^{-1}) and the middle erythrocyte lifespan (63 days), the value for the area under the curve (AUC) of each person can be calculated. Multiplied by the elimination rate constant (E_k) in humans of 0.15 h^{-1} (*39*) and the volume of distribution (VD) of 0.38 L/kg (*33*), the daily intake of AA can be estimated (*24, 39*).

$$\text{AA } [\mu\text{g/kg of bw/day}] = \left[\frac{\text{AAVal [pmol/g of globin]}}{k \times \text{erythrocyte lifespan} \times \frac{1}{2}}\right] \times E_k \times \text{MW acrylamide} \times \text{VD} \quad (1)$$

For this study group a median daily intake of 0.43 μg/kg of bw/day, in the range from 0.21 to 1.04 μg/kg of bw/day, was calculated. The median dialy intake is significantly higher in the group of children, with 0.56 μg/kg of bw/day, in comparison to the median daily intake in the group of adults, with 0.42 μg/kg of bw/day.

From the respective mercapturic acid levels, the daily intake was calculated using the urinary excretion factor (F_{UE}), which considers that about 50% of the AA intake is excreted within 24 h (*9*). The 24 h AA excretion was calculated via the sum of the creatinine-based mercapturic acid levels, which is multiplied by the amount of creatinine excreted per day. This 24 h creatinine excretion ($CE_{smoothed}$) is dependent on muscle amount and was calculated according to the body weight (mmol kg^{-1} day^{-1}) (*40*) of the children with levels between 0.3 and 0.8 g/day and in the adolescents with levels between 0.6 and 1.9 g/day. For adults, average 24 h creatinine excretion amounts for women of 1.16 g/day and for men of 1.79 g/day were used (*41*) for the calculation.

$$\text{AA } [\mu\text{g/kg of bw/day}] = \{\text{AAMA} + \text{GAMA } [\mu\text{mol/g of creatinine}] \times \text{CE}_{\text{smoothed}} \times \text{MW acrylamide}\}/F_{UE} \times \text{bw} \quad (2)$$

Using this formula, a daily AA intake of 0.51 μg/kg of bw/day was estimated, in the range from <LOD to 2.32 μg/kg of bw/day. With this calculation the differences in the urine excretion (urine volume and creatinine amount) according to age are considered. The estimated daily intake in the group of young children (6−10 years) was estimated to be 0.74 μg/kg of bw/day. The median intake in the group of adults (21−80 years) is lower, with 0.50 μg/kg of bw/day, although this difference is not statistically significant.

Despite using different parameters of exposure and two completely different models for the calculation of the daily intake, the results are fairly the same. The median intake in this collective of the nonsmoking population in Germany is between 0.43 μg/kg of bw/day (calculated form AAVal) and 0.51 μg/kg of bw/day (calculated from sum AAMA + GAMA).

The intake levels calculated from the hemoglobin adduct represent the internal burden during the past 4 months. The calculated intake must be considered as an individual mean intake over this time period. It varies from 0.21 to 1.04 μg/kg of bw/day. Calculated from the short-term parameter, the mercapturic acids in the urine, the daily intakes vary between <LOD and 2.32 μg/kg of bw/day. Comparing the results from using the different parameters we can show that there is a high interindividual variability in the AA intake of the general population. The "life style", meaning the chosen diet, is thereby the important factor. Looking at the short-term urinary metabolite excretion, we may deduce that there is even a greater variation between day-to-day exposure. This can be easily explained as AA contamination occurrs in only certain foodstuffs. If these are avoided, there might be days with low intake, or on a day with increased AA intake through French fries, crisps, and coffee, a consumer will have an intake of up to 2−3 μg/kg of bw/day.

These results are in excellent accordance with the intake estimations that were made in different European countries and were summarized by the WHO/FAO. The estimates at national level ranged from 0.2 to 2.0 μg/kg of bw/day. The committee concluded that an intake of 1 μg/kg of bw/day of AA could be taken to represent the average for the general population and that an intake of 4 μg/kg of bw/day could be taken to represent high consumers (*2*).

The median dietary intake of acrylamide is in line with the estimates of Bjellaas (*16*), who used 24 h urine. He estimated a daily increment of 0.47 (range = 0.17−1.16) μg/kg of bw/day (*16*).

From the results of this study we may conclude that the interpretation of the urinary metabolite concentration is more complex as the volume- and creatinine-based concentraions are highly influenced by multiple factors such as lifestyle and age. Against this background, the accordance of the daily intake estimations from urine metabolites and the blood adduct is really remarkable.

According to both models, the median daily intake is higher in the group of children. In relation to the median intake of the adults, the children take in approximately 1.3 times (estimation based on AAVal) to 1.5 times (estimation based on the sum of AAMA and GAMA) the amount of AA per body weight and day. This relationship between internal burden and age is a consequence of the fact that children consume more food per kilogram of body weight, as a function of their surface-to-volume ratio and hence higher caloric requirements.

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