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Sustained Human Background Exposure to Acrolein Evidenced by Monitoring Urinary Exposure Biomarkers

*Meike Ruenz, Katharina Goerke, Tamara Bakuradze, Klaus Abraham, Alfonso Lampen, Gerhard Eisenbrand, and Elke Richling**

Scope: This study investigates a potential correlation between the intake of heat-processed food and the excretion of the acrolein (AC) biomarkers ***N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine (HPMA) and *N*-acetyl-*S*-(carboxyethyl)-L-cysteine (CEMA) based on two human studies.**
Methods and Results: Human exposure to AC is monitored using the AC-related mercapturic acids HPMA and CEMA in the urine of a) non-smoking volunteers under defined living conditions and b) of non-smoking volunteers on unrestricted or vegan diet under free living conditions. Free living volunteers in part show markedly enhanced urinary excretions of HPMA and CEMA. The intake of heat-processed food does not influence AC-related biomarker excretion. Incidentally enhanced urinary exposure biomarker levels appear to suggest AC exposure possibly from open fire, barbecuing, or tobacco smoke. However, kinetics of urinary biomarkers related to tobacco and other potential smoke exposure, do not correlate with those observed for HPMA and CEMA.
Conclusion: This study is the first to convincingly show a sustained and substantial background exposure to AC in non-smoking humans, clearly independent from uptake of heat-processed foods. The data strongly point to endogenous AC generation by pathways of mammalian and/or microbial metabolism as yet not taken into consideration.

1. Introduction

Acrolein (prop-2-enal, AC) is a highly reactive α,β-unsaturated carbonyl compound that forms Michael adducts with nucleophilic groups of amino acids, peptides, structural, and functional proteins and other biomolecules of biological relevance. Such AC-related modifications have been found to often affect biochemical functions associated with cellular and tissue physiology, including enzyme activity, signal transduction, transcription, DNA repair, and more. Furthermore, under specific conditions, AC may form DNA adducts and thus has been suspected to exert genotoxic potential. Conjugation with glutathione (GSH) is a major metabolic detoxification pathway of AC. The primary glutathione adduct is metabolically trimmed by splitting off glutamic acid and glycine and by subsequent acetylation of the resulting cysteine adduct to give *N*-acetyl-*S*-(3-oxopropyl)-L-cysteine. Oxidation of the latter produces *N*-acetyl-*S*-(carboxyethyl)-L-cysteine (CEMA). The major AC-derived metabolite in urine is, however the reductive metabolite *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine (HPMA) which has been utilized as a biomarker of human exposure to tobacco smoke derived AC.[1–5] Further (minor) products of direct AC metabolism include glyceraldehyde, oxalic acid, malonic acid, and 3-hydroxypropionic acid.[1]

AC is formed during combustion of organic material and has been found to occur in outdoor (<0.05–2.47 µg m^{-3}) and indoor (<0.05–8.1 µg m^{-3}) air.[6] Increased concentrations were found in cigarette smoke and car exhausts.[7] It was also reported that frequent wok cooking leads to elevated exposure to AC (supposedly by inhalation of cooking fumes), as monitored in the urine of Chinese women in Singapore.[5] Furthermore, heating of fats and oils entails AC formation, resulting in AC concentrations of up to 242 mg kg^{-1}.[8] However, in fried food like French fries, potato crisps, and donuts only minor contents of free AC (8–26 µg kg^{-1}) have been determined.[9,10]

AC-related urinary mercapturic acids, HPMA and CEMA, have been utilized as biomarkers of exposure in animals and humans.[2,11–14] The excretion of HPMA and CEMA reflects direct exposure to AC as well as exposure to AC as an intermediate

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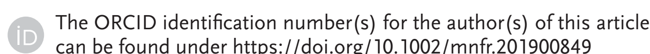
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metabolite formed from exogenous industrial compounds or endogenous sources.[14]

In the urine of non-smokers, mean and median HPMA levels were reported in several studies to range from 43.5 to 493 μg g^{-1} creatinine (cr) (0.20–2.23 μmol g^{-1} cr).[2,11]–[14] For CEMA, a mean level of 42 μg g^{-1} cr (0.18 μmol g^{-1} cr) was reported for non-smokers.[14]

AC exposure has been assessed, based on the daily HPMA excretion (200–1000 μg/24 h) in urine of non-smokers or former smokers on abstinence and on about 20% assumed urinary excretion of ingested AC, in analogy to data obtained from rats.[15–17] This resulted in an estimate of total AC exposure of 300–1400 μg d^{-1}, suggesting an intake of 5–24 μg kg^{-1} bodyweight (bw) d^{-1}.[15]

Data on endogenous formation of AC in the human organism are limited. In general, AC may be formed from carbohydrates, triacylglycerides, and certain amino acids.[1] Non-microbial endogenous formation routes of AC include myeloperoxidase catalyzed formation of AC from threonine,[18] amine oxidase catalyzed formation from spermine and spermidine,[19] and formation as a byproduct of lipid peroxidation.[20]

In addition, microbial generation of AC in the human gut from glycerol has received enhanced attention.[21–23] Glycerol is supposed to be abundant in the gut as nutritional component, as a product of microbial fermentation and of digestion of luminal lipids and membrane constituents of desquamated epithelial cells. Certain gut microbiota can metabolize glycerol into 3-hydroxypropanal (3-HPA), catalyzed by vitamin B12 dependent bacterial glycerol/diol dehydratases.[23–26] The metabolite 3-HPA can spontaneously convert into AC. In aqueous media 3-HPA is equilibrating with its hydrate, 1,1,3-propanetriol and its dimer, 2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane. For this multicomponent system the term "reuterin" has been introduced, after *Lactobacillus reuteri*, which is known to be a major microbial source of 3-HPA.[22,23]

Indications for a potential association of heated food intake with an increased excretion of HPMA and CEMA have previously been reported.[27,28] In a study with 13 male volunteers, at baseline a mean urinary HPMA concentration of 11.6 ± 28.8 μmol g^{-1} cr was reported following a fasting period of 11 h. The intake of 150 g self-prepared potato crisps with an unknown AC content entailed a mean urinary HPMA excretion of 66.6 ± 44.1 μmol g^{-1} cr.[27] In a study with five male volunteers, at baseline mean urinary concentrations of 0.18 ± 0.02 μmol g^{-1} cr HPMA and of 0.06 ± 0.01 μmol g^{-1} cr CEMA were observed after a fasting period of 11 h. Consumption of 175 g commercially available potato crisps with a reported AC content of 26.5 ± 2.1 μg kg^{-1} resulted in an increased excretion of HPMA (0.47 ± 0.15 μmol g^{-1} cr) and CEMA (0.35 ± 0.11 μmol g^{-1} cr).[27] Likewise, in three further studies with healthy volunteers excretion of HPMA was found to increase from 0.53–0.67 to 1.16–1.47 μmol g^{-1} cr (c_{max} at 12 h) after consumption of fried foods including fried chicken and French fries.[28]

In the present investigation, the potential correlation between the intake of heat-processed food and the excretion of the AC biomarkers HPMA and CEMA was monitored in the urine of volunteers from two human studies. At variance to studies previously performed, in the present studies heat-processed foods were ingested as part of the normal diet under realistic living conditions. During study I volunteers consumed a controlled diet for 9 days in a controlled environment whereas study II was performed under free-living conditions. The volunteers (omnivores and vegans) protocolled their diet during the 10-day study period. Both studies represent state-of-the-art duplicate diet studies.

2. Experimental Section

2.1. Chemicals

Chemicals were of analytical grade. The mercapturic acids, *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine (HPMA), *N*-acetyl-d3-*S*-(3-hydroxypropyl)-L-cysteine (D_3-HPMA), *N*-acetyl-*S*-(carboxyethyl)-L-cysteine (CEMA), *N*-acetyl-d3-*S*-(carboxyethyl)-L-cysteine (D_3-CEMA), *N*-acetyl-*S*-(2-cyanoethyl)-L-cysteine (CYMA), (±)-1-(methyl)-5-(2-pyridinyl)-2-pyrrolidinone (cotinine) and (±)-1-(methyl-D_3)-5-(2-pyridinyl)-2-pyrrolidinone (D_3-cotinine) were purchased from Toronto Research Chemicals (Toronto, Canada). The cr(urinary) colorimetric assay kit was obtained from Cayman Chemical Company (Ann Arbor, USA).

2.2. Design of Study I

Study I was a 9-day duplicate diet human intervention study in a controlled environment with a controlled diet. Study details have been reported earlier.[29] Briefly, the study was performed with 14 non-smoking male volunteers under tightly controlled environmental and nutritional living conditions, excluding any potential inadvertent acrylamide (prop-2-enamide, AA) and AC exposure such as exhaust gases, open fire, or tobacco smoke. A scheme of the study design is given in **Figure 1**. The study included three washout phases (days: 1–3; 5–6; 8–9) characterized by intake of diets established to be devoid of noteworthy AA contents, ascertaining low or absent thermal processing. Diets consisted of unheated or mildly heated foods (≤100 °C) such as yoghurt, fruits, vegetables, boiled meat, potatoes, noodles, and rice. On days 4 and 7, diets were ingested containing heat-processed food items (>100 °C). Thermal processing was confirmed by measuring AA contents in duplicates of servings as consumed allowing exact dosimetry of dietary AA intakes. On day 4, the diet consisted of pan-fried chicken breast, breakfast cereals, crisp bread, biscuits, and coffee and on day 7, pan-fried sausages, pan-fried steak, French fries, fried potatoes, breakfast cereals, crisp bread, potato crisps, biscuits, and coffee. Total urine was collected during the whole study period to monitor the excretion of urinary AA and AC biomarkers.

2.3. Design of Study II

Study II was a 10-day duplicate diet study under free-living conditions with protocolled diet. Details of the study were reported elsewhere.[30] Briefly, volunteers (ten males, ten females) were instructed at study onset to maintain their normal lifestyle and usual dietary habits. Group 1 (five males, five females), without dietary preferences, consumed an unrestricted diet (volunteers U1–U10, U = unrestricted diet). Group 2 (five males, five

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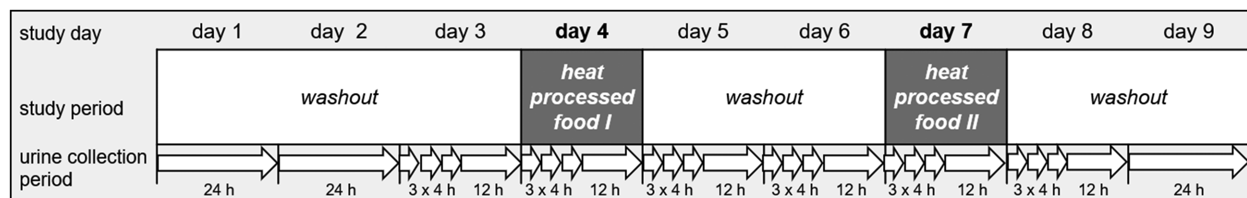

Figure 1. Study design of study I: 9-day study with controlled diet (n = 14), washout: diet included not and marginally heated food (≤100 °C), heat-processed food I/heat-processed food II: diet included not and marginally heated food as well as heat-processed food (>100 °C)

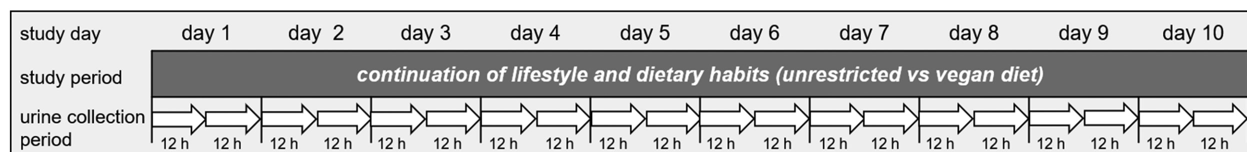

Figure 2. Study design of study II: 10-day study with protocolled unrestricted diet (n = 10) versus vegan diet (n = 10)

females) consisted of vegans who were instructed to follow their vegan habits during the study (volunteers V1–V10; V = vegan diet) (**Figure 2**). The food intake was protocolled by the volunteers in a dietary diary, recording time and amount of the consumed foods. Duplicates of servings as consumed were prepared by the volunteers for analysis and total urine was collected during the whole study period. After finishing food and urine analysis, results suggested a more detailed consideration of the volunteers living conditions during the study period. Volunteers therefore were asked to report on living conditions to the best of their recalling (about 1 year later), especially with respect to potential sources of non-dietary AA and AC exposure, such as inadvertent exposure to open fire and/or tobacco smoke. In addition, based on an amendment to the study, the biomarkers cotinine and CYMA, associated with tobacco smoke and other potential smoke exposure, were monitored in the urine of 16/20 volunteers, the ones who provided consent to the amendment.

2.4. Determination of HPMA and CEMA

The mercapturic acids HPMA and CEMA were determined in urine by a stable isotope dilution method (SIDA). As internal standards D_3-HPMA (study I: 50 µL, 10 µg mL^{-1}; study II: 20 µL, 10 µg mL^{-1}) and D_3-CEMA (study I: 10 µL, 10 µg mL^{-1}; study II: 5 µL, 10 µg mL^{-1}) were added to the urine samples. Sample preparation using solid phase extraction (SPE) was performed as described elsewhere.[29,30] HPLC–MS/MS analysis was carried out using a 1290 HPLC (Agilent, Santa Clara, USA) combined with a QTRAP 5500 (AB Sciex, Darmstadt, Deutschland). The methods applied for the samples of the two studies differed slightly. Study I: injection volume: 5 µL; HPLC column: Luna C8(2),150 mm × 4.6 mm, 3 µm with HPLC guard column (Phenomenex, Torrance, USA); flow: 0.6 mL min^{-1}; solvent A: 0.1% aqueous acetic acid; solvent B: acetonitrile; gradient: 0–9 min: 4% of solvent B, 9–11 min: increased up to 10% solvent B; ESI–MS/MS analysis: negative electrospray ionization, multiple reaction monitoring (MRM) mode. Optimized MS parameters are given in Table S1, Supporting Information. Calibration plots of peak area versus concentration ratios (HPMA/D_3–HPMA or CEMA/D_3–CEMA) were linear with an R^2 value of at least 0.999. Limits of detection (LOD) and of quantification (LOQ) were 1.0/3.5 fmol for HPMA and 3.3/11.0 fmol for CEMA (absolute amounts). Study II: injection volume: 2 µL; HPLC column: Zorbax Eclipse XDB-C18, 50 mm × 4.6 mm, 1.8 µm with UHPLC guard column (Agilent); solvent A: 0.1% aqueous acetic acid; solvent B: acetonitrile; flow: 0.6 mL min^{-1}; gradient: 0–2 min: 1% of solvent B, 2–3 min: increased up to 10% solvent B; ESI-–MS/MS analysis: negative electrospray ionization, scheduled multiple reaction monitoring (sMRM). Optimized MS parameters are given in Table S2, Supporting Information. Calibration plots of peak area versus concentration ratios (HPMA/D_3–HPMA or CEMA/D_3–CEMA) were linear with an R^2 value of at least 0.999. LOD/LOQ were 0.5/0.9 fmol for HPMA and 2.6/8.5 fmol for CEMA (absolute amounts).

2.5. Determination of Smoke Exposure Associated Biomarkers

Biomarkers associated with inhalative exposure to tobacco or other smoke, cotinine, and CYMA, were determined in the SPE eluates prepared for the mercapturic acid analysis of 16 volunteers who had given their written consent to this additional testing. The determination of cotinine and CYMA was performed via HPLC–MS/MS as described elsewhere.[30]

2.6. Determination of Creatinine

Urinary creatinine (cr) was determined using a cr (urinary) assay kit following the manufacturer's information.

2.7. Determination of Urine Volumes

Total urine volumes were obtained by converting the determined urine weights into total urine volume, assuming a density of 1 kg m^{-3}.

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2.8. Data Analysis

MS data were evaluated by Analyst 1.6 Software (AB Sciex, Darmstadt, Germany). Further data analysis and preparation of the respective plots were carried out using Origin 9.1 Software (Origin Lab Corporation, Northampton, USA). All statistical hypotheses were tested with a 5% significance level against a two-sided alternative. Normal distribution was checked using the Anderson Darling test. For data of study I, a paired *t*-test was applied when normality could not be rejected, otherwise the Wilcoxon signed rank test was used. For data of study II, an unpaired *t*-test was applied when normality could not be rejected, otherwise (normality not proven) a Mann–Whitney *U* test was performed.

2.9. Compliance with Ethical Standards

Both human studies have been approved by the ethics commission of Rhineland-Palatinate, Mainz (no. 837.449.12 (8600-F) and no. 837.029.15 (9797)) and performed in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki). Informed consent was obtained from all individual participants included in the studies.

3. Results

3.1. Study I

This study was performed as a 9-day human intervention study in non-smoking male volunteers ($n = 14$) under tightly controlled living and nutritional conditions. Monitoring of daily AA intake and AA related biomarker excretion served to confirm consumption of heat-processed food. Results as reported previously demonstrated a) an association of urinary biomarker excretion with nutritional AA uptake and b) a sustained background of AA related biomarker excretion.[29] With the onset of the present study, a dosimetry of the nutritional AC intake, similar to the nutritional AA dosimetry was initially attempted. A gas chromatography–mass spectrometry (GC–MS) method originally established for AC determination in fats and oils[8] was applied with slight modifications to determine the AC contents in the collected duplicate diet meals. However, this method did not yield reliable data for AC contents in meals, since labeled standard recoveries were erratic and not reproducible and thus results appeared not meaningful. Supposedly, the high reactivity of AC towards food constituents (including water) may have prevented reliable dosimetry of AC contents in meals by the methodology applied. Similar experience has been noted elsewhere.[28] In contrast to the unsuccessful attempts to determine genuine AC contents in the foods consumed, monitoring urinary biomarkers of AC exposure provided clear indications for substantial exposure to AC (**Figure 3**).

The average daily HPMA excretion of the volunteers was 1.95 ± 0.35 µmol (0.98 ± 0.18 µmol g^{-1} cr). During the washout days, the HPMA excretion stayed comparable between individual days, ranging from 1.36 to 2.30 µmol d^{-1} (0.77–1.09 µmol g^{-1} cr). The consumption of heat-processed food (>100 °C) on day 4 and day 7 did not result in HPMA excretions deviating from the range observed during the washout days (Figure 3a,c).

In analogy to HPMA, CEMA excretion on day 4 and day 7 did not deviate from background during washout days, resulting in a mean daily CEMA excretion of 0.65 ± 0.26 µmol (0.32 ± 0.13 µmol g^{-1} cr), with a range from 0.46 to 0.85 µmol (0.25–0.40 µmol g^{-1} cr) (Figure 3b,d). Overall, the CEMA excretion amounted to about one-third of the HPMA excretion, reflecting an average CEMA/HPMA ratio of 0.33 ± 0.13. The ratio varied considerably between individuals (standard deviation: 38%), whereas the intraindividual variation was significantly lower (standard deviation: 5–16%).

AC exposure may be assessed, assuming that within 24 h about 20% of the AC intake is excreted as HPMA, in analogy to the excretion rate reported for rats.[15] This would indicate an AC exposure of the volunteers of 547 ± 99 µg d^{-1} (7.2 ± 1.4 µg kg^{-1} bw d^{-1}). For humans there is only very limited data from an experiment with one male volunteer available, showing about 26% of ingested AC to become excreted as HPMA and CEMA within 24 h.[27] Based on this reported human excretion rate a very similar AC exposure estimate of 561 ± 112 µg d^{-1} (7.4 ± 1.6 µg kg^{-1} bw d^{-1}) would result.

AC indoor air concentration was not measured concomitantly during the study. In a later analysis carried out about one year after study termination, AC in the indoor atmosphere was undetectable (LOQ: 1 µg m^{-3}; SGS-TÜV Saar GmbH). A default consideration of putative inhalative AC exposure from indoor atmosphere, assuming the LOQ of 1 µg m^{-3} as virtual exposure level would result in a worst case inhalative AC exposure of 20 µg d^{-1} (0.24–0.33 µg kg^{-1} bw d^{-1}), based on a respiratory volume of 20 m^3 d^{-1}. This would contribute less than 4% to the estimated total AC exposure and thus does not appear to be relevant.

3.2. Study II

A 10-day study was performed with non-smoking male and female volunteers ($n = 20$) who maintained their lifestyle and their dietary habits (unrestricted diet vs vegan diet) during the whole study period. Results concerning the correlation of dietary AA intake and AA biomarker excretion have been published previously. In brief, although limited in terms of volunteer numbers, the study indicated that vegans ingested more AA (0.38 ± 0.23 µg kg^{-1} bw d^{-1}) than volunteers who did not follow dietary restrictions (0.26 ± 0.10 µg kg^{-1} bw d^{-1}). Excretion kinetics of urinary AA-related mercapturic acids *N*-acetyl-*S*-(2-carbamoylethyl)-L-cysteine (AAMA) and *N*-acetyl-*S*-(2-hydroxy-2-carbamoylethyl)-L-cysteine (GAMA) were essentially concordant with the respective dietary AA intake. Disproportionately enhanced AA-related biomarker excretion could be traced back to reportedly inadvertent, passive exposure to tobacco and/or fire smoke, as evidenced by the respective urinary exposure biomarkers, cotinine, and CYMA. Some additional contribution of endogenous background AA exposure was demonstrated as well individually.[30]

The average absolute daily excretion of the AC-related biomarkers HPMA and CEMA monitored in volunteers of study II is displayed in **Figure 4**. Cr-adjusted values exhibited the same trends and are therefore not shown.

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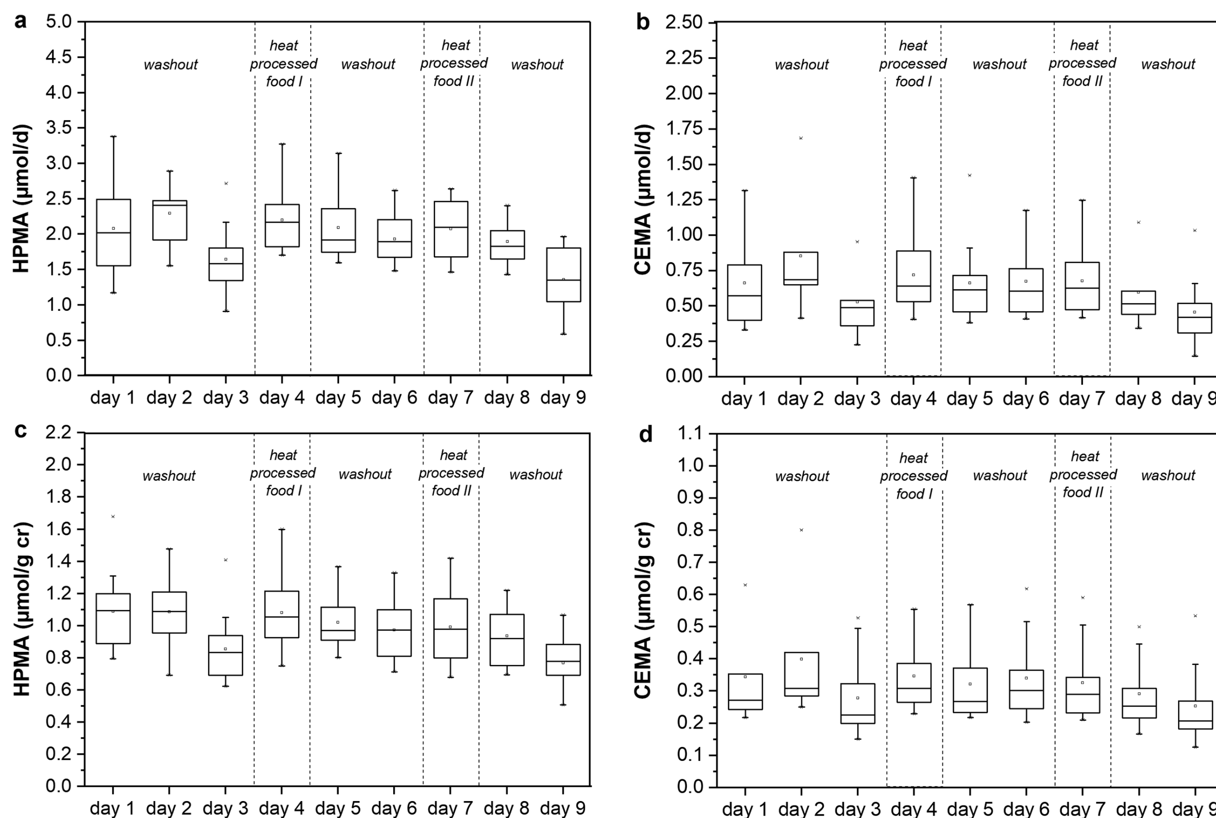

Figure 3. Urinary excretions of HPMA and CEMA during study I given as a,b) absolute amounts in µmol d^{-1} and c,d) as cr-adjusted concentrations in µmol g^{-1} cr. Study I was a 9-day study with fourteen male volunteers consuming a controlled diet. On days 1, 2, 3, 5, 6, 8, and 9 washout diets with not or only marginally heated food (≤100 °C) were consumed. The diet plan of days 4 and 7 contained heat-processed food (>100 °C). Boxes represent the interquartile range (IQR), the maximum length of each whisker is 1.5 times the IQR, crosses are outliers according to the 1.5 times the IQR criterion, squares represent mean values, horizontal lines within boxes represent median values. HPMA, *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine; CEMA, *N*-acetyl-*S*-(carboxyethyl)-L-cysteine; cr, creatinine

Daily HPMA excretion of the volunteers was 2.41 ± 0.97 µmol (1.45 ± 0.58 µmol g^{-1} cr) with 2.08 ± 0.56 µmol d^{-1} (1.13 ± 0.23 µmol g^{-1} cr) for the volunteers on unrestricted diet and 2.73 ± 1.20 µmol d^{-1} (1.76 ± 0.66 µmol g^{-1} cr) for volunteers on vegan diet. The cr-adjusted excretion showed a significant difference ($p < 0.05$) between these diets, whereas no statistically significant difference was observed regarding the absolute amounts.

Examination of the CEMA kinetics showed a mean daily excretion of 0.71 ± 0.40 µmol (0.44 ± 0.30 µmol g^{-1} cr) with no statistically significant difference between volunteers on unrestricted diet (0.59 ± 0.26 µmol/0.31 ± 0.08 µmol g^{-1} cr) versus volunteers on vegan diet (0.84 ± 0.48 µmol/0.58 ± 0.37 µmol g^{-1} cr).

The CEMA excretion was about one-third of the HPMA excretion with a mean CEMA/HPMA ratio of 0.33 ± 0.21. The daily CEMA/HPMA ratio differed between the volunteers (standard deviation: 63%) and showed considerable intraindividual variation (standard deviation: 12–53%).

As can be seen from Figure 4, the daily excretion of HPMA and CEMA varied significantly between volunteers. Some volunteers showed a quite constant excretion of HPMA (range of about 0.8–3.0 µmol d^{-1}) and CEMA (range of about 0.2–0.75 µmol d^{-1}). For other volunteers, enhanced HPMA and CEMA concentrations of about 10.0 and 2.5 µmol d^{-1}, respectively were recorded. The absolute daily HPMA and CEMA excretion of four representative volunteers (U1, V1, U5, V5) is given in **Figure 5**. Cr-adjusted values showed the same trends and are therefore not displayed.

As can be seen in Figures 4a,b and 5a,b, volunteer U1 on unrestricted diet, and volunteer V1 on vegan diet, exhibited a quite constant daily mercapturic acid excretion and a HPMA/CEMA ratio with relatively low intraindividual variation. The average HPMA/CEMA ratio of volunteer U1 was 0.27 ± 0.03 (= ± 13%), that of volunteer V1 was 0.21 ± 0.03 (= ± 12%). This relatively constant HPMA and CEMA excretion appeared uninfluenced by variations in heated food intake, although diets varied from day to day. For example, volunteer U1 consumed noodles with sauce and soup, sandwiches, pudding, soft drinks, coffee, and beer on day 1 and an increased amount of heat-processed food like bakery products, pizza, a gratinated meal and coffee on day 2. The increased intake of heat-processed food on day 2 compared to day 1 did not result in an enhanced excretion of HPMA and CEMA (Figure 5a). Similar results were seen for volunteer V1 (Figure 5b) and other volunteers (not shown in detail), arguing for a quite constant HPMA and CEMA excretion despite varying diets.

By contrast, other volunteers on unrestricted (volunteer U5) or on vegan diet (volunteer V5) displayed high mercapturic acid excretion at several days (Figure 5c,d). Volunteer U5 showed an enhanced HPMA and CEMA excretion on days 1, 2, 5, and 6

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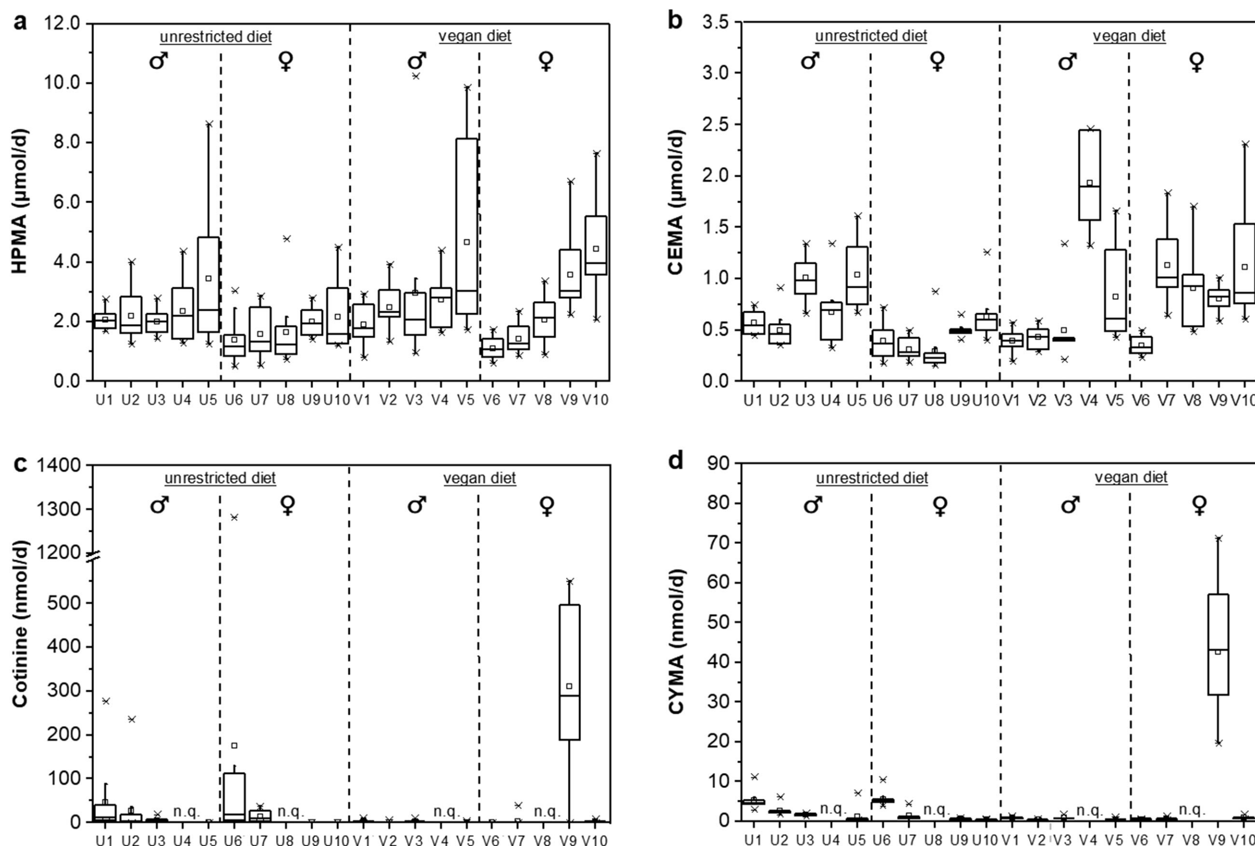

Figure 4. Urinary excretion of a) HPMA, b) CEMA, c) cotinine, and d) CYMA during study II. HPMA and CEMA excretion are given as absolute amounts in µmol d^{-1}. Cotinine and CYMA excretion are given as absolute amounts in nmol d^{-1}. Study II was a 10-day study performed with twenty volunteers under free-living conditions with protocolled diet. Ten volunteers (5 ♂, 5 ♀) consumed an unrestricted diet, ten volunteers (5 ♂, 5 ♀) consumed a vegan diet. In the figure, the volunteers of each group are sorted according to increasing range of absolute HPMA excretion. Boxes represent the interquartile range (IQR), the maximum length of each whisker is 1.5 times the IQR, crosses are outliers according to the 1.5 times the IQR criterion, squares represent mean values, horizontal lines within boxes represent median values. HPMA, *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine; CEMA, *N*-acetyl-*S*-(carboxyethyl)-L-cysteine; CYMA, *N*-acetyl-*S*-(2-cyanoethyl)-L-cysteine; n.q, not quantified (missing consent)

(Figure 5c). For the other days the average HPMA and CEMA excretion was comparable to the HPMA and CEMA excretion of volunteer U1 (Figure 5a) and V1 (Figure 5b). The CEMA/HPMA ratio was 0.40 ± 0.19 (= ± 48%) on average, but decreased to 0.34, 0.29, 0.10, and 0.27 on the days with high HPMA excretion (days 1, 2, 5, and 6, respectively). As observed for volunteers U1 and V1, HPMA and CEMA excretion of volunteers U5 and V5 was not found associated with an increased intake of heat-processed food. For instance, volunteer U5 showed the highest AC-related mercapturic acid excretion on day 5 (Figure 5c) despite a rather low intake of heat-processed food (a roll, baguette and a sausage), whereas on days 2 and 9 his diet included a higher proportion of heat-processed foods (day 2: French fries, Wiener Schnitzel, roll, sausage; day 9: potato croquettes, steak and pizza). Yet, on those days the excretion of HPMA and CEMA was clearly lower than on day 5 (Figure 5c).

Volunteer V5, following a vegan diet, was the second representative volunteer who exhibited high mercapturic acid excretion at several days. He showed markedly enhanced HPMA and CEMA excretions on days 1, 3 and 6, as compared to the other study days (Figure 5d). The average CEMA/HPMA ratio of volunteer V5 was 0.19 ± 0.04 (± 19%), slightly decreasing during the days with enhanced mercapturic acid excretion (day 1: 0.18, day 3: 0.13, day 6: 0.17). Volunteer V5 consumed heat-processed foods (potato croquettes, bakery products and a pan-fried meal), at a higher intake on day 2 than on day 1. Urinary biomarkers however behaved in an opposite way (Figure 5d). In summary, biomarker kinetics of volunteers did not reflect at all the intake of heat-processed food.

The incidentally high excretion of AC-related urinary biomarkers, observed in individual volunteers on specific days in study II (free-living conditions) was not observed during study I (controlled environment). Disproportionately enhanced AC exposure appeared to indicate exposure to further AC sources like tobacco and/or barbecue smoke. Therefore, volunteers were retrospectively interviewed concerning potential non-dietary AC exposure sources they might have been exposed to during study II. Interviews with questionnaires were performed and evaluated about 1 year after completion of the study. Although subject to recall bias, they suggested inhalative exposure from sources like open fire, barbecues and/or tobacco smoke (possibly second hand) for some volunteers. Such reported exposure for some volunteers at first glance appeared associated with enhanced AC biomarker excretion. For instance, volunteer U5 who showed an enhanced

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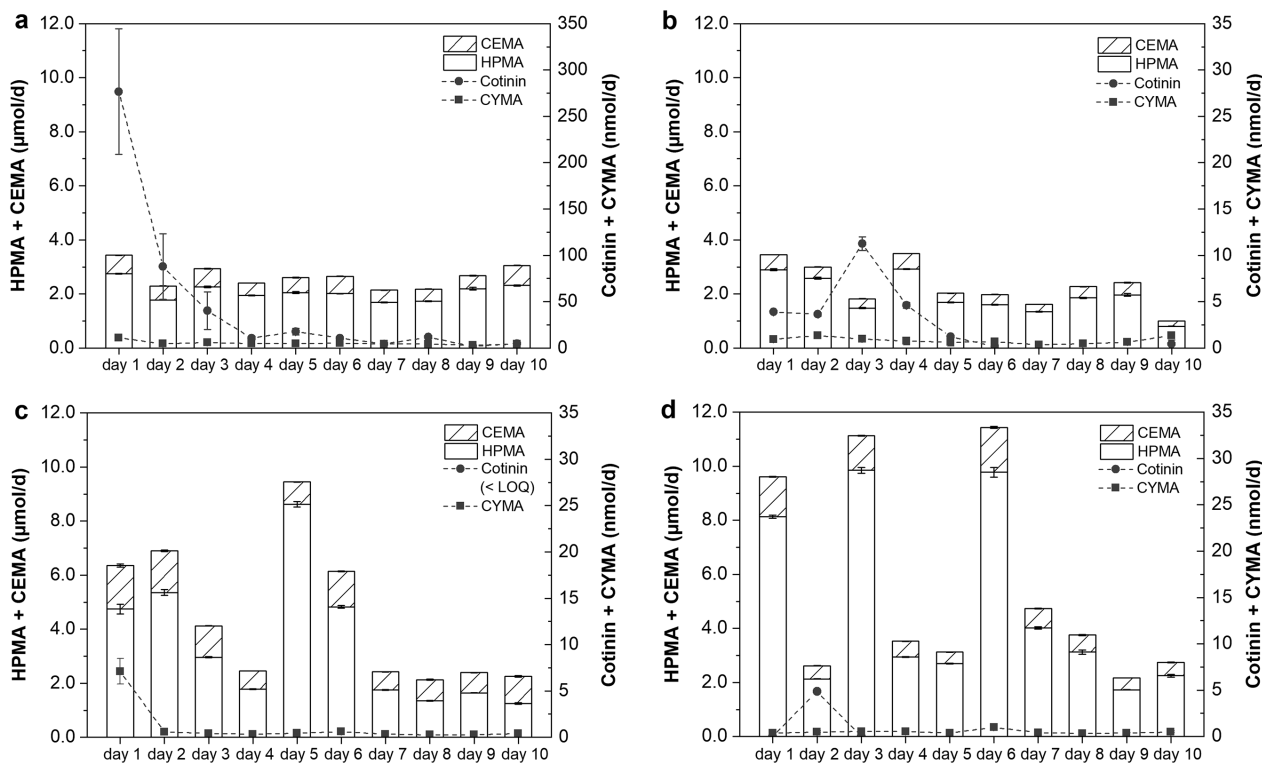

Figure 5. Urinary excretion of HPMA, CEMA, cotinine and CYMA of four representative volunteers (a: U1, b: V1, c: U5, d: V5) during study II given. HPMA and CEMA excretion are given as absolute amounts in µmol d^{-1}. Cotinine and CYMA excretion are given as absolute amounts in nmol d^{-1}. Study II was a 10-day study performed with twenty volunteers under free-living conditions with protocolled diet. Ten volunteers (5 ♂, 5 ♀) including volunteer U1 and U5 consumed an unrestricted diet, ten volunteers (5 ♂, 5 ♀) including volunteer V1 and V5 consumed a vegan diet. HPMA and CEMA values are given as mean ± range. Cotinine and CYMA values are given as mean ± standard deviation. Values below the limit of quantification (LOQ) were not displayed. HPMA: *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine, CEMA: *N*-acetyl-*S*-(carboxyethyl)-L-cysteine, CYMA: *N*-acetyl-*S*-(2-cyanoethyl)-L-cysteine

HPMA and CEMA excretion (days 1, 2, 5, and 6) reported to have participated in a barbecue on the same or the preceding days (days 1, 2, and 5). To verify the retrospective recalling, cotinine and CYMA, associated with tobacco smoke and/or potential other smoke exposure, were determined in the urine samples of 16 out of 20 volunteers. However, the excretion of these biomarkers did not correlate with the HPMA and CEMA excretion (Figures 4 and 5). Volunteers who showed enhanced HPMA (and CEMA) excretion levels did not show increased cotinine or CYMA levels. Likewise, enhanced HPMA and CEMA excretion of individual volunteers on single days were not associated with increased cotinine or CYMA excretion (Figure 5). For instance, volunteer U5 showed enhanced HPMA and CEMA excretion on days 1, 2, 5, and 6, whereas cotinine was absent in the urine on these days and only minute amounts of CYMA were detected, despite a reported participation in barbecuing. Likewise, HPMA and CEMA excretion of volunteer V5 was enhanced on days 1, 3, and 6, however cotinine and CYMA levels were not increased on those days.

On the assumption that 20% of the AC intake is excreted as HPMA,[15] average AC exposure may be assessed to be 668 ± 272 µg d^{-1}, corresponding to 10.1 ± 4.1 µg kg^{-1} bw d^{-1} (unrestricted diet: 8.6 ± 2.0 µg kg^{-1} bw d^{-1}, vegan diet: 11.5 ± 5.2 µg kg^{-1} bw d^{-1}, difference not statistically significant). Exposure assessment based on the assumption that 26% of the AC intake is excreted as HPMA and CEMA[27] would indicate a total AC exposure of 666 ± 258 µg d^{-1} corresponding to 10.1 ± 4.1 µg kg^{-1} bw d^{-1} (unrestricted diet: 8.4 ± 2.0 µg kg^{-1} bw d^{-1}; vegan diet: 11.7 ± 5.1 µg kg^{-1} bw d^{-1}, difference not statistically significant).

4. Discussion

AC-related mercapturic acids, HPMA and CEMA, and tobacco smoke exposure related biomarkers, cotinine and CYMA, were monitored in the urine of non-smoking volunteers under various dietary regimes. Study I was a 9-day human intervention study performed with fourteen volunteers under strictly controlled conditions. In contrast, study II was performed under free-living conditions. Twenty volunteers maintained their lifestyle and dietary habits (unrestricted diet vs vegan diet) during a 20-day study period. The comparison between volunteers on unrestricted diet and those on vegan diet was expected to reflect differential exposure to process-related contaminants. Vegans were supposed to preferentially consume less heat-treated vegetable food, yet to incidentally ingest highly processed vegetable-based food such as certain meat substitutes. Retrospective recall of potential exposure to further exogenous AC sources was contrasted with results of monitoring biomarkers of inadvertent passive exposure to tobacco smoke, cotinine and CYMA, in the urine of 16 volunteers of study II.

The average AC-related biomarker excretion in both studies was comparable to the range of urinary HPMA and CEMA

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published for non-smokers in several prior studies.[2,11–14] Volunteers of study I showed an average HPMA excretion of 1.95 ± 0.35 µmol d^{-1} (0.98 ± 0.18 µmol g^{-1} cr d^{-1}) and volunteers of study II an average HPMA excretion of 2.41 ± 0.97 µmol d^{-1} (1.45 ± 0.58 µmol g^{-1} cr d^{-1}). Previously determined average HPMA excretion levels in non-smokers were similar, ranging from 0.20 µmol g^{-1} cr (43.5 µg g^{-1} cr)[14] to 2.23 µmol g^{-1} cr (493 µg g^{-1} cr).[13] For CEMA the average excretion of the volunteers was 0.65 ± 0.26 µmol d^{-1} (0.32 ± 0.13 µmol g^{-1} cr d^{-1}) during study I and 0.71 ± 0.40 µmol d^{-1} (0.44 ± 0.30 µmol g^{-1} cr d^{-1}) during study II. In non-smokers an average CEMA excretion of 42 µg g^{-1} cr (0.18 µmol g^{-1} cr) has been reported elsewhere.[14]

4.1. Inter- and Intraindividual Variations

The average CEMA/HPMA ratio of the volunteers was 0.33 ± 0.13 (study I) and 0.33 ± 0.21 (study II). The interindividual variation of the CEMA/HPMA ratio was lower during study I (standard deviation: 38%) than during study II (standard deviation: 63%). Interindividual differences between volunteers may reflect differences in the metabolism, for example due to polymorphisms of enzymes involved in the AC metabolism. The intraindividual variation was clearly lower during study I (standard deviation: 5–16%), performed in a controlled environment and with restricted physical exercise, at variance to study II (standard deviation: 12–53%) which was performed under free-living conditions. This may suggest some influence of nutritional and lifestyle variability.

4.2. Lack of Correlation Between Intake of Heat-Processed Food and Excretion of AC-Related Biomarkers

Both studies did not indicate a correlation between intake of heat-processed food and excretion of the urinary AC biomarkers HPMA and CEMA.

Study I encompassed strictly controlled diet intake and living conditions with defined washout and intervention phases. Substantial and sustained HPMA (≈ 2.0 µmol d^{-1}) and CEMA (≈ 0.6 µmol d^{-1}) excretion of the volunteers remained largely constant during the whole study period. No noteworthy modulation of urinary mercapturic acid excretion was observed, irrespective of whether heat-processed food was consumed or not (Figure 3).

Observations of study II were in part in line with those of study I, showing volunteers on unrestricted and on vegan diet to excrete on average HPMA in the range of about 0.8–3.0 µmol d^{-1} and CEMA in the range of about 0.2–0.75 µmol d^{-1} (Figure 4). However, other individuals showed markedly enhanced HPMA and CEMA excretions (about 10.0 µmol d^{-1} and 2.5 µmol d^{-1}, respectively) again without a detectable association with food intake, irrespective of whether they consumed heat-processed food or not. In contrast, their AA-related biomarker excretion had previously indeed been found to correlate with the intake of heat-processed foods under controlled[29,31] as well as free-living conditions.[30]

Associations between intake of heat-processed food and an increased excretion of urinary AC biomarkers observed in other studies[27,28] could not be confirmed in the here presented studies. This discrepancy may result from marked differences in the design of the studies. In the study of Watzek et al.[27] after 12 h fasting, commercially available (AC: 4.6 µg/175 g) or self-prepared potato crisps (AC: not determined) were consumed at one defined time point, with subsequent urine collection in short intervals (2 h to max. 12 h intervals). Similar short urine collection intervals were used in the study of Wang et al.[28] after consumption of fried foods including fried chicken and French fries. In the present investigation food was consumed within a normal daily regimen, with no prior fasting. In addition, the urine collection periods during study I (4–24 h intervals) and study II (12 h intervals) were comparatively longer. In view of the observed excretion kinetics of HPMA ($n = 1$, $t_{1/2} = 8.9$ h, $t_{max} = 2$ h) and CEMA ($n = 1$, $t_{1/2} = 11.8$ h, $t_{max} = 2$ h),[27] the longer collection intervals in the present studies may have obscured the detection of rapid and small variations of AC-related urinary biomarker excretion, especially given their already substantial background. In addition, it is conceivable that 12 h fasting not only reduced or minimized AC exposure from dietary sources but also from various pathways of endogenous metabolism due to reduced dietary energy uptake. The apparent correlation with putative AC dietary uptake as reported by Watzek et al.[27] may thus, at least in part, just be reconciled with enhanced endogenous AC formation associated with energy metabolism subsequent to food uptake.

4.3. Relevance of Mercapturic Acid Background Levels

The observed absence of a correlation between intake of heat-processed food and excretion of the urinary AC biomarkers is at variance to the observations made earlier for the process-related contaminant AA. The AA-related biomarker excretion previously was found to correlate with the intake of heat-processed foods under controlled conditions[29,31] as well as under free-living conditions.[30] This may be reconciled with a much lower background of urinary AA biomarkers as compared to those associated with AC exposure. In the present investigation, a substantial and rather constant HPMA and CEMA excretion was observed for all volunteers of study I and in part for volunteers of study II, despite the fact that their diets varied considerably between study days. This relatively constant excretion of AC-related exposure biomarkers indicates a sustained and substantially higher (at least about factor 10) overall exposure to AC as compared to AA. Moreover, even though adduct formation of AC with peptides and some release of AC from those adducts had previously been observed under physiological conditions,[32,33] results of the studies reported here render it not very likely that food intake or other exogenous sources of AC exposure were major contributors to the observed AC biomarker excretion. Rather, these observations point to a substantial contribution from endogenous, as yet underexplored sources within the organism.

4.4. Supposed Sources of Enhanced HPMA and CEMA Excretion

Potential sources for the observed markedly enhanced incidental excretion of AC-related biomarkers in study II could not be identified unequivocally in the present study. The HPMA

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and CEMA excretion did not correlate with an increased intake of heat-processed food. Furthermore, no association with other potential exogenous AC exposure sources was observed, especially not between the excretion of tobacco smoke related biomarkers monitored after approval of an amendment to the study, and AC exposure biomarkers. Since not all volunteers originally participating could be contacted or responded, the number of volunteers evaluated with regard to (inadvertent) smoke exposure was reduced (16/20 originally participating volunteers). Of note, a suspected exposure to tobacco smoke, or fumes from barbecues or open fires, as primarily suggested from evaluation of retrospective questionnaire was not verified by results of monitoring urinary biomarkers associated with (tobacco) smoke exposure. Some confounding, considered of minor importance, may potentially result from differential physical activity of volunteers, since in study I all participants were required to reduce their physical activities to a minimum, whereas in study II physical activities of the volunteers were not monitored.

At present, the observed variations in biomarker excretion at several days remain largely unexplained and require further research into additional exogenous or endogenous factors of potential influence. Microbiological generation of AC or AC equivalents is well established and has been shown to also occur in the gastrointestinal tract. A predominant AC precursor appears to be glycerol, an intrinsic food constituent predominantly bound in tri- and other glycerides, liberated during lipid digestion.[22,23] The bacterial glycerol metabolite 3-HPA spontaneously converts into AC and, equilibrating with its hydrate and oligomeric forms constitutes the multicomponent system "reuterin". The AC in the latter has been reported to convert process-related contaminants, such as certain heterocyclic aromatic amines (PHIP) into AC derived adducts (PHIP-M1).[23] In humans *L. reuteri* has been found in the gastrointestinal and in the urinary tract, on skin and in breast milk.[21] Its abundance appears to vary between individuals, which may in part be reconciled with the differential AC-related biomarker excretion found in the present study, but which may also reflect differential dietary glycerol exposure. It thus appears possible that the human microbiome may substantially contribute to endogenous AC exposure.

This study is the first to convincingly show a sustained and substantial background exposure to AC in nonsmoking humans, clearly independent from uptake of heat-processed foods. The data strongly point to endogenous AC generation by pathways of mammalian and/or microbial metabolism as yet not taken into consideration. As can be estimated from the data presented here, total exposure to AC may be more than tenfold higher than exposure to AA, a process-related food contaminant of public concern. The biological consequences of such sustained AC exposure are far from being established and require further detailed research.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

G.E. served as scientific advisor of ISIC. All the other authors declare no conflict of interest.

Author Contributions

M.R. performed the experimental and analytical work and, in close collaboration with K.G. and T.B., organized and carried out the human intervention studies. The investigation was conceptualized by E.R., G.E., K.A., and A.L. It was supervised by E.R. and G.E. Results were evaluated and interpreted by M.R., E.R., and G.E., with the support of K.A. and A.L. All authors contributed to the preparation of the manuscript.

Keywords

acrolein, biomarkers of exposure, mercapturic acids, *N*-acetyl-*S*-(3-hydroxypropyl)-L-cysteine (HPMA), *N*-acetyl-*S*-(carboxyethyl)-L-cysteine (CEMA)

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