Acrylamide: A Cooking Carcinogen?
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Exposure to acrylamide (AA) has been monitored by mass spectrometric detection of the adduct, N-(2-carbamoylethyl)valine (CEV), to the N-termini of hemoglobin (Hb), according to the N-alkyl Edman method. In these studies, a conspicuous background level, about 40 pmol/g of globin, of apparently the same adduct was regularly observed in Hb from persons without known exposure to AA. For testing of the hypothesis that this adduct originates from AA formed in cooking, rats were fed fried animal standard diet for 1 or 2 months. These animals exhibited a strong increase of the level of the studied Hb adduct, compared to control rats fed unfried diet. By gas chromatography/tandem mass spectrometry, the identity with CEV was confirmed by the concordance of the product ion spectrum of the studied adduct with that of a verified standard and by interpretation of the fragment ions. Further support of the chemical structure, at the same time pinpointing AA as the causative reactive factor, was obtained through the demonstration that AA is formed in the heating of the feed and that the level of AA in the fried feed is compatible with the measured levels of the CEV adduct. The raised CEV adduct levels observed in experimental animals are of a magnitude that is similar to the background level in nonsmoking humans. These data render it likely that cooking of food is a major source of the background dose of AA also in humans. An evaluation of cancer tests of AA and available data for its metabolism leads to the estimation that the background dose of AA is associated with a considerable cancer risk.

Introduction

Adducts to hemoglobin (Hb)1 have been identified and quantified for the purpose of in vivo dose monitoring of reactive chemicals or metabolites. Through the development of a sensitive analytical technique, the N-alkyl Edman method (1, 2), the measurement of adducts to the N-termini, valines, of globin chains has become a powerful tool for the dosimetry of genotoxic chemical carcinogens. This method has been applied for dose monitoring in persons occupationally exposed to acrylamide (AA) (3). AA reacts by Michael addition with the terminal NH2 groups of globin chains during formation of N-(2-carbamoyl-ethyl)valine (CEV) (Figure 1). AA is metabolized to glycidamide, an epoxide (Figure 2) which is reactive toward DNA (4) and is assumed to be the mutagenic and cancer-initiating species, whereas AA is probably the main cause of neurotoxic effects of AA exposure (5, 6).

In studies of occupational exposure to AA, a conspicuous background level of an adduct that seems to be identical with the CEV adduct from AA is regularly observed in control persons without known exposure to AA (7). The average level of this adduct in Hb from nonsmoking controls is about 40 pmol/g of globin.2 Preliminary estimation of the lifetime cancer mortality risk increment associated with an AA exposure leading to the observed background CEV adduct level yields unexpectedly high values (8). It is for this reason important to clarify whether the causative factor really is AA and, if so, to identify its sources.

The assessment of an adduct to the α-amino group of the N-terminal amino acid permits the conclusion that the adduct is formed in reaction with the protein and that it is not due to incorporation of a modified amino acid, as might be the case with, for example, cysteine-S adducts (9). One characteristic of background CEV is that the level is appreciably lower in wild animals and grazing cows than in humans.3 Furthermore, observed adduct levels in nonsmokers exhibit a relatively narrow inter-individual variation around the mean value (20–60 pmol/g of globin). These observations would signify a

Footnotes:
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1 Abbreviations: AA, acrylamide; CEV, N-(2-carbamoyl-ethyl)-L-valine; d7CEV, N-(2-carbamoyl-ethyl)[2H7]-L-valine; d3CEV, N-(2-carbamoyl-ethyl)[2H3]-L-valine; GC/MS/MS, gas chromatography/tandem mass spectrometry; Hb, hemoglobin; NICI, negative ion chemical ionization; PFPTH, pentafluorophenylthiohydantoin.
2 M. Törnqvist et al., unpublished work.

References:
(1) G. M. Toor, et al., unpublished work.
(2) R. J. Toor, et al., unpublished work.
factor that differs between wild animals and humans, and it is generally spread in human populations. The occurrence of AA in tobacco smoke (with raised CEV levels in Hb from smokers; 7) indicates that AA is formed in heating of biological matter.

It was hypothesised that intake of fried diet might lead to exposure to AA and be causative in the buildup of the background level of the CEV adduct. Therefore, an experiment was carried out with rats with studies of the influence of intake of fried diet on the level of the monitored adduct. Furthermore, the identity of the adduct was verified in different, complementary, ways. Acrylamide concentrations in the diets that were used were measured after modification of a standard method. The levels of the observed adduct in the rats were compared with the level of intake of AA in the fried diet.

Materials and Methods

Caution: Acrylamide, N,N-dimethylacrylamide, and pentafluorophenyl isothiocyanate are hazardous and should be handled with care.

Chemicals. Acrylamide (AA) and N,N-dimethylacrylamide were obtained from Sigma-Aldrich AG. Pentafluorophenyl isothiocyanate (> 96%) was obtained from Fluka (Buchs, Switzerland) and was purified on a Sep-Pak silica cartridge (2). The pentafluorophenylthiodydantoin of N-(2-carboxamidoethyl)-L-valine (CEV-PFPTh) and N-(2-carboxamidoethyl)(2,3-H2)-L-valine (d3CEV-PFPTh) were obtained from E. Bergmark (cf. refs 3 and 7). The structure of the CEV had been verified by 1H NMR (Я3). The PFPTh of N-(2-carboxamido(2,3-H2)-L-valine (d3CEV-PFPTh) was prepared via reaction of [2,3,3,3H]acrylamide (from Cambridge Isotope Laboratories, Andover, MA) with hemolysates as described by Törnqvist (2) and detachment of the d3CEV-PFPTh by derivatization with the N-alkyl Edman method (see below). Globin alkylated with AA to be used as a standard and for calibration was prepared and characterized as previously described (7). Myoglobin, salt-free, from horse skeletal muscle was obtained from ICN Biomedicals (Aurora, OH). Formamide was washed twice with pentane. All other chemicals and solvents were analytical grade. The water used was Millipore-filtered (Milli-Q).

Instrumentation for Analysis of Hb Adducts. The adducts were analyzed, after derivatization of samples (see below), by gas chromatography/tandem mass spectrometry (GC/MS/MS) on a Finnigan TSQ 700 instrument. The operating procedures for the gas chromatography were as follows: helium gas carrier at a constant gas pressure of 8 psi (55 kPa) and samples injected on a Finnigan TSQ 700 instrument. The operating procedures for gas chromatography were as follows: injection temperature of 250 °C and splitless injection of 2 μL on a HP PAS 1701 fused silica capillary column (25 m × 0.32 mm i.d.; 0.25 μm film thickness). The temperature program was as follows: isothermal for 1 min at 65 °C, increased at a rate of 15 °C/min to 250 °C, and isothermal for 10 min. Quantification was performed by comparison of the peak area ratio of 2,3-dibromopropionamide with the internal standard 2,3-dibromo-N,N-dimethylpropionamide, monitored by using electron ionization (70 eV) and selected ion monitoring (m/z 106, 108, 150, and 152 and m/z 178 and 180, respectively).

The graphitized carbon black columns (carbograph 4, 7 mm × 12 mm i.d.) were obtained from Laboratory Analyticals (LARA, Rome, Italy). The mixtures were prepared in a Warri model 700 G blender. Filtration was performed on a glass-fiber prefiter (90 mm i.d.) and an SRP25 PTFE filter (45 μm, Mini-Sart), both obtained from Sartorius (Göttingen, Germany). Purification via gel permeation chromatography was performed on Bio-Rad SX-3 gel (Hercules, CA), in a glass column prepared according to SLV (10) (400 mm × 25 mm i.d.).

Animals and Diets. Sprague-Dawley rats, 1 month old, were obtained from B&K Universal AB (Sollentuna, Sweden). Two experiments were carried out. In the first experiment (expt I), there were six males with three in each diet group, and in expt II, the two diet groups consisted of eight rats, four of each sex. The weight of each animal was controlled regularly (Table 1). All animals were kept under the same external conditions (three animals/cage, temperature of 20 °C, and a 12 h light–dark cycle). At the end of the experiments, blood samples, for Hb adduct assessment, were collected by heart puncture.

Rats were fed B&K Rat and Mouse Standard Diet in unfried or fried form; 1 kg of the standard diet was soaked in 750 mL of water and formed as a pancake (2–3 mm thickness), and then dried at room temperature (unfried) or fried on both sides to a brown color in expt I and a moderately brown color in expt II in a hot Teflon pan without fat. In expt I, the pancakes were heated to 200–220 °C (surface temperature) for about 2 min, and in expt II, the surface temperature was 180–200 °C for about 5 min. The temperature during frying was measured with a CIE model 307 digital thermometer, connected with a probe (accuracy of ±2.2 °C; Clas Ohlson, Stockholm, Sweden). The residual water content measured after preparation was about 15% of the total weight of the food in both diets. The diets were freshly prepared twice a week; one portion was provided immediately, and the rest was stored at 4 °C until it was used.

Hb Adduct Assessment. (1) Preparation and Derivatization of Blood Samples. Erythrocytes, separated from plasma and washed twice with saline, were hemolyzed by addition of 1.5 volumes of Millipore-filtered water. Globin was then precipitated with ethyl acetate from a 2-propanol/HCl solution of the hemolysate (2, 11).

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Table 1. Weight of Rats (grams ± SD) during the Experiments

<table>
<thead>
<tr>
<th>Expt I</th>
<th>group (n)</th>
<th>day 1</th>
<th>day 41</th>
<th>day 62</th>
<th>day 72</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (3)a</td>
<td>91.9 ± 2.9</td>
<td>384 ± 10</td>
<td>456 ± 22</td>
<td>492 ± 34</td>
<td></td>
</tr>
<tr>
<td>fried diet (3)a</td>
<td>89.9 ± 4.6</td>
<td>368 ± 13</td>
<td>432 ± 4</td>
<td>488 ± 13</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Expt II</th>
<th>group (n)</th>
<th>day 1</th>
<th>day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (4)a</td>
<td>91.8 ± 2.2</td>
<td>357 ± 11</td>
<td></td>
</tr>
<tr>
<td>fried diet (4)a</td>
<td>92.4 ± 10.8</td>
<td>345 ± 11</td>
<td></td>
</tr>
<tr>
<td>control (4)b</td>
<td>92.1 ± 0.6</td>
<td>250 ± 7</td>
<td></td>
</tr>
<tr>
<td>fried diet (4)b</td>
<td>91.9 ± 1.1</td>
<td>251 ± 13</td>
<td></td>
</tr>
</tbody>
</table>

a Male rats. b Female rats.
Samples of globin were dissolved in formamide and derivatized with pentafluorophenyl isothiocyanate, leading to detachment of alkylated N-terminal valines as PFPTHs according to the N-alkyl Edman method (1, 2). For the analysis of the CEV adducts, the internal standard, d7CEV-PFPTH, was added prior to the extraction of the PFPTHs with diethyl ether, and water was added before the second extraction according to modifications as described by Bergmark (7). The samples were washed as previously described.

(2) Quantification of Adduct Levels. A calibration curve for the determination of levels of the CEV adduct in Hb was prepared by addition of different amounts of standard globin, corresponding to 0–1 nmol/g of the CEV adduct, and internal standard (d7CEV-PFPTH) to horse muscle myoglobin used as “blank” globin (2). The calibration samples were derivatized and analyzed by the N-alkyl Edman method in the same way as the rat Hb samples.

The quantification was achieved with GC/MS/MS, NICI, and based on the ratio of the peak areas of the analyte and internal standard. The major mass fragment m/z 375 [M – 20] and its product ions m/z 303, 304, and 319 were used for quantification of the CEV adducts and compared with product ions m/z 310 and 326 of the major fragment m/z 382 [M – 20] of the internal standard d7CEV-PFPTH (7).

(3) Identification of the Adducts. The structure of the adduct in rat Hb, studied because of its similarity with the adduct from AA, the CEV adduct, with respect to retention time and measured product ions (m/z 303, 304, and 319) was further verified. The total product ion spectrum of the precursor ion m/z 375 of the observed adduct was compared with that of a standard of CEV-PFPTH with deuterium substitution in the valine residue of N-(2-carbamoyl-ethyl)[2H7]-L-valine (d7CEV-PFPTH) or in the AA moiety of N-(2-carbamoyl[2H3]ethyl)-L-valine-PFPTH (d3CEV-PFPTH) (Figure 3).

Identification of Acrylamide in the Diets. For the analysis of AA content in the diets, samples were prepared essentially as the diets used in feeding exp II. Unfried B&K Rat and Mouse Standard Diet was divided into two portions. One portion (10.5 g) was mixed with Millipore-filtered water (7.5 mL), and a small pancake was made of the resulting mixture. The pancake was fried in a Teflon pan on both sides to a brown color. The other portion (10 g) served as a blank. Samples were mixed with water (100 mL) until they were homogeneous (ca. 1 min), and the resulting mixture was filtered (Sartorius glass-fiber filter) by suction. The filtrate (ca. 40 mL) was passed through a graphitized carbon black column retarding large and hydrophobic molecules. The eluate was collected, and N,N-dimethylacrylamide was added to the eluate as an internal standard. The analytes and standard were brominated to 2,3-dibromopropionamide and 2,3-dibromo-N,N-dimethylpropionamide, respectively, by using KBr (7.5 g), HBr (acidification to pH 1–3), and saturated bromine water according to the method of Castle et al. (12, 13). After 18 h at 0 °C, the excess bromine was decomposed by adding 1 M sodium thiosulfate as drops until the yellow color disappeared. Sodium sulfate (15 g) was added, and the solution was extracted with an ethyl acetate/hexane solution [2 × 10 mL, 1:4 (v/v)]. The two organic fractions were combined, filtered through a Sartorius Mini-Sart filter, and purified by gel permeation chromatography (retarding large molecules, e.g., lipids) with an ethyl acetate/cyclohexane solution [1:1 (v/v)] as the eluent at a flow rate of 5 mL/min. One fraction was collected between 16 and 40 min, and the eluate was concentrated to 100 µL and transferred to GC insert vials. Whenever precipitation of impurities occurred, centrifugation was performed. Standards of 2,3-dibromopropionamide, with addition of AA to water and in the final analysis unfried feed as a matrix, were prepared in the same way. Quantification was performed by GC/MS as described above.
Results

Animals. The weights of the animals during the experimental periods, from age 30 days to age 102 or 60 days in expt I or II, respectively, are given in Table 1. A slight tendency of relatively slower growth of the males fed fried diet, compared to the controls, is not statistically significant.

Identification of the Hb Adduct. A peak in the MS analysis of the blood samples from rats was found to agree with regard to retention time and monitored product ions (m/z 303, 304, and 319) with the PFPTH of the adduct N-(2-carbamoylethyl)valine (CEV) (for the structure, see Figure 3), i.e., the product of the reaction of acrylamide (AA) with the N-terminal valines in hemoglobin. It was further shown that the product ion spectra of precursor ion m/z 375 of standard CEV-PFPTH and of the corresponding peak from a sample from a rat fed fried diet were concordant (panels a and b of Figure 4). A further identification follows from interpretation of the mass fragments of the analyte and the synthetic standard. The interpretation was obtained by comparison with standards of CEV-PFPTH that were deuterium-substituted either in the valine residue ([2H7]isopropyl) or in the adducted group (2-carbamoyl[2H6]ethyl) (d9CEV-PFPTH) (cf. Figure 3 and Table 2).

Hb Adduct Levels from Acrylamide. The levels of the CEV adducts in Hb from rats are summarized in Table 3. The groups of rats fed fried feed exhibited, as compared to the control animals (Table 3), an approximately 10 times higher level of this adduct. The difference between the two experiments can partly be explained by the difference in feeding time and by the fact that the frying method varied somewhat between batches. In expt II, the feeding was discontinued after 30 days, i.e., before steady-state levels of adducts were reached, which occurs after about 60 days, the life span of rat erythrocytes. In expt I, the duration of exposure to adduct-generating material in fried or control food exceeded this time (cf. ref 14).

Acrylamide Identification in Fried Food. A modification of a standard method was worked out for the identification of AA in feed. Increased levels of AA were observed in the fried diet. In two analyses that were carried out, the AA content in the unfried feed was below the detection limit, 10 μg/kg. In a first exploratory analysis of freshly fried feed, an AA content of 200 μg/kg was determined. In a second analysis of freshly fried feed, an AA content of 110 μg/kg was found. This value was determined in comparison with a standard sample containing 100 μg of AA/kg of feed; it allows for the matrix effect and should therefore be given a larger weight in the mean value. As the frying procedure was not standardized, the mean value which may be given as 150 μg/kg may be uncertain by a factor of 2.

Discussion

Feeding rats with fried feed is shown to lead to a large increase in the level of the Hb adduct concluded to be N-(2-carbamoylethyl)valine (CEV), i.e., the adduct formed in the reaction of acrylamide (AA) with the N-termini of the globin chains, and which occurs as a background adduct in humans. In the animals fed fried diet, the CEV level approached a steady-state level some 10 times higher than that of the control animals. In GC/MS/MS analysis, the identity with CEV of the monitored adduct was confirmed by the concordance of its product ion spectrum with that of a verified standard and by the interpretation, with the aid of deuterium-substituted standard compounds, of the fragment ions. Further support for the chemical structure, at the same time pinpointing AA as the causative reactive factor, was obtained through the demonstration that AA is formed in the heating of the feed and that by and large the level of AA in the fried feed is compatible with the measured levels of the CEV adduct in rats.

It may in this context be noted that heating of the feed does not provoke a general increase in adduct levels, as it was shown in the study presented here that the levels in rat Hb of N-(2-hydroxyethyl)valine and N-(2-hydroxypropyl)valine remained unaffected or are even somewhat decreased following heating of the rat feed (data not shown). The background levels of these adducts have in previous studies been shown to have determinants other than food heating (15, 16).

The quantitative comparison of the uptake of AA and adduct level was based on the assumption that the daily food consumption by the rats was 10% of the body weight, a figure that would be approximately valid at the low

Table 3. Level of the N-(2-Carbamoylethyl)valine Adduct in Hb from Rats Fed the Unfried Control Diet Compared to Fried Diet (picomoles per gram ± SD)

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>fried diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>males</td>
<td>19.6 ± 2.6 (n = 3)</td>
<td>157.3 ± 9.4 (n = 3)</td>
</tr>
<tr>
<td>females</td>
<td>4.8 ± 0.8 (n = 4)</td>
<td>65.9 ± 15.5 (n = 4)</td>
</tr>
</tbody>
</table>

Table 2. GC/MS/MS Fragmentations (NCI) of the Precursor Ions (Pul) [M – 20] from the Standards CEV-, d9CEV-, and d9CEV-PFPTH, with Given Relative Intensities (ris) of Precursor Ions and Product Ions

<table>
<thead>
<tr>
<th>Compd (M)</th>
<th>Pul m/z (ri)</th>
<th>P – I m/z (ri)</th>
<th>P – II m/z (ri)</th>
<th>P – III m/z (ri)</th>
<th>P – IV m/z (ri)</th>
<th>P – V m/z (ri)</th>
<th>P – VI m/z (ri)</th>
<th>P – VII m/z (ri)</th>
<th>P – VIII m/z (ri)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEV-PFPTH</td>
<td>375 (48)</td>
<td>360 (9)</td>
<td>340 (6)</td>
<td>319 (35)</td>
<td>303/304 (87/100)</td>
<td>284 (44)</td>
<td>256 (8)</td>
<td>226 (10)</td>
<td>187 (32)</td>
</tr>
<tr>
<td>d9CEV-PFPTH (398)</td>
<td>378 (71)</td>
<td>363 (9)</td>
<td>343 (6)</td>
<td>319 (22)</td>
<td>303/304 (76/100)</td>
<td>284 (38)</td>
<td>256 (9)</td>
<td>229 (17)</td>
<td>187 (59)</td>
</tr>
<tr>
<td>d9CEV-PFPTH (402)</td>
<td>382 (69)</td>
<td>364 (10)</td>
<td>344 (6)</td>
<td>319 (120)</td>
<td>290 (44)</td>
<td>262 (9)</td>
<td>233 (12)</td>
<td>187 (39)</td>
<td></td>
</tr>
</tbody>
</table>
body weight during the first month of the dietary experiments (days 1–30) (Table 1; cf. ref 17). With these assumptions, the expected adduct levels on days 30 (expt II) and 72 (expt I) following intake of food containing 150 (75–300) \( \mu \)g of acrylamide/kg were estimated (14). This calculation (cf. ref 8) was based on data for the life span of Hb in the rat (60 days), the in vivo half-life of AA in the rat (1.4 h), and the rate constant for the reaction of AA with the N-termini of Hb (4.4 \( \times \) 10^{-6} L g^{-1} h^{-1}) (3). From the data summarized in Table 4, it appears that the measured adduct level in expt i is higher than expected, whereas in expt II, a better agreement between found and expected levels is obtained. The frying temperature was higher in the preliminary expt I than in expt II, and the frying for analysis of AA was carried out in accordance with expt II.

The background level of CEV in the control rats fed unfried standard feed is probably partially due to heating of certain components in the manufacture of laboratory animal feed, with formation of AA as a result (demonstrated in preliminary analyses; data not shown).

The raised CEV adduct levels observed in experimental animals are of a magnitude that is similar to the background level in nonsmoking humans, 40 (20–60) pmol/g of globin. These data render it likely that cooking of food and heating in food manufacture is one major source of the background dose of AA also in humans.\(^5\) Awaiting studies of CEV adduct levels in humans with specific food habits, this suggestion is supported by the observation that in coelenterates patients a significant (P < 0.01) drop in this adduct level occurs during the first month after operation, a period dominated by "mild" feeding.\(^2\) Furthermore, it has been shown that the CEV adduct level is independent of the intestinal flora.\(^2\)

In two experimental 2 year studies of F344 rats fed AA via their drinking water, the relationship between the administered dose and tumor incidence has been established (18, 19). From these experimental studies, the U.S. Environmental Protection Agency (20) derived a unit risk factor for human exposure, amounting to 4.5 \( \times \) 10^{-3} at a life-long daily uptake of 1 \( \mu \)g of AA/kg of body weight. From known kinetic parameters, the human background CEV level is computed to be due to a daily uptake of 1.6 \( \mu \)g of AA/kg, corresponding to a cancer risk of 7 \( \times \) 10^{-3} from life-long exposure. Application of a linear multiplicative risk model found to be adaptable to the experimental data (21) indicates that the cancer risk may be even higher. In view of this high risk, the following measures are needed: removal of the present uncertainty of the risk estimate, primarily through a critical comparative validation of the risk models and improved determination of parameter values, in particular, the dose of the putatively carcinogenic metabolite, glycylamide, per absorbed amount of AA; studies of factors, for example, temperature, heating time, and food composition, that are determinants of AA formation; and identification of the origin and mechanism of formation of AA.

In recent years, a range of background adducts to Hb and/or DNA in knowingly unexposed humans have been demonstrated (22–24). In the study presented here, an observed background adduct is demonstrated experimentally to be a true adduct; it is structurally identified and quantitatively related to an identified environmental factor.

### Acknowledgment

We are grateful to Dr. Emma Bergmark for generous sharing of standard compounds, Mr. Ioannis Athanassiadis for skilful assistance with mass spectrometric studies, Mr. Jarl Olsson for assistance with animal experiments, and Prof. Lars Ehrenberg and Dr. Ulf Olsson for valuable viewpoints. This work was partly supported financially by the Swedish Association for Graduate Engineers, by the Foundation for Strategic Environmental Research, and by the Centre for Research on Natural Resources and the Environment at Stockholm University.

### References


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\(^5\) In ongoing work, it has been shown that frying of meat leads to the formation of acrylamide.

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### Table 4. Observed and Expected Increase of N-(2-Carbamoylvaleryl)Valine Levels in Hb after Intake of Feed Containing 150 (75–300) \( \mu \)g of Acrylamide/kg

<table>
<thead>
<tr>
<th>expt</th>
<th>feeding time (days)</th>
<th>observed (Table 3) (pmol/g) (95% confidence interval)</th>
<th>expected (pmol/g) (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 3)</td>
<td>72</td>
<td>138 (96–180)</td>
<td>56 (28–112)</td>
</tr>
<tr>
<td>2 (n = 8)</td>
<td>30</td>
<td>61 (25–99)</td>
<td>42 (21–84)</td>
</tr>
</tbody>
</table>


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