Dietary and lifestyle determinants of acrylamide and glycidamide hemoglobin adducts in non-smoking postmenopausal women from the EPIC cohort

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ABSTRACT

Purpose: Acrylamide was classified as 'probably carcinogenic' to humans in 1994 by the International Agency for Research on Cancer. In 2002, public health concern increased when acrylamide was identified in starchy, plant-based foods, processed at high temperatures. The purpose of this study was to identify which food groups and lifestyle variables were determinants of hemoglobin adduct concentrations of acrylamide (HbAA) and glycidamide (HbGA) in 801 non-smoking postmenopausal women from 8 countries in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort.

Methods: Biomarkers of internal exposure were measured in red blood cells (collected at baseline) by HPLC/MS/MS (high-performance liquid chromatography/tandem mass spectrometry). In this cross-sectional analysis four dependent variables were evaluated: HbAA, HbGA, sum of total adducts (HbAA+HbGA), and their ratio (HbGA/HbAA). Simple and multiple regression analyses were used to identify determinants of the four outcome variables. All dependent variables (except HbGA/HbAA), and all independent variables were log-transformed (log_2) to improve normality. Median (25^{th}-75^{th}percentile) HbAA and HbGA adducts levels were 41.3 (32.8-53.1) pmol/g Hb and 34.2 (25.4-46.9) pmol/g Hb, respectively.

Results: The main food group determinants of HbAA, HbGA, HbAA+HbGA were biscuits, crackers, and dry cakes. Alcohol intake and body mass index were identified as the principal determinants of HbGA/HbAA. The total percent variation in HbAA, HbGA, HbAA+HbGA, and HbGA/HbAA explained in this study was 30%, 26%, 29%, and 13%, respectively.

Conclusions: Dietary and lifestyle factors explain a moderate proportion of acrylamide adduct variation in non-smoking postmenopausal women from the EPIC cohort.

Key words (4 to 6): acrylamide; glycidamide; hemoglobin adducts; biomarkers; diet; nutrition.

INTRODUCTION

Acrylamide was identified in food in 2002, and is mainly formed through the Maillard reaction whereby a carbonyl compound (a reducing sugar, such as glucose or fructose) reacts with the amino group of asparagine processed at high temperatures (>120 ºC; i.e. frying, baking, or roasting) [1, 2]. Nevertheless, acrylamide has also been found in foods cooked at temperatures lower than 100ºC (e.g. prune juice) [3]. Thus, levels of acrylamide in foods depend on factors such as temperature and length of cooking time, water content, and the amount of both reducing sugars and asparagine levels present in foods [4]. Freisling et al. assessed the principal food group determinants of acrylamide intake based on a 24-h dietary recall (24hDR) in 13,486 men and 23,508 women from the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, and identified bread, crisp bread, rusks, coffee, and potatoes [5].

Once acrylamide is consumed, it is absorbed in the gastrointestinal tract and, via the circulation, is distributed to peripheral tissues [6, 7]. In the body, acrylamide is metabolized to glycidamide mainly by the cyp2e1 enzyme complex, and is conjugated with reduced glutathione for elimination. Acrylamide is neurotoxic in animals and in humans, but only glycidamide is considered to have mutagenic and genotoxic properties [1, 8]. As a consequence of animal and *in vitro* studies, the International Agency for Research on Cancer (IARC) classified acrylamide as 'probably carcinogenic' to humans [9].

Acrylamide and glycidamide can bind to N-terminal valine of hemoglobin (Hb) in red blood cells, and form adducts, both of which are considered valid biomarkers that reflect human internal exposure within the last 120 days (the average lifespan of erythrocytes) [1, 10]. Tobacco smoking is an important source of acrylamide exposure, and smokers have been observed to have mean Hb adducts levels three to four times higher than non-smokers [11–13].

Two previous EPIC studies evaluated biomarkers of acrylamide measured as acrylamide and glycidamide Hb-adducts (HbAA and HbGA, respectively). The first study, published by Vesper et al., aimed to determine acrylamide exposure variability (both at the individual- and group/country-level)

in 240 men and 270 women, and at the same time, determine which non-dietary factors could play a role in this variability [14]. The second study, published by Ferrari et al., was conducted with the intention to compare HbAA and HbGA levels with total estimated dietary acrylamide intakes assessed using dietary questionnaires (DQs), and a 24hDR in 240 men and 270 women to estimate the validity of the EPIC dietary acrylamide assessment [15]. The main objective of the present study, which differed from the two former EPIC studies, was to identify which food groups and lifestyle factors (assessed through country-specific DQs and lifestyle questionnaires) were determinants of HbAA and HbGA concentrations in a subgroup of non-smoking postmenopausal women from the EPIC cohort. The relation between intakes of several food items using DQs, and HbAA and HbGA adducts levels has been previously evaluated in three different studies [16–18].

MATERIALS AND METHODS

Study population and data collection

The EPIC study comprises 23 research centers in 10 European countries, and was designed to evaluate the relation between nutrition and lifestyle factors and the incidence of cancer and other chronic diseases. The present study includes 8 of the 10 participating EPIC countries: France, Germany, Spain, the United Kingdom (UK), Greece, Italy, The Netherlands, and Sweden (Umeå). Norway and Denmark did not participate in this analysis (EPIC-Denmark published their results separately as the Diet, Cancer, and Health cohort) [17].

The methodology of the EPIC study has been previously described [19]; all local ethics committees, and/or the IARC ethical review boards approved the study. Briefly, recruitment started between 1992-1998, and all EPIC participants provided information on habitual diet through country-specific validated DQs, referring to the year before recruitment. Information on tobacco smoking, physical activity, and education were assessed using country-specific questionnaires. Anthropometric measures (height, weight, and waist or hip circumference) were obtained at baseline by trained personnel; however, participants from France and Oxford (UK) cohorts self-reported their height and weight.

Umeå (Sweden), did not collect information on waist or hip circumference, and only 29% participants from France had information on these anthropometric measures.

Blood samples (serum, plasma, white blood cells, and erythrocytes) were collected at recruitment for 385,747 of the over 500,000 EPIC participants, and were stored in liquid nitrogen (-196ºC) at the central biological bank located at IARC; blood samples from Umeå were kept in freezers (-80ºC) at local repositories [19]. The present study population comprises control women from two published nested case-control studies of acrylamide hemoglobin adducts levels and ovarian and endometrial cancers risk in EPIC [20, 21]. The selection of cases and controls for these two nested-case control studies followed the protocol that has been previously described by Cust et al. and Peeters et al. [22, 23]. Briefly, two controls (free of cancer, with the exception of non-melanoma skin cancer) were randomly selected using an incidence density sampling protocol, for each case subject (ovarian or endometrial cancer case) at the time of diagnosis. Matching criteria for both cases and controls included study center, menopausal status (premenopausal, postmenopausal, 'undefined'), age at recruitment, (±6 months), time of day of blood draw (±1 hour), fasting status (<3, 3-6, >6 hours). For the present study, only women who were postmenopausal at blood draw, and non-smokers at recruitment were selected, since it has been postulated that acrylamide may disrupt hormonal balance, and it is known that smoking contributes to Hb-adducts levels. Postmenopausal status refers to women who reported having had the last menstrual period more than 1 year before recruitment, or when they were more than 55 years old [23]. The category of non-smoking women includes those women who reported being never smokers or having quit smoking 5 or more years before recruitment.

Thus, a total of 802 non-smoking postmenopausal control women (416 and 386 controls from the ovarian and the endometrial nested case-control studies, respectively) were available for the present study. One participant was excluded from analyses because she did not have information on HbGA adduct level, leaving a total of 801 observations

Assessment of dietary acrylamide intake

The methodology followed to create the EPIC acrylamide database has been previously described [15, 24]. In brief, the EPIC acrylamide database was assembled using information on average acrylamide levels in foods from an EU monitoring database (maintained by the European Community Institute for Reference Materials and Measurements; IRMM), and the frequency of consumption of these foods using country-specific DQs and the EPIC-Soft food classification.

Measurement of acrylamide and glycidamide hemoglobin adducts

The methodology to measure HbAA and HbGA in EPIC has been previously described [14, 15]. Briefly, 300 μL of hemolysed erythrocytes were used to measure HbAA and HbGA, and were analyzed by HPLC/tandem mass spectrometry (HPLC/MS/MS) as has been published elsewhere [12, 25]. All blood samples were measured and analyzed in a randomized and blinded manner. Two independent adduct measures per sample were performed. Hemoglobin adduct concentrations were reported as the average of these two measurements relative to the amount of hemoglobin. The detection limits for this method were 3 and 4 pmol/g Hb for HbAA and HbGA, respectively. Additionally, 42 (5%) blood samples from the same participants were sent in duplicate to evaluate the reproducibility of the hemoglobin adducts measurements.

Statistical methods

All continuous variables included in the analysis were assessed for normality using the Kolmogorov-Smirnov test, and were log-transformed (log_2) in order to reduce skewness. To account for zeroes in dietary and lifestyle variables, a $log_2(x + 0.1)$ transformation was applied. Regarding adduct values, four outcomes were evaluated: log-transformed HbAA adducts (log_2HbAA), log_2HbGA, sum of total adducts [log_2(HbAA + HbGA)], and HbGA/HbAA ratio. Simple and multiple linear regression analyses were used to assess the associations between each of the four outcome variables and food consumption and lifestyle data.

The following dietary variables and food groups were evaluated to build the HbAA, HbGA, and HbAA+HbGA final models: total energy, total carbohydrates, total fat, total fiber, total proteins, starch, potatoes, ‘vegetables’, ‘fruits, nuts and seeds’, ‘cereal and cereal products’, ‘meat and meat

223 products'[26], 'cakes and biscuits', 'flour, flakes, starches, and semolina', 'pasta, rice, and other
224 grains', 'bread, crisp bread, and rusks', 'breakfast cereals', 'salty biscuits, aperitif biscuits, and
225 crackers', 'dry cakes and biscuits', 'bread', 'pastries', 'olives', 'deep frying fats', 'chocolate, candy,
226 paste, confetti', 'snacks', 'bread, and pizza dough', 'olive oil', 'coffee', 'decaffeinated coffee', and
227 'tea'. Then, a correlation matrix was performed to identify interdependency between dietary variables.
228 Variables that were not correlated ($r < 0.6$), that were matching factors for both nested case-control
229 studies (country was used instead of center due to the number of observations), and 'type of control'
230 (endometrial, ovarian control) were selected for building the final models. Lifestyle variables such as
231 alcohol intake (g/day) and body mass index (kg/m^2; BMI) were also investigated as they may affect
232 the activity of Cyp2e1 [5, 27].

233 Stepwise selection was used to build models for HbAA, HbGA, and HbAA+HbGA adduct outcome
234 variables. Matching factor and 'type of control' variables were forced to be included in the stepwise
235 selection, and covariates were included in the model if they met the 0.10 significance level. Stepwise
236 selection was also performed with all food items energy-adjusted using the residual method [28], but
237 according to the Akaike Information Criterion (AIC) [29], these models were not optimal compared to
238 those without energy-adjusted food items.

239 Lifestyle variables such as physical activity using the Cambridge index [30], education level (none,
240 primary, technical/professional, secondary, and higher education), history of diabetes (yes, no,
241 unknown), ever use of oral contraceptives (OCs), and ever use of hormone replacement therapy (HRT)
242 were also evaluated; however, were not included in HbAA, HbGA, and HbAA+HbGA final models
243 because they did not have an effect on β-estimates.

244 The HbGA/HbAA ratio model only included lifestyle variables described above (BMI, physical
245 activity, education level, history of diabetes, OCs use, HRT use, and alcohol intake), since the ratio of
246 HbGA/HbAA may reflect the metabolism of acrylamide to glycidamide.

247 Intraclass correlation coefficients (ICC) were estimated to evaluate the reproducibility of acrylamide
248 measurements using 42 duplicate blood samples from the same participants [31]. Analyses stratified

by alcohol intake (never drinkers, drinkers), by BMI (<25, 25-30, ≥30 kg/m^2), and by European region (Northern countries: the UK, The Netherlands, Germany, Sweden; Southern countries: France, Italy, Spain, Greece) were also performed. A Wilcoxon signed-rank test was used to assess differences in Hb-adducts levels by alcohol intake, BMI, and second-hand smoke exposure (SHS). Variables for SHS were not evaluated in final models due to the large number of missing values (>50%). R square (R^2) values were used to describe the percent variation in Hb-adduct levels explained by the independent variables. Partial R^2 values for each of the selected variables in the models were estimated using Type II sums of squares. Pearson's correlation coefficients for continuous variables were also estimated. To test whether the slope of a regression line differed significantly from zero, Student's *t* statistics and the corresponding *P*-values were used. All statistical tests were evaluated at α-level 0.05.

All analyses were performed using SAS v. 9.1 (Cary, North Carolina, USA). Graphics were created using R v. 3.1.

RESULTS

Correlation Matrix

Total carbohydrates was correlated with total fiber, total proteins, starch, and 'cereal and cereal products'. Total fat was correlated with total proteins. Starch was correlated with 'cereal and cereal products'. Thus, total carbohydrates, total fat, and starch were excluded from the analyses because these were larger groups of foods.

Dietary acrylamide intake and baseline characteristics

The median (25^{th}-75^{th} percentile) acrylamide intake at baseline based on DQ information was 20.3 (13.5-29.9) μg/day. The median (25^{th}-75^{th} percentile) estimated dietary acrylamide in relation to body weight was 0.3 (0.2-0.5) μg/kg-body weight/day. The highest median intakes were found in the UK, the Netherlands, and Germany, whereas Italy had the lowest median intake (Table1).

The median (25^{th}-75^{th} percentile) age in the present subcohort of women was 59 (54-63) years. Means and standard deviations, as well as medians and 25^{th}-75^{th} percentiles are presented for all dietary and lifestyle variables that were used in all analyses (Supplemental Table1).

Hemoglobin adducts of acrylamide and glycidamide

The ICC for the present study based on 42 duplicates was 0.96 for HbAA and 0.95 for HbGA.

The median (25^{th}-75^{th} percentile) HbAA and HbGA adducts level were 41.3 (32.8-53.1) and 34.2 (25.4-46.9) pmol/g of Hb, respectively (Table1). The highest median HbAA adduct level was observed in the UK, The Netherlands, and Spain, while Greece had the lowest HbAA levels, followed by Italy. Regarding HbGA adduct levels, the highest medians were found in the UK, Spain, and The Netherlands, and the lowest median was also found in Greece and Italy (Table1). The geometric means for HbAA and HbGA adducts in the total dataset were 5.3 and 5.1 pmol/g Hb respectively (data not shown). Values for HbAA+HbGA, and HbGA/HbAA by EPIC country are also presented in Table 1.

Regarding differences in Hb-adducts levels by alcohol intake and by BMI, only the ratio HbGA/HbAA differed (never vs ever drinkers, *P*-value <0.0001; <25, 25 to <30, ≥30 kg/m^2, *P*-value <0.0001) (Figure 1, and Figure 2 respectively). No statistically significant differences in Hb-adduct levels were observed between women who reported being exposed to SHS (n=95) and who were not exposed (n=149) (data not shown).

Simple linear regression analyses

The crude Pearson's correlation coefficient between log_2-acrylamide intake and log_2HbAA was 0.36, between log_2-acrylamide intake and log_2HbGA was 0.35, between log_2-acrylamide intake and log_2(HbAA+HbGA) was 0.37, and between log_2HbAA and log_2HbGA was 0.86 (all *P-values* <0.0001). Log_2HbAA was inversely associated with BMI (*P-value* <0.0001) and positively associated with alcohol intake (*P-value*= 0.04). A statistically significant inverse association was found between

HbGA/HbAA ratio and alcohol intake (*P-value* <0.0001), whereas a positive association was observed between HbGA/HbAA ratio and BMI (*P-value* <0.0001) (Table 2).

Multiple linear regression analyses

Four multivariable linear regression analyses (all models included country, age at recruitment, date of blood donation, fasting status, and type of control) were performed for each of the four outcome variables with the aim to identify independent determinants of log_2HbAA, log_2HbGA, log_2(HbAA+HbGA), and HbGA/HbAA (Table 3). Analyses stratified by alcohol intake and by BMI were performed, but no major differences were observed, thus, only overall results are presented.

The HbAA model explained 30% of the variation in HbAA levels, and the food groups 'salty biscuits, aperitif biscuits, crackers', 'dry cakes, biscuits', and 'vegetables' were statistically significant positively associated with log_2HbAA values, whereas 'tea' was inversely associated .

The HbGA model explained 26% of the variation of HbGA levels, and the following groups were statistically significantly associated with HbGA-log levels: 'salty biscuits, aperitif biscuits, crackers', 'dry cakes, biscuits', and 'deep frying fats'. Alcohol intake was inversely associated with log_2HbGA values.

The HbAA+HbGA model explained 29% of the variation of the sum of total adducts levels, and only 'salty biscuits, aperitif biscuits, crackers', 'dry cakes, biscuits', and 'deep frying fats' were significantly associated with the sum of total adducts levels.

The HbGA/HbAA model explained 13% of the variation in HbGA/HbAA ratio, and BMI was positively associated, whereas alcohol consumption at recruitment was inversely associated with the HbGA/HbAA ratio. No other lifestyle variable explained variation in the ratio.

Multiple linear regression analyses were also performed by European region (data not shown). The three different models from the northern countries explained a higher variation in HbAA, HbGA, and HbAA+HbGA levels than the southern countries; however, the food groups identified as dietary

determinants of Hb-adducts levels by European region were, in general, similar to those presented in table 3.

DISCUSSION

The present study was carried out with the aim to identify independent determinants of HbAA, HbGA, HbAA+HbGA, and HbGA/HbAA. We investigated these associations in a cross-sectional study of 801 non-smoking postmenopausal women from the EPIC cohort. The most important determinants of HbAA, HbGA, and HbAA+HbGA adduct levels were 'salty biscuits, aperitif biscuits, and crackers', and 'dry cakes and biscuits'; whereas alcohol intake and BMI (inversely and positively associated, respectively) were identified as the two main determinants of HbGA/HbAA ratio.

To our knowledge, there are only three published studies that evaluated the relation between specific food group determinants and Hb-adducts of acrylamide and glycidamide, and all of them obtained dietary information through DQs [16–18]. The first study was based on a Norwegian population, and included 19 men and 31 women (*n*=50*)* of which 14% of the subjects were smokers. The second study comprised 296 female, non-smoking, pre- and postmenopausal women from the Nurses' Health Study II (NHS-II). The Danish study, similar to the present study, was based on postmenopausal women who reported being non-smokers at baseline (*n*=537).

The current study had the highest estimated intake of acrylamide compared to the Norwegian and the NHS-II studies. The Norwegian study reported a median acrylamide intake of 12.8 µg/day among non-smoking women (in EPIC, 20.3 µg/day), and the NHS-II study reported a mean energy-adjusted intake of 19.3 µg/day (in EPIC, 21.9 µg/day). The Danish study did not report information on overall dietary acrylamide intake.

Blood samples from both EPIC and NHS-II studies were measured in the same laboratory using the same protocol. Likewise, the Norwegian and the Danish studies shared the same methodology [16, 17]. The median adduct levels for HbAA and HbGA in the present study (41.3 and 34.2 pmol/g of Hb, respectively) were higher than the values reported in the Norwegian (36.8 and 18.2 pmol/g of Hb), and the Danish (35 and 21pmol/g of Hb) studies; however, the NHS-II study reported the highest values of

346 Hb-adducts (43.9 and 49.4 pmol/g of Hb). The NHS-II study also had the highest median values of
347 HbGA/HbAA (1.10), compared to the Norwegian (0.49), and the present (0.8) study.

348 The correlation between estimated acrylamide intake (based on DQs), and Hb-adducts of acrylamide
349 and glycidamide was low (ranging from 0.08 to 0.43) in most studies, including EPIC [15, 16, 32–34].
350 The NHS-II study reported moderate correlations between acrylamide intake and HbAA or HbGA
351 (0.19-0.35). These differences could be due to errors in dietary assessment methods, and to incomplete
352 data on acrylamide content in food composition databases. The NHS-II acrylamide database was
353 mainly based on values from the FDA's Exploratory Analysis of Acrylamide in Foods, but specific
354 foods that were frequently consumed in the study were further analyzed, such as different brands of
355 breakfast cereals, dried food, bread, and potatoes chips among others [18].

356 The main food group determinants of Hb-adducts (HbAA, HbGA, and HbAA+HbGA) in the present
357 study were 'salty biscuits, aperitif biscuits, and crackers', and 'dry cakes, and biscuits'; whereas
358 Freisling et al. reported that 'bread, crisp bread, and rusks', 'coffee', and 'potatoes', were the main
359 determinants of dietary acrylamide intake (based on a single 24 hDR) in a different subgroup of EPIC
360 men and women [5]. Coffee was selected in the stepwise procedure as one of the food determinants of
361 HbGA, and HbAA+HbGA in the present study, but was not statistically significant. Inverse
362 associations between 'tea' and Hb-adduct levels (HbAA, HbGA, and HbAA+HbGA) were observed in
363 the present study. This result may be explained by the possible effect of tea polyphenols, which have
364 been observed to decrease HbAA levels in animals [35]. The variable 'vegetables' was selected as a
365 determinant of log2HbAA; however, this result might has been confounded by other acrylamide
366 containing foods, since 'vegetables' includes all forms of cooking methods (including frying and
367 baking). The food groups 'bread, crisp bread, rusks', and 'potatoes' were not selected in any of the
368 models presented in this study; however the possible effect of 'potatoes', especially fried potatoes,
369 might have been represented by the variable 'deep frying fats'. It is worth noting that the present study
370 was evaluating adducts levels, whereas the Friesling et al. study was evaluating acrylamide intake
371 based on 24 hDR. Further, differences in the results of these two EPIC analyses may reflect
372 differences in the subpopulation studied (e.g, age distribution, sex, menopausal status).

373 The Norwegian study identified as dietary determinants of HbAA levels 'potatoes', 'chips/snacks',
374 'crisp bread', and 'jam/preservatives. Furthermore, 'chips/snacks', and 'crisp bread' were also
375 recognized as determinants of HbAA+HbGA. Similar to EPIC, the NHS-II study also identified
376 dietary determinants of HbAA, HbGA, and HbAA+HbGA; but the food groups were different from
377 the determinants observed in the present study. The Danish study reported 'coffee' to be associated
378 with HbAA and HbGA, 'chips' to HbAA, and 'biscuits/crackers' to HbGA levels. Dietary habits are
379 different between the US and European countries, so direct comparison of food groups as determinants
380 of HbAA levels may not be possible.

381 The proportion of response variation (R^2) explained by food groups and/or lifestyle variables in the
382 present study varied from 13% in the HbGA/HbAA model to 30% for HbAA. The Danish study
383 obtained a response variation of 17% and 12% in the HbAA and HbGA models respectively; however
384 the highest response variations explained were obtained in the Norwegian study (48% in the HbAA,
385 and 37% in the HbAA+HbGA model) [16, 17]. The NHS-II study did not report this information [18].

386 The main difference between our study and the other three published studies is that prior analyses only
387 included dietary variables that were suspected to be sources of acrylamide intake. The current study
388 included both known sources of dietary acrylamide together with lifestyle variables, such as alcohol
389 intake and BMI, with the intention to better describe the independent determinants of Hb-adducts and
390 their ratio in non-smoking individuals. Diet is complex, and interactions between acrylamide and food
391 ingredients are possible (i.e, acrylamide uptake in humans has been hypothesized to be impaired by a
392 diet rich in proteins) [26]. Likewise, it has been suggested that alcohol intake and BMI may influence
393 the activity of Cyp2e1, the enzyme complex that metabolizes acrylamide to glycidamide. This enzyme
394 is involved in alcohol metabolism mainly when alcohol concentrations in the blood are high. It has
395 been hypothesized that alcohol may compete with acrylamide as a substrate [36]. This could partially
396 explain the results observed in the present study; in which higher alcohol intake was inversely
397 associated with HbGA and HbGA/HbAA, as has been reported in other studies [14, 36]. The
398 mechanism by which BMI may influence acrylamide metabolism is still unclear, but similar to the
399 present study, other studies have observed that BMI was positively associated with the ratio

HbGA/HbAA, and negatively associated with HbAA [14, 37, 38]. Recently, statistically significant differences in the ratio of HbGA/HbAA between vegetarians and non-vegetarians have been reported, suggesting that dietary factors may also contribute to acrylamide/glycidamide metabolism [39].

The present study had the largest sample size compared to the previous published papers, and the laboratory methods used to quantify both acrylamide and glycidamide hemoglobin adducts were standardized and followed rigorous quality assurance/quality control [12]. Tobacco is one of the most important sources of acrylamide, and it is well-established that cigarette smoke influences Hb-adducts levels (smokers have mean HbAA levels at least three to four times higher than non-smokers) [11, 14]. Moreover, it has been hypothesized that acrylamide may influence hormonal homeostasis [40–42]. Thus, the present study was designed to reduce confounding by these factors, and only those women who reported being postmenopausal and never smokers were included in the study. Further, limitations derived from using estimates of dietary acrylamide intake, as described above, were avoided in the present analysis of food groups and biomarkers levels.

There are some limitations that should be acknowledged: a) Hb-adducts of acrylamide and glycidamide are valid biomarkers for acrylamide exposure and internal dose; however, these adducts reflect levels of acrylamide and glycidamide during the average lifespan of erythrocytes, which is around 120 days [1, 10], and all dietary and lifestyle variables in EPIC were assessed through DQs that referred to the previous year before recruitment [19]. b) Cooking methodology, which influences acrylamide levels in food, was not recorded in some EPIC centers; however, relevant information on food preparation was available for potatoes (except in Italy), bread, and breaded meats [15, 24]. c) Only one baseline blood sample was collected for each participant. d) Acrylamide content in foods may vary seasonally [43], which was not accounted for in our analyses; nevertheless, all models were adjusted for date of blood extraction to minimize this effect. (e) Ferrari et al. described variations in dietary patterns across EPIC countries [15], and this may have influenced Hb-adducts levels and our prediction models, although all models included country to adjust for country-level effects (i.e. questionnaire design, dietary habits). f) SHS could not be evaluated in our statistical models due to the large number of missing values (>50%); however, in a subset of women from the current study, no

statistically significant differences in Hb-adduct levels were observed between women who were exposed to SHS and who were not exposed to SHS.

To conclude, dietary food group and lifestyle variables explain a moderate proportion of HbAA and HbGA adduct level variation in 801 postmenopausal non-smoking women in the EPIC cohort. The main food groups determinants of HbAA, HbGA, and HbAA+HbGA were ‘Salty biscuits, aperitif biscuits, and crackers’, and ‘dry cakes, and biscuits’. Alcohol intake and BMI were identified as the principal determinants for the ratio HbGA/HbAA levels. In this regard, future studies assessing associations between acrylamide and disease risk should take into consideration the use of both biomarkers of acrylamide exposure (HbAA and HbGA), in addition to alcohol intake and BMI.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Table 1. Estimated dietary acrylamide intake and hemoglobin acrylamide and glycidamide adducts levels by EPIC country in a subgroup of non-smoking postmenopausal women

EPIC country	Total observations n (%)	Endometrial controls n (%)	Ovarian controls n (%)	Acrylamide intake (µg/day) Median (QR)	Acrylamide[a] intake (µg/day) Median (QR)	Acrylamide intake µg/ kg-body weight/day Median (QR)	HbAA pmol/g of Hb Median (QR)	HbGA pmol/g of Hb Median (QR)	HbAA+HbGA pmol/g of Hb Median (QR)	Ratio of HbGA/HbAA Median (QR)
France	65 (8)	35 (9)	30 (7)	20.0 (14.5- 25.6)	17.6 (13.5- 21.6)	0.3 (0.2- 0.4)	38.5 (34.2- 47.3)	29.4 (24.6- 36.8)	66.4 (60.1- 82.9)	0.7 (0.7- 0.8)
Italy	126 (16)	74 (19)	52 (12)	8.6 (5.5- 11.5)	7.8 (4.1- 10.7)	0.1 (0.1- 0.2)	35.8 (26.7- 44.9)	28.9 (21.9- 38.2)	64.7 (49.8- 87.7)	0.8 (0.7- 0.9)
Spain	127 (16)	72 (19)	55 (13)	16.6 (9.8- 25.9)	19.0 (13.0- 25.4)	0.2 (0.1- 0.4)	42.4 (33.8- 53.8)	39.8 (28.6- 48.6)	82.4 (62.9- 101.5)	0.9 (0.8- 1.0)
United Kingdom	153 (19)	59 (15)	94 (23)	31.4 (23.8- 40.4)	30.5 (24.2- 39.5)	0.5 (0.3- 0.6)	55.6 (44.4- 69.0)	47.1 (35.2- 61.3)	102.9 (83.1- 133.4)	0.8 (0.7- 1.0)
The Netherlands	116 (14)	38 (10)	78 (19)	28.8 (19.4- 40.0)	30.0 (22.1- 40.4)	0.4 (0.3- 0.6)	45.3 (37.2- 54.4)	38.6 (29.5- 51.7)	80.9 (68.0- 105.2)	0.9 (0.7- 1.0)
Greece	59 (7)	16 (4)	43 (10)	17.7 (14.4- 21.9)	19.1 (17.5- 22.8)	0.2 (0.2- 0.3)	26.4 (21.1- 35.3)	22.0 (16.5- 31.8)	50.4 (40.1- 67.1)	0.9 (0.7- 1.0)
Germany	102 (13)	56 (15)	46 (11)	22.8 (18.5- 29.5)	24.9 (19.9- 29.2)	0.3 (0.2- 0.5)	36.2 (29.7- 43.8)	29.3 (23.1- 38.5)	67.2 (53.0- 78.5)	0.8 (0.7- 0.9)
Sweden	53 (7)	34 (9)	19 (5)	18.3 (14.8- 21.0)	22.6 (20.5- 25.0)	0.3 (0.2- 0.3)	38.5 (34.9- 46.9)	34.6 (25.9- 42.2)	72.8 (62.9- 87.8)	0.9 (0.8- 1.0)
Total	**801**	**384**	**417**	**20.3 (13.5-29.9)**	**21.9 (14.6-29.2)**	**0.3 (0.2-0.5)**	**41.3 (32.8-53.1)**	**34.2 (25.4-46.9)**	**75.5 (58.5-100.0)**	**0.8 (0.7-1.0)**

EPIC, European Prospective Investigation into Cancer and Nutrition; QR, quartile range, HbAA, hemoglobin adducts of acrylamide; HbGA, hemoglobin adducts of glycidamide; Hb, hemoglobin

a Energy-adjusted using the residual method

Table 2. Simple linear regression with β-estimates for the association between dietary and lifestyle variables and log-transformed (log_2) HbAA, HbGA, HbAA+HbGA, and HbGA/HbAA.

	Log_2HbAA		Log_2HbGA		HbAA+HbGA		HbGA/HbAA	
Variables	**β**	***P*-value**	**β**	***P*-value**	**β**	***P*-value**	**β**	***P*-value**
Acrylamide intake (µg/day)	0.20	<.0001	0.23	<.0001	0.21	<.0001	0.02	0.02
Age at recruitment (y)	-0.003	0.26	0.001	0.79	-0.002	0.60	0.002	0.03
Body mass index (kg/m^2)	-0.32	<.0001	-0.01	0.91	-0.18	0.02	0.17	<.0001
Alcohol intake (g/day)	0.01	0.04	-0.01	0.09	0.002	0.79	-0.01	<.0001
Total dietary fiber (g/day)	0.17	<.0001	0.15	0.002	0.16	0.0001	-0.01	0.53
Total proteins (g/day)	0.15	0.0003	0.18	0.0003	0.17	0.0002	0.01	0.30
Meat and meat products (g/day)	-0.02	0.20	0.00	0.78	-0.01	0.58	0.01	0.01
Potatoes and other tubers (g/day)	0.04	<.0001	0.05	<.0001	0.05	<.0001	0.01	0.16
Vegetables (g/day)	0.05	0.01	0.03	0.17	0.04	0.03	-0.01	0.10
Fruits, nuts, and seeds (g/day)	-0.02	0.21	-0.02	0.32	-0.02	0.25	0.002	0.78
Olives (g/day)	-0.05	<.0001	-0.05	<.0001	-0.05	<.0001	0.001	0.79
Cereal and cereal products (g/day)	-0.02	0.51	-0.02	0.47	-0.02	0.51	0.00003	1.00
Flour, flakes, starches, semolina (g/day)	-0.07	<.0001	-0.06	<.0001	-0.06	<.0001	0.004	0.25
Pasta, rice, other grains (g/day)	-0.02	0.02	-0.03	0.01	-0.02	0.01	-0.002	0.48
Bread, crisp bread and rusks (g/day)	-0.04	0.01	-0.03	0.10	-0.04	0.03	0.007	0.19
Breakfast cereals (g/day)	0.04	<.0001	0.03	<.0001	0.04	<.0001	-0.002	0.34
Salty biscuits, aperitif biscuits, crackers (g/day)	0.04	<.0001	0.04	<.0001	0.04	<.0001	-0.003	0.21
Dry cakes, biscuits (g/day)	0.02	0.0003	0.03	<.0001	0.03	<.0001	0.005	0.02
Pastries (g/day)	-0.02	0.06	-0.04	0.0001	-0.03	0.004	-0.01	<.0001
Cakes, biscuits (g/day)	0.03	0.0003	0.04	<.0001	0.03	<.0001	0.004	0.14
Chocolate, candy, paste, confetti (g/day)	0.03	<.0001	0.03	<.0001	0.03	<.0001	0.001	0.51
Confectionery non-chocolate, candied fruits (g/day)	0.02	0.004	0.03	0.004	0.02	0.003	0.002	0.41
Snacks (g/day)	0.05	<.0001	0.06	<.0001	0.05	<.0001	0.002	0.35
Olive oil (g/day)	-0.04	<.0001	-0.04	<.0001	-0.04	<.0001	0.002	0.33
Deep frying fats (g/day)	0.03	0.04	0.03	0.03	0.03	0.03	0.004	0.36
Tea (ml/day)	0.02	<.0001	0.02	0.0001	0.02	<.0001	-0.002	0.06
Coffee (ml/day)	0.02	0.001	0.02	0.01	0.02	0.002	-0.0001	0.98
Decaffeinated coffee (ml/day)	0.02	<.0001	0.03	<.0001	0.03	<.0001	0.002	0.24
Energy intake (kcal/day)	0.13	0.01	0.13	0.02	0.13	0.01	-0.0001	0.99

HbAA, hemoglobin adducts of acrylamide, HbGA, hemoglobin adducts of glycidamide

All independent variables were log-transformed (log_2) to improve normality.

Table3. Multiple linear stepwise regression with β-estimates and standard errors for the association between dietary and lifestyle variables and log-transformed (log2) HbAA, HbGA, HbAA+HbGA, and HbGA/HbAA.

Hemoglobin adducts	Dietary and/or life style variables	β (SE)	*P-value*	Partial R^2	Model R^2
Log_2HbAA	Salty biscuits, aperitif biscuits, crackers	0.03 (0.01)	0.0001	0.01	0.3
	Dry cakes, biscuits	0.02 (0.01)	0.003	0.01	
	Tea	-0.01 (0.005)	0.02	0.01	
	Vegetables	0.05 (0.02)	0.02	0.005	
	BMI	-0.15 (0.07)	0.05	0.004	
	Deep frying fats	0.04 (0.02)	0.08	0.003	
Log_2HbGA	Dry cakes, biscuits	0.03 (0.01)	0.0001	0.02	0.26
	Salty biscuits, aperitif biscuits, crackers	0.03 (0.01)	0.0002	0.01	
	Alcohol at recruitment	-0.02 (0.01)	0.01	0.01	
	Deep frying fats	0.07 (0.03)	0.02	0.01	
	Coffee	0.01 (0.01)	0.06	0.003	
	Tea	-0.01 (0.01)	0.07	0.003	
Log_2(HbAA+HbGA)	Salty biscuits, aperitif biscuits, crackers	0.03 (0.01)	0.0001	0.01	0.29
	Dry cakes, biscuits	0.02 (0.01)	0.0005	0.01	
	Tea	-0.01 (0.005)	0.02	0.005	
	Deep frying fats	0.05 (0.02)	0.03	0.004	
	Fiber	0.07 (0.04)	0.05	0.003	
	Coffee	0.01 (0.01)	0.08	0.003	
HbGA/HbAA	BMI	0.14 (0.03)	<0.0001	0.03	0.13
	Alcohol at recruitment	-0.01 (0.002)	0.00002	0.02	
	Education level			0.006	
	None	reference	-		
	Primary school completed	-0.01 (0.02)	0.73		
	Technical/professional school	-0.02 (0.03)	0.51		
	Secondary school	-0.02 (0.03)	0.61		
	Higher education	-0.06 (0.03)	0.05		
	Not specified	0.02 (0.05)	0.70		
	Missing	-0.04 (0.07)	0.53		
	Ever use of OCs			0.003	
	No	reference	-		
	Yes	-0.02 (0.01)	0.12		
	Missing	0.04 (0.06)	0.53		
	Physical activity			0.003	
	Inactive	reference	-		
	Moderately inactive	0.02 (0.02)	0.28		
	Moderately active	-0.02 (0.02)	0.29		
	Active	0.01 (0.02)	0.77		
	Missing	-0.03 (0.09)	0.74		
	Ever use of HRT			0.002	
	No	reference	-		
	Yes	0.004 (0.02)	0.81		
	Missing	-0.05 (0.04)	0.22		
	Diabetes				

No	reference	-	
Yes	-0.02 (0.03)	0.47	0.001
Missing	-0.03 (0.07)	0.64	

SE, standard error; HbAA, hemoglobin adducts of acrylamide; HbGA, hemoglobin adducts of glycidamide; BMI, body mass index (kg/m2); HRT, hormonal replacement therapy; OC, oral contraceptive.

All models are adjusted for country, age at recruitment (y), date of blood donation, fasting status (no, in between, yes), and type of control (endometrial, ovarian control). All independent continuous variables were log-transformed (log_2) to improve normality.

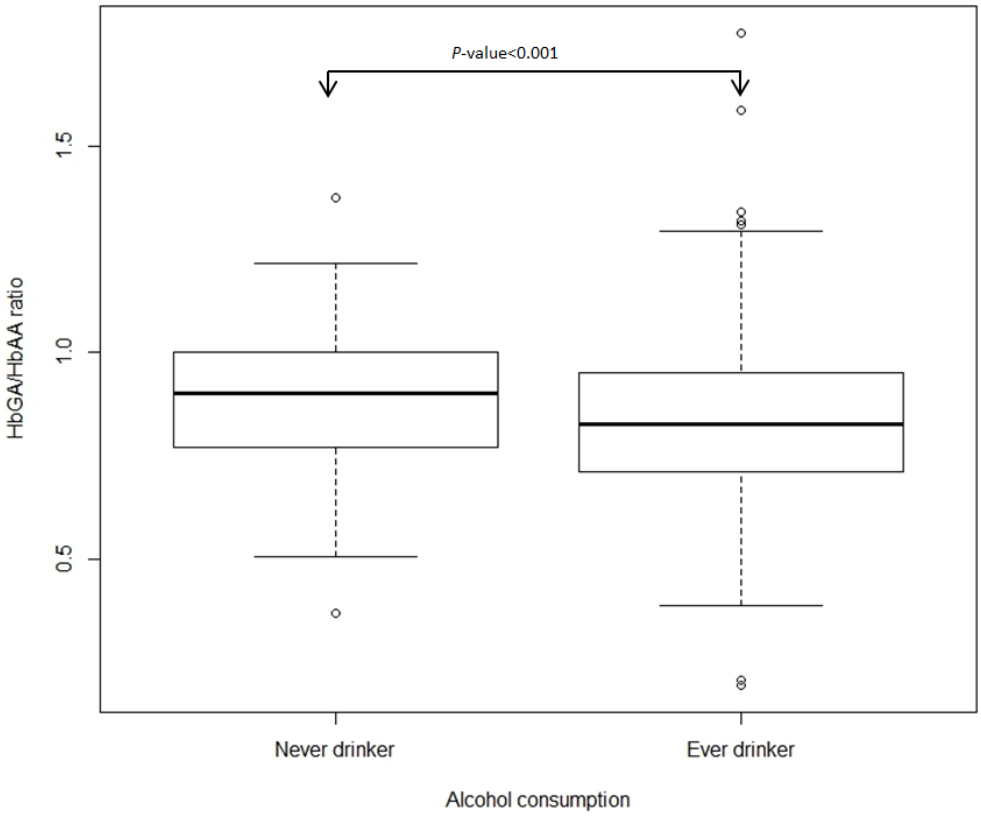

Figure 1. Box-and-whisker plot of HbGA/HbAA ratio versus alcohol consumption (never drinkers, ever drinkers). Arrow marks significant differences between groups. *P*-value based on a Wilcoxon rank sum test.

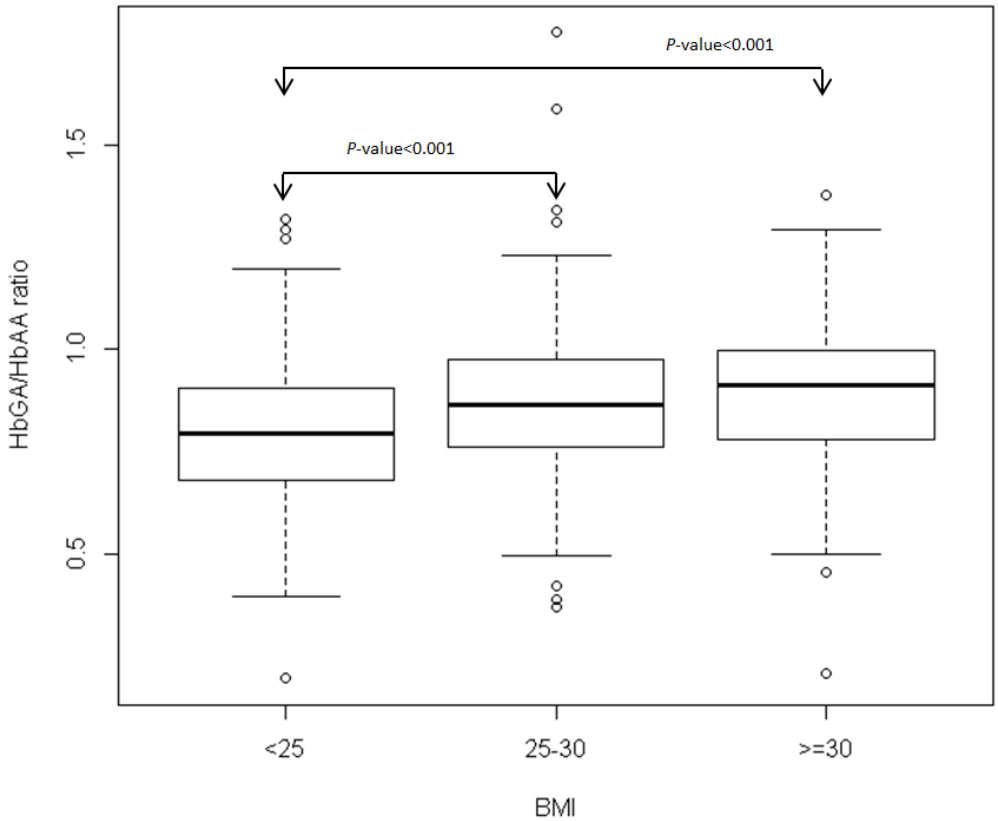

Figure 2. Box-and-whisker plot of HbGA/HbAA ratio versus body mass index (BMI; <25, 25-30, ≥30 kg/m^2).Arrow marks significant differences between groups. *P*-values based on a Wilcoxon rank sum test.

Supplemental table 1. Baseline characteristics of the 801 non-smoking postmenopausal women

Variables	Mean	SD	P5	P25	Median	P75	P95
Lifestyle variables							
Age at recruitment (y)	58.79	6.30	49.29	54.33	58.83	63.06	69.44
Body Mass Index (kg/m^2)	26.73	4.90	20.07	23.21	25.96	29.41	36.07
Alcohol at recruitment (g/d)	6.04	8.63	0.00	0.18	2.13	8.87	24.26
Dietary variables (g/day)							
Total dietary fiber	22.45	7.02	12.28	17.76	21.73	26.28	34.70
Total proteins	83.02	25.34	45.70	65.42	81.00	97.23	128.04
Meat and meat products	90.88	48.77	21.05	56.34	84.03	121.54	175.53
Potatoes and other tubers	78.00	58.92	7.09	32.90	67.11	114.85	178.94
Vegetables	215.85	139.33	56.35	114.68	183.18	292.20	472.48
Olives	1.30	4.44	0.00	0.00	0.00	0.03	8.57
Fruits, nuts, seeds	289.73	179.12	69.58	161.15	259.53	376.37	628.21
Flour, flakes, starches, semolina	0.56	2.13	0.00	0.00	0.00	0.16	2.54
Pasta, rice, other grains	58.64	56.09	3.36	20.03	41.86	80.12	176.62
Cereal and cereal products	198.09	93.95	79.97	135.42	182.42	241.91	375.86
Bread, crisp bread, rusks	116.16	65.97	28.80	69.50	104.77	152.00	234.88
Breakfast cereals	17.25	45.85	0.00	0.00	0.00	11.17	115.10
Salty biscuits, aperitif biscuits, crackers	2.14	5.10	0.00	0.00	0.22	2.14	11.43
Dry cakes, biscuits	9.63	20.88	0.00	0.00	3.44	12.32	38.93
Pastries	0.91	2.00	0.00	0.00	0.00	0.99	4.62
Cakes and biscuits	46.57	47.92	0.00	15.35	34.43	63.76	128.57
Chocolate, candy bars, paste, confetti	6.26	11.21	0.00	0.00	2.00	8.04	25.00
Confectionery non-chocolate, candied fruits	2.57	7.10	0.00	0.00	0.13	1.67	12.90
Snacks	2.29	9.26	0.00	0.00	0.00	0.21	10.01
Olive oil	9.00	14.06	0.00	0.00	0.47	15.22	39.51
Deep frying fats	0.32	1.04	0.00	0.00	0.00	0.00	2.03
Tea	220.49	318.36	0.00	0.00	41.42	375.00	855.00
Coffee	282.75	262.19	0.00	70.00	191.90	475.00	750.00
Decaffeinated coffee	38.39	110.55	0.00	0.00	0.00	3.57	250.00
Energy intake (kcal/day)	1,913.10	550.34	1,132.44	1,529.36	1,842.57	2,214.45	2,861.80

SD, Standard deviation; P5, 5th percentile, P25, 25th percentile; P75, 75th percentile; P95, 95th percentile