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Dietary intake of meat and meat-derived heterocyclic aromatic amines and their correlation with DNA adducts in female breast tissue

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It was the aim of this study to examine the association of the consumption of meat in general, meat prepared by different cooking methods and the dietary intake of heterocyclic aromatic amines (HCA) with the level of DNA adducts in the breast tissue of women undergoing reduction mammoplasty. Dietary intake of meat and HCA were assessed via questionnaire in 44 women undergoing reduction mammoplasty. DNA adduct analysis in breast tissue was performed by ^{32}P-postlabelling analysis. Spearman rank correlation coefficients (r) were calculated to examine the association of meat consumption and dietary HCA intake with tissue DNA adduct levels. A median DNA adduct level of 18.45 (interquartile range 12.81–25.65) per 10^9 nucleotides in breast tissue was observed; median HCA intake was 40.43 ng/day (interquartile range 19.55–102.33 ng/day). Total HCA intake (r = 0.33, P = 0.03), consumption of fried meat (r = 0.39, P = 0.01), beef (r = 0.32, P = 0.03) and processed meat (r = 0.51, P = 0.0004) were statistically significantly correlated with the level of DNA adducts in breast tissue. The detected DNA adducts could not be confirmed to be specific HCA-derived DNA adducts by comparison with external standards, using the ^{32}P-postlabelling assay. We observed strong correlations of dietary HCA intake and consumption of fried and processed meat with DNA adduct levels in breast tissue of 44 women. Since the detected DNA adducts were not necessarily specific only for HCA, it is possible that HCA intake is a surrogate of other genotoxic substances, such as polycyclic aromatic hydrocarbons, in meat prepared at high temperatures.

Introduction

Breast cancer is the most common cancer among women in industrialized countries and diet is thought to be related to breast cancer risk. Meat consumption was associated with an increased risk of pre- (1,2) and post-menopausal (3) breast cancer in some studies but not others (4). Meat, especially processed or cooked red meat, is a source of carcinogens, such as heterocyclic aromatic amines (HCA), polycyclic aromatic hydrocarbons (PAH) and endogenously formed N-nitroso compounds (ENOC). HCA are formed at parts-per-billion levels in fried or grilled meats as products of protein pyrolysis or Maillard reactions (5) and have been shown to be carcinogenic in rodent bioassays (6). Specifically, the amino imidazo-derivatives 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) have been shown to be potent mammary carcinogens in female rats (7). Furthermore, it has been demonstrated *in vitro* that HCA are metabolically activated to form DNA adducts in human mammary epithelial cells (8,9). PhIP–DNA adducts have been detected in female human breast tissue using accelerator mass spectrometry following an oral dose of ^{14}C-labelled PhIP (10) and, most recently, DNA adducts in human breast cells have been tentatively identified as PhIP-derived adducts by means of immunohistochemistry (11). These results led to the hypothesis that HCA may be implicated in human mammary carcinogenesis.

Assessing intake of HCAs from meat by means of a dietary questionnaire may be inaccurate because of differences in the use of cooking methods, the preferred degree of browning/doneness and the measurement error implicated by dietary questionnaires. These factors are likely to have an impact on the exact long-term exposure compared with the calculated intake from dietary questionnaires (12). Additionally, the individual cancer risk is likely to be determined by both, exposure to xenobiotics and host factors such as toxicokinetics, metabolic capacity and DNA repair. Thus, biomonitoring the burden of chemical carcinogens by analysis of covalent DNA adducts formed by their metabolically activated intermediates determines the dose at the target molecule DNA. Furthermore, analysis of DNA adducts in the target organ, the mammary gland, and specifically in the enriched target cell population, the epithelial cells, that have been shown to be the cells that most often mammary tumours originate from reflects both exposure level and host factors (13).

It was the aim of this study to examine the association of the consumption of meat in general, meat prepared by different cooking methods and the dietary intake of HCA with the level of DNA adducts in the target organ, mammary gland, in females undergoing reduction mammoplasty.

Materials and methods

Patients, tissue sample collection and dietary assessment

Forty-four Caucasian women were recruited for participation in our study. These women had undergone surgery for elective reduction mammoplasty at the Krankenhaus Alteneichen, Hamburg, Germany. During this surgery, samples of human mammary tissue (ranging from 81 to 2227 g per breast) had been removed. The use of human tissue samples was approved by the local ethics committee. Tissues from patients of 30 years or older were examined macroscopically by a pathologist at the Department of Pathology, General Hospital Altona, Hamburg, Germany.

All patients gave their informed written consent and completed a questionnaire on food consumption over the past 12 months as used in the German cohorts of the European Prospective Investigation into Cancer and Nutrition (14,15). In addition, detailed questions on meat preparation habits and the preferred degree of browning were included as described in detail elsewhere (16,17). Briefly, participants were

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asked how often they consumed 16 types of meat and which cooking methods they prefer for each type of meat. With the help of four pictures, subjects stated which degree of browning they favoured. Total HCA concentration and concentration of the most abundant HCA (PhIP, DiMeIQx and MeIQx) were estimated using published data of their content in different types of meat (5,18–20). HCA intake from gravy was assessed by asking about the use of meat drippings to prepare gravy and was automatically added to a specific meat item's intake. By combining information on degree of browning, cooking method and the amount of meat intake, the mean daily dietary intake of HCA was estimated. In addition to HCA intake, we estimated ENOC formation from meat-derived haeme iron as in detail described previously (21). All participants completed a questionnaire on anthropometrics, reproductive history, medication use and smoking history. Body mass index (BMI) was calculated from weight and height.

Tissue workup

Breast tissue samples were maintained ice cooled and processed on the day of surgery according to the described procedures (22,23). Briefly, samples were minced and hydrolysed overnight at 37°C with magnetic stirring in Dulbecco's modified Eagle's medium with 5% foetal calf serum containing 0.5% (w/v) collagenase (Sigma-Aldrich, Hamburg, Germany). The resulting emulsion was centrifuged, lipid removed and the pellet re-suspended in fresh medium (5 ml). Erythrocytes were haemolysed by addition of distilled water and following centrifugation and re-suspension in medium cells and organoids were allowed to settle under refrigeration. Epithelial organoids were enriched by fractionated filtration through 140- and 53-μm nylon mesh and finally stored at −80°C until DNA was isolated by phenol extraction according to the published protocols (24).

DNA adduct analysis

DNA adduct analysis was performed by ^{32}P-postlabelling analysis using the solid-phase extraction procedure (24). Each sample was analysed two to four times (standard deviations between measurements were within 15%). According to the published procedures (24–26), the labelling mix was applied to polyethylene imine cellulose plates (Macherey and Nagel, Düren, Germany; 10 × 10 cm) with a wick (10 × 7 cm) of Whatman no. 17 paper stapled to the top. Chromatography in the D1 direction was performed overnight with sodium phosphate buffer (1 M, pH 6.0), development in D2 (5.3 M lithium formate, 8.5 M urea, pH 3.5) was performed in the opposite direction to D1 (bottom to top in Figure 1). This was followed by development in D3 (1.2 M lithium chloride, 0.5 M Tris–HCl, 8.5 M urea, pH 8.0; perpendicular to D2, from left to right). Finally, the plates were developed with 1.7 M sodium phosphate (pH 6.0). Autoradiography was performed at −80°C using intensifying screens. Quantification of adduct levels was accomplished by Cerenkov counting of excised adduct spots or areas and published calculation procedures (24). The external standard consisted of a DNA sample modified with PhIP isolated from the pancreas of male rats that had been maintained on a diet fortified with PhIP (400 p.p.m.) for 2 weeks (27).

Statistical analysis

Spearman correlation coefficients were calculated to examine the association of meat consumption, dietary HCA intake and ENOC exposure with breast tissue DNA adduct level. We computed partial Spearman correlation coefficients adjusting for BMI to account for body fat mass and used linear regression models to examine the association of dietary HCA intake, ENOC, BMI, energy intake, age and cigarette smoking with DNA adduct levels.

Results

Information on dietary HCA intake and measurement of breast tissue DNA adducts were available for 44 women. Baseline characteristics of these women are shown in Table I. Mean age of these women was 36.5 years, ranging from 16 to 67 years.

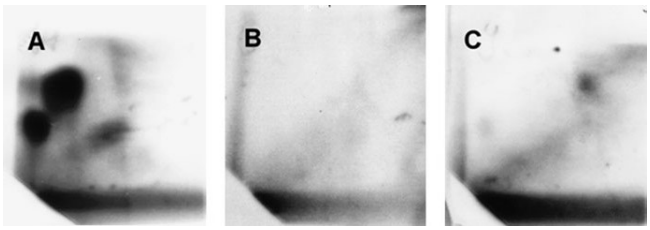

Fig. 1. Autoradiographic representations of ^{32}P-postlabelling/ion-exchange thin-layer chromatography analyses of DNA isolated from pancreas of rats treated with PhiP (**A**, positive control) and from individual samples of human mammary tissue (**B** and **C**).

DNA samples isolated from breast tissue were analysed using the ^{32}P-postlabelling technique. Adduct levels (relative adduct labelling, RAL) were up to 66.40 DNA adducts per 10^9 nucleotides with one sample below the detection limit of

Table I. Baseline characteristics of study participants (n = 44)

Age (years)	Mean ± SD (range)	36.5 ± 12.8 (16–67)
BMI (kg/m^2)	Mean ± SD (range)	25.7 ± 3.2 (19.5–32.5)
Smoking status	Never (%)	50.0
	Former (%)	11.4
	Current (%)	38.6
Mother or sister with breast cancer	No (%)	79.1
	Yes (%)	11.6
	Do not know (%)	9.3
Ever pregnant	No (%)	30.2
	Yes (%)	69.8
Ever use of oral contraceptives	No (%)	35.7
	Yes (%)	64.3

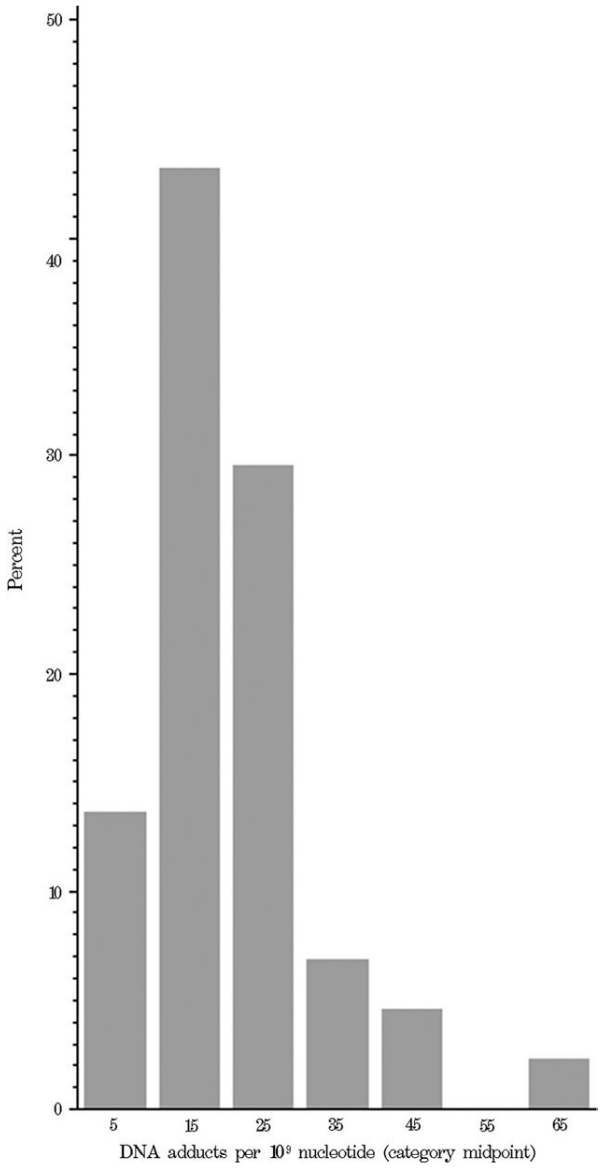

Fig. 2. Distribution of DNA adduct level (DNA adducts per 10^9 nucleotide) in breast tissue of 44 women.

5.0 DNA adducts per 10^9 nucleotides. The median was 18.45 (interquartile range 12.81–25.65) DNA adducts per 10^9 nucleotides (Figure 2). The detected DNA adducts could not be confirmed to be specific HCA-derived DNA adducts by comparison with the external standard based on different autoradiographic fingerprints upon multidirectional thin-layer chromatography (Figure 1). Thus, the exact structure of these lipophilic adducts remains undetermined. The adduct level correlated significantly with BMI (Spearman correlation coefficient $r = 0.47$, $P = 0.002$), but less with age ($r = 0.27$, $P = 0.06$) and not with smoking status ($r = 0.01$, $P = 0.93$) or total energy intake ($r = 0.19$, $P = 0.20$).

Poultry and processed meat contributed most to the meat consumption in this group of women (Table II). We observed a high correlation of beef (Figure 3) and processed meat (Figure 4), but less for pork and none for poultry consumption with DNA adduct level. Most meat was consumed fried followed by stir-fried (Table II). The amount of meat consumed by these two cooking methods, especially by frying, was highly correlated with the DNA adduct level in breast tissue. The correlation with stir-frying was stronger after we excluded poultry consumption because poultry consumption was not associated with DNA adduct levels. We also computed partial Spearman correlation coefficients, which were adjusted for BMI. Doing so, slightly improved the correlations of poultry, pork and processed meat intake with DNA adduct concentrations as well as the correlation between the consumption of stir-fried meat and DNA adduct concentrations.

Median total HCA intake was 40.34 ng/day with PhIP being the most abundant HCA, followed by MeIQx and DiMeIQx (Table III). We observed statistically significant correlations between total HCA, PhIP and MeIQx intake and the extent of DNA adducts in breast tissue (Table III). These results were confirmed by linear regression models, in which we observed a strong association between total HCA intake and DNA adduct levels in breast tissue (beta-coefficient = 2.52, $P = 0.03$). The observed correlations were stronger when calculating a partial Spearman correlation coefficient adjusted for BMI. Age at surgery, smoking status and energy intake were not statistically significantly associated with adduct levels in linear regression models (data not shown). Total HCA intake from pork, processed meats and beef were positively correlated with the amount of DNA adducts in the breast tissue of the women included in our study (Table III). However, there was no correlation with HCA intake from poultry. For any type of meat, MeIQx intake was most strongly associated with the DNA adduct level in breast tissue (data not shown).

In addition to the intake of HCA, we also estimated the formation of ENOC. The median ENOC exposure in this group of women was 71.06 μg/day (interquartile range 56.02–91.38 μg/day). The exposure was significantly correlated with the DNA adduct level in breast tissue, but the association was slightly attenuated after taking BMI into account. We further examined whether the correlation between total HCA intake and DNA adduct level was attenuated after taking ENOC exposure into account. The partial Spearman correlation coefficient between total HCA intake and DNA adduct level was 0.30 ($P = 0.06$) (adjusted for ENOC and BMI). Vice versa, the correlation between ENOC and DNA adduct level was very strongly attenuated after taking total HCA intake and BMI into account (partial Spearman $r = 0.08$, $P = 0.61$).

Discussion

In this study, we examined whether the level of DNA adducts in breast tissue of healthy women undergoing reduction mammoplasty was related to the dietary HCA intake We observed statistically significant correlations between total HCA, PhIP and MeIQx intake and level of covalent DNA modification. However, the detected DNA adducts could not be confirmed to be specific HCA-derived DNA adducts by chromatographic comparison with external standards.

The estimates of intake of HCA indicate that the consumption of HCA (mean intake of 40.3 ng/day) was relatively low in the study population but data were comparable to other German women (28). Most prominent source of HCA was beef and processed meat. Especially beef contributed most to total HCA intake although the consumption of beef was lower than the consumption of poultry and processed meat. In

Table II. Meat consumption by subgroups and correlation between meat consumption and DNA adduct level[a] in breast tissue of 44 women

	Intake (g/day)		Spearman correlation coefficient (P-value) between DNA adduct level and meat intake (by meat type or cooking method, respectively)	Partial Spearman correlation coefficient (P-value)[b] between DNA adduct level and meat intake (by meat type or cooking method, respectively)
	Median	Interquartile range		
Meat intake by meat type[c]				
Poultry	12.48	6.90–22.31	0.004 (0.98)	0.014 (0.93)
Beef and veal	5.31	1.70–13.44	0.32 (0.03)	0.29 (0.07)
Pork	5.67	1.79–14.75	0.14 (0.37)	0.25 (0.11)
Processed meat[d]	10.73	4.55–22.33	0.51 (0.0004)	0.56 (0.0002)
Meat intake by cooking method[e]				
Steaming/boiling	6.10	1.24–10.15	0.10 (0.50) [0.21 (0.17)][f]	0.11 (0.49) [0.23 (0.15)][f]
Stir-frying	12.14	3.83–22.46	0.15 (0.33) [0.27 (0.08)]	0.19 (0.23) [0.31 (0.05)]
Frying	14.02	7.80–32.65	0.39 (0.01) [0.33 (0.03)]	0.34 (0.03) [0.29 (0.06)]
Grilling/barbecuing	4.03	0.36–8.63	−0.09 (0.56) [0.18 (0.24)]	−0.04 (0.81) [0.17 (0.29)]

[a]Median DNA adduct level 18.45 (interquartile range 12.81–25.65) DNA adducts per 10^9 nucleotides.
[b]Adjusted for BMI.
[c]Daily intake of poultry, beef and veal, pork and processed meat as derived from the respective food frequency questionnaire (FFQ) items.
[d]Processed meat intake was calculated by adding up the consumption of sausages, bacon, ham, hamburger and meatballs.
[e]Meat intake by cooking method was calculated by adding up the consumption of all meat items assessed in the FFQ that were prepared by steaming/boiling, stir-frying, frying or grilling/barbecuing, respectively.
[f]In brackets: results after excluding poultry.

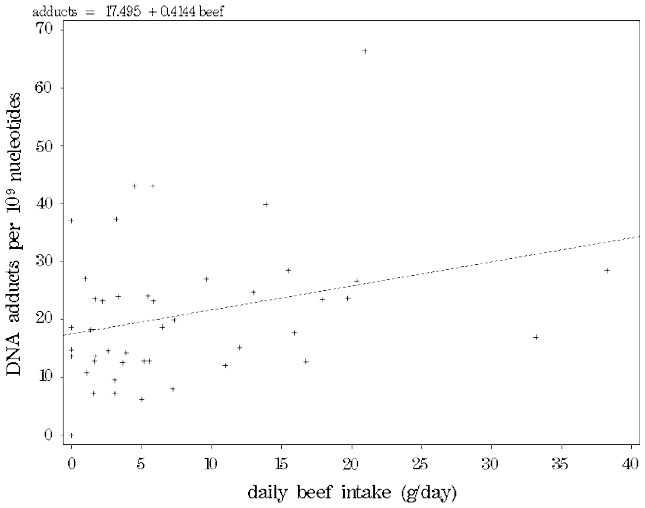

Fig. 3. Correlation between beef consumption (g/day) and DNA adduct level (DNA adducts per 10^9 nucleotide) in breast tissue of 44 women.

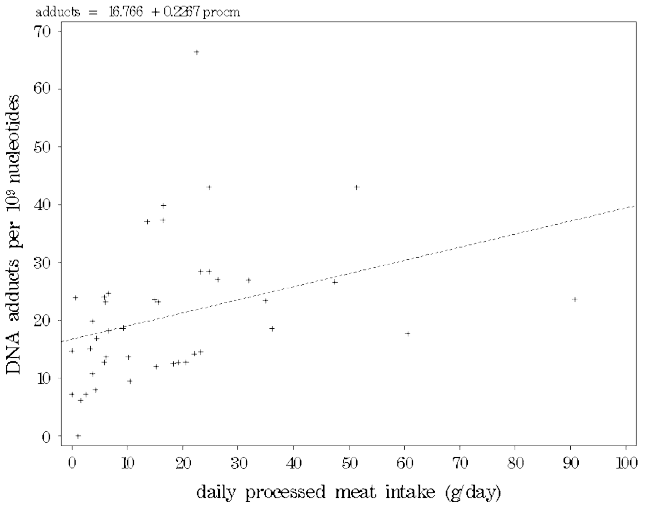

Fig. 4. Correlation between processed meat consumption (g/day) and DNA adduct level (DNA adducts per 10^9 nucleotide) in breast tissue of 44 women.

a Swedish study that used a similar approach to assess HCA intake, median total HCA intake was 77 ng/day, which also included HCA intake from fish (29). In US studies, the estimated HCA intake is generally higher (mean PhIP intake 78.1 ng/day; mean MeIQx intake 21.9 ng/day) than in European studies (30), which might be explained by differences in total consumption of beef and preferences for specific cooking methods and a higher degree of doneness/browning.

The determination of DNA adducts has been conducted in mammary tissue enriched in epithelial cells, the cell type in the mammary gland from which most breast tumours originate. A number of studies reported elevated DNA adduct levels in breast tissues from mammary cancer patients as compared to reduction mammoplasty patients (11,31–33). The highly sensitive ^{32}P-postlabelling technique has proven useful in the study of DNA adduct level especially when exposure to complex mixtures and unknown carcinogens occurs (13). While other methods such as immunochemical detection may be more specific for the detection of adducts derived from a specific genotoxic agent, the ^{32}P-postlabelling technique allows detection of a broad spectrum of unknown adducts (13,31). Recently, Pfau *et al.* (27) described an association between DNA adduct level in human mammary tissue and N-acetylator genotype of the patients, and HCA-induced DNA adduct formation in human mammary cells has been shown to be influenced by polymorphisms in genes encoding for phase I enzymes (34). Polymorphisms of glutathione transferase but not sulphotransferase were associated with adduct level in breast tissue of cancer patients (35,36). Also, DNA adducts have been detected in exfoliated cells isolated from human milk (37). However, in these studies, no information regarding the intake of HCA was available for the patients.

The consumption of beef and processed meat in Germany is high (38,39). In our study, beef and especially processed meat intake was correlated with DNA adducts in breast tissue. When looking at cooking methods, most meat is fried or stir-fried. These two cooking methods were also correlated with DNA adduct level and are both known to lead to HCA formation in meat. In contrast, only small amounts of meat were grilled in our study and we did not observe a statistically significant correlation between grilled meat consumption and DNA adducts. Two case–control studies reported a higher risk of breast cancer in women who consumed meat well done compared with women who did not (40,41). Zheng *et al.* observed that women who consumed hamburger, beef steak and bacon consistently very well done had a 4.62 times higher risk (95% confidence interval = 1.36–15.70) than women who consumed the meats rare or medium done (34). However, other studies did not observe associations of meat cooking, meat doneness and breast cancer (42,43).

Several hypotheses might explain associations of meat consumption in general and meat cooked with specific methods with breast cancer. In discussion are not only HCA but also endogenous N-nitroso compounds and PAH. Grilling can form both PAH and HCA, whereas frying forms predominantly HCA (44). Carcinogenicity of different HCA has been proven in animal studies (45,46); specifically PhIP, MeIQx and MeIQ have been demonstrated to induce mammary tumours in female rats. Conflicting results arose from epidemiologic studies. Women with a high PhIP exposure were more likely to have breast cancer in an US (47) and an Uruguayan case–control study (48), but Delfino *et al.* (49) did not see an increased risk of breast cancer with increasing intake of HCA.

We observed improved Spearman correlation coefficients between pork, processed meat, stir-fried meat as well as the intake of single HCA and the concentration of DNA adduct levels in breast tissue after adjusting for BMI. The Spearman correlation coefficient between BMI and DNA adduct concentration was 0.44 (P = 0.006). Previously, a positive association between BMI and aromatic DNA adducts in pancreatic tumour tissue has been reported (50), whereas for BMI and the DNA adduct concentration in peripheral blood lymphocytes inverse correlations have been seen in two studies (51,52). It has been hypothesized that increased body fat changes adduct concentrations by affecting the distribution and storage of lipophilic carcinogens.

Here, we report to the best of our knowledge for the first time on the analysis of DNA adduct levels in the breast epithelial cells of women for whom intake of HCA was estimated based on a dedicated questionnaire. We observed a strong association between intake of several HCA and DNA adduct level. However, the DNA adducts could not be

Table III. Correlation of HCA intake with DNA adduct level[a] in breast tissue in 44 women

HCA intake (ng/day)[b]			Spearman correlation coefficient (P-value) between DNA adduct level and HCA intake	Partial Spearman correlation coefficient (P-value)[c] between DNA adduct level and HCA intake
	Median	Interquartile range		
Total HCA	40.34	19.55–102.33	0.33 (0.03)	0.41 (0.008)
PhIP	23.07	8.52–67.55	0.31 (0.04)	0.39 (0.01)
MeIQx	11.90	4.93–22.92	0.37 (0.01)	0.41 (0.007)
DiMeIQx	1.69	0.41–4.68	0.18 (0.25)	0.23 (0.15)
Total HCA from				
Poultry	4.01	0.23–19.80	0.06 (0.71)	0.06 (0.70)
Beef	11.43	0.92–24.48	0.28 (0.06)	0.27 (0.08)
Pork	2.34	0.15–7.77	0.28 (0.06)	0.39 (0.01)
Processed meat	4.55	1.83–25.76	0.44 (0.003)	0.49 (0.001)

[a]Median DNA adduct level 18.45 (interquartile range 12.81–25.65) DNA adducts per 10^9 nucleotides.
[b]Daily intake of total HCA as well as MeIQx, MeIQ and PhIP were estimated by combining information on degree of browning, cooking method and the amount of meat intake.
[c]Adjusted for BMI.

confirmed to be specific HCA-derived adducts. Cooking of meat at high temperature not only leads to the formation of HCA but also to other components such as PAH (53) or especially in processed meat to N-nitrosamines (54) that are thought to be related to cancer risk. It might well be that HCA are simply a surrogate marker of the compounds that arise from cooking at high temperature. Red meat consumption leads to a dose-dependent increase in ENOC formation in humans (55). Indeed, we also observed a significant, although less strong, association of ENOC exposure with DNA adduct level. However, when mutually adjusting total HCA intake and ENOC exposure, the partial Spearman correlation coefficient for ENOC, but not HCA intake was strongly attenuated. This supports the finding that HCA intake (or correlates of) is strongly associated with DNA adduct level in breast epithelial cells. PAH intake, which we did not compute in our study, increases with increasing consumption of not only grilled meats but also smoked meats (56).

HCA intake was estimated employing a questionnaire with photographs of meat dishes with different levels of doneness and using a database of HCA levels in several types of meat at different levels of doneness. While these questionnaires have been shown to be more accurate than other dietary questionnaires, they also have limitations in predicting HCA intake.

In conclusion, consumption of processed meat as well as fried and stir-fried meat and HCA intake were correlated with the level of DNA adducts in breast tissue in this group of women. However, the observed DNA adducts could not be determined to be specific HCA adducts. This may indicate that not only HCA might be a risk factor for breast cancer but also other compounds in fried or grilled meat are, and HCA are simply surrogates for other eventually genotoxic substances in meat.

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