Effects of Perinatal Exposure to Acrylamide and Glycidamide on Intestinal Tumorigenesis in *Min*/+ Mice and Their Wild-type Litter Mates

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Abstract. *The tumorigenic capacity of acrylamide (AA) in the intestine of C57BL/6J Min/+ mice, as well as in their wild-type (wt) litter mates was investigated. In Experiment 1, the mice were s.c. injected with 10 or 50 mg/kg body weight (bw) of AA or glycidamide (GA) at week 1 and 2 after birth. In Experiment 2, the mice were given 50 mg/kg bw/injection of AA or GA 1 week before birth to the dam, alone or in combination with exposure of the pups at week 1 and 2 after birth. Following GA exposure at week 1 and 2, the formation of small intestinal tumors in Min/+ mice increased in a dose-dependent manner (r_s=0.32, p=0.008): a 1.3-fold increase in the number of tumors with 50 mg/kg bw GA compared to the controls (p<0.05). In the wt litter mates, there was a dose response relationship between the GA exposure and the frequency of animals with one or more intestinal neoplasm (intestinal tumors + aberrant crypt foci) (p=0.018): at 50 mg/kg bw of GA an 8-fold induction was found compared to the controls (p=0.017). In Experiment 2, Min/+ mice exposed to GA in utero had fewer small intestinal tumors than the controls (p<0.05). However, following GA exposure the number of intestinal tumors correlated positively with the number of injections (small intestine: r_s=0.32, p=0.002; colon: r_s=0.27, p=0.01). When exposed early in life, GA is a weak intestinal tumorigen in Min/+ mice and their wt litter mates.*

Abbreviations: AA, acrylamide; GA, glycidamide; ACF, aberrant crypt foci; *APC*/*Apc*, adenomatous polyposis coli gene (human/murine); FAP, familial adenomatous polyposis; *Min*/+ mice, multiple intestinal neoplasia mice; wt, wild-type.

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Acrylamide (AA), which is metabolised to the genotoxic derivate glycidamide (GA) in both rodents and humans (1, 2), induces testicular mesotheliomas and mammary gland adenomas in rats (3, 4) and initiates skin and lung cancer in mice (5-7). Since AA is also converted to GA in humans, the International Agency for Research on Cancer (IARC) has classified it as a probable human carcinogen (Class 2A) (8). Worldwide concern was caused when it was found that AA may occur in significant amounts in heat-processed carbohydrate-rich foods and coffee (9-11). These foods are important elements of the basic diet consumed by the general population.

Human colorectal cancer has been associated with environmental and lifestyle factors, including lack of physical activity and particular food habits (12). Even though mutagens and carcinogens, such as heterocyclic amines, polycyclic aromatic hydrocarbons, *N*-nitroso compounds and AA are found in various foods, their potential role in human colorectal carcinogenesis is not clear. Nevertheless, colorectal cancer is associated with red meat consumption and has, to some degree, been linked to heterocyclic amines, as well as unidentified meat mutagens formed in the heating process (13).

Because AA has been found to cross the human placenta (14, 15) and is detected in human breast milk (14, 16), humans are also exposed to AA during foetal life and infancy. Similar transplacental transfer of AA has also been observed in mice, rats, rabbits, dogs and pigs (17-20), and AA was observed to be distributed uniformly in the foetus (18-20). However, risk assessments of AA have, so far, only been based on tumor responses following exposure in adult rodents (3-7). Therefore, it is important to provide information on the risk associated with exposure at earlier and vulnerable phases of life (21-23).

The multiple intestinal neoplasia (C57BL/6J *Min*/+) mouse is, like humans with the familial adenomatous polyposis (FAP) syndrome, heterozygous for a mutation in the tumor suppressor gene *Apc*. This mutation leads to the

development of numerous neoplastic lesions in the intestines, mainly in the small intestine of the *Min*/+ mice and in the colon of FAP patients. Complete somatic inactivation of *Apc*/*APC* in discrete crypts of the intestinal epithelium seems to be the initial event of the tumorigenesis in *Min*/+ mice (24, 25), human FAP and, importantly, in 80% of human sporadic colorectal cancer (26-28). Therefore, the *Min*/+ mouse provides a good carcinogenesis model in which the initial molecular pathogenesis is the same in both humans and mice. We have found that neonatally exposed *Min*/+ mice are particularly susceptible to intestinal carcinogens (21, 29). Previously, we used this model to test several possible human carcinogens such as heterocyclic amines (24, 30-32) and chlorination by-products in drinking water (33), as well as the experimental carcinogen azoxymethane (AOM) (23, 25).

The genotoxicity of AA occurs predominantly through the action of its epoxide GA, which has a much higher reactivity with DNA bases than AA (1, 34). Cytochrome P450 2E1 (CYP2E1) is the enzyme that catalyses the oxidation of AA to GA (35). In adult mice approximately 80% of the AA is converted into GA (36). Neonatal mice are deficient in CYP2E1, resulting in only a very low conversion rate of AA to GA (1). The reduced concentration of CYP2E1 has also been shown in humans prior to and after birth (37).

The main objective of the present study was to examine whether AA or GA may act as intestinal tumorigens, using perinatal exposure in *Min*/+ mice as a particularly susceptible test model. In addition, we wanted to examine the carcinogenicity of AA and GA in the intestine of their wild-type (wt) litter mates. Pre- and/or postnatal exposure of the animals to AA or GA was conducted and the intestinal lesions scored at the age of 8-12 weeks. In the colon, the score included flat and classical aberrant crypt foci (ACF), the smallest observable lesions (38).

Materials and Methods

Mouse breeding. C57BL/6J-*Min*/+ males and C57BL/6J-+/+ (wt) females were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The males were housed singly in plastic cages and the females were housed 2 animals per cage. The animal room was maintained on a 12-hour light/dark cycle, with controlled humidity (55±5%) and temperature (20-24˚C). Water and feed were given *ad libitum*.

In Experiment 1, both the mothers and the pups were given SDS RM3 feed (Special Diets Services, Witham, UK). From week 12, the feed for the wt mice was changed to the less energy containing SDS RM1 (Special Diets Services). In Experiment 2, the SDS RM1 feed was used, except during the 6 weeks between mating and weaning when SDS RM3 was used.

When 3-weeks-old, the *Min*/+ and wt pups were identified by an allele-specific PCR-assay, as described previously (31). Both genotypes were used in the experiment.

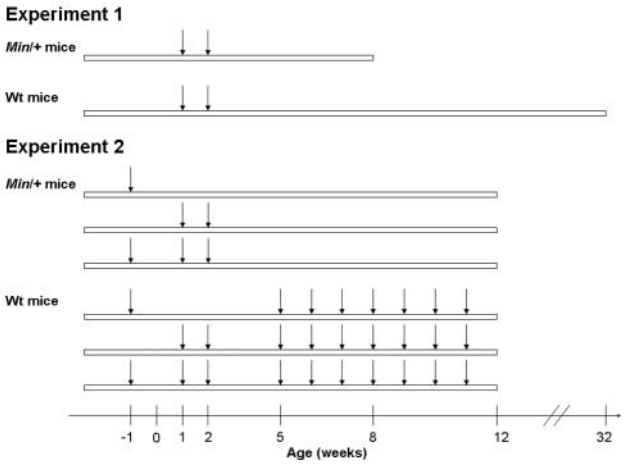

Figure 1. *Experimental design. In Experiment 1, Min/+ mice and their wt litter mates were treated with 10 or 50 mg/kg bw acrylamide (AA) or glycidamide (GA) at week 1 and 2 after birth. The Min/+ mice were sacrificed at week 8 after birth, and the wt mice at week 32. In Experiment 2, all mice were exposed to 50 mg/kg bw/injection AA or GA, with the first group exposed 1 week before birth, the second group 1 and 2 weeks after birth, and the third group 1 week before birth and again at week 1 and 2 after birth. In addition, the wt mice were exposed once a week from week 5 to 11. The mice in Experiment 2 were sacrificed at week 12 after birth.*

Treatment. AA (Sigma-Aldrich, St. Louis, MO, USA; ≥99% purity) and GA (Toronto Research Chemicals, North York, Canada; 98% purity) were dissolved in 0.9% NaCl. Dosing solutions were prepared on the day of administration. Both AA and GA were found to be stable in saline for at least 1 week under cool and dark conditions (HPLC-UV; unpublished results). The compounds were administered by *s.c.* injections. The experimental protocols are shown in Figure 1.

In Experiment 1, the mice were injected with 10 or 50 mg/kg body weight (bw) AA (*Min*/+ mice: n=19, 8 females and 11 males; and n=17, 12 females and 5 males, respectively, and wt mice: n=24, 16 females and 8 males; and n=12, 3 females and 9 males, respectively) or GA (*Min*/+ mice: n=26, 19 females and 7 males; and n=21, 14 females and 7 males, respectively, and wt mice: n=24, 10 females and 14 males; and n=24, 11 females and 13 males, respectively) once a week at week 1 and 2 after birth (10 μl/g bw). The *Min*/+ mice were sacrificed by cervical dislocation at 8 weeks of age, and the wt mice at 32 weeks (Figure 1).

In Experiment 2, the groups were exposed prenatally to 50 mg/kg bw AA (*Min*/+ mice: n=28, 11 females and 17 males; wt mice: n=33, 10 females and 23 males) or GA (*Min*/+ mice: n=17, 8 females and 9 males; wt mice: n=11, 4 females and 7 males) by administration to the dams 1 week before birth. A second set of groups was exposed neonatally to 50 mg/kg bw AA (*Min*/+ mice: n=36, 20 females and 16 males; wt mice: n=16, 11 females and 5 males) or GA (*Min*/+ mice: n=30, 15 females and 15 males; wt mice: n=11, 5 females and 6 males) once at week 1 and again at week 2 after birth. In a third set of groups, 50 mg/kg bw AA (*Min*/+ mice: n=11, 6 females and 5 males; wt mice: n=20, 12 females and 8 males) or GA (*Min*/+ mice: n=18, 8 females and 10 males; wt mice: n=16, 7 females and 9 males) were given once *via* the dam 1

week before birth and then directly to the pups once at week 1 and again at week 2 after birth (perinatal exposure). In addition, in all three sets of groups, the wt animals were given 50 mg/kg bw AA or GA once a week from week 5 to week 11 after birth. At 12 weeks of age, all the mice were sacrificed by cervical dislocation (Figure 1).

In both experiments, control dams and pups were given 10 μl/g bw 0.9% NaCl (experiment 1: n=19, 11 females and 8 males; experiment 2: n=23, 12 females and 11 males). In none of these experiments did any of the mice look sick or discomforted due to the treatments.

Scoring of ACF and tumors. The small intestine and the colon were removed separately and rinsed in ice-cold phosphate-buffered saline (PBS) before they were longitudinally incised. The small intestine was divided into proximal, middle and distal parts. They were then fixed flat between wet (PBS) filter papers for a minimum of 24 h in 10% neutral buffered formalin. After a 10 second stain in 0.2% methylene blue (George T. Gurr Ltd., London, UK) dissolved in 10% neutrally buffered formalin, the intestines were kept in 10% neutrally buffered formalin for a minimum of 24 h before the mucosa was examined by transillumination using an inverse light microscope. The number, size and location of the lesions in the small intestine and colon were scored. The sizes of the tumors were scored as mm^2, and the location in the small intestine was described as cm distally from the ventricle. Classical and flat ACF in the colon were scored, as described in Paulsen *et al.* (39). Briefly, the flat ACF are likely to be the precursors of colorectal adenomas, while the classical ACF are not directly related to tumorigenesis, but are an expression of general mutagenesis in the colon epithelium (29, 38-40). The size of an ACF was scored as the number of crypts/lesion. A colonic tumor was defined as a lesion with >35 aberrant crypts.

Statistical analysis. Kruskal-Wallis One Way ANOVA on Ranks (SigmaStat software; Jandel Scientific, Erkrath, Germany) was used to test for the differences between the groups. For isolation of the groups that differed from the control group, Dunn's Method was used. Differences in proportions between the groups were assessed statistically by the Fischer Exact probability test. Correlations between the tumor parameters and the doses tested were evaluated by the non-parametric Spearman's rank order correlation coefficient r_s. Logistic regression with the dose treated as a continuous variable was used to examine trends between the dose and the induction of lesions in the wt litter mates (SPSS, Chicago, IL, USA). A p-value of <0.05 was considered significant.

Results

Effects of neonatal exposure to AA and GA (Experiment 1)

Final body weight. None of the groups differed from their respective controls in final body weight, with the exception of the male *Min*/+ mice treated with 10 mg/kg bw GA, which had a significantly lower final body weight (8 weeks) than that of their male controls (22.6 g ±0.9 g *vs.* 24.7 g ±1.5 g; Dunn's Method, $p<0.05$). Of 113 mice, 4 animals died before the scheduled sacrifice: 1 *Min*/+ and 1 wt male in the control group, and 2 wt males in the GA50 group.

Induction of intestinal lesions. Variance analysis showed that the number of small intestinal tumors differed significantly among the groups of *Min*/+ mice exposed to AA, GA, or vehicle (females + males and females, ANOVA on Ranks, $p<0.005$; Figure 2A). However, the only group that significantly differed from the controls was that of the *Min*/+ mice exposed to 50 mg/kg bw/injection of GA, showing 1.3-fold increase in the number of small intestinal tumors (females + males, $p<0.05$, Dunn's test; Figure 2A). In addition, considering all the animals treated with GA or vehicle, a dose-dependent induction of the small intestinal tumors was revealed ($r_s=0.32$, $p=0.008$, N=66). In addition, in the females alone, a dose response relationship was evident ($r_s=0.35$, $p=0.019$, N=44). The distribution profile of the tumors along the small intestine was not affected by AA or GA treatment (Figure 3).

No statistical differences between the treatment groups with respect to the number of tumors, flat ACF or classical ACF were observed in the colons of *Min*/+ mice (Figure 2 B-D).

In the wt litter mates, the total number of intestinal lesions/mouse was generally low (Table I). However, also in these animals, the group exposed to 50 mg/kg bw/injection of GA differed from the other groups. The frequency of animals with one or more intestinal lesions (small intestinal tumors + colonic tumors + flat ACF + classical ACF) was significantly higher in this group than in the control group (Fisher Exact test, 1-sided, $p=0.017$). In the same group, there was also an apparent dose-dependent effect of GA. In animals treated with 10 or 50 mg/kg bw GA or vehicle there was a positive trend between frequency of animals with any intestinal lesion and dose (Logistic regression with the dose treated as a continuous variable; $p=0.018$). No colonic tumors were seen in the wt animals and classical ACF was only observed in the group treated with 50 mg/kg bw/injection of GA (one ACF in each of 2 of the 24 animals).

Growth of intestinal lesions. None of the treatments affected the size of the small intestinal or colonic tumors, neither in *Min*/+ mice nor in wt mice, and the tumor load (total tumor area) of the treated animals did not differ from that of the controls. The number of crypts/foci of flat and classical ACF was similar in all the groups (data not shown).

Effects of prenatal and neonatal exposure to AA and GA (Experiment 2)

Final body weight. The final body weight of the *Min*/+ mice did not differ significantly from that of the controls. However, the wt animals exposed both pre- and neonatally to 50 mg/kg bw/injection of GA (GA 3) showed a significantly lower final body weight in comparison with that of the controls with the females 19.8 ±1.1 g *vs.* 21.9 ±1.0 g and the males 25.4 ±2.0 g *vs.* 28.3 ±2.3 g (Dunn's Method, $p<0.05$). The additional weekly exposure of wt animals from

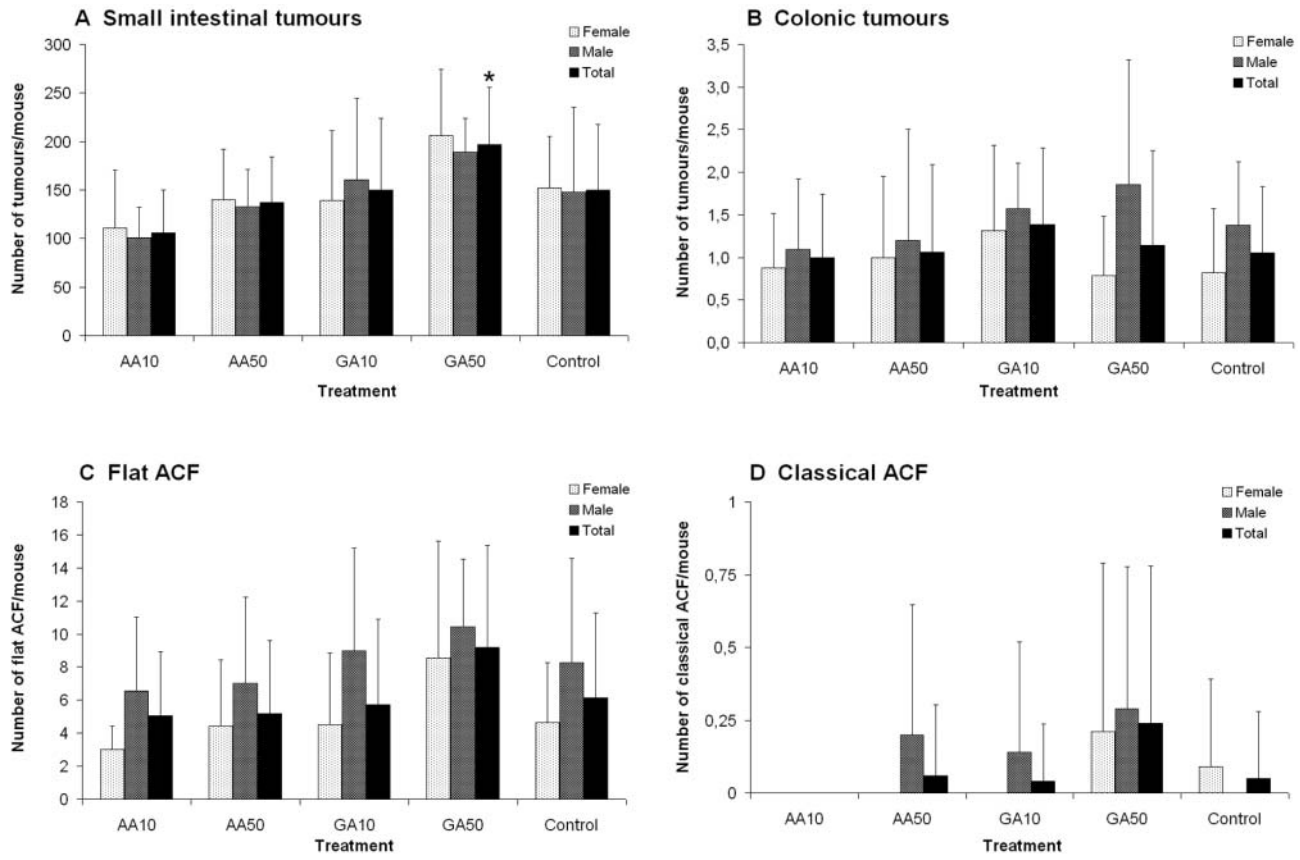

Figure 2. *Number of intestinal lesions in Min/+ mice treated with 10 or 50 mg/kg bw acrylamide (AA) or glycidamide (GA) at week 1 and 2 after birth (Experiment 1). A: Small intestinal tumors. B: Colonic tumors. C: Flat ACF in the colon. D: Classical ACF in the colon. Columns and bars indicate mean±SD. Asterisks (*) indicate statistically significant difference from the control group ($p<0.05$, Dunn's test). In these 113 Min/+ mice, 15,924 lesions were scored.*

week 5-11 did not appear to have any effect on the weight. Of the 163 mice, 4 died before the scheduled sacrifice: 1 *Min*/+ and 1 wt female in the AA 2 group, 1 *Min*/+ female in the GA 1 group and 1 *Min*/+ male in the AA 3 group.

Induction of intestinal lesions. The number of small intestinal tumors varied significantly among the groups of *Min*/+ mice exposed prenatally/neonatally to AA, GA or vehicle (ANOVA on Ranks, $p<0.001$; Figure 4A). Surprisingly, the only group that differed from the control group was GA 1, *Min*/+ mice treated with 50 mg/kg bw GA only before birth, which showed a statistically significant reduction of small intestinal tumors (Dunn's Method, $p<0.05$). However, applying trend analysis to all the animals treated with GA or vehicle, a dose-dependent (number of injections of 50 mg/kg bw GA) induction of small intestinal tumors was revealed ($r_s=0.32$, $p=0.002$, N=89). A similar statistically significant dose response relationship was not found after AA injections.

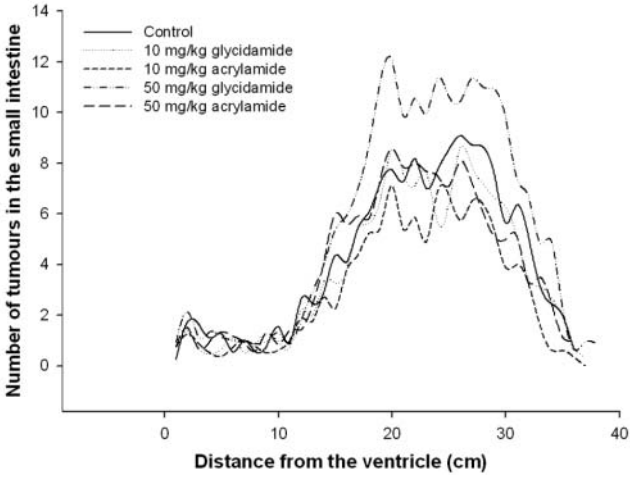

Figure 3. *Distribution of tumors along the small intestine in female Min/+ mice treated with acrylamide and glycidamide. The tumor position is given as distance from the ventricle in cm. The mean number of tumors/cm intestine/mouse was scored.*

Table I. *Number of intestinal lesions in each group and incidence of lesions/group in wild-type C57BL/6J mice after neonatal exposure (week 1 and 2 after birth) of acrylamide (AA) or glycidamide (GA) (Experiment 1) or after perinatal exposure (1 week before birth, week 1 and 2 after birth, or 1 week before birth + week 1 and 2 after birth) of 50 mg/kg bw/injection of AA or GA (Experiment 2).*

Treatment	N	Small intestinal tumors	Colonic tumors	Flat ACF	Classical ACF	Incidence (% animals with lesions[§])
Experiment 1						
AA10[a]	24	0	0	2	0	4
AA50[b]	12	0	0	1	0	8
GA10[a]	24	1	0	1	0	8
GA50[b]	24	4	0	1	2	25*
Control	31	1	0	0	0	3
Experiment 2						
AA 1[c]	33	1	0	6	0	15
AA 2[d]	16	0	0	1	0	6
AA 3[e]	20	1	0	2	0	15
GA 1[c]	11	0	0	1	0	9
GA 2[d]	11	1	0	1	0	18
GA 3[e]	16	2	0	1	0	19
Control	26	1	0	1	0	8

*Statistically significant differences from the respective control ($p=0.017$; 1-sided Fisher Exact test). In the AA10 group, both lesions were found in one animal. In the GA50 group, one animal had two small intestinal tumors. In the AA 1 group, two animals had two flat ACF each. [a]10 mg/kg bodyweight, [b]50 mg/kg bodyweight, [c]injection 1 week before birth, [d]injection week 1 and 2 after birth, [e]injection 1 week before birth and week 1 and 2 after birth, [§]small intestinal tumors, colonic tumors and flat and classical ACF pooled.

In the colon, the number of tumors differed significantly among the groups of *Min*/+ mice exposed prenatally/neonatally to AA, GA or vehicle (males, ANOVA on Ranks, $p<0.001$; Figure 4B). In the males treated with 50 mg/kg bw GA both before and after birth (GA 3), there was a statistically significant 3.7-fold increase in the number of colonic tumors (Dunn's Method, $p<0.05$). In terms of the number of injections the induction of colonic tumors following GA exposure was dose-dependent (females + males; $r_s=0.27$, $p=0.01$, N=88).

A statistically significant difference in the number of flat ACF was observed among the groups of *Min*/+ mice exposed prenatally/neonatally to AA, GA or vehicle (females + males, females or males, ANOVA on Ranks, $p<0.02$; Figure 4C). The groups treated with 50 mg/ kg bw AA only before birth or only after birth (AA 1 and AA 2), had significantly higher numbers of flat ACF when compared with the controls (Dunn's Method, $p<0.05$). However, none of the treatment groups differed significantly from the control groups with respect to the number of classical ACF, although there was a tendency for more classical ACF in the groups treated with AA (2.9-fold increase when exposed to AA both pre- and postnatally (AA 3); Figure 4D).

When compared with the control group, the increased incidences of wt animals with intestinal lesions in the groups treated with AA or GA did not reach statistical significance (Table I).

Growth of intestinal lesions. In *Min*/+ males, there was a statistically significant difference in the size of the small intestinal tumors (ANOVA on Ranks, $p=0.049$; Figure 5A) and also in the size of colonic tumors (ANOVA on Ranks, $p=0.041$; Figure 5C) among the groups exposed prenatally/neonatally to AA, GA or vehicle. However, Dunn's Method could not isolate any group that differed significantly from the controls. Nevertheless, in terms of the number of exposures (number of injections of 50 mg/kg bw), a positive weak, but statistically significant trend in the size of the small intestinal tumors, was revealed (AA: $r_s=0.20$, $p=0.043$, N=99; GA: $r_s=0.26$, $p=0.013$, N=89). In the colon, there was a negative correlation between the size of the colonic tumors and the number of doses within both the AA- and GA-treated groups when compared to the controls (AA: $r_s=-0.24$, $p=0.026$, N=84; GA: $r_s=-0.24$, $p=0.041$, N=76).

Among the groups, the small intestinal tumor load (total area of tumors) in the *Min*/+ mice differed significantly

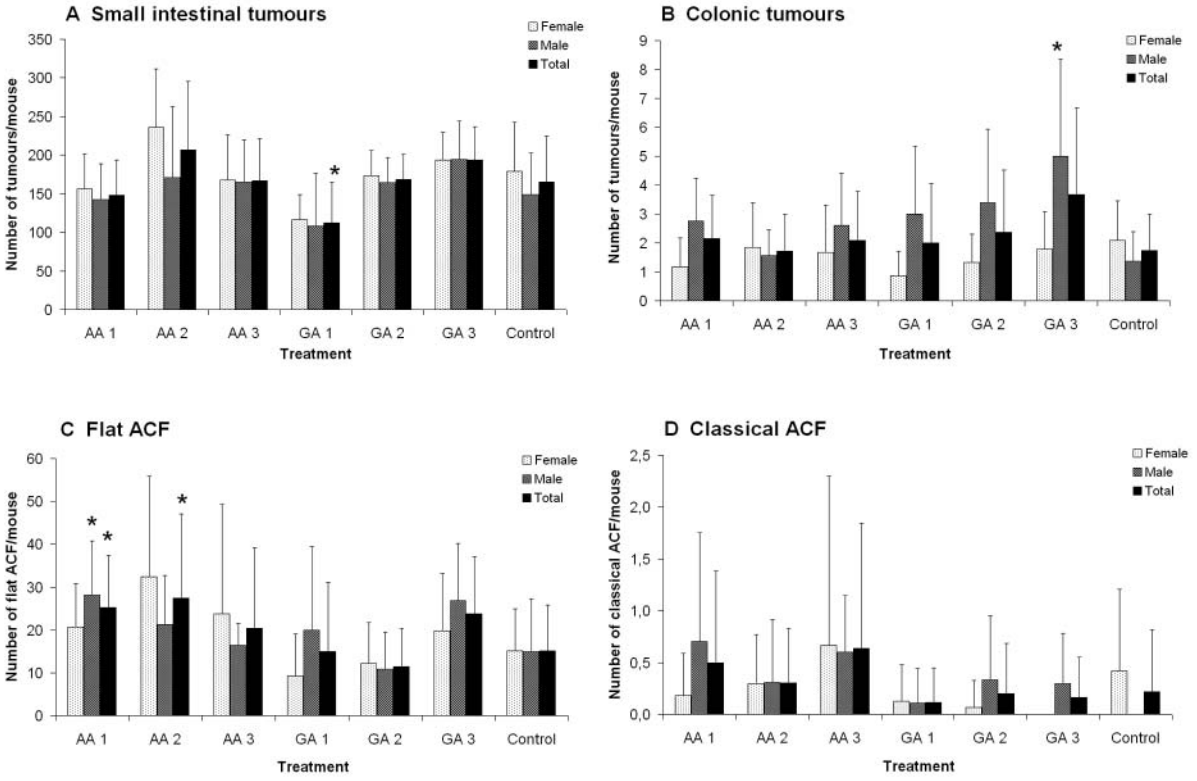

Figure 4. *Number of intestinal lesions in Min/+ mice treated with 50 mg/kg bw acrylamide (AA) or glycidamide (GA) 1 week before birth (AA 1 and GA 1), week 1 and 2 after birth (AA 2 and GA 2), or both (AA 3 and GA 3) (Experiment 2). A: Small intestinal tumors. B: Colonic tumors. C: Flat ACF in the colon. D: Classical ACF in the colon. Columns and bars indicate mean±SD. Asterisks (*) indicate statistically significant difference from the control group ($p<0.05$, Dunn's test). In 163 Min/+ mice, 31,746 lesions were scored.*

(females + males, females or males, ANOVA on Ranks, $p<0.011$; Figure 5B); however, Dunn's Method could not isolate any group that differed significantly from the controls. In the small intestine, however, a dose-dependent (number of injections of 50 mg/kg bw) increase in the tumor load was observed following GA treatment ($r_s=0.39$, $p<0.001$, N=89). In the colon, there were no differences between the groups regarding the tumor load.

The number of crypts/foci of flat and classical ACF did not differ between the groups. As in Experiment 1, the majority of ACF were detected in the distal half of the colon.

In the wt mice, none of the treatment groups differed significantly from the controls with respect to the size of the intestinal lesions.

Discussion

Acrylamide is reported to be a weak carcinogen in adult rodents, inducing tumors in the testes, mammary glands, thyroid gland, CNS, oral cavity, clitoral gland, uterus, skin and lungs (5-7), while other organs, such as the intestine, were not affected. In the present study, we tested the tumorigenic potential of AA, and its epoxide GA, in the small intestine and colon after perinatal exposure of *Min*/+ mice and their C57BL/6 wt litter mates. The mice were exposed to AA or GA at week 1 and 2 after birth (Experiment 1), or 1 week before birth, alone or in combination with exposure at week 1 and 2 after birth (Experiment 2). The most consistent findings were the effects caused by the active metabolite, GA. This is in agreement with the low conversion rate of AA to GA observed in neonatal mice (1).

In Experiment 1, GA exposure induced a dose-dependent increase in the number of small intestinal tumors in *Min*/+ mice (1.3-fold increase with 50 mg/kg bw GA). In the wt litter mates, there was a dose response relationship between GA and the frequency of animals with one or more intestinal lesions (8-fold induction compared with controls). No effects were observed in the colon or on the growth of the intestinal lesions, or with AA.

In Experiment 2, although GA given prenatally suppressed the tumorigenesis, there was a positive trend in the number of small intestinal and colonic tumors in *Min*/+ mice with the number of GA injections. The relative weak

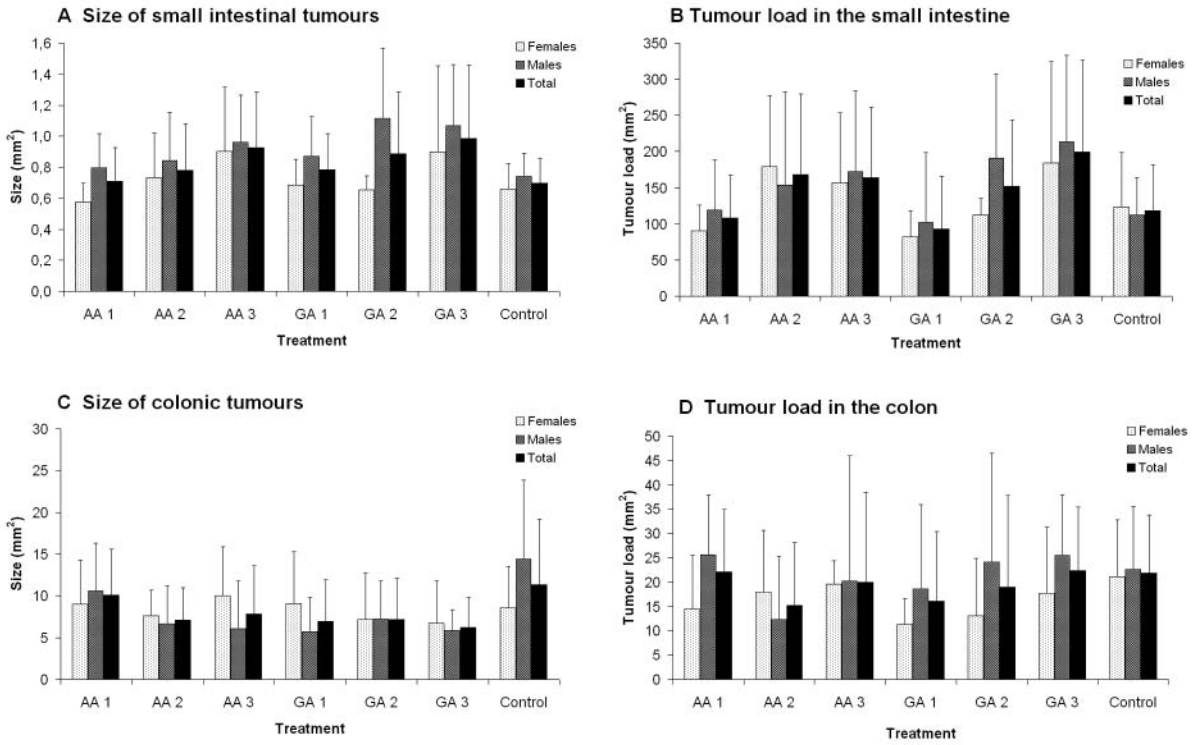

Figure 5. *Mean size (mm^2) of the small intestinal (A) and colonic (C) tumors and tumor load (total area of tumors, mm2) in the small intestine (B) and the colon (D) in Min/+ mice treated with 50 mg/kg bw acrylamide (AA) or glycidamide (GA) 1 week before birth (AA 1 and GA 1), week 1 and 2 after birth (AA 2 and GA 2), or both (AA 3 and GA 3) (Experiment 2). Columns and bars indicate mean ± SD. Asterisks (*) indicate statistically significant difference from the control group ($p<0.05$, Dunn's test).*

effect of GA 3 (injections of 50 mg/kg bw GA 1 week before birth in addition to at week 1 and 2 after birth) was probably due to the inhibitory effect observed with GA 1 (injection of 50 mg/kg bw GA 1 week before birth). It can be speculated that, at this early stage GA is cytotoxic to intestinal cells, causing cell death instead of tumor initiation. However, a combined pre- and postnatal exposure to GA produced more lesions in comparison with postnatal exposure alone, showing that prenatal exposure could be of importance in the final outcome. There was a 1.7- to 1.8-fold statistically significant increase of flat ACF in *Min*/+ mice exposed to AA 1 (injections of 50 mg/kg bw AA 1 week before birth) and AA 2 (injections of 50 mg/kg bw AA week 1 and 2 after birth). The reason for this effect following prenatal and postnatal exposure, but not after combined pre- and postnatal treatment, is not known.

The observed AA- and GA-induced enhancement of tumor size in the small intestine, and the opposite reduction of tumor size in the colon in *Min*/+ mice in Experiment 2, was dependent on the number of injections. The increase in the size of the small intestinal tumors may be due to the induction of tumors at an earlier point in time to those formed spontaneously, to faster growth, or to both. The reduction of the tumor size in the colon indicates either an induction at a later point in time, slower growth, or both, when compared to the tumors occurring spontaneously. A similar pattern has been observed earlier in *Min*/+ mice exposed to the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine (PhIP) postnatally on day 3 and 10 after birth (41). The pronounced correlation between the number of GA injections and the increase in the small intestinal tumor load is related to the observed increase in both the number and size of the tumors (Experiment 2).

Neonatal exposure to GA resulted in a statistically significant lower final body weight of the male *Min*/+ mice in comparison with the controls. This is consistent with previous studies both on mice (42) and rats (43, 44), showing that pups exposed perinatally to AA had a significant decrease in body weight, which was dose-dependent. Moreover, when the wt litter mates (females and males) were perinatally exposed to GA, the final body weight decreased significantly. However, this small reduction, though statistically significant, will probably have little biological consequence.

The biological variation in tumor number was high in all groups. Even though the variance analysis showed a statistically significant difference between the groups, it was frequently impossible to isolate the groups that differed from the controls using the post-hoc test. On occasions, statistically significant differences between the treated groups and the controls were only observed in one of the genders. However, the trend analyses, generally, revealed statistically significant dose response relationships for both sexes. We do not know why the significant effect of GA exposure in Experiment 1 was not reproduced in Experiment 2. However, the test conditions in these experiments were different.

In the present study, the control *Min*/+ mice had a higher number of tumors than in our previous studies. We do not know whether this is due to genetic drift or environmental changes in our animal department. The routine health controls do not indicate any pathological events occurring in the animal rooms. Although we found statistically significant tumorigenic activity of AA and GA in this study, these effects might well be more pronounced in *Min*/+ mice with a background containing a lower number of tumors.

In this study we used high doses of AA or GA to determine whether or not they could act as intestinal tumorigens. Furthermore, by using 50 mg/kg bw of AA or GA we could directly compare the effect with those observed in our previous studies using 50 mg/kg bw of the known intestinal carcinogen PhIP in the same mouse models (21, 24, 41). Compared with PhIP, AA and GA appear rather weak in terms of their intestinal tumor inducing potency in the *Min*/+ mouse. While the exposure to PhIP increases the number of the small intestinal tumors 3- to 9-fold in C57BL/6J *Min*/+ mice (21, 24, 41), exposure to an equal dose of GA only increased the number of small intestinal tumors in these animals 1.3-fold (*i.e.* PhIP is 2.3- to 6.9-fold more potent than GA). This is in agreement with the results of Husøy *et al.* (45), who found that the frequency of DNA damage (micronucleus induction in red blood cells) in C57BL/6J *Min*/+ mice following exposure to 50 mg/kg bw PhIP was 2.5 times higher than that following 50 mg/kg bw AA (6.43 ‰ *vs.* 2.54‰). Both PhIP and AA are quickly absorbed, widely distributed across tissues and rapidly excreted (46, 47). Although AA is less potent than PhIP with respect to intestinal tumorigenesis, it might still represent a similar or greater risk than PhIP, since the intake of AA is 100-1000 times higher than that of PhIP (AA approximately 0.5 μg/kg bw/day (48, 49) and PhIP approximately 1.1-2.4 ng/kg bw/day (50, 51)).

In conclusion, we have demonstrated a weak but significant tumorigenic activity of AA and GA in the intestine of *Min*/+ mice and their wt litter mates. The main effects were observed following GA exposure. The tumorigenicity observed may be related to the perinatal exposure, since such effects have never been observed in rodents exposed later in life.

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References

1 Gamboa da Costa G, Churchwell MI, Hamilton LP, Von Tungeln LS, Beland FA, Marques MM and Doerge DR: DNA adduct formation from acrylamide *via* conversion to glycidamide in adult and neonatal mice. Chemical Res Toxicol *16*: 1328-1337, 2003.

2 Fennell TR, Sumner SC, Snyder RW, Burgess J, Spicer R, Bridson WE and Friedman MA: Metabolism and hemoglobin adduct formation of acrylamide in humans. Toxicol Sci *85*: 447-459, 2005.

3 Johnson KA, Gorzinski SJ, Bodner KM, Campbell RA, Wolf CH, Friedman MA and Mast RW: Chronic toxicity and oncogenicity study on acrylamide incorporated in the drinking water of Fischer 344 rats. Toxicol Appl Pharmacol *85*: 154-168, 1986.

4 Friedman MA, Dulak LH and Stedham MA: A lifetime oncogenicity study in rats with acrylamide. Fundam Appl Toxicol *27*: 95-105, 1995.

5 Bull RJ, Robinson M and Stober JA: Carcinogenic activity of acrylamide in the skin and lung of Swiss-ICR mice. Cancer Lett *24*: 209-212, 1984.

6 Bull RJ, Robinson M, Laurie RD, Stoner GD, Greisiger E, Meier JR and Stober J: Carcinogenic effects of acrylamide in Sencar and A/J mice. Cancer Res *44*: 107-111, 1984.

7 Robinson M, Bull RJ, Knutsen GL, Shields RP and Stober J: A combined carcinogen bioassay utilizing both the lung adenoma and skin papilloma protocols. Env Health Perspect *68*: 141-145, 1986.

8 International Agency for Research on Cancer. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. IARC 60, pp. 389-433, Lyon, France, 1994.

9 Rosen J and Hellenas KE: Analysis of acrylamide in cooked foods by liquid chromatography tandem mass spectrometry. Analyst *127*: 880-882, 2002.

10 Tareke E, Rydberg P, Karlsson P, Eriksson S and Tornqvist M: Analysis of acrylamide, a carcinogen formed in heated foodstuffs. J Agric Food Chem *50*: 4998-5006, 2002.

11 Tareke E, Rydberg P, Karlsson P, Eriksson S and Tornqvist M: Acrylamide: a cooking carcinogen? Chem Res Toxicol *13*: 517-522, 2000.

12 Willett WC: Diet and cancer. Oncologist *5*: 393-404, 2000.

13 Wu K, Giovannucci E, Byrne C, Platz EA, Fuchs C, Willett WC and Sinha R: Meat mutagens and risk of distal colon adenoma in a cohort of U.S. men. Cancer Epidemiol Biomarkers Prev *15*: 1120-1125, 2006.

14 Sorgel F, Weissenbacher R, Kinzig-Schippers M, Hofmann A, Illauer M, Skott A and Landersdorfer C: Acrylamide: increased concentrations in homemade food and first evidence of its variable absorption from food, variable metabolism and placental and breast milk transfer in humans. Chemotherapy *48*: 267-274, 2002.
15 Schettgen T, Kutting B, Hornig M, Beckmann MW, Weiss T, Drexler H and Angerer J: Trans-placental exposure of neonates to acrylamide -a pilot study. Int Arch Occup Environ Health *77*: 213-216, 2004.
16 Fohgelberg P, Rosen J, Hellenas KE and Abramsson-Zetterberg L: The acrylamide intake *via* some common baby food for children in Sweden during their first year of life -an improved method for analysis of acrylamide. Food Chem Toxicol *43*: 951-959, 2005.
17 Edwards PM: The insensitivity of the developing rat foetus to the toxic effects of acrylamide. Chemico-Biological Interactions *12*: 13-18, 1976.
18 Ikeda GJ, Miller E, Sapienza PP, Michel TC, King MT, Turner VA, Blumenthal H, Jackson WE III and Levin S: Distribution of 14C-labelled acrylamide and betaine in foetuses of rats, rabbits, beagle dogs and miniature pigs. Food Chem Toxicol *21*: 49-58, 1983.
19 Ikeda GJ, Miller E, Sapienza PP, Michel TC, King MT and Sager AO: Maternal-foetal distribution studies in late pregnancy. II. Distribution of [1-14C]acrylamide in tissues of beagle dogs and miniature pigs. Food Chem Toxicol *23*: 757-761, 1985.
20 Marlowe C, Clark MJ, Mast RW, Friedman MA and Waddell WJ: The distribution of [14C]acrylamide in male and pregnant Swiss-Webster mice studied by whole-body autoradiography. Toxicol Appl Pharmacol *86*: 457-465, 1986.
21 Paulsen JE, Steffensen IL, Andreassen A, Vikse R and Alexander J: Neonatal exposure to the food mutagen 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine *via* breast milk or directly induces intestinal tumors in multiple intestinal neoplasia mice. Carcinogenesis *20*: 1277-1282, 1999.
22 Rice JM: Perinatal period and pregnancy: intervals of high risk for chemical carcinogens. Env Health Perspect *29*: 23-27, 1979.
23 Paulsen JE, Steffensen IL, Namork E, Eide TJ and Alexander J: Age-dependent susceptibility to azoxymethane-induced and spontaneous tumorigenesis in the *Min*/+ mouse. Anticancer Res *23*: 259-265, 2003.
24 Andreassen A, Mollersen L, Vikse R, Steffensen IL, Mikalsen A, Paulsen JE and Alexander J: One dose of 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine (PhIP) or 2-amino-3-methylimidazo[4, 5-f]quinoline (IQ) induces tumors in *Min*/+ mice by truncation mutations or LOH in the *Apc* gene. Mutation Res *517*: 157-166, 2002.
25 Mollersen L, Paulsen JE and Alexander J: Loss of heterozygosity and nonsense mutation in *Apc* in azoxymethane-induced colonic tumors in *Min*/+ mice. Anticancer Res *24*: 2595-2599, 2004.
26 Kinzler KW and Vogelstein B: Lessons from hereditary colorectal cancer. Cell *87*: 159-170, 1996.
27 Smith KJ, Johnson KA, Bryan TM, Hill DE, Markowitz S, Willson JK, Paraskeva C, Petersen GM, Hamilton SR, Vogelstein B and Kinzler KW: The *APC* gene product in normal and tumor cells. Proc Natl Acad Sci USA *90*: 2846-2850, 1993.
28 Miyoshi Y, Nagase H, Ando H, Horii A, Ichii S, Nakatsuru S, Aoki T, Miki Y, Mori T and Nakamura Y: Somatic mutations of the *APC* gene in colorectal tumors: mutation cluster region in the *APC* gene. Hum Mol Gen *1*: 229-233, 1992.
29 Steffensen IL, Schut HA, Paulsen JE, Andreassen A and Alexander J: Intestinal tumorigenesis in multiple intestinal neoplasia mice induced by the food mutagen 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine: perinatal susceptibility, regional variation, and correlation with DNA adducts. Cancer Res *61*: 8689-8696, 2001.
30 Mollersen L, Vikse R, Andreassen A, Steffensen IL, Mikalsen A, Paulsen JE and Alexander J: Adenomatous polyposis coli truncation mutations in 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine (PhIP)-induced intestinal tumors of multiple intestinal neoplasia mice. Mutation Res *557*: 29-40, 2004.
31 Steffensen IL, Paulsen JE, Eide TJ and Alexander J: 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine increases the numbers of tumors, cystic crypts and aberrant crypt foci in multiple intestinal neoplasia mice. Carcinogenesis *18*: 1049-1054, 1997.
32 Steffensen IL, Paulsen JE and Alexander J: The food mutagen 2-amino-9H-pyrido[2, 3-b]indole (AalphaC) but not its methylated form (MeAalphaC) increases intestinal tumorigenesis in neonatally exposed multiple intestinal neoplasia mice. Carcinogenesis *23*: 1373-1378, 2002.
33 Steffensen IL, Paulsen JE, Engeset D, Kronberg L and Alexander J: The drinking water chlorination by-products 3, 4-dichloro-5-hydroxy-2[5H]-furanone (mucochloric acid) and 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone do not induce preneoplastic or neoplastic intestinal lesions in F344 rats, balb/ca mice or C57bl/6J-*Min*/+ mice. Pharmacol Toxicol *85*: 56-64, 1999.
34 Doerge DR, Gamboa da Costa G, McDaniel LP, Churchwell MI, Twaddle NC and Beland FA: DNA adducts derived from administration of acrylamide and glycidamide to mice and rats. Mutat Res *580*: 131-141, 2005.
35 Sumner SC, Fennell TR, Moore TA, Chanas B, Gonzalez F and Ghanayem BI: Role of cytochrome P450 2E1 in the metabolism of acrylamide and acrylonitrile in mice. Chem Res Toxicol *12*: 1110-1116, 1999.
36 Sumner SC, Selvaraj L, Nauhaus SK and Fennell TR: Urinary metabolites from F344 rats and B6C3F1 mice coadministered acrylamide and acrylonitrile for 1 or 5 days. Chem Res Toxicol *10*: 1152-1160, 1997.
37 Johnsrud EK, Koukouritaki SB, Divakaran K, Brunengraber LL, Hines RN and McCarver DG: Human hepatic CYP2E1 expression during development. J Pharmacol Exp Ther *307*: 402-407, 2003.
38 Paulsen JE, Steffensen IL, Loberg EM, Husoy T, Namork E and Alexander J: Qualitative and quantitative relationship between dysplastic aberrant crypt foci and tumorigenesis in the *Min*/+ mouse colon. Cancer Res *61*: 5010-5015, 2001.
39 Paulsen JE, Loberg EM, Olstorn HB, Knutsen H, Steffensen IL and Alexander J: Flat dysplastic aberrant crypt foci are related to tumorigenesis in the colon of azoxymethane-treated rat. Cancer Res *65*: 121-129, 2005.
40 Nucci MR, Robinson CR, Longo P, Campbell P and Hamilton SR: Phenotypic and genotypic characteristics of aberrant crypt foci in human colorectal mucosa. Hum Path *28*: 1396-1407, 1997.
41 Steffensen IL and Alexander J: Impact of genetic background on spontaneous or 2-amino-1-methyl-6-phenylimidazo[4, 5-b]pyridine (PhIP)-induced intestinal tumorigenesis in *Min*/+ mice. Cancer Letters *240*: 289-296, 2006.

42 Field EA, Price CJ, Sleet RB, Marr MC, Schwetz BA and Morrissey RE: Developmental toxicity evaluation of acrylamide in rats and mice. Fundam Appl Toxicol *14*: 502-512, 1990.
43 Wise LD, Gordon LR, Soper KA, Duchai DM and Morrissey RE: Developmental neurotoxicity evaluation of acrylamide in Sprague-Dawley rats. Neurotoxicol Teratol 17: 189-198, 1995.
44 Garey J, Ferguson SA and Paule MG: Developmental and behavioral effects of acrylamide in Fischer 344 rats. Neurotoxicol Teratol *27*: 553-563, 2005.
45 Husoy T, Abramsson-Zetterberg L, Olstorn HB, Paulsen JE and Alexander J: Adenomatous polyposis coli influences micronuclei induction by PhIP and acrylamide in mouse erythrocytes. Mutation Res *580*: 111-118, 2005.
46 Dearfield KL, Abernathy CO, Ottley MS, Brantner JH and Hayes PF: Acrylamide: its metabolism, developmental and reproductive effects, genotoxicity, and carcinogenicity. Mutation Res *195*: 45-77, 1988.
47 King RS, Kadlubar FF and Turesky RJ: *In vivo* metabolism of heterocyclic amines. *In*: Food Borne Carcinogens: Heterocyclic Amines. Nagao M and Sugimura T (eds.). Wiley, West Sussex, pp. 90-111, 2000.
48 Dybing E and Sanner T: Risk assessment of acrylamide in foods. Toxicol Sci *75*: 7-15, 2003.
49 Svensson K, Abramsson L, Becker W, Glynn A, Hellenas KE, Lind Y and Rosen J: Dietary intake of acrylamide in Sweden. Food Chem Toxicol *41*: 1581-1586, 2003.
50 Augustsson K, Skog K, Jagerstad M and Steineck G: Assessment of the human exposure to heterocyclic amines. Carcinogenesis *18*: 1931-1935, 1997.
51 Zimmerli B, Rhyn P, Zoller O and Schlatter J: Occurrence of heterocyclic aromatic amines in the Swiss diet: analytical method, exposure estimation and risk assessment. Food Addit Contam *18*: 533-551, 2001.

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