Timing of solid food introduction is associated with urinary F_2-isoprostane concentrations in childhood

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BACKGROUND: Timing of solid food introduction in infancy has been associated with several chronic diseases. To explore potential mechanisms, we investigated the relationship between timing of solid food introduction and F_2-isoprostanes—a marker of oxidative stress.

METHODS: Urinary F_2-isoprostanes were assessed in 336 healthy children aged less than 11.5 y with 1,266 clinic visits (mean = 3.8 visits per child) in the Diabetes Autoimmunity Study in the Young. We analyzed the association between F_2-isoprostane concentrations and infant diet exposures using linear mixed models adjusted for age, age^2, HLA-DR3/4,DQB1*0302 genotype, first-degree relative with type 1 diabetes, maternal age, maternal education, sex, and exposure to *in utero* cigarette smoke.

RESULTS: Later solid food introduction was associated with lower F_2-isoprostane concentrations in childhood (on average, 0.10 ng/mg per month of age at introduction; estimate: −0.10 (95% confidence interval (CI): −0.18, −0.02) *P* value = 0.02). Moreover, childhood F_2-isoprostane concentrations were, on average, 0.24 ng/mg lower in individuals breastfed at solid food introduction (estimate: −0.24 (95% CI: −0.47, −0.01) *P* value = 0.04) compared with those who were not. Associations remained significant after limiting analyses to F_2-isoprostanes after 2 y of age.

CONCLUSION: Our results suggest a long-term protective effect of later solid food introduction and breastfeeding at solid food introduction against increased F_2-isoprostane concentrations throughout childhood.

Timing of complementary food introduction has been implicated in the development of a number of chronic diseases, such as obesity (1,2), celiac disease (3,4), type 1 diabetes (T1D) (5,6), and atopic disorders (7,8). Previous guidelines established by the American Academy of Pediatrics recommended introduction of solid foods at 4 to 6 mo of age, exclusive breastfeeding for the first 4 to 6 mo of age, continued breastfeeding to the first birthday and beyond if possible, and the use of infant formula for the first year of life for those infants who are not breastfed (9). In 2012, the American Academy of Pediatrics updated their recommendations to include exclusive breastfeeding for about 6 mo, followed by continued breastfeeding as complementary foods are introduced, with continuation of breastfeeding for 1 y or longer as mutually desired by mother and infant (10). The European Society of Pediatric Gastroenterology, Hepatology and Nutrition recommends not introducing solid foods before 4 to 6 mo of age (11). However, a study by Clayton *et al.* (2013) recently showed 40.4% of US mothers introduced solid foods before age 4 mo (12). It is important to understand the short- and long-term effects of infant feeding in order to better educate mothers and inform recommendations.

One possible long-term effect of early introduction of solid foods is increased oxidative stress, which results from an imbalance between the production of free radicals and reactive oxygen species, and the natural antioxidant capacity of the body that blocks and scavenges these radicals (13). Early life exposures have been shown to increase oxidative stress levels, such as exposure to maternal smoke *in utero* (14,15) and infant formula feeding compared to breastfeeding (16,17). Oxidative stress can damage lipids, proteins, and nucleic acids and has been implicated in the pathogenesis of many diseases, including pediatric diseases, such as asthma, cystic fibrosis, and juvenile rheumatoid arthritis (13,18,19). A well-studied biomarker of *in vivo* oxidative stress is F_2-isoprostanes, which are formed during the nonenzymatic oxidation of arachidonic acid by free radicals, including reactive oxygen species. Measurement of urinary F_2-isoprostanes is an index of systemic oxidative stress in humans.

We previously investigated factors associated with urinary F_2-isoprostanes in a cohort of healthy children at increased genetic risk for developing T1D (20). Being female, having the HLA-DR3/4 genotype, a higher plasma γ-tocopherol:total lipids ratio, and a lower α-carotene:total lipids ratio were associated with higher F_2-isoprostane concentrations (20). In the current analysis, we explore whether timing of infant diet exposures is associated with increased urinary F_2-isoprostanes.

RESULTS

Table 1 describes the 328 children in the subcohort with F_2-isoprostane measurements and the individual associations

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between these characteristics and creatinine adjusted urinary F_2-isprostane concentrations. Increasing age of the child, greater than 12 y of maternal education, and increasing maternal age at the birth of the child were associated with decreased urinary F_2-isoprostane concentrations. Having the high T1D-risk HLA-DR3/4,DQB1*0302 genotype, being female, and being exposed to maternal cigarette smoke *in utero* were associated with increased F_2-isprostane concentrations throughout childhood.

Table 1. Association between population characteristics and creatinine-adjusted urinary F_2-isoprostane concentrations in the 328 children in the subcohort of the Diabetes Autoimmunity Study in the Young

Characteristic	*n*	Unadjusted estimate (95% CI)
Child's age, mean (SD), years	4.2 (2.4)	−0.24 (−0.28, −0.20)*
HLA-DR3/4, DQB1*0302	95 (28.3%)	0.29 (0.04, 0.54)*
First-degree relative with T1D	110 (32.7%)	0.19 (−0.08, 0.46)
Female	162 (48.2%)	0.30 (0.07, 0.53)*
Race/ethnicity, non-Hispanic white	248 (73.8%)	−0.04 (−0.31, 0.22)
Maternal education, >12 y	259 (77.5%)	−0.38 (−0.66, −0.10)*
Maternal age, mean (SD), years	29.7 (5.5)	−0.02 (−0.04, −0.003)*
Exposure to maternal cigarette smoke *in utero*	41 (12.4%)	0.39 (0.03, 0.75)*
Exposure to environmental tobacco smoke	362 (29.1%)	0.10 (−0.14, 0.34)

CI, confidence interval; T1D, type 1 diabetes. *$P < 0.05$.

Table 2 displays the associations between infant diet predictors and F_2-isoprostane concentrations. Adjusting for age, age^2, HLA-DR3/4,DQB1*0302, first-degree relative with T1D, sex, maternal education, maternal age, and exposure to maternal cigarette smoke *in utero*, the later a child was introduced to solid foods, the lower their F_2-isoprostane concentrations in childhood (on average, 0.10 ng/mg per month of age at introduction) (estimate: −0.10 (95% CI: −0.18, −0.02) *P* value: 0.02). The full final model for the association between age at first exposure to solid foods and F_2-isoprostane concentrations is displayed in **Supplementary Table S1** online where in addition to age at first exposure to solid foods, age, age^2, and sex remained significant. We then examined individual components of the solid foods variable and found that lower childhood F_2-isoprostane concentrations were associated with later introduction to any cereal (wheat/barley/oats/rice) (on average, 0.10 ng/mg per month of age at introduction to any cereal, estimate: −0.10 (95% CI: −0.17, −0.02) *P* value: 0.02) and meat (0.04 ng/mg per month of age at introduction to meat, estimate: −0.04 (95% CI: −0.07, −0.005) *P* value: 0.03).

The associations between breastfeeding at complementary food introduction and F_2-isoprostane concentrations throughout childhood are also described in **Table 2**. Adjusting for age, age^2, HLA-DR3/4,DQB1*0302, first-degree relative with T1D,

Table 2. Infant diet predictors of creatinine-adjusted urinary F_2-isoprostane concentrations in healthy DAISY children (1,246 observations, 328 subjects)

Infant diet characteristic	Mean duration or age at first exposure (SD)	Adjusted estimate at all ages[a] (95% CI)	Adjusted estimate after age 2[a] (95% CI)
Exclusive breastfeeding duration (months)	1.4 (1.8)	−0.02 (−0.09, 0.04)	−0.04 (−0.11, 0.03)
Breastfeeding duration (months)	6.5 (7.0)	−0.01 (−0.02, 0.01)	−0.01 (−0.03, 0.005)
Breast milk months	4.2 (3.4)	−0.02 (−0.05, 0.02)	−0.03 (−0.06, 0.01)
Age at first exposure to cow's milk (months)	4.0 (3.6)	−0.005 (−0.04, 0.03)	−0.01 (−0.04, 0.02)
Age at first exposure to any solid food (months)	4.5 (1.4)[b]	−0.10 (−0.18, −0.02)*	−0.11 (−0.19, −0.03)*
Age at first exposure to any cereal (wheat/barley/oats/rice) (months)	4.6 (1.4)	−0.10 (−0.17, −0.02)*	−0.10 (−0.18, −0.03)*
Age at first exposure to foods containing wheat/barley (months)	6.9 (1.9)	−0.04 (−0.10, 0.02)	−0.04 (−0.10, 0.01)
Age at first exposure to foods containing rice/oat (months)	4.7 (1.5)	−0.07 (−0.14, 0.01)	−0.08 (−0.15, −0.005)*
Age at first exposure to fruit, excluding fruit juice (months)	5.8 (1.5)	−0.06 (−0.13, 0.01)	−0.06 (−0.13, 0.02)
Age at first exposure to vegetables (months)	6.0 (1.4)	−0.02 (−0.09, 0.06)	−0.03 (−0.10, 0.05)
Age at first exposure to meat (months)	9.3 (3.1)	−0.04 (−0.07, -0.005)*	−0.04 (−0.07, -0.003)*
Breastfeeding at food introduction variables	*n* (%) breastfed at food introduction		
Breastfeeding at introduction of solid foods	188 (56.5%)	−0.24 (−0.47 −0.01)*	−0.26 (−0.50, −0.03)*
Breastfeeding at introduction of cereal (wheat/barley/oats/rice)	186 (55.9%)	−0.22 (−0.45, 0.01)	−0.24 (−0.47, −0.01)*
Breastfeeding at introduction of wheat/barley	147 (44.1%)	−0.12 (−0.35, 0.10)	−0.13 (−0.36, 0.09)

CI, confidence interval.
[a]Adjusted for age, age^2, HLA-DR3/4,DQB1*0302, first-degree relative with T1D, sex, maternal education, maternal age, and exposure to maternal cigarette smoke *in utero*. [b]The mean age at first exposure to any solid food is less than the means of its components because 13 children were introduced to cheese before any cereal. *$P < 0.05$.

sex, maternal education, maternal age, and exposure to maternal cigarette smoke *in utero*, childhood F_2-isoprostane concentrations were, on average, 0.24 ng/mg lower in those who were breastfed at introduction of solid foods (estimate: −0.24 (95% CI: −0.47, −0.01) *P* value: 0.04) compared with those who were not breastfed at introduction of solid foods. The full final model for breastfeeding at introduction of solid foods is displayed in **Supplementary Table S1** online where in addition to breastfeeding at introduction of solid foods, age, age^2, and sex remained significant.

To further investigate the relationship between F_2-isoprostane concentrations, timing of introduction of solid foods, and breastfeeding at the introduction of solid foods, a categorical variable with six levels was created. It represented whether the child was introduced to solid foods early (<4 mo of age), according to previous recommendations by the American Academy of Pediatrics (4–5 mo of age), or late (≥6 mo of age) and whether or not the child was breastfed when introduced to solid foods. We chose being introduced to solid foods between 4 and 5 mo of age and breastfed at introduction of solid foods as the reference group. Being introduced to solid foods before 4 mo of age and not being breastfed at the time of introduction was significantly associated with increased F_2-isoprostane concentrations compared with being introduced to solid foods between 4 and 5 mo of age and breastfed at introduction of solid foods (**Figure 1**).

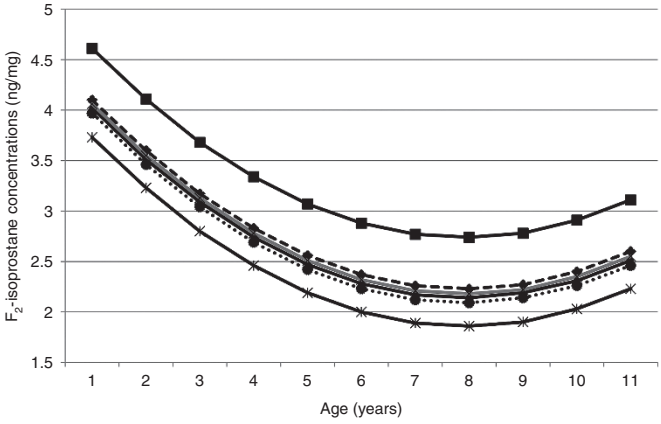

Figure 1. Urinary F_2-isoprostane concentrations are related to age at first exposure to any solid food and being breastfed at introduction of solid foods in DAISY children. DAISY children introduced to solid foods before 4 mo of age and not breastfed at introduction of solid foods (solid black line with square symbols) had, on average, significantly higher F_2-isoprostane concentrations compared with children introduced to solid foods between 4 and 5 mo of age and breastfed at introduction of solid foods (solid black line with triangle symbols) ($P = 0.0004$) after adjusting for age, age^2, HLA-DR3/4,DQB1*0302, first-degree relative with T1D, sex, maternal education, maternal age, and exposure to maternal cigarette smoke *in utero*. The lowest F_2-isoprostane concentrations were seen in children introduced to solid foods at or after 6 mo of age and breastfed at introduction of solid foods (solid black line with asterisk symbols). The additional groups are represented by the following lines: introduced to solid foods before 4 mo of age and breastfed at introduction of solid foods (dashed black line with diamond symbols), introduced to solid foods between 4 and 5 mo of age and not breastfed at introduction of solid foods (solid gray line with x symbols), introduced to solid foods at or after 6 mo of age and not breastfed at introduction of solid foods (dotted black line with circle symbols).

To see if age at solid food introduction had an impact on F_2-isoprostane concentrations long after the initial exposure, we ran the same analyses as described in the preceding paragraph, but limited F_2-isoprostane measurements to those collected after 2 y of age (**Table 2**). Later introduction to solid foods, including any cereal (wheat/barley/oats/rice) and meat, as well as being breastfed at introduction of solid foods were still associated with decreased F_2-isoprostane concentrations in childhood after limiting F_2-isoprostane measurements to those after 2 y of age. Additionally, breastfeeding at introduction of cereal (wheat/barley/oats/rice) and later introduction to rice/oat were associated with decreased F_2-isoprostane concentrations in childhood after limiting F_2-isoprostane measurements to those after 2 y of age. The final full models for first exposure to solid foods and breastfeeding at introduction of solid foods limited to F_2-isoprostane measurements after 2 y are given in **Supplementary Table S1** online.

DISCUSSION

This longitudinal analysis in a prospective cohort demonstrates a novel association between timing of solid food introduction and F_2-isoprostane concentrations, a measure of oxidative stress. Oxidative stress plays an influential role in the development of many chronic diseases. Understanding how early life exposures affect oxidative stress throughout childhood may help to prevent the long-term effects oxidative stress appears to have on development of chronic disease both in childhood and later in life.

One of the strengths of this study is its prospective design. Infant diet data were collected prospectively at 3-mo intervals for the first 15 mo of life, increasing accuracy of the data. Another strength of this study is that F_2-isoprostane measurements collected at multiple time points throughout childhood allowed us to estimate the effect of timing of infant diet exposures on F_2-isoprostane concentrations throughout early childhood. To our knowledge, this is the first study to examine the effect of timing of infant diet exposures on F_2-isoprostane concentrations.

Previous studies examining oxidative stress in relation to infant feeding type have found higher F_2-isoprostane values in children who were formula fed compared with children who were breastfed (16,17). Human colostrum has been shown to exhibit antioxidant and anti-inflammatory properties (21). Additionally, scavengers of free radicals, including α-tocopherol, cysteine, and ascorbate are higher in breast milk than cow's milk (22). We did not find an association between breastfeeding duration and F_2-isoprostane concentrations. However, our results suggest that breastfeeding at solid food introduction is associated with decreased F_2-isoprostane concentrations and average F_2-isoprostane concentrations were highest in children who were introduced to solid foods before 4 mo of age and not breastfed at introduction of solid foods.

Breastfeeding at introduction of solid foods has been explored as a protective factor for inflammatory diseases, such as celiac disease, T1D, and food allergies. A number of studies have observed a protective association between breastfeeding

while introducing gluten and celiac disease risk (4,23–25). Previously, the Diabetes Autoimmunity Study in the Young (DAISY) showed reduced islet autoimmunity (IA) risk if cereals were introduced while the child was still breastfeeding (26), and more recently, a protective association between breastfeeding at introduction of solid foods and T1D risk was shown (5). A nested case–control study exploring introduction of complementary foods and its relationship to developing food allergies found that infants diagnosed with food allergies before 2 y of age were less likely to be breastfed when cow's milk protein was first introduced (8). The results of our current analyses provide another reason for breastfeeding while introducing solid foods—reducing urinary F_2-isoprostane concentrations in childhood.

Regarding timing of introduction to solid foods, much of the literature supports introducing solid foods after 4-mo of age. Before 4 mo of age, the child has a relatively immature gut immune system that may have an abnormal response to solid food antigens. Grimshaw *et al.* (8) found that infants diagnosed with a food allergy before 2 y of age were introduced to solid foods earlier (≤16 wk of age). Early introduction to gluten-containing foods has been associated with celiac disease autoimmunity (3). Early introduction to solid foods (5), gluten-containing foods (6), and dairy products (27) have been associated with increased T1D risk, and early introduction to cereals (26,28), gluten-containing foods (6,29), fruits and berries (30), root vegetables (28), and egg (28) have all been associated with increased risk of IA. Early introduction to solid foods has also been associated with childhood obesity (1,2).

The results of this study suggest that an increase in urinary F_2-isoprostane concentrations may be a potential mechanism through which timing of solid food introduction increases risk of childhood diseases, such as T1D, celiac disease, and allergies. The gut microbiota may play a role in this pathway with infant diet leading to increased oxidative stress, changing the gut microbiota, and eventually leading to chronic disease as shown in a murine model (31). This murine model showed a long-term high-fat diet induced oxidative stress that then influenced gut microbiota based on different strains of intestinal bacteria having varying degrees of growth sensitivity to oxidative stress, believed to eventually lead to metabolic syndrome (31). A study examining the influence of milk-feeding type and HLA-genotype on intestinal microbiota in infants with a family history of celiac disease found that while both milk-feeding type and HLA-genotype influenced intestinal microbiota, breastfeeding reduced the differences in microbiota composition attributed to HLA-genotype (32). This is encouraging for those with the increased risk HLA genotypes, as the effect these genotypes have on the microbiota associated with increased risk of celiac disease, can be reduced through a modifiable exposure, early infant diet.

In conclusion, our results suggest a long-term protective effect of later solid food introduction and breastfeeding at solid food introduction against increased urinary F_2-isoprostane concentrations throughout childhood. Future research that investigates pathways between infant diet, gut microbiota, oxidative stress, and chronic disease in human populations may inform new strategies for chronic disease prevention starting in infancy.

METHODS

Study Population

DAISY recruited two groups of children between 1993 and 2004 who are at increased risk for T1D, and followed them prospectively for IA and T1D development. One group is composed of first-degree relatives of patients with T1D, identified through the Barbara Davis Center for Childhood Diabetes and recruited mainly between birth and 2 y of age. The second group is composed of infants born at St. Joseph's Hospital in Denver, CO, whose umbilical cord blood was screened for T1D-susceptibility HLA-DR,DQ genotypes and were recruited if they had these genotypes (33). Details of the newborn screening, sibling and offspring recruitment, and follow-up of both cohorts have been published previously (34). All study protocols were approved by the Colorado Multiple Institutional Review Board, and informed consent was given by parents of all participating children.

The DAISY cohort is composed of 2,547 children at increased genetic risk for developing T1D. F_2-isoprotanes were investigated in a representative sample of 380 children (i.e., subcohort) selected from the DAISY cohort via stratified sampling based on HLA-DR genotype and family history of T1D. F_2-isoprostane measurements were obtained on 336 children in the subcohort between the ages of 6 mo and 11.5 y from August 1997 to December 2005. Of the 336 with F_2-isoprostane measurements, 1 child was missing maternal education, 3 children were missing exposure to maternal smoke *in utero*, 3 children were missing first exposure to solid food, and 1 child was missing both maternal education and exposure to maternal smoke *in utero*, which results in 328 children with complete prospective exposure data and 1,246 F_2-isoprostane measurements (mean 3.8 measurements per child) used for analysis (**Figure 2**). There were 14 children who developed IA and the measurements at clinic visits at which a child was determined to be islet autoantibody positive are not included in these analyses.

F_2-Isoprostane Measurement

Urine specimens were stored at −70 °C until shipment to the Vanderbilt University Eicosanoid Core Laboratory for the analysis of F_2-isprostanes. F_2-isoprostanes were measured as previously described (35,36). Briefly,

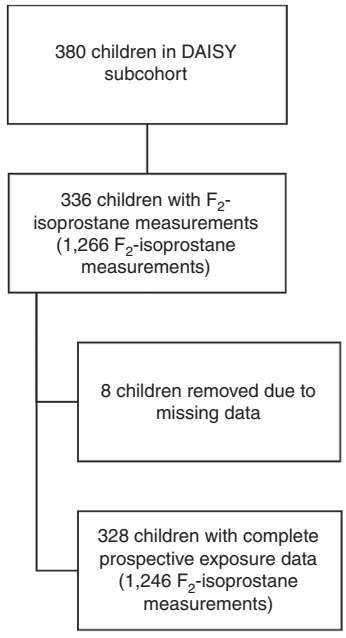

Figure 2. Flow chart for formation of analysis cohort.

after addition of the internal standard, [^{2}H4]-8-iso-$PGF_{2\alpha}$, F_2-IsoPs were extracted with C18 and silica Sep-Pak cartridges. The extracted F_2-isoprostanes were then converted to the corresponding pentafluorobenzyl esters and purified by thin-layer chromatography. They were then converted to the trimethylsilyl ether derivatives and subsequently analyzed by gas chromatography/negative ion chemical ionization–mass spectrometry (37,38). Creatinine is measured to correct for the concentration of the urine. Analysis of blinded duplicated pairs sent to the Eicosanoid Core Laboratory showed an intraclass coefficient of 0.93. F_2-isoprostane concentrations are reported as nanograms per milligram (ng/mg) of urine creatinine. For children who were not toilet-trained, we collected urine samples by placing cotton balls in the diaper. This mode of urine collection (cotton ball vs. direct stream catch) had no effect on F_2-isoprostane measurements (39).

Infant Diet Measurement

Data on infant diet were collected during telephone or face-to-face interviews at 3, 6, 9, 12, and 15 mo of age. At each interview, mothers were asked to report the date of introduction and frequency of intake (i.e., number of servings per day) of all milks, formulas, and foods the infants consumed during the previous 3 mo. Exclusive breastfeeding duration was determined by the reported age at which the infant was exposed to any foods or liquids other than breast milk or water. Breast milk months is a relative quantity of breast milk based on the proportion of breast milk to formula over the first 9 mo of life. For example, for infants exclusively breastfed for the first 9 mo, the proportion of breast milk to formula for each month was 1.0, and the number of breast milk months summed to 9.0 breast milk months. For infants who received both breast milk and formula, the total number of servings of breast milk for each month was divided by the total number of servings of formula and breast milk for that month, and these were summed over the first 9 mo to arrive at the number of breast milk months. Based on previous work showing a protective effect of breastfeeding when introducing cereals (for IA) (26) or gluten (for celiac disease) (23), we created three additional breastfeeding variables to represent whether the child was breastfed at the time of introduction to any solid foods, cereals, and foods containing gluten (wheat/barley).

We created an overall variable of age at first exposure to any solid foods, as well as six variables that were components of this solid foods variable that included age at exposure to cereal (wheat/barley/oats/rice), wheat/barley, rice/oats, fruit (not including fruit juice), vegetables, and meat. There were no reports of introducing rye in the infant diet in DAISY children. Juices were not included in the fruit variable because we were interested in solid food introductions. The study was an observational study; therefore, no dietary advice was given to the participating families.

Environmental Tobacco Smoke

Exposure to maternal smoke *in utero* was collected via a mailed questionnaire when the child was 8–12 wk old. The child's exposure to environmental tobacco smoke (ETS) was assessed via questionnaire every 3 mo during the first 15 mo of life and annually starting at age 2 y. Children who had a mother or father who smoked or were exposed at least once per week to other adults who smoked were coded as "exposed" to ETS. Seifert *et al.* (2002) demonstrated a correlation of 0.8 between responses to this ETS questionnaire and urine cotinine concentrations (a marker of tobacco smoke inhalation) in the DAISY population (40).

Statistical Analysis

We analyzed the relationship between F_2-isoprostane concentrations and timing of infant diet exposures using a linear mixed modeling approach in the DAISY subcohort. We tested each infant diet exposure for association with F_2-isoprostane concentrations in separate models adjusted for age, age^2, HLA-DR3/4,DQB1*0302, first-degree relative with T1D, sex, maternal education, maternal age, and exposure to maternal cigarette smoke *in utero*. As seen previously (20), the relationship between age and F_2-isoprostanes was nonlinear, and the best fit of the model required the inclusion of both age and age^2. The following linear mixed models were tested for each of the individual predictors for the best fit based on the lowest Akaike information criteria: a random intercept only; a random slope for age only; a random intercept and a random slope for age; and a random intercept, random slope for age, and a random slope for age^2. Linear mixed models with both a random intercept and a random slope for age represented the best fit to the data. The mixed model provides a regression coefficient, a SE, and a *P* value for each variable to indicate its contribution toward explaining variation in F_2-isoprostane concentrations.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

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