Serum Dioxin Concentrations and Age at Menarche
Marcella Warner,1 Steven Samuels,1,2 Paolo Mocarelli,3 Pier Mario Gerthoux,3 Larry Needham,4 Donald G. Patterson Jr.,4 and Brenda Ekenazi1

1School of Public Health, University of California at Berkeley, Berkeley, California, USA; 2Division of Occupational/Environmental Medicine and Epidemiology, University of California at Davis, Davis, California, USA; 3Department of Laboratory Medicine, University of Milano-Bicocca, School of Medicine, Hospital of Desio, Desio-Milano, Italy; 4Division of Environmental Health Laboratory Science, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) constitute a group of polyhalogenated aromatic hydrocarbons that are persistent, widespread environmental contaminants, frequently detected at parts-per-trillion levels (lipid basis) in animals and humans throughout the industrialized world (Zook and Rappe 1994). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic congener within this group of compounds and has been shown to cause a wide variety of effects in animals, including altered reproductive development [Birnbaum 1994, 1995; International Agency for Research on Cancer (IARC) 1997]. Increasing evidence suggests that exposure to TCDD during earlier stages of development is particularly hazardous to reproductive development (Chaffin et al. 1996). In utero and lactational TCDD exposure in rodents has been associated with delays in pubertal development in animal studies. On 10 July 1976, as a result of a chemical explosion, residents of Seveso, Italy, experienced the highest levels of TCDD exposure experienced by a human population. Twenty years later, we initiated the Seveso Women’s Health Study (SWHS), a retrospective cohort study of female residents of the most contaminated areas, to determine whether the women were at higher risk for reproductive disease. We examined the association of TCDD serum levels, based on measurements in serum collected soon after the explosion, with reported age at menarche among the 282 SWHS women who were premenarcheal at the time of the explosion. We found no change in risk of onset of menarche with a 10-fold increase in TCDD (e.g., 10–100 ppt; hazard ratio = 0.95; 95% confidence interval, 0.83–1.09; p-value for trend = 0.46). When TCDD levels were categorized, there was also no evidence of a dose-response trend (p = 0.65). In summary, we found that individual serum TCDD measurements are not significantly related to age at menarche among women in the SWHS cohort. The women in this study experienced substantial TCDD exposure during the postnatal but prepubertal developmental period. Given that animal evidence suggests in utero exposure has the most significant effect on onset of puberty, continued follow-up of the offspring of the SWHS cohort is important.

Keywords: dioxin, endocrine disruptors, environmental exposures, epidemiology, menarche, puberty, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Environmental Health Perspectives • VOLUME 112 | NUMBER 13 | September 2004 1289
who declined to participate was not significantly different from those who did participate. For this analysis, we included all women who were premenarcheal on 10 July 1976, the date of the explosion (n = 282).

**Procedure.** The institutional review boards of the participating institutions approved the study. Details of the study have been presented elsewhere (Eskenazi et al. 2000). Briefly, participation included signed informed consent, blood draw, personal interview, and for most women, a gynecologic examination and ultrasound. The interview was conducted by a trained nurse-interviewer who was blinded to serum TCDD levels and zone of residence. Age at menarche was determined from the question, "At what age did you get your first menstrual period?"

**Laboratory analyses.** TCDD was measured in archived sera by high-resolution gas chromatography/high-resolution mass spectrometry methods (Patterson et al. 1987). Values are reported on a lipid-weight basis in parts per trillion (Akins et al. 1989).

Details of serum sample selection have been presented elsewhere (Eskenazi et al. 2000). For the 282 women in this analysis, we measured TCDD in sera collected between 1976 and 1977 for 257 women, between 1978 and 1981 for 23 women, and in 1996 for two women whose earlier samples had insufficient volume. For women with detectable post-1977 TCDD measurements (n = 20), the TCDD exposure level was back-extrapolated to 1976 using the Filser model (Kreuzer et al. 1997). For nondetectable values (n = 22), a serum TCDD level equal to one-half the detection limit was assigned (Hornung and Reed 1990).

**Statistical analyses.** We considered serum TCDD both as a continuous variable (log_{10} TCDD) and a categorical variable. TCDD was first categorized into quartile groups (≤ 55.9, 56.0–140.2, 140.3–300, > 300 ppt). Because the lower limit of the serum TCDD level was relatively high, the lowest group was subdivided into women with levels ≤ 20.0 and 20.1–55.9 ppt. We selected 20 ppt (body burden ~ 4 ng/kg) as the cutoff point because this was the average TCDD level of 1976 serum pools collected from Italian women living in an unexposed area (Eskenazi et al. 2004). For additional analyses, we categorized preexposure experience as "unexposed."

Statistical analyses were performed using Cox survival models in Stata 7 (Stata Corporation, College Station, TX, USA). We did not censor data because for each woman age of menarche was observed. Each woman was entered into the denominator ("risk set") for her year-group at the date of the accident or on her seventh birthday, whichever was later.

The Cox model assesses effects on age-specific probabilities of beginning menstruation by the relative hazard, or hazard ratio (HR), the ratio of probabilities computed for each categorized level of exposure versus the reference group or for the effect of a 10-fold increase in TCDD (log_{10} TCDD). For the categorical analysis, we used the highest dose group (> 300 ppt) as the reference group because the lowest dose group (≤ 20 ppt) had the smallest sample size. We report model-free standard errors, which are valid even when conventional assumptions for regressions are violated (Huber 1967). We examined the effect of potential confounders and effect modifiers, including height, weight, body mass index (BMI), and report of participation in athletic training at the time of interview (we did not obtain this information for early time periods), and smoking and alcohol consumption habits between 10 and 14 years of age.

The Cox model with constant HR may not be plausible when there is an inevitable event and the age-specific rates increase to 100%. We therefore also considered parametric regression survival-time models in which the natural log of the age at menarche is expressed as a linear function of the covariates.

The youngest age at menarche reported by the women who were premenarcheal at the time of the explosion was 8 years. We addressed the possibility of bias associated with the inclusion of women who, relative to their birth cohort, might already be at risk for late age at menarche at the time of the explosion (e.g., a woman who was 14 years of age but still premenarcheal in 1976). We therefore repeated the analysis on the subset of 158 women who were < 8 years of age at the time of the explosion and who were presumably not yet at risk for menarche.

To further assess the possibility of bias, we added to the analysis data 153 women who were in the same birth cohort as the 282 women in the analysis sample (birth years 1959–1976) but who had begun menstruating before the explosion date, 10 July 1976. These 153 women would have been at risk for menarche after the explosion had they not reached menarche before the explosion; their premenarcheal ages are all "unexposed." For the enlarged sample of 435 (153 + 282) women, we repeated the analysis with this additional "unexposed" exposure category (unexposed, ≤ 20, 20.1–55.9, 56.0–140.2, 140.3–300, and > 300 ppt) and each "unexposed" woman entered the denominator on her seventh birthday.

### Results

Demographic characteristics of the 282 women who were premenarcheal at exposure are presented in Table 1. On 10 July 1976, the age of the 282 women was 6.9 ± 3.7 years (mean ± SD; range = 0–17 years), and 158 (56%) were < 8 years of age. The mean age at menarche

| Characteristic | All premenarcheal women (No. [%]) | Women < 8 years of age in 1976 (No. [%]) | Age at menarche (years) |
|---------------|-----------------------------------|------------------------------------------|------------------------|
|               | Total                             |                                          |                        |
|               | 282 (100)                         | 158 (56)                                 | 12.8 ± 1.6             |
|               | Age at exposure (years)           |                                          | 12.5 ± 1.5             |
|               | 0–4                               | 84 (30)                                  | 12.6 ± 1.5             |
|               |                                   | 84 (53)                                  | 12.6 ± 1.5             |
|               | 5–7                               | 74 (26)                                  | 12.4 ± 1.6             |
|               |                                   | 74 (47)                                  | 12.4 ± 1.6             |
|               | 8–10                              | 69 (24)                                  | 12.4 ± 1.3             |
|               |                                   | 0 (0)                                    | —                      |
|               | 11–17                             | 55 (20)                                  | 13.8 ± 1.5             |
|               |                                   | 0 (0)                                    | —                      |
|               | Year of birth                     |                                          |                        |
|               | 1959–1966                         | 93 (33)                                  | 13.3 ± 1.5             |
|               |                                   | 0 (0)                                    | —                      |
|               | 1967–1970                         | 90 (32)                                  | 12.4 ± 1.6             |
|               |                                   | 58 (37)                                  | 12.6 ± 1.7             |
|               | 1971–1976                         | 99 (35)                                  | 12.5 ± 1.4             |
|               |                                   | 99 (63)                                  | 12.5 ± 1.4             |
|               | Zone of residence                 |                                          |                        |
|               | A                                 | 58 (21)                                  | 13.0 ± 1.8             |
|               | B                                 | 224 (79)                                 | 12.7 ± 1.5             |
|               |                                   | 121 (77)                                 | 12.5 ± 1.6             |
|               | Current BMI (kg/m²)               |                                          |                        |
|               | < 19.8                            | 93 (33)                                  | 13.2 ± 1.6             |
|               |                                   | 63 (40)                                  | 13.1 ± 1.6             |
|               | 19.8–26                           | 166 (60)                                 | 12.6 ± 1.5             |
|               |                                   | 87 (55)                                  | 12.2 ± 1.4             |
|               | 26–29                             | 12 (4)                                   | 12.5 ± 2.5             |
|               |                                   | 5 (3)                                    | 11.4 ± 1.9             |
|               | > 29                              | 8 (3)                                    | 12.4 ± 1.1             |
|               |                                   | 2 (1)                                    | 12.0 ± 1.4             |
|               | Physical activity                 |                                          |                        |
|               | No                                | 117 (42)                                 | 12.7 ± 1.5             |
|               |                                   | 55 (35)                                  | 12.1 ± 1.5             |
|               | Yes                               | 185 (58)                                 | 12.8 ± 1.5             |
|               |                                   | 103 (65)                                 | 12.8 ± 1.5             |
|               | Alcohol use at 10–14 years        |                                          |                        |
|               | No                                | 271 (96)                                 | 12.8 ± 1.5             |
|               |                                   | 153 (97)                                 | 13.0 ± 1.5             |
|               | Yes                               | 11 (4)                                   | 11.8 ± 1.7             |
|               |                                   | 5 (3)                                    | 12.0 ± 2.5             |
|               | Cigarette smoking at 10–14 years  |                                          |                        |
|               | No                                | 273 (97)                                 | 12.9 ± 1.6             |
|               |                                   | 153 (97)                                 | 12.7 ± 1.5             |
|               | Yes                               | 9 (3)                                    | 12.6 ± 1.4             |
|               |                                   | 5 (3)                                    | 12.2 ± 1.5             |

[—, No observations.]
reported for the 282 women was 12.8 ± 1.6 year. The mean age at follow-up (1996–1998) was 27.3 ± 3.8 years and the mean BMI was 21.4 ± 3.1 mg/kg². Women who had higher current BMIs or who consumed alcohol or smoked regularly between 10 and 14 years of age reported earlier ages of menarche.

Serum TCDD levels are presented in Table 2 by reported age at menarche for all premenarcheal women (n = 282) and for women who were < 8 years of age (n = 158) at exposure. The median serum TCDD level was 140.3 ppt (range, 3.6–56,000 ppt) for all premenarcheal women and 205.0 ppt (range, 3.6–56,000 ppt) for those who were < 8 years of age at exposure. Serum TCDD levels did not vary by reported age of menarche for either group (p > 0.5, analysis of variance (ANOVA)).

Results of Cox models are presented in Table 3. When we examined the effect of potential confounders and effect modifiers, we found no variables to confound (i.e., change potential confounders and effect modifiers, we report unadjusted results. The median serum TCDD level was 140.3 ppt (range, 3.6–56,000 ppt) for all women who were < 8 years of age at the time of the explosion. The conclusion of no association of age at menarche and TCDD persisted when we applied alternative models (log-normal, log-logistic) in which the mean of the log of age at menarche was the response (results not shown).

Finally, the analysis of all 435 women in the 1959–1976 birth cohort, with preexposure ages categorized as “unexposed” also showed no association of TCDD level and age-specific hazard of menarche (data not shown).

Discussion

The results of this study of women residing in Seveso, Italy, in 1976 at the time of an explosion that released high levels of TCDD provide little evidence of an association of exposure and age of menarche. That is, we found no evidence that the association between TCDD levels measured in serum collected near the time of exposure among the 282 women who were premenarchal at the time of the explosion, the subset of 158 women who were < 8 years of age at the time of the exposure, or the 435 women who belonged to the 1959–1976 birth cohort.

A limitation of our study is the retrospective recall of age of menarche. However, moderate to high correlations between actual and recalled menarche have been reported for females up to 19 years of age after the event (Must et al. 2002). In our study, the time between onset of menarche and study interview ranged from 5 to 19 years. The women in the SWHS reported age at menarche in whole years, presumably age at last birthday, and age was not rounded to the nearest “biological age.” Such nondifferential measurement error would reduce precision and would tend to bias our findings toward no effect.

A second limitation of the present study is that members of the lowest TCDD exposure group (≤ 20 ppt) experienced relatively high serum levels in comparison with contemporary levels reported for this area (≥ 2 ppt) (Warner M, unpublished data). If there is a threshold for TCDD effects on age of onset of menarche but it is ≤ 20 ppt, we would not be able to detect it in this population. However, we also found no association in analyses that counted preexposure experience as “unexposed.”

Another limitation of this study is that, although the explosion resulted in exposure specifically to TCDD, analyses of pooled serum from residents of an unexposed zone suggest there was substantial background exposure to other PCDDs, PCDFs, and PCBs during this time period (80 ppt TCDD toxic equivalents (TEQ), on average) (Eskenazi et al. 2004). Therefore, individuals with TCDD levels < 20 ppt might still have had substantial total TEQ exposure. Because we considered only TCDD in this study, our results may have underestimated an effect due to total TEQ exposure.

An advantage of this study over previous studies is that we were able to measure TCDD levels in individual serum samples collected near the time of exposure. Previous studies have used cross-sectional exposure measures (Den Hond et al. 2002) or had to rely upon alternative exposure assessment methods including ecologic measures (Guo and Kao 2003) or modeling (Blanck et al. 2000).

Our finding of no association of TCDD with age at menarche is consistent with results reported in studies with postnatal exposure to other dioxin-like compounds, including PCBs and PCDFs (Den Hond et al. 2002; Guo and Kao 2003). However, our results differ from those of Blanck et al. (2000), which, in contrast to animal studies, showed an earlier rather than later age of menarche with in utero and perinatal PBB exposure. There are several reasons why our results may differ. The PBB studied by Blanck et al. (2000), 2.2',4,4',5,5'-hexabromobiphenyl, which was the main congener (60–80%) in the Fire Master mixture, is not a dioxin-like.
congener and does not bind to the aryl hydrocarbon receptor, unlike coplanar PCBs (Darnerud 2003). Second, the PBB-exposed cohort was exposed in utero and via lactation, unlike the SWHS cohort, in whom no exposure occurred in utero and only three women reported having been breast-fed postexplosion. It is possible that the fetus is more sensitive to the effects of exposure to dioxin-like compounds in utero. In fact, although in utero and lactational TCDD exposure in animal studies has been associated with significant effects on onset of puberty (Gray and Ostby 1995; Wolf et al. 1999) and ovarian function (Gray and Ostby 1995; Heimler et al. 1998), the evidence for these adverse effects after only postnatal exposure is limited, based on studies using the immature intact and immature hypophysec-tomized rat models (Gao et al. 1999; Li et al. 1995, 1997; Son et al. 1999). Thus, postnatal (but prepubertal) TCDD exposure experienced by the SWHS cohort, although substantial in dose, likely missed the critical window for exposure effects.

In summary, we have shown that individual serum TCDD measurements are not significantly related to age at menarche among women in the SWHS cohort. The women in this study experienced substantial TCDD exposure during the postnatal but prepubertal developmental period. Given that animal evidence suggests in utero exposure has the most significant effect on onset of puberty, continued follow-up of the offspring of the SWHS cohort is important.

REFERENCES

Akins J, Waldrep K, Benten T. 1989. The estimation of total serum lipids by a completely enzymatic summation method. Clin Chim Acta 184:219–228.

Birnbaum L. 1984. The mechanism of dioxin toxicity: relationship to risk assessment. Environ Health Perspect 102:157–167.

Birnbaum L. 1995. Developmental effects of dioxins and related endocrine disrupting chemicals. Toxicol Lett 82/83:743–756.

Blanch H, Marcus M, Tolbert PE, Rubin C, Henderson AK, Hertzberg VS, et al. 2000. Age at menarche and Tanner stage in girls exposed in utero and postnatally to polybrominated biphenyls. Epidemiology 11:641–647.

Chaffin C, Peterson R, Hutz R. 1998. In utero and lactational exposure of female Holtzman rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin: modulation of the estrogen signal. Biol Reprod 59:62–67.

Darnerud PO. 2003. Toxic effects of brominated flame retardants in man and in wildlife. Environ Int 29:841–853.

Den Hond E, Reels HA, Hoppenbrouwers K, Nawrot T, Thijs L, Vandermeulen C, et al. 2002. Sexual maturation in relation to polychlorinated aromatic hydrocarbons: Sharpe and Skakkebaek’s hypothesis revisited. Environ Health Perspect 110:771–776.

di Domenico A, Silano V, Viviano G, Zapponi G. 1980. Accidental release of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) at Seveso, Italy. II. TCDD distribution in the soil surface layer. Ecotoxicol Environ Saf 4:298–320.

Eskesen B, Mocarelli P, Warner M, Needham L, Patterson D, Samuels S, et al. 2004. Relationship of serum TCDD concentrations and age at exposure of female residents of Seveso, Italy. Environ Health Perspect 112:22–27.

Eskesen B, Mocarelli P, Warner M, Samuels S, Vercellini P, Olive D, et al. 2000. Seveso Women’s Health Study: a study of the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on reproductive health. Chemosphere 40:1247–1253.

Eskesen B, Warner M, Mocarelli P, Samuels S, Needham LL, Patterson DG Jr, et al. 2002. Serum dioxin concentrations and menstrual cycle characteristics. Am J Epidemiol 156:383–392.

Faqi AS, Dalsenter PR, Merker HJ, Chahoud I. 1998. Effects on developmental landmarks and reproductive capability of 3,3’,4,4’-tetrachlorobiphenyl and 3,3’,4,4’,5-pentachlorobiphenyl in offspring of rats exposed during pregnancy. Hum Exp Toxicol 17:365–372.

Gao X, Son DS, Terranova PF, Rozman KK. 1999. Toxic equivalence of polychlorinated dibenzo-p-dioxins in an ovulation model: validation of the toxic equivalency concept for polychlorinated dibenzo-p-dioxins and dibenzofurans. Toxicol Sci 51:259–264.

Gray L, Ostby J. 1995. Estrous cyclicity and ovarian follicles in female rats after prenatal exposure to 3,3’,4,4’-pentachlorobiphenyl. Toxicol Lett 142:271–277.

Hornung R, Reed L. 1990. Estimation of average concentration in the presence of non-detectable values. Appl Occup Environ Hyg 5:48–51.

Huber PJ. 1967. The behavior of maximum likelihood estimates under non-standard conditions. In: Proceedings of the Fifth Berkeley Symposium on Mathematical Statistics and Probability, Vol 1 (LeCam LM, Neyman J, eds). Berkeley, CA:University of California Press, 221–233.

IARC (International Agency for Research on Cancer). 1997. Polychlorinated dibenz-para-dioxins and polychlorinated dibenzofurans. IARC Monogr Eval Carcinog Risks Hum 69:33–343.

Kreuzer PE, Csányi GA, Baur C, Kessler W, Pöpke O, Greim H, et al. 1997. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and congeners in infants. A toxicokinetic model of human lifetime body burden by TCDD with special emphasis on its uptake by nutrition. Arch Toxicol 71:383–400.

Li X, Johnson D, Rozman K. 1995. Reproductive effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in female rats: ovulation, hormonal regulation, and possible mechanisms(s). Toxicol Appl Pharmacol 133:321–327.

Li X, Johnson D, Rozman K. 1997. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) increases release of lutetizing hormone and follicle-stimulating hormone from the pituitary of immature female rats in vivo and in vitro. Toxicol Appl Pharmacol 142:284–289.

Must A, Phillips SM, Naumova EN, Blum M, Harris S, Dawson-Hughes B, et al. 2002. Recall of early menstrual history and menarcheal body size: after 30 years, how well do women remember? Am J Epidemiol 155:672–679.

Muto T, Imano N, Nakahara K, Takahashi H, Hano H, Wakui S, et al. 2003. Estrus cyclicity and ovarian follicles in female rats after prenatal exposure to 3,3’,4,4’-pentachlorobiphenyl. Toxicol Lett 142:271–277.

Patterson D, Hampton L, Lapeza C, Beiser W, Green V, Alexander L, et al. 1987. High-resolution gas chromatographic/high-resolution mass spectrometric analysis of human serum on a whole-weight and lipid basis for 2,3,7,8-tetrachlorodibenzo-p-dioxin. Anal Chem 59:2000–2005.

Sager DB, Girard DM. 1994. Long-term effects on reproductive parameters in female rats after transplacental exposure to PCBs. Environ Res 66:52–76.

Son DS, Ushinohama K, Gao X, Taylor CC, Roby KF, Rozman KK, et al. 1999. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) blocks ovulation by a direct action on the ovary without alteration of ovarian steroidogenesis: lack of a direct effect on ovarian granulosa and thecal-interstitial cell steroidogenesis in vitro. Reprod Toxicol 13:521–530.

Wolf CJ, Ostby JS, Gray LE Jr. 1999. Gestational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) severely alters reproductive function of female hamster offspring. Toxicol Sci 51:259–264.

Zook D, Rapp C. 1994. Environmental sources, distribution, and fate. In: Dioxins and Health (Schechter A, ed). New York:Plenum Press, 79–113.