HORMONALLY ACTIVE ENVIRONMENTAL AGENTS AND REPRODUCTIVE DEVELOPMENT

1. Meta Hypothesis

Prenatal and postnatal (including peripubertal) exposure to hormonally active environmental agents can alter development of the reproductive system resulting in multiple outcomes that can occur at various stages of development and may result in cumulative effects over time.

2. Specific Hypotheses

1. Prenatal and postnatal exposure to hormonally active environmental agents can alter early childhood development with direct implications for reproductive health and function (e.g., hypospadias, hypothyroidism, obesity).

    1.1 Male exposure to phthalates in the prenatal period is associated with hypospadias.

    1.2 Polybrominated diphenyl ether exposure during the prenatal period is associated with hypothyroidism that leads to altered reproductive development among highly exposed children.

    1.3 Exposure to bisphenol A increases the risk of obesity in genetically susceptible children.

2. Prenatal and postnatal exposure to endocrine-active environmental agents can alter sexual maturation and reproductive function (e.g., timing of puberty, polycystic ovarian disease) that are dependent both on earlier changes in reproductive development and on more proximal exposures and effects.

    2.1 Exposure to bisphenol A in early childhood is associated with an acceleration in age of onset of puberty in girls.

    2.2 Exposure to phthalates during early childhood is associated with the development of polycystic ovarian syndrome in adolescent females.

    2.3 Critical time windows for exposure to lead and associated delays in age of onset of puberty in girls are both in early childhood and around the time of sexual maturation.

    2.4 Genetic polymorphisms can alter sensitivity to phthalate exposures in childhood.

3. Background and Justification

Development of the reproductive system begins early in gestation and continues through infancy, childhood, adolescence, and into adulthood. A number of adverse outcomes can occur as the result of interference with development of this complex system that includes the reproductive organs, endocrine system, and hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal axis that control their development and function. Exposure to environmental agents that are hormonally active agents (HAAs), which are also called endocrine disruptors (EDs), has been shown to affect the reproductive system in both animals and humans. Early outcomes include birth defects such as hypospadias and cryptorchidism in boys, as well as hormonal changes such as hypothyroidism in boys

and girls, which interfere with optimal reproductive health. Later outcomes from the same exposures may include alterations in growth, timing, and progression of puberty, and disease states such as polycystic ovary disease (PCOS) and endometriosis in females and testicular dysgenesis syndrome in males. Such changes may be cumulative; that is, adverse outcomes at early ages may predispose an individual to be at greater risk for additional adverse effects (e.g., cryptorchidism and later changes in fertility [Lee, 2005] or early menarche and breast cancer [Vihko & Apter, 1986]). Recent studies in animals suggest exposure to certain HAAs during fetal life may cause multiple abnormalities after birth, (e.g., abnormal reproductive organs, increased tumors), and once adulthood is reached, an increase in the incidence of infections. These changes appear to be transgenerational, that is, effects that are carried into subsequent generations because of changes in DNA methylation patterns that are transmitted in the male germline to the next generation (Anway, Cupp, Uzumcu, & Skinner, 2005; Chang, Anway, Rekow, & Skinner, 2006).

Increasing trends in hypospadias (Paulozzi, Erickson, & Jackson, 1997; Paulozzi, 1999) and cryptorchidism (Paulozzi, 1999) have been reported in the United States and other countries. Secular trends of decreasing age at menarche in girls have been reported in Europeans, North Americans, and Australians during the last century. Recent data suggest decreasing age for menarche as well as in measures of puberty onset (e.g., onset of breast and pubic hair development) in the United States (Herman-Giddens et al., 1997; Herman-Giddens, 2006; Kaplowitz, Slora, Wasserman, Pedlow, & Herman-Giddens, 2001; Lee, Guo, & Kulin, 2001). Data are fewer for timing of puberty in boys and some of the male pubertal markers may not be as reliable as in females, but age at genital growth and pubic hair development appears to be earlier in recent years (Herman-Giddens, 2006). These changes may result from better (and over) nutrition, greater and earlier growth, increasing incidence of obesity (Lee et al., 2007; Wang, 2002), and environmental or socioeconomic factors.

A variety of environmental chemicals have been cited in the literature as potential HAAs, including insecticides and herbicides (e.g., dichlorodiphenyltrichloroethane [DDT], atrazine); pharmaceuticals (drug estrogens); chemicals associated with consumer goods/household products (e.g., bisphenol A, phthalates, nonylphenol, polybrominated diphenyl ethers [PBDEs], perfluorinated compounds [PFOA, PFOS]); industrial chemicals (e.g., polychlorinated biphenyls [PCBs], dioxins, polycyclic aromatic hydrocarbons [PAHs]); heavy metals (e.g., arsenic, lead, mercury, and cadmium); and natural hormones such as the phytoestrogens (Gray, Ostby, Cooper, & Kelce, 1999; Howdeshell, Hotchkiss, Thayer, Vandenbergh, & Vom Saal, 1999; Rubin, Murray, Damassa, King, & Soto, 2001; Schonfelder et al., 2002; Ashby, Tinwell, Stevens, Pastoor, & Breckenbridge, 2002; Wolf, Ostby, & Gray, 1999; Fenton, Hamm, Birnbaum, & Youngblood, 2002; Kuriyama, Talsness, Grote, & Chahoud, 2005; Talsness et al., 2005; McDonald, 2005; Lilienthal, Hack, Roth-Harer, Grande, & Talsness, 2006; Ceccatelli, Faass, Schlumpf, & Lichtensteiger, 2006; Eriksson, Fischer, & Fredriksson, 2006). Recent studies of exposure to some environmental agents suggest they may accelerate (Blanck et al., 2000; Krstevska-Konstantinova et al., 2001) or delay (Den Hond et al., 2002; Wu, Buck, & Mendola, 2003; Selevan et al., 2003) pubertal development in girls. Data on the effects of HAAs on age at puberty in boys are fewer (Den Hond et al., 2002) but indicate an association between PCB and polychlorinated dibenzofuran (PCDF) exposures with delayed puberty and decreased penile length (Den Hond & Schoeters, 2006). These observations are concordant with laboratory data on the effects of HAAs (Gray et al., 1999; Yu et al., 2004). Because there are only limited data on specific critical windows for chemical exposures in relation to timing of puberty, the entire prepubertal period, including in utero growth and development and the peripubertal period, should be considered as critical times for exposures.

3.1 Public Health Importance

Prevalence/incidence

Exposure to phthalates and other HAAs is widespread in American children (Centers for Disease Control and Prevention [CDC], 2003), and animal studies increasingly suggest the potential for toxicity at current levels of exposure (Vom Saal & Hughes, 2005). Exposure to HAAs in the residential environment can occur from sources such as drinking contaminated water, breathing polluted air, ingesting food, and contacting or ingesting contaminated soil or dust, as well as using certain commercial products containing synthetic HAAs (e.g., cleaners, pesticides, cosmetics and food additives) (National Research Council [NRC], 1999). Rudel Camann, Spengler, Korn, & Brody (2003) investigated potential indoor exposures to numerous endocrine disruptors found in consumer uses. Analyses of indoor air and dust found 52 HAA compounds in air including phthalates (plasticizers, emulsifiers), o-phenylphenol (disinfectant), 4-nonylphenol (detergent metabolite), and 4-tert-butylphenol (adhesive). Sixty-six endocrine disrupting compounds were present in dust samples taken from homes, with frequent detections of penta- and tetrabrominated diphenyl ethers (flame retardants) and numerous pesticides in dust. An intermediate of a flame retardant banned in 1977 (2,3-dibromo-1-propanol), as well as the banned pesticides heptachlor, chlordane, methoxychlor, and DDT were also frequently detected in dust and air (Rudel et al., 2003).

One common congenital anomality, hypospadias affects 27-55 out of 10,000 births in the United States (Paulozzi, Erickson, & Jackson, 1997; Paulozzi 1999) or 0.8 percent of male live births (Pohl, Joyce, Wise, & Cilento, 2007). Cryptorchidism rates vary by gestational age and birth weight, affecting 3 percent of full-term male newborns (up to 7.7 percent in low birth weight males), decreasing to about 1 percent by age 1 due to spontaneous descent (Pohl et al., 2007). In utero exposure to phthalates has been associated with decreased anogenital distance. This suggests phthalate exposure may cause this structural change (Swan et al., 2005). Polycystic ovarian syndrome (PCOS) is the most common endocrine abnormality of premenopausal women, affecting 6.6 percent of a sample of 400 women (age 18-45 years) seeking a pre-employment physical (Azziz et al., 2004). Symptoms of PCOS may emerge during late puberty and shortly thereafter (Jeffrey Chang & Coffler, 2007).

According to the CDC (2003), most girls reach puberty between 8 and 13, and most boys reach puberty between 9 and 14. Approximately 37 percent of 7-year-old and 52 percent of 8-year-old African-American girls showed signs of precocious (early onset) puberty, while corresponding numbers for Caucasian girls were 6 percent and 16 percent, respectively (Herman-Giddens et al., 1997).

Economic and/or social burden

The total cost of evaluating and providing care to reproductive-aged women with PCOS in the United States is $4.36 billion (Azziz, Marin, Hoq, Badamgarav, & Song, 2005). There is a 15-40 fold increased risk for testicular cancer and an increased risk for infertility in men with a history of cryptorchidism, but data are too sparse to estimate economic costs (Pohl et al., 2007).

Very early or very late puberty has been associated with conditions that sometimes arise during adolescence and more often during adulthood that carry increased health care costs. Early menarche is reported to be a risk factor for breast cancer, underscoring the role of early developmental milestones as indicators for adult onset disease (Vihko & Apter, 1986). Certain girls with premature adrenarche are at risk of developing functional ovarian hyperandrogenism, PCOS, and hyperinsulinism (Vuguin, Linder, Rosenfeld, Saenger, & Dimartino-Nardi, 1999; Banerjee et al., 1998). Psychological and psychosocial disturbances are also associated with precocious puberty. Central precocious puberty often

leads to lower self-esteem, and early menarche has been associated with comorbid depression and substance abuse (Stice, Presnell, & Bearman, 2001).

Delayed puberty is associated with short stature and lack of sexual development, characteristics that may lead to emotional and social difficulties. Bone mass gain is rapid during puberty. Recent data suggest a delay in pubertal maturation may cause prolonged, possibly irreversible defects in bone mineralization that alters peak bone mass and interferes with normal bone accretion process and causes osteoporosis (Rakover et al., 2000; Moreira-Andres et al., 1998).

Preventability/malleability

Pohl et al. (2007) suggest changes in incidence of hypospadias and associated costs of medical treatment could best be affected by identification of environmental factors that may be responsible for the increase that has occurred over the past several decades. Changes in timing of puberty have been associated with increasing obesity in the U.S. population, which may also be related to environmental factors. The National Children's Study (NCS) is well positioned to evaluate the contribution of HAAs and other environmental factors to these problems, which will provide information on possible preventive measures and interventions that may be effective in reducing the incidence of adverse reproductive development and disease outcomes.

3.2 Justification for a Large Prospective Longitudinal Study

Lack of accurate information on the level and timing of past exposures to HAAs has been the principal limitation of most previous studies of the potential human impacts of known and suspected HAAs. This limitation will be addressed by the prospective design of the National Children's Study in that exposures to chemicals will be measured during pregnancy, in breast milk, and in the perinatal period before the appearance of health effects. Measurement of multiple outcomes related to single and multiple exposures and continuous or repeated exposures is possible with a large longitudinal study. The potential for cumulative effects on the reproductive system can only be discerned through the use of a large longitudinal sample that allows repeated measures of exposure and evaluation of reproductive outcomes over time. Measures of exposure or biomarkers of exposure are available for most HAAs of interest and will link exposures at specific life stages with early or late reproductive outcomes. Methods for measuring gene prevalence and expression will permit examination of genetic polymorphisms that may influence gene-environment interactions to allow assessment of genetically determined interindividual differences in susceptibility to HAAs.

Since the effects of HAAs are gender specific, it will be necessary to study exposure-outcome links separately in males and females, effectively reducing the sample size for each case to approximately 50,000. Susceptible subgroups related to genetic polymorphisms may require additional subgroup studies.

Need to study interactions

Transcriptional regulation of the Fas/FasL pathway by phthalates has been identified as critical to Sertoli cell injury, and polymorphisms in this group of genes are likely to result in differential toxicity (Yao, Lin, Sawhney, & Richburg, 2007). There is recent evidence for polymorphisms in CYP19 (aromatase) influencing age at menarche in girls (Guo et al., 2006) and several of the CYPs are affected by HAAs. Other studies have shown associations between Wnt 7a deregulation in development of the

female reproductive tract and exposure to DES (Sassoon, 1999) or PCBs (Ma & Sassoon, 2006). Changes in expression of several genes have been associated with DES exposure in a mouse model (Newbold et al., 2007). It is likely that many more potential gene-environment interactions will be revealed before children in the NCS reach the age of onset of puberty.

3.3 Scientific Merit

HAAs are chemicals that can interfere with hormonal signaling systems. HAAs may mimic, block, or modulate the synthesis, release, transport, metabolism, binding, or elimination of natural hormones. They may temporarily or permanently alter feedback loops in the brain, pituitary, gonads, thyroid, and other components of the endocrine system (Gore, 2001, 2002). Compounds with estrogenic activity were the focus of most initial concern about HAAs. Chemicals with antiestrogenic, progestogenic, androgenic, antiandrogenic, antithyroid, hypothalamic, and other effects have also come to be recognized (National Research Council [NRC], 1999). The results of endocrine disruption are often not easily detected. They may be subtle and delayed in onset and may have intergenerational effects.

History gives some notable examples of human populations exposed to high levels of HAA compounds that resulted in adverse outcomes for the exposed or their offspring. Herbst et al. (1971) observed several cases of clear cell adenocarcinoma (CCA) of the vagina in young women who had been exposed in utero one to two decades earlier to DES, a synthetic estrogen prescribed to pregnant women in the 1950s and 1960s to prevent miscarriage. In 1976 in Seveso, Italy, community-wide exposure to high concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) occurred after an industrial explosion (Mocarelli et al., 1996, 2000) causing substantially lower male-female sex ratios (0.38 at birth in offspring of the most highly exposed fathers versus 0.56 in unexposed). Sex ratio was also altered among the offspring of men exposed occupationally to TCDD in a pesticide manufacturing plant in Ufa, Bashkortostan, Russia (Ryan, Amirova, & Carrier, 2002). Accidental contamination of the Michigan food chain in 1973 by FireMaster, a fire retardant containing polybrominated biphenyls (PBBs), led to exposure of more than 4,000 persons who ingested contaminated meat and milk. Breast-fed girls exposed to high levels of PBBs in utero had an earlier age of menarche than did either breast-fed girls exposed to lower levels of PBBs in utero or girls who were not breast fed (Blanck et al., 2000).

Synthetically produced HAAs also include pesticides (e.g., DDT and endosulfan), certain plastics (e.g., bisphenol A), and other industrial chemicals (e.g., PCBs, PBDEs, and phthalates) (NRC, 1999). The United States banned some of these substances because they can interfere with endocrine function, including DDT, diethylstilbestrol, and PCBs. Evidence of the ability of HAAs to affect development and reproductive capacity came from studies of wildlife but more recently has been buttressed by in vitro studies that have begun to elucidate some of the molecular mechanisms of action of HAAs. Additionally, clinical and epidemiologic studies have begun to explore the possibility that human exposures to hormonally active compounds, particularly in utero and during early childhood, may be responsible, at least in part, for changes in semen quality, increasing incidence of congenital malformations of the reproductive organs, increasing rates of testicular cancer, and an apparent increase in incidence of precocious puberty.

Research on the human health effects of HAAs is still new. The ability to investigate the possible impacts of HAAs on human health has been limited because most previous studies have employed case-control designs with retrospective assessments of exposure. Except for studies of pharmacologic agents such as DES, most studies have been limited in their ability to accurately assess either the amount or the timing of exposure to putative HAAs. Studies to examine the possible etiologic contributions of HAAs to these trends in the case of hypospadias and cryptorchidism (Toppari et al.,

1996), testicular cancer (Carlsen, Giwercman, Keiding, & Skakkebaek, 1995), and timing of onset of puberty in young girls (Blanck et al., 2000) are needed.

Further study of the potential for genetic effects and the role of genetic polymorphisms will advance our understanding of the effects of HAAs on reproductive development and potential gene-environment interactions. The possibility of epigenetic changes in DNA following HAA exposure that may lead to transgenerational reproductive effects (Anway et al., 2005; Anway, Leathers, & Skinner, 2006; Chang et al., 2006) can be evaluated in samples collected in the National Children's Study. A number of other modes of action for HAAs have recently been reviewed by Tabb and Blumberg (2006), including several which may be investigated in adjunct or follow-up studies to the NCS.

3.4 Potential for Innovative Research

New findings

The strengths of the study include new knowledge to be generated in the following areas:

- Determine which endocrine active chemicals, at what doses, and at what times in development, contribute to the genesis of reproductive and other health problems in children and adults.
- Assess impacts of combinations of HA chemicals.
- Examine modification of HA effects by genetic polymorphisms.
- Study later impacts of early exposures and the cumulative nature of reproductive effects; for example, birth defects of the reproductive organs, alterations in onset of puberty, disease states including PCOS and endometriosis, fertility, and increased risk of breast and testicular tumors.

3.5 Feasibility

Access at critical periods of exposures/outcomes

Evaluation of children at birth, at 6-month intervals during the first 2 years of life, and at regular intervals beyond that will allow assessment of birth defects and anthropometric measures. The proposed approach can be applied successfully to study the impact of HAAs in a sample of 100,000 children with serial assessment of reproductive outcomes at birth, childhood, puberty, and adulthood; access to medical records for disease states such as PCOS; collection of maternal breast milk and maternal and child blood and urine samples at multiple time points; and serial questionnaires to assess pathways of exposure.

4. Exposure Measures

4.1 Individuals Targeted for Measurement

Primary/maternal

- Bisphenol A
- Atrazine

- Organochlorines
- Lead, cadmium, mercury, arsenic
- Dioxins
- PCBs
- PBDEs

Primary/child

- Bisphenol A
- Atrazine
- Organochlorines
- Lead, cadmium, mercury, arsenic
- Dioxins
- PCBs
- PBDEs

Secondary/paternal (pilot studies/adjunct study)

- Bisphenol A
- Atrazine
- Organochlorines
- Lead, cadmium, mercury, arsenic
- Dioxins
- PCBs
- PBDEs
- Semen quality

4.2 Methods

Primary/maternal

- Blood sample
- Urine sample

- Breast milk sample

Primary/child

- Blood sample
- Urine sample

Secondary/paternal

- Blood sample
- Urine sample
- Semen sample

4.3 Life Stage

Primary/maternal

- Preconception, prenatal, and throughout nursing

Primary/child

- Birth and throughout nursing, through adolescence

Secondary/paternal

- At time of first positive pregnancy test in mother

5. Outcome Measures

5.1 Outcomes Targeted for Measurement in Child

- Physical/morphologic malformation
- Hormonal status, particularly thyroid status
- Tanner stage exam (male and female)
- Menstrual history (female)
- Spermarche (male)
- Semen quality (male) (pilot study/adjunct studies)

5.2 Methods

- Urine samples

- Blood samples
- Interview
- Direct observation by a medical professional or via medical record review
- Physical exam (or self exam)
- Semen sample

5.3 Life Stage

- Birth through age 21for birth defects and growth
- Yearly, starting at ages 6 for girls and 7 for boys until age 18 for pubertal assessments
- 18-21 years for semen sample

6. Important Confounders, Mediators, and Effect Modifiers

- **Obesity, diet, and nutrition measures:** Higher percentage of body fat increases the risk of precocious puberty; later onset in underdeveloped nations is often attributed to poor nutrition (Anderson, Dallal, & Must, 2003).
- **Smoking status of parents; urine cotinine:** Smoking, prenatal and postnatal, may reduce the age of onset of puberty (Windham, Bottomley, Birner, & Fenster, 2004). Urine cotinine is measured to examine active/passive smoking exposures.
- **Mother's menstrual and reproductive history:** Generally, the mother's menstrual history is considered the biggest predictor of age of puberty in both male and female children. Some of this effect may be seen in ethnic differences (Blanck et al., 2000).
- **Father's reproductive history:** There are genetic components for hypospadias, cryptorchidism, spermarche, and semen quality that may be related to the father's reproductive history (Pohl et al., 2007).
- **Genetic factors:** 5-alphareductase type 2 gene mutations (Silver & Russell, 1999) and androgen receptor mutations (Silver, 2000) are risk factors for hypospadias.
- **Gestational age at birth:** There is some evidence that a younger gestational age at birth is associated with greater incidence of hypospadias and cryptorchidism (Pohl et al., 2007) and is a predictor of an earlier age at menarche; however, evidence points to small for gestational age (SGA) as the predictor of precocious puberty (Adair, 2001).
- **Mother's alcohol consumption during pregnancy:** Some studies have reported an association between alcohol consumption and hypospadias (Carbone et al., 2007) and an association between later onset of puberty and maternal alcohol use, while other recent studies have reported no effect (Blanck et al., 2000).

- **Socioeconomic status and stress:** The impact of stressful sociologic factors has been related to precocious pubertal development.

- **Certain diseases or conditions:** Obesity and precocious puberty has been associated with conditions such as neurofibromatosis, hypothyroidism, polycystic ovary syndrome, etc. Delayed puberty has been associated with conditions such as sickle cell disease, thalassaemia, Celiac disease, Gaucher disease type 1, Cushing's disease, and other endocrine deficiencies.

7. Power and Sample Size

Birth defects of the reproductive system, growth, body weight, endocrine status (e.g., hypothyroidism), and puberty timing, progression, and completion will be examined in girls and boys. Subgroups include: those in rural communities exposed chronically or seasonally to pesticides; African-American girls (who usually demonstrate an earlier entrance into puberty than other races, implying unique genetic and/or environmental factors); children with certain diseases or conditions (for precocious puberty: neurofibromatosis, hypothyroidism, polycystic ovary syndrome, etc; for delayed puberty: sickle cell disease, thalassaemia, Celiac disease, Gaucher disease type 1, Cushing's disease, etc.); and populations consuming foods such as fish with high concentrations of bioaccumulative endocrine active chemicals.

In terms of gene-environment interactions, assuming 50 percent gene prevalence of polymorphisms in, for example, the Fas/FasL pathway in controls, a control-case ratio of 1, and five exposure quantiles, a sample size of 8,583 is required to detect a 50 percent increase in risk at 90 percent power. If gene prevalence is 10 percent, then 20,556 participants are required. Under these same assumptions, a sample size of 106,661 is required to detect a 20 percent increase in risk at 90 percent power (Spiegelman & Logan, 2001).

Sample size estimates based on existing methods for monitoring puberty and information about the timing of puberty available in the literature generally range between 5,000 and 20,000 children. These were based on estimates of exposure to relatively high levels of chemicals of concern set at 10 percent or 20 percent of the study population. For the most basic assessment of differences in proportions of children reaching puberty early, the NCS has sufficient power (alpha for a two-sided test of 0.05; beta = 0.20) to detect a 1.2 percent difference in the proportion of girls or boys with early pubertal attainment, assuming the prevalence of exposure is 20 percent and the proportion of children with early puberty in the controls is approximately 14 percent. Looking at subgroup analyses with a sample size of 5,000 children where approximately 15 percent are exposed, there is sufficient power to detect a 4 percent difference in the proportion of children attaining puberty early under similar assumptions. In another example, if the rates of hypospadias are approximately 30 per 10,000 among unexposed boys and 50 per 10,000 in exposed boys, the NCS has sufficient power to observe a difference when about 10 percent of the population is exposed.

Refinements in current methods for assessing puberty (e.g., by developing more objective and sensitive indicators, including biochemical and molecular biomarkers) would be expected to improve the power.

8. Other Design Issues

Ethical/ burden considerations:

- The study will need to have a formal strategy and process for effectively communicating results of physiological and biochemical measures to the child's parents and to a responsible health care provider. Any abnormalities identified in blood or anthropometric measurements would be noted and appropriate follow-up medical care recommended.
- The study also will also need to have a have a formal strategy and process for effectively communicating results of environmental monitoring to the child's parents along with appropriate and feasible recommendations regarding the correction of any unhealthful environmental findings.
- Potential embarrassment about pubertal issues.
- The influence of DNA collection for genetic evaluation and RNA for gene expression profiling presents ethical questions.
- Some minimally invasive procedures are possible.
- Repeated tests are potentially burdensome.

Cost/complexity of data collection:

- It is important to have good retention rates to examine pubertal progression and completion.

9. References

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