Biomarkers for Assessing Reproductive Development and Health: Part 1—Pubertal Development

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The proposed National Children's Study has helped raise awareness of the issues related to children's health and the importance of monitoring the growth and development of children from preconception through adulthood. Many genetic predispositions can adversely impact the normal development process, and various environmental exposures have been linked to adverse reproductive health in rodent models and a small number of accidental human exposures. To monitor reproductive health and identify adverse effects at the earliest possible juncture, investigators must develop a network of biomarkers covering all stages and aspects of reproductive development and function. Biomarkers are biological indicators that can be measured repeatedly and are informative on one or more aspects of biological development or function. They can range from the anatomical level down to the molecular level and may provide information on the nature of an exposure, the effect of an exposure, or the susceptibility of individuals or populations to the toxic effects of an exposure. In theory, biomarkers can be used to monitor a wide variety of conditions and responses ranging from abnormal development to early indicators of late-onset disease. The main stumbling block with this theory has been finding appropriate biomarkers for particular conditions and exposures. Such biomarkers must be easily accessible, robust, and sensitive. Ideally, they will be expressed across a large section of the population, and can be monitored quickly, easily, conveniently, and with minimal cost. In this review, we discuss some of the current and emerging biomarkers of human pubertal development. *Key words:* **biomarker, development, human, longitudinal cohort study, puberty.** *Environ Health Perspect* **112:105–112 (2004). doi:10.1289/ehp.6265 available via *http://dx.doi.org/* [Online 24 September 2003]**

A recent surge of reports from pediatricians in industrialized countries indicates that many girls are presenting with secondary sex characteristics at a younger age than has previously been considered normal (Herman-Giddens et al. 1997, 2001; Papadimitriou 2001). For example, current medical texts generally state that only 1% of girls show signs of puberty (breast development or growth of pubic hair) before 8 years of age. However, a study by Herman-Giddens et al. (1997) indicates that a substantial portion of American girls are presenting with one or both of these characteristics by age seven, and that 1% of girls have one or both of these pubertal markers by age three. Studies have also shown that the rate of growth for children and adolescents in the United States and other countries is significantly greater than in previous years (Freedman et al. 2000; Karpati et al. 2002). Some countries have even recorded an overall increase in final height (Padez 2002), although a recent review on the phenomenon concluded that the trends in final height and pubertal development are not strongly connected and that more longitudinal studies are needed for investigators to understand the short- and long-term consequences of the trends before we can interpret their importance (Karlberg 2002).

The phenomena of earlier puberty and increased final height have commonly been referred to as "the secular trend in growth." It is believed that the trend may have been in place for as many as 150 years in certain parts of the world (Samaras and Storms 2002), and it has been attributed largely to better child care—primarily as a result of improved nutrition, increased food supply, and improved health and sanitation services. A second hypothesis is that in some cases the mechanism of precocious puberty might involve environmental exposure to estrogenic endocrine disruptors (Teilmann et al. 2002). This has been suggested after studies indicating that a relatively high proportion of children (primarily girls) who have emigrated from developing to developed countries suffer precocious puberty and that their blood serum contains elevated levels of estrogenic pesticides (Krstevska-Konstantinova et al. 2001). Environmental exposures have also been associated with delayed puberty. Data from the Third National Health And Nutrition Examination Survey (NHANES III) supported a significant negative relation between blood lead levels and delayed attainment of menarche and pubic hair among U.S. girls 10–16 years of age (Wu et al. 2003). Among girls 8–18 years of age in the NHANES III, blood lead concentrations were significantly associated with delays in growth and pubertal development (Selevan et al. 2003). Although these and other examples provide evidence that endocrine disruptors may disturb pubertal development, there has been little research in this area, and therefore we do not yet have any clear cause–effect relationships in humans.

One reason for the confusion surrounding the secular trend in growth is that puberty is a complex and multifaceted process that does not have a single identifiable trigger. A large, longitudinal study such as the National Children's Study (NCS 2003) offers an ideal opportunity to further investigate the causes of the secular growth phenomenon, as it would enable researchers to obtain samples from multiple individuals at multiple time points of development. This would permit a thorough characterization of pubertal and other growth processes. In this way the NCS also affords an unprecedented and unparalleled opportunity to study the true sensitivity and accuracy of current biomarkers of pubertal development, to further characterize and develop and refine emerging biomarkers, and to identify previously unknown biomarkers. Furthermore, the length of the study and the fact that indicators of pubertal development in children are unlikely to be measured for at least 5–10 years provides ample time to develop and refine emerging biomarkers and techniques for biomarker identification. The purpose of this review is to discuss current and emerging biomarkers of male and female pubertal development that might be of use in a longitudinal children's study such as the NCS.

Biomarkers

The term "biomarker" has been defined in many ways. For the purposes of this review, a

This article is part of the mini-monograph "Understanding the Determinants of Children's Health."

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We thank S. Fenton (U.S. EPA), M. Klebanoff (NICHD), and members of the Fertility and Early Pregnancy Working Group of the National Children's Study for critically reviewing this manuscript prior to submission.

The information in this document has been subjected to review by the National Health and Environmental Effects Research Laboratory of the U.S. EPA and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

The authors declare they have no competing financial interests.

Received 6 February 2003; accepted 2 July 2003.

biomarker is any biological index capable of being measured that is associated with or indicative of a defined biological end point such as a developmental or disease stage. Biomarkers can be informative on several different levels: *a*) Biomarkers of developmental or disease stage—measurable indices that indicate a specific stage in a normal or disease development process. These are the focus of this article as they pertain to puberty. *b*) Biomarkers of exposure—measurable changes in biological appearance or function that indicate exposure to a particular stimulus, which may be chemical, biological, or physical in nature. Such biomarkers may be useful in identifying potentially toxic exposures. Biomarkers of exposure need not be a direct result of the exposure, although in many examples this is the case. *c*) Biomarkers of effect—environmental exposures, either chemical or physical, that can produce a multitude of effects. These may be systemic or localized, and measurable at the clinical, cellular, or molecular level. *d*) Biomarkers of susceptibility—it is increasingly recognized that genetic markers can be used to identify predisposition or increased risk of developing certain conditions. Some individuals have genetic makeups that make them more or less susceptible to developing certain diseases, whether genetically or environmentally induced or a combination of both.

Biomarkers can include a variety of measurable targets, including biochemical, molecular, cellular, genetic, immunological, or physiological changes. Some of those described to date include the presence of a parent compound or metabolite (Hecht 2002); proliferation and differentiation indices (Iatropoulos and Williams 1996); apoptotic end points (Samaha et al. 1997); formation of DNA adducts or damage (Poirier and Weston 1996); chromosomal abnormalities (Lucas 1997); micronuclei formation (Schoket et al. 1999); expression of specific genes (Riggins 2001); changes in gene expression profile (single or multiple genes) (Thomas et al. 2001); and measurement of enzyme activity (Chen et al. 1999), to name but a few. In some cases different biomarkers can be used to measure the same indicator. At the gross, physiological level, for example, genital or breast size and the growth of pubic hair can be used to stage pubertal development. Other biomarkers of puberty are cellular in nature, including serum levels of pituitary–gonadal axis hormones and other proteins.

In the context of a large epidemiologic study such as the NCS, biomarkers must be easy to obtain and measure. This means that they are obtainable with noninvasive or minimally invasive measures, and that the assay(s) used to measure the biomarker(s) are robust, sensitive, and, optimally, adaptable to a high throughput format. The biomarkers requiring the least effort to measure are those apparent in gross anatomy. Perhaps the best example of this is the use of Tanner scales as biomarkers of pubertal developmental stage (Tanner 1962).

Tanner Scales: Anatomical Markers for Staging Pubertal Development

Puberty is a developmental period associated with tremendous change. The timing of the onset of puberty varies among adolescents as does, most likely, the interval between onset of pubertal markers. Changes in onset of markers correlate more strongly with the presence of secondary sex characteristics than they do with chronological age (Marshall and Tanner 1969, 1970; Wu et al. 2002). Although recent studies suggest that differences appear to exist in the ordering of pubertal development across different racial groups (de Muinich Keizer and Mul 2001; Herman-Giddens et al. 1997; Wu et al. 2002), the order in which secondary sex characteristics appear and their subsequent stages of development had previously been considered to be relatively uniform (Tanner 1986). On the basis of this earlier knowledge of pubertal development, Tanner (1962) developed a standard for assessing pubertal development (sexual maturity scale; SMS) that has been used widely in clinical practice for many years. In girls this scale encompasses age at breast and pubic hair developmental stage and age at menarche. Not all girls (women) are able to accurately recall their exact age at menarche. Investigators have handled such missing data in a number of ways, either by substituting the mean age for girls with known ages, or using grade in school and presumed chronological age assuming the girl entered school at 5 years of age and did not skip or repeat a grade. Other crude proxies of pubertal development in girls include presence/absence of axillary hair (Lindgren 1996). Although there is no direct analogy of age at menarche for boys, there is a Tanner scale for assessing pubertal stage in boys. In addition, some investigators have used proxy markers such as axillary hair, voice changes, testicular size, (3–4 mL) or age at ejaculation. It is important to note that these changes do not occur simultaneously but follow growth of the testes and penis at varying time intervals (Tanner 1986).

With respect to the Tanner method, a limitation for use in some populations or under certain circumstances is that it requires nude children and adolescents to be visually inspected by a trained clinician for the appearance of secondary sex characteristics. The Tanner SMS is often not practical for the purposes of population-based research, as many adolescents (and their parents) are uncomfortable with the idea of being undressed in the presence of a stranger. Given this limitation, many investigators have attempted to develop alternative methods of obtaining this information.

Duke and colleagues (1980) were the first to develop a method to determine pubertal development via self-assessment. Adolescents were shown sex-specific sets of the SMS photographs (with accompanying descriptive phrases developed by the investigators) and were asked to indicate which photograph in each series (breast, penis, and pubic hair growth, as appropriate) most closely resembled their current stage of development. Participants were also examined and independently rated by one of the investigators. There was considerable agreement between the assessment of secondary sex characteristics by adolescent girls and the assessment by investigators, ranging from $\kappa = 0.81$ for breast development to $\kappa = 0.91$ for pubic hair distribution. Among males the concordance was also high, with a kappa coefficient of 0.88 for penis development and pubic hair distribution combined. In instances where there was disagreement between the adolescent and the investigator, the ratings never differed by more than one Tanner stage.

Since the pioneering work of Duke et al. in 1980, several investigators have evaluated their approach in other populations. One of the criticisms of Duke's work is that the study population was predominantly white and of upper socioeconomic status. Neinstein (1982) set out to test the method in an ethnically diverse middle-income population. Among females he found a correlation of 0.86 for breast development and a correlation of 0.73 for pubic hair distribution. For males he reported correlations of 0.73 and 0.69 for penis development and pubic hair distribution, respectively. The correlation between the adolescent rating and the physician rating of overall pubertal development was higher among females than among males ($p < 0.05$); however, there were no significant differences with respect to race or ethnicity. This is an important finding, as age at puberty milestones are reported in some populations to vary by race/ethnicity (Wu et al. 2002).

Another concern raised regarding the use of the method of Duke and colleagues is that it may not be appropriate for use in adolescents with mental and/or physical problems. To address this issue, Hardoff and Tamir (1993) asked both learning-disabled and non–learning-disabled adolescents of normal intelligence to indicate which Tanner photograph most closely resembled their current stage of pubic hair development. Among the learning-disabled adolescents, only 58% were able to accurately identify their current pubertal stage ($\kappa = 0.43$). Ten percent of learning-disabled adolescents overestimated their current pubertal

stage, compared with only 2% of non–learning-disabled adolescents.

How well can puberty be measured in subgroups of children with underlying medical problems that act either directly or indirectly on the hypothalamus–pituitary–gonadal (HPG) axis? One answer comes from studies of children with cystic fibrosis (CF). Children with CF are often smaller and thinner than those without the disease, a fact that can lead them to become self-conscious about their appearance. Boas and colleagues (1995) compared the accuracy of self-reported pubertal development in a sample of both male adolescents with CF and those without the disease. After being examined by a physician, the participants were asked to identify which of the Tanner photographs looked most like their current stage of development. For pubic hair they reported kappa coefficients of 0.802 and 0.732 for the adolescents with CF and those without CF, respectively. Penis development was reported less accurately, with a kappa coefficient of 0.489 for males with CF and a kappa of 0.345 for those without the disease. Similarly, Schall and colleagues (2002) demonstrated that self-assessment of pubertal status is valid and reliable for use among adolescents with Crohn disease. Among female adolescents, they reported kappa coefficients of 0.74 and 0.81 for breast development and pubic hair distribution, respectively. The agreement was slightly better among males, with a kappa coefficient of 0.85 for both penis development and pubic hair stage.

At approximately the same time that Duke and associates were working on their self-assessment method, Morris and Udry (1980) were developing a similar tool. The method of Morris and Udry, however, was based on illustrations made from the original Tanner photographs. In addition to having short descriptions under each drawing, they also asked several general questions regarding pubertal development, such as "Do the clothes you wore last year still fit?" Adolescent subjects were asked to complete the sex-specific questionnaire and return it to an assistant in a sealed envelope. The subjects were then examined by a physician who was unaware of their responses. They reported Pearson correlations for overall pubertal development of 0.57 for males and 0.52 for females. The investigators suggested that the correlations might improve if adolescents were given the opportunity to examine their bodies in private prior to completing the questionnaire.

Like the technique of Duke and colleagues, the work of Morris and Udry has been tested in many populations. Hergenroeder et al. (1999) examined the performance of Morris and Udry's drawings in a racially diverse sample of adolescent females. Overall, they found very little agreement between the adolescent self-rating of pubertal development and the physician assessment. The kappa coefficient for breast development was higher among African-American females ($\kappa = 0.42$) than among European-American females ($\kappa = 0.35$); however, for pubic hair distribution, agreement with the physician was higher among European Americans ($\kappa = 0.44$ vs. $\kappa = 0.26$.)

There is some concern that adolescents with anxiety disorders (e.g., body dysmorphic syndrome) at the extremes of body size or those with eating disorders might have difficulty accurately assessing their pubertal development. Williams and associates showed both the Morris and Udry drawings and the Tanner photographs to a sample of adolescents with diverse levels of fatness (determined by skinfold thickness) (Williams et al. 1988). None of the participants were morbidly obese nor did they have anorexia. They reported correlations (Kendall's $\tilde{\tau}\beta$) ranging from 0.65 for penis development to 0.82 for male pubic hair distribution. No significant differences in self-assessment ability were found with respect to fatness. In contrast, Bonat and colleagues tested the Tanner stage line drawings in a racially diverse sample of children from suburban Maryland, 41% of whom were obese. Although they found no differences with respect to pubic hair ratings, they reported that obese females were more likely than nonobese females to overestimate their Tanner breast development stage (0.5 ± 1.1 stages) (Bonat et al. 2002.)

Hick and Katzman (1999) evaluated the use of Morris and Udry's drawings in a sample of adolescent females with anorexia nervosa. The subjects were asked to complete two separate sets of forms, one in which they selected their current level of pubertal development, and the other in which they indicated their desired level. For breast development, there was 30% agreement between the adolescent rating and that of the physician. The adolescent self-assessment of pubic hair distribution was more accurate (50% agreement). Female adolescents with anorexia nervosa tended to underestimate their breast development and overestimate their pubic hair distribution. The majority of the adolescents desired secondary sex characteristics that were equal to or more mature than their current Tanner stage.

Several issues might affect the accuracy of an adolescent's self-assessment of pubertal maturation: the adolescent's actual Tanner stage, where the instrument is administered (school vs. clinic setting), and whether he/she is aware that a physical examination is imminent. To study these issues, Schlossberger and colleagues (1992) asked a sample of adolescents from an urban public middle school to complete Morris and Udry's instrument. The students were unaware that they would later receive a physical examination. Two months later, the investigators invited the students to their clinic for a physical examination, where they were again asked to complete the self-assessment instrument. Not surprisingly, they reported that adolescents were more accurate in their self-assessment in the clinic setting than they were in the school setting. Whether this effect was due to the setting itself or to the knowledge of the impending physical exam is unknown. In general, adolescents were found to overestimate their Tanner stage when they were early in pubertal development and tended to underestimate their development at later stages.

In addition to the photograph and drawing-based methods of pubertal self-assessment, several teams of investigators have developed verbal and written instruments. These assessment tools are thought to be more acceptable in a classroom setting, as school officials might find visual depictions to be inappropriate (Petersen et al. 1988). Petersen and colleagues tested their pubertal assessment interview (puberty development scale; PDS) on two successive cohorts of middle school students who were interviewed twice yearly from sixth to eighth grade. The internal consistency of the questions in the interview was high, with Cronbach alpha coefficients ranging from 0.68 to 0.83. In addition, most of the adolescents' responses followed the expected pattern, as few of them experienced regression of their pubertal indicators over time. Although the validity of the PDS was not examined (the students were not examined by a physician), a different study reported correlations with physician assessments in the range of 0.61–0.67 (Brooks-Gunn et al. 1987).

Several years after the development of the PDS, Carskadon and Acebo (1993) adapted it for use on a written questionnaire. The investigators found the validity of the written adaptation of the PDS to be high, with Spearman correlations ranging from 0.84 and 0.87. The items on the questionnaire were also internally consistent, with Cronbach alpha coefficients ranging from 0.67 to 0.70.

In an attempt to simplify the assessment of pubertal development even further, Berg-Kelly and Erdes (1997) tested the use of a global question. Adolescents were asked, "Considering your bodily development, how do you rate yourself compared to your classmates: very late, somewhat late, similar to most of your classmates, somewhat early, or very early?" Using this question, they reported a very high concordance with physician assessments: 95% for males and 93.5% among females.

Overall, many adolescents appear to be able to provide a reasonably accurate assessment of their pubertal maturation based on the presence of secondary sex characteristics. Although the use of Tanner's SMS pictures with accompanying written explanations

seems to perform more favorably than other self-assessment methods, many of the methods yield correlations in the range of those reported for inter-rater agreement of pubertal assessments conducted by health professionals (Berg-Kelly and Erdes 1997). It should be noted, however, that self-assessment instruments are still not ideal for use in population-based research, as they perform less well in special populations (e.g., certain racial/ethnic groups and those with clinical diagnoses such as anorexia nervosa). Furthermore, measurement of puberty in boys and girls is most likely associated with error because of differences between raters, despite training. Still, this error is unlikely to be systematic (bias) with respect to potential environmental exposures. The magnitude of this error may vary with respect to other characteristics of the population (e.g., chronic disease, socioeconomic status, fatness) but is likely to be a conservative estimate of effect. However, if this error is systematic, the potential for bias arises with respect to pubertal outcomes. To address this methodologic concern, continued investigation into sensitive biomarkers is needed if we are to estimate more precisely the effect of environmental exposures or other factors on human fecundity as measured by the onset and attainment of puberty. As such, the development of new methods for pubertal assessment, is urgently needed.

Other Physical Biomarkers of Pubertal Development

Skeletal growth. Skeletal growth is one of the most striking characteristics of puberty. A commonly used marker of skeletal growth is linear growth velocity (height increase per year), and this correlates with the Tanner stages of sexual development [see Family Practice Notebook.com (2003a, 2003b) for further details of male and female Tanner stages). In females the pubertal growth spurt starts at Tanner breast stage 2 (the start of puberty) and peaks at stage 3. Linear-growth velocity begins to increase in males at genital stage 3 and pubic-hair stage 2, but peak height velocity is not attained until Tanner stage 4.

Body composition. Significant changes in body composition (body mass index and lean body mass) also occur during puberty and show distinct and important gender differences. Lean body mass, which primarily reflects muscle mass, begins to increase during early puberty in both boys and girls. As pubertal stage advances in boys, their body fat mass increases while the percentage of body fat decreases. Among girls both body fat mass and percentage of body fat increases with advancing pubertal stage (Saetung et al. 2000).

The chief advantages of using height, weight, and fat mass as biomarkers of pubertal stage are that the methodology to obtain the data is noninvasive, socially acceptable, rapid, and simple to use. Equipment is relatively inexpensive in most cases, and there is a good selection of commercial products available for measuring height and body fat (Healthchecksystems.com 2003).

Fundamental voice frequency. It is well known that among males the pitch of the voice, or the fundamental voice frequency, lowers substantially during puberty. Using laryngography, Harries and colleagues (1997) demonstrated that the most abrupt change in the adolescent male voice occurs during the transition from Tanner stage 3 to Tanner stage 4 in pubertal development. Recent advances in technology have made it possible to identify changes in fundamental voice frequency by recording adolescents reading standardized passages of text at regular intervals (i.e., every 3 months) (Campisi et al. 2002). Although the technique appears to be less subjective than the various methods of pubertal self-assessment, additional work is needed to verify its reliability.

Molecular and Cellular Biomarkers of Puberty

Secondary sex characteristics determined using Tanner scales provide a relatively useful measure of pubertal stage and have formed the cornerstone of many studies into pubertal development. However, gross anatomical categorization is relatively limited in its applicability to a large and diverse population and is also limited in that it only informs on the physiological end point. That is, if puberty-associated problems exist, then these observational measurements of pubertal stage are usually of little use in diagnosis and prognosis. If pubertal or other developmental problems are occurring or might be expected to occur (given, for example, a certain genetic makeup of an individual), then methods to observe and characterize such problems at an early (preclinical) stage are required. Such methods require monitoring molecular and cellular changes, and accessible tissues (e.g., urine, blood) are the normal source of material for such studies. Indeed, these specimens are used extensively to measure numerous indicators including chemical, hormone and metabolite levels, the expression levels of genes and proteins, and the integrity of genetic material (DNA). Characterization of the normal molecular and cellular changes that occur in the prepubescent and pubescent body may offer the best approach to achieve this aim, and recent studies have uncovered a number of promising biomarkers of normal and abnormal pubertal development that may prove useful in longitudinal human studies.

Genetic markers. One of the main benefits of postgenomic methodologies and bioinformatics is that they have increased our ability to identify the genetic factors primarily or partly responsible for developmental problems. At least one ongoing longitudinal study (Avon Longitudinal Study of Parents and Children 2003) has been specifically designed to determine ways in which an individual's genotype combines with environmental pressures to influence health and development (Golding et al. 2001). Such studies enable researchers to identify genetic biomarkers of susceptibility, enhancing our ability to diagnose potential reproductive problems early on and resulting in the instigation of appropriate preventive measures. For example, recent work by Witchel et al. (2001) suggests that in some cases the development of premature pubarche (PP) can be associated with the occurrence of multiple sequence variants at various susceptibility loci, especially steroidogenic enzyme genes such as cytochrome P45021 (CYP21). Another steroidogenic enzyme, CYP19 (P450 aromatase), is of central importance in pubertal development (MacGillivray et al. 1998). The aromatase enzyme complex catalyzes the conversion of androgens to estrogens in a wide variety of tissues. Aromatase deficiency (reviewed in Bulun 2000) usually results from autosomal recessive inheritance of mutations in the *CYP19* gene. Females with this condition have ambiguous genitalia and fail to develop secondary sexual characteristics. Sexual development appears to progress normally in males, although the pubertal growth spurt and skeletal maturation are adversely affected. In contrast to the effects observed with *CYP19* mutations, certain *CYP19* splice variants can lead to increased extraglandular aromatization, producing an excess of estradiol that leads to PP in females and feminization of males (Stratakis et al. 1998).

Other genes have also been identified that have splice variants, polymorphic variants or mutations that could serve as possible biomarkers of susceptibility to precocious or delayed puberty in one or both sexes. Mutations in *Dax1*, for example, have been associated with PP in males (Domenice et al. 2001). Mutations in type II 3-beta hydroxysteroid dehydrogenase have been associated with PP in girls (Marui et al. 2000). Point mutations in luteinizing hormone (LH) receptor have been associated with PP in boys (Cocco et al. 1996), but no effect has been seen in girls. Thus, identification and characterization of these and other such biomarkers could facilitate the development of genetic screens capable of indicating the likelihood or cause of abnormal pubertal development.

Biomarkers of bone growth and mineralization. During puberty, bone growth and mineralization, as well as bone turnover, increase dramatically. Current data suggest biochemical markers of bone remodeling may be useful in the clinical investigation of bone turnover in children in health and disease.

van Coeverden et al. (2002) demonstrated that bone metabolism markers are good predictors of bone mass in boys and of bone mass increase in both sexes. Previously, Sen et al. (2000) showed that during the male pubertal growth spurt, there is a relationship between bone remodeling and increasing serum osteocalcin and alkaline phosphatase levels. Peak levels occur when sexual maturation reaches Tanner stage 4 and are associated with the rapid growth in height. As sexual maturation reaches stage 5, levels gradually decrease with growth maturation, and their levels decline to the level of adults. Other studies have examined urinary excretion of specific bone resorption markers as a function of adolescent growth stages. In a prospective longitudinal study of urinary excretion of a bone resorption marker [collagen type I N-telopeptides (NTx)] in adolescents, Bollen (2000) found that for both males and females, the excretion of NTx was correlated with the changes in growth rate during adolescence. The expression of other collagens, including procollagen type I C-terminal propeptide (PICP), the cross-linked C-terminal telopeptide of type I collagen (ICTP), and procollagen type III N-terminal propeptide (P3NP), also demonstrates some correlation with pubertal development (Crofton et al. 1997).

Hormonal biomarkers of puberty. Puberty has been defined as

> . . . a maturational process of the hypothalamus-pituitary-gonadal axis, resulting in growth and development of the genital organs and, concomitantly, in physical and psychological changes towards adulthood leading to the capacity to reproduce. (Delemarre-van de Walle 2002)

This definition highlights the importance of the sex steroids and other hormones in the process and the consequent utility of these hormones as biomarkers of pubertal development.

Increased secretion of adrenal androgens occurs in the earliest stages of puberty under the control of the hypothalamus–pituitary–adrenal axis. These hormones cause pubic and armpit hair to develop and sensitize the androgen receptors of the hypothalamus and the pituitary, eventually leading to the activation of the HPG axis and the initiation of puberty. The main hormone involved in the regulation of puberty is gonadotrophin-releasing hormone (GnRH). Produced in the hypothalamus, GnRH stimulates the production and release of both LH and follicle-stimulating hormone (FSH) from the pituitary. However, it is difficult to measure levels of GnRH, as it secreted into portal circulation and transported directly to the pituitary. Furthermore, it has a short half-life of only 4–8 min (Redding et al. 1973a, 1973b). Thus, the onset of pubertal development is usually measured through hormones regulated directly or indirectly by GnRH, including the gonadotrophins (LH, FSH) and sex steroid hormones (testosterone and estrogen). The onset of puberty sees an increase in the levels of LH. At first this only occurs during the night and is associated with increases in testosterone (boys) and estrogen (girls) the following morning. Because all three of these hormones can be robustly and inexpensively measured in urine, these initial increases can be used as early biomarkers of pubertal onset (Wu et al. 1993). In addition to the gonadotrophins and sex steroid hormones, there are a number of other hormones whose regulation and level of expression appear to be closely linked with pubertal development. These include the following:

Mullerian inhibiting substance. Mullerian inhibiting substance (MIS) (also known as anti-Mullerian hormone), a gonadal peptide hormone, is a member of the transforming growth factor-beta family and an important factor for male sex differentiation. It is produced by Sertoli cells from the time of fetal sex differentiation to puberty and is among the best characterized of Sertoli cell products, making it a good biomarker for the pathophysiological state of such cells. Because of the thorough characterization of MIS levels, it is possible that serum MIS could be used to evaluate pubertal onset. MIS values for males rise rapidly during the first year of life (uniformly measurable in all prepubertal boys), are highest during late infancy, then gradually decline until puberty (Lee et al. 1996). In normal testes the switchoff of MIS expression is usually associated with the terminal differentiation of Sertoli cells and the appearance of primary spermatocytes (Rajpert-De Meyts et al. 1999) make it a good candidate for staging pubertal development in boys. MIS levels correlate better with developmental age than with chronological age, and males with delayed puberty have elevated levels. In contrast MIS is undetectable in most prepubertal female subjects and is expressed in the ovary only until the end of fetal life. The main drawback with using MIS as a measure of pubertal development is that current assays have only been successfully tested on blood serum. Given that sampling frequency of blood in a large longitudinal study is unlikely to be high enough to provide a detailed developmental timeline, it appears that a urine-based assay needs to be developed if this test is to be seriously considered for application as a biomarker of pubertal development. To date there has been only one published study involving the measurement of MIS in urine (Hudson et al. 1990), and levels were found to be 20–140 times less than those found in serum.

Inhibins. The inhibins are peptides, mainly of gonadal origin, that suppress FSH production. Like MIS they offer a potential measure of reproductive development (see review by Raivio and Dunkel 2002). This has been determined through characterization of their normal expression levels. Inhibin B expression, for example, is high in infant boys but declines in concert with the increase in gonadotrophins, reaching a nadir at 6–10 years of age (Crofton et al. 2002). Byrd et al. (1998) consequently suggested that inhibin B could potentially be used as a biomarker in the diagnosis of cryptorchidism and precocious puberty. Studies by Brugo-Olmedo et al. (2001) suggested that serum inhibin B levels might also be a reliable marker of the presence of testicular spermatozoa in patients with nonobstructive azoospermia. If this is confirmed, it could be used as another biomonitoring approach to assessing pubertal onset in boys. In boys between Tanner stages 1 and 2, serum inhibin B levels again increase but then plateau. Serum inhibin A levels in human males are below detection limits, but both inhibin A and B are measurable in girls. Inhibin A and B are found at different levels in girls depending on pubertal stage (Foster et al. 2000; Sehested et al. 2000), suggesting that significant changes in serum concentrations of these and other FSH-regulatory peptides accompany the onset and progress of puberty and should be investigated as possible alternative staging markers for pubertal development. Although inhibins, like MIS, are promising biomarkers of pubertal development, they suffer from the same disadvantage—almost all studies on inhibins have relied on blood serum as the biological source material. This is because only inhibin A has been detected in urine, and then only in pregnant women (Wang et al. 1999).

Leptin. Leptin, an adipocyte hormone important in regulating energy homeostasis, interacts with the reproductive axis at multiple sites, with stimulatory effects at the hypothalamus and pituitary, and inhibitory action on the gonads. Leptin appears to be a pleiotrophic hormone affecting many different tissues in the body. Evidence is accumulating that it potentially affects the regulation of GnRH and LH secretion, puberty, pregnancy, and lactation (reviewed by Brann et al. 2002). Normal leptin secretion is necessary for normal reproductive function to proceed, and leptin may be a signal allowing for the point of initiation of and progression toward puberty (Mantzoros et al. 1997). Both prepubertal boys and girls show a progressive increase of leptin levels until Tanner stage 2. At the initiation of puberty there is a divergence in serum leptin concentrations between boys and girls. In boys, concentrations increase and then markedly decrease to prepubertal concentration levels. In girls, however, levels continue to increase (Roemmich and Rogol 1999). Although leptin thus appears to offer a possible biomarker of pubertal onset and development, further studies are first required to clarify its role and relationship.

Antisperm autoantibodies. Another possible target for biomarker investigation in adolescent males is antisperm autoantibodies (ASA). Although few human studies have been conducted in this area, it appears that prepubertal presence of ASA often occurs among patients with urogenital pathology. For example, Kurpisz et al. (1996) found significant antibody activity to human sperm in sera samples from prepubertal boys with testicular failures (cryptorchid or mobile testis). Of 26 ASA-positive boys examined by Sinisi et al. (1997), 24 had genital tract abnormalities (cryptorchidism, testicular torsion, hypospadias), and two had leukemia with testicular infiltration. However, genital tract abnormalities do not always lead to the production of ASA. In a study of 159 prepubertal boys suffering from various testicular pathologies, Lenzi et al. (1991) found only 21% of the patients' sera showed antibody activity against antigens on the sperm of healthy fertile donors. The presence of serum antisperm antibodies is normally considered problematic, as it is a good indicator that fertility will be or is compromised. Indeed, Check et al. (2002) suggested that this test be performed as part of routine semen analysis. However, we retain a poor understanding of the profile of ASA that leads to antibody-mediated male infertility, thus warranting further studies into this area.

Spermaturia. The evaluation of sperm in urine (spermaturia) has been used previously to assess the age of onset of spermarche. Spermatogenesis can begin before any other signs of puberty (Nysom et al. 1994). This conclusion was obtained from spermaturia studies in two normal boys with testicular volumes of 3 mL and no other signs of puberty and indicates that the definition of start of puberty as testicular volumes of 4 mL or more may be too rigorous. Mol et al. (2002) discovered highly significant associations between spermaturia and both Tanner stage and testicular size, hence supporting the validity of spermaturia as a useful indicator of puberty. They did, however, find that a substantial rate of false negatives are likely to be included using this method. Spermaturia is a more common and regular event during early and midpuberty than in more mature subjects, but one problem preventing its regular use as an indicator of puberty is the intermittent occurrence of sperm-negative urine samples. The incorporation of spermaturia as a test for puberty in a large sample group would give this test more power to determine the distribution of the age of onset. Research is needed to develop a practical approach for doing this. Conceptually, it should be possible to develop a home urine collection system that would include a filter upon which sperm would be trapped. The system could be used at regular intervals and the filters saved for later extraction and identification of sperm.

Specimen Collection

There are many molecular and cellular biomarkers that can potentially be used to measure pubertal development. However, it is also clear that the nature of these markers is such that biospecimens must be obtained from study participants. Biospecimen collection is thus one of the main issues to consider in determining the molecular and cellular biomarkers that can be measured in a study. The use of biospecimens in an epidemiologic study, although often necessary, can be confounded by many different events. For example, sample harvest timing and procedures, as well as storage and extraction protocols, may affect the expression or integrity of some biomarkers, particularly those that are cellular or molecular in nature. Furthermore, the level of certain biomarkers, such as serum hormones, may naturally fluctuate on a daily or even hourly basis. Time is also a factor when measuring certain analytes in blood and urine. Because of instability, some analytes must be measured within a few hours of collection if the measurement is to be useable. In addition, the appropriate use of preservatives, storage media, and storage and transportation temperatures must also be considered. Sampling procedures must therefore be planned and conducted to reduce the variation that can arise from the use of different protocols. This will require the development of standardized protocols for collection, storage, and transportation of samples. For example, samples might be obtained during school hours under appropriate medical supervision and with parental approval. This would save travel to the clinic by parents accompanying their child and enable study workers to access large groups of children at about the same time of day. Another approach is to develop methods for home collection of biospecimens (Rockett et al. 2004).

Where indicators of puberty are being measured, it is clear that the entire cohort will comprise (pre-) adolescents in varying stages of development. As such, study participation and compliance with specimen collection are key issues underlying the success of such a study. This necessitates the identification of biomarkers that can be derived through noninvasive methods such as anatomical measurements or readily accessible biospecimens such as urine, saliva, and blood. Many robust methods are already available, such as the measurement of urinary hormones (Lasley et al. 1994) or DNA analysis from buccal cells (Lum and Marchand 1998). An emerging paradigm for biomonitoring studies in children is the use of surrogate tissue analysis (STA) (Rockett 2002; Rockett et al. 2002). This approach has yet to be verified but may prove useful in 5–10 years' time. In the STA approach an easily obtained tissue (i.e., biospecimen) is used to provide information about an inaccessible target tissue. For example, one might examine gene or protein expression in a patient's peripheral blood lymphocytes to determine if there is altered development or function in their uterus (Reddy et al. 2001; Rockett et al. 2002). Much work is still required to verify this approach, but many see it as a valid and useful new paradigm.

Conclusions

Over the past 15 years or so, there has been a surging interest in children's health and development. This has led to the formation and increasing expansion of a pediatric research network (American Academy of Pediatrics 2003) and the instigation of a large number of longitudinal and cross-sectional cohort studies: the Northern California Childhood Leukemia study, 1995–2003 (2003); the U.S.–Mexico Border Children's Pesticide Exposure study, 1996–2004 (2003); the Minnesota Children's Pesticide Exposure study, 1997–present (2003); the NCS, 2000–present (2003); and the National Health and Nutrition

Table 1. Summary of biomarkers for assessing pubertal onset and progression in males.

Biomarker of pubertal onset	Biomarker of pubertal progression
Body composition	Body composition
	Bone metabolism markers
	Bone resorption markers
	Fundamental voice frequency
	FSH
Inhibin B	Inhibin B
Leptin	Leptin
	LH
MIS	
Pubic hair type and extent	Pubic hair type and extent
Penis size	Penis size
	Skeletal growth rate
Spermaturia	
Testis size	Testis size
Testosterone	Testosterone

Table 2. Summary of biomarkers for assessing pubertal onset and progression in females.

Biomarker of pubertal onset	Biomarker of pubertal progression
Body composition	Body composition
Bone resorption markers	Bone resorption markers
Estrogen	Estrogen
FSH	FSH
	Inhibin A
Inhibin B	Inhibin B
Leptin	Leptin
LH	LH
	Menarche
Pubic hair type and extent	Pubic hair type and extent
Skeletal growth rate	Skeletal growth rate
Thelarche	Thelarche

Examination Survey (2003). Data from these research centers and studies have provided increasing evidence for links between environmental exposures and pubertal development (e.g., blood lead and alterations in pubertal development in girls (Seleven et al. 2003; Wu et al. 2003). It is likely that over the next 20 years or so we will see a shift toward even more intense monitoring of child health, especially as the high-profile NCS gains momentum and begins to generate data.

Puberty is the most dramatic process in child development. Unfortunately, the multiple characteristics of puberty have made it difficult to determine an all-encompassing definition in terms of the biochemical and physiological changes that occur. Defining puberty has also been confounded by the secular trend in growth. Although most agree that puberty is the time at which onset of sexual maturation occurs, there are clearly various biomarkers (anatomical and biochemical) that can be used to define this onset (Tables 1 and 2). Puberty also occurs at different rates and in different orders in different people, depending on genetic and environmental components. For example, in African-American girls, pubic hair, on average, seems to appear slightly ahead of breast development. In contrast the first manifestation of puberty in white girls is usually breast development. Thus, an accurate clinical definition of puberty must be developed that acknowledges any of the various biomarkers that can appear first. This need not be specific but might include the appearance of any one or small number of carefully selected biochemical and/or anatomical biomarkers. In many cases these will be different between males and females, as they are physiologically distinct in many respects. Identification and verification of such biomarkers across the span of human diversity can only be determined through the longitudinal study of large cohorts.

We are still far from completely understanding most normal development processes and the bounds within which "normality" falls. Thus, before we can understand a disease processes, we must first determine what constitutes normal pubertal development in today's society, namely, what is an acceptable range for normal development and what constitutes developmental "unevenness" versus pathology. This educational process, encompassing laboratory research and human epidemiologic studies, should reveal new biomarkers that are both robust and sensitive.

In some cases it seems unlikely that current biomarkers of reproductive development and health will be displaced, at least in the near to mid-future. For example, the use of Tanner staging is a simple and inexpensive method of assessing pubertal development. Though it has its faults, such as being a subjective method, it is probably still the best choice for use in large longitudinal cohort studies such as the NCS. However, given the multiple and extensive biochemical and anatomical changes that occur during puberty, it is highly likely that molecular and cellular markers, which can be measured without subjectivity, will play an increasingly important role in assessing pubertal development. Only a few such biomarkers are known today, but it seems likely that many more are awaiting discovery and characterization. It is our expectation that at least some of these will enable health care professionals to better follow the pubertal process in future generations of children and ultimately pave the way for new approaches in disease identification and management.

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