

PAULIINA UTRAINEN

Premature Adrenarche

Clinical and Metabolic Features

Doctoral dissertation

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Series Editors: Professor Raimo Sulkava, M.D., Ph.D.
School of Public Health and Clinical Nutrition

Professor Markku Tammi, M.D., Ph.D.
Institute of Biomedicine, Department of Anatomy

Author's address: Clinical Research Center
Pediatrics
University of Kuopio
FI-70211 KUOPIO
FINLAND
Tel. +358 40 355 2270
Fax +358 17 172 410
E-mail: pauliina.utriainen@uku.fi

Supervisors: Professor Raimo Voutilainen, M.D., Ph.D.
Institute of Clinical Medicine, Pediatrics
University of Kuopio

Docent Jarmo Jääskeläinen, M.D., Ph.D.
Institute of Clinical Medicine, Pediatrics
University of Kuopio

Reviewers: Docent Päivi Tapanainen, M.D., Ph.D.
Department of Pediatrics and Adolescence
University of Oulu

Docent Tiina Laine, M.D., Ph.D.
Hospital for Children and Adolescents
University of Helsinki

Opponent: Professor Stefan A. Wudy, M.D., Ph.D.
Justus Liebig University
Giessen, Germany

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ABSTRACT

Adrenarche means the maturational increase in the adrenocortical production of androgens that gradually begins in mid-childhood. Premature adrenarche (PA), defined as the appearance of signs of adrenarche before the age of 8 yrs in girls and 9 yrs in boys, has recently been connected with adverse metabolic features and low birth weight. Thus far, studies on PA have almost exclusively concentrated on girls with premature pubarche (=premature appearance of pubic hair).

The purpose of this cross-sectional study was to describe the typical clinical features of PA and their correlation with circulating adrenal androgen (AA) concentrations in a sample of prepubertal Finnish children with (n=73) and without (n=98) signs of PA. Another aim was to study the associations of PA with prepubertal growth, body composition and components of the metabolic syndrome.

Among the prepubertal children with suspected PA, there were nine girls in whom the serum AA concentrations were normal for age. Thus, biochemically confirmed PA was found in 64/73 subjects. In the control group, there were 36 children whose serum androgen concentrations exceeded the cut-off level for biochemical adrenarche (DHEAS ≥ 1 $\mu\text{mol/l}$) at the time of examination. Serum AA concentrations were highest in the subjects with pubic and/or axillary hair but also higher in children with other signs of androgen action than in controls ($P < .02$ for serum DHEA, DHEAS and androstenedione concentration).

A higher percentage of the girls with clinical signs of adrenarche (n=63) than of the control girls (n=80) were obese (BMI ≥ 95 percentile; 30 vs. 16%, $P = .05$) and had “childhood metabolic syndrome (cMBS)” (modified WHO MBS criteria; 16 vs. 5%, $P = .03$). Of the metabolic risk factors, only serum insulin concentrations during the 2-h oral glucose tolerance test and insulin sensitivity indices differed significantly between the study groups after adjustment for weight. The metabolic alterations were more frequent in the PA girls with pubic or axillary hair than in those with other androgenic signs.

The girls with biochemically confirmed PA (n=54) had experienced more rapid early childhood growth than the nonPA control girls (n=52). Girls with PA were taller at the current examination (median age 7.6 yr; height SDS 1.2 vs. 0.0, $P < .001$), and they were significantly taller already at the age of 1 yr. The differences in height were significant even when adjusted for BMI SDS and parent-specific expected height. There were no significant differences in the median birth weight or length SDS between the PA and control girls (-0.19 vs. -0.09, $P = .63$ and -0.12 vs. 0.23, $P = .13$, respectively).

The children with PA (n=64) had higher areal bone mineral density in femoral neck (FN) and in lumbar spine (LS) compared with their nonPA controls (n=62) as measured by dual-energy X-ray absorptiometry. However, the differences in bone mineral density were not significant when adjusted for the bone size or the height of the child. In the PA group, age, gender, height SDS, BMI SDS and soft lean mass (%) but not DHEAS or 25-hydroxyvitamin D concentrations were independently associated with areal bone density in LS in a linear regression model.

In conclusion, there is a wide phenotypic variability in PA. Some children have AA concentrations inconsistent with the signs of androgen action, reflecting individual sensitivity to androgens. Many of the children with PA are overweight and have weight-associated adverse metabolic features. The metabolic consequences may vary between the subgroups of children with signs of PA. Girls with PA have enhanced early growth, suggesting an early beginning of the phenomenon. Low birth weight does not seem to play a significant role in the pathogenesis of PA in our population.

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Medical Subject Headings: Adrenarche/physiology; Androgens; Birth Weight; Body Composition; Body Height; Body Weight; Bone Density; Child; Child Development; Glucose Tolerance Test; Dehydroepiandrosterone; Cross-Sectional Studies; Growth/physiology; Insulin/blood; Metabolic Syndrome X; Metabolism; Phenotype; Puberty, Precocious/physiopathology; Risk Factors; Vitamin D/analogs & derivatives

Great are the works of the Lord;
They are studied by all who delight in them
Psalm 111:2

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Kuopio, September 2009

Pauliina Utriainen

LIST OF ABBREVIATIONS

AA	Adrenal androgen
$\Delta 4A$	Androstenedione
ACTH	Adrenocorticotrophic hormone
ALP	Alkaline phosphatase
BA	Bone age
BL	Birth length
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BP	Blood pressure
BW	Birth weight
CAH	Congenital adrenal hyperplasia
CI	Confidence interval
cMBS	Childhood metabolic syndrome
CV	Coefficient of variation
CVD	Cardiovascular disease
CYP	Cytochrome P450
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DNA	Deoxyribonucleic acid
11DOF	11-Deoxycortisol
DXA	Dual-energy X-ray absorptiometry
E	Epinephrine
F	Cortisol
FN	Femoral neck
FOH	Functional ovarian hyperandrogenism
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment for insulin resistance
HPLC	High performance liquid chromatography
3 β -HSD	3 β -hydroxysteroid dehydrogenase
IGF-I	Insulin-like growth factor I
IS	Insulin sensitivity
ISI _{comp}	Insulin sensitivity index

LC-MS	Liquid chromatography–mass spectrometry
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LRP5	Low-density lipoprotein (LDL) receptor-related protein 5
LS	Lumbar spine
LSD	Least significant difference
MBS	Metabolic syndrome
MCR _{est}	Metabolic clearance rate of glucose
NCEP ATPIII	National Cholesterol Education Program, Adult Treatment Panel III
NC21OHD	Non-classical 21-hydroxylase deficiency
NE	Norepinephrine
nonPAH	Subject(s) with neither pubic nor axillary hair
21OHD	21-hydroxylase deficiency
17OHP	17-hydroxyprogesterone
25-OHD	25-hydroxyvitamin D
OGTT	Oral glucose tolerance test
PA	Premature adrenarche
PAH	Subject(s) with pubic or axillary hair
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PP	Premature pubarche
PSEH	Parent-specific expected height
QCT	Quantitative computed tomography
RIA	Radioimmunoassay
SAA	Signs of androgen action
SD	Standard deviation
SDS	Standard deviation score
SGA	Small for gestational age
SHBG	Sex hormone binding globulin
SNP	Single nucleotide polymorphism
SULT2A1	DHEA sulfotransferase
TC	Total cholesterol
TG	Triglycerides
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

This work is based on the “Premature adrenarche” - study project with the following original publications:

- I** Utriainen P, Voutilainen R, Jääskeläinen J. Continuum of phenotypes and sympathoadrenal function in premature adrenarche. *Eur J Endocrinol* 2009; 160:657-665.
- II** Utriainen P, Voutilainen R, Jääskeläinen J. Girls with premature adrenarche have accelerated early childhood growth. *J Pediatr* 2009; 154:882-887.
- III** Utriainen P, Jääskeläinen J, Romppanen J, Voutilainen R. Childhood metabolic syndrome and its components in premature adrenarche. *J Clin Endocrinol Metab* 2007; 92:4282-4285.
- IV** Utriainen P, Jääskeläinen J, Saarinen A, Vanninen E, Mäkitie O, Voutilainen R. Body composition and bone mineral density in children with premature adrenarche, and the association of LRP5 gene polymorphisms with bone mineral density. *J Clin Endocrinol Metab* 2009; 94, in press.

In addition, some unpublished data are presented.

CONTENTS

1. Introduction	15
2. Review of the literature	17
2.1. Adrenal androgen production and action	17
2.1.1. Adrenal cortex	17
Adrenocortical zone	17
Development from fetal to adult adrenal cortex	17
Steroid synthesis in the adrenal cortex	19
Interaction between the adrenal cortex and medulla	22
2.1.2. Androgen receptor and the peripheral regulation of androgen action	23
2.1.3. Androgens in the target tissues	25
2.1.4. Adrenarche	28
Definition of adrenarche	28
Clinical signs of adrenarche	29
Initiators and regulators of adrenarche	30
2.2. Premature adrenarche	31
2.2.1. Definition, clinical findings and prevalence	31
2.2.2. Differential diagnosis	33
2.2.3. Growth and timing of puberty	35
2.2.4. Association of premature adrenarche with metabolic characteristics	37
The metabolic syndrome	37
What is the metabolic syndrome?	37
What is insulin resistance?	39
Components of the metabolic syndrome in premature adrenarche	40
Ovarian and adrenal hyperandrogenism	42
Definition of functional ovarian hyperandrogenism and polycystic ovary syndrome	42
Ovarian and adrenal hyperandrogenism in premature adrenarche	43
Insulin-like growth factor system in premature adrenarche	45
Genetic variation and metabolic sequelae in premature adrenarche	45
2.2.5. Bone mineral density in premature adrenarche	46
3. Aims of the study	47
4. Methods and design	48
4.1. Study population	48
4.1.1. Subjects with signs of androgen action (SAA group)	48
4.1.2. Subjects with premature adrenarche (PA group)	48
4.1.3. Control group	49
4.1.4. Study settings	49
4.2. Clinical evaluation	51
4.3. Growth assessment	52
4.4. Biochemical analyses	52
4.4.1. Blood sampling, glucose tolerance test and ACTH test	52
4.4.2. Assays	53
4.4.3. Determination of insulin sensitivity and definition of metabolic syndrome	55

4.4.4. Assessment of steroidogenic enzyme activities	57
4.4.5. Low density lipoprotein (LDL) receptor related protein 5 (<i>LRP5</i>) genotyping	57
4.5. Measurement of bone mineral density and body composition	58
4.6. Data analysis	59
5. Results	60
5.1. Family and patient history	60
5.1.1. Family	60
5.1.2. Gestation and birth	60
5.1.3. Appearance of signs of androgen action	62
5.2. Clinical characteristics	62
5.3. Growth	64
5.4. Serum androgen concentrations and their correlation with clinical characteristics	69
5.5. Other steroids and steroid ratios	71
5.6. Components of the metabolic syndrome	71
5.7. Other endocrine-metabolic characteristics	76
5.8. Bone mineral density and biochemical bone markers	78
6. Discussion	82
6.1. Subjects	82
6.2. Signs of androgen action and androgen concentrations	83
6.3. Growth and body composition	86
6.4. Features of the metabolic syndrome	89
6.5. Other biochemical findings	94
6.6. Bone mineral density	95
6.7. General aspects	98
6.8. Future perspectives	99
7. Summary and conclusions	100
8. References	102
9. Original publications	119

1. INTRODUCTION

Adrenarche means the maturational change in the adrenal cortex that leads to increasing production of adrenal androgens usually in mid-childhood. Adrenarche was first discovered by Albright and Talbot already in the 1940s, when they measured 17-ketosteroids in urinary samples and connected the increase in androgen metabolites to the emerging signs of androgen action (Albright 1947). Premature adrenarche, on the other hand, refers to precocious timing of adrenarche, which can manifest as the appearance of adult type body odor, oily hair, acne and pubic or axillary hair before the age of 8 years in girls and 9 years in boys.

The term premature adrenarche has been inconsistently used. Some have considered it a synonym for premature pubarche – the most prominent androgenic sign in prepubertal children - while others have used the term for all prepubertal hyperandrogenic signs. Still other authors suggest that premature adrenarche refers to the biochemical finding, i.e. increased adrenal androgen production. Most studies on premature adrenarche have examined only children with premature pubarche, whereas little is known about other signs of androgen action in prepubertal children.

Although the existence of adrenarche has been known for decades, the initiators of the increased androgen production during adrenarche remain poorly defined. Adrenarche is known to be regulated independently of gonadarche, but no specific trigger has been identified. Accordingly, the underlying mechanisms that lead to the premature timing of adrenarche are also obscure. In 1998, Ibáñez and co-workers reported that premature pubarche is linked to low birth weight (Ibáñez *et al.* 1998a). A few years later, an Australian study group found that premature pubarche was associated with small birth weight but also with rapid weight gain and overweight in childhood (Neville and Walker 2005).

Premature adrenarche has traditionally been regarded as a benign variation of normal development. However, recent studies have connected premature adrenarche with several

unfavorable metabolic features: increased risk of ovarian hyperandrogenism (Ibáñez *et al.* 1993), hyperinsulinism and decreased insulin sensitivity (Ibáñez *et al.* 1996, Oppenheimer *et al.* 1995), increased triglyceride and decreased HDL cholesterol concentrations (Ibáñez *et al.* 1998b). Most of those studies have been conducted by a single study group with a Spanish female population, in which all the subjects have presented with premature pubarche. Based on their studies, the Spanish authors have also suggested that premature adrenarche and the related hyperinsulinism could be treated by insulin sensitization with metformin to prevent the unfavorable metabolic consequences of premature adrenarche (Ibáñez *et al.* 2000a).

The purpose of the present study was to describe the clinical features of an unselected group of prepubertal children with signs of premature adrenarche, and to uncover the possible associations of premature adrenarche with birth measures, childhood growth, body composition, bone mineral density and metabolic indices.

2. REVIEW OF THE LITERATURE

2.1. Adrenal androgen production and action

2.1.1. Adrenal cortex

Adrenocortical zones

The adrenal gland consists of two separate organs: the medulla and cortex. The medulla produces catecholamines and is a part of the peripheral sympathetic system. The adult adrenal cortex contains three layers with distinct histology and function (reviewed in Miller *et al.* 2008). The outer layer is called the zona glomerulosa (ZG) and it produces mainly the mineralocorticoid aldosterone. The innermost layer the zona reticularis (ZR) and the middle layer the zona fasciculata (ZF) produce androgens and glucocorticoids. ZF is the main source of cortisol, whereas ZR produces adrenal androgens dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS) and androstenedione. Mineralocorticoids regulate Na-K balance and blood pressure, and their production is under the control of renin-angiotensin system. The production of glucocorticoids is regulated by pituitary adrenocorticotropin (ACTH). ACTH stimulus is also required for adrenal androgen production, but otherwise the regulation of their synthesis remains poorly identified.

Development from fetal to adult adrenal cortex

The fetal adrenal cortex differs from the adult adrenal cortex in structure and function (reviewed in Seron-Ferre and Jaffe 1981, Mesiano and Jaffe 1997). It is comprised of three layers: outer definitive zone (DZ), innermost fetal zone (FZ) and transitional zone (TZ). Functionally, these three layers have distinct steroidogenic enzyme expression profiles with distinct roles in fetal adrenal steroidogenesis. DZ is similar to the adult ZG; it expresses the enzymes needed for the

synthesis of mineralocorticoids. The steroidogenic enzyme expression patterns of TZ and FZ resemble those of ZF and ZR in the adult adrenal cortex, respectively (Mesiano *et al.* 1993). The main difference between the fetal and post-natal adrenal cortex is the predominance of the androgen-producing FZ in the fetal adrenal gland both functionally and in size. During the fetal period, the adrenal cortex produces high amounts of DHEAS, an androgen specific for adrenals, for placental estrogen production.

After birth, FZ atrophies, and the production of androgens in the adrenal cortex declines rapidly within few months (FIGURE 1) (Bech *et al.* 1969). The regulation and mechanism of this fetal zone involution is poorly understood. The production of DHEAS remains low through infancy and early childhood. It has been considered that at approximately 6 to 8 years of age the production of adrenal androgens begins to increase again (Smith *et al.* 1975, de Peretti and Forest 1976, Korth-Schutz *et al.* 1976a, Reiter *et al.* 1977), and peak DHEAS concentrations are reached during early adulthood (FIGURE 1). Recent studies suggest that the maturational process of adrenal cortex is more gradual, as they showed that both serum adrenal androgen (AA) concentrations and the urinary excretion rate of androgen metabolites rise consistently from the age of 3 years on (Palmert *et al.* 2001, Remer *et al.* 2005). According to histological and immunohistochemical findings, there is only a thin non-continuous layer of ZR in the adrenal cortex between 4 months and 5 years of age (Gell *et al.* 1998). In contrast, ZG and ZF are well developed soon after birth and they produce mineralocorticoids and glucocorticoids at steady rate throughout childhood and adulthood (Kenny *et al.* 1966). ZR develops gradually. This zone becomes continuous usually between 6 and 8 years of age, paralleling the increase in adrenal androgen production. The total volume of the adrenal cortex increases continuously through the childhood, the highest increase occurring at around 13 years of age (Dhom 1973). Along with the appearance of continuous ZR, the expression of some key steroidogenic enzymes changes: 3β -HSD expression decreases, and the 17α -hydroxylase and

17,20-lyase activities of P450c17 (CYP17) enzyme increase (Schiebinger *et al.* 1981, Gell *et al.* 1998, Suzuki *et al.* 2000).

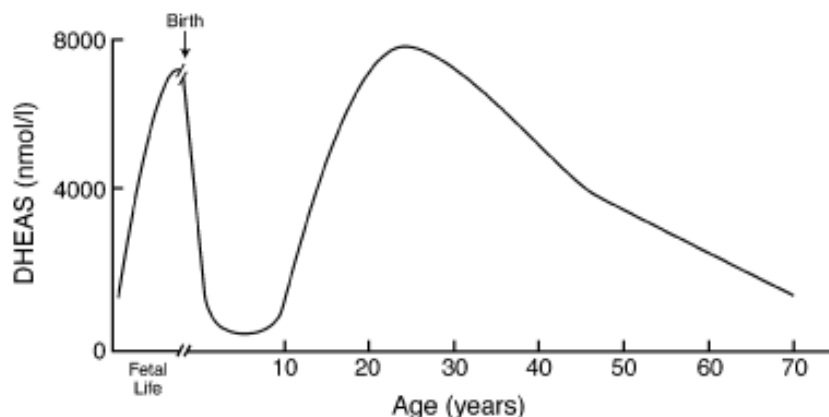


FIGURE 1. Variation in the circulating dehydroepiandrosterone sulfate (DHEAS) concentrations throughout human life.

[Reproduced from Auchus and Rainey (2004) with the permission of Wiley Blackwell]

Steroid synthesis in the adrenal cortex

The pathways of steroid synthesis are depicted in FIGURE 2, and thoroughly reviewed by Miller (1988 & 2009). The steroid production profile of each adrenocortical zone is determined by the expression pattern of the steroidogenic enzymes.

A total of 5 oxidative cytochrome P450 enzymes that regulate steroidogenesis are expressed in the adrenal cortex. These include P450scc, also called CYP11A, isoenzymes P450c11 α and P450c11AS (CYP11B1 and CYP11B2), P450c17 (CYP17), P450c21 (CYP21) and P450aro (CYP19; aromatase). In addition to these cytochrome P450 enzymes, 3 β -hydroxysteroid dehydrogenase (3 β -HSD) type 2 is expressed in adrenal cortex and is involved in the regulation of steroid synthesis. The mitochondrial P450scc enzyme catalyzes the three reactions needed to convert cholesterol to pregnenolone. The P450c11 enzymes, also found in the mitochondria, has the

11 β -hydroxylase, 18-hydroxylase and 18-methyl oxidase activities, enabling the formation of aldosterone from 11-deoxycorticosterone solely in ZG, and cortisol from 11-deoxycortisol. CYP21 catalyzes the 21-hydroxylation of progesterone and 17 α -hydroxyprogesterone (17OHP).

Adrenal androgen production is elicited by the 17 α -hydroxylase and 17,20-lyase activity of P450c17, which catalyze the conversion of pregnenolone to 17 α -hydroxypregnenolone and further to DHEA, respectively. Low activity of 3 β -HSD, another enzyme expressed in the smooth endoplasmic reticulum (SER), also theoretically favors androgen production because of higher availability of steroid precursors for DHEA production (FIGURE 2). High 3 β -HSD activity facilitates the formation of Δ^4 ketosteroids from Δ^5 steroids by catalyzing the conversion of the hydroxyl group to a keto group and the isomerisation of B ring (Δ^5) to A ring (Δ^4), enabling the production of cortisol and aldosterone. Immunohistochemical studies have shown that 3 β -HSD expression decreases in ZR as a child matures and that the expression is low in ZR when compared with that in ZF and ZG in adult adrenal cortex (Gell *et al.* 1998, Suzuki *et al.* 2000).

P450c17 is encoded by a single gene on chromosome 10q24.3. It catalyzes both 17 α -hydroxylase and 17,20-lyase reactions; the absolute and relative activities of these two enzymatic functions determine the rate of cortisol and DHEA production. Thus, P450c17 is a key regulator of adrenocortical steroidogenesis (FIGURE 2). P450c17 is expressed in both ZF and ZR but not in ZG, which produces only 17-deoxy C21 steroids such as progesterone and aldosterone. In ZF, 17 α -hydroxylase activity is present, whereas the 17,20-lyase activity is low. Instead, both of the P450c17-catalyzed reactions occur in ZR, enabling the production of the 17-hydroxy C19 steroid DHEA. The differential regulation of these two catalytic activities in a single enzyme has not been unequivocally defined despite intensive research. First, the abundance of P450 oxidoreductase in relation to P450c17 was suggested to favor 17,20-lyase activity (Lin *et al.* 1993). Then it was demonstrated that phosphorylation of certain Ser/Thr residues on P450c17 protein promotes 17,20-lyase activity (Zhang *et al.* 1995). Strong evidence exists that cytochrome b5 increases 17,20-

activity, probably by facilitating the interaction between P450 oxidoreductase and P450c17 (Katagiri *et al.* 1995, Auchus *et al.* 1998, Suzuki *et al.* 2000). A further support to this view was provided by a recent study in which the increase in the expression rate of b5 paralleled that in the activity of 17,20-lyase in rhesus macaque adrenals, and a co-transfection of a non-steroidogenic cell line with a construct encoding P450c17 and b5 induced a b5 dose-dependent increase in DHEA production (Nguyen *et al.* 2009).

The last step in the synthesis of DHEAS is the sulfonation of DHEA by the sulfotransferase (SULT2A1) enzyme (FIGURE 2). Unlike other enzymes involved in the adrenal steroid synthesis, SULT2A1 is expressed in the adrenals, but not in the gonads. The expression of SULT2A1 increases in ZR from 5 years of age onward (Suzuki *et al.* 2000).

Interaction between the adrenal cortex and medulla

The adrenal cortex and medulla have traditionally been regarded as two separately functioning organs because of their differential embryonic origin and role in physiology. However, these two adrenal organs are now known to interact by paracrine and endocrine mechanisms (Bornstein *et al.* 1997). The basis for this view are the immunohistochemical studies showing a close contact between chromaffin and cortical cells in the adrenal gland and thereby providing a direct route for their interaction (Bornstein *et al.* 1994). In patients with congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency, the synthesis of gluco- and mineralocorticoids is reduced, and that of androgens is increased in the adrenal cortex. These patients also have low adrenomedullary epinephrine production, and the medullary hypofunction is most pronounced in those with most severe deficits in cortisol production (Merke *et al.* 2000). Furthermore, both adrenocortical and adrenomedullary function may be augmented in children born small for gestational age (SGA), as measured by increased circulating DHEAS and epinephrine concentrations, respectively (Tenhola *et al.* 2002). Thus, both *in vitro* and *in vivo* studies suggest cross-talk between the adrenal cortex and medulla.

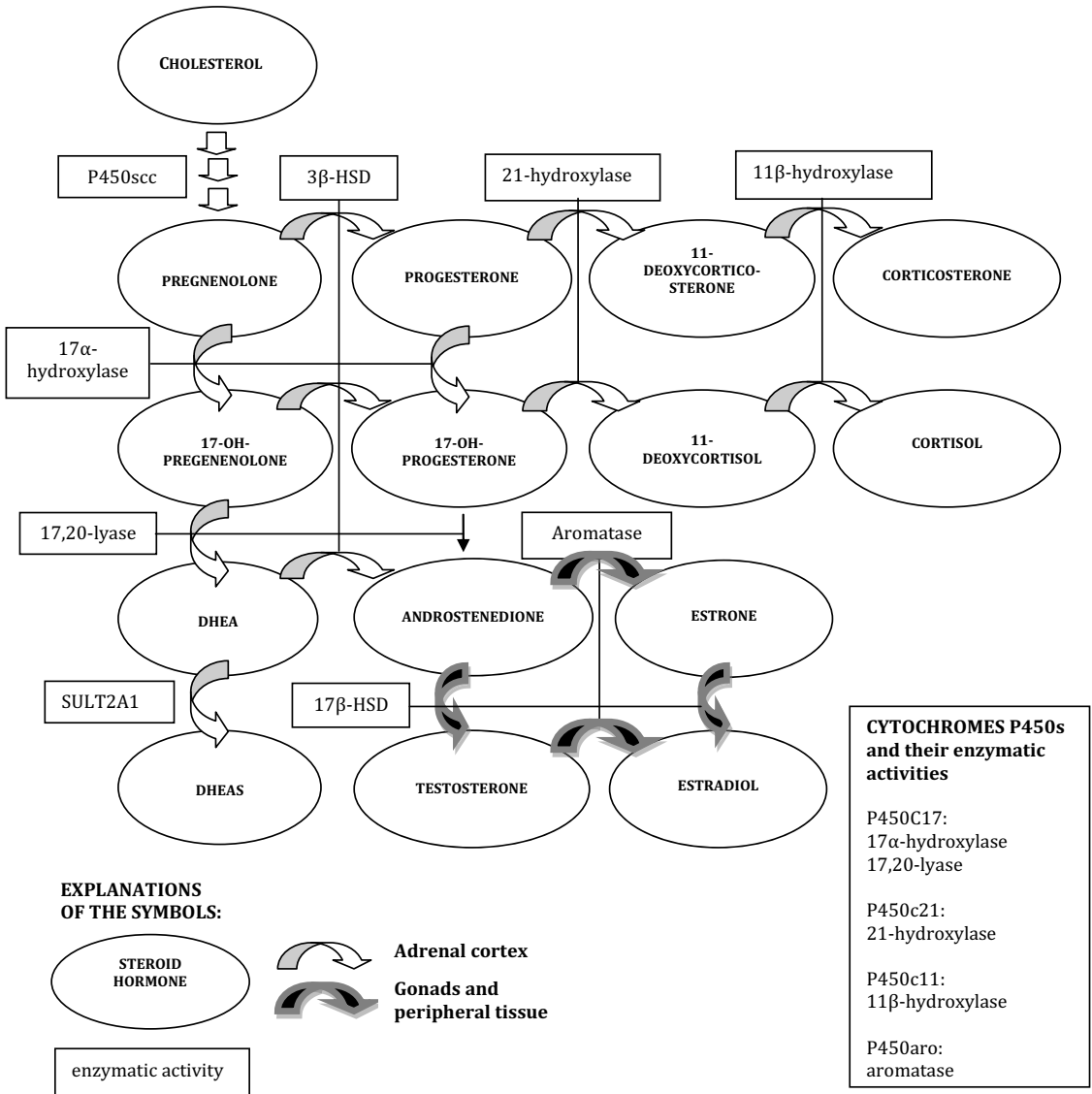


FIGURE 2. Steroid synthesis pathways in the adrenal cortex. (Modified from Miller 2009)

2.1.2. Androgen receptor and the peripheral regulation of androgen action

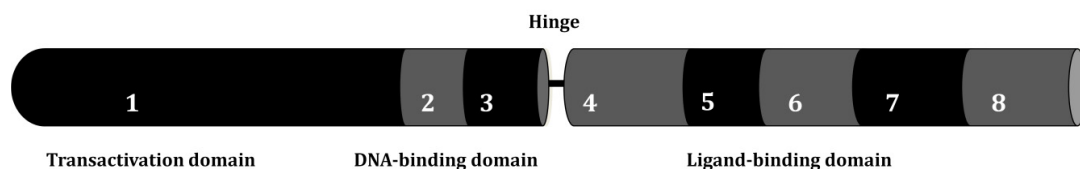


FIGURE 3. Androgen receptor (AR). Numbers 1 to 8 refer to AR gene exons.

The effect of androgens is mediated via androgen receptor (AR) in target tissues. AR is expressed in several organs, including bone, muscle, fat, brain and skin (Blauer *et al.* 1991, Choudhry *et al.* 1992, Abu *et al.* 1997, Beier *et al.* 2005). AR is a ligand-dependent transcription factor that interacts directly with the target genes like the other members of the steroid hormone receptor superfamily. The AR gene is located on the X-chromosome at Xq11-12 (Brown *et al.* 1989) and consists of the N-terminal transactivating domain, DNA-binding domain and C-terminal ligand binding domain (Brinkmann *et al.* 1989). Functional AR is essential for androgen action as demonstrated in complete androgen insensitivity syndrome (CAIS), where loss-of-function mutations of the AR gene cause a female phenotype in XY-karyotype subjects (Hughes and Deeb 2006). The responsiveness of each target tissue to androgens is determined by the relative and absolute abundance of functional AR. The sensitivity of AR can also vary. Shorter CAG repeat number in AR gene has been linked with increased sensitivity of AR in both *in vitro* studies and in humans with different hyperandrogenic conditions (Chamberlain *et al.* 1994, Mifsud *et al.* 2000, Ibáñez *et al.* 2003a). Also the preferential inactivation of the longer, and thus less active AR allele on the X-chromosome (Vottero *et al.* 1999) and the lower rate of methylation of the AR gene (Vottero *et al.* 2006) can increase the responsiveness to androgens by modulating both the sensitivity and abundance of AR.

The adrenal androgens, DHEA, DHEAS and androstenedione ($\Delta 4A$), are also called weak androgens, because they need to be converted to more potent AR ligands such as testosterone and dihydrotestosterone (DHT) to activate AR. In the skin, 17β -HSD and 5α -reductase enzymes catalyze the conversion of DHEA and $\Delta 4A$ to testosterone and DHT by 3β -HSD. Thus, the expression and activity of these enzymes can modulate the effect of androgen action in the target organs. In general, 5α -reductase activity is high in the apocrine sweat glands. In patients with excessive odor, the concentration of DHT is higher than that of testosterone in the apocrine sweat glands (Kurata *et al.* 1990). In men, 90% of the circulating testosterone is produced by the testes, but in women, half of the testosterone is derived from peripheral conversion of adrenal 17-ketosteroids (DHEA, DHEAS and $\Delta 4A$) by 17β -HSD enzyme.

Adipose tissue may modify androgen metabolism by several mechanisms (reviewed by Kershaw and Flier 2004, Pasquali 2006). Firstly, adipose tissue expresses many steroidogenic enzymes, e.g. aromatase, 17β -HSD, 3β -HSD and 5α -reductase, with a partly differential expression pattern in the visceral and subcutaneous fat. Secondly, adipose tissue secretes several adipokines with endocrine properties and expresses many steroid receptors, including AR. It is well-established that obesity is associated with increased serum concentrations of androgens in women. Similarly, obese children have higher testosterone and DHEAS concentrations than the age-matched normal-weight peers (Reinehr *et al.* 2005). Furthermore, the expression of 17β -HSD in relation to aromatase is high in visceral and low in subcutaneous fat, implicating preferential androgen production in visceral fat. Accordingly, women with central obesity have higher testosterone production rate than those with the same weight, but more subcutaneous fat (Kirschner *et al.* 1990). Finally, obesity is characterized by low serum sex hormone binding globulin (SHBG) concentrations, particularly in those with excess central fat (Pasquali *et al.* 1990).

Taken together, there are several mechanisms by which the androgen action can be regulated at the pre-receptor and receptor level.

2.1.3. Androgens in the target tissues

In general, the main function of androgen steroids is to stimulate the development and maintenance of male characteristics. Together with sex chromosomes, androgens are responsible for the sexual differentiation during fetal development. They also maintain spermatogenesis and induce the male secondary sexual characteristics: taller stature, higher muscle mass and bone density, voice deepening and growth of androgenic hair.

In females, androgens induce the appearance of pubic and axillary hair. The significance of androgens in other physiologic processes in women is not fully understood as reviewed by Auchus and Rainey (2004). An excess amount of androgens in women can cause virilization, hirsutism, alopecia, anovulation and secondary amenorrhea, as seen in the polycystic ovary syndrome (PCOS) (Revised PCOS Consensus Workshop Group 2004).

The most apparent signs of androgen effect during normal early pubertal development are the skin manifestations: acne, comedones and the appearance of pubic and axillary hair. Immunohistochemical studies have shown that AR is expressed widely in the skin, e.g. in the sebaceous glands and the secretory epithelium of axillary apocrine sweat glands, providing a direct mechanism for the above mentioned effects of androgens in the skin (Blauer *et al.* 1991, Choudhry *et al.* 1992, Beier *et al.* 2005). It is well-established that the function of sebaceous glands and the development of hair follicles are androgen dependent. Firstly, pubic and axillary hair can only develop in the presence of androgens. For example, as pubic and axillary hair normally appears during pubertal development characterized by increasing androgen production, and castration before puberty prevents axillary hair growth (Hamilton 1951). Moreover, patients with androgen insensitivity syndrome typically present with no or only scant pubic and axillary hair (Hughes and Deeb 2006). Secondly, acne and hirsutism, disorders of the sebaceous glands and hair follicles, do not manifest before the beginning of pubertal development (Rosenfield and Lucky 1993).

Furthermore, serum levels of DHEAS have been associated with sebaceous gland activity in prepubertal children (Stewart *et al.* 1992), and androgen concentrations are higher in girls and women with hirsutism or acne than in those without (Lucky *et al.* 1983, Vexiau *et al.* 1990, Rosenfield and Lucky 1993, Slayden *et al.* 2001).

Interestingly, the effect of a given androgen concentration on the pilosebaceous unit varies not only between individuals but also within each individual. In certain skin areas, androgens stimulate the development of vellus hairs to terminal hairs and in other areas like face, androgens cause the enlargement of sebaceous glands (reviewed in Rosenfield 2005). Moreover, even with constant androgen concentrations, acne usually fades away by the mid-twenties. The degree of androgen excess does not correlate with the severity of acne, hirsutism or alopecia in the hyperandrogenic women. On the other hand, some women present with acne and/or hirsutism despite normal androgen levels (Reingold and Rosenfield 1987, Deplewski and Rosenfield 2000, Azziz 2003). Thus, skin manifestations of androgen action are dependent on the production of androgens, but there are also other factors modulating them.

AR is abundantly expressed in bone tissue (Abu *et al.* 1997), and androgens stimulate bone formation during growth. Androgens are responsible for the sexual dimorphism in bone growth and body dimensions, but the mechanisms of their effect on bone are not thoroughly known. Their influence on bone density accrual is even more unclear. The growth promoting role of androgens is indicated by the increased prepubertal growth velocity in untreated 21-hydroxylase deficiency patients (Jääskeläinen and Voutilainen 1997). In girls with androgen insensitivity syndrome, low bone mineral density is a common finding (Soule *et al.* 1995). Thus, both bone linear growth and bone mass accrual are modified by androgens. At least part of the anabolic effect of androgens on bones is mediated via local aromatization to estrogens. However, animal studies have revealed that both estrogens and androgens are needed for proper bone growth and bone mass attainment (Goulding and Gold 1993, Vanderschueren *et al.* 1997, Lea *et al.* 1998, Gaumet-Meunier *et al.*

2000, Venken *et al.* 2006). This view is also supported by a study with a male patient with both aromatase deficiency and mild hypogonadism (Rochira *et al.* 2007). However, androgens mainly increase the bone volume and to a lesser extent the volumetric mineral density (reviewed in Vanderschueren *et al.* 2008).

It is well established that an adequate amount of androgens protects against bone loss in aging women and men, but the effect on bone density accrual during growth has been less evident. A recent study on healthy Caucasian children found a relationship between prepubertal androstenediol excretion (measured by urinary metabolites) and bone mineral content and strength strain index in late puberty (Remer *et al.* 2009). Androstenediol is a direct metabolite of the most abundant adrenocortical steroid, DHEA. This conversion is catalyzed by 17β -HSD. The same study group has previously reported a connection between DHEA secretion, as estimated by the sum of urinary DHEA and DHEAS metabolites, and proximal radial diaphyseal bone mineral content and density in children before the appearance of pubic hair (Remer *et al.* 2003).

2.1.4. Adrenarche

Definition of adrenarche

Adrenarche refers to the gradual increase in adrenocortical androgen production in mid-childhood. The term "adrenarche" was first introduced by Talbot and Albright in 1940s (Talbot *et al.* 1943, Albright 1947). This maturational biochemical process is associated with morphological development of the ZR in the adrenal cortex, which becomes continuous normally after around 6 to 8 years of age (Dhom 1973, Ibáñez *et al.* 2000b, Auchus and Rainey 2004, Havelock *et al.* 2004). Increasing circulating androgen concentrations lead to the appearance of the clinical signs of adrenarche: adult type body odor, oily hair, acne and comedones, pubic and axillary hair. In some children, also a mild increase in growth velocity occurs. The term adrenarche can be used to

describe these clinical findings, in which case 'biochemical adrenarche' is used to refer to the increase in adrenal androgen production.

The adrenarcheal signs appear when a certain level of circulating adrenal androgens - DHEAS, DHEA or $\Delta 4A$ – has been attained. The serum DHEAS concentration of approximately 1 $\mu\text{mol/l}$ (40 $\mu\text{g/dl}$) has been considered a hallmark of biochemical adrenarche (Rosenfield 2007). The appearance time of adrenarcheal signs also depends on individual sensitivity to androgens in the androgen target tissues.

At adrenarche, the production of adrenal androgens (C19 steroids) increases while the production of cortisol remains rather stable (Kenny *et al.* 1966, Korth-Schutz *et al.* 1976a, Wudy *et al.* 2007). This suggests that adrenarche results from separate augmentation of the 17,20-lyase activity of the P450c17 enzyme in the adrenal cortex (see FIGURE 1). Thereafter, weak adrenal androgens need to be converted to more potent androgens in the skin or other peripheral tissues before clinical signs of adrenarche can be detected. The appearance of adrenarcheal signs is regulated at several levels of androgen metabolism as already discussed in the previous chapters (2.1.2 and 2.1.3.).

Clinical signs of adrenarche

Clinical signs of adrenarche are normally manifested concomitantly with those of central puberty, and these cannot always be separated from each other. The appearance of pubic hair or that accompanied by axillary hair is the most pronounced, and sometimes erroneously regarded even as the only, sign of androgen action in adrenarche. The increasing circulating androgen concentrations also cause the appearance of adult type body odor, acne and comedones, and oily hair. A transient increase in linear growth velocity and bone maturation may also occur. Within the pilosebaceous unit, vellus hairs develop into terminal hairs in response to an androgen stimulus in hair-growth prone areas. In other skin areas, androgens induce the maturation of the sebaceous glands of the

pilosebaceous unit, enabling the appearance of comedones and acne (Rosenfield *et al.* 1998, Deplewski and Rosenfield 2000). In girls, the first terminal hairs usually develop along the labia, and can thus be easily missed in routine examination (Marshall and Tanner 1969).

Adrenarcheal symptoms typically appear when DHEAS and other adrenal androgen concentrations reach a certain level. However, the appearance of the signs of adrenarche may vary depending on the peripheral androgen sensitivity (discussed in more detail in the previous chapters). Furthermore, there seems to be ethnic differences in the normal appearance time of adrenarcheal signs. This could be partly explained by the corresponding differences in the circulating androgen concentrations between ethnic groups at prepubertal age (Girgis *et al.* 2000).

In the careful longitudinal examinations by Tanner and co-workers in 1960s, the commencement of pubic hair - termed as pubic hair stage 2 in the Tanner's ratings - was on average reached at the age of 11.7 years and pubic hair stage 3 at a mean age of 12.4 years in Caucasian girls (Marshall and Tanner 1969). In a recent study with mixed ethnic population the estimated median age of the appearance of pubic hair stage 3 was 11.6 years in girls (Rosenfield *et al.* 2009).

Initiators and regulators of adrenarche

Adrenarche is regulated independently of gonadarche (Sklar *et al.* 1980, Auchus and Rainey 2004). Up to date, the exact triggering mechanisms of adrenarche are not completely understood, and no specific promoter of adrenal androgen production has been found (Auchus and Rainey 2004). Absent adrenarche in children with familial glucocorticoid deficiency due to ACTH receptor gene mutations indicates that ACTH is needed for adrenal androgen production (Weber *et al.* 1997). However, the role of ACTH in C19 steroid synthesis seems to be permissive rather than regulatory, as ACTH concentrations remain constant during adrenarche.

Cytochrome P450c17 enzyme catalyzes steroid 17 α -hydroxylation and 17,20-lyase reactions leading to the synthesis of DHEA, which can then be further converted to DHEAS and

$\Delta 4A$ (discussed in more detail in the chapter 2.1.1). In adrenarche, separate augmentation of 17,20-lyase activity of CYP17 could explain the increase in DHEA, DHEAS and $\Delta 4A$ without similar changes in cortisol production (Havelock *et al.* 2004). Thus, enhancers (P450 oxidoreductase and cytochrome b5) and post-translational modifications like serine phosphorylation of CYP17 seem to be involved (Zhang *et al.* 1995). However, the triggering mechanisms are still unidentified.

In addition to the circulating concentrations of adrenal androgens, their peripheral metabolism and tissue-specific androgen sensitivity, other factors presumably affect the clinical presentation and timing of adrenarche. *In vitro* studies have demonstrated an inverse relationship between the length of the polymorphic CAG repeat in the first exon of the AR gene and the transcriptional activity of AR (Chamberlain *et al.* 1994). Studies in both Finnish (Lappalainen *et al.* 2008a) and Spanish (Ibáñez *et al.* 2003a) children have found that the CAG repeat sequence is shorter in children with premature adrenarche than in control children. These findings indicate that the increased sensitivity of AR may be involved in the premature timing of adrenarche. They also provide a possible explanation to the notion that some children present with signs of adrenarche but with low prepubertal androgen concentrations.

Several studies have connected low birth weight (BW) with increased circulating DHEAS concentrations at prepubertal and pubertal age (Francois and de Zegher 1997, Dahlgren *et al.* 1998, Ibáñez *et al.* 1999a, Ghirri *et al.* 2001, Tenhola *et al.* 2002, Veening *et al.* 2004). Rapid early weight gain also correlates with prepubertal DHEAS levels (Ong *et al.* 2004). These observations suggest that adrenal development is programmed during fetal and early postnatal life. However, the association of low BW with DHEAS levels has not been seen in all SGA populations (Boonstra *et al.* 2004, Radetti *et al.* 2004).

Overweight in childhood seems to predispose to premature adrenarche since obesity is more common in children with PA than in normal child population (Charkaluk *et al.* 2004, Neville and Walker 2005). Moreover, overweight is associated with increased circulating androgen

concentrations in healthy prepubertal child populations (Ong *et al.* 2004, Reinehr *et al.* 2005). These observations, and the recently recognized active endocrine role of adipose tissue in general, suggest a similar role for fat accumulation in the initiation or promotion of adrenarche as it has in central puberty (Kaplowitz 2008). Potential adipose tissue associated stimulators of adrenal androgen synthesis include increased insulin and leptin concentrations.

While the mechanisms of the initiation of adrenarche remain obscure, genetic factors most probably are involved in the timing of adrenarche. Several gene polymorphisms involving mainly with steroid metabolism and insulin-IGF system have been associated with PA and PP, and with the metabolic features of children with PA or PP (Ibáñez *et al.* 2001a & 2002 & 2003a, Tomboc and Witchel 2003, Witchel *et al.* 2001 & 2003, Petry *et al.* 2005 & 2006, Roldan *et al.* 2007, Lappalainen *et al.* 2008a & 2008b). Also epigenetic modulation of the AR gene has been suggested to play a role in the clinical presentation of PA (Vottero *et al.* 2006, Lappalainen *et al.* 2008a).

2.2. Premature adrenarche

2.2.1. Definition, clinical findings and prevalence

The first descriptions of premature adrenarche (PA) came up from the recognition of precocious appearance of pubic and axillary hair, at that time called "sexual hair", by Albright and the group of Wilkins. Premature sexual hair was connected with the previous findings of increasing urinary excretion of 17-ketosteroids in mid-childhood (Talbot *et al.* 1943), and the term adrenarche was first introduced by Albright (1947). Soon after this, the first report of a series of children with "precocious adrenarche" was published (Silverman *et al.* 1952). The term "PA" was thereafter established when further studies convinced that adrenal androgen production is the cause of the

phenomenon (Visser 1966, Visser and Degenhart 1966, Rosenfield 1971, Korth-Schutz *et al.* 1976b).

Originally premature pubarche (PP), the appearance of pubic hair before the age of 8 years in girls or 9 years in boys, was considered the main or only clinical manifestation of PA (Silverman *et al.* 1952) and PP is still often erroneously considered a synonym of PA. Even in the early reports on PA and PP, it was acknowledged that axillary hair may accompany the appearance of pubic hair (Silverman *et al.* 1952). Later studies have revealed also several other signs of androgen action in PA (Korth-Schutz *et al.* 1976a, Voutilainen *et al.* 1983, Kaplowitz *et al.* 1986, Charkaluk *et al.* 2004).

Adrenarche is regarded premature if the clinical signs – oily hair, acne and comedones, pubic or axillary hair, adult type body odor - occur before the age of 8 years in a girl or before 9 years in a boy and the signs are accompanied by increased circulating adrenal androgen concentrations for age (Ibáñez *et al.* 2000b). However, the term PA has also been used to describe the clinical phenomenon - premature signs of adrenarche (Voutilainen *et al.* 1983, Kaplowitz *et al.* 1986, Pere *et al.* 1995). Several authors have used the term PA even as a synonym for PP (Likitmaskul *et al.* 1995, Banerjee *et al.* 1998, Vuguin *et al.* 1999, Ghizzoni and Milani 2000). On the other hand, some authors would reserve the term adrenarche for the biochemical event, defined by increase in circulating adrenal androgen concentrations (Rosenfield 2007). Thus, the use of the term 'PA' is not clearly established. In any case, central puberty, congenital virilizing adrenal hyperplasia (CVAH) and virilizing tumors have to be excluded before the diagnosis of PA can be made.

In most studies, children with PA have had circulating androgen concentrations appropriate for the Tanner stage of pubic hair or even below that (Rosenfield 1971, Korth-Schutz *et al.* 1976a, Rosenfield *et al.* 1982, Voutilainen *et al.* 1983, Lashansky *et al.* 1991, Pere *et al.* 1995). Some studies, however, suggest that PA is accompanied by markedly increased serum adrenal androgen

concentrations described as "exaggerated adrenarche" (Likitmaskul *et al.* 1995, Banerjee *et al.* 1998). Studies in Catalan (Northern-Spanish) PP girls also suggest that PA is followed by both adrenal and ovarian hyperandrogenism after puberty, indicating that the PA associated hyperandrogenism would not be transient (Ibáñez *et al.* 1993 & 1997a & 2000c).

PA is almost tenfold more common in girls than boys (Rosenfield 1994), and its prevalence seems to differ between ethnic groups. However, the prevalence of PP and PA has remained poorly described. A recent American study exploring the attainment of pubertal hallmarks at population level, reported that only 0.01% of non-hispanic white girls had pubic hair at stage 3 or more by the age of 8 years. In contrast, 3.0% of black and 1.3% of Mexican-American girls had attained stage 3 pubic hair at that age (Rosenfield *et al.* 2009).

2.2.2. Differential diagnosis

Before PA can be diagnosed, other conditions causing hyperandrogenic signs must be ruled out. These include precocious central puberty, virilizing tumors and late onset congenital adrenal hyperplasia (CAH).

When evaluating a patient with apparent PA, the occurrence of precocious central puberty must be considered. PA can be distinguished from precocious central puberty by careful clinical assessment including Tanner staging. The hallmarks of central puberty are the beginning of breast development in girls (Tanner stage M2 or more) and testis volume exceeding 3 to 4 ml in boys (Tanner stage G2 or more). Central puberty can be ascertained by the GnRH test, where stimulated LH-FSH ratio exceeds 1.

In prepubertal children, virilizing adrenal tumors can present with similar symptoms as PA: premature appearance of pubic and axillary hair, acne and accelerated growth in height. However, the appearance of androgenic signs is usually more rapid in virilizing tumors than in

idiopathic PA, and growth in height is typically pronouncedly accelerated. Virilizing tumors can be diagnosed by imaging with ultrasound or computed tomography.

The most important condition to be ruled out before the diagnosis of PA can be made is late onset CAH, where the first sign may be PP or other prepubertal androgenic sign (New 2006). The diagnosis of CAH is based on baseline and ACTH-stimulated 17-hydroxyprogesterone (17-OHP) concentration. The exact diagnosis is made by genetic analysis.

CAH is an inherited disorder where there is a defect in one of the five steroidogenic enzymes in the cortisol production pathway (see FIGURE 1; reviewed in New 2004, Speiser and White 2003). This leads to decreased cortisol production and to the accumulation of cortisol precursors. These precursors can be diverted to the production of androgens, which may then be secreted in excess. In 90 to 95% of the cases, the CAH causing defect is in the 21-hydroxylase (P450c21) enzyme. The three forms of 21-hydroxylase deficiency (21OHD) are the classic salt-wasting, the classic simple-virilizing and the non-classic form. In both of the classic forms, a newborn girl can manifest with ambiguous genitalia because of the exposure to high androgen concentrations during fetal life. In the salt-wasting form, there is also a lack of aldosterone production, which can lead to severe hypovolemia and failure to thrive. Several mutations with variable effects on 21-hydroxylase activity can cause 21OHD, and they explain about 80 to 90 percent of the phenotypic variability.

The non-classical form of 21OHD (NC21OHD) is caused by mild mutations resulting in 20-50% of normal 21-hydroxylase activity (reviewed in New 2006). Accordingly, NC21OHD is characterized by only a mild increase in adrenal androgen production and it does not lead to virilization in female fetuses. As in the classic forms, the clinical presentation in NC21OHD shows a wide variation. Typical manifestations of NC21OHD are accelerated prepubertal growth, PP, acne, hirsutism, alopecia, amenorrhea, anovulation and infertility. Therefore, NC21OHD must be considered in the differential diagnosis of PA and PCOS.

The incidence of classic 21OHD in Finland is approximately 1 in 15 000 live births, similar to the world wide incidence. The prevalence of the non-classic 21-hydroxylase deficiency is very low in Finland and other Northern European populations (Jääskeläinen *et al.* 1997, Therrell *et al.* 1998).

The prevalence of 21-hydroxylase deficiency and other mild or non-classical steroidogenic defects have been investigated in several small studies in children with apparent PA or PP with somewhat inconsistent findings (Morris *et al.* 1989, Siegel *et al.* 1992, Balducci *et al.* 1994, Dacou-Voutetakis and Dracopoulou 1999, Accetta *et al.* 2004). Variable frequencies of CAH are probably partly explained by the differences in the PA inclusion criteria, but also by ethnic differences.

Non-classic 3 β -HSD deficiency can also cause PP and needs to be considered while diagnosing PA (Witchel *et al.* 2001). It is noteworthy that the biochemical diagnosis of 3 β -HSD deficiency can be challenging and correct hormonal criteria (Lutfallah *et al.* 2002) should be carefully used. Furthermore, heterozygous mutations of the 21-hydroxylase gene may present with mild hyperandrogenic signs as seen in PA (Witchel *et al.* 2001, New 2004). Rarer steroidogenic enzyme defects causing CAH, like 11 β -hydroxylase or 11 β -HSD deficiency, may also be considered.

2.2.3. Growth and timing of puberty

Until recently, the prevailing view has been that adrenarche is separate from gonadarche, and that PA has no effect on the timing of central puberty or final height. However, a study by Ibáñez and co-workers on Catalan (Northern-Spanish) PP girls suggested that particularly girls born SGA may enter puberty earlier than normal, and that this could lead to diminished adult height (Ibáñez *et al.* 2006). On the other hand, several other studies have indicated that prepubertal growth in height is accelerated in children with PA compared with the controls (TABLE 1) (Voutilainen *et*

al. 1983, Ibáñez *et al.* 1992, Ghizzoni and Milani 2000, Accetta *et al.* 2004, Zukauskaite *et al.* 2005). Advanced bone age has also been repeatedly shown in subjects with PA (Rosenfield *et al.* 1982, Voutilainen *et al.* 1983, Ibáñez *et al.* 1992, Accetta *et al.* 2004, Diaz *et al.* 2008).

Low BW has been linked with PA in two separate populations (Ibáñez *et al.* 1998a, Neville and Walker 2005), whereas yet another study showed no difference in the BW measures between Caucasian girls with a history of PP and their controls (Meas *et al.* 2002).

TABLE 1. Studies on growth in height and weight in subjects with premature pubarche (PP) and premature adrenarche (PA).

Reference	Subjects	Population	Findings
Voutilainen <i>et al.</i> 1983	18 girls with "clinical PA", no controls	Caucasian/Finnish	Increased height SDS
Kaplowitz <i>et al.</i> 1986	24 PP children, 17 controls	African-American & Caucasian	Advanced HA/CA in PP subjects, but no significant difference between PP and control groups
Ibáñez <i>et al.</i> 1992	127 PP girls, no controls	Northern-Spanish & -Italian	Tall stature (increased 'HA/CA')
Pere <i>et al.</i> 1995	34 children with "clinical PA", no controls	Caucasian/Finnish	Increased height SDS at examination, height acceleration from 2.3 years onward (retrospective analysis)
Ghizzoni and Milani 2000	38 girls with PA, no controls	Caucasian/Italian	Accelerated prepubertal growth (increased height SDS); normal final height
Accetta <i>et al.</i> 2004	28 girls with PP (6 with CAH), no controls	Brazilian	Height percentiles above average, advanced bone age in 43%
Zukauskaite <i>et al.</i> 2005	20 girls with PA, 13 controls	Caucasian/Lithuanian	Increased height SDS

HA/CA, Height age/chronological age

2.2.4. Association of premature atherosclerosis with metabolic characteristics

The metabolic syndrome

What is the metabolic syndrome?

The metabolic syndrome (MBS) is a cluster of metabolic risk factors that predispose to cardiovascular diseases (CVD) and are thought to share a common underlying pathophysiological process. The existence of such a cluster of unfavorable metabolic features was first described by Reaven in 1988. In his constellation, labelled originally as syndrome X, insulin resistance was the key feature (Reaven 1988). Subsequently, different organizations including World Health Organization (WHO) (Alberti and Zimmet 1998), the European Group for the study of Insulin Resistance (EGIR) (Balkau and Charles 1999), the National Cholesterol Education Program -Adult Treatment Panel III (NCEP-ATP III) (Third report of NCEP ATP III, 2002, Grundy *et al.* 2005), the American Association of Clinical Endocrinologists (AACE) (Einhorn *et al.* 2003) and the International Diabetes Federation (IDF) (Alberti *et al.* 2005), have proposed their own criteria for MBS. Features generally included in the MBS constellation are insulin resistance or glucose intolerance, dyslipidemia, hypertension and abdominal obesity. However, the MBS criteria by the NCEP-ATP III and IDF do not include direct insulin sensitivity assessment. The WHO and EGIR definitions consider insulin resistance as a key feature of the syndrome as was the case in the original “syndrome X”. There is still ongoing debate about the components to be included in the MBS entity. However, MBS has been associated with an increased risk of type 2 diabetes and CVD in large population-based studies (Galassi *et al.* 2006, Gami *et al.* 2007).

General consensus has been that insulin resistance is central in the pathophysiology of the metabolic disturbances in MBS (Eckel *et al.* 2005). On the other hand, accumulation of excess abdominal fat (central obesity) has been suggested to play a major role in the development of

insulin resistance and MBS (Despres and Lemieux 2006), and its relation to insulin resistance and other components of MBS has been well documented. However, it remains uncertain, which comes first: central obesity or insulin resistance, and the pathophysiology of MBS is still not well understood. Obesity obviously predisposes to MBS but not all obese people develop MBS. To simplify, MBS develops when a susceptible individual acquires excess body weight.

During the last two decades, extensive research has revealed new components related to MBS. These components include low grade inflammation characterized by mildly increased circulating CRP, TNF- α , IL-6 and other cytokines, impaired function of adipose tissue characterized by altered secretion of adipocytokines such as leptin, adiponectin, resistin, ghrelin and visfatin, and increased sympathetic tone (Eckel *et al.* 2005, Grassi 2006).

It has been proposed that MBS may originate already during the fetal period and early life (Barker *et al.* 1989 & 1993, Phillips *et al.* 1994, Hales and Barker 2001). Although children rarely show full-blown MBS, metabolic disturbances can be identified already in childhood. The prevalence of childhood obesity has reached epidemic proportions and obesity-related unfavorable metabolic features are common in obese child populations (Ten and Maclaren 2004, Weiss *et al.* 2004, Goodman *et al.* 2005, Cali and Caprio 2008). To assess the later risk of MBS, some modifications of MBS criteria for child populations have been proposed and used (Cook *et al.* 2003, Cruz *et al.* 2004, de Ferranti *et al.* 2004, Weiss *et al.* 2004, Ford *et al.* 2005, Reinehr *et al.* 2007a). However, there is even less consensus on the definition of MBS in children than in adults.

Interestingly, a recent study with a multi-ethnic pediatric sample found that obese children and adolescents have increased CRP and IL-6 and decreased adiponectin concentrations (Weiss *et al.* 2004). There are also other reports showing that non-traditional risk factors of CVD are found in obese children, especially in those classified as having metabolic syndrome (Invitti *et al.* 2006).

What is insulin resistance?

Insulin sensitivity means the capability of insulin to lower blood glucose by stimulating glucose uptake and decreasing its production. Insulin resistance, on the other hand, refers to decreased response to insulin in muscles, liver and adipose tissue. Insulin resistance is considered a key feature in MBS (Reaven 1988, Eckel *et al.* 2005), and it is strongly related to obesity. Decreased insulin sensitivity is also common in obese children and youth (Weiss *et al.* 2004, Chiarelli and Marcovecchio 2008).

Several methods have been constructed for assessing insulin sensitivity and β -cell function (Pacini and Mari 2003). Because of the complex nature of insulin sensitivity, any method is only an estimate of the genuine insulin sensitivity. The euglycemic clamp (DeFronzo *et al.* 1979) by which glucose uptake can be reliably measured is considered the golden standard method for assessing insulin sensitivity. Because of the complexity of conducting euglycemic clamp studies, it is not suitable for screening. Moreover, the euglycemic clamp measures mainly insulin sensitivity in skeletal muscle, whereas other tissues, such as the liver and adipose tissue, also play a role as determinants of insulin sensitivity. Therefore, several other methods for evaluating insulin sensitivity have been constructed. These methods can be divided into two groups: those with the index structurally related to that of euglycemic clamp and those only correlating with it but based on different principles (Pacini and Mari 2003). Like euglycemic clamp studies, also the glucose tolerance tests, intravenous (IVGTT) and oral (OGTT), provide real estimates of insulin sensitivity (Pacini and Mari 2003). Fasting measurements, like HOMA-IR (Matthews *et al.* 1985) and QUICKI (Katz *et al.* 2000), are widely used because they are easy to perform. Because of their simplicity, they are useful in epidemiology and screening, but they are only crude measures of insulin resistance.

Components of the metabolic syndrome in premature adrenarche

PA has traditionally been regarded as a benign variant of normal development. However, fairly recent studies have linked PA with several unfavorable metabolic features (reviewed in Ibáñez *et al.* 2009). Studies on Catalan girls with a history of PP have shown reduced insulin sensitivity, unfavorable lipid profiles and increased central fat mass independently of weight in these girls (Ibáñez *et al.* 1997b & 1998b & 2003b). The connection between PA and hyperinsulinism was, however, first discovered in American-Hispanic girls with PP (Oppenheimer *et al.* 1995). Soon thereafter, the Spanish study group reported hyperinsulinemia along with functional ovarian hyperandrogenism in girls with a PP history (Ibáñez *et al.* 1996). Subsequently, they found that their PP girls also had increased triglyceride and decreased HDL cholesterol concentrations, and suggested that PA might be connected with an increased risk of cardiovascular disease (Ibáñez *et al.* 1998b). Thereafter, they have linked the unfavorable metabolic features of the PP girls with low BW (Ibáñez *et al.* 1998a & 1999b). They have also shown that their PP girls with normal weight have increased fat mass and excess central fat compared with controls matched for pubertal stage (Ibáñez *et al.* 2003b). Interestingly, in that study, prepubertal and mid-pubertal PP girls did not have altered lipid concentrations compared with their peers, disagreeing with the previous findings by the same group (Ibáñez *et al.* 1998b). Increased insulin concentrations and reduced insulin sensitivity have also been reported in few small studies in other populations in both prepubertal and pubertal subjects (see TABLE 2).

Several studies suggest that on average, children with PA weigh more, and are more often overweight than their peers (Dimartino-Nardi 1999, Neville and Walker 2005). Two studies have also reported increased leptin concentrations in PP girls (Ibáñez *et al.* 2000d, Güven *et al.* 2005). However, there was no significant difference in leptin concentrations between premature and on-time adrenarche groups at prepuberty in a third study (Dorn *et al.* 2008). Interestingly, a recent small study reported increased total/HDL cholesterol ratio, android fat distribution and increased

tumor necrosis factor alpha (TNF- α) concentrations in children with PP (Mathew *et al.* 2008). In that study, no difference was found in the adiponectin concentrations between the study groups. Furthermore, increased neutrophil count has previously been found in PP girls with born SGA (Ibáñez *et al.* 2005). Thus, those two studies have linked PP with alterations in the inflammatory factors, which nowadays are known to play an important role in the metabolic syndrome.

TABLE 2. Previous studies on components of metabolic syndrome in premature pubarche (PP) and premature adrenarche (PA).

Reference	Subjects	Population	Findings
Oppenheimer <i>et al.</i> 1995	12 girls with PP, no controls	African/Mexican-American	Low insulin sensitivity
Ibáñez <i>et al.</i> 1996	Girls with history of PP	Catalan (Northern-Spanish)	High serum insulin concentrations during OGTT
Ibáñez <i>et al.</i> 1998b	Girls with history of PP	Catalan (Northern-Spanish)	Unfavorable lipid profiles: high TG, low HDL at pubertal age
Potau <i>et al.</i> 1999	Boys with PP	Catalan (Northern-Spanish)	No difference in serum insulin concentrations
Vuguin <i>et al.</i> 1999	35 PP girls, no controls	Caribbean-Hispanic/African-American	Low insulin sensitivity
Denburg <i>et al.</i> 2002	11 PA boys and 8 controls	U.S./Mixed ethnicity	High serum insulins, reduced insulin sensitivity
Meas <i>et al.</i> 2002	27 girls with history of PP	Caucasian	No difference in insulin concentrations at post-menarche
Ibáñez <i>et al.</i> 2003	67 girls with history of PP	Catalan (Northern-Spanish)	Higher central fat mass
Teixeira <i>et al.</i> 2004	25 girls with PP, 14 controls	Brazilian	No difference in lipids or insulin concentrations
Güven <i>et al.</i> 2005	24 PA girls, 13 controls	Caucasian/Turkish	Higher BP, TC, LDL and LDL/HDL ratio
Mathew <i>et al.</i> 2008	10 PP children, 10 controls	African-American & Caucasian	Higher TC/HDL ratio, android fat distribution

OGTT, oral glucose tolerance test; TG, triglycerides; HDL, high-density lipo-protein cholesterol; BP, blood pressure; TC, total cholesterol; LDL, low density lipoprotein cholesterol

It has been postulated that PA is associated with adverse metabolic consequences (Ibáñez *et al.* 2000b & 2009). There is convincing evidence that prepubertal subjects with PP have relative hyperinsulinism. The evidence of the association between PA and the other components of metabolic syndrome mainly relies on the studies by one group investigating Catalan PP girls (see TABLE 2). There is only one other follow-up study that has investigated the metabolic features of PA subjects after prepubertal age (Meas *et al.* 2002). In that study, there was no difference in the insulin sensitivity measures or lipid concentrations between the PP and control girls at 17 years of age.

Ovarian and adrenal hyperandrogenism

Definition of functional ovarian hyperandrogenism and polycystic ovary syndrome

Functional ovarian hyperandrogenism (FOH) refers to overproduction of androgens in the ovaries. It is diagnosed by augmented responses of 17OHP and androstenedione to GnRH-analog stimulation. FOH can be considered a component of polycystic ovary syndrome (Revised PCOS Consensus Workshop Group 2004).

PCOS is a syndrome in which the following features are noted in combination: (1) signs of hyperandrogenism and/or increased circulating androgen levels, (2) oligo- or anovulation and (3) polycystic ovaries (Revised PCOS Consensus Workshop Group 2004). PCOS is the most common cause of hyperandrogenism in women, and one of the most frequent endocrine disorders in women at reproductive age, with a prevalence of 5-10% (Asuncion *et al.* 2000). The common clinical findings of PCOS are hirsutism, acne and androgenic alopecia along with menstrual disturbances. In addition to the characteristics included in the present PCOS criteria (Revised PCOS Consensus Workshop Group 2004), many PCOS women are also obese and insulin resistant, and have abnormalities in gonadotropin production and lipid metabolism. Women with PCOS are at an

increased risk of type 2 diabetes mellitus (Dahlgren *et al.* 1992, Legro *et al.* 1999, Morin-Papunen *et al.* 2000), and probably also for CVD. The pathophysiology of PCOS remains poorly understood, and the definition is still under debate. However, the prevailing view is that PCOS is above all a hyperandrogenic disorder with ovarian dysfunction (Revised PCOS Consensus Workshop Group 2004).

Functional ovarian and adrenal hyperandrogenism in premature adrenarche

Based on a series of studies with Catalan PP girls, it has been postulated that girls with PA have an increased risk for functional ovarian hyperandrogenism (FOH) already at early pubertal stages. In 1993, it was reported that many post-pubertal girls with history of PP have hirsutism, oligomenorrea and ovarian hyper-responsiveness to the gonadotropin releasing hormone (GnRH)-analog leuprolide acetate (Ibáñez *et al.* 1993). A couple of years later the same group found that girls with history of PP have higher basal and leuprolide acetate -stimulated 17OHP, Δ 4A, DHEA and DHEAS concentrations at different pubertal stages (Ibáñez *et al.* 1997a). Furthermore, the degree of ovarian hyperandrogenism (defined by 17OHP response to GnRH agonist) correlated with hyperinsulinism during adolescence in the PP girls identified as having FOH (Ibáñez *et al.* 1996), and the constellation of PP and FOH has subsequently been connected with low BW (Ibáñez *et al.* 1998a & 1998c). The same study group also found that post-pubertal girls with a history of PP had an excessive response of DHEA and androstenedione to ACTH (Ibáñez *et al.* 2000c). Based on these findings they suggested that PA predisposes to both functional adrenal and ovarian hyperandrogenism in adolescence. Among the 47 adolescent PP girls in that study, 17 girls (36%) fulfilled the criteria of FOH. It is noteworthy that there was no control group in that study and the ACTH responses were compared with the reference values from previous studies by the same authors. Based on these studies, Ibáñez and co-workers have postulated a common pathogenetic

pathway from low BW to PA, and further to hyperandrogenism and PCOS at adulthood (FIGURE 4).

In addition to the above-mentioned findings by the Spanish group, a study with African-American and Caribbean-Hispanic PP girls indicated ovarian hyperresponsiveness in girls with PP (Banerjee *et al.* 1998). Another study group did not find evidence of FOH in the leuprolide stimulation test in their prepubertal PP girls but the connection between PA and adrenal hyperandrogenism was supported by the augmented steroid responses in ACTH test (Mathew *et al.* 2002). The authors thus concluded that in children with PP and PA, adrenal hyperandrogenism comes first, and if FOH is to develop, it cannot be discovered at the time of diagnosis of PA when ovarian activity is still minimal. Unlike in PCOS, adrenal hyperandrogenism would thus be the first event and FOH a secondary one in the sequelae of PA.

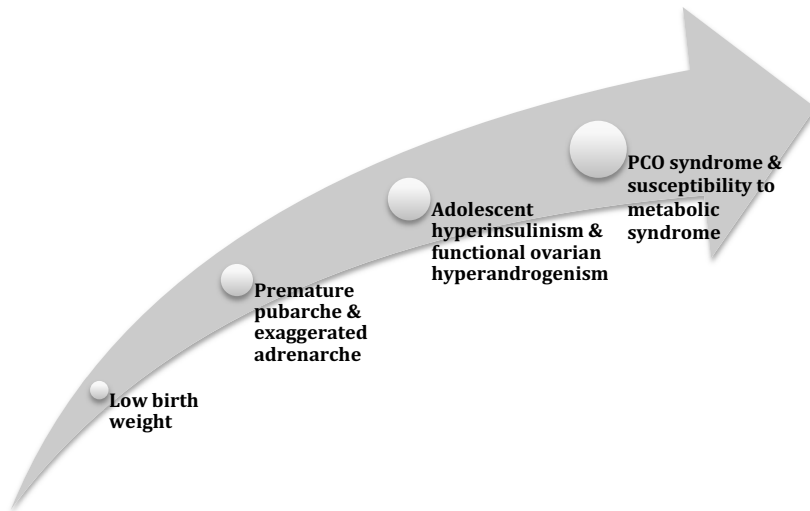


FIGURE 4. Schematic model of the postulated pathway connecting low birth weight (fetal growth restriction), premature pubarche, hyperinsulinism and adulthood polycystic ovary syndrome (PCOS). (Suggested in: Ibáñez *et al.* 1996, 1998a, 1998c & 2009, and de Zegher and Ibáñez 2006)

Insulin-like growth factor system in premature adrenarche

Two separate studies have shown increased circulating concentrations of insulin-like growth factor I (IGF-I) in children with PA. First, high IGF-I levels in combination with increased insulin and low IGF-I binding protein I (IGFBP-I) concentrations were reported in 32 prepubertal Spanish PP girls compared with their 21 prepubertal controls (Ibáñez *et al.* 1997b). In that study, mid- and post-pubertal PP girls did not have increased IGF-I concentrations compared with their healthy peers. Another study group has subsequently found increased IGF-I concentrations in prepubertal Hispanic American PP girls (Silfen *et al.* 2002). They have also reported similar findings in prepubertal PP boys (Denburg *et al.* 2002).

Genetic variation and the metabolic sequelae in premature adrenarche

Several common genetic polymorphisms have been studied in PA. The AR gene CAG repeat polymorphism has been linked with the risk of PP and ovarian hyperandrogenism after PP (Ibáñez *et al.* 2003a). Also genetic variation in aromatase gene and the G972R variant of the insulin receptor substrate-1 gene have been connected with FOH in post-pubertal girls with history of PP (Ibáñez *et al.* 2002, Petry *et al.* 2005). The distribution of insulin gene variable number of tandem repeat (INS VNTR) genotypes was similar in girls with history of PP and their controls. However, the PP girls carrying the INS VNTR class I allele had lower BW, reduced insulin sensitivity and less favorable lipid profile than those with allele III homozygosity (Ibáñez *et al.* 2001a).

2.2.5. Bone mineral density

There is some evidence that adrenal androgens have a positive influence on bone mineral accrual and density. Two relatively small studies have also reported increased bone mineral density (BMD) in PA subjects (Ibáñez *et al.* 2000d, Sopher *et al.* 2001). In a Spanish study, PP girls had BMD measures higher than the respective population reference values (Ibáñez *et al.* 2000d). There was no control group in that study. Another study with 14 PA subjects and 16 controls revealed that total body BMD was higher in the PA than control girls at a prepubertal age (Sopher *et al.* 2001). The researchers did not report whether PA was biochemically confirmed. Thus, the evidence of the influence of PA on bone mass accrual remains inconclusive.

3. AIMS OF THE STUDY

The purpose of this study was to describe the clinical features of premature adrenarche in detail, and to study their correlation with circulating androgen concentrations in prepuberty. Another aim was to uncover the associations of premature adrenarche with metabolic parameters, body composition and prepubertal growth.

The specific aims were:

1. To describe the clinical presentation of premature adrenarche in a prepubertal sample of Finnish children.
2. To investigate the correlation between the clinical signs of adrenarche and circulating androgen concentrations in prepubertal children.
3. To study birth measures and prepubertal growth in subjects with premature adrenarche.
4. To determine whether premature adrenarche influences body composition and bone mineral density in prepuberty.
5. To study the correlation between premature adrenarche and the components and indicators of the metabolic syndrome at a prepubertal age.

4. METHODS AND DESIGN

4.1. Study population

4.1.1. *Subjects with signs of androgen action (SAA group)*

The inclusion criteria of the study group were the appearance of any of the following clinical sign(s) before the age of 8 years in girls and 9 years in boys: adult type body odor, oily hair, acne, pubic or axillary hair. In addition, the clinical and biochemical evaluation had to be performed before the age of 9 years in girls and 10 years in boys. Children with any known endocrine disorder and long-term medication, including oral and inhaled corticosteroids, were excluded. All children meeting these criteria between October 2004 and January 2006 in Northern Savo, a region in Eastern Finland with a population of 250 000, were invited to participate in the study. Study subjects were collected among the patients admitted to the pediatric outpatient clinic of Kuopio University Hospital due to the hyperandrogenic symptoms. In addition, information letters were sent through the health care centers to the well baby and school clinics and announcements were published annually in main local newspapers. Seventy-six eligible children with prepubertal signs of androgen action (SAA) were found of whom 73 (96.1%) were willing to participate. An informed written consent was obtained from all the parents and from the subjects who were at least 7 years of age.

4.1.2. *Subjects with premature adrenarche (PA group)*

At recruitment, inclusion was based solely on the androgenic signs and no biochemical criterion was used. Of the 73 SAA subjects in the original cohort, sixty-four children had premature adrenarche (PA) defined by both clinical signs of androgen action and biochemical evidence of adrenarche (DHEAS ≥ 1 $\mu\text{mol/l}$), and they formed the PA group (FIGURE 5).

4.1.3. Control group

The original control cohort consisted of 99 healthy children born between 1995 and 1999 and living in the same area as the SAA subjects. A list of a random sample of child citizens was obtained from the Finland's population register. Invitation letters were sent to the families of the children selected from the list by order sampling in each age and gender group, in order to yield a matched control group for the SAA subjects. Approximately 20% of the control child population invited were willing to participate. Children with any known endocrine disorder or long-term medication (including oral or inhaled corticosteroids) were excluded. One girl in the control group refused blood sampling, and her results were excluded from the analyses. Thus, the control group comprised 98 children. For the PA group, a smaller control group was selected, including only the control children who had no biochemical evidence of adrenarche (DHEAS $<1 \mu\text{mol/l}$) (n=64) (FIGURE 5). An informed written consent was obtained from all the parents and from the subjects who were at least 7 years of age.

4.1.4. Study settings

Analyses were performed in four different settings (See also FIGURE 5):

- (1) The entire originally recruited cohort with 73 prepubertal children with signs of androgen action (SAA) and their controls (n=98). (**STUDY I**)
- (2) Children with both clinical and biochemical evidence of adrenarche (DHEAS $\geq 1 \mu\text{mol/l}$) (PA group, n=64) and their controls with neither clinical nor biochemical signs of adrenarche (DHEAS $<1 \mu\text{mol/l}$) (nonPA control group, n=62). (**STUDY IV**)
- (3) Separate analyses in girls with PA (n=54) and their nonPA control girls (n=52). (**STUDY II**)
- (4) Separate analyses in girls in the original cohort (SAA, n=63 vs. control girls, n=80) (**STUDY III**)

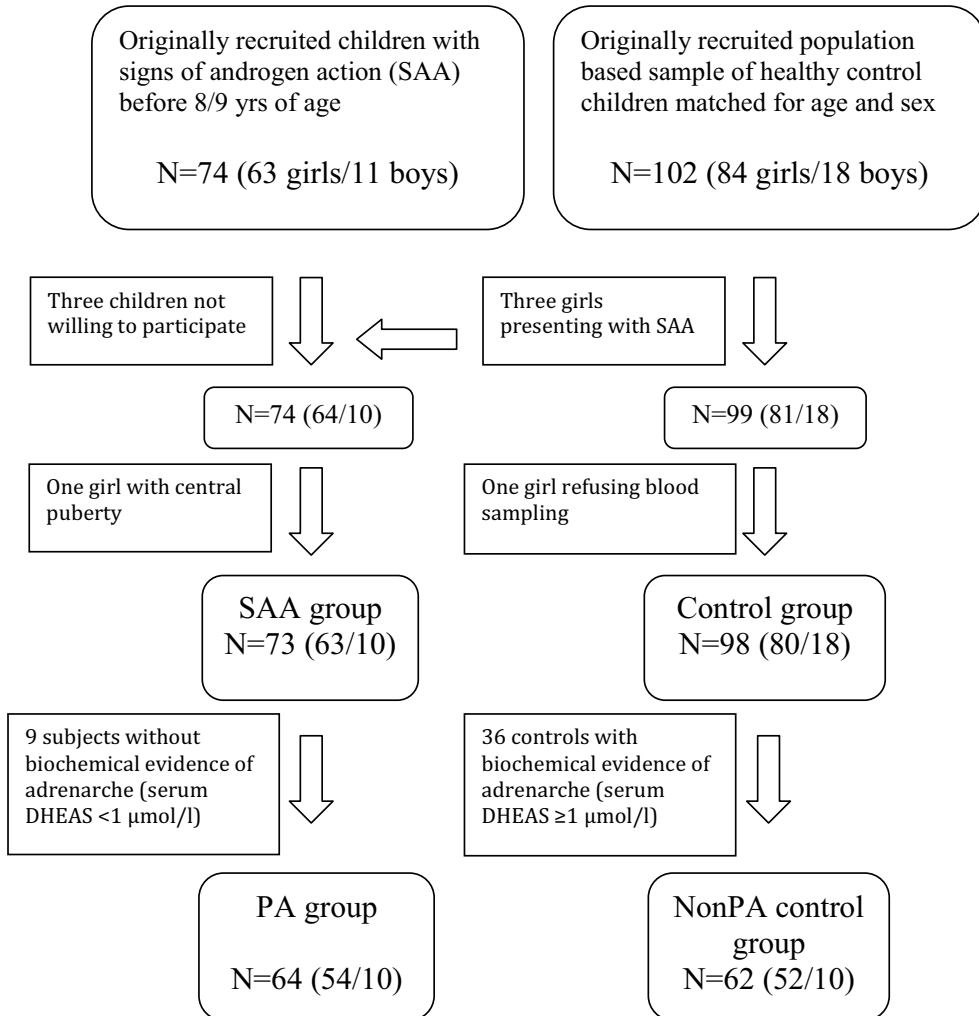


FIGURE 5. Flow chart of the study groups.

4.2. Clinical evaluation

All the subjects and their parents were interviewed using a structured questionnaire to obtain the occurrence and appearance time of androgenic signs. Adult-type body odor was recorded if the parents or the child reported a change in the type of axillary odor or a need for antiperspirant. If the parents or the child complained about greasiness of hair or need for daily hair wash, "oily hair" was recorded. All subjects were thoroughly examined with special emphasis on androgenic and pubertal signs: Tanner pubertal stage, axillary hair, acne and comedones were evaluated in a systematic physical examination. Axillary odor and wetness were also recorded when evident in the clinical examination.

Height was measured with a calibrated Harpenden stadiometer (Holtain Ltd, Crymch, UK) and recorded to the nearest 0.1 cm as the mean of three repeated measurements and converted to SDS, according to the current Finnish growth charts. Weight was measured after an overnight fast and recorded to the nearest 0.1 kg, and converted to weight-for-height according to the current Finnish growth charts. BMI [$\text{weight}(\text{kg})/\text{height}^2(\text{m})$] was calculated, and BMI SD score (SDS) determined by British reference values (Cole *et al.* 1995). Bone age (BA) determination was not included in the study protocol for ethical reasons. However, the BA scans were analyzed in the 26 girls with premature pubarche in whom it had been taken as part of a standard clinical evaluation. BA was evaluated by two pediatric endocrinologists independently and the mean of the two assessments was recorded. The Greulich and Pyle method (Greulich and Pyle 1959) was used. Abdominal ultrasonography was performed on each subject with pubic or axillary hair; no adrenal tumor was found in any of the subjects.

Blood pressure (BP) was measured with a standard sphygmomanometer after a 30 minutes rest in bed in the afternoon. The proper cuff was chosen to fit to the arm length of each subject. BP was measured three times from the left arm of the subject who was in a supine position. Each

measurement was recorded to the nearest 2 mmHg, and the average of the three repeated readings was used for all analyses.

4.3. Growth assessment

Growth analyses were performed in the girls with PA and their controls. Birth weight (BW), length (BL) and duration of gestation were obtained from hospital records. Birth size data were converted to standard deviation scores (SDSs) according to the Finnish growth charts adjusted for duration of gestation and gender (Pihkala *et al.* 1989). Ponderal index was calculated with formula: $BW(g)/BL(cm)^3 \times 100$. For those born preterm (<37 week, n=6), corrected length measures (according to the gestational age) were applied until the age of 2 years. One PA girl was born as a twin, and she was excluded from the analyses concerning birth size and 1 year measurements. Also two very preterm girls with gestational ages of 29 and 31 weeks were excluded from these analyses.

Height and weight were measured annually in well baby and school clinics by trained nurses as part of the routine Finnish well baby clinic system. In case of a lacking exact annual measurement, the value was obtained by extrapolating from the individual growth chart of the respective child. At each measurement, height was converted to SDS, and weight to weight-for-height according to the current Finnish growth charts (Sorva *et al.* 1990). Parent-specific expected height (PSEH) was calculated as previously described (Sorva *et al.* 1989; Pere *et al.* 1995).

4.4. Biochemical analyses

4.4.1. Blood sampling, glucose tolerance test and ACTH test

An intravenous cannula was placed in the right antecubital vein, and basal samples for blood hemoglobin (Hb) and glycated hemoglobin (HbA1c), plasma glucose, lipids, alkaline

phosphatase (ALP) and ACTH, and serum insulin, SHBG, IGF-I, 25-hydroxyvitamin D (vitamin D; 25-OHD), cortisol (F), 11-deoxycortisol (11DOF), 17-hydroxyprogesterone (17OHP), androstenedione (Δ 4A), dehydroepiandrosterone (DHEA), and its sulfate (DHEAS) were drawn between 0900 and 1000 hours, after an overnight fast. A standard 2-hour oral glucose tolerance test (OGTT) was performed by administering 1.75 g/kg glucose (max 75 g) to each subject. Blood samples for plasma glucose (heparinized syringe) and serum insulin analyses were taken at 30, 60, 90 and 120 minutes. Then a low-dose ACTH test was performed by administering 1 μ g/1.73 m² of synthetic ACTH (Synacthen®, Novartis Pharma GmbH, Nürnberg, Germany) intravenously to each subject. A serum sample for F, 11DOF, 17OHP, Δ 4A and DHEA measurements was taken after 30 minutes. In the early afternoon, after one-hour rest in bed, a plasma sample for norepinephrine (NE; noradrenaline) and epinephrine (E; adrenaline) analyses was drawn into a specific tube. All subjects underwent a GnRH-test (Relefact® 3.5 μ g/kg intravenously, Hoechst, Frankfurt am Main, Germany) with LH and FSH sampling at 0, 30, 60 and 90 minutes. Plasma glucose concentration was analyzed within 30 minutes of sampling, blood HbA1c, hemoglobin, plasma lipids and ALP within the same day. Serum samples were separated within an hour of sampling, immediately frozen and stored at -80 C until assayed. All plasma samples were taken into chilled tubes, separated within 15 minutes, and stored at -80 C until assayed.

4.4.2. Assays

Plasma glucose concentrations were analyzed by a blood gas analyzer using a glucose oxidase method (Clarke electrode, Rapidlab 865 or Rapidlab 1265, Bayer, Tarrytown, NY, USA), in which the intra-assay coefficient of variation (CV) was 2.0% and inter-assay CV 2.0%. Blood HbA1c was analyzed with automated cation exchange liquid chromatography (Tosoh G7, Tosoh Corporation, Minato-ku, Tokyo, Japan), with intra-assay CV of 0.4% and inter-assay CV of 1.8%. Serum insulin and SHBG concentrations were analyzed with specific time-resolved

fluoroimmunoassays by AutoDelfia (PerkinElmer Life and Analytical Sciences Wallac Oy, Turku, Finland). The intra-assay CV was 2.0% and the inter-assay CV was 2.7% in the insulin assay, while those for the SHBG assay were 4.0% and 2.6%, respectively.

Serum DHEAS and $\Delta 4A$ concentrations were determined with specific Coat-A-Count radioimmunoassays (RIAs) (Diagnostic Products Corporation, Los Angeles, CA, USA). In the DHEAS assay, the intra-assay coefficient of variation (CV) was 3.8 - 5.3% and the inter-assay CV was 6.3 - 11%. For the $\Delta 4A$ assay, the intra-assay CV was 3.2 - 9.4% and inter-assay CV 4.1 - 15.6%. Serum DHEA concentrations were measured with an in-house RIA derived from a previously described method (Apter *et al.* 1979). Serum 11DOF concentrations were determined with liquid chromatography-mass spectrometry (LC-MS) (PE Sciex, Foster City, CA, USA) and serum 17OHP concentrations with a previously described LC-MS method (Turpeinen *et al.* 2005), both with a detection limit of 1 nmol/l. Serum F concentrations were measured with the Immulite 2000 Cortisol chemiluminescence immunoassay (Diagnostic Products Corporation), with intra-assay CV of 5.2 - 7.4% and total variation of 6.8 - 9.4%. Specific RIAs for serum testosterone (Diagnostic Products Corporation) and estradiol (DiaSorin, Saluggia, Italy) measurements had detection limits of 0.35 and 0.02 nmol/l, respectively. Serum LH and FSH concentrations were determined with the time-resolved fluoroimmunoassays by AutoDelfia (PerkinElmer Lifesciences, Turku, Finland). Total CV of the LH assay was 2.5% and that of the FSH assay 2.8%. In the first 125 subjects, plasma ACTH concentrations were analyzed by an immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) with inter-assay CV of 6.4 - 8.4 %. For the samples of the remaining 46 subjects, Immulite chemiluminescent immunoassay (Diagnostic Products Corporation) was used. The intra-assay CV for the Immulite ACTH assay was 3.1 - 9.6 % and inter-assay CV 5.1 - 9.4%. Plasma E and NE concentrations were analyzed with high performance liquid chromatography (HPLC) (Chromsystems GmbH, München, Germany). The intra-

and inter-assay CV for E were 6.0 and 8.8%, and those for NE measurements 3.5 and 8.4%, respectively.

Plasma total cholesterol (TC) and triglyceride (TG) concentrations were determined using colorimetric enzymatic methods, and cholesterol in high-density (HDL) and low-density (LDL) lipoproteins with direct enzymatic methods (Thermo Electron Co, Vantaa, Finland). The inter-assay CV was 2.7% for the TC measurement, 3.3% for HDL cholesterol at 1.31 mM, 4.3% for LDL cholesterol at 2.69 mM, and 4.5% for TG at 1.04 mM. Serum IGF-I concentration was determined with an immunochemiluminometric assay on the IMMULITE 2000 analyzer (Diagnostic Products Corporation), with the detection limit of 3 nmol/l, the inter-assay CV less than 4%, and the total CV less than 9% in the range of 10-180 nmol/l.

4.4.3. Determination of insulin sensitivity and definition of metabolic syndrome

To determine insulin sensitivity (IS), the following indices were calculated using the fasting and OGTT plasma glucose and serum insulin measurements:

- (1) Homeostasis model assessment for insulin resistance (HOMA-IR): $[\text{Fasting insulin } (\mu\text{U/ml}) * \text{Fasting glucose (mmol/l)}] / 22.5$ (Matthews *et al.* 1985),
- (2) Insulin sensitivity index (ISI_{comp}): $10\ 000 / \sqrt{[\text{Fasting glucose (mg/dl)} * \text{Fasting insulin } (\mu\text{U/l}) * \text{Mean glucose (mg/dl)} * \text{Mean insulin } (\mu\text{U/l})]}$ (Matsuda and DeFronzo, 1999),
- (3) Metabolic clearance rate of glucose (MCR_{est}): $[18.8 - 0.271 * \text{BMI (kg/m}^2) - 0.0052 * \text{Insulin at 120 min (pmol/l)} * 0.27 * \text{Glucose at 90 min (pmol/l)}]$ (Stumvoll *et al.* 2000).

The “childhood metabolic syndrome” (cMBS) was assessed by two previously described definitions: the U.S. National Cholesterol Education Project Adult Treatment Panel III (NCEP ATP III) MBS criteria modified for adolescents and the World Health Organisation (WHO) criteria with child specific cut-off values (TABLE 3). In the ATP III

definition, we used the BMI (>75th percentile on the Finnish BMI chart) instead of waist circumference (>75th percentile of the U.S. reference values in the original ATP III) for assessing overweight, and determined plasma instead of whole blood glucose. In the WHO definition, high fasting serum insulin concentration (>90th percentile of the controls) was used as a marker of reduced IS.

TABLE 3. Definitions of metabolic syndrome modified for children (cMBS) according to the Third National Health and Nutrition Examination Survey (U.S.) Adult Treatment Panel III (NCEP ATPIII) and according to the World Health Organisation (WHO).

Definition	Risk factor	Defining level
Modified ATP III*	Body mass index [†]	>75 th percentile for age/gender ^a
	Plasma triglycerides	>1.1 mmol/l
	Plasma HDL cholesterol	<1.3 mmol/l
	Systolic or diastolic BP	>90 th percentile for gender, age and height ^b
	Fasting plasma glucose	>5.6 mmol/l ^c
cMBS = any 3 criteria met		
WHO*	Body mass index	>75 th percentile for age and gender ^a
	Plasma triglycerides	>90 th percentile of the control group (>0.97 mmol/l)
	Plasma HDL cholesterol	<10 th percentile of the control group (<1.1 mmol/l)
	Systolic or diastolic BP	>90 th percentile for gender, age and height ^b
	Fasting serum insulin	>90 th percentile of the control group (>7.81 mU/l)
cMBS = high fasting serum insulin + any 2 other criteria met		

*The original NCEP ATPIII criteria in de Ferranti *et al.*, (2004), and the original WHO criteria in Alberti and Zimmet (1998)

[†]BMI percentile instead of weight circumference percentile

^aFinnish national BMI charts (Childhood Obesity, Current Care Summary 26.10.2005. Working group appointed by the Finnish Pediatric Society; BMI charts based on the growth data in Sorva *et al.*, 1990)

^bU.S. normative BP tables by the National High Blood Pressure Education Program Working Group on Hypertension Control in Children and Adolescents (Update on the task force report, 1996)

^cCorresponding B-Glucose >6.1 mmol/l

HDL, high-density lipoprotein; BP, blood pressure

4.4.4. Assessment of steroidogenic enzyme activities

The activities of steroidogenic enzymes were estimated by calculating serum steroid concentration ratios. ACTH stimulated (st) values were used for the ratios including 11DOF or 17OHP because of their immeasurably low baseline concentrations. The following steroid ratios were calculated:

- (1) DHEAS/DHEA: a marker of SULT2A1 (DHEA sulfotransferase) activity,
- (2) Δ 4A/DHEA: 3 β -HSD activity,
- (3) (st11DOF+stF)/st17OHP: 21-hydroxylase activity, and
- (4) stF/st11DOF: 11 β -hydroxylase activity.

4.4.5. Low density lipoprotein (LDL) receptor-related protein 5 (LRP5) genotyping

DNA was isolated from peripheral blood samples using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). All the 23 coding exons and the flanking intronic, 5' and 3' untranslated regions of the *LRP5* gene were sequenced. Primer sequences for PCR and sequencing were generated from the genomic sequence (NM_002335) using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). To prevent non-specific annealing of the primers, Blast (<http://www.ncbi.nlm.nih.gov/blast>) was used.

The PCRs for the sequencing reactions were performed in a final volume of 20 μ l containing 10 ng of genomic DNA, 8 pmol of forward and reverse primers, 16 mM dNTP (Finnzymes, Espoo, Finland), and 1 unit of AmpliTaq Gold DNA polymerase with 10x AmpliTaq Gold Buffer I (Applied Biosystems, Foster City, CA, USA). The reactions for exons 1, 4, and 5 also contained 10% dimethyl sulphoxide (Sigma-Aldrich, St. Louis, MO, USA). Amplifying was performed in Tetrad Peltier Thermal Cycler-225 (MJ Research, Waltham, MA, USA) with the following cycling profile: (1) denaturation (10 min; 95°C), (2) 35 cycles of denaturation (95°C for 45 s), annealing (60–65°C for 45 s), and extension (72°C for 45 s), (3) final extension (72°C; 10

min). The PCR products were run on a 1.6% SeaKem LE agarose gel (BioWhittaker Molecular Applications, Rockland, ME, USA) and visualized with ethidium bromide staining. The PCR products were purified with enzymatic ExoI/SAP treatment (USB, Cleveland, OH, USA), labelled with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and sequenced (ABI 3730 DNA analyzer; Applied Biosystems). Sequence chromatograms were analyzed with Sequencher 4.7 program (Gene Codes Corporation, Ann Arbor, MI, USA).

4.5. Measurement of bone mineral density and body composition

Bone mineral content (BMC, g) and areal density (BMD_{areal} ; g/cm^2) of lumbar spine (L2-4; LS) and left femoral neck (FN) were measured by dual-energy X-ray absorptiometry (DXA) using Lunar DPX (Lunar Radiation Corporation, Madison, WI). All subjects were measured with the same scanner by trained personnel in Kuopio University Hospital. Quality assurance tests have shown an inter-assay variation of 0.8% for the LS and 2.3% for the FN measurements in children by the previous Lunar DPX scanner (Kröger *et al.* 1993). To minimize the effect of bone size, measures of volumetric BMD (BMD_{vol} ; g/cm^3) were calculated as follows: ¹⁾ $LS\ BMD_{\text{vol}}\ (\text{g}/\text{cm}^3) = LS\ BMD_{\text{areal}}\ (\text{g}/\text{cm}^2) \times [4/\pi \times \text{width of measurement area in LS (cm)}]$ and ²⁾ $FN\ BMD_{\text{vol}}\ (\text{g}/\text{cm}^3) = FN\ BMD_{\text{areal}}\ (\text{g}/\text{cm}^2) \times 4/[\pi \times \text{height (cm) of the measurement area} / \text{measurement area (cm}^2\text{) in FN}]$ (Kröger *et al.* 1993). The width of vertebrae L2-L4 and the width of FN were obtained from the scan analysis reports.

Body composition was assessed by eight-tactile bioelectrical, multifrequency and segmental impedance analysis (BIA) with Inbody 3.0[®] (Biospace, Seoul, Korea). This device measures volume of water based on the body's resistance to electric current, and thereby derives fat free mass and fat mass, and calculates various indirect measures of body composition for a given height of the subject.

4.6. Data analysis

SPSS 14.0 software statistical package (SPSS Inc., Chicago, IL) was used for all statistical analyses. All continuous parameters were first tested for normality. The independent samples *t*-test was performed to analyze the differences between the study groups. In the case of nonnormally distributed parameters, logarithmic (ln) transformation was performed prior to analyses or the non-parametric Mann-Whitney test was used. In the analyses comparing the three subgroups among the SAA girls, ANOVA with least significant difference (LSD) post hoc test with 2 comparisons (LSD multiplied by two) was applied (*STUDY III*). In the other subgroup analyses, ANOVA with Bonferroni correction was used. Correlations between variables were analyzed with the Pearson or Spearman correlation test, as appropriate. Univariate and multivariate linear regression models were used to test correlations independent of confounding factors. Logistic regression models were also constructed to study independent associations between growth and PA, and to determine independent predictors of cMBS in girls. To compare prevalences of prematurity, SGA, MBS components and other conditions between the study groups, Pearson Chi-Square and Fischer's exact test was used as appropriate. Data are presented as mean (95 % confidence interval) or median (inter-quartile [25th to 75th percentile] range or total range). $P \leq 0.05$ was considered statistically significant in all analyses.

5. RESULTS

5.1. Family and patient history

5.1.1. Family

In the entire cohort, the mothers of the SAA subjects had earlier menarche than mothers of the control children (median 12 vs. 13 years, $P=0.021$). This difference was statistically more significant between the SAA subjects with biochemically ascertained PA ($n=64$) and their controls in whom adrenarche was biochemically excluded (nonPA; $n=62$) ($P=0.003$). The mother of one control girl and 2 girls with SAA (both with PP and PA) had PCOS. PA was not reported in the parents of either SAA or control children. The average number of siblings was 1.8 among the control children and 1.4 among the children with SAA ($P=0.06$). The difference in the number of siblings was more evident between the children with PA and their nonPA controls (mean difference 0.74; $P=0.007$).

5.1.2. Gestation and birth (STUDY II)

Three out of the 73 SAA subjects (4.1%) and 3/98 (3.1%) of the controls had been born SGA (defined as BW or BL <-2.0 SDS) ($P=0.51$). There was no significant difference in the frequency of prematurity (gestational age <37 weeks) (SAA 5/73 vs. controls 4/98, $P=0.33$) or maternal preeclampsia (SAA 7/73 vs. controls 4/98, $P=0.15$) between the study groups. The gestation was affected by mother's gestational diabetes mellitus (GDM) in 6 SAA subject (8.2%) and in 5 controls (5.1%) ($P=0.30$). There were no significant differences in the BW or BL SDS between the SAA and control groups, although the children with SAA were slightly shorter (in cm) than the controls (TABLE 4). The very preterm subjects excluded from the birth size and early growth analyses (2 SAA and 2 control subjects) were born at 29 ($n=2$) and 31 ($n=2$) weeks gestational ages with the BWs of 1020, 1190, 1330 and 1490 grams. Inclusion of these subjects in

the birth size analyses yielded similar results: there were no significant differences in BW and BL SDS between the PA and control groups.

When comparing the girls with PA (n=54) with their nonPA controls (n=52), the frequencies of SGA (1.9% [1/54] vs. 3.8% [2/52], $P=0.49$) and prematurity (7.4% [4/54] vs. 3.8% [2/52], $P=0.36$) did not differ significantly. Four mothers of PA girls (7.4%) suffered from preeclampsia during pregnancy, whereas none of the mothers of the nonPA control girls had preeclampsia. This difference was statistically not significant ($P=0.06$). There was no significant difference in the prevalence of the mother's GDM between these groups either (PA 5/54 vs. control girls 2/52, $P=0.23$).

At birth, the girls with PA were slightly shorter (in cm) than the nonPA controls (TABLE 5). However, there was no significant difference in the BL SDS between the PA and control group (median -0.12 vs. 0.23, $P=0.13$). Neither did BW, BW SDS or ponderal indices differ significantly between the PA and control girls (TABLE 5).

TABLE 4. Characteristics of the original study groups. Values presented as median (range).

Variable	Controls (n=98)	SAA (n=73)	P value (t test)
Girls / boys	80/18	63/10	0.53 (Chi-Square)
Age	7.6 (5.1, 8.9)	7.5(4.8, 9.9)	0.73
Gestational age (week)	40+2 (29+1, 42+3)	39+6 (29+0, 42+1)	0.08*
Birth weight (g) [†]	3580 (2320, 5050)	3540 (2060, 5458)	0.08
Birth weight SDS [†]	0.01 (-2.32, 2.99)	0.04 (-2.09, 3.85)	0.41
Birth length (cm) [†]	51.0 (46.0, 56.0)	50.0 (45.0, 56.0)	0.008*
Birth length SDS [†]	0.23 (-2.69, 2.44)	0.00 (-2.17, 2.89)	0.06
Height (cm)	126 (107, 147)	131 (108, 146)	<0.001
Height SDS	0.2 (-2.9, 2.9)	1.2 (-1.3, 4.2)	<0.001
Weight (kg)	26.6 (16.5, 50.1)	31.7 (16.7, 56.8)	<0.001*
Weight-for-height (%)	103 (81, 159)	108 (89, 178)	0.003
BMI (kg/m ²)	16.3 (12.9, 25.0)	17.6 (13.9, 29.4)	0.001*
BMI SDS	0.26 (-2.12, 3.11)	0.79 (-1.16, 3.59)	0.002*
Mother's height	165 (155, 177)	165 (150, 180)	0.35
Father's height	179 (163, 197)	178 (159, 197)	0.88
PSEH	0.2 (-1.5, 1.5)	0.2 (-1.6, 1.7)	0.49

SAA, children with prepubertal signs of androgen action; PSEH, Parent-specific expected height

*Mann-Whitney test; [†]twins (n=3) and very premature (gestational age <33 weeks'; n=4) subjects excluded

5.1.3. Appearance of signs of androgen action (STUDY I)

In the SAA subjects, the first sign of androgen effect had occurred at a median age of 6.4 years (range 2.0-8.8 years). In most cases, the first sign had been adult type body odor. Pubic and axillary hair was usually preceded by other signs of androgen action.

In the original control cohort, there were 3 girls who turned out to have SAA by clinical examination or careful interview. They were included in the SAA group in all analyses.

5.2. Clinical characteristics (STUDY I)

In the SAA subjects, the most prevalent sign of androgen action was adult type body odor (65/73 [89%]). Thirty-five out of the 73 children (48%) had pubic or axillary hair, typically in combination with other signs. The frequencies of all the adrenarcheal signs and their different combinations in the SAA group are depicted in TABLE 6. The subjects with pubic or axillary hair (PAH group) were older than those with other symptoms only (nonPAH group) (median age 7.9 vs. 7.2 years, $P=0.002$). Despite a trend towards higher weights in the PAH than nonPAH SAA group, there were no significant differences in the BMI SDS (median 0.88 vs. 0.68, $P=0.11$) or weight-for-height (median 111% vs. 107%, $P=0.11$) between the subgroups. Oily hair was more often reported in the girls than boys with SAA (75% vs. 40%, $P=0.04$). Axillary hair was observed in 17/63 SAA girls (27%) but in none of the ten SAA boys ($P=0.06$). Other androgenic signs occurred at similar frequency in the SAA girls and boys. Mild acanthosis nigricans was observed in 9 SAA subjects and in 4 control children ($P=0.02$). None of the SAA subjects had hirsutism or clitoromegaly. All boys had testicular volume ≤ 3 ml and all the girls had breasts at the M1 Tanner stage.

TABLE 5. Demographic, anthropometric and metabolic features of girls with premature adrenarche (PA) and their nonPA control girls. Medians (inter-quartile ranges) are shown.

	PA (n=54)	non PA controls (n=52)	P (t test)
<i>At birth</i>			
Birth weight (g) [^]	3495 (3110, 3740)	3535 (3270, 3910)	0.30
Birth length (cm) [^]	50.0 (48.0, 51.0)	50.3 (49.1, 52.0)	0.04*
Birth weight SDS [^]	-0.19 (-0.74, 0.65)	-0.09 (-0.48, 0.66)	0.63
Birth length SDS [^]	-0.12 (-0.69, 0.72)	0.23 (-0.30, 1.03)	0.13
Ponderal index (g/cm ³) [^]	2.80 (2.69, 2.90)	2.78 (2.61, 3.02)	0.57
Gestational age (week)	40.3 (38.9, 41.0)	40.4 (39.4, 41.1)	0.63*
PSEH SDS	0.27 (-0.20, 0.83)	0.12 (-0.26, 0.57)	0.16
<i>At examination</i>			
Age (year)	7.6 (7.1, 8.1)	7.5 (6.8, 8.0)	0.32
Height (cm)	131 (126, 136)	125 (120, 128)	<0.001 [†]
Weight (kg)	31.8 (26.3, 37.0)	24.6 (22.4, 27.8)	<0.001*
Height SDS	1.2 (0.6, 1.8)	0.0 (-0.6, 0.6)	<0.001 [†]
Sitting/total height (%)	53.7 (53.1, 54.3)	53.7 (53.1, 54.4)	0.95
BMI SDS	0.76 (-0.02, 2.31)	-0.11 (-0.61, 0.82)	<0.001
Weight for height (%)	111 (99, 135)	101 (95, 111)	0.002*
Mean S-insulin (mU/l)	35.6 (28.6, 43.0)	23.1 (19.6, 25.6)	0.006 [‡]
S-IGF-I (nmol/l)	24 (19, 30)	19 (16, 24)	0.031 [†]
S-DHEAS (μmol/l)	2.1 (1.4, 2.6)	0.6 (0.5, 0.7)	<0.001 [†]

*Mann-Whitney test, [†]Covariance analysis adjusted for current BMI SDS and age, [‡]for current BMI SDS SDS, SD score; S, serum; Mean S-insulin, mean serum insulin concentration during the 2-h OGTT; DHEAS, dehydroepiandrosterone sulfate; PSEH, parent-specific expected height

[^]Very preterm girls (n=2) and a girl born as a twin excluded

TABLE 6. Clinical signs of androgen action in the 73 prepubertal children.

Number of subjects with each combination of signs	Adult type body odor (n=65)	Oily hair (n=51)	Acne and/or comedones (n=41)	Pubic hair (n=28)	Axillary hair (n=17)
SAA subjects with pubic or axillary hair (n=35)					
n=8	X	X	X	X	
n=6	X	X	X	X	X
n=3	X	X		X	
n=2	X	X		X	X
n=2	X	X	X		X
n=2	X		X		X
n=2		X	X	X	
n=2	X			X	X
n=2	X			X	
n=2		X		X	
n=1	X	X			X
n=1	X		X	X	
n=1		X			X
n=1			X		X
SAA subjects with no pubic or axillary hair (n=38)					
n=12	X	X	X		
n=11	X	X			
n=8	X				
n=5	X		X		
n=1		X	X		
n=1			X		

5.3. Growth (*STUDY II*)

To study the effect of adrenarche on prepubertal growth, growth parameters were compared between the children with PA (fulfilling both clinical and biochemical criteria of PA) and the controls, who had neither signs nor biochemical evidence of adrenarche (DHEAS <1µmol/l). These analyses were performed separately in girls and boys, and only the results of the girls are presented here.

The girls with PA were significantly taller than their controls (median height SDS 1.2 vs. 0.0, $P<0.001$; TABLE 5). This difference remained significant when the possible confounding effects of parental heights (PSEH) and weight (BMI SDS) were accounted for by using them as covariates in the univariate linear model ($P<0.001$). Compared with their controls, the girls with PA

had enhanced growth in height already during the first year of life. Despite their slightly, but not significantly, lower BL SDS, they were significantly taller at the age of 1 year than the control girls ($P=0.008$) (FIGURE 6). This difference was significant also after adjustment for PSEH and BMI SDS at 1 year of age ($P=0.028$). Mean growth velocity was higher in the PA than control girls also during the second year of life (Δ length SDS from 1 to 2 years of age: PA girls + 0.46 SD vs. control girls +0.16 SD, $P=0.005$). In the PA group, there was a significant mean change in length SDS from birth to 2 years of age (+0.75; $P<0.001$). There was no significant difference in the PSEH between the PA and control groups.

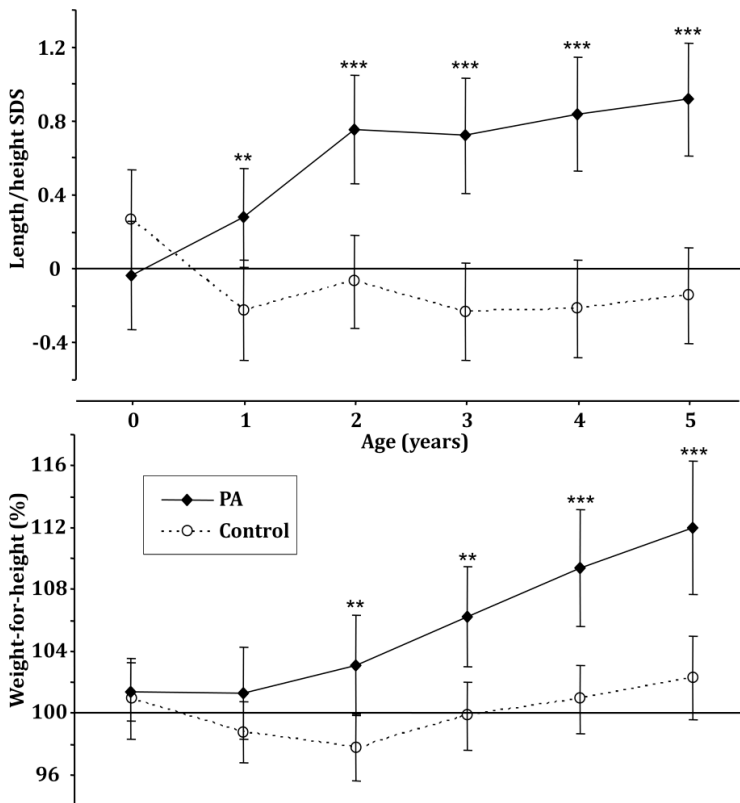


FIGURE 6. The growth pattern of girls with premature adrenarache (PA) and in their non-PA controls. Means and 95% confidence intervals are shown. Independent samples *t* test: ** $P\leq 0.01$; *** $P\leq 0.001$ between PA and controls.

Girls with PA had significantly higher IGF-I concentrations than the controls ($P=0.001$). The difference in IGF-I concentration remained significant after adjustment for BMI SDS and age (TABLE 5). Serum IGF-I concentration correlated with current height SDS when the PA and control girls were analyzed together ($r=0.37$, $P<0.001$), also when adjusted for age and BMI SDS in linear regression ($P=0.01$). No significant correlation was found between height SDS and IGF-I in the PA and nonPA groups when analyzed separately.

There was a trend towards more rapid prepubertal growth in the PA girls with pubic or axillary hair (PAH-PA) than in the other PA girls (nonPAH-PA), but the differences in height SDS between these PA subgroups were not statistically significant at any annual measurement point. The growth patterns in height according to these subgroups of PA girls are illustrated in FIGURE 7.

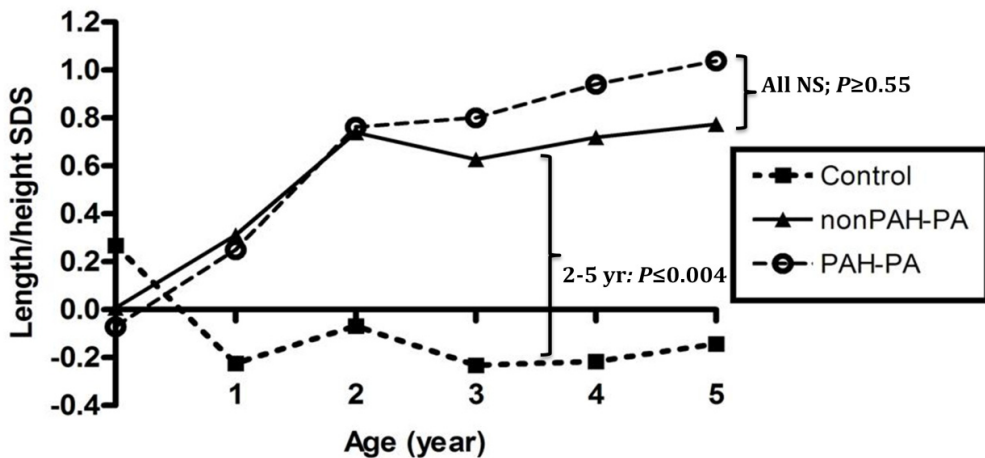


FIGURE 7. Growth pattern in length/height in the PA girls with (PAH-PA, $n=29$) and without (nonPAH-PA, $n=25$) pubic or axillary hair, and in the nonPA control girls ($n=52$). Mean length/height SDS at each annual measurement is shown. Oneway ANOVA with Bonferroni correction between the subgroups.

The girls with PA weighed more than their controls (median BMI SDS 0.76 vs. -0.11, $P<0.001$); the difference in weight was significant at the 2-year measurement point and thereafter (TABLE 5, FIGURE 6). In a multiple linear regression model including both the PA girls and nonPA controls, height SDS was independently associated with BL, PSEH, current BMI SDS, DHEAS and IGF-I concentration. Among the PA girls, height SDS was independently associated only with BMI SDS and PSEH SDS (TABLE 7).

TABLE 7A. Multiple linear regression model depicting the relationship of height SDS with antropometric and biochemical factors in the girls with PA (n=51*).

	Stand. coeff.	P value	Regression coeff. (95% CI)
Birth length SDS	0.19	0.12	0.21 (-0.06, 0.47)
PSEH SDS	0.52	<0.001	0.90 (0.52, 1.28)
Mother's age at menarche (year)	-0.15	0.17	-0.12 (-0.29, 0.05)
Age (year)	-0.12	0.29	-0.18 (-0.52, 0.16)
BMI SDS	0.32	0.006	0.35 (0.09, 0.54)
S-DHEAS ($\mu\text{mol/l}$)	0.19	0.09	0.24 (-0.04, 0.52)
S-IGF-I (nmol/l)	0.19	0.09	0.03 (-0.00, 0.05)
Mean S-insulin (mU/l)	-0.05	0.70	-0.002 (-0.01, 0.01)

Stand. coeff, standardized regression coefficient; PSEH, parent-specific expected height

† Coefficient of determination, $R^2=0.52$

*Very preterm girls (n=2) and a girl born as a twin excluded

Mean S-insulin, mean serum insulin concentration during the 2-h OGTT

TABLE 7B. Multiple linear regression model depicting the relationship of height SDS with antropometric and biochemical factors in the entire group of girls with PA and their nonPA controls (n=106).

	Stand. coeff.	P value	Regression coeff. (95% CI)
Birth length SDS	0.17	0.01	0.20 (0.05, 0.36)
PSEH SDS	0.44	<0.001	0.81 (0.58, 1.05)
Mother's age at menarche (year)	-0.18	0.005	-0.16 (-0.26, -0.05)
Age (year)	-0.03	0.68	-0.04 (-0.24, 0.15)
BMI SDS	0.32	<0.001	0.31 (0.17, 0.44)
S-DHEAS ($\mu\text{mol/l}$)	0.23	0.001	0.22 (0.09, 0.34)
S-IGF-I (nmol/l)	0.16	0.02	0.02 (0.00, 0.04)
Mean S-insulin (mU/l)	0.02	0.79	0.001 (-0.01, 0.01)

PSEH, parent-specific expected height

† Coefficient of determination, $R^2=0.64$

Mean S-insulin, mean serum insulin concentration during the 2-h OGTT

The girls with PA were further divided into tertiles according to BW SDS. The growth pattern of girls in each subgroup is illustrated in FIGURE 8. Expectedly, BL SDS differed significantly between the BW SDS tertile groups ($P<0.001$). There were no significant differences in the length/height SDS at or after 1 year of age between these subgroups of PA girls (FIGURE 8).

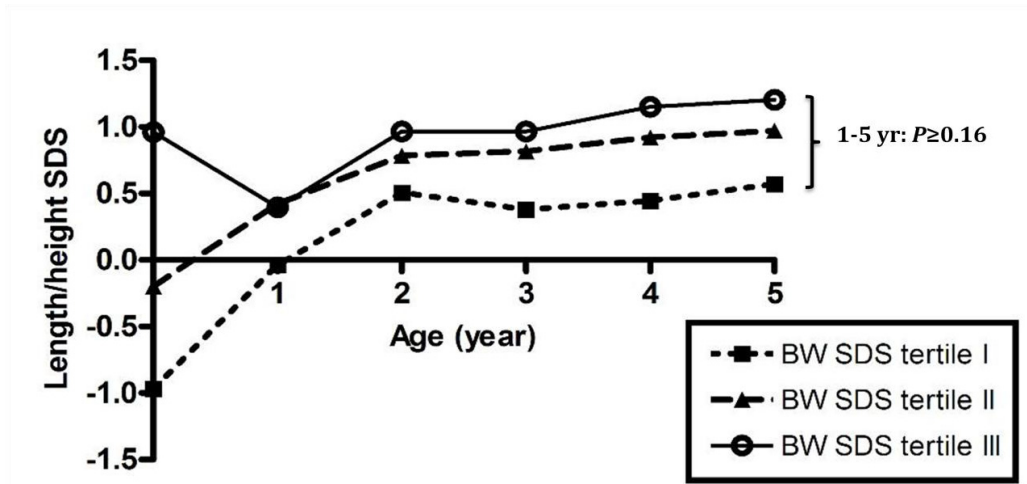


FIGURE 8. Growth pattern of the girls with PA ($n=54$) according to the birth weight SDS (BW) tertiles. Mean length/height SDS at each annual measurement is presented. At birth: $P<0.001$; 1-5 yr: $P\geq 0.16$ by oneway ANOVA.

Among the 26 girls with PA in whom the bone age (BA) was determined, BA was advanced (≥ 2.0 SDS) in 4 subjects (15%). On average, BA was 1.0 years ahead of the chronological age (CA). There was a positive linear relationship between the bone age advancement (BA-CA) and IGF-I concentration ($r=0.51$, $P=0.008$), which remained statistically significant after adjustment for PSEH, age, BMI SDS, estradiol, insulin and DHEAS concentrations ($P=0.019$, multiple regression analysis).

5.4. Serum androgen concentrations and their association with clinical characteristics (STUDY I)

Among the SAA children, the serum concentrations of DHEA, DHEAS and Δ 4A were higher in the SAA children with pubic or axillary hair (PAH group; n=35) than in those without (nonPAH; n=38). Compared with the controls, also the nonPAH SAA subjects had increased adrenal androgen concentrations (FIGURE 9). There were 64 children with SAA in whom serum DHEAS concentration was at least 1 μ mol/l, which is generally regarded a biochemical hallmark of adrenarche. Thus, nine children in our non-selected cohort presented with prepubertal signs of androgen action but without biochemically diagnosable adrenarche. Three of them had pubic and/or axillary hair. In these 9 girl subjects, DHEA [median 4.3 (range 2.5-5.7) vs. 7.6 (2.9-22.4) nmol/l, $P<0.001$] and Δ 4A [1.5 (0.6-1.9) vs. 2.9 (0.7-6.6) nmol/l, $P<0.001$] were also lower than in the other SAA children.

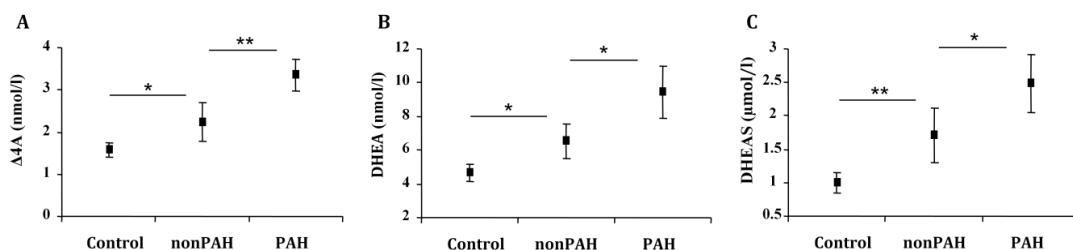


FIGURE 9. Serum concentrations of androstenedione (Δ 4A) dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEAS) in children with prepubertal signs of androgen action with (PAH, n=35) and without pubic or axillary hair (nonPAH; n=38), and in the control children (n=98). Means and 95% confidence intervals are shown. One-way ANOVA with Bonferroni correction: * $P<0.01$, ** $P\leq 0.001$.

In the entire study population (n=171), baseline and stimulated Δ 4A, DHEA and DHEAS concentrations correlated positively with BMI SDS ($P\leq 0.004$ for all). In the SAA subjects, serum Δ 4A but not DHEAS or DHEA concentrations were related to BMI SDS (FIGURE 10).

Accordingly, overweight SAA subjects (weight $\geq 90^{\text{th}}$ percentile; $n=31$) had higher $\Delta 4\text{A}$ concentrations than the normal-weight subjects (weight $< 90^{\text{th}}$ percentile; $n=42$) (median baseline $\Delta 4\text{A}$ 3.4 vs. 2.3 nmol/l, $P=0.001$; stimulated $\Delta 4\text{A}$ 4.4 vs. 3.0 nmol/l, $P<0.001$). There were no significant differences in the DHEAS, or baseline or stimulated DHEA concentrations between the overweight and normal weight SAA subgroups. Higher BMI SDS was associated with higher $\Delta 4\text{A}$ concentrations also in the controls (FIGURE 10).

In the entire study cohort, ($n=171$) BW SDS correlated inversely with DHEAS ($r=-0.18$, $P=0.02$). This correlation was found separately in the SAA group ($r=-0.34$, $P=0.004$) but not in the control children. There was also a significant inverse correlation of BW SDS with DHEA ($r=-0.27$, $P=0.02$) and $\Delta 4\text{A}$ ($r=-0.30$, $P=0.01$) concentrations in the SAA group. The relationship between BW SDS and AA concentrations remained significant ($P\leq 0.001$ for DHEA, DHEAS and $\Delta 4\text{A}$) when adjusted for current BMI SDS, age and sex in linear regression in the SAA group. When analyzed separately in the girls with PA (biochemically confirmed), there was also a significant inverse relationship between BW SDS and DHEA ($P=0.02$), DHEAS ($P=0.009$) and $\Delta 4\text{A}$ ($P=0.006$) independently of age and current weight (BMI SDS).

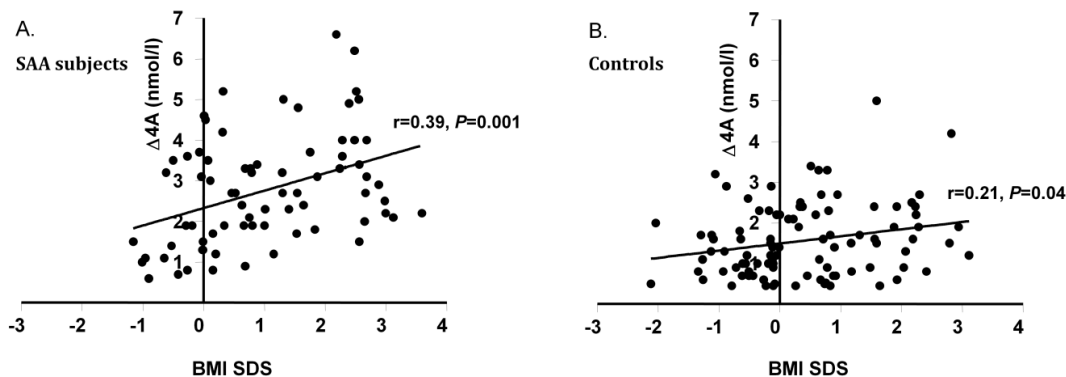


FIGURE 10. Correlation of serum androstenedione ($\Delta 4\text{A}$) concentrations with BMI SDS. Spearman correlation coefficients (r) and P values are presented.

5.5. Other steroids and steroid ratios (*STUDY I*)

All the subjects had normal baseline and stimulated 17OHP concentrations (<3.0 and <30 nmol/l, respectively) (New *et al.* 1983). Thus, none of the children in the SAA or control group had NC21OHD by biochemical criteria. Heterozygosity for 21OHD mutations could not be ruled out, as DNA sequencing was not performed. The stimulated 17OHP concentrations of all the SAA subjects were within the range of the controls (range: SAA <1-8.2 nmol/l; controls <1-10.1 nmol/l). However, the median stimulated 17OHP was slightly higher in the SAA than control group (median 4.1 nmol/l vs. 3.3 nmol/l, $P=0.03$). There was no significant difference in the plasma ACTH, serum basal/stimulated cortisol or stimulated 11-deoxycortisol (11DOF) concentrations between the children with SAA and the controls (*STUDY I*: Table 4).

Children with SAA had a higher DHEAS/DHEA ratio than the controls (250 vs. 200, $P=0.004$), suggesting increased SULT2A1 activity in the SAA children. They also had higher stimulated cortisol/11DOF (median 302 vs. 201, $P=0.032$) and a trend towards lower (11DOF+cortisol)/17OHP ratio (149 vs. 176, $P=0.051$). These hormone ratios were used as surrogate markers of 11 β -hydroxylase and 21-hydroxylase activities, respectively. There was no difference in the median cortisol/ACTH or $\Delta 4A$ /DHEA ratio (reflecting ACTH sensitivity and 3 β -HSD activity, respectively).

5.6. Components of the metabolic syndrome (*STUDY III*)

The prevalence of the “childhood metabolic syndrome” (cMBS) as assessed by the modified WHO and NCEP ATP III definitions (TABLE 3) was higher in the girls with SAA than in the control girls (FIGURE 11). The difference was even more pronounced when the girls with PA were compared with their nonPA controls; ten of 54 (18.5%) PA girls fulfilled the WHO cMBS

criteria and 24.1% the ATP III criteria compared with the frequencies of 0% and 3.9% in the non-PA control girls (n=52) ($P=0.001$ and $P=0.003$, respectively). In addition, there was a trend towards higher prevalence of cMBS among the SAA girls with pubic or axillary hair than among the other SAA girls (19% vs. 13% by the WHO and 28% vs. 19% by the ATP III definition).

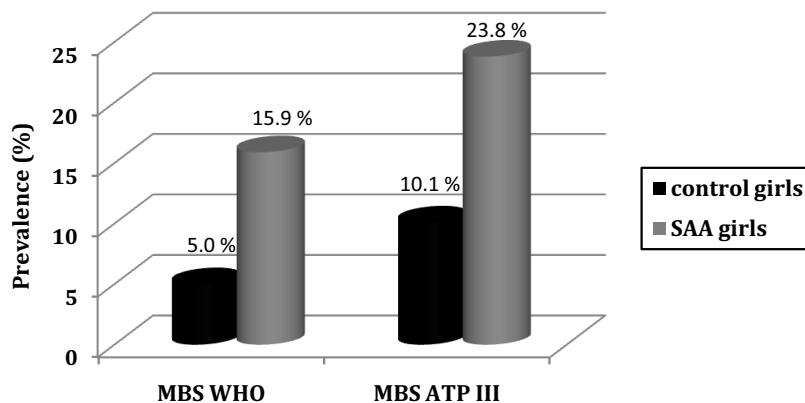


FIGURE 11. The prevalence of metabolic syndrome (MBS) in the prepubertal girls with signs of androgen action (SAA; n=63) and in the control girls (n=80), according to WHO and ATP III MBS criteria modified for children (described in TABLE 3). SAA vs. control girls: $P=0.03$ for both by Chi-square test.

In the entire girl population, the most prevalent component of cMBS according to the criteria used was overweight (BMI >75th percentile). This was the only component that separately analyzed was more prevalent in the girls with SAA than in the controls (59% vs. 41%, $P=0.03$). There were no significant differences in the frequencies of the other components between the SAA and control girls. However, a higher percentage of the SAA girls with pubic or axillary hair (PAH subgroup) fulfilled the criteria of high fasting insulin than of the control girls (28% vs. 10%, $P=0.02$; *STUDY III*: Table 2). cMBS by both criteria was strongly predicted by weight. The prevalence of cMBS by the BW SDS, BMI SDS and fasting insulin tertiles in the SAA girls is displayed in FIGURE 12.

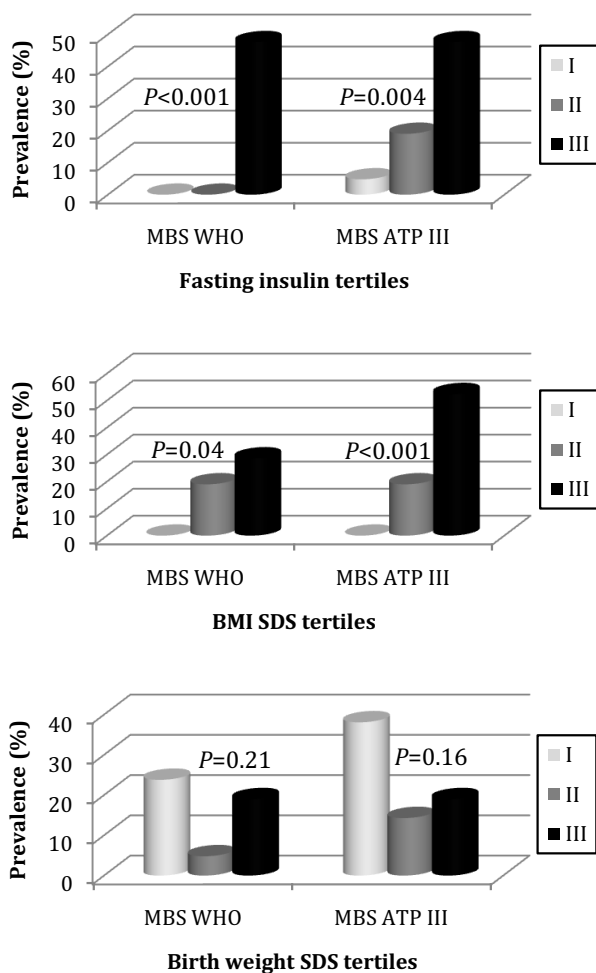


FIGURE 12. The prevalence of metabolic syndrome (MBS) by the modified WHO and NCEP ATP III criteria by the tertiles of birth weight SDS, BMI SDS and fasting insulin in the girls with signs of androgen action (n=63). *P* values obtained from Chi-Square test.

Compared with controls, the girls with SAA had higher weight-for-height, BMI SDS and fat percent (TABLE 8). Insulin concentrations and IS indices were strongly associated with weight. Only one normal-weight (BMI percentile <75th percentile) girl with SAA had a high mean insulin concentration during OGTT (defined as >90 percentile of the controls), one had high fasting plasma

insulin (defined as >90 percentile of the controls, TABLE 3) and none of the normal-weight SAA subjects had cMBS by either definition. There were no significant differences in the prevalence of any cMBS component between the normal-weight SAA and control girls.

TABLE 8. Components and indicators of metabolic syndrome in the girls with SAA and in the control girls. Values are expressed as median (inter-quartile range = 25th, 75th percentile).

Variable	SAA (n=63)	Controls (n=80)	P value
Weight-for-height (%)*	108 (99, 130)	106 (95, 113)	0.010
BMI SDS (kg/m²)*	0.75 (-0.02, 2.24)	0.18 (-0.52, 0.90)	0.003
Fat percent (%)*	21 (16, 31)	17 (13, 24)	0.004
Systolic BP (mmHg)†	105 (97, 111)	99 (93, 104)	0.08
Diastolic BP (mmHg) †	63 (58, 69)	60 (56, 67)	0.58
fP-Glucose (mmol/L) †	4.9 (4.6, 5.1)	4.8 (4.6, 5.0)	0.97
2-h P-Glucose (mmol/L) †	6.0 (5.0, 6.6)	5.8 (5.1, 6.6)	0.73
fS-Insulin (mU/L) †	5.6 (3.6, 7.2)	4.0 (3.1, 5.2)	0.068
Mean S-Insulin (mU/L) †	35 (28, 43)	24 (21, 30)	0.001
fB-HbA_{1c} (%)†	5.2 (5.1, 5.4)	5.3 (5.1, 5.5)	0.34
HOMA-IR*	1.18 (1.05, 1.52)	1.06 (0.93, 1.19)	0.04
ISI_{comp}†	0.71 (0.59, 0.96)	1.04 (0.83, 1.33)	0.004
MCR_{est} (ml/kg/min)*	11.2 (10.0, 12.1)	12.1 (11.1, 12.5)	<0.001
fP-TC (mmol/L) †	4.2 (3.8, 4.8)	4.2 (3.8, 4.7)	0.87
fP-TG (mmol/L) †	0.62 (0.49, 0.77)	0.57 (0.43, 0.72)	0.31
fP-LDL C (mmol/L) †	2.6 (2.2, 3.0)	2.4 (2.1, 2.9)	0.81
fP-HDL C (mmol/L) †	1.36 (1.13, 1.62)	1.47 (1.22, 1.69)	0.37
fS-SHBG (nmol/l) †	70 (51, 98)	103 (82, 124)	0.001

BMI, body mass index; S, serum; f, fasting; P, plasma; B, blood; Mean S-Insulin, mean serum insulin concentration during the OGTT; HbA_{1c}, glycated hemoglobin; HOMA-IR, homeostasis model assessment for insulin resistance; ISI_{comp}, insulin sensitivity index; TC, total cholesterol; TG, triglycerides; LDL C, low-density lipoprotein cholesterol; HDL C, high density lipoprotein cholesterol; SHBG, sex hormone binding globulin

HOMA-IR = [fasting insulin (μU/ml)*fasting glucose (mmol/l)]/22.5

ISI_{comp} = 10 000/√[fasting glucose (mg/dl)*fasting insulin (μU/l)*mean glucose (mg/dl)*mean insulin (μU/l)]

Metabolic clearance rate (MCR): [18.8 - 0.271 * BMI (kg/m²) - 0.0052 * Insulin at 120 min (pmol/l) * 0.27 * Glucose at 90 min (pmol/l)]

*Mann-Whitney test, †Analysis of variance; weight-for-height as covariate

In multivariable logistic regression, current weight-for-height, but not BW SDS or DHEAS concentration correlated independently with cMBS (defined by the modified WHO criteria) in the SAA girls and in the entire girl cohort (TABLE 9). When applying a similar regression model for the modified NCEP ATP III cMBS definition, weight-for-height ($P<0.001$) and age ($P=0.03$) were also the only independent predictors of cMBS in the entire group of girls.

TABLE 9. Logistic regression model depicting the relationship of birth weight, current weight and DHEAS with metabolic syndrome (Modified WHO MBS definition for children; TABLE 3) in the prepubertal girls (n=143)(A), and separately in girls with signs of androgen action (n=63)(B).

A.

	Regression coefficient	P value	Odds ratio (95% CI)
Birth weight SDS	0.30	0.28	1.35 (0.78, 2.34)
Weight-for-height (%)	0.11	<0.001	1.11 (1.05, 1.17)
S-DHEAS ($\mu\text{mol/l}$)	-0.12	0.76	0.89 (0.40, 1.97)
Age (year)	1.16	0.02	3.20 (1.18, 8.68)

† Coefficient of determination, $R^2=0.37$

B.

	Regression coefficient	P value	Odds ratio (95% CI)
Birth weight SDS	0.03	0.92	1.03 (0.56, 1.90)
Weight-for-height (%)	0.08	0.006	1.08 (1.02, 1.14)
S-DHEAS ($\mu\text{mol/l}$)	-0.65	0.22	0.52 (0.19, 1.46)
Age (year)	1.11	0.07	3.04 (0.90, 10.3)

† Coefficient of determination, $R^2=0.25$

DHEAS, dehydroepiandrosterone sulfate

5.7. Other endocrine-metabolic characteristics (*STUDIES I, II and III*)

Biochemical findings of the study groups are shown in TABLES 8 and 10. Girls with SAA had higher mean insulin concentrations during the OGTT than the control girls (TABLE 8). This difference remained significant when adjusted for weight-for-height. The subgroup of SAA girls with pubic or axillary hair (PAH; n=32) had also higher fasting insulin concentration compared with the control girls (6.5 vs. 4.6 mU/l, $P<0.001$), even after adjustment for weight-for-height ($P=0.012$). No differences were found in the fasting or mean OGTT glucose concentrations between the study groups. As defined by HOMA-IR, ISI_{comp} and MCR_{est} , the girls with SAA had lower insulin sensitivity (IS) than the control girls (TABLE 8). In the subgroup analyses, the differences in the IS indices were significant between the PAH girls and the controls ($P<0.002$ for all), but not between the other SAA girls (nonPAH) and the controls. There was also a trend towards higher plasma TG (0.70 vs. 0.62 mmol/l, $P=0.09$) and lower HDL cholesterol concentrations (1.39 vs. 1.49 mmol/l, $P=0.09$) in the SAA than control girls, but these trends became weaker when the comparisons were adjusted for weight (TABLE 8). Plasma total, LDL or HDL cholesterol or TG concentration was not related to BW SDS in either SAA or control children. Neither was there any significant correlation between BW SDS and glucose or insulin concentrations.

The mean serum SHBG concentration was significantly lower in the SAA than control girls (79 vs. 103 nmol/l, $P<0.001$). SHBG concentration correlated negatively with fasting ($r=-0.60$, $P<0.001$) and mean OGTT insulin ($r=-0.41$, $P<0.001$), and androstenedione concentrations ($r=-0.47$, $P<0.001$), and positively with ISI_{comp} index ($r=0.55$, $P<0.001$) among the SAA girls.

In the entire study cohort, the mean plasma NE concentration was slightly higher in the SAA than control group, whereas there was no difference in the plasma adrenaline concentrations (*STUDY I*; Figure 1). Among the children with SAA, plasma NE concentrations correlated

significantly only with E concentrations ($r=0.32$, $P=0.006$). There was no significant linear correlation between NE concentration and BMI SDS, weight-for-height, or age. However, the SAA children with NE in the highest quartile ($n=18$) had a lower mean BW SDS (-0.32 vs. 0.33 SDS, $P=0.04$) and ISI_{comp} (0.73 vs. 1.02 , $P=0.004$), and a higher mean diastolic blood pressure (67 vs. 61 mmHg, $P=0.01$), body fat percent (24 vs. 19% , $P=0.02$), serum TG (0.71 vs. 0.49 mmol/l, $P=0.01$), peak (73 vs. 49 mU/l, $P=0.005$) and mean OGTT insulin (41 vs. 26 mU/l, $P=0.001$) concentration than those in the lowest NE quartile ($n=18$). There were no significant differences in BMI SDS or weight-for-height between these NE quartiles. In addition to the mentioned metabolic parameters, mean stimulated $\Delta 4A$ concentration was also higher in the highest NE quartile than in the lowest quartile among the SAA children (4.2 vs. 3.1 nmol/l, $P=0.02$).

There were no significant differences in the ACTH or cortisol concentrations between the SAA and control groups (TABLE 10). All our prepubertal subjects had low basal and GnRH-stimulated LH and FSH, and low basal estradiol and testosterone concentrations (*STUDY I*: Table 3). Compared with the control group, serum IGF-I and ALP concentrations were higher in the children with SAA (TABLE 10).

TABLE 10. Biochemical findings in children with SAA and in the prepubertal control children. Median (inter-quartile range= 25^{th} , 75^{th} percentile).

Variable	SAA (n=73)	Controls (n=98)	P value (t test)
S-IGF-I (nmol/l)	24 (20, 31)	19 (15, 24)	<0.001
S-SHBG (nmol/l)	72 (53, 100)	101 (81, 121)	<0.001
P-ACTH (nmol/l)	3.6 (2.3, 6.0)	3.9 (2.4, 5.4)	0.68
S-Cortisol (nmol/l)	231 (159, 316)	220 (177, 293)	0.71
P-Norepinephrine (nmol/l)	1.45 (1.17, 1.86)	1.31 (1.09, 1.63)	0.03
P-Adrenaline (nmol/l)	0.19 (0.13, 0.29)	0.18 (0.13, 0.29)	0.90*
S-25-OHD (nmol/l)	53 (45, 67)	58 (50, 70)	0.054*
P-ALP (U/l)	614 (538, 774)	548 (468, 621)	<0.001

S-25-OHD, serum 25-hydroxyvitamin D; P-ALP, plasma alkaline phosphatase

*Mann-Whitney test

5.8. Bone mineral density and biochemical bone markers (*STUDY IV*)

Bone mineral densities (BMD) were compared between the children with PA and their nonPA controls. Children with PA had higher bone mineral content (BMC) and BMD_{areal} in both LS and FN as depicted in the TABLE 11. In contrast, there were no significant differences in BMD_{vol} at either site. Both LS and FN BMD_{areal} correlated strongly with height SDS in the PA ($r=0.45$ and $r=0.43$, respectively; $P<0.001$ for both) and control group ($r=0.62$ and $r=0.63$, respectively; $P<0.001$ for both). Differences in the LS and FN BMD_{areal} between the PA and control group were no longer significant when adjusted for height SDS.

Among the children with PA, FN BMD_{areal} (g/cm^2) was also positively correlated with BMI SDS ($r=0.33$, $P=0.008$) and age ($r=0.38$, $P=0.002$), and negatively with SHBG concentrations ($r=-0.31$, $P=0.01$). LS BMD_{areal} (g/cm^2) correlated positively with BMI SDS ($r=0.37$, $P=0.003$), IGF-I ($r=0.27$, $P=0.03$) and $\Delta 4A$ concentrations ($r=0.31$, $P=0.01$), and negatively with SHBG concentrations ($r=-0.29$, $P=0.02$) in the PA group. BMD_{areal} s were not correlated with 25-OHD, ALP or DHEAS concentrations in the PA group. In the control group, FN BMD_{areal} (g/cm^2) correlated positively with BMI SDS ($r=0.29$) and IGF-I concentrations ($r=0.28$) ($P=0.03$ for both), and LS BMD_{areal} (g/cm^2) correlated positively with BMI SDS ($r=0.39$, $P=0.002$) and 25-OHD concentrations ($r=0.34$, $P=0.009$), and negatively with DHEAS concentrations ($r=-0.33$, $P=0.009$). Serum estradiol concentration (measured only in girls) did not correlate significantly with BMD values in either PA or control group.

In the PA group, FN BMD_{areal} was independently associated with age, BMI SDS, height SDS, *LRP5* SNPs E644E and F549F and LS BMD_{areal} was associated with age, sex, BMI SDS, height SDS, soft lean mass percent and *LRP5* SNP E644E in linear regression models (TABLE 12). Applying testosterone instead of DHEAS into otherwise similar model yielded comparable results. Like DHEAS, testosterone was not independently associated with LS or FN BMD_{areal} . Similar

models in the control group showed that height SDS ($P=0.002$) and physical activity ($P=0.045$) were the only parameters that were independently associated with LS BMD_{areal}. Height SDS ($P<0.001$), age ($P<0.001$) and male sex ($P=0.036$) were positively associated with FN BMD_{areal} in the nonPA control group. Among the PA subjects, LS BMD_{areal} was greater in the girls than boys (0.73 vs. 0.70 g/cm², $P=0.02$).

TABLE 11. Bone mineral densities in children with premature adrenarche (PA) and their controls. Means \pm 95% confidence intervals are shown.

<i>Bone mineral density parameters</i>	PA (n=64)	Control (n=62)	<i>P (t test)</i>
<i>All subjects (Z-scores)</i>			
BMD areal femoral neck	+0.56 \pm 0.25	-0.09 \pm 0.23	<0.001
BMD volumetric femoral neck	+0.28 \pm 0.25	+0.08 \pm 0.24	0.26
BMD areal lumbar spine	+0.20 \pm 0.26	-0.31 \pm 0.29	0.009
BMD volumetric lumbar spine	+0.07 \pm 0.25	+0.14 \pm 0.28	0.73
<i>Girls (n=106)</i>			
	n=54	n=52	
BMC femoral neck (g)	2.83 \pm 0.12	2.42 \pm 0.10	<0.001
BMD areal femoral neck (g/cm ²)	0.74 \pm 0.02	0.67 \pm 0.02	<0.001
BMD volumetric femoral neck (g/cm ³)	0.38 \pm 0.01	0.36 \pm 0.01	0.07
BMC lumbar spine (g)	17.3 \pm 0.94	13.4 \pm 0.87	<0.001
BMD areal lumbar spine (g/cm ²)	0.74 \pm 0.02	0.66 \pm 0.02	<0.001
BMD volumetric lumbar spine (g/cm ³)	0.30 \pm 0.01	0.29 \pm 0.01	0.30
<i>Boys (n=20)</i>			
	n=10	n=10	
BMC femoral neck (g)	3.05 \pm 0.39	2.7 \pm 0.24	0.17*
BMD areal femoral neck (g/cm ²)	0.78 \pm 0.08	0.76 \pm 0.05	0.57*
BMD volumetric femoral neck (g/cm ³)	0.38 \pm 0.03	0.40 \pm 0.02	0.29*
BMC lumbar spine (g)	16.9 \pm 2.34	15.6 \pm 1.80	0.23*
BMD areal lumbar spine (g/cm ²)	0.70 \pm 0.02	0.70 \pm 0.04	1.00*
BMD volumetric lumbar spine (g/cm ³)	0.28 \pm 0.02	0.29 \pm 0.02	0.23*

*Mann-Whitney test

BMC, bone mineral content; BMD, bone mineral density

TABLE 12. A linear regression model showing the independent associations of biochemical and body composition parameters with femoral neck (FN) and lumbar spine (LS) bone mineral density (BMD) in prepubertal children with premature adrenarache (n=64).

	FN BMD _{areal}			LS BMD _{areal}		
	Stand. coeff.	Regr. coeff. (95% CI)	P	Stand. coeff.	Regr. coeff. (95% CI)	P
Age	0.54	0.057 (0.04,0.08)	<0.001	0.22	0.023 (0.00,0.05)	0.04
Gender (boys vs. girls)	0.029	0.007 (-0.05,0.06)	0.79	-0.33	-0.076 (-0.14,0.01)	0.02
DHEAS (µmol/l)	0.051	0.004 (-0.01,0.02)	0.54	0.065	0.004 (-0.01,0.02)	0.53
S-25-OHD (nmol/l)	-0.027	0.000 (-0.00,0.00)	0.75	0.00	-0.001 (-0.00,0.00)	0.11
BMI SDS	0.56	0.040 (0.00,0.08)	0.04	0.79	0.055 (0.01,0.011)	0.02
Height SDS	0.36	0.027 (0.01,0.04)	0.001	0.30	0.022 (0.00,0.04)	0.02
Soft lean mass (%)	0.48	0.510 (-0.02,1.04)	0.06	0.63	0.650 (0.00,1.29)	0.05
LRP5 SNP E644E	-0.26	-0.072 (-0.12,-0.03)	0.006	-0.33	-0.087 (-0.14,0.03)	0.003
LRP5 SNP F549F	0.26	0.060 (0.02,0.10)	0.003	-0.13	-0.030 (-0.08,0.02)	0.25
Physical activity	0.08	0.009 (-0.02,0.03)	0.43	-0.18	-0.022 (-0.05,0.01)	0.14
S-Mean insulin (mU/l)	0.13	0.000 (-0.00,0.00)	0.18	-0.04	0.000 (-0.00,0.00)	0.73

[†]Coefficient of determination (R²; the estimated size of effect of all variables in the models) 0.60 for FN BMD_{areal} and 0.44 for LS BMD_{areal}

Stand. coeff., standardized coefficient; Regr. coeff., regression coefficient; CI, confidence interval; S, serum; DHEAS, dehydroepiandrosterone sulfate; ALP, alkaline phosphatase; 25-OHD, 25-hydroxyvitamin D; Mean insulin, serum mean insulin during 2-h oral glucose tolerance test

The children with PA had significantly lower serum 25-OHD and higher ALP concentration compared with the nonPA controls (TABLE 13). Serum ALP and 25-OHD concentrations were not intercorrelated. There was a weak negative correlation between 25-OHD and absolute fat mass ($r=-0.26$, $P=0.04$) among the PA children, but otherwise neither ALP nor 25-OHD was related to measures of body composition or age in the PA or control group. Accordingly, the difference in 25-OHD concentration between the PA and control children was no longer significant when adjusted for body fat mass ($P=0.08$), but remained significant after adjustment for age, sex and the season of sampling (winter/summer) ($P=0.02$). ALP correlated positively with

DHEAS ($r=0.35$, $P=0.007$), $\Delta 4A$ ($r=0.29$, $P=0.03$) and testosterone concentrations ($r=0.37$, $P=0.004$) in the children with PA but not in their non-PA controls.

Serum PTH concentrations were higher in the PA than control group, but the difference was not significant when adjusted for age and gender (TABLE 13). Serum calcium, phosphate, osteocalcin or ICTP concentrations did not differ between the PA and non-PA control groups. Serum PTH and 25-OHD concentrations were inter-correlated in the PA ($r=-0.36$, $P=0.004$) but not in the non-PA control group ($P=0.87$). Serum ALP, ICTP, calcium or phosphate concentration did not correlate significantly with BMD parameters in either study group, whereas serum PTH and osteocalcin concentrations correlated positively with FN BMD_{areal} in the PA subjects ($r=0.35$, $P=0.004$ and $r=0.25$, $P=0.05$, respectively) and serum 25-OHD concentration with LS BMD_{areal} in the non-PA controls ($r=0.33$, $P=0.009$).

TABLE 13. Biochemical markers of bone formation and resorption in prepubertal children with premature adrenarche (PA, n=64) and in the controls without premature adrenarche (nonPA, n=62). Means \pm 95% confidence intervals are shown.

<i>Bone marker</i>	PA (n=64)	nonPA (n=61)	<i>P (t test)</i>	<i>P (univariate model)</i>
S-ALP (U/l)	643 \pm 39	549 \pm 36	0.001	0.001
S-25-OHD (nmol/l)	53 \pm 3.7	59 \pm 4.0	0.024*	NM
S-ICTP (μ g/l)	11.9 \pm 1.02	11.7 \pm 0.69	0.83	0.71
S-Osteocalcin (μ g/l)	43.3 \pm 3.1	41.1 \pm 2.6	0.28	0.36
S-Calcium (mmol/l)	2.37 \pm 0.01	2.37 \pm 0.02	0.84	0.99
S-Phosphate (mmol/l)	1.36 \pm 0.03	1.38 \pm 0.04	0.28	0.42
S-PTH (ng/l)	34.6 \pm 2.6	31.1 \pm 2.02	0.04	0.06

P values taken from t test, univariate linear model adjusted for gender and age and Mann-Whitney test*

S, serum; DHEAS, dehydroepiandrosterone sulfate; ALP, alkaline phosphatase; 25-OHD, 25-hydroxyvitamin D; NM, not measured

* Blood sample was not obtained in one control girl (n=61 in serum parameters)

6. DISCUSSION

The main findings of this cross-sectional study were the followings: (1) In the prepubertal children with signs of androgen action (SAA subjects), circulating adrenal androgen (AA) concentrations correlated with the clinical signs of adrenarche. The highest AA concentrations were measured in the subjects with pubic or axillary hair. (2) There were, however, surprisingly many prepubertal subjects with very low serum AA concentrations despite evident signs of androgen action. (3) Compared with the control girls, a higher percentage of the girls with SAA, particularly of those with biochemically confirmed premature adrenarche (PA), were overweight and had “childhood metabolic syndrome” (cMBS). Of the adverse metabolic features, only insulin sensitivity was reduced in the girls with SAA independently of weight. (4) Girls with PA had accelerated early growth compared with their peers; they were significantly taller already at the age of 1 year.

6.1. Subjects

In the original cohort of the present study, all prepubertal children presenting with sign(s) of androgen action (SAA), but without central puberty were included. Thus, there should be no selection bias. The inclusion criteria for the SAA group did not include any biochemical markers of adrenarche; they included clinical signs only. In some analyses, when the role of adrenal androgens was regarded particularly important, only the SAA children with biochemical evidence of adrenarche (DHEAS ≥ 1 $\mu\text{mol/l}$) were included. This also made the comparisons between this and previous studies more reliable, as many of the previous studies have used biochemical criteria. By symptom-orientated inclusion criteria we were able to reach a representative sample of prepubertal children with PA, whereas most of the previous studies have concentrated on highly selected

populations. On the other hand, the present findings in the whole group of SAA children may not always be applicable to more selected patient groups. It should be kept in mind that it is the appearance of androgenic symptoms (not necessarily an increase in circulating androgens) that brings a child to see the physician, and knowledge of the whole clinical spectrum of the phenomenon is thus important.

The control children were invited using a list obtained from the Finnish population register. From the list, control subjects were selected by order sampling. Separate sampling was performed in each sex- and age group to yield a matched group for the SAA study subjects. Because the control cohort was community-based, it should have been a representative sample of the normal child population. In the analyses of some metabolic parameters, such as insulin and cholesterol concentrations, it might have been better to have a control group matched for weight. That was impossible in the present study setting. On the other hand, the population-based control cohort enabled us to compare the prevalence of overweight and cMBS between the subjects with SAA and the healthy controls.

6.2. Signs of androgen action and androgen concentrations

In this study, the varying signs of androgen action in prepubertal children were described. It is noteworthy that the appearance of pubic or axillary hair was usually the latest sign of increasing adrenal androgen concentrations during the commencement of adrenarche. Thus, careful symptom history and clinical examination could reveal PA already before the appearance of pubic or axillary hair in many cases. This is important to keep in mind when a child is examined *e.g.* because of accelerated growth. In the prepubertal subjects, the androgenic signs were found in several different combinations and they had appeared in varying order. Among the SAA subjects, children with pubic or axillary hair typically had higher AA concentrations than those with other

androgenic signs only. This indicates that less androgen is usually needed to produce adult type body odor than to induce pubic and axillary hair in prepubertal children.

A somewhat surprising finding was that there were 9 girls with evident signs of adrenarche but low DHEAS concentrations. On the other hand, even a higher number of control children exceeded the biochemical hallmark of adrenarche (defined as DHEAS ≥ 1 $\mu\text{mol/l}$) although they had no signs of androgen action. These findings support the previous notion that sensitivity to androgens differs between individuals and between the target organs within each individual (Toscano *et al.* 1993, Rosenfield 2005). Especially the finding of low DHEAS concentrations in some of the prepubertal subjects with androgenic signs is in line with previous similar reports in children with PP (Rosenfield *et al.* 1982, Mathew *et al.* 2002). Variable responses to similar androgen concentrations may be due to different expression levels of AR and steroid metabolizing enzymes like 5 α -reductase and aromatase, among target organs and among individuals. The sensitivity of AR also varies and is determined by genetic differences in the AR gene (Chamberlain *et al.* 1994). Both the abundance and sensitivity of AR may also be regulated by epigenetic modifications (Vottero *et al.* 1999 & 2006). These genetic and epigenetic mechanisms may actually be involved in the pathogenesis of PA (or clinical signs of PA) in some children (Vottero *et al.* 1999, Ibáñez *et al.* 2003a, Lappalainen *et al.* 2008a).

Expectedly, PA seems to present as a continuum: some children present with higher AA production and more pronounced signs of androgen action, while the others have only slightly increased circulating AA concentrations and “mild” symptoms. This study showed that also the “milder symptoms” – other than pubic and axillary hair – of androgen action at prepubertal age are usually caused by adrenal androgens, as the SAA subjects with these “mild” signs had significantly higher AA concentrations than their age-matched peers without any androgenic signs. Serum LH and FSH concentrations were at a prepubertal level, and testosterone and estrogen concentrations low in all the subjects. Thus, gonadal contribution to androgens was excluded.

In this non-selected group of prepubertal children with signs of androgen excess, none had NC21OHD by hormonal criteria (New *et al.* 1983). This is in line with the low prevalence of NC21OHD in Finland (Jääskeläinen *et al.* 1997). Deficiency of 3 β -HSD or 11 β -hydroxylase was also not suggested by the hormonal profiles in any of the subjects. It is noteworthy that we used the low-dose ACTH test (1 μ g) for excluding steroidogenic enzyme defects. The previously published hormonal 21OHD criteria (New *et al.* 1983) are based on the standard-dose ACTH test (250 μ g). However, low-dose ACTH tests have been proved reliable in evaluating the function of the hypothalamic-pituitary-adrenal axis (Arvat *et al.* 2000, Tordjman *et al.* 2000, Panamonta *et al.* 2003). In this study, low-dose ACTH caused a significant increase in all adrenal steroids measured. All the SAA subjects had their stimulated 17OHP and 11DOF concentrations within the range of the controls, speaking against any adrenal steroidogenic enzyme deficiencies. Moreover, a recent study suggests that baseline 17OHP measurement recognizes NC21OHD among patients with PP with 100% sensitivity and 99% specificity (Armengaud *et al.* 2009).

Unfortunately, we did not have an opportunity to measure 17-hydroxypregnenolone (17OH-Preg) and could thus not calculate the 17OHP/17OH-Preg ratio, which would better reflect the activity of 3 β -HSD than the Δ 4A/DHEA ratio (Lutfallah *et al.* 2002). The availability of the 17OH-Preg data would also have allowed one to estimate 17,20-lyase activity by the DHEA/17OH-Preg ratio. Human P450c17 catalyzes the conversion of 17OHP to Δ 4A at only 3% rate for the conversion of 17OH-Preg to DHEA (Miller 2009), and therefore the Δ 4A/17OHP ratio is not a reliable marker of the 17,20-lyase activity. The low-dose test has not been validated for diagnosing heterozygous carriers of the 21-hydroxylase gene mutations and genetic analyses of that gene were not performed. Thus, potential heterozygous carriers of 21-hydroxylase gene mutations could not be identified in the present study. Defining the 24-hour excretion rate of major urinary steroidogenic metabolites could have been useful in identifying all possible steroidogenic enzyme deficiencies.

Biochemical changes during normal adrenarche could be explained by increasing 17,20-lyase and SULT2A1, and decreasing 3 β -HSD activities in the ZR of the adrenal cortex. Based on the serum steroid ratios, children with SAA had higher SULT2A1 activity and a trend towards lower 21-hydroxylase activity than the prepubertal controls. The difference in the surrogate marker of 21-hydroxylase activity [(st11DOF+stF)/st17OHP] was mostly due to the higher stimulated 17OHP and lower (non-significantly) stimulated 11DOF concentrations. A previous study with normal child population did not show any significant changes in stimulated 17OHP or 11DOF during normal pubertal development (Lashansky *et al.* 1991).

6.3. Growth and body composition

It has been noted before that many children with PA are tall for age at the time of diagnosis (Voutilainen *et al.* 1983, Ibáñez *et al.* 1992, Ghizzoni and Milani 2000, Accetta *et al.* 2004). The novel finding in the present study was that the girls with PA had experienced greater growth velocity already during the first 2 years of life. In fact, most of the difference in height SDS observed at the time of diagnosis between the PA and control girls had developed by the age of 2 years. Although many of the girls with PA were overweight or obese, the more rapid growth in height could not be explained by the weight gain only. Firstly, the difference in height between the study groups became significant before that in weight. Secondly, the differences in height SDSs between the PA and control girls remained significant when adjusted for the corresponding BMI SDS. Moreover, not all tall PA subjects were overweight.

The present study setting did not allow one to draw firm conclusions about the cause of the early growth enhancement in the PA girls. Nevertheless, the high early growth rate in the PA girls suggests that the pathogenetic process of PA begins earlier than conventionally considered. An earlier beginning of adrenarche in general has actually been suggested also by Remer and co-

workers, who found that there is a continuous increase in urinary androgen metabolite excretion from 3 years onward in healthy children (Remer *et al.* 2005). The present study confirmed the previous findings of increased insulin and IGF-I concentrations in prepubertal girls with PA (Ibáñez *et al.* 1997b, Vuguin *et al.* 1999). Given the growth promoting effect of IGF-I and insulin, it is tempting to speculate that the insulin-IGF- system had a role in the early growth enhancement of the PA children, already before the appearance of any other signs of adrenarche. The underlying mechanisms of the early growth pattern in PA remain to be uncovered by population-based follow-up studies. These studies should begin at early post-natal life and include regular androgen, insulin and growth factor measurements.

The function of the hypothalamic-pituitary-adrenal (HPA) axis may be modulated during fetal life (Clark *et al.* 1996, Phillips *et al.* 2000, Kajantie *et al.* 2002). Several case-control studies have shown that children born SGA have increased prepubertal and pubertal DHEAS concentrations (Francois and de Zegher 1997, Ibáñez *et al.* 1999a, Tenhola *et al.* 2002, Veening *et al.* 2004). In the present study, no significant differences were found in the mean BW or BL SDS between the PA and control girls. Neither did the frequency of SGA differ between the study groups. These findings disagree with the study by Ibáñez and co-workers who found lower BW SDS in girls with a history of PA (all with PP) (Ibáñez *et al.* 1998a). However, there was an inverse linear correlation between BW SDS and DHEAS, DHEA and $\Delta 4A$ concentrations in the PA girls of the present study. Likewise, a population-based study revealed an inverse relationship between BWs and prepubertal AA concentrations in the general population (Ong *et al.* 2004). On the other hand, the same group later found no correlation between BW and urinary androgen metabolites at the age of 8 years, when the current weight was allowed for (Honour *et al.* 2007). In both of those studies, androgen production was related with higher current weight and early weight gain. No significant correlation between BWs and AA concentrations were found in the control group in the current study. There was a trend towards lower BLs and gestational ages in the girls with PA, and

the history of pre-eclamptic pregnancy was slightly more prevalent in the PA than control girls. Taken together, low BW (fetal growth restriction) does not seem to play any significant role in the pathogenesis of PA in our population, but prenatal factors may slightly affect the timing of adrenarche or AA concentrations in childhood.

The concept of fetal programming postulates that fetal growth restriction predisposes to several unfavorable metabolic features in later life (reviewed in Hales and Barker 2001). Birth size has also been connected with earlier pubertal development; menarche occurs at earlier age particularly when thinness at birth is followed by overweight during prepuberty (Adair 2001, Sloboda *et al.* 2007). The PA girls in this study had only a trend towards lower birth length, but increased current weight and more rapid weight gain in infancy compared with the controls. Among the PA girls, no significant differences in the height SDSs were found after 1 year of age between the BW tertiles. Thus, low BW alone does not seem to explain the increased current height in these PA girls. The ponderal indices were very similar in the PA and control groups. This is important to note because many of the associations between birth size and later metabolic disturbances have previously been attributed to the thinness at birth (Phillips *et al.* 1994, Hales and Barker 2001).

On average, children with PA weighed more, and had a higher fat mass and fat percentage than the controls. Increased prevalence of overweight in the present sample is in line with several previous studies that have connected PA and PP with increased weight (Dimartino-Nardi 1999, Vuguin *et al.* 1999, Neville and Walker 2005). One study showed that girls with PA had increased central fat mass despite normal weight (Ibáñez *et al.* 2003b). In the present study, no separate measurement of visceral and subcutaneous fat compartments was performed. As androgens have anabolic properties, one could assume that children with PA have increased muscle mass. In the body composition analysis, absolute muscle mass was higher in the PA than control group, but there was no significant difference in the muscularity between the study groups when total body weight

was accounted for. Hence, the slight increase in circulating adrenal androgens in PA does not seem to have any major anabolic effect on muscle mass at a prepubertal age.

6.4. Features of the metabolic syndrome

The girls with SAA had higher prevalence of cMBS and reduced insulin sensitivity compared with the control girls. The differences in the insulin concentrations during the OGTT and in the IS indices remained significant between the SAA girls and their controls even when adjusted for weight. This is in agreement with previous studies in which circulating insulin concentrations were increased in Catalan (Northern-Spanish) PP girls independently of weight (Ibáñez *et al.* 1997b & 1998b). The PP girls in those studies were normal weight, whereas our girls with SAA had higher mean weight compared with the healthy control girls. Moreover, the prevalence of cMBS and relative hyperinsulinemia in this study were strongly related to overweight: only one normal-weight girl with SAA had high insulin concentrations during the OGTT (defined as $\geq 90^{\text{th}}$ percentile of the controls), and none fulfilled the cMBS criteria. Compared with the Spanish study, the subjects with SAA in this study had lower insulin concentrations in general. This is apparently partly due to the different selection criteria of the subjects. In the present study, prepubertal girls with all signs of androgen action – not only those with PP – were included and no biochemical criteria were used, whereas all the Spanish female subjects had PP and increased adrenal androgen concentrations. The differences in the insulin levels may also reflect ethnic differences. In population-based studies, moderately and severely obese white children had lower insulin concentrations than their black and Hispanic peers (Weiss *et al.* 2004).

In addition to the studies by the Spanish group, there are a few other previous reports connecting PA with hyperinsulinemia. In two studies with African American and Hispanic American PP girls and boys the PA subjects typically presented with overweight or obesity

(Oppenheimer *et al.* 1995, Denburg *et al.* 2002). Our finding that lowered insulin sensitivity in PA children is strongly related with overweight is supported by a recent study in which PA and overweight had an additive effect on serum insulin concentrations in prepubertal subjects (Jean *et al.* 2009).

In our current study, girls with pubic or axillary hair had lower IS and higher insulin concentrations than those with other signs of PA. Of note, our study showed for the first time that mild hyperinsulinism is characteristic for PA throughout its clinical spectrum, not only for those with PP.

There were no significant differences in the fasting total, HDL or LDL cholesterol, or triglyceride concentrations between the study groups. The girls with SAA had a trend towards unfavorable HDL and triglyceride levels compared with their peers, but it seemed to be explained by their higher weight. Spanish PP girls were reported to have increased TG and decreased HDL concentrations (Ibáñez *et al.* 1998b). The PP subjects in that study were not overweight and the differences between their PP and control subjects remained significant after adjustment for weight. In that study, PP subjects of all pubertal stages were included, and the number study subjects in each Tanner stage was rather small. Moreover, the differences in TG and HDL between PP and control groups were not congruent in the all pubertal stages. However, alterations in lipid metabolism might fully develop only during puberty. This could explain why no significant differences between the SAA and control groups were seen in our prepubertal subjects. Follow-up studies from childhood until adulthood are apparently needed to elucidate this.

In the SAA girls, low BW did not seem to be related with unfavorable metabolic features. This disagrees with the suggested cascade from fetal growth restriction to PA, hyperinsulinism and ovarian hyperandrogenism (Ibáñez *et al.* 1998a & 1998c & 1999b & 2001b). These contradictory findings may have several explanations. First, the present study population was not as selected as in the previous studies: also children with “mild” signs of adrenal androgen action were included and

on average, our SAA subjects had rather mildly increased androgen concentrations. Possible ethnic differences cannot be excluded either. Furthermore, the possible effect of low BW could be covered by other stronger factors, like current weight, childhood weight gain, and genetic and nutritional factors. Moreover, being born large for gestational age and several maternal factors, e.g. obesity and weight gain during pregnancy, may also correlate with metabolic features in childhood (Boney *et al.* 2005, Mingrone *et al.* 2008) and confound the association between BW and metabolic indices. In any case, our study does not indicate any strong connection between low BW and the metabolic features in PA.

The prevalence of cMBS was significantly higher in the SAA than control group, but it remains unclear whether the mildly increased adrenal androgen concentrations in SAA/PA children have any role in this. The results indicated that overweight together with hyperinsulinemia are the best predictors of adverse metabolic features in both the SAA and control girls. Based on the present study setting, it is impossible to judge whether girls with SAA are at increased risk of MBS, type II diabetes mellitus or cardiovascular diseases in adulthood. The most striking difference between the SAA and control girls was in the insulin concentrations and in the insulin sensitivity indices. Because there were no differences between the study groups in the glucose concentrations even during the OGTT, lower insulin sensitivity based on the HOMA-IR and ISIcomp was determined only by higher insulin levels. Considering the physiologic decrease in insulin sensitivity during puberty (Bloch *et al.* 1987, Caprio *et al.* 1989), one cannot predict whether the differences in insulin concentrations between the prepubertal SAA and control girls remain after puberty. There are no large-scale follow-up studies that have investigated that. However, one study examining girls at different pubertal stages implied that relative hyperinsulinism in PP girls continues during puberty (Ibáñez *et al.* 1998b). Likewise, a recent small study with Turkish girls with history of PP found that the relative insulin resistance in PP girls persisted during adolescence (Livadas *et al.* 2009). On the other hand, in a study with 27 post-menarcheal Caucasian girls with history of PP, no

alterations in insulin sensitivity were found compared with the age-matched control girls (Meas *et al.* 2002).

It is noteworthy that the prevalence of cMBS was high in both the study and control group. This may be partly because the cMBS definitions that we used could have been too sensitive. Particularly the cut point used to define overweight ($\geq 75^{\text{th}}$ percentile on the national BMI charts) was rather low. As the main goal in this study was to compare the study groups, this limitation should not have confounded the main results, *i.e.* differences between the study groups. On the other hand, the high frequency of cMBS in the healthy control cohort was related to the high prevalence of overweight. It thus reflects the epidemic of childhood overweight that is also seen in Finland, and points out the strong correlation between overweight and metabolic disturbances already in childhood (Weiss *et al.* 2004, Goodman *et al.* 2005, Cali and Caprio 2008).

Interestingly, plasma NE was slightly higher in the SAA than control children, and high NE concentrations were associated with lower BW SDS and higher serum TG and insulin concentrations in the children with SAA. All these measures are related to MBS. Moreover, NE itself as a marker of sympathetic activity has been associated with components of MBS. Stress-stimulated plasma NE concentration predicted systolic BP and insulin resistance (high HOMA-IR index) in an 18-year follow-up study in healthy young Norwegian men (Flaa *et al.* 2008a & 2008b). On the other hand, high NE levels were interpreted to reflect resistance of fat tissue to NE in women with PCOS (Ek *et al.* 1997). Weight loss in obese adults has also been shown to improve metabolic indices and decrease NE concentrations (Straznicky *et al.* 2005). Furthermore, hyperinsulinemia can increase sympathetic nervous activity (Berne *et al.* 1992), and many of the SAA subjects were overweight and had relatively high insulin concentrations. Thus, NE could be a biochemical marker of increased risk of metabolic disturbances in some children with SAA. However, the number of subjects in each NE category was rather small and the differences in the

NE concentrations between the groups were marginal. Therefore, this interesting finding needs to be replicated in larger studies.

At present, there are several different definitions of MBS for adult populations (Alberti and Zimmet 1998, Balkau and Charles 1999, Einhorn *et al.* 2003, Alberti *et al.* 2005, Grundy *et al.* 2005). For children, there are even more definitions and criteria for MBS, and these criteria may classify different individuals as having MBS (Golley *et al.* 2006, Reinehr *et al.* 2007a, Pirkola *et al.* 2008). This undoubtedly complicates the comparisons between different studies and reduces the usefulness of these definitions in general. More importantly, there may be differences between the definitions in their association with the development of type 2 diabetes and cardiovascular consequences. Based on the present and some previous studies, fasting glucose concentrations usually remain unchanged in overweight or obese prepubertal children (Sinha *et al.* 2002, Golley *et al.* 2006). The MBS definitions that include only fasting glucose without any insulin measures are thus insensitive to alterations in insulin sensitivity in prepubertal subjects.

In this study, waist circumference was not measured which is a limitation with respect to definitions of cMBS. Increased visceral fat accumulation is currently considered a key feature in MBS. The correlation between waist circumference and unfavorable metabolic features has been noted also in children, and the use of waist circumference measure in defining MBS is recommended by several authors (Third Report of NCEP ATPIII 2002, Kahn *et al.* 2005, Watts *et al.* 2008). On the other hand, no national normative values exist for waist circumference in children. Moreover, there is a strong correlation between weight measures (*e.g.* BMI) and waist circumference (Golley *et al.* 2006) and some researchers have preferred using BMI instead of waist circumference in their MBS criteria for children (Weiss *et al.* 2004).

6.5. Other biochemical findings

Children with PA had lower mean 25-OHD concentration than the controls. This is presumably due to their higher mean weight and fat mass. In obese subjects, circulating vitamin D levels are lower than in normal-weight men and women, presumably because vitamin D is stored in subcutaneous fat compartments, and because of decreased exposure to sunlight (Compston *et al.* 1981, Liel *et al.* 1988, Wortsman *et al.* 2000, Arunabh *et al.* 2003). Low vitamin D concentrations have been noted also in obese children (Reinehr *et al.* 2007b).

Serum ALP concentrations were higher in the PA than control children, but they were not correlated with BMD measures in either study group. As the PA subjects were taller than the controls and the ALP concentrations of the PA subjects were independently associated with height SDS, the higher ALP concentrations seemed to reflect the increased growth velocity in the PA children. Serum ALP concentrations in PA subjects have not been reported previously.

The mean plasma epinephrine concentration was similar in the SAA and control groups. Unlike NE, which is also secreted by the sympathetic nerve endings, epinephrine is produced solely in the adrenal medulla. Epinephrine is the most abundant catecholamine of the adrenal medulla, and thus a reliable measure of adrenomedullary function (Eisenhofer *et al.* 1995). There are no previous reports on circulating catecholamine concentrations in PA subjects.

Like PA, classic 21OHD is characterized by increased circulating androgen concentrations. In patients with 21OHD, adrenomedullary secretion of epinephrine is reduced and the structural development of adrenal medulla may be incomplete (Merke *et al.* 2000). In addition, epinephrine levels seem to be lowest in the 21OHD patients with the most severe cortisol deficiency (Merke *et al.* 2000). The differences between these two conditions are obvious. Patients with 21OHD have diminished adrenocortical cortisol secretion, whereas in children with PA, cortisol production is normal. Presumably it is the low intra-adrenal cortisol concentration, rather than high androgen

levels, that causes the hypofunction of adrenal medulla in 21OHD. However, normal adrenomedullary function in the SAA and PA children in this study suggests that the development of adrenal medulla is intact in this patient group.

6.6. Bone mineral density

During pubertal growth, higher androgen concentrations in boys than in girls contribute to the sexual dimorphism in the skeleton, *i.e.*, greater bone length and larger bone volume in men. On the other hand, several studies have shown a positive relationship between sex steroid concentrations and bone strength measures in both men and women (Steinberg *et al.* 1989, Slemenda *et al.* 1996, Greendale *et al.* 1997). Because of the anabolic properties of androgens, one could assume that increased adrenal androgen production in PA might have a positive influence on bone mass. Indeed, children with PA had higher BMD_{areal} in both LS and FN than the controls without clinical or biochemical evidence of adrenarche did. However, volumetric BMDs did not differ significantly between the study groups. Higher BMD was also found in 14 Hispanic PP girls compared with their 16 healthy controls (Sopher *et al.* 2001). In that study, BMD_{areal} was significantly higher also when adjusted for body size. Another study with PP girls reported high BMD scores compared with reference values (Ibáñez *et al.* 2000d). In the study by Sopher and co-workers, the number of subjects was rather low. Moreover, they measured the total body BMD, as compared to LS and FN BMD measures in our study. The discrepancy in the results of the present and the previous studies may also be explained by differences in the selection criteria of the subjects and by ethnic differences. However, no consistent differences in BMD values between Caucasian and Hispanic children have been detected in general (Bachrach *et al.* 1999, Weaver *et al.* 2007).

The children with PA were taller than their nonPA controls. The differences in the BMD_{areal} measures between the study groups vanished when adjusted for height. Given that bone

age is typically advanced in children with PA (Voutilainen *et al.* 1983, Ibáñez *et al.* 1992, Accetta *et al.* 2004, Diaz *et al.* 2008), it seems that higher BMD_{areal} values observed in this study mostly reflected advanced skeletal growth. This was supported by the linear regression model analysis, in which height and weight were the main determinants of BMD_{areal} in the entire child population.

The mechanisms of bone mass accrual in children remain somewhat unclear. The present study attempted to assess the independent contribution of adrenal androgens and some other potential factors to BMDs in prepubertal children. Our results indicate that growth in height and bone size, and to lesser extent body weight, are more important determinates of bone mass accrual than increased circulating adrenal androgen concentrations in prepubertal children. The concentrations of DHEAS, 25-OHD or insulin, or the physical activity score were not independently associated with BMD_{areal} in either LS or FN in either PA or control children. The important role of the size of the child in BMD is in line with previous studies (Lu *et al.* 1994, Yilmaz *et al.* 2005). Also the positive relationship between muscularity (measured as soft lean mass percent) and BMD is consistent with other studies (Remer *et al.* 2003, Vicente-Rodriguez *et al.* 2005, Remer *et al.* 2009). Male gender was independently associated with higher FN BMD_{areal} in the nonPA controls, whereas LS BMD_{areal} was higher in the PA girls than boys. These gender differences are in accordance with previous findings in prepubertal children (Boot *et al.* 1997, Jones and Dwyer 1998, Garnett *et al.* 2004).

The lack of independent correlation between BMD and the concentrations of DHEAS, testosterone and estradiol may be explained by the low hormone concentrations in this prepubertal sample in general. A recent study with prepubertal subjects also found no relationship between BMD and DHEAS concentrations (Garnett *et al.* 2004). It is possible that the effect of increased androgen action at prepubertal age becomes detectable in bone scans during later stages of pubertal development. This view is supported by the recent finding that in healthy children, prepubertal urinary androstendiol excretion rate predicted bone mineral content and diaphyseal bone strength at

adolescence (Remer *et al.* 2009). Furthermore, an Estonian study with boys at different pubertal stages found a positive correlation between BMD and testosterone concentration (Pomerants *et al.* 2007). Based on that study on pubertal boys and the present study on prepubertal children, one could infer that male pubertal levels of testosterone are needed to promote bone mineral accrual. On the other hand, women with hyperandrogenism have had greater BMD values than healthy control women in several study populations (Buchanan *et al.* 1988, Dagogo-Jack *et al.* 1997, Glinborg *et al.* 2005). Thus, even rather small increases in androgen production can have a positive effect on bone mass at least in adults.

In this study, we did not find any association between the *LRP5* A1330V polymorphism and bone characteristics. This is probably explained by the small sample size. In previous studies, this polymorphism has been repeatedly associated with lower BMD values (Tran *et al.* 2008, Lee *et al.* 2009). On the other hand, two other *LRP5* single nucleotide polymorphisms (SNPs), E644E and F549F, were associated with FN BMD_{areal}. These findings need to be confirmed in larger studies.

Limitations of the present study with respect to BMD measurements include the lack of data concerning bone age and the exact consumption of dairy products. Milk consumption has been associated with BMD in children (Boot *et al.* 1997). Also physical activity has a positive effect on bone mineral accrual in children (Boot *et al.* 1997, Jones and Dwyer 1998, Bailey *et al.* 1999). In this study, we attempted to take this potentially confounding factor into account. The rough estimates of the amount of physical activity enabled a separation of the subjects into 3 categories. There were no differences in the physical activity scores between PA and control children. However, more accurate measures of actual physical activity might have improved the reliability of the analyses. Furthermore, direct measures of volumetric bone density were not obtained. Quantitative computed tomography (QCT) is a more sensitive and accurate method for analyzing volumetric BMD than the DXA method used in this study. Particularly, QCT is less sensitive to differences in body size. Finally, bone strength is not determined by bone density alone, and

possible differences in the actual bone strength are impossible to assess by the traditional BMD analyses. Measuring bone characteristics is particularly difficult in children, as reviewed in depth by Petit and co-workers (Petit *et al.* 2005).

6.7. General aspects

This study confirmed that there is a spectrum of clinical manifestation of PA, and that signs of androgen action appear in several different combinations and order in prepubertal children. Moreover, a relatively large group of children presented with androgen concentrations that were inconsistent with the clinical signs. This indicates that there is high inter-individual and intra-individual variability in the sensitivity to androgens. In this study with an unselected group of SAA children, no typical medical history predisposing to SAA/PA could be identified. Despite a trend towards lower absolute BL, there were no significant differences in BL or BW SDS between PA and control children. Moreover, ponderal indices were very similar in the PA and control groups. Overweight was more common in the SAA than control subjects, but there were also thin and normal weight SAA and PA subjects. Both birth sizes and current anthropometric measures overlapped considerably in the two study groups.

Thus, the pathogenesis of PA appears to be multifactorial. There are presumably several subgroups among prepubertal children with hyperandrogenic signs with differential underlying mechanisms of the androgen abundance or action. It is noteworthy that the metabolic-endocrine prognosis of PA may differ according to the etiology.

6.8. Future perspectives

To further elucidate the mechanisms underlying the pathogenesis of PA, a population-based follow-up study from early infancy through puberty with regular laboratory and clinical assessments would be needed. The possible roles of the insulin-IGF system and other biochemical factors in the development of PA could be studied by regular laboratory screening in such a cohort. On the other hand, there are several candidate genes that may predispose to PA or modify the consequences of PA. Active research is ongoing in this field. These studies have already revealed some genetic factors that are associated with PA. Another future research goal is to clarify the long-term prognosis of PA. Thus far, no large-scale follow-up studies including PA subjects and controls exist. With such a follow-up study one could reliably investigate whether the relative hyperandrogenism and hyperinsulinism in prepubertal PA subjects persists through adulthood as has been suggested. Similarly, the proposed increased risk of ovarian hyperandrogenism, PCOS and MBS (Ibáñez *et al.* 1993 & 1998b & 1998c) in PA subjects could be evaluated by comparing the incidence numbers between PA and control subjects within a cohort. Reliable evidence of the long-term sequelae of PA is needed before the use of insulin sensitization (*e.g.* with metformin) (Ibáñez *et al.* 2000a) could be considered for insulin resistant PA subjects in our population.

7. SUMMARY AND CONCLUSIONS

1. In the present study, it was discovered that prepubertal hyperandrogenic signs can appear in various combinations and in varying order. Usually the appearance of pubic and axillary hair was the last clinical sign of adrenarche. This suggests that more androgens are needed to induce pubic and axillary hair than other androgenic signs, *e.g.* adult type body odor. Active recognition of the variable signs might improve early diagnosis of PA.
2. This study confirmed that there is a wide phenotypic variability in PA. There was no single typical feature in the patient history or current characteristics by which the PA subjects could have been reliably identified. It is probable that there are subgroups among the children with PA with different underlying mechanisms of androgen excess or action. Thus, findings in one subgroup of PA subjects may not be valid for all children with signs of PA.
3. Unlike in some previous studies, there were no significant differences in the BW or BL SDSs, or in the prevalence of SGA between the PA and control girls. Thus, low BW does not seem to play any significant role in the pathogenesis of PA in our population.
4. This study revealed a distinct prepubertal growth pattern in the girls with PA. These girls had higher growth rate during the first two years of life than the control girls, and they were taller already at the age of 1 year. At the median age of 7.6 years, the difference in height SDS between the PA and control girls averaged 1.2 SDS. The enhanced growth was not exclusively explained by weight, although weight gain was also more rapid in the PA than control girls. This finding of early growth acceleration in PA girls suggests that the pathogenetic processes underlying PA begin earlier than previously considered.

5. A higher percentage of the girls with SAA (and PA) than of the controls was classified as having the “childhood metabolic syndrome” (cMBS) according to the modified MBS definitions. Of the MBS components, serum insulin concentrations were higher and insulin sensitivity indices lower in the SAA than control girls, independently of weight. Otherwise, cMBS was strongly determined by weight, in both the SAA and control girls. Thus, girls with SAA/PA may be at an increased risk of developing metabolic disturbances, but this seems to be caused mainly by their higher weight. Routine measurement of insulin sensitivity in overweight SAA/PA girls could be recommended. More importantly, weight management should be specially emphasized in these children.

6. Children with PA had a higher areal BMD in FN and LS than the control children as measured by DXA. However, the differences between the study groups were not significant when adjusted for either the size of the bone or the height of the child. There was no difference in the muscle/total mass ratio between the study groups either. These findings suggest that the slight increase in adrenal androgens during adrenarche has no major anabolic effect on bone density or muscularity at a prepubertal age. Nevertheless, more accurate measurements of bone characteristics might better elucidate the influence of androgens on bone. Follow-up studies are needed to reveal potential later effects of PA on bone density and strength.

7. The most important take-home messages for clinicians of this study are the following:

- 1) PA may explain early accelerated growth in height in prepubertal girls.
- 2) Adult type body odor is the most common and typically the first sign of premature adrenarche. It should be actively inquired from the parents and assessed in the physical examination of prepubertal children in whom PA is possible.
- 3) In PA girls, low insulin sensitivity is mainly seen in those with concomitant obesity.

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