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SAILA LAAKSO (NÉE LAPPALAINEN)

Genetic Variation in Premature Adrenarche

Association Studies on Candidate Genes in a Case-Control Cohort

Doctoral dissertation

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Series Editors: Professor Raimo Sulkava, M.D., Ph.D.
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Professor Markku Tammi, M.D., Ph.D.
Institute of Biomedicine, Department of Anatomy

Author´s address: Clinical Research Centre, Pediatrics
University of Kuopio
P.O. Box 1627
FI-70211 KUOPIO
FINLAND

Supervisors: Docent Jarmo Jääskeläinen, M.D., Ph.D.
Institute of Clinical Medicine, Pediatrics
University of Kuopio and Kuopio University Hospital

Professor Raimo Voutilainen, M.D., Ph.D.
Institute of Clinical Medicine, Pediatrics
University of Kuopio and Kuopio University Hospital

Reviewers: Docent Päivi Miettinen, M.D., Ph.D.
Hospital for Children and Adolescents
Helsinki University Central Hospital and Biomedicum Helsinki
University of Helsinki

Docent Riitta Veijola, M.D., Ph.D.
Department of Pediatrics
University of Oulu and Department of Pediatrics and Adolescence
Oulu University Hospital

Opponent: Dr. John Achermann, F.R.C.P.C.H., M.D.
Institute of Child Health
University College London
United Kingdom

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ABSTRACT

Premature adrenarche (PA) is defined as adrenarcheal levels of adrenal androgens before the age of 8 yrs in girls and the age of 9 yrs in boys leading to androgenic signs ranging from pubarche to oily skin and adult type body odor. PA has been connected with adverse metabolic features and increased risk for ovarian hyperandrogenism. The pathogenesis of PA is considered polygenic. However, underlying genetic factors remain largely unknown.

We aimed to determine the role of genetic variation of PA candidate genes in a case-control cohort of prepubertal PA children (63 girls and 10 boys) and their age- and gender-matched controls (79 girls and 18 boys). The following candidate genes with previously described polymorphisms were selected based on the current knowledge of PA: ACTH receptor (*MC2R*), androgen receptor (*AR*), low density lipoprotein receptor-related protein 5 (*LRP5*), transcription factor 7-like 2 (*TCF7L2*), and fat mass and obesity associated gene (*FTO*). We compared genotype distributions between the PA and control groups, and used single marker association analyses to relate genetic variants with clinical phenotype.

The minor variant of the single nucleotide polymorphism (SNP) MC2R -2 T>C was more frequent in subjects with premature pubarche than in children with milder signs of PA and controls. The minor variant was associated with a higher ratio of ACTH to cortisol in the control group, in agreement with previous studies that have shown decreased ACTH sensitivity due to the polymorphism. In children with PA, the minor variant associated with higher androstenedione level and ratio of androstenedione to cortisol, suggesting shifting of steroidogenesis from corticosteroids to androgens. The length of CAG_n at X-chromosomal *AR* correlates inversely with the activity of AR. Children with PA had a shorter CAG_n repeat than the controls, and the difference became even stronger when we took the X-chromosome inactivation into account. The lean PA children with a BMI below the median of the group had a shorter CAG_n than the PA children with higher BMI or the controls with the same BMI. More active AR may have a significant role in the pathogenesis of PA in these lean children. Minor variants at SNPs A1330V and N740N of *LRP5* were associated with higher dehydroepiandrosterone sulfate and cholesterol levels in control children, but no association between genetic variants at *LRP5* and clinical parameters was observed in the children with PA. The minor variant at rs7903146 of *TCF7L2* was more frequent in lean PA children. The minor variant at rs9939609 of *FTO* was not more frequent in children with PA, suggesting that this genetic variant in *FTO* has no major role in the increased BMI of PA children. The power of the study was limited, and the results need to be confirmed in different populations. However, the value of the study lies in the use of unbiased controls and in the precise phenotyping of all the children from a homogenous population.

In conclusion, MC2R -2 T>C may have a role in the pathogenesis of premature pubarche. Lean PA children show a different genotype, with a shorter CAG_n repeat, indicating a more active AR, and increased frequency of the minor variant at rs7903146 of *TCF7L2* in comparison to PA children with higher BMI.

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Medical Subject Headings: Adrenarche/genetics; Adrenocorticotropic Hormone; Androstenedione; Body Mass Index; Body Weight; Case-Control Studies; Cholesterol; Dehydroepiandrosterone Sulfate; Genes; Genetic Markers; Genetics; Genetic Variation; Genotype; Phenotype; Polymorphism, Genetic; Receptors, Androgen; Receptors, Corticotropin; LDL-Receptor Related Proteins; Proteins/genetics; TCF Transcription Factors/genetics; Polymorphism, Single Nucleotide/ genetics; Puberty, Precocious/genetics; X Chromosome

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Saila Laakso
Espoo, September 2009

ABBREVIATIONS

A	Adenine
ACTH	Adrenocorticotropic hormone
Apo	Apolipoprotein
AR	Androgen receptor
BMI	Body mass index
BP	Blood pressure
C	Cytosine
CAG_n	Cytosine-adenine-guanine repeat length
CAH	Congenital adrenal hyperplasia
cAMP	3',5'-cyclic adenosine monophosphate
CI	Confidence interval
CP	Cerebral palsy
CRH	Corticotropin releasing hormone
DAX-1	Dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X-chromosome, gene-1
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
FOH	Functional ovarian hyperandrogenism
FTO	Fat mass and obesity-associated gene
G	Guanine
GAD2	Glutamate decarboxylase 2
GnRH	Gonadotropin-releasing hormone
GWA	Genome wide association
HDL	High-density lipoprotein
HSD17B5	Hydroxysteroid 17β-dehydrogenase 5
HOMA-IR	Homeostasis model assessment for insulin resistance
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IGF-1R	Type 1 IGF receptor
ISI_{comp}	Insulin sensitivity index
LDL	Low-density lipoprotein
LD	Linkage disequilibrium
LRP5	Low density lipoprotein receptor-related protein 5
MAF	Minor allele frequency
MC2R	Melanocortin-2 receptor
mRNA	Messenger ribonucleic acid
$mwCAG_n$	Methylation weighted biallelic means of CAG_n
OGTT	Oral glucose tolerance test
PA	Premature adrenarche
PAI-1	Plasminogen activator inhibitor-1
PCOS	Polycystic ovary syndrome
PP	Premature pubarche

SDS	Standard deviation score
SERKAL	Sex reversal, female, with dysgenesis of kidneys, adrenals and lungs
SF-1	Steroidogenic factor-1
SHBG	Sex hormone-binding globulin
SNP	Single nucleotide polymorphism
SORBS1	Sorbin and SH3 domain containing 1 gene
SULT2A1	DHEA sulfotransferase
T	Thymine
TCF7L2	Transcription factor 7-like 2
T2DM	Type 2 diabetes mellitus
UGT2B	Uridine diphospho-glucorunosyltransferase 2B
Vmax	Maximum velocity of the enzyme catalytic activity
VNTR	Variable number of tandem repeats
Δ4-A	Androstenedione
17OHP	17-hydroxyprogesterone
3βHSD	3β-hydroxysteroid dehydrogenase
3' UTR	3' untranslated region

LIST OF ORIGINAL PUBLICATIONS

This study is based on the following articles, which are referred to in the text by the corresponding Roman numerals (I-IV)

I Lappalainen S, Utriainen P, Kuulasmaa T, Voutilainen R, and Jääskeläinen J. ACTH receptor promoter polymorphism associates with the severity of premature adrenarche and modulates hypothalamo-pituitary-adrenal axis in children. Pediatr Res 2008;63:410-4.

II Lappalainen S, Utriainen P, Kuulasmaa T, Voutilainen R, and Jääskeläinen J. Androgen receptor gene CAG repeat polymorphism and X-chromosome inactivation in children with premature adrenarche. J Clin Endocrinol Metab 2008;93:1304-9.

III Lappalainen S, Saarinen A, Utriainen P, Voutilainen R, Jääskeläinen J, and Mäkitie O. *LRP5* in premature adrenarche and in metabolic characteristics of prepubertal children. Clin Endocrinol (Oxf). 2009;70:725-31.

IV Lappalainen S, Voutilainen R, Utriainen P, Laakso M, and Jääskeläinen J. Genetic variation of *FTO* and *TCF7L2* in premature adrenarche. Metabolism 2009;58:1263-9.

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1 INTRODUCTION

Adrenarche is unique to humans and higher primates in whom the production of adrenal androgens follows an age- and gender-dependent pattern (Parker 1999). During fetal development, the fetal cortex of the adrenal gland secretes large amounts of androgens which act as precursors for the estrogen production in the placenta (Siiteri and MacDonald 1966). After birth, the fetal cortex disappears and the levels of adrenal androgens decrease. Adrenal androgens stay low until adrenarche, the rise in adrenal androgen levels after the age of 6 yrs, preceding the activation of central puberty (de Peretti and Forest 1976, Mesiano and Jaffe 1997). At puberty, the adrenal androgen levels rise to a higher level in men than in women, and dehydroepiandrosterone sulfate (DHEAS) has the highest circulating plasma level of all steroid hormones in adults with a slow decline during aging (Rosenfield et al. 1982, Labrie et al. 1997, Nafziger et al. 1998). Adrenocorticotropic hormone (ACTH) stimulates adrenal androgen secretion, but no change in circulating ACTH levels is seen during adrenarche (Nieschlag et al. 1973, Apter et al. 1979). It is not known which factors awaken the reticular zone of the adrenal cortex to secrete androgens at adrenarche.

Premature adrenarche (PA) is defined as the adrenarcheal levels of adrenal androgens (DHEAS above 1 μmol/l) in girls before the age of 8 yrs and in boys before the age of 9 yrs, resulting in androgenic signs ranging from growth of pubic hair (premature pubarche, PP) and axillary hair (Silverman et al. 1952, Thamdrup 1955) to oily hair and skin (Rosenfield et al. 1982), adult-type sweating and body odor (Voutilainen et al. 1983, Kaplowitz et al. 1986, Likitmaskul et al. 1995). PA was long considered a benign variant of pubertal development until it was connected in the '90s with adverse metabolic features and a possible risk for ovarian hyperandrogenism (Rosenfield 1994, Dimartino-Nardi 1999, Ibáñez et al. 2000a). The pathogenesis of PA is considered polygenic, and possible associations have been investigated in genes participating in steroidogenesis (Witchel et al. 2001, Petry et al. 2005), androgen action (Ibáñez et al. 2003b, Vottero et al. 2006), and in the functions of insulin and insulin-like growth factors (IGF) (Ibáñez et al. 2002, Roldan et al. 2007). However, the pathogenesis of PA remains largely unknown.

Predispoding genetic factors for adrenarche remain to be identified in the human genome. The development of genetic analyses made it possible to construct the sequence of the whole human

genome, also revealing the millions of single nucleotide polymorphisms and other structural variants within the genome (International Human Genome Sequencing Consortium 2004). Genotype-phenotype associations have been studied, but the mechanisms between genetic variants and clinical phenotype are difficult to solve. By searching for associations between genetic variants and phenotype, we can gain insight into the mechanisms behind PA. This was the aim of the thesis.

2 REVIEW OF THE LITERATURE

2.1 ADRENARCHE

2.1.1 Physiology of adrenal androgen production

The adrenal cortex secretes androgens androstenedione (Δ4-A), dehydroepiandrosterone (DHEA) and its sulfated form DHEAS in age- and gender-dependent pattern (Parker 1999). During the fourth week of gestation, coelomic epithelial cells and underlying mesonephric mesenchymal cells migrate to form the adrenogonadal primordium, of which the primitive adrenal and gonadal primordial cells separate by the eight week of gestation (Mesiano and Jaffe 1997). The key regulators of adrenal development are the orphan nuclear receptors DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X-chromosome, gene-1) and steroidogenic factor-1 (SF-1). Mutations in these transcription factors have also been found in patients with adrenal hypoplasia (Muscatelli et al. 1994, Zanaria et al. 1994, Achermann et al. 1999, Lin et al. 2006). During fetal development, the fetal cortex of the adrenal gland secretes huge amounts of adrenal androgens for the placental estrogen production (Siiteri and MacDonald 1966). Soon after birth, the fetal cortex regresses by apoptosis and adrenal androgen levels decrease, remaining low until the time of adrenarche, the reactivation of adrenal androgen production (de Peretti and Forest 1976, Mesiano and Jaffe 1997, Lashansky et al. 1991). Adrenarche occurs slightly earlier in girls than in boys (Sizonenko and Paunier 1975, Ducharme et al. 1976), but DHEAS levels in boys exceed those in girls around the age of 20 years (Rosenfield et al. 1982). DHEAS has the highest circulating plasma level of all steroid hormones in adults. With aging, DHEAS levels decline on average 2% per year from the highest levels in the twenties through the sixties (Labrie et al. 1997, Nafziger et al. 1998)

Adrenal androgens are considered weak androgens, which can be converted to stronger androgens and estrogens by target tissues, and their effects are mediated by nuclear hormone receptors. The visible signs of adrenarche include growth of pubic and axillary hair, oily hair, comedones, acne, and the development of adult type sweat secretion and body odor in children.

Adrenal androgens also participate in growth and bone maturation. As adrenal androgens are also neurosteroids (Goodyer et al. 2001), adrenarche may have a role in brain development (Suzuki et al. 2004). It has been proposed that adrenarche promotes changes in behavior and cognition preparing children for the challenges of puberty (Campbell 2006, Hochberg 2008).

Adrenarche results from the formation of continuous innermost layer of adrenal cortex called zona reticularis which secretes mainly DHEA and DHEAS (Dhom 1973, Reiter et al. 1977). The medullary capsule separating the cortex and medulla of the adrenal gland breaks down, and the focal development of zona reticularis starts at the age of 5 yrs. A continuous zone is usually present by the age of 8 yrs (Dhom 1973). The first appearance of zona reticularis cells is detected at the age of 3 yrs, and some studies have shown a gradual rise in adrenal androgen levels already from that age (Palmert et al. 2001, Remer et al. 2005). The outer layers of the adrenal cortex, the zona glomerulosa and fasciculata, secrete mineralocorticoids and glucocorticoids, respectively. The adrenal medulla secretes catecholamines. The zones of the adrenal cortex are formed by migration of undifferentiated cells from the gland periphery under the gland capsula toward the medulla. As the cells migrate, they differentiate to have zone specific steroidogenic capacities (Kim and Hammer 2007).

Like all steroid hormones, also adrenal androgens are produced from cholesterol (Figure 1). Cholesterol enters the mitochondria with the assistance of the steroidogenic acute regulatory protein (StAR). Within the mitochondria, cholesterol is converted to pregnenolone by the cholesterol side chain cleavage enzyme (P450scc). Pregnenolone undergoes 17α-hydroxylation by microsomal P450c17, and 17-hydroxypregnenolone is further converted to DHEA by the 17,20-lyase activity of the same P450c17 enzyme (Miller 2002). Sulfotransferase (SULT2A1) catalyzes the sulfonylation of DHEA to DHEAS, which has a more stable plasma level due to the longer half-life in blood and less fluctuating secretion (Rosenfeld et al. 1975). DHEAS is secreted by the adrenal cortex only and not by the gonads (Nieschlag et al. 1973).

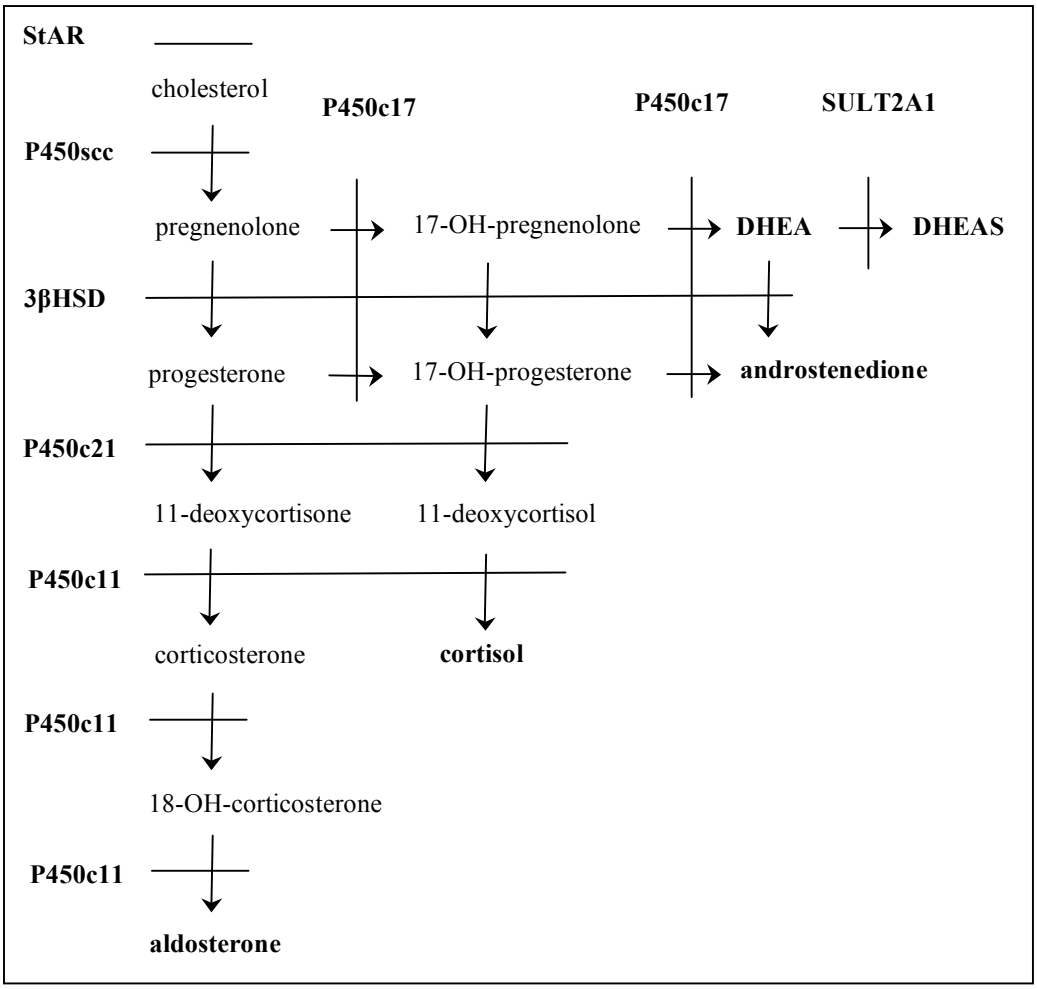

Figure 1. Steroidogenesis in the human adrenal cortex. Cholesterol enters the mitochondia with assistance of the steroidogenic acute regulatory protein (StAR). The horizontal and vertical lines represent enzymes that are expressed in a zone-specific pattern. Arrows indicate the direction of the metabolic pathway. P450scc, cholesterol side-chain cleavage enzyme; P450c17, 17α-hydroxylase/17,20-lyase; SULT2A1, dehydroepiandrosterone sulfotransferase; 3βHSD, 3β-hydroxysteroid dehydrogenase; P450c21, 21-hydroxylase; P450c11, 11β-hydroxylase/18-hydroxylase/18-oxidase; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate. Modified from (Miller 1988).

The levels of enzymes needed for steroid production directly regulate the secretion of adrenal androgens, which cannot be stored as lipid-soluble steroids in the adrenal cortex (Miller 2008). P450c17 has both 17α-hydroxylation and 17,20-lyase activities, of which only 17α-hydroxylation is required for glucocorticoid synthesis, and neither of them is needed for mineralocorticoid production (Figure 1). The discrimination between 17α-hydroxylation and 17,20-lyase activities is regulated by serine phosphorylation of P450c17 (Zhang et al. 1995) and the allosteric action of cytochrome b_5 (Auchus et al. 1998, Akhtar et al. 2005), both of which act to optimize the interaction of P450c17 with its electron donor, P450 oxidoreductase. The abundant expression of P450 oxidoreductase and cytochrome b_5 increases the 17,20-lyase activity in the zona reticularis, whereas the low expression of 3β-hydroxysteroid dehydrogenase (3ßHSD), which competes for substrates with P450c17 diverts the steroidogenesis further to the production of androgens (Kelnar and Brook 1983, Endoh et al. 1996, Gell et al. 1998, Dardis et al. 1999, Suzuki et al. 2000). The activity of SULT2A1 also increases in the zona reticularis by the time of adrenarche (Suzuki et al. 2000). In vitro studies on mouse Y-1 adrenocortical cells have shown that SF-1 regulates the expression of several steroidogenic genes, e.g. *P450scc*, *StAR* and *3ßHSD* (Parker and Schimmer 1997), whereas DAX-1 represses their expression (Zazopoulos et al. 1997, Lalli et al. 1998).

2.1.2 Regulation of adrenarche

Adrenarche precedes gonadarche, i.e., the activation of gonadal hormone secretion, and these events are regulated separately (Dhom 1973, Rosenfield et al. 1982, Apter and Vihko 1985). Patients with precocious puberty exhibit gonadarche in the absence of adrenarche (Sklar et al. 1980, Counts et al. 1987), and adrenarche progresses normally despite the treatment aiming at the pituitary-gonadal suppression in these children (Wierman et al. 1986, Palmert et al. 2001). In addition, patients with gonadal dysgenesis or isolated gonadotropin deficiency have a normal onset of adrenarche (Albright et al. 1942, Sizonenko and Paunier 1975, Sklar et al. 1980, Counts et al. 1987). The regulation of adrenal androgen production has been summarized in simplified form in figure 2.

The pituitary gland secretes ACTH, which stimulates adrenal glucocorticoid and androgen production in a circadian rhythm (Nieschlag et al. 1973, de Peretti and Forest 1976). Without

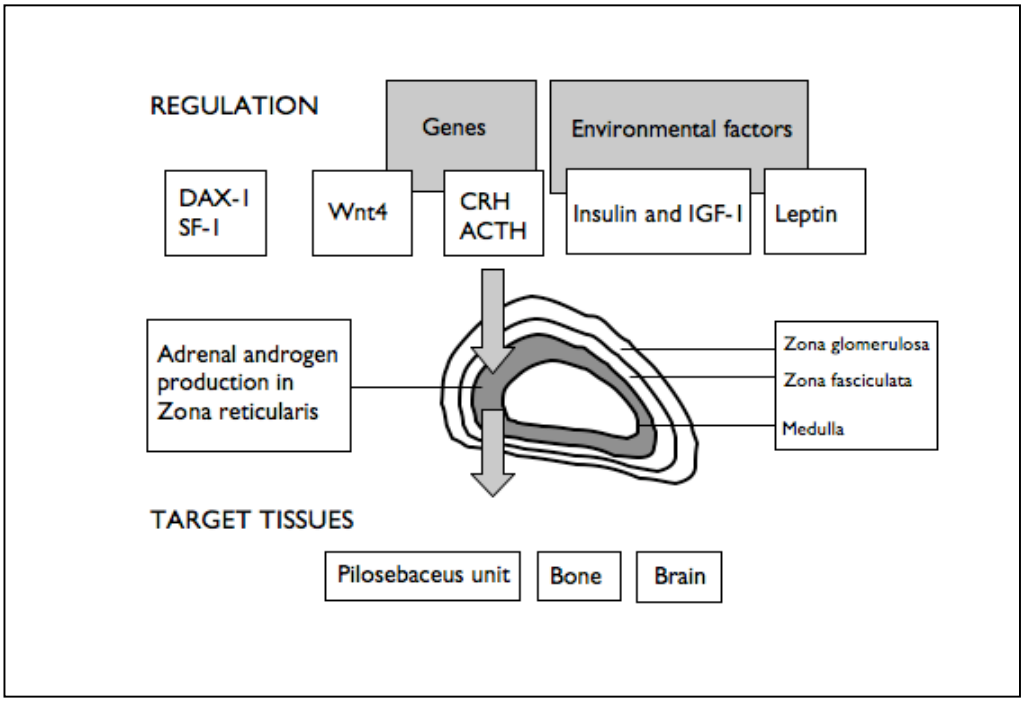

Figure 2. The regulation of adrenal androgen production from the zona reticularis and the target tissues of circulating adrenal androgens. The transcription factors DAX-1 and SF-1 are essential for the development of the adrenal gland. Genes and environmental factors form the basis for the regulation of adrenal androgen production, in which factors such as Wnt4, adrenocorticotropic hormone (ACTH), insulin, insulin-like growth factor 1 (IGF-1) and leptin participate. The innermost layer of the adrenal cortex, called the zona reticularis, secretes adrenal androgens that have effects on e.g. the pilosebaceus unit, bone and brain.

the action of ACTH, a rise in the adrenal androgen secretion cannot happen. The lack of adrenarche in the patients with familial glucocorticoid deficiency syndrome due to ACTH resistance provides evidence for a significant role of ACTH in the regulation of adrenarche (Sizonenko and Paunier 1975, Weber et al. 1997). ACTH mediates the effects on steroidogenesis through a membrane receptor called melanocortin-2 receptor (MC2R), the activation of which increases intracellular cAMP level, leading to the stimulation of synthesis and activity of StAR and the steroidogenic enzymes:P450c17, 3βHSD, SULT2A1 (McAllister and Hornsby 1988, McCarthy and Waterman 1988). Besides these effects on steroidogenesis,

ACTH is essential for the maintenance and growth of steroidogenic cells in the adrenal cortex (Dallman 1984). The hypothalamo-pituitary-adrenal axis regulates its own functions by a long feedback; glucocorticoids inhibit the secretion of corticotropin releasing hormone (CRH) in the hypothalamus, and this inhibits the secretion of ACTH from pituitary gland (Watts 2005). However, there are no significant changes seen in the circulating cortisol and ACTH levels during adrenarche (Apter et al. 1979). In addition, DHEA and DHEAS levels are normal for both chronological and bone age in most children and adolescents with Cushing's disease (Hauffa et al. 1984). It has been proposed that factors like prolactin, estrogens, or CRH could modulate the actions of ACTH in the zona reticularis cells (Ibáñez et al. 1999b, Baquedano et al. 2007), but no convincing mechanisms for these hypotheses have been found. Intra-adrenal factors such as the sympatho-adrenal system and cytokines have also been suggested to participate in the initiation of adrenarche (Ehrhart-Bornstein et al. 1998, l'Allemand and Biason-Lauber 2000).

An interaction between adrenal androgens and serum cytokines, IGF-1, and insulin has been postulated in adrenarche (Belgorosky et al. 2009), as all these factors have gender-dependent changes during puberty. In cultured fetal adrenocortical cells, IGF-2 is expressed in response to ACTH, and it promotes the production of cortisol and DHEAS by increasing the expression of P450scc and P450c17 (Voutilainen and Miller 1987, Mesiano et al. 1997), whereas IGF-1 increases the expression of P450c17 and 3βHSD in cultured adult adrenocortical cells (l'Allemand et al. 1996, Kristiansen et al. 1997). Serum DHEAS levels correlate positively with serum IGF-1 in prepubertal girls, whereas no correlation has been found in girls during puberty or in boys before and during puberty (Guercio et al. 2002, Guercio et al. 2003). These results have been suggested to indicate sexual dimorphism in the regulation of adrenarche, in which IGF-1 may regulate adrenal progenitor cell proliferation and migration (Baquedano et al. 2005). On the other hand, insulin resistance and compensating hyperinsulinemia occur during puberty (Moran et al. 1999), and plasma insulin concentrations correlate positively with IGF-1 levels (Bloch et al. 1987). Plasma insulin levels also correlate with serum DHEAS levels in pubertal children, but not in prepubertal children with adrenarche (Bloch et al. 1987, Smith et al. 1989). Decreased insulin sensitivity is related to serum growth hormone concentrations and body fat during puberty (Amiel et al. 1986, Travers et al. 1995, Moran et al. 1999). The possible role of

insulin sensitivity and BMI in adrenarche is not straightforward and may be gender-dependent (Guercio et al. 2002, Guercio et al. 2003).

Obese children have elevated adrenal androgen levels compared to lean children (Denzer et al. 2007), and body weight correlates positively to adrenal androgen levels in normal-weighted prepubertal children (Ong et al. 2004). The timing of adrenarche has been connected with the most rapid rise in BMI during longitudinal follow-up (Remer and Manz 1999). Leptin stimulates 17,20-lyase activity of P450c17 *in vitro*, possibly by affecting on the phosphorylation of the enzyme (Biason-Lauber et al. 2000), but no relationship between leptin and DHEAS levels are found in boys during puberty (Mantzoros et al. 1997). In contrary to the current body weight, adrenal androgen levels are inversely related to birth weight in both boys and girls. It has been suggested that higher adrenal androgen secretion could contribute to the links between early catch-up growth and adult disease risks, possibly by enhancing insulin resistance and central fat deposition (Ong et al. 2004).

Many studies have indicated a role of genetic regulation in adrenal androgen secretion. A significant genetic component has been determined with a heritability of 58% in the weight-adjusted adrenal androgen excretion rate in a study on monozygotic and dizygotic twins with the mean ages of 11.3 and 8.7 yrs, respectively. Environmental factors account for 17% of the variation in the adrenal androgen production, and their role may be more important in girls than in boys (Pratt et al. 1994). Besides the age- and gender-dependent variation of adrenal androgen levels, there is a significant genetic component in the residual variation of serum DHEAS levels in adults (Rotter et al. 1985, Yildiz et al. 2006). In addition, there is significant heterogeneity in the secretion of DHEA in response to ACTH, whereas there is little inter-subject variability in the cortisol secretion (Azziz et al. 2001).

It may be speculated that the expression patterns of many genes are different between the zona fasciculata and reticularis. The first microarray study on 750 genes found 17 genes whose expression differed significantly between the two zones. Several genes that are expressed at higher levels in the zona reticularis encode components of the major histocompatibility complex and enzymes involved in peroxide metabolism. The same study confirmed earlier results: *3βHSD* is expressed at a very low level in the zona reticularis, whereas the expression of *SULT2A1* is higher in the zona reticularis than in the zona fasciculata (Wang et al. 2001). In comparison of the adult adrenal cortex with the fetal cortex, the microarray study on thousands

of transcripts showed higher expression of *IGF-1* and *3βHSD* in the adult cortex, in addition to many genes with an unknown role in the adrenocortical function (Rainey et al. 2001). The search for factors regulating the expression of steroidogenic enzymes is continuing. For example, transcription factors such as the orphan nuclear receptor called estrogen related-receptor α, SF-1 and GATA-6 have been found to enhance the expression of *SULT2A1* (Saner et al. 2005, Seely et al. 2005).

From an evolutionary perspective, genes must have a central role in the regulation of adrenarche. Adrenarche is a recent event in human evolution, as only the chimpanzee exhibits adrenarche comparable to that of man (Cutler et al. 1978). Rhesus macaques experience morphological changes parallel to fetal zone regression during the first three months of life, resulting in the differentiation of the innermost zona reticularis which lacks 3ßHSD, but exhibits increased cytochrome b_5 expression (Nguyen et al. 2008). Interestingly, female rhesus macaques exposed in utero to exogenous androgen excess developed features of hyperandrogenism and metabolic disorders that are similar to polycystic ovary syndrome (PCOS) in humans (Abbott et al. 2005). Variation in the *CYP17* gene encoding P450c17 has been examined as an explanation of the evolution of adrenarche in higher primates, but such variation does not exist (Arlt et al. 2002). The lack of appropriate animal models has hampered the research on the regulation of adrenarche (Abbott and Bird 2009).

2.2 *PREMATURE ADRENARCHE*

2.2.1 Definition, clinical features and long-term sequelae

The clinical phenotype varies in subjects with PA. The androgenic signs include premature pubarche and axillary hair (Silverman et al. 1952, Thamdrup 1955), oily hair and skin (Rosenfield et al. 1982), adult-type sweating and body odor (Voutilainen et al. 1983, Kaplowitz et al. 1986, Likitmaskul et al. 1995) (Table 1). Most studies have shown only transient effects of PA on growth and maturity. The growth of children with PA may be accelerated, which can be seen already before other androgenic signs (Silverman et al. 1952, Likitmaskul et al. 1995, Pere et al. 1995). The bone age is often slightly advanced (Silverman et al. 1952, Ibáñez et al.

1992, Balducci et al. 1994, Likitmaskul et al. 1995), and PA subjects have higher bone mineral content and density than controls when adjusted for age, weight, height, and fat mass (Sopher et al. 2001). The final height of PA subjects is not significantly reduced, however (Pere et al. 1995), and it may be even above midparental height (Ibáñez et al. 1992). PA is followed by normal-timed gonadarche (Silverman et al. 1952, Ibáñez et al. 1992), or PA girls may reach menarche somewhat earlier than the maternal and population menarcheal age (Pere et al. 1995).

Table 1. Clinical features of premature adrenarche.

Symptoms	Growth of pubic or axillary hair
	Oily hair
	Comedones and acne
	Adult-type sweating and body odor
Age	Girls < 8 yrs and boys < 9 yrs
Findings	Adrenal androgens elevated for chronological age
	e.g. serum DHEAS ≥ 1 μmol/l
	Growth may be accelerated
	Bone age may be slightly advanced
Prevalence	0.8 – 2.8%
Differential diagnosis	Adrenal tumors
	Steroidogenic enzyme defects
	Precocious central puberty

PA is caused by the early maturation of zona reticularis resulting in increased adrenal androgen secretion for chronological age (Dhom 1973). The adrenarcheal minimum level of DHEAS has been set at 1 μmol/l (40 μg/dl) (Rosenfield et al. 1982). However, pubic hair appears at variable DHEAS levels above that and even at lower levels, while the other adrenal androgens may be elevated (Rosenfield et al. 1982, Kaplowitz et al. 1986, Lashansky et al. 1991, Likitmaskul et al. 1995). Some investigators have defined exaggerated adrenarche by the basal or ACTH-stimulated adrenal androgen levels above the normal adrenarcheal levels (Granoff et al. 1985, Lucky et al. 1986, Likitmaskul et al. 1995, Rosenfield 2007).

The incidence of premature adrenarche is unknown. It can be assumed that many children with PA do not visit the doctor or child welfare clinics. The prevalence of pubic hair before the age of 8 yrs is 2.8% in white American girls (Herman-Giddens et al. 1997), whereas it is only 0.8% in Lithuanian girls (Zukauskaite et al. 2005). The prevalence of PA is higher among girls than boys with a ratio around 10:1 (Silverman et al. 1952, Thamdrup 1955). The prevalence varies in different populations, and PA is more common in black people (Kaplowitz et al. 1986). African-Americans also enter puberty earlier than white children. Black girls reach Tanner stage II for breast development at the mean age of 8.9 yrs, compared with white girls at 10.0 yrs, and boys reach Tanner stage 2 for genital growth at the mean age of 9.5 and 10.1 yrs, respectively. Similarly, the mean ages at stage II for pubic hair development in Africa-American and white girls are 8.8 yrs and 10.5 yrs, respectively, and in boys, 11.2 yrs and 12.0 yrs (Herman-Giddens et al. 1997, Herman-Giddens et al. 2001).

In the differential diagnosis of PA, adrenal tumors, steroidogenic enzyme defects and central precocious puberty are to be kept in mind (Silverman et al. 1952). The first papers describing PA reported a high proportion of PA children having cerebral dysfunction (Silverman et al. 1952, Thamdrup 1955, Rosenfield et al. 1982), while later reports have concentrated on otherwise healthy children. Children with moderate to severe cerebral palsy (CP) have earlier pubarche (at a mean age of 8.2 yrs) than healthy girls (10.5 yrs), and to a lesser extent this can also be seen in boys (10.7 yrs *vs*. 11.9 yrs, respectively). Advanced sexual maturation associates with high body fat in girls with CP, but with low body fat in boys with CP (Worley et al. 2002).

In last decades, PA has been connected with adverse features. Children with PA have been found to have hyperinsulinemia, alterations in the IGF system, an unfavorable lipid profile and higher BMI. Lean Catalan girls with PP have higher mean serum insulin values during an oral glucose tolerance test (OGTT) before and throughout puberty than controls. Furthermore, increased IGF-1 levels and decreased SHBG and IGF binding protein 1 (IGFBP-1) levels have been reported in most of these girls (Ibáñez et al. 1997a). A study on Caribbean Hispanic and African-American girls with PA showed nearly half of them to have reduced insulin sensitivity in response to an intravenous glucose tolerance test with tolbutamide. ACTH-stimulated androgen levels were higher in those girls with reduced insulin sensitivity, most of whom were also obese and had acanthosis nigricans (Vuguin et al. 1999). In line with these two studies, predominantly obese Hispanic prepubertal PA girls had elevated free IGF-1 levels, that

correlate with adrenal androgens in the insulin-resistant subset of these girls (Silfen et al. 2002a). Many studies have indicated clearly increased BMI in PA children in comparison to age-matched healthy controls (Vuguin et al. 1999, Silfen et al. 2002a, Charkaluk et al. 2004), and a study on normal weighted Catalan PP girls has shown increased central fat mass in comparison to selected controls with similar BMI (Ibáñez et al. 1998a, Ibáñez et al. 2003a). Independently of BMI, Catalan PP girls had higher triglycerides levels and higher ratio of low-density lipoprotein (LDL) to high-density lipoprotein (HDL) (Ibáñez et al. 1998a). Other studies, however, have failed to find significant differences in the lipid pattern (Meas et al. 2002). Furthermore, prepubertal PA children have differences in their psychological and cognitive functions compared with children who have normal-onset adrenarche, which suggest them to be more vulnerable to various psychopathologies (Dorn et al. 1999).

Already in the 1960s, PA was described in the literature to precede PCOS (Wilkins 1965). This concept was supported by studies on postmenarcheal PP girls with oligomenorrhea and increased incidence of functional ovarian hyperandrogenism (FOH) determined by higher gonadotropin-releasing hormone (GnRH) agonist-stimulated 17-hydroxyprogesterone (17OHP) levels (Ibáñez et al. 1993). In addition, increased 17-hydroxypregnenolone and DHEA responses to GnRH agonist stimulation have been observed during pubertal development in Catalan PP girls, suggesting increased ovarian activity of P450c17 (Ibáñez et al. 1997b). On the other hand, increased 17-hydroxypregnenolone response to ACTH stimulation has been found in premenarcheal Caribbean Hispanic and African American girls with PA (Banerjee et al. 1998), as well as in a small group of white and black American PP girls, in whom no difference in the response to GnRH agonist stimulation was observed (Mathew et al. 2002). The results have been interpreted to mean that PA increases the risk for PCOS, suggesting common pathogenic mechanisms (Kousta 2006, Witchel 2006, Ibáñez et al. 2009). In a heterogeneous group of PA girls, exaggerated adrenarche was suggested to carry an increased risk for PCOS, although the risk may vary with the clinical and hormonal characteristics of the particular study population (Rosenfield 2007). No large longitudinal studies with efficient power have been conducted to confirm the theory that PA precedes PCOS.

Endocrine programming during fetal life may play a role in the regulation of adrenarche. There is an inverse correlation between birth weight and prepubertal adrenal androgen levels in healthy children, whereby the catch-up growth is correlated with serum adrenal androgens

(Francois and de Zegher 1997, Ong et al. 2004). The mean birth weight of Catalan PP girls has been reported to be about 1 SDS lower than in healthy controls, and the difference is even larger in postmenarcheal PP girls with and without FOH (Ibáñez et al. 1998b). A retrospective study on Australian PA girls revealed an increased proportion of subjects with a history of prematurity or being born small for gestational age (SGA) (Neville and Walker 2005). Studies on SGA children show higher DHEAS levels at the age of 12 yrs and after menarche (Ibáñez et al. 1999c, Tenhola et al. 2002). A continuum of adverse development rabging from prenatal programming to premature adrenarche and later on to PCOS has been postulated (van Weissenbruch 2007, Ibáñez et al. 2009). However, a French study on post-menarcheal PP girls failed to find a link between PP and either low birth weight or insulin resistance, although a higher risk for hirsutism and modest hyperandrogenism in girls with PP was shown (Meas et al. 2002).

The incidence of PA is lower in boys, and the clinical outcomes seem to differ in comparison to girls with PA. A small study on Hispanic boys with PP did not find any difference in insulin sensitivity, IGF-1 or SHBG levels or in birth weight between PP boys and their bone age- and pubertal stage-matched controls before and during puberty (Potau et al. 1999). However, a small study on an ethnically heterogeneous group of prepubertal boys found higher IGF-1, but lower SHBG levels and decreased insulin sensitivity in PA boys independent of BMI (Denburg et al. 2002).

It has been debated whether PA children should be followed routinely into adulthood and would they benefit from therapeutic interventions (Rosenfield 2007, Ibáñez et al. 2009). PA children have some metabolic characteristics that imply a higher risk for the impaired glucose metabolism and cardiovascular complications. Furthermore, PA girls may be at a higher risk for developing PCOS and infertility. Both anti-androgen flutamide and insulin-sensitizing metformin have been used with success in preliminary studies on PA girls during and after puberty to reduce androgen levels, fat mass, hyperinsulinemia and cholesterol levels, and further improve the features of FOH (Ibáñez et al. 2000b, Ibáñez et al. 2000c, Ibáñez et al. 2008). There are no studies on the benefits of interventions such as exercise and diet in children with PA, which could be essential in preventing the increase of BMI and the worsening of glucose metabolism.

2.3 GENETIC VARIATION

The genetic code is stored in genomes, which vary between species and between individuals, and are differently regulated from cell to cell. Resolving the whole genome sequences of different species has made it possible to determine how evolution has shaped the human genome. Determining the genetic variation between individuals has opened up the possibility to associate genotype with phenotype. Finding out the mechanisms behind the regulation of gene expression is opening our eyes to the complexity of gene expression in different organs, tissues and cells at different time points in the lifespan in response to different stimuli from the inner and outer world.

2.3.1 Variation of the human genome

The structure of the human genome offers the platform for genetic variation. The size of the human genome reaches three billion base pairs, and only around one percent of it represents the 22500 genes coding proteins (International Human Genome Sequencing Consortium 2004). When the sequence of nearly the whole human genome was reported for the first time in 2001, a surprising amount of non-coding elements was revealed (Lander et al. 2001, Venter et al. 2001). The factors that have separated humans from other eukaryotes, multicellular organisms and vertebrates lie not in the number of base pairs or in the number of protein coding genes, but in the non-coding sequences, including introns, regulatory elements and transposable elements. Segmental duplications, gene duplications, recombinations and mutations during replication have been driving forces in genetic evolution.

Genetic variation makes every human genome individual, leading to an ever-different phenotype. The human genome project and the sequencing of three individual human genomes have revealed up to four million single nucleotide polymorphisms (SNPs) and a huge amount of structural variants (Levy et al. 2007, Bentley et al. 2008, Wheeler et al. 2008). A SNP is defined as a change of one nucleotide to another of the three possibilities in at least 1% of all humans. Common SNPs have a minor allele frequency (MAF) of more than 5%. The density of SNPs varies in the sequence. On average, human genome has a SNP in every 1–1.9 kb (Sachidanandam et al. 2001, Bentley et al. 2008). Structural variation covers insertions,

deletions, inversions, duplications and translocations, encompassing copy-number variants. Structural variants range from small insertion/deletion events to segmental duplications, and from more than one base pair to a few million base pairs in length. An integrated map of genetic variation for eight human genomes defines the location of 1695 sites of structural variation (> 6 kb in length) and 796273 small insertion/deletions (1-100 bp in size) (Kidd et al. 2008). Structural genetic variation covers a much larger proportion of the whole human genome than SNPs. SNPs result in different amino acids of encoded proteins, different regulation of gene expression and different splicing sites for RNA processing, or they may have no effect (Hull et al. 2007). Structural genetic variation can confer phenotypes through many mechanisms, including gene dosage and unmasking functional SNPs on the remaining allele (Human Genome Structural Variation Working Group et al. 2007). The goal of mapping all the sequence variation in the human genome is the understanding of the genotype-phenotype associations, the mechanisms of diseases and the individual responses to treatments.

Candidate gene analyses have been used to determine a possible association between the phenotype or disease and variation in a gene that is important for the condition. However, many reported associations have never been replicated after the first reports (Lohmueller et al. 2003). The international HapMap project was established in 2003 to determine the amount and linkage of common human sequence variation (International HapMap Consortium 2003). Since then, most of the SNPs have been described, and information on linkage disequilibrium patterns of the SNPs has been collected in the public databases. Tagging SNPs are used to locate genetic variation behind complex traits in genome wide association studies (GWA), which have successfully discovered loci and genes that could have never been suspected to be associated with disease when using the candidate gene approach based on the biochemical and molecular knowledge of the day. Population isolates, like the Finns, have higher linkage disequilibrium with less variation in the genome between subjects, offering advantages for GWA studies on complex traits (Varilo and Peltonen 2004). New biochemical and bioinformatics methods are needed not only to determine more precisely the biochemical basis of mechanisms between genetic variation and molecular networks, but also to evaluate gene-environment interconnections.

2.3.2 Variation in gene expression

Every somatic cell has the same genome with all the variants in an individual combination characteristic in each human being. Gene expression is regulated in every cell in a tissue-specific manner. Thus, genetic variants may have different effects in different cells based on the cell-specific regulation.

All the steps of gene expression are regulated by multiple mechanisms (Orphanides and Reinberg 2002). Gene expression begins with chromatin structure modifications, enabling transcription initiation and ends with posttranslational modifications of encoded proteins. Humans have around 3000 transcription factors that participate in the regulation of transcription and interact with each other by silencing and enhancing the transcription. The promoter regions for the binding of transcription factors may be large and located far from the coding exons (Levine and Tjian 2003). For example, steroid hormones bind to nuclear receptors, and these complexes works as transcription factors by binding with DNA response elements and altering the transcription rate of the target genes (Perissi and Rosenfeld 2005). Transcribed mRNA is processed before translation, introns are spliced out, and alternative splicing sites may be used to get different proteins. At this stage, microRNA molecules can silence gene expression by degradation of mRNA molecules.

In addition, epigenetics involve heritable mechanisms that regulate gene expression. Epigenetic mechanisms are not stored in the genomic sequence, but in the chromatin structures and histone modifications (Bjornsson et al. 2004). X-chromosome inactivation involves epigenetic mechanisms that mostly silence one of the two X-chromosomes into transcriptionally inactive highly condensed heterochromatin in each female cell to compensate for gene dosage. X-inactivation occurs shortly after the implantation of female embryos or during the induction of cell differentiation, and the maintenance of stable X-inactivation requires synergistic actions of several epigenetic mechanisms: coating of the X-chromosome by *Xist* RNA, DNA methylation and histone modifications (Heard and Disteche 2006).

Interaction of promoter region, genes, microRNAs, chromatin remodeling and other factors form complex networks (Phillips 2008). We are still at the beginning on our way to understand the genetic regulation of complex traits and polygenic diseases.

2.4 GENES IN PREMATURE ADRENARCHE

The pathogenesis of PA was discussed in the literature before physiological adrenarche was described. It is unequivocal nowadays that premature adrenarche results from the early development of the zona reticularis, secreting increased amounts of androgens for the chronological age (Silverman et al. 1952, Thamdrup 1955, Conly et al. 1967). Like the regulation of physiological adrenarche, the pathogenesis of premature adrenarche remains obscure. Factors such as obesity, hyperinsulinemia and increased IGF-1 levels may participate in the regulation of PA, and the process may begin with prenatal programming.

Genes have been demonstrated to have an essential role in PA, with polygenic effects on heterogeneous phenotypes. A linkage analysis study on three families with either PP or adolescent hyperandrogenism found difficulties in classifying family members as affected or unaffected, and ended with conclusions that the condition is multifactorial, with several genes contributing to the condition and distinct susceptibility genes may be present in various families (Sanders et al. 2002). Several candidate gene studies have searched for susceptibility variants in genes involved in steroidogenesis, androgen action and metabolism (Table 2).

2.4.1 Genes in steroidogenesis and androgen action

The genes encoding steroidogenic enzymes have been tempting targets as candidates for the genetic regulation of PA. Defects in *CYP21* and *CYP11* encoding P450c21 and P450c11, and in *3βHSD* can cause a PA-like condition with variable signs of virilization (Marui et al. 2000). Before the development of sequencing methods, enzyme activities were calculated from responses to ACTH stimulation, and many more defects in steroidogenesis were found than could be confirmed by sequencing the coding regions of *CYP21* and *3βHSD* later on (August et al. 1975, Morris et al. 1989, Oberfield et al. 1990, del Balzo et al. 1992, Hawkins et al. 1992, Siegel et al. 1992, Balducci et al. 1994, Chang et al. 1995, Sakkal-Alkaddour et al. 1996). Non-classical congenital adrenal hyperplasia (CAH) due to mutations in *CYP21* and *3βHSD* can be ruled out in PA children by measurements of basal 17OHP and 17-hydroxypregnenolone levels, respectively, and if those are moderately elevated, by performing an ACTH stimulation test (Leite et al. 1991, Likitmaskul et al. 1995, Mermejo et al. 2005). The frequency of *CYP21*

Table 2. Heterozygote mutations and polymorphisms studied in association with premature adrenarche and clinical measures.

Gene	Mutation/ polymorphism	Number of PA subjects (girls/boys)	Number of control subjects	Heterozygote frequency or MAF (%)	P*	Association with minor variant	Reference
CYP21	Heterozygous mutations (n=12)	48 (40/8)	50	8/48 vs. 1/50 (V281L)			(Dacou-Voutetakis and Dracopoulou 1999)
CYP21	Mutations (n=9)	40 (34/6)	15 (15/0)	14/40 vs. 1/15	NS		(Witchel et al. 2001)[b]
CYP21	Mutations (n=14)	53 (35/0)	35 (35/0)	13/53 vs. 8/35	NS		(Potau et al. 2002)[a]
HSD3B2	Conformational polymorphisms	18 (17/1)	100	3/18 vs. 0/100			(Nayak et al. 1998)[b]
HSD3B2	Conformational polymorphisms	40 (34/6)	15 (15/0)	3/40 vs. 0/15	NS		(Witchel et al. 2001)[b]
HSD17B5	3 SNPs	190 (190/0)	71 (71/0)		NS		(Petry et al. 2007)[a]
CYP19	4 SNPs in coding region	186 (186/0)	71 (71/0)	26% vs. 44% (SNP50)	0.001	T, DHEAS, IS (SNP50)	(Petry et al. 2005)[a]
CYP19	6 SNPs in promoter	190 (190/0)	71 (71/0)		NS	T (SNP43)	(Petry et al. 2006)[a]
AR	CAG repeat	181 (181/0)	124 (124/0)	0.7 shorter CAG_n	0.003	FOH	(Ibáñez et al. 2003b)[a]
AR	CAG repeat	25 (25/0)	33	0.9 shorter CAG_n	<0.05		(Vottero et al. 2006)
UGT2B	Y85D	69 (69/0)	88 (88/0)		NS		(Tomboc and Witchel 2003)[b]
IGF-1R	E1013E (A→G)	69 (63/6)	92 (31/61)	60.9% vs. 48.9%	0.037		(Roldan et al. 2007)[b]
Insulin	VNTR	141 (141/0)	140 (140/0)	27% vs. 29%	NS	BW, IS	(Ibáñez et al. 2001)[a]
IRS-1	G972R	54 (54/0)	79 (79/0)	17/54 vs. 15/79	0.074	SHBG	(Ibáñez et al. 2002)[a]
IRS-1	G972R	40 (34/6)	15 (15/0)	11/40 vs. 1/15	NS		(Witchel et al. 2001)[b]
SORBS1	T228A	79 (79/0)	50 (50/0)	6% vs. 8%	NS		(Witchel et al. 2003)
PAI-1	-675 4G/5G indel	182 (182/0)	115 (115/0)	47% vs. 50%	0.85	Insulin resistance	(Lopez-Bermejo et al. 2007)[a]
GAD2	-243A→G	87 (87/0)				BMI	(Witchel et al. 2009)[b]
GR	N363S	40 (34/6)	15 (15/0)	5/40 vs. 2/15	NS		(Witchel et al. 2001)[b]
ADRB3	W46R	40 (34/6)	15 (15/0)	7/40 vs. 2/15	NS		(Witchel et al. 2001)[b]
MC4R	V1031I	75 (69/6)	95		NS		(Martin et al. 2004)[b]

MAF, minor allele frequency; NS, non-significant; T, testosterone; DHEAS, dehydroepiandrosterone; IS, insulin sensitivity; AR, androgen receptor; FOH, functional ovarian hyperandrogenism; UGT2B, UDP-glucuronyltransferase 2B; IGF-1R, insulin-like growth factor-1 receptor; BW, birth weight; IRS-1, insulin receptor substrate-1; SORBS1, sorbin and SH3-domain-containing-1 gene; PAI-1, plasminogen activator inhibitor-1; GAD2, glutamate decarboxylase 2; BMI, body mass index; GR, glucocorticoid receptor; ADRB3, β_3-adrenergic receptor; MC4R, melanocortin-4 receptor

[a] Cohort of Catalan PP girls studied before, during and after puberty in comparison to healthy short-normal children.

[b] Cohort of American PA girls and boys with different ethnic origin compared with in genotype distribution analysis to healthy adults.

* P value is missing, if no significance was reported in the reference.

mutations leading to non-classical CAH varies between populations, and is relatively high in Hispanics and Italians (Speiser et al. 1985). In Finland, non-classical CAH due to *CYP21* mutations is rare (Jääskeläinen et al. 1997).

All the polymorphisms and mutations reported in children with PA have been shown in Table 2. Heterozygote carriers of *CYP21* mutations have been reported with an increased frequency varying from 35% to 37.5% in PA subjects of American and Hellenic origin, whereas no increased frequency has been reported in Catalan PP girls. However, no clinical parameter has been linked to the heterozygosity of *CYP21* mutations in subjects with PA (Dacou-Voutetakis and Dracopoulou 1999, Witchel et al. 2001, Potau et al. 2002). Heterozygous carriers of *3βHSD* mutations have been found in 7.5% of the American PA children, but the mutations did not associate with the phenotype, either. Forty two % of these American PA children had mutations in either or both of the *CYP21* and *3βHSD* genes, whereas only 6.6% of a sample of women without PA had a variant in these genes. Surprisingly, five of the six PA boys were heterozygous carriers of *CYP21* mutations (Witchel et al. 2001).

Adrenal steroids are synthesized in the zona reticularis, but peripheral target tissues metabolize them. DHEAS can be converted back to DHEA and further to Δ4-A, testosterone and dihydrotestosterone (DHT), and from Δ4-A to estrogens. 17β-Hydroxysteroid dehydrogenase (HSD17B5) catalyzes reactions including the conversion of Δ4-A to testosterone and DHEA to androstenediol. No *HSD17B5* genotype was associated with PP or testosterone levels, when one SNP in the promoter region of *HSD17B5* and three exonic SNPs were analyzed (Petry et al. 2007). *CYP19* encodes aromatase enzyme that catalyzes the conversion of androgens to estrogens. The genotype distribution is different according to SNP50 in the coding region of *CYP19* between Catalan PP girls and controls. The major variant homozygote A/A is more frequent in the PP girls, in whom the A/A genotype is associated with higher testosterone and DHEAS levels and decreased insulin sensitivity when adjusted for pubertal stage (Petry et al. 2005). However, the haplotype with G allele in the SNP50 was associated with an increased probability of PP and further development of FOH in the haplotype analysis that was based on four tagging SNPs, suggesting that another variant in the haplotype may be the causal variant (Petry et al. 2005). In the distal promoter region of *CYP19*, SNP43 was associated with testosterone levels in the same Catalan cohort, but no genotype was associated with an increased risk for PP (Petry et al. 2006). Increased 5α-reductase and 11β-

hydroxysteroid dehydrogenase activities could influence adrenal androgen levels in target tissues by producing DHT from testosterone and by reducing the conversion of cortisol to inactive cortisone, respectively, but no differences in their activities based on urinary steroid metabolites have been observed in prepubertal PA girls (Silfen et al. 2002b). Glucuronidation by the UDP-glucuronyltransferase 2B (UGT2B) is one mechanism through which androgens are inactivated, but no differences have been found between PP children and healthy controls in the allele frequencies of *UGT2B* variant associated with lower V_{max} of the enzyme (Tomboc and Witchel 2003). Recently, Noordam et al. identified heterozygous inactivating mutations in the gene encoding human 3'-phosphoadenosine-5'-phosphosulfate (PAPS) synthase 2 in a girl with PP, hyperandrogenic anovulation, very low DHEAS levels, and increased androstenedione and testosterone levels. PAPS is required for the catalytic activity of SULT2A1 that converts DHEA to DHEAS, and the observations on the patient highlighted the crucial role of DHEA sulfation as a gatekeeper to human androgen synthesis (Noordam et al. 2009).

Increased sensitivity of hair follicles to adrenal androgens has been postulated as a possible pathogenic mechanism for PA since Silverman *et al.* reported in the first paper on PA that PA subjects have variable levels of adrenal androgens, overlapping with those in normal children (Silverman et al. 1952). The androgen receptor gene (*AR*) contains a highly polymorphic region with a variable number of CAG repeats (CAG_n) encoding a polyglutamine tract, the length of which has an inverse relationship with the transcriptional activity of AR (Chang et al. 1988, Lubahn et al. 1988, Chamberlain et al. 1994, Beilin et al. 2000). Two studies have demonstrated that Mediterranean girls with PP have a mean CAG_n about one repeat shorter than healthy controls, indicating that they have more active ARs (Ibáñez et al. 2003b, Vottero et al. 2006). In addition, the shorter *AR* gene CAG_n has been associated with an increased risk of subsequent FOH in the Catalan PP girls (Ibáñez et al. 2003b). Vottero *et al*. studied *AR* gene methylation and found that the methylation pattern in the pubic hairs of Italian prepubertal PP girls was similar to girls with Tanner stage II (Vottero et al. 2006), but the role of *AR* gene methylation in the receptor activation and androgen sensitivity has not been examined in more detail.

2.4.2 Genes in metabolism

Children with PA have hyperinsulinemia, higher BMI and an impaired lipid profile, and their first-degree relatives often have cardiovascular risk factors. The parents of Catalan PP girls have an increased prevalence of type 2 diabetes mellitus (T2DM) and impaired glucose tolerance accompanied with an unfavorable lipid profile, compared with the overall prevalence of T2DM and impaired glucose tolerance in Catalonia. Furthermore, hyperandrogenism and gestational diabetes mellitus are frequent among the mothers of PA girls (Ibáñez et al. 1999a). Interesting theories about the connection between hyperinsulinemia and hyperandrogenemia suggest that hyperinsulinemia may precipitate hyperandrogenemia in vulnerable individuals by unmasking latent abnormalities in the regulation of steroidogenesis, or it may be a marker of a more fundamental abnormality that affects multiple systems (Rosenfield 1996).

Genetic variation in several genes participating in insulin-IGF signaling or body weight regulation has been studied mainly in two cohorts of PA children: Catalan PP girls during and after puberty, and American PP girls and boys. Variation has been explored in genes encoding proteins at various steps of insulin-IGF-signaling: insulin, insulin receptor substrate-1 (IRS-1), sorbin and SH3-domain-containing protein (SORBS1) involved in insulin-mediated glucose uptake, and type 1 IGF receptor (IGF-1R). Genetic variation associating with insulin resistance and higher BMI has been studied in genes encoding plasminogen activator inhibitor-1 (PAI-1), glutamate decarboxylase 2 (GAD2), glucocorticoid receptor, and β_3-adrenergic receptor and melanocortin-4 receptor (Table 2). A polymorphism in *IGF-1R* has a different genotype distribution between PA children and controls. Variants in the insulin gene and *IRS-1* have been associated with differences in the PA phenotype, but variation in *SORBS1* has not been associated with PA or phenotype of PA (Ibáñez et al. 2001, Witchel et al. 2001, Ibáñez et al. 2002, Tomboc and Witchel 2003, Roldan et al. 2007).

The minor variant G at SNP E1013E in *IGF-1R* has been associated with higher circulating IGF-1 levels (Bonafe et al. 2003). In PP children, the frequency of this minor variant is increased (Roldan et al. 2007). The variant does not relate to clinical measures, however, although IGF-1 was not measured in the study (Roldan et al. 2007). Interestingly, all six PP boys studied were either heterozygotes or homozygotes for the minor variant at SNP E1013E (Roldan et al. 2007).

A variable number of tandem repeats (VNTR) in the insulin gene relates to the transcription levels of the insulin gene (Le Stunff et al. 2000). In Catalan PP girls, the class I genotype of VNTR has been associated with lower birth weight and reduced insulin sensitivity in agreement with previous studies, but there was no difference in the genotype distribution between PP girls and age- and BMI-matched controls. VNTR class I allele and low birth weight had additive effects on hyperinsulinemia and dyslipidemia in these PP girls (Ibáñez et al. 2001).

The minor variant at G927R of *IRS-1* impairs insulin signaling (Almind et al. 1993). Among the Catalan PP girls heterozygous for the minor variant of G927R, SHBG concentrations were lower than in those homozygous for the major variant. The frequency of the minor variant was twice as high in PP girls who later developed FOH as in healthy control girls, although *IRS-1* genotype was not a significant predictor for the development of FOH in the regression model with significant effects of age, insulin, LDL cholesterol and IGFBP-1 levels (Ibáñez et al. 2002). In the American PP cohort, the G927R variant in *IRS-1* and T228A at *SORBS1* have not shown significantly different genotype distributions from controls, nor have there been associations with clinical parameters (Witchel et al. 2001, Tomboc and Witchel 2003).

The 4G/5G insertion/deletion polymorphism in the promoter of *PAI-1* has been related to circulating PAI-I levels and inconsistently with insulin resistance and cardiovascular disease (Eriksson et al. 1995, Panahloo et al. 1995, Viitanen et al. 2001). The genotype distribution of the 4G/5G polymorphism did not differ between Catalan PP girls and controls. In post-menarcheal PP and control girls, the 5G allele was associated with insulin resistance (Lopez-Bermejo et al. 2007). The minor variant of rs2236418 in the promotor of *GAD2* was associated with morbid obesity in the initial case-control study of 1200 subjects (Boutin et al. 2003), but further studies have not been able to replicate the result (Swarbrick et al. 2005, Groves et al. 2006). In the American PP girls, the minor G variant was associated with an increased BMI at both initial and follow-up visits, but the genotype distribution of PP girls was not compared with that of healthy controls (Witchel et al. 2009). Genetic variations in the genes encoding the glucocorticoid receptor, β_3-adrenergic receptor, and melanocortin-4 receptor have not shown an association with PA (Table 2) (Witchel et al. 2001, Martin et al. 2004).

Taken together, the minor variants of SNP E1013E at IGF-1R and G927R at IRS-1 may underlie PP by increasing IGF-1 levels and decreasing SHBG levels, respectively, whereas the

VNTR genotype of insulin gene may provide a link between low birth weight and cardiovascular risk factors in PP girls.

2.4.3 Candidate genes in the current study

The hypotheses for associations of PA with following candidate genes were based on the knowledge of PA and earlier described genetic variants in these genes (Figure 3).

2.4.3.1 *MC2R*

ACTH has an essential role in the regulation of adrenarche (Weber et al. 1997). Genetic factors may explain heterogeneity in adrenal secretion of DHEA in response to ACTH (Azziz et al. 2001). The ACTH receptor is a G protein-coupled membrane receptor that belongs to the melanocortin receptor family and is called the melanocortin-2 receptor (MC2R) (Buckley and Ramachandran 1981, Gantz and Fong 2003). In the human *MC2R* gene, one intron separates the coding exon 2 from the upstream untranslated exon 1 (Naville et al. 1997). A SNP has been described within the transcription initiation site at position – 2 bp, altering the consensus sequence from T to C (MC2R -2 T>C). The minor variant of MC2R -2 T>C results in lower promoter activity *in vitro* due to changes in transcription initiation, and it is associated with lower cortisol and DHEA secretion in response to ACTH stimulation *in vivo* (Slawik et al. 2004, Reisch et al. 2005).

2.4.3.2 *AR*

The development of pubic hair is dependent on the interplay between the plasma androgen level and other biologic factors that affect the response of the pilosebaceous unit to androgens (Rosenfield 1994). One of the suggested biologic factors is the androgen receptor (AR), through which the androgens mediate their effects. The ligand-bound AR binds to regulatory DNA elements in the promoter region of target genes to influence the transcription rate through interaction with cofactors and transcription machinery (Perissi and Rosenfeld 2005). The length

of the CAG_n repeat in exon 1 of the X-chromosomal *AR* gene has an inverse relationship with the transcriptional activity of AR (Chamberlain et al. 1994, Beilin et al. 2000). Previous studies have demonstrated that girls with PP have a shorter mean CAG repeat in the AR than healthy controls (Ibáñez et al. 2003b, Vottero et al. 2006), but none of these studies has taken X-chromosome inactivation into account. Methylation of *Hpa*II sites close to the *AR* gene CAG_n region correlates with X-inactivation (Allen et al. 1992).

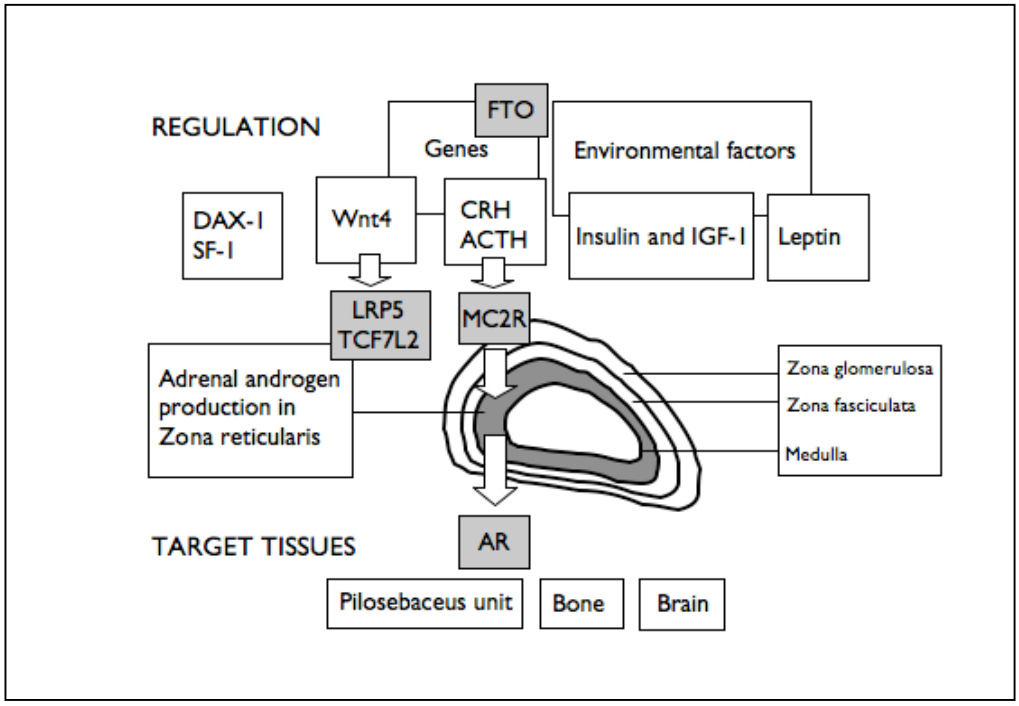

Figure 3. The positions of the candidate genes in the regulation of adrenarche. The melanocortin 2-type receptor (MCR2R) mediates the effects of adrenocorticotropin (ACTH). The androgen receptor (AR) mediates the effects of adrenal androgens on target tissues. Low-density liporotein receptor-related protein 5 (LRP5) and transcription factor 7-like 2 (TCF7L2) participate in Wnt signaling. The fat mass and obesity associated (FTO) gene has been connected with obesity, and body weight is correlated with increased circulating adrenal androgen levels.

2.4.3.3 *LRP5* and *TCF7L2*

Both low density lipoprotein receptor-related protein 5 (LRP5) and transcription factor 7-like 2 (TCF7L2) are factors in WNT signaling that is essential for embryogenesis, postnatal development and tissue homeostasis (He et al. 2004), and which has an active role in the development and function of adrenal glands (Kim et al. 1998, Suwa et al. 2003, Kim et al. 2008). In humans, loss-of-function mutations in *WNT4* have been described in the autosomal recessive SERKAL syndrome with female to male sex reversal and renal, adrenal and lung dysgenesis (Mandel et al. 2008), and also in women with the Mayer-Rokitansky-Küster-Hauser-like syndrome associated with Müllerian-duct regression, virilization and high androgen levels (Biason-Lauber et al. 2004, Philibert et al. 2008). Wnt4 represses steroidogenesis in the adrenal cortex and gonads based on the studies on transgenic *Wnt4* mice (Jordan et al. 2003, Heikkilä et al. 2005).

LRP5 is a single-span transmembrane receptor belonging to the low-density lipoprotein receptor family (Hey et al. 1998). LRP5 and its homologue LRP6 act as Wnt co-receptors (He et al. 2004). Common polymorphic variants in *LRP5* have been shown to contribute to bone density in different populations and various age groups (Koay et al. 2004, Mizuguchi et al. 2004, Ferrari et al. 2004, Kiel et al. 2007, Koay et al. 2007, Saarinen et al. 2007). In the canonical WNT signaling, Wnt molecules bind to their receptors co-acting with LRP5 on cell membrane, which leads to the inactivation of β-catenin degradation intra-cellularly, resulting in increased the amounts of β-catenin. Downstream in the canonical WNT signaling pathway are TCF transcription factors that together with β-catenin activate WNT target gene expression. In the absence of β-catenin, TCFs bind to target gene promoters and repress their expression (Jin 2008). The genetic variants of *TCF7L2* have been previously associated with type 2 diabetes (Grant et al. 2006, Cauchi et al. 2007, Wang et al. 2007) and an increased risk for early impairment of glucose metabolism in obese children (Körner et al. 2007).

2.4.3.4 *FTO*

PA is associated with increased BMI (Vuguin et al. 1999, Silfen et al. 2002a, Charkaluk et al. 2004), and it is well known that healthy obese children have elevated adrenal androgen levels

compared with lean children (Denzer et al. 2007). Genetic variants in the fat mass and obesity associated gene (*FTO*) have been associated consistently with obesity (Dina et al. 2007, Frayling et al. 2007, Grant et al. 2008). Each risk allele increases weight around 1.5 kg in adults, and the homozygote carriers of the risk alleles have 1.67-fold increased odds of obesity (Frayling et al. 2007). The association reflects an increase in fat mass that is observed from early infancy on, but not at birth (Frayling et al. 2007, Grant et al. 2008, Lopez-Bermejo et al. 2008). The *FTO* risk genotype correlates with dysregulation of glucose and lipid metabolism to an extent consistent with its effect on BMI (Frayling et al. 2007, Do et al. 2008, Freathy et al. 2008). The effect of genetic variants at *FTO* can be compensated by regular sports (Rampersaud et al. 2008), whereas low physical activity accentuates the effect of the risk variant on body fat accumulation (Andreasen et al. 2008). In addition, *FTO* is expressed in the human hypothalamus, pituitary and adrenal gland, suggesting a potential role in the hypothalamic-pituitary-adrenal axis implicated in body weight regulation (Su et al. 2004, Dina et al. 2007). However, the function of *FTO* remains largely unknown.

3 AIMS OF THE STUDY

The study was conducted to determine the role of genetic variation of PA candidate genes in a case-control cohort of prepubertal PA children and their age- and gender-matched controls. Genotype distributions between the groups were compared and single marker association analyses were used to relate genetic variants with the clinical phenotype.

Five candidate genes were studied:

1) *MC2R* gene encoding the ACTH-receptor, which has an essential role in adrenarche and the individual response of adrenal androgen production to ACTH (I)

2) *AR* gene and X-chromosome inactivation, because low adrenal androgen levels have been observed in PA children, suggesting higher androgen sensitivity (II)

3) *LRP5* and *TCF7L2* genes, because of their role in Wnt signaling in adrenal cortex and in metabolic functions (III, IV)

4) *FTO* gene, because it is associated with obesity, and body weight correlates positively with adrenal androgen levels in healthy prepubertal children (IV).

4 SUBJECTS AND METHODS

This study on genetic variation in prepubertal PA is a part of a longitudinal follow-up study on a case-control cohort of PA children with the entire clinical spectrum of androgenic signs and their age- and gender-matched controls collected by Pauliina Utriainen, MD, Jarmo Jääskeläinen, MD, PhD, and Raimo Voutilainen, MD, PhD (Utriainen et al. 2007, Utriainen et al. 2009a, Utriainen et al. 2009b). The clinical evaluation of study subjects, hormonal assessments and genotyping methods have been described in detail in the original publications (I-IV).

4.1 CASE-CONTROL COHORT

The study group was comprised of 171 prepubertal Caucasian children from the Northern Savo district in Eastern Finland. For the subjects with PA, the criteria for entry into the study were any clinical sign(s) of adrenarche, including pubic or axillary hair, acne, adult-type body odor or oily hair before the age of 8 yrs in girls and 9 yrs in boys independent from the DHEAS levels. All eligible children were invited to the study between October 2004 and January 2006. Seventy-six eligible children were found, and 74 (97.4%) of them were willing to participate (64 girls and 10 boys). One girl with signs of central puberty in addition to PA was included in the analysis of genotype distribution of the polymorphism in *MC2R* promoter (I), but excluded from all the other analyses (63 girls and 10 boys; I, II, III and IV). Steroidogenic enzyme defects and virilizing tumors were excluded biochemically and by adrenal ultrasonography. Altogether 97 healthy age- and gender-matched healthy controls (79 girls and 18 boys) were a random sample of children from the same district, obtained from the Finnish population register, and approximately 20% of the control children invited were willing to participate. At examination, girls in both groups had to be less than 9 yrs and boys less than 10 yrs of age. Children with central puberty, any endocrine disorder or long-term medication were excluded from both groups. The study protocol was approved by the Research Ethics Committee of Kuopio University Hospital. Informed written consent from parents and assent from children

were obtained for participation in the study, including collection and genotyping of DNA samples.

The appearance time of the adrenarcheal signs was obtained by interviewing the parents. Clinical examination of all the children was performed by the same physician (Pauliina Utriainen) to determine the androgenic signs and exclude centrally activated puberty. Birth weight, birth length and gestational age data were obtained from hospital records. The birth measures were converted to SD scores (SDS) by plotting them on the growth charts and adjusting the birth measures for gender and duration of gestation (Pihkala et al. 1989). Height was measured with a calibrated Harpenden stadiometer to the nearest 0.1 cm. Weight was measured in thin underwear to the nearest 0.1 kg and converted to percentages in relation to the median weight-for-height according to the national reference values (Sorva et al. 1990). BMI was calculated according to the formula [weight (kg) / height^2 (m)], and BMI SDS was determined by British reference values (II) (Cole et al. 1995). Blood pressure (BP) was measured with a standard sphygmomanometer from the left arm in supine position after a 30-min rest in bed and recorded as the average of three repeated measurements.

For the endocrine-metabolic assessment, baseline levels of plasma glucose, triglycerides, total cholesterol, LDL and HDL cholesterol, and serum insulin, cortisol, DHEAS, DHEA, Δ4-A, and SHBG were measured after an overnight fast between 0900 and 1000 h from all subjects. For all the children, ACTH stimulation tests were performed administering synthetic ACTH 1-24 (Synacthen; Novartis Pharma GmbH, Nürnberg, Germany) 1 ug/1.73 m^2 i.v. Serum samples for cortisol, DHEA, Δ4-A, and 17OHP were taken 30 min after the ACTH administration. An oral glucose tolerance test (OGTT) was performed by administering 1.75 g/kg glucose (max 75 g) to each subject with samples for plasma glucose and serum insulin analyses taken at 30, 60, 90 and 120 min. Hormonal assays are described in the original publications (I and II). For the evaluation of insulin sensitivity, homeostasis model assessment for insulin resistance (HOMA-IR) was calculated according to the formula: fasting plasma glucose (mmol/l) * fasting serum insulin (mU/l) / 22.5 (Allard et al. 2003), and insulin sensitivity index (ISI_{comp}) was calculated according to the formula: 10 000 / √ [fasting glucose (mg/dl) * fasting insulin (µU/l) * mean glucose (mg/dl) * mean insulin (µU/l)] (Matsuda and DeFronzo 1999).

4.2 GENOTYPING METHODS

DNA was isolated from whole blood samples using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The genetic variants were genotyped using restriction fragment length analysis (I), TaqMan Allelic Discrimination Assays® (IV) and by direct sequencing of the gene (III). The variants and genotyping methods are described in Table 3.

Table 3. Genetic polymorphisms analyzed and the methods used in genotyping.

Gene	Chromo-somal location	Polymorphism	Position	Genotype	Genotyping method	Orig. publ.
MC2R	18p11.2	MC2R -2 T>C	-2 from the transcription initiation site	T/C	RFLP	I
AR	Xq11.2	CAG_n	Exon 1	CAG_n	PCR, automated electrophoresis	II
LRP5	11q13.4	19 SNP	Exons, introns, 3'UTR		Direct sequencing	III
TCF7L2	10q25.3	rs7903146	Intron 3	C/T	TaqMan Assay®	IV
TCF7L2	10q25.3	rs12255372	Intron 4	G/T	TaqMan Assay®	IV
FTO	16q12.2	rs9939609	Intron 1	T/A	TaqMan Assay®	IV

MC2R, melanocortin-2 receptor; *LRP5*, lowdensity lipoprotein receptor-related protein 5, *TCF7L2* transcription factor 7-like 2; *FTO*, fat mass and obesity associated gene; SNP, single nucleotide polymorphism; UTR, untranslated region; RFLP, restriction fragment length polymorphism based on polymerase chain reaction (PCR) and *Sac*I enzyme digestion.

CAG repeat length was determined by automated fluorescence detection, and the X-chromosome inactivation assay was based on the *AR* gene methylation (II) (Allen et al. 1992). *Hpa*II sites close to the *AR* gene CAG_n are methylated on the inactive X-chromosome. Methylation-sensitive restriction enzyme *Hpa*II digests only the unmethylated (active X-chromosome) DNA, which is thereby unavailable for the following PCR amplification. Both digested and undigested DNA samples were amplified by PCR, and the PCR products were run on denaturing gel as in the genotyping analysis. To compensate for the unequal amplification of alleles, values for the digested samples were normalized with those for the undigested samples with calculations for shorter allele with formula (p1d/p1u) / (p1d/p1u + p2d/p2u), in which p1d and p2d represent the peak areas (p) of *Hpa*II-digested (d) alleles (1 and 2), and p1u and p2u are the corresponding peak areas of the undigested (u) alleles (Lau et al. 1997). Methylation weighted biallelic means of CAG_n ($mwCAG_n$) were achieved by multiplying each allele in a

genotype pair by its percentage of total expression (100 minus inactivity %) and summing the two adjusted repeat values (Hickey et al. 2002). Individuals homozygous at the *AR* gene CAG_n locus (9 controls and 1 PA subject) and boys were included in the $mwCAG_n$ analyses, because variation in the allele expression would not alter the mean value of alleles of equivalent repeat number or the mean of one allele. Skewing of X-chromosome inactivation was determined as 80% or higher for the activity of one allele (Naumova et al. 1996).

4.3 STATISTICAL ANALYSES

Fisher's exact test (I-III) and test for equality of proportions (IV) were used to test for differences in genotype groups and MAFs between the control and PA groups. For single marker association analyses Student's t-test (II, IV), Mann-Whitney U-test (I-IV), ANOVA (IV) and Kruskall-Wallis (IV) tests were used to compare clinical parameters between genotype groups, while univariate linear model (III, IV) and multiple linear regression (II) were used to test for differences adjusted for age and gender, or other factors. The difference between the arithmetic mean of CAG_n and the $mwCAG_n$ was tested with paired samples t-test (II). The strength of the relationship between $mwCAG_n$ and the clinical measurements was estimated by Kendall's rank correlation, because it offers both a meaningful estimate of the strength of the relationship and the statistical significance (II). The results are presented as means with 95% confidence intervals (CI), if not otherwise stated. In the case of skewed distribution, raw data were log-transformed prior to using the tests with assumptions of normal distribution, and results are presented as geometric means with 95% CI. For measures remaining non-normally distributed after log-transformation, non-parametric tests were used. No corrections for multiple testing were performed. We tried to limit the problem of multiple testing by restricting the analyses to the most important measures only. $P < 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS 14.0 statistical package (SPSS Inc., Chicago, IL) and test for equality of proportions and power calculations were conducted with R statistical program, version 2.7.2 (IV) (http://www.r-project.org/). Hardy-Weinberg equilibrium was calculated according to standard procedures using χ^2 test (I, III, IV). Linkage disequilibrium between the *LRP5* or *TCF7L2* polymorphisms was analyzed with Haploview4.0 (III, IV) (Barrett et al. 2005).

5 RESULTS AND DISCUSSION

5.1 CHARACTERISTICS OF THE CASE-CONTROL COHORT

Pauliina Utriainen *et al.* have reported the clinical data of the cohort (Utriainen et al. 2007, Utriainen et al. 2009a, Utriainen et al. 2009b). Thirty-five (48%) of the 73 PA children were recorded to have PP with the presence of pubic or axillary hair (PP), whereas 38 PA children (52%) had other androgenic signs (nonPP). Nine PA subjects had serum DHEAS concentrations below 1 μmol/l, and 36 of the 97 control subjects had DHEAS at or above 1μmol/l at the time of evaluation. Only one PA subject had exaggerated adrenarche defined as basal serum DHEAS concentration exceeding 6 μmol/l. None of the PA subjects had non-classical 21-hydroxylase deficiency based on the baseline and ACTH-stimulated 17OHP levels (Utriainen et al. 2009a).

Table 4. Clinical and hormonal characteristics in children with premature adrenarche (PA) as compared to healthy age- and gender-matched controls.

Variable	Mean (95% CI)		*P* value*
	Control n = 97	PA n = 73	
Gender (boys/girls)	18/79	10/63	
Age (yr)	7.5 (7.3-7.7)	7.5 (7.2-7.7)	0.8
Birth weight (SDS)	0.2 (-0.04-0.4)	-0.03 (-0.3-0.2)	0.2
Birth length (SDS)	0.3 (0.1-0.5)	-0.05 (-0.3-0.2)	0.04
Weight-for-height (%)	102 (93-111)**	108 (93-123)**	0.004**
BMI (SDS)	0.4 (0.2-0.6)	1.0 (0.7-1.3)	0.001
Height (SDS)	0.3 (0.1-0.5)	1.2 (0.9-1.4)	<0.001
DHEAS (μmol/L)†	0.8 (0.7-0.9)	1.7 (1.5-2.0)	<0.001
Δ4-A (nmol/L)†	1.3 (1.2-1.5)	2.4 (2.1-2.7)	<0.001
SHBG (nmol/L) †	102 (96-108)	82 (73-90)	<0.001
Cholesterol (mmol/L)	4.2 (4.1-4.4)	4.2 (4.1-4.4)	0.9
HOMA-IR†	0.9 (0.8-1.0)	1.1 (1.0-1.2)	0.002
ISI_{comp}	1.1 (1.0-1.2)	0.8 (0.7-0.9)	<0.001
Systolic BP (mmHg)	100 (98-102)	105 (102-107)	0.004

CI, confidence interval; SDS, SD score; BMI, body mass index; DHEAS, dehydroepiandrosterone sulfate; Δ4-A, androstenedione HOMA-IR; homeostasis model assessment for insulin resistance according to the formula: fasting plasma glucose (mmol/l) x fasting serum insulin (mU/l) / 22.5; ISI_{comp}, insulin sensitivity index according to the formula: 10 000 / √ [fasting glucose (mg/dl) x fasting insulin (μU/l) x mean glucose (mg/dl) x mean insulin (μU/l)]; BP, blood pressure

* Values taken from Student's t-test unless indicated otherwise

** Median (interquartile range), analyzed with Mann-Whitney U-test

† Raw data were log-transformed prior to using the t-test, and results are presented as geometric means with 95% CI.

Gender distribution and mean age were similar between the PA and control groups (Table 4). PA subjects had higher weight-for-height, BMI and current height, whereas there were no significant differences in the birth measures. Adrenal androgens were significantly higher in the PP subjects and nonPP subjects than in the control subjects (Utriainen et al. 2007). There was no difference in the lipid profile between the groups. The differences in the androgen and SHBG levels and in ISI_{comp} remained significant when adjusted for BMI, whereas the significance of the difference in HOMA-IR and systolic BP disappeared (P = 0.06 and 0.2, respectively).

5.2 GENOTYPE DISTRIBUTIONS

5.2.1 *MC2R*, *LRP5*, *TCF7L2* and *FTO*

SNP MC2R -2 T>C, SNPs rs7903146 and rs12255372 in *TCF7L2* and rs9939609 in *FTO* were genotyped in the 73 PA children and the age- and gender matched 97 control subjects (I, IV, Table 5). Nineteen SNPs were found in *LRP5* in the total group of 170 children. Eleven SNPs in *LRP5* were synonymous and four were non-synonymous exonic variants, of which five SNPs had MAF over 5% in either group (Figure 1 in III, Table 5). Three intronic SNPs and one SNP in the 3' untranslated region of *LRP5* were also identified. Eleven SNPs in *LRP5* had MAF lower than 5% in both control and PA group, but none were found to be a likely disease-causing mutation (III). All of the genotyped SNPs were in Hardy-Weinberg equilibrium with a *P*-value > 0.05 (I, III, IV).

There were no statistically significant differences in the genotype distributions or in the MAFs of the SNPs in *MC2R, LRP5, TCF7L2* and *FTO* between the PA and control groups (I, III, IV, Table 5). However, the minor variant in MC2R -2 T>C was more frequent in the PP children than in nonPP and control children, suggesting its role in the development of PA [controls n = 97, nonPP n = 38 *vs*. PP n = 36; combined T/C&C/C frequency; 10%, 11% *vs*. 28%, P = 0.04, I]. The obesity risk-conferring variant at rs9939609 of *FTO* was not more frequent in the PA children despite their higher BMI, compared with healthy controls. Furthermore, the 95% CI for differences in the minor allele proportions showed a tendency for

the risk variant of rs9939609 to be less frequent in the PA children than in the population-based age-matched prepubertal controls (IV, Table 5).

Table 5. The genotype distributions and differences in minor allele frequencies of common single nucleotide polymorphisms (SNP) among prepubertal children with premature adrenarche (PA) and healthy age- and gender-matched controls.

Gene	SNP	Genotype distribution (A/A, A/a, a/a; %)		Difference in MAF (95% CI)	*P*
		Control (*n* = 97)	PA (*n* = 73)		*/**
MC2R	MC2R -2 T>C	90, 10, 0	81, 18, 1[#]	0.05 (-0.01 - 0.11)	0.2/0.1
LRP5	F549F	94, 6, 0	86, 14, 0	0.04 (-0.02 - 0.09)	0.1/0.2
	E644E	89, 11, 0	90, 10, 0	-0.01 (-0.06 - 0.04)	0.8/0.9
	N740N	91, 9, 0	86, 14, 0	0.02 (-0.03 - 0.08)	0.5/0.5
	V1119V	74, 25, 1	64, 36, 0	0.05 (-0.03 - 0.13)	0.2/0.3
	A1330V	91, 9, 0	88, 12, 0	0.02 (-0.04 - 0.07)	0.6/0.7
TCF7L2	rs7903146	73, 26, 1	67, 30, 3	0.03 (-0.05 - 0.12)	0.5/0.4
	rs12255372	72, 26, 2	70, 27, 3	0.01 (-0.07 - 0.10)	0.9/0.8
FTO	rs9939609	32, 50, 18	41, 45, 14	-0.06 (-0.18 - 0.05)	0.5/0.3

MC2R, melanocortin-2 receptor; *LRP5*, low density lipoprotein receptor-related protein 5, *TCF7L2* transcription factor 7-like 2; *FTO*, fat mass and obesity associated gene.
*, *P* value from Fischer's exact test on the differences in the genotype distributions; **, *P* value for the difference in minor allele proportions; [#], total *n* = 74.

Reliable haplotype analyses expanding the whole *LRP5* gene could not be performed, because the haploview analysis revealed only one small LD block (D' 1.000, r^2 0.30) between the intronic rs4988322 and the exonic V1119V. However, the control children with the minor variant at A1330V also had the minor variant at N740N, whereas there was one PA subject with the minor variant at N740N but not at A1330V (III). LD between the rs7903146 and rs12255372 at *TCF7L2* was high (r^2 = 0.82, D' 0.90). Therefore, we tested only rs7903146 in the single marker association analyses (IV).

Although our PA group is among the largest ones reported, the power of our study to demonstrate small differences in the genotype distributions was limited. The two groups with a total of 170 children provided only 14% and 11% power to detect the observed differences in the proportions of minor alleles at rs9939609 of *FTO* and rs7903146 of *TCF7L2*, respectively (α = 0.05). To show a statistical difference at the observed differences in the proportions of

minor alleles at rs9939609 and rs7903146 with 80% power ($\alpha = 0.05$), we would have needed 892 and 1383 subjects in the PA and control groups, respectively (IV).

5.2.2 Length of CAG repeat in the androgen receptor gene and X-inactivation

Children with PA had an arithmetic mean *AR* CAG_n 0.72 repeats shorter than the controls (95% CI for the difference; 0.09-1.34, $P = 0.025$), and the mean difference in $mwCAG_n$ was even larger [0.76 (0.14-1.38), $P = 0.017$), II]. None of the PA subjects had $mwCAG_n$ longer than 25 repeats, whereas nine controls had (Figure 1 in II). The arithmetic mean and $mwCAG_n$ differed significantly from each other in the whole group of children, indicating the importance of considering X-inactivation in the analyses of X-chromosomal genes (II). The results confirmed the finding of a similar difference in the arithmetic mean of CAG_n between the Catalan PP girls and controls (Ibáñez et al. 2003b). Although the difference of 0.8 repeats in *AR* gene $mwCAG_n$ between the PA and control groups is small, its clinical relevance may be considerable. The meta-analysis of the studies on the association of male infertility with *AR* gene CAG_n has revealed a statistically significant difference of 0.3 repeats between the cases and controls (Davis-Dao et al. 2007). The longitudinal follow-up of our PA girls will show whether the length of *AR* gene CAG_n is inversely related to the risk of ovarian hyperandrogenism as observed in the Catalan PP girls (Ibáñez et al. 2003b).

We did not find any differences between our control and PA groups in the incidence of nonrandom X-inactivation (proportion of subjects with either maternal or paternal allele being inactive in ≥ 60% of cells; 35% controls *vs*. 39% PA, $P = 0.7$) or skewed X-inactivation (inactive ≥ 80%; 1.7% controls *vs*. 3.3% PA, $P > 0.9$), suggesting no significant role of X-inactivation itself in the pathogenesis of PA (II). In contrast, a small previous study in Italian women with idiopathic hirsutism has shown skewing of X-inactivation, leading to preferential expression of the *AR* gene with shorter CAG_n (Vottero et al. 1999). The results on Italian women with hirsutism also conflict with a larger Spanish study demonstrating neither skewing of X-inactivation nor difference in mean CAG_n length between women with hyperandrogenic or idiopathic hirsutism and healthy controls (Calvo et al. 2000). We were not able to investigate X-inactivation in peripheral target tissues, but an excellent study comparing the X-inactivation

between different tissues has shown a similar X-inactivation pattern in blood cells and other tissues (Bittel et al. 2008).

We observed a weak correlation between the mwCAG$_n$ and subject's age in the PA group (τ = 0.27, P = 0.026, II). X-inactivation offers a possible epigenetic mechanism through which environmental factors may influence gene expression (Heard and Disteche 2006), but the mechanisms leading to secondary skewing of X-inactivation are not fully understood. A previous study has shown greater variability in X-inactivation in women older than 60 yr (Bittel et al. 2008). In addition, X-inactivation patterns have been shown to differ between sister pairs having the same genotype, but different clinical presentation in the families with PCOS (Hickey et al. 2006).

5.3 ASSOCIATIONS BETWEEN GENOTYPE AND PHENOTYPE

The associations between genotype and phenotype were searched by single marker association analyses on the common genetic variants at *MC2R*, *AR*, *LRP5*, *TCF7L2* and *FTO* in the PA and control groups. In the following, the results will be presented and discussed according to adrenocortical functions, anthropometric data and metabolic parameters. Of the SNPs in *LRP5*, SNP E644E was not associated with any clinical measures in the control or the PA group (III). As the control children with SNP A1330V also had the SNP N740N, their results for the SNP N740N were the same as for SNP A1330V that leads to an amino acid change (III).

The value of these studies lays in the precise phenotyping of PA subjects and unbiased controls, that all come from a homogenous population. However, our results should be confirmed in other populations with different genetic backgrounds and different environments, and by large genome-wide association studies. It is possible that some of the observed associations are by chance only, as the number of children in our study was relatively small. Further studies are required to determine the functional significance of the polymorphisms genotyped. The biochemical mechanisms are not fully understood for them, i.e., how they affect the gene expression or the functions of encoded proteins.

5.3.1 Adrenocortical function

The circulating levels of adrenocortical hormones and SHBG of the PA children have been summarized according to genotype groups in Table 6. There was no difference in the age between the genotype groups (I, III, IV).

5.3.1.1 MC2R

PA children with minor variant at MC2R -2 T>C had higher baseline plasma ACTH, serum DHEA and Δ4-A levels in comparison to the PA subjects with the T/T genotype (I). The statistically significant difference remained in the baseline Δ4-A level when the difference was adjusted for age and gender (P = 0.02, Table 6) Among the controls, the baseline hormone levels did not differ between the MC2R genotype groups. As an indicator of ACTH sensitivity, the baseline ACTH/cortisol ratio was calculated and it was significantly higher in the subjects with the T/C or C/C genotype than in the T/T subgroup of control children (I), which is in accordance with the previously reported higher ACTH/cortisol ratio among healthy adult men with C allele (Slawik et al. 2004). Recently, the minor variant at MC2R -2 T>C was shown with increased frequency in patients with infantile spasms, and it was associated with poor response to the ACTH therapy (Liu et al. 2008).

As an indicator of shifting steroidogenesis from glucocorticoids to adrenal androgens, we calculated the ratio of cortisol level to Δ4-A level, which was lower in the children with the T/C or C/C genotype than in those with the T/T genotype when all children were analyzed together [T/T n = 147 *vs*. T/C&C/C n = 23; ratio of cortisol level to Δ4-A level; mean (95% CI); 169 (152-185) *vs*. 114 (86-142), P = 0.008, I]. Indicated by the altered cortisol/Δ4-A ratios in the children with the C allele, it is possible that decreased ACTH sensitivity due to MC2R -2 T>C polymorphism shifts the adrenal steroidogenesis from the cortisol synthesis to the androgen pathway. It has been postulated that in times of critical or chronic illness, steroid synthesis is diverted from adrenal androgens to glucocorticoids to allow maintenance of high glucocorticoid levels that are crucial for coping with the illness (Parker et al. 1985, Bornstein and Chrousos 1999). Thus, the mechanism in PA would be the opposite to the stressful states in critical and chronic illness, and even in functional hypothalamic amenorrhea, in which plasma adrenal

androgens are decreased and cortisol levels are increased (Luppa et al. 1991, Beishuizen et al. 2002, Bomba et al. 2007, Dimopoulou et al. 2007).

5.3.1.2 *AR* mwCAG$_n$

One of our hypotheses was that increased androgen sensitivity would be a mechanism for PA in children with adrenal androgen levels normal for their age. However, we did not find any inverse correlation between *AR* mwCAG$_n$ and androgen or SHBG levels, nor did we find any significant difference in mwCAG$_n$ between the children with DHEAS level below 1μmol/l or above that level, representing biochemical adrenarche (II). Our results are in contrast to the study on post-menarcheal Catalan PP girls in whom higher testosterone levels and more pronounced signs of hyperandrogenism were observed in girls with a mean CAG$_n$ of less than 20 repeats (Ibáñez et al. 2003b). Our results suggest that *AR* gene CAG$_n$ has no role in the feedback regulation of adrenal androgen secretion in prepubertal children with PA.

5.3.1.3 *LRP5*, *TCF7L2* and *FTO*

No minor variant of the SNPs at *LRP5*, *TCF7L2*, or *FTO* were associated with adrenal androgen levels in the PA children when adjusted for age and gender. The baseline serum cortisol level was lower in the PA subjects with the minor variant of F549F at *LRP5* than in those without, but no differences were found in serum cortisol concentrations measured after ACTH stimulation, and no similar association was observed in the control group (III). In the control children, the minor variants of A1330V and N740N at *LRP5* were associated with higher DHEAS level (A/A *vs.* A/a; mean, 0.8 *vs.* 1.4 μmol/l, $P = 0.01$), but no association with other androgen levels or the cortisol level was observed (III). Even though the control children with both minor variants of SNPs A1330V and N740N did not have clinical signs of PA, their mean serum DHEAS concentration was > 1.0 μmol/L, which can be considered an adrenarcheal level.

The association between the minor variants at A1330V and N740N and serum DHEAS level suggests that LRP5 in the lipid metabolism of adrenocortical cells or in the canonical Wnt signaling may modulate the adrenal hormone profile during childhood. LRP5 in the lipid intake

of adrenocortical cells has not been studied, while previous studies have confirmed its role in the metabolism of lipoproteins (Kim et al. 1998, Magoori et al. 2003, Fujino et al. 2003). Wnt signaling has been suggested to play a role in the regulation of adrenal steroidogenesis. ACTH increases *WNT4* expression in primary cultures of adrenocortical cells (Kuulasmaa et al. 2008), and adenovirus mediated *WNT4* expression increases steroidogenesis and the expression of steroidogenic genes in cultured adrenocortical cells (Chen and Hornsby 2006). Adipocytes secrete Wnt molecules which induce the transcription of *StAR* and stimulate the steroidogenesis of adrenocortical cells *in vitro* (Schinner et al. 2007). Furthermore, the adrenal development is significantly compromised in the embryos with SERKAL syndrome due to a *WNT4* mutation, indicating an important role of Wnt signaling in the adrenal development (Mandel et al. 2008). In transgenic mice, targeted disruption of ß-catenin impairs development and maintenance of adrenal cortex (Kim et al. 2008).

Although we did not find any association between *LRP5* sequence variants or the *TCF7L2* variants and PA, Wnt signaling may nonetheless participate in the regulation of adrenarche. Recently, LRP5 has been shown to participate in the regulation of serotonin synthesis in duodenal heterochromatin cells via Wnt signaling, and serotonin acts in an endocrine fashion to regulate bone cell metabolism in mice (Yadav et al. 2008). On the other hand, WNT4 may antagonize the canonical Wnt signaling in the adrenal cortex, as *Wnt4* over-expression inhibits testosterone synthesis in mouse testes by repressing synergism between ß-catenin and SF-1 (Jordan et al. 2003). SF-1, in turn, is essential for the adrenal development (Luo et al. 1994, Achermann et al. 1999). The mechanisms of Wnt signaling may be more complicated in the regulation and development of adrenocortical cells than known today.

Table 6. Adrenocortical hormones and SHBG according to genotype groups in children with premature adrenarche.

Gene Polymorphism	Genotype (*n*)	The mean levels of circulating hormones (95% confidence interval)					
		Age (yr)	DHEAS (µmol/L)*	Δ4-A (nmol/L)*	Cortisol (mmol/L)*	Stim. cortisol (mmol/L)*	SHBG (nmol/L)
MC2R	T/T (60)	7.4 (7.2-7.7)	1.7 (1.4-2.0)	2.3 (2.0-2.6)	224 (198-254)	593 (567-620)	81 (72-90)
MC2R -2 T>C	T/C&C/C (13)	7.6 (6.8-8.3)	2.2 (1.8-2.7)	3.3 (2.5-4.3)	275 (214-353)	617 (554-688)	85 (56-114)
	*P***	0.6	0.2	**0.02**	0.2	0.5	0.6
LRP5	C/C (63)	7.5 (7.3-7.7)	1.7 (1.5-2.0)	2.4 (2.1-2.8)	249 (224-278)	604 (579-630)	83 (74-92)
F549F	C/T (10)	7.2 (6.5-7.8)	1.9 (1.4-2.5)	2.3 (1.5-3.3)	149 (104-214)	556 (489-633)	75 (40-110)
	*P***	0.3	0.4	>0.9	**0.002**	0.2	0.3
V1119V	A/A (47)	7.5 (7.2-7.7)	1.7 (1.4-2.0)	2.4 (2.1-2.9)	247 (215-284)	610 (580-640)	83 (73-93)
	A/G (26)	7.4 (7.1-7.8)	1.9 (1.5-2.4)	2.4 (2.0-3.0)	208 (172-251)	575 (535-619)	80 (62-97)
	*P***	>0.9	0.3	>0.9	0.1	0.2	0.8
A1330V	C/C (64)	7.4 (7.2-7.6)	1.7 (1.4-1.9)	2.4 (2.1-2.7)	228 (202-258)	602 (576-628)	84 (74-94)
	C/T (9)	7.8 (7.2-8.3)	2.5 (1.5-4.1)	2.9 (1.9-4.3)	266 (213-332)	565 (496-643)	65 (53-78)
	*P***	0.3	0.1	0.5	0.5	0.3	0.2
TCF7L2	C/C (49)	7.5 (7.2-7.8)	1.7 (1.4-2.0)	2.5 (2.1-2.9)	225 (196-259)	591 (563-620)	82 (70-93)
rs7903146	C/T&T/T (24)	7.4 (7.0-7.8)	1.8 (1.4-2.4)	2.3 (1.8-2.9)	248 (205-300)	609 (564-658)	82 (68-96)
	*P***	0.5	0.5	0.7	0.3	0.5	>0.9
FTO	T/T (30)	7.3 (7.1-7.6)	1.6 (1.3-2.0)	2.5 (2.1-3.0)	248 (211-290)	603 (567-642)	87 (72-101)
rs9939609	T/A (33)	7.5 (7.2-7.9)	1.7 (1.4-2.1)	2.3 (1.9-2.8)	240 (205-281)	586 (552-623)	80 (67-94)
	A/A (10)	7.5 (6.6-8.4)	2.3 (1.4-3.8)	2.7 (1.7-4.2)	173 (109-274)	615 (529-716)	70 (49-92)
	P#	0.7	0.2	0.7	0.06	>0.9	0.4

MC2R, melanocortin-2 receptor; *LRP5*, low density lipoprotein receptor-related protein 5, *TCF7L2* transcription factor 7-like 2; *FTO*, fat mass and obesity associated gene; *, Raw data were log-transformed prior to testing, and results are presented as geometric means with 95% confidence interfals; **, *P* values from univariate linear model adjusted for age and gender, except for age from Student's t-test; #, *P* values from general linear regression adjusted for age and gender, except for age from One-Way ANOVA.

5.3.2 Anthropometric data

The birth measures and the current weight adjusted for height of the children with PA according to various genotype groups have been summarized in Table 7. None of polymorphisms in *LRP5* was associated with either birth measures or current weight-for-height (Table 7, III).

5.3.2.1 *MC2R* and birth measures

The minor variant at MC2R -2 T>C was associated with lower birth weight SDS in the children with PA (I, Table 7). Previous studies have found an inverse correlation between birth weight and adrenal androgen levels in healthy children (Francois and de Zegher 1997, Ong et al. 2004), whereas PA and subsequent FOH have been connected with low birth weight (Ibáñez et al. 1998b, Neville and Walker 2005). The PA children with the minor variant at MC2R -2 T>C had higher ACTH stimulated cortisol levels than the control children with the same genotype (I). A meta-analysis has demonstrated an inverse connection between birth weight and circulating cortisol levels at adulthood (Montfoort et al. 2005). Birth weight can be considered to picture the intrauterine milieu, in which some factors have restricted the intrauterine growth and well being of developing fetus. It can be speculated that the minor variant at MC2R -2 T>C may have had an influence on the stress responses of the PA children during fetal development. According to the Barker hypothesis, intrauterine growth restraint reprograms the development of cardiovascular disease risk profile at adulthood (Barker et al. 1993, Eriksson et al. 1999). The follow-up of our PA children will show whether the minor variant at MC2R -2 T>C is associated with adverse metabolic features later in life. The association between birth weight and MC2R -2 T>C may also reflect the higher proportion of PP children in the group of PA children that have the minor variant, although no statistically significant differences in birth weight SDS between PP, nonPP and control girls were found (Utriainen et al. 2007).

Table 7. Anthropometric data according to various genotype groups in the children with premature adrenarche.

Gene Polymorphism	Genotype (*n*)	Mean (95% CI)		Median (IQR)
		Birth weight (SD score)*	Birth length (SD score)*	Weight-for-height (%)**
MC2R	T/T (60)	0.1 (-0.2 - 0.4)	0.1 (-0.2 - 0.4)	111 (94 - 128)
MC2R -2 T>C	T/C&C/C (13)	-0.6 (-1.0 - -0.2)	-0.6 (-1.1 - -0.02)	104 (89 - 119)
	P	**0.04**	0.06	0.5
LRP5	C/C (63)	-0.1, (-0.4 - 0.1)	-0.1 (-0.3 - 0.2)	108 (95 - 122)
F549F	C/T (10)	0.4 (-0.8 - 1.7)	0.1 (-1.2 - 1.3)	121 (105 - 138)
	P	0.2	0.8	0.3
V1119V	A/A (47)	-0.05 (-0.4 - 0.3)	-0.1 (-0.4 - 0.2)	108 (93 - 123)
	A/G (26)	-0.02 (-0.6 - 0.5)	0.1 (-0.4 - 0.6)	110 (94 - 126)
	P	>0.9	0.4	0.9
A1330V	C/C (64)	0.01 (-0.3 - 0.3)	-0.1 (-0.3 - 0.2)	108 (91 - 125)
	C/T (9)	-0.4 (-1.2 - 0.4)	0.1 (-0.6 - 0.9)	111 (101 - 121)
	P	0.3	0.6	0.8
TCF7L2	C/C (49)	-0.04 (-0.4 - 0.3)	0.04 (-0.4 - 0.3)	112 (94 - 130)
rs7903146	C/T&T/T (24)	-0.02 (-0.5 - 0.4)	-0.05 (-0.5 - 0.4)	103 (93 - 113)
	P	>0.9	>0.9	0.2
FTO	T/T (30)	-0.1 (-0.5 - 0.3)	-0.1 (-0.5 - 0.3)	108 (94 - 122)
rs9939609	T/A (33)	-0.1 (-0.4 - 0.3)	-0.1 (-0.4 - 0.3)	107 (94 - 120)
	A/A (10)	0.4 (-0.8 - 1.6)	0.3 (-0.8 - 1.3)	130 (116 - 144)
	P	0.4	0.6	0.2

CI, confidence interval; IQR, interquartile range; *MC2R*, melanocortin-2 receptor; *LRP5*, low density lipoprotein receptor-related protein 5, *TCF7L2* transcription factor 7-like 2; *FTO*, fat mass and obesity associated gene. *P* values from *Student's t-test or One-Way ANOVA, **Mann-Whitney U-test or Kruskall-Wallis non-parametric test.

5.3.2.2 *TCF7L2* and weight-for-height

No difference in birth measures or weight-for-height between genotype groups according to rs7903146 of *TCF7L2* was shown (Table 7). However, the frequency of minor variant at rs7903146 of *TCF7L2* was higher in the lean PA subjects (weight-for-height under the median of the PA group) than in the controls with the same BMI [weight-for-height <108% respecting BMI < 0.79 SDS; controls, $n = 65$ *vs.* PA, $n = 37$; difference in MAF (95%CI); 0.12 (-0.001, 0.23), $P = 0.038$, IV]. A recent GWA study on nearly 2000 diabetic patients and 3000 controls has found the risk variants at *TCF7L2* to associate with increased risk for T2DM in the subjects with a lower BMI than the median BMI of the cases, whereas the risk ratio was lower in the

subjects with a higher BMI (Timpson et al. 2009). The study demonstrates a substantial etiological heterogeneity within T2DM, which is also evident within PA.

5.3.2.3 *FTO* and weight-for-height

Increased weight-for-height was evident in our PA children compared with healthy age- and gender matched controls. Of all genetic factors found so far, the polymorphisms at *FTO* have been connected most strongly with higher BMI (Li and Loos 2008). The difference in the current weight-for-height between *FTO* rs9939609 genotype groups was significant only in our healthy prepubertal children [mean weight-for-height; T/T, T/A *vs*. A/A; 99%, 109% *vs*. 112%, $P = 0.001$], but not in the children with PA (IV). The lack of association in the PA group may result from insufficient group size, but may also suggest that the role of genetic variation in *FTO* is minor in the regulation of increased weight-for-height in PA children. A previous study has found an association between increased BMI and the minor variant at rs2236418 of *GAD2* in PP children, but whether the association was present in healthy subjects was not tested (Witchel et al. 20098).

The role of *FTO* can be considered to be primary in the accumulation of weight, affecting eating behavior. In the pathogenesis of PA, the increased weight-for-height may be secondary or just one factor among others. The obesity-associated genetic variation at *FTO* has been connected with reduced satiety responsiveness (Wardle et al. 2008) and increased energy intake in prepubertal children (Cecil et al. 2008, Wardle et al. 2009). In mice, *FTO* is expressed most abundant in hypothalamic nuclei governing energy balance, and the expression is down-regulated by fasting (Gerken et al. 2007). *FTO* shares sequence motifs with non-heme dioxygenases with a potential role in nucleic acid demethylation and epigenetic regulation (Gerken et al. 2007, Sanchez-Pulido and Andrade-Navarro 2007). Although associated with increased energy intake, the risk variants in *FTO* may not be connected with energy expenditure (Berentzen et al. 2008, Cecil et al. 2008, Speakman et al. 2008). Nonetheless, a recent extensive metabolic study on healthy non-obese young men showed increased energy efficiency in oxidative muscle fibers after exercise in homozygous carriers of the obesity-related risk allele at rs9939609 of *FTO*, suggesting that increased energy efficiency with potential mitochondrial

coupling could represent a key defect linking *FTO* to obesity (Grunnet et al. 2009). Furthermore, the expression of *FTO* is reduced in the adipocytes of transgenic obese *db*/*db* mice (Qi et al. 2008) and the risk variants have been associated with signs of cerebro-cortical insulin resistance (Tschritter et al. 2007), suggesting that other possible mechanisms in the actions of FTO. In contrast, the retrospective analysis of the growth pattern of our PA children has revealed increased height velocity during the first two years of life, which precedes the accumulation of weight compared with controls. These findings suggest that increased weight-for-height is not a primary event in PA (Utriainen et al. 2009b).

5.3.2.4 *AR* mwCAG$_n$ and BMI

In our PA children, mwCAG$_n$ correlated positively to BMI SDS (II, Figure 3). After dividing the PA group by the median BMI (0.79 SDS), the PA children with lower BMI had a shorter mwCAG$_n$ than those with higher BMI, with a mean difference of 1.13 repeats (95% CI, 0.38-1.87, P = 0.004). A similar difference in mwCAG$_n$ was found between the PA children with lower BMI compared with controls having the same BMI SDS (II). The results demonstrate that PA children with lower BMI have more active AR, which offers a tempting mechanism to explain PA in lean children. Hyperinsulinemia is more evident in the PA subjects with higher weight-for-height, and may be the key inducer of PA in them.

In previous studies, a positive correlation between the CAG$_n$ and body fat mass has been found in healthy males (Zitzmann et al. 2003, Stanworth et al. 2008), whereas no correlation between mwCAG$_n$ and BMI has been seen in the Australian women with PCOS (Hickey et al. 2002). Furthermore, treatment with low-dose flutamide in combination with metformin has achieved a greater reduction in the adiposity of post-menarcheal PP girls with a shorter mean CAG$_n$ than in subjects with a mean CAG$_n$ longer than 20 repeats (Ong et al. 2007). A recent study on over 3300 men with age of 40-79 yrs, showed a positive correlation between CAG$_n$ and serum testosterone and estradiol levels. Weaker transcriptional activity of AR was interpreted as compensation for higher testosterone levels, and the residual phenotypic differences in e.g. body weight were postulated to reflect differences in the estrogen levels after aromatization of higher testosterone levels (Huhtaniemi et al. 2009). We cannot state whether adrenal androgens are metabolized to estrogens in our children with PA.

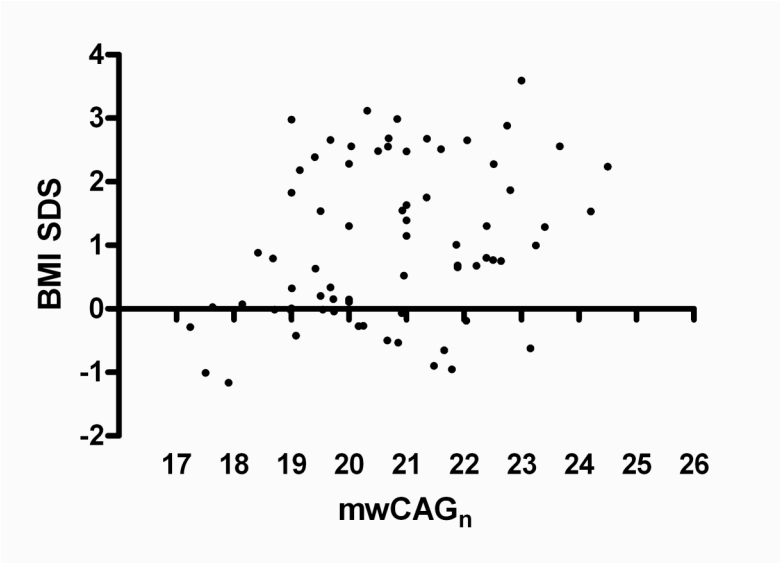

Figure 3. Scatter plot of BMI SDS by $mwCAG_n$. As can we see from scatter plot figure, there was a positive correlation between methylation-weighted biallelic mean of the CAG repeat number ($mwCAG_n$) in androgen receptor gene and body mass index SD scores (BMI SDS) with a coefficient of 0.19 in prepubertal children with premature adrenarche (n = 71, τ = 0.19, P = 0.02 with Kendall's rank correlation test).

5.3.3 Metabolism

Insulin and IGF-1 functions have been postulated to participate in the pathogenesis of PA. Adverse metabolic features have been shown in prepubertal PA children and in PA subjects after puberty (Ibáñez et al. 1998a, Vuguin et al. 1999, Silfen et al. 2002a, Ibáñez et al. 2003a, Charkaluk et al. 2004). We tested whether the polymorphisms at *AR*, *LRP5*, *TCF7L2* and *FTO* are associated with measures of glucose metabolism, lipid profile and BP in prepubertal PA children and in healthy age- and gender-matched controls (II-IV). We did not find any associations between *FTO* rs9939609 and metabolic parameters either in the PA or the control group (IV).

5.3.3.1 *AR* mwCAG$_n$ and insulin sensitivity

An inverse correlation between the insulin sensitivity index ISI$_{comp}$ and *AR* gene mwCAG$_n$ was shown in the PA subjects (τ = -0.30, P = 0.014), but the statistical significance disappeared when corrected for BMI (II). The previous study in Catalan postmenarcheal PP girls has shown greater hyperinsulinemia in response to an OGTT in girls with a biallelic mean of CAG$_n$ shorter than 20 in than in those with a mean CAG$_n$ longer than 20, but no difference in HOMA was observed between the groups. (Ibanez et al. 2003b). In healthy adult men, CAG$_n$ has been positively associated with fasting insulin concentrations, independently of BMI, suggesting a protective role of short CAG$_n$ for cardiovascular risk factors (Zitzmann et al. 2003). The discrepancy between the studies may reflect population differences and the difference in age and sex among the studies.

5.3.3.2 *TCF7L2* and glucose metabolism

In the lean PA subjects, there were weak trends for higher insulin resistance index HOMA-IR and lower ISI$_{comp}$ in the subjects with the diabetes-related risk allele at rs7903146 of *TCF7L2*, suggesting a possible role of the risk variant in glucose metabolism (IV). The genetic variants at *TCF7L2* have been associated with decreased insulin secretion, which is linked to impaired incretin effects and β-cell proliferation (Jin 2008). The biochemical mechanisms by which the genetic variation in the intron region influences the expression of *TCF7L2* or WNT signaling in glucose metabolism remain largely unknown. The risk allele of rs7903146 was associated with higher fasting and 120-min blood glucose in OGTT in a cohort of 283 obese children with a mean age of 11.9 yr and mean BMI 2.8 SDS (Körner et al. 2007). In children and young adults with age at the onset of diabetes less than 25 yrs, the risk variants at rs7903146 and rs12255372 of *TCF7L2* were more frequent in the autoantibody-negative subjects than in autoantibody-positive diabetic patients (Yu et al. 2009). The risk variant at rs7903146 was also more frequent in the glutamate acid decarboxylase antibody (GADA) -negative subjects with age of 15-34 yrs at the onset of type 1 diabetes mellitus, whereas no difference in the genotype distribution was found between older GADA-negative and -positive patients (Bakhtadze et al. 2008). Neither of the studies on autoantibody-negative and -positive diabetes patients found associations between

the risk variants of *TCF7L2* and clinical measures at the onset of diabetes mellitus (Bakhtadze et al. 2008, Yu et al. 2009).

5.3.3.3 *LRP5*, blood pressure and lipid profile

The weight-for-height-adjusted BP and lipid levels did not differ between our girls with PA and the control girls (Utriainen et al. 2007). The previous reports on lipoprotein levels and lipid pattern in PA come from different populations with different ages (Ibáñez et al. 1998a, Meas et al. 2002, Guven et al. 2005, Andiran and Yordam 2008). The only associations between genetic polymorphisms and systolic BP in our series were found with the minor variant at V1119V of *LRP5* in the PA children [A/A n = 47 *vs*. A/G n = 26; mean systolic BP (95% CI); 103 mmHg (100-105) *vs*. 108 mmHg (104-112), P = 0.02] and the minor variant at F549F of *LRP5* in the control children [C/C n = 91 *vs*. C/T n = 6; 100 mmHg (98-102) *vs*. 108 mmHg (95-121), P = 0.04, III]. *LRP5* is expressed in the muscular component of large developing blood vessels, and the canonical Wnt signaling through LRP5 has been suggested to play a unique role in cardiovascular development (Wang et al. 2005). In a previous Japanese study, homozygosity for A1330V was associated with lower diastolic and mean BP in males (Suwazono et al. 2006b), but we found no association between A1330V and systolic BP in prepubertal children.

Higher total and LDL cholesterol levels were associated with the minor variants at A1330V, N740N and V1119V of *LRP5* in the healthy control children (III). This finding is in line with the previous study in a Japanese population suggesting that the risk variant at A1330V is an independent risk factor for hypercholesterolemia (Suwazono et al. 2006a). *LRP5* knockout mice display diet-induced hypercholesterolemia due to decreased hepatic clearance of chylomicron remnants (Fujino et al. 2003). Our results show that the association between the minor variant at A1330V and plasma cholesterol levels is already apparent in prepubertal children. In the PA children, the single marker analysis with *LRP5* SNPs showed no similar associations with lipid profile. It is likely that the determinants of metabolic characteristics and lipid profile in children with PA are more complex than in healthy children.

5.3.4 Future perspectives and associations between androgen action, body weight and glucose metabolism

In children with PA, androgen levels, body weight and glucose metabolism may influence each other. Overweight contributes to androgen levels possibly via hyperinsulinemia. On the other hand, androgens may alter adipose tissue mass deposition through site-specific modulation of preadipocyte differentiation as well as lipid synthesis and lipolysis in mature adipocytes (Blouin et al. 2008). In PCOS patients, who resemble PA children, a vicious circle has been suggested to exist whereby androgen excess favoring the abdominal deposition of fat further facilitates androgen secretion by ovaries and adrenals (Escobar-Morreale and San Millan 2007). Besides being a target of hormonal actions, adipose tissue is a major site for metabolism of sex steroids, including adrenal androgens (Kershaw and Flier 2004, Blouin et al. 2009). The adrenal androgens in PA children may be metabolized by adipose tissue to stronger androgens or estrogens with more potent local effects. .

Analysis of gene expression patterns of adipose cells in PA children would give insight to the role of increased weight-for-height in the pathogenesis of PA. Unfortunately, the techniques of adipose tissue biopsy do not currently offer ethically suitable methods to study dozens of prepubertal children. Recently, new gene loci associated with BMI were identified. Likely causal genes with unknown function are highly expressed in the central nervous system, e.g. transmembrane protein 18 (*TMEM18*) and glucosamine-6-phosphate deaminase 2 (*GNPDA2*) genes (Willer et al. 2009, Zhao et al. 2009). It could be interesting to clarify possible regulation of PA and adrenarche through genetic factors acting on the central nervous system. Until now, there have been no candidate gene studies on the role of genes acting primarily in the brain in children with PA.

Androgen action, weight regulation and insulin action do not all need to be disturbed for the clinical manifestation of PA. There are also lean PA subjects with higher androgen sensitivity and milder hyperinsulinemia (II). The retrospective analysis of the growth pattern of our girls with PA showed accelerated linear growth before the rise in weight-for-height when compared with healthy controls. At the time of PA presentation, the IGF-1 levels in these girls were increased compared with controls (Utriainen et al. 2009b). It may be speculated that PA children have hyperinsulinemia and higher IGF-1 levels already before the rise in weight-for-height and adrenal androgen levels, but no prospective study on the development of PA has

been conducted. The mechanism for development of androgenic signs in children with PA, but with DHEAS levels below the adrenarcheal level remains to be identified. We did not find any difference in androgen sensitivity as estimated by *AR* gene mwCAG$_n$ between subjects with DHEAS level below and above the adrenarcheal level (II).

Genotype-phenotype associations may be different between girls and boys. A study on monozygotic and dizygotic twins revealed a significant genetic component in the weight-adjusted adrenal androgen excretion rate. The study also suggested that the role of genetic regulation may be bigger in boys, whereas the role of environmental factors may be bigger in girls (Pratt et al. 1994). The number of boys with PA in our series is insufficient to test gender-dependent differences in the regulation of adrenarche, but we did adjust all the associations for gender. Androgens are known to have gender-dependent effects, for example on adipose tissue in pubertal children and adults. The only longitudinal study, investigating the effects of weight loss on the adrenal androgen levels within 1 yr on obese children suggested that the role of body weight is possibly larger in girls. In that study, obese prepubertal girls losing substantial weight demonstrated no significant change in DHEAS levels, whereas girls without weight loss showed an increase. Obese prepubertal boys demonstrated a significant increase of DHEAS levels regardless of their weight change over the 1-yr study period (Reinehr et al. 2005).

Different settings and research methods are needed to clarify the regulation of PA and the genetic and environmental factors behind it. Larger study groups and international collaboration could confirm our results and define more precisely genotypes behind different phenotypes of PA. It is important to study the whole clinical spectrum of PA and to use unbiased controls. Otherwise, significant results could be missed, and wrong conclusions could be drawn. Each polymorphism has only a minor role in the polygenic pathogenesis, and the necessary group sizes will be at least ten times bigger than in our study. The large GWA studies have shown that the combined power of several genetic variants, including *TCF7L2* and *FTO*, to predict the development of diabetes is only minor (van Hoek et al. 2008, Lyssenko et al. 2008). Furthermore, genetic variation explained less than 10% of the phenotypic variation in T2DM (Ruchat et al. 2008).

Although genetic studies on PA may not find strong predictors for the development of PA, they may be useful tools to identify factors in the genetic regulation of adrenarche. GWA studies and case reports of extreme types of adrenarche may find new genes behind adrenarche.

In addition to SNPs, structural variants and RNA interference are possible factors in the genetic regulation of adrenarche. To define more precisely the nature of on-time adrenarche, healthy children with varying age, adrenal androgen levels and androgenic signs as well as children with no adrenarche due to e.g. ACTH resistance may provide fruitful cohorts to explore. On the other hand, the pathways of steroid and glucose metabolism, the regulation of growth and the central regulation of puberty will be defined in more detail revealing possible factors in the regulation of adrenarche. Although the lack of appropriate animal model has hampered the research on adrenarche, the future may offer us new approaches e.g. with genetic engineering and stem cell cultures.

The clinical significance of our results on genetic variation in candidate genes *MC2R*, *AR*, *LRP5*, *TCF7L2* and *FTO* in PA is minor, because the results do not help clinicians to estimate the risk of developing PA or to make decisions about the follow-up or treatments of PA children. Even the clinical relevance of PA itself is not clear. Although in previous studies PA has been associated with risks of cardiovascular disease and hyperandrogenism, large follow-up studies are needed to confirm the long-term sequelae. However, we have succeeded in defining some factors behind different phenotypes of PA. For example, the results in PA subjects with BMI less than the group median showed that higher androgen sensitivity and a higher prevalence of a diabetes-related risk variant in *TCF7L2* may play a role in the pathogenesis of PA. The role of genetic factors in the pathogenesis may be larger in lean PA subjects.

Behind different phenotypes of PA, there are different genetic factors to be found together with different environmental factors. Follow-up studies on PA children will show how the different genotypes and phenotypes are related with risks of consequent disturbances in hormonal functions or metabolism in adulthood.

6 CONCLUSIONS

We genotyped ten polymorphisms in the *MC2R*, *AR*, *LRP5*, *TCF7L2* and *FTO* genes, and determined X-chromosome inactivation patterns in 73 PA children and 97 age- and gender-matched controls. Based on the differences in genotype distributions and single marker association analysis, we can make the following conclusions about the role of genetic variation in adrenarche, bearing in mind the limited power of the study.

1) The minor variant of MC2R -2 T>C is more frequent in PP children and is associated with higher Δ4-A/cortisol ratio, suggesting a shift in adrenal steroidogenesis from corticosteroids to androgens (I).
2) X-chromosome inactivation plays no major role in the pathogenesis of PA (II).
3) PA children with a BMI less than the median of the group have a shorter $mwCAG_n$ in *AR*, indicating higher androgen sensitivity (III).
4) PA children with a lower BMI than the median of the group have a higher frequency of the diabetes risk-conferring variant of rs7903146 in *TCF7L2* (IV).
5) The obesity-related risk variant of rs9939609 in *FTO* was found in similar frequency in PA and control children, and was not associated with increased weight-for-height in PA children (IV).
6) Genetic variation in *LRP5* was not associated with PA, but the minor variants were associated with serum DHEAS levels and lipid measures in healthy prepubertal children (III).

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