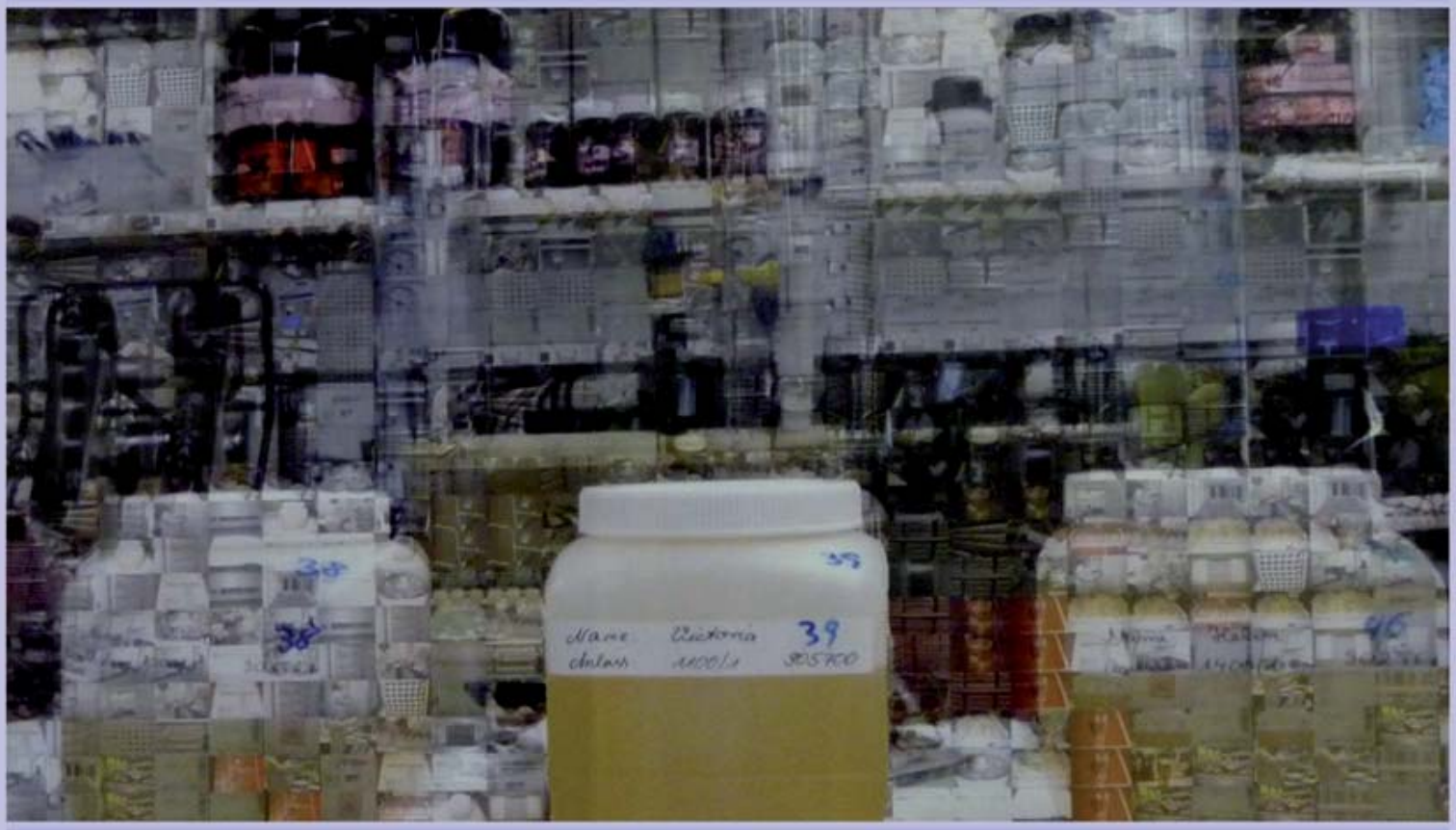
Lijie Shi

Interplay of nutrition and prepubertal steroid hormones: role in the timing of puberty



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Interplay of nutrition and prepubertal steroid hormones: role in the timing of puberty

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Summary

Interplay of nutrition and prepubertal steroid hormones: role in the timing of puberty

Background and aim

Adrenarche [the increase in adrenal androgen (AA) secretion] and puberty are two important events in human's growth and maturation. Children with premature adrenarche or precocious puberty are at a risk for the development of hormone-related cancers or chronic disease later in life. Childhood nutritional status has been suggested to be associated with pubertal timing, whether it is also relevant for adrenarche is not clear. Therefore, ***the first aim*** of this thesis was to examine whether body composition and certain dietary intakes may influence AA production in children.

Excess adrenal steroid hormone [especially AA and glucocorticoid (GC)] exposures can influence somatic growth and sexual maturation - two important physiological signs of pubertal development. The evidence for the role of them in the physiological range in pubertal timing is limited. Accordingly, ***the second and the third aims*** were to examine the association of prepubertal AA and GC status respectively with early and late pubertal markers, independent of nutritional status.

Evidence of growth promoting properties of low circulating concentration of estrogens during childhood also exists; however, further elucidation of the role of estrogens in the pubertal timing has been hampered so far by the lack of a sensitive measurement method. ***The last aim*** was to investigate prepubertal urinary estrogen (E) excretion level (using a recently developed isotopic dilution/gas chromatography-mass spectrometry method) and its association with pubertal markers. Data came from the prospective DOrtmund Nutritional and Anthropometric Longitudinally Designed (DONALD) Study, which observes diet, growth, and metabolism in healthy children from birth until young adulthood.

Results

Four consecutive analyses (Studies I-IV) were performed. **In Study I** (n=137), among all the examined anthropometric (fat mass and fat free mass) and dietary (protein, fat and fiber intake, glycemic index and load) factors, only fat mass and animal protein intake were positively associated with AA-secretion and explained 6% of total AA variation even after accounting for the indices of major steroidogenic enzyme activities. Although animal protein intake and AA secretion were associated with each other, **Study II** (n=109) showed that the negative associations of AA secretion with ages at Tanner stage 2 for breast (B2, girls), genital (G2, boys) and pubic hair (PH2, boys and girls) development were independent of animal protein intake and the negative associations of animal protein intake with the start of pubertal growth spurt (ATO) and age at peak height velocity (APHV) were independent of AA. Children with a higher AA secretion had a 1.5 yrs earlier begin of PH2, a 0.8 yrs earlier begin of B2_G2, and 0.2 yr shorter duration of pubertal growth acceleration than those with a lower one. In contrast to AA, GC secretion [**Study III** (n=110)] was positively associated with pubertal timing and this association seemed to be sex specific, i.e. only in girls. Girls with higher prepubertal GC secretion reached ATO 0.7 yrs later and menarche 0.9 yrs later; age at B2, PH2 and PHV tended also to occur later. In **Study IV** (n=120), urinary E excretions were not associated with ATO, APHV, or age at PH2, but with duration of pubertal growth acceleration in both sexes. Girls with higher E levels reached B2 0.9 yr and menarche 0.3 yrs earlier than girls with lower one. Prepubertal E production was not associated with age at G2 in boys.

Conclusions

Body fat mass may relevantly influence prepubertal adrenarchal androgen status and animal protein intake may also make a small contribution to children's AA-secretion levels. Prepubertal variations of steroid hormones (AA, GC, and E) in physiological range appear to be differently involved in the modulation of pubertal timing in healthy children. Higher AA and E secretion may not be critical for the earlier onset of pubertal growth spurt and APHV, but precipitate a shorter pubertal growth spurt in boys and girls. In girls, higher AA or E production predicts a notably earlier onset of breast development; however age at menarche appears to be only determined by E. In boys, AA but not E is relevant for genital development. GC may influence pubertal timing of girls but not of boys. Higher GC secretion, as a marker of higher stress activity, predicts notably later onset of pubertal growth spurt and menarche. Breast and pubic hair development as well as APHV may also tend to occur later.

Zusammenfassung

Zusammenspiel von Ernährung und präpuberalen Steroidhormonen: Bedeutung für das Pubertätstiming

Hintergrund und Ziel

Adrenarche [Anstieg der adrenalen Androgen(AA)-sekretion nach dem dritten Lebensjahr] und Pubertät sind zwei wichtige Ereignisse in der Wachstums- und Entwicklungsphase des Menschen. Kinder mit vorzeitiger Adrenarche oder früher Pubertät sind gefährdet, im späteren Leben hormon-assoziierte Tumore oder chronische Erkrankungen zu entwickeln. Es wird angenommen, dass das Pubertätstiming mit dem Ernährungsstatus in der Kindheit assoziiert ist, inwieweit der Ernährungsstatus auch für die Adrenarche relevant ist, ist weitgehend ungeklärt. Aus diesem Grund war **das erste Ziel** dieser Arbeit, zu untersuchen, ob die Körperzusammensetzung und der Verzehr ausgewählter Nährstoffe die AA Produktion in der Kindheit beeinflussen können.

Ein Überschuss an adrenalen Steroidhormonen [insbesondere AA and Glucocorticoide (GC)] kann sich auf das körperliche Wachstum und die sexuelle Reifung – zwei wichtige physiologische Zeichen der puberalen Entwicklung – auswirken. Die Evidenz hinsichtlich ihrer Bedeutung für das Pubertätstiming im physiologischen Hormonschwankungsbereich ist bislang limitiert. Folglich waren **das zweite und das dritte Ziel**, die Assoziationen des präpuberalen AA und GC Status mit frühen und späten Pubertätsmarkern zu untersuchen, unabhängig vom Ernährungsstatus.

Wachstumsfördernde Eigenschaften werden auch geringen zirkulierenden Östrogenkonzentrationen während der Kindheit zugeschrieben. Eine weitergehende Charakterisierung der Bedeutung von Östrogenen (E) für das Pubertätstiming war aufgrund des Fehlens sensitiver Analysemethoden nur eingeschränkt möglich. Somit war **das letzte Ziel**, das präpuberale renale E-Ausscheidungslevel zu untersuchen (anhand einer kürzlich entwickelten Isotopenverdünnungs-gaschromatographischen-massenspektrometrischen Methode*)* und seine Assoziation mit Pubertätsmarkern zu bestimmen. Die in dieser Arbeit verwendeten Daten stammten aus der prospektiven DOrtmund Nutritional and Anthropometric Longitudinally Designed (DONALD) Study, die Ernährung, Wachstum und Stoffwechsel in gesunden Kindern von Geburt an bis ins junge Erwachsenenalter untersucht.

Ergebnisse

Vier aufeinanderfolgende Analysen (Studien I-IV) wurden durchgeführt. In Studie I (n=137) zeigten unter allen untersuchten anthropometrischen (Fett- und fettfreie Masse) und Ernährungsfaktoren (Protein-, Fett- und Ballaststoffzufuhr, Glykämischer Index und Glykämische Last) nur die Fettmasse und die Zufuhr von tierischem Eiweiß eine positive Assoziation mit der AA-Sekretion. Beide zusammen erklärten 6% der gesamten AA Variation, auch nach Berücksichtigung der Indizes der wichtigsten steroidogenen Enzymaktivitäten. Obwohl die Zufuhr von tierischem Eiweiß und die AA-Sekretion miteinander korreliert waren, zeigte Studie II (n=109), dass die negativen Assoziationen der Zufuhr von tierischem Protein mit dem Beginn des puberalen Wachstumsschubs (ATO) und dem Alter zur maximalen Wachstumsgeschwindigkeit (APHV) unabhängig von AA waren. Ebenso waren die deutlichen Assoziationen zwischen höherer AA Sekretion und einem früheren Alter zu Tannerstadium 2 für Brust- (B2, Mädchen) bzw. Genitalentwicklung (G2, Jungen) und Pubesbehaarung (PH2, Jungen und Mädchen) unabhängig von der Zufuhr von tierischem Eiweiß. Kinder mit einer höheren AA Sekretion hatten einen um 1,5 Jahre früheren Beginn von PH2, einen um 0,8 Jahre früheren Beginn von B2_G2 und eine um 0,2 Jahre kürzere Dauer der puberalen Wachstumsakzeleration als solche Kinder, die eine niedrigere AA-Sekretion aufwiesen. Im Gegensatz zu AA war die GC-Sekretion [Studie III (n=110)] positiv mit dem Pubertätstiming assoziiert, diese Assoziation erwies sich als geschlechtsspezifisch und konnte nur bei Mädchen beobachtet werden. Mädchen mit einer höheren präpuberalen GC Sekretion erreichten ATO um 0,7 Jahre, die Menarche um 0,9 Jahre später; B2, PH2 und PHV traten ebenfalls tendenziell später auf. In Studie IV (n=120) war die renale E Exkretion nicht mit ATO, APHV oder dem Alter zu PH2 assoziiert, aber in beiden Geschlechtern mit der Dauer der

puberalen Wachstumsakzeleration. Mädchen mit höheren E Leveln erreichten B2 um 0,9 Jahre und die Menarche um 0,3 Jahre früher als Mädchen mit geringeren E Leveln. Bei Jungen war die präbuberale E Produktion nicht mit dem Alter zu G2 assoziiert.

Schlussfolgerungen

Die Körperfettmasse hat einen relevanten Einfluss auf den präpuberalen adrenalen Androgenstatus. Auch der Verzehr tierischen Proteins könnte einen moderaten Beitrag zu den AA-Sekretionsleveln von Kindern leisten. Präpuberal variierende Steroidhormonlevel (AA, GC und E) im physiologischen Bereich, scheinen unterschiedlich an der Regulation des Pubertätstimings bei gesunden Kindern beteiligt zu sein. Eine höhere Sekretion sowohl von AA als auch von E ist wahrscheinlich eher unbedeutend für einen früheren Beginn des puberalen Wachstumsschubs und APHV, begünstigt allerdings einen kürzeren puberalen Wachstumsschub bei Jungen und Mädchen. Bei Mädchen sagt eine höhere AA oder E Produktion eine merklich frühere Brustentwicklung voraus; wobei das Alter zu Beginn der Menarche nur durch E beeinflusst zu sein scheint. Bei Jungen sind höhere AA, nicht aber höhere E bedeutsam für einen früheren Beginn der Genitalentwicklung. GC könnten das Pubertätstiming von Mädchen, nicht aber von Jungen beeinflussen. Eine höhere GC-Sekretion, als Marker einer höheren Stress Aktivität, ist vor allem ein Prädiktor eines später einsetzenden puberalen Wachstumsschubs und einer späteren Menarche. Damit erweisen sich sowohl modifizierbare Ernährungsfaktoren, wie die Proteinzufuhr, als auch nur teilweise beeinflussbare Steroidhormone als bedeutsame unabhängige Modulatoren des Pubertätstimings. Inwieweit andere Ernährungseinflüsse auch unabhängig von Steroideffekten den Pubertätseintritt verändern bedarf weiterer Untersuchungen.

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List of Abbreviations

AA(s)	Arenal androgen(s)
ACTH	Adrenocorticotropic hormone
Androstenediol	5-androstene-3β,17β-diol
ATO	Age at take-off
APHV	Age at peak height vecolity
B2_G2	Tanner stage 2 for breast and genital development
%BF	Percent body fat
BMI	Body mass index
BMR	Basal metabolic rate
BSA	Body surface area
CAH	Congenital adrenal hyperplasia
CNS	central nervous system
CV	Coefficients of variation
∑C19	Sum of major urinary AA metabolites
∑C21	Sum of major urinary cortisol metabolites
CRH	Corticotropin-releasing hormone
CYB5	Cytochrome b5
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DONALD	DOrtmund Nutritional and Anthropometric Longitudinally Designed
E(s)	Estrogen(s)
E1	Estrone
E2	Estradiol
E3	Estriol
E-sum3	Sum of E1, E2, and E3
E-sum5	Sum of estrogen metabolites
EGF	Epidermal growth factor
FFM	Fat free mass
FM	Fat mass
FMI	Fat mass index
FOD	Frequency of detection
GABA	Gamma-aninobutyric acid

GC-MS	Gas chromatography/mass spectrometry
GC(s)	Glucocorticoid(s)
GH	Growth hormone
GI	Glycemic index
GL	Glycemic load
GnRH	Gonadotropin –releasing hormone
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HSD(s)	Hydroxysteroid dehydrogenase(s)
ID/GC-MS	Isotopic dilution/GC-MS
IGF	Insulin-like growth factor
LOD	Limit of detection
MR	Mineralocorticoids receptor
OATs	Organic anion transporters
OATPs	Organic anion transporting polypeptides
P450scc	Side-chain cleavage enzyme desmolase
P450aro	Aromatase
PA	Premature adrenarche
PB	Preece and Baines model
PCOS	Polycystic ovarian syndrome
PH	Pubic hair
PH2	Tanner stage 2 for pubic hair development
RIA	Radioimmunoassay
SDS	Standard deviation score
SHBG	Sex-hormone-binding globulin
5a-THF	5α-tetrahydrocortisol
THF	5β-tetrahydrocortisol
THE	5β-tetrahydrocortisone
UFF	Urinary free cortisol
UFE	Urinary free cortisone
yr(s)	Year (s)
ZF	Zona fasciculata
ZG	Zona glomerulosa
ZR	Zona reticularis

1. Introduction

Adrenarche and puberty are two important events in human growth and biological maturation. Puberty is the transitional period between the juvenile state and adulthood during which the adolescent growth spurt occurs, secondary sexual characteristics appear, fertility is achieved, and profound psychological changes take place (1). Adrenarche is the process of increase of adrenal androgen (AA) production beginning several years before the onset of puberty (2, 3). Children with premature adrenarche or precocious puberty are at a risk for the development of hormone related cancers (4-6) or chronic disease (7) later in life.

The influence of childhood nutritional status, especially with regard to body fat mass (8, 9) and animal protein intake (10), on pubertal timing has been intensively discussed, however the evidence for the role of body composition and dietary intakes in the modulation of AA production or adrenarche is limited. Therefore, ***the first aim*** of this thesis was to examine whether body composition and certain dietary intakes may influence AA production in healthy children.

The link between nutritional status and the physiological variations in timing of puberty can be significant but it is not particularly strong, suggesting that this relationship may be mediated by one or more growth-, energy balance-, or sexual maturation-related hormones, such as insulin, IGF (Insulin-like growth factor)-1 , leptin, glucocorticoids (GCs), adrenal or gonadal sex steroid hormones. The prospective DOrtmund Nutritional and Anthropometric Longitudinally Designed (DONALD) Study, which observes diet, growth, and metabolism in healthy children, provides a good data basis for the investigation of the potential roles of several of these candidate hormones for the pubertal development. The DONALD Study is entirely observational and non-invasive until the participants are 18 years old. Therefore in this thesis the assessments of hormone status were based on 24-h urine data. which are regularly collected in yearly intervals by the DONALD participants. Since to date, the measurement of peptide hormones in urine samples has not yet been satisfactorily established, this thesis concentrated exclusively on steroid hormones.

Excess adrenal steroid hormone [i.e. AA (11, 12) and GC (13-16)] exposures can influence somatic growth and sexual maturation - two important physiological signs of pubertal development. The evidence for a role of steroid hormone variations in the physiological range in the pubertal timing is limited. Accordingly, ***the second and the third aim*** was to examine the association of prepubertal AA secretion and GC status (as a marker of stress activity) respectively with early and late pubertal markers, independent of

nutritional status. Few evidence of growth promoting properties of low circulating concentration of estrogens (Es) during childhood also exists; however, further elucidation of the role of Es in the pubertal timing has been hampered, so far by the lack of sensitive measurement method. ***The last aim*** was to investigate prepubertal urinary E excretion level [using a recently developed isotopic dilution/gas chromatography-mass spectrometry (ID/GC-MS) method] and its association with pubertal markers.

Outline

The thesis begins with a Theoretical Background (**Chapter 2**). Firstly, the biosynthesis, metabolism, assessment and biological role of major steroid hormones during growth are introduced. Secondly, the current knowledge about the potential influences of nutritional status, hypothalamic and peripheral signals for the pubertal development, especially regarding the role of steroid hormones, is summarized. Based on this overview, several research questions are formulated (**Chapter 3**).

A General Methodology Section (**Chapter 4**) describes the DONALD Study as well as methodological considerations relevant to all, or the majority, of the analyses. This Chapter also includes a study as preliminary methodological work to examine the urine volume or water load dependency of AA and GC metabolites in healthy children (Chapter 4.8). The background for this is that observational and experimental studies have suggested that urine volume should be considered as a confounder when using urinary free cortisol (UFF) and cortisone (UFE) to assess GC status. However, to date the potential influence of water load on urinary excretion rates of other steroid hormone metabolites has never been investigated.

The major research questions will be addressed in a series of analyses of DONALD sub-samples which are referred to as Studies I-IV. They are presented in **Chapter 5** and comprise study-specific Introduction, Methods, Results, and Discussion Sections. A General Discussion (**Chapter 6**) brings the study results into a broader context and provides also overall conclusions and ideas for future research.

2. Theoretical Background

2.1 Adrenocortical hormones

There are three major groups of hormones produced by the adrenal cortex: mineralocorticoids, GCs, and sex steroids. GCs, primarily produced by the zona fasciculata (ZF), have an important role in metabolism of carbohydrate, lipid, and protein and multiple effects on immune, circulatory, skeletal, renal, and central nervous system. Mineralocorticoids, normally solely produced by the zona glomerulosa (ZG) are essential for the maintenance of blood volume and sodium balance (17). The major sex steroids, produced primarily in the zona reticularis (ZR), are AAs, which contribute to appearance of auxiliary and pubic hair and a transient physical maturation of children (18).

2.1.1 Biosynthesis

The precursor of steroid hormones is cholesterol, which has a 17-carbon steroid nucleus. The cells of the steroidogenic tissues can de novo synthesize cholesterol from acetate, but most of its supply comes from cholesterol derived from the plasma low density lipoproteins, which are influenced by dietary fats including dietary cholesterol. Cholesterol is converted to steroid hormones by steroidogenic enzymes in the mitochondria and smooth endoplasmic reticulum. Most steroidogenic enzymes are members of the cytochrome P450 group of oxidases, which are formally referred to with a common nomenclature consisting of “CYP” followed by a unique designator (19, 20). The hydroxysteroid dehydrogenases (HSDs) are another group of enzymes involved in the steroidogenic reactions. The zone specific steroid biosynthesis pathways of the adrenal gland are shown in the **Figure 1**.

Adrenal androgens

Steroidogenic cells deliver large amounts of cholesterol to the cholesterol side–chain cleavage enzyme, termed P450scc, which catalyzes the first and rate-limiting step in steroidogenesis, converting cholesterol to pregnenolone. Once pregnenolone is produced from cholesterol, it may undergo 17α-hydroxylation by P450c17 to yield 17α-hydroxypregnenolone, or it may be converted by 3β-hydroxysteroid dehydrogenase (3βHSD) to progesterone - the first biologically important steroid. 17α-hydroxypregnenolone can be converted to 17α-hydroxyprogestrone by 3βHSD or to a C19 steroid, dehydroepiandrosterone (DHEA) by 17,20-lyase, the second isoform of P450c17. DHEA can also be converted by DHEA-sulphotransferase to DHEA-sulfate (DHEA-S) or by 3βHSD to androstenedione (21).

Glucocorticoids and Mineralocorticoids

Because of the existence of 21-hydroxylase (CYP21) in the ZG and ZF, progesterone or 17α-hydroxyprogesterone can be hydroxylated at the 21-position, producing 11-deoxycorticosterone and 11-deoxycortisol, respectively. In the next step, the two isoforms of 11β-hydroxylase (CYP11B1 and B2), catalyze the conversion of 11-deoxycorticosterone and 11-deoxycortisol to the GCs, corticosterone and cortisol respectively. The biosynthetic pathway of the major mineralocorticoids, aldosterone initially parallels that of cortisol, except that 17-hydroxylation does not occur because of the absence of P450c17 in the ZG. On the other hand, only the ZG has the ability to catalyze the 18-oxidation of corticosterone to aldosterone, the major Mineralocorticoid (21)

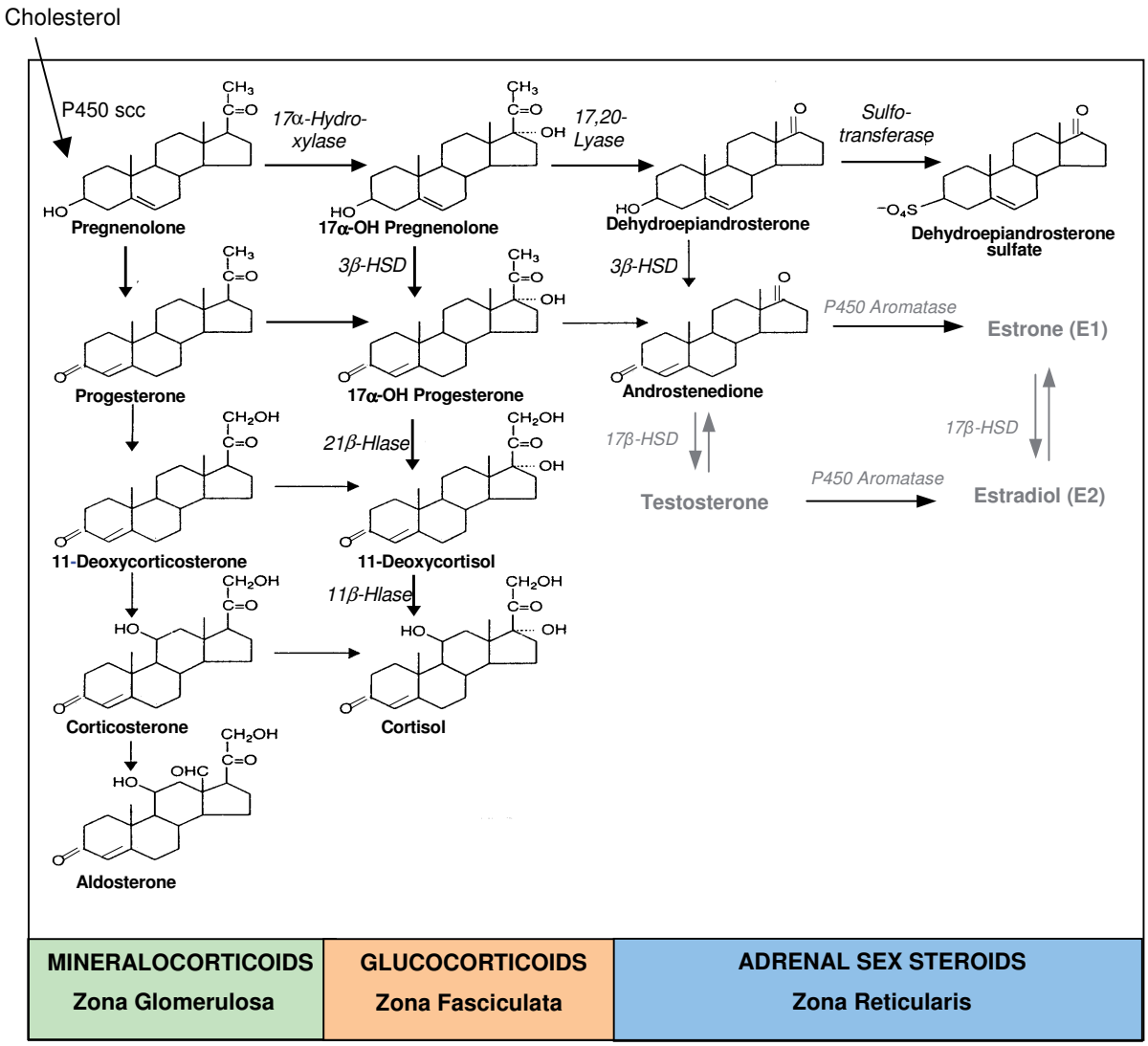

Figure 1. Major steroid biosynthesis pathways

Mineralocorticoids, glucocorticoids, adrenal steroids are produced by the zona glomerulosa, fasciculata, and reticularis respectively. The major enzymes, which catalyze the steroidogenic reactions, are side-chain cleavage enzyme desmolase (P450scc), CYP17 (17α-Hydroxylase and17, 20-lyase), 3β-hydroxysteroid dehydrogenase (3βHSD), 21-Hydroxylase (21β-Hlase), and 11β-hydroxylase (11β-Hlase).

2.1.2 Development during growth

During childhood, the adrenal cortex changes in size, cell distribution, and function. This is impressively reflected by the progressively increase of AA production occurring several years before the onset of puberty (2, 3). As opposed to AAs, the production of mineralocorticoids and GCs does not follow such a clear age-associated pattern.

The onset of AA production from the adrenal ZR leads to the phenomenon of adrenarche, which occurs only in higher primates (22). Most researchers believe that adrenarche begins in mid-childhood at about 6-8 years (17). In contrast, two longitudinal studies – one performed in healthy children collecting 24-h urine samples at yearly intervals (3) and another in girls (with idiopathic central precocious puberty during long-term pituitary-gonadal suppression) providing blood samples at 3- to 6-month intervals (2) – suggest that adrenarche is not the result of sudden, rapid increase of AA production, but a gradual maturational process that begins in early childhood around 3 years. The AA secretion continues to increase during and after puberty and reaches maximum values in young adulthood. Thereafter follows a slow gradual decrease in AA levels in the elderly (adrenopause).

2.1.3 Abnormal adrenal androgen secretion

Premature adrenarche (PA) is defined as the appearance of pubic and/or axillary hair in the absence of breast or testicular development before the age of 8 yrs in girls and 9 yrs in boys. This condition associated with increased AA production occurs more commonly in girls than in boys with an unexplained sex ratio of nearly 10:1 (23). Hyperinsulinemia, insulin resistance, and unfavorable lipid profiles have been demonstrated in girls with PA (24, 25). Girls with PA are also at an increased risk for the development of polycystic ovarian syndrome (PCOS) (26). PCOS is characterized by hyperandrogenism and chronic anovulation with complications including hyperinsulinemia, insulin resistance, early onset of type 2 diabetes mellitus, dyslipidemia, cardiovascular disease and, infertility (27).

Congenital adrenal hyperplasia (CAH) is a group of inherited diseases caused by defective activity in enzymes that contribute to the synthesis of cortisol from cholesterol in adrenal cortex. 21-hydroxylase deficiency is the most common form of CAH. Patients with 21-hydroxylase deficiency can not adequately synthesize cortisol. To compensate this deficit of cortisol, more adrenocorticotropic hormone (ACTH) is secreted, resulting in the subsequent overproduction of AAs. The features of such patients include inappropriately rapid linear growth in childhood, early epiphyseal fusion of the long bones, short final stature, development of sexual hair, apocrine body odor, and penile or clitoral enlargement, and, infertility (28).

2.1.4 Regulation of adrenal androgen secretion

During adrenarche, the increase in AA production is closely associated with the morphological changes of adrenal zones (the increase of ZR's thickness) and is accompanied by the changes of zone-specific expression of characteristic steroidogenic enzymes, which have been suggested to be the primary cause of adrenarche (21, 29, 30).

As mentioned above AA biosynthesis requires CYP17 (17α-hydroxylase and 17,20-lyase). Activity of 3βHSD regularly acts to decrease AA production through competition with CYP17. Decreased amounts of 3βHSD (21, 29, 30) (**Figure 2A**) and increased expression of CYP17 (30) (**Figure 2B**) with increasing age in the ZR of the adrenals from children undergoing adrenarche have been demonstrated by immunohistochemistry and gene expression studies. However, the in vivo metabolic evidence for the contribution of the above steroidogenic enzymes to adrenarchal androgen increases in healthy children is missing. Especially, their combined effects on AA secretions have not yet been examined.

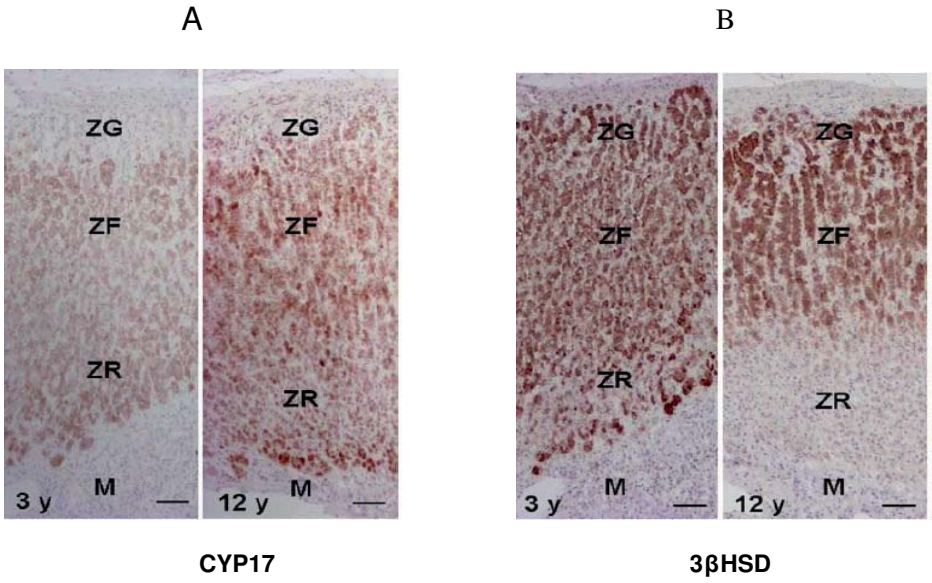

Figure 2. Immunohistochemistry for CYP17 (A) and 3βHSD (B)

(**A**) Immunoreactivity of CYP17 is weakly detected in the zona fasciculata (ZF) and zona reticularis (ZR), whereas it is not detected in the ZG or medulla (M) in the adrenal at age 3 years. Immunoreactvity of CYP17 is strongly detected in the ZF and ZR in the adrenal at 12 years. (**B**) Immunoreactivity of 3βHSD is detectable in the ZF, ZR, and ZG, whereas it is not detected in the M of the adrenal at age 3 yrs. In the 12 yrs adrenal, Immunoreactvity of 3βHSD is marked in the ZG and ZF, but not present in M, and has almost vanished in ZR (30).

The regulation of adrenarche is a complex event with involvement of many factors from multiple levels. These factors can either influence the expressions and actions of adrenal

enzymes or influence the growth of ZR (31). An unifying theory satisfactorially explaining the adrenarchal process and its regulation has not been put forward until today.

Regulation by pituitary and hypothalamus hormones

The neuroendocrine system that regulates the secretion of adrenocortical hormones is the HPA axis. Both pituitary ACTH and corticotrophin releasing hormone (CRH) (32) from hypothalamic neurons have been suggested to be able to regulate AA secretion. However, it is problematic to indentify an independent role of CRH in *in vivo* studies due to the concomitant stimulation of ACTH secretion (17). Effects of ACTH on steroid biosynthesis include promotion of the synthesis of steroidogenic enzymes and general actions on the adrenocortical synthesis of protein, RNA and DNA for the cell growth (33, 34). ACTH can also increase the availability of cholesterol to CYP11A1, resulting in an increased pregnenolone synthesis (35). However, several experiments and clinical observations have shown that while circulating concentrations of DHEA(S) rise progressively, ACTH concentration do not change significantly with increasing age (36). This indicates that although a certain level of ACTH secretion is required for AA production, adrenarche is not simply a global activation of HPA axis.

Regulation by non-hypophysische hypothalamus hormones

Obesity, increased insulin secretion, insulin resistance and hyperandrogenism are common features in women with PCOS (37). Experimentally induced weight gain (38) or weight loss (39) in adults has been shown to result in an elevated or reduced AA secretion, respectively. In addition, one longitudinal study has shown that individual changes of body mass inex (BMI) are associated with changes of urinary DHEA-S output during adrenarche (40). Accordingly, candidate hormones related to body mass especially fat mass, such as insulin (41), IGF-1 (41, 42) , and leptin (43) have been suggested to play a role in AA production or adrenarche.

Recently, a dose-dependent increase in the production of AA with increasing leptin in physiological amount (43) has been reported. Correspondingly, in a observational study, I'Allemand et al, (44) found a weak association between circulating free leptin and DHEA-S in prepubertal children. However, another study in adult women with PCOS did not show any effect of leptin on circulating sex hormone levels (45). In vitro, cell culture and immunohistochemical studies have reported the expression of insulin receptors in human adrenal tissues (46, 47) with the similar amount as the IGF-1 receptors (46). However, it is not clear whether insulin modulate AA secretion through binding with its own or IGF-1's receptors. The involvement of IGF or insulin on AA production is, at least in part, due to changes in the expression of specific steroidogenic enzymes. In line with this, physiological

levels of insulin and IGF-1 have been shown to be able to induce steroidogenic enzymes in cultured human adrenocortical cells (41, 48). Observational study in healthy girls showed that serum DHEA-S was associated with insulin resistance throughout prepuberty and puberty and with serum IGF-1 only in prepuberty (49). The result from the same group showed that in normal prepubertal boys, neither serum IGF-1 nor insulin sensitivity was associated with serum DHEA-S levels; however, changes in insulin sensitivity might be involved in AA synthesis between late prepuberty and early puberty (50).

In conclusion, *in vitro* studies and observational studies suggest that insulin and/or IGF-1 are involved in the regulation of adrenal androgen production. Further studies are called for to differentiate the role of insulin and IGF-1 and to examine whether the level of insulin and/or IGF-1 are especially critical for adrenarche in girls.

2.1.5 Dietary factors and adrenal androgen secretion

Several studies have suggested that dietary protein and high glycemic index (GI) or glycemic load (GL) exert insulin or IGF-1 stimulating effects. Clinical trial in women with PCOS using oral-glucose-tolerance tests showed that glucose loads not only increase hyperinsulinemia but also stimulate AA secretion (51). Smith et al. (52, 53) have illustrated the various interactions between glycemic load, insulin sensitivities, IGF-1 and androgens in subjects with acne. Regular consumption of foods with a high GI elevates serum insulin concentrations, which may raise androgen (including DHEA-S and testosterone) concentration and contribute to acne (54). Conversely low-GI foods have been shown to reduce androgen levels (53).

Dietary protein intake , in particular animal protein, can also induce insulin and/or IGF-1 secretion (55, 56). However, to our knowledge, no study has directly examined the association between dietary protein intake and AA secretion. Indirectly, two reports (57, 58) have found that acne (influenced by androgens) prevalence increased as populations adopt a relative fat and animal product-rich (western) diet through migration or cultural changes. In addition, low-fat, high fiber diets tend to decrease the concentration of IGF-1 (59) and androgens (60).

Therefore, dietary intakes seem to be also involved in the modulation of the individual AA production and it would be of great interest to examine which dietary factors influence AA secretion in healthy children.

2.1.6 Metabolism of adrenocortical hormones

The liver and the kidney are the principal organs involved in the clearing the steroid hormones from the circulation. Although most tissues can metabolize steroids, the liver is

the primary site of steroid hormone metabolism, and the kidney is the primary site of steroid hormone excretion (61). Hepatic metabolism accomplishes two functions: a decrease in the biological activity of the hormones; and an increase in their water solubility. Metabolites can be easier excreted in urine after they are converted to the hydrophilic form (18).

The pathways involved in metabolism of the steroids yield more than 50 different metabolic products formed by about a dozen distinct enzymatic reactions. The general reactions involved in the metabolism of steroid hormones include reduction (in ring A, C20 and C17 position), oxidation, hydroxylation, and conjugation (18). The major metabolic pathways of GCs and AAs are shown in **Figure 3** and **Figure 4,** respectively.

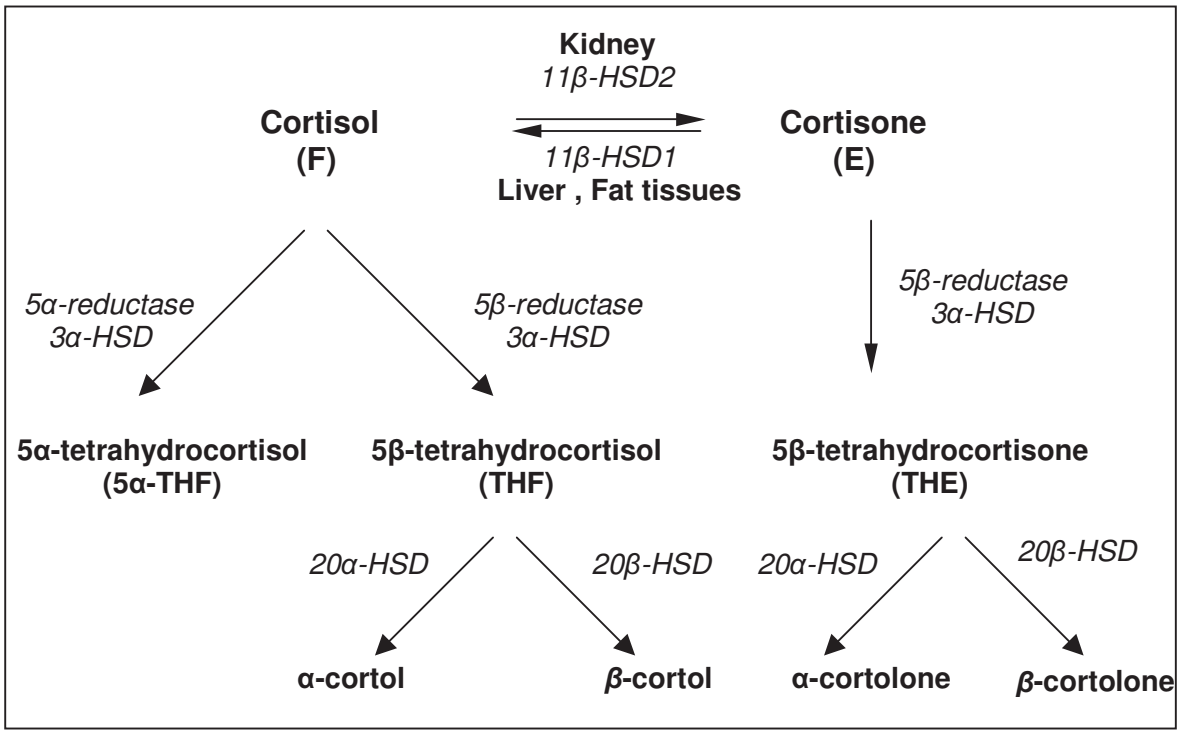

Figure 3. Metabolic pathways of glucocorticoids

Although the liver is the major site of steroid hormone metabolizing, metabolic processing of active hormones also occur in the kidney. It is well established that the intrarenal conversion of active cortisol to inactive cortisone by 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 is crucial in prevention of cortisol induced blood pressure increases by protecting the non-selective mineralocorticoids receptor (MR) from occupation by cortisol (62, 63). There is also evidence for the renal conversion of cortisol to 6β-hdydroxycortisol, which may provide an additional pathway to protect MR from occupation by cortisol and might also be relevant in blood pressure regulation (64). Similar

to cortisol, progesterone is also a strong MR agonist *in vitro* due to its high binding affinity to the MR (65). However, the anti-mineralocorticoid effect of progesterone in vivo seems to be moderate. This could be due to the fact that progesterone is effectively metabolized by enzymes in MR target tissues to its downstream metabolites with lower affinity to the MR.

In conclusion, although only a small part of the steroid hormones are metabolized in the kidney, these active metabolic processes may play important physiological roles, especially in the regulation of blood pressure.

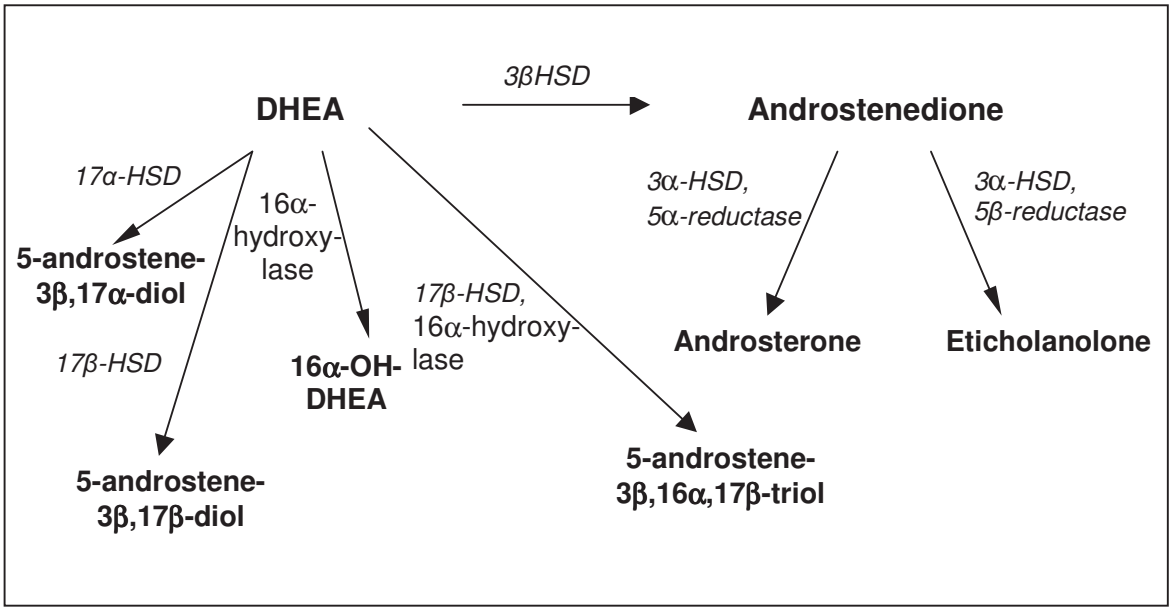

Figure 4. Metabolic pathways of adrenal androgens

2.2 Estrogens and testosterone in children

Biosynthesis

Compared to AAs, Es and testosterone exert stronger sexual hormone effects; however their productions in prepubertal stage are very low. Small fractions of prepubertal Es and testosterone are secreted by adrenal gland, ovary, and testes; most of the circulating amounts are derived from extraglandular conversion of AAs. The AAs, DHEA and androstenedione, can be converted by 17β-hydroxysteroid dehydrogenase (17βHSD) into androstenediol and testosterone, respectively. Es are produced by the aromatization of androgens by a series of reactions catalyzed by *P450aro* (aromatase), which is encoded by a single gene CYP19 (**Figure 1**). Aromatase is present in a number of extraglandular tissues, such as bone (66), brain (67), and adipose tissues (68). The the distribution of 17ßHSD in extragonadal tissues is less characterized (69).

Biological role of estrogen and testosterone

E and testosterone play key roles in the pubertal developmental and reproductive functions. E is well acknowledged for its essential role in the dramatic changes occurring throughout female puberty, such as breast development and establishment of fertility (70). In addition, estradiol (E2), the most potent estrogenic steroid, has been shown to be essential for the normal pubertal growth and epiphyseal maturation in both sexes (71-73). T on the other hand appears to play a relatively diminutive role in the pubertal growth spurt (74) and is primarily responsive for the development of secondary sexual characteristics of males.

Metabolizing of estrogens

Usually only E2, sometimes also together with estrone (E1), is measured in blood samples. E2 is the most potent estrogenic hormone, whereas E1 is less active, however it can be transported in serum and, in a wide range of extraglandular tissues, converted to the more potent E2 by the enzyme 17βHSD (75). E1 and E2 can be hydroxylated at position C-2 and C-4 to form catechol estrogens. Another alternative metabolic pathway of E1 and E2 is hydroxylation at position C-16, whereby 16α- or 16β hydroxylated E1 can be generated, as well as estriol (E3) and 16-epiestriol (16-epiE3) (**Figure 5**). After hydroxylation, estrogens can be converted to methyl ethers, glucuronides and/or sulfates (76). 16α-hydroxlyated (77) and 4-hydroylated estrogens (78) have been suggested to be linked to carcinogenesis, especially breast cancer.

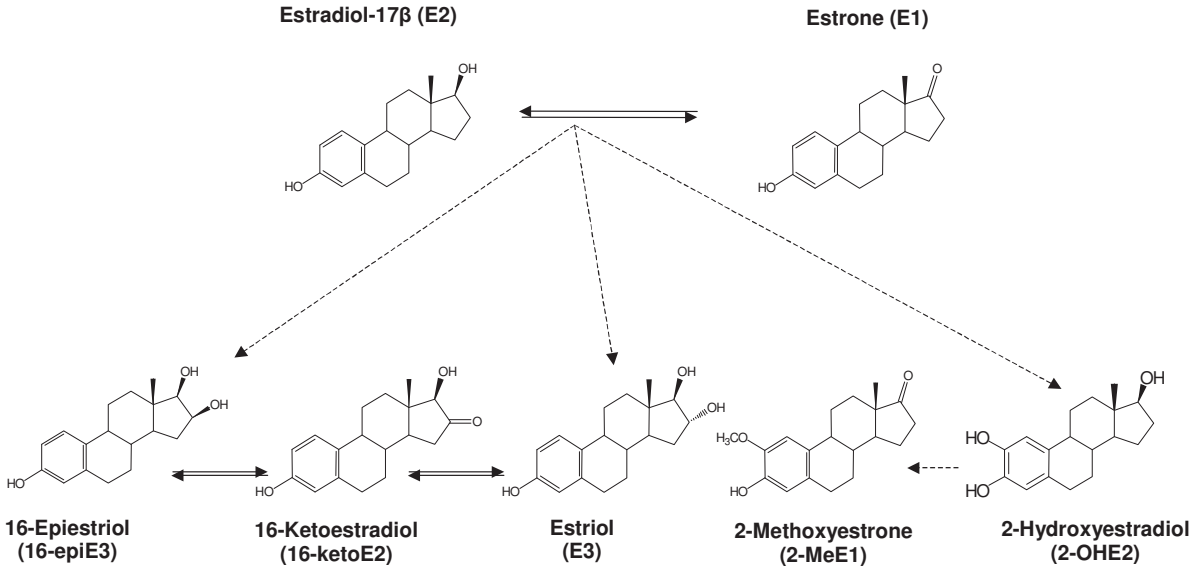

Figure 5. Metabolic pathways of estrogens

2.3 Methodological considerations – measurement and assessment of steroid hormones

In the present thesis, all of the analyses regarding steroid hormones were based on urine data, because most of the metabolized products of steroid hormones can be measured in the urine and there are several advantages using 24-h urine samples compared with using blood samples. Firstly, measurements of 24-h urinary steroid metabolites can provide better estimates of hormone secretion rates than a single measurement of plasma levels. They integrate the produced metabolites over a 24-h period and are neither affected by short-term fluctuations and sudden secretion bursts nor by varying plasma protein binding capacities. In addition, some physiological and pathophysiological relevant steroidogenic enzyme activities can be estimated from specific urine metabolite ratios. Finally, the non-invasive character of urine measurements is suitable for studies carried out in healthy population especially children (79).

2.3.1 Assessment of urinary glucocorticoids and adrenal androgens

The method for the assessment of GC and AA secretion rates through measuring steroid profiles in 24-h urine samples using GC-MS has been established. The quantitatively most important urinary GC metabolic products are tetrahydrocortisone (THE), tetrahydrocortisol (THF), 5α-tetrahydrocortisol (5α-THF), α-cortolone, β-cortolone, α-cortol, and β-cortol, which are all C21-steroids. The sum of these 7 metabolites (∑C21) is usually employed to assess overall daily adrenal cortisol secretion (80) (Figure 3). The major AA metabolites include androsterone, etiocholanolone, 5-androstene-3β,17α-diol, 5-androstene-3β,17β-diol (androstenediol), DHEA, 16α-hydroxy-DHEA, and 5-androstene-3β,16α,17β-triol, which are all C19-steroids. The sum of these 7 metabolites (∑C19) is used to assess overall daily AA secretion (3) (Figure 4).

Because of methodological limitations, profiles of important endogenous urinary Es in prepubertal 24-h urine samples have not yet been explicitly examined. A reasonable marker for the assessment of E production rates in children is lacking.

2.3.2 Measurement challenge of estrogen determination in prepubertal children

Few evidence for growth promoting properties of low circulating concentration of estrogens during childhood also exists (81). However, further elucidation of the biological significance of prepubertal Es has been hampered by the fact that conventional immunoassays are not sensitive enough to assess low levels of Es. Despite continuous improvement of immunoassays, e.g. recombinant cell bioassays (82), a certain degree of

cross-reactivity cannot be eliminated. The recent development of more sensitive and specific techniques such as ID/GC-MS (83) and Liquid chromatography-tandem mass spectrometry assay (84) are important steps forward in the struggle toward more sensitive and valid estimation of true endogenous sex steroid levels in prepubertal children. To date, in children these techniques have been only used to measure sex steroid hormone levels in serum. However, the urinary excretion process leads to a aggregating effect rendering the determination of metabolites with low serum concentrations or high metabolic clearance possible. Therefore, it would be very challenging to apply these techniques after sophistically modified work up for simultaneous determination “profiling” of important endogenous urinary Es in prepubertal 24-h urine samples.

In order to correctly assess and interpret the urinary metabolite data, only a precise measurement method is not enough. Factors that can confound the assessment of urinary excretion rate of steroid hormone metabolites must be considered. Since the alterations of the excretion rates of one steroid hormone or its metabolites are not only dependent on the production of them, but also on their renal handling, it has been considered helpful to learn about the possible renal transport mechanisms of them**.**

2.3.2 Renal handling of steroid hormone metabolites

The major role of the kidney is the elimination of hepatic metabolites. Free steroids that are filtered by the kidney are largely reabsorbed; therefore their urinary excretions are low and composed of only about 1% of urinary steroid metabolites. Until now there is no evidence for a specific transport system for free steroid hormones. The reabsorption and excretion of them may most probably base on solute-solvent coupling effect, i.e. passive diffusion (85) .

Over 90% of steroid metabolites are excreted as the sulfate or glucuronide conjugates, which are more polar and more readily excreted in the urine. Since the available pool of sulfate is rather limited and rapidly depleted, the sulfate conjugates of steroid hormones constitute of a clearly smaller fraction of conjugated metabolites. The transport of steroid conjugates is mostly mediated by carrier. Several new isoforms of organic anion transporters (OATs) and organic anion transporting polypeptides (OATPs) have been reported to be able to mediate the transepithelial transport of estrogen sulfate (86), DHEA-S (86) and estradiol 17β-D-glucuronide (87) at the apical membrane of the proximal (86) and/or distal tubule (88). These results suggest an important role of OATs in the excretion and/or reabsorption of steroid conjugates in the kidney.

As shown on a theoretical basis by Walser (89), the passive or even active reabsorption of certain substances can be influenced by urine flow. Although a large fraction of the water and the resolved substances are reabsorbed in the proximal tubule, the fine

adjustment of the reabsorption and excretion of them happens in the distal part of the kidney. The epithelial permeability of the collecting duct and distal tubule has been shown to be associated with the degree of flow-dependent excretion of solute (90). The differences of epithelial permeability can be attributed, at least in part, to differences in lipid solubility, if the transport of the substance (e.g. free steroid hormones) is by passive diffusion. If the transport of the substances must be mediated by transporters, the binding affinity to the respective transporters may play a relevant role regarding the epithelial permeability.

Experimental studies (91, 92) and observational studies (93) suggest that UFF and UFE are dependent on urine volume after water loading in healthy adults (91) and children (93). Until now, the potential influence of water load on urinary excretion rates of other steroid hormone metabolites has not yet been investigated. For a reasonable evaluation of the physiological role of them, a preliminary methodological work is needed to elucidate this aspect. If a clear water load effect on urinary excretion rates of major steroid hormone metabolites is identified, urine volume should be considered as a confounder in the statistical analysis.

2.4 Puberty

Entering puberty is one of the most important milestones in life. It not only represents the step into maturity, but also involves important body and physiological changes. A clear definition of pubertal onset is the prerequisite for the subsequent research concerning the exploration of mechanisms.

2.4.1 Definition of pubertal timing

The pubertal growth spurt and the appearance of secondary sex characteristics are the most visible manifestations of puberty (94).

Definition according to adolescent growth spurt

The pattern of postnatal growth is well documented: a high growth rate is observed immediately after birth, with rapid deceleration until 3 years of age; after that the velocity is slowly decreased to the nadir termed the *minimal prespurt (take-off) velocity* just before the pubertal growth spurt (95). Typically, pubertal growth consists of a phase of acceleration, followed by a phase of deceleration, and eventual cessation of growth with the closure of epiphyseal. The growth process is controlled by the endocrine system. It is generally accepted that growth hormone (GH) is one of the components responsible for growth during childhood, given a normal thyroid hormone secretion. Growth during adolescence is related both to GH and sex steroids (94). Due to the lack of sensitive sex steroid

measurement methods, there is not enough evidence concerning the role of sex steroids in prepubertal growth and the subsequent earlier onset of growth spurt.

Definition according to secondary sexual characteristics

Secondary sexual characteristics are commonly chosen to describe the timing or stages of pubertal maturation. In girls, the first external sign of sexual maturation is either the appearance of pubic hair or the initiation of breast development (96). Internal markers such as the increase of uterine volume, a visible layer of uterine mucosa, and the increase of ovary cysts can be detected by ultrasonography (95). Menarche is usually a late event in the sequence of pubertal changes. In boys, pubertal development begins with an increase of testicular size. Over the further course of puberty, penis size increases, pubic hair appears, and the voice breaks. The detection of sperm in the first morning urine specimen is a phenomenon that probably reflects the onset of spermatogenesis, named spermarche (97). Breast, genital, and pubic hair development have been assessed using a progressive scale developed by Tanner and Marshall (96, 98), which have been widely used throughout the world. Tanner staging is based on a scale of 5 progressive stages described and depicted in photographs in which stage 1 is prepubertal, stage 2 is onset, and stage 5 reflects adulthood. The age at which such stages are reached comes in a sequential fashion, but there is still large individual variation. As an example, the time sequences of different pubertal markers according to the First Zurich Longitudinal Study (1954-1980) (99, 100) are presented in **Figure 6** for both boys (**A**) and girls (**B**).

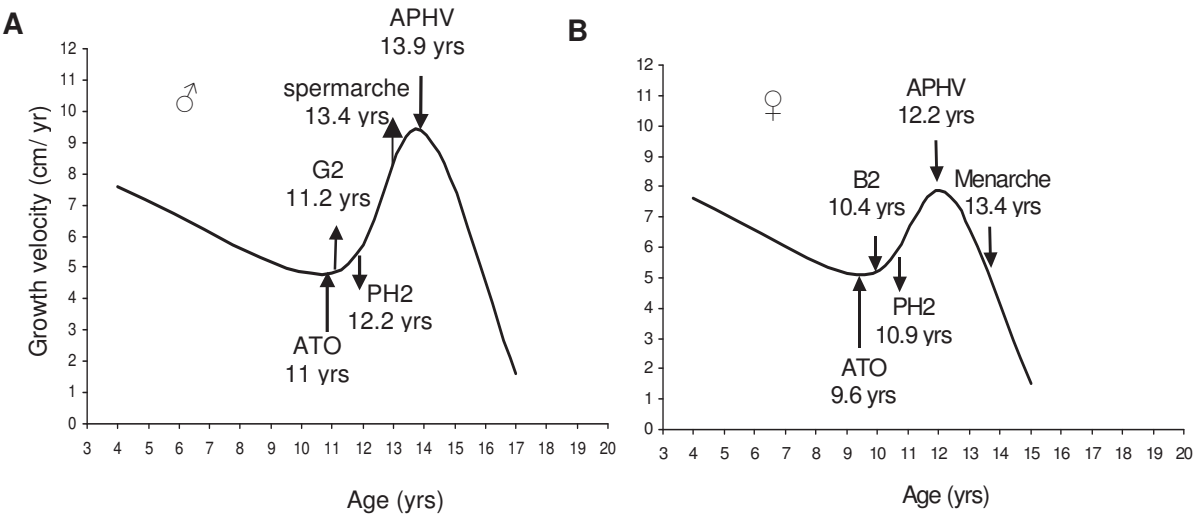

Figure 6. Growth velocity curves of a typical male (A) and a female (B) with commonly used early and late pubertal markers (data based on the First Zurich Longitudinal Study (99, 100)

The timing of puberty is highly variable and determined by multiple factors. Investigating the association of environmental factors and childhood hormone exposures with pubertal physical changes may provide some clue to the understanding of the mechanisms of pubertal onset modulation. A schema of the potential environmental, peripheral, and hypothalamic signals for the regulation of pubertal timing are summarized and illustrated in Figure 7.

2.4.2 Hypothalamic signals and pubertal onset

Although various determinants of the pubertal process and their network-like interrelations are yet to be identified and specified, it is clear that the amplification of pulsatile GnRH secretion in the central nervous system (CNS) is one of the key elements required for puberty onset (101, 102). The precise mechanisms leading to disinhibition of GnRH production, and the subsequent gonadotropin secretion, remain unknown. Several mechanisms have been suggested: i) During childhood, the GnRH neurosecretory neurons ("gonadostat") of the nucleus arcuatus are exquisitely sensitive to the suppressive effect of small amount of circulating sex steroids. Coincident with the onset of puberty, the "gonadostat" becomes progressively less sensitive to the inhibitory effects of sex steroids upon gonadotropin –releasing hormone (GnRH) release, which results in the increased release of GnRH in a pulsatile pattern (1). Apart from this gonadal steroid-dependent negative-feedback mechanism, "intrinsic" CNS control

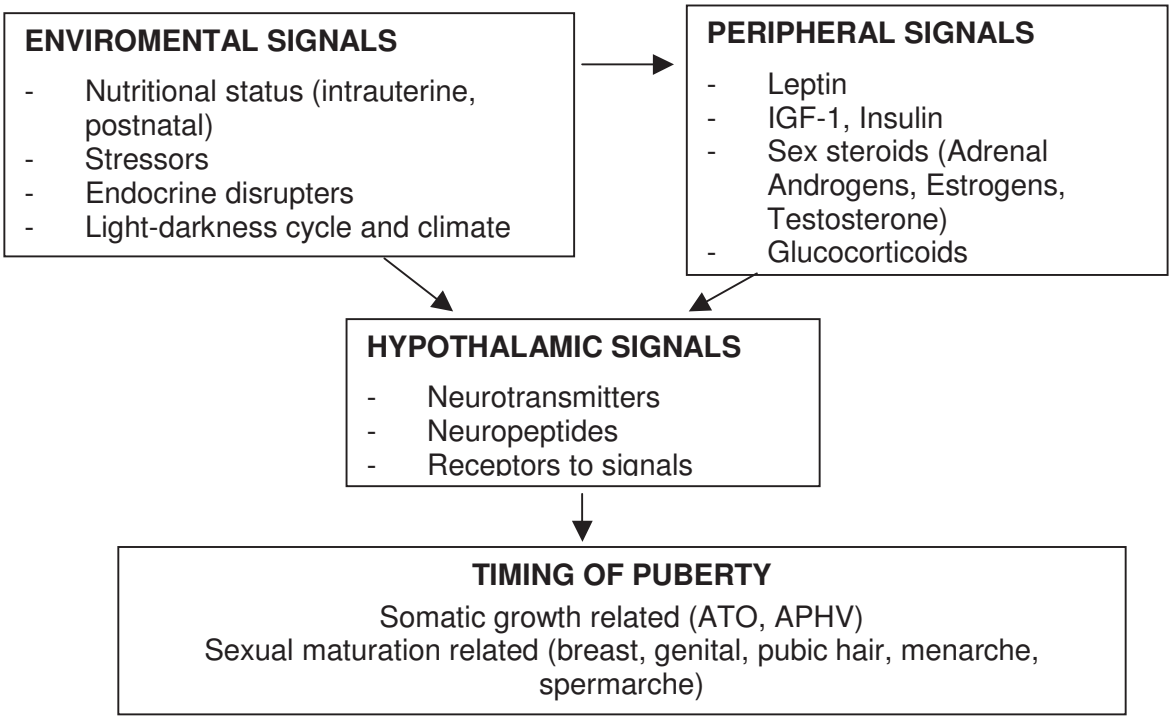

Figure 7. Schema of the potential environmental, peripheral, and hypothalamic signals for the regulation of pubertal timing

mechanisms have also been discussed. ii) GnRH neurons can be regulated by both stimulatory and inhibitory signals, which are released by different neurons in hypothalamus. For examples, Kisspeptin is released as stimulatory (103, 104) and gamma-aminobutyric acid (GABA) (105) as inhibitory neurotransmitters. The attenuation of GABA or amplifying of kisspeptin signal principally triggers the pubertal resurgence of pulsatile GnRH release. iii) Cell culture studies suggested that molecules of astrocyte origin like epidermal growth factor (EGF) (106) and transforming growth factor (TGF) -β (107, 108) may also influence GnRH release.

2.4.3 Nutritional status and pubertal onset

The currently identified and intensively discussed environmental factors are mainly about prenatal, postnatal and childhood nutritional status. Birth weight and rapid growth or weight gain after birth reflect partly the prenatal (intrauterine) and postnatal nutritional status. Some studies found that children who had a low birth weight or were small for gestational age were more likely to have advanced timing of puberty (109, 110); however, other results are controversial (111). A recent study (112) demonstrated a possible additive effect a 'low' birth weight and rapidly weight gain between birth and 24 month on earlier onset of pubertal growth spurt (age at take-off, ATO), age at peak height velocity (APHV) and age at menarche. Although the variation of body fat or size throughout childhood is partly genetically determined, it is a surrogate indicator of children's nutritional status. The relationship of body fat and/or BMI with pubertal timing has been observed in several studies and is reviewed by Kaplowitz (113). Most studies have concluded that a higher BMI especially body fat mass (FM) in the prepubertal stage may predict an earlier pubertal development (113).

Apart from nutritional status reflected by anthropometrical assessment, the direct influence of dietary intakes of certain nutrients during childhood has also been suggested: higher intakes of fat (114, 115) or animal protein (115) were associated with earlier menarche, whereas higher intakes of carbohydrate (116) and fiber (117, 118) were related to later menarche. Recently, it has been shown that animal protein intake during childhood predicts not only age at menarche, but also somatic growth related pubertal markers, i.e., ATO and APHV (10). As suggested by Berkey et al. (115) a phytoestrogen-rich diet might delay puberty by interfering with endogenous steroid hormone activity (119). Regarding the general diet quality, Cheng et al. suggest that children with lower diet quality in prepuberty, as indicated by the nutrient density-based index NQI, experience their pubertal growth spurt at an earlier age, independently of body composition (120).

The link between nutritional status and physiological variations in timing of puberty can be significant but it is not particularly strong, suggesting that this relationship is indirect and

may be mediated by one or more potentially nutrition- and puberty-related hormones, such as leptin, insulin, IGF-1, GCs or sex steroid hormones. Nutritional factors may affect the availability of the circulating hormone levels or affinity of these hormones to receptors in peripheral target tissues. They can also influence the production of these hormones through interfering with GH/IGF-1 axis, HPG axis, or HPA axis. So firstly, the potential roles of some these candidate hormones (as peripheral signals) on different pubertal markers should be explored herein.

2.4.4 Peripheral signals and pubertal onset

Leptin, GH/IGF-axis, insulin

Prepubertal and peripubertal changes in circulation endogenous hormones which are related to nutrition and nutritional status, such as leptin, insulin, and IGF-1, appear to be involved in the alterations of the pubertal timing. For leptin a permissive role in the acceleration of puberty onset has been demonstrated, but it is not the crucial element or "trigger" in the timing of puberty (reviewed by (121)). Appropriate IGF-1 and GH concentrations are required for a normal pace of pubertal maturation. Deprivation of IGF-1 delays the timely onset of puberty and slows the tempo of pubertal progression (reviewed by (122). The various possibly underlying mechanisms for these IGF-1 effects have been explicitly discussed by Veldhuis et al.(123). Insulin resistance in obese subjects is associated with compensatory hyperinsulinemia and decreased levels of sex-hormone-binding globulin (SHBG), resulting in increased sex steroid bioavailabity (124). Therefore, insulin could be also relevant for the regulation of timing of puberty.

Glucocorticoids

Excessive exercise, inadequate protein-calorie nutrition (either because of unavailability, CNS-related anorexia, or chronic illness), psychiatric illness such as anoreia nervosa and other forms of emotional or physical stress are associated with hypogonadotropic status (125). In stress situations, cortisol secretion can increase 10-fold (125). Therefore, the stress-associated hypogonadotropic status might be mediated by GCs. GCs can inhibit gonadotropin secretion both in the basal state and in response to gonadotropin releasing hormones, resulting in a decrease in gonadal sex steroid production. In addition, GCs, in excess, inhibit linear growth and skeletal maturation in children (15, 126). Most of the previous findings regarding relationships between cortisol secretary activity and pubertal linear growth and sexual maturation were based on experimental studies in animals or clinical studies in subjects with excessive emotional or physical stress; only a small number of studies have been undertaken in healthy children and adolescents. These studies can only answer the questions, whether cortisol levels are cross-sectionally associated with

growth rate (127) and pubertal stage (128, 129) or menarche status (130, 131). Due to the methodological inconsistencies between theses studies, the results are also controversial.

Sex steroids

Sex steroids, especially Es (132), as peripheral signals have been suggested to interact with the neurobiological machinery that controls pulsatile release of GnRH and may be thereby involved in the onset of puberty (133). However, before central puberty or the advent of increased GnRH, the amount of Es of gonadal origin is very small and the variation of Es are mostly attributable to the peripheral conversion from its precursor AAs. Therefore, the increase of AA secretion (adrenarche) before any discernible physical sign of puberty has been proposed to be a harbinger or promoter of pubertal onset (12, 134-137). Until now, most of the conclusions are based on studies of pathological states, such as PA (138, 139), CAH (140), precocious puberty (141), and various forms of delayed puberty (142). These studies do not show a clear and consistent link between adrenarche and puberty onset, therefore most of the experts are of the opinion that gonadarche occurs independently of increase of AAs during adrenarche. However, it seems quite reasonable that in a relevant number of normal individuals, AAs, along with other factors, may contribute to the onset of breast and genital development either directly by interacting with hypothalamic neurotransmitters or indirectly as important substrates for E production.

Interim conclusion

The evidence for the physiological role of prepubertal steroid hormones (AAs, Es, and GCs) in modulating the onset of different pubertal events is still limited. Firstly, although the role of AA increases (adrenarche) in pubic hair development, i.e. pubarche (143-145) has been established, the information on the strength of the temporal acceleration of pubarche by a stronger versus a less pronounced adrenarche in healthy children is lacking. Secondly, in children with PA, gonadarche can occur at a normal rate or earlier than average population data. However, the physiological link between adrenarche and gonadarche is still not clear. Thirdly, although Es (146-148), AAs and GCs (15, 126) show associations with skeletal development and maturation in adolescents (146-148), their roles in childhood for prepubertal growth and the subsequent onset and progression of pubertal events have not yet been extensively examined. Finally, previous studies on the role of perinatal and childhood nutritional status (including birth weight, rapid weight gain, body composition and dietary intakes) in pubertal timing have been mostly performed without considering sex steroid hormone status. Therefore, it is not clear whether the observed effects of certain nutritional factors on pubertal timing may be directly mediated by sex steroids.

3. Research questions

As summarized in **Interim conclusion in chapter 2.4.4**, several open questions remain. To address some of these issues, the following five research questions have been formulated for this thesis:

Research question 1 – Nutritional status and adrenarche

As explained in Chapters 2.1.4, 2.1.5, and 2.4.2, prenatal and childhood nutritional status, which are potentially associated with pubertal timing, in the form of low birth weight, childhood obesity and typical "Western diet pattern" (high animal protein and high fat intakes), may also be relevant for adrenarche, an important maturation event before the pubertal onset. Therefore before the relationship between adrenarche and pubertal timing is examined, it has been tried to identify ***which nutritional factors are relevant for the regulation of adrenarche or adrenarchal androgen production?***

Research question 2 – Adrenarche and pubertal timing

We aimed to investigate the influence of Adrenarche (indicated by AA production before pubertal growth spurt) on early and late pubertal markers in healthy children. Concrete queries are:

1) ***How strong is the adrenarche effect on timing of pubarche?***
2) ***Dose a stronger adrenarche favor an earlier begin and/or rapid progression of pubertal growth spurt?***
3) ***Is the variation of adrenarche with the variation of gonadarche in physiological range associated?***

Research question 3 – Animal protein intake and pubertal timing

Most of the previous studies about the influence of dietary factors on pubertal timing concentrated on menarche status, which occurs in a later pubertal stage and only in girls. Two important early pubertal markers - gonadarche and pubarche – have not yet been explicitly examined. A newly published study from our group showed that only higher animal protein (among all macronutrients) intake, may be relevant for the earlier onset of pubertal growth spurt. In this thesis, we sought to answer the questions:

1) ***Is animal protein intake also relevant for gonadarche and pubarche?***
2) ***Is the potential effect of animal protein intake mediated by AAs.***

Research question 4 – "Stress" and pubertal timing

Delayed pubertal development has been observed in subjects with excessive emotional or physical stress. Our question is: ***Does the increased prepubertal GC levels (as marker of higher stress activity) in the physiological range predict a later onset of pubertal development?***

Research question 5 – Estrogens and pubertal timing

Research on the biological role of prepubertal estrogens in pubertal timing has been hampered by the lack of sensitive measurement methods. We have applied the recently developed ID/GC-MS to measure urinary estrogen metabolites in prepubertal 24-h urine samples and have tried to characterize the ***prepubertal estrogen production levels of healthy children***. Based on these data, we further investigate the question: ***Are prepubertal 24-h E productions associated with somatic growth-related and/or sexual maturation-related pubertal markers?***

This thesis aimed to address these questions using data from the comprehensive DONALD Study which provides repeated assessments of dietary intake, growth and metabolism in healthy, free-living children from birth until young adulthood (see Chapter 4.1). Four studies have been carried out and will be presented subsequently (Chapter 5.1 to Chapter 5.4). The research questions 2 and 3 were addressed in study II and questions 1, 4, and 5 in study I, III, and IV respectively.

4. General Methodology

In order to assess the role of prepubertal steroid hormone exposures on the timing of puberty, an accurate and well designed longitudinal study in healthy free-living children with concomitant measurements of anthropometry, dietary intakes and hormone status is necessary. The DONALD Study (the central research project at the Research Institute of Child Nutrition) supplied us a good platform for answering the research questions listed in Chapter 3.

4.1 Study population and design

4.1.1 Population and design of the DONALD Study

The DONALD Study is a longitudinal, open cohort study that was started in 1985 in the area of Dortmund, Germany, and it is still ongoing (149). The study's purpose is to examine the relations between diet, metabolism, growth and development in free-living children. To achieve this, an average of 40-50 healthy infants from Dortmund and surroundings are newly recruited each year and first examined at the age of 3 months. Their parents must be prepared to participate in a longitudinal study and be eligible for participation, provided that at least one of the parents has sufficient knowledge of the German language. The DONALD Study is entirely observational and non-invasive until the participants are 18 years old. It was approved by the Ethics Committee of the University of Bonn, and all assessments are performed with written consent of the parents or the participants themselves, depending on their age.

From the first examination onwards, detailed data on nutrition, growth, metabolism and health status are collected at regular intervals between infancy and young adulthood, i.e. up to three further visits in the first year of life (at 6, 9 and 12 months), two in the second (at 18 and 24 months), and one per year thereafter. Depending on age, each visit involves a 3-day weighed dietary record, a 24-h urine sample, anthropometric measurements, a medical examination, and a comprehensive interview on socio-demographic, lifestyle, and health issues. From the age of 18 years onwards, fasting blood samples are taken. This invasive component has been introduced since January 2005. Furthermore, parental information including weight and height is repeatedly assessed. The design of the DONALD Study is summarized in **Figure 8**.

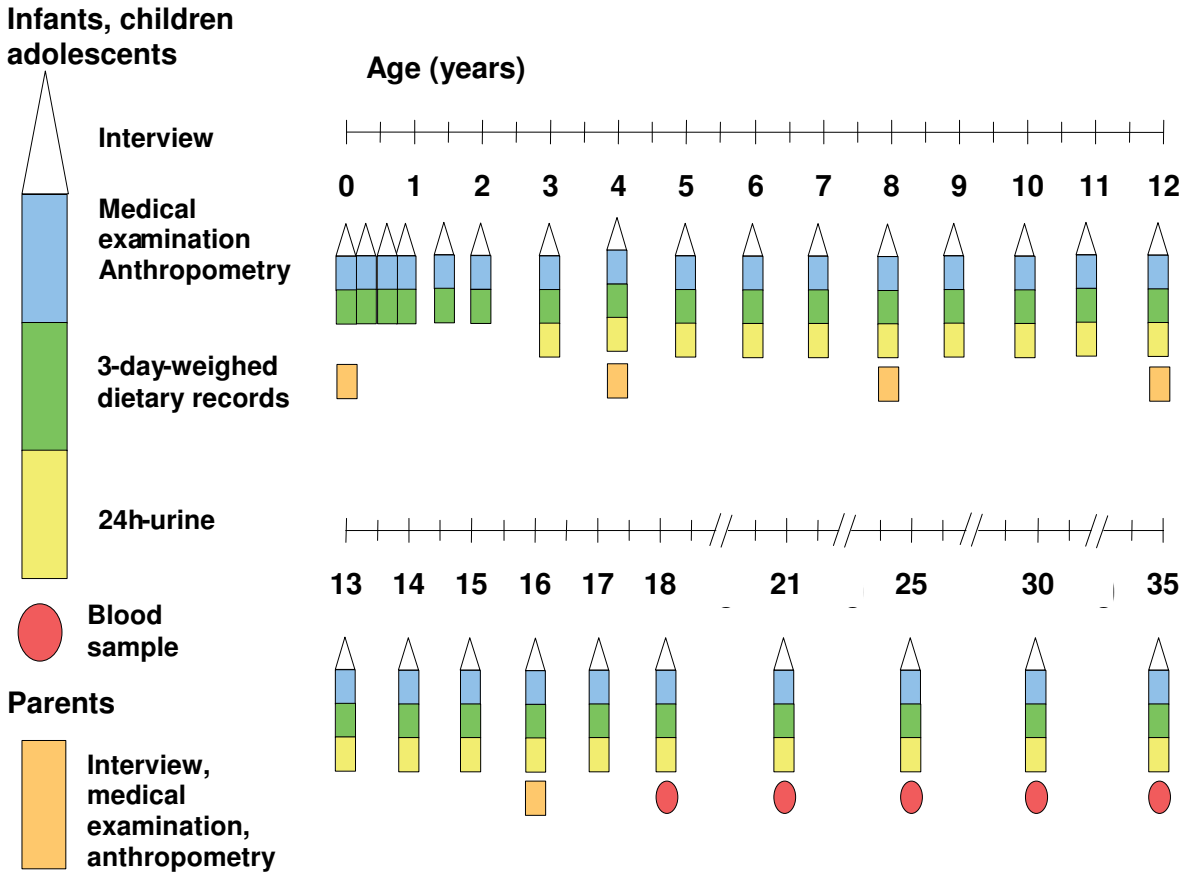

Figure 8. Design of the DONALD Study [modified from (150)].

4.1.2 DONALD subpopulations of the current thesis

The sample size for the analysis of each research question was essentially determined by the DONALD Study design. More than 1,200 children have participated in the DONALD Study so far, but when the study was started in 1985, the ages at the initial visit were quite variable. Besides, many children have not yet left the toddler years. In this thesis, eligible participants of each study had to provide information on the respective influencing and outcome variables, as well as important confounders, which depended on the purpose of the analysis.

The population of Study I (n=137) was the subsample of 400 children, who were initially selected for the establishment of reference values for urinary markers of adrenarche (3). The sample of **Study II** (n=109), **III** (n=110), **and IV** (n=120) were derived from another subpopulation (n=132) of the DONALD Study, in whom plausible data of pubertal variables i.e. ATO, APHV, and Tanner stages as well as 24-h urine samples at both time points (i.e. 2 yrs and 1 yr) before ATO were available. Depending on the covariates or confounding

variables included in the respective analyses, sample size varied (for details see Chapters 5.2.3, 5.3.3.and 5.4.3).

4.2 Dietary assessment

In the DONALD Study, dietary intake is assessed by 3-day weighed dietary records. Parents are asked to weigh all foods and beverages consumed by their children, including leftovers, to the nearest 1 g over three consecutive days with the help of regularly calibrated electronic food scales (initially Soehnle Digita 8000, Leifheit SG, Nassau, Germany; now WEDO digi 2000, Werner Dorsch GmbH, Muenster/Dieburg, Germany). Parents are instructed by trained dieticians, and semi-quantitative recording (e.g. number of teaspoons, tablespoons, cups, and pieces) is allowed when exact weighing is not possible, e.g. foods eaten away from home or snacks. Information on recipes or the types and brands of food items is also requested, and packaging or labels are collected when convenience foods are consumed. At the end of the 3-day period, a dietician visits the participants at home to collect the completed records. During this visit the dietician checks the plausibility of the record using a structured questionnaire and enquires about unusual events which might have affected eating behaviour during the data collection period. The dietary records are analysed using the continuously updated in-house nutrient database LEBTAB (151). It includes any food item ever recorded in DONALD and is based on information from standard nutrient tables, product labels or recipe simulation based on the labeled ingredients and nutrients (e.g. commercial weaning foods). Currently, LEBTAB contains energy and 30 nutrients contents for more than 6,970 entries (920 basic food items, 5,830 food products, and 220 dietary supplements) (150).

For Studies I-III, only dietary records considered plausible were included. The reported energy intake was used as a surrogate measure for the total quality of the dietary records by relating it to the basal metabolic rate (BMR). BMR was estimated using the equations of Schofield (152) that include, age, sex, weight and height. Age- and sex-specific cut-offs for the ratio of energy intake to BMR were used to identify implausible records. These cut-offs were calculated for children by Sichert-Hellert et al. (153) for three recording days. They are based on age-specific coefficients of variation of energy intake and levels of light physical activity and range from 0.97 in boys and girls up to 5 years of age to 1.04 and 1.01 for boys and girls between 6 and 13 years, respectively.

4.3 Anthropometric assessment

In the DONALD Study, anthropometric measurements are performed by nurses who have been trained according to standard procedures (154), with the children dressed in underwear

only. The nurses undergo an annual quality control check in which intra- and inter-observer agreement is carefully monitored. In addition, all instruments are routinely calibrated. The number of the anthropometric measurements that are performed depends on the age of the participant. Those that were relevant for this thesis are weight, height, and skinfold thickness. **Body weight** is assessed to the nearest 100 g with an electronic scale for subjects in standing position (Seca 753 E, Seca GmbH & Co KG, Hamburg, Germany). **Standing height** is measured to the nearest 0.1 cm with a digital telescopic wall-mounted stadiometer. **Skinfold thickness** is measured on the right side of the body at the biceps, triceps, subscapular and suprailiac sites to the nearest 0.1mm using a Holtain calliper (Holtain Ltd., Crymych, UK).

For this thesis, **BMI** was calculated using the formula weight/height² (kg/m²). Body surface area (**BSA**) was calculated according to the formula of Du Bois and Du Bois (155) as follows: BSA (m²) 0.007184 x height (cm) $^{0.725}$ x weight (kg) $^{0.425}$. Equation of Slaughter et al. for prepubertal children (156) was used to estimate **percent body fat (%BF)**. Fat mass (**FM**) in kg was obtained by multiplying percent body fat by body weight. Fat free mass (**FFM**) was calculated by subtracting FM from body weight. The definition of the International Obesity Task Force (IOTF) generally formed the basis for assessing the proportion of overweight children (157), while 25 kg/m² served as the BMI cut-off for parental overweight (158).

4.4 Pubertal stage assessment

4.4.1 Somatic growth-related pubertal markers – ATO and APHV

Height data were analyzed using the parametric Preece and Baines model 1 (PB1) (159). The parameters of each child's growth curve were estimated by a nonlinear regression model (PROC NLIN in SAS). It has been previously shown that the lower limit of the age range offered to the model should not be under 2 years of age, and that the fit of the adolescent growth curve is better if the age range includes data from not more than a few years before ATO (160). PB1 was therefore fitted on various sex-specific age ranges of the height-for-age data, beginning with age 2, in order to determine the optimal range for our data. **ATO** was defined as the age at minimal height velocity (zero acceleration) at the onset of the pubertal growth spurt (161). Goodness of fit was determined by: i) graphical inspection of each child's individual growth curve, ii) by a comparison of the residual standard deviations (random error had to be smaller than the expected measurement error for height), and by considering iii) the plausibility and 4) the distribution of the pubertal parameters estimated (8). Consequently, all available measurements from age 5 onwards for girls, and from age 6 onwards for boys were used. The mean number of measurements per child was

17.7 (range 9-21) in girls and 15.7 (range 8-20) in boys. The PB1 model also produced estimates of velocity at take-off, age at peak height velocity (APHV) and peak height velocity.

4.4.2 Sexual maturation-related pubertal markers – Tanner stages and Menarche/voice break

Breast, genital, and pubic hair were examined by one of the DONALD study pediatricians once at age 3 months and 1yr, then annually from age 5 yrs onward. In addition, children and/or their parents are asked at each visit (during children's puberty) whether **menarche** (in girls) **or voice break** (in boys) has already occurred, and if so, in which month and year.

Breast (B) and pubic hair (PH) staging were determined in DONALD by visual inspection using a progressive scale according to Marshall and Tanner i.e. Tanner staging (96, 98). The details, as taken from the literatures (96, 98), are described as follows: **B1:** the prepubertal stage; includes no palpable breast tissues. Papillae may be slightly raised from the chest contour; **B2:** the breast bud stage; elevation of breast and papilla as a small mound, enlargement of areola diameter: The development of this appearance is the first indication of pubertal development of the breast; **B3:** Further enlargement of breast and areola, with no separation of their contours. **B4:** Projection of areola and papilla to form a secondary mound above the level of the breast; **B5:** Mature stage; projection of papilla only, due to recession of the areola to the general contour of the breast. The basis of **pubic hair (PH)** staging is the quantity and distribution. **PH1** is characterized by the nonsexual general body hair, which may be more concentrated in the pubic area and may be present throughout childhood. There is no long, coarse, or heavily pigmented hair. **PH2** is pigmented straight or slightly curled hair, which is noticeably longer than the fine prepubertal hair and usually appears first along the labia majora. **PH3** is a greater concentration of dark, coarse, curly hair extending onto the mons pubis. **PH4** is hair that is adult in texture and thickness but not yet distributed in the full typical adult manner. Adult pubic hair, **PH5**, is mature in quantity and type. Distribution is, at minimum, an inverse triangle. Its extension laterally is variable but is usually not toward the umbilicus. The assessment of pubic hair development in males is similar with in females, for further details see literature (98).

The male **genital development** was determined by measuring testis size and development of penis and scrotum according to Tanner staging (98). **Testis volume** was measured by using the Prader orchidometer, which is a series of models of know volume with the shape of ellipsoids (162). They are mounted on a string in order of increasing size, and each model in numbered according to its volume in mL. Since the onset of male genital development can be estimated more accurately by the increase of testis size than by penis

and scrotum development (163-165). Testis volume ≥ 4ml was used as the cut-off for the onset of male genital development as suggested in the literature (162).

4.5 Urinary measurements

4.5.1 24-h urine samples

Starting at the age of 3-4 years, 24-h urine collections are performed on the 3rd day of the 3-day weighed dietary record according to standardized procedures. Parents and children are carefully instructed on how to collect complete 24-h urine samples. The first micturition in the morning is discarded, defining the start of the-collection, which ends with the first micturition the following morning. Additionally, any omitted collection is specified. All micturitions are stored immediately in preservative-free, Extran-cleaned (Extran, MA 03; Merck Darmstadt, Germany) 1 l plastic containers at < –12 °C before they are transported to the research institute by the dietician. The exact time of each urination as well as any other additional information, is noted on a special urine collection form. At the institute, the containers are stored at ≤ –20 °C before being analyzed. After thawing and stirring, all urine samples undergo routine check using a commercial test strip (combur 9, Roche Diagnostics GmbH, Mannheim, Germany) and total 24-h urine volume is determined. Aliquots of the urine samples are then frozen for potential future analysis at –22°C. Completeness of the 24-h urines was ascertained via the criteria, daily creatinine excretion rates >0.1 mmol $kg^{-1} \cdot d^{-1}$ (166). Creatinine was measured by the Jaffé method with the use of a creatinine analyzer (Beckman-2; Beckman Instruments Inc, Fullerton, CA).

4.5.2 Short description of glucocorticoid, adrenal androgen, and estrogen measurements

UFF and UFE

UFF and UFE were measured by specific radioimmunoassays (RIA) with the use of tritiated steroids (Amersham Pharmacia Biotech, Freiburg, Germany) and specific antibodies, raised and characterized in the steroid laboratory of the department of pharmacology (University of Heidelberg, Germany), as described elsewhere (167). Before radioimmunoassay, UFF and UFE were extracted from the urine with dichloromethane and chromatographically purified by using Celite columns (Celite columns 545 AW; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Intra- and interassay coefficients of variation (CVs) were < 10% and < 13%, respectively.

Other GC and AA metabolites

Urinary steroid profiles were determined using quantitative data produced by GC-MS analysis according to the method described previously (3, 80) in Steroid Research and Mass Spectrometry Unit (Justus-Liebig-University, Giessen, Germany). In short, free and conjugated urinary steroids were extracted by solid phase extraction (Sep-Pak C18 cartridge, Waters Associates, Mil-ford, MA), and the conjugates were enzymatically hydrolyzed (type Ipowdered Helix pomatia, Sigma-Aldrich Corp., St. Louis, MO). The hydrolyzed steroids were recovered by Sep-Pak extraction. Known amounts of three internal standards (5α-androstane-3α,17α-diol, stigmasterol, and cholesteryl butyrate) were added to a portion of each extract before formation of methyloxime-trimethylsilyl ethers. GC was performed using an Optima-1 fused silica column (Macherey-Nagel, Dueren, Germany). Helium was used as carrier gas at a flow rate of 1 ml/min. The gas chromatograph (Agilent 6890 series gas chromatography, Agilent 7683 Series Injector, Agilent Technologies, Waldbronn, Germany) was directly interfaced to a mass selective detector (Agilent 5973N MSD, Agilent Technologies) operated in selected ion monitoring mode. Calibration of the GC-MS was achieved by analysis of a reference mixture containing known amounts of all separation compounds. The injections took place with an 80 C (2min) GC oven; the temperature was then increased by 20 C/min to 190 C (1 min). For separation of steroids, it was increased by 2.5 C/min to 272 C. After calibration, values for the excretion of individual steroids were determined by measuring the selected ion peak areas against the internal standards.

Estrogen metabolites

Urinary Es were determined by using ID/GC-MS (168), which is based on benchtop GC-MS. The recently modified and validated method (by Steroid Research and Mass Spectrometry Unit, Justus-Liebig-University, Giessen, Germany) consisted of equilibration of urine (5 ml) with a cocktail containing stable isotope labeled analogs of the analytes as internal standards ([2,4-$^2H_2$] E1, [2,4,16,16-$^2H_4$] E2, [2,4,17-$^2H_3$] E3, [1,4,16,16-$^2H_4$] 2-MeOE$_1$, [1,4,16,16,17-$^2H_5$]2-OHE$_2$, [2,4,15,15,17-$^2H_5$]16-OE2 and [2,4-$^2H_2$]16-epiE3). Then, solid phase extraction (C_{18} cartridges), enzymatic hydrolysis (Sulfatase from Helix pomatia Type H-1), re-extraction, purification by anion exchange chromatography and derivatisation to trimethylsilyl ethers followed. The samples were analyzed by GC-MS (Agilent GC 6890N / 5975MSD; fused silica capillary column 25 m x 0.2 mm i.d., film 0.10 µm). Calibration plots were linear and showed excellent reproducibility with coefficients of determination (r^2) between 0.999 and 1.000. Intra-assay CV ranged between 1.00% for 16-epiE3 and 1.92% for E1. The inter-assay CVs lay between 0.65% for T and 2.21% for E3. Sensitivity was highest for 2-OHE2 (0.25 pg per absolute injection: Signal to noise ratio (S/N) = 3) and lowest for 16-epiE3 (2 pg per absolute injection: S/N = 2.6). Accuracy – determined in a two level spike experiment – showed

relative errors ranging between 0.15% for 16-OE2 and 11.63% for 2-OHE2. Chromatography showed clear peak shapes for the components analyzed.

4.5.3 Influence of storage duration

As a preparatory work, we checked, whether the frequently examined C19 steroids as quantified by GC-MS in 24-h urine samples might be relevantly affected by the duration of storage of the urine samples at –20 °C. For this, a total of 90 urine samples (45 stored for 3 years, and another 45 stored for 14 years) of 8 years old children were analyzed. A reasonable stability was observed for the metabolites (**Table 1**), demonstrating that at least for these steroids, no statistical adjustment for varying storage duration is required in the respective data analyses.

Table 1. Comparison of GC-MS measurement values of single urinary androgen metabolite and derived adrenal androgen secretion markers between 3 and 14 years of storage duration [1]

	Storage duration	
n (boys)	3 years 45 (22)	14 years 45 (22)
16α-DHEA (µg/d)	72 (49, 95)	63 (28, 96)
Androstentriol-16α (µg/d)	17 (10, 42)	24 (13, 45)
Androsterone (µg/d)	110 (66, 143)	111 (86, 189)
Etiocholanolone (µg/d)	83 (56, 127)	99 (66, 126)
DHEA&M [3] (µg/d)	109 (68, 151)	108 (41, 161)
ΣC19 [4] (µg/d)	306 (221, 451)	315 (205, 467)

[1] Differences between 2 non-dependent samples (8 years old children) were tested with Wilcoxon rank sum test. There was no significant difference (P > 0.05) between 3 and 14 years storage duration for all the variables. Values are Median (p25, p75).
[2] Sum of urinary DHEA, and its direct metabolites (16α-DHEA, 5-androstene-3β,16α,17β-triol)
[3] Sum of urinary C19 metabolites (see Chapter 2.3.1)

4.6 Parental information and children's birth data

At several occasions during the course of children's participation, parents are asked to provide information about family characteristics, their educational status and employment. Parental weight and height are measured at regular intervals by the same trained nurses who assess the anthropometrics of the participating children. Information on birth weight and length as well as gestational age is abstracted from the "Mutterpass", a standardized document given to all pregnant women in Germany.

4.7 Statistical analyses

All statistical analyses were carried out using the Statistical Analyses System SAS (versions 9.1.3, SAS Institute Inc., Cary, NC). A p-value <0.05 was considered statistically significant. The exposure and outcome variables together with sample size, study design and major statistical method of the respective studies are summarized (**Table 2).** tudy II-IV shared the same outcome variables i.e. markers of pubertal timing. Details on the modelling of the exposure variables can be found in the Method Sections of Chapters 4.8-5.4.

Confounders and mediators

A confounder distorts the effect that is of direct interest, because the observed association can, in part or totally, be attributed to this factor. In order to qualify as a confounder, the following criteria have to be met (**Figure 9**) (169): i) The variable must be causally associated with the outcome (i.e. even in the absence of the exposure of interest); ii) it must be associated with the exposure of interest (non-causally or causally), and iii) the confounder must not lie on the causal pathway between exposure and the outcome (as an intermediate variable or mediator). In the present thesis, potential confounders were generally evaluated individually as well as in full models. Details on the statistical modelling for each research question can be found in the Method Sections of Chapters 4.8-5.4.

A mediator is an intermediate variable lying on the pathway between exposure and outcome and can thus serve to explain the mechanisms of their association (**Figure 9**) (170). For this reason, adjusting for intermediary variables has to be treated with caution and should only be conducted in a separate step. If an association is eliminated by adjustment for a mediator, this suggests a potential mechanism involving the mediator, but does not question the result obtained before (as in the case of a confounder).

An example for confounder and mediator from the current thesis is given as following: In Study I, we identified that animal protein intake might influence AA secretion, which itself is one of the most important candidate hormones that may modulate pubertal timing. Thus, in Study II it was examined whether AAs are the mediator between animal protein intake and pubertal timing. In this case, body fat mass is one of the confounders (**Figure 9**).

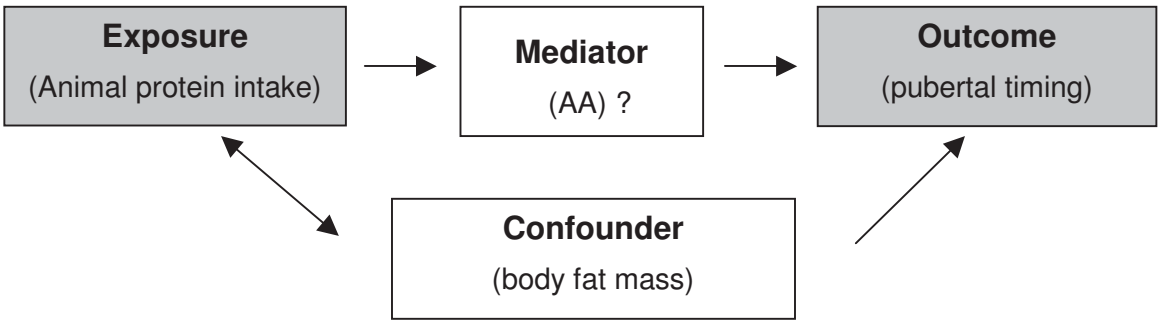

Figure 9. Definition of confounder (169) and mediator (170) with an example from the current thesis

Table 2. Overview of the statistical analysis

Study	Sample size	Design	Exposures	Outcomes	Statistical method
Preliminary Methodological work	120 (60 boys)	Cross-sectional	Urine volume	selected AA and GC metabolites or metabolite sums	linear regression models (proc glm)
Study I	137 (68 boys)	Cross-sectional	• Body composition • Dietary intakes	AA secretion marker[1]	stepwise multiple regression models (proc reg)
Study II	109 (54 boys)	longitudinal	• AA secretion marker[1] • Animal protein intake	Pubertal timing markers: • ATO • APHV • duration of pubertal growth acceleration[3] • age at Tanner stage B2_G2 • age at Tanner stage PH2 • age at menarche/voice break	linear regression models (proc glm)
Study III	110 (55 boys)	longitudinal	Markers of GC status[2]		
Study IV	120 (64 boys)	longitudinal	Markers of estrogen status[4]		

[1] ΣC19 , sum of the 7 quantitatively most important urinary AA metabolites (see Chapter 2.3.1)
[2] Include UFF+UFE (potentially bioactive free glucocorticoids); and ∑C21 [GC secretion marker (see Chapter 2.3.1)]
[3] Duration between APHV und ATO (APHV-ATO)
[4] Include E-sum3 (sum of E1, E2, and E3, major estrogens) and E-sum5 (sum of E-sum3, 16-epiE3, and 16-ketoeE2, total estrogens) (see Chapter 5.4.3)

4.8 Preliminary methodological work – Urine volume dependency of renally excreted glucocorticoid and adrenal androgen metabolites in healthy children

4.8.1 Summary

Observational and experimental studies suggested that urine volume should be considered as a confounder when using UFF and UFE to assess glucocorticoid (GC) status. We aimed to examine whether AA metabolites may be also affected by urine volume in healthy children. Further, to compare the flow dependence of GC and AA metabolites, selected GC metabolites were also examined. In 24-h urine samples of 120 (60 boys), 4-10 yrs old healthy children, urinary steroid profiles were determined by GC-MS analysis; UFF and UFE by radioimmunoassay. To assess daily GC and AA secretion rates, the 7 quantitatively most important urinary GC and AA metabolites were summed and denoted as ∑C21 and ∑C19, respectively. Association of urine volume with AA (∑C19, DHEA, 16α-hydroxy-DHEA) and GC (∑C21, UFF, UFE, 6β-hydroxycortisol, 20α-dihydrocortisoloutcome) outcome variables were examined in linear regression models adjusted for body surface area. Among the examined AA metabolites, 16α-hydroxy-DHEA (β=0.54, p<0.0001) and DHEA (β=0.42, p=0.05) showed relative strong association with urine volume and ∑C19 (β=0.23, p=0.08) showed positive trend. Regarding GC metabolites, urine volume showed a stronger association with cortisol's direct metabolites, i.e. cortisone, 6β-hydroxycortisol and 20α-dihydrocortisol (β=0.4-0.5, p<0.01) than with cortisol itself (β=0.28, p<0.05). ∑C21 was not associated with urine volume. In conclusion, the water load-dependency of urinary DHEA,16α-hydroxy-DHEA, 6β-hydroxycortisol and 20α-dihydrocortisol were comparable with the known water load-dependency of UFE and stronger than that of UFF in healthy children. Total AA secretion marker (∑C19) was moderately confounded by urine volume, but total GC secretion marker (∑C21) not.

4.8.2 Introduction

UFF is frequently used as an index for functional glucocorticoid activity in clinical laboratories. Recent findings suggested that UFE may be a useful complementary analyte to UFF for a more meaningful assessment of functional glucocorticoid activity (79, 171-173). Observational and experimental studies suggested that both UFF and UFE are dependent on urine volume after water loading in healthy children (93, 174) and adults (91, 92) with a prominent stimulation of UFE excretion. Accordingly, it has been argued that urine volume should be considered as a confounder when using UFF and/or UFE to assess glucocorticoid

status. Furthermore, since water loads affect UFF and UFE differently, the ratio of UFE and UFF as an estimate of renal 11ß-hydroxysteroid dehydrogenase type 2 (11ß-HSD2) activity (175), may also be confounded.

As mentioned in Chapter 2.3, apart of UFF and UFE, a wide spectrum of steroid hormone metabolites can be measured in the urine and used to assess hormone status. In clinical laboratories, the urinary precursor/product ratios have also been used to globally estimate different enzyme activities, which can be used as diagnostic tools to confirm the existence of certain inborn endocrine disorders (176). Similar to UFF and UFE and their ratio, if other steroid metabolites also have varying flow-dependent characteristics, not only the excretion rates of these metabolites, but also the ratios of them (possibly used as estimates for certain enzyme activities) would be confounded by urine volume.

Until now, the potential influence of water load on urinary excretion rates of the major AAs - DHEA and its metabolites - has not yet been investigated. Therefore, the first aim of the present study was to examine whether daily DHEA, 16α-hydroxy-DHEA, and the total AA secretion marker (∑C19) are associated with 24-h urine volume in healthy children. Further, in order to compare the flow dependency of some major steroid hormones and their metabolites with each other, the association of urine volume with urinary free glucocorticoids (UFF and UFE) and additional glucocorticoid metabolites were also systematically examined.

4.8.3 Methods

Subjects

The present sample comprised a subgroup of 169, 4-10 yrs old children participating in the DONALD study, in whom both analysis of urinary steroid profiles by GC-MS and measurements of UFF and UFE by specific radioimmunoassay have been performed. Of those 169 children, 49 children were excluded, in whom DHEA and 16α-hydroxy-DHEA were under detection limit (12.5 µg/L) of GC-MS analysis. Hence, the study population finally examined consisted of 120 children (60 boys, 60 girls).

Measurement and assessment

Body weight and height was measured and body surface area was calculated (for details see chapter 4.3). Procedures for the 24-h urine collection have been described (chapter 4.5.1). Urinary steroid profiles were determined by GC-MS analysis (chapter 4.5.2). To assess daily GC and AA secretion rates, the 7 quantitatively most important urinary glucocorticoid and AA metabolites were summed and denoted as ∑C21 and ∑C19 respectively (chapter 2.3.1). Apart from DHEA and 16α-hydroxy-DHEA, the sum of the other 5 androgen metabolites was calculated as follows: ∑C19 – (DHEA +16α-DHEA) and it was indicated as $\sum C19\_{non\ DHEA}$ in this study. UFF and UFE were measured by specific radioimmunoassays (chapter 4.5.2).

Statistical analysis

Descriptive data are given as means ± SD or median with interquartile range when appropriate. Sex differences for anthropometric and urinary characteristics were tested by using unpaired t-test. Preliminary analysis of covariance was used to test for sex-by-urine volume interactions. No interactions were significant for any of the outcome variables (*P* > 0.1*)*, thus the subsequent analyses were performed with boys and girls combined.

Association of urine volume with outcome variables - ∑C19, DHEA, 16α-hydroxy-DHEA, $\sum C19\_{non\ DHEA}$, ∑C21, UFF, UFE, 6β-hydroxycortisol and 20α-dihydrocortisol - were examined in linear regression models (proc glm) adjusted for body surface area. All variables used were also checked for normality and where required, natural log transformation was performed prior to the entering of the variables into the models. Additionally, to avoid errors arising from urine collection the daily excretion rates were not determined conventionally. Instead they were determined as follows: each individually calculated 24-h analyte/creatinine ratio was multiplied by individual body weight and by published constant sex- and age-specific body weight-related creatinine reference values (166) and this yields the corresponding creatinine-standardized 24-h analyte excretion rate (79, 177).

4.8.4 Results

A general description of the study sample with respect to anthropometic and urinary characteristics is given in **Table 3**. There was no sex difference for age and all the anthropometric and urinary variables. **Table 4** gives the results for the association of urine volume with urinary GC (∑C21) and AA (∑C19) secretion markers and selected steroid metabolites. Since all the outcome variables have the same unit and all of them are $\log_e$ transformed, we could compare the association strength (β) between them directly. Among the examined urinary androgen metabolites, 16α-hydroxy-DHEA showed the strongest association with urine volume (β=0.54, p<0.0001). DHEA (β=0.43, p=0.05) and adrenal androgen secretion marker, ∑C19 (β=0.23, p=0.08) showed a positive trend. The sum of other C19 metabolites apart from DHEA and 16α-hydroxy-DHEA ($\sum C19\_{non\ DHEA}$) was not associated with urine volume. Regarding the glucocorticoid metabolites, urine volume showed a relative stronger association with direct metabolites of cortisol, i.e. 6β-hydroxycortisol, 20α-dihydrocortisol and cortisone (UFE) (β=0.44-0.51, p<0.01) than with cortisol (UFF) itself (β=0.28, p<0.05). Total cortisol secretion marker, ∑C21 was not associated with urine volume. In the present analysis, a parameter estimation (β) of 0.30-0.50 means that with 10% increase of urine volume, daily excretion of the respective outcomes (metabolites and metabolite sums) increase by approximately 3-5%

Table 3. Characteristics of the study sample (n=120)*

	Boys n=60	girls n=60
Age (yrs)	8.0 ± 1.3	7.7 ± 2.0
Weight (kg)	29.4 ± 6.5	27.4± 7.7
Height (cm)	132.7± 8.9	129.3 ± 12.8
Body surface area (m^2)	1.04 ± 0.4	0.99 ± 0.18
Urine volume (mL/d)	610 ± 216	616 ± 238
Creatinine (mmol/d)	4.7 ± 1.1	4.3 ± 1.7
∑C19 (µg/d)	389 (254, 553)	295 (209, 499)
DHEA (µg/d)	15.0 (11.2, 26.3)	12.9 (9.1, 20.7)
16α-hydroxy-DHEA (µg/d)	86.2 (62.7, 123.2)	61.8 (40.9, 98.4)
$\sum C19_{\_nonDHEA}$ (µg/d)	288 (202, 417)	234 (157, 406)
∑C21 (µg/d)	3757 (3145, 4570)	3396 (2694, 4530)
6β-hydroxycortisol (µg/d)	61.6 (39.5, 88.6)	47.8 (34.5, 84.1)
20α-dihydrocortisol (µg/d)	18.1 (15.1, 24.3)	17.5 (11.9, 13.3)
UFF (µg/d)	13.1 (9.4, 17.9)	10.5 (8.0, 15.1)
UFE (µg/d)	25.7 (21.3, 32.3)	23.5 (16.2, 30.1)

*Values are means ± SD or medians (P25, P75); Differences between boys and girls were tested with unpaired t-test. P>0.05 for all the examined variables; $\sum C19_{\_nonDHEA} = \sum C19 - (DHEA + 16\alpha\text{-hydroxy-DHEA})$;

Table 4. Association of 24-h urine volume with urinary glucocorticoid (∑C21) and adrenal androgen (∑C19) secretion markers as well as selected glucocorticoid and adrenal androgen metabolites in 120 healthy children*

	β	***SE***	***P***
∑C19	0.22	0.13	0.09
DHEA	0.42	0.22	0.06
16α-hydroxy-DHEA	0.54	0.12	<0.0001
$\sum C19_{\_nonDHEA}$	0.16	0.13	0.22
∑C21	0.07	0.07	0.31
6β-hydroxycortisol	0.43	0.14	0.003
20α-dihydrocortisol	0.45	0.11	<0.0001
UFE	0.53	0.09	<0.0001
UFF	0.28	0.12	0.02

* result of multiple regression models adjusted for body surface area; all the outcome variables and urine volume were nature log transformed

4.8.5 Discussion

Our results indicate that apart from certain urinary GC metabolites, excretions of major AA metabolites were also dependent on urine volume in healthy children. Daily AA secretion marker (∑C19) showed a weak (not significant) renal flow dependency. The relative strong association of urine volume with the two important components of ∑C19, i.e., DHEA and its direct metabolite 16α-hydroxy-DHEA, seem to be responsible for the observed trend in flow rate-dependency of ∑C19, since the sum of the other 5 components of ∑C19 did not show any association with urine volume. In addition, we found not only UFF and UFE, but also urinary excretion rates of 6β-hydroxycortisol and 20α-dihydrocortisol were dependent on daily water load, howewer GC secretion marker (∑C21) was not.

Until now, the underlying mechanisms responsible for the different flow-dependent characteristics of various steroid hormones and their metabolites are unknown. As shown on a theoretical basis by Walser (89), the passive and even active reabsorption of certain substances can be influenced by urine flow. Although a large amount of the water and the resolved substances are reabsorbed in the proximal tubule, the fine adjustment of the reabsorption and excretion occurs in the distal part of the kidney. Thus, a potential

mechanism could be that, with increased urine flow rate, less of the resolved substances would be reabsorbed in the distal tubular or collection duct (90).

Urinary steroid hormones and their metabolites are excreted in different forms in the urine. Some are excreted principally in free form such as cortisol, cortisone, 6β-hydroxycortisol and 20α-dihydrocortisol; some dominantly as sulfate conjugates such as DHEA and 16α-hydroxy-DHEA; and others mostly as glucuronide conjugates such as the major C21 metabolites THE, THF and 5α-THF (178). To date, there is no evidence for a specific transport system for the free forms of steroids. The epithelial transport of these substances may be most probably by passive diffusion. According to Walser's theory (89), the degree of flow-dependence of passively reabsorbed solutes would be higher for those with a higher epithelial permeability and vice versa.. Since epithelial permeability for cortisol is greater than for its more polar metabolites 6β-hydroxycortisol and 20α-dihydrocortisol, it should be theoretically more dependent on urine flow than 6β-hydroxycortisol and 20α-dihydrocortisol. However, our study has shown a reverse result. Therefore, we speculated that apart from the "escape" mechanism of substances from reabsorption (91) (**FIG.2 Model 1**), other potential mechanisms must exist.

It is well known that, the conversion of cortisol to cortisone by 11β-HSD2 (mainly in the distal part of the tubule) is crucial in prevention of cortisol induced blood pressure increases, due to the non-selective MR occupancy by cortisol (179, 180). There is also an evidence for the renal conversion of cortisol to 6β-hdydroxycortisol (181). This may provide an additional pathway to protect MR from occupation by cortisol and might be also relevant for blood pressure regulation (64, 182). Since the expression of 20α-hydroxysteroid dehydrogenase has been found in human kidney samples (183, 184), part of the excreted 20α-hdydroxycortisol could be also produced in the renal tubular cells. We suppose that the relative stronger flow dependence of cortisone, 6β-hydroxycortisol and 20α-dihydrocortisol compared to cortisol might be, at least in part, explained through their intrarenal tubular production. Renally produced cortisone, 6β-hydroxycortisol and 20α-dihydrocortisol, might be easier to excreted against less fluid influx in the distal tubular and collecting duct cells with increased urine flow rate due to higher water intake (93) (**Figure 10. model II**). 16-α hydroxylation of DHEA is catalyzed preferentially by CYP3A7 (185, 186), which is consistently expressed in normal kidney (187). So, similar with direct cortisol metabolites, the possible renal production of 16α-hydroxy-DHEA may explain its stronger flow dependence than that of DHEA.

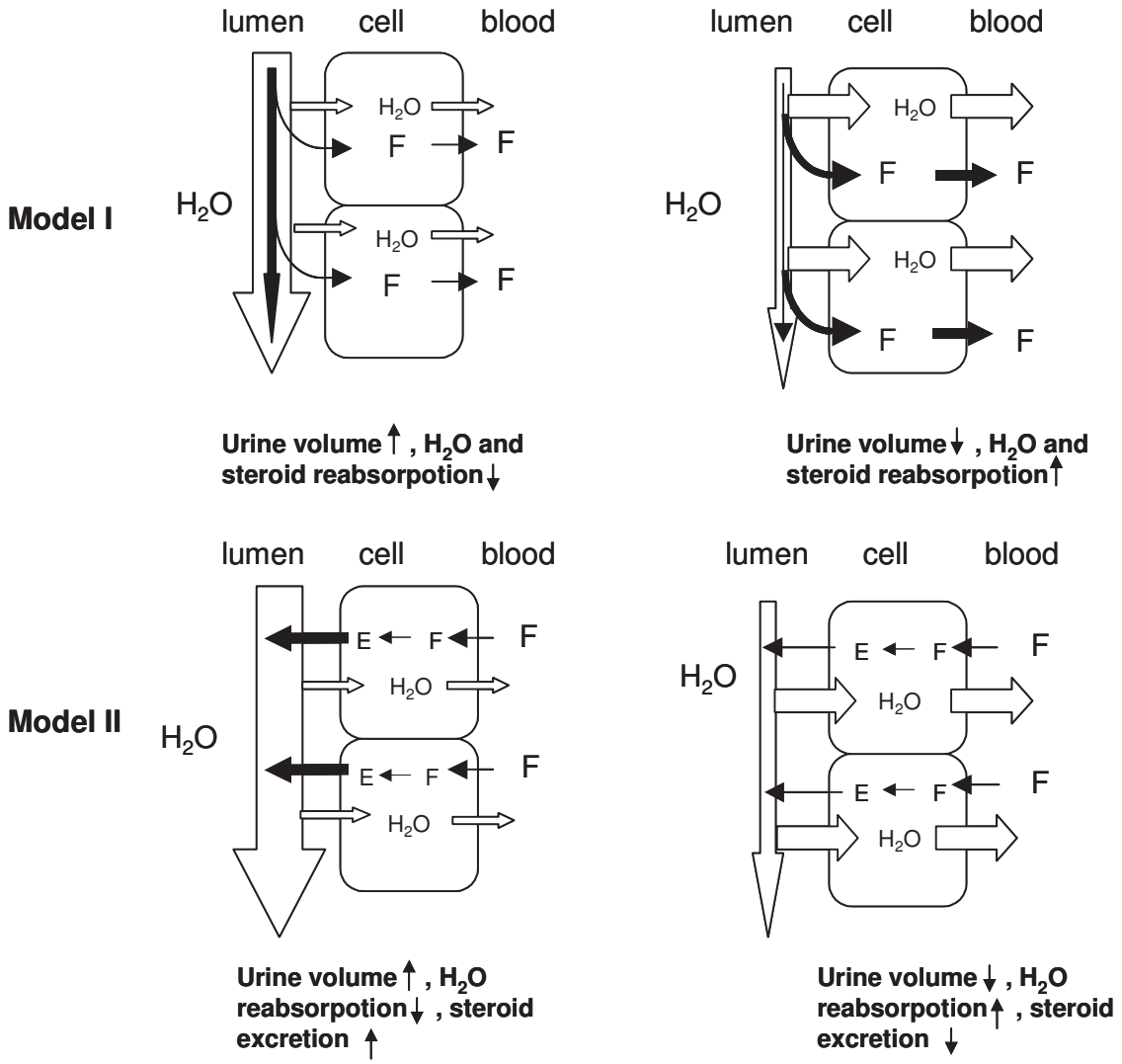

Figure 10. Two possible mechanisms of flow-dependent excretion of steroid hormones and their metabolites [with cortisol (F) and cortisone (E) as examples]
Model I : with increased water flow in the distal tubular and/or collecting duct**,** less solute (like cortisol and DHEA, which are not produced in the kidney) would be reabsorbed; **Model II :** with increased water flow in the distal tubular and/or collecting duct, more solute (like cortisone, 6β-hydroxycortisol and 16α-hydroxy-DHEA, intracellular produced in the tubule), would be excreted against less fluid influx.

In line with the published data (93), we did not find any association between the total GC secretion marker and urine volume. In addition, we found that apart from DHEA and 16α-hydroxy-DHEA, the sum of the other 5 C19 metabolites was not associated with urine volume either. A large fraction of the C21 and C19 metabolites excreted in the urine is in the form of steroid conjugates, which are generally less active, more polar and more readily excreted in the urine (188). These strong water soluble steroid conjugates are difficult to permeate cell membranes through diffusion; secretion and reabsorption of them are primarily

mediated by OATs (86) and/or organic anion transporting polypeptides (OATPs) (189, 190). Since the fine adjustment of the reabsorption and excretion of water occurs in the distal part of the kidney, the existence of transporters in that part and the affinity of the transporters to the respective steroid conjugates might determine the epithelial permeability for these conjugates and their subsequent flow-dependent character. To our knowledge, to date only 3 transporters – mouse (m) /rat (r)OAT5 (86, 191-193), rOAT8 (194) and hOATP1A2 (87) – were reported to be expressed on the apical membrane of the distal tubule or cortical collecting duct and may be responsible for the distal reabsorption of steroid conjugates. Although, both sulfate and glucuronide conjugates may be candidate substrate for these OATs, until now only DHEA-S and estrogen sulfate were actually identified to have a high affinity to these transporters. It can be assumed that this transporters may most probably have a selective affinity to those metabolites (e.g. DHEA-S) that are believed to play a potential biological role after reabsorption. Most of the conjugated metabolites without bioactive potential are rather eliminated from the body than reabsorbed. Thus, the flow-dependent characteristic of DHEA, which is principally excreted as DHEA-S, may be due to the selective reabsorption of it in the distal tubule and/or collecting duct.

Because of the limited age range of our subjects (4-10 years) and the cross-sectional study design, we can not give clinical advice in detail. But we have been able to show with a 10% increase of urine volume, daily excretion of DHEA and 16α-hydroxy-DHEA increased by 4% and 5% respectively. Therefore, the confounding of water load on DHEA and 16α-hydroxy-DHEA might be of relevance for clinical diagnosis as well as physiological and pathophysiological research. Especially for the statistical analysis, urine volume should be considered as a potential confounder in regression models with GC or AA metabolites or the ratios of their metabolites as predictors or outcomes.

In conclusion, water load-dependent renal excretion of urinary DHEA,16α-hydroxy-DHEA, 6β-hydroxycortisol and 20α-dihydrocortisol was comparable with that of UFE in healthy children. Total AA secretion marker (∑C19) appears only moderately confounded by urine volume, and total GC secretion marker (∑C21) not. Whether the intra-renal production of 16α-hydroxy-DHEA, cortisone, 6β-hydroxycortisol, and 20α-dihydrocortisol could be the potential mechanism for their relative stronger water flow-dependency needs to be investigated in the further studies.

5. Studies

In this Chapter, the study-specific summaries, introductions, methods, results, and discussions of Study I to Study IV are firstly presented. In addition, the results of Study II to Study IV are summarized and presented in the end.

5.1 Study I – Body fat and animal protein intake in addition to steroidogenic enzymes affect adrenarchal androgen secretion

5.1.1 Summary

Adrenarche is the increase in AA production starting in childhood. Until now, it has been unknown whether or not nutritional factors modulate adrenarche. We aimed to examine whether body composition and certain dietary intakes may associated with the AA production in children after accounting for urinary indicators of major adrenarche-related steroidogenic enzymes. Androgen and glucocorticoid metabolites were profiled by GC-MS in 24-h urine samples of 137 healthy prepubertal children aged 3-12y, for whom birth characteristics, growth velocity data, and 3-d weighed-diet record information are available. Associations of the sum of C19 metabolites (reflecting daily AA-secretion) with nutritional factors [FM, FFM, nutrient intakes, glycemic index, glycemic load] and AA-relevant estimates of steroidogenic enzyme were examined in stepwise multiple regression models adjusted for age, sex, urine volume, and total energy intake. Enzyme activity estimates were calculated by specific urinary steroid metabolite ratios. Among the nutrition-relevant predictors, FM ($p<0.0001$) explained most of the variation of AA-secretion ($R^2=5\%$). Animal protein intake was also positively associated with AA-secretion ($p<0.05$), explaining 1% of its variation. FFM ($p=0.1$) and total protein intake ($p=0.05$) showed positive trends. The difference in daily AA-secretion between the lowest and highest quartile of FM was comparable to that between the lowest and highest estimated activity of one of the major steroidogenic enzymes. In conclusions, body fat mass may relevantly influence prepubertal adrenarchal androgen status. In addition, animal protein intake may also make a small contribution to children's AA-secretion levels.

5.1.2 Introduction

Adrenarche is defined as the onset of increasing adrenal secretion of C19 steroids - principally DHEA and its sulfate ester – in children at about 6-8 yrs (17). Recent longitudinal data suggest that adrenarche is a gradual process of increasing AA secretion from early childhood through adolescence (2, 3). To date, the exact mechanism regulating adrenarche is not clear, although many factors potentially involved have been postulated.

Numerous data have strongly suggested that alterations of the steroidogenic enzyme activities within the adrenal zona reticularis (ZR), which is primarily responsible for AA formation, are the primary cause of adrenarche (21, 29, 30). AA biosynthesis requires cytochrome P450c17 (CYP17), which as a complex enzyme, converts pregenolone to 17-OH-pregenolone (17α-hydroxylase activity) and then converts 17-OH-pregenolone to DHEA (17,20-lyase activity). Activity of 3β-hydroxysteroid dehydrogenase (3βHSD) regularly acts to decrease AA production through competition with CYP17. Decreased amounts of 3βHSD (29, 30, 195) and increased expression of CYP17 (30) with increasing age in the ZR of the adrenals from children undergoing adrenarche have been demonstrated by immunohistochemistry and gene expression studies. However, the in vivo metabolic evidence for the contribution of the above steroidogenic enzymes to adrenarchal androgen increases in healthy children is missing. Especially, their combined effects on AA secretion have not yet been examined.

Recently, influences of birth weight and body size on AA secretion have been also suggested in a number of studies. AA secretion was found to be increased in children with lower birth weight (196, 197) and BMI (44, 198). Higher body mass and lower birth weight are also associated with higher insulin and/or IGF-1 levels (199-201) in childhood. Cell culture and immunohistochemical studies have reported the expression of IGF-1 and insulin receptors (46, 47) in human adrenal tissues. Therefore, it has been suggested that IGF-1 may be the modulators of AA secretion. Whether dietary protein (55, 56) and GI or GL (202), which have been shown to affect insulin and/or IGF-1 secretion, also influence AA secretion is not clear.

Therefore, we aimed to investigate whether a relevant part of variation of 24-h AA secretion may be also explained by body composition and dietary intakes in healthy children. In doing so, we have taken, the estimated activities of adrenarche-relevant steroidogenic enzymes and further potential influencing factors, e.g. birth characteristics, into account. Steroidogenic enzyme activities in vivo were estimated using product/precursor ratios of specific urinary steroid metabolites profiled by GC-MS.

5.1.3 Methods

Subjects

The present sample comprised a subgroup of 400, 3-18y old children and adolescents participating in the DONALD study, in whom analysis of urinary steroid profiles (3, 80) have been performed by GC-MS (3, 80, 203). Among the 382 DONALD participants with available information on pubertal development stage, all prepubertal children (Tanner stage=1) were selected (n = 186) to ensure that steroid profiles reflect primarily adrenal and not gonadal

hormone production. Tanner stages of puberty in boys (external genitalia: scrotum and penis; G) and in girls (breast; B) were assessed by a study paediatrician. Exclusion criteria were: i) incomplete anthropometric data (weight, height, skinfold thicknesses) at the time of hormone measurements or one year before the hormone measurements (anthropometrics necessary for growth velocity calculation) (n = 18); ii) lacking information on birth characteristics (n = 3); and iii) lacking 3-d dietary record at the visit, for which the hormone measurements were available (n = 2). In addition, 26 children without measurable androstenediol concentration (age ≤ 5y) (3) were excluded. Androstenediol data is required for the estimation of the 17,20–lyase activity within the Δ5-pathway of AA biosynthesis (204). Hence, the study population finally examined, consisted of 137 children (68 boys, 69 girls) (**Table 5**).

Measurement and assessment

Body weight, height and Skinfold thickness were measured (see chapter 4.3). BMI, percent body fat, FM, and FFM was calculated. For each child, age- and sex-independent BMI SD-scores were calculated by using the German reference curves (205). We assessed the proportion of overweight children according to the definition of the International Obesity Task Force (157). Growth velocity was calculated as the height gain during the year before the hormone measurements. Information on birth weight, birth length, and gestational age were abstracted from the Mutterpass. We used the 3-day dietary record collected at the visit for which steroid measurements were available, to calculate the mean intake of total energy (kcal/d), carbohydrate (g/d), total protein (g/d), animal and vegetable protein (g/d), and the dietary GI and GL (g/d) (206) for each participant. 24-h urine collections were carried out on the 3rd dietary recording day. Procedures for the 24-h urine collection have been described previously (166). Urea was analyzed photometrically with Urease-Berthelot method (Randox Laboratories Ltd, Crumlin, UK). Urinary steroid profiles were determined by GC-MS analysis according to the method described previously (chapter 4.5.2) (3, 80). Since previous studies (207) have suggested that ACTH stimulation is required for AA production and can modulate its levels independent of age, we assessed ACTH-dependent cortisol secretion as an indicator for ACTH-stimulus. To assess daily GC and AA secretion rates, the 7 quantitatively most important urinary glucocorticoid and AA metabolites were summed and denoted as ∑C21 and ∑C19 respectively (chapter 2.3.1).

Estimation of steroidogenic enzyme activities

The estimation of 3βHSD, 17α-hydroxylase (17-OH) (204), and 17,20-lyase activities (204) is based on product/precursor ratios as presented in **Table 2**. For diagnostic purposes 3βHSD activity is conventionally estimated using the following ratio: sum of the androstenedione metabolites androsterone and etiocholanolone divided by the sum of specific DHEA metabolites (3). Since androsterone and etiocholanolone (in the numerator) and the DHEA

metabolites (in the denominator) are also the major constituents of our outcome ΣC19, the inclusion of this ratio as a covariate in multiple regression models would lead to an inappropriate co-linearity between predictor and outcome. Therefore, for the current analysis we used an alternative estimate based on the major specific intra-adrenal precursor and product metabolites of 3βHSD (for details see FIG. 1). The conventional diagnostic estimate of 3βHSD (3), on the other hand, reflects primarily the peripheral conversion of DHEA to androstenedione. Also the ratio of 17,20-lyase to 17-OH activity (17,20-lyase / 17α-OH) was calculated. This was done because evidence suggests that the post-translational regulators of CYP17 (208, 209) e.g. the presence of cytochrome b5 (CYB5) and serine phosphorylation of CYP17 promote AA production through increasing the activity of 17,20-lyase relative to 17-OH (17, 21, 30).

Another essential enzyme of glucocorticoid pathway, 21-hydroxylase (21-OH), is not directly involved in the production of AA, however clinical studies (210) showed that a deficiency of 21-OH can cause excessive production of adrenal C19 steroids, therefore we also estimated its activity using product/precursor ratio from the sum of urinary major cortisol metabolites and the sum of 17-hydroxyprogesterone metabolites **(Table 6)** (3).

Statistical analysis

Descriptive data are given as means ± SD or median with interquartile range when appropriate. Sex differences for anthropometric, hormonal, and dietary characteristics were tested by using chi square or unpaired t-test as appropriate. Preliminary analysis of covariance was used to test for sex-by-fat mass, sex-by-protein intake, and sex-by-enzyme activity interactions. No interactions were significant for the outcome variable ∑C19 ($P > 0.1$), thus the subsequent analyses were performed with boys and girls combined.

Associations of urinary ∑C19 with nutritional factors and other AA secretion predictors in prepubertal children were examined in stepwise multiple regression models preadjusted for age, sex, urine volume, and total energy intake. In a first analyses, FM and FFM were entered as the major independent variables and estimates of 3βHSD, 17-OH, 17,20-lyase/17α-OH, 21-OH, ∑C21, growth velocity, as well as birth weight as continuous covariates and full-term birth (≥ 37 wk gestational age = 1, otherwise = 0) as an categorical covariate. Because cortisol secretion (∑C21) (an indicator for the ACTH-dependent adrenocortical activity) is elevated in healthy children with higher body fat (211), we adjusted ∑C21 for fat mass before entering it in the regression models. In a second set of analyses (with only the significant of the above covariates remaining in the model), the relevance of the following dietary factors was examined: total or animal protein intake, GI or GL, fat and fiber intake. Further, in an additional model we included urinary urea (as a biomarker for

protein intake) in place of the total protein intake derived from dietary records. All variables used were checked for normality and where required, the natural log transformation was performed prior to analysis. Multicollinearity tests were performed to check independence of the variables. For none of the covariate combinations multicollinearity was observed.

Further, to illustrate and compare the magnitude of the association of AA secretion with nutritional and enzymatic factors more clearly, z-scores of body composition, animal protein intake, urinary urea, and steroidogenic enzymes were grouped into 3 categories respectively: low (< 25^{th} percentile), medium (≥ 25^{th} percentile and ≤75^{th} percentile), and high (>75^{th} percentile). The respective z-scores were obtained by internal standardization to the sample in this analysis (mean = 0, SD = 1; by chronological age and sex). Linear regression models were used adjusting for potential confounders identified in the stepwise regression analyses. The adjusted geometric means of ∑C19 by categories of respective independent variables were predicted by the model when the other variables were held at their mean values. P for trend refers to the P values obtained in linear regression models with respective variables as continuous variables.

5.1.4 Results

A general description of the study sample with respect to birth, anthropometric and dietary characteristics is given in **Table 5**. The urinary characteristics of the study sample are presented in **Table 6.** There was no sex difference for our outcome variable ∑C19. With respect to the nutritional factors, percentage body fat was significantly higher in girls than in boys without any differences in GI, the densities (per MJ) of GL and protein intakes. Further, activity estimates of 17-OH and 21-OH as well as 17,20-lyase/17-OH differed between the sexes.

Table 7 reports the associations of urinary ∑C19 with nutritional factors and further AA secretion predictors in prepubertal children by stepwise multiple regression analysis adjusted for age, sex, urine volume, and total energy intake. FM was positively associated with AA secretion and explained 5% of its variation. Only a trend (p =0.1) was seen for the association between FFM and ∑C19 ($\beta_{standard}$ =0.15), which was much weaker than that between FM and ∑C19 ($\beta_{standard}$ =0.24, p<0.0001). Animal protein intake was significantly directly (p =0.03) associated with AA secretion. When total protein was offered in place of animal protein intake, total protein intake showed a trend (p =0.05, data not shown).

Table 5. Birth, anthropometric and dietary characteristics of the study sample (n=137)[1]

	Boys n=68	girls n=69	*P* for differences[2]
Age (yrs)	7.1 ± 2.1	6.9 ± 2.3	n.s.
Birth weight (kg)	3.6 ± 0.5	3.3 ± 0.4	<0.01
Gestational age (wk)	40 (39, 41)	40 (39. 40)	n.s.
Weight (kg)	25.8 ± 7.1	24.4 ± 7.7	n.s.
Height (cm)	125.6 ± 13.0	122.3 ± 14.0	n.s.
BMI (kg/m²)	16.0 ± 1.7	16.0 ± 2.0	n.s.
BMI-SDS[3] (SD)	-0.06 ± 0.88	-0.05 ± 0.96	n.s.
Fat mass (kg)	3.5 (2.8, 4.5)	3.9 (3.0, 4.6)	n.s.
Fat-free mass (kg)	21.1 (18.0, 25.9)	18.9 (16.2, 22.9)	<0.05
Percentage body fat (%)	13.9 (12.3, 17.4)	15.9 (14.0, 20.0)	<0.05
Growth velocity[4] (cm/y)	6.3 (5.7, 7.3)	6.7 (5.7, 7.3)	n.s.
Overweight[5] [n (%)]	5 (3.7)	8 (5.9)	n.s.
Energy intake (MJ/d)	6.8 ± 1.4	5.9 ± 1.1	<0.0001
Protein intake (g/d)	51 ± 13	45 ± 10	<0.01
Protein intake (% of energy)	12.5 ± 1.7	12.7 ± 1.9	n.s.
Animal protein intake (g/d)	33 (24, 40)	27 (20, 35)	<0.05
Animal protein intake (% of energy)	8.1 (6,8, 9.3)	8.2 (6.5, 9.3)	n.s.
Carbohydrate intake (g/d)	207 ± 46	183 ± 42	<0.01
Carbohydrate intake (% of energy)	50.9 ± 4.7	51.6 ± 5.8	n.s.
Fat intake (g/d)	67 ± 16	59 ± 14	<0.01
Fat intake (% of energy)	37 ± 4	36 ± 5	n.s.
Fiber intake (g/d)	17 ± 5	15 ± 5	n.s.
Fiber intake (% of energy)	1.0 ± 0.3	1.1 ± 0.3	n.s.
GI[6]	55 ± 3	55 ± 4	n.s.
GL[7] g carbodydrate/d	113 ± 27	102± 27	<0.05
GL[7] (g/MJ)	16.7 ± 2.1	17.1 ± 2.6	n.s.

[1] Values are means ± SD or medians (P25, P75)
[2] Differences between sexes were tested with unpaired t-test for continuous variables; chi-square test for categorical variables; n.s., not significant
[3] SDS, standard deviation scores according to the German reference curves for BMI (205)
[4] 1 year before the hormone measurements
[5] Defined by using the international Obesity Task Force definition (157)
[6] Glycemic Index
[7] Glycemic load

Table 6. Urinary characteristics of the study sample (n=137)[1]

	Boys n=68	Girls n=69	*P* for differences[2]
Urine volume (mL)	637 (461, 835)	543 (435, 753)	n.s.
Creatinine (mmol/d)	4.1 (2.8, 5.4)	3.1 (2.6, 4.3)	<0.05
Urea (mmol/d)	205 (154, 286)	177 (144, 219)	<0.01
∑C19[3] (µg/d)	288 (173, 432)	277 (165, 385)	n.s.
∑C21[4] (mg/d)	3.3 (2.5, 3.8)	3.0 (2.3, 3.4)	n.s.
3βHSD[5]	0.85 (0.57, 1.42)	1.13 (0.86, 1.73)	<0.05
17-OH[6]	2.93 (2.19, 3.98)	3.81 (2.72, 4.62)	<0.01
17,20-lyase[7]	1.75 (1.49, 2.06)	1.72 (1.47, 2.08)	n.s.
17,20-lyase / 17-OH[8]	0.65 (0.41, 0.90)	0.47 (0.32, 0.66)	<0.01
21-OH[9]	18.2 (14.8, 22.9)	16.7 (13.9, 20.0)	<0.05

[1] Values are medians (P25, P75).
[2] Differences between sexes were tested with unpaired t-test; n.s., not significant.
[5] Activity of 3βHSD (3β-hydroxysteroid dehydrogenase) was estimated as product/precursor ratio from the sum of urinary progesterone metabolites and the sum of urinary Pregnenolone metabolites: (5β-Pregnan-3α,20α-diol + 5β-Pregnan-3α,17α-diol-20-on) / (5-Pregnen-3β,20α-diol +5-Pregnen-3β,17α,20α-triol) **(Figure 11).**
[6] Activity of 17-OH (17α-hydroxylase) was estimated as product/precursor ratio (Δ5 and Δ4 pathway). from the sum of the 17-hydroxy-pregenolone and 17-hydroxy-progesterone metabolites and the sum of the pregnenolone and progesterone metabolites: (5-Pregnen-3β,17α,20α-triol + 5β-Pregnan-3α,17α,20α-triol) / (5-Pregnen- 3β,20α-diol + 5β-Pregan-3α,20α- diol) (204).
[7] Activity of 17,20-lyase was estimated as product/precursor ratio (Δ5 and Δ4 pathway) from sum of the major C19 metabolites and the sum of the 17-hydroxy-pregenolone and 17-hydroxy-progesterone metabolites: (5-Androsten-3β,17β-diol + Androsteron + Etiocholanolon) / (5-Pregnen-3β,17α,20α-triol + 5β-Pregnan-3α,17α,20α-triol) (204).
[8] Ratio of 17,20-lyase and 17α-hydroxylase.
[9] Activity of 21-OH (21-hydroxylase) was estimated as product/precursor ratio from the sum of urinary major cortisol metabolites and the sum of urinary 17-hydroxyprogesterone metabolites: (tetrahydrocortisone + tetrahydrocortisol + 5α-tetrahydrocortisol) / (17-hydroxypreganolones + pregnanetriol + 11-oxopregnanetriol) (3)

Furthermore, when we used urinary urea excretion as a marker for dietary protein intake, this biomarker was also associated with AA secretion too (p = 0.03, categorical data given in **Figure 12**). Other nutritional factors were not significant. Among all the estimates of steroidogenic enzyme activities, 3βHSD showed the highest explanatory contribution (R^2 = 0.11) to AA secretion. In addition, AA secretion was also explained by 21-OH, 17-OH, 17,20-lyase/17-OH, cortisol secretion (∑C21) and birth weight. 17-OH, 17,20-lyase/17-OH and ∑C21 were positively and 3βHSD, 21-OH and birth weight negatively associated with AA secretion.

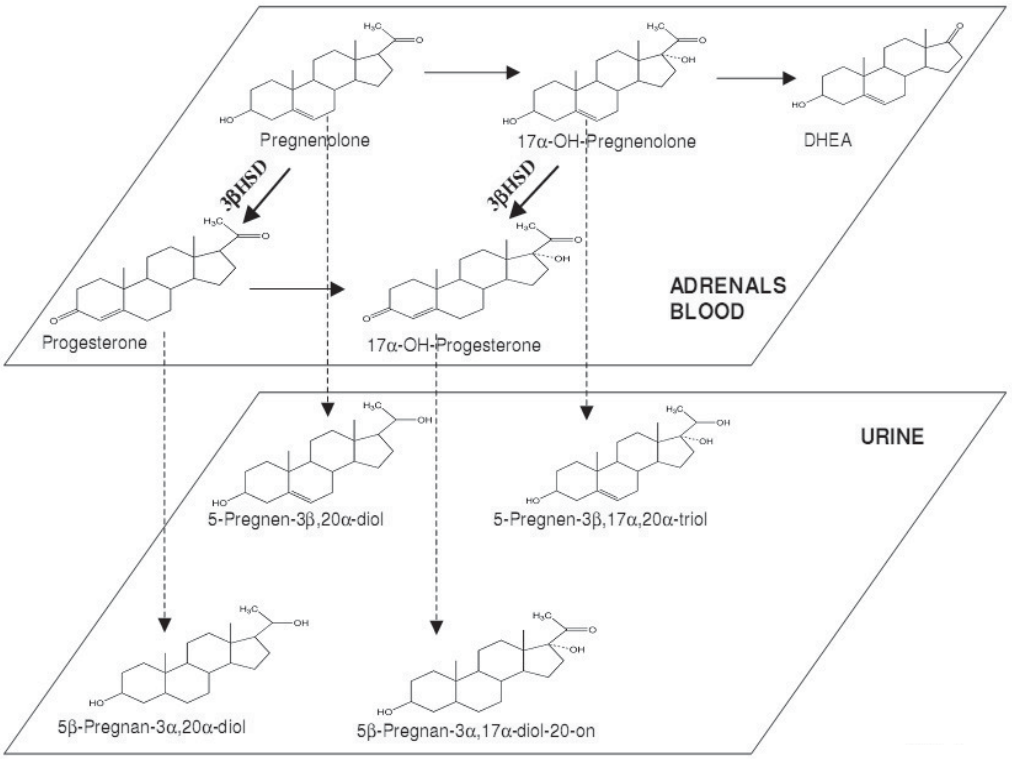

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Figure 11. Presentation of circulating precursors and products of 3βHSD in blood and their corresponding metabolites excreted in the urine.

Activity of 3βHSD was calculated as product/precursor ratio from the sum of urinary progesterone metabolites (5β-Pregnan-3α,20α-diol + 5β-Pregnan-3α,17α-diol-20-on) and the sum of urinary Pregnenolone metabolites (5-Pregnen-3β,20α-diol +5-Pregnen-3β,17α,20α-triol)

Figure 12 shows the adjusted means of the AA secretion marker ∑C19 for different z-score categories of FM (**A**), animal protein intake and urinary urea (**B**), estimate of 21-OH activity (**C**), and estimate of 3βHSD activity (**D**) in our 137 healthy prepubertal children. Those with a high FM or low 21-OH z-score have an approximately 120 µg higher daily AA secretion than those with a low FM or high 21-OH z-score. Those with a high animal protein intake or a low 3βHSD z-score have respectively an approximately 50 µg and 70 µg/d higher daily AA secretion than those with a low animal protein intake or high 3βHSD z-score .

Table 7. Associations of urinary ∑C19 with nutritional factors and further adrenal androgen secretion predictors in 137 prepubertal children[1]

Predictors	β	**SE**	β_{stand}	**R²**	***P***
A priori adjusted variables[2]	-	-		0.53	-
3βHSD	-0.22	0.02	-0.20	0.11	<0.0001
21-OH	-0.82	0.04	-0.37	0.06	<0.0001
Fat mass	0.40	0.03	0.28	0.05	<0.0001
17,20-lyase / 17-OH [3]	0.82	0.04	0.63	0.04	<0.0001
17-OH	0.73	0.05	0.47	0.04	<0.0001
∑C21	0.76	0.04	0.31	0.04	<0.0001
Animal protein intake (g)	0.006	0.001	0.09	0.01	0.03
Birth weight (kg)	-0.10	0.02	-0.07	0.004	0.04
Fat free mass	0.41	0.25	0.15	0.003	0.1
Model R²				**0.88**	

$\beta_{stand,}$ standardized parameter estimate; R², coefficient of determination; R² x 100, indicates the percentage of variation of the dependent variable being explained by the independent variable.
[1] Results of stepwise multiple regression; fat mass, fat free mass, 3β-hydroxysteroid dehydrogenase (3βHSD), 17α-hydroxylase (17-OH), 17,20-lyase, ∑C21 and 21-hydroxylase (21-OH) were $\log_e$ transformed.
[2] Age, sex, urine volume, and total energy intake.
[3] Ratio of 17,20-lyase and 17α-hydroxylase.

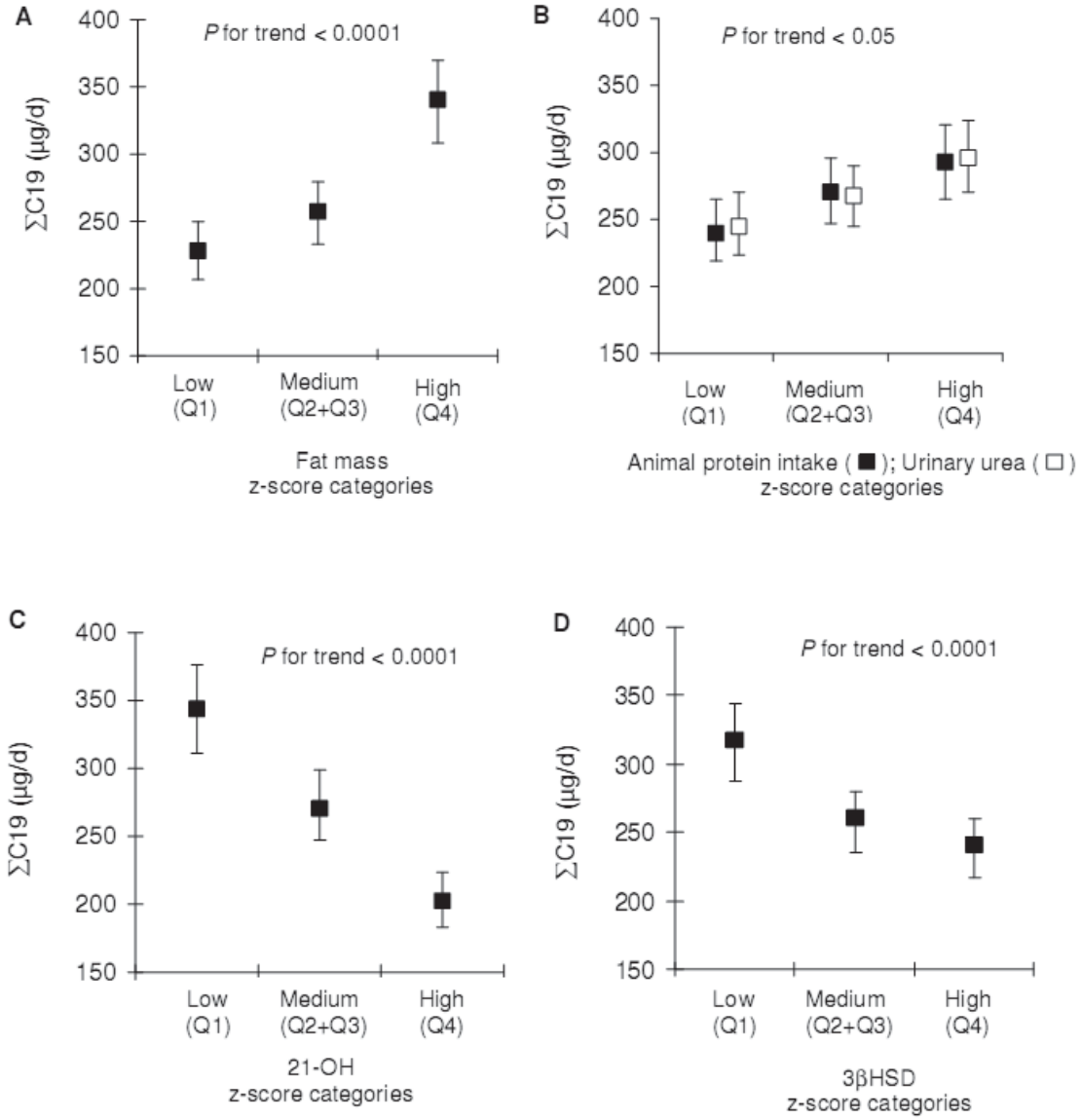

Figure 12. ∑C19 (marker of AA secretion) by categories of fat mass (A), animal protein intake and urinary urea (B), 21-hydroxylase (21-OH) (C), and 3β-hydroxysteroid dehydrogenase (3βHSD) (D) z-scores in healthy prepubertal children (n = 137).

Data are geometric means (95% confidence intervals) of ∑C19 for quartiles (Q)of the respective variables [adjusted for age, sex, urine volume, total energy intake, ∑C21 (marker of cortisol secretion), 17-hydroxylase, ratio of 17,20-lyase to 17-hydroxylase and 21-OH activities]. *P* for continuous trend refers to the *P* values obtained in linear regression models with the respective independent variables as continuous variables.

5.1.5 Discussion

In the present study we examined the cross-sectional association of markers of AA secretion with body composition and dietary intakes after taking estimates of the major adrenarche-related steroidogenic enzyme activities, ACTH-dependent adrenocortical activity, and birth

characteristics into account. Our data indicate that body fat mass is independently associated with AA secretion and to a minor degree probably also animal protein intake.

The association of body mass and AA secretion has been examined in numerous studies (212, 213). Most cross-sectional (44, 214) and case-control studies (215) found that children with higher BMI levels also had higher plasma levels of DHEA-S. A longitudinal study in healthy children reported increases in BMI to be associated with increases in urinary DHEA-S output (215), but this was not observed for plasma DHEA-S in young girls with idiopathic central precocious puberty (2). All the previous studies in healthy children did not take data of other relevant metabolic predictors like steroidogenic enzyme activities into account. In addition, BMI does not precisely represent FM, but also mirrors FFM. Until now, only few studies (118, 198, 213) investigated the association between body composition instead of body weight or BMI with AA secretion markers. The two studies of de Ridder et al. (118, 213) included only pubertal girls. Another longitudinal study (198) did not find any clear associations between urinary changes of DHEA-S and individually varying increases in FM or FFM. However, urinary DHEA-S represents only a relative small part of the AA secretion compared to ∑C19, which is regarded as a more appropriate AA secretion marker (3, 216). The current study is, to our knowledge, the first to analyze the association between body composition and AA secretion in prepubertal boys and girls accounting also for important endocrine enzymatic influences as estimated from urine steroid metabolite ratios. We found that higher body FM contributed to higher AA secretion in prepubertal children. Although body fat explained only 5% of the variation of total adrenarchal androgen secretion, the strength of its association with AA secretion – comparable to the effect sizes of 21-OH – suggested that it may modify children's AA levels.

In addition, lower birth weight showed also a significant, but small contribution to AA secretion, which confirmed previous studies (2, 196). Body mass development and variation in birth weight (196, 199, 201) have been shown to be associated with changes of insulin and/or IGF-1 bioactivity in childhood. *In vitro* and immunohistochemical studies demonstrated the expression of insulin and IGF-1 receptors in human adrenal tissues (46, 47) and the activation of these receptors on adrenal cortical cells may play a pivotal role in adrenal androgen production. It has therefore been suggested that insulin and/or IGF-1 might be the intermediate factors explaining the association of birth weight and body mass with AA secretion. A recent study (217) suggests that the IGF-I receptor signaling pathway may directly modulate the proliferation of adrenal progenitor cells to stimulate the development of adrenal zones, but no evidence of a direct action of IGF-1 on zona reticularis was reported. Furthermore, circulating IGF-1 correlates more closely with FFM than with FM in prepubertal children (44), and we observed that the association between FFM and AA secretion was attenuated and no longer significant once FM entered into the model. Therefore, it can be

speculated that factors, which are more clearly associated with fat mass, such as insulin and leptin, may play a more specific role on AA secretion. The negative correlation of insulin sensitivity with serum DHEA-S frequently observed (49, 50) supposes a possible role of insulin on the regulation of AA secretion. Furthermore, a dose-dependent increase in the production of AA with increasing leptin in physiological amounts has been recently reported (43). *In vitro* data on the effects of insulin, IGF-1 (41, 42) and leptin (43), on adult human adrenocortical cells indicate that these hormones may be involved in alterations of the biosynthesis of AA, at least in part, by changing gene expression of specific steroidogenic enzymes. In line with this, the contribution (partial R^2) of body fat to AA became lower after entering the steroidogenic enzymes into the model.

Dietary intakes of protein, in particular animal protein (56) and GI/GL (202) have been shown to induce insulin and/or IGF-1 secretion and may subsequently influence AA secretion (51). The current study is – to our knowledge - the first to examine the association of dietary protein intake and GI/GL with AA secretion. Of course, the inclusion of blood samples with IGF-1 and insulin measurements would also have been advantageous to confirm hypotheses on the underlying mechanisms, but are unfortunately not available during childhood and adolescence in the DONALD Study. In the present study, we did not find significant contributions of GI or GL to AA secretion. However, we found that dietary protein intake may modestly influence AA secretion in healthy prepubertal children, and especially animal protein seems to be responsible for this association. In addition, our findings on protein intake and AA secretion were supported by urea excretion rates quantified in the concomitantly collected 24-h urine samples. In view of the fact that dietary protein intake is a modifiable factor, the small possible influence of animal protein intake on prepubertal total androgen level could be of relevance. Nevertheless, the biological meaning of 1 % variation in androgen secretion that we observed remains questionable as long as it is not further substantiated by more specific studies.

As mentioned above, one strength of our study is that we attempted to account for the influences of various steroidogenic enzymes (known or assumed to be involved in the regulation of AA secretion) in the form of specific urine metabolite ratios. With this approach we could provide estimates for possible partial and combined contributions of different enzymes to total AA production in vivo. This allowed us to determine the relevance of body composition and dietary intakes for AA secretion more specifically than without accounting for the important enzymatic factors. The cross-sectional design of study is a clear study limitation.

In conclusion, we have demonstrated that body fat mass may relevantly influence prepubertal adrenarchal androgen status. In addition, animal protein intake may also make a

small contribution, of which biological meaning needs to be examined more specifically in future studies.

5.2 Study II – Prepubertal adrenarchal androgens and animal protein intake independently and differentially influence pubertal timing

5.2.1 Summary

Whether adrenarche impacts on pubertal development is controversial. We aimed to examine the associations of AA secretion with early and late pubertal markers, independent of potential influences of dietary animal protein intake. Daily AA excretion rates were quantified by GC-MS in 109 prepubertal healthy free-living children (54 boys), for whom 24-h urine samples, 3-day weighed dietary records and anthropometrical data 1 and 2 yrs before the start of pubertal growth spurt (ATO) as well as information on Tanner stages and birth characteristics were available. Multitivariate regression analysis (adjusted for sex, fat mass and total energy intake) was performed to examine the independent associations of AA and animal protein intake (mean of 1 and 2 yrs before ATO) with outcomes: ATO, APHV, age at menarche/voice break, duration of pubertal growth acceleration, and ages at Tanner stage 2 for breast (girls) and genital (boys) development (B2_G2) and pubic hair (PH2). Higher adrenarchal C19 steroids predicted earlier ages at PH2 ($P<0.0001$) and B2_G2 ($P=0.009$), as well as a shorter pubertal growth acceleration period ($P=0.001$), independently of animal protein intake. Children with a higher AA secretion had a 1.5 yrs earlier begin of pubarche and a 0.8 yrs earlier begin of B2_G2 than those with a lower AA excretion. Furthermore, animal protein intake was independently negatively associated with ATO and APHV ($P<0.05$ each), and tended to be negatively associated with age at menarche/voice break ($P=0.07$). In conclusion, while higher animal protein intake may be involved in an earlier attainment of ATO and APHV, a more intensive adrenarchal process may precipitate a shorter pubertal growth spurt and a notably earlier onset of breast and genital development in girls and boys, respectively.

5.2.2 Introduction

Whether adrenarche, the increase in AA secretion during mid-childhood, may be implicated in the timing of puberty has not been longitudinally examined in healthy children until now. Based on available mostly cross-sectional clinical data, this question has been debated controversially for decades. A number of authors assume that this developmental rise of the principal adrenal 17-ketosteroids DHEA and DHEA-S several years before puberty onset may rather represent a marker of body maturation than a factor influencing the begin of puberty (95, 218, 219).

During normal growth adrenarche and gonadarche are temporally closely linked, but in pathological situations adrenarche can occur without subsequent gonadarche, and gonadarche without preceding adrenarche (218). Despite this apparent causal independence of both developmental processes, the appearance of pubic hair in girls is usually attributed to the influence of adrenarchal C19 steroids on the androgen-dependent groin area (17). Accordingly, in normal girls pubic hair occurs as one of the first indications of approaching puberty (220) and in girls with premature pubarche (premature pubic hair development) circulating DHEA or DHEA-S levels are usually elevated for the respective chronological age (221). In normal developing boys the appearance of pubic hair rarely precedes the beginning of genital development and appears to depend more closely on gonadal testosterone secretion (222). However, boys with precocious pubarche are – like girls – also characterized by an elevated DHEA or DHEA-S level (144).

Recently, Binder et al. have shown that daily replacement with 25 mg of DHEA orally to adolescent girls with central adrenal insufficiency induces a significant progress in pubic hair growth (145). These data together with the hormonal findings in (i) children with precocious pubarche (17, 143, 144, 222) and (ii) infants with a combined occurrence of genital hair and elevated 25 DHEA-S (but without any other signs of androgen excess) (223) underscore that AAs are causally involved in the appearance and growth of pubic hair. However, information on the strength of the temporal acceleration of pubarche by a stronger versus a less pronounced adrenarche in healthy children is lacking.

Intensity of adrenal androgen secretion appears to be influenced by a number of lifestyle- related and early life factors. Body fatness (224), dietary protein intake (224) as well as the combination of low birth weight and a high current weight have been associated with a more pronounced adrenarche (225).

There is convincing evidence that exaggerated adrenarche with or without precocious pubarche in low birth weight girls can be followed by an early onset of puberty and early menarche (143, 226). In a recent review on adrenarche and the polycystic ovary syndrome, Nader (12) renewed the hypotheses that adrenarche is not only a promoter of pubarche, but also of gonadarche, suggesting that sexual maturation can occur earlier. Therefore, the first aim of the present paper was to examine this hypothesis in healthy children.

Previous studies suggest that animal protein intake influence the onset of menarche (115), which occurs in a later pubertal stage. Recently, animal protein intake in mid-childhood has been reported to potentially trigger an earlier onset of growth-related – puberty markers, i.e. ATO and APHV (10). However, two of the most important early pubertal markers - gonadarche and pubarche – have not yet been explicitly examined. Thus, our second aim was to investigate whether dietary animal protein intake might be also relevant for gonadarche and pubarche. Finally, since animal protein appears to modulate the intensity of

AA secretion (224), we also aimed to examine whether the contribution of animal protein intake to an altered timing of at least some pubertal markers is mediated by AAs.

5.2.3 Methods

Subjects

The number of children included in this analysis was derived as follows: A total of 376 subjects of the DONALD cohort had sufficient height measurements to allow plausible estimation of the puberty marker age at take-off of the pubertal growth spurt (ATO) (8). Of these, 111 children, who had not refused regularly assessment of Tanner stages, had also collected 24-h urine samples as well as dietary data at both time points (2 yrs and 1 yr) before ATO. In two children not all information on potential confounding variables (birthweight, gestational age, breast feeding, maternal overweight) were available. Hence, the subcohort analyzed herein included 109 prepubertal healthy children (54 boys). However, information on age at menarche or voice break were available only for n=100 (49 boys).

Measurement and assessment

The growth-dependent puberty variable ATO was determined using the parametric Preece and Baines model 1 (159) which also produces estimates of velocity at take-off, age at peak height velocity (APHV), and peak height velocity (PHV) (chapter 4.4.1). From the derived data, we have calculated the duration of pubertal growth acceleration (APHV minus ATO) which can be used as an index for growth spurt duration and puberty duration. Tanner stages for pubic hair (PH) and either breast (girls) or genital (external genitalia, boys) development (B_G) are assessed by one of the DONALD study pediatricians (chapter 4.4.2). In addition, children and/or parents are asked at each visit whether menarche or voice break has already occurred and the respective month and year are recorded. We have used age at testis volume ≥ 4 mL to define the onset of genital development in boys (Tanner stage G2). Herein, we denote Tanner stage 2 for breast and genital development in girls and boys, respectively as B2_G2.

Body weight, height and Skinfold thickness were measured (see chapter 4.3). BMI and fat mass index (FMI = fat mass / height²) was calculated. Urinary steroid profiles were determined by GC-MS analysis (chapter 4.5.2). To assess daily GC and AA secretion rates, the 7 quantitatively most important urinary glucocorticoid and AA metabolites were summed and indicated by ∑C21 and ∑C19 respectively (chapter 2.3.1). The mean 3-day intakes of total energy (kcal/d) and animal protein (g/d) at 2 yrs and 1 yr before puberty onset were also calculated.

Statistical analysis

Descriptive data are given as means ± SD or median with interquartile range when appropriate. Sex differences for pubertal, anthropometric, hormonal, and dietary characteristics were tested by using chi square or unpaired t-test.

Multiple linear regression analyses were used to analyze the associations of hormone excretion rates and animal protein intake with pubertal variables. The hormonal and nutritional predictors were included as arithmetic mean values of the respective data from one yr and two yr before ATO. For this, 24-h excretion rates of ∑C19 (log-transformed) were expressed as standard deviation score (SDS) of published GC-MS reference values (3) and animal protein consumption as % of daily energy intake. Analyses of covariance were performed to test for sex interactions. No sex-by-animal protein intake and no sex-by-∑C19 interactions ($p > 0.1$) were observed for the outcomes ATO, APHV, duration of pubertal growth acceleration, age at B2_G2, age at PH2, and age at menarche/voice break. Accordingly, the respective multiple regression analyses were performed with boys and girls combined.

Potential confounding factors considered in the models with ∑C19 as predictors were: sex, FMI, total energy intake-SDS, animal protine intake (% energy), urine volume related to body surface area, gestational age, birthweight, breastfeeding ≥ 2weeks, and maternal overweight (BMI ≥ 25 kg/m2). FMI and total energy intake were expressed as SDS of the steroid GC-MS reference sample (21). To exemplify that the associations between C19 steroids and puberty onset were comparable in both sexes, a sex-stratified subanalysis was done for the puberty marker B2_G2. In this subanalysis two models were run which differed only with regard to inclusion mode of FMI-SDS. In model 2, FMI-SDS was preadjusted for overall daily cortisol secretion (adrenocortical activity), i.e., residuals were obtained by regressing FMI-SDS on the sum of urinary glucocorticoid metabolites (for the glucocorticoid parameter, see urinary measurements). This preadjustment of FMI-SDS accounts for the fact that cortisol secretion (adrenocortical activity) and body fatness are associated in healthy children (211). As a consequence, body fatness variation (via covariation with adrenocortical activity) partly reflects the potential C19 influence on the puberty outcomes (i.e., the part of C19 variation caused by adrenocortical activity). Removal of the latter influence from the FMI-SDS variable by preadjustment reduces inappropriate attribution of parts of the C19 influence to FMI and should hence improve specificity of the variation explained by C19.

Potential confounding factors considered in the models with animal protein intake as predictors were: sex, FMI, total energy intake-SDS, gestational age, birthweight, breastfeeding ≥ 2weeks, and maternal overweight (BMI ≥ 25 kg/m2). AA was considered as a potential mediator in an attempt to address that AA might lie on the pathway between animal protein intake and pubertal timing.

Additionally, animal protein intake (% energy) and ∑C19-SDS were each grouped into 3 categories: low (<25th percentile), medium (≥ 25th and ≤ 75th percentiles), and high (≥ 75th percentiles). Least-squares regression analyses, adjusted for sex, FMI-SDS, birthweight, and urine volume or total energy intake-SDS, were used to predict the adjusted means of puberty variables by categories of ∑C19-SDS or animal protein intake (% energy). P for trend refers to the P values obtained in linear regression models with respective variables as continuous variables.

5.2.4 Results

Anthropometric, urinary, and nutritional characteristics of the study sample (at average growth curve-derived biological age of 1.5 yrs before ATO) are presented in **Tables 8 and 9**. For most variables – e.g., fat-free mass index, daily C19 excretion rates, and daily energy intake – the absolute values were significantly higher in boys, whereas the corresponding SDS or % energy-related data, used in the regression analyses, showed no longer sex differences. Boys showed significantly longer pubertal growth acceleration than girls. ATO, APHV, ages at B2_G2 and PH2 occurred earlier in girls than in boys. Only for B2_G2 the sex difference was not significant (**Table 9**).

In the fully adjusted model (allowing for animal protein intake and other covariates) with ∑C19-SDS as predictor, higher adrenarchal C19 steroids predicted earlier ages at B2_G2 (P=0.009) and PH2 (P<0.0001) as well as shorter pubertal growth acceleration (P =0.002) (**Table 10**). An example for boys and girls with a pronounced adrenarche and a short pubertal growth acceleration period (as well as a weak adrenarche and the corresponding growth acceleration period) is given in **Figure 13**. The sex-stratified subanalysis given in **Table 11** shows, as an example for B2_G2, that the C19-puberty associations were comparable in both sexes. Age at G2 and B2 was negatively associated with C19 in boys and girls, respectively and this association was significant after FMI-SDS was preadjusted for overall daily cortisol secretion (adrenocortical activity) (for details see statistical analysis).

Animal protein intake was negatively associated with ATO ($P < 0.05$) and with APHV and menarche/voice break ($P = 0.05$, as trend) but not associated with duration of pubertal growth acceleration, age at B2_G2, or age at PH2 in the model without adjusting for C19 (**Table 12, Model 1**). After adjusting for C19, the association of animal protein intake with ATO did not change, with APHV became more significant (p=0.04), and with menarche/voice break became less significant (p=0.07) (**Table 12, Model 2**).

Table 8. Anthropometric and pubertal characteristics of the study sample (n=109)

	Boys n=54	girls n=55	P for difference[1]
Age (yrs)[2]	8.8 ± 0.9[3]	7.3 ± 0.7	<0.0001
Anthropometric[2]			
Weight (kg)	30.9 ± 5.0	25.3 ± 4.8	<0.0001
Height (cm)	137.1 ± 7.0	126.2 ± 6.0	<0.0001
Body surface area (m^2)	1.09 ± 0.11	0.94 ± 0.10	<0.0001
BMI (kg/m^2)	16.3 ± 1.6	15.7 ± 1.8	NS
BMI-SDS [4]	-0.16 ± 0.76	-0.15 ± 0.86	NS
Fat mass index (kg/m^2)	2.2 (1.8, 2.8)[6]	2.2 (2.0, 2.8)	NS
Fat mass index –SDS[6]	-0.46 ± 0.76	-0.17 ± 1.0	NS
Fat-free mass index (kg/m^2)	13.8 ± 0.9	13.1 ± 0.8	<0.0001
Fat-free mass index –SDS[6]	-0.06 ± 0.86	0.05 ± 0.77	NS
Pubertal			
ATO (yrs)	10.4 ± 0.9	8.8 ± 0.6	<0.0001
APHV (yrs)	13.6 ± 0.9	11.8 ± 0.7	<0.0001
Duration of pubertal growth acceleration (APHV - ATO) (yrs)	3.2 ± 0.3	2.9 ± 0.4	<0.05
Age at Tanner (B2_G2) (yrs)	10.9 ± 1.0	10.6 ± 0.9	NS
Age at Tanner (PH2) (yrs)	11.6 ± 1.1	10.8 ± 1.1	<0.05
Age at menarche/voice break (yrs)[7]	13.7 ± 1.1	13.1 ± 0.8	<0.05

[1] Sex differences were tested with unpaired t test
[2] values were derived from each individual´s arithmetic mean from one yr and two yrs before age at take off (ATO), i.e., data represent average values approximately 1.5 yrs before ATO
[3] Mean ± SD (all such values)
[4] SDS, SD scores according to the German reference values for BMI
[5] Median: 25th and 75th percentile in parentheses (all such values)
[6] calculation based on the data of children with C19 steroid GC-MS reference values (3)
[7] no information on age at menarche for 4 girls and age at voice break for 5 boys.

Table 9. Urinary, nutritional and early-life related characteristics of the study sample (n=109)

	Boys n=54	girls n=55	P for difference[1]
Urinary[2]			
Urine volume (mL/d)	700 (579, 1017)[3]	617 (501, 734)	<0.05
∑C19 (µg/d)	444 (313, 609)	243 (167, 346)	<0.05
∑C19-SDS[4]	-0.20 ± 1.04[5]	-0.23 ± 0.91	NS
∑C21[6] (µg/d)	4220 (3646, 4725)	3421 (2864, 3783)	<0.0001
∑C21-SDS[6]	0.30 ± 0.91	0.64 ± 0.87	NS
Nutritional[2]			
Energy intake (MJ/d)	7.3 ± 1.0	6.3 ± 0.9	<0.0001
Vegetable Protein intake (g/d)	20 ± 5	17 ± 3	<0.05
[% of energy]	[4.6 ± 0.8]	[4.6 ± 0.9]	NS
Animal protein intake (g/d)	37 ± 9	30 ± 8	<0.05
[% of energy]	8.5 ± 1.7	8.0 ± 1.7	NS
Early life-related			
Birthweight (g)	3609 ± 466	3405 ± 355	<0.05
Gestational age (wk)	40 (40, 41)	40 (39, 40)	NS
Maternal overweight [n (%)]	17 (31.5)	15 (27.3)	NS
Full breast feeding for ≥ 2 wk [n (%)]	43 (79.7)	47 (85.5)	NS

[1] Sex differences were tested with unpaired t test for continuous variables and chi-square test for categorical variables
[2] values were derived from each individual´s arithmetic mean from one yr and two yrs before age at ATO, i.e., data represent average values approximately 1.5 yrs before ATO
[3] Median: 25th and 75th percentile in parentheses (all such values)
[4] Calculation based on the data of children with C19 steroid GC-MS reference values (3)
[5] Mean ± SD (all such values)
[6] SDS calculation based on the data of children with glucocorticoid reference values (80).

Table 10. Association of AAs (∑C19-SDS) with pubertal variables in 109 healthy children[1]

Outcomes	*β*	*SE*	*P*
ATO	-0.002	0.09	1.0
APHV	-0.13	0.10	0.20
Duration of pubertal growth acceleration	-0.12	0.03	0.002
Age at Tanner (B2_G2)	-0.29	0.11	0.009
Age at Tanner (PH2)	-0.65	0.12	<0.0001
Age at menarche /voice break[2]	-0.07	0.11	0.6

[1] Final multiple regression model (adjusted for sex, FMI-SDS, body surface related urine volume, total energy intake-SDS, animal protein intake (%), birthweight, gestational age, full breastfeeding ≥ 2wk, and maternal overweight) showing the independent associations of both predictors C19-SDS and animal protein intake (as % of energy intake)
[2] n=100 (50 girls).

Table 11. Sex stratified analysis for the association of AAs (∑C19-SDS) with age at Tanner stage 2 for breast (girls, n=55) and external genitalia (boys, n=54) development[1]

	Outcomes	β	SE	*P*
Model 1 [2]	Age at B2	-0.31	0.16	0.06
	Age at G2	-0.32	0.16	0.06
Model 2 [3]	Age at B2	-0.32	0.15	0.03
	Age at G2	-0.33	0.15	0.03

[1] Final multiple regression model (adjusted for FMI-SDS, body surface related urine volume, total energy intake-SDS, birthweight, gestational age, maternal overweight, and full breastfeeding ≥ 2wk) showing the independent associations of both predictors C19-SDS and animal protein intake (as % of energy intake)
[2] Conventional adjustment for the standard deviation score of fat mass index (FMI-SDS)
[3] FMI-SDS preadjusted for overall daily cortisol secretion (adrenocortical activity; for further details see statistical analysis).

The adjusted means of the ages at Tanner stage 2 (B_G and PH) for ∑C19-SDS categories and ages at take-off and peak height velocity for animal protein intake (%) categories are shown in **Figure 14**. Children with a higher AA secretion level had a 0.8 yrs earlier begin of breast or genital development and a 1.5 yrs earlier begin of pubarche than those with a lower AA excretion. Additionally, peak height velocity and the begin of the pubertal growth spurt occurred approximately 0.4 or 0.5 yrs earlier in high animal protein consumers.

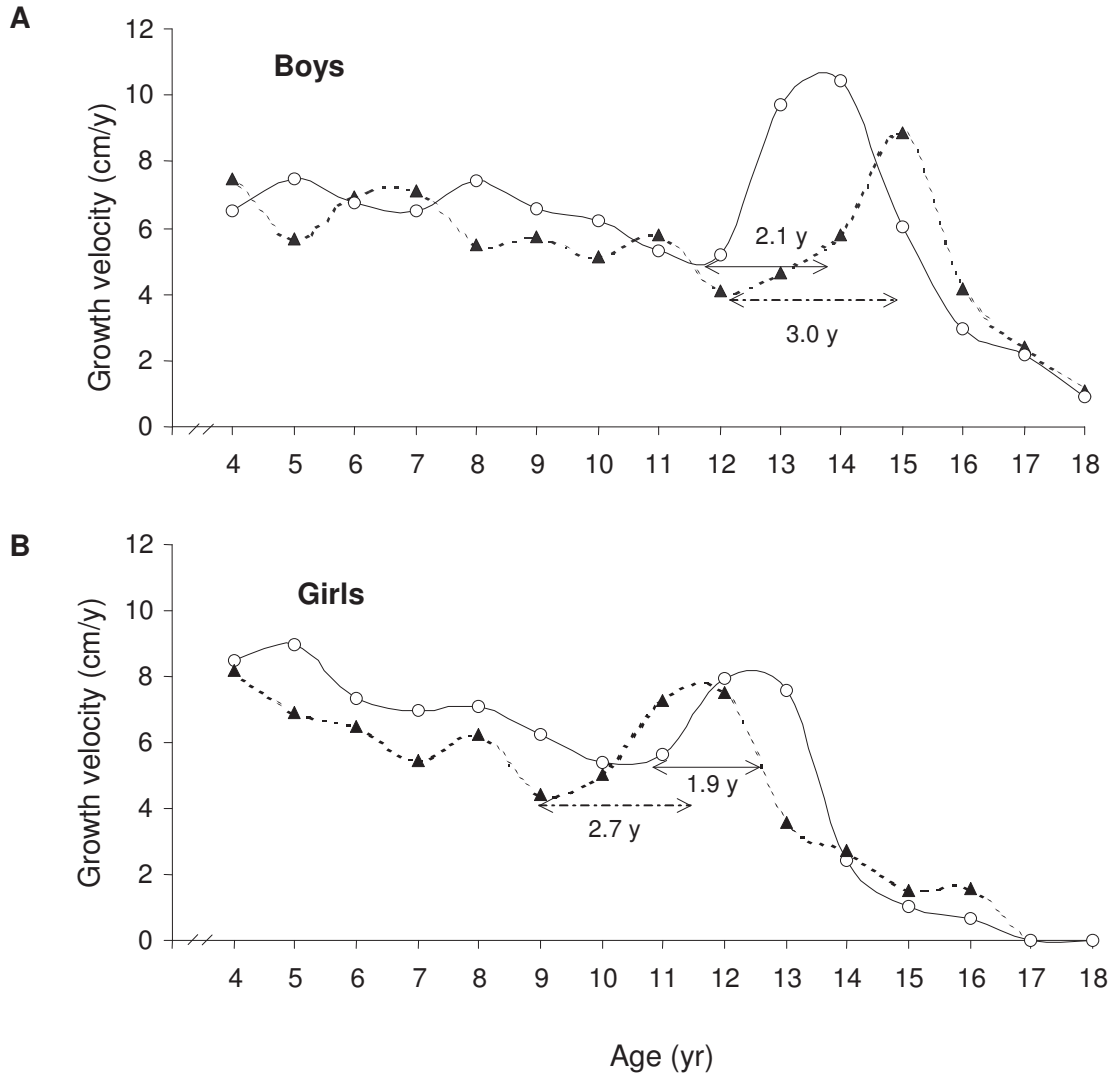

Figure 13. Individual growth velocity curves for children (A, boys; B, girls) with strong (—) and weak (- - -) adrenarche and corresponding durations of pubertal growth acceleration.
∑C19-SDS, boys: 1.01 vs. -1.92; girls: 1.73 vs. -1.53.

Table 12. Association of animal protein intake with pubertal variables in 109 healthy children

Outcomes		*β*	*SE*	*P*
ATO	Model 1[1]	-0.10	0.04	0.02
	Model 2[2]	-0.10	0.05	0.02
APHV	Model 1	-0.10	0.05	0.05
	Model 2	-0.10	0.05	0.04
Duration of pubertal growth acceleration	Model 1	-0.001	0.02	1.0
	Model 2	-0.01	0.02	0.7
Age at Tanner (B2_G2)	Model 1	-0.06	0.06	0.3
	Model 2	-0.08	0.06	0.1
Age at Tanner (PH2)	Model 1	-0.04	0.07	0.6
	Model 2	-0.07	0.06	0.3
Age at menarche/ voice break[3]	Model 1	-0.12	0.06	0.05
	Model 2	-0.12	0.06	0.07

[1] Model 1 was adjusted for sex, FMI-SDS, total energy intake-SDS, birthweight, gestational age, full breastfeeding ≥ 2wk, and maternal overweight)
[2] In Model 2, ∑C19-SDS was additionally adjusted apart from the covariates in Model1
[3] n=100 (50 girls).

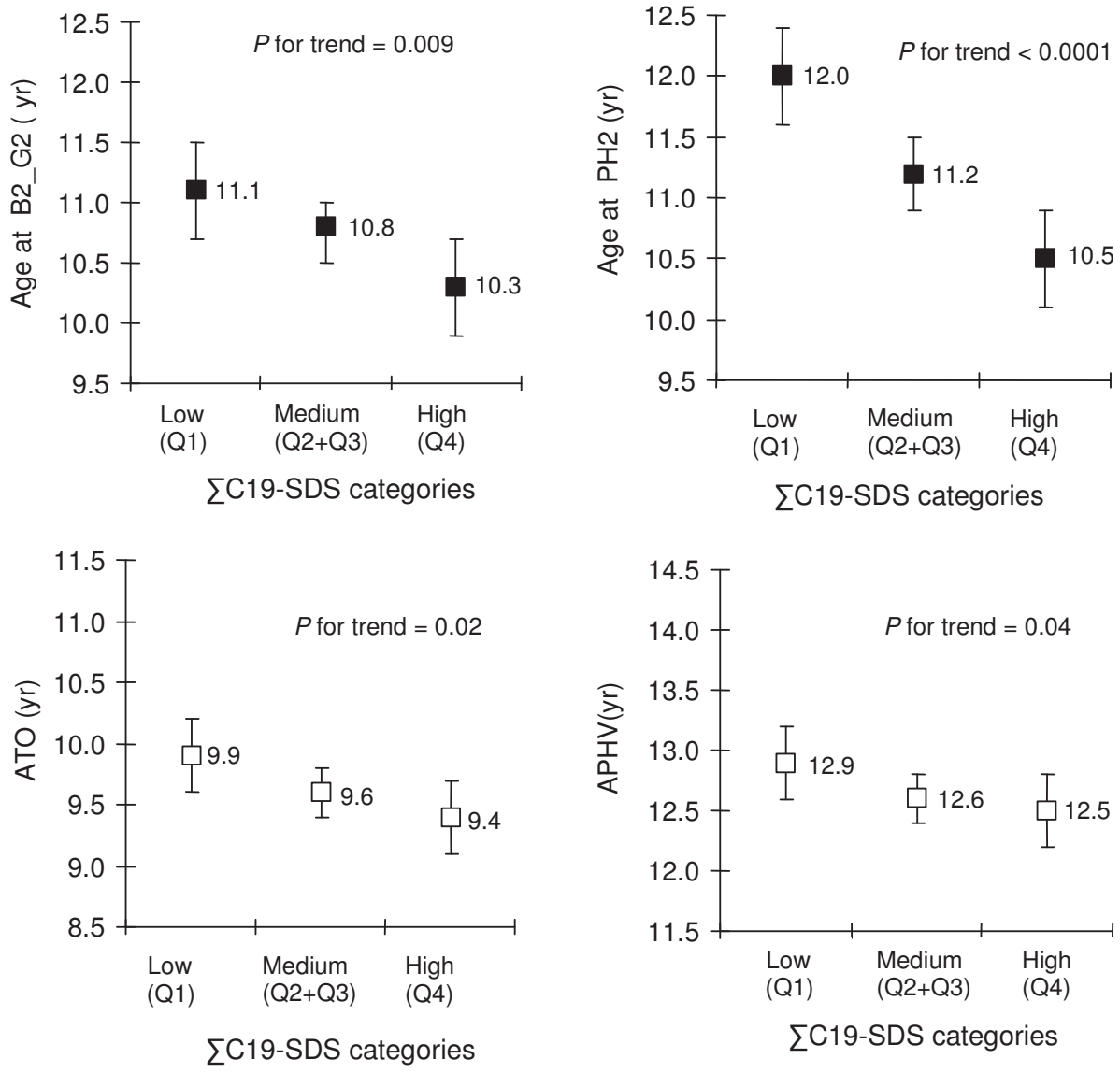

Figure 14. Ages at Tanner stage B2_G2 (A) and PH2 (B) by categories of SDS of urinary 24-h C19 steroid excretion rates; ATO (C) and APHV (D) by categories of animal protein intake.

Data are means (95 % CIs) adjusted for FMI, birthweight, sex animal, and protein intake (A, B) or C19-SDS (C, D). *P* for continuous trend refers to the *P* values obtained in linear regression models with C19-SDS and animal protein intake as continuous variables (Q, quartile; n = 109)

5.2.5 Discussion

Our prospective analysis of adrenarchal and nutritional influences on the timing of early and late pubertal markers provides observational evidence that adrenarche and dietary animal protein intake are independently and differentially involved in the modulation of pubertal development in healthy non-obese children. Higher prepubertal AAs may precipitate a shorter pubertal growth acceleration period and an earlier onset of breast and genital development in girls and boys, respectively.

In line with our earlier observation that the mid growth spurt (onset) around age 6-7 yrs is obviously not due to a preceding (or parallel) increase in AA secretion (227), AAs did not show a predictive association with the start of the pubertal growth spurt (ATO) either. However, children in the highest quartile of animal protein intake appear to experience ATO 0.5 yrs and APHV 0.4 yrs earlier than children with the lowest animal protein intake (Figure 2). These results extend our previous analyses (10) in that the associations of protein intake with the pubertal markers are obviously independent, i.e., not mediated by AAs.

Although dietary animal protein intake may increase adrenarchal androgen secretion in children (224) and urinary DHEA-S and 3α-androstanediol glucuronide excretion in adults (228), it appears evident that the significant associations of 24-h AA excretion rates with duration of pubertal growth acceleration and ages at B2_G2 and PH2 constitute independent AA influences and no pathway effects arising from higher dietary protein intakes. This can be deduced since (i) the association between animal protein intake and AAs is only modest (224) and (ii) animal protein intake was not (neither alone nor together with C19) significant in the respective regression models for the outcomes: duration of pubertal growth acceleration, age at B2_G2, and age at PH2.

It is generally assumed that adrenarchal androgens impact on pubic hair (17, 144, 229) and that premature pubarche is usually due to premature adrenarche, i.e., due to prepubertally markedly elevated AA levels (23, 226). In accord herewith, oral DHEA-S or DHEA substitution therapy in patients with atrichia pubis led to accelerated pubic hair growth (145, 230). However, to our knowledge, the impact of physiological adrenarche on the timing of pubarche has not yet been examined in healthy children. Accordingly, our present findings provide novel physiologically based information on the effect strength of AAs regarding the approximate time span between earlier and later onset of pubarche due to the influence of AAs. Children with a more pronounced adrenarche experience their first pubic hair growth, on average, one and a half yr earlier than children with only a moderate adrenarche.

The timing of puberty is highly variable and apart from genetic influences (231) also environmental factors including nutrition add to this variability. As explicitly discussed by Veldhuis et al. (232), among the nutritionally influenceable hormones, appropriate concentrations of IGF-I (and growth hormone) are required for a normal pace of maturation of at least some of the puberty variables. Since animal protein intake stimulates the growth promoting peptide IGF-I (55) in circulation, it is thus intelligible why this dietary component may be associated particularly with the growth-related puberty markers ATO and APHV.

Some of the authors who have favored the hypothesis that a higher level of AA may precipitate an earlier sexual maturation have proposed that here an increased peripheral aromatization of adrenal androgens to estrogens could be involved (11). Actually, in the male (pubertal and adult) – as in the female – estrogens constitute the primary determinant of the sex steroid negative feed back to the HPG axis. Accordingly, for an appropriate gonadal function in men a preceding conversion of testosterone to estradiol is essential (233). These data suggest that also before puberty onset and peripubertally, androgens – including AAs – are aromatized to estrone and estradiol in both sexes. How these estrogens and/or adrenarchal secretion products might interact with the neurobiological machinery that controls pulsatile GnRH release and thereby accelerate puberty onset is not known.

There is compelling evidence that various neuropeptides and neurotransmitters like kisspeptin and GABA are involved in the neurobiological mechanism holding and/or releasing the check on GnRH pulsatility during juvenile development (104). The GABA synapses and their receptors on GnRH neurons principally exert an inhibitory action on hypothalamic GnRH release (105). Thus, the peripubertal activation of the HPG axis requires attenuation of the GABA signal to allow for an increase in pulsatile LHRH release. In this regard the neurosteroid effects reported for DHEA-S (the quantitatively most important adrenarchal secretion product) may also play a relevant role for the GnRH neuronal network. DHEA-S has been shown to act as an noncompetitive antagonist of the GABA receptor complex in a number of studies summarized by Campbell (234). Campbell put forward the hypothesis that this inhibitory action on the GABA system may be one cause for the changes in social behavior, anxiety, and learning abilities seen from childhood onward (234). We would like to extend this hypothesis by suggesting that the physiological adrenarchal increase in secretion of weak sex steroids, especially DHEA-S, may provide one of the extra-hypothalamic signals that – probably via successive GABA inhibition – could accelerate puberty onset and modulate puberty duration.

Our findings that higher AAs predict an earlier pubertal development in healthy children are in agreement with the reports of a higher risk for early puberty onset in (i) girls with precocious pubarche (143, 235), (ii) patients with nonclassical 21-hydroxylase deficiency

(236), and (iii) patients with classical congenital adrenal hyperplasia (237). The fact that we found a significant variation of puberty timing due to physiologically varying AA levels in healthy children, who attained the pubertal milestones at normal pace (in accordance with other European studies (13, 238-241)) underscores the role of adrenarche as one physiological modulator that fine-tunes pubertal development.

It appears that this fine-tuning is primarily related to breast or genital development and pubarche and to a lesser degree to growth related puberty markers. Unfortunately, only few studies – largely focusing on children with premature adrenarche (242) – have examined the relationship between adrenarche and pubertal growth. Also studies directly examining the association between ATO and the onset of breast or genital development are widely lacking. However, analyses of longitudinal data from birth cohort groups of the Fels Longitudinal Study revealed an almost constant ATO in girls born 1929-1983 (243), whereas a convincing trend toward an earlier breast development onset has been reported for the corresponding time interval (244). Our findings of possible differential effects of AA and dietary protein intake on these developmental landmarks could be part of the explanation for the above probable dissociation between ATO and breast development.

A major strength of our present analysis is that we could examine prospectively collected 24-h urine samples and weighed dietary records (regarded as the most accurate technique for assessing dietary intake) that refer to a biological (1 yr and 2 yrs before ATO) and not a chronological age. A limitation may be the relatively small sample size, but it was sufficient to detect differences of 0.4 yrs in pubertal timing between 2 groups (high vs. low level of the respective predictor). The clinical relevance of smaller effect sizes may be questionable.

In conclusion, nutrition and the intensity of the adrenarchal process appear to be independently and differentially involved in the modulation of pubertal timing. While higher animal protein intake may be involved in an earlier attainment of ATO and APHV, higher AA levels may precipitate a shorter pubertal growth spurt and a notably earlier onset of breast and genital development in girls and boys, respectively.

5.3 Study III – Prepubertal glucocorticoid status and pubertal timing

5.3.1 Summary

Whether glucocorticoid (GC) impacts on the timing of puberty is not clear. We aimed to examine the associations of prepubertal GC status with early and late pubertal markers in 110 healthy children (55 boys), for whom 24-h urine samples, 3-day weighed dietary records and anthropometrical data 1 and 2 yrs before the start of pubertal growth spurt (age at take-

off; ATO) were available. GC status was assessed with the sum of the 7 quantitatively most important urinary GCs, indicated by ∑C21 (reflecting daily overall cortisol secretion level, determined by GC-MS analysis) and with the sum of urinary free cortisol and cortisone, indicated by UFF+UFE (reflecting potentially bioactive free GCs, measured by specific radioimmunoassay). Multitivariate regression analysis was performed to examine the associations of ∑C21 or UFF+UFE (mean of 1 and 2 yrs before ATO) with outcomes: ATO, age at peak height velocity (APHV), duration of pubertal growth acceleration, age at menarche/voice break, and ages at Tanner stage 2 for breast (girls) and genital (boys) development (B2_G2) and pubic hair (PH2). Girls with higher ∑C21 reached ATO 0.7 yrs later (p=0.01) and menarche 0.9 yrs later (p=0.007). ∑C21 tended to be also positively associated with age at B2 (p=0.1), PH2 (p=0.09), and PHV (p=0.07) in girls, but not in boys. UFF+UFE was not significant for any of the pubertal variables. In conclusion, prepubertal variation of GC secretion levels even in physiological range may already influence pubertal timing of girls but not of boys. Higher GC secretion level predicted notably later onset of pubertal growth spurt and menarche. Breast and pubic hair development as well as APHV tended also to occur later.

5.3.2 Introduction

Previous studies suggested that prolonged glucocorticoid (GC) treatment inhibits gonadal axis function (13, 14, 245), the changes of which can influence pubertal development. Regarding the influence of GC on pubertal timing in humans, only indirect evidence is available. Delayed pubertal development has been demonstrated in highly trained male and female runners (246, 247), female ballet dancers (248), and girls with anorexia nervosa (249), who have usually increased GC levels induced by chronic activation of the HPA axis (250, 251). Accordingly, GCs have been suggested to be a potential mediator for the delayed pubertal sexual maturation in such populations. In addition, both endogenous and exogenous GC, in excess, inhibit GH secretion (252), linear growth and skeletal maturation in children (15, 253) and might influence the subsequent onset or progression of pubertal growth spurt. To date, studies regarding the role of physiological variation of endogenous GCs on pubertal timing in healthy children are lacking.

Therefore we aimed to investigate whether a higher prepubertal GC level (assessed by using 24-h urinary GC measurements) predicted a later pubertal development (indicated by both somatic growth-related and sexual maturation-related pubertal markers) in healthy boys and girls. In addition, the potential influences of birth characteristics (112), fat mass (8, 9), adrenarchal androgens (254), and protein intakes (10, 254) were also considered.

5.3.3 Methods

Subjects

The number of children included in this analysis was derived as follows: A total of 376 subjects of the DONALD cohort had sufficient height measurements to allow plausible estimation of the puberty marker age at take-off of the pubertal growth spurt (ATO) (112). Of these, 111 children, who had not refused regularly assessment of Tanner stages, had also collected 24-h urine samples as well as dietary data at both time points (2 yrs and 1 yr) before ATO. One boy was excluded as outlier because of his extremely high cortisol excretion level. Hence, the subcohort analyzed herein included 110 prepubertal healthy children (55 boys). Information on age at menarche and voice break were available only for 51 girls and 50 boys repectively.

Measurement and assessment

ATO and APHV were estimated (chapter 4.4.1). From the derived data, we have calculated the duration of pubertal growth acceleration (APHV minus ATO), which can be used as an index for growth spurt duration and puberty duration (8, 238) Tanner stages for pubic hair (PH) and either breast (girls) or genital (external genitalia, boys) development (B_G) are assessed by one of the DONALD study pediatricians (chapter 4.4.2). In addition, children and/or parents are asked at each visit whether menarche or voice break has already occurred and the respective month and year are recorded. We have used age at testis volume ≥ 4 mL to define the onset of genital development in boys (Tanner stage G2). Herein, we denote Tanner stage 2 for breast and genital development in girls and boys, respectively as B2_G2.

Body weight, height and skinfold thickness were measured (see chapter 4.3). BMI and fat mass index (FMI = fat mass / height^2) were calculated. For each child, age- and sex-independent BMI SD-scores (SDS) were calculated by using the German reference curves (205). Total energy (kcal/d) intakes were derived from the mean of 3 days of dietary recording. Urinary nitrogen excretions (as biomarker of dietary protein intake) were measured by the method of Kjeldahl (255). Urinary steroid profiles were determined by GC-MS analysis (chapter 4.5.2). To assess daily GC and AA secretion rates, the 7 quantitatively most important urinary glucocorticoid and AA metabolites were summed and indicated by ∑C21 and ∑C19 respectively (see chapter 2.3.1). UFF and UFE were measured by specific radioimmunoassays (see chapter 4.5.2). The sum of UFF and UFE (UFF+UFE) was used to assess potentially bioactive free glucocorticoids (79, 172).

Statistical analysis

Descriptive data are given as means ± SD or median with interquartile range when appropriate. The anthropometric, urinary, and dietary variables were arithmetic mean values of the respective data from one yr and two yrs before ATO. Sex differences for sample characteristics were tested by using unpaired t-test.

Multiple linear regression analyses were used to analyze the associations between daily GC secretion rates (∑C21) or potentially bioactive free glucocorticoids (UFF+UFE) with pubertal variables (ATO, APHV, duration of pubertal growth acceleration, age at B2_G2, age at PH2, and age at menarche/voice break). In order to obtain age independent values, ∑C21 and ∑C19 were expressed as SDS of the published GC-MS reference values (3, 80). Accordingly, SDS of FMI and total energy intake were also calculated using the same reference population. Since to date, no established reference values for UFF+UFE of healthy children over a continuous age period are available, BSA-corrected UFF+UFE i.e. the ratio of UFF+UFE and BSA was calculated and included in the model. Confounding factors included in the fully adjusted regression models were: sex, birth weight, total energy intake-SDS, FMI-SDS, ∑C19-SDS, and nitrogen excretion residue (adjusted for total energy intake with residue method). In model with UFF+UFE as major predictor, urine volume (related to BSA) was additionally adjusted (93).

Analyses of covariance were performed to test for sex interactions. No sex-by-UFF+UFE interaction was observed for any of the outcomes, thus the multiple regression analyses with UFF+UFE as the major predictor were performed with boys and girls combined. Since sex-by-∑C21 interactions were observed for outcomes ATO and age at menarche/voice break ($p < 0.1$), an additional sex-stratified analysis was done when ∑C21 was included as major predictor.

5.3.4 Results

Anthropometric, urinary, and nutritional characteristics of the study sample are presented in **Table 13**. Boys and girls - at a biologically comparable prepubertal stage (on average 1.5 yrs before ATO) - showed approximately 1.5 yrs difference in chronological age. ATO, APHV, ages at B2_G2 and PH2 occurred earlier in girls than in boys. Only for B2_G2 the sex difference was not significant.

In models with boys and girls combined, neither UFF+UFE nor $\sum C21$ was significant for any of the pubertal outcome variables (**Table 14**). The fully adjusted models (**Table 15**, M4) of sex-stratified analysis for the association between $\sum C21$ and pubertal variables showed that, in girls apart from “duration of pubertal growth acceleration”, ∑C21 was positively associated with all the other pubertal variables. For outcomes ATO ($p = 0.01$) and age at menarche ($p = 0.007$), the associations were significant; for outcomes APHV ($p=0.07$), age at

B2 (p =0.1) and PH2 (p=0.09), positive trends were observed. For most of the outcome variables, these associations could not be identified until the confounding variables (especially biomarker of protein intake, i.e. nitrogen excretion) were considered (**Table 15**). In boys, ∑C21 was not associated with any of the pubertal variables.

Further, for girls, to illustrate the mean differences of ATO (**Figure 15, A**), APHV (**Figure 15, B**), age at B2 (**Figure 15, C**), and age at menarche (**Figure 15, D**) by low (<25th percentile), medium (≥ 25th and ≤ 75th percentiles), and high (≥ 75th percentiles) categories of ∑C21-SDS, least-squares regression analyses (adjusted for birth weight, total energy intake-SDS, FMI-SDS, ∑C19-SDS, and nitrogen excretion) were done. Girls with a higher GC secretion level showed 0.7 yrs later begin of pubertal growth spurt (ATO) and 0.9 yrs later begin of menarche than those with a lower GC secretion level.

Table 13. Characteristics of the study sample (n=110)

	Boys n=55	girls n=55	P for difference[1]
Age (yrs)[2]	8.8 ± 0.8[3]	7.3 ± 0.7	<0.0001
Anthropometric[2]			
Weight (kg)	30.8 ± 4.9	25.3 ± 4.8	<0.0001
Height (cm)	136.9 ± 6.8	126.2 ± 6.0	<0.0001
Body surface area (m²)	1.09 ± 0.11	0.94 ± 0.10	<0.0001
BMI-SDS [6]	-0.12 ± 0.76	-0.15 ± 0.86	0.9
Fat mass index (kg/m²)	2.2 (1.8, 2.9)[4]	2.2 (2.0, 2.8)	0.6
Fat mass index –SDS[7]	-0.42 ± 0.75	-0.17 ± 1.0	0.1
Urinary[2]			
Urine volume (mL/d)	700 (577, 1012)	617 (501, 734)	0.002
∑C21(µg/d)	4197 (3413, 4725)	3421 (2864, 3783)	<0.0001
∑C21-SDS[7]	0.30 ± 0.91	0.64 ± 0.87	0.05
UFF (µg/d)	15.7 (12.4, 18.0)	12.3 (9.1, 15.7)	<0.0001
UFE (µg/d)	30.7 (25.1, 35.3)	25.2 (20.8, 30.2)	0.001
UFF+UFE (µg/d)	45.6 (38.2, 52.7)	37.9 (30.4, 45.5)	0.0001
∑C19-SDS[7]	-0.18 ± 1.05	-0.23 ± 0.91	0.8
Nitrogen (mmol/d)	516 (455, 571)	411 (348, 457)	<0.0001
Dietary			
Total energy intake (MJ/d)	7.3 ± 0.9	6.3 ± 0.9	<0.0001
Total energy intake-SDS[7]	-0.30 ± 0.69	-0.06 ± 0.72	0.08

Early life-related			
Birth weight (g)	3593 ± 478	3405 ± 355	0.02
Gestational age (wk)	40 (40, 41)	40 (39, 40)	0.6
pubertal			
ATO (yrs)	10.4 ± 0.8	8.8 ± 0.6	<0.0001
APHV (yrs)	13.5 ± 0.9	11.8 ± 0.7	<0.0001
Duration of pubertal growth acceleration (APHV - ATO) (yrs)	3.1 ± 0.3	2.9 ± 0.4	0.0008
Age at Tanner stage (B2_G2) (yrs)	10.8 ± 0.9	10.6 ± 0.9	0.2
Age at Tanner stage (PH2) (yrs)	11.6 ± 1.1	10.8 ± 1.1	0.0003
Age at menarche/voice break (yrs)[7]	13.7 ± 1.1	13.1 ± 0.8	0.002

[1] Sex differences were tested with unpaired t test
[2] Values were derived from each individual´s arithmetic mean from one yr and two yrs before ATO, i.e., data represent average values approximately 1.5 yrs before ATO
[3] Mean ± SD (all such values)
[4] Median: 25th and 75th percentile in parentheses (all such values)
[5] SDS, SD scores according to the German reference curve for BMI (205).
[6] Calculated based on the data of children with C19 steroid GC-MS reference values (3, 80)
[7] No information on age at menarche for 4 girls and age at voice break for 5 boys

Table 14. Association of 24-h urinary glucocorticoid secretion marker (∑C21) and potentially bioactive free glucocorticoids (UFF+UFE) with early and late pubertal markers in 110 healthy children[1]

	UFF+UFE			∑C21		
Outcomes	***β***	***SE***	***P***	***β***	***SE***	***P***
ATO	0.02	0.28	0.9	0.12	0.09	0.2
APHV	0.20	0.31	0.5	0.06	0.10	0.5
Duration of pubertal growth acceleration	-0.48	0.44	0.3	-0.02	0.05	0.7
Age at Tanner stage (B2_G2)	0.48	0.40	0.2	0.20	0.12	0.1
Age at Tanner stage (PH2)	0.44	0.40	0.3	0.18	0.14	0.2
Age at voice break/menarche[2]	-0.46	0.43	0.3	0.03	0.15	0.8

[1]Results of multiple regression models adjusted for sex, birth weight, total energy intake-SDS, FMI-SDS, ∑C19-SDS, nitrogen excretion (as residual of total energy intake); in model with UFF+UFE, body surface related urine volume was additionally adjusted.
[2] n=101 (51 girls).

Table 15. Sex stratified analysis for the association between 24-h urinary glucocorticoid secretion marker (∑C21) and pubertal variables in 55 boys and 55 girls

Outcomes		boys			girls		
		β	SE	*P*	β	SE	*P*
ATO	M1[1]	-0.06	0.12	0.6	0.23	0.10	0.02
	M2 (M1+ FMI)[2]	-0.17	0.13	0.2	0.21	0.11	0.06
	M3 (M1+FMI+∑C19) [3]	-0.15	0.13	0.3	0.18	0.12	0.1
	M4 (M1+FMI+ ∑C19+N) [4]	**-0.02**	**0.14**	**0.9**	**0.34**	**0.13**	**0.01**
APHV	M1[1]	-0.08	0.13	0.5	0.03	0.11	0.8
	M2 (M1+ FMI)[2]	-0.16	0.14	0.2	0.10	0.13	0.5
	M3 (M1+FMI+∑C19) [3]	-0.11	0.14	0.4	0.12	0.14	0.4
	M4 (M1+FMI+ ∑C19+N) [4]	**-0.08**	**0.16**	**1.0**	**0.28**	**0.15**	**0.07**
Age at Tanner stage B2_G2	M1[1]	-0.13	0.14	0.3	-0.10	0.15	0.5
	M2 (M1+ FMI)[2]	-0.12	0.15	0.5	0.05	0.17	0.8
	M3 (M1+FMI+∑C19) [3]	-0.06	0.16	0.7	0.19	0.17	0.3
	M4 (M1+FMI+ ∑C19+N) [4]	**0.13**	**0.17**	**0.4**	**0.29**	**0.20**	**0.1**
Age at Tanner stage PH2	M1[1]	-0.22	0.16	0.2	-0.05	0.19	0.8
	M2 (M1+ FMI)[2]	-0.17	0.18	0.3	-0.01	0.22	0.9
	M3 (M1+FMI+∑C19) [3]	-0.05	0.17	0.8	0.31	0.20	0.1
	M4 (M1+FMI+ ∑C19+N) [4]	**0.05**	**0.19**	**0.8**	**0.39**	**0.23**	**0.09**
Age at Voice break /menarche[5]	M1[1]	-0.17	0.19	0.4	0.16	0.13	0.2
	M2 (M1+ FMI)[2]	-0.34	0.21	0.1	0.24	0.15	0.1
	M3 (M1+FMI+∑C19) [3]	-0.32	0.22	0.2	0.29	0.16	0.07
	M4 (M1+FMI+ ∑C19+N) [4]	**-0.30**	**0.25**	**0.3**	**0.51**	**0.18**	**0.007**

[1] Basic multiple regression model, adjusted for sex, birth weight, and total energy intake-SDS
[2] FMI-SDS was additional adjusted
[3] ∑C19-SDS was additional adjusted
[4] Final model. Nitrogen excretion (as residual of total energy intake) was additional adjusted
[5] 50 boys and 51 girls

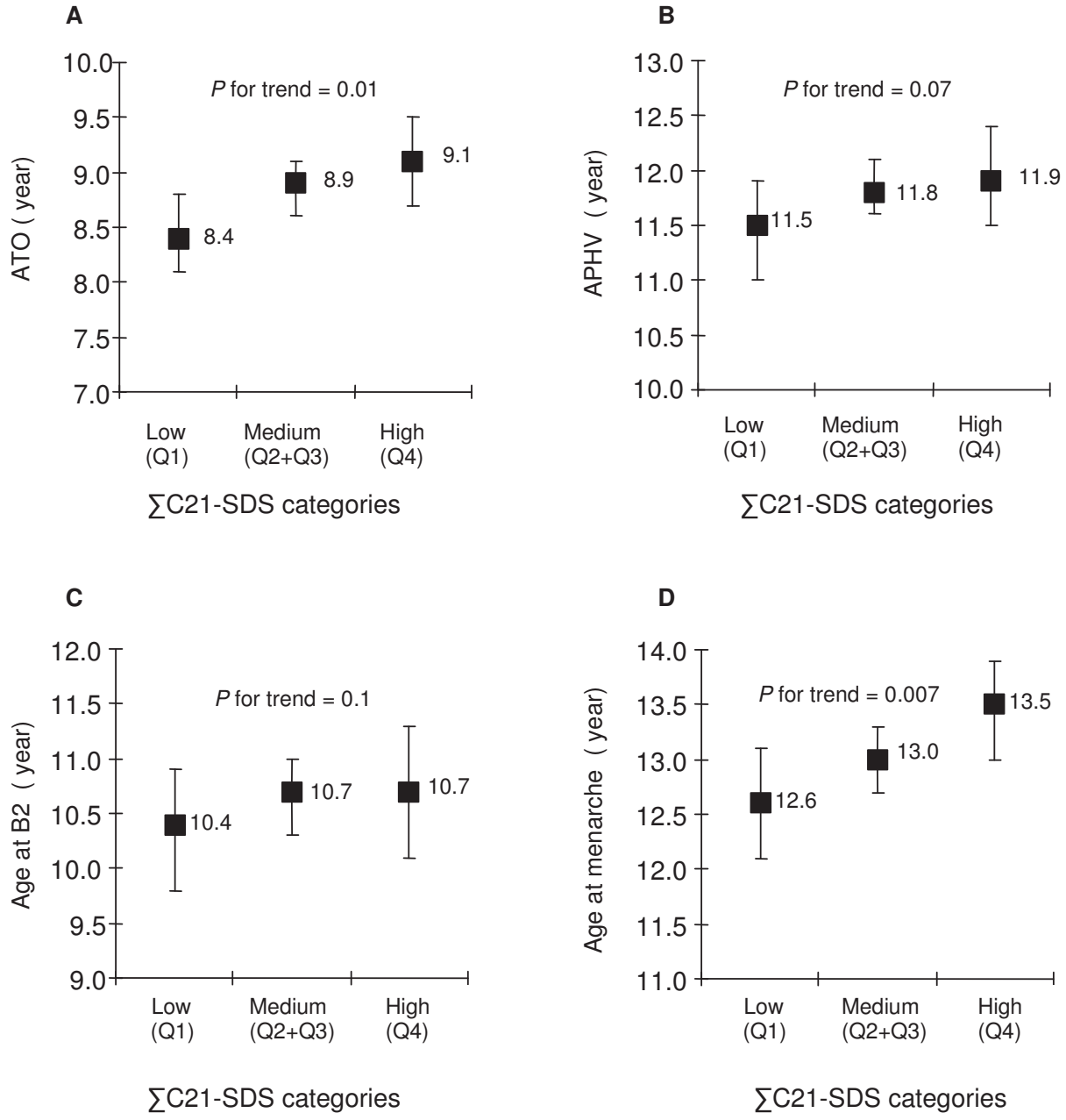

Figure 15. ATO (A), APHV (B), ages at Tanner stage B2 (C), and age at menarche (n=51) (D) of 55 girls by categories of SDS of urinary 24-h glucocorticoid secretion marker (∑C21)

Data are means (95 % CIs) adjusted for sex, birth weight, total energy intake-SDS, FMI-SDS, ∑C19-SDS, nitrogen excretion (as residual of total energy intake); *P* for continuous trend refers to the *P* values obtained in linear regression models with ∑C21-SDS as continuous variables (Q, quartile).

5.3.5 Discussion

This study is the first prospective study to investigate whether the variation of prepubertal GC status may be associated with the timing of somatic growth-related and/or sexual maturation-

related pubertal markers in healthy children. The GC status was assessed with two parameters derived from 24-h urinary GC metabolites: i) ∑C21, reflecting daily overall cortisol secretion of the adrenal gland (80) or adrenocortical activity; ii) UFF+UFE, the sum of urinary free cortisol and cortisone, reflecting potentially bioactive free GCs (172). We found that only ∑C21 was associated with pubertal timing in girls, but not in boys. Girls with higher prepubertal cortisol secretion levels reached ATO 0.7 yrs later and age at menarche 0.9 yrs later. In these girls, breast and pubic hair development as well as APHV tended also to occur 0.3-0.4 yrs later.

Cortisol, the main GC In humans, is one of the most principal effectors of the stress system. Chronic activation of the stress system may lead to an increased and prolonged secretion of cortisol and lead to suppression of growth hormone and/or IGF-I secretion (16, 252, 253). Delayed or arrested growth has been observed in children with Cushing's syndrome (253), or with stress-related mood disorders, such as chronic anxiety (256) and eating disorders (257-260). Delayed pubertal growth spurt (indicated by age at peak height velocity) was observed also in children with long-term oral GC therapy (261, 262). All these studies have been undertaken in ill children and their ages at onset of pubertal growth spurt (ATO) have not yet been examined or reported. Regarding the regulating role of GC for the pubertal growth spurt, no comparable study was available in healthy children. Our prospective study shows that even in the physiological range of GC variability, a higher prepubertal (exactly 1 and 2 yrs before ATO) endogenous cortisol secretion (∑C21) can already predict a later onset of the pubertal growth spurt in healthy girls, in whom APHV tended also to occur somewhat later.

Another adverse role of excess GC is its inhibitory effect on reproduction. GC act at multiple levels of the HPG axis, suppressing the gonadotropin- releasing hormone (GnRH) neurons (263), the pituitary gonadotropin secretion (245), and the gonadal hormone production (14). Rat experiment studies showed that GC can even repress the estrogen-stimulated growth of female sex hormone target tissues, i.e. inhibit uterine growth (13). In humans, suppression of gonadal function caused by chronic activation of the HPA axis has been demonstrated in highly trained runners (246, 247), ballet dancers (248), and girls with anorexia nervosa (249). Delayed menarche were observed in female balled dancers (248) and girls with anorexia nervosa (264), who demonstrated also elevated plasma concentration of cortisol. In the present study, we found that in healthy girls, higher GC secretion levels tend to predict a later occurrence of breast and pubic hair development; however the effects were not significant. The strongest effect of GCs was observed for age at menarche. Menarche, usually a late event in the sequence of pubertal changes is preferentially regulated by estrogens directly produced in the gonads. Therefore GCs, which may directly

inhibit HPG axis or the gonadal hormone production (14), showed also a relative strong effect on the age at menarche. On the other hand, pubic hair development in girls is generally considered to be regulated by adrenal androgens (∑C19) (254), which are, to the most part, independent of the activation of HPG axis. Breast development reflects the effect of estrogens, which may originate from the local conversion of adrenal androgens by aromatase highly expressed in breast tissues (265). Therefore, onset the breast development might rather be dependent on the overall adrenal androgen level and the local aromatase activity in breast tissues than ovary estrogen production.

Nevertheless, the effects of GCs on pubertal timing in girls were not observed when using UFF+UFE, an estimate of potentially bioactive free GCs, instead of ∑C21. We assume that UFF+UFE might rather reflect a more short-term stress response or GC activity than an overall adrenal GC secretion. The latter might rather be a result of a long-term adaptation process of HPA axis, especially if short-term determinants of GC activity like protein intake (266) have been controlled for. Prolonged or chronic stress results in suppressed gonadotropin secretion and inhibition of growth and reproduction but when the duration of the stress response is transient or acute, the effects are less clear (250). Previous studies have suggested that cortisol metabolic clearance may be enhanced in obese adults (267, 268) and children with higher body fat (211) and consequently their total cortisol secretion rates (∑C21) may also be increased. In the present study, the observed effects of ∑C21 on pubertal variables were independent of body fat mass, therefore here ∑C21 could be considered as an appropriate marker for individual adrenocortical activity.

Another interesting finding of the present study was that prepubertal cortisol secretion rates were only associated with pubertal events in girls but not in boys. Studies using animal models have clearly demonstrated that males and females respond differently to stress, which may be due, at least in part, to the different predominance of particular sex steroid influences in particular life periods (269). Various observations in female rhesus monkeys indicate that the ovaries, and particularly estradiol secretion, influence the effects of stress on LH secretion (reviewed by (270). In addition, it could be speculated that the higher androgen status already discernible in boys (271) might partly counteract some of the GC actions of males. However, sex comparison of the effects of stress on reproduction has not yet been made in humans. Whether the difference of sex steroid status between boys and girls (272) in prepubertal stage could explain the different response of somatic growth and sexual maturation to the variation of adrenal cortical activity in healthy children should be investigated in further studies.

The strength of the present study is that in the statistical analysis, most of the potentially important anthropometric, hormonal, and nutritional confounding factors could be considered.

The adjustment of them is necessary, since the specific effect of GC on pubertal timing could not be identified until we took these confounders, especially protein intake, into account (see model construction process shown in TABLE 3). A limitation may be the relatively small sample size, but it was sufficient to detect significant differences > 0.4 yrs in pubertal timing between 2 groups (high vs. low level of cortisol secretion).

In conclusion, prepubertal variation of endogenous glucocorticoid secretion even in the physiological range may already be relevant for pubertal timing of girls, but not of boys. A higher prepubertal glucocorticoid secretion level predicts notably later onset of pubertal growth spurt and menarche. Breast and pubic hair development as well as APHV also tend to occur later. Whether the difference of sex steroid status between boys and girls (272) in prepubertal stage could explain the different responses of somatic growth and sexual maturation to the variation of adrenocortical activity in healthy children should be investigated in the further study. For long-term outcomes like markers of pubertal timing, the rather short-tem GC activity parameter, i.e. UFF+UFE appears to be less predictive than the overall cortisol secretion index ∑C21.

5.4 Study IV – Prepubertal urinary estrogen excretion and its relationship with pubertal timing

5.4.1 Summary

Whether prepubertal estrogen production impacts on the timing of puberty is not clear. We aimed to investigate prepubertal 24-h estrogen excretion levels and their association with early and late pubertal markers. Daily urinary excretion rates of estrogens were quantified by stable isotope dilution/GC-MS in 132 prepubertal healthy children who provided 24-h urine samples 1 and 2 yrs before the start of the pubertal growth spurt (Age at take-off, ATO). E-sum3 (estrone+estradiol+estriol) was used as a marker for potentially bioactive estrogen metabolites and E-sum5 (E-sum3+16-epiestriol+16-ketoestradiol) for total estrogen production. Pubertal outcomes were ATO; age at peak height velocity (APHV); duration of pubertal growth acceleration (APHV-ATO), age at Tanner stage 2 for pubic hair (PH2), genital (G2, boys) and breast (B2, girls) development; age at menarche. Prepubertal urinary estrogen excretions (E-sum3 and E-sum5) were not associated with ATO, APHV, and age at PH2, but with duration of pubertal growth acceleration ($p < 0.01$) in both sexes. Girls with higher E-sum3 reached B2 0.9 yrs ($p=0.04$) and menarche 0.3 yrs earlier ($p=0.04$) than girls with lower E-sum3. E-sum3 was not associated with age at G2 in boys ($p=0.6$). For most pubertal variables the associations with E-sum3 were stronger than with E-sum5. In

conclusion, prepubertal estrogens may not be critical for the onset of the pubertal growth spurt, but influence its duration in both boys and girls. Prepubertal estrogen levels may already predict the timing of girl's menstruation and breast development, but do not appear to affect sexual maturation in boys.

5.4.2 Introduction

Children with early puberty are at risk for the development of hormone related cancers [e.g., breast (4, 5) and testicular cancer (6)] later in life. Recent studies (272, 273) suggest that the increased prepubertal sexual hormone exposures may account for the earlier onset of puberty.

Sensitive gonadotropin assays have shown that the pulsatile nature of LH and FSH secretion, which traditionally characterizes the onset of puberty, is present already before the appearance of pubertal physical signs with a relative low pulse amplitude (273-275). At the same time, a diurnal variation of circulating estradiol (E2) in prepubertal children has also been suggested (273). Increasing evidence points at a physiological importance of even low estrogen concentrations on somatic growth (81, 276) and sexual maturation (277, 278).

Until now, most studies, which address directly (273) or indirectly (277, 279, 280) the association between prepubertal endogenous estrogen concentration and pubertal onset – are based exclusively on measurements of prepubertal serum concentrations of E2. Although E2 is the most potent estrogenic steroid, less potent estrogens, estrone (E1) (70) and estriol (E3) as well as some of their metabolites (281, 282) also contribute to estrogenic activity. Additionally, E2 level measured in a single blood sample may not be representative of its 24-h secretion. Using stable ID/GC-MS, we could determine the urinary estrogen metabolites in 24-h urine samples of prepubertal children. Therefore, the first aim of the present study was to determine the daily estrogen metabolite excretion levels of healthy free-living boys and girls at a biologically comparable prepubertal stage i.e. 1 and 2 yrs before the onset of pubertal growth spurt (one of the earliest pubertal markers).

The second aim of this study was to investigate the association of prepubertal 24-h estrogen production with both somatic growth-related and sexual maturation-related pubertal markers in boys and girls, which had not yet been systematically investigated. In addition, the potential influences of birth characteristics (112), body mass (8, 9) and adrenarchal androgens (254) were also considered.

5.4.3 Methods

Subjects

A plausible estimation of ATO was achieved in a subset of 376 participants of DONALD study with sufficient height measurements [for details see reference (8, 112)]. Of these, 132

children had not refused regular assessment of Tanner stages and had also collected 24-h urine samples at both time points (2 yrs and 1 yr) before ATO. Complete information on birth characteristics were also available in all these children. Hence, the subcohort analyzed herein included 132 prepubertal healthy children (69 boys).

Measurement and assessment

ATO and APHV were estimated (chapter 4.4.1). From the derived data, we have calculated the duration of pubertal growth acceleration (APHV minus ATO) which can be used as an index for growth spurt duration and puberty duration (8, 238). Tanner stages for pubic hair (PH) and either breast (girls) or genital (external genitalia, boys) development were assessed by one of the DONALD study pediatricians (chapter 4.4.2). Testis volume was determined by palpation using the Prader orchidometer. We used age at testis volume ≥ 4 mL to define the onset of genital development in boys. Herein, we denoted Tanner stage 2 for breast and genital development in girls and boys, respectively as B2 and G2. In addition, girls or their parents were asked whether menarche has occurred since the previous visit, and if so, in which month and year. This information was available for 51 out of the 63 girls in this analysis.

Urinary estrogen and its metabolites were measured by stable ID/GC-MS method (168) (chapter 4.5.2) The detailed measurement method for adrenal androgens (3) and glucocorticoid metabolites (80) by GC-MS have been described previously. To assess daily GC and AA secretion rates, the 7 quantitatively most important urinary glucocorticoid and AA metabolites were summed and indicated by ∑C21 and ∑C19 respectively (chapter 2.3.1).

Statistical analysis

Percentiles of 24-h urinary estrogen metabolite excretion rates and the respective frequency of detection 1 yr and 2 yrs before ATO were calculated. Two metabolites 2-OHE2 and 2MeOE1 with a frequency of detection below 25% at both collection time points were not further considered in the following statistical analyses. Subjects with missing values at both time points were excluded. In subjects with one measurable value either at 1 yr or 2 yrs before ATO, the corresponding metabolite concentration of the respective other time point was set at the value equal to the limit of detection (LOD) divided by the square root of 2 (283, 284). Paired t-test was used to examine the differences of estrogens and their metabolites between 1 yr and 2 yrs before ATO.

To examine the association between prepubertal estrogen levels and pubertal timing, the sum of E1, E2, and E3 (E-sum3) was used as a marker for potentially bioactive estrogen

metabolites and the sum of E-sum3, 16-epiE3, and 16-OE2 (E-sum5) for total estrogen production. Preliminary analysis (**Figure 17**) showed that 120 children of our original sample (n=132), had applicable E-sum3 and E-sum5 data at 1 and 2 yrs before ATO, i.e., at least one of these E-sums' components had measurable concentrations > LOD. Thus, the subsequent analyses were performed in these 120 children.

For description of the sample characteristics, means ± SD or median with interquartile range are given. To obtain more stable individual's hormone values, means of 1 yr and 2 yrs before ATO were calculated and used in the following analyses. Sex differences for sample characteristics were tested with unpaired t-test.

To illustrate the relationships between E-sum3, E-sum5, ∑C19, and BMI as well as their associations with chronological age, a preliminary Pearson's correlation was run. Non-normally distributed E-sum3, E-sum5 and ∑C19 were (natural) Log transformed.

Analyses of covariance were performed to test for sex-by-hormone interactions. No sex-by-E-sum3 or sex-by-E-sum5 interactions ($p > 0.1$) were observed for any of the outcomes: ATO, APHV, duration of pubertal growth acceleration, age at Tanner stage B2_G2, and age at Tanner stage PH2. Accordingly, all the subsequent analyses were firstly performed with boys and girls combined. While somatic growth and pubic hair development are physical signs occurring in both sexes, breast development, menarche, and external genitalia development are sex specific. Therefore, sex stratified analyses were done with age at menarche and age at B2 as outcomes for girls and age at G2 as outcome for boys.

Least-squares regression analyses were used to analyse the associations of urinary estrogens with pubertal variables. The distribution of E-sum3 or E-sum5 was grouped into 3 categories: low (<25th percentile), medium (≥ 25th and ≤ 75th percentiles), and high (≥ 75th percentiles). The adjusted means were the least square means predicted by the model when the other variables were held at their mean values. In basic models (model 1), the respective pubertal variables were adjusted for sex only. Since BMI (8, 9) as well as adrenal androgens (11, 135, 254, 271) may modulate pubertal timing and both of them are also associated with estrogen levels, BMI-SDS and ∑C19–SDS [calculation based on reference (3)] were separately included as adjustment variable in two additional regression models. As suggested by previous studies (285), part of the variation of BMI is associated with the variation of adrenocortical activity, which itself is an important determinant of adrenal androgen secretion (207). As a consequence, body mass variation (via covariation with adrenocortical activity) partly reflects the potential C19 influence on the puberty outcomes (i.e., the part of C19 variation caused by adrenocortical activity). Therefore, we removed the influence of adrenocortical activity on BMI by preadjustment of BMI-SDS with total cortisol

secretion (∑C21, as an indicator for adrenocortical activity) before entering BMI-SDS in the regression models. By this way, we should be able to better identify the association of adrenal androgens and their converted products - estrogens with pubertal timing. Trend testing was performed with E-sum3 or E-sum5 as continuous independent variables. In addition, gestational age and birth weight were also considered. Since they did not modify the association of E-sum3 or E-sum5 with the respective pubertal outcomes, they were not retained in the models.

5.4.4 Results

Urinary estrogen metabolites –measurement assessment

Percentiles of daily urinary estrogen metabolite excretion rates and the respective frequency of detection 1 yr and 2 yrs before ATO are presented in . The detection frequencies of 3 major estrogens E1, E2 and E3 ranged between 30 and 50%. Among all the **Table 16** strogen metabolites, 16-epiestriol had the highest detection frequency (approximately 75%) and quantitatively, it was also the most predominant estrogen metabolite, which made up > 50% of total urinary estrogen excretion in healthy children before ATO. For most of the single estrogen metabolites as well as E-sum3 and E-sum5, values of 1 yr before ATO were significantly higher than 2 yrs before ATO (**Figure 17**).

Table 16. Percentiles (95% confidence interval) of detectable urinary estrogen metabolites (ng/d) 1 and 2 years before age at take-off (ATO) in 132 healthy children*

Variables	P10	P25	P50	P75	P90	FOD (%)
Estrone (E1)						
2y before ATO	<LOD	<LOD	<LOD	87 (19–202)	272 (217–485)	39
1y before ATO	<LOD	<LOD	<LOD	286 (194–462)	670 (599–1287)	44
Estradiol (E2)						
2y before ATO	<LOD	<LOD	<LOD	70 (<LOD–131)	191 (148–284)	28
1y before ATO	<LOD	<LOD	<LOD	64 (<LOD–127)	171 (148–259)	27
Estriol (E3)						
2y before ATO	<LOD	<LOD	<LOD	193 (154– 294)	393 (334–569)	41
1y before ATO	<LOD	<LOD	133 (<LOD–188)	261 (322– 408)	424 (559–670)	51
E-sum3						
2y before ATO	<LOD	<LOD (<LOD–11)	155 (90–205)	318 (449– 536)	569 (701–813)	68
1y before ATO	<LOD	77 (<LOD–171)	324 (258–454)	711 (566– 886)	1159 (909–2069)	77
16-epiestriol						
2y before ATO	<LOD	71 (<LOD–638)	879 (768–1004)	1183 (1085– 1293)	1459 (1331–1665)	75
1y before ATO	<LOD	485 (<LOD–753)	972 (882–1067)	1263 (1185– 1481)	1655 (1516–1831)	77

16-ketoestradiol						
2y before ATO	<LOD	<LOD	<LOD	139 (<LOD–244)	337 (256–1207)	27
1y before ATO	<LOD	<LOD	<LOD	247 (190–287)	540 (356–1327)	39
E-sum5						
2y before ATO	<LOD (<LOD–169)	612 (222–276)	1152 (979–1287)	1678 (1470– 1909)	2307 (2378–3298)	89
1y before ATO	282 (<LOD–550)	969 (612–1159)	1512 (1309–1649)	2187 (1920– 2568)	2903 (2628–3749)	92
2-hydroxyestradilol						
2y before ATO	<LOD	<LOD	<LOD	<LOD	183 (107–485)	17
1y before ATO	<LOD	<LOD	<LOD	<LOD (<LOD–109)	168 (137–281)	20
2-methoxyestrone						
2y before ATO	<LOD	<LOD	<LOD	<LOD	62 (<LOD–212)	11
1y before ATO	<LOD	<LOD	<LOD	<LOD	<LOD (<LOD–171)	9
E-total						
2y before ATO	<LOD (<LOD–191)	612 (232–841)	1196 (1041–1375)	1828 (1628– 2149)	2496 (2307–3667)	89
1y before ATO	301 (<LOD–607)	1071 (775–1233)	1574 (1385–1724)	2251 (1964– 2635)	3153 (2820–3855)	93

*FOD, frequency of detection; Esum3 , sum of E1, E2, and E3; E-sum5, sum of E-sum3, 16-epiestriol and 16-ketoestradiol; E-otal , sum of E-sum5, 2-hydroxyestradilol, and 2-methoxyestrone.
LOD, limit of detection; LOD for E1, E2, 2-hydroxyestradilol, and 16-ketoestradiol is 0.025 ng/mL; for E3 is 0.25 ng/mL; for 16-epiestriol is 0.2 ng/mL; for 2-methoxyestrone is 0.05 ng/mL

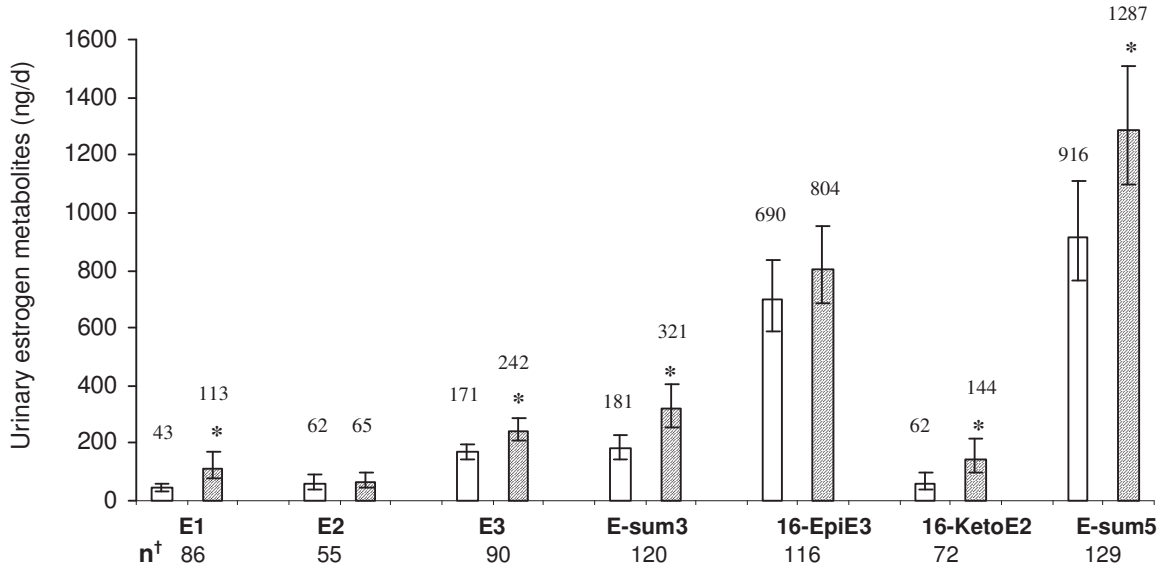

Figure 16. Differences of estrogen metabolites between1 and 2y before ATO.

Data are geometric means (95% CIs) of respective estrogen metabolites. E1, estrone; E2, estradiol; E3, estriol; E-sum3, E1+E2+E3; 16-EpiE3, 16-epiestriol; 16-ketoE2, 16-ketoestradiol; E-sum5 , E-sum3 +16-EpiE3+16-ketoE2. * Significant ($p<0.05$) differences between 1 and 2 y before ATO (tested with paired *t* test). † **number** of subjects with at least one measurable value in 1 or 2 y before ATO. For concentration below the limits of detection (LOD), a value equal to the LOD divided by the square root of 2 was used.

Association between estrogens and pubertal timing

Sample characteristics for investigating the associations of estrogens with early and late pubertal markers are presented in **Table 17**. Boys and girls, at a biologically comparable prepubertal stage (on average 1.5 yrs before ATO), showed approximately 1.5 yrs difference in chronological age, but no difference for E-sum3 and E-sum5. Boys showed a significantly longer "duration of pubertal growth acceleration" than girls. ATO, APHV, age at B2_G2, and age at PH2 occurred earlier in girls than in boys.

BMI, ∑C19, E-sum3 and E-sum5 were significantly positively correlated with each other (**Table 18**). BMI correlated stronger with ∑C19 (r=0.54) than with E-sum3 (r=0.33) or E-sum5 (r=0.22). E-sum3 and E-sum5 were not significantly correlated with chronological age in the present sample.

Associations of E-sum3 or E-sum5 with ATO, APHV, age at B2_G2, and age at PH2 are presented in **Table 19**. There were no associations between E-sum3 or E-sum5 with ATO. and APHV. Children with higher E-sum3 tended to reach B2_G2 earlier (p=0.07), and had an earlier age at PH2 (p=0.02) independent of BMI. After adjusting for ∑C19, these associations were attenuated and significance was no longer discernible. However, even after the adjustments for both BMI and ∑C19, E-sum3 was still associated with "duration of pubertal growth acceleration" (p= 0.002). Children with higher E-sum3 (before ATO) had a 0.3 yrs shorter "duration of pubertal growth acceleration". For most of the pubertal variables (but not for APHV) the associations with E-sum3 (marker of potentially bioactive estrogen metabolites) were stronger than those with E-sum5. The latter was only significantly associated with "duration of pubertal growth acceleration".

Sex-stratified analyses (after adjustment for BMI), showed that girls with higher E-sum3 reached Tanner stage B2 0.9 yrs earlier (p= 0.04) than girls with lower E-sum3 (**Figure 18A**). After adjusting for ∑C19-SDS, significance existed no longer (p= 0.2). Girls experienced their menarche 0.3 yrs earlier (p= 0.04) than girls with a lower E-sum3 (**Figure 18B**) independent of BMI and ∑C19. However, E-sum3 was not associated with age at G2 for boys (p= 0.6) (**Figure 18C**).

Table 17. Characteristics of the study sample for examination of association between daily urinary estrogen excretion and pubertal timing (n=120)

	Boys n=64	Girls n=56	P for difference[1]
Age (yrs)[2]	8.8 ± 0.9[3]	7.3 ± 0.7	<0.0001
Anthropometric[2]			
Weight (kg)	31.8 ± 6.2	26.2 ± 5.5	<0.0001
Height (cm)	137.0 ± 6.9	126.9 ± 6.3	<0.0001
Percentage body fat (%)	14.4 (12.1, 18.9)	15.3 (13.2, 19.7)	0.4
BMI (kg/m²)	16.8 ± 2.2	16.1 ± 2.1	0.1
BMI-SDS [5]	-0.09 ± 1.13	-0.13 ± 1.1	0.3
Urinary[2]			
∑C19 (µg/d)	451 (330, 624)[4]	249 (187, 398)	<0.0001
∑C19-SDS[5]	-0.06 ± 1.15	-0.10 ± 0.88	0.8
∑C21 (µg/d)	4246 (3669, 4760)	3453 (3092, 3956)	<0.0001
∑C21-SDS[5]	0.33 ± 0.90	0.75 ± 0.87	0.01
E-sum3 (ng/d)[6]	389 (244, 644)	346 (186, 525)	0.4
E-sum5 (ng/d)[7]	1566 (1028, 1964)	1424 (931, 1712)	0.3
Pubertal			
ATO (yrs)	10.4 ± 0.8	8.9 ± 0.6	<0.0001
APHV (yrs)	13.5 ± 0.9	11.7 ± 0.7	<0.0001
Duration of pubertal growth acceleration (yrs)[8]	3.1 ± 0.3	2.9 ± 0.4	0.0002
Age at B2_G2 (yrs)	10.8 ± 1.1	10.5 ± 1.0	0.1
Age at PH2 (yrs)	11.5 ± 1.2	10.7 ± 1.1	0.0004
Age at menarche (yrs)[9]	-	13.0 ± 0.8	-
Early life-related			
Birth weight (g)	3609 ± 448	3427 ± 352	0.02
Gestational age (wk)	40 (40, 41)	40 (39, 40)	0.7

[1] Sex differences were tested with unpaired t test for continuous variables
[2] values were derived from each individual´s arithmetic mean from one yr and two yrs before age at take off (ATO), i.e., data represent average values approximately 1.5 yrs before ATO
[3] Mean ± SD (all such values)
[4] Median: 25th and 75th percentile in parentheses (all such values)
[5] Calculated based on the data of children with C19 (3) and C21 (80) steroid GC-MS reference values
[6] Sum of estrone, estradiol, and estriol
[7] Sum of E-sum3, 16-epiestriol, and 16-ketoestradiol
[8] Duration between APHV and ATO
[9] No information on age at menarche for 5 girls

Table 18. Cross correlation between age, BMI, urinary adrenal androgen and estrogen secretion in 120 prepubertal healthy children

	Age (yrs)	BMI	∑C19	E_{sum-3}
BMI (kg/m²)	0.26*			
∑C19 (log e)	0.55***	0.54***		
E-sum3 (log e)	0.12	0.33**	0.46***	
E-sum5 (log e)	0.10	0.22*	0.26*	0.51***

BMI, body mass index; ∑C19, marker of urinary adrenal androgen secretion; E-sum3, sum of estrone, estradiol, and estriol; E-sum5, sum of E-sum3, 16-epiestriol, and 16-ketoestradiol.
*P <0.05
**P <0.001
***P <0.0001

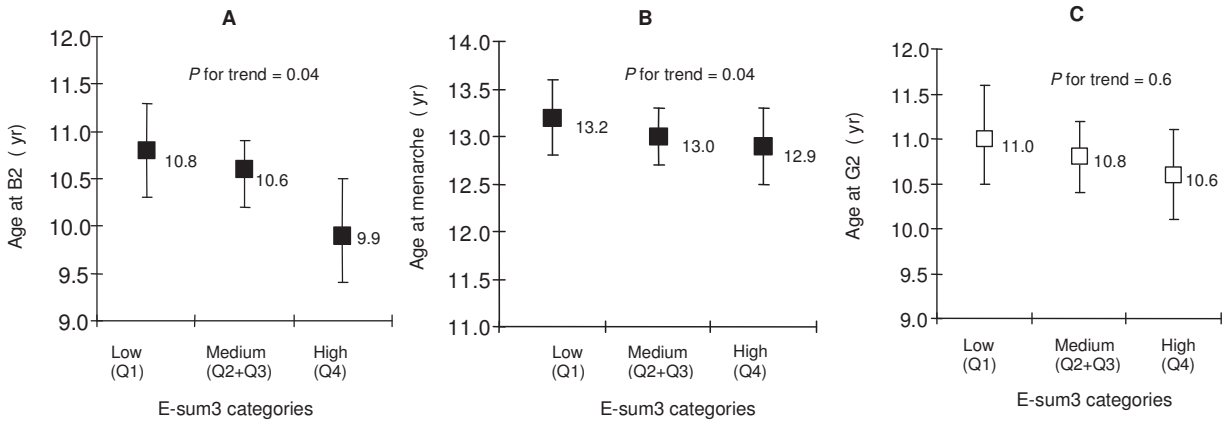

Figure 17. (A) Age at B2 (55 girls) and (B) Age at menarche (51 girls) (C) Age at G2 (62 boys) by categories of E-sum3 [mean of 1 and 2 yrs before ATO] in healthy children.

Data are means (95% CIs) adjusted for sex and BMI-SDS. *P* for trend refers to the *P* value obtained in linear regression models with E-sum3 as continuous variable, Q, quartile. Medians of E-sum3 in low, medium, and high group were 143, 371 and 750 ng/d respectively.

Table 19. Association of 24-h urinary estrogen excretions with early and late pubertal markers in healthy prepubertal children[1]

	Categories of E-sum3[1]				Categories of E-sum5[1]			
	low	**normal**	***high***	*P for trend*[2]	**low**	**normal**	***high***	*P for trend*[2]
ATO (n=120)								
Model 1 (M1)[3]	9.5 (9.2, 9.8) [4]	9.6 (9.5, 9.8)	9.6 (9.4, 9.9)	0.4	9.5 (9.3, 9.8)	9.6 (9.4, 9.8)	9.7 (9.4, 9.9)	0.7
M1+BMI-SDS[5]	9.5 (9.2, 9.8)	9.7 (9.5, 9.9)	9.6 (9.3, 9.9)	0.6	9.5 (9.3, 9.8)	9.6 (9.4, 9.8)	9.6 (9.4, 9.9)	0.8
M1+∑C19-SDS	9.5 (9.2, 9.8)	9.6 (9.5, 9.8)	9.6 (9.3, 9.9)	0.5	9.5 (9.3, 9.8)	9.6 (9.4, 9.8)	9.6 (9.4, 9.9)	0.8
APHV (n=118)								
Model 1 (M1)[3]	12.8 (12.5, 13.1)	12.6 (12.4, 12.8)	12.5 (12.2, 12.8)	0.1	12.7 (12.4,13.0)	12.6 (12.4, 12.8)	12.5 (12.2, 12.8)	0.09
M1+BMI-SDS[5]	12.8 (12.5, 13.1)	12.6 (12.4, 12.8)	12.5(12.2, 12.8)	0.2	12.7 (12.4,13.0)	12.6 (12.4, 12.8)	12.6 (12.4, 12.9)	0.1
M1+∑C19-SDS	12.7 (12.4, 13.0)	12.6 (12.4, 12.8)	12.6 (12.2, 12.9)	0.5	12.7 (12.4,13.0)	12.6 (12.4, 12.8)	12.6 (12.3, 13.0)	0.2
Duration of pubertal growth acceleration (n=118)								
Model 1 (M1)[3]	3.3 (3.1, 3.4)	3.0 (2.9, 3.1)	2.8 (2.7, 3.0)	<0.0001	3.2 (3.0, 3.3)	3.0 (2.9, 3.1)	2.9 (2.7, 3.0)	<0.0001
M1+BMI-SDS[5]	3.2 (3.1, 3.3)	2.9 (2.9, 3.0)	2.9 (2.8, 3.0)	<0.0001	3.1 (3.0, 3.3)	3.0 (2.9, 3.1)	2.9 (2.8, 3.0)	0.0001
M1+∑C19-SDS	3.2 (3.1, 3.3)	3.0 (2.9, 3.0)	2.9 (2.8, 3.1)	0.0008	3.1 (3.0, 3.2)	3.0 (2.9, 3.1)	3.0 (2.9, 3.1)	0.001
Age at B2/G2 (n=117)								
Model 1 (M1)[3]	10.9 (10.6, 11.3)	10.7 (10.4, 11.0)	10.2 (9.8, 10.6)	0.02	10.7 (10.4,11.1)	10.7 (10.5,11.0)	10.3 (9.9,10.7)	0.1
M1+BMI-SDS[5]	10.9 (10.5, 11.3)	10.7 (10.4, 10.9)	10.3 (9.9, 10.7)	0.07	10.7 (10.4,11.1)	10.7 (10.5,11.0)	10.4 (10.0,10.7)	0.2
M1+∑C19-SDS	10.8 (10.4, 11.2)	10.7 (10.4, 11.0)	10.3 (9.9, 10.7)	0.3	10.7 (10.3,11.0)	10.7(10.4,11.0)	10.5 (10.1,10.9)	0.4
Age at PH2 (n=116)								
Model 1 (M1)[3]	11.5 (11.1, 12.0)	11.1 (10.8, 11.4)	10.7 (10.3, 11.2)	0.02	11.2 (10.8,11.6)	11.2 (10.9,11.5)	10.7 (10.3,11.1)	0.3
M1+BMI-SDS[5]	11.5 (11.1, 11.9)	11.0 (10.7, 11.3)	10.9 (10.5, 11.3)	0.06	11.2 (10.8,11.6)	11.2 (10.9,11.5)	10.8 (10.4,11.7)	0.4
M1+∑C19-SDS	11.3 (10.9, 11.7)	11.0 (10.8, 11.3)	11.1 (10.6, 11.4)	0.6	11.1 (10.7,11.5)	11.2 (10.9,11.4)	11.0 (10.6,11.4)	0.9

[1] Categories of E-sum3 or E-sum5 were defined as follows: low (E-sum3< 25 percentile*),* normal (E-sum3≥ 25th percentile and ≤ 75th percentile), and high (E-sum3>75th percentile);
[2] From linear regression models with E-sum3 (log e) or E-sum5 (log e) as continuous variables;
[3] Model1 was adjusted for sex*;*
[4] All such values are least-squares means (95% CIs); [5] Adjusted for cortisol secretion (∑C21-SDS)

5.4.5 Discussion

This study is the first to determine the estrogen excretion levels in 24-h urine samples of healthy free-living boys and girls using ID/GC-MS at a biologically comparable prepubertal stage, i.e. 1 and 2 yrs before the onset of one of the earliest pubertal markers (ATO). Despite the relative low detection frequencies for some metabolites, we could identify significant increases for most of the estrogenic steroids from a younger biological age (2 yrs before ATO) to an older one (1 yr before ATO), which confirmed the biological validity of our measurements. Based on these measurements, we found that higher prepubertal urinary estrogen excretions (indicated by E-sum3 and E-sum5) were not associated with earlier ATO and APHV, but with a shorter duration between them, i.e. a shorter duration of pubertal growth acceleration. Girls with higher E-sum3 showed a 0.9 yrs earlier onset of breast development and 0.3 yrs earlier onset of menstruation than girls with lower E-sum3. Prepubertal estrogens did not appear to be related to the onset of genital development of boys and pubes hair development in both boys and girls.

Most of the previous studies on prepubertal estrogens (70, 81, 273) have examined blood samples. To our knowledge, only one study (286) has measured estrogen profiles in overnight urines of girls (8-14 yrs, n=20) and the estimated 24h total urinary estrogen excretions were 2.85-10.68 µg. In agreement with this data, 63 girls in our study at 1 yr before ATO (7-9 yrs), i.e. at a clearly younger average age, had correspondingly also a lower total estrogen excretion (E-sum5) ranging from 0.3 to 3.2 µg/d. The total estrogen excretion level of our 69 boys (1.6 µg/d) was nearly the same as that of girls (1.4 µg/d) of the same biological age. Chronologically boys were 1.5 yrs older than girls. Therefore, although we did not measure the estrogen excretion levels of boys and girls at the same chronological age, we can conclude that prepubertal girls have a higher estrogen production (on a chronological age basis) than boys. This is in line with the suggestions from the published blood data (82, 272, 286).

When comparing the 24-h excretion pattern of urinary estrogen metabolites with that of healthy premenopausal women, the relative amounts of E2 of prepubertal children appeared somewhat lower [also illustrated by Maskarinec et al. (286)] whereas 16-EpiE3 was obviously much higher (ratio of 16-EpiE3 to E2 was 10:1 in children vs. 1:2 in adult). The reason could be that in children, the bioactive estrogens like E2 may be preferentially metabolized to less active compounds and the metabolic pathway to 16-EpiE3 appears to dominate in children.

Although the role of such metabolites as 16α-hydroxylated estrogens with potentially breast carcinogenic effects (281, 282) on pubertal timing would have been particularly interesting, the low detection frequency limited the examination of the effects of these single metabolites. However, we could use the sum of 3 major estrogens E1, E2, and E3 (E-sum3)

to estimate daily excretion of the potentially bioactive estrogen fraction and the sum of the 5 major estrogen metabolites (E-sum5) as a surrogate for 24-h total estrogen production in a relatively large number of healthy children (n=120). This allowed us to investigate the potential roles of childhood estrogen production on the onset and progression of pubertal growth spurt as well as on the early and late sexual maturation markers.

E2 has been shown to be essential for the increase in growth velocity and epiphyseal fusion during pubertal growth in both sexes (74, 287). Nevertheless, it is not clear whether the elevation of estrogen levels in childhood may already contribute to or predict the increase of growth velocity and the subsequent earlier onset of pubertal growth spurt. In the present study, we did not find a significant association between prepubertal urinary estrogen excretions and ATO, which was not determined by adrenal androgen secretion either (254). Therefore, it seems possible that the start of the pubertal growth spurt may not be primarily determined by total endogenous sex hormone production. Whether a specific sex steroid metabolite, e.g. androstenediol (288-290) which can act as an estrogen or an androgen receptor agonist, or certain neuropeptide like kisspeptin (104) may play a role in the initiation of the pubertal growth spurt need to be further examined.

In this study, both E-sum3 and E-sum5 showed modest associations with a later growth spurt marker APHV, however after considering adrenal androgens and body mass, these associations vanished. These results suggest that, at that early prepubertal stage, the variations of adrenal androgen levels and body mass (8) may play a more import role in predicting APHV. Nevertheless, in our view, it does not mean that estrogen is not necessary for pubertal growth. On the contrary, adequate reserve of AA and body fat at early prepubertal stage may predict a higher estrogen level at a later prepubertal stage and during puberty, since adrenal androgens are important substrates converted to estrogens by aromatase, i.e., by the enzyme largely expressed in adipose tissues (291). The good correlation between ∑C19 and E-sum3 (r=0.46) also suggests that adrenal androgens could be the major sources of estrogens in prepubertal stage.

Clinical studies (71, 292) have shown that males with syndromes of estrogen deficiency experienced normal prepubertal growth and were able to sustain linear growth, but had no epiphyseal fusion. Therefore, it appears that one important physiological contribution of estrogens is not attributed to their stimulative but repressive function on linear growth due to their role in the closure of the epiphyseal plate (287). A shorter duration of growth spurt acceleration may indicate an earlier maturation of the epiphysis. Thus, based on our findings –(children with higher estrogen excretions before ATO had a shorter duration of growth spurt acceleration) we speculate that prepubertal estrogen levels may already predict the timing of epiphyseal maturation in puberty.

E-sum3 showed a significant association with age at PH2, however after including BMI or ΣC19, the association was no longer significant. This result together with recent longitudinal findings in healthy children (254) confirms clinical observations (222) that not estrogens but rather androgens are causally involved in the appearance and growth of pubic hair (pubarche). While somatic growth and pubic hair development are physical signs occurring in both sexes, breast development, menarche, and external genitalia development are sex specific. In boys, prepubertal urinary estrogen excretions did not appear to affect onset of their genital development. In girls, E-sum3 but not E-sum5 (reflecting total estrogen production) was associated with age at B2 and menarche. A reason could be that the quantitatively most dominant component of E-sum5 (i.e.,16-epiE3), may be a peripherally catabolized product without prior action in the breast and other estrogen-dependent tissues. Therefore, we propose that the estrogen effect on breast development and menstruation of girls might be better identified by using E-sum3 than E-sum5. Estrogen effects (at least in the early prepubertal stage) on breast development appear to be partly attributed to the local conversion from adrenal androgens, since after inclusion of adrenal androgens in the model, E-sum3 was no longer significant. However, the predictive effect of E-sum3 on the timing of menarche was independent of adrenal androgens, which did not show any association with age at menarche (254).

Nevertheless, the observed estrogen effects were weaker than we had expected. One reason could be the sample size (n=51-56 healthy girls), which is rather large compared with previous studies (273, 286), but may be not large enough to clearly differentiate the effects between two relatively closely related variables such as adrenal androgens and estrogens. Another reason could be that the time window which we investigated i.e. 1.5 yrs before ATO is relatively far away from the onset of breast development (nearly 3 yrs) and especially menarche (nearly 5.5 yrs). So, it would be interesting to know whether the estrogen effect is more pronounced in a later prepubertal stage i.e. directly before the start of breast development or menstruation. In this early prepubertal stage, even our very sensitive isotope GC-MS method could not detect all metabolites; therefore the frequency of undetectable hormone values may have introduced a correlation bias and also limited our ability to investigate the physiological role of single metabolites. The values of the pubertal markers are in accordance with values reported from other European studies (238-240, 293)

In conclusion, we found that daily estrogen production of boys is comparable with that of girls at the same biological developmental stages, i.e., 1 and 2 yrs before the onset of pubertal growth spurt. Higher prepubertal estrogen production may not be critical for the initiation of the pubertal spurt, but may increase the rate of growth spurt progression in both boys and girls. Prepubertal estrogen levels may already predict the age at menarche. However, the

effect of estrogens on the onset of breast development might be partly attributed to the conversion from adrenal androgens. Prepubertal estrogens do not appear to influence sexual maturation in boys.

5.5 Summarized results of Studies II-IV

The samples of **Studies II-IV** were derived from the same subpopulation of the DONALD Study and had the same outcome variables i.e. earlier and late pubertal markers. The major exposures were AAs (Study II), GCs (Study III), and Es (Study IV). The results showed: A higher AA secretion or E production was not associated with an earlier onset of pubertal growth spurt (ATO) or APHV, but with a shorter pubertal growth spurt in boys and girls. In girls, higher AA secretion or E production predicted a notably earlier onset of breast development; however age at menarche was only determined by Es. In boys, AA but not E was associated with genital development. GC may influence pubertal timing of girls, but not of boys. Higher GC secretion predicted notably later ATO and menarche. Breast and pubic hair development as well as APHV tended also to occur later. To discuss these results in a broader context in Chapter 6, they are summarized and presented in **Table 20**. In addition, the mean ages of the onset of pubertal events from the DONALD, Zürich Longitudinal (99, 100), and two cross-sectional representative German studies (240, 294) are summarized and presented in **Table 21** for boys (**A**) and girls (**B**). The comparison between these studies will be further discussed in Chapter 6.1.

Table 20. Influence of prepubertal GCs, AAs, and Es on pubertal timing (summarized results of Studies II-IV)

Outcomes	Girls			Boys		
	GCs	AAs	Es	GCs	AAs	Es
ATO	↑[1]	NS[2]	NS	NS	NS	NS
APHV	↑[3] trend	NS	NS	NS	NS	NS
Duration of pubertal growth acceleration	NS	↓[4]	↓	NS	↓	↓
Age at Tanner stage (B2_G2)	↑ trend	↓	↓	NS	↓	NS
Age at Tanner stage (PH2)	↑ trend	↓	NS	NS	↓	NS
Age at voice break/menarche	↑	NS	↓	NS	NS	NS

[1] Significantly ($p<0.05$) positively associated with outcomes
[2] Not significant
[3] Positively associated with outcomes with trend ($p<0.1$)
[4] Significantly ($p<0.05$) negatively associated with outcomes

Table 21. Mean ages of onset of pubertal events in a subgroup of children with hormone measurements from the DONALD Study: comparison with the Zürich Longitudinal Study and two cross-sectional representative German studies

A. Boys

	DONALD	**Zürich**[1]	**Former GDR**[2]	**KiGGS**[3]
	1985 begin (ongoing)	(1954-1980)	1985	2003-2006
	(n=54)	(n=45)	(n=8675)	(n=3932)
	Germany Longitudinal	Switzerland Longitudinal	Germany Cross-sectional	Germany Cross-sectional
ATO	10.4 ± 0.9 [4]	10.6 ± 0.8 [5]	ND	ND
APHV	13.6 ± 0.9 [4]	13.9 ± 0.8 [5]	13.0 –13.5[6]	ND
Age at G2 [Testicular volume(TV)]	10.9 ± 1.0 (TV ≥ 4mL)	11.8 ± 0.9 (TV ≥ 3mL)	ND	ND
Age at G2 (penis/scrotum)	10.7 ± 1.0	11.2 ± 1.5	11.1 ± 1.32	ND
Age at PH2	11.6 ± 1.1	12.2 ± 1.5	11.8 ± 1.40	10.9 (10.8-11.1)[7]

B. Girls

	DONALD	**Zürich**[1]	**Former GDR**[2]	**KiGGS**[3]
	1985 begin (ongoing)	(1954-1980)	1985	2003-2006
	(n=55)	(n=45)	(n=8689)	(n=3776)
	Germany Longitudinal	Switzerland Longitudinal	Germany Cross-sectional	Germany Cross-sectional
ATO	8.8 ± 0.6 [4]	9.0 ± 0.7 [5]	ND	ND
APHV	11.8 ± 0.7 [4]	12.1 ±1.0 [5]	11.0-11.5 [6]	ND
Age at B2	10.6 ± 0.9	10.9 ±1.2	10.9 ± 1.25	ND
Age at PH2	10.8 ± 1.1	10.4 ±1.2	11.4 ± 1.09	10.8 (10.6-10.9)[7]
Age at Menarche	13.1 ± 0.8	13.4 ±1.1	12.7 ± 1.09	12.8 (12.8-12.9)

[1] Zürich Longitudinal Study conducted between 1954-1989 in Switzerland (99, 100)
[2] A multicenter study of a cohort (8-17 yrs) derived from former German democratic Republic, initiated in 1985 y (294)
[3] nationwide German Health Interview and Examination Survey for Children and Adolescents (10-17 yrs) (240)
[4] estimated by using Preece-Baines 1 model
[5] estimated by using Preece-Baines 3 model
[6] average age range
[7] mean (95% CI), self-assessed by children and adolescents (n=3739 in girls

6. General Discussion

In this thesis, firstly the association of body composition and certain dietary intakes with AA production in healthy children was examined (**Study I)**. The result showed that among all the examined anthropometric (fat mass and fat free mass) and dietary (protein, fat and fiber intake, glycemic index and load) factors, only fat mass and animal protein intake may be relevant for the modulation of adrenarchal androgen secretion. Thereafter, the associations of prepubertal steroid hormones, i.e. AAs (Study II), GCs (Study III), or Es (Study IV), with early and late pubertal markers were investigated. The corresponding results have been summarized in Chapter 5.5.

Below, firstly, general methodological strengths and limitations concerning the DONALD study will be discussed. Subsequently, the central findings of the Studies I-IV will be debated. Finally, an overall conclusion and ideas for future research will be provided.

6.1 Methodological strengths and limitations

DONALD Study population

The DONALD Study population is not representative of Germany, displaying higher education and a generally high interest in nutrition and health-related topics (149). This is probably a result of its demanding design and the high number of follow-up visits. Because of the non-representativeness, extremes of dietary behavior or anthropometric development did not exist in the present analyses. On the one hand, this may have decreased statistical power to detect associations. On the other hand, the relative homogeneity of the DONALD Study makes confounding due to unmeasured socio-economic, lifestyle or environmental factors less likely. This possible advantage is rarely discussed, but has been noted by other researchers working with similarly homogeneous data sets (295). Also, it should be emphasized that representativeness is of minor importance when subject-related biological exposure-outcome associations are investigated, as it does not necessarily affect physiological relationships. With respect to generalization of the results to other populations, there would only be limitations if the DONALD participants were biologically different from other pediatric age groups (296). However, this is not assumed in this context.

Pubertal marker assessments

Differences in measurement or assessment methods for pubertal markers can affect findings and thus, comparability of findings between studies. Several approaches (such as graphic, mathematical-fitting, and smoothing-polynomial splines) are available for identification of the

timing and the magnitude of the adolescent growth spurt. They generally provide a description of the human growth curve, from which parameters such as ATO and APHV can be derived. The PB1 model (159), one of the mathematical-fitting methods used in Studies II-IV, has been questioned as it assumes a distinct form of the growth curve that may not fit children who differ from standard growth patterns. Therefore, applying a non-structural method, a smoothing spline function that provides a curve adapted to the individual height trajectories, was investigated in advance. This method does have, however, the disadvantage that a high measurement frequency is necessary (297). Intensive method comparison showed that the PB1 model in the DONALD population yielded better agreement between the estimated ATO and the raw height data (the plots of the yearly increments in height) as well as other puberty markers than the non-structural method. Until now, no reference ATO in the nationwide of Germany is available. The estimated ATO in the DONALD Study is similar with that of the Zürich Longitudinal Study in Switzerland (1954-1980 yr). APHV of the DONALD population is comparable with the general population of former German Democratic Republic (GDR) derived from a multicenter study of a cohort initiated in the mid-1980s (294) (**Table 21**).

Although the exact determination of age at menarche relies on valid recall by the parents or the adolescent, the average age at menarche of the DONALD population is very similar to the data seen in the German Health Interview and Examination Survey for Children and Adolescents (KiGGS population) (240). For girls, age at PH2 of the DONALD population is the same as that of the KiGGS population; for boys, it is 0.7 yrs later. This might be due to the different assessment methods. In the DONALD Study the PH staging was assessed by trained personnel. In doing so, significant variations between observers may be involved. However, this method provides generally more reliable information than self-assessment (298), as conducted in KiGGs Study. Self-assessment in boys and girls for Tanner staging tended to overestimate stage during the early puberty stages and underestimate in the later stages compared with the staging by a physician (244). Unfortunately, no data of breast and genital stages are available in KiGGs Study. The breast stage assessment of the DONALD Study was conducted by visually inspection, which is less accurate than the palpation method. The mean age at B2 of our study population (10.6 yr) is 0.3 yrs earlier than the GDR population (294). For boys, we used testis volume ≥ 4ml as cut-off for the onset of male genital development, which is more accurate than penis and scrotum stages (163-165) as determined in GDR population (294). The onset of male genital development of our study population 10.9 yr is 0.2 earlier than the GDR reference population (11.1 yr). Apart from the different measurement method, different time period of data collection can also contribute to these small differences of breast and genital stages between the DONALD and the GDR population (reference **Table 21**. in Chapter 5.5).

Urinary hormone assessments

As shown in the preliminary methodological work (Chapter 4.8), the excretion rates of certain steroid hormone metabolites (e.g. DHEA, 16α-hydroxy-DHEA, UFF, UFE) are dependent on urine volume. Total AA secretion marker (∑C19) appears only moderately confounded by urine volume, and total GC secretion marker (∑C21) dose not. Therefore, when only ∑C21 or ∑C19 is used in the analysis, the result would be little biased. However, when like in Study I, several steroidogenic enzyme activities are estimated using certain steroid metabolite ratios, urine volume should be a priori examined using statistical models. Although the urine-flow dependency of those single metabolites used in the metabolite ratios have not yet been explicitly examined. During the process of model constructions (Study I), we observed a notably variation of results with and without considering urine volume (data not shown). Based on the results of previous studies (92, 93) and the preliminary methodological work, we speculated that the strength of the urine flow-dependency of certain steroid hormone metabolites may be, at least in part, dependent on the proportion of the intra-renal production of them (details see Chapter 4.8.5). To date, the expressions of a spectrum of steroidogenic enzymes, such as 20α-HSD, 17β-HSD type 5, 3α-HSD type 3 for 20α-reduction and 3α-HSD, 3β-HSD type 2, 5α-reductase type 1, and 5β-reductase for ring A (C3, C5) reduction, have been identified in the kidney. The fraction of renally produced steroid metabolites is perhaps so small that it can be disregarded for the assessment of total AA and GC production. However, when using single metabolites or the ratio of them to estimate certain hormone or enzyme activities, the influence of urine volume should be considered.

Anthropometrical assessment

The detailed anthropometric data in the DONALD Study is collected according to standard procedures (154) by trained and quality-monitored personnel, who have been working for the DONALD Study for many years. A high degree of continuity and reduced inter- and intra-observer variability can thus be guaranteed. The comparison of the BMI distribution in DONALD with the German reference percentiles did not suggest major deviations (299). Prevalence of overweight as based on the German reference curves (205), were somewhat lower in the population of Study II-IV (aged 7-11 yrs, 9%) than in the representative KiGGS survey (7-10 yrs, 15%) (300).

Fat mass was estimated through skinfold measurements, which are more susceptible to measurement error than specialized, research-based techniques, such as DEXA, and may underestimate body fatness (301). But rigorous training and regular experience, as is the case in the DONALD Study personnel, can reduce intra- and inter-observer variability

notably. Besides, in prepubertal DONALD participants, strong associations were observed between FFM estimated from skinfold measurements and independent measures of muscularity such as 24-h creatinine excretion, grip force, and forearm muscle area as assessed by peripheral quantitative computer tomography analysis (302).

The usage of BF% has been criticized because it adjusts for weight, but could still be confounded by height and is thus not an independent index of body fatness (303). Instead, it has been suggested to adjust FM for fat free proxies of body size such as height (303). In **Study I**, FM as one of the important explanatory variables was not prior adjusted for height; however it was indirectly adjusted through including age as a covariate. In this study age was strongly associated with body height, therefore to avoid multicollinearity, in the final model we included only age, of which the adjustment is obligatory for the outcome AA secretion. In studies **II-IV**, the outcome ATO (and APHV) was itself derived from height measurements, the adjustment for height in multivariate analyses would hence be inappropriate. Therefore, FMI (= FM/ body height^2 , in kg/m^2) introduced by Maynard et al (304) was calculated and included in the model.

Quality of dietary assessment

The quasi-gold standard within “traditional” dietary assessment methods, i.e. 3-day weighed dietary records, is part of the DONALD schedule. The recent published validation study (305) confirmed that the weighed dietary records in DONALD provide solid estimates of total protein intake. Previous analyses of DONALD sub-samples, albeit not explicitly designed for validation purposes, had already indicated good validity of the dietary data. In one paper, Remer et al. examined the contributions of recorded intakes of protein-rich animal food groups (milk, eggs, meat, fish) to 24-h urinary iodine excretion and observed contributions (per 100 g or 1 liter of intake) which reflected almost precisely the average iodine content of these food groups as reported in food tables or after chemical analysis (216). For example, in 6-12 yrs old DONALD children a consumption of 1 liter of milk explained an increase in daily iodine excretion of 70 µg and in the same time period average iodine content of 70 µg/L was directly quantified in milk samples.

6.2 Interpretation and implication of study results

6.2.1 Nutritional status and adrenarche

In Study I, we have demonstrated that body fat mass may relevantly influence prepubertal adrenarchal androgen status. In addition, animal protein intake may also make a small contribution. As mentioned in chapter 5.1.5, one strength of our study is that we attempted to

account for the influences of various steroidogenic enzymes in the form of specific urine metabolite ratios. Although metabolite ratios as global indicator for enzyme activities are not accurate enough to identify tissue-specific pathway enzymes, through which nutrition or nutrition associated hormones may act, the inclusion of the enzyme activity estimates of them in the multivariate analysis can reduce the genetic-related inter-individual variability of AAs. In doing so, the relative small contribution of dietary factors could be better identified also with cross-sectional design. For example, in Study I, the effect of animal protein intake was not identified until inclusion of the estimate of 17α-hydroxylase, one of the most important steroidogenic enzymes.

In Study I, we have only examined the contribution of nutritional status on AA secretion in prepubertal children, because it is difficult to apply the same model in pubertal children. One reason is that after the pubertal onset, ΣC19 comprises also the increasing amount of gonadal steroid and therefore is not a specific indicator for androgens originated from adrenal gland. Another reason is that, for the estimation of 3β-HSD and 17α-hydroxylase activities, we had to use particular progesterone metabolites, which could be confounded by the phase of menarche cycle of pubertal girls.

In nutritional epidemiology, it is common that rather small contribution or effect size is obtained. However, in view of the fact that dietary factors, in contrast to genetically determined factors, can be easier modified, the potential contribution of them should not be disregarded. The biological meaning of animal protein intake on AA secretion should be examined more specifically by using diet experiments. When the effect of it is confirmed by experimental studies, a reduced consumption of animal products could be recommended for example for individuals with higher androgen- induced acne vulgaris.

The contribution of body fat on AA secretion that we observed in Study I, was much greater than that of animal protein intake. However, based on the available observational studies, we could still not conclude, whether the increased FM induces the increased AA secretion or the increased AA secretion causes the fat accumulation. Experimentally induced weight loss in adults results in decreased urinary AA excretion (39) and DHEA (S) replacement therapy causes no change in body weight (306, 307). These evidences support that, to some extent, FM may be one of the “causes” and not the ”effect” of AA increases. Nevertheless, a differentiation of body weight into FM and FFM is required.

Altogether, prepubertal animal protein intake and body fat mass appear to be not only relevant for pubertal development but also for adrenarche. As summarized in Chapter 2.4.3, steroid hormones (AA, GC, or E) as peripheral signals may mediate the link between

nutritional status and the timing of puberty. The DONALD Study provided a good data basis for the investigation of the potential roles of steroid hormones on different pubertal markers.

6.2.2 Prepubertal steroid hormones and pubertal timing

Associations of prepubertal AAs, GCs, and Es with early and late pubertal markers were examined in **Study II, III, and IV,** respectively. According to the results (summarized in **Table 20** in Chapter 5.5), we could not make a general statement about the role of prepubertal steroid hormones in the timing of puberty, because the effects of them appear not only to be vary between sexes, but also between different pubertal markers.

Somatic growth-related pubertal markers

In study III, we found that GC may influence the timing of pubertal growth spurt of girls, but not of boys. The mechanism of this sex dimorphism is not clear. In girls, higher GC secretion, as a marker of higher stress activity, was associated with later onset of pubertal growth spurt (ATO) and APHV (with trend), but not with duration of growth acceleration. This suggests that the regulating role of GCs on longitudinal bone growth before ATO and between ATO and APHV are in the same direction. On the contrary, higher AA or E levels do not appear to significantly influence ATO and APHV, but may be associated with a shorter duration of growth acceleration. This perhaps reflects the integrative biphasic actions of AAs or Es in different growth stages. Before ATO, AAs or Es may promote longitudinal growth indirect through interfering with GH/IGF-1 axis; however the effect may be so small that it can not be identified in a small sample of a healthy population. Once pubertal growth spurt begins, the repressive function of Es on linear growth due to their direct role in the closure of the epiphyseal plate might be dominant and this may result in reduced growth acceleration potential. Until now, no direct growth repressive function of AAs have been suggested, the effect of AAs on pubertal spurt duration might be mediated by their conversion products Es. However, this reduced growth acceleration potential or duration does not necessarily affect final height, owing to the considerable variation of growth before ATO (308).

Sexual maturation-related pubertal markers

Although the concerns about sexual precocity appear to be much greater in girls than in boys, these issues must be addressed in both sexes, in a comprehensive and comparative perspective. Appearance of pubic hair is a common secondary sexual characteristic of males and females. The role of increase of AAs (adrenarche) on pubic hair development, i.e. ***pubarche*** (143-145) has been established. The strength of the temporal acceleration of pubarche by a stronger versus a less pronounced adrenarche in healthy children was quantified in the current thesis (Study II) for the first time. We found that children with a higher

AA secretion had a 1.5 yrs earlier begin of pubic hair development. In boys, the first external sign of sexual maturation is usually not the appearance of pubic hair, but an increase in testicular volume ≥ 4 mL (162). In the current thesis, we found that only AAs may be relevant for genital development of boys; GC and E may be not. It has been suggested that androgens secreted from adrenal gland could act on GnRH neurons via binding with their receptors expressed in type 1 astrocytes (133), resulting in the activation of HPG axis.

The commonly used markers of female pubertal timing are ***Tanner stage B2*** and ***menarche***. Since the age at menarche is relatively easy to ascertain and least affected by observational errors (309), it is often considered to be a crude indicator for the puberty onset of female. In fact there exists dissociation between Tanner stage B2 and menarche (121). Menarche is the endpoint of a complex sequence of maturational events and primarily controlled by hypothalamic-pituitary-ovarian maturation, whereas many peripheral signals can stimulate breast development independently of hypothalamic-pituitary-ovarian maturation (121). Therefore, these two events might show different responses with different sensitivity to the influences of environmental or peripheral signals. In line with this theory, we observed in the current thesis that age at menarche occurred earlier in girls with higher E (Study IV) and lower GC levels (Study III) in prepubertal stage, but that it was not dependent on AA secretion (Study II). Although breast budding is induced by estrogenic action, the association between Es and age at B2 appeared to be dependent on AA levels and body fat mass. This is plausible, since AAs as substrates and fat tissue as the location for aromatase (68) provide the important sources for the local production of estrogens in breast tissues. Thus, the origin of the Es that initiate the breast development may not primarily originate from ovary production. Similarly, although breast development tended also to occur somewhat later in girls with higher GC secretion levels, the effect was rather moderate and not significant. Hence, factors that influence age at menarche are not necessarily relevant for breast development, too.

With respect to the predictive effects of prepubertal i) AAs on public hair, breast, and genital development in Study II; ii) GCs on ATO (girls) and age at menarche in Study III; and iii) Es on breast development and age at menarche in Study IV, differences of 0.3~1.5 years between the highest and the lowest quantile of the respective steroid hormones are in fact large enough to indicate biological meaningfulness. However, because of the lack of comparable studies to date, further clinical implications should be confirmed in other populations.

Issues about the studies design of Study II-IV

The study populations of Study II-IV were derived from the same subpopulation of DONALD Study, in whom plausible data of pubertal variables i.e. ATO, APHV, and Tanner stages as well as 24-h urine samples at both time points (2 yrs and 1 yr) before ATO were available. These hard criteria have both advantages and limitations for the data analysis as well as for the interpretation of the study results.

On one hand, the mean levels of two 24-h urine measurements (i.e. 1 and 2 yrs before ATO) provided a more stable assessment of individual hormone levels than that obtained from a single urine sample. In addition, the concomitant examination of both somatic growth-related and sexual maturation-related pubertal markers in the same study sample makes the direct comparison of hormones' effects on different pubertal markers possible.

On the other hand, although the sample size is large enough to identify the significance of the relative strong predictors of certain pubertal markers, it is difficult to compare the effects between the examined steroid hormones, body mass, and dietary factors in a combined analysis, especially when sex stratification is obligatory. In addition, the complexity of the physiological relationship between these steroid hormones and their interplay with body fat mass makes it difficult to conclude that the role of them are independent with each other. Further, in order to examine the role of steroid hormones also for the onset of one of the earliest pubertal markers (i.e. ATO), we had selected the time windows for the hormone measurements at 1 and 2 yrs before ATO, which is relatively far away (nearly 5 yrs) from the onset of the later puberty markers such as APHV, age at menarche or voice break. This may reduce the probability to identify the role of sex steroid hormones, since the further relevant increases of them occur at and after ATO. For example, in this thesis, we did not consider the potential role of testosterone, one of the most important gonadal androgens. The reason is that the detection frequency of it in this early prepubertal stage is even lower than that of Es. Thus, an additional consideration of testosterone would reduce the sample size and consequently the power to identify the effects of other predictors of interest.

6.3 Conclusions and Perspectives

Body fat mass may relevantly influence prepubertal adrenarchal androgen status and animal protein intake may also make a small contribution to children's AA-secretion levels. Prepubertal variations of steroid hormones (AAs, GCs, and Es) in physiological range appear to be differently involved in the modulation of pubertal timing in healthy children. Higher AA and E secretion may not be critical for the earlier onset of pubertal growth spurt and APHV, but precipitate a shorter pubertal growth spurt in boys and girls. In girls, higher AA or E production predicts a notably earlier onset of breast development; however age at menarche

appears to be only determined by Es. In boys, AAs but not Es are relevant for genital development. GCs may influence pubertal timing of girls but not of boys. Higher GC secretion, as a marker of higher stress activity, predicts notably later onset of pubertal growth spurt and menarche. Breast and pubic hair development as well as APHV may also tend to occur later. It appears that GCs may counteract the role of Es in the sexual maturation of girls, especially regarding age of menarche. In addition, the negative associations of animal protein intake with ATO, APHV, and age at menarche were not mediated by AA secretion.

The public health relevance of the puberty timing mainly stem from the fact that it is potential intermediary factor of the association between childhood influences (e.g. steroid hormone status) and later adult disease, such as diabetes (310), breast cancer (4), and cardiovascular disease (311). Therefore, it will be crucial for future analyses to evaluate whether prepubertal steroid hormone levels are associated with additional outcomes other than pubertal markers, such as cardiovascular disease markers (e.g. blood pressure) or insulin sensitivity, in order to see whether any effect persists beyond puberty. In addition, since higher prepubertal GC level in physiological range may predict later pubertal growth spurt in girls, it is a challenge to know whether the final height of such girls could also be affected.

7. References

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List of Publications and Presentations

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Shi L, Wudy SA, Buyken AE, Hartmann MF, Hoffmann P**,** Remer T
Prepubertal urinary estrogen excretion and its relationship with pubertal timing
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Remer T, Shi L, Buyken AE, Marser-Gluth C, Hartmann MF, Wudy SA.
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Daily urinary free cortisol and cortisone excretion is associated with urine volume in healthy children.
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Shi L, Wudy SA, Buyken AE, Hartmann MF, Remer T.
Adrenarchal androgens, prepubertal macronutrients intake and pubertal timing.
47. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung (DGE), 11.12.03.2010 Jena, Deutschland, Proc Germ Nutr Soc 14: 5.

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