Association of childhood DHEAS concentration, pubertal development and DNA methylation at puberty-related genes

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Abstract

Objective. High concentrations of DHEAS often precede premature puberty, and sometimes polycystic ovary syndrome (PCOS). We hypothesized that the underlying mechanisms might involve DNA methylation. As an indicator of the downstream effects of DHEAS, we looked for associations between prepubertal DHEAS concentration, pubertal-progression, and DNA methylation at puberty-related genes in blood cells.

Design. Blood methylome and DHEAS concentration at 7.5 and 8.5 years, respectively, were analyzed in 91 boys and 82 girls. Pubertal-development data were collected between 8.1 – 17 years (all from UK birth cohort, Avon Longitudinal Study of Parents and Children; ALSPAC).

Methods. Correlation between DHEAS and pubertal measurements was assessed by Spearman correlation. DHEAS association with methylation at individual CpGs or regions was evaluated by linear regression, and nearby genes examined by enrichment analysis and intersection with known puberty-related genes.

Results. Boys and girls with higher childhood DHEAS concentrations had more advanced pubic hair growth throughout puberty; girls also had advanced breast development, earlier menarche and longer menstrual cycles. DHEAS concentration was associated with methylation at individual CpGs near several puberty-related genes. In boys, 14 genes near CpG islands with DHEAS-associated methylation were detected, and in girls there were 9 which included *LHCGR* and *SRD5A2*; *FGFR1* and *FTO* were detected in both sexes.

Conclusions. The association between DHEAS and pubertal development, as reported previously, suggests a physiological connection. Our novel findings showing that DHEAS concentration correlates negatively and linearly with DNA methylation levels at regulatory regions of key puberty-related genes, provides a mechanism for such a functional relationship.

Significance statement: Premature adrenarche is often associated with early puberty, and increased likelihood of girls developing PCOS. While interaction with the reproductive axis is strongly indicated, adrenarche is not essential for sexual maturation, and these connections are poorly understood. Our analysis of ALSPAC cohort data confirmed association between DHEAS concentration at 8.5 y, and subsequent pubertal development in girls. Taking a novel approach to focus on DNA methylation at genomic regions most likely to impart function, we found negative linear correlation between DHEAS and methylation levels at key puberty-related genes. Beyond indicating that adrenarcheal DHEAS concentrations might predict rates of pubertal development in girls, and possibly comprise an early indicator for PCOS, our study provides an underlying mechanism connecting these developmental milestones.

Introduction

Prior to puberty, through apparently distinct regulatory mechanisms, adrenarche signals development of the adrenal gland *zona reticularis* and its production of androgens, primarily dehydroepiandrosterone, (DHEA) and the sulfated, DHEAS. DHEAS and DHEA concentrations are correlated and both reflect adrenal development and adrenarcheal progression[1]. Adrenal androgen synthesis starts as early as 3 y, can be measured in the circulation by ~6 y, and peaks during early adulthood[2–6]. The manifestations of adrenarche result from this increase in androgens, and include growth of pubic ("pubarche") and axillary hair, body odor, oily skin and acne. If these clinical signs are evident before 8 y in girls and 9 y in boys, or DHEAS levels are >40 µg/dl at these ages, adrenarche is considered premature[7].

Patients with various pathologies have demonstrated that adrenarche is not essential for sexual maturation[8–12], although advanced puberty often occurs in congenital adrenal hyperplasia[13,14]. Furthermore, healthy girls with premature adrenarche characteristically experience earlier puberty[15–24]. This coordinated timing might be due to common activators, such as obesity, birth size and accelerated early-life growth[17,25–27], or because these androgens facilitate maturation of

the HPG axis. In support of the latter, the very high concentrations of DHEA and DHEAS allow their binding to steroid receptors, even though their affinity is much lower than that of the cognate ligands[28]; they can also be converted to more potent sex steroids[28]. Girls with premature adrenarche are at higher risk for developing polycystic ovarian syndrome (PCOS)[21,29–31], and postnatal administration of DHEA is a well-established animal model for PCOS[32]. The adrenal androgens thus appear to facilitate activity of the HPG axis, though the underlying mechanisms are unclear.

One possible consequence of high adrenal androgens in early life might be through altering DNA methylation. DNA methylation patterns change throughout childhood and adolescence[33–36], and following DHEA treatment in mice[37,38] and cultured cells[39]. Further, a cohort-based study revealed differential methylation at individual CpGs in girls whose DHEAS concentrations were defined as "high" or "low"[40], without examining differences across the spectrum of concentrations normally present in the population. Still, any function for altered methylation at individual CpGs is ambiguous, given that genomic regions regulating gene activity are likely subject to similar modification[41,42].

We hypothesized that DHEAS concentrations in early childhood are associated with: (i) pubertal timing and/or progression, and (ii) altered DNA methylation at specific genes involved in this developmental transition. We looked for correlations across the entire range of DHEAS concentrations in children of adrenarcheal age, and considered the association between DHEAS and methylation both at specific CpGs and in regulatory regions of puberty-related genes. Our approach uncovered variation in DNA methylation of likely biological significance comprising a possible molecular mechanism underlying the connection between adrenarche and pubertal development.

Materials and Methods

ALSPAC cohort

The original Avon Longitudinal Study of Parents and Children (ALSPAC) cohort[43,44] consists of pregnant women resident in Avon, UK with expected delivery dates between 1/4/1991-31/12/1992, who were invited to take part in the study. Of the initial pregnancies, 14,676 fetuses resulted in 14,062 live births and 13,988 children alive at 1 year of age. When the oldest children were ~7 years old, the sample was bolstered with eligible cases who had failed to join the study originally. The total sample size using any data collected after the age of seven is 15,447 pregnancies, resulting in 15,658 fetuses, of whom 14,901 were alive at 1 year of age.

This cohort comprises questionnaires, clinical measurements and biochemical measurements ("variables") from mother-child pairs at several timepoints; the study website contains details of all available data through a searchable data dictionary and variable search tool (http://www.bristol.ac.uk/alspac/researchers/our-data/). We used puberty-related questionnaires (publicly available at ALSPAC data dictionary:

https://www.bristol.ac.uk/alspac/researchers/our-data/questionnaires/puberty-questionnaires) filled by mother or child; fasting blood measurements at 8.5 years; behavioral questionnaires filled by teachers of the children at 6 years; and other variables derived from these and other data (exact variables used and form of collection in Table S1; more details are publicly available in ALSPAC data dictionary). Some data were missing.

The genome-wide DNA methylation data was from fasting blood samples of the same children at 7.5 years of age, measured using Illumina Infinium HumanMethylation450 BeadChip (450 K) arrays as part of the Accessible Resource for Integrated Epigenomic Studies (ARIES) project[45]. From this cohort, 91 boys and 82 girls had both DNA methylation data and DHEAS measurements and were analyzed in this study.

Ethical approval and informed consent

Ethical approval was obtained from the ALSPAC Ethics and Law Committee and Local Research Ethics Committees (NHS Haydock REC: 10/H1010/70). Consent for biological samples was collected in accordance with the Human Tissue Act (2004). Informed consent for use of data collected via questionnaires and clinics was obtained from participants following recommendations of the ALSPAC Ethics and Law Committee at the time. At age 18, study children were sent 'fair processing' materials describing ALSPAC's intended use of their health and administrative records and were given clear means to consent or object via a written form. Data were not extracted for participants who objected, or who were not sent fair processing materials. The study was performed in accordance with the principles of the Declaration of Helsinki.

Analysis of variables correlated with DHEAS concentrations

Variables provided by ALSPAC with only missing values, or less than three unique values (nominal) are not suitable for correlation analysis and were removed. The remaining 227 variables in girls and 223 in boys were analyzed by Spearman correlation which, together with rho, p-value and n (number of samples), were calculated for each variable pair using the "rcorr" function in the "Hmisc" package in R. The full list of variables analyzed and their method of collection are in Table S1. Spearman correlation was chosen since it is robust for variables not normally distributed and for ordinal parameters which are prevalent in the data.

CpG site and region methylation analysis

We adjusted beta values for cell-type composition using a regression-based approach[46]. We used annotations from the Illumina manifest "IlluminaHumanMethylation450kanno.ilmn12.hg19" to include only CpGs located in CpG island (CpGI) shores and shelves which are known to be most dynamic[47]. We performed linear regression between beta values of each CpG site methylation (dependent variable) and the log-transformed DHEAS concentration (independent variable), using "cpg.assoc" function from the R package "CpGassoc", and adjusted p-values by the Benjamini & Hochberg method. Enrichment

analysis for methylation of CpGs with p-value <0.01 was performed using the "gometh" function in the "missMethyl" package in R and the -log10(p-value) was calculated.

We also grouped CpGs located in shores and shelves of the same island, based on the "Island_Name" annotation from the Illumina manifest, and looked for linear associations between methylation in these groups and log-transformed DHEAS concentration. Groups with the number of CpGs higher than 20% of the number of samples (n-value) were excluded to avoid overfitting and spurious correlations. Linear regression between methylation at each CpG (dependent) within each group, and the log-transformed DHEAS concentration (independent) was performed. Groups were chosen if methylation of >20% of their CpGs was significantly associated with DHEAS (p-value <0.05), and for at least half of these significant sites, the correlation was in the same direction (positive or negative) (Fig. S1). Enrichment analysis for genes annotated near the most significant regions was performed using "gometh", and -log10(p-value) calculated, as above.

Functional analysis of annotated genes with known puberty-related function

The nearest gene to each CpGI was assigned based on the Illumina manifest. For islands with more than one annotated gene, all genes were taken for analysis. These genes were intersected with a list of puberty-related genes, generated using "Geneshot", and a PubMed search for the term "puberty", to create a literature-based list of relevant genes, where "AutoRIF" was chosen as the resource[48].

Results

DHEAS concentration and BMI at age 8.5 y were similar in girls and boys

In 8.5-year-olds, mean DHEAS concentrations were similar (p>0.05) in boys (27.12 ± 2.45 mcg/dl; n=91) and girls (27.59 ± 2.99 mcg/dl; n=82: Fig. S2A). Mean BMI at 8.1 y was also similar (p>0.05) between sexes (16.84 ± 0.44 in boys and 16.64 ± 0.46 in girls: Fig. S2B). There were 15 boys who, according to World Health Organization (WHO) guidelines[49], were overweight (BMI >17.5), six of whom were obese (BMI >19.7), while two were underweight (BMI <13.3). In girls, eight were overweight (BMI >17.8), two of them obese (BMI >20.6), and two underweight (BMI <12.9). BMI data are partial (57/91 boys, 42/82 girls) and therefore BMI was not accounted for in our linear regression models.

Known and novel variables were found to correlate with DHEAS concentration

We examined all variables to identify traits associated with DHEAS concentration at 8.5 y. In females there were 36, and in males 32 variables that correlated significantly with DHEAS (p-value <0.05); 12 were common to both sexes (Table S2). Androstenedione was the most

significantly correlated in both sexes and, as previously reported[50,51], SHBG was negatively correlated (Table S2).

In both sexes, DHEAS concentration was negatively correlated with age at peak height velocity, and positively correlated with height at 11.6 y (in girls also at 8.1, 9.6, 10.6 and 13.1 y). It was positively correlated with weight at 9.6, 10.6, 11.6 and 13.1 y (in boys also at 8.1 y); and BMI in girls at 9.6 and 11.6 y, and in boys at 10.6 and 17 y. This accords with previous reports that children with higher DHEAS are more frequently overweight[18], and the finding that level of boys' participation in vigorous activities was negatively correlated. DHEAS also correlated positively with early-life caloric intake at 3, 7 and 13 y in both sexes, and with triglyceride levels. In boys, it correlated negatively with birthweight and IGFBP1, and positively with IGF1 and proinsulin, and in girls, with leptin (Table S2).

The number of traumas experienced between ages 0-17 y was correlated with DHEAS concentration at 8.5 y, though this was positive in boys and negative in girls. For girls, this included number of traumas between 0-5 y, degree to which the child had many fears at Year 6 in school, and the Adverse Childhood Experience (ACE)[52] extended score (0-13) and categories (low, low-mid, mid-high, high). These findings suggest that traumatic experiences might affect adrenarcheal timing, and/or that DHEAS might impact fearfulness and anxiety.

Verbal ability at age 6 y was positively correlated with DHEAS only in boys. Boys also had more social difficulties associated with higher DHEAS concentrations, being negatively correlated with "degree to which the child was generally liked by his peers", and "degree to which he shared readily with others"; and a positive correlation with "degree to which the child was bullied" (from the Strengths and Difficulties Questionnaire (SDQ) peer problems score: Table S2). These parameters are consistent with reported DHEAS effects on childhood social interactions and aggression[53,54], and brain development[55–58].

Girls with higher DHEAS concentration at 8.5 y proceeded to have more advanced pubertal development

At 8.1 y, 92.6% of girls were Tanner Stage 1 for pubic hair growth; the rest were Stage 2. Pubic hair growth at 8.1 y was correlated with DHEAS at 8.5 y (Fig 1A,B). Breast development was Tanner Stage 1 for 83.6% of girls at 8.1 y and the rest were Tanner Stage 2 (Fig 1A); this was not correlated with DHEAS (Fig 1F). None of the girls reported menarche at this age.

DHEAS concentration at 8.5 y also correlated with pubic hair growth at later timepoints (11.7, 13.1, 14.6 y; Figs 1C-E), and with subsequent breast development (at 9.6, 10.7, 11.7 y; Figs 1G-I). It was negatively correlated with age of menarche (Fig 1J), as reported previously[16,17,19,20,22,59], and positively correlated with length of menstrual cycle at 15.5 y (Fig 1K).

Boys with higher DHEAS concentration at 8.5 y had more advanced pubic hair growth at later ages

At 8.1 y, 62/63 boys were Tanner Stage 1 for pubic hair development (Fig 2A). DHEAS at 8.5 y correlated with pubic hair measured at later timepoints (10.7, 11.7, 15.5, 16 y; Figs 2B-F). Tanner stages of testis and penis development reported at 8.1 y were surprisingly advanced (Fig 2A), perhaps due to inconsistency in reporting this measurement[60,61], and no correlation with DHEAS was evident at any age examined (Figs 2G-I).

Methylation levels of many individual CpGs showed some correlation with DHEAS concentration

Linear regression analysis identified 10,752 individual CpGs in boys and 5,505 in girls where methylation levels appeared to correlate with DHEAS concentration (p-value <0.05; Table S3). The most significant CpGs with nearby annotated genes (Table 1) included *LGR4* whose sequence variants are linked to delayed puberty[62]. Other genes have connections to puberty (e.g. *PTPRN2, DIO1*), spermatogenesis (*CLU, LMTK2, SPAG8*), or are involved in neuronal activity (*PRRT1, C11ORF9, IQSEC, LMTK2*), growth and metabolism (*RPH3AL, SHOX2*).

For functional understanding of the methylation sites that were most significantly correlated (p-value <0.01), we performed enrichment analysis of their associated genes. In girls, "hormone-protein receptor activity" was amongst the most enriched molecular functions (Fig S3A), while in boys, the most enriched biological processes included "positive regulation of steroid metabolic process" (Fig S3B). However, none of these individual CpGs remained significant following multiple comparison adjustment (FDR <0.05).

Regional analysis reveals CpGIs with DHEAS-associated changes in methylation

The above analysis examined individual CpGs, but functional changes in methylation comprising part of a regulatory mechanism are expected to be coordinated and occur primarily at CpGI shores and shelves[47]. We thus performed region-based analysis using linear regression to model the relationship in the context of CpGI shores and shelves.

There were 1,824 and 3,627 islands that met our criteria in girls and boys, respectively, with 8,529 shore or shelf CpGs in girls, and 17,722 in boys (Table S4). Of these CpGs, methylation of 2,766 in girls and 8,588 in boys was positively correlated with DHEAS, the remaining being negatively correlated. Among the islands where methylation correlated with DHEAS, 1,286 in girls and 2,726 in boys had at least one annotated gene in their proximity (Table 2). We intersected these genes between boys and girls, and 449 genes had methylation associated with DHEAS in both sexes (Table S5).

Analysis of these genes in girls, revealed enriched molecular function terms related to activity of ion channels, and biological processes relating to cell adhesion and morphogenesis (Fig 3A). In boys, the most enriched molecular function term was transcriptional activity, and biological processes included regulation of I/NF-kappaB-signaling, axon myelination, RNA metabolic process, Wnt signaling and growth (Fig 3B).

CpGIs with DHEAS-associated differential methylation are located near puberty-related genes

We next intersected the full list of genes (i.e. with DHEAS-associated CpGI shore/shelf methylation), and a set of 248 puberty-related genes (Table S6). This revealed 12 puberty-related genes with DHEAS-associated levels of methylation evident only in boys, 7 only in girls, and two common to both sexes (Fig 4; Table S7 lists these CpGs, their location relative to the gene and correlation with DHEAS).

Methylation of the promoter/5'end of *FGFR1* was strikingly negatively correlated with DHEAS in both sexes, at three CpGs in boys and two in girls (Figs 5A-C; Table S6). These sites are located in putative regulatory regions: in boys, they are 400-800 bp upstream of the first transcriptional start-site (from FANTOM5 CAGE data), two are in a predicted promoter region (Genehancer[63]), and 400-800 bp upstream of an androgen receptor (AR) binding site (Remap ChIP-seq[64]). In girls, the affected CpGs are in *FGFR1* first intron, 200-400 bp from a distinct AR binding site and a peak in CAGE reads, suggesting another regulatory element, and mechanism mediating the DHEAS effect (Fig 5C).

The methylation level at the promoter/5' end of *RPGRIP1L;FTO* was also associated with DHEAS concentrations in both sexes: though positively correlated in males, in females one of these same sites was negatively correlated (Figs 5D,E), leaving any functional connection unclear.

In girls, the negative correlation between DHEAS and methylation of a CpG at the promoter of *LHCGR* (Fig 5F) was particularly notable, given its location and the role of LH receptor in reproductive function. There was also a negative correlation between DHEAS and methylation at a site 891 bp upstream of *SRD5A2* which encodes 5α-reductase-2, the enzyme catalyzing synthesis of dihydrotestosterone. This genomic region binds many proteins (ReMAP: ChIP-seq data; Fig 5G), indicating a regulatory region and functional consequences of methylation.

We further detected a site near *INHBB* where changes in methylation met the criteria for association with DHEAS, though it was not listed among the puberty-related genes. It has, however, a recognized role in reproductive function and is associated with pubertal timing[65,66]. The CpG methylation, which was negatively correlated with DHEAS (Fig 5H), occurs 2240 bp upstream of *INHBB* near (~350 bp) an estrogen receptor α (ERα) binding site (ChIP-seq)[67–69]. This region is transcribed (CAGE peak) and enriched with H3K4me1 (ENCODE), suggesting function as a distal enhancer (Fig 5I).

Discussion

Our findings confirm previous reports connecting childhood DHEAS with various aspects of physical and psychosocial development, metabolic risk factors in both sexes, and pubertal development in girls[16–20,27,70–77]. Although DHEAS might affect pubertal development indirectly by altering metabolic status[15], our novel findings that its concentrations correlate negatively and linearly with DNA methylation at regulatory regions of key puberty-related genes, provide a mechanism for a direct functional relationship.

Our initial approach revealed several genes with known roles in sexual maturation or reproduction near individual CpGs with DHEAS-correlated methylation. However, because none of these remained significant following multiple comparison adjustment, we analyzed regions where functional changes in methylation most likely occur. This selective approach identified *FTO* and *FGFR1* in both sexes. *FTO* encodes an α-ketoglutarate-dependent dioxygenase associated with obesity[78,79], PCOS[80] and pubertal timing[81]. However, the methylation patterns at this locus differed between sexes, perhaps reflecting the association between increased childhood weight and precocious puberty which is more prominent in girls[82], though the correlations are not strong enough to reach clear conclusions. In contrast, the affected CpGs near *FGFR1,* in both sexes, are located in putative regulatory regions near AR binding sites. Sequence variation in *FGFR1* is associated with delayed puberty and congenital hypogonadotropic hypogonadism[83–87]. Methylation at the *FGFR1* promoter, including at one of the CpGs that we identified, was found negatively associated with *FGFR1* expression[88], pointing to a role for DHEAS-regulated demethylation on its expression, and likely effects on pubertal onset.

The association between DHEAS and methylation at *LHCGR* and *SRD5A2*, both of which play roles in androgen synthesis, is also in line with likely effects on pubertal timing and possibly PCOS. Variants of *LHCGR* are associated with PCOS[89,90], and its expression in granulosa cells is higher in PCOS women[91,92]. Furthermore, the *LHCGR* promoter is hypomethylated in women with PCOS and in the DHEA-induced PCOS mouse model[37,93,94]. Activity of 5α reductases, one of which is encoded by *SRD5A2*, is elevated in PCOS women and their daughters, suggesting epigenetic regulation[95,96], and we reported previously that the related *SRD5A1* is epigenetically-regulated in accordance with early-life environments[97,98]. Premature adrenarche has been suggested as a risk factor for PCOS[31,99], and our findings suggest that DHEAS might facilitate the drop in *LHCGR* and *SRD5A2* promoter methylation, leading to their increased expression. Elevated levels of LHCGR could promote more ovarian steroidogenesis to increase DHEAS further, while SRD5A2 would increase androgen potency, driving PCOS progression and severity, supporting this connection.

DHEAS-associated changes in DNA methylation were also apparent upstream of *INHBB. INHBB* encodes the βB subunit of Inhibin B, Activin B and Activin AB which play multiple roles in reproductive function, including driving growth of ovarian granulosa cells[100]. Levels of Inhibin B increase throughout puberty[101], and were suggested to serve as a marker for precocious puberty[65,66]. The most affected CpG is found in a locus that carries several markers of a

transcriptional enhancer. This CpG is located near an ERα binding site, and *INHBB* expression is regulated by estrogen[102–104], suggesting DHEAS-regulated demethylation possibly via, or impacting, ERα-mediated regulation of this gene.

In summary, our study supports a positive correlation between DHEAS concentration in 8.5 y old girls and their subsequent pubertal development, and indicates that it might serve as an early indicator for PCOS which is characterized by irregular cycles, high androgen concentration and metabolic dysfunction[99,105–111]. Correlation between pubertal development and DHEAS in boys was less clear, perhaps due to shortcomings associated with some of the self-assessed data[112]. Still, the accessibility of this approach to children and adolescents allows establishment of larger cohorts and more accurate statistical analysis.

Our findings also provide a mechanistic basis for these observations through DHEAS linearly-associated changes in DNA methylation at regulatory regions of key puberty-related genes. This might contribute to development of methylome-based diagnostic tools for early puberty. The relevance of such modifications in peripheral blood cells is not always clear and serves only as a proxy to indicate similar methylation in the inaccessible hypothalamic and pituitary tissues that regulate the reproductive axis. However, a greater response might be expected in functional tissues which contain the complete machinery involved in regulating their expression. Still, the correlations observed may well not signify simple cause-effect relationships, and these findings will need to be examined further in suitable animal or cell models to clarify the complex and multifactorial connection between adrenarche and pubertal development.

Conflict of Interest The authors have nothing to disclose.

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Data Availability: All data used in this study were taken from and are available through ALSPAC.

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Figure legends

Figure 1. DHEAS concentration in 8.5-year-old girls is correlated with more advanced pubic hair growth and breast development, earlier menarche and longer menstrual cycles during and after puberty. (A) Percentage of girls in each Tanner stage at 8.1 y for breast development and pubic hair growth. (B-E) Correlation between DHEAS concentration in girls at 8.5 y, and pubic hair growth Tanner stage at several timepoints: (B) 8.1, (C) 11.7, (D) 13.1, (E) 14.6 y. (F-I) Correlation between DHEAS concentration in girls at 8.5 y and breast development Tanner stage at several timepoints: (F) 8.1, (G) 9.6, (H) 10.7, (I) 11.7 y. (J-K) Correlation between DHEAS concentration in girls at 8.5 y and: (J) age of menarche reported at age 17 years (in months); (K) length of menstrual cycle (days) at age 15.5 y. (B-K) Spearman correlation with ρ and p values reported at the top of each figure.

Figure 2. DHEAS concentration in 8.5-year-old boys is correlated with more advanced pubic hair growth, but not testis and penis development. (A) Percentage of boys in each Tanner stage at 8.1 y for testis and penis development and pubic hair growth. (B-F) Correlation between DHEAS concentration in boys at 8.5 y and pubic hair growth Tanner stage at several timepoints: (B) 8.1, (C) 10.7, (D) 11.7, (E) 15.5, (F) 16 y. (G-I) Correlation between DHEAS concentration in boys at 8.5 y and testis and penis development Tanner stage at several timepoints: (G) 8.1, (H) 10.7, (I) 15.5 y. (B-I) Spearman correlation with ρ and p values reported at the top of each figure.

Figure 3. Top 10 most enriched GO terms of genes near CpGIs whose changes in methylation levels were linearly correlated with DHEAS concentration. The top 10 highest -log10(p-value) molecular functions and biological processes of genes near islands with DHEAS-associated methylation levels, for (A) girls and (B) boys. The shade indicates the number of genes intersecting with the GO term genes.

Figure 4. Puberty-related genes intersecting with genes located near CpGIs with DHEAS-associated methylation levels. Intersection of genes associated with the differential methylation in females, males, and a set of puberty-related genes. The nine intersecting genes

between the female set and the puberty-related gene set are listed on the left and the 12 in the males are on the right. *FGFR1* and *FTO* are found in all groups.

Figure 5. DHEAS-associated methylation near puberty-related genes. Correlation between DHEAS concentration at 8.5 y and methylation at CpG sites near (A,B) *FGFR1*, (D,E) *FTO*, (F) *LHCGR,* (G) *SRD5A2* and (H) *INHBB*, in both sexes, or just girls, as marked. Also shown are UCSC genome browser snapshots of the (C) *FGFR1* and (I) *INHBB* loci with the CpG sites significantly associated with DHEAS concentration (marked in girls with vertical orange lines, and in boys [in C] with vertical blue lines). The tracks shown from top to bottom are: Genome base position, GENCODE V44 depicting the gene location, CpGIs, FANTOM5 data including: TSS peaks and CAGE total and max counts, ENCODE H3K27Ac, H3K4me1 and H3K4me3 markers, GeneHancer Regulatory Elements and Gene Interactions (DE: distal enhancer), ReMap ChIP-seq filtered to show only the (C) androgen receptor or (I) estrogen receptor; and Infinium probe set position.

Table 1. The top 15 individual CpG sites associated with annotated genes, for which the methylation levels are linearly associated with DHEAS concentration.

	CpG site	P.value	Nearest gene	Relation to gene	Enhancer
Females	cg02593958	6.50E-05	MEGF11	5'UTR	
	cg26849331	7.26E-05	PRRT1	3'UTR	
	cg16970748	1.75E-04	FAM155B	TSS1500	
	cg23634928	1.95E-04	PTPRN2	Body	TRUE
	cg17315281	2.09E-04	SORCS2	TSS1500	
	cg04764584	2.48E-04	SLC9A3	Body	TRUE
	cg02238136	2.85E-04	ZNF620	Body;5'UTR	
	cg16290399	3.26E-04	RAD17;TAF9	5'UTR;TSS200	
	cg20996314	3.33E-04	HR	TSS1500	TRUE
	cg07195126	3.40E-04	VANGL2	5'UTR	
	cg09374648	3.41E-04	PNRC1	TSS1500	
	cg14346046	3.44E-04	LGR4	TSS1500	
	cg17091610	3.48E-04	L3MBTL	TSS1500	
	cg03585598	3.56E-04	LYRM4;FARS2	Body;TSS1500	
	cg04426862	3.68E-04	PAX9	5'UTR	
Males	cg10503298	1.55E-05	CTBP1	Body	
	cg11783834	1.77E-05	CLU	Body;TSS200;TSS1500	TRUE
	cg09584521	1.92E-05	CD58	Body	
	cg18171955	2.97E-05	C11orf9	Body	
	cg25051248	2.98E-05	CASKIN2	Body	

	cg21193975	3.02E-05	WDR5	3'UTR	
	cg18276808	3.23E-05	MUC2	Body	
	cg03886898	3.29E-05	DIO1	TSS1500	
	cg05897163	4.50E-05	PPP1R1B	TSS1500	TRUE
	cg14666369	5.25E-05	ACADS	Body	
	cg24871089	5.42E-05	RPH3AL	5'UTR	
	cg09437283	5.93E-05	IQSEC1	Body	
	cg20501518	6.33E-05	SHOX2	Body	TRUE
	cg17279887	6.74E-05	LMTK2	TSS1500	
	cg14353508	7.79E-05	SPAG4	Body	

Table 2. Number of islands whose methylation correlated with DHEAS concentration, and the number of CpGs in their shores and shelves.

	Total		Near at least one annotated gene	
	M	F	M	F
Islands associated with DHEAS concentration	3627	1824	2726	1286
CpGs included in associated islands	17722	8529	13846	6146
Positively associated CpGs	8588	2766	6929	1978
Negatively associated CpGs	9134	5763	6917	4168

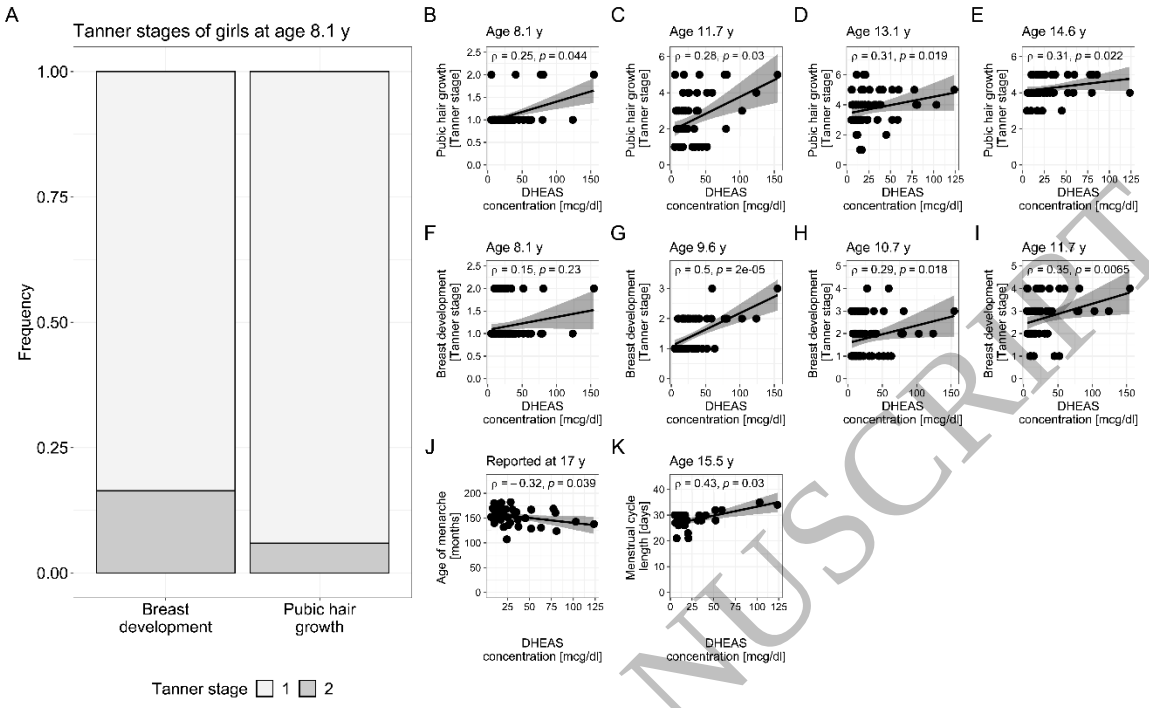

Figure 1
400x250 mm (DPI)

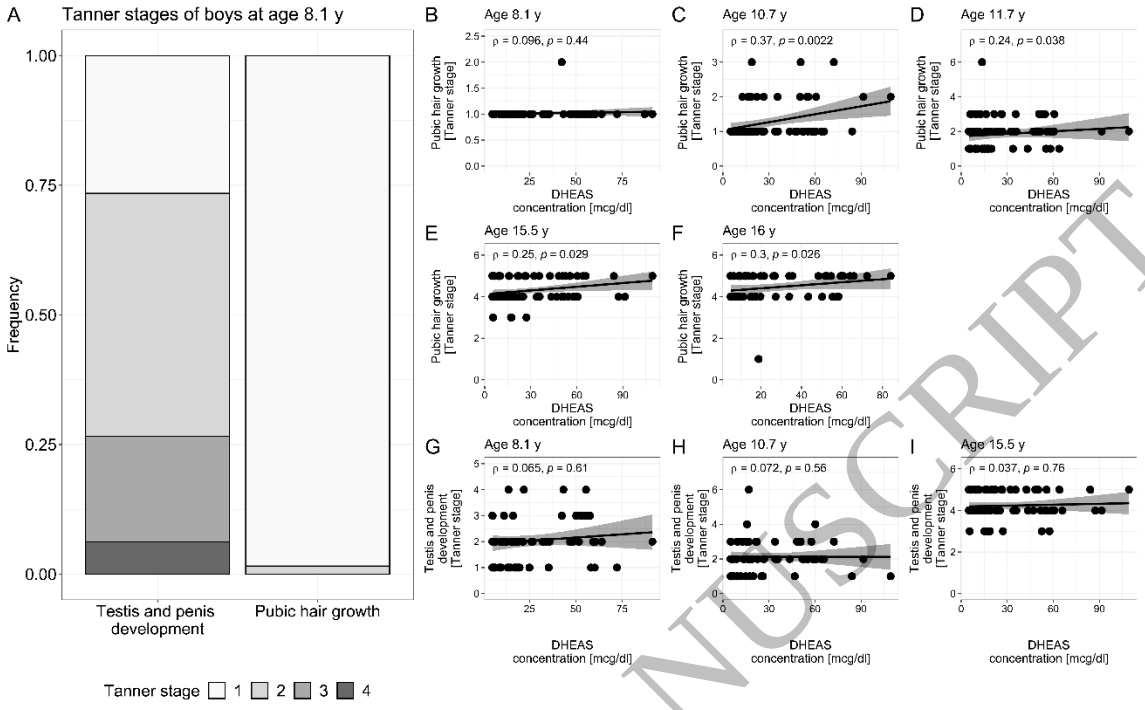

Figure 2
400x250 mm (DPI)

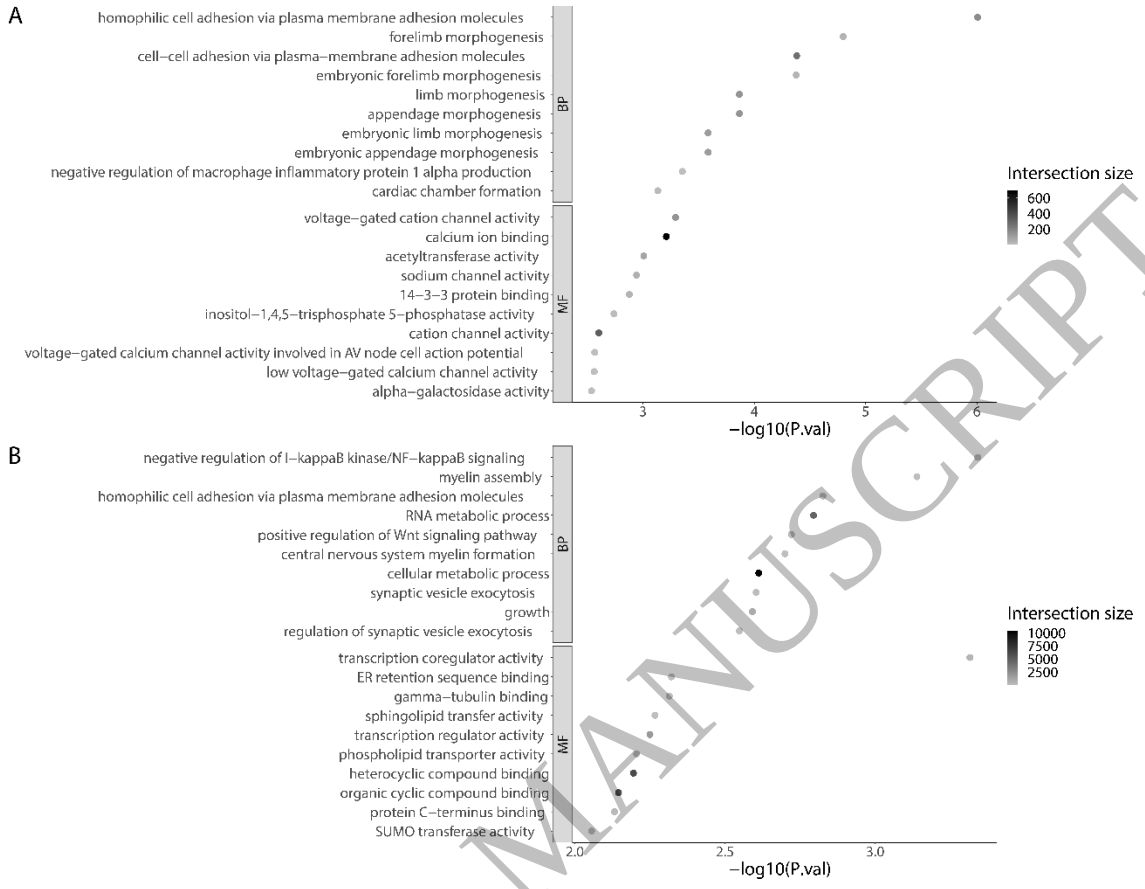

Figure 3
559x430 mm (DPI)

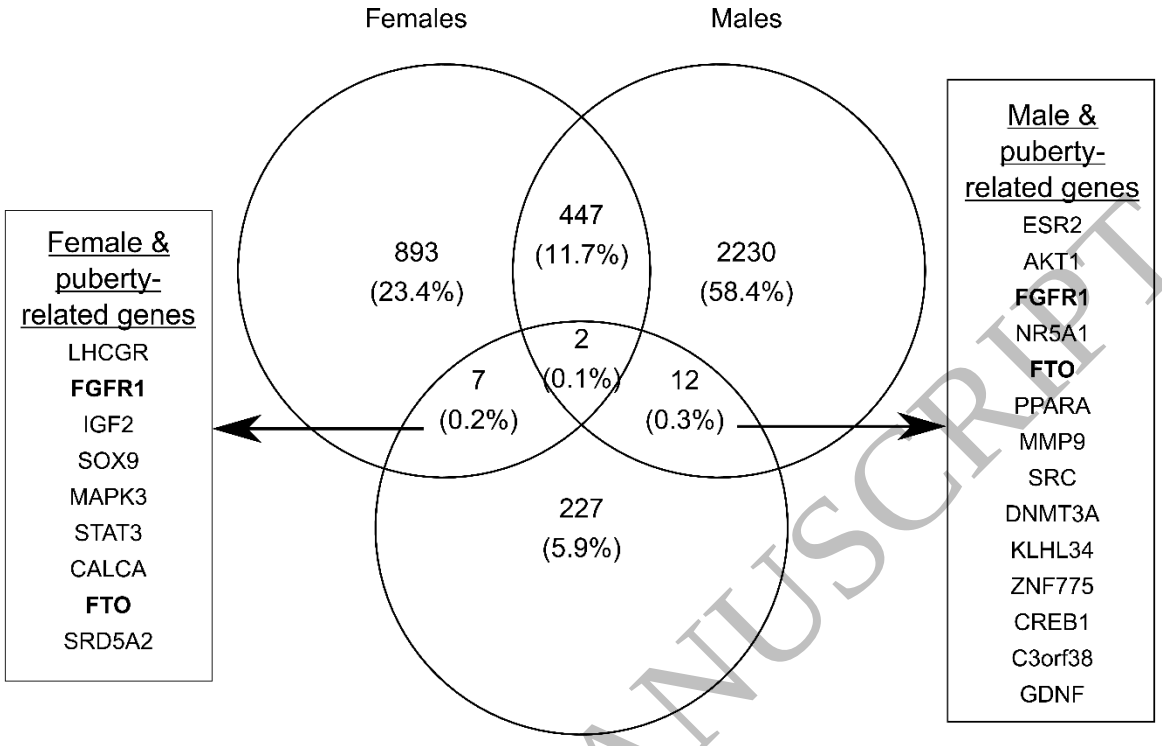

Figure 4
423x289 mm (DPI)

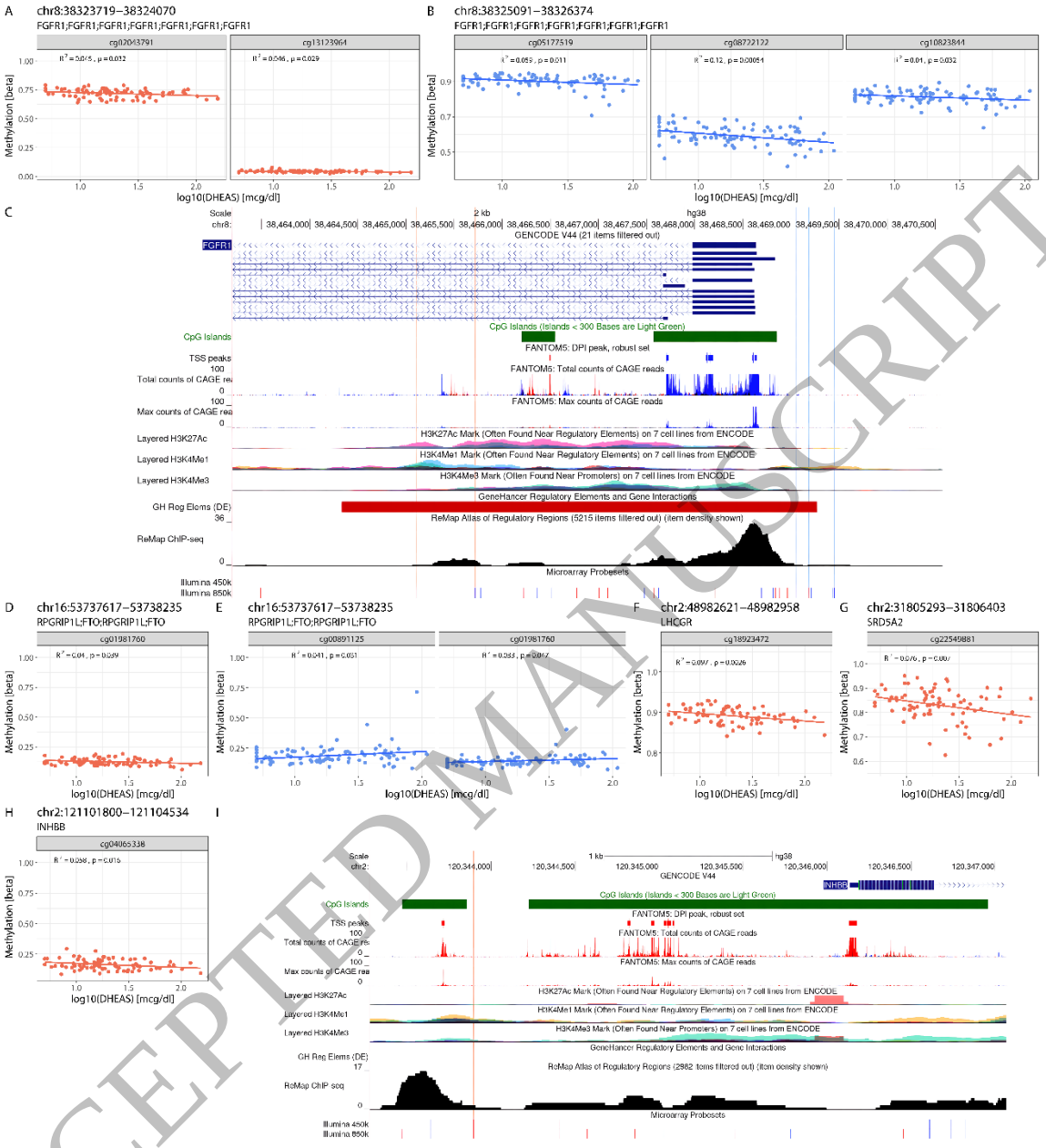

Figure 5
512x559 mm (DPI)