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The role of gonadotropins in testicular and adrenal androgen biosynthesis pathways -insights from males with congenital hypogonadotropic hypogonadism on hCG/rFSH and on testosterone replacement-

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SUMMARY

Objective: To delineate the role of gonadotropins in male androgen biosynthesis pathways

Design: Case-control study

Patients and measurements: 25 males with congenital hypogonadotropic hypogonadism (CHH) underwent hCG/rFSH and testosterone treatment sequentially. Serum steroid hormone profiles (testosterone precursors and metabolites) on both replacement regimens were analyzed, using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and compared to those of healthy controls, matched by age, BMI and serum testosterone.

Results: On testosterone replacement, serum concentrations of the classic Δ4 pathway hormones progesterone and 17-hydroxy-progesterone (17-OHP), and the marker steroid of an alternative pathway of testosterone synthesis (androstenediol) were decreased, compared to controls. Androstanediol, a marker of the backdoor pathway of dihydrotestosterone (DHT) synthesis, was increased. 17-OH-pregnenolone, androstenedione and DHEAS (Δ5 pathway), three 11-oxygenated C19 androgens (11-keto-A4, 11-keto-T and 11-keto-DHT) and the testosterone (T) metabolites DHT and 17ß-estradiol (E2) were similar to controls.

On gonadotropin replacement, 17-OHP, 17-OH-pregnenolone, DHEAS and androstenedione, as well as DHT, androstenediol, and all 11-oxygenated C19 androgens were normal. Progesterone (Δ4 pathway) was slightly decreased, androstanediol (backdoor DHT pathway) and E2 (T metabolite) were increased.

Conclusions: In males with CHH, serum steroid hormone profiles resemble those of healthy men, if hCG/rFSH is used for substitution. Gonadotropins contribute to steroid hormone production along the classic Δ4 pathway and co-activate an alternative pathway of testosterone biosynthesis via androstenediol. Backdoor DHT biosynthesis, Δ5 17-OH-pregnenolone, DHEA(S) and androstenedione synthesis and 11-oxygenated C19 androgen production are activated *in*dependently of gonadotropins. The androgen replacement modality used for treatment of hypogonadal males with absent or reduced endogenous LH/FSH- secretion may impact on long term health and quality of life.

Introduction

Androgens are a family of steroid hormones important for male sexual development, for reproductive health and metabolic homeostasis. The "classic" androgens, testosterone (T) and dihydrotestosterone (DHT) are C19 carbon steroid hormones, synthesized in the *testes* from cholesterol via different steroidogenic pathways. Androgens are also produced in the *adrenal* zona reticularis or locally, in *target tissues*, by conversion from circulating C21 androgen precursor steroids 1. Steroidogenic enzymes, involved in the formation of T and DHT and differentially expressed and activated in gonadal and adrenal tissues include 3ß-hydroxysterid-dehydrogenase (HSD3B2), 17α-hydroxylase/C17-20-lyase (CYP17A1), 17ß-hydroxysteroid-dehydrogenase (HSD17B3 and 5) and 5α-reductase (SRD5A2) (Figure1). While androgen production in Leydig cells is dependent on stimulation by luteinizing hormone (LH), produced by gonadotropic anterior pituitary cells, adrenal steroid secretion is regulated by adrenocorticotropic hormone (ACTH) from corticotropic pituitary cells.

The biological effects mediated by androgens are conveyed through the androgen receptor (AR; NR3C4), a ligand- activated nuclear receptor that functions as a transcription factor, and regulates gene expression in target tissues, and that is present in reproductive organs, brain, bone, heart, liver, skin and larynx 2. Differential androgenic effects are conveyed by the relative concentrations of the respective androgenic steroids in circulation and are further modulated by other factors (their binding to SHBG or albumin in serum with consequences on clearing rates 3, passive influx or active transport into target cells, specific affinities to the AR, distinct promotor activation profiles 4. Further regulation of AR activity is conveyed by variation in length of a glutamine repeat region (encoded by a polymorphic (CAG)n repeat in exon 1) present at the NH2-terminal domain of the AR 5.

In the human male, the virilizing properties of T and DHT cause internal and external sexual differentiation during fetal life, resulting in the "male primary sexual characteristics" 6. The pubertal increase of circulating testosterone and its maintenance at adult male concentrations in serum is responsible for both virilizing and anabolic effects, resulting in male secondary sexual characteristics, muscle accrual, bone mineralization and stimulation of erythropoiesis.

Beyond T and DHT, *precursor steroids and metabolites* are able to elicit androgenic action. The role of these androgenic steroids has been established in conditions accompanied by androgen excess, such as congenital adrenal hyperplasia 7, polycystic ovary syndrome 8 and castration-resistant prostate cancer 9; however, their contribution to androgenic action in the healthy male is as yet unclear. The impact of gonadotropins on the secretion of bioactive androgenic steroids and their precursors is equally unresolved.

Males with congenital hypogonadotropic hypogonadism (CHH) display central hormone deficiency, with disturbed secretion or action of hypothalamic GnRH on the anterior pituitary gland. As a consequence, LH and FSH stimulation of the gonads is deficient. CHH can thus be viewed as a naturally occurring human

"knock down" of gonadotropin stimulation of the gonads, with uncompromised ACTH secretion. To delineate the role of gonadotropins in *testicular and adrenal androgen biosynthesis pathways*, we analyzed steroid hormone profiles in serum of CHH males twice, once, while patients were on testosterone and once, while they were on gonadotropin replacement.

We hypothesized that the hormonal replacement modality currently used in males with CHH affects the quantity of *bioactive precursor* steroid hormones and *metabolites of testosterone*, measurable in the patient´s serum.

Patients and methods

The study was performed at the Centre for Reproductive Medicine and Andrology, University of Münster in collaboration with the Children's Hospital Kiel, Department of Pediatric Endocrinology and Diabetes, University of Schleswig-Holstein, Germany.

Patients and controls

Twenty-five males (age range: 18-41 years) with congenital GnRH and gonadotropin deficiency (congenital hypogonadotropic hypogonadism, CHH) were included. Eleven were normosmic or hyposmic and 14 were anosmic, indicating Kallmann syndrome (KS).

CHH was confirmed by absent or arrested pubertal maturation by or after age 14 years, with inadequately low serum levels of LH, FSH and T, a blunted LH surge <5 U/l following GnRH agonist (busereline) stimulation, and/or absent rise in testes volumes 3-6 months after priming with low-dose exogenous T over 3-6 months, or by inadequate spontaneous pubertal virilization at age ≥18 years.

Fifty-six healthy young partners of women with proven female infertility were recruited from the infertility clinic, to serve as controls. All had normal testicular volumes, normal serum T levels and normal semen parameters (according to WHO 2010 criteria). Controls were matched by age (+/- 2 years), BMI (+/-3 kg/m^2) and by serum testosterone levels (+-8nmol/l), to avoid bias.

Treatment protocols

Subcutaneous hCG and rFSH injections were administered to CHH males for 2-4 years, according to a protocol as described previously 10, until testicular maturation was accomplished.

Specifically, in pre-pubertal patients, a starting dose of 500 IU hCG was injected subcutaneously twice weekly. Incremental increases every 3-6 months to a maximum of 2-3 x 1500 IU hCG s.c./week were administered, if necessary, to achieve serum T levels in the mid-normal adult range (T>3.5 ng/ml, [12 nmol/l]) by one year after start of hCG. In post-pubertal men who had previously received exogenous T, a starting dose of 2 x 1000-1500 IU hCG s.c./week was applied, aiming at adult serum T levels by 3 months. After 3(-6) months of hCG treatment, rFSH was added at a standard dose of 3x 150 IE s.c./week. This combined replacement was continued until gonadal maturation was achieved, as indicated by plateauing sperm concentrations in semen and achievement of maximal testicular volumes. Thereafter, males were

switched to exogenous T substitution, either by application of T gel or by i.m. injections of T enanthate or T undecanoate. Doses were adjusted after 3 months of treatment, to achieve serum T levels in the mid - normal adult range.

Hormonal measurements

The circulating steroid hormone precursors and metabolites of T in each CHH male were investigated once while patients were being treated with combined human chorionic gonadotropin (hCG) + recombinant FSH (rFSH) for at least two years, and another time, while they were on replacement with exogenous T for at least 6 months. Blood samples from CHH males were collected between 8 and 12 a.m. and serum was frozen at -20 °C. Serum steroid hormone profiles, including steroid hormone members of the Δ5 and Δ4 classic pathways of T biosynthesis, the alternative T pathway, the backdoor DHT pathway and the 11-oxygenated C19 androgen pathway (Figure 1) were analyzed simultaneously in both serum samples of each patient and all control samples (Figure 2), using liquid chromatography-tandem mass spectrometry (LC-MS/MS), based on previously published methods [11,12]. In brief, aliquots of serum samples, calibrator and controls (with a volume of 0.1 mL) were combined with the internal standard mixture to monitor recovery. All samples were extracted using Oasis MAX SPE System Plates (Waters, Milford, MA, USA). LC-MS/MS was performed using a Waters Quattro Premier/Xe triple-quadrupole mass spectrometer connected to a Waters Acquity (Waters, Milford, MA, USA).

Ethics

All procedures (Figure 2) were approved by the ethics committee of the State Medical Board (Approval code 4 I Nie). Informed consent was obtained by adults, and assent by minors was provided with consent of their parents.

Statistics

Statistical analysis and drawing of graphs was performed using Graph Pad Prism 5.0 (GraphPad Software Inc. La Jolla, CA, USA). Where normality of distribution was determined, parametric t-tests for independent samples were conducted; otherwise Mann-Whitney-U test was used. Significance was defined as $p < 0.05$. Results are displayed as means (±SD).

Results

Combined treatment of CHH males with hCG and rFSH resulted in steroid hormone profiles similar to those of healthy men, but this was not the case, while exogenous testosterone was used for replacement. Serum steroid hormone levels on the different treatment modalities in CHH males and matched controls are summarized in Table 1 and plotted individually in Figure 3 and Figure 4.

While CHH patients were *on T substitution*, decreased serum concentrations of some members of the classical Δ4 pathway of androgen biosynthesis (progesterone ($p=0.0104$), 17-OH-progesterone (17 OHP) ($p<0.0001$)) and of the alternative T pathway steroid androstenediol ($p=0.004$)) were observed, compared

to controls. The marker steroid of the backdoor DHT pathway androstandiol (p=0.025), was slightly increased.

The testosterone metabolites DHT and 17-ß estradiol (E2), the Δ5 steroid 17-pregnenolone, the sulfated form of the Δ5 pathway steroid dehydroepiandrosterone DHEA, i.e. dehydroepiandrosterone sulfate (DHEAS), androstenedione (A4) and all measured 11-oxygenated C19 androgens (11-keto-testosterone (11-K-T), 11-keto-dihydro-testosterone (11-K-DHT) and 11-keto-androstenedione (11-K-A4)) were comparable to those of controls.

By contrast, normal concentrations were found for most steroid hormones in serum of CHH males, while they were *on hCG/rFSH replacement*. Specifically, steroid profiles resembled those of healthy male controls, regarding the Δ 4 pathway of androgen biosynthesis (17-OHP) and the metabolite DHT, the marker steroid of an alternative T pathway via androstenediol, the Δ5 pathway steroids Δ5 steroid 17-pregnenolone , DHEAS and A4 and all aforementioned 11-oxygenated C19 androgens (11-K-A4, 11-K-T, 11-K-DHT). Serum progesterone was slightly decreased (p=0.0104), the testosterone metabolite E2 and the backdoor DHT pathway steroid androstanediol were increased (both p<0.0001).

Discussion

An optimal endocrine replacement strategy for hypogonadotropic hypogonadal males aims at normalizing all aspects of deficient androgenic action. While testosterone replacement has been used in clinical practice to solely convey androgenic effects, gonadotropins have been employed for the purpose of additionally initiating testicular growth and spermatogenesis 13.

The present study provides data on steroid hormone profiles of males with CHH, in which these two different replacement regimens were applied sequentially. The hormone concentrations in serum reflect the overall production of hormone that is contributed to the blood stream by each hormone-producing tissue. The naturally occurring “knock down condition” of central hormonal stimulation of gonads, with uncompromised ACTH secretion that is present in CHH males, was used as a model, to enable delineation of gonadotropin effects on testicular and adrenal steroidogenic pathways involved in male androgen biosynthesis. Specifically, hCG/rFSH-effects on serum steroid hormone concentrations of the classic Δ5 pathway of steroid biosynthesis, on concentrations of steroids of the Δ4 steroidogenic pathway, the alternative pathway of testosterone biosynthesis, the backdoor pathway of DHT synthesis and on concentrations of the 11-oxygenated C19 androgen pathway were investigated. In addition, the serum levels of testosterone metabolites were investigated.

Our results indicate that treatment of CHH males with gonadotropins results in steroid hormone profiles similar to those of healthy men, with few exceptions (E2, progesterone). However, this is not the case using a regimen based on exogenous testosterone. If testosterone is applied, steroidogenic pathways in testicles of CHH males remain unstimulated. By contrast, if hCG +rFSH are used, the LHCG receptor in Leydig cells

is activated 14. In response, multiple steroids of the classical steroidogenic cascade are synthesized by the gonads, including the classic potent androgens T and DHT. This explains the differences observed in serum steroid levels in CHH males on the two different replacement regimens

Δ4 pathway

Progesterone, 17-OH-progesterone (17 OHP)

One major finding of this study is that hCG/rFSH replacement stimulates and thus normalizes some important steroid hormones belonging to the Δ4 pathway of steroidogenesis in hypogonadotropic hypogonadal males. 17-OHP levels were significantly decreased on treatment with testosterone, but not while the males were on gonadotropin replacement, indicating that a major proportion of this precursor steroid is produced in the gonads and requires gonadotropin stimulation for secretion, while a minor part of it stems from other sources. It is likely that ACTH- stimulated production in the adrenal gland contributes to it.

17-OHP is recognized to have anti-mineralocorticoid 15 and glucocorticoid potency 16,17, without direct androgenic properties. Since cross-reactivity among steroids is a recognized phenomenon, due to the high degree of homology at the DNA-binding domains of nuclear receptors within the steroid hormone receptor superfamily 18, the measured differences in serum 17-OHP concentrations may have clinical implications.

Surprisingly, progesterone serum levels in CHH males were decreased on both hCG+rFSH and testosterone substitution, when compared to controls, but the decrease was more pronounced on testosterone replacement. This indicates that the gonadotropins used for replacement do enhance progesterone serum production in males, albeit to a lower extent than in healthy subjects. The use of hCG instead of LH for CHH replacement may explain this phenomenon. Progesterone acts via intracellular progesterone receptors (PRs) A and B, thereby exerting classical genomic action. In addition, it has non-genomic effects. In males, progesterone is involved in sperm capacitation/acrosome reaction, it influences LH receptor expression and subsequent testosterone biosynthesis in Leydig cells; it interacts with the $GABA_A$ receptor complex in the CNS, thereby eliciting effects on sleep, mood and cognition, and also exert effects in adipose tissue and kidneys 19. Progesterone is a high-affinity antagonist of the mineralocorticoid receptor (NR3C2; MR)15, thus eliciting anti-mineralocorticoid effects 20-23. Progesterone is able to transactivate the glucocorticoid receptor (NR3C1; GR) 16. A lack of progesterone and 17-OHP in men could therefore have an impact on water and electrolyte balance and systemic blood pressure regulation.

Δ5 pathway

17-OH-pregnenolone, androstenedione

The observation that androstenedione serum concentrations were mildly decreased on testosterone replacement, compared to the situation on replacement with hCG/rFSH, although not different from those of controls, indicates that a neglectable part of the steroid production stems from gonadotropin stimulation.

In females, it has been previously suggested that 50% of circulating plasma androstenedione is of adrenal origin [24]. Our data indicate that in males, only a very minor part of androstenedione is produced on gonadotropin-stimulation, thus in gonads.

The gonadotropin-independent production of androstenedione is unlikely to result from Δ4 progesterone via 17-OHP, as the human CYP17A1 17,20-lyase is known to be 50 times less efficient to cleave C20/21 off 17-OHP, compared to 17-pregnenolone [25]. Therefore, androstenedione is likely generated through the conversion of DHEA by 3ß-hydroxysteroid dehydrogenase type 2 or 1 (HSD3B2/1) and, thus, a consequence of Δ5activity.

In present study, the Δ5 steroid 17-pregnenolone concentrations in serum were not significantly higher on hCG than during T replacement, although there was a trend towards lower 17-pregnenolone concentrations on T compared to the situation on hCG/rFSH, making interpretation of these findings difficult. The observations could either be interpreted as a confirmation that this part of Δ5 pathway steroidogenesis is gonadotropin-independent. Alternatively, it could also be, that enzymatic activities of CYP17A1 17,20-lyase and HSD3B2 are enhanced in the presence of gonadotropins, with faster conversion of 17-pregnenolone to A4 and T in Leydig cells, and potentially "suppressed", when men are on T replacement.

The enzyme 3ß-hydroxysteroid dehydrogenase type 2 (HSD3B2), required for the synthesis of androstenedione from DHEA, and the co-factor CYB 5A (cytochrome b5), required to support CYP17A1 17,20-lyase activity to generate DHEA from 17-pregnenolone are expressed in both testicular Ledig cells [26] and a fraction of adrenal cells, at the interface between zona fasciculata and reticularis [27] and in non-steroidogenic tissues, such as liver, skin, adipose and kidney [28]. Androstenedione has only mild androgenic potency [29], but it is converted to testosterone in testes by 17ß hydrosysteroid dehydrogenase type 3 (HSD17B3) and type 5 (HSD17B5 = aldo-keto-reductase type 1C3 =AKR1C3)) and in adipose tissues by 17ß hydrosysteroid dehydrogenase type 5 (HSD17B5) [30].

DHEA(S)

The serum concentration of DHEAS was comparable on both gonadotropin and testosterone replacement and similar to controls, indicating that gonadotropins are not responsible for increasing DHEAS serum levels in CHH males. DHEAS is the sulfated storage form of DHEA and serum concentrations are of both steroids are tightly correlated [31]. Our observations increase the existing evidence that Δ5 pathway DHEA synthesis is almost exclusive to the adrenal gland. The role of ACTH as the driver of DHEA has previously been established, based on the observation that dexamethasone suppresses the nocturnal rise of DHEA and cortisol, but not of testosterone [32], and that DHEA is secreted synchronously with cortisol [33]. Although LHCG receptors have been found in a few adrenal cells of the zona reticularis and the deeper layer of the zona fasciculata [34], the data of this study suggest, that hCG (or LH)-stimulation of adrenal cells via LHCG receptors does not contributes in a relevant way to the DHEAS serum pool. No additional stimuli seem to

be involved in driving DHEA synthesis, as DHEA and DHEAS serum levels were undetectable in ACTH-deficient men with panhypopituitarism, lacking both gonadotropins and ACTH 35. ACTH acts via adrenal melanocortin type 2 receptors (MC2R) in cells of the adrenal cortex 36. Although the *MC2R* gene expression has been observed also in human fetal and adult Leydig cells, ACTH action on testicular tissues seem to be clinically relevant only in situations with ACTH hypersecretion 37-39.

Thus, ACTH-stimulated DHEA of adrenal Δ5 pathway origin could serve as a precursor for LH-driven testicular androgen biosynthesis 40: After leaving the adrenal gland in form of its sulfate ester DHEAS, 41 and after subsequent gonadotropin-independent conversion to androstenedione in testes, liver, skin, adipose and kidney, the final step of "classic" testicular testosterone synthesis from androstenedione could then be regulated by LH stimulation.

Backdoor pathway of DHT synthesis

Androstenedione can alternatively be converted to DHT via 5-α-androstandione through the backdoor pathway of DHT synthesis, thereby bypassing testosterone 42. Other backdoor DHT pathways use progesterone and 17-OHP for DHT production. In the present study, androstanediol was chosen as a marker steroid of these three converging pathways (Figure 1). Activity of the backdoor DHT pathways is known to be relevant for sexual differentiation during fetal life and for most androgen-mediated events at male puberty. Backdoor pathways are also active to ensure production of potent androgens in conditions with deficient frontdoor androgen synthesis pathways 43. Androstanediol has been shown to be clinically relevant in prostate cancer for its AR-activating properties in these cancer cells 44.

Variable androstanediol concentrations were found in serum of CHH males on either replacement regimen, illustrating gonadotropin independence of the backdoor DHT-pathways and thus regulation via other mechanisms. In view of our observation that serum 17-OHP biosynthesis is stimulated by gonadotropins, thus partially originating form gonadal production, this precursor could also serve for backdoor DHT production via a17-OH-allopregnanolone, in addition to frontdoor (classic) T and DHT synthesis via androstenedione.

Alternative pathway of T biosynthesis

An alternative pathway of testosterone biosynthesis uses DHEA to enhance testosterone production via production of androstenediol (in contrast to the classic pathway via androstenedione). Until now it was unclear, whether ACTH or gonadotropic stimulation is necessary for its initiation. In the present study, androstenediol was decreased on testosterone replacement, but not, if gonadotropins were applied. These data indicate that normal androstenediol serum levels are achieved only if gonadotropins are present, while a fraction of around 50 % of serum androstenediol concentrations results independently of hCG/rFSH stimulation.

Testosterone metabolites

DHT

DHT serum levels were comparable to controls in CHH males during hCG/rFSH replacement, but were slightly increased after the patients were switched to exogenous testosterone, indicating that the pathways of DHT production are more active if exogenous testosterone is provided. DHT is known to result mainly from 5-alpha reduction of testosterone [45] and from the aforementioned backdoor pathway in adrenal and prostatic tissues. DHT exerts the strongest bioactivity on the AR [46].

17ß-estradiol

17ß-estradiol (E2), a major metabolite of testosterone, was found to be slightly elevated as compared to controls while patients were undergoing gonadotropin replacement. About 50-75 % of circulating E2 is derived from extragonadal aromatization of testosterone by fat, bone, brain, testes and other tissues [47]. Although we matched for serum T in both cohorts, mean serum T levels were slightly higher during gonadotropin application, compared to the situation during T treatment. This explains why T was aromatized to a greater extent during hCG/rFSH substitution. Alternatively, the higher E2 levels could be interpreted as an enhancement of aromatase activity by hCG/rFSH.

E2 acts on estrogen receptors alpha and beta (ERα and ERβ) that are present in reproductive organs, brain, bone, blood vessels, liver, and skin and breast tissue. Many biologic effects, hitherto attributed to testosterone are conveyed by estradiol: estrogen action is important for the regulation of spermatogenesis; E2 is involved in the regulation of the somatotropic axis, it mediates epiphyseal closure in bones, improves bone mineralization and bone microarchitecture, decreases fat mass, thereby favoring lean body mass. In addition, it positively affects glucose metabolism by enhancing insulin sensitivity, and conveys vasomotor stability [48-50].

11-oxygenated C19 androgen pathway

Another important finding of this study is that, serum androgens resulting from the 11-oxygenated C19 pathway, namely 11-keto-androstenedione (11-K-A4), 11-keto-testosterone (11-K T) and 11-keto-dihydro-testosterone (11-K-DHT) in CHH males are similar to those of controls, independent of the replacement strategy. This indicates that C19 oxygenated products are synthesized independently of gonadotropins. We deduce from this, that 11-oxyentated androgens stem exclusively from non-gonadal sources; accordingly the concentrations of these androgens should not be altered in subjects with hypogonadism, irrespective of the origin of gonadal failure.

The capacity of adrenals to produce 11-OH androstenedione (11-OH-A4), the most abundant unconjugated C19 adrenal steroid, was already recognized in 1972 [51]. Recently, it has been observed that 11-oxygenated C19 androgens are able to activate the androgen receptor (AR) to an extent which is comparable to classic androgens, and that they play a role in congenital adrenal hyperplasia (CAH) and prostate cancer [7,52].

11K-T is a partial AR agonist with biologic action comparable to T, and results from conversion of T via 11ß-hydroxy-testosterone (11-OH-T) or from A4, via 11ß-hydroxy-androstenedione (11-OH-A4) and further via 11ß-keto-androstenedione (11-K-A4) [41,53] (Figure 1). 11-K-DHT, the 5α-reduced product of 11-K-T, acts as a full AR agonist, comparable to DHT [46]. The metabolites 11-keto-A4, 11-OH -T and 11-hydroxy-dihydrotestosterone (11-OH -DHT) also have partial agonist activity, albeit less than that of 11-K-T.

As there is no relevant role of gonadotropins on the secretion of the 11 ox C19 androgenic steroids, ACTH seems to be the exclusive driver for their adrenal production in humans. This conclusion is supported by the observation of other investigators, that only trace amounts of 11 oxygenated C19 steroids were present in serum of patients with adrenal insufficiency [7]. It also agrees with the knowledge that 11-OH androstenedione, the precursor of the 11-oxygenated androgen pathway, can be enhanced by exogenous ACTH [41] and that CYP11B1 is predominantly expressed in the zona fascicula of the adrenal cortex, where it also catalyzes the final step in cortisol biosynthesis [54].

Beyond the pathophysiology of hypogonadism, existence of these non-Leydig cell produced and non-gonadotropin-stimulated androgens explains mechanisms of progression of castration-resistant prostate cancer, specifically the failure of GnRH agonist treatment to deplete all relevant androgens promoting cancer progression and the efficacy of P450 CYP17A1 inhibitors in improving survival in this condition.

Clinical observations

In the present study, testosterone and DHT reached normal adult serum levels during both hCG + rFSH and testosterone replacement in CHH males, enabling virilisation and/or its maintenance. After 2 years of treatment with gonadotropins, sperm was present in semen > 90 % of CHH males. Anabolic effects, *i.e.* body muscle and bone mass accrual, were observed during both regimens, but not investigated in more detail. Although an increased incidence of gynecomastia has been described as an adverse feature occurring with gonadotropin therapy in CHH males, breast budding was observed predominantly in obese subjects and independently of the replacement strategy (despite slighty higher E2 concentrations during gonadotropin-treatment). Obviously, the use of individualized (and minimized) hCG and T dosages was successful in reducing this unpleasant side effect in lean subjects.

All AR transactivating androgens may exert additional effects by interaction with other receptors such as glucocorticoid (GC) and mineralocorticoid receptors (MR) with influences on glucose and lipid metabolism and salt and water balance. However, measurement of these effects was not within the scope of this study.

Limitations

One limitation of the present study, regarding the investigation of gonadotropin-effects is that hCG was used to replace LH. This substance may have slightly different properties regarding activation of LHCG receptors [55]. However, at present, rLH is not licenced for clinical use in males.

Summarized results and conclusions

These biochemical studies of serum steroid hormone patterns in CHH males on two different androgenic replacement regimens contribute to our knowledge of human steroidogenesis, specifically androgen production and its regulation. Gonadotropins contribute to steroid production along the classic Δ4 pathway, by stimulation of 17-OHP production. In addition, gonadotropins co-activate an alternative pathway of T biosynthesis from DHEA via androstenediol.

However, Δ5 biosynthesis of 17-OH-pregnenolone, DHEA(S) seems fully gonadotropin-independent, and the production of androstenedione is largely gonadotropin-independent. Thus, an "adrenal-peripheral tissues-testicular collaboration" regarding androgen synthesis by classic or alternative pathways seems possible.

The 11-oxygenated C19 androgen pathway are activated independently of gonadotropins. The activity of the three DHT backdoor pathways (converging in androstanediol biosynthesis) is not increased by gonadotropins.

A replacement regimen with combined hCG/rFSH mimics physiologic steroid hormone profiles better than a substitution with exogenous testosterone. The documented differences in steroid profiles on testosterone replacement in hypogonadal males with absent or severely reduced endogenous LH and FSH secretion may have long term consequences for health and wellbeing. Specifically, body composition, bone health, glucose and lipid metabolism, salt and water balance, cognition, mood, sleep and sexual function could be affected. The steroidogenic differences could also be relevant for gonadotropin-suppressive treatments with long-acting testosterone preparations in males with primary hypogonadism. To what extent this hypothesis is true, should be addressed in future clinical studies.

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Figure legends

Figure 1

Pathways of human androgen biosynthesis

-The classic pathways, proceeding parallel for Δ5 and Δ4, convert steroidogenic precursors and lead to formation of T, which can be further converted to DHT.

-The alternative pathway of T formation proceeds via androstenediol.

-The backdoor pathway proceeds via androstanediol to generate DHT.

-The 11-oxygenated C19 androgen pathway generates 11K T and 11 K DHT.
The steroids measured in the present study are indicated in bold.

Figure 2

Patients, interventions and outcome measures

Figure 3

-Serum steroid hormone concentrations from Δ5 and Δ4 pathways of CHH males (including testosterone metabolites) analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), once, while patients were undergoing hCG/rFSH treatment and again, while they were on T replacement, compared to those of healthy matched controls.

Figure 4

-Serum steroid hormone concentrations of CHH males from alternative / backdoor pathways of androgen biosynthesis
-Serum androstenediol concentrations, representing the alternative pathway of testosterone formation, in CHH males on hCG/rFSH and T replacement, compared to those of healthy controls.
-Serum androstanediol concentrations, representing the backdoor pathway of DHT formation in CHH males, on gonadotropin and T replacement and in healthy controls.
-Serum 11K T and 11 K DHT concentrations, representing the 11-oxygenated C19 androgen pathway in CHH males, on gonadotropin and T replacement and in healthy controls.

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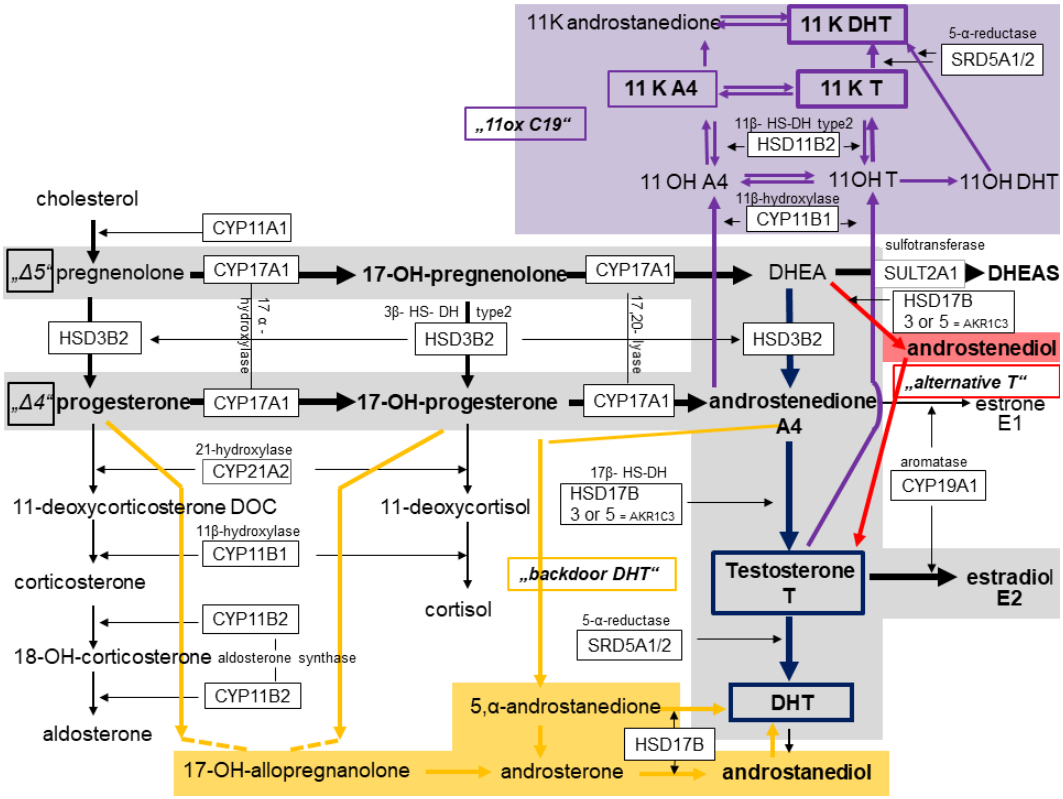
	CHH on hCG/rFSH	CHH on T	control (no hormone replacement)
	n= 25		n=56
Classic pathway			
Testosterone [nmol/l]	27.55 ± 9.40 27.05 (9.20-47.20)	22.12 ± 8.94 20.55 (7.2-41.1)	23.97 ± 7.84 21.9 (10.2-44.0)
Progesterone [nmol/l]	0.20 ± 0.13 0.17 (0.02-0.45)	0.16 ± 0.10 0.13 (0.02-0.36)	0.28 ± 0.13 0.27 (0.07-0.59)
17-OHP [nmol/l]	7.61 ± 4.95 6.93 (0.69-20.25)	1.65 ± 2.24 6.93 (0.69-20.25)	6.07 ± 2.69 5.70 (2.88-18.22)
17 OH-Preg [nmol/l]	5.50 ± 5.09 3.69 (0.36-20.31)	7.29 ± 4.36 6.98 (0.88-19.47)	9.75 ± 6.99 6.97(2.08-29.65)
DHEAS [nmol/l]	6027 ± 3123 5475 (2486-17134)	5329 ± 2417 5392 (1555-10693)	6456 ± 2628 6829 (689-11964)
Androstenedione [nmol/l]	3.29 ± 1.03 3.26 (1.42-5.74)	2.69 ± 0.81 2.72 (1.29-4.56)	3.09 ± 1.25 2.96 (1.51-7.42)
DHT [nmol/l]	1.77 ± 0.96 1.60 (0.57-4.13)	2.55± 1.86 2.17 (0.35-7.87)	1.59 ± 0.73 1.47 (0.36-4.65)
Estradiol [nmol/l]	0.18 ± 0.11 0.18 (0.04-0.50)	0.10 ± 0.06 0.08 (0.03-0.27)	0.11 ± 0.04 0.11 (0.03-0.20)
Cortisol [nmol/l]	290 ± 98 269 (172-571)	286 ± 119 258 (138-538)	318 ± 122 272 (113-615)
11-oxygenated androgen pathway			
11-K-T [nmol/l]	0.83 ± 0.51 0.82 (0.29-1.95)	0.81 ± 0.64 0.70 (0.00-2.44)	1.16 ± 0.97 0.51 (0.00-5.90)
11-K-DHT [nmol/l]	0.57 ± 0.42 0.48 (0.00-1.52)	0.60 ± 0.27 0.60 (0.00-1.09)	0.58 ± 0.44 0.51 (0.00-2.3)
11-K-A4 [nmol/l]	0.48 ± 0.19 0.35 (0.09-1.13)	0.50 ± 0.48 0.43 (0.06-2.35)	0.41 ± 0.23 0.35 (0.09-1.13)
Alternative pathway			

Androstenediol	2.56 ± 2.17	1.03 ± 1.17	2.31 ± 1.95
[nmol/l]	1.98 (0.00-9.09)	0.78 (0.00-5.71)	2.04 (0.00-9.25)
Backdoor pathway			
Androstanediol	0.46 ± 0.50	0.29 ± 0.49	0.11 ± 0.89
[nmol/l]	0.31 (0.00-1.46)	0.00 (0.00-1.86)	0.00 (0.00-0.84)



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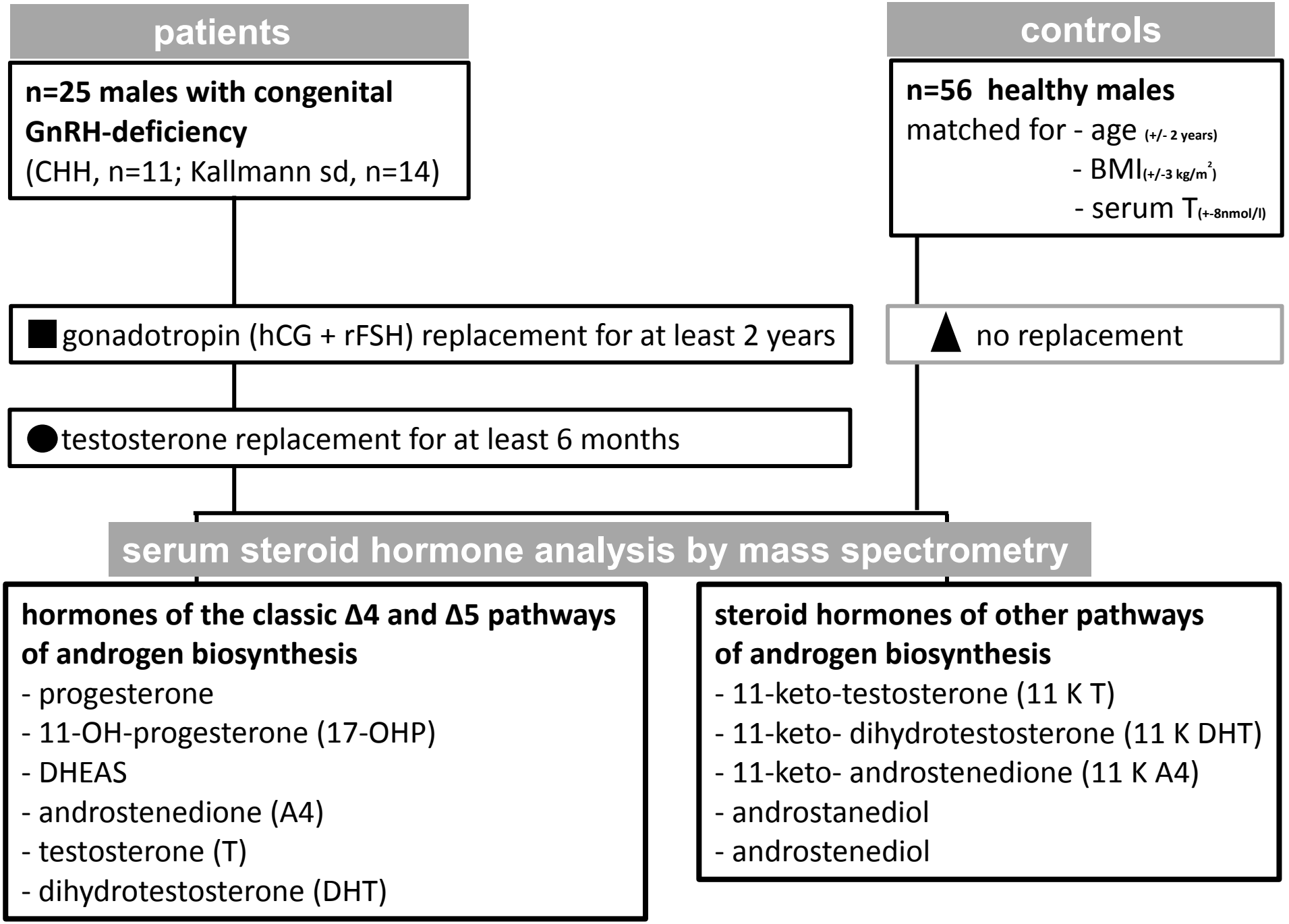
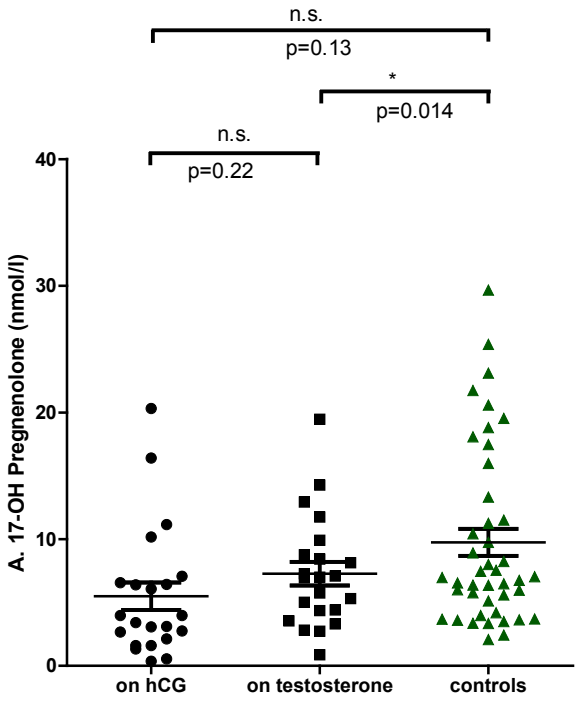
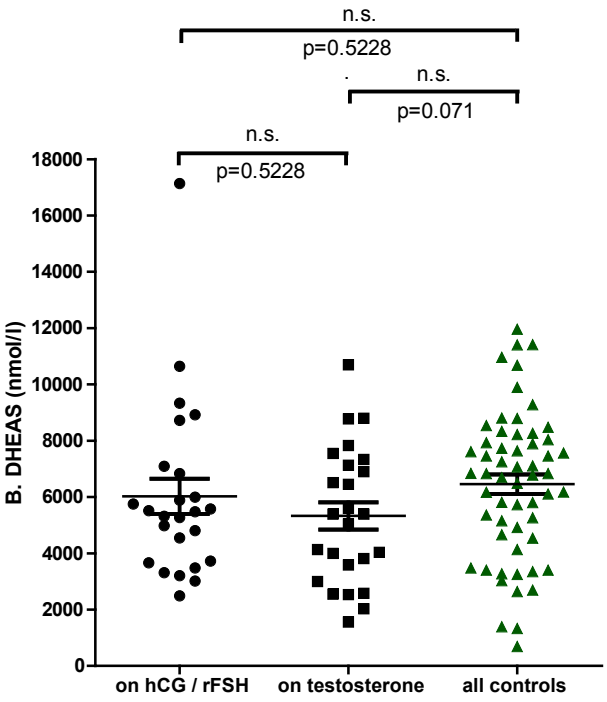
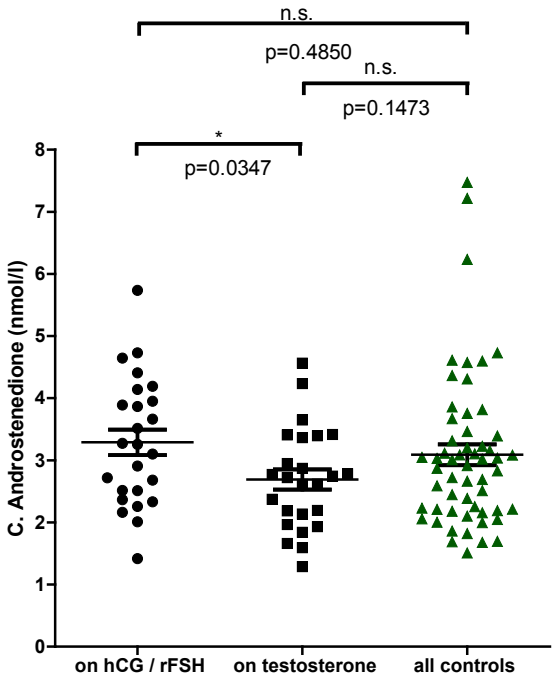
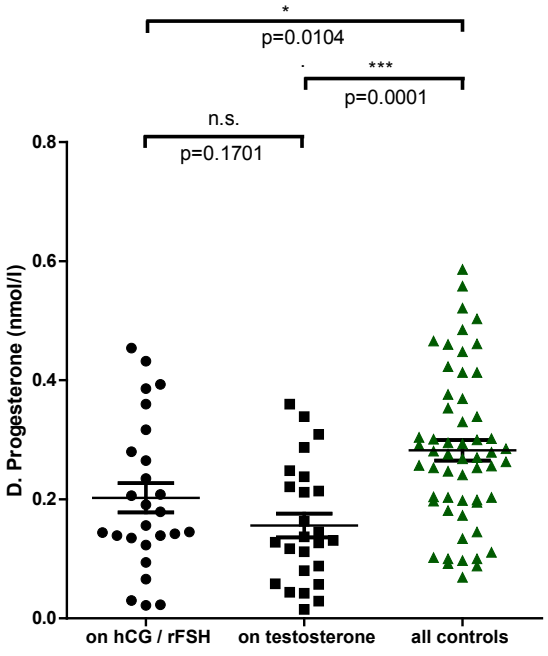
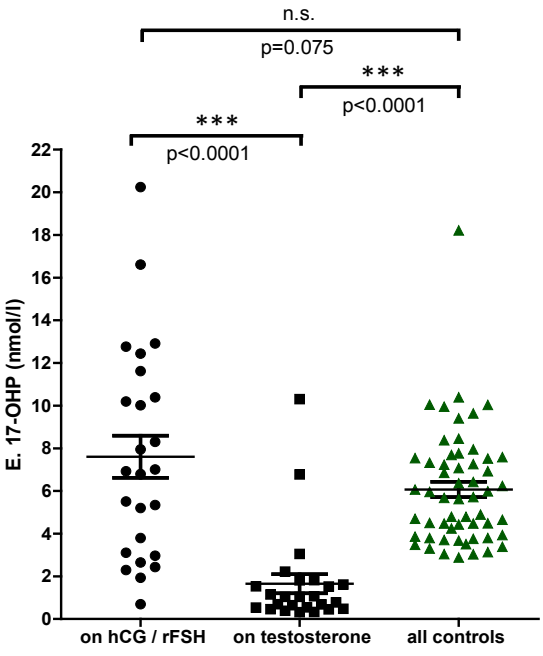

patients
n=25 males with congenital GnRH-deficiency
(CHH, n=11; Kallmann sd, n=14)
controls
n=56 healthy males
matched for - age (+/- 2 years)
- BMI (+/-3 kg/m²)
- serum T (+-8nmol/l)
■ gonadotropin (hCG + rFSH) replacement for at least 2 years
▲ no replacement
● testosterone replacement for at least 6 months
serum steroid hormone analysis by mass spectrometry
hormones of the classic Δ4 and Δ5 pathways of androgen biosynthesis
- progesterone
- 11-OH-progesterone (17-OHP)
- DHEAS
- androstenedione (A4)
- testosterone (T)
- dihydrotestosterone (DHT)
steroid hormones of other pathways of androgen biosynthesis
- 11-keto-testosterone (11 K T)
- 11-keto- dihydrotestosterone (11 K DHT)
- 11-keto- androstenedione (11 K A4)
- androstanediol
- androstenediol

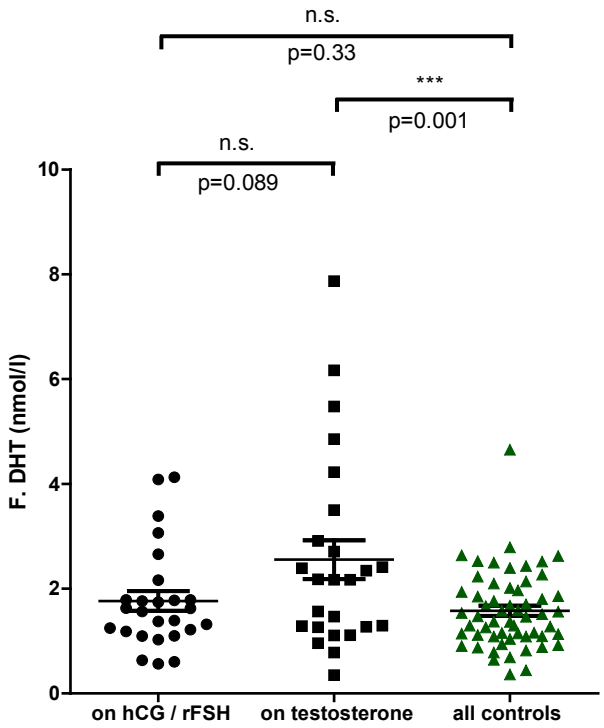

n.s.
p=0.13
*
p=0.014
n.s.
p=0.22
A. 17-OH Pregnenolone (nmol/l)
0
10
20
30
40
on hCG
on testosterone
controls

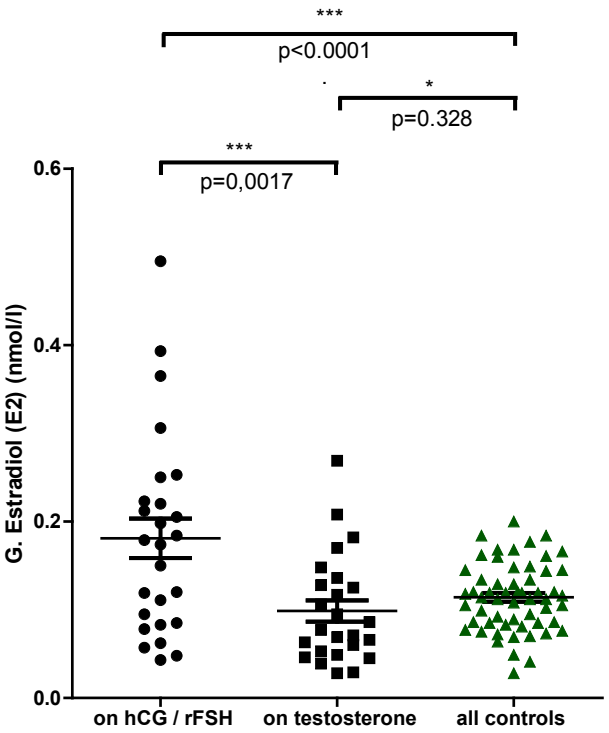

n.s.
p=0.5228
.
n.s.
p=0.071
n.s.
p=0.5228
B. DHEAS (nmol/l)
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
on hCG / rFSH
on testosterone
all controls

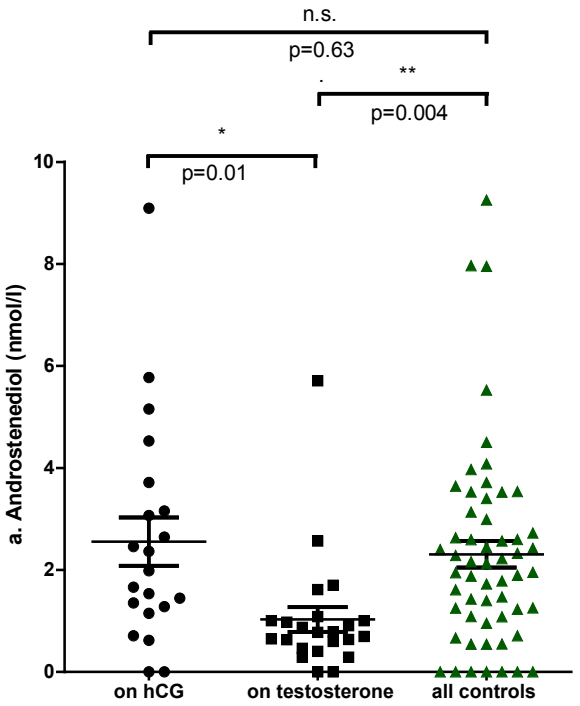

n.s.
p=0.4850
n.s.
p=0.1473
*
p=0.0347
C. Androstenedione (nmol/l)
0
1
2
3
4
5
6
7
8
on hCG / rFSH
on testosterone
all controls

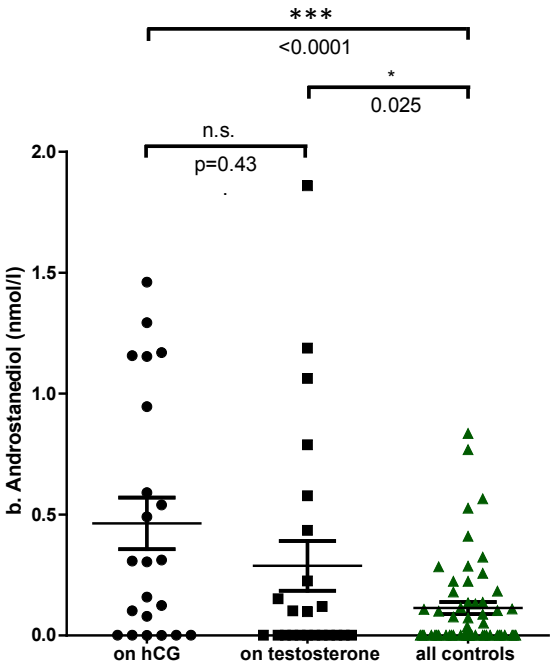

*
p=0.0104
.
***
p=0.0001
n.s.
p=0.1701
D. Progesterone (nmol/l)
0.0
0.2
0.4
0.6
0.8
on hCG / rFSH
on testosterone
all controls

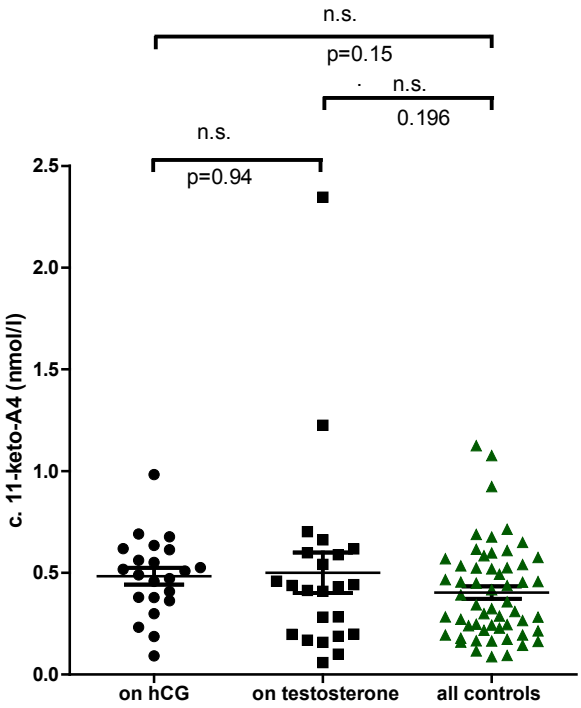

n.s.
p=0.075
***
p<0.0001
***
p<0.0001
E. 17-OHP (nmol/l)
0
2
4
6
8
10
12
14
16
18
20
22
on hCG / rFSH
on testosterone
all controls

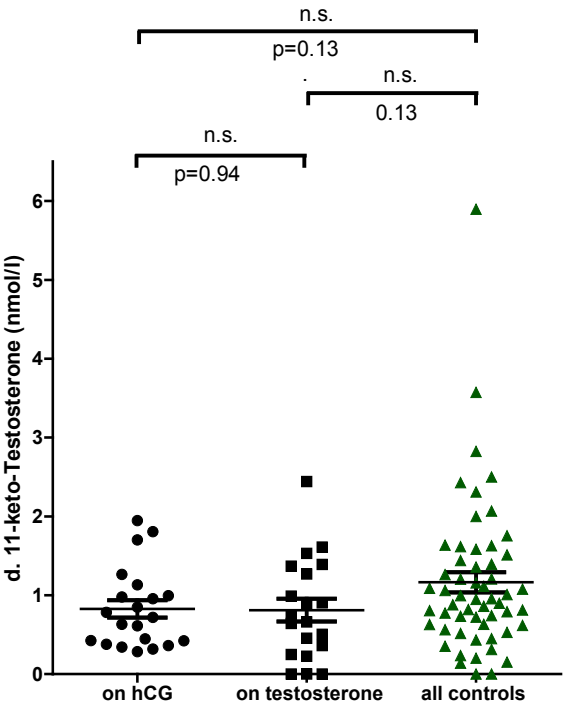


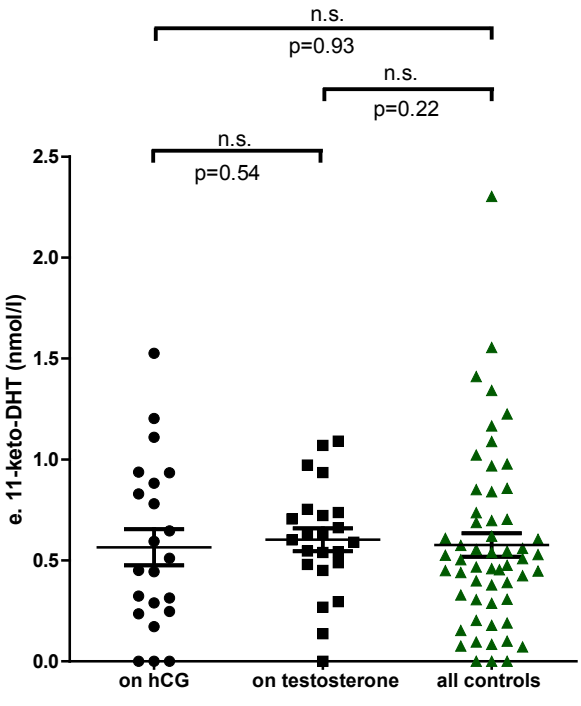



n.s.
p=0.63
**
p=0.004
*
p=0.01
a. Androstenediol (nmol/l)
10
8
6
4
2
0
on hCG
on testosterone
all controls


***
<0.0001
*
0.025
n.s.
p=0.43
b. Androstanediol (nmol/l)
2.0
1.5
1.0
0.5
0.0
on hCG
on testosterone
all controls


n.s.
p=0.15
n.s.
0.196
n.s.
p=0.94
c. 11-keto-A4 (nmol/l)
2.5
2.0
1.5
1.0
0.5
0.0
on hCG
on testosterone
all controls


n.s.
p=0.13
n.s.
0.13
n.s.
p=0.94
d. 11-keto-Testosterone (nmol/l)
6
5
4
3
2
1
0
on hCG
on testosterone
all controls


n.s.
p=0.93
n.s.
p=0.22
n.s.
p=0.54
e. 11-keto-DHT (nmol/l)
2.5
2.0
1.5
1.0
0.5
0.0
on hCG
on testosterone
all controls