RESEARCH

The *AMH* genotype (rs10407022 T>G) is associated with circulating AMH levels in boys, but not in girls

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Abstract

Objective: Fetal anti-Müllerian hormone (AMH) is responsible for normal male sexual differentiation, and circulating AMH is used as a marker of testicular tissue in newborns with disorders of sex development. Little is known about the mechanism of action in postnatal life. A recent genome wide association study (GWAS) reported genetic variation of AMH affecting AMH levels in young men. This study investigated the effect of genetic variation of AMH and AMH type II receptor (AMHR2) (*AMH*rs10407022 T>G and *AMHR2*rs11170547 C>T) on circulating reproductive hormone levels and pubertal onset in boys and girls.

Design and methods: This study is a combined longitudinal and cross-sectional study in healthy Danish boys and girls from the general population. We included 658 boys aged 5.8–19.8 years and 320 girls aged 5.6–16.5 years. The main outcome measures were genotyping of *AMH* and *AMHR2*, pubertal staging and serum levels of reproductive hormones.

Results: *AMH*rs10407022T>G was associated with higher serum levels of AMH in prepubertal boys (TT: 575 pmol/L vs TG: 633 pmol/L vs GG: 837 pmol/L, $P = 0.002$) and adolescents (TT: 44 pmol/L vs TG: 58 pmol/L vs GG: 79 pmol/L, $P < 0.001$). Adolescent boys carrying the genetic variation also had lower levels of LH (TT: 3.0 IU/L vs TG: 2.8 IU/L vs GG: 1.8 IU/L, $P = 0.012$). Hormone levels in girls and pubertal onset in either sex did not seem to be profoundly affected by the genotypes.

Conclusion: Our findings support recent GWAS results in young adults and expand our understanding of genetic variation affecting AMH levels even in boys prior to the pubertal decline of circulating AMH.

Introduction

Anti-Müllerian hormone (AMH) is produced by immature Sertoli cells in the male and in granulosa cells in preantral and small antral follicles in the female (1, 2). AMH is a glycoprotein hormone belonging to the TGF-β superfamily (3) and is encoded by the *AMH* gene. AMH binds to the specific AMH type II receptor (AMHR2) (4).

In the male fetus, AMH is essential for regression of the Müllerian ducts (5, 6). In a 46,XY fetus, mutations of *AMH* or *AMHR2* cause persistent Müllerian duct syndrome (PMDS) often including bilateral cryptorchidism but otherwise normal virilization of external genitalia (7). In postnatal life, serum levels of AMH are high during

childhood but decline rapidly during puberty when the Sertoli cells differentiate and start to express androgen receptors (8, 9). The function of AMH in adult men is not known. Circulating AMH varies widely between male individuals at levels comparable with levels in young adult females (10). No strong associations between serum AMH levels, and reproductive parameters have been reported in adult men (11). However, AMH concentrations in seminal fluid have been positively associated with sperm motility and spermatogenesis (12, 13, 14).

In women, AMH seems to be a gatekeeper of estradiol and inhibin B production by inhibition of FSH-induced follicle growth (15) and aromatase activity in granulosa cells (16, 17, 18). Circulating levels of AMH reflect the number of antral follicles as well as the number of resting primordial follicles (19). Thus, age-specific AMH levels appear to predict age at menopause (20). Healthy girls tend to maintain their individual AMH levels throughout childhood and puberty with minor fluctuations at the onset of puberty (21).

The biological actions of AMH depend on the individual AMH production and AMHR2 sensitivity. The non-synonymous single-nucleotide polymorphism (SNP) rs10407022 (*AMH* rs10407022 T>G) found in the coding region of *AMH* causes amino acid substitution (Ile[49]Ser), and minor allele carriers seem to have impaired AMH signaling/function (22). The SNP is associated with higher follicular phase estradiol levels in normo-ovulatory women and thereby supporting the notion of AMH regulating FSH-sensitivity in the ovary (23).

The non-coding rs11170547 SNP (*AMHR2* rs11170547 C>T) is located in a putative enhancer of the gene encoding AMHR2 (24). Thus, a reduced expression of AMHR2 on cell surfaces is expected in minor allele carriers. Accordingly, rs11170547 T is positively associated with circulating estradiol levels in adult women (22). Interaction between *AMH* rs10407022 T>G and *AMHR2* rs11170547 C>T is associated with age at menopause (25).

In a genome wide association study (GWAS) of British adolescents, a considerable part of inter-individual variation of circulating AMH levels was explained by genetic variation of *AMH* in young men but not in women (26). The present study is to our knowledge the first to assess the effect of genetic variation of AMH signaling (*AMH* rs10407022 T>G and *AMHR2* rs11170547 C>T) on circulating hormone levels in boys and girls during pubertal development.

Our findings support recent GWAS results in young adults and expand our understanding of genetic variation

affecting AMH levels even in boys prior to the pubertal decline of circulating AMH.

Subjects and methods

Healthy boys and girls

Participants were recruited from The Copenhagen Puberty Study (27, 28), a population-based combined cross-sectional and longitudinal cohort study of healthy Danish children and adolescents. The study was conducted at ten schools in the Copenhagen area, 2006–2014. All pupils were invited, 3102 girls and 3101 boys of whom 35% girls and 25% boys chose to participate.

We included all boys (658) and girls (320) with available DNA analyses on *AMH* rs10407022 T>G and *AMHR2* rs11170547 C>T as well as Tanner stage, and serum levels of AMH or inhibin B. No children had a history of endocrine, gynecological or cerebral illness.

Cross-sectional part of the study: 558 boys and 236 girls, median (range) age; boys: 11.6 (6.1–19.8) and girls: 10.7 (6.0–14.9). Longitudinal part of the study, 84 girls and 100 boys from two of the included schools were examined twice a year. Median (range) age at baseline; girls: 9.2 (5.6–12.9) years and boys: 9.4 (5.8–15.6) years. The children were followed for an average of 4.6 years (0.5–7.6) for the girls and 5.5 (0.4–7.6) for the boys.

We have previously described reproductive hormone levels (21) and genotypes (*FSH* and *FSHR*) in the same cohorts (29). In the present study, we report novel data concerning *AMH* rs10407022 T>G and *AMHR2* rs11170547 C>T, and their associations with circulating reproductive hormone levels.

Blood samples were drawn and a thorough clinical examination was performed in all participating children at every visit, including Tanner staging of breast (B1-B5) and genital development (G1-G5) in girls and boys, respectively (30). The total or median (range) number of blood samples varied between gender and study design: Girls, cross-sectional part 236, longitudinal part 8 (2–14) per girl; boys, cross-sectional part 558, longitudinal part 9 (2–14) per boy.

The total number of children and blood samples available for hormone analyses: AMH (320 girls: 931 samples; 658 boys: 1408), FSH (318 girls: 929; 658 boys: 1406), LH (318 girls: 929; 658 boys: 1406), inhibin B (316 girls: 910; 658 boys: 1405), estradiol (318 girls: 920; 658 boys: 1405) and testosterone (319 girls: 930; 658 boys: 1405).

Genotyping

Peripheral blood (EDTA-preserved) was used for isolation of genomic DNA using the QuickGene-810 Nucleic Acid Isolation System (Fujifilm, Life Science Products, Tokyo, Japan) and quantified on a NanoDrop ND-1000 spectrophotometer (Saveen Werner, Limhamn, Sweden).

The SNPs were analyzed using KASP SNP genotyping assays (LGC Genomics, Hoddesdon, UK), which facilitates bi-allelic discrimination through a competitive PCR and incorporation of a fluorescent resonance energy transfer quencher cassette. KASP genotyping assays were designed by LGC Genomics toward the following sequences: rs10407022, CTCCAGGCA[T/G]CCCACAAGAGC and rs11170547, GTTCTCCCTTTCAC[C/T]TACTAACACTAATTTG. A standard touch-down PCR program, as advised from the manufactures, was used to discriminate alleles. The annealing temperature was decreased by 0.6°C in 10 cycles from 61°C to 55°C for *AMHR2* rs11170547 C>T and from 68°C to 62°C for *AMH* rs10407022 T>G.

Reproductive hormone assays

All blood samples were drawn between 8:00 h and 13:00 h from an antecubital vein, clotted and centrifuged, and serum was stored at −20°C until hormone analyses were performed. Blood samples were analyzed after maximum 8 years of storage in a freezer at −20°C. All samples were analyzed in the same laboratory blinded for the technician for age and pubertal stage.

Serum AMH levels were determined using the Beckman Coulter enzyme immunometric assay (Immunotech, Beckman Coulter, Marseilles, France) with a detection limit of 2.0 pmol/L. The intra- and inter-assay coefficients of variation (CVs) were less than 7.8% and 10.8%, respectively. Serum levels of FSH and LH were measured by time-resolved immunofluorometric assays (Delfia; PerkinElmer) with detection limits of 0.06 and 0.05 IU/L, respectively. Intra-assay and inter-assay CVs were less than 5%. Serum concentrations of inhibin B were measured using the Beckman Coulter GenII assay with a detection limit of 3 pg/mL and inter-assay CV <11%. Serum estradiol was measured by radioimmunoassay (Pantex, Santa Monica, CA, USA), detection limit 18 pmol/L and intra- and inter-assay CVs were <8 and 13%, respectively. Testosterone was quantified using a newly developed and validated TurboFlow-liquid chromatography-tandem mass spectrometry (LC–MS/MS) method (31). Detection limit was 0.10 nmol/L, and relative standard deviation for the

quality control sample at the low and high concentration level was 10.0% and 5.7%, respectively. Hormone levels below the detection limit were assigned the value of 0.5 times the detection limit.

Statistical analyses

To evaluate if genotypes affected hormone levels as puberty progressed, longitudinal hormone levels stratified for genotypes were visualized according to age.

Our study contains data from the longitudinal part of The Copenhagen Puberty study (*n* = 84 girls and 100 boys). The raw dataset of each individual contains data collected in several examinations as puberty progressed. To evaluate the effect of SNPs on reproductive hormone levels, observations were grouped according to Tanner stage for each individual. To obtain sufficient group sizes for analyses, we formed subgroups according to merged Tanner stages as follows: Prepubertal girls: B1 and boys: G1; mid-pubertal girls: B2+B3 and boys: G2+G3; late pubertal girls: B4+B5 and boys: G4+G5 boys. If multiple observations within a specific Tanner subgroup were available from a given individual, a mean value was calculated. This mean value was introduced into the model and used as the single-occasion observations of the cross-sectional part of the study when performing statistical analyses. No girl contributed with more than one observation per statistical analysis.

Associations between genotypes (*AMH* rs10407022 T>G and *AMHR2* rs11170547 C>T) and circulating hormone levels were evaluated (*AMH* rs10407022 TT vs TG vs GG and *AMHR2* rs11170547 CC vs CT vs TT) using the Kruskal–Wallis test. A *P* value ≤0.05 was considered statistically significant.

To determine if the genotypes influenced the onset of puberty (B2 or G2), we performed probit analyses (29).

Ethical considerations

The Copenhagen Puberty Study (ClinicalTrials.gov ID: NCT01411527) was carried out in accordance with the Second Helsinki Declaration and in accordance with the protocols approved by the scientific ethical committee at The Capital Region of Denmark (KF 01 284; V200.1996/90, KF 01 030/97/KF 01276357/H-1-2009-074) as well as the Danish Data Protection Agency (2010-41-5042). All children and parents received written information, and informed consent was obtained from all participants.

Results

The children and adolescents had the following allele distributions: *AMH* rs10407022 T>G (TT 684, TG 263, GG 24, minor allele frequency (MAF) 16%) and *AMHR2* rs11170547 C>T (CC 777, CT 188, TT 12, MAF 11%). Distributions were consistent with Hardy–Weinberg equilibrium (Pearson's $\chi^2 = 0.05$, $P = 0.830$ and $\chi^2 = 0.03$, $P = 0.860$, respectively).

Boys

Boys carrying *AMH* rs10407022 G had higher serum levels of AMH prior to – as well as after – the pubertal decline of AMH levels; prepubertal boys (G1); Median (Range) AMH TT: 575 (120–1883) pmol/L vs TG: 633 (66–2075) pmol/L vs GG: 837 (363–1468) pmol/L, $P = 0.002$ and adolescents (G4+G5): TT: 44 (13–159) pmol/L vs TG: 58 (13–631) pmol/L vs GG 79 (50–119) pmol/L, $P < 0.001$ (Fig. 1 and Supplementary Table 1, see section on supplementary data given at the end of this article).

Adolescent boys carrying *AMH* rs10407022 G had lower serum levels of LH (G4+5): LH TT 3.0 (0.4–9.3) IU/L vs TG 2.8 (1.0–7.3) IU/L vs GG 1.8 (0.6–3.6) IU/L, $P = 0.012$. No associations with FSH, inhibin B, testosterone or estradiol, respectively, were found (Supplementary Table 1).

We found a weak association between *AMHR2* rs11170547 C>T and lower serum levels of AMH in the mid-pubertal boys (G2+3): Median (Range) CC: 232 (20–1580) pmol/L vs CT: 150 (36–1086) pmol/L vs TT: 62 (49–75) pmol/L, $P = 0.047$. *AMHR2* rs11170547 C>T was not systematically associated with any other of the circulating reproductive hormone levels (Supplementary Table 1).

Girls

Adolescent carriers of *AMH* rs10407022 G had lower serum levels of estradiol (B4+5): TT 171 (31–681) pmol/L vs TG 174 (56–420) pmol/L vs GG 78 (37–113) pmol/L, $P = 0.048$. This was mirrored by a nearly significant association with higher LH and lower testosterone in B4+5. No associations between *AMH* rs10407022 T>G and circulating levels of AMH, FSH or inhibin B, respectively, were found (Supplementary Table 1).

AMHR2 rs11170547 C>T was associated with lower serum estradiol levels in adolescent (B4+5) girls; CC 153 (31–562) pmol/L vs CT 194 (32–681) pmol/L vs TT 160 (121–198) pmol/L, $P = 0.037$.

No associations between *AMHR2* rs11170547 C>T and AMH, inhibin B, FSH, LH or testosterone, respectively, were found (Supplementary Table 1).
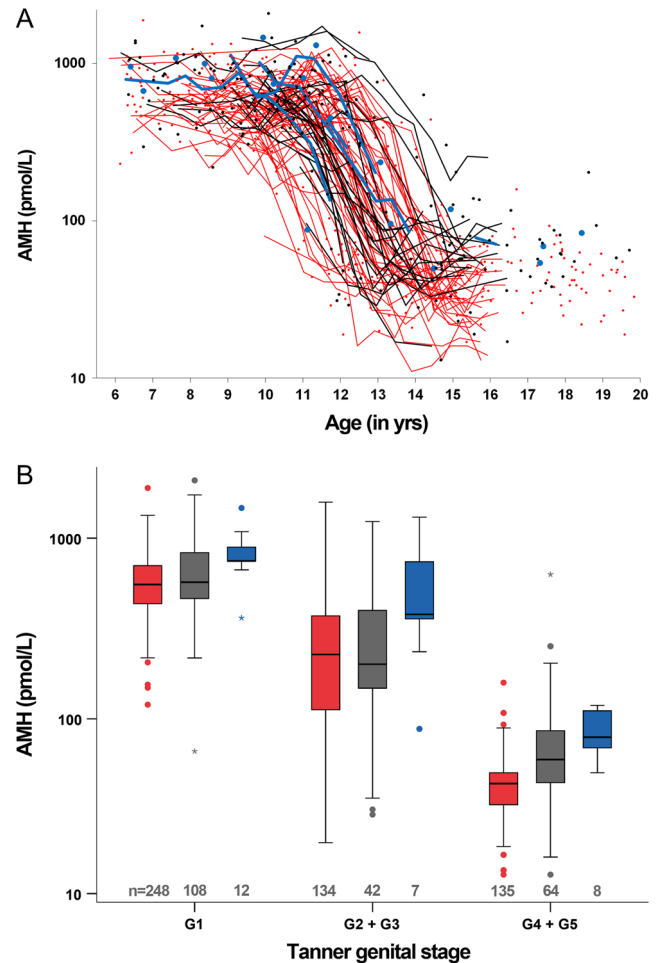

Figure 1

Boys. (A) Serum AMH levels according to age stratified by *AMH* rs10407022 T>G, TT red; TG black; GG blue. (B) Serum AMH levels according to Tanner subgroups stratified by *AMH* rs10407022 T>G, TT red; TG black; GG blue. If multiple observations within a specific Tanner subgroup were available for the same boy, a mean value was calculated. In the boxplots medians are marked by thick black lines; boxes contain 50% of the distribution. Whiskers mark 2.5th–97.5th percentile.

Age at pubertal onset

Neither genotype affected age at pubertal onset in boys nor girls; i.e. boys: mean age at testis volume ≥ 4 mL (95% CI): *AMH* rs10407022 TT 11.8 (11.6–11.9) years vs TG 11.8 (11.6–12.1) years vs GG 11.4 (10.7–12.1) years, $P = 0.670$; *AMHR2* rs11170547 CC 11.8 (11.6–11.9) years vs CT 11.9 (11.6–12.2) years vs TT 11.1 (9.7–12.5) years, $P = 0.786$; girls: mean age at thelarche (95% CI): *AMH* rs10407022 TT 10.0 (9.8–10.2) years vs TG 9.9 (9.5–10.3) years vs GG 10.0 (8.7–11.3) years, $P = 0.554$; *AMHR2* rs11170547 CC 10.0 (9.8–10.3) years vs CT 9.7 (9.2–10.2) years vs TT 9.7 (8.0–11.5) years, $P = 0.183$ (data not shown).

Discussion

This is the first study to evaluate the effect of genetic variation affecting AMH signaling on reproductive hormones in healthy children before and during puberty. The genetic variation of *AMH* (*AMH* rs10407022 T>G) was associated with serum levels of AMH in boys, but not in girls.

Males

We observed a significant effect of the selected SNP in the gene encoding *AMH* on circulating AMH levels. The minor allele, *AMH* rs10407022 G, was strongly associated with higher AMH levels in prepubertal (G1) and adolescent boys (G4+G5). Individual AMH levels are relatively stable in prepubertal boys and adolescents (8). However, we speculate that any genetic effect in the mid-pubertal boys (G2+G3) may have been blunted by the drastic pubertal decrease of serum AMH level (8) resulting in a greater variation of AMH levels depending on the specific timing of examination during pubertal onset. This wide dispersion of AMH levels in mid-pubertal boys may have been aggravated by the merging of Tanner stages (i.e. boys of G2+G3 treated as one group), which was necessary to perform reliable statistical analyses. There is a risk that the extreme decline of AMH is also responsible for the weak association observed in the mid-pubertal boys (G2+3) between the *AMHR2* rs11170547 C>T minor alleles and lower circulating AMH levels. We would expect the reverse association indicating compensatory increased levels of AMH due to impaired signaling through the receptor.

In the present study, the effect on AMH levels in adolescence was not caused by an effect on age at pubertal onset. In support of our findings, a recent GWAS study of 1360 male and 1455 female British adolescents described that three SNPs in and around the *AMH* gene had a strong influence on circulating AMH levels in males but not in females (26). Our findings add novel information on the impact of genetic variation in AMH signaling on circulating AMH levels in childhood and puberty.

The biological importance of the postnatal secretion of AMH and its large inter-individual variation of serum levels is still unknown. During the mid-childhood quiescence of the hypothalamic-pituitary-gonadal (HPG) axis, high AMH is a marker of testicular tissue (32). Low levels of AMH can be found in boys with bilateral cryptorchidism due to Sertoli cell dysfunction (33). In patients with Klinefelter syndrome (47,XXY), AMH declines to sub-normal levels simultaneously with the pubertal hyalinization of seminiferous tubules (34). Thus, healthy adolescents with AMH levels in the low part of the normal range may have compromised Sertoli cell function. However, studies on semen from healthy males and patients have not showed associations between circulating AMH and reproductive parameters (11, 35). *AMH* rs10407022 G was associated with lower levels of LH in the adolescent boys. This is in line with our previous report on an association between circulating AMH and LH in boys from the same cohort (8). Our present genetic findings suggest that AMH regulates LH levels. Whether regulation is exceeded directly on the hypothalamic-pituitary level (36) or a result of secondary effect of AMH function in the testes remains to be elucidated (37).

In support of GWAS findings (26), we did not observe systematic associations between *AMHR2* rs11170547 C>T and reproductive serum levels in boys.

Females

The negative association between *AMH* rs10407022 G and estradiol levels in late adolescent girls was based on very few observations and may thus be a random finding. In addition, the here-employed estradiol assay is insensitive in ranges near the detection limit. We also did not time our blood sampling in girls who had experienced menarche to a specific cycle day, which may explain the large variation in many reproductive hormones, i.e. LH, inhibin B and estradiol in this late pubertal group. Furthermore, due to the putative inhibitory effect on aromatase activity, we would expect the opposite effect; i.e. higher estradiol levels in homozygote minor alleles with reduced AMH function.

The positive association with estradiol observed in *AMHR2* rs11170547 C>T heterozygotes seems more physiologically relevant. However, low levels of estradiol in the few homozygote minor alleles question this finding. In support of a true positive association is other data from healthy adult women, in whom minor allele carriers of *AMH* rs10407022 T>G and *AMHR2* rs11170547 C>T had higher serum levels of estradiol, which did not affect their numbers of antral follicles (23). These findings support *in vitro* data of AMH-suppressing aromatase activity in granulosa cells (38, 39).

We found a statistically significant and positive effect of the *AMH* SNP on circulating testosterone in the prepubertal girls. This was, however, not consistent across the alleles; i.e. testosterone was lower in the small homozygote group. We would expect that an impaired

AMH signaling increased the aromatase activity and thereby decreased the testosterone level. Additionally, in prepubertal girls, a major part of circulating testosterone is derived from the adrenal gland.

Our findings are also in line with studies of adult women, where the two investigated SNPs did not affect circulating AMH levels (23). In a cross-sectional study of healthy women, *AMH* rs10407022 T>G affected age at menopause only when interaction with *AMHR2* rs11170547 C>T was considered, however, the effect size was limited (25). Consequently, when serum levels of AMH are used to counsel individual women about their reproductive lifespan, genetic variation of AMH signaling does not seem to be relevant to account for.

We speculate if the striking gender difference regarding the selected *AMH* SNP in exon 1 could be explained by different splice variants expressed in the testes and ovaries. It is known that the testes primarily express the splice variant of the *AMH* gene with exon 1, which is not the case in the ovaries and pituitary gland, respectively (https://www.gtexportal.org/home/). Mamsen and coworkers (40) have shown that the levels of precursor molecules of AMH differ in the testes compared to the ovaries, which further points at gender difference in the genetic regulation of AMH levels.

Strengths and limitations

The children in this population-based cohort study are from ten schools in the affluent part of Copenhagen, and they may therefore not be fully representable for the entire background population. Girls were not examined according to their menstrual cycle. A major challenge for the interpretation of our data is the low minor allele frequency and thus low number of homozygotes in both sexes. Time interval from blood sampling to hormone assay analysis was independent from genotype. Thus, identified differences in hormone level between genotypes were not attributable to storage duration. Possible risk of blood sample damage resulting in low or false hormone level results is deemed highly unlikely, as repeated analysis of the same sample after re-freezing and thawing has produced similar hormone levels in our laboratory.

In addition to the main hypothesis of AMH polymorphisms affecting circulating AMH levels, we have tested a number of secondary associations with other hormone levels. Thus, there is a risk of type I error. We have therefore approached weak associations with critical interpretations.

In conclusion, genetic variation of AMH signaling was associated with circulating AMH levels in boys at specific stages of pubertal development. Further studies are necessary to elucidate whether low AMH in minor allele carriers of *AMH* rs10407022 T>G could potentially reflect compromised adult testicular function.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contribution statement
I K G, A S B and C P H carried out analyses and drafted the article. M G M, L A and C P H collected data. K A carried out genetic analyses, K M M and A J designed the study. All authors critically revised the manuscript and approved its final version.

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