Supplemental Materials

for

Comparison of detection of normal puberty in girls by a hormonal sleep test and a gonadotropin-releasing hormone agonist test

Robert L Rosenfield, Brian Bordini, Christine Yu

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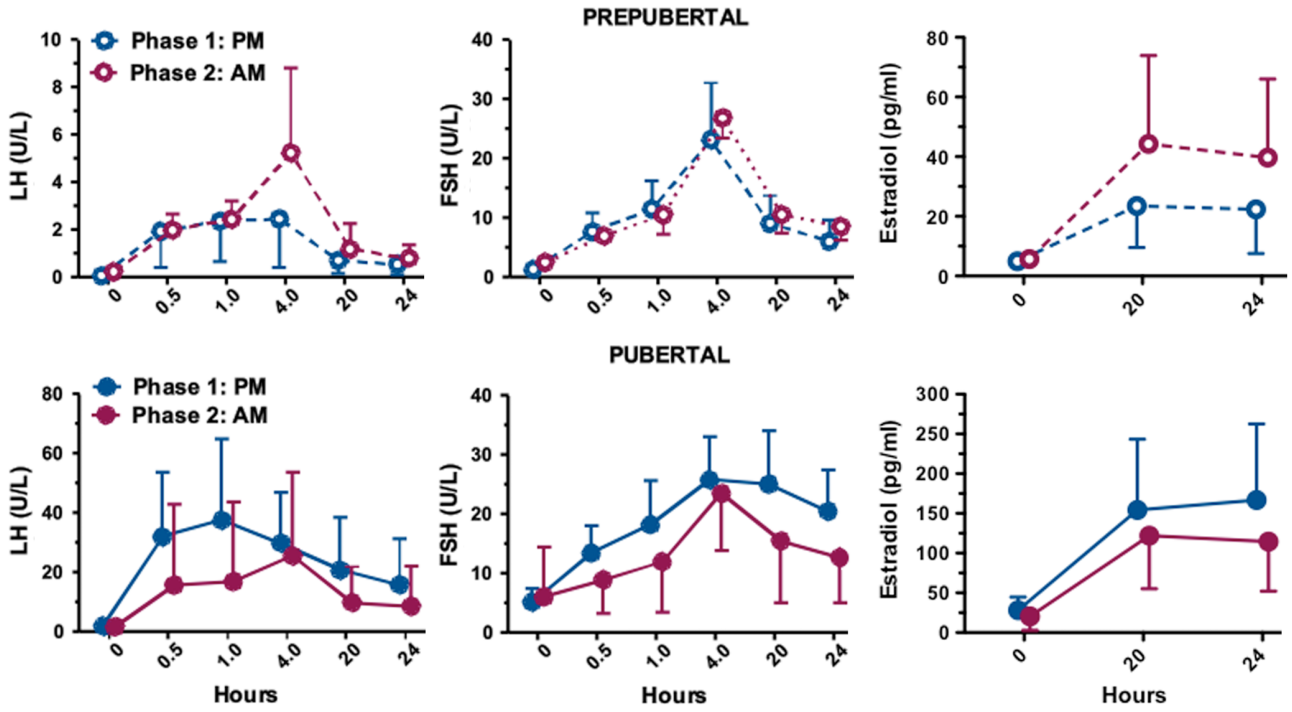
Supplemental Figure 1. Comparison of Morning and Evening GnRH Agonist Testing

Supplemental Figure 1. Comparison of responses to GnRHag administered in the evening after low-dose dexamethasone (Phase 1) to that administered in the morning (Phase 2). Baseline (0) is that for early morning. Upper panels prepubertal, lower panels pubertal. Note discontinuous scale.



Prepubertal phase 1 girls (n=20) were significantly younger than prepubertal phase 2 girls (n=7) (7.9 ± 1.1 vs 9.5 ± 0.6, SD, yr) and had a significantly lower bone age (7.9 ± 1.3 vs 9.8 ± 0.7 yr), both p = 0.0002. Pubertal phase 1 girls (n=20) had a significantly higher breast stage than pubertal phase 2 girls (n=13) (3.0 ± 0.5 vs 2.3 ± 0.5 yr; p = 0.001. Otherwise there were no significant clinical differences between study phases in stage-matched girls.

Repeated measures 2-way ANOVA demonstrated no significant differences between morning and evening gonadotropin responses to GnRHag, and there were no differences in estradiol responses to GnRHag after controlling for age, pubertal stage, and body mass index. This study had 80% power to detect a 1.3 SD (prepubertal) to 1.0 SD (pubertal) difference at any given time point between groups.

Supplemental Table 1. Sleep test descriptive statistics in girls. Mean ± SD (5-95th percentile).

GROUP	WAKE		SLEEP			
	Mean Level	Peak Level	Mean Level	Peak Level	Mean Level Rise	Peak Level Rise
LH (U/L)						
Prepubertal (n = 27)	≤0.15 ± 0.02 (≤0.15)	0.15 ± 0.02 (≤0.15-0.20)	0.25 ± 0.16 (≤0.15 - 0.5)	0.49 ± 0.52 (≤0.15 - 1.25)	0.11 ± 0.16* (0 - 0.35)	0.35 ± 0.52* (0 - 1.1)
Pubertal (n=34)	2.1 ± 2.0*a* (0.15 - 6.3)	3.6 ± 2.7 (≤0.15 - 8.0)	3.5 ± 2.7 (0.15 - 8.7)	6.0 ± 4.0 (0.15 - 12.6)	1.4 ± 1.4† (0.0 - 4.6)	3.3 ± 2.4† (0.0 - 7.8)
p Pre vs Pub	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*
FSH (U/L)						
Prepubertal (n = 27)	1.3 ± 0.6 (0.5 - 2.0)	1.3 ± 0.7 (0.4 - 2.4)	1.8 ± 1.0 (0.5 - 3.1)	2.0 ± 1.2 (0.5 - 3.7)	0.57 ± 0.77** (-0.4 - 1.5)	0.75 ± 0.82** (-0.2 - 1.9)
Pubertal (n=34)	4.0 ± 2.1 (1.0 - 8.1)	4.1 ± 2.1 (1.0 - 8.3)	4.9 ± 2.5 (0.9 - 9.1)	5.5 ± 2.8 (1.1 - 10.2)	0.9 ± 1.5* (-0.6 - 3.9)	1.4 ± 2.0** (-0.6 - 5.9)
p Pre vs Pub	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*NS*	*NS*
Estradiol (pg/ml)						
Prepubertal (n = 27)	4.9 ± 2.9 <5 - 8	4.9 ± 2.0 <5 - 9	5.1 ± 1.6 <5 - 8	5.3 ± 1.6 <5 - 9	0.0 ± 2.4 -4.0 - 3.0	0.4 ± 2.2 -3.0 - 3.0
Pubertal (n=34)	20 ± 14 (<5 - 44)	22 ± 16 (<5 - 55)	20 ± 15 (<5 - 47)	26 ± 18 (<5 - 53)	-0.1 ± 8.5 (-14 - 14)	4.2 ± 16 (-19 - 37)
p Pre vs Pub	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*NS*	*NS*

p Sleep vs Wake:
** <0.02*
*** <0.001*
† <0.0001

Supplemental Table 2. LH pulse analysis descriptive statistics in girls. Mean ± SD (5-95th percentile).

GROUP	WAKE		SLEEP		
	Pulse Frequency (per 3hr)	Pulse Amplitude Mean (U/L)	Pulse Frequency (per 3hr)	Pulse Amplitude Mean (U/L)	Pulse Amplitude Mean Rise (U/L)
Prepubertal (n = 27)	0.05 ± 0.27 (0-0)	0.01 ± 0.04 (0-0)	0.6 ± 0.6* (0-1.8)	0.34 ± 0.4 (0-1.1)	0.34 ± 0.4** (0-1.1)
Pubertal (n=34)	1.2 ± 1.1 (0-3.2)	2.5 ± 2.5 (0-7.4)	1.2 ± 0.7 (0.0-2.0)	5.3 ± 3.6 (0 - 11.8)	2.8 ± 2.1† (0 - 6.6)
p Pre vs Pub	*<0.0001*	*<0.0001*	*<0.001*	*<0.0001*	*<0.0001*

p Sleep vs Wake:
** <0.01*
*** <0.001*
† <0.0001

Supplemental Table 3. GnRH agonist test descriptive statistics in girls. Mean ± SD (5-95th %ile).

GROUP	0 hr (7-10AM)	0.5 hr	1.0 hr	4 hr	20 hr	24 hr
LH (U/L)						
Prepubertal	0.12 ± 0.1	2.0 ± 1.3	2.4 ± 1.5	3.2 ± 2.7	0.8 ± 0.7	0.6 ± 0.5
(n = 27)	(<0.1 - 0.3)	(0.8 - 3.1)	(0.9 - 4.1)	(0.9 - 8.9)	(0.3 - 2.2)	(0.2 - 1.9)
Pubertal	2.0 ± 2.1	26 ± 25	30 ± 29	29 ± 22	17 ± 16	13 ± 15
(n=33)	(0.1 - 7.2)	(1.7- 81)	(2.0 - 99)	(2.9 - 85)	(1.1 - 59)	(0.9 - 51)
p Pre vs Pub	*0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*0.004*	NS
FSH (U/L)						
Prepubertal	1.7 ± 0.9	7.5 ± 2.8	11 ± 4.4	24 ± 8.5	9.5 ± 4.3	6.6 ± 3.5
(n = 27)	(0.5 - 2.9)	(4.2 - 12)	(5.7 - 18)	(9.3 - 37)	(4.3 - 14)	(2.5 - 11)
Pubertal	4.6 ± 2.5	12 ± 5.5	16 ± 8.3	25 ± 8.3	21 ± 11	17 ± 8.2
(n=33)	(1.1 - 9.0)	(5.2 - 25)	(7.2 - 38)	(14 - 39)	(7.6 - 43)	(5.8 - 32)
p Pre vs Pub	*<0.0001*	*<0.001*	*0.010*	NS	*<0.0001*	*<0.0001*
Estradiol (pg/ml)						
Prepubertal	5.2 ± 2.0	-	-	-	29 ± 20	26 ± 19
(n = 27)	(<5 - 8)	-	-	-	(8 - 51)	(7 - 51)
Pubertal	25 ± 17	-	-	-	142 ± 82	146 ± 87
(n=33)	(6 - 53)	-	-	-	(30 - 343)	(33 - 354)
p Pre vs Pub	*<0.0001*				*<0.0001*	*<0.0001*

Supplemental Analyses of the Pubertal Transition: Effects of Adiposity in Girls and Sexual Dimorphisms in Pituitary-Gonadal Function

Previous reports indicate that excessive adiposity affects pubertal hormonal responses to sleep in pubertal (PUB), but not prepubertal (PRE), girls (1, 2). Overweight (OW) PUB girls were found to have blunted sleep-related LH and FSH rises compared to those of normal-weight (NW) PUB girls, although their average gonadotropin and estradiol levels were similar. In addition, the estradiol responses to GnRHag-induced LH release was reported to be lower in OW-PUB than in NW-PUB girls, compatible with decreased gonadotropin biopotency (1). Preliminary comparisons within the 2007-2010 cohort showed that sleep-related LH rises were also lower in OW-PUB (n=6) than in NW-PUB (n=8) ($p < 0.02$).

The pubertal transitions of girls and boys were found to differ in previously unreported ways.

This Supplement reports secondary analyses carried out to document these findings.

Comparison of Overweight to Normal-Weight Girls

Subjects & Methods

Subjects and statistical considerations. Forty-eight percent of the PRE and 41% of PUB girls were overweight (body mass index $\geq 85^{th}$ percentile). These studies had 80% power to detect 1 SD differences between groups. Results are presented as mean ± SD.

Supplemental laboratory methods. Plasma free estradiol was computed from plasma total estradiol and percent free testosterone with a sensitivity of 0.7 pg/ml and precision of 12% (3).

Results

Baseline. NW and OW girls of like stage were similar in chronologic and bone age. The PRE groups differed significantly only in BMI percentile (52 ± 30 vs 94 ± 4.7). The PUB groups differed significantly in BMI percentile (50 ± 20 vs 94 ± 5.3), breast stage (2.5 ± 0.5 vs 3.0 ± 1.5, $p = 0.028$), and SHBG (32 ± 14 vs 22 ± 10 nM, $p < 0.05$); plasma free testosterone tended to be higher in OW-PUB (range <3- 8 vs <3-10 pg/ml, $p = 0.07$).

Sleep tests. Prepubertal girls had nominally significant sleep-related gonadotropin rises ($p < 0.05$) that were of similar magnitude irrespective of BMI status (**Table S1**).

Table S1. Sleep-related gonadotropin levels in OW and NW girls (mean ± SD).

	Wake LH	Sleep Rise LH	Wake FSH	Sleep Rise FSH
	Mean (U/L)	Mean (U/L)	Mean (U/L)	Mean (U/L)
NW-PRE (n=14)	≤0.15 ± 0.02	0.08 ± 0.10	1.3 ± 0.68	0.56 ± 0.82
OW-PRE (n=13)	≤0.15 ± 0.00	0.13 ± 0.21	1.2 ± 0.55	0.59 ± 0.75
NW-PUB (n=20)	1.9 ± 2.1	1.8 ± 1.6	3.9 ± 1.9	1.3 ± 1.7
OW-PUB (n=14)	2.3 ± 2.0	0.77 ± 1.1*	4.0 ± 2.4	0.36 ± 0.87

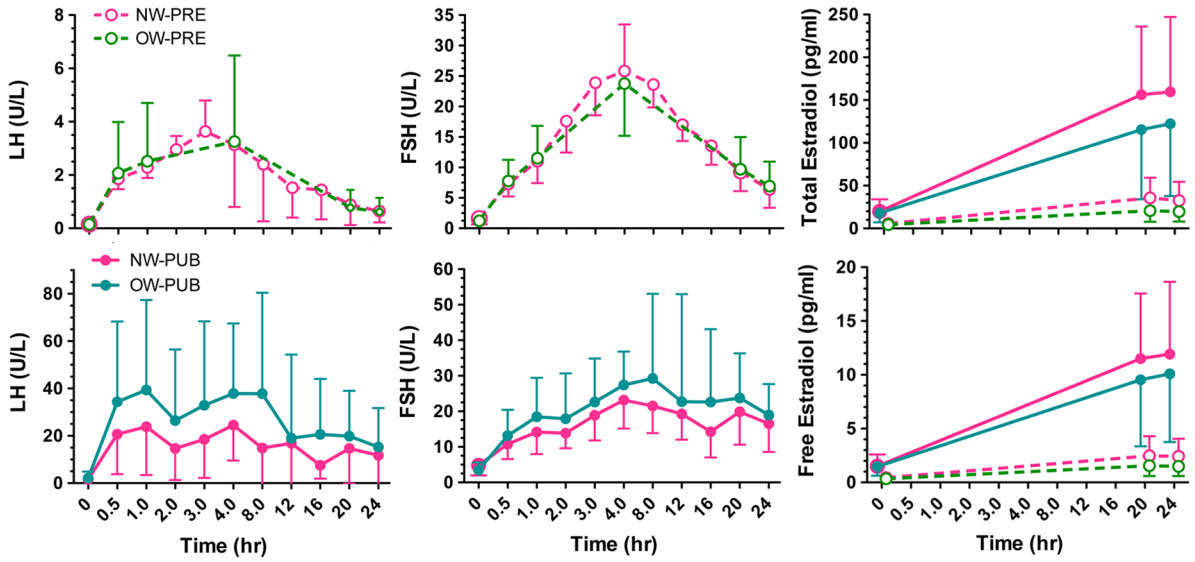
* $p < 0.05$ vs NW-PUB rise

Wake and sleep gonadotropin *levels* were not significantly different between stage-matched OW and NW groups, nor were estradiol levels or sleep-related rises. However, OW-PUB girls had significantly lower sleep-related *rises* in mean LH than NW-PUB though the OW-PUB were slightly more advanced in breast stage (**Table S1)**. The LH changes were attributable to decreased sleep-related rises in mean pulse amplitude ($p=0.035$). OW-PUB girls' sleep-related FSH rises were also blunted in that their sleep-related rise in mean FSH was not significant ($p=0.2$), unlike NW-PUB.

GnRHag tests. Prepubertal OW (n = 12) and NW (n = 14) girls had virtually identical responses to GnRHag (**Figure S1**). OW-PUB (n = 12) tended to have higher LH and FSH responses and had significantly lower estradiol responses to GnRHag than NW-PUB (n = 21) (main effect after controlling for the more advanced breast stage of OW-PUB, $p=0.01$); no time-points differed significantly between groups (**Fig. S1**). The ratio of

peak estradiol/peak FSH was significantly lower in OW-PUB than NW-PUB girls (4.2 ± 2.8 vs 7.7 ± 5.0, p=0.025), and the comparable ratio involving free estradiol was marginally significant (p=0.05).

Figure S1. Responses to GnRHag of normal-weight and over-weight girls (mean ± SD). Prepubertal data shown in open symbols, pubertal data in closed symbols. Datasets were incomplete (n ≤7) at intermediate timepoints (2, 3, 8,12, and 16 hr).



Discussion

Sleep-related gonadotropin rises are confirmed to be blunted in overweight premenarcheal pubertal girls, though average gonadotropin and estradiol levels are normal, as previously reported in a subset of these study groups (1). This expanded dataset shows a blunting of LH pulse amplitude, but not of pulse frequency; this finding is compatible with an obesity effect on LH metabolism (4), but does not exclude an obesity effect on hypothalamic rhythmicity. Such OW effects were not seen in boys (5). However, the possibility cannot be excluded that LH pulsation was blunted because slow-wave sleep was less in OW-PUB girls (6).

Estradiol responses to GnRHag are shown by these additional data to be significantly lower in the OW-PUB although data at the individual time-points did not differ significantly. Gonadal hypo-responsiveness to FSH in OW-PUB is suggested by a significantly decreased ratio of peak total estradiol to peak FSH post-GnRHag. However, this abnormality is partially attributable to the lower SHBG of obesity since the comparable ratio computed from free estradiol is only marginally decreased. Boys had similar patterns, though the more complete data at intermediate time-points revealed their LH to be transiently significantly increased 16-hr post-GnRHag (5). While these data are compatible with the evidence that gonadotropin clearance from blood is accelerated in obese girls, which diminishes gonadotropin bioactivity (4), the significantly low estradiol in OW-PUB girls is greatly attributable to their low plasma SHBG, which itself accelerates estradiol clearance (7).

Comparison of Pituitary-Gonadal Function in Girls and Boys

Subjects & Methods

Subjects and statistical analyses. Girls were compared to a subset of boys from a contemporaneous companion study (PRE, n = 18; early PUB, n = 13) (5). PUB boys were primarily defined by 8AM testosterone ≥20 ng/dl. Analyses were controlled for the early PUB stage of girls (breast stage ≥2, but premenarcheal) by excluding PUB boys who had adult 8AM testosterone levels. Results are expressed as mean ± SD.

Results

Boys were older than girls of comparable pubertal status, as expected. PRE age (yr) was 10.1± 0.8 (boys) and 8.3 ± 1.2 (girls, p <0.0001); PUB age was 12.1 ± 1.0 (boys) and 11.2 ± 1.1 (girls, p = 0.01).

PRE boys had significantly higher evening wake (p = 0.03), sleep and early morning LH (p <0.01) than PRE girls. PRE boys also released significantly more LH in response to GnRHag than PRE girls (p <0.05 at all time-

points from 0.5-20 hr). Whereas a sleep peak LH ≥0.5 U/L was associated with the appearance of puberty in most girls, 95% of boys remained prepubertal until the sleep peak LH reached a much higher level (≥3.7 U/L) (5). Similarly, whereas a 4-hr LH ≥4.5 U/L was associated with puberty in most girls, 95% of boys remained prepubertal until ≥14.8 U/L was achieved. Subsequently, early pubertal boys and girls had similar wake, sleep and morning LH levels as well as LH responses to GnRHag.

Although the ambient FSH level critical for the onset of puberty seemed similar in boys and girls (approximating 1.0 U/L, see text), FSH levels were significantly lower in PRE boys than girls (e.g., baseline 1.1 ± 0.6 vs 1.7 ± 0.9 U/L, $p = 0.025$). Though early PUB boys' nocturnal FSH remained significantly lower ($p < 0.025$), morning baseline FSH was similar between the sexes. However, both PRE and PUB boys released significantly less FSH than girls of comparable pubertal status at all post-GnRHag time-points ($p < 0.0001$).

Estradiol levels did not rise significantly during sleep in PUB girls. This contrasts with PUB boys' testosterone levels, which rose significantly during sleep ($p < 0.0001$). However, we found significantly higher morning than sleep estradiol levels, consistent with most published data, which suggests that the spontaneous estradiol peak of puberty occurs mid-day in girls, rather than at night as testosterone does in boys (8-11).

In spite of their higher LH levels, only 6% of PRE boys had a significant detectable gonadal steroidogenic (testosterone) response to GnRHag ($p < 0.0001$) (5). This contrasts with PRE girls, in 73% of whom estradiol rose significantly from morning baseline levels that were below the assay functional sensitivity (<10 pg/ml) into the pubertal range (>15 pg/ml) post-GnRHag.

The relationship between LH secretion during sleep and in response to GnRHag was more variable in girls than boys (e.g., sleep peak vs 4-hr post-GnRHag, r=0.807 vs 0.964, resp., $p < 0.01$)**.** This contributed to our inability to identify a single sleep or post-GnRHag LH level in girls that provided both ≥95% sensitivity and specificity for the detection of puberty, which contrasts to boys, in whom sleep peak LH ≥3.7 U/L and LH 4-hr post-GnRHag ≥14.8 U/L each met this criterion (5).

Discussion

Our 6-10 yr-old prepubertal girls had lower spontaneous and GnRHag-stimulated LH levels, but higher FSH spontaneous and stimulated levels, than prepubertal 9-12 yr-old boys. Our frequent sampling protocol thus demonstrated a sexual dimorphism in LH responsiveness to GnRHag that was not seen prepubertally in a previous report (12). The higher FSH/LH ratio of girls has been fairly well established to begin during fetal life and to persist into childhood (13-15). The LH differences seem related to congenital/perinatal androgen-programming of LH pulsatility (16), whereas FSH differences seem related to negative feedback effects of the lower inhibin-B and higher activin-A serum levels of girls than boys (17, 18).

Notably, GnRHag-stimulated gonadotropins elicited pubertal sex steroid levels in prepubertal girls, but not in prepubertal boys: prepubertal girls' estradiol rose significantly in response to GnRHag. This evidence of exquisite prepubertal ovarian gonadotropin-sensitivity is consistent with the evidence that prepubertal girls have higher estrogen levels than prepubertal boys (19). Though it is uncertain whether ovarian steroidogenesis is more sensitive to gonadotropin stimulation than testicular steroidogenesis, in view of the greater potency of estradiol and the greater sensitivity of the estradiol assay, the ovary seems *functionally* more sensitive: puberty appears to begin at a lower LH level in girls than boys. Boys remained prepubertal at sleep-related LH levels found only in pubertal girls; puberty did not develop in boys until the sleep peak LH reached a level considerably higher than in girls. The characteristic capacity of mature females to generate a large LH surge in response to GnRH or GnRHag seems to emerge only in the perimenarcheal stage of puberty (12, 20) and, thus, seems to be related to attainment of the critical level of estrogen production required to sensitize the gonadotrope to GnRH and promote positive feedback (21).

Cyclic pituitary-ovarian activity becomes apparent during the late prepubertal years (22, 23). Peripubertal gonadotropin secretion is expected to be highly sensitive to negative feedback by the modest estradiol secretion it engenders, initiating subclinical cycles of activity/attenuation of pituitary function and follicular growth. We postulate that this cyclicity underlies the significantly lesser relationship between LH secretion during sleep and in response to GnRHag in girls than boys and our inability to identify a single sleep or post-GnRHag LH level in girls that provided both ≥95% sensitivity and specificity for detecting puberty.

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