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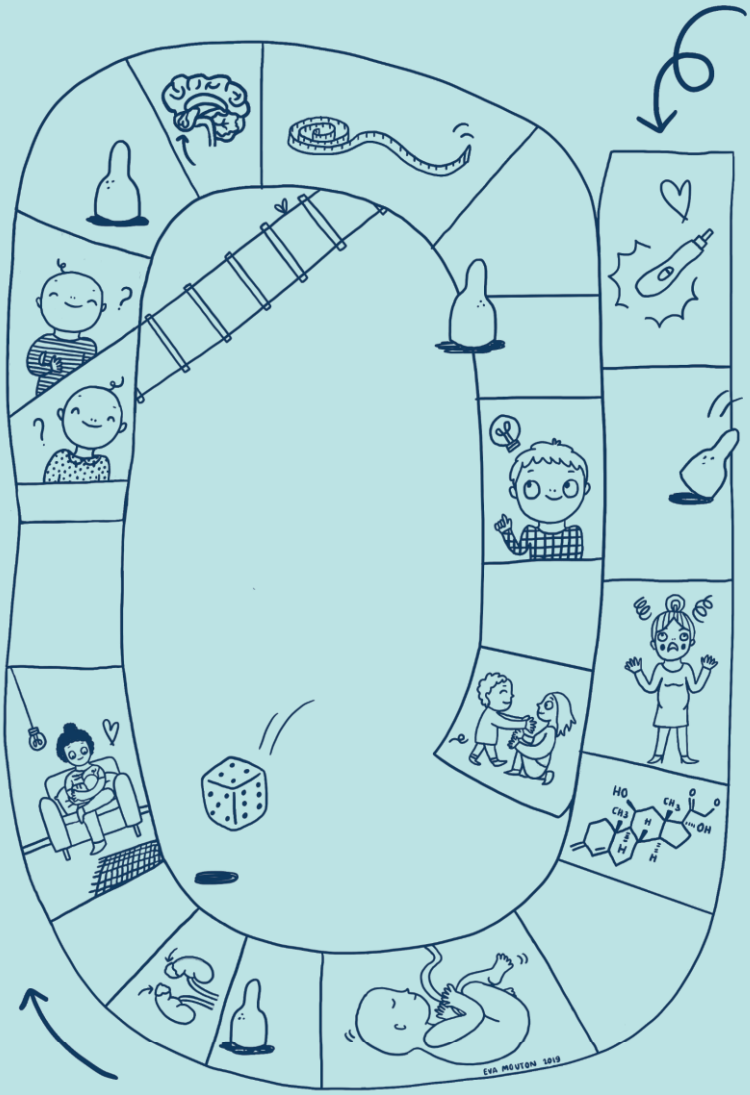
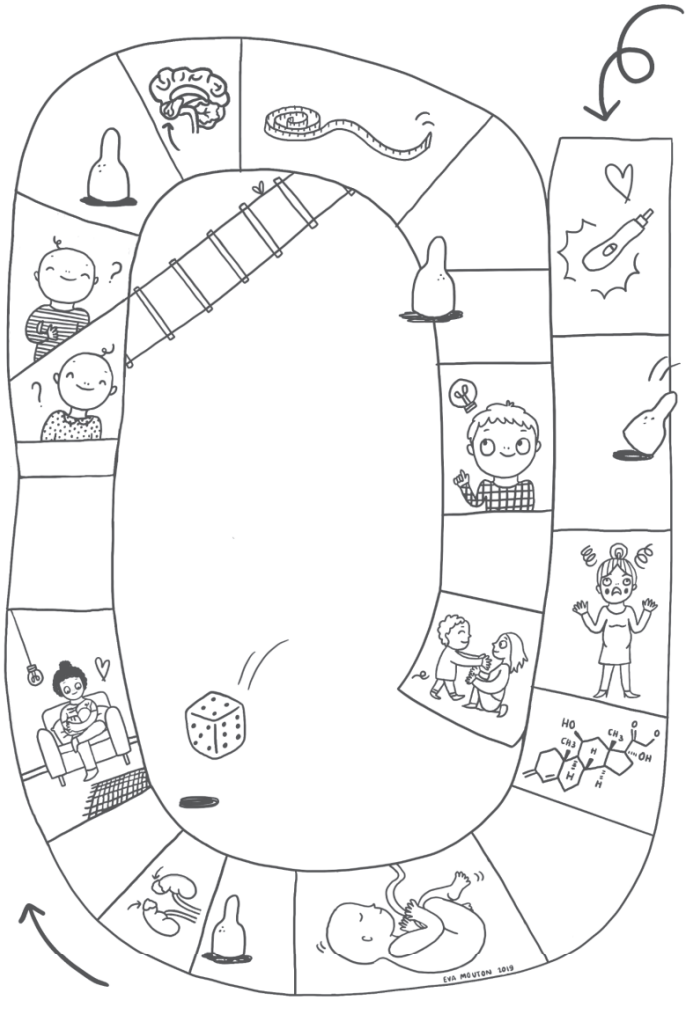
Part 2

Glucocorticoid regulation and sex

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Gender-specific differences in hypothalamus–pituitary–adrenal axis activity during childhood: a systematic review and meta-analysis

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Biology of Sex Differences (2017) 8:3.

ABSTRACT

Background

Gender-specific differences in HPA axis activity have been postulated to emerge during puberty. We conducted a systematic review and meta-analysis to test the hypothesis that gender-specific differences in HPA axis activity are already present in childhood.

Methods

From inception to January 2016, PubMed and Embase.com were searched for studies that assessed non-stimulated cortisol in serum or saliva, or cortisol in 24h-urine in healthy males and females aged ≤18yr. Studies were reported conform the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement. Standardized mean differences (95%CIs) were calculated and analyzed using fixed-effect meta-analysis stratified for age: <8yr (prepubertal) and 8-18yr (peri-/postpubertal). For comparison, we ran the same analyses using random-effects models.

Results

Two independent assessors selected 413 out of 6,158 records (7%) for full-text screening, of which 79 articles were included. Of these, 58 (with data on 16,551 subjects) were included in the meta-analysis. Gender differences in cortisol metabolism differed per age group. Boys aged <8yr had 0.18 (0.06 to 0.30) nmol/L higher serum and 0.21 (0.05 to 0.37) nmol/L higher salivary cortisol levels, while between 8-18yr, boys had 0.34 (0.28 to 0.40) nmol/L lower serum and 0.42 (0.38; 0.47) nmol/L lower salivary cortisol levels. In 24h-urine, cortisol was consistently higher in boys, being 0.34 (0.05 to 0.64) and 0.32 (0.17 to 0.47) µg/24h higher in the <8yr and 8-18yr groups, respectively. However, gender-differences in serum cortisol <8 yr and between 8-18 yr were absent when using random-effects models.

Conclusions

Gender differences in cortisol metabolism are already present in childhood, with higher salivary cortisol in boys aged <8yr compared to girls. This pattern was reversed after age 8 yr. In contrast, the gender-specific difference in cortisol production as assessed through 24h-urine did not change with age. Although differences were small, and analyses of gender differences in serum cortisol were inconclusive, they might contribute to gender-specific origins of health and disease.

BACKGROUND

The hypothalamus-pituitary-adrenal (HPA) and hypothalamus-pituitary-gonadal (HPG) axes are closely connected. Animal studies demonstrated that CRH inhibits the HPG axis at all levels, while testosterone inhibits the HPA axis at the hypothalamic level. Additionally, estrogens stimulate the HPA axis at both the hypothalamic and adrenal levels. Moreover, CRH levels were dependent on the phase of the menstrual cycle, with the highest concentrations occurring during the follicular phase.[1,2]

Human studies suggested that estrogens decrease the hepatic A-ring reduction of cortisol, albeit not in the short term[3] and increase the production of CBG, thereby affecting the bioavailability of cortisol.[1,4,5] The latter being enhanced by the use of oral contraceptives. Furthermore, HPA axis responses to acute psychological stress were different depending on the phase of the menstrual cycle.[2,4]

Due to an increase in sex steroid concentrations, gender differences in HPA axis activity have been postulated to emerge during puberty.[6,7] However, more recent evidence suggests that gender differences in HPA axis activity are already present early in life.[1,8,9] Putative mediators of these prepubertal gender differences are the postnatal reproductive hormone surge, also known as mini-puberty,[10] and sex-specific effects of styles in parental care, such as psychosocial stress reactivity to maternal over-controlling behavior.[11] However, physiological gender differences in cortisol concentrations during childhood have not been studied yet.

Therefore, the question was raised whether gender differences in unstimulated HPA axis activity emerge during puberty or whether they are already present earlier in life. Accordingly, we conducted a systematic review and meta-analysis with the hypothesis that gender-specific differences in unstimulated HPA axis activity are present in early life and are subsequently influenced by puberty.

METHODS

Search strategy

From inception up to 14 January 2016, PubMed and Embase.com were searched (by BvdV and JCFK) for studies that reported non-stimulated cortisol in serum or saliva, or cortisol in 24h-urine for healthy boys and girls aged ≤18 yr separately. Appendix 1 presents the full search strategy, which was based on the following index terms or free-text words: 'cortisol' or 'glucocorticoid', and 'sex difference' or 'sexual characteristics', and 'child' or 'adolescent'. Studies in children with (psycho)pathology, on synthetic glucocorticoids, or with risk for abnormal HPA axis activity (e.g., a history of maltreatment) were excluded. An English language restriction was applied for abstracts of published

articles. No restrictions for year of publication or study design, apart from reviews and case reports, were applied. The review protocol was based on the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement.

Data collection

Two independent assessors (BvdV and JJH) screened 6,158 titles and abstracts without consideration of outcomes. Studies were not assessed blindly. Disagreement between assessors was discussed until consensus was reached. When gender differences were analyzed without reporting on cortisol levels for boys and girls separately or when data were only presented in graphs, authors were requested for additional quantitative data. Data were stratified into two age groups: <8 yr (prepubertal) and between 8–18 yr (peri-/postpubertal). Ideally, stratification would have been based on pubertal staging according to Tanner. Unfortunately, only a minority of the included studies reported on the subjects' Tanner stages. Because pubertal onset before age 8 years is considered to be pathologic,[12] we chose 8 yr as cut off for stratification. When articles reported on serial cortisol measurements, we included only data on the youngest assessment age. When cortisol levels were reported prepubertally as well as peri-/postpubertally within the same individual, we included one sampling moment for each stratified group. When articles reported on the same study population, we included the article with the lowest bias risk. When articles reported on dynamic tests of HPA axis activity, we only included baseline cortisol. We only included the control subjects of case-control studies. If known, we excluded female subjects on oral contraceptives. When gender differences were described but not quantified, the articles were included in the descriptive analysis rather than the meta-analysis.

Meta-analysis

When necessary, we converted serum and salivary cortisol levels into nmol/L, and 24h-urine cortisol levels into µg/24h. When means ± SDs were not reported, the SD was calculated based on the following assumptions: the 95% CI is 3.92 SDs wide (2 × 1.96); the inter-quartile range is 1.35 SDs wide; the range is 4 SDs wide; the SD is the SE multiplied by the square root of the sample size.[13] To assess parametricity, we assumed that a normal distribution extends no more than 2 SDs from the mean,[14] i.e., when normally distributed, the mean minus 2 SDs should be >0 nmol/L. Data analyses were performed using Review Manager (RevMan) version 5.3.5, 2014. For each study, the standardized mean gender difference (95% CI) in cortisol concentration was calculated by combining the SD with the sample size. Subsequently, fixed-effect meta-analyses were performed first, which assumes that the effect estimate of the group differences was fixed across studies. Second, the results of these analyses were compared with random-effects meta-analysis, which weigh studies of variable sample sizes more equally. We reported any

source of bias from each included article conform the PRISMA statement and assessed selection, performance, detection and other biases. (Figure 1, Appendix 2) Bias was assessed as low, unclear or high. A sensitivity analysis was done by excluding studies that had ≥ 1 high bias risks. Heterogeneity of the data was assessed by the I^2 statistic, with significance defined as I^2 >50%. Publication bias was assessed through funnel plots.

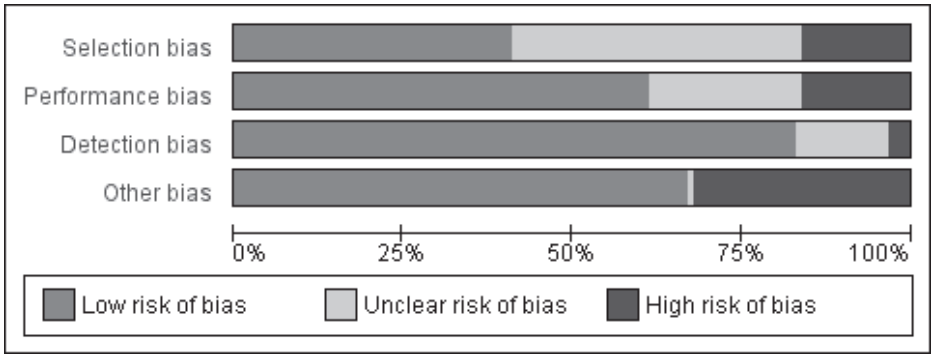


Figure 1: Risk of bias graph presenting a summary of the judgements of the assessors concerning risk of bias across all studies included in the meta-analysis. Bias risk is presented as percentage of total studies (n = 58).

RESULTS

Figure 2 shows the flowchart of the descriptive analysis and meta-analysis. Of the 6,158 titles and abstracts, 414 (7%) were eligible for full-text screening, from which 79 articles (19%) were included. Thirty-one authors of articles with insufficient quantitative data were contacted, of whom 12 responded: six provided the necessary quantitative data, five did not have access to the raw data anymore and one was not willing to participate. Two articles reported the cortisol production rate assessed through 24h-serum sampling, which hampered inclusion in the meta-analysis. The authors of 27 articles that only provided gender-specific data in figures were contacted, but could not be reached. Subsequently, these articles were excluded. Finally, 21 articles were included only in the descriptive analysis, and 58 articles (with data on 16,551 subjects) had sufficient data for inclusion in the meta-analysis.

Description of included studies

Studies were conducted in Europe (n = 36), North-America (n = 37), Asia (n = 3), South-America (n = 2) or Africa (n = 1), and were published between 1973 and 2016. Sample sizes ranged from 11 to 2,824 subjects, with seven studies having a sample size >500 subjects. Study designs were as follows: randomized placebo-controlled (n = 2), pro-

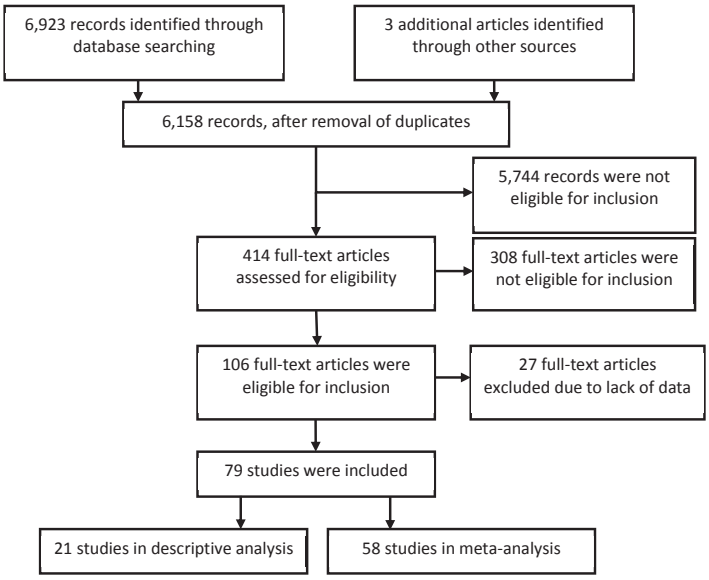


Figure 2: This flowchart presents the different phases of the systematic review and meta-analysis, conform the PRISMA-statement. (www.prisma-statement.org)

spective observational (n = 29), non-randomized intervention, i.e., stress tests (n = 15), cross-sectional (n = 16), longitudinal (n = 11) and case-control (n = 6). All studies that assessed serum or salivary cortisol used immunoassays, except for one that used high-performance liquid chromatography (HPLC). Studies that assessed 24h-urine cortisol used immunoassays (n = 4), gas chromatography–mass spectrometry (n = 3), HPLC (n = 1), and liquid chromatography-UV detection (n = 1). Twenty-two studies (28%) did not collect morning samples, of which 11 did not report the time of collection and 11 described specifically that samples were collected in the afternoon. Online Supplementary File 1 presents the data extracted from the articles included in the meta-analysis. Three out of 21 studies (14%) included in the descriptive analysis had no high bias risk (Table 1), while 16 out of 58 studies (28%) included in the meta-analysis had no high bias risk (Figure 1).

Gender-specific differences

Descriptive analysis

Table 1 summarizes the data on the 21 studies included in the descriptive analysis. The majority (90%) of these studies reported no significant gender differences in cortisol levels. Before age 8 yr, one study [15] found significantly lower salivary cortisol levels for boys at awakening. Between ages 8-18 yr, one study [8] found significantly lower morning salivary cortisol levels in boys.

Table 1: Summary of studies included in the descriptive analysis.

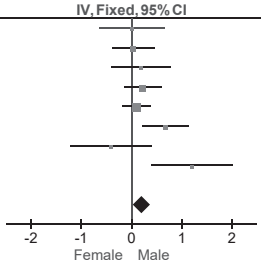
Group	First author (year)	N (%girls)	Age (yr)	Sample protocol	Assay	Result	Bias*
Saliva <8 yr	Klug (2000) [35]	119 (46%)	0	3 point day curve	Immunoassay	No gender differences	2
	Eiden (2015) [36]	257 (?)	0.75	Laboratory Temperament Assessment	Immunoassay	No gender differences	3
	Plusquellec (2011) [37]	466 (?)	1.6 ± 0. 1	Morning sample	Immunoassay	No gender differences	2
	Spinrad (2009) [38]	84 (49%)	4.5	Preschool Laboratory Assessment	Immunoassay	No gender differences	2
	Hatzinger (2007) [15]	102 (42%)	4.9 ± 0. 4	CAR	Immunoassay	Cortisol levels were lower in boys at awakening ($p<0.1$)	1
Saliva 8–18 yr	Safarzadeh (2005) [39]	100 (58%)	6 – 14	Morning sample	Immunoassay	No gender differences	1
	Isaksson (2015) [40]	68 (50%)	9	Morning sample	Immunoassay	No gender differences	2
	Kjölhede (2014) [41]	231 (50%)	9.5 ± 1.5	Morning sample	Immunoassay	No gender differences	1
	Vaillancourt (2008) [8]	154 (52%)	12.3 ± 0.8	Six samples standardized across time and day	Immunoassay	On Saturday morning boys had significantly lower morning levels. On Monday and Thursday no gender differences were found.	1
	Gunnar (2009) [42]	82 (49%)	9 – 15	TSST	Immunoassay	No gender differences	1
Serum <8 yr	Fadalti (1999) [43]	72 (49%)	0 – 2	Morning sample	Immunoassay	No gender differences	0
	Ballerini (2010) [44]	319 (45%)	0 – 5	Surplus serum	Immunoassay	No gender differences	2
	Parker (1978) [45]	106 (43%)	2 – 12	Morning sample	Immunoassay	No gender differences	2
Serum 8–18 yr	Kulasingam (2010) [46]	419 (?)	0 – 15	Surplus serum	Immunoassay	No gender differences	3
	Soldin (2005) [47]	376 (?)	0 – 18	Surplus serum	Immunoassay	No gender differences	1
	Karbasy (2015) [48]	711 (?)	0 – 19	?	Immunoassay	No gender differences	1
	Fadalti (1999) [43]	82 (49%)	6 – 18	Morning sample	Immunoassay	No gender differences	0
	Barra (2015) [49]	120 (45%)	12.4 ± 3	Morning sample	Immunoassay	No gender differences	1
	Chalew (1997) [50]	15 (73%)	12.7 ± 2.2	24h-blood withdrawal	Immunoassay	No gender differences	1
	Linder (1990) [51]	82 (58%)	8 – 17	24h-blood withdrawal	HPL	No gender differences .	0
Urine <8 yr	-						
Urine 8–18 yr	Dorn (1996) [52]	20 (55%)	15.2 ± 1.1	24h-urine sample	Immunoassay	No gender differences	1

* number of high risks of bias out of 4 bias categories (selection, performance, detection and other biases)

A

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
0 yr (Davis 1995)	30.1	36.4	18	29.5	32.8	18	6.1%	0.02 [-0.64, 0.67]
3 yr (Gunnar 2010)	4.03	4.58	37	3.89	3.01	54	14.9%	0.04 [-0.38, 0.46]
3.3 yrs (De Bruijn 2009)	2.82	1.86	19	2.45	1.82	27	7.5%	0.20 [-0.39, 0.79]
4 yr (Pérez-Edgar 2008)	11.9	4.7	51	10.8	5	60	18.6%	0.22 [-0.15, 0.60]
4.1yr (Mills 2008)	3.03	2.76	115	2.76	2.76	85	33.1%	0.10 [-0.18, 0.38]
4.3 yr (Tout 1998)	5.79	2.48	38	4.41	1.38	37	12.0%	0.68 [0.21, 1.14]
4yr (Gunnar 2010)	2.92	1.35	11	4.47	4.88	13	3.9%	-0.40 [-1.22, 0.41]
7.3yr (Törnhage 2002)	10.5	5.2	19	5.2	1.88	11	4.0%	1.19 [0.38, 2.00]
Total (95% CI)			**308**			**305**	**100.0%**	**0.21 [0.05, 0.37]**

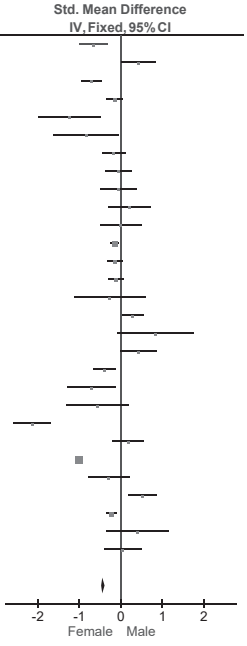
Heterogeneity: Chi² = 13.34, df = 7 (P = 0.06); I² = 48%
Test for overall effect: Z = 2.53 (P = 0.01)



B

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
8yr (Jones 2006)	9.58	1.51	68	10.59	1.49	72	1.6%	-0.67 [-1.01, -0.33]
8 yr (Azurmendi 2016)	8	4.14	49	6.35	3.59	41	1.0%	0.42 [-0.00, 0.84]
8yr (Martikainen 2013)	6.8	1.7	126	8	1.7	132	2.9%	-0.70 [-0.96, -0.45]
8.4 yr (Michels 2012)	12.66	6.12	183	13.84	8.96	202	4.6%	-0.15 [-0.35, 0.05]
9 yr (Alghadir 2009) active	0.13	0.026	25	0.16	0.017	12	0.3%	-1.25 [-2.00, -0.50]
9yr (Alghadir 2009) sedentary	0.18	0.014	12	0.2	0.028	16	0.3%	-0.84 [-1.62, -0.05]
9.2 yr (Cicchetti 2001)	8.64	5.16	124	9.52	5.21	80	2.3%	-0.17 [-0.45, 0.11]
9.4 yr (Turan 2015)	5.2	3.2	79	5.6	10.8	74	1.8%	-0.05 [-0.37, 0.27]
9.9 yr (Osika 2007)	5.34	2.1	34	5.5	3.33	50	1.0%	-0.05 [-0.49, 0.38]
10yr (Törnhage 2002)	11	4.08	28	8.9	13.03	31	0.7%	0.21 [-0.30, 0.72]
10.4±0.4yr (Cieslak 2003)	3	0.5	29	3	0.3	32	0.7%	0.00 [-0.50, 0.50]
11.1±0.55yr (Dietrich 2013)	11.2	4.7	818	11.9	4.73	786	19.0%	-0.15 [-0.25, -0.05]
11.9±0.6yr (Chen 2014)	4.69	5.52	213	5.52	5.24	212	5.0%	-0.15 [-0.34, 0.04]
11.9 yr (Portnoy 2015)	4.06	3.72	224	5.79	22.18	214	5.2%	-0.11 [-0.30, 0.08]
12 yr (Tzortzi 2009)	9.05	3.63	11	10.24	4.86	10	0.2%	-0.27 [-1.13, 0.59]
12.7 ± 2.9yr (Allen 2009)	0.17	0.18	119	0.13	0.09	116	2.8%	0.28 [0.02, 0.54]
12.7 yr (Yu 2009)	6.22	0.92	10	4.83	2.04	10	0.2%	0.84 [-0.08, 1.76]
13.4 yr (Mrug 2016)	3.43	2.83	40	2.31	2.5	39	0.9%	0.42 [-0.03, 0.86]
14.0±0.5yr (Belva 2013)	24.6	10.6	115	29.2	13.2	108	2.6%	-0.38 [-0.65, -0.12]
14.9yr (Alghadir 2009) active	0.22	0.015	20	0.24	0.034	30	0.5%	-0.70 [-1.29, -0.12]
14.9 yr (Alghadir 2009) sedentary	0.16	0.018	25	0.17	0.014	10	0.3%	-0.57 [-1.32, 0.17]
14 -16yr (Fransson 2014)	6.6	2	43	10.9	2	89	0.9%	-2.14 [-2.59, -1.69]
15.3 ± 1.1yr (Covelli 2012)	27	11	49	25	11.2	57	1.2%	0.18 [-0.20, 0.56]
15.5 yr (West 2010)	10.64	1.02	1418	11.67	1.02	1406	29.8%	-1.01 [-1.09, -0.93]
16 ±1yr (Georgopoulos 2011)	9.1	8.3	35	11.6	8.3	29	0.7%	-0.30 [-0.79, 0.20]
16.1 ±1.0yr (Daughters 2013)	3.22	2.13	59	2.3	1.38	73	1.5%	0.52 [0.17, 0.87]
16.6 ± 0,5yr (Reynolds 2013)	25.93	14.1	573	29.5	19	362	10.5%	-0.22 [-0.35, -0.09]
17.9 yr (Minckley 2012)	10.47	6.29	14	8.42	2.84	14	0.3%	0.41 [-0.34, 1.16]
18 yr (Colomina 1997)	13.7	7.7	37	13.4	7.6	36	0.9%	0.04 [-0.42, 0.50]
Total (95% CI)			**4580**			**4343**	**100.0%**	**-0.42 [-0.47, -0.38]**

Heterogeneity: Chi² = 481.05, df = 28 (P < 0.00001); I² = 94%
Test for overall effect: Z = 19.37 (P < 0.00001)



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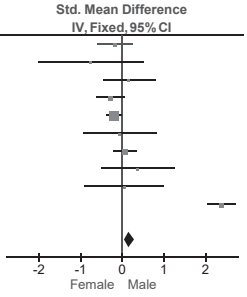
Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
0 yr (Elmlinger 2002)	253	95.7	42	271	97.4	44	7.7%	-0.18 [-0.61, 0.24]
0 yr (Forest 1978)	312	168	15	448	187	3	0.9%	-0.76 [-2.03, 0.51]
0 yr (Tennes 1973)	435.9	313.6	23	389.8	227.6	17	3.5%	0.16 [-0.47, 0.79]
0 yrs (Garagorri 2008)	249.41	99.32	80	280.31	124.16	58	12.0%	-0.28 [-0.62, 0.06]
0-2 yr (Bailey 2013)	159.8	135.3	261	190.2	186.2	208	41.6%	-0.19 [-0.37, -0.01]
0.4 yr (Lashansky 1991)	342	146	13	353	196	8	1.8%	-0.06 [-0.94, 0.82]
2-8 yr (Bailey 2013)	134.4	75.6	110	129.9	71.5	86	17.4%	0.06 [-0.22, 0.34]
3 yr (Lashansky 1991)	337	160	14	281	113	8	1.8%	0.37 [-0.51, 1.25]
4-10 yr (Tsvetkova 1977)	518.69	193.1	9	510.42	157.3	8	1.5%	0.04 [-0.91, 1.00]
6.8 yr (Soriano-Rodriguez 2010)	441.4	13.8	106	408.3	13.8	117	11.6%	2.39 [2.05, 2.74]
Total (95% CI)			**673**			**557**	**100.0%**	**0.17 [0.05, 0.29]**

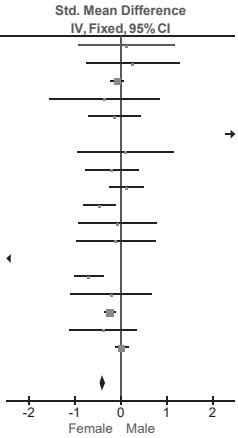
Heterogeneity: Chi² = 186.39, df = 9 (P < 0.00001); I² = 95%
Test for overall effect: Z = 2.79 (P = 0.005)



D

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
8 yr (Apter 1979)	240	80.3	7	226.2	109.5	7	0.4%	0.13 [-0.91, 1.18]
8yr (Lashansky 1991)	254	86	8	229	94	7	0.4%	0.26 [-0.76, 1.28]
8yr (Ong 2004)	210	58	423	215	69	347	22.4%	-0.08 [-0.22, 0.06]
9 yr (Ilias 2009)	112.97	49.14	5	153.26	131.05	6	0.3%	-0.36 [-1.56, 0.84]
6-15 yr (Ross 1986)	74.5	35.9	23	80	44.1	24	1.4%	-0.13 [-0.71, 0.44]
10yr (Stroud 2011)	164.4	5.9	39	146.5	5.99	30	0.9%	2.98 [2.28, 3.68]
11-14 (Tsvetkova 1977)	424.89	171.1	7	408.3	160	7	0.4%	0.09 [-0.95, 1.14]
11y (Elmlinger 2002)	341	113.5	22	364	117.3	23	1.3%	-0.20 [-0.78, 0.39]
12.5 yr (Susman 1991)	364.7	149.5	56	344.3	148.7	52	3.2%	0.14 [-0.24, 0.51]
13 yr (Hackney 2003)	397.3	126.9	62	466.3	162.8	63	3.6%	-0.47 [-0.82, -0.11]
13yr (Lashansky 1991)	223	74	10	229	94	11	0.6%	-0.07 [-0.92, 0.79]
14.6 yr (Ghaziuddin 2003)	1,757.5	1,191.9	12	1,914.7	1,658.2	9	0.6%	-0.11 [-0.97, 0.76]
14.7 yr (Huybrechts 2014)	299.5	1.6	297	305.6	1.7	426	7.8%	-3.67 [-3.91, -3.43]
15 yr (Syme 2008)	546.3	140.7	77	714.6	306.24	82	4.4%	-0.70 [-1.02, -0.38]
15.5yr (Lashansky 1991)	262	80	13	279	77	8	0.6%	-0.21 [-1.09, 0.68]
16.6 ± 0,5yr (Reynolds 2013)	551	154	653	590.98	202.79	401	29.1%	-0.23 [-0.35, -0.10]
17 yr (Stupnicki 1995)	347	130	14	397	125	15	0.8%	-0.38 [-1.12, 0.35]
8-18yr (Bailey 2013)	218.3	98.9	370	215.7	110.7	369	21.7%	0.02 [-0.12, 0.17]
Total (95% CI)			**2098**			**1887**	**100.0%**	**-0.39 [-0.46, -0.32]**

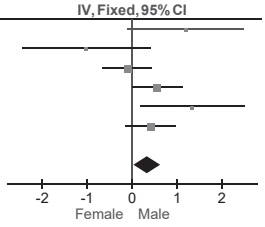
Heterogeneity: Chi² = 876.34, df = 17 (P < 0.00001); I² = 98%
Test for overall effect: Z = 11.36 (P < 0.00001)



E

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
3yr (Lundberg 1981)	3.35	1.12	4	2.46	0.44	9	5.2%	1.19 [-0.11, 2.49]
4.5yr (Lundberg 1983)	8.5	1.8	8	11.9	5.5	3	4.3%	-1.02 [-2.45, 0.41]
3-4 yr (Wudy 2007)	20.5	6.63	25	21.2	6.49	25	28.6%	-0.11 [-0.66, 0.45]
5-6 yr (Wudy 2007)	30.5	10.2	25	25.4	7.52	25	27.4%	0.56 [-0.01, 1.13]
5-10 yr (Nakamura 1984)	14.8	3.6	7	9.98	3.2	8	6.6%	1.34 [0.18, 2.50]
7-8 yr (Wudy 2007)	36.9	12.9	25	31.9	10.9	25	28.0%	0.41 [-0.15, 0.97]
Total (95% CI)			**94**			**95**	**100.0%**	**0.34 [0.05, 0.64]**

Heterogeneity: Chi² = 11.11, df = 5 (P = 0.05); I² = 55%
Test for overall effect: Z = 2.28 (P = 0.02)



F

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
8yr (Honour 2007)	2,826.86	774.81	244	2,557.71	655.56	188	57.7%	0.37 [0.18, 0.56]
9-10 yr (Wudy 2007)	35.5	9.26	25	34.9	12.5	25	6.9%	0.05 [-0.50, 0.61]
4-15yr (Canalis 1982)	15.2	6.3	14	13	5.6	15	3.9%	0.36 [-0.38, 1.09]
11-12 yr (Wudy 2007)	54.1	13.8	25	43.6	12.2	25	6.4%	0.79 [0.22, 1.37]
13 ±3yr (Vaindirlis 2000)	94	32	12	69	33	10	2.8%	0.74 [-0.13, 1.61]
13-14 yr (Wudy 2007)	53.2	21.4	25	49.5	15.1	25	6.9%	0.20 [-0.36, 0.75]
14-19yr (Nakamura 1984)	40.9	23	6	33.3	3.1	7	1.7%	0.45 [-0.66, 1.56]
15-16 yr (Wudy 2007)	68.5	21	25	65.1	22	25	6.9%	0.16 [-0.40, 0.71]
17-18 yr (Wudy 2007)	75	25.3	25	81.1	32.3	25	6.9%	-0.21 [-0.76, 0.35]
Total (95% CI)			**401**			**345**	**100.0%**	**0.32 [0.17, 0.47]**

Heterogeneity: Chi² = 8.67, df = 8 (P = 0.37); I² = 8%
Test for overall effect: Z = 4.31 (P < 0.0001)

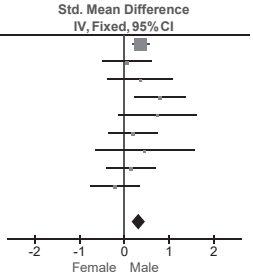


Figure 3: Forest plots of gender differences per subgroup (Fixed effect analyses)
A Salivary cortisol (nmol/L) <8 yr of age **B** Salivary cortisol (nmol/L) 8–18 yr of age **C** Serum cortisol (nmol/L) <8 yr of age **D** Serum cortisol (nmol/L) 8–18 yr of age **E** 24h-urine cortisol (µg/24h) <8 yr of age **F** 24h-urine cortisol (µg/24h) 8–18 yr of age

Meta-analysis

Nine articles (16%) did not report mean and SD values, which were therefore calculated. Figure 3 shows the results of the fixed-effect meta-analysis. Compared to girls, boys <8 yr had 0.21 (0.05 to 0.37) nmol/L ($P = 0.01$, $I^2 = 48\%$) higher salivary and 0.18 (0.06 to 0.30) nmol/L ($P < 0.01$, $I^2 = 94\%$) higher serum cortisol levels. Between ages 8-18 yr, boys had 0.42 (0.38 to 0.47) nmol/L ($P < 0.01$, $I^2 = 94\%$) lower salivary and 0.34 (0.28 to 0.40) nmol/L ($P < 0.01$, $I^2 = 97\%$) lower serum cortisol levels. In contrast, free cortisol in 24h-urine was 0.34 (0.05 to 0.64) µg/24h ($P = 0.02$, $I^2 = 55\%$) higher in boys aged <8 yr and 0.32 (0.17 to 0.47) µg/24h ($P < 0.01$, $I^2 = 8\%$) higher in boys between ages 8-18 yr. The sensitivity analyses did not significantly change the results, although it decreased the heterogeneity: boys <8 yr had 0.40 (0.11 to 0.69) nmol/L ($P < 0.01$, $I^2 = 55\%$) higher salivary, 0.45 (0.30 to 0.61) nmol/L ($P < 0.01$, $I^2 = 94\%$) higher serum and 0.28 (-0.04 to 0.61) µg/24h ($P = 0.08$, $I^2 = 33\%$) higher 24h-urine cortisol; boys 8–18 yr had 0.20 (0.13 to 0.26) nmol/L ($P < 0.01$, $I^2 = 47\%$) lower salivary, 0.10 (0.02 to 0.18) nmol/L ($P = 0.01$, $I^2 = 33\%$) lower serum and 0.24 (0.02 to 0.47) µg/24h ($P = 0.04$, $I^2 = 24\%$) higher 24h-urine cortisol.

Appendix 3 shows the results of the random-effects meta-analyses. When analyzed by the random-effects method, the effect estimates of serum cortisol <8 yr and between 8-18 yr became non-significant ($P = 0.46$ and $P = 0.62$, respectively). This also applied to salivary cortisol <8 yr ($P = 0.06$) and urinary cortisol <8yr ($P = 0.12$), although trends in the same direction were observed.

Funnel plots showed no evidence of publication bias. (Appendix 4)

DISCUSSION

The results from this meta-analysis suggest that gender-specific differences in HPA axis activity are already present early in life. They also support previous observations which show that cortisol metabolism diverges between genders at pubertal age. Before age 8 yr, cortisol in both serum and saliva was higher in boys compared to girls, at least in fixed-effect meta-analysis. These patterns were reversed after age 8 yr. In contrast, gender differences in 24h-urine cortisol remained consistent with age, with higher cortisol levels in urine for boys before and after age 8 yr.

Total serum cortisol and free salivary cortisol reflect the balance between cortisol production and degradation, i.e., the bioavailability. Our meta-analysis suggests that puberty induces gender-specific changes in the bioavailability of cortisol, as reflected by similar changes in both total serum and free salivary cortisol levels, at least in fixed-effect models. Even though associations were absent for total serum cortisol in random-effects models, the change in free salivary cortisol could not be explained by an estrogen-induced increase in the production of CBG.[4] Moreover, the gender differ-

ence in cortisol in 24hr-urine (i.e., non-metabolized, free cortisol, representing cortisol production rate) remained consistent with age. Consequently, sex-hormone dependent effects on the hepatic metabolism of cortisol are more likely to explain our observations. Cortisol is metabolized reversibly by 11βHSD type 2, and irreversibly by α- and β-ring reductases, and CYP3A. Animal studies showed a lower bioavailability of glucocorticoids in females due to decreased 11βHSD type 1 [16-18] and relatively increased 11βHSD type 2 activity,[18] as compared to males. In addition, previous observations in humans suggest that estrogens could alter hepatic cortisol metabolism through increased CYP3A activity,[19,20] and decreased A-ring reduction.[3,21] In contrast, sex-specificity in the activities of 11βHSD isozymes is debated in humans.[3,21,22] Since analyses of gender-specific differences in total serum cortisol were inconclusive in random-effects models (Appendix 3) and only one of the included studied had assessed CBG levels next to cortisol, we cannot exclude a gender-specific influence of CBG [4] on the serum cortisol level.

The HPA axis set point can be modified through an altered balance between mineralocorticoid and glucocorticoid receptor expression.[23] Animal studies have suggested that patterns in receptor expression develop in a gender-specific manner from birth onwards.[24] In humans, behavioral patterns that impact a child's stress vulnerability have been associated with gender-specific changes in cortisol levels from age 1.5 yr onwards.[11,25] Therefore, even in our sample of normal children, gender-specific effects of stress exposure could be an explanation for our results.[9]

Even subtle disturbances in HPA axis activity have been associated with cardiovascular disease and its risk factors.[26-28] Cardiovascular disease susceptibility is gender-specific,[7,29] which has been suggested to be due to gender differences in HPA axis activity, stress vulnerability and responsivity.[4,30-32] Early in life, developmental plasticity offers the child the capacity to change his HPA axis set point based on stress experiences.[9,33] This ability offers opportunities to withstand early-life challenges, but it has also been suggested to affect disease risk later in life. Accordingly, although the gender differences found in our study were small, these patterns might contribute to gender-specific origins of health and disease.[9]

The major strength of this study is our systematic approach and the effort to contact all authors of eligible publications, enabling us to include the data on 16,551 healthy children. Moreover, articles with a lack of quantitative data were included in our descriptive analysis with the aim to be as complete as possible. The large sample size enabled us to perform a sensitivity analysis, which decreased the heterogeneity between studies. Furthermore, we accounted for this heterogeneity by calculating standardized mean differences, based on the intervention effects relative to the variability observed.[13] Additionally, we chose fixed-effect meta-analysis, because the studies with a large sample size were most likely conducted with greater methodological accuracy.[13] Fixed-effect meta-analysis has the advantage of increasing the impact of large studies on the effect

estimate. For comparison, results of random-effects meta-analyses, which put more weight on studies with small sample sizes, were also included. (Appendix 3).

A limitation of this study is that only a subset of studies (16%) considered gender differences as the primary outcome. In addition, in 22 studies (28%) samples were not collected specifically during mornings. Both could have led to a selection or performance bias, which we accounted for in our sensitivity analysis. Furthermore, 21 articles with data on 3,985 subjects could not be included in the meta-analysis due to lack of gender-stratified quantitative data, while most of these articles reported no significant gender differences. However, funnel plots of the articles included in the meta-analysis were not suggestive of publication bias. Instead, the plots seem to indicate that most articles reported on the nonexistence of gender differences, which might be a result of the common idea that gender differences are nonexistent at this early age. Nonetheless, our meta-analysis shows that significant gender differences are already present early in life. Another limitation is that almost all studies that reported on salivary or serum cortisol used immunoassays. Due to its superior specificity, liquid chromatography-tandem mass spectrometry is the method of choice for steroid hormone analysis.[34] Furthermore, we stratified studies based on the mean age or age range of the study group. Since study samples differed in age range, we have probably included some subjects < 8 yr of age in the 8-18 yr groups, and vice versa. An overview of the age ranges of studies included in the meta-analysis is presented in Appendix 5. Moreover, only a minority of the included studies assessed Tanner pubertal staging. Therefore, we were unable to address the question at which maturational stage the direction of the gender-specific dimorphism in cortisol changes.

CONCLUSIONS

In conclusion, gender differences in HPA axis activity are present early in life, with higher salivary cortisol concentrations in boys. A gender-specific evolution of cortisol metabolism is suggested to be induced by puberty, resulting in lower bioavailability of cortisol in boys. Although results from random-effects analyses were inconclusive for serum cortisol, the gender difference in cortisol production seems to be consistent between genders with age. Future research should take gender differences in HPA axis activity into account, regardless of age. Whether gender differences in stress-induced cortisol levels also exist is unknown and remains to be explored.

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APPENDIX 1

Appendix 1A: Search strategy for PubMed (14 January 2016)

Search	Query	Records (n)
#1	"Hydrocortisone"[Mesh] OR "Glucocorticoids"[Mesh] OR "11-beta-Hydroxysteroid Dehydrogenases"[Mesh] OR "Tetrahydrocortisone"[Mesh] OR "Tetrahydrocortisol"[Mesh] OR cortisol*[tiab] OR hydrocortison*[tiab] OR epicortisol*[tiab] OR cortifair*[tiab] OR cortril*[tiab] OR glucocorticoid*[tiab] OR beta hydroxysteroid dehydrogenase*[tiab] OR 11 oxoreductase*[tiab] OR 11 oxidoreductase*[tiab] OR 11 hydroxysteroid dehydrogenase*[tiab] OR 11b hydroxysteroid dehydrogenase*[tiab] OR 11 reductase*[tiab] OR 11beta hydroxysteroid dehydrogenase*[tiab] OR tetrahydrocortiso*[tiab] OR "hsd11b2"[tiab] OR "11bhsd2"[tiab] OR "11betahsd2"[tiab] OR 11beta hsd*[tiab] OR hydroxycortisol*[tiab] OR "Circadian Rhythm"[Mesh] OR "twenty four hour"[tiab] OR circadian*[tiab] OR diurnal*[tiab] OR nyctohemeral*[tiab]	**250,920**
#2	child*[tw] OR schoolchild*[tw] OR infan*[tw] OR adolescen*[tw] OR pediatri*[tw] OR paediatr*[tw] OR neonat*[tw] OR boy[tw] OR boys[tw] OR boyhood[tw] OR girl[tw] OR girls[tw] OR girlhood[tw] OR youth[tw] OR youths[tw] OR baby[tw] OR babies[tw] OR toddler*[tw] OR "Mental Disorders Diagnosed in Childhood"[MeSH] OR teen[tw] OR teens[tw] OR teenager*[tw] OR newborn*[tw] OR postneonat*[tw] OR postnat*[tw] OR perinat*[tw] OR puberty[tw] OR preschool*[tw] OR suckling*[tw] OR picu[tw] OR nicu[tw] OR "Arthritis, Juvenile"[Mesh] OR "Myoclonic Epilepsy, Juvenile"[Mesh] OR "Leukemia, Myelomonocytic, Juvenile"[Mesh] OR "Xanthogranuloma, Juvenile"[Mesh] OR "Juvenile Delinquency"[Mesh] OR "Corneal Dystrophy, Juvenile Epithelial of Meesmann"[Mesh]	**3,664,351**
#3	"Sex Characteristics"[Mesh] OR "Sex Factors"[Mesh] OR sex characteristic*[tiab] OR sex difference*[tiab] OR sex dimorphism*[tiab] OR sexual dimorphism*[tiab] OR sexual difference*[tiab] OR sexual characteristic*[tiab] OR sex factor*[tiab] OR sexual factor*[tiab] OR sexual dimorphi*[tiab] OR sex influenc*[tiab] OR sexual influenc*[tiab] OR gender*[tiab]	**451,894**
#4	(#1 AND #2 AND #3)	**2,643**

Abbreviations: Mesh = Medical subject headings; tiab = words in title OR abstract; tw = words in title, abstract, MeSH and other content related fields

Appendix 1B: Search strategy for Embase.com (14 January 2016)

Search	Query	Records (n)
#1	'hydrocortisone'/exp OR 'glucocorticoid'/de OR '11beta hydroxysteroid dehydrogenase'/exp OR 'tetrahydrocortisone'/exp OR 'tetrahydrocortisol'/exp OR cortisol*:ab,ti OR hydrocortison*:ab,ti OR epicortisol*:ab,ti OR cortifair*:ab,ti OR cortril*:ab,ti OR glucocorticoid*:ab,ti OR ('beta hydroxysteroid' NEAR/3 dehydrogenase*):ab,ti OR (11 NEXT/1 oxoreductase*):ab,ti OR (11 NEXT/1 oxidoreductase*):ab,ti OR ('11 hydroxysteroid' NEXT/1 dehydrogenase*):ab,ti OR ('11b hydroxysteroid' NEXT/1 dehydrogenase*):ab,ti OR (11 NEXT/1 reductase*):ab,ti OR ('11beta hydroxysteroid' NEXT/1 dehydrogenase*):ab,ti OR tetrahydrocortiso*:ab,ti OR 'hsd11b2':ab,ti OR '11bhsd2':ab,ti OR '11betahsd2':ab,ti OR (11beta NEXT/1 hsd*):ab,ti OR hydroxycortisol*:ab,ti OR ('trier social stress' NEXT/1 test*):ab,ti OR tsst:ab,ti OR (stress NEAR/3 hormone*):ab,ti OR (stress NEAR/3 marker*):ab,ti	**235,221**
#2	adolescen*:ab,ti OR 'adolescence'/exp OR 'adolescent coping orientation for problem experiences'/exp OR 'adolescent development'/exp OR 'adolescent disease'/exp OR 'adolescent health'/exp OR 'adolescent parent'/exp OR 'adolescent pregnancy'/exp OR 'adolescent smoking'/exp OR 'adolescent'/exp OR 'adolescent-family inventory of life events and changes'/exp OR babies:ab,ti OR baby:ab,ti OR 'birth weight'/exp OR boy:ab,ti OR boyhood:ab,ti OR boys:ab,ti OR 'brazelton neonatal behavioral assessment scale'/exp OR 'child abuse'/exp OR 'child advocacy'/exp OR 'child behavior checklist'/exp OR 'child behavior'/exp OR 'child care'/exp OR 'child death'/exp OR 'child health care'/exp OR 'child health'/exp OR 'child nutrition'/exp OR 'child parent relation'/exp OR 'child psychology'/exp OR 'child restraint system'/exp OR 'child safety'/exp OR 'child welfare'/exp OR child*:ab,ti OR 'child'/exp OR 'childhood disease'/exp OR 'childhood mortality'/exp OR 'childhood'/exp OR girl:ab,ti OR girlhood:ab,ti OR girls:ab,ti OR 'high risk infant'/exp OR infan*:ab,ti OR 'infant disease'/exp OR 'infant mortality'/exp OR 'infant nutrition'/exp OR 'infant welfare'/exp OR 'infanticide'/exp OR 'infantile diarrhea'/exp OR 'infantile hypotonia'/exp OR 'juvenile delinquency'/exp OR neonat*:ab,ti OR 'neonatal weight loss'/exp OR 'newborn disease'/exp OR 'newborn morbidity'/exp OR 'newborn period'/exp OR newborn*:ab,ti OR 'newborn'/exp OR nicu:ab,ti OR 'only child'/exp OR paediatr*:ab,ti OR pediatri*:de,ab,ti OR 'pediatric advanced life support'/exp OR 'pediatric anesthesia'/exp OR 'pediatric cardiology'/exp OR 'pediatric hospital'/exp OR 'pediatric intensive care nursing'/exp OR 'pediatric nurse practitioner'/exp OR 'pediatric nursing'/exp OR 'pediatric rehabilitation'/exp OR 'pediatric surgery'/exp OR 'newborn hypoxia'/exp OR 'pediatric ward'/exp OR 'pediatrics'/exp OR perinat*:ab,ti OR 'perinatal development'/exp OR 'perinatal period'/exp OR 'persistent hyperinsulinemic hypoglycemia of infancy'/exp OR picu:ab,ti OR postnat*:ab,ti OR 'postnatal care'/exp OR 'postnatal development'/exp OR 'postnatal growth'/exp OR postneonat*:ab,ti OR preschool*:ab,ti OR puberty:ab,ti OR 'runaway behavior'/exp OR 'school child':ab,ti OR schoolchild*:ab,ti OR 'severe myoclonic epilepsy in infancy'/exp OR suckling*:ab,ti OR teen:ab,ti OR teenager*:ab,ti OR teens:ab,ti OR toddler*:ab,ti OR 'transient hypogammaglobulinemia of infancy'/exp OR youth:ab,ti OR youths:ab,ti	**4,477,134**
#3	'sex difference'/exp OR 'sex ratio'/exp OR ('boy'/exp AND 'girl'/exp) OR (sex NEAR/3 characteristic*):ab,ti OR (sex NEAR/3 difference*):ab,ti OR (sex NEAR/3 dimorphism*):ab,ti OR (sexual NEAR/3 dimorphism*):ab,ti OR (sexual NEAR/3 difference*):ab,ti OR (sexual NEAR/3 characteristic*):ab,ti OR (sex NEAR/3 factor*):ab,ti OR (sexual NEAR/3 factor*):ab,ti OR (sexual NEAR/3 dimorphi*):ab,ti OR (sex NEAR/3 influenc*):ab,ti OR (sexual NEAR/3 influenc*):ab,ti OR gender*:ab,ti OR (boy*:ab,ti AND girl*:ab,ti) OR sex:ab,ti	**1,014,014**
#4	(#1 AND #2 AND #3)	**4,280**

/exp = EMtree keyword with explosion; /de = EMtree keyword without explosion; :ab,ti = words in title or abstract; NEXT/x = words in that order next to each other, x places apart; NEAR/x = words near to each other, x places apart

APPENDIX 2

Risk of bias of studies included in the meta-analysis (See for argumentation Online Supplementary File 2)

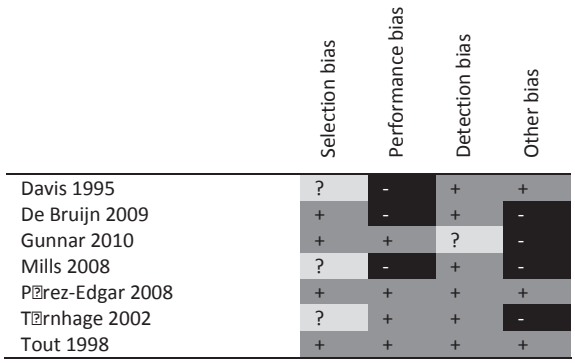
Risk of selection bias included: participants' age range, and sex-specific differences in participation or baseline characteristics. Risk of performance bias included: time of sample collection, protocol transparency, and sex-specific differences in protocol compliance. Risk of detection bias included: sex-specific differences in assay methods. Non-parametric distribution of the data was recorded as a risk of other biases. Bias could be assessed as low (i.e., unlikely to alter the results), unclear (i.e., raises doubt about results) or high (i.e., weakens confidence in results). Colored squares indicate: ■ = Low risk ■ = Unclear risk, ■ = High risk of bias.

	Selection bias	Performance bias	Detection bias	Other bias
Bailey 2013	+	-	+	+
Elmlinger 2002	?	?	+	+
Forest 1978	?	?	+	+
Garagorri 2008	+	+	+	+
Lashansky 1991	?	+	?	+
Soriano-Rodriguez 2010	+	?	+	+
Tennes 1973	?	+	-	-
Tsvetkova 1977	?	+	+	+

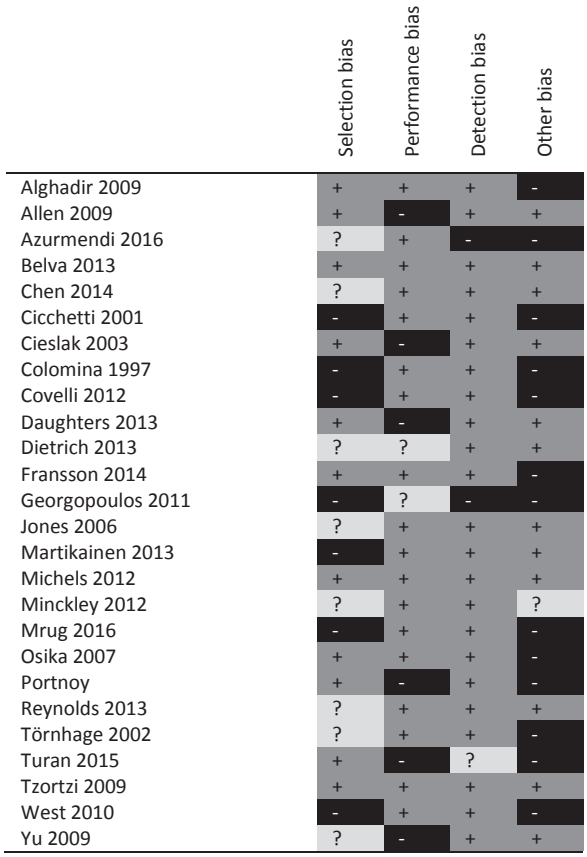
A. Serum <8 yr

	Selection bias	Performance bias	Detection bias	Other bias
Apter 1979	?	?	+	-
Bailey 2013	+	-	+	+
Elmlinger 2002	?	?	+	+
Ghaziuddin 2003	+	+	+	-
Hackney 200	-	+	+	+
Huybrechts 2014	-	+	+	+
Ilias 2009	-	+	+	-
Lashansky 1991	?	+	?	+
Ong 2004	+	+	+	+
Reynolds 2013	?	+	+	+
Ross 1986	-	-	+	-
Stroud 2011	+	-	+	+
Stupnicki 1995	-	-	+	+
Susman 1991	?	+	+	+
Syme 2008	-	?	?	+
Tsvetkova 1977	?	+	+	+

B. Serum 8–18 yr



C. Saliva <8 yr



D. Saliva 8–18 yr

	Selection bias	Performance bias	Detection bias	Other bias
Lundberg 1981	?	+	+	-
Lundberg 1983	?	+	?	-
Nakamura 1984	-	?	+	+
Wudy 2007	+	+	+	+

E. Urine <8 yr

	Selection bias	Performance bias	Detection bias	Other bias
Canalis 1982	?	?	?	+
Honour 2007	+	+	+	-
Nakamura 1984	-	?	+	+
Vaindirlis 2000	?	+	?	+
Wudy 2007	+	+	+	+

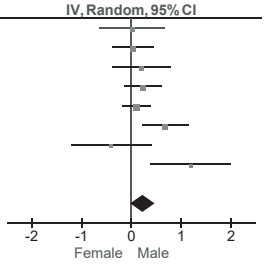
F. Urine 8–18 yr

APPENDIX 3

Forest plots of gender differences per subgroup (Random effect analyses)

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Random, 95% CI
0 yr (Davis 1995)	30.1	36.4	18	29.5	32.8	18	9.2%	0.02 [-0.64, 0.67]
3 yr (Gunnar 2010)	4.03	4.58	37	3.89	3.01	54	15.4%	0.04 [-0.38, 0.46]
3.3 yrs (De Bruijn 2009)	2.82	1.86	19	2.45	1.82	27	10.6%	0.20 [-0.39, 0.79]
4 yr (Pérez-Edgar 2008)	11.9	4.7	51	10.8	5	60	16.9%	0.22 [-0.15, 0.60]
4.1yr (Mills 2008)	3.03	2.76	115	2.76	2.76	85	20.6%	0.10 [-0.18, 0.38]
4.3 yr (Tout 1998)	5.79	2.48	38	4.41	1.38	37	13.8%	0.68 [0.21, 1.14]
4yr (Gunnar 2010)	2.92	1.35	11	4.47	4.88	13	6.7%	-0.40 [-1.22, 0.41]
7.3yr (Törnhage 2002)	10.5	5.2	19	5.2	1.88	11	6.8%	1.19 [0.38, 2.00]
Total (95% CI)			**308**			**305**	**100.0%**	**0.23 [-0.01, 0.48]**

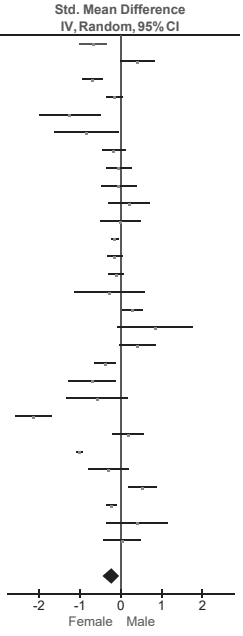
Heterogeneity: Tau² = 0.05; Chi² = 13.34, df = 7 (P = 0.06); I² = 48%
Test for overall effect: Z = 1.90 (P = 0.06)



A. Serum <8 yr

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Random, 95% CI
8yr (Jones 2006)	9.58	1.51	68	10.59	1.49	72	3.7%	-0.67 [-1.01, -0.33]
8 yr (Azurmendi 2016)	8	4.14	49	6.35	3.59	41	3.5%	0.42 [-0.00, 0.84]
8yr (Martikainen 2013)	6.8	1.7	126	8	1.7	132	3.9%	-0.70 [-0.96, -0.45]
8.4 yr (Michels 2012)	12.66	6.12	183	13.84	8.96	202	4.0%	-0.15 [-0.35, 0.05]
9 yr (Alghadir 2009) active	0.13	0.026	25	0.16	0.017	12	2.6%	-1.25 [-2.00, -0.50]
9yr (Alghadir 2009) sedentary	0.18	0.014	12	0.2	0.028	16	2.5%	-0.84 [-1.62, -0.05]
9.2 yr (Cicchetti 2001)	8.64	5.16	124	9.52	5.21	80	3.8%	-0.17 [-0.45, 0.11]
9.4 yr (Turan 2015)	5.2	3.2	79	5.6	10.8	74	3.8%	-0.05 [-0.37, 0.27]
9.9 yr (Osika 2007)	5.34	2.1	34	5.5	3.33	50	3.5%	-0.05 [-0.49, 0.38]
10yr (Törnhage 2002)	11	4.08	28	8.9	13.03	31	3.3%	0.21 [-0.30, 0.72]
10.4±0.4yr (Cieslak 2003)	3	0.5	29	3	0.3	32	3.3%	0.00 [-0.50, 0.50]
11.1±0.55yr (Dietrich 2013)	11.2	4.7	818	11.9	4.73	786	4.1%	-0.15 [-0.25, -0.05]
11.9±0.6yr (Chen 2014)	4.69	5.52	213	5.52	5.24	212	4.0%	-0.15 [-0.34, 0.04]
11.9 yr (Portnoy 2015)	4.06	3.72	224	5.79	22.18	214	4.0%	-0.11 [-0.30, 0.08]
12 yr (Tzortzi 2009)	9.05	3.63	11	10.24	4.86	10	2.4%	-0.27 [-1.13, 0.59]
12.7 ± 2.9yr (Allen 2009)	0.17	0.18	119	0.13	0.09	116	3.9%	0.28 [0.02, 0.54]
12.7 yr (Yu 2009)	6.22	0.92	10	4.83	2.04	10	2.2%	0.84 [-0.08, 1.76]
13.4 yr (Mrug 2016)	3.43	2.83	40	2.31	2.5	39	3.5%	0.42 [-0.03, 0.86]
14.0±0.5yr (Belva 2013)	24.6	10.6	115	29.2	13.2	108	3.9%	-0.38 [-0.65, -0.12]
14.9yr (Alghadir 2009) active	0.22	0.015	20	0.24	0.034	30	3.1%	-0.70 [-1.29, -0.12]
14.9 yr (Alghadir 2009) sedentary	0.16	0.018	25	0.17	0.014	10	2.6%	-0.57 [-1.32, 0.17]
14 -16yr (Fransson 2014)	6.6	2	43	10.9	2	89	3.4%	-2.14 [-2.59, -1.69]
15.3 ± 1.1yr (Covelli 2012)	27	11	49	25	11.2	57	3.6%	0.18 [-0.20, 0.56]
15.5 yr (West 2010)	10.64	1.02	1418	11.67	1.02	1406	4.1%	-1.01 [-1.09, -0.93]
16 ±1yr (Georgopoulos 2011)	9.1	8.3	35	11.6	8.3	29	3.3%	-0.30 [-0.79, 0.20]
16.1 ±1.0yr (Daughters 2013)	3.22	2.13	59	2.3	1.38	73	3.7%	0.52 [0.17, 0.87]
16.6 ± 0,5yr (Reynolds 2013)	25.93	14.1	573	29.5	19	362	4.1%	-0.22 [-0.35, -0.09]
17.9 yr (Minckley 2012)	10.47	6.29	14	8.42	2.84	14	2.6%	0.41 [-0.34, 1.16]
18 yr (Colomina 1997)	13.7	7.7	37	13.4	7.6	36	3.4%	0.04 [-0.42, 0.50]
Total (95% CI)			**4580**			**4343**	**100.0%**	**-0.23 [-0.43, -0.03]**

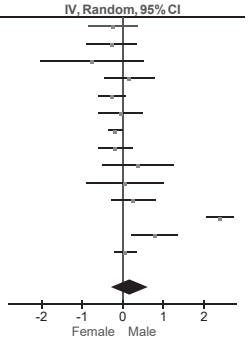
Heterogeneity: Tau² = 0.25; Chi² = 481.05, df = 28 (P < 0.00001); I² = 94%
Test for overall effect: Z = 2.23 (P = 0.03)



B. Serum 8–18 yr

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Random, 95% CI
1-7d (Elmlinger 2002)	524	190.82	28	569	184.44	17	7.2%	-0.23 [-0.84, 0.37]
8-15d (Elmlinger 2002)	311	99.75	20	342	119.39	20	7.1%	-0.28 [-0.90, 0.35]
0 yr (Forest 1978)	312	168	15	448	187	3	5.0%	-0.76 [-2.03, 0.51]
0 yr (Tennes 1973)	435.9	313.6	23	389.8	227.6	17	7.1%	0.16 [-0.47, 0.79]
0 yrs (Garagorri 2008)	249.41	99.32	80	280.31	124.16	58	7.8%	-0.28 [-0.62, 0.06]
0.4 yr (Lashansky 1991)	342	146	13	353	196	420	7.3%	-0.06 [-0.61, 0.50]
0-2 yr (Bailey 2013)	159.8	135.3	261	190.2	186.2	208	8.1%	-0.19 [-0.37, -0.01]
16d-3y (Elmlinger 2002)	253	95.66	42	271	97.44	44	7.7%	-0.18 [-0.61, 0.24]
3 yr (Lashansky 1991)	337	160	14	281	113	8	6.3%	0.37 [-0.51, 1.25]
4-10 yr (Tsvetkova 1977)	518.69	193.1	9	510.42	157.3	8	6.1%	0.04 [-0.91, 1.00]
4-6y (Elmlinger 2002)	253	143.88	28	218	117.86	23	7.3%	0.26 [-0.29, 0.81]
6.8 yr (Soriano-Rodriguez 2010)	441.4	13.8	106	408.3	13.8	117	7.8%	2.39 [2.05, 2.74]
7-8y (Elmlinger 2002)	400	141.58	26	294	123.47	24	7.2%	0.78 [0.21, 1.36]
2-8 yr (Bailey 2013)	134.41	75.57	110	129.91	71.48	86	7.9%	0.06 [-0.22, 0.34]
Total (95% CI)			**775**			**1053**	**100.0%**	**0.17 [-0.28, 0.63]**

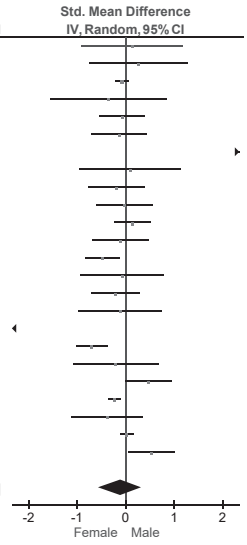
Heterogeneity: Tau² = 0.67; Chi² = 194.87, df = 13 (P < 0.00001); I² = 93%
Test for overall effect: Z = 0.75 (P = 0.46)



C. Saliva <8 yr

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Random, 95% CI
8 yr (Apter 1979)	240	80.3	7	226.2	109.5	7	3.6%	0.13 [-0.91, 1.18]
8yr (Lashansky 1991)	254	86	8	229	94	7	3.7%	0.26 [-0.76, 1.28]
8yr (Ong 2004)	210	58	423	215	69	347	4.5%	-0.08 [-0.22, 0.06]
9 yr (Ilias 2009)	112.97	49.14	5	153.26	131.05	6	3.4%	-0.36 [-1.56, 0.84]
9-10y (Elmlinger 2002)	317	165.82	31	328	153.32	40	4.3%	-0.07 [-0.54, 0.40]
6-15 yr (Ross 1986)	74.5	35.9	23	80	44.1	24	4.2%	-0.13 [-0.71, 0.44]
10yr (Stroud 2011)	164.4	5.9	39	146.5	5.99	30	4.1%	2.98 [2.28, 3.68]
11-14 (Tsvetkova 1977)	424.89	171.1	7	408.3	160	7	3.6%	0.09 [-0.95, 1.14]
11y (Elmlinger 2002)	341	113.5	22	364	117.3	23	4.2%	-0.20 [-0.78, 0.39]
12y (Elmlinger 2002)	386	114.29	22	388	117.35	23	4.2%	-0.02 [-0.60, 0.57]
12.5 yr (Susman 1991)	364.7	149.5	56	344.3	148.7	52	4.4%	0.14 [-0.24, 0.51]
13y (Elmlinger 2002)	430	131.12	21	442	103.32	25	4.2%	-0.10 [-0.68, 0.48]
13 yr (Hackney 2003)	397.3	126.9	62	466.3	162.8	63	4.4%	-0.47 [-0.82, -0.11]
13yr (Lashansky 1991)	223	74	10	229	94	11	3.9%	-0.07 [-0.92, 0.79]
14y (Elmlinger 2002)	440	109.18	32	466	131.63	30	4.3%	-0.21 [-0.71, 0.29]
14.6 yr (Ghaziuddin 2003)	1,757.5	1,191.9	12	1,914.7	1,658.2	9	3.9%	-0.11 [-0.97, 0.76]
14.7 yr (Huybrechts 2014)	299.5	1.6	297	305.6	1.7	426	4.5%	-3.67 [-3.91, -3.43]
15 yr (Syme 2008)	546.3	140.7	77	714.6	306.24	82	4.5%	-0.70 [-1.02, -0.38]
15.5yr (Lashansky 1991)	262	80	13	279	77	8	3.9%	-0.21 [-1.09, 0.68]
16y (Elmlinger 2002)	444	111.99	31	388	116.58	40	4.3%	0.48 [0.01, 0.96]
16.6 ± 0,5yr (Reynolds 2013)	551	154	653	590.98	202.79	401	4.6%	-0.23 [-0.35, -0.10]
17 yr (Stupnicki 1995)	347	130	14	397	125	15	4.1%	-0.38 [-1.12, 0.35]
8-18yr (Bailey 2013)	218.3	98.9	370	215.7	110.7	369	4.5%	0.02 [-0.12, 0.17]
17-19y (Elmlinger 2002)	465	124.74	30	403	103.57	42	4.3%	0.54 [0.07, 1.02]
Total (95% CI)			**2265**			**2087**	**100.0%**	**-0.11 [-0.56, 0.33]**

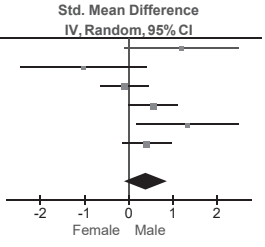
Heterogeneity: Tau² = 1.12; Chi² = 906.47, df = 23 (P < 0.00001); I² = 97%
Test for overall effect: Z = 0.50 (P = 0.62)



D. Saliva 8–18 yr

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Random, 95% CI
3yr (Lundberg 1981)	3.35	1.12	4	2.46	0.44	9	9.9%	1.19 [-0.11, 2.49]
4.5yr (Lundberg 1983)	8.5	1.8	8	11.9	5.5	3	8.6%	-1.02 [-2.45, 0.41]
3-4 yr (Wudy 2007)	20.5	6.63	25	21.2	6.49	25	23.4%	-0.11 [-0.66, 0.45]
5-6 yr (Wudy 2007)	30.5	10.2	25	25.4	7.52	25	23.1%	0.56 [-0.01, 1.13]
5-10 yr (Nakamura 1984)	14.8	3.6	7	9.98	3.2	8	11.6%	1.34 [0.18, 2.50]
7-8 yr (Wudy 2007)	36.9	12.9	25	31.9	10.9	25	23.3%	0.41 [-0.15, 0.97]
Total (95% CI)			**94**			**95**	**100.0%**	**0.39 [-0.10, 0.87]**

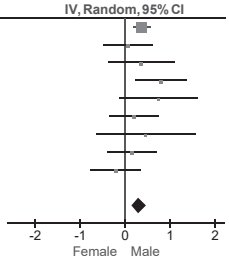
Heterogeneity: Tau² = 0.18; Chi² = 11.11, df = 5 (P = 0.05); I² = 55%
Test for overall effect: Z = 1.55 (P = 0.12)



E. Urine <8 yr

Study or Subgroup	Male Mean	Male SD	Male Total	Female Mean	Female SD	Female Total	Weight	Std. Mean Difference IV, Random, 95% CI
8yr (Honour 2007)	2,826.86	774.81	244	2,557.71	655.56	188	47.0%	0.37 [0.18, 0.56]
9-10 yr (Wudy 2007)	35.5	9.26	25	34.9	12.5	25	8.6%	0.05 [-0.50, 0.61]
4-15yr (Canalis 1982)	15.2	6.3	14	13	5.6	15	5.0%	0.36 [-0.38, 1.09]
11-12 yr (Wudy 2007)	54.1	13.8	25	43.6	12.2	25	7.9%	0.79 [0.22, 1.37]
13 ±3yr (Vaindirlis 2000)	94	32	12	69	33	10	3.6%	0.74 [-0.13, 1.61]
13-14 yr (Wudy 2007)	53.2	21.4	25	49.5	15.1	25	8.5%	0.20 [-0.36, 0.75]
14-19yr (Nakamura 1984)	40.9	23	6	33.3	3.1	7	2.3%	0.45 [-0.66, 1.56]
15-16 yr (Wudy 2007)	68.5	24	25	65.1	22	25	8.5%	0.15 [-0.41, 0.70]
17-18 yr (Wudy 2007)	75	25.3	25	81.1	32.3	25	8.5%	-0.21 [-0.76, 0.35]
Total (95% CI)			**401**			**345**	**100.0%**	**0.31 [0.14, 0.48]**

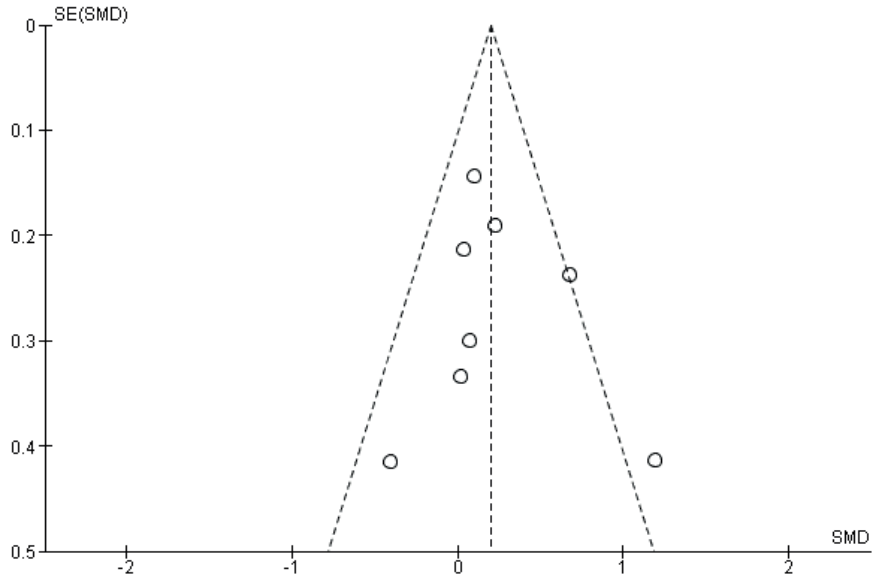
Heterogeneity: Tau² = 0.01; Chi² = 8.71, df = 8 (P = 0.37); I² = 8%
Test for overall effect: Z = 3.59 (P = 0.0003)



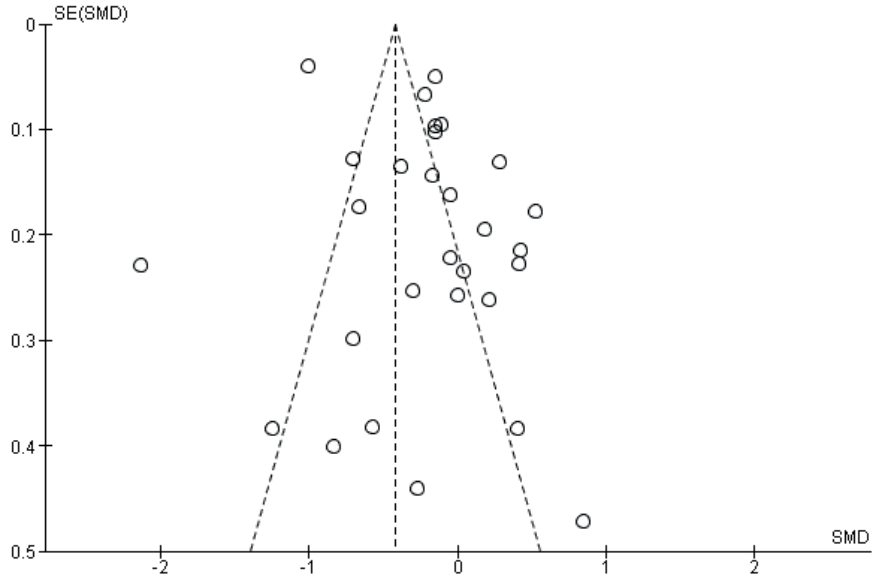
F. Urine 8–18 yr

APPENDIX 4

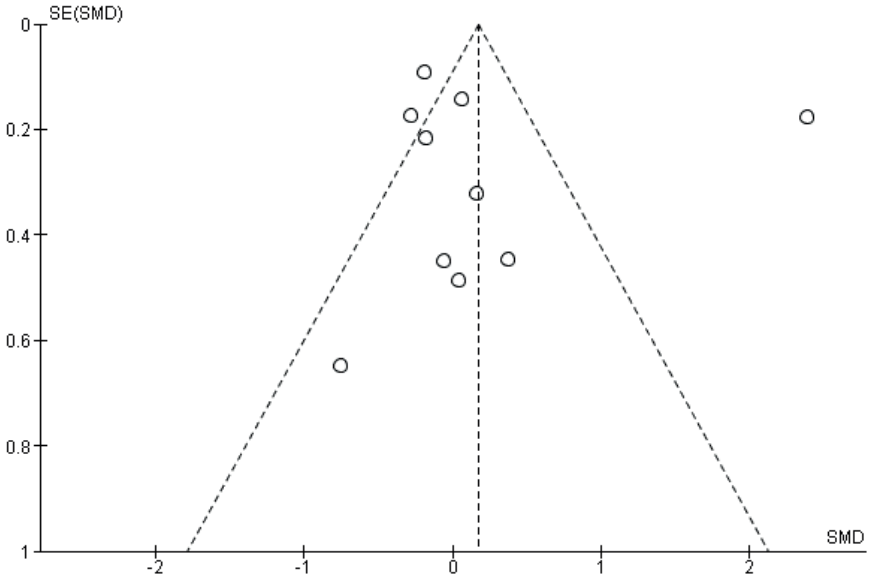
Funnel plots



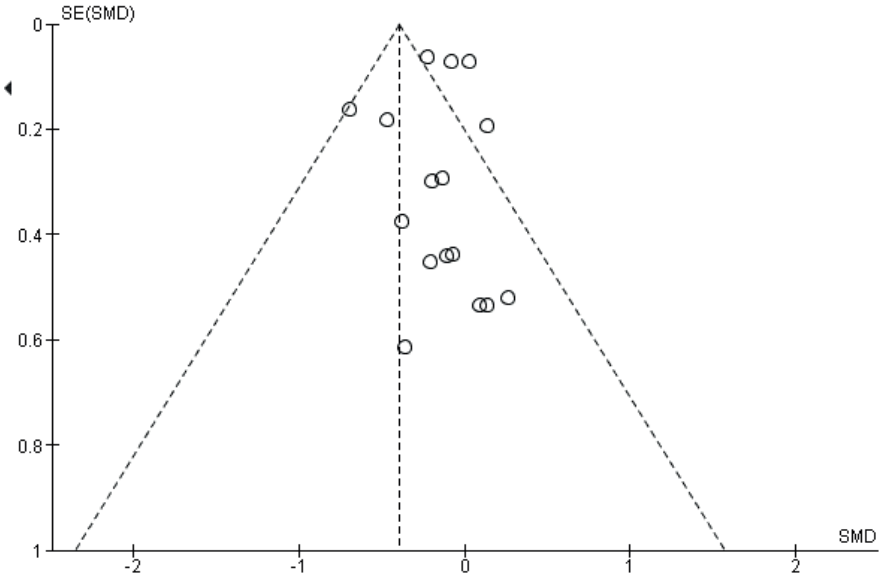
A. Serum <8 yr



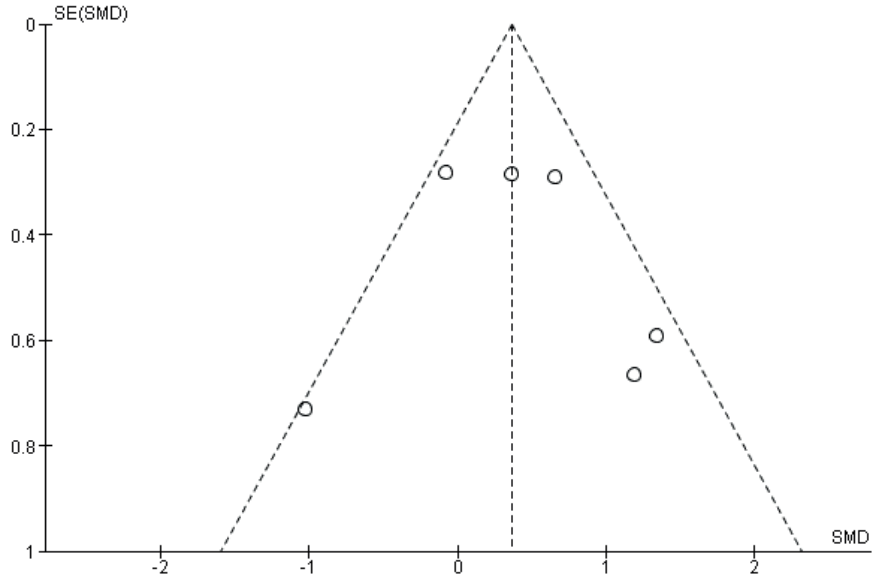
B. Serum 8–18 yr



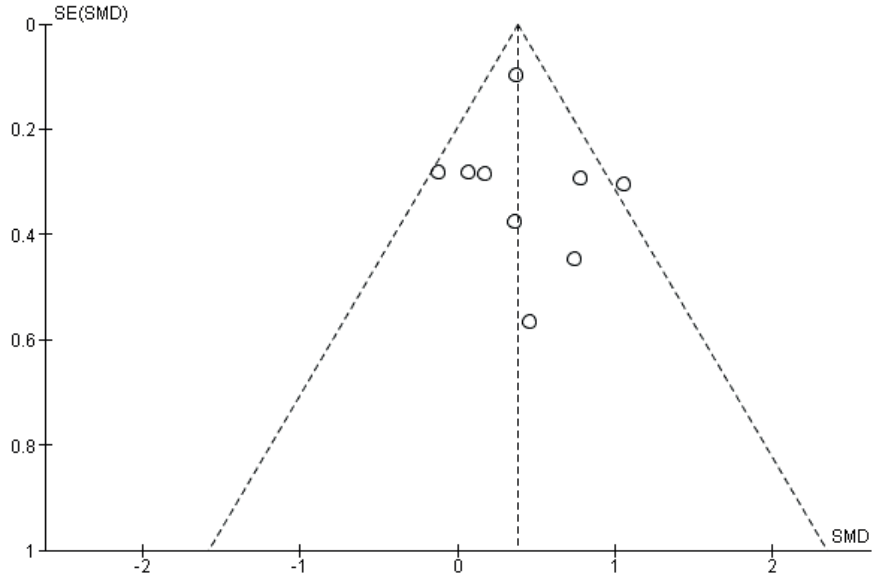
C. Saliva <8 yr



D. Saliva 8–18 yr



E. Urine <8 yr



F. Urine 8–18 yr

APPENDIX 5.

Overview of age ranges of studies included in meta-analysis

Study	Mean age ± SD*	Study	Mean age ± SD*
Davis 1995	2 days	Mrug 2016	13.36 ± 0.95 yr
Forest 1978	115.3 ± 120.1 days	Vaindirlis 2000	13 yr ± 3.5 yr
Tennes 1973	3 days	Hackney 2003	13.4 ± 0.9 yr
Garagorri 2008	3 days	Belva 2013	girls 14.0 ± 0.5 yr, boys 14.0 ± 0.4 yr
De Bruijn 2009	38.61 ± 9.4 months	Ghaziuddin 2003	14.6 ± 1.5 yr
Lundberg 1981	3 yr	Huybrechts 2014	14.7 ± 1.2 yr
Gunnar 2010	3.81 ± 0.23 yr	Fransson 2014	ranges 14 - 16 yr
Mills 2008	4.14 ± 0.24 yr	Nakamura 1984	5 – 10 yr and 14 - 19 yr
Tout 1998	mean 4.3 yr	Covelli 2012	15.3 ± 1.1 yr
Lundberg 1983	boys mean 52.3 months, girls 54.9 months	West 2010	15.4 ± 0.4 yr
Soriano-Rodriguez 2010	6.8 ± 0.19 yr	Syme 2008	boys 14.4 ± 1.7 yr, girls 14.4 ± 1.9 yr
Michels 2012	boys 8.44 ± 1.18 yr, girls 8.39 ± 1.20 yr	Daughters 2013	16.1 yr ± 1.0 yr
Apter 1979	range 7.5 – 8.5 yr	Reynolds 2013	16.6 yr ± 0.5 yr
Azurmendi 2016	8 yr	Georgopoulos 2011	boys 15.3 ± 2.0 yr, girls 16.0 ± 1.4 yr
Honour 2007	range 8.2 – 8.4 yr	Minckley 2012	17.86 ± (S.E.M.) 0.096 yr
Jones 2006	range 7- 9 yr	Stupnicki 1995	boys 17.3 ± 0.8 yr, girls 16.4 ± 0.6 yr
Martikainen 2013	boys 8.2 ± 0.3 yr, girls 8.1 ± 0.3 yr	Colomina 1997	range 17.5 – 18.5 yr
Ong 2004	8.2 ± 0.1 yr	Elmlinger 2002	ranges 16 days – 3 yr, 11 yr
Cicchetti 2001	9.24 ± 2.33 yr	Tsvetkova 1977	ranges 4-10 yr and 11-14 yr
Turan 2015 Osika 2007 Ilias 2009 Cieslak 2003	9.38 ± 0.62 yr 9.9 ± 0.6 yr boys 9.5 ± 1.9 yr, girls 9.1 ± 1.3 yr 10.4 ± 0.4 yr	Lashansky 1991	Boys 0.42 ± 0.24, 3.2 ± 1.6, 7.4 ± 1.8, 13.1 ± 1.2, 15.2 ± 1.4 yr Girls 0.42 ± 0.2,2.5 ± 1.5, 9.3 ± 2.2, 12.5 ± 0.9, 15.9 ± 0.7 yr
Stroud 2011	10.5 ± 1.7 yr	Bailey 2013	0.41 ± 0.37, 5.29 ± 1.74, 13.48 ± 3.03 yr
Dietrich 2013 Chen 2014	11.1 yr ± 0.55 yr 11.87 ± 0.60 yr	Wudy 2007	ranges 3-4, 5-6, 7-8, 9-10, 11-12, 13-14, 15-16, 17-18 yr
Portnoy 2015 Susman 1991	11.92 ± 0.59 yr mean boys 12.72 yr, girls 11.99 yr	Alghadir 2009	boys 9.3 ± 1.5 and 14.9 ± 3.7 yr girls 8.96 ± 1.8 and 14.82 ± 4.6 yr
Allen 2009 Yu 2009	12.7 yr ± 2.9 yr 12.6 ± 1.8 yr	Törnhage 2002	median girls 7.4 and 10.3 yr, boys 7.1 and 10.2 yr
Ross 1986 Canalis 1982	range 6-15 yr range 4-15 yr	Tzortzi 2009	ranges boys 10 yr and 3 months – 13 yr and 7 months, girls 10 yr and 3 months – 13 yr and 3 months

*Unless otherwise indicated

ONLINE SUPPLEMENTARY FILES

1. Extracted data of studies included in the meta-analysis (https://doi.org/10.6084/m9.figshare.11013239.v1)
2. Risk of bias of studies included in the meta-analysis (https://doi.org/10.6084/m9.figshare.11014217.v1)