Morning Salivary Cortisol in Young Children: Reference Values and the Effects of Age, Sex, and Acute Bronchiolitis

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Objective To identify morning salivary cortisol reference values in infancy and at 2 years of age and to investigate the influence of age, sex and acute bronchiolitis.

Study design In this South-East Norwegian cohort study, 308 children hospitalized with moderate to severe acute bronchiolitis in infancy in 2010-2011 were compared with 223 healthy controls included in 2012 by measuring morning salivary cortisol levels at inclusion and at 2 years of age. Samples were collected shortly after awakening after 6 AM. The influences of age, sex, and acute bronchiolitis were assessed by regression analysis.

Results In infancy, cortisol values were higher in acute bronchiolitis, with an age- and sex-adjusted weighted mean group difference of 13.9 nmol/L (95% CI 8.1-19.7; $P < .0001$). The median level in reference group was 23.7 nmol/L (95% CI 9.7-119.6). At 2 years of age, sex but not inclusion groups differed, with significantly higher values in girls. The weighted mean of all boys' cortisol levels was 32.4 nmol/L, (95% CI 30.5-34.3), and all girls' levels were 36.9 nmol/L (95% CI 34.7-39.2; $P < .003$).

Conclusions Salivary cortisol levels were higher at 2 years of age than in infancy in the reference group, were higher in girls than in boys at 2 years of age, and were higher in infants at the time of acute bronchiolitis than in healthy infants. *(J Pediatr 2017;184:193-8).*

Trial registration ClinicalTrials.gov: NCT00817466

Cortisol levels normally show a circadian rhythm with physiologically increased levels in the morning, with an additional cortisol awakening response.[1,2] Cortisol levels can be analyzed in blood, urine, and saliva.[3-5] Salivary samples are noninvasive, and do not induce the trauma, stress, and potentially higher cortisol compared with serum sampling.[6] However, a potential shortcoming of salivary measurement is the issue of spot sampling of a biomarker with known diurnal variation.[7] There are few reports on reference values of morning salivary cortisol levels in infants and toddlers, reflecting the biologically active, free fraction of serum cortisol.[8-10]

Reduced morning cortisol has been associated with allergic diseases such as asthma and allergic rhinitis in young and older children, pointing to an involvement of adrenocortical function.[11-14]

Links between stress, cortisol levels, and asthma in early childhood or later asthma development have been proposed,[14-16] and we recently showed that being hospitalized for acute bronchiolitis in infancy increased the risk for reduced health-related quality of life.[17] However, investigation of possible causal associations between infant salivary cortisol levels and later asthma requires relevant reference values of morning salivary cortisol levels in early childhood.

Our primary aim was to describe reference values for morning salivary cortisol levels during infancy and at 2 years of age. Second, we sought to investigate whether age, sex, or acute moderate to severe bronchiolitis in infancy influenced morning salivary cortisol levels.

Methods

The present study included 531 children with at least 1 (total 762) salivary cortisol level measurement in infancy, when they were recruited into the study and/or at the 2-year follow-up (**Figure 1**; available at www.jpeds.com). The source population included 404 infants hospitalized with moderate to severe acute bronchiolitis in 8 pediatric departments of southeast Norway. Additionally, 240 infants were recruited by postal invitation to 3000 randomly selected children 0-12 months of age from the municipalities of Oslo and Fredrikstad[17] from March 22, 2012 to July 2, 2012, who were included in the Bronchiolitis ALL SE-Norway study

RSV Respiratory syncytial virus

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L.R. is employed as a fellow paid by the Innlandet Hospital Trust's Research Fund (150189), which also refunded the costs for the salivary cortisol analyses. The authors declare no conflicts of interest.

http://dx.doi.org10.1016/j.jpeds.2017.01.064

(hereafter referred to as the Bronchiolitis study).[18] As previously reported, respiratory syncytial virus (RSV) was identified in 83% and human rhinovirus in 34% of participants; 44% received oxygen therapy and 7.4% received ventilatory support.[18,19] The follow-up investigation at 2 years, performed from September 27, 2011, to December 14, 2011, September 11, 2012 to December 18, 2012, and October 7, 2013 to January 22, 2014, was attended by 499 of the initial 644 infants (77.5%).

Inclusion criteria for all infants were age 0-12 months, and for inclusion into the Bronchiolitis study, moderate to severe bronchiolitis, with a clinical score of at least 4 on a scale from 0 to 10 (most severe).[18] Exclusion criteria for all infants were severe underlying disease, and for the Bronchiolitis study, more than one episode of either bronchopulmonary obstruction or cough lasting for longer than 4 weeks before recruitment.

Procedures

Clinical investigations, morning cortisol sampling, and parental structured interviews were conducted at inclusion and at 2 years of age.

The study was approved by the Regional Committee for Medical and Health Research Ethics and The Norwegian Data Protection Authority and was registered in the Norwegian bio bank registry as well as ClinicalTrials.gov number, NCT00817466. Written informed consent was obtained from caregivers of all children.

Saliva Sampling

Parents were instructed to sample saliva in the morning as soon as possible after the child's awakening (after 6:00 a.m.) and before the children's first meal. Two small (0.7 × 1.8 mm), tasteless, arrowhead-shaped Sorbette (hydrocellulose, Salimetrics Europe Ltd, Suffolk, UK) microsponges attached to plastic applicator shafts were inserted into the child's mouth, preferably under the tongue, and kept there for a total of 60-90 seconds, until the microsponges were soaked with saliva.[20] The salivary samples in their respective standard containers were brought to the investigation site, and thereafter frozen at −86°C until transferal to Karolinska Institutet, Stockholm, for analysis. Radioimmunoassay was performed according to the manufacturer's instructions using kits from Cisbio Bioassays (Codolet, France) with monoclonal rabbit antibodies binding cortisol. For further description, see the **Appendix** (available at www.jpeds.com). The assay is standardized against the reference method, mass spectrometry.

Main Outcome

Reference values were defined as salivary cortisol levels (nmol/L) ranging from the 5th to the 95th percentile in infancy (at inclusion) and at 2 years of age. For comparison with other studies, geometric means were estimated and reported. Secondary outcomes for assessing potential influence of age, sex, and acute bronchiolitis were weighted mean salivary cortisol levels (nmol/L) with 95% CI.

Statistical Analyses

Background characteristics are given as means with SD, mean with minimum and maximum, or numbers with percentages, as appropriate. Neither morning salivary cortisol levels nor their natural logarithms were normally distributed. Percentiles including 95% CI for the 5th and 95th percentiles were used for estimating reference values.

To assess the potential impact of age, sex, and hospitalization for acute bronchiolitis on morning salivary cortisol levels, associations with cortisol were examined by the Huber M method of regression analysis,[21] whereas associations between dichotomous variables were analyzed by Pearson χ^2 test. Weighted means were calculated by Huber M regression methods, applying groups as categorical values, and estimating intercepts as the weighted mean. The significance level was set at .05. Interaction between age, sex, and morning salivary cortisol was tested by 2-way robust regression. Percentile analyses and robust regression analyses were done with NCSS 2007 (NCSS Statistical Software, Kaysville, Utah); otherwise, IBM SPSS (SPSS Inc, Chicago, Illinois) version 22.

Results

Salivary samples were obtained from January 15, 2010, to May 20, 2011, from 383 infants at a mean age of 5.1 months (range 0.2-13.4) and from 379 children at a mean age of 24.2 months (range 17.2-34.7; **Table I**), with samples at both time points in 231 children and on 1 occasion in the remaining 300 children (**Figure 1**). Background characteristics were similar between children from the reference group and bronchiolitis group with respect to sex, age at 2 years, parental asthma, ethnicity, and breast feeding, but significantly different with respect to weight and length, parental education, and use of inhaled corticosteroids (**Table I**). No interaction was found between age, sex, or morning salivary cortisol at the 2 time points.

Morning salivary cortisol ranged from 1.9 to 691.4 nmol/L in infancy and from 2.5 to 189.0 nmol/L at 2 years of age.

Reference Values

In infancy, the reference group had a geometric mean of 26.8 (95% CI 24.0-30.0) nmol/L with the reference values given by percentiles (**Table II**). The bronchiolitis group had significantly higher cortisol values (**Figures 2-4**; **Figures 2** and **3** available at www.jpeds.com), with a geometric mean of 37.0 nmol/L (95% CI 33.0-41.4) and a median of 39.9 nmol/L.

At 2 years of age, the weighted mean cortisol values were similar in the control and bronchiolitis groups. Reference values were therefore based on values including all children (**Table II**; **Figures 5** and **6**, available at www.jpeds.com), with a geometric mean of 32.1 nmol/L (95% CI 30.4-33.9).

Cortisol levels were above 3 SD in 1.5% and in 1.9% of the children at inclusion and at 2 years of age, respectively. By robust regression, we found no association or individual

Table I. Background characteristics of the 531 children with cortisol results during infancy, at 2 years of age, at either or both times

	Reference group	Bronchiolitis group	*P* value
All subjects (n = 531)	223	308	
Boys, n (%)	125 (56.1%)	186 (60.4%)	.317
Age (mo), mean (min–max)			
Inclusion (n = 531)	6.5 (1.0-13.4)	4.1 (0.2-11.9)	<.001
Two years of age (n = 453)	24.2 (17.2-34.7)	24.3 (18.8-34.7)	.667
Time (mo) between visit 1 and 2 (n = 379)	17.7 (1.2)	20.0 (1.2)	<.001
Weight (kg), mean (SD)			
Inclusion (n = 518)	7.9 (1.8)	6.5 (1.8)	<.001
Two years of age (n = 433)	12.9 (1.6)	13.2 (1.7	.05
Length (cm), mean (SD)			
Inclusion (n = 371)	67.8 (6.4)	62.9 (6.8)	<.001
Two years of age (n = 434)	88.6 (4.4)	87.1 (4.1)	<.001
Parental asthma (n = 520), n (%)	86 (29.6%)	94 (31.6%)	.632
Parental allergic rhinitis (n = 527), n (%)	84 (37.7%)	89 (29.3%)	.049
High education, n (%)*			
Mothers (n = 483)	199 (89.2%)	71 (63.3%)	<.001
Fathers (n = 487)	179 (81.4%)	150 (56.2%)	<.001
Caucasian, n (%)			
Mothers (n = 495)	211 (94.6%)	254 (93.4%)	.566
Fathers (n = 491)	206 (92.4%)	252 (94.0%)	.466
Breastfed, n (%)			
Inclusion (n = 453)	148 (75.1%)	187 (73.0%)	.617
Two years of age (n = 439)	10 (5.4%)	7 (2.8%)	.161
Second-hand smoke, n (%)			
Inclusion (n = 485)	9 (4.1%)	44 (16.5%)	<.001
Two years of age (n = 446)	1 (0.5%)	6 (2.4%)	.124
Boys with asthma at 2 years of age	5 (4.7%)	39 (22.9%)	<.001
Girls with asthma at 2 years of age	3 (3.6%)	13 (11.6%)	.042
Current inhaled corticosteroids at 2 years of age (n = 453)	9 (4.6%)	46 (18.0%)	<.001
Gestational age at birth < $37^{0/7}$ weeks (n = 443)	8 (3.8%)	28 (11.9%)	.002

*Defined as completed at least 3 years of schools after secondary school.

repeatability between salivary cortisol at inclusion and at 2 years of age in any of the groups, with a regression coefficient of 0.001 ($P = .93$) in the reference group and a regression coefficient of −0.010 ($P = .83$) in the bronchiolitis group.

The Role of Age and Sex

The weighted mean cortisol in the reference group was significantly higher at age 2 years (34.9 nmol/L; 95% CI 32.6-37.2) than in infancy (28.7 nmol/L; 95% CI 25.1-32.4) with a difference of 7.8 nmol/L (95% CI 2.4-13.1; $P = .004$), whereas the reverse was found in the bronchiolitis group at age 2 years (33.8 nmol/L; 95% CI 31.8-35.7) vs infancy (41.5 nmol/L; 95% CI 37.6-45.4), with a difference of −6.4 nmol/L (95% CI −11.0, −1.8; $P = .006$). Sex-stratified results are shown in **Figures 3** and **6**.

However, increasing age within infancy or at 2 years of age was not associated with cortisol levels in regression analyses (**Table III**; available at www.jpeds.com).

At 2 years of age (but not in infancy), girls had significantly higher weighted mean and geometric mean morning salivary cortisol compared with boys (**Figures 6** and **7**

Table II. Reference values: morning salivary cortisol percentile levels (nmol/L) at inclusion*

	Percentiles								
	2.5	5	10	25	50	75	90	95	97.5
Inclusion									
Reference	4.5	9.7	12.5	18.2	23.7	40.1	58.3	119.6	222.1
95% CI		3.1-12.1						64.9-258.6	
Two years									
All	11.8	13.6	16.2	24.6	34.1	43.9	56.6	65.2	80.2
95% CI		12.1-15.2						60.5-73.1	
Boys	9.0	12.7	15.5	23.7	31.8	42.2	51.7	62.8	74.8
95% CI		8.3-14.8						55.6-77.6	
Girls	12.9	14.4	16.9	26.7	38.5	45.6	60.3	69.7	112.0
95% CI		12.6-16.5						62.0-162.1	

*From the 199 infants in the reference group (0-13 months of age), and at the 2-year follow-up from all 379 children, stratified by sex (boys n = 220, girls n = 159).

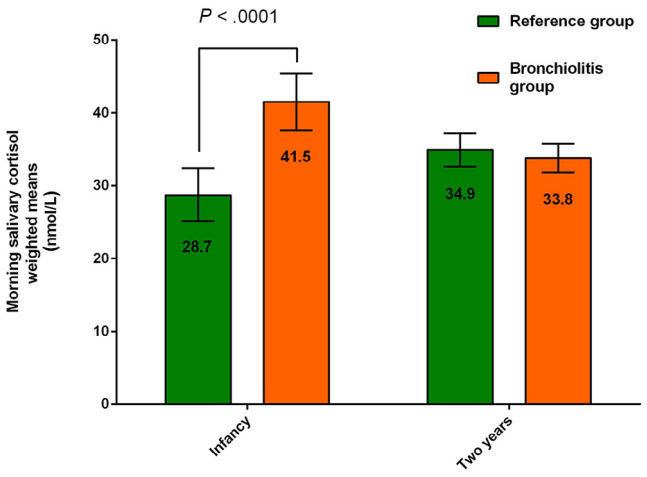

Figure 4. Comparison of mean salivary cortisol levels, control and bronchiolitis groups. Morning salivary cortisol (weighted mean [95% CI]) levels given for children in the reference group and bronchiolitis group at inclusion and at 2 years of age. The cortisol levels in infants with acute bronchiolitis were significantly higher than levels in the reference group in infancy and the bronchiolitis follow-up group at 2 years ($P < .0001$). Weighted mean within the bars.

[available at www.jpeds.com]; weighted mean 36.9 nmol/L [95% CI 34.7-39.2] vs 32.4 nmol/L [95% CI 30.5-34.3] and geometric mean of 35.1 nmol/L [95% CI 32.4-38.0] vs 30.1 nmol/L [95% CI 28.0-32.3], respectively; $P < .003$). Reference values are therefore presented stratified for sex (**Table II**). The sex difference in cortisol levels was not associated with the higher frequency of a doctor's diagnosis of asthma in boys (results not shown).

The Role of Acute Moderate-to-Severe Bronchiolitis

Morning salivary cortisol levels were significantly higher in infants with acute bronchiolitis than in infants in the reference group (**Figures 4** and **6**, with a weighted mean difference of 12.8 nmol/L [95% CI 7.4-18.1] that remained significant after adjusting for age and sex (13.9 nmol/L [95% CI 8.1, 19.7]; $P < .0001$).

At 2 years of age, however, the weighted mean cortisol levels were similar in the bronchiolitis (33.8 nmol/L; 95% CI 31.8-35.7) and reference group (34.9 nmol/L,; 95% CI 32.6-37.2), and are thus presented for all 379 children (34.3 nmol/L; 95% CI 32.8-35.8).

The predictability of cortisol at inclusion for assignment to the bronchiolitis or reference group is illustrated by a receiver operating characteristic diagram (**Figure 8**). The area under the curve was 0.634 (95% CI 0.577-0.690). The cutoff value of cortisol giving the highest sum of sensitivity and specificity is 36.9 nmol/L, giving a sensitivity of 51.6% and a specificity of 68.3%.

Neither breastfeeding, parental education, second-hand smoke, weight, nor length was associated significantly with salivary cortisol level at inclusion or at 2 years of age (results not shown). Subgroup analyses of non-Caucasian vs Caucasian parents could not be performed owing to low numbers of non-Caucasian parents.

Discussion

Reference values based on the 5th-95th percentiles were estimated in 199 children from a general population in infancy, and at 2 years of age in 379 children including the same reference population as well as children who in infancy were hospitalized with acute bronchiolitis. Salivary cortisol levels were significantly higher at 2 years of age than in infancy in the reference group, were significantly higher in infants with bronchiolitis compared with the reference group, and in girls at 2 years compared with boys. However, age was not associated significantly with morning salivary cortisol at either of the 2 time points.

We found generally higher values than salivary cortisol levels reported as quartiles by Ivars et al[10] in a Swedish infant population. Although our median value of 23.7 nmol/L (at a mean age of 6 months) was similar to the mean value of 24.6 nmol/L at 6 months of age reported by Ivars et al, their median cortisol levels varied from 5.1 to 10.9 nmol/L during infancy. Similar to our reference group, the Swedish study recruited 130 infants from a general population, with 95-120 samples collected each month through infancy, and both studies using the same radioimmunoassay method.[10,22] The differences in cortisol levels may be related to the timing of salivary sampling. In our study, parents sampled saliva as soon as possible after first awakening after 6 AM, before feeding, in contrast with the Swedish parents who collected saliva samples at least 1 hour after solid food, sleep, or crying and riding a car, and 30 minutes after intake of liquids. In line with a circadian rhythm, Ivars et al observed that early morning samples (between 7:30 and 9:30 a.m.) were higher than evening samples (between 6:30 and 9:30 p.m.).[10] Using the same analysis protocol, values between those found in the present study, and those found by Ivars et al were reported by Stenius et al[23] in 6-month-old infants (geometric mean of 14.9 nmol/L) with salivary sampling within one-quarter of an hour after awakening in the morning, and before the first meal. Higher cortisol levels in samples taken as soon as possible after awakening are supported by the decreasing cortisol levels observed in samples taken hourly from 8 to 10 a.m. in children older than 2 years of age participating in a Japanese study of 57 healthy 0.5 to 4.0-year-old children.[9] Compared with the higher reference values observed in the present study, their defined lowest and upper limits of the reference range in micrograms/dL—0.076 (equals 2.1 nmol/L) and 0.827 (equals 22.8 nmol/L), respectively—may reflect that the samples were taken later during the day. An awakening cortisol response, described as the period of cortisol secretory activity in the immediate 45-60 minutes after awakening,[24] reaches a maximum about 30 minutes after wakefulness.[1] An awakening response is supported by a study in infants at a mean age of 2 months, reporting a mean value of 0.31 μg/dL (8.6 nmol/L) immediately after awakening and

0.60 μg/dL (16.6 nmol/L) 30 minutes after awakening from at least 30 minutes of sleep.[25] Thus circadian rhythm, with highest peaks in the morning, as well as awakening response, as described by Michels et al[26] in only 52% of children 5-11 years of age, may explain the higher values in the present study.

The higher cortisol values among girls at 2 years of age in our study is a novel finding, and may have several causes. Higher cortisol levels in girls may be related to a more pronounced cortisol awakening response compared with boys, as described by Pruessner et al[27] in 12 year-old children, or may be explained partially by a prolonged "mini-puberty" in girls compared with boys.[28] Because salivary cortisol reflects the free fraction of cortisol, our finding cannot be explained by a higher level of corticosteroid-binding globulin. Similarly, sex differences in cortisol levels could not be explained by the higher frequency of a doctor's diagnosis of asthma among boys.

Higher morning salivary cortisol in infants with acute bronchiolitis suggests possible pathophysiological involvement of cortisol in acute bronchiolitis, although cortisol levels could not be used to classify infants into bronchiolitis or control groups owing to the overlapping values, as illustrated by the receiver operating characteristic curve and dot plots. The infants had their first samples taken during moderate to severe acute bronchiolitis,[18] in line with higher cortisol values found with severe disease that possibly reflects suppression of the Th1 response, as described in RSV infection in infants.[29]

A possible limitation of our study was the skewed study population, where approximately 89% of mothers in the reference group and 63% in the bronchiolitis group had higher education compared with the national average of 48% of all women between 25 and 50 years of age, according to Statistics Norway.[30] We found no association between parental education and salivary cortisol levels. Another potential limitation is the low proportion of successful collection of samples at both study points, especially in the bronchiolitis group. However, the high probability of a difference between ill and control groups at inclusion and between the sexes at the follow-up when both groups are analyzed together makes it likely that they reflect true differences.

Owing to the high proportion of infants with detected RSV during acute bronchiolitis, it was not possible to perform robust analyses into the potential specific impact of RSV on salivary cortisol level.

A potential shortcoming of salivary measurement is the representativeness of a spot sample of a biomarker with known diurnal variation.[7] However, measuring morning salivary cortisol may provide information about the hypothalamic-pituitary-adrenal axis and capacity for reaching high peak values that may be blunted by measuring a 24-hour or several hour urinary value. Another potential source of variation, particularly for the highest values, could be blood contamination. However, other studies have shown that blood contamination has little impact on salivary cortisol measurements.[31,32]

Greater variations at the upper end of salivary vs serum cortisol levels may be explained by the fact that salivary cortisol is an ultrafiltrate of the free fraction of serum cortisol. If acute stress or other factors lead to a surge of cortisol exceeding the corticosteroid globulin binding capacity, it is possible that we may find a higher relative increase in salivary vs total plasma cortisol.[33]

Sampling time is likely to be crucial for salivary measurements. We standardized the sampling to the best of our ability, but cannot rule out potential deviations from the time of sampling. However, our approach of asking parents to sample as soon as possible after awakening reduces potential variation in time. Also, we did only 1 sampling per child on each occasion; thus, repeatability could not be assessed. However, Nagakura et al[9] found no significant day-to-day variation between 3 samples taken at 4- to 8-day intervals.

Reference morning cortisol values in infants were 9.7 to 119.6 nmol/L (5th-95th percentile), compared with 11.8 to 80.2 nmol/L at 2 years. Sex-specific reference values may be necessary, because girls at 2 years of age in the present study had higher cortisol values than boys. Acute bronchiolitis in infancy was associated with higher morning salivary values during hospitalization, but seemed not to influence morning cortisol levels at 2 years of age. ■

We thank all children and caregivers, participants in the Bronchiolitis Study Group, the several hundred hospital staff that were involved in recruiting patients and running the study, and involved members of the Oslo Research group of Asthma, Allergy in Children, the Lung and Environment (ORAACLE).

Submitted for publication Sep 4, 2016; last revision received Jan 9, 2017; accepted Jan 26, 2017

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Appendix

Methods: In each tube was a known amount of ^{125}I-labeled cortisol in Tris buffer, competing with the salivary cortisol to be attached to antibody binding sites. The saliva was kept within the tubes in a water bath at 37°C for 30 minutes before it was poured out. Then, the tubes were rinsed with a predefined amount of water and left upside down for a period until they were put into a gamma counter. A standard curve for comparison was produced from a cortisol calibrating sample in the kit. The working range for the method is 0-2000 nmol/L and 150 μL of saliva is required. The analytical sensitivity is 3.0 nmol/L.

Results: At 2 years of age, the geometric mean values of morning salivary cortisol were 31.6 nmol/L (95% CI 29.5-33.9) in the bronchiolitis group and 32.8 nmol/L (95% CI 30.1-35.6) in the reference group.

Table III. Morning salivary cortisol (nmol/L)

	Reference group		Bronchiolitis group	
Cortisol levels	**Unadjusted weighted mean**	**Age increase**	**Unadjusted weighted mean**	**Age increase**
Infancy	28.7	0.7	41.5	–0.6
95% CI	25.1 to 32.4	–0.2 to 1.6	37.6 to 45.4	2.4 to 1.2
Two years	34.9	0.0	33.8	–0.4
95 % CI	32.6 to 37.2	–0.6 to 0.6	31.8 to 35.7	–1.0 to 0.3

*Given as unadjusted weighted mean for reference group and bronchiolitis group, sexes together, in infancy and at 2 years of age and changes by monthly increases in age at salivary cortisol sampling.

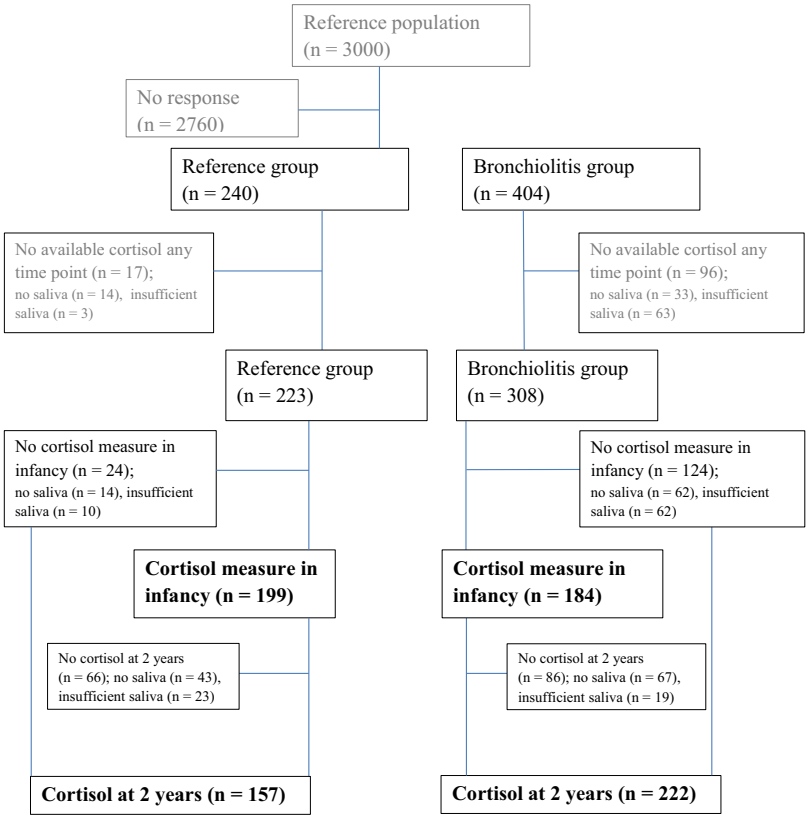

Figure 1. Flowchart of inclusion. Entrance of subjects with available cortisol measurements.

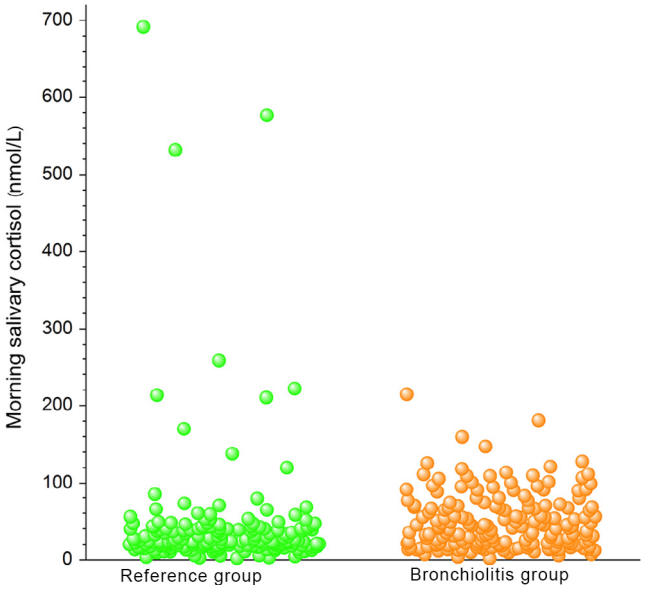

Figure 2. Morning salivary cortisol at inclusion, dot plot; each dot representing an individual.

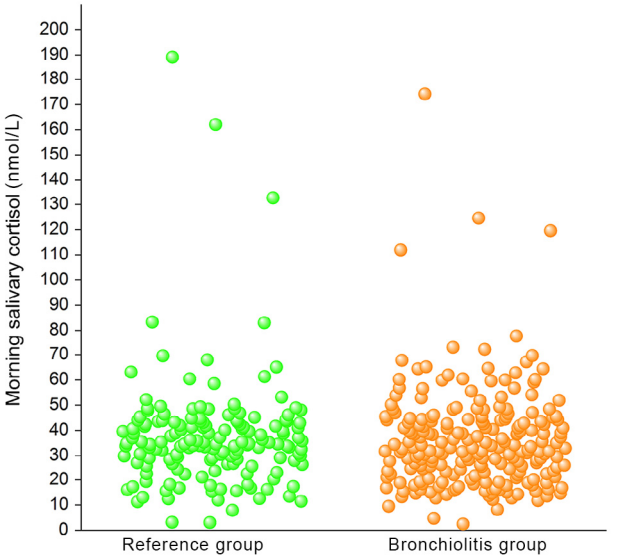

Figure 5. Morning salivary cortisol at 2 years of age, dot plot; each dot representing an individual.

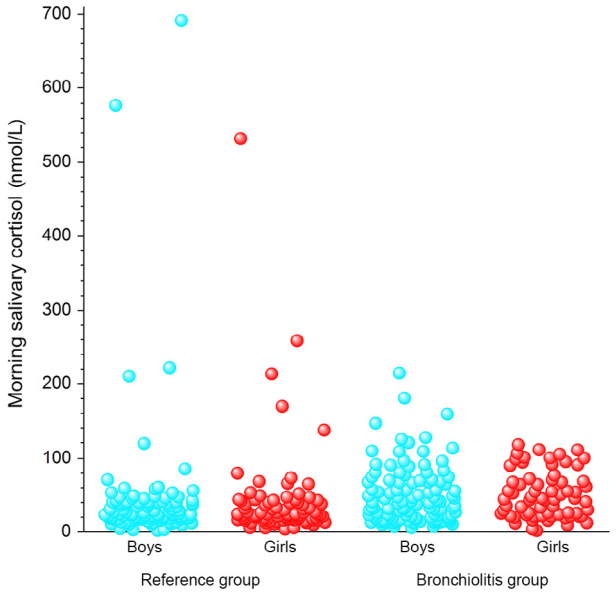

Figure 3. Sex distribution, morning salivary cortisol at inclusion dot plot; each dot representing an individual.

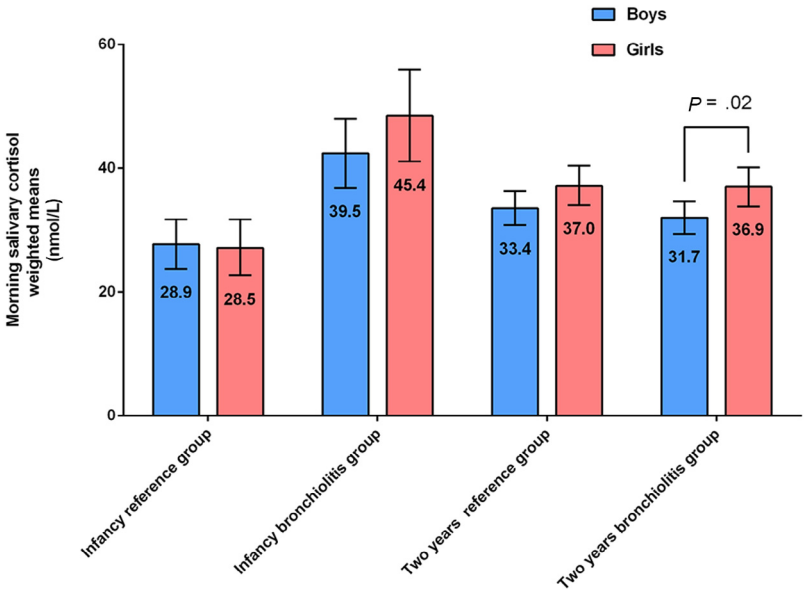

Figure 6. Morning salivary cortisol (weighted mean (95% CI)) are given for boys (*blue bars*) and girls (*red bars*) in infancy and at 2 years of age. P-value is shown for difference between sexes when <0.05. Weighted mean are shown within the bars. Cortisol levels were significantly higher for girls at 2 years of age.

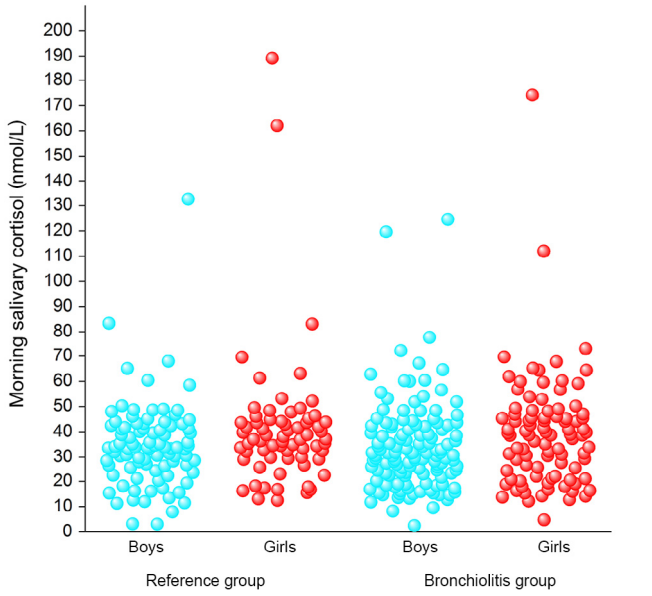

Figure 7. Sex distribution, morning salivary cortisol at 2 years of age, dot plot; each dot representing an individual.

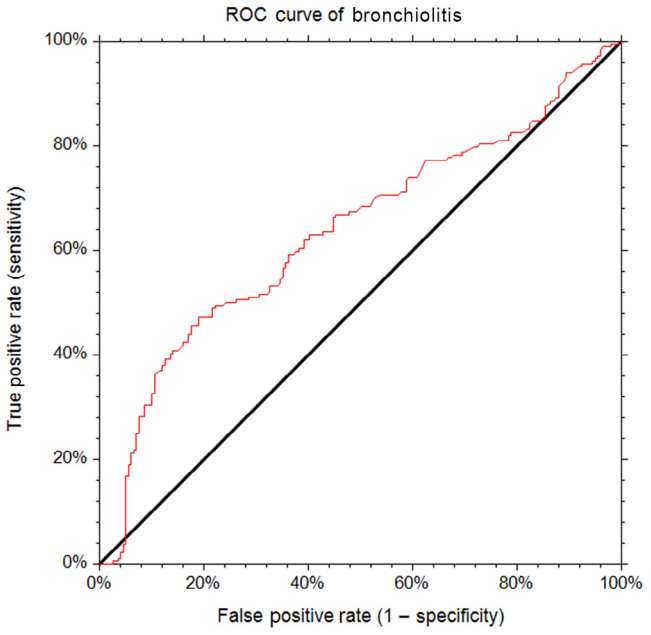

Figure 8. Receiver operating characteristic (ROC) curve, showing predictability for bronchiolitis by morning salivary cortisol at inclusion.