Author’s response to reviews

Title: High end of normal ACTH and cortisol levels are associated with specific cardiovascular risk factors in pediatric obesity: a cross-sectional study

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Author’s response to reviews: see over
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Title: High end of normal ACTH and cortisol ranges are associated with specific cardiovascular risk factors in pediatric obesity: cross-sectional study

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Author's response to reviews: see over
High end normal ACTH and cortisol ranges are associated with specific cardiovascular risk factors in pediatric obesity: cross sectional study” by F. Prodam at al.

Answer to the Referees’ comments

Referee 1

1) The manuscript could benefit from a careful reading by a native English speaker – there are instances of awkward phrases and incorrect grammar throughout. For example, “strictly associated” isn’t really well defined in English – presumably the authors mean associated even after accounting for covariates, but that isn’t clear.
Response: The paper has been revised by the suggested Edanz service.

2) Last sentence in abstract: As these findings are cross-sectional and far from definitive on the causal direction, revise sentence to read: “These specific associations suggest complex mechanisms through which the HPA axis may contribute to metabolic impairments in obesity.”
Response: The sentence has been modified as follows “Understanding the specific role of ACTH and cortisol in the pathogenesis of metabolic alterations in obesity merits further investigations.”

3) Ref: Specify the IFG and IGT cut points used in Methods
Response: We have inserted the cut-offs in the Methods as suggested.

4) Ref: Page 6: At what time, exactly, were cortisol and ACTH measured? As the authors certainly know, these variables are very time-sensitive. “Morning” is not sufficiently precise. Did the time of day for phlebotomy vary significantly among the three tertiles of ACTH/cortisol? This variable should have been taken into account in the analysis.
Response: Blood sampling was performed at 8 a.m. Children had to be present at 7.30 a.m. in the clinic to enable the preparation of the setting. ACTH and cortisol samples were the first to be collected. As already described, 10 children who presented an excessive level of distress during the blood sampling, were excluded. Moreover, phlebotomies that were particularly difficult to obtain, were excluded (more than 5 minutes to collect ACTH and cortisol samples) despite the fact that the children were not distressed (13 out of 27 did not satisfy inclusion criteria). These aspects have been better described in Methods and results, as suggested.

5) Ref: Page 8: It is unclear in Methods how you will use the blood pressure cut-offs of 95th and 90th percentiles. Please indicate here that 95th was used to define hypertension and 90th was used for your definition of one criterion of metabolic syndrome.
Response: Hypertension was evaluated according to two specific cut-offs. The first was the cut-off suggested by the National High Blood Pressure Education Program Working Group and hypertension was defined as a blood pressure > 95th percentile for age, gender and height. However, in the literature some Authors and many NCEP ATPIII derived definitions set the cut-off for hypertension at 90th percentile for age, gender and height. Indeed, we classified all the children by using both cut-offs to explore whether ACTH and cortisol were differently associated with blood pressure and to minimize differences with respect to other published papers. We have now better explained the concept in the paper.
Moreover, we did not divide the children for metabolic syndrome. Our aim was to define an association between ACTH, or cortisol and cardiovascular risk factors. Being that a worldwide accepted definition of each cardiovascular risk factor investigated in childhood is absent, we
chose the most used cut-offs derived by a pediatric NCEP ATPIII classification of the metabolic syndrome.

6) Ref: Page 7: Does “diet-naïve” mean “weight-stable”? If so for how long?
Response: Children and adolescents were included if they were diet naïve, that is they had not ever been engaged in a diet program to decrease their weight in our center or in any other institution (other hospitals, school, paediatrician, self-made etc). We have now better explained this aspect.
All the subjects were weight-stable in sense of a long-history of obesity. However, it is not possible to know for how long because a structured plan of surveillance and classification by paediatrician is still lacking in our country. Self-reported data by parents, in particular with respects to obesity, were too imprecise to be used in this study.

7) Ref: Page 8: As part of the metabolic syndrome evaluation, was waist circumference measured? If measured, please include in the manuscript.
Response: As previously discussed, we did not classify our subjects for metabolic syndrome, but we used its cut-offs for the definition of cardiovascular risks because this definition is well accepted.
On the other hand, we measured the waist circumference of this cohort and we have added these data, as suggested.

8) Ref: “Triglycerides and HDL-cholesterol percentiles were referred to Lipid Research Clinic Pediatric Prevalence Study [21]” – does this mean you used the cut-points for Total and LDL-cholesterol specified in Table 1 of this document (total >200, LDL >130)? Please be more exact.
Response: Not exactly. Because our cohort was also composed by very young children and of both sexes, we used the percentiles of Table 2 of that paper. Table 2 showed the percentiles according to sex and stratified by age subgroups (we used ≥ 90th percentile for triglycerides and ≤ 10th percentile for HDL-cholesterol). We have better pointed out this aspect.

9) Ref: Page 9: Minor point: “Analysis of covariance was used to determine differences in those with an without....” should be “Analysis of covariance was used to determine differences in those with and without...”
Response: We improved the sentence.

10) Ref: Was a power calculation performed for this study? Regardless, please indicate in Results the power of the study for detecting differences in the studied variables.
Response: The Hospital covers an area of approximately 500,000 population of the North-East Piedmont. The sampling proportion was based on the age structure of the community and of the general paediatric population referring to the Division and on sample size calculation with respect to mean cortisol variation, as inserted in the methods.

11) Ref: Page 9: If the authors truly meant what I believe the phrase “near tertiles” means, then they wouldn’t have cut points like 16.10 and 26.93 for ACTH – these would be 16 and 27. It seems likely that exact tertiles were used.
Response: We used the expression “near tertiles” because they were rounded up to one decimal for cortisol and to two decimal for ACTH. However, because differences are really minimal, we cut the adjective, as suggested.

Response: We used BMI instead of BMISDS as a covariate because we also corrected for age. In fact BMISDS is calculated on age and sex distribution. To correct in the same model for both age
and BMISDS (BMI + age) can underestimate the results. However, when we repeated the analysis by introducing BMISDS with and without age, the significance did not change.

13) Ref: Minor point – the authors likely mean “sex” when they use “gender” – please correct throughout the manuscript.
Response: We corrected this word as suggested.

14) Ref: Hypertension was diagnosed in a remarkably large percentage of these subjects – these data are far from what is reported generally. Is there some referral bias that could account for this strange finding? Please also specify what the American Academy of Pediatrics criteria are, either here or in Methods. The sentence “Only 1 subject fulfilled all the cardiovascular criteria, while 63 (15.5%) failed to meet the criteria” is very unclear at present.
Response: We were also surprised for the prevalence, but is real. We recorded a similar prevalence in a cohort evaluated for another paper on prevalence of metabolic syndrome (awaiting for final decision). It has to be underlined that our population is likely more complicated, it is followed in a tertiary care center and hypertension was defined if also systolic or diastolic blood pressure are elevated. The percentage for both systolic and diastolic hypertension is quite lower. All measurements were performed according to guidelines. In particular, blood pressure was measured three times at 2-min intervals by a mercury sphygmanometer with an appropriate cuff size after participants were seated quietly for at least 5 minutes with their right arm supported at the level of the heart and feet flat on the floor. The mean was used in the analysis. Because of a large prevalence, we compared the measurements in the day of analysis with respect to the day of enrolment in which blood samplings were not performed, to confirm the diagnosis of hypertension. We have better described this, as suggested.
In many of these children, although the data are not included in this study because it is ongoing, we suggested a domiciliary blood pressure evaluation which resulted in similar data. Many factors could be hypothesized: a diet rich in salt and/or fructose, family history, a stressful environment etc. We are now investigating how factors could be involved and if these blood pressure values are associated with organ damages.
The sentence “only 1 subject…” has been improved.

15) Ref: Page 10 – It appears, from the legend in Table 2, that the paragraph starting with “ACTH and cortisol levels were positively associated with…” should indicate that the first results are unadjusted for covariates – please revise to: “In unadjusted analyses, ACTH and cortisol levels were positively associated with…” Probably preferable is not to present the unadjusted results at all – simply remove the unadjusted discussion and remove Table 2, which appears to show unadjusted data, from the manuscript.
Response: We corrected the sentence as suggested to clarify our results. We proposed Table 2, as supplemented Table. We have decided to not completely cut it because of some observations by referee 2.

16) Ref: The phrase “positivity for cardiovascular risk factors” is not proper English – please revise throughout the manuscript to read “presence of cardiovascular risk factors”
Response: The sentence has been modified throughout the manuscript and the abstract.

17) Ref: For the analysis of ACTH and cortisol among those with metabolic abnormalities, again, only the adjusted results should be discussed.
Response: We have modified the discussion, as suggested. On the other hand, we did not cut the part on BMI because the second reviewer asked us to deepen this part.
18) Ref: Discussion – for this paper, an explication of prior findings in adults would actually be quite useful to place these results in perspective. 
Response: Prior findings on adults have been expanded at the start of the discussion.

19) Ref: Please carefully rephrase the sentence, “We showed that both hormones were continuously and directly associated with glucose, triglycerides, and blood pressure, while cortisol also with LDL-cholesterol independently by confounders including gender, age, puberty, BMI and insulin resistance.” 
Response: We have rephrased the sentence as suggested.

20) Ref: Page 14: “but not HDL-cholesterol” should be “but not lower HDL-cholesterol” 
Response: We have improved the sentence as suggested.

21) Ref: Discussion seems a bit too directed towards the notion that high ACTH and cortisol are causing the metabolic abnormalities observed – the resolution of high ACTH and cortisol after weight reduction suggests HPA axis dysregulation is more frequently a consequence, rather than a cause, of obesity-associated abnormalities. Most of the references to Cushing syndrome could thus profitably be removed. 
Response: Thank you, we previously discussed this aspect in the limitations of the study. Now, we have better focused this concept in discussion and in the abstract, as suggested. Some references have been removed.

22) Ref: Another significant limitation not mentioned is the absence of true body fat measurements from DEXA. Socioeconomic status, which has been found to affect stress/cortisol levels, was not accounted for. Also absent is any normal weight group. There is also no statistical control for the many comparisons being made in the paper. 
Response: Thank you. We have inserted all the other limitations. In particular, unfortunately, body composition was not measured because our Ethic Committee did not allow us to perform radiological techniques to measure fat mass. However, many studies demonstrated that BMI is a specific indicator of body fatness in children. We chose only BMI and BMISDS according to the evidence that almost all comparisons indicated that BMI was at least as strongly associated with levels of the various cardiovascular risk factors, in particular lipids, in the Bogalusa Heart study (Freedman DS, Katzmarzyk PT, Dietz WH, Srinivasan SR, Berenson GS. Relation of body mass index and skinfold thicknesses to cardiovascular disease risk factors in children: the Bogalusa Heart Study. Am J Clin Nutr 2009; 90:210-6). In addition, if BMI was poorly correlated with DXA in normal weight children, but in obesity, regression models including BMI, age, and race ethnicity as predictor variables have been found to be good predictors of body fatness, accounting for about 79% (boys) to 81% (girls) of the variability in percentage body fat (Freedman DS, Wang J, Thornton JC, Mei Z, Sopher AB, Pierson Jr RN, Dietz WH, Horlick M. Classification of body fatness by body mass index-for-age categories among children. Arch Pediatr Adolesc Med 2009). Because we had data on waist circumference, we inserted them as previously discussed. Moreover, we excluded data on socioeconomic status because we have limited data. In fact, many parents of obese children refused to give us this information in regards to privacy. Furthermore, we lack of a control group. When we designed the study, we excluded all patients followed in our Service because nobody satisfied our criteria to be a good control; in fact our pediatric population is composed by subjects with inflammatory diseases, short stature, celiac disease etc, all condition that can modulate HPA axis. One choice could be the enrolment of a healthy population of schoolchildren, but we believe that a population which is followed in a tertiary care center is not completely comparable with a school population in terms of chronic stress.
23) Ref: Table 1 would be improved by dividing the children into the two available groups (overweight, and obese) - and indicating how many children in each group had abnormal results for the metabolic parameters. Give pubertal stage information separately for boys and girls.
Response: Table 1 has been modified according to suggestion of both referees. We have inserted a new Table 2 in which we stratified subjects for overweight and obesity.

Referee 2.

1) Ref: Abstract … cardiovascular risk factors ….and hypertension …can not be “measured” (blood pressure can be measured).
Response: We modified the verb, as suggested.

2) Ref: Abstract. What do the author mean by: “Obese children and adolescents …. have high normal morning ACTH and cortisol levels leading to the hypothesis that HPA hyperactivity comes out early in life with an association already present for values in the higher range of normality”? Do higher ACTH and cortisol levels, that are still in the normal range, represent HPA hyperactivity?
Response: The first observation was that the association between ACTH, cortisol and metabolic alteration is present in children and not only in adolescents, but also in early life. We improved the sentence.
Secondly, we observed an association for ACTH and cortisol values in the highest tertile. Indeed, we can speculate that this is because the association is present and a HPA hyperactivity could be present also in the highest part of the normal range. Many studies have recorded higher cortisol levels in adults with metabolic syndrome or some of its components, but cortisol are often in the normal range and the alteration was an increased cortisol response to some stimuli (food intake, low-dose ACTH-test etc) or loss of diurnal cortisol variation. However, we have no data to demonstrate it. We have better discussed this aspect. Moreover, it has to be underline that ACTH and cortisol cut-off values for the normal range are usually calculated on an adult population without providing reference ranges for children. A recent published paper shows how general reference ranges would elicit false-positive, and more critically false negative results (Brossaud J et al Clin Chem Lab Med. 2012 Apr 21;50(5):901-3. doi: 10.1515/cclm-2012-0142.). In fact, they analysed cortisol levels in a pediatric population and detected 26% of subjects with low basal levels and 31% of subjects with “adrenal insufficiency” in a cohort of apparently unaffected children.

3) Ref: What were signs suggestive for Cushing’s syndrome, please state criteria.
Response: We used Endocrine Society Guidelines. We have inserted symptoms, as suggested (decreased height and increased weight).

4) Ref: Why did the authors use the SEM. This is misleading, they probably did not want to show us (the reader) the uncertainty of how the sample mean represents the population mean. Refer to: http://bja.oxfordjournals.org/content/90/4/514.full Please provide the SDs for the arithmetic mean values or the median values with (e.g.) the 25th and 75th percentiles.
Response: We have provided SD as suggested.

5) Ref: “ACTH and cortisol were also categorized into near tertiles.” What are near tertiles?
Response: We used the expression “near tertiles” because they were rounded up to one decimal for cortisol and to two decimal for ACTH. However, because differences are really minimal, we cut the adjective.
6) Ref: The authors wrote that 27 subjects were excluded “because they did not satisfy inclusion criteria”. These criteria should be clearly stated.
- What were the criteria for the diagnosis of congenital adrenal hyperplasia?
- ... urines ... were incomplete in 20 of 31 patients”. Spot samples?, 24-h samples?
- Was the diagnosis “Cushing’s Syndrome” only done in those with “complete” urine collection?
Response:
a) Criteria have been clearly stated as suggested and reorganized as also suggested by the first reviewer.
b) Late-onset congenital hyperplasia was diagnosed according to Endocrine Society Guidelines (Speiser PW et al J Clin Endocrinol Metab, September 2010, 95(9):4133–4160).
c) Urines for urinary free cortisol were 24-h samples. We pointed out the information.
d) Nobody had a Cushing’s Syndrome. All suspected subjects performed a low dose dexamethasone test and all subjects completely inhibited cortisol levels. Twenty subjects also correctly collected 24-h samples for urinary free cortisol for two days. The last 11 subjects collected partial samples because some spots of one collection were lost during the school day. However, urinary free cortisol levels were normal in both samples (complete and incomplete collection). All of these subjects are still followed for obesity and nobody has had a late diagnosis of a Cushing’s Syndrome.

7) Ref: I did not understand the sentence: “Analyzing subjects positive to each alteration with respect to the cut-offs chosen, revealed higher ACTH levels in those with ....”
Response: The sentence was modified. These results are referred to subjects divided for cardiovascular risk factors as yes or no. Otherwise, the ahead point describes correlations between continuous variables.

8) Ref: Tables and text: why not presenting result in SI units? If there are strong reasons to do so, please provide at least the corresponding conversion factors.
Response: Results are now all represented in SI.

9) Ref: A Table with mean values of age, BMI, lipids, hormones etc. given for an overall age range from 4-18 years makes no sense. Provide all parameters according to useful age ranges, Tanner stages, or at least prepuberty vs. puberty (and in that case sex-stratified).
Response: Table 1 has been modified according to suggestion of both referees. Moreover, we have inserted a second Table stratified for weight status according to referee 1 suggestions.

10) It appears to me that the HOMA-IR and Glc0 changes are possibly more pronounced between tertile 1 and tertile 2 (cortisol tertiles, Table 2) compared to tertile 2 vs. tertile 3. Since means of glucose and HOMA are lower in tertile 1 compared to tertile 2, could it not be that a potential positive association between plasma cortisol and HOMA or glucose may exist particularly in the ranges of normal-to-moderately high HOMA or Glc0 levels? In this range of not so strong insulin increases hepatic CGB production should be less strongly suppressed, implying that more of the Cortisol secreted by the adrenals can circulate as total cortisol CBG-bound. Please check the corresponding HOMA-IR and Glc0 ranges for their partial correlations with cortisol.
Response: Thank for your observation. We checked partial correlations and mistakes are not present, with just a correlation for fasting glucose and not for HOMA. We have also performed partial correlation including tertile 1 and 2 (without tertile 3 cortisol levels) but we were not able to demonstrate any significance. Because your idea is really interesting and because CGB is directly produced also by adipose tissue we will evaluate CBG levels in future further studies respect to puberty and glucose metabolism in children and adolescents.
Furthermore, the Table 2 of the first version has been proposed as Supplemental Table 1, according to referee 1 suggestions.

11) Table 3, Title should read: Partial correlations for ACTH (....) and cortisol (....) with cardiovascular risk factors.
Response: We have modified the title as suggested.

12) Page 12, “... the link between HPA dysregulation in obesity ...“ How do the authors know that this is a dysregulation? Could it not be a metabolic adaptation?
Response: We removed the term as suggested. We believe that HPA alteration is a metabolic adaptation of obesity which could, however, contribute to its comorbidities when the alteration starts. On the other hand, our study is not able to resolve this issue but it only suggests and reinforce the link. We have better discussed this aspect.

13) Page 13 “... cardiovascular risk factors remains positive in unselected obese child and / or obese adolescent populations [9,13].” Please add.
Response: We improved the sentence as suggested.

14) Page 13 The authors regarded one of their findings as unexpected, namely the observed lack of an association between BMI and cortisol. They provided as an explanation that the population they examined was a “homogenous population in terms of obesity“.
- What does this exactly mean for 4-18 years olds?
- How low were the standard deviations or the ranges for body fatness or BMI in the different age groups? [see also SEM vs. SD, (4)]
Response: BMI and BMISDS mean ± SD are presented in Table 1 and Suppl. Table 1. Their distribution is homogenous in the cohort as 4-18 years old or if the cohort was divided for Tanner stage or ACTH and cortisol tertiles. Similar data are also recorded for waist circumference. In particular, the distribution of SD of BMI and BMISDS is similar according to ACTH and cortisol tertiles. However, the association between BMI or BMISDS is only present for ACTH, but not for cortisol.

15) Page 13, Data interpretation: Similar to the lack of an association between BMI and plasma cortisol in the obese population, a lack of association between BMI or body fatness and urinary free cortisol & free cortisone (in 24-h urines) has been demonstrated in non-obese children [Dimitriou T et al., Am J Clin Nutr. 2003;77:731-6]. However, major glucocorticoid metabolites in 24-h urine samples (reflecting ACTH-driven adrenocortical activity or cortisol secretion) were significantly associated with fatness in these non-obese children [Dimitriou T et al.]. The latter findings together with the present of Prodam et al., strongly suggest that adrenocortical activity (driven by ACTH, i.e., ACTH itself) is related to body composition – during growth – whether children are lean/normal-weight [Dimitriou] or obese [Prodam]. In other words the associations observed by Prodam et al between ACTH and BMI on the one hand and the non-association between circulating cortisol [in the Dimitriou paper: urinary free cortisol and/or cortisone] and BMI on the other hand appear not to be restricted to obese children.
This important issue must not be ignored in the Discussion.
Response: Thank you very much for your observation which strongly reinforce our data. We inserted data on this work in our discussion.

16) Cortisol secretion (even after correcting for body surface area) varies markedly with age and differs between sexes at least after the start of puberty [Wudy SA et al. Am J Physiol Endocrinol Metab. 2007;293:E970-6]. Furthermore, literature data suggest that there is only
very little variation in total plasma cortisol levels in lean/normal-weight children with age (this is no contradiction, since metabolic clearance rate of cortisol probably varies correspondingly). However, the question emerges: is this also the case in obese children? Some vague literature hints “say no”. Prodam et al. can contribute to (perhaps) solve this question by showing a scatter of all of their obese children’s plasma cortisol measurements on the Y-axis and age on X-axis. Such a Figure would be very interesting and helpful.

Response: Thank you very much for your suggestion. We have analysed ACTH and cortisol levels with respect to age by dividing subjects in 2-year breaks, according to Wudy et al paper. We failed to demonstrate a difference in ACTH and cortisol levels across Tanner stages or age subgroups in our obese cohort as a whole, or with respect to gender distribution. We observed higher cortisol levels in Tanner 4-5 stages with respect to Tanner 1 in the whole group and ACTH levels in males than in females in Tanner 4-5 and in 14.0-15.9 years. However, the significance was lost when we adjusted for BMI or BMISDS. In the other words, our adolescents in late puberty, in particular males, had higher ACTH and cortisol levels because they were more obese. A figure has been inserted, as suggested.

17) The authors should shortly discuss or comment on the probable interplay of CBG, total plasma cortisol, free (bioavailable) cortisol, cortisol metabolism/clearance and ACTH responses.

Response: Thank you very much for your suggestion. We have inserted a brief paragraph on CGB regulation in obesity and stress.

18) Overall discussion is quite long, in parts very speculative, and several conclusions are not clear. Only two (out of several more) examples:
- “The characteristics of this association suggest that ACTH could precede a decreased clearance of steroid hormones, another mechanism discussed as a player in the state of functional hypercortisolism of obesity [8].”
- “However, only ACTH increased the risk to be positive for altered glucose levels. This could be due, as previously discussed, to the fact that in the pediatric age a transient suppression of cortisol with higher ACTH is an adaptive response of obesity to prevent comorbidities, in particular type 2 diabetes [27].”

Thus, Discussion has to be rewritten and should focus on clearly described hypotheses and argumentations.

Response: We have rewritten and modified the discussion as suggested.