

# 1 Leukocyte Telomere Length in Children with Congenital Adrenal Hyperplasia

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1 **Abstract**

2 **Context** Exposure to chronic stress and hypercortisolism is associated with decreased leukocyte  
3 telomere length (LTL), a marker for biological aging and cardiovascular disease. Children with congenital  
4 adrenal hyperplasia (CAH) are treated with glucocorticoids.

5 **Objective** To investigate LTL in children with CAH.

6 **Design** Prospective observational cohort study. Patients were followed-up at two visits (mean 4.1±0.7  
7 months apart).

8 **Setting** Four academic Pediatric Endocrinology Outpatient Clinics.

9 **Patients** Children aged 0-18 years with genetically confirmed CAH.

10 **Main outcome measures** At each visit, LTL was determined by quantitative real-time PCR. All subjects  
11 underwent detailed clinical and endocrinologic evaluation and were classified as undertreated, optimally  
12 treated or overtreated, accordingly. The influence of clinical factors on LTL was investigated using linear  
13 mixed models adjusted for age, sex, and BMI-z.

14 **Results** We studied 76 patients, of whom 31 (41%) were girls, 63 (83%) had classic CAH, 67 (88%) received  
15 hydrocortisone and 8 (11%) prednisolone. Median age at first visit was 12.0 years (IQR 6.3–15.1), and median  
16 BMI-z was 0.51 (IQR -0.12–1.43). LTL was shorter in patients with classic compared to non-classic CAH (-0.29,  
17 P=0.012), in overtreated than in optimally treated patients (-0.07, P=0.002), and patients receiving  
18 prednisolone compared with hydrocortisone (-0.34, P<0.001). LTL was not associated with undertreatment or  
19 daily HC-equivalent dose (P>0.05).

20 **Conclusions** LTL is shorter in patients with classic than non-classic CAH, as well as those who are  
21 overtreated with hydrocortisone or treated with long-acting glucocorticoids. These findings may be  
22 attributed to chronic exposure to supraphysiologic glucocorticoid concentrations, and indicate that LTL  
23 may be used as a biomarker for monitoring glucocorticoid treatment.

## 1 Introduction

2 Telomeres are tandem repeats of a non-coding hexameric nucleotide sequence (5'-TTAGGG-3') located  
3 at the ends of human chromosomes. Together with their binding proteins, telomeres form complex  
4 structures that protect the chromosomes during cell division from nucleolytic degradation (1-3). The  
5 regulation of telomere length is a dynamic process involving the enzyme telomerase. Cell divisions,  
6 including normal aging, as well as various environmental factors, such as oxidative or inflammatory  
7 stress, lead to shortening of telomere length (4-6). Reduced leukocyte telomere length (LTL) in adults has  
8 been identified as a biomarker for accelerated biological aging and cardiovascular diseases, such as  
9 myocardial infarction, stroke, diabetes mellitus type 2, and obesity (7-9). Moreover, reduced LTL has  
10 been associated with chronic stress and hypercortisolism (10-12). This raises the question whether  
11 reduced LTL might be relevant for patients on life-long treatment with glucocorticoids (GC), such as  
12 patients with congenital adrenal hyperplasia (CAH).

13 CAH is an autosomal recessive disorder characterized by a genetic defect in adrenal steroid biosynthesis.  
14 This results in primary adrenal insufficiency accompanied by chronic stimulation of the adrenal cortex,  
15 which leads to increased production of adrenal androgens (13-15). Children with classic CAH due to 21-  
16 hydroxylase deficiency require life-long treatment with GC, most often hydrocortisone (HC), from the  
17 time of diagnosis onwards. Milder forms of the disease (non-classic, late-onset CAH), may not require  
18 long-term GC treatment. When inadequately treated, patients present with accelerated growth during  
19 childhood with advanced skeletal maturation, which results in reduced final height (16), signs of  
20 virilization and disturbances of the menstrual cycle in females (17), and testicular adrenal rest tumors  
21 (TART) in males, which may impair testicular development and male fertility (18). Current treatment of  
22 classic CAH aims to provide adequate glucocorticoid and, when necessary, mineralocorticoid substitution  
23 to prevent adrenal crises, and to suppress the excessive secretion of CRH and ACTH, thereby reducing  
24 circulating concentrations of adrenal androgens and steroid precursors prior to the enzymatic defect.

1 However, achieving and maintaining adrenal androgen suppression is far more challenging than  
2 preventing adrenal crises, and in a significant number of patients it has proven impossible to control  
3 hyperandrogenism without employing supraphysiologic doses of glucocorticoid. Overtreatment with  
4 glucocorticoids bears the risk of compromising growth in childhood and affects long-term bone health  
5 and metabolic health (13,19,20). Current treatment monitoring relies largely on specialists' experience,  
6 integrating clinical and laboratory parameters (20). Long-term outcomes of CAH show adverse effects on  
7 growth, pubertal development, development of polycystic ovary syndrome, obesity and metabolic  
8 syndrome (21,22), suggesting chronic overtreatment with glucocorticoids.

9 It is currently not known whether chronic glucocorticoid treatment and treatment quality  
10 (undertreatment, optimal treatment, or overtreatment) influences LTL in CAH. Therefore, we aim to  
11 investigate LTL in children with CAH and its association with treatment quality, CAH subtype, use of long-  
12 acting glucocorticoids, and daily glucocorticoid dose.

## 14 **Methods**

15 This prospective, observational cohort study was part of a larger study investigating novel potential  
16 treatment monitoring tools for children with CAH. The study was performed in accordance with the  
17 principles set out in the Declaration of Helsinki and was approved by the Institutional Review Boards of  
18 the four participating centers: 'Aghia Sophia' Children's Hospital, National and Kapodistrian University of  
19 Athens Medical School (Athens, Greece), University Hospital Inselspital (Bern, Switzerland), Sophia  
20 Children's Hospital, Erasmus Medical Center (Rotterdam, The Netherlands), and Willem-Alexander  
21 Children's Hospital, Leiden University Medical Centre (Leiden, The Netherlands). Written informed  
22 consent was provided by all parents/guardians according to national laws; in addition, children gave their  
23 assent.

## 1 *Patients*

2 Children aged 0-18 years with CAH were recruited from the Pediatric Endocrinology Outpatient Clinics of  
3 the participating centers. Inclusion criteria were: (1) genetically confirmed CAH due to 21-hydroxylase  
4 deficiency (21-OHD), 3-beta-hydroxysteroid dehydrogenase deficiency (3 $\beta$ -HSD) or 11-beta-hydroxylase  
5 deficiency (11 $\beta$ -HSD); (2) Tanner stage 1 or pubertal (Tanner stage  $\geq$ 2 or post menarche. Exclusion  
6 criteria were (1) intercurrent illnesses at or within one week before visit; (2) poor understanding of  
7 language, leading to insufficient ability to understand the written study information.

## 8 *Assessments*

9 All subjects underwent a detailed clinical and biochemical evaluation following a standardized protocol  
10 at two consecutive outpatient clinic visits (mean time:  $4.1 \pm 0.7$  months apart). At each visit, height and  
11 weight were measured rounded to the nearest decimal by trained outpatient clinic assistants. BMI was  
12 calculated and converted to z-scores using World Health Organization (WHO) reference charts (23).  
13 Pubertal status was assessed by a Pediatric Endocrinologist according to Tanner staging, with  
14 prepubertal defined as Tanner stage 1 and pubertal as Tanner stage of breast development  $\geq$ 2 for girls  
15 and testicular volume  $\geq$ 4 mL for boys. For all patients who received glucocorticoid treatment  
16 (hydrocortisone [HC], prednisolone, or both), daily HC-equivalent dose in mg/m<sup>2</sup>/day was calculated  
17 using a factor 4.2 to convert prednisolone to HC-equivalent. Biochemical evaluation included  
18 measurement of serum 17-hydroxyprogesterone (17OHP) and androstenedione (A4) concentrations  
19 according to local laboratory protocols. At the second visit, bone age (BA) was assessed by the treating  
20 Pediatric Endocrinologist using radiography of the left hand according to Greulich & Pyle, and a testicular  
21 ultrasound was performed in boys to rule out the presence of testicular adrenal rest tumors (TART). At  
22 both visits, the treating Pediatric Endocrinologist classified patients as being optimally treated,  
23 undertreated or overtreated by expert opinion based on the detailed clinical and hormonal evaluation  
24 following the current standard-of-care as set out by the most recent international guidelines (20). The

1 following parameters were evaluated during each study visit and taken into consideration by the treating  
2 Pediatric Endocrinologist's to assess treatment quality:

- 3 - Serum 17OHP. Target for optimal treatment: slightly above upper limit of reference interval of  
4 local laboratory (local reference intervals available from the corresponding author upon  
5 request.) Overtreatment considered in case of suppressed values or values within reference  
6 interval; undertreatment considered in case of values clearly above reference interval.
- 7 - Serum A4. Target for optimal treatment: within reference interval of local laboratory (local  
8 reference intervals available from the corresponding author upon request). Overtreatment  
9 considered in case of suppressed values; undertreatment considered in case of values above  
10 upper limit of reference interval.
- 11 - Daily HC-equivalent dose. Target for optimal treatment: 8-12 mg/m<sup>2</sup>/day.  
12 Overtreatment/undertreatment considered in case of daily HC-equivalent dose >12/<8  
13 mg/m<sup>2</sup>/day, respectively.
- 14 - Delta BA minus calendar age. Target for optimal treatment: delta value of 0.  
15 Overtreatment/undertreatment considered in case of delta <0/>0, respectively.
- 16 - Delta height SDS. Target for optimal treatment: delta value of 0. Overtreatment/undertreatment  
17 considered in case of delta <0/>0, respectively.
- 18 - Delta weight and BMI SDS. Target for optimal treatment: delta value of 0.  
19 Overtreatment/undertreatment considered in case of delta >0/<0, respectively.
- 20 - Use of stress dosing. Target for optimal treatment: no or sporadic use of stress dosing.  
21 Undertreatment considered in case of frequent and/or prolonged use of stress dosing.
- 22 - Absence of adrenal crises. Undertreatment considered in case of adrenal crisis.

1 - Absence of TART. Undertreatment considered in case of newly developed TART or progression  
2 of existing TART. As TARTs may persist despite current optimal treatment, persisting known  
3 TARTs were not taken into consideration of possible undertreatment.

4 In case of undertreatment or overtreatment, glucocorticoid treatment dose was adjusted. For  
5 patients with non-classic CAH, treatment duration in years was recorded; the patients with classic  
6 CAH were treated from birth onwards.

### 7 *LTL measurements*

8 LTL was determined at both consecutive outpatient clinic visits. Genomic DNA was extracted from  
9 human peripheral blood samples using QIAamp DNA Mini Kit (Qiagen, Prismet, Aarhus, Denmark)  
10 according to the instructions of the manufacturer. Telomere Length (TL) was measured using Multiplex  
11 Monochrome Quantitative Real-Time PCR (MMQPCR), as previously described (24, 25). Quantitative real-  
12 time PCR reactions were carried out in triplicates in a total volume of 12  $\mu$ L using 20-60 ng of template  
13 DNA, with final concentrations of 1 $\times$  Kapa SYBR<sup>®</sup> FAST qPCR Master Mix (Kapa Biosystems, Wilmington,  
14 MA, USA), 1 $\times$  ROX Low Reference Dye, 900 nm of telg and telc primers, and 900 nm of single-copy gene  
15 (albd and albu) primers (24). All PCR reactions were carried out in clear 96-well plates on a QuantStudio™  
16 5 Real-Time PCR System (Applied Biosystems LLC, South San Francisco, CA, USA). Five serial dilutions of a  
17 reference sample spanning 6.25-100ng were run in duplicate on each plate. The data were analyzed  
18 using the QuantStudio™ 5 Design and Analysis Software v1.5.1 (Applied Biosystems). Two standard  
19 curves for each plate were generated from the reference sample DNA dilutions, one for the telomere  
20 amplicon signal (T) and one for the single-copy gene (albumin) amplicon signal (S). Ratios of telomere to  
21 single-copy gene (T/S) signals were calculated for each sample and this ratio was proportional to the  
22 average telomere length of each sample. Moreover, the two DNA samples of each patient (at study visit  
23 1 and after on average 4 months at study visit 2) were run in the same plate.



1 The thermal cycling profile was Stage 1: 15 min at 95°C; Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C; and  
2 Stage 3: 36 cycles of 15 s at 94°C, 10 s at 62°C, 15 s at 74°C with signal acquisition, 10 s at 84°C, 15 s at  
3 88°C with signal acquisition. The 74°C reads provided the Ct values for the amplification of the telomere  
4 template (in early cycles when the single-copy gene signal is still at baseline); the 88°C reads provided  
5 the Ct values for the amplification of the single-copy gene template (at this temperature there is no  
6 signal from the telomere PCR product, because it is fully melted).

7 The primer sequences (ThermoFisher Scientific, Invitrogen) were as follows:

8 telg AACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT

9 telc TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA

10 albu CGGCGGCGGGCGGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG

11 albd GCCCGGCCCGCCGCGCCCGTCCCGCCGAAAAGCATGGTCGCCTGTT

12 In 26 samples from 22 patients, LTL could not be measured as there was not enough DNA available, e.g.  
13 due to the child's distress during venipuncture. Patients for whom DNA was not available from both  
14 study visits (n=5) were excluded from further analyses (Figure 1).

### 15 *Statistical analyses*

16 The distribution of continuous data was evaluated by visual inspection of histograms and the Shapiro-  
17 Wilk test; data are presented as median (interquartile range, IQR) or mean (standard deviation, SD)  
18 accordingly. Patients with CAH due to 21-OHD were further subdivided into patients with classic CAH  
19 (salt-wasting or simple virilizing) and patients with non-classic CAH. Differences in clinical and treatment  
20 characteristics between patients with classic and non-classic CAH were compared using Mann-Whitney  
21 tests for continuous variables and  $\chi^2$ -tests for categorical variables. With regard to LTL, we excluded  
22 patients with outlying values of  $\Delta$ LTL based on a median absolute deviation (MAD)  $\geq \pm 3$  from further

1 analysis (26) if the difference between LTL at the first vs. second visit could not be accounted for by  
2 changes in BMI z-score and age (24). The influence of clinical characteristics (puberty, CAH subtype) and  
3 treatment characteristics (HC vs. prednisolone treatment, daily HC-equivalent dose, treatment quality,  
4 and years of treatment for patients with non-classic CAH) on LTL were assessed using linear mixed-  
5 models analyses with robust standard errors. All models included random effects for unique patients and  
6 visits. Additionally, all multivariate analyses included fixed effects for age, sex, and BMI z-score; for each  
7 additional variable, we ran a separate model to prevent overfitting. All statistical analyses were  
8 performed in SPSS version 25 (IBM Corp., Armonk, NY) or STATA version 16 (Statacorp., College Station,  
9 TX) with a two-sided  $\alpha$  of 0.05. We used GraphPad Prism version 8.0.0 (GraphPad Software, San Diego,  
10 CA) for creating bar charts and RStudio (Version 1.1.383, Boston, Massachusetts) for visualizing the  
11 coefficients from the mixed model regressions.

12

### 13 **Results**

14 We included 81 patients with CAH, of whom 78 (96%) patients had CAH due to 21-OHD, 2 (3%) patients  
15 had CAH owing to 11 $\beta$ -HSD and 1 (1%) patient had CAH due to 3 $\beta$ -HSD (Figure 1). The results section will  
16 focus on the patients with CAH due to 21-OHD; the 3 patients with non-21-OHD are presented separately  
17 at the end of the results section. Two out of the 78 patients with CAH due to 21-OHD were excluded  
18 from further analyses due to outlying values in  $\Delta$ LTL between the two visits (absolute  $\Delta$ LTL of 0.199 and  
19 0.459, respectively) that could not be explained by changes in age or BMI z-score. Follow-up was  
20 complete for all patients except for one with classic CAH who was lost to follow-up between the first and  
21 second visit.

#### 22 *Clinical characteristics*

1 Of the 76 children with CAH due to 21-OHD who were included in the analyses, 31 (41%) were girls, 63  
2 (83%) had classic CAH and 13 (17%) had non-classic CAH (Table 1). At the first visit, median age was 12.0  
3 years (IQR 6.3–15.1) and median BMI z-score 0.51 (IQR -0.12–1.43); 30/76 (40%) patients were  
4 prepubertal, of whom 28/63 (44%) patients with classic CAH and 2/13 (15%) patients with non-classic  
5 CAH (P=0.051). At the second visit, median age was 12.3 years (IQR 6.6–15.4) and median BMI z-score  
6 was 0.59 (IQR -0.04–1.44); 28/75 (37%) patients were prepubertal, of whom 27/62 (44%) patients with  
7 classic CAH and 1/13 (8%) with non-classic CAH (P=0.024). Ultrasound testing for TART was performed in  
8 32/44 (73%) boys and 9/31 (29%) girls at the second visit and yielded abnormal findings in 6 patients:  
9 known TARTs in 2 boys, newly developed hypo-echogenic lesions suggestive of progression of TARTs in 2  
10 boys, varicocele in one boy and hydrometrocolpos in one girl. None of the patients with abnormal  
11 ultrasound findings were considered undertreated by the Pediatric Endocrinologist at the time of  
12 ultrasound testing.

### 13 *Treatment characteristics*

14 One child with non-classic CAH did not receive glucocorticoid treatment during the study. Of the  
15 remaining 75 patients, 67 (89%) received hydrocortisone (HC) treatment, 4 (5%) patients received  
16 prednisolone and 4 (5%) combination of prednisolone and hydrocortisone (Table 2). The median HC-  
17 equivalent dose at the first visit was 12.7 mg/m<sup>2</sup>/day (IQR 10.0–14.6) and did not differ between children  
18 with classic and non-classic CAH (P=0.891). Fifty-five out of 63 (87%) children with classic CAH  
19 additionally were treated with fludrocortisone. At the first visit, optimal GC treatment was achieved in  
20 46/76 (61%) patients, while 22/76 (29%) patients were undertreated and 7/76 (9%) patients were  
21 overtreated. At the second visit, optimal GC treatment was achieved in 41/75 (55%) patients, while  
22 31/75 (42%) patients were undertreated, and 2/75 (3%) patients were overtreated. The treating  
23 Pediatric Endocrinologists' rationale for considering patients as overtreated or undertreated are  
24 presented in Table 3.

### 1 *LTL in children with CAH due to 21-OHD*

2 Leukocyte telomere length was available for 60 patients at both visits, for 8 patients at the first visit only  
3 and for 9 patients at the second visit only. Median LTL was 1.18 (IQR 1.04–1.39) at the first visit and 1.21  
4 (IQR 1.06–1.39) at the second visit. Mean  $\Delta$ LTL was  $0.017 \pm 0.083$ . The results of the linear mixed-model  
5 analyses are presented in Table 4. In univariate analyses, LTL in children with CAH was associated with  
6 age (coefficient -0.015, 95% CI -0.0256 to -0.004,  $P=0.009$ ), but not with sex and BMI z-score (both  
7  $P>0.05$ , Table 4). In multivariate analyses, adjusting for age, sex, and BMI z-score, children with classic  
8 CAH had shorter LTL than children with non-classic CAH (coefficient -0.253, 95% CI -0.474 to -0.034,  
9  $P=0.024$ ). Children using prednisolone had shorter LTL than children using hydrocortisone (coefficient -  
10 0.304, 95% CI -0.469 to -0.140,  $P<0.001$ ) (Figures 2 and 3). Treatment quality influenced LTL (global  
11  $P=0.008$ ). When compared to children on optimal treatment, overtreated children had shorter LTL  
12 (coefficient -0.072, 95% CI -0.1117 to -0.032,  $P=0.002$ ), while undertreated children had similar LTL  
13 (coefficient -0.006, 95% CI -0.044 to 0.032,  $P=0.756$ ) (Figures 2 and 3). Daily HC-equivalent dose was not  
14 associated with LTL (Table 4). In patients with non-classic CAH, treatment duration was not associated  
15 with LTL (coefficient 0.044, 95% CI -0.055 to 0.142,  $P=0.383$ ).

### 16 *LTL in children with CAH due 11 $\beta$ -HSD and 3 $\beta$ -HSD*

17 LTL was available at both visits for 1 female patient with 3 $\beta$ -HSD aged 7.2 years at the first visit, and  
18 available at one visit in two male patients with 11 $\beta$ -HSD aged 15.0 and 17.6 years. Their LTL ranged from  
19 0.87–1.51 and were in the similar range of values as the patients with CAH due to 21-OHD.

## 20 **Discussion**

21 In this cohort of children and adolescents with CAH, LTL was shorter in patients with classic CAH  
22 compared to patients with non-classic CAH, in overtreated patients, and in patients treated with  
23 prednisolone, a long-acting glucocorticoid. These findings may be attributed to chronic exposure to

1 supraphysiologic glucocorticoid concentrations and indicate that LTL may play a potential role as an  
2 additional biomarker for monitoring long-term glucocorticoid therapy in patients with CAH.

3 Telomere length in childhood is influenced by a large number of biologic and environmental factors, such  
4 as ethnicity, weight status, physical activity, chronic stress and traumatic events (1). Over the past  
5 decade, several studies have linked hypercortisolism to reduced telomere length. *In vitro* studies have  
6 shown that hydrocortisone exposure reduces telomerase activity in human T lymphocytes (27), whereas  
7 treatment with a GC receptor antagonist resulted in longer telomeres in human mesenchymal stem cells  
8 (28). In rodents, telomere length in lymphocytes, as well as hippocampal cells, is reduced after exposure  
9 to chronic stress or prolonged administration of corticosterone (11). In healthy human subjects,  
10 telomere length has recently been shown to correlate negatively with salivary bedtime cortisol  
11 concentrations (11). However, studies investigating LTL in humans with adrenal insufficiency on GC  
12 therapy are rare. Only one study reports LTL in a subgroup of 52 patients with non-functioning pituitary  
13 adenomas with adrenal insufficiency for which HC treatment was given. This study found a shorter LTL in  
14 patients with daily HC dose >20mg versus <20mg, but the association between daily HC dose on a  
15 continuous scale and telomere length did not reach statistical significance (29). Similarly, daily HC dose  
16 was not associated with LTL in our current study. This might reflect interindividual differences in  
17 pharmacokinetics influencing hydrocortisone uptake, metabolism and clearance (30,31), however, it  
18 might also reflect the relatively small sample size of both studies.

19 Our findings in patients with CAH on chronic GC treatment indicate that LTL is negatively associated with  
20 prolonged exposure to supraphysiologic GC concentrations, and concur with the previous studies. It is  
21 likely that our findings will be replicated in other cohorts treated with exogenous glucocorticoids, but  
22 these studies have yet to be published. Interestingly, we found the largest effect size with regard to LTL  
23 shortening in patients treated with prednisolone compared to hydrocortisone. Prednisolone is a more  
24 potent and longer-acting glucocorticoid than hydrocortisone. Its prolonged use in children with CAH is

1 associated with less favorable metabolic profile and growth outcomes. Given that reduced LTL is also  
2 associated with obesity and cardiovascular disease (7-9), our findings reinforce the importance of  
3 avoiding prolonged use of prednisolone in children with CAH, as suggested in the current international  
4 guidelines (20). We did not find a direct association between undertreatment and reduced LTL. This  
5 might indicate that chronically elevated androgen concentrations *per se* might not be associated with  
6 accelerated shortening of telomeres. This finding concurs with a recently published meta-analysis  
7 showing no difference in LTL between women with polycystic ovary syndrome (PCOS), a disease  
8 characterized by androgen excess, vs. women without PCOS, and the absence of a relation between  
9 serum dehydroepiandrosterone sulfate (DHEAS) within women with PCOS. *In vitro* and epidemiologic  
10 studies show that androgens might upregulate telomerase expression and/or activity and enhance  
11 telomere length through aromatization to estradiol. However, further research studies are needed to  
12 elucidate the relation between the chronic androgen burden in CAH due to 21-hydroxylase deficiency  
13 and changes in LTL.

14 LTL was not associated with BMI z-score in children with CAH. The negative association between LTL and  
15 BMI has been established in adults (7,9,32), and has also been described in some studies investigating  
16 children (24,33). However, other studies in children did not find a significant association (32), in line with  
17 our results, or only an association with waist circumference (34), a measure of central adiposity. The  
18 absence of an association of LTL with BMI z-score in our patients with CAH may be attributed to the  
19 strong association of LTL with overtreatment with glucocorticoids, which itself is associated with  
20 increased BMI and especially central adiposity (19,21).

21 It is important to emphasize that LTL is a dynamic biomarker, which is reflected by the positive mean  
22  $\Delta$ LTL value in patients with two measurements of LTL in our current study. As an example, it has been  
23 shown that a comprehensive, multidisciplinary lifestyle intervention is associated with increased LTL in  
24 children and adolescents with overweight and obesity (25). Similarly, glucocorticoid exposure is

1 dynamically associated with LTL. A case-control study comparing adults with Cushing syndrome, a  
2 disease characterized by endogenous hypercortisolism, with healthy controls matched for sex, age and  
3 smoking status, unexpectedly found no differences in telomere length between patients with Cushing  
4 syndrome and controls in cross-sectional analyses (10). However, in longitudinal analyses in a subgroup  
5 of 15 patients, patients with active Cushing syndrome had shorter LTL, which became longer after  
6 effective treatment of their hypercortisolism. These results were confirmed by another recent study  
7 reporting a lower telomere length in adults with active Cushing syndrome than healthy controls, but  
8 similar telomere length in cured Cushing syndrome (11). It is hypothesized that the shift towards  
9 accelerated shortening of LTL due to prolonged exposure to supraphysiologic GC concentrations can be  
10 shifted back to the steady-state level when GC exposure returns to physiologic concentrations (11).  
11 Therefore, LTL might have merit in monitoring long-term exposure to supraphysiologic GC  
12 concentrations as in the treatment of patients with CAH.

13 Although treatment of CAH has been available for decades, optimal treatment and its assessment  
14 remains challenging in daily clinical practice, where the fine balance between overtreatment and  
15 undertreatment is continuously sought. In spite of existing treatment guidelines (20), monitoring  
16 remains unsatisfactory and relies largely on the specialist's experience, integrating clinical and single  
17 laboratory parameters that are known to reflect a momentary status and depend on the timing of  
18 sample collection and its relation to the time of day and drug intake. The inability to replicate physiologic  
19 cortisol concentrations with administration of hydrocortisone, which is the preferred glucocorticoid  
20 during childhood and adolescence, is primarily due to the pharmacokinetic properties of this medication.  
21 More specifically, hydrocortisone tablets have almost complete bioavailability, which leads to  
22 supraphysiologic cortisol concentrations within 1-2 hours after administration, but very short half-life, so  
23 cortisol concentrations decline monoexponentially and become undetectable 6 hours later (35). This is  
24 more evident in females, given that they have significantly shorter half-life of cortisol than males (35,36).

1 Therefore, patients are often at risk for developing in tandem hypercortisolism or hyperandrogenism  
2 (13). Indeed, studies investigating cortisol, 17OHP and A4 in hair, a matrix that reflects long-term  
3 exposure to these hormones, i.e. over a period of months, found simultaneously increased hair cortisol  
4 and hair androgen concentrations in children and adults with CAH (37,38). Moreover, chronic  
5 overtreatment of patients with CAH is related to the occurrence of metabolic morbidity from an early  
6 age onwards (39). As a consequence, identification of novel (bio)markers for better monitoring of CAH  
7 over the long-term is warranted. It is unlikely that LTL assessment as a single parameter will have merit  
8 in the treatment monitoring of CAH, while further studies are needed to investigate whether LTL could  
9 be an additional useful biomarker in association with the current routine clinical and biochemical  
10 parameters in the long-term treatment monitoring of CAH.

11 Strengths of our study include the standardized protocol for clinical evaluation at each study center,  
12 thereby minimizing treatment differences between centers and treating Pediatric Endocrinologists.  
13 Furthermore, we used a comprehensive treatment monitoring protocol, including extensive  
14 endocrinologic, radiographic and ultrasound evaluation, and measured LTL twice, which enabled us to  
15 investigate LTL and treatment quality dynamics. Our study also has several limitations. These include the  
16 small sample size of the subgroups of overtreated patients and patients treated with prednisolone,  
17 which might have led to imprecise estimates in the linear mixed models. We nevertheless found  
18 statistically significant shorter LTL in these patients, in line with previous studies investigating  
19 hypercortisolism and LTL. Furthermore, we did not measure telomere length directly, but measured  
20 relative telomere length in leukocytes using quantitative PCR, which is a widely used and validated  
21 method (40-42). Finally, our study was not designed to include a cohort of healthy control patients for  
22 comparison of absolute LTL values. The findings of the current study (median LTL of 1.18 at median age  
23 12 years) match with those of a population-based cohort of healthy children in Greece in whom we  
24 previously measured LTL using the same methods (mean LTL of 1.13 in children with obesity up to a



1 mean LTL of 1.33 in children with normal BMI; both groups at mean age 11 years) (43). However, direct  
2 comparison of absolute telomere length of children with CAH compared to healthy children should be  
3 further investigated.

4 In conclusion, in our study we determined the LTL in children with CAH and demonstrated that LTL is  
5 shorter in patients with classic CAH compared with patients with non-classic CAH, in overtreated patients  
6 compared to optimally treated patients, and in patients using prednisolone versus patients using  
7 hydrocortisone. Altogether, these findings indicate that LTL in children with CAH reflects chronic  
8 exposure to supraphysiologic glucocorticoid concentrations. Therefore, LTL might play an important role  
9 as an add-on to current long-term treatment monitoring tools in patients with CAH.

10

#### 11 **Acknowledgements**

12 The authors would like to thank Ulrike Halbsguth and Sara Metzger for their help with the data  
13 collection.

14

#### 15 **Data availability**

16 The datasets generated during and/or analyzed during the current study are not publicly available but  
17 are available from the corresponding author on reasonable request.

18

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- 37  
38

## 1 **Legends for Figures and Tables**

2 **Figure 1.** Study flow chart.

3 Abbreviations: LTL, leukocyte telomere length; 11 $\beta$ -HSD; 11-beta-hydroxylase deficiency; 3 $\beta$ -HSD; 3-beta-  
4 hydroxysteroid dehydrogenase deficiency.

5  
6 **Figure 2.** Scatter/bar plot showing the distribution of LTL stratified on patient and treatment  
7 characteristics. The dots represent the individual patients. The bars represent the median + interquartile  
8 range. The asterisks marks statistically significant differences in the multivariate linear mixed models  
9 analyses ( $P < 0.05$ ). For patients in whom LTL was available at both study visits, their median LTL is  
10 depicted in the bar charts of sex, pubertal status, CAH subtype and medication. Abbreviations: CAH,  
11 congenital adrenal hyperplasia.

12  
13 **Figure 3:** Coefficient plot showing the association between clinical and treatment characteristics with LTL in  
14 patients with CAH due to 21-OHD. This figure shows the results of the linear mixed models analyses adjusted  
15 for unique patients, visits, age, sex, and BMI z-score. The white diamonds indicate the reference categories.  
16 The asterisks marks statistically significant associations ( $P < 0.05$ ).  
17 Abbreviations: LTL, leukocyte telomere length; CAH, congenital adrenal hyperplasia; 21-OHD, 21-hydroxylase  
18 deficiency; HC, hydrocortisone; CI, confidence interval.

19  
20 **Table 1.** Characteristics of the study population

21 All values are median (IQR) or count (%). Abbreviations: CAH, congenital adrenal hyperplasia; 21-OHD,  
22 21-hydroxylase deficiency; IQR, interquartile range.

23

1 **Table 2.** Treatment characteristics of the 75 CAH patients receiving glucocorticoid treatment during the  
2 study.

3 \*Data available for n = 74 patients (one patient with classic CAH lost to follow-up between first and  
4 second visit). All values are median (IQR) or count (%). Abbreviations: CAH, congenital adrenal  
5 hyperplasia; 21-OHD, 21-hydroxylase deficiency; IQR, interquartile range.

6  
7 **Table 3. Rationale for adapting glucocorticoid dose in children with CAH during the study.**

8 All displayed values are count (percentage). NB: as more than one reason to adapt dose could be  
9 present, the numbers in the columns add up to a higher number than the number of visits.

10 <sup>a</sup> Other reasons taken into consideration for evaluating the patient as being undertreated included: poor  
11 compliance; refusal of parents to increase the dose; acne; advanced bone age; hyperpigmentation and  
12 relative weight loss; too late timing of morning dose; vomiting; start of oral contraceptives.

13 <sup>b</sup> Other reasons taken into consideration for evaluating the patient as being overtreated included:  
14 parents having increased dose in-between visits on their own initiative; increased hydrocortisone dose  
15 (in patient treated concomitantly with prednisolone).

16 Abbreviations: CAH, congenital adrenal hyperplasia; HC, hydrocortisone; SDS, standard deviation score.

17 **Table 4.** Outcomes of linear mixed models analyses for LTL as dependent variable

18 \*Adjusted for age, sex, BMI z-score. The coefficients for age, sex, and BMI z-score are derived from a  
19 multivariate model including these 3 parameters as fixed effects and unique patients and visits as  
20 random effects. For each of the other listed variables, a separate multivariate model was run to prevent  
21 overfitting. Abbreviations: CI, confidence interval; CAH, congenital adrenal hyperplasia; HC,  
22 hydrocortisone; LTL, leukocyte telomere length.

## 1 Tables

## 2 Table 1. Characteristics of the study population

	All 21-OHD CAH patients (n=76)	Classic CAH patients (n=63)	Non-classic CAH patients (n=13)	P-value
Sex, female	31 (41)	25 (40)	6 (46)	0.666
Age at first visit in years	12.0 (6.3; 15.1)	11.1 (5.9; 15.1)	13.8 (11.8; 15.3)	0.045
BMI z-score at first visit	0.51 (-0.12; +1.46)	0.50 (-0.17; +1.42)	0.65 (0.03; +1.84)	0.530
Pubertal status at first visit				0.051
Prepubertal	30 (40)	28 (44)	2 (15)	
Pubertal	46 (60)	35 (56)	11 (85)	
All values are median (IQR) or count (%). Abbreviations: CAH, congenital adrenal hyperplasia; 21-OHD, 21-hydroxylase deficiency; IQR, interquartile range.				

3

## 4 Table 2. Treatment characteristics of the 75 patients with CAH receiving glucocorticoid treatment

## 5 during the study

	All patients 21-OHD CAH patients (n=75)	Classic CAH patients (n=63)	Non-classic CAH patients (n=12)	P-value
First visit				
Medication - glucocorticoids				1.000
Hydrocortisone only	67 (89)	56 (89)	11 (92)	
Prednisolone	8 (11)	7 (11)	1 (8)	
Daily HC-equivalent dose, mg/m <sup>2</sup>	12.7 (10.0; 14.6)	12.7 (9.9; 14.9)	13.3 (10.1; 14.0)	0.891
Treatment quality				0.198
Undertreatment	22 (29)	20 (32)	2 (17)	
Optimally treated	46 (61)	36 (57)	10 (83)	
Overtreatment	7 (9)	7 (11)	0 (0)	
	All patients 21-OHD CAH patients (n=74)	Classic CAH patients (n=62)	Non-classic CAH patients (n=12)	P-value
Second visit*				
Medication - glucocorticoids				1.000
Hydrocortisone only	66 (89)	55 (89)	11 (92)	
Prednisolone	8 (11)	7 (11)	1 (8)	
Daily HC-equivalent dose, mg/m <sup>2</sup>	12.5 (10.2; 15.4)	12.4 (10.2; 15.4)	13.2 (10.0; 15.3)	0.982
Treatment quality				0.377
Undertreatment	31 (42)	27 (44)	4 (33)	
Optimally treated	41 (55)	34 (55)	7 (58)	
Overtreatment	2 (3)	1 (2)	1 (8)	
*Data available for n = 74 patients (one patient with classic CAH lost to follow-up between first and second visit). All values are median (IQR) or count (%). Abbreviations: CAH, congenital adrenal hyperplasia; 21-OHD, 21-hydroxylase deficiency; IQR, interquartile range.				

1 **Table 3. Rationale for adapting glucocorticoid dose in children with CAH during the study.**

<b>Feature</b>	<b>Undertreatment (n=50 visits)</b>	<b>Overtreatment (n=9 visits)</b>
<b>HC-equivalent glucocorticoid dose</b> <i>Target: 8 – 12 mg/m<sup>2</sup>/day</i>	16 (32%)	2 (22%)
<b>Weight change</b> <i>Target: <math>\Delta</math>Weight/BMI SDS of 0</i>	10 (20%)	2 (22%)
<b>Lab results</b> <i>Target: 17OHP slightly above upper limit of reference interval; A4 within reference interval</i>	34 (68%)	6 (67%)
<b>Growth change</b> <i>Target: <math>\Delta</math>Height SDS of 0</i>	10 (20%)	0
<b>Other reasons</b>	10 (20%) <sup>a</sup>	2 (22%) <sup>b</sup>
<p>All displayed values are count (percentage). NB: as more than one reason to adapt dose could be present, the numbers in the columns add up to a higher number than the number of visits.</p> <p><sup>a</sup> Other reasons taken into consideration for evaluating the patient as being undertreated included: poor compliance; refusal of parents to increase the dose; acne; advanced bone age; hyperpigmentation and relative weight loss; too late timing of morning dose; vomiting; start of oral contraceptives.</p> <p><sup>b</sup> Other reasons taken into consideration for evaluating the patient as being overtreated included: parents having increased dose in-between visits on their own initiative; increased hydrocortisone dose (in patient treated concomitantly with prednisolone).</p> <p>Abbreviations: CAH, congenital adrenal hyperplasia; HC, hydrocortisone; SDS, standard deviation score.</p>		

2

3



1 **Table 4. Outcomes of linear mixed models analyses for LTL as dependent variable**

	Univariate analyses			Multivariate analyses*		
	Coefficient	95% CI	P-value	Coefficient	95% CI	P-value
<b>Age in years</b>	-0.0146	-0.0256; -0.0036	0.009	-0.0145	-0.0255; -0.0035	0.010
<b>Sex, female vs male</b>	0.0479	-0.1032; 0.1990	0.534	0.0491	-0.0956; 0.1938	0.506
<b>BMI z-score</b>	0.0051	-0.0461; 0.0562	0.846	0.0046	-0.0450; 0.0542	0.855
<b>Puberty, pubertal vs pre-pubertal</b>	-0.0732	-0.1730; 0.0266	0.151	-0.0002	-0.0958; 0.0954	0.996
<b>CAH subtype, classic vs non-classic</b>	-0.1834	-0.4094; 0.0426	0.112	-0.2526	-0.4735; -0.0336	0.024
<b>Medication, prednisolone vs HC</b>	-0.3305	-0.4564; -0.2045	<0.001	-0.3042	-0.4685; -0.1399	<0.001
<b>Daily HC-equivalent dose, mg/m<sup>2</sup></b>	-0.0012	-0.0063; 0.0039	0.642	0.0014	-0.0041; 0.0070	0.607
<b>Treatment quality</b>			0.025			0.008
<b>Undertreatment vs optimally treated</b>	-0.0054	-0.0419; 0.0311	0.772	-0.0060	-0.0436; 0.0317	0.756
<b>Overtreatment vs optimally treated</b>	-0.0627	-0.1079; -0.0175	0.007	-0.0719	-0.1172; -0.0267	0.002
*Adjusted for age, sex, BMI z-score. The coefficients for age, sex, and BMI z-score are derived from a multivariate model including these 3 parameters as fixed effects and unique patients and visits as random effects. For each of the other listed variables, a separate multivariate model was run to prevent overfitting. Abbreviations: CI, confidence interval; CAH, congenital adrenal hyperplasia; HC, hydrocortisone; LTL, leukocyte telomere length						

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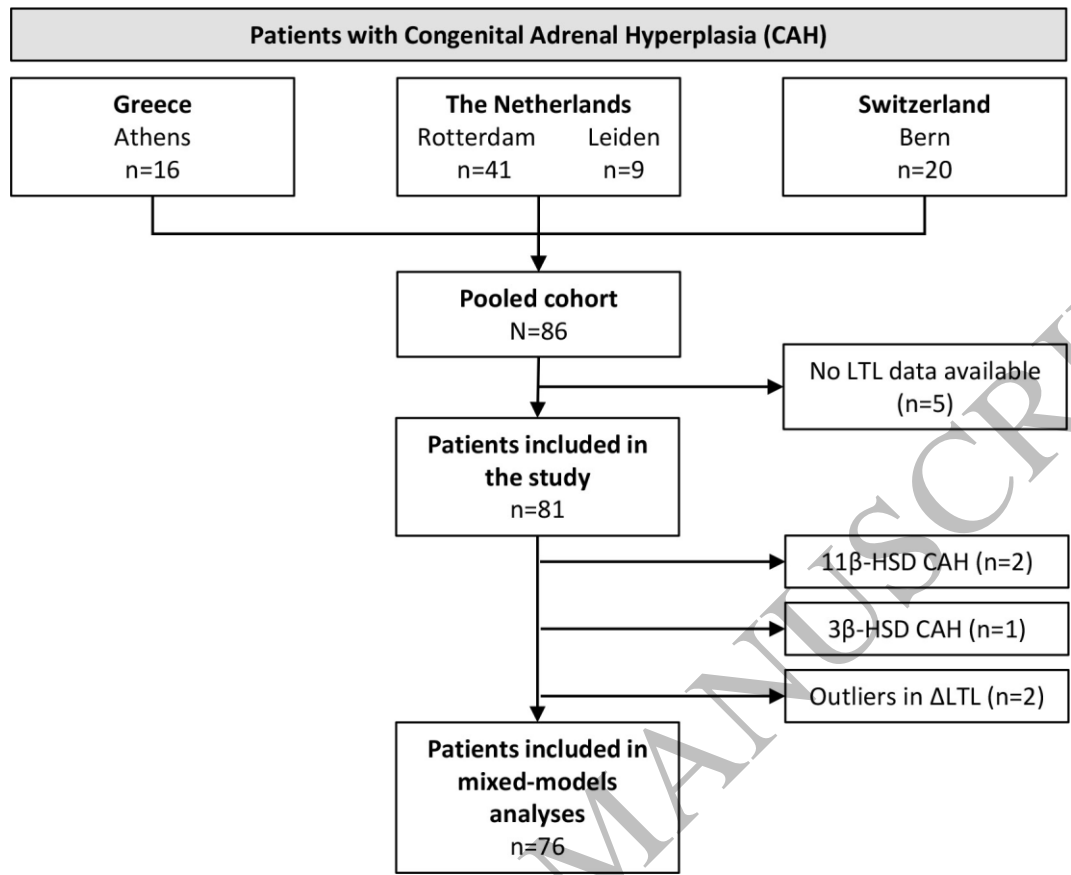


Figure 1  
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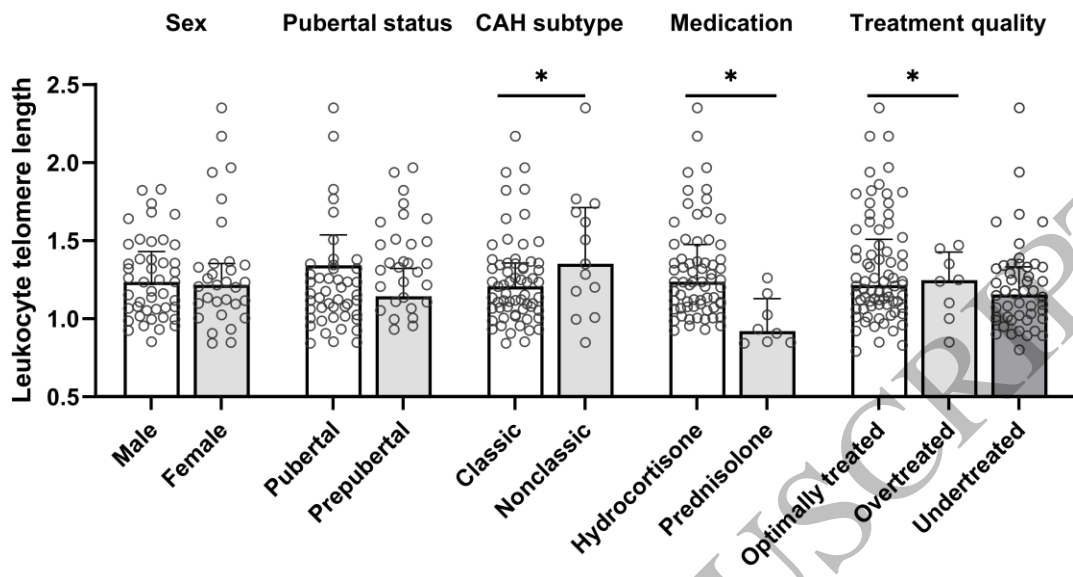
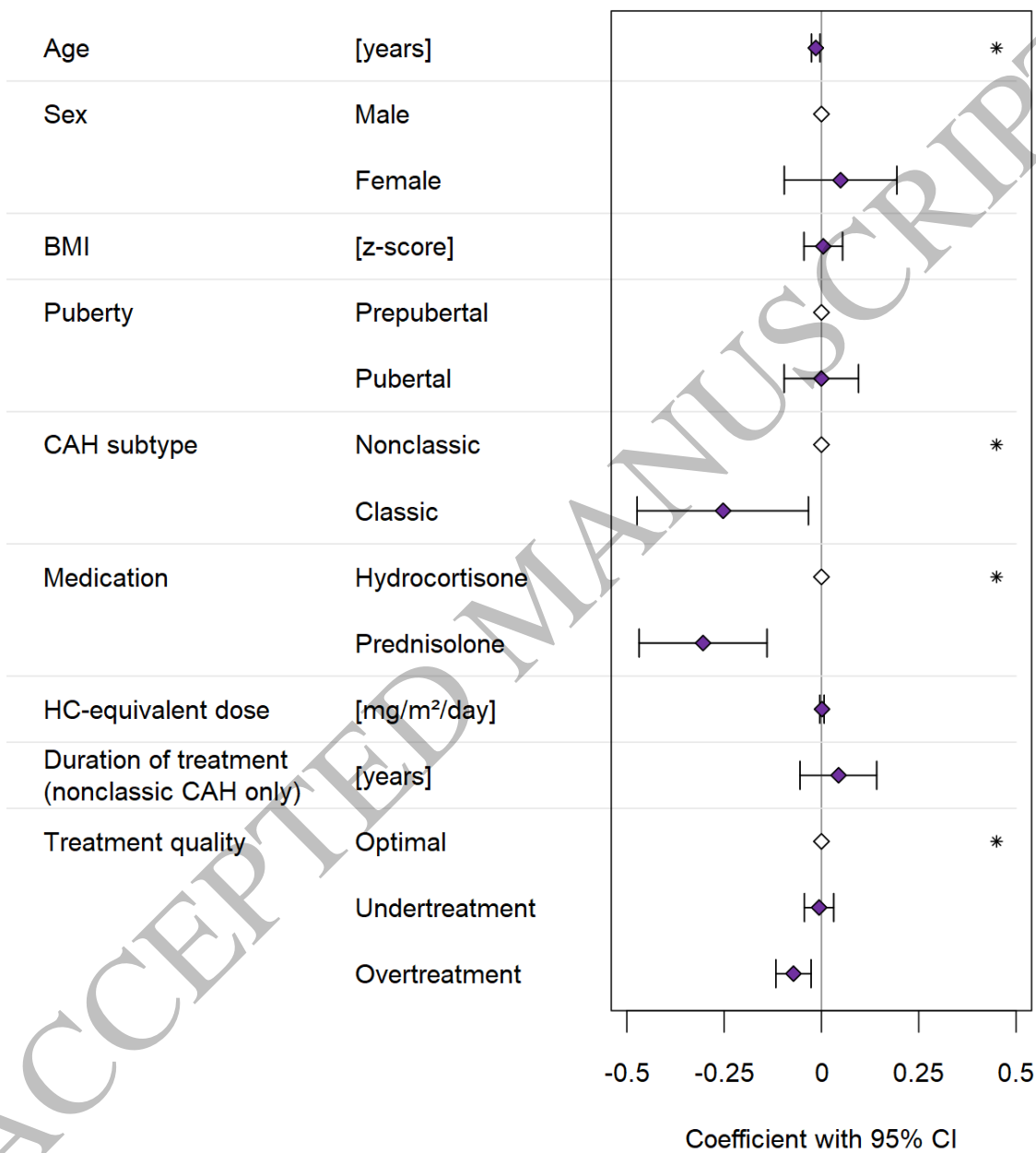


Figure 2  
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