Leukocyte Telomere Length in Children with Congenital Adrenal Hyperplasia

Christina Raftopoulou*[1,2], Ozair Abawi*[3], Grit Sommer[4], Maria Binou[1,2], George Paltoglou[1,2], Christa E. Flück[4], Erica L.T. van den Akker[3], E. Charmandari[1,2]

* shared authorship

[1] *Division of Endocrinology, Metabolism and Diabetes, First Department of Pediatrics, National and Kapodistrian University of Athens Medical School, 'Aghia Sophia' Children's Hospital, Athens, Greece*

[2] *Division of Endocrinology and Metabolism, Center for Clinical, Experimental Surgery and Translational Research, Biomedical Research Foundation of the Academy of Athens, Athens, Greece*

[3] *Division of Endocrinology, Department of Pediatrics, Erasmus MC-Sophia Children's Hospital, University Medical Center Rotterdam, Rotterdam, The Netherlands*

[4] *Pediatric Endocrinology, Diabetology and Metabolism, Department of Pediatrics, Bern and Department of BioMedical Research, University Hospital Inselspital, University of Bern, Bern, Switzerland*

Short title: Telomere length in children with CAH

Key words: Congenital adrenal hyperplasia, telomeres, LTL, 21-hydroxylase, adolescents, glucocorticoid

Corresponding author:

Evangelia Charmandari, MD, MSc, PhD, MRCP(UK), CCT(UK)

Professor of Pediatric and Adolescent Endocrinology

Division of Endocrinology, Metabolism and Diabetes, First Department of Pediatrics,

National and Kapodistrian University of Athens Medical School, "Aghia Sophia" Children's Hospital

Thivon and Papadiamantopoulou Street, Athens, 11527, Greece

&

Division of Endocrinology and Metabolism,

Center for Clinical, Experimental Surgery and Translational Research,

Biomedical Research Foundation of the Academy of Athens

4 Soranou tou Efessiou Street, Athens, 11527, Greece

Tel/Fax: +30-213 2013384; Mobile: +30-6947-904369

Email: evangelia.charmandari@googlemail.com

ORCHID ID: https://orcid.org/0000-0002-0851-6998

Reprints should be addressed to:

Evangelia Charmandari, MD, MSc, PhD, MRCP(UK), CCT(UK)

Professor of Pediatric and Adolescent Endocrinology

Division of Endocrinology, Metabolism and Diabetes, First Department of Pediatrics, National and

Kapodistrian University of Athens Medical School, "Aghia Sophia" Children's Hospital

Thivon and Papadiamantopoulou Street, Athens, 11527, Greece

Grants or fellowships supporting the writing of the paper: This study was funded by a European Society for Pediatric Endocrinology (ESPE) research unit grant and the Elisabeth Foundation, a Dutch non-profit foundation supporting academic research.

Disclosure summary: The authors have nothing to disclose.

Abstract

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

Objective To investigate LTL in children with CAH.

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

Setting Four academic Pediatric Endocrinology Outpatient Clinics.

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

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

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

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

Introduction

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

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

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

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

Methods

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

Patients

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

Assessments

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

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

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

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

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

LTL measurements

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

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 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 88°C with signal acquisition. The 74°C reads provided the Ct values for the amplification of the telomere template (in early cycles when the single-copy gene signal is still at baseline); the 88°C reads provided the Ct values for the amplification of the single-copy gene template (at this temperature there is no signal from the telomere PCR product, because it is fully melted).

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

telg ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT

telc TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA

albu CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG

albd GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT

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

Statistical analyses

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

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

Results

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

Clinical characteristics

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

Treatment characteristics

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

LTL in children with CAH due to 21-OHD

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

LTL in children with CAH due 11β-HSD and 3β-HSD

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

Acknowledgements

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

Data availability

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

References

1. Gorenjak V, Petrelis AM, Stathopoulou MG, Visvikis-Siest S. Telomere length determinants in childhood. *Clin Chem Lab Med*. 2020;58(2):162-177.
2. Lu W, Zhang Y, Liu D, Songyang Z, Wan M. Telomeres-structure, function, and regulation. *Exp Cell Res*. 2013;319(2):133-141.
3. Shay JW, Wright WE. Telomeres and telomerase: three decades of progress. *Nat Rev Genet*. 2019;20(5):299-309.
4. Epel E. How "reversible" is telomeric aging? *Cancer Prev Res (Phila)*. 2012;5(10):1163-1168.
5. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell*. 2013;153(6):1194-1217.
6. von Zglinicki T. Oxidative stress shortens telomeres. *Trends Biochem Sci*. 2002;27(7):339-344.
7. Cheng F, Carroll L, Joglekar MV, Januszewski AS, Wong KK, Hardikar AA, Jenkins AJ, Ma RCW. Diabetes, metabolic disease, and telomere length. *Lancet Diabetes Endocrinol*. 2021;9(2):117-126.
8. Haycock PC, Heydon EE, Kaptoge S, Butterworth AS, Thompson A, Willeit P. Leucocyte telomere length and risk of cardiovascular disease: systematic review and meta-analysis. *Bmj*. 2014;349:g4227.
9. Lee M, Martin H, Firpo MA, Demerath EW. Inverse association between adiposity and telomere length: The Fels Longitudinal Study. *Am J Hum Biol*. 2011;23(1):100-106.
10. Aulinas A, Ramírez MJ, Barahona MJ, Valassi E, Resmini E, Mato E, Santos A, Crespo I, Bell O, Surrallés J, Webb SM. Telomere length analysis in Cushing's syndrome. *Eur J Endocrinol*. 2014;171(1):21-29.
11. Lee RS, Zandi PP, Santos A, Aulinas A, Carey JL, Webb SM, McCaul ME, Resmini E, Wand GS. Cross-species Association Between Telomere Length and Glucocorticoid Exposure. *J Clin Endocrinol Metab*. 2021;106(12):e5124-e5135.
12. Aulinas A, Ramírez MJ, Barahona MJ, Mato E, Bell O, Surrallés J, Webb SM. Telomeres and endocrine dysfunction of the adrenal and GH/IGF-1 axes. *Clin Endocrinol (Oxf)*. 2013;79(6):751-759.
13. Claahsen-van der Grinten HL, Speiser PW, Ahmed SF, Arlt W, Auchus RJ, Falhammar H, Flück CE, Guasti L, Huebner A, Kortmann BBM, Krone N, Merke DP, Miller WL, Nordenström A, Reisch N, Sandberg DE, Stikkelbroeck N, Touraine P, Utari A, Wudy SA, White PC. Congenital Adrenal Hyperplasia-Current Insights in Pathophysiology, Diagnostics, and Management. *Endocr Rev*. 2022;43(1):91-159.
14. Flück CE, Achermann JC, Miller WL. The Adrenal Cortex and Its Disorders. In: Sperling MA, ed. Pediatric Endocrinology. Vol 4th ed. . Philadelphia: Saunders; 2014.
15. Leger J. Congenital adrenal hyperplasia Vol 2022: Orphanet; 2012.
16. Speiser PW. Growth and development: congenital adrenal hyperplasia-glucocorticoids and height. *Nat Rev Endocrinol*. 2010;6(1):14-15.

17. Goto M, Piper Hanley K, Marcos J, Wood PJ, Wright S, Postle AD, Cameron IT, Mason JI, Wilson DI, Hanley NA. In humans, early cortisol biosynthesis provides a mechanism to safeguard female sexual development. *J Clin Invest*. 2006;116(4):953-960.

18. Engels M, Span PN, van Herwaarden AE, Sweep F, Stikkelbroeck N, Claahsen-van der Grinten HL. Testicular Adrenal Rest Tumors: Current Insights on Prevalence, Characteristics, Origin, and Treatment. *Endocr Rev*. 2019;40(4):973-987.

19. Speiser PW. Medical treatment of classic and nonclassic congenital adrenal hyperplasia. *Adv Exp Med Biol*. 2011;707:41-45.

20. Speiser PW, Arlt W, Auchus RJ, Baskin LS, Conway GS, Merke DP, Meyer-Bahlburg HFL, Miller WL, Murad MH, Oberfield SE, White PC. Congenital Adrenal Hyperplasia Due to Steroid 21-Hydroxylase Deficiency: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab*. 2018;103(11):4043-4088.

21. Paizoni L, Auer MK, Schmidt H, Hübner A, Bidlingmaier M, Reisch N. Effect of androgen excess and glucocorticoid exposure on metabolic risk profiles in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J Steroid Biochem Mol Biol*. 2020;197:105540.

22. Reisch N. Review of Health Problems in Adult Patients with Classic Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency. *Exp Clin Endocrinol Diabetes*. 2019;127(2-03):171-177.

23. de Onis M, Garza C, Onyango AW, Borghi E. Comparison of the WHO child growth standards and the CDC 2000 growth charts. *J Nutr*. 2007;137(1):144-148.

24. Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. Nucleic Acids Res. 2009; 37(3): e21.

25. Paltoglou G, Raftopoulou C, Nicolaides NC, Genitsaridi SM, Karampatsou SI, Papadopoulou M, Kassari P, Charmandari E. A comprehensive, multidisciplinary, personalized, lifestyle intervention program is associated with increased leukocyte telomere length in children and adolescents with overweight and obesity. *Nutrients*. 2021;13(8).

26. Leys C, Ley C, Klein O, Bernard P, Licata L. Detecting outliers: Do not use standard deviation around the mean, use absolute deviation around the median. *Journal of Experimental Social Psychology*. 2013;49(4):764-766.

27. Choi J, Fauce SR, Effros RB. Reduced telomerase activity in human T lymphocytes exposed to cortisol. *Brain Behav Immun*. 2008;22(4):600-605.

28. Wei N, Yu Y, Joshi V, Schmidt T, Qian F, Salem AK, Stanford C, Hong L. Glucocorticoid receptor antagonist and siRNA prevent senescence of human bone marrow mesenchymal stromal cells in vitro. *Cell Tissue Res*. 2013;354(2):461-470.

29. Athanasoulia-Kaspar AP, Auer MK, Stalla GK, Jakovcevski M. Shorter telomeres associated with high doses of glucocorticoids: the link to increased mortality? *Endocr Connect*. 2018;7(11):1217-1226.

30. Melin J, Parra-Guillen ZP, Michelet R, Truong T, Huisinga W, Hartung N, Hindmarsh P, Kloft C. Pharmacokinetic/Pharmacodynamic Evaluation of Hydrocortisone Therapy in Pediatric Patients with Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab*. 2020;105(3).

31. Sarafoglou K, Zimmerman CL, Gonzalez-Bolanos MT, Willis BA, Brundage R. Interrelationships among cortisol, 17-hydroxyprogesterone, and androstenendione exposures in the management of children with congenital adrenal hyperplasia. *J Investig Med*. 2015;63(1):35-41.

32. Zannolli R, Mohn A, Buoni S, Pietrobelli A, Messina M, Chiarelli F, Miracco C. Telomere length and obesity. *Acta Paediatr*. 2008;97(7):952-954.

33. Ooi DSQ, Dorajoo R, Gurung RL, Dehghan R, Lim YY, Ho CWL, Tay V, Karuppiah V, Loke KY, Lim SC, Liu JJ, Sng AA, Lee YS. Association of leukocyte telomere length with obesity-related traits in Asian children with early-onset obesity. *Pediatr Obes*. 2021;16(8):e12771.

34. Garfein J, Flannagan KSJ, Mora-Plazas M, Oliveros H, Marín C, Villamor E. Prospective associations between leukocyte telomere length and adiposity in childhood. *Pediatr Obes*. 2021:e12868.

35. Charmandari E, Johnston A, Brook CG, Hindmarsh PC. Bioavailability of oral hydrocortisone in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. J Endocrinol. 2001; 169(1): 65-70.

36. Charmandari E, Hindmarsh PC, Johnston A, Brook CG. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency: alterations in cortisol pharmacokinetics at puberty. J Clin Endocrinol Metab. 2001; 86(6): 2701-8.

37. Auer MK, Krumbholz A, Bidlingmaier M, Thieme D, Reisch N. Steroid 17-Hydroxyprogesterone in Hair Is a Potential Long-Term Biomarker of Androgen Control in Congenital Adrenal Hyperplasia due to 21-Hydroxylase Deficiency. *Neuroendocrinology*. 2020;110(11-12):938-949.

38. Noppe G, de Rijke YB, Koper JW, van Rossum EF, van den Akker EL. Scalp hair 17-hydroxyprogesterone and androstenedione as a long-term therapy monitoring tool in congenital adrenal hyperplasia. *Clin Endocrinol (Oxf)*. 2016;85(4):522-527.

39. Torky A, Sinaii N, Jha S, Desai J, El-Maouche D, Mallappa A, Merke DP. Cardiovascular Disease Risk Factors and Metabolic Morbidity in a Longitudinal Study of Congenital Adrenal Hyperplasia. *J Clin Endocrinol Metab*. 2021;106(12):e5247-e5257.

40. Eastwood JR, Mulder E, Verhulst S, Peters A. Increasing the accuracy and precision of relative telomere length estimates by RT qPCR. *Mol Ecol Resour*. 2018;18(1):68-78.

41. Montpetit AJ, Alhareeri AA, Montpetit M, Starkweather AR, Elmore LW, Filler K, Mohanraj L, Burton CW, Menzies VS, Lyon DE, Jackson-Cook CK. Telomere length: a review of methods for measurement. *Nurs Res*. 2014;63(4):289-299.

42. Lin J, Smith DL, Esteves K, Drury S. Telomere length measurement by qPCR - Summary of critical factors and recommendations for assay design. *Psychoneuroendocrinology*. 2019;99:271-278.

43. Lamprokostopoulou A, Moschonis G, Manios Y, Critselis E, Nicolaides NC, Stefa A, Koniari E, Gagos S, Charmandari E. Childhood obesity and leucocyte telomere length. *Eur J Clin Invest*. 2019;49(12):e13178.

Legends for Figures and Tables

Figure 1. Study flow chart.

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

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

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

Table 1. Characteristics of the study population

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

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

*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.

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

[a] 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.

[b] 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).

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

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

Tables

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.				

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

First visit	All patients 21-OHD CAH patients (n=75)	Classic CAH patients (n=63)	Non-classic CAH patients (n=12)	P-value
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^2	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)	
Second visit*	**All patients 21-OHD CAH patients (n=74)**	**Classic CAH patients (n=62)**	**Non-classic CAH patients (n=12)**	**P-value**
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^2	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.				

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

Feature	Undertreatment (n=50 visits)	Overtreatment (n=9 visits)
***HC-equivalent* glucocorticoid dose** *Target: 8 – 12 mg/m2/day*	16 (32%)	2 (22%)
Weight change *Target: ΔWeight/BMI SDS of 0*	10 (20%)	2 (22%)
Lab results *Target: 17OHP slightly above upper limit of reference interval; A4 within reference interval*	34 (68%)	6 (67%)
Growth change *Target: ΔHeight SDS of 0*	10 (20%)	0
Other reasons	10 (20%)[a]	2 (22%)[b]

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.

[a] 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.

[b] 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).

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

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**
Age in years	-0.0146	-0.0256; -0.0036	0.009	-0.0145	-0.0255; -0.0035	0.010
Sex, female vs male	0.0479	-0.1032; 0.1990	0.534	0.0491	-0.0956; 0.1938	0.506
BMI z-score	0.0051	-0.0461; 0.0562	0.846	0.0046	-0.0450; 0.0542	0.855
Puberty, pubertal vs pre-pubertal	-0.0732	-0.1730; 0.0266	0.151	-0.0002	-0.0958; 0.0954	0.996
CAH subtype, classic vs non-classic	-0.1834	-0.4094; 0.0426	0.112	-0.2526	-0.4735; -0.0336	0.024
Medication, prednisolone vs HC	-0.3305	-0.4564; -0.2045	<0.001	-0.3042	-0.4685; -0.1399	<0.001
Daily HC-equivalent dose, mg/m^2	-0.0012	-0.0063; 0.0039	0.642	0.0014	-0.0041; 0.0070	0.607
Treatment quality			0.025			0.008
Undertreatment vs optimally treated	-0.0054	-0.0419; 0.0311	0.772	-0.0060	-0.0436; 0.0317	0.756
Overtreatment vs optimally treated	-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

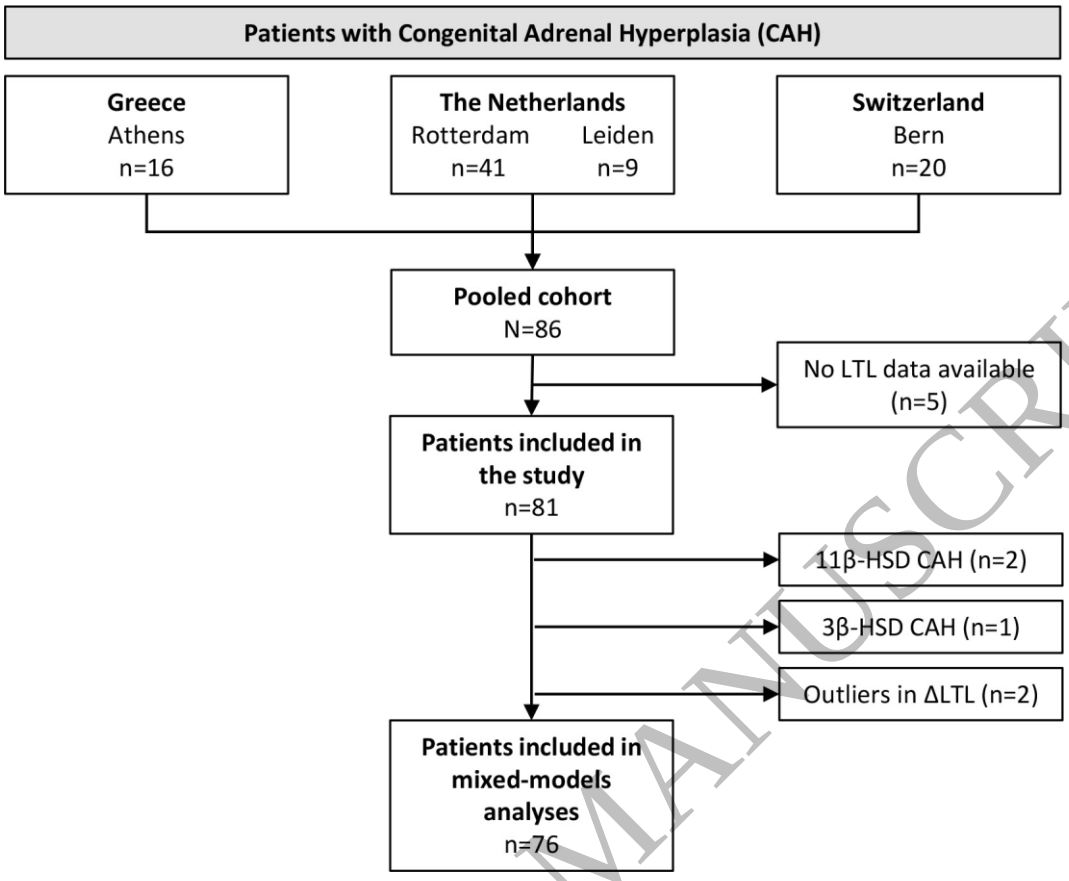

Figure 1
140x115 mm (x DPI)

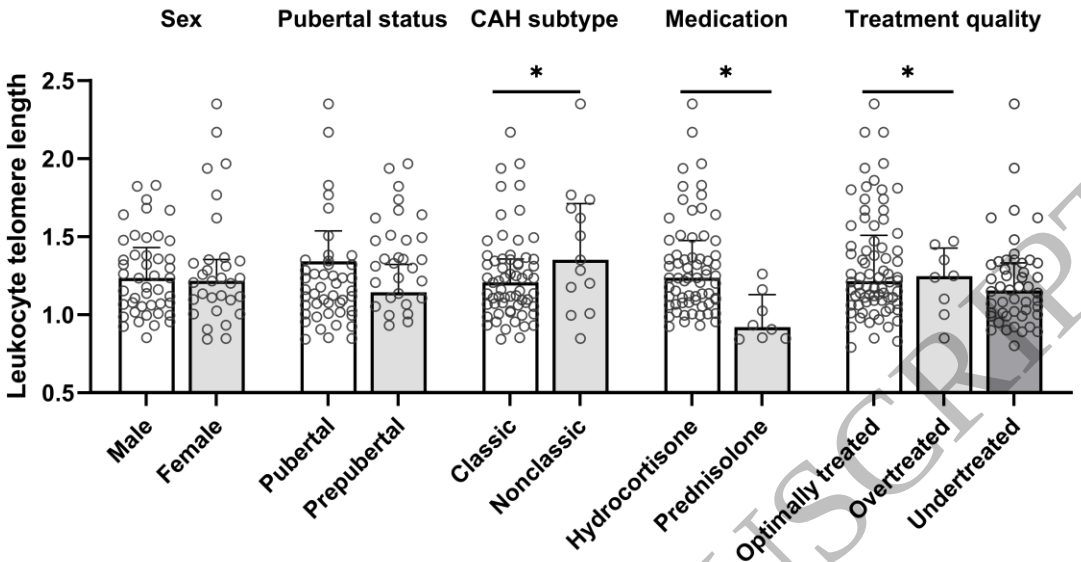

Figure 2
160x84 mm (x DPI)

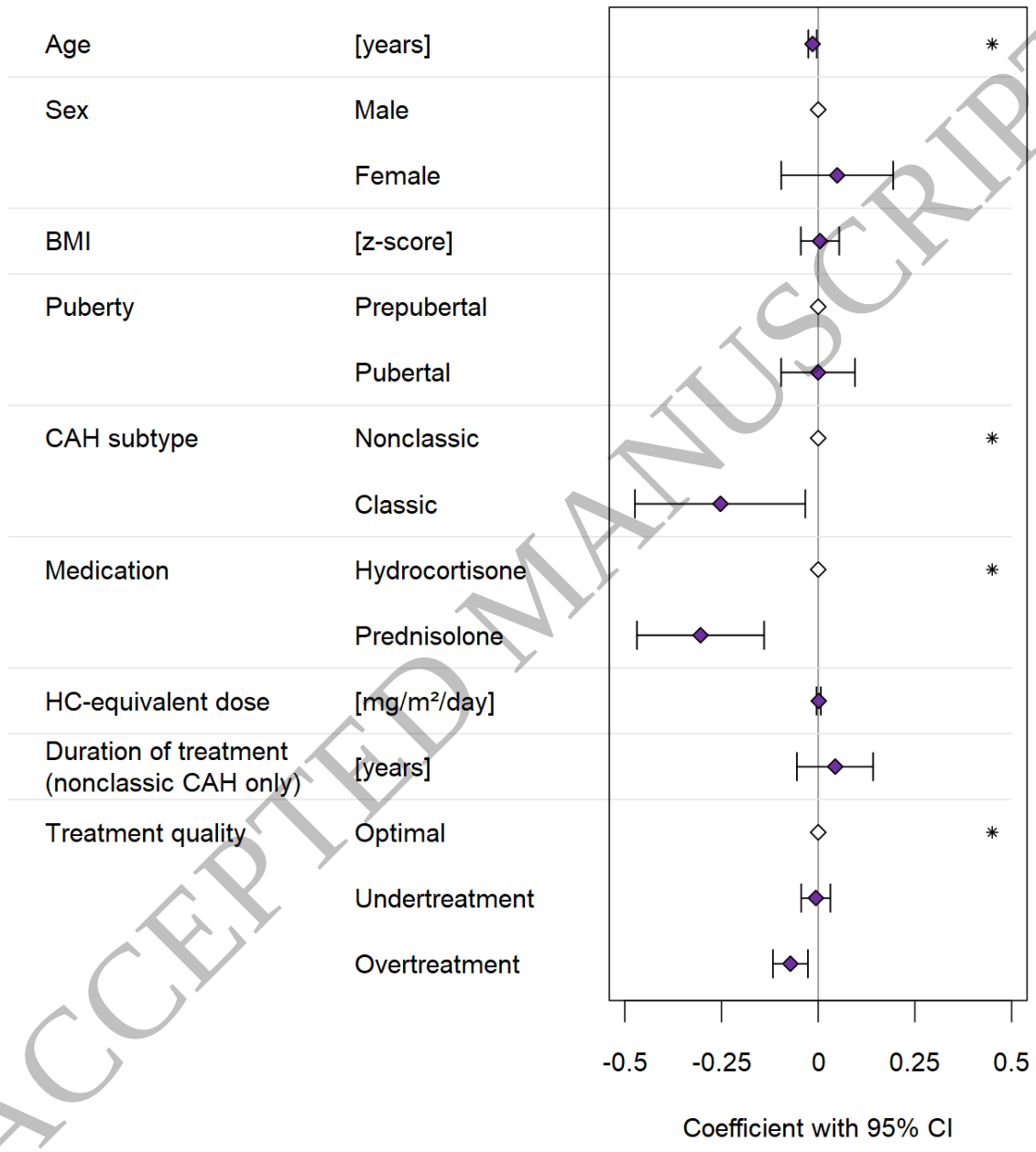

Figure 3
160x200 mm (x DPI)