Title: Telomere length analysis in Cushing's syndrome

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Abstract:

Introduction: Hypercortisolism in Cushing's syndrome(CS) is associated with increased morbility and mortality. Hypercortisolism also occurs in chronic depressive disorders and stress, where telomere length(TL) is shorter than in controls. We hypothesized that telomere shortening might occur in CS and contribute to premature aging and morbidity.

Aim: investigate TL in CS compared to controls.

Methods: Seventy-seven CS patients (14 males, 59 pituitary, 17 adrenal, 1 ectopic; 21 with active disease) were compared to 77 gender-, age- and smoking- matched controls. 15 CS were evaluated longitudinally, during active disease and after remission of hypercortisolism. Leukocyte TL was measured by TRF-Southern technique. Clinical markers were included in a multiple linear regression analysis to investigate potential predictors of TL.

Results: Mean TL in CS and controls was similar (7667 base pairs-bp- vs 7483,NS). After adjustment for age, in the longitudinal evaluation, TL was shorter in active disease than after remission (7273 vs 7870,$p<0.05$). Age and dyslipidemia were negative predictors($p<0.05$), and total leukocyte count a positive predictor for TL($p<0.05$). As expected, a negative correlation was found between TL and age (CS r-0.4 and controls r-0.292,$p<0.05$). No correlation was found between circulating cortisol, duration of exposure to hypercortisolism or biochemical cure and TL.

Conclusion: Even though in the cross-sectional comparison of CS and controls no difference in TL was found, in the longitudinal evaluation, patients with active CS had shorter TL than after biochemical cure of hypercortisolim. These preliminary results suggest that hypercortisolism might negatively impact on telomere maintenance. Larger group of patients are needed to confirm these finding.

Introduction

Cushing's syndrome (CS), a rare disease due to excessive cortisol secretion, is associated with increased mortality and severe morbidity (increased cardiovascular risk and fatigability, osteopenia, neuropsychological alterations and impaired health-related quality of life- HRQoL), not completely reversible after biochemical control (1). The mechanisms by which these abnormalities do not recover completely appear to be complex and are not currently well understood. Hyperstimulation of the hypothalamic-pituitary-adrenal axis also resulting in hypercortisolism may also occur in psychiatric diseases like acute and chronic stress and post-traumatic stress disorder (2,3). These situations are associated with poor health indexes and telomere length (TL) has been found to be shorter than in matched controls (4).

Telomeres are repetitive DNA sequences, located at the end of linear chromosomes, essential to maintain genomic stability. Without telomeres, genetic material could be lost after every cell division; thus, when telomeres are critically short, cell division stops and senescence and apoptosis are induced (5). To avoid telomere attrition and to maintain TL, germ-line cells and a few somatic cells produce an enzymatic complex called telomerase. Telomerase function can be regulated by genetic, epigenetic, environmental and hormonal factors (5). These include mainly stress hormones such as cortisol, catecholamines, estrogens and growth factors.

In this line, accelerated telomere shortening, higher levels of urinary catecholamines and free urinary cortisol have been observed in situations with high perceived psychological stress (in sisters of patients with cancer, in acute mental stress) (6). In vitro studies have shown a 50% reduction of telomerase activity in lymphocytes after exposure to high levels of hydrocortisone (7) and a rapid and dynamic loss of telomeric sequences after exposure of mice thymocytes to dexamethasone (8). Shorter leukocyte TL has been described associated with elevated cortisol responses and dysregulated patterns of daily cortisol secretion in women who are patient caregivers (9). Recently, a longitudinal study evaluating the association between coexisting changes in cortisol and telomerase activity in peripheral blood mononuclear cells (PBMCs) has been published (10). The authors examined whether participation in mindfulness-based interventions and improvements in psychological distress and metabolic factors were associated with increases in telomerase activity. They observed that serum cortisol levels were negatively correlated with changes in telomerase activity, suggesting that changes in stress-related cortisol might be one of the signals regulating telomerase

levels in humans.

This evidence led us to hypothesize that telomere shortening may be behind the increased morbidity and features of premature ageing in patients with CS. Hypercortisolemia could contribute to premature ageing by inducing accelerated telomere shortening, which in turn could be implied in the persistent morbidity and clinical consequences associated with CS, even years after biochemical remission. Since TL is an indicator of chromosome stability, proliferative capacity and cellular ageing, measuring TL could contribute to the understanding of its clinical and biological significance. To the best of our knowledge, telomere dysfunction has not been evaluated in CS patients before.

The aim of this study was to investigate TL in patients diagnosed with CS compared to sex-, age- and smoking- matched healthy controls and to evaluate whether normalization of the hypothalamic-pituitary-adrenal axis after treatment reverses possible abnormalities.

SUBJECTS AND METHODS

Subjects

In this case-control study, patients with endogenous CS followed in our institution since 1982 were eligible. Patients with adrenal carcinoma were excluded. Seventy-seven CS patients and 77 controls, matched for gender, age and smoking participated in the study. Fourteen were men (18.2%) and 63 women (81.8%). Mean age at the time of the study was 48.6±12.8 years. Fifty-nine patients were of pituitary origin (76.6%), 17 of adrenal origin (adrenal adenoma or bilateral macronodular hyperplasia) and in one patient the origin was unknown (ectopic ACTH secretion of unknown source). Twenty-one patients (27.3%) had active disease at the time of the study and 56 (72.7%) were cured; mean time of remission of hypercortisolism was 6.4±7.2 years. Eight active CS patients (38%) were treated with metyrapone, 6 (28.5%) with ketoconazole and 3 (14.2%) with both drugs. Mean duration of endogenous hypercortisolism was 72 months (range 11-264). Duration of hypercortisolism was considered as the period between onset of symptoms (as referred by the patients) and remission of hypercortisolism (in patients in remission) or the time of current analysis (in active patients). The period between onset of symptoms and biochemical diagnosis of CS was 34 months (range 3-120). Twenty-two patients (28.6%) had received pituitary radiotherapy and 71 (92.2%) had undergone surgery. Fifty-three % (n=41) were cured after initial treatment and had no recurrence and 19.5% (n=15)

were cured after further therapies for recurrent disease. Fifteen cured patients (19.5%) were adrenal insufficient at the time of telomere analysis and required substitution therapy with hydrocortisone (mean dose 17.6±3.7 mg, range 10-20). Nine (11.7%) patients were GH-deficient (4 of which were replaced with recombinant human GH); 8 women (10.4%) were gonadotropin-deficient (all on estrogen/progesterone hormone replacement therapy), and 15 patients (19.4%) were hypothyroid, 10 due to TSH deficiency and 5 due to primary hypothyroidism (all on L-thyroxine replacement). CS was considered in remission if either adrenal insufficiency was demonstrated (basal morning cortisol < 100 nmol/l [<4µg/dl] and/or undetectable 24-h free urinary cortisol) or morning cortisol suppression (<50 nmol/l, < 1.8 µg/dl) after 1 mg dexamethasone overnight was observed. Twenty-five patients (32%) were on antihypertensive medication, 17 (22%) on statin treatment for dyslipidemia, and 12 (16%) were treated with calcium and vitamin-D.

In a subgroup of 15 CS (all women) patients studied initially with active disease, a second analysis of TL was performed once they were in remission. In this longitudinal study, 3 were of adrenal origin and 12 of pituitary origin. Mean age at the time of active disease was 43.5±12.1 years and at remission was 46.6±11.3 years. The time elapsed between both analyses was 40.1±15.6 months and mean time of remission was 28.5±14.1 months. Three cured patients (20%) were adrenal insufficient at the time of telomere analysis and required substitution therapy with hydrocortisone (mean dose 18.3±2.2mg, range 10-20); 4 patients (26.6%) were hypothyroid, 2 due to TSH deficiency and 2 due to primary hypothyroidism (all on L-thyroxine replacement). None of the cured patients were GH-deficient; 7 women (46.6%) were postmenopausal at remission but no gonadotropin-deficiency was observed (n=8).

Seventy-seven controls selected from the blood bank donor's database or from healthy volunteers recruited among hospital employees were matched for gender, age and smoking status, three features known to affect TL. Namely, age is an important determinant of TL, typically decreasing with advancing age (11). Females usually present longer TL than males, since estrogens stimulate telomerase activity and protect DNA from reactive oxygen species (ROS)-induced damage (12). Cigarette smoke constituents increase cumulative and systemic oxidative stress and inflammation, which induce increased white blood cell turnover, resulting in accelerated TL shortening (13). Medical history and physical examination excluded any who reported glucocorticoid exposure, severe and/or acute diseases and severe psychiatric alterations (however, anxiety

and mild depression were not exclusion criteria). Four controls (5.7%) were on antihypertensive therapy, another 4 (5.7%) were receiving statin treatment for dyslipidemia, and 3 (4.3%) were treated with calcium and vitamin-D.

Anthropometry (weight, height, body mass index and waist/hip ratio) was measured in patients and controls. Hypertension was defined as systolic blood pressure > 140 mmHg or diastolic blood pressure > 90 mmHg or the use of antihypertensive medications. Dyslipidemia was defined as total cholesterol (TC) >220 mg/dl, low-density lipoprotein (LDL) >130 mg/dl, triglyceride levels ≥150 mg/dl or treatment with lipid-lowering medication. Diabetes mellitus was confirmed with fasting glucose levels >126 mg/dL in two consecutive determinations or 2-hour glucose after OGTT >200 mg/dL. Adult patients were considered osteopenic when T score was <-1 and >-2.5 or osteoporotic when T score was <-2.5 SD.

All participants provided a blood sample for DNA extraction and gave their informed consent. The study was approved by the hospital ethics committee.

Methods

Genomic DNA extraction from total leukocytes was performed using an adapted Proteinase K and Phenol protocol (14). Blood samples from the patients were collected in EDTA tubes to reduce DNA degradation. Genomic DNA was isolated from blood buffy coats. The buffy coat and white blood cell pellets were stored frozen at -80°C prior to processing. The white bood cell layers were harvested and digested with buffer containing 0.1 M MgCl2, 0.02 M EDTA, 0.5% SDS, 0.01 M Tris, pH 8.0, and 1 mg/mL of proteinase K at 37°C overnight. The lysates were homogenized by passes through a blunt 20-gauge needle (0.9 mm diameter) at 4ºC temperature and DNA was purified by phenol:chloroform:isoamilic alcohol (25:24:1) extraction, and ethanol precipitation. Finally, genomic DNA was dissolved in Tris-EDTA buffer and was quantified by spectrophotometric analysis. The quality of genomic DNA was checked for high molecular weight by 1% agarose gel electrophoresis.

TL measurements were performed by the telomere restriction fragment assay (TRF) using the Telo TAGGG Telomere Length Assay Kit (Roche 12209136001). Briefly, 1 µg of DNA was digested with 20 units of Rsal

and HinfI for 2 h at 37ºC. Samples were loaded on a 0.5% Seakem® Gold Agarose gel and were run for 21 h at 35 V. Gels were treated with HCl, denaturalized and neutralized, and then transferred to a nylon membrane by capillarity for 12-18 h. After fixation with UV, hybridization was carried out with a DIG-labelled telomeric probe (3 h at 42ºC). Finally, restriction washes, incubation with anti-DIG-AP antibody and detection by chemiluminiscence was carried out. Images were analysed with the program Quantity One. TRF mean was calculated using the formula: TRF mean = $\sum OD_i / \sum (OD_i / L_i)$, where OD_i is the chemiluminiscent signal and L_i is the length of the TRF fragment at position *i* (15). The accuracy of Southern Blot technique is up to ± 300 base pairs (16). A control sample, 2 µg of digested DNA derived from a single batch of Hela cells, was run on each gel to minimize interassay variation. The mean TL for Hela cells was 4113bp with a standard deviation of ±210bp, which is in the range of the accuracy of Southern Blot technique.

Biochemical, hormone and bone analyses

Routine serum determinations were performed by standard automated laboratory methods: fasting glucose, total cholesterol, high and low-density lipoprotein (HDL/LDL) cholesterol and triglyceride levels. Blood counts were performed using automated cell counters. Twenty-four-hour urinary free cortisol was measured with a commercial RIA with prior extraction with an organic solvent. Plasma ACTH, serum cortisol and IGF-1 levels were measured using a commercial chemiluminiscent immunometric assay. Lumbar spine and whole body bone mineral density and content (BMD and BMC) were measured by DXA scanning (Delphi QDR 4500; Hologic); the mean precision error (CV) was 1%.

Statistical analysis

Statistical analyses were performed using the SPSS 19.0 statistical package for Windows (SPSS Inc, Chicago Illinois). Initially a descriptive analysis of all variables was performed in order to verify correct introduction of data in the database. Quantitative data are expressed as mean and SD (Gaussian distribution) or as median and range (non-Gaussian distribution), and categorical data are expressed as percentages. Data distribution was analyzed by the Kolmogorov-Smirnov test. TL variable was normally distributed. Logarithmic transformations were performed where necessary to normalize the distribution of a particular measure. Comparison between 2 groups was performed using Student's t (Gaussian distribution) or Mann-Whitney's

U (non-Gaussian distribution) tests. A Chi-square test was performed for categorical variables. Fisher exact test was performed when appropriate. Pearson's correlation coefficient was used to estimate linear association between two quantitative variables. Analysis of covariance (ANCOVA) was performed to evaluate TL after adjustment for age and for total leukocyte count (as covariates). Multiple linear regression analysis including age, gender, body mass index, T2DM, dyslipidemia, hypertension, psychiatric history, duration of hypercortisolism, current hypercortisolism, total leukocytes and 24 hour urinary free cortisol as potential predictive factors for TL (as dependent variable) was performed.

P values < 0.05 were considered significant.

RESULTS

Comparison between CS and matched controls (Tables 1 and 2).

Main baseline characteristics of CS patients and controls are summarized in table 1. CS patients had more hypertension, diabetes, dyslipidemia and osteoporosis than their matched controls ($p<0.05$). Mean TL values in CS and controls are summarized in Figure 1. No differences were observed between males and females (7732±1242 vs. 7540±1361 bp, respectively). TL did not differ between CS and controls (7667±1260 vs. 7483±1214, respectively, ns). TL did not differ between active CS, cured CS (with or without secondary adrenal insufficiency) and their matched controls (Figure 1).

As expected, a negative linear correlation between age and TL in the whole sample was observed ($R = -0.341$, $p <0.001$). When both groups were evaluated separately, this negative correlation was maintained in CS patients ($R = -0.400$, $p < 0.001$) and in controls ($R = -0.292$, $p < 0.01$) (Figure 2). A positive correlation was found between IGF-1 and TL in CS patients ($R = 0.331$, $p < 0.05$), but was not correlated with the presence or absence of GH deficiency or rhGH replacement therapy. No differences in TL were observed related to the presence of pituitary deficiencies and/or replacement therapies either. No correlation was observed between duration of hypercortisolism and TL ($R = -0.025$, p NS), or between morning serum cortisol ($R = 0.047$, p NS), 24 hour urinary free cortisol ($R=0.072$, p NS) or plasma ACTH ($R=0.192$, p NS) and TL. In active CS patients, we did not observed differences in TL according to steroidogenesis inhibitors we used (metyrapone 8258±1178 vs ketoconazole 7896±1432, NS).

In the multiple linear regression analysis performed to identify potential predictive factors of TL, we

observed that age and dyslipidemia were negative predictive factors for TL shortening (p=0.006 and p 0.017, respectively), while total leukocyte count was a positive predictor for TL (p=0.043) (R^2 0.23), indicating that more leukocytes were associated with longer TL. The main leukocyte cell subtypes count (neutrophils and lymphocytes) differed between active CS patients and controls (Table 2), but not between cured CS patients and their healthy controls. After adjustment for total leukocyte count as covariate, no differences in TL between the 21 active CS and their controls were observed either (7600±1197 vs 7450±1274, p NS).

Longitudinal analysis in CS patients evaluated both during active disease and in remission

As expected, patients were older once remission was attained. Ten patients (66%) clearly showed an increment of TL upon remission of CS. In 5 (33%) TL decreased after remission (Figure 3), but was minimal in 2 and of doubtful relevance, since it was around the detection limit of 300 bp (around 4%) TL's variation in our population (20). Moreover, after adjustment for age as covariate, TL was shorter in active disease than after remission (7273±1263 vs. 7870±1039, respectively, $p<0.05$) in the same patients (figure 3), in sharp contrast with TL shortening usually observed as age increases. No significant differences in the presence of hypertension, dyslipidemia, diabetes or use of medications were observed between the group of patients who increased their TL during remission and those who did not increase TL. Patients who incremented TL, also decreased their body mass index more after remission than those who did not increase TL (-2.3 kg/m^2 vs. -0.8 kg/m^2) although due to the small group size, it did not reach statistical significance (p = 0.19). A trend for a positive correlation between TL at remission and duration of remission was also seen (R = 0.494, p=0.061).

DISCUSSION

To the best of our knowledge, this is the first study to evaluate TL in this rare disease and with a relatively large series of CS patients. When investigated longitudinally, our preliminary data show that patients with active CS have a shorter TL, which become longer after hypercortisolism disappeared with effective treatment. However, in the cross-sectional case-control study comparing all patients with CS and matched controls, no differences in TL were found. This was also the case when patients with active hypercortisolism, and those considered in remission (with or without concomitant adrenal insufficiency) were compared with their respective matched controls.

CS patients provide a unique opportunity to examine the effects of hypercortisolism on telomere maintenance. CS determines increased morbidity and mortality, especially in the untreated state but also after therapy when compared to background population (1, 17). Severe morbidities are also increased even in the 3 years prior to diagnosis when compared to normal population, and are not completely reversible after endocrine cure (17). The mechanisms by which CS patients do not recover completely after biochemical remission are still unknown. It is possible that telomere dysfunctions partially contribute to these abnormalities. In other situations where hypercortisolim is often present such us chronic stress and some psychiatric conditions, TL has been found to be shorter than in matched controls (6,9). These previous evidences took us to hypothesize that TL shortening could contribute to the increased morbidity and features of premature ageing observed in endogenous hypercortisolism of CS. Thus, we planned this study in order to investigate the telomere system in these patients.

We have evaluated a significant number of CS patients (n=77), a rare disease with an incidence ranging from 0.7 to 2.4 cases per million inhabitants per year (18). They were carefully matched for age, gender and smoking status with controls. These relatively small groups may contribute to explain why no differences in TL were observed between CS and controls. Furthermore, many other factors apart from hypercortisolism may affect TL, both individual and environmental (genetic, epigenetic, socio-economic status, lifestyle, growth factors, etc) (5). Additionally, TL may be affected by what is known as a "pseudolengthening" mechanism (19); specifically, TL of lymphocytes becomes increasingly shorter than those of granuylocytes over the years (20). And since a redistribution of leukocyte cell type is often seen in hypercortisolism (lymphopenia and neutrophilia) this may also affect the measured TL obtained from the total leukocyte count (21). In fact, we did find that in active disease total leukocyte and neutrophil counts were higher and lymphocytes lower than in matched controls. We observed that total white blood cell counts in each individual blood sample also affected TL, and CS patients had higher total leukocyte counts compared to healthy controls, similar to other series (21). However, after adjustment for total leukocyte count (as a covariate) no differences in TL between CS and their healthy controls were identified.

In the multiple regression analysis, leukocytes count together with age and the presence of dyslipidemia were predictive factors for TL, explaining 23 percent of the TL present in our CS patients. Not surprisingly, age was a negative predictive factor for TL, in the whole sample and in the different subgroups analysed. A

positive correlation was also seen between IGF1 levels and TL, as described in healthy population (11, 22). Both findings support the reliability and validity of our results and the methodology used, since similar correlations have been described in much larger populations (but not in CS patients)(14); namely TL was positively correlated with serum IGF1 and negatively associated with age in a cohort of 476 healthy Caucasians aged 16-104 years (22). We also observed a negative correlation between TL and dyslipidemia as described in other paradigms, where cholesterol has been associated with faster biological aging (23).

As expected, some baseline characteristics differed between CS and controls, such as serum morning cortisol and 24 hour urinary free cortisol, certain cardiovascular risk factors and psychiatric conditions (anxiety and depression), which were more prevalent in CS patients. Most of these features have recently been related to telomere dysfunctions (9, 24), although not all results published in the literature are concordant (25). Even though in the case-control regression analysis they did not seem to have impacted on TL with the exception of dyslipidemia which negatively affected TL, we can not rule out that in much larger studies some of these clinical features could determine TL in some way or another. We did not find any influence of medical treatment to reduce cortisol during active disease or glucocorticoid replacement in patients with adrenal insufficiency after CS therapy on TL.

The longitudinal analysis of 15 patients evaluated both during hypercortisolism and in remission, adjusting for age (as a covariate), confirmed our initial hypothesis, since patients with hypercortisolism during active disease did have shorter telomeres than later in remission (average 596 bp). In spite of being 40.1±15.6 months older at remission, TL was longer and positively associated with duration of remission. Although this finding is very preliminary based on a small number of patients, which makes difficult to reach firm conclusions, it would support our initial hypothesis of a negative effect of a hyperactive hypothalamic-pituitary-adrenal axis on TL and cell senescence observed in other studies. Accelerated telomere shortening was observed in a group of 647 women (who had a sister with breast cancer) with higher perceived stress and higher levels of urinary free cortisol and cathecolamines (6). Similarly, shorter buccal cell TL was observed in children exposed to laboratory stressors with higher levels of salivary cortisol and higher autonomic reactivity (26). Greater cortisol responses and dysregulated patterns of daily cortisol secretion were associated with shorter leukocyte TL in 14 postmenopausal women caregivers of a partner with dementia compared to matched noncaregiver controls (27). Consistent with this and with our longitudinal results, one

in vitro study observed how exposure to high hydrocortisone levels comparable to those that might be reached in vivo during stress, reduced telomerase activity in lymphocytes (7). As the major pathway for telomere lengthening seems to be through telomerase activation, this could explain why a patient could have shorter TL during hypercortisolism. It is probably that when cortisol normalizes, a recovery of telomerase activity takes place, increasing TL or lowering attrition rates.

Contrary to this evidence and to our results, a recent publication showed telomere shortening associated with hypocortisolism was observed in patients with high levels of chronic stress exposure or high degrees of inflammation which could lead to an exhaustion of the HPA axis. It is difficult to identify the mechanism responsible for accelerated telomere shortening in hypocortisolism, often preceded by a hypercortisolaemic phase in long-term chronic stress exposure, suggesting that TL could be a measure of cumulative stress (28). We found no differences in TL in our hypocortisolaemic patients compared to cured patients without secondary adrenal insufficiency; an explanation could be that all adrenal insufficient patients were correctly replaced with hydrocortisone.

Lifestyle modifications like increased physical activity after remission may also increase TL, as reported in some studies, by inducing changes in telomerase activity. The mean fall in BMI in patients who increased TL was greater than in those who decreased TL after remission (-2.3 kg/m^2 vs. -0.8 kg/m^2), but did not reach statistical significance, probably due to the small sample size in the longitudinal evaluation. This change in BMI may contribute to explain the increase in TL in cured patients, similarly that seen in a recent longitudinal intervention study with Mediterranean diet, where BMI was inversely correlated with changes TL (29).

A model of dynamic telomere balance under stress has been suggested, in which severe stress first would lead to increased turnover and depletion of circulating cells followed by a compensatory re-population when stress ends (in short stress conditions). This model could also be present in CS patients, but has to be confirmed. It would appear to be important to distinguish between true reversal of telomere shortening and replenishment by younger cells ("pseudo-lengthening") that probably takes place in CS after remission (19).

The study has several limitations. The sample size, although respectable considering that CS is a rare disease, precludes any analysis in different etiological subgroups of CS. This also did not allow to control for all potential confounders especially medical treatment during active disease, physical activity, current stress, etc.

Especially in hypocortisolemic patients after surgery for CS a perfect cortisol replacement is an elusive goal. Although the results of the longitudinal evaluation are the opposite to what is expected by increasing age and it is an interesting result, this finding is certainly preliminary based on a small group of patients. We could not include the remaining 6 active patients, because 4 of them still present active disease and we lost the follow up in two patients. A larger group of patients, as well as a larger group of patients followed longitudinally would clearly strengthen the conclusion of our preliminary findings. White blood cells, the most characterized tissue source for telomere studies, easily obtainable from peripheral blood, may vary in their cell type's distribution in blood as seen in CS patients. TL variability even in the same cell and for individuals of similar age complicates any conclusions on telomere biology in CS patients (30). Most studies on telomere biology and ageing are much larger and cross-sectional but large scale, longitudinal, prospective and well-designed studies are lacking. It would be interesting to evaluate TL in other tissues such as the pituitary or the adrenal in CS, since glucocorticoids induce changes in the immune system; however, this would be even more difficult than obtaining peripheral leukocytes for TL evaluation. As well as, we could not measure telomerase activity, which probably could provide a more direct approach on both telomere system and its dynamics.

The main conclusion of this study is that in individual CS patients in whom hypercortisolism is controlled after successful treatment, TL increases despite being on average 3 years older. It would appear, therefore, that telomerase activity would be induced once hypercortisolism disappears, and this could be one of the mechanisms by which increased morbidity, mortality and biological ageing improve when disease is controlled. However, in the entire group of CS patients no difference in TL was observed when compared to healthy controls, pointing to the fact that many other factors determine TL apart from age, including dyslipidemia, healthier life-styles or differences in leukocyte subsets cell counts. Larger prospective studies are required to confirm these changes in TL in CS and investigate implications of these abnormalities further.

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383 **FIGURE LEGENDS:**

384 **Figure 1**. Telomere length (TL) in the whole group of Cushing's syndrome (CS) patients and controls
385 (7667±1260 vs 7483±1214 bp.), as well as in patients with active CS (7943±1309 vs 7230±1591 bp.), cured
386 CS without (7510±1219 vs 7639±1335 bp.) or with adrenal insufficiency (AI) (7727±1323 vs 7394±1411
387 bp.) compared with their respective matched controls. No differences were observed. * Abbreviations: CS,
388 Cushing's syndrome; AI, adrenal insufficiency; TL, telomere length.

389

390 **Figure 2.** Telomere length in relation to age in patients with Cushing's syndrome (•) and controls (◦).
391 Telomere length is shortened with advancing age in both CS ($R = -0.400$, $p < 0.001$) and controls ($R = -0.292$,
392 $p < 0.01$). *Abbreviations: bp. base pairs.

393

394 **Figure 3. 3A**: Changes in telomere length (TL) in 15 patients in whom samples were obtained both during
395 active hypercortisolism (7273±1263 bp.) and after remission (7870±1039 bp.). **3B**: TL increased in 10/15
396 patients, increasing age. The dotted line shows the detection limit of the Southern Blot technique.
397 *Abbreviations: bp. base pairs; CS. Cushing's syndrome

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403 **TABLES:**

404 **Table 1.** Baseline characteristics of patients with Cushing's syndrome (CS) and controls. Data are presented

405 as % and mean ± SD.

	CS (n=77)	Controls (n=77)	p
Age (years)	48.6± 12.8	48.4± 12.6	NS
Smokers	24.7%	19.4%	NS
Alcohol consumption	26%	27.3%	NS
Diabetes mellitus (type 2)	14.3%	1.4%	<0.05
Arterial hypertension	57.1%	12.9%	<0.001
Dyslipidemia	45.5%	20.0%	<0.05
Osteoporosis	29.9%	2.9%	<0.001
Psychiatric history	37.7%	11.4%	<0.001
Body mass index (kg/m2)	28 ± 5.6	26.4 ± 4.9	<0.05
Waist to hip ratio	0.92±0.07	0.85±0.07	<0.05
24h urinary free cortisol (nmol/24 hours)	266±180	132±59	<0.001
Morning serum cortisol (nmol/l)	450±259	375±120	<0.05
Leukocytes ($x10^9$/l)	7.3±2.3	5.8±1.7	<0.05
Neutrophils ($x10^9$/l)	4.4±2.0	3.5±1.2	<0.05
Lymphocytes ($x10^9$/l)	2.1±0.8	1.9±0.4	NS

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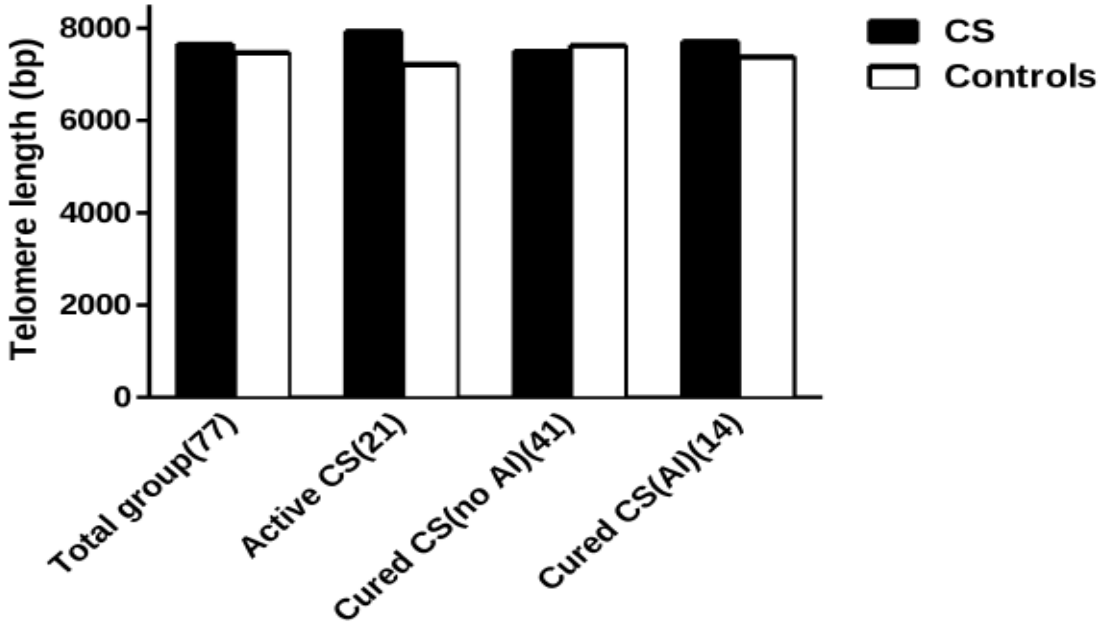
407 **Table 2.** Total leukocyte counts and leukocyte main subsets distribution (neutrophils and lymphocytes) of
408 Cushing's syndrome (CS) patients during active disease and remission and their matched controls. Data are
409 expressed as mean±SD.

	CS	Controls	p
-Leukocytes in active disease ($x10^9$/l) (n=21):	8.8 ± 2.3	5.9 ± 1.4	<0.01
.neutrophils (%)	64.7 ± 11.0	55.5 ± 6.1	<0.05
.lymphocytes (%)	24.5 ± 9.1	32.1 ± 7.8	<0.05
-Leukocytes in cured patients without adrenal insufficiency ($x10^9$/l) (n=41):	6.7 ± 2.1	5.8 ± 1.8	<0.05
.neutrophils (%)	57.1± 8.2	54.9 ± 13.8	NS
.lymphocytes (%)	31.1 ± 6.6	30.9 ± 7.1	NS
-Leukocytes in cured patients with adrenal insufficiency ($x10^9$/l) (n=15):	6.6 ± 1.5	6.2 ± 2.1	NS
.neutrophils (%)	58.3 ± 8.7	52.5 ± 7.7	NS
.lymphocytes (%)	29.6 ± 9.6	34.5 ± 6.6	NS

410 *Abbreviations: bp. base pairs

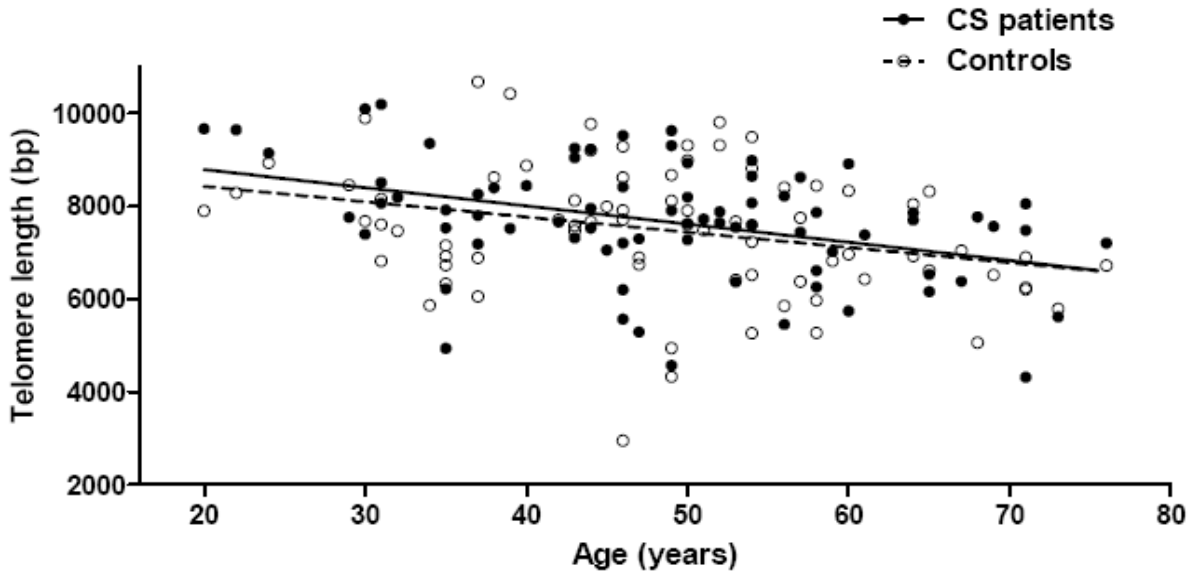
411

Figure 1. Telomere length (TL) in the whole group of Cushing's syndrome (CS) patients and controls (7667±1260 vs 7483±1214 bp.), as well as in patients with active CS (7943±1309 vs 7230±1591 bp.), cured CS without (7510±1219 vs 7639±1335 bp.) or with adrenal insufficiency (AI) (7727±1323 vs 7394±1411 bp.) compared with their respective matched controls. No differences were observed.



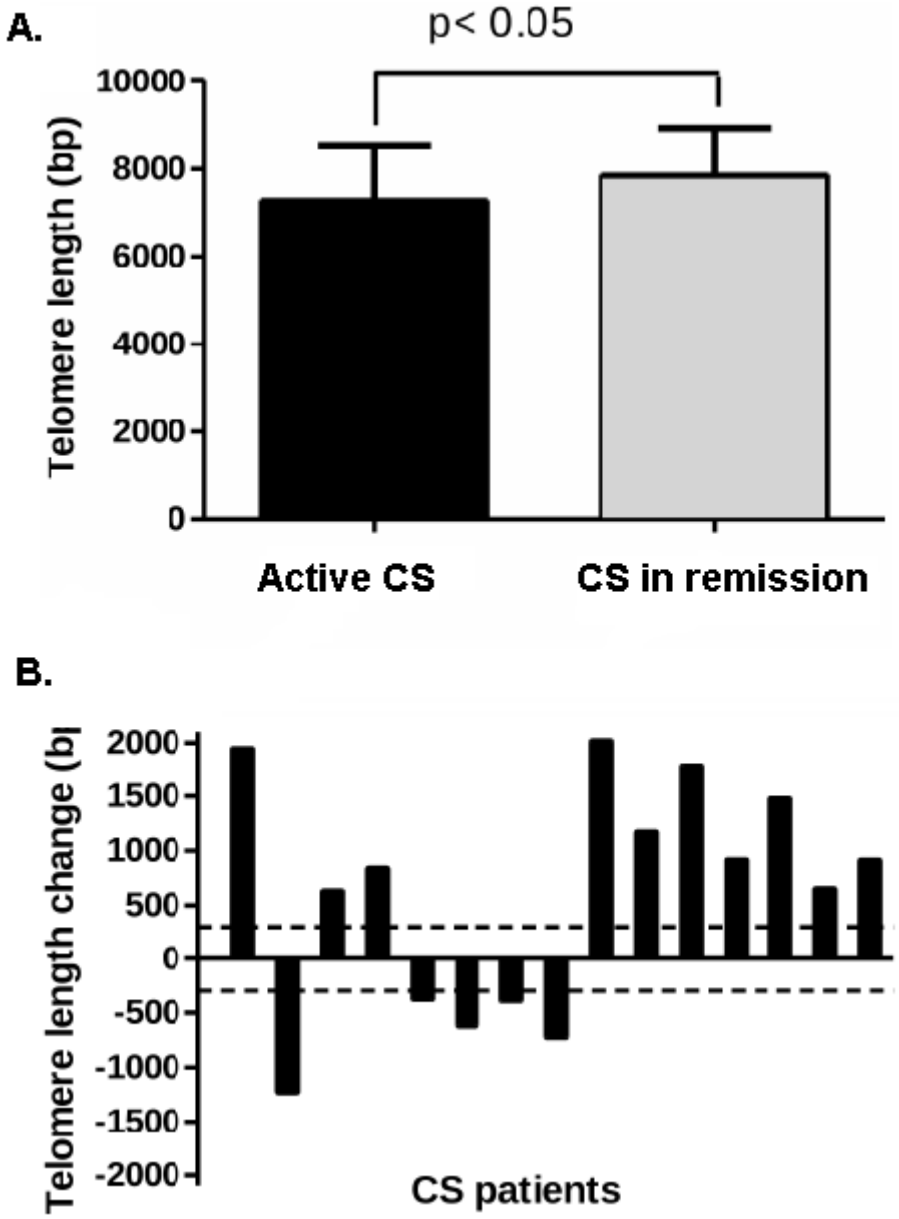
* Abbreviations: CS, Cushing's syndrome; AI, adrenal insufficiency; TL, telomere length

Figure 2. Telomere length in relation to age in patients with Cushing's syndrome (•) and controls (◦). Telomere length is shortened with advancing age in both CS (R = -0.400, p <0.001) and controls (R = -0.292, p <0.01).



*Abbreviations: bp. base pairs.

Figure 3. A: Changes in telomere length (TL) in 15 patients in whom samples were obtained both during active hypercortisolism (7273±1263 bp.) and after remission (7870±1039 bp.). 3B: TL increased in 10/15 patients, increasing age. The dotted line shows the detection limit of the Southern Blot technique.



*Abbreviations: bp. base pairs; CS. Cushing's syndrome

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